The role of biofilm in wounds

A thesis submitted to the University of Wales, Cardiff in candidature for the degree of

DOCTOR OF PHILOSOPHY

By

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The research was undertaken under the auspices of the University of Wales Institute, Cardiff (UWIC) at the Cardiff school of Health Sciences,
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DECLARATION

This work has not been previously been accepted in substance for any degree and is not being submitted in candidature for any degree.

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List of publications and presentations

This work has been the subject of the following publications and presentations

Publications

Cooper RA and Okhiria O (2008). The issue of biofilms in wounds. Chapter 12; In Advances in Wound Care. Ed. White R. Healthcare Comm. UK Limited: Aberdeen pp189-204.

Rose Cooper and Olusola Okhiria (2006). Biofilms, wound infection and the issue of control. Wounds UK; 2 (3) 48-57.

Posters and oral Presentations

Okhiria OA, Cooper R.A. and Peters A. (2009) Poster presentation of „‟Detection of biofilm markers in wound dressings‟‟‟ at the Society for General Microbiology (SGM) spring meeting, Harrogate, UK March 30 - April 2, 2009.

Okhiria OA (2009) Oral presentation of „‟ Prevalence of biofilm in wounds" at the Research Seminar Series of Cardiff School of Health Sciences, UWIC on the 25th of February, 2009.

Okhiria O. A (2009) Poster presentation of „‟A survey of used wound dressings for the detection of biofilm markers" at the Annual Academic Associate Symposiums UWIC, January 15, 2009.

Okhiria OA, Cooper RA, Peter A (2007) Poster presentation of „‟Developing an assay method for screening honey for quorum sensing inhibition of biofilm organisms‟‟‟ at the 4th American Society for Microbiology conference on Biofilms in Quebec, Canada March 25-29, 2007.

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Okhiria OA (2006) Oral presentation of „‟ Developing an assay method for quorum sensing inhibition by honey" at the Cardiff School of Health Sciences Research Colloquiums, Gregynog, UK on the 16th of May, 2006.

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Abstract

Biofilms have long been implicated in persistent infections and have recently been associated with chronic wounds. The role of bacteria in wounds is not yet fully understood and their ability to form biofilm is yet to be fully elucidated. Biofilms are associated with phenotypic features such as the signalling molecules for regulation of activities within biofilm and secretion of extracellular polymeric substances (EPS). The presence of biofilm may be confirmed by specialised microscopy techniques or by detecting biofilm markers. Routine methods are not yet available for detecting biofilms in wounds. The aims of this project were to investigate the role of biofilm in wounds, by examining the ability of wound isolates to form biofilm and produce signalling molecules and by developing a wound model; to relate laboratory findings with *in vivo* activity by exploring the possibility of detecting biofilm markers in dressings removed from chronic wounds.

Biofilm characteristics of 97 *Pseudomonas aeruginosa* strains isolated from wounds were investigated. Their antibiotic susceptibility to commonly used antibiotics was determined. The isolates were examined for ability to form biofilm and to produce acyl-homoserine lactone (AHL) signalling molecules in batch culture and were characterised using PCR. A wound model was developed for biofilm continuous culture using gauze as the substratum for biofilm attachment and culture effluent was examined for AHL production and detached fragments of biofilm. Gauze removed from the culture at 30 hours was examined with scanning electron microscopy (SEM). Thirty five dressings removed from chronic wounds were investigated for the presence of biofilm markers including AHL and EPS. Organisms from the wound dressings were isolated and examined for biofilm formation, AHL and EPS. Thirty hour biofilm of *P. aeruginosa* wound isolate was exposed to 40 % (w/v) honey in wound model continuous culture. The potential of some honeys to inhibit the growth and quorum sensing (QS) of a biofilm forming organism, *Chromobacterium violaceum* was investigated.

Over 90% of the 97 cultures of *P. aeruginosa* were resistant to 3 antibiotics while resistance to 4 others ranged between 3 – 19%. Eighty eight of the isolates (90.72%) formed biofilm while 78 (81.4%) produced AHL. PCR characterisation of the isolates showed that 82 (84.53%) have 100% genetic similarity linkage to the cohort, 3 (3.09%) have 75 - 99% while 12 (12.37%) are of 50 - 75% linkage. Examination of stained culture effluent smear from wound model revealed biofilm embedded in EPS and AHL was detected in sterilised culture effluent. SEM examination of gauze removed from wound model after 30 hours culture confirmed biofilm structures. Eleven (31.4%) wound dressings tested positive for AHL, 28 (80%) contained EPS (4 not tested for EPS). Organisms that formed biofilm were isolated from 32 (91.4%) dressings while Gram negative bacteria that produced AHL were isolated from 13 dressings. Two selected Gram negative bacteria from wound dressing that were cultured in wound model showed progressive biofilm formation with EPS and AHL production. The 30 hr biofilm exposed to honey was dislodged within 6 hours and no viable organism was recovered from culture. Honey inhibited the growth and QS of *C. violaceum* in a dose dependent manner. Of the 10 honeys examined, 8 (80%) inhibited the growth and quorum sensing of *C. violaceum*, 1 slightly inhibited quorum sensing while 1 showed no inhibitory effect.

Indicators of biofilms detected in used wound dressings have the potential to be used in the diagnosis of biofilms in chronic wounds. The antimicrobial effect of honey on biofilm and quorum sensing as shown in this study suggests that application of honey in wound management will provide effective treatment for wounds with biofilm.

Chapter 1

Introduction and Literature Review

1.1 Introduction

The ability of a microorganism to establish an infection depends on a number of factors which include those of the host and the pathogen. The pathogenic factors are as numerous as the host factors and diverse in their functions. The number of organisms present at the site of infection could overwhelm the host defence system (Gardner *et al*, 2004; Sibbald *et al*, 2003) and to a large extent possession and expression of virulence factors (Tang *et al*, 1996) by the organisms often determine the progression of microbial colonisation to infection (Jensen *et al*, 2007; Williams *et al*, 2000) even in immune competent individuals.

Infection occurs when colonisation of a host tissue by organisms results in invasion from where the organisms are disseminated to the surrounding tissues causing diseases locally or in remote parts of the body (Kadioglu *et al*, 2008). The attachment of organisms to the cells or mucosal surfaces is mediated by adhesins which are usually located at the tip of pili or fimbriae (Lasaro *et al*, 2009; Choy *et al,* 2007; Wu *et al,* 2007; Coutte *et al,* 2003; Johnson and Stell, 2000). The studies of host pathogen relationship have shown that pathogenic organisms employ diverse strategies to circumvent the innate defence mechanism of the host to establish infections (Cote *et al*, 2006; Leid *et al,* 2005). These strategies include the production of cytotoxic enzymes and toxins to breakdown the host tissue for growth and other metabolic activities and formation of protective shields against host defence and antimicrobial interventions. Organisms like *Pseudomonas aeruginosa* produces enzymes like hyluronidase and gelatinase (Naglik *et al*, 2003), and exotoxins (Johnson and Stell, 2000; Caldwell *et al,* 2009; Hamood *et al*, 1996) aggregation factors (clumping proteins) (Hall-Stoodley *et al*, 2004; Kayaoglu and Ørstavik, 2004) which have profound damaging effect on host tissues sometimes leading to organ failure or death. Pathogenic effects on the host are often times affected by the organism‟s ability to secrete protective shields such as capsules (Cortés *et al,* 2002; Fritz *et al,* 2000), cysts (Fukasawa *et al,* 2006; Gebbers

and Marder, 2005), spore (Cote *et al*, 2006; Hoa *et al,* 2001), extracellular polysaccharides (Feldmesser *et al,* 2001) and biofilm (Leid *et al,* 2005; Boyd A and Chakrabarty, 1995). Studies have shown that protections offered by shields like biofilm enhance the ability of pathogens to establish chronic infections (Brady *et al*, 2008; Wolcott and Ehrlich, 2008; Donlan and Costerton, 2002). The roles of adhesins as an important factor in chronic infections have been studied (Mahdavi *et al*, 2002) and association with biofilm formation has been demonstrated (Peng *et al*, 2008; Bu *et al*, 2008; Froeliger and Fives-Taylor, 2001). The genetic units that are responsible for the virulence capability of an organism are usually located within the genome at the pathogenic islands (Schmidt H and Hensel, 2004; Hansen-Wester and Hensel, 2002).

Regulation of these wide varieties of virulence factors by several species of pathogenic organisms has been associated with the production of diffusible chemical substances known as quorum sensing molecules (QSMs) which are population density dependent (Williams *et al,* 2007; Fuqua *et al,* 1996 and 1994). It has been shown that QSMs known as autoinducer -1 are mainly utilised amongst the same species whereas autoinducer - 2 bring about universal signal for interspecies communications amongst several organisms (Atkinson and Williams, 2009; Williams, 2007; Sun *et al*, 2004).

The role of quorum sensing molecules in modulating virulence factors in pathogenic organisms have been widely studied and reviewed; and include pigment production (Wang *et al*, 2008; Fothergill *et al*, 2007), conjugation (Fuqua *et al,* 1994; Zhang *et al,* 1993), sporulation (Bassler and Losick, 2006; Camilli and Bassler, 2006; Enjalbert and Whiteway, 2005) production of enzymes (Zhu *et al*, 2002 b) and toxins (Ohtani *et al,* 2002; Jensen *et al,* 2007) and biofilm formation (Waters *et al*, 2008; Christensen *et al*, 2007).

1.2 Biofilm

Biofilm is a complex, heterogeneous and integrated community of surface attached microorganisms of either single or multiple species that are encased within the extracellular polymeric matrix produced by them (Chang *et al,* 2007; Sutherland, 2001; Costerton *et al,* 1999). They have been found attached to solid (abiotic) surfaces including industrial water systems as well as medical environments and devices (Jacobsen *et al*, 2008; Donlan and Costerton, 2002) and mucosal surfaces in humans (biotic) (Hall-Stoodley *et al*, 2004; Costerton *et al,* 1987).

In the natural environment where it has been postulated that 99.9% of bacteria grow in biofilms and in human infections biofilm communities are often characterized by genetic diversity involving multiple microbial species (Donlan, 2002; Donlan and Costerton, 2002). The intriguing fact about the population diversity of biofilm was said to have been revealed when the dental plaque from the oral cavity of a volunteer was found to contain up to 800 phylotypes (Palmer, 2009).

The major reasons for biofilm formation in the host include protection from the harmful effects of the host defence system, effective colonisation and confiscation of a nutrient-rich environment, utilization of cooperative benefits amongst the biofilm organisms and innate ability of organisms to exist as biofilm (Jefferson, 2004). Protection of biofilm against immune mechanisms has been shown (Cerca *et al*, 2006; Leid *et al*, 2005; Davey and O'Toole, 2000).

Although biofilms are beneficial to mankind in processes such as biodegradation (Mor and Sivan, 2008; Zhang *et al,* 1995) and sewage treatment (Wu *et al*, 2006; Tsuno, *et al*, 2001) various problems have been associated with biofilms, including corrosion of pipes (Teng *et al*, 2008; Keevil, 2004) and involvement with persistent infections in animals and humans particularly in cystic fibrotic, surgical site, orthopaedic and wound infections (Driffield *et al*, 2008; James *et al*, 2008; Gilbert *et al,* 1997; Anderson and O'Toole, 2008; Costerton *et al*, 1987). The phenotypic and biochemical properties of

biofilm organisms are unique and distinct from their planktonic counterparts and enable the organisms to greatly resist body immune system and antimicrobials hence biofilm is associated with persistent infections (Costerton *et al,* 1999; Costerton, 2001).

1.2.1 Biofilm Formation

Most bacteria grow in a free-living planktonic state, but some are able to exhibit different phenotypes which differ in physiological characteristics including structural and metabolic changes (Sauer *et al*, 2002). Sauer and colleagues (2002) observed *P. aeruginosa* converted from planktonic to sessile form and grew as biofilm, a complex process that requires coordinated activities. The main stages of biofilm formation are reversible attachment, irreversible attachment, maturation-1, maturation-2, and dispersion (Figure 1. 1). These various stages of biofilm formation are initiated when the planktonic organisms transform to the sessile form (O'Toole *et al,* 2000). Each of the stages has distinguishing characteristic features (Sauer *et al*, 2002) and requires regulation by quorum sensing molecules (Pearson *et al*, 1994).

1.2.1.1 Attachment

Motile (planktonic) bacteria transform to the sessile form prior to biofilm formation as they adhere to a favourable surface; such as a medical device or the host tissue. In some cases initial adhesion of biofilm forming microorganisms is achieved by means of adhesins located on specialised organelles such as fimbriae (pili) (Lasaro *et al,* 2009; Sauer *et al,* 2002). There are 2 stages of attachment; the reversible attachment occurs when the organisms are able to revert back to the planktonic form and move away from the surface of attachment but at the irreversible stage the organisms are attached and biofilm formation is initiated.

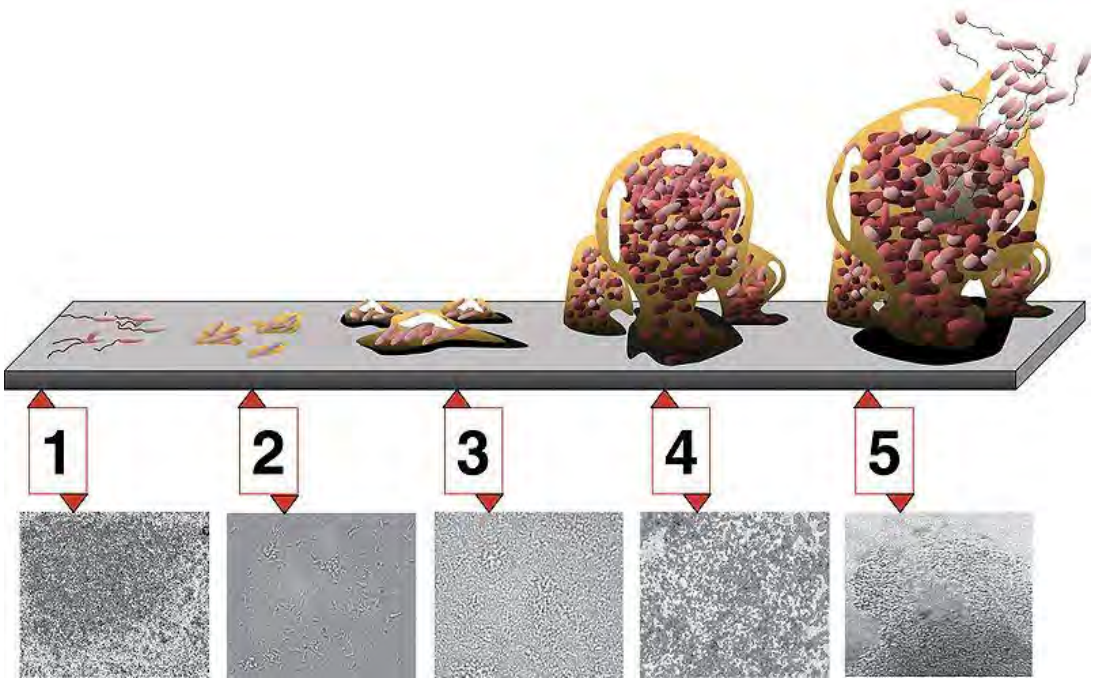

Figure 1.1 The 5 stages of biofilm formation (Monroe, 2007)**.** The diagram and the corresponding microscopic examination of biofilm growth showing (1) Initial attachment; (2) irreversible attachment; (3) biofilm maturation I (4) biofilm maturation II (5) dispersion. Each stage of development in the diagram is paired with a micrograph of a developing *P. aeruginosa* biofilm.

1.2.1.2 *Formation of Microcolonies*

The cells aggregate as they divide on adhesion to a surface but the daughter cells multiply outward and upward from the point of attachment to form cell clusters. The dividing cells produce quorum sensing molecules and extracellular polymeric substances (EPS), or polymer matrix. The matrix houses the aggregating cells in microcolonies and also attaches the biofilm to the surface on which it is formed (Watnick and Kolter, 1999; McKenney *et al*, 1998). Microcolonies become larger as the number of organisms increase and the quantity of EPS produced also increases. More signalling molecules and EPS are produced by the organisms within the microcolonies at this stage. It has been found that microorganisms are usually not distributed evenly throughout the biofilm but in aggregates of microcolonies although

flagellum - driven movement within biofilm have been observed amongst bacteria in biofilm *in vitro* (Malic *et al*, 2009; Tolker - Neilsen *et al,* 2000). The fully mature biofilm structure comprises of bacterial cells, the polymer matrix, and interstitial water channels that facilitate the exchange of nutrients and wastes in and out of the biofilm into the surrounding environment (Stoodley *et al*, 2002; Costerton *et al*, 1995; Sauer *et al*, 2007). Expressions of some genes such as the one for alginate biosynthesis (algC) in *P. aeruginosa* (Davies and Geesey, 1995; Davies *et al*, 1993) and wca locus in *Escherichia coli* are usually upregulated during biofilm formation while certain genes like those for the syntheses of flagellin (fliC) in *E. coli* are repressed (Prigent-Combaret *et al* 1999) and expression of several genes have been observed during biofilm formation (Goldsworthy 2008; Schembri *et al*, 2003; Sauer *et al*, 2002). It was found that the average difference in detectable protein regulation between each of the stages of development was 35% which were approximately 525 proteins and when planktonic cells were compared with maturation-2 stage biofilm cells, more than 800 proteins were upregulated at least six fold the level of planktonic cells, an expression level which was over 50% of the proteome. *P aeruginosa* displayed multiple phenotypes during biofilm development and biofilm cells at dispersion were found to be similar to the planktonic cells in phenotypic expression. The results obtained for *P. putida* biofilm was higher than those of *P. aeruginosa* showing the uniqueness of each organism (Sauer *et al*, 2002). In a similar study of the gene expression of *E. coli* biofilms, Schembri and his colleagues (2003) observed 206 genes were upregulated and 27 down regulated in *E. coli* biofilm than exponential phase while 389 were upregulated and 192 down regulated in biofilm than during the stationary phase of *E. coli* culture. In summary, biofilm formation is a multifactorial process which is a collective product of a variety of interactions and adaptive responses of the organisms within the biofilms.

1.2.1.3 *Detachment and dispersal of biofilm organisms*

The biofilm environment is innately regulated and studies have shown that high population density within a mature biofilm induced programmed detachment of bacteria from biofilm through the secretion of chemical substances by the organisms (O'Toole *et al,* 2000). Studies have shown that detachment occurs when the organisms respond to chemical substances secreted by them such as signalling molecules (Stoodley *et al*, 2005), proteins and degradative enzymes (Barraud *et al,* 2006; Boyd and Chakrabarty 1995) and oxidative or nitrosative stress-inducing molecules such as nitric oxide (NO) produced as a result of metabolic processes within a biofilm (Rice *et al*, 2005; Wood *et al*, 2007; Schlag *et al*, 2007). It has also been shown that alginate lyase; a degradative enzyme produced by biofilm organisms cleaves the polymer matrix into short oligosaccharides. The cleavage antagonises the attachment characteristics of alginate leading to increased detachment of biofilm organisms (Barraud *et al,* 2006; Boyd and Chakrabarty, 1995). The presence of optimal amounts of nutrients has been observed as an inducing factor for dispersal of biofilm organisms by increasing the growth of organisms and production of autoinducers which usually aid the dispersal processes within the biofilm (Rice *et al,* 2005). However other studies have shown detachment of biofilm due to nutrient starvation (Hunt *et al,* 2004; Thormann *et al*, 2001; Delaquis *et al*, 1989). Detachment processes enhance the sloughing of biofilm and switching of sessile organisms within the biofilm to the planktonic, free-swimming phenotypes which leave the biofilm. The detached organisms disperse to other locations to recommence biofilm formation (Barraud *et al,* 2006); a process that aids the spread of biofilm infections within a host and sometimes this might cause thromboembolism which could lead to death (Wenzel, 2007). Intriguingly it has been observed that biofilms with thick extrapolysaccharides have less chance of detachment which tends to reduce the spread of infections (Xavier and Bassler, 2003).

1.2.2 Biofilm physiology

Biofilm has a complex physiology due to the collective effect of the activities of the various organisms within the biofilm. The knowledge about biofilm physiology was highly important in understanding the activities of biofilm organisms for effective control and has become available through the use of various methods to examine the ultra-structure including electron microscope (Davis *et al*, 2008; Stickler *et al*, 1998), staining and examination with light microscope (Davis *et al*, 2008; Harrison – Balestra *et al*, 2003; Serralta *et al*, 2001) epifluorescent microscope (Davis *et al*, 2008; Tolker - Neilsen *et al*, 2000) confocal laser scanning microscope (CLSM) (Percival *et al*, 2008 a) and detection of chemical products such as the quorum sensing molecules (Nakagami *et al,* 2008; Singh *et al*, 2000; Stickler *et al*, 1998). The architecture is mainly influenced by the extracellular polymeric substances (EPS) produced by the individual residents and have been used in the study of biofilm. The EPS is often composed of polysaccharides (alginate and levan) (Laue *et al*, 2006), membrane vesicles (MVs) (Schooling and Beveridge**,** 2006)**,** proteins, lipids, enzymes and nucleic acids (Steinberger and Holden**,** 2005). Although EPS aid the adhesion of biofilm to surfaces and tissues but the main function is to serve as the niche within which biofilm organisms inhabit. The EPS form the bulk of the biofilm architecture (Chang *et al,* 2007; Laue *et al*, 2006) forming 75 -90 % of biofilm while only 10 - 25% are cells. Although alginates influence the architecture of biofilms by facilitating the non-specific attachment of organisms to surfaces, holding together biofilm organisms during initial development and reducing loss of water within the biofilm. However, it has been shown that EPS such as alginate might not be necessary for biofilm formation (Stapper *et al* 2004; Wozniak *et al,* 2003). Biofilms of different species exhibit unique arrangements of the cellular and extracellular structural components. *Pseudomonas* biofilms cells were found to be most dense at the attachment surfaces and became increasingly diffuse

near the outer regions, whereas *Vibrio* biofilms exhibited the opposite trend (Lawrence *et al*, 1991).

In general, biofilms are usually highly hydrated, with the open structures comprising of up to 73 to 98% extracellular materials and space (channels) (Lawrence *et al,* 1991). Chemical gradients (pH, redox potential, and ions) are known to occur usually within the biofilm due to the various degrees of diffusion of nutrients, metabolic products, and oxygen in all parts of the biofilm (Hunter and Beveridge, 2005). The rate of growth and development of biofilm organisms are greatly influenced by chemical gradient, the organisms at the biofilm-liquid interface having the fastest growth as against the much slower rate of those in the interior of the microcolonies, possibly due to limited access to nutrients (Møller *et al,* 1996). The cells within biofilm are able to maintain intracellular pH homeostasis thus enhancing their physiological condition and acid tolerance (McNeill and Hamilton, 2004). Transportation of nutrients within biofilm is achieved through the water channels which allow intake of nutrients into the cells and excretion of waste products into the surrounding environment (deBeer *et al,* 1994 and 1996; De Beer and Stoodley; 1995). Biofilms constituents are diverse as the microbes within them especially the multispecies biofilms. Production of quorum sensing molecules sometimes depends on the site of infection (Favre-Bonte *et al,* 2007) and quorum sensing dependent phenotypes also vary according to the site where the organisms are isolated. Various differences have been observed amongst the biofilms from the wound, the dental plaque and those from the natural ecology like the rocks or water especially in their microbial population (Reardon *et al*, 2004) as well as their extracellular matrix (Sutherland, 2001; Branda *et al*, 2005) and production of virulence factors (Favre-Bonte *et al,* 2007).

1.2.3 Quorum sensing

Microorganisms are capable of communicating with each other to coordinate complex activities like multicellular organisms in addition to the basic role of nutrients metabolism, growth and multiplication. Adaptation to an environment by microorganisms involves response to a series of changes via signal transduction systems, comprising of protein kinase that phosphorylates itself using adenosine triphosphate and response regulator which accepts the phosphoryl groups (Stock *et al*, 1989; Barrett and Hoch, 1998) (Fig. 1.2).

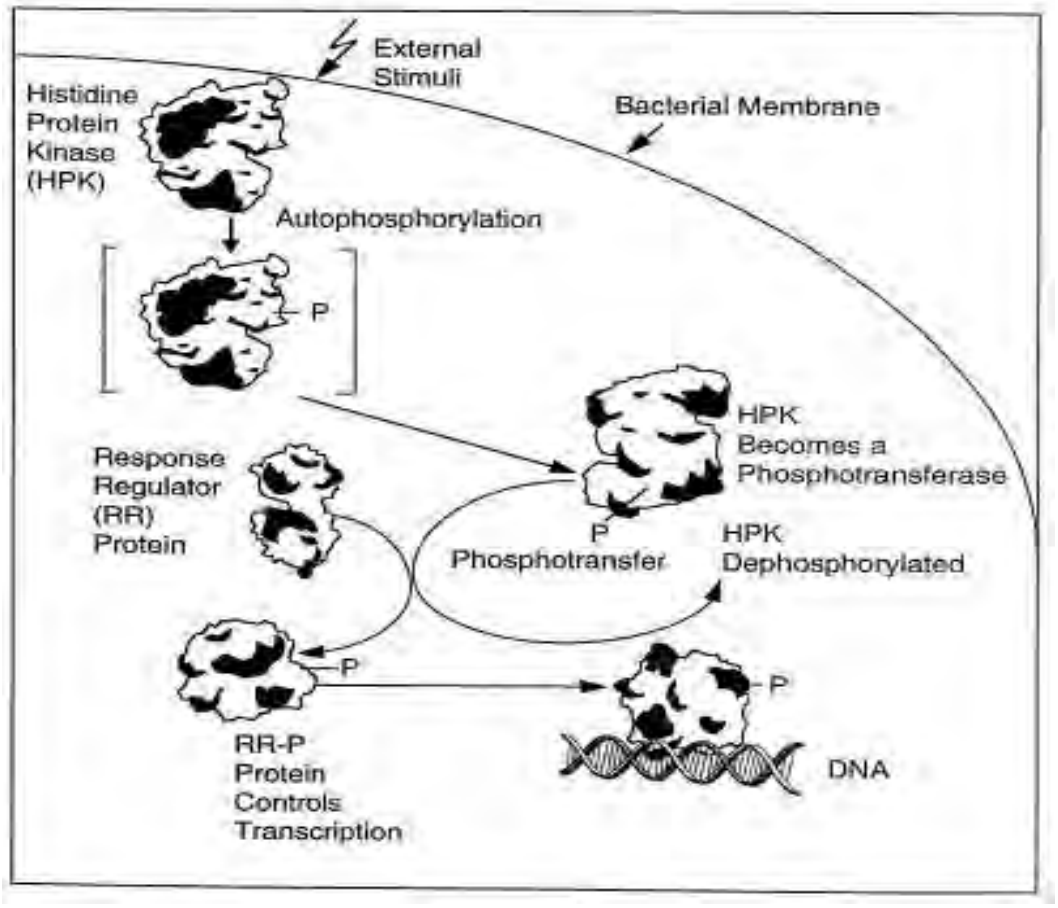

Figure 1.2: Networking in 2- component signal transduction (Barrett and Hoch, 1998).

These coordinated activities are achieved through cell - cell communications signal molecules (autoinducers) (Parsek and Greenberg, 2005; Williams *et al*, 2007), which are produced by each of the organisms involved in biofilm formation (Yao *et al*, 2007; Mcdougald *et al*, 2006; Rice *et al,* 2005).

Quorum sensing is a process by which organisms monitor and respond to the presence of other organisms within the environment through the production of signalling molecules. In biofilm, regulation of metabolic activities and population density is controlled by a cell density-dependent gene expression as a result of accumulation of signalling molecules in the medium. The autoinducer molecules produced by individual microorganisms diffuse through the cell and accumulate in the medium and once a threshold level is attained due to large numbers of organisms, the entire population of microorganisms within the environment respond to the critical cell mass via transcriptional regulation of various target genes (Sauer *et al,* 2002; de Kievit and Iglewski 2000). Studies have shown that QS controls the processes of biofilm formation in microorganisms and detection of other bacteria is through the secretion and detection of autoinducer molecules that regulate mRNA production for specific genes in response to the population density signal (Williams *et al*, 2007; Fuqua *et al*, 1996). In Gram-negative bacteria such as *P. aeruginosa*, there are two types of autoinducers (AIs); AI-1 and AI-2. AI-1 molecules are N-acyl-homoserine lactones (AHL) and AI-2, a furanosyl borate diester. The AHL regulatory system consists of two structural genes; the luxI that encodes the AI-1 synthase and luxR that encodes the AI-1 response regulator. LuxI and LuxR homologues are present in a wide variety of Gram-negative bacteria and control several genes processes including production of virulence factors such as toxins, enzymes, biofilm formation and antibiotic resistance (Passador *et al***,** 1993; Xu *et al*, 2000 & 2006; Patel, 2005; Sakuragi and Kolter, 2007; Kendall and Sperandio**,** 2007). The gene responsible for AI-2 production (luxS) is highly conserved across numerous species and it also has the ability to regulate gene expression in other bacterial species (inter-species communication) and it has been correlated with pathogenicity of several organisms whereas the AI-1 such as AHL is typical of intra-species communication (de Kievit and Iglewski, 2000). In general quorum sensing plays

a key role in the regulation of biofilm architecture (Liu *et al*, 2007); dispersion of organisms (Rice *et al*, 2005) and virulence factors (Schuster *et al,* 2003; Fuqua *et al*, 2001; Rumbaugh *et al,* 1999). The importance of QS in pathogenicity was demonstrated in burn wounds of mice infected with *P. aeruginosa* (wild and mutant strains). The colony forming units (CFU) of organisms recovered from the livers, spleens and skins of mice with infected mutants were significantly lower than those of the wild type (Rumbaugh *et al*, 1999). In another study, mice with implants were infected with *P. aeruginosa* and on examination of the implants at days 4, 7, 14 and 21 post infection, the CFU of organisms on the implants were significantly lower in mutant strains than the wild type suggesting that the clearance of organisms from the implants by the immune system of the mice was QS dependent (Christensen *et al,* 2007).

Although quorum sensing molecules are required for biofilm formation, interestingly some strains of pathogenic organisms such as *P. aeruginosa* have been found to produce biofilm independent of quorum sensing (Favre – Bonte *et al,* 2007; Schaber *et al*, 2004 and 2007) but QS is required for full biofilm formation (Favre – Bonte *et al,* 2003). It was also found that these QS deficient strains were able to cause infections and are less susceptible to antimicrobials (Favre- Bonte *et al*, 2007 and 2003) indicating some virulence factors do not depend on quorum sensing molecules (Schaber *et al,* 2007 b) and that QS dependent phenotypes vary according to the site of isolation of the organisms (Favre-Bonte *et al*, 2007).

Because of the role of QS in the regulation of virulence factors including biofilm formation, blockage of quorum sensing in pathogenic organisms has therefore been suggested as a novel treatment strategy especially in the control of biofilm infections (Bjarnsholt and Givskov, 2007). It has been shown that attenuation of *P. aeruginosa* QS by garlic resulted in rapid clearing of *P. aeruginosa* from the lungs of mice models (Bjarnsholt *et al,* 2005a) and susceptibility of *P. aeruginosa* biofilms to antimicrobials

(Bjarnsholt *et al,* 2005b; Rasmussen *et al,* 2005a) and reduced the killing of *Caenorhabditis elegans* cells (Rasmussen *et al,* 2005a) and rapid clearance from the lungs of infected mice (Rasmussen *et al,* 2005b).

1.2.4 Factors that influence Biofilm formation

Biofilm formation is influenced by various factors ranging from the microbial physiology and phenotypic characteristics, environmental stress, nutritional requirements to the presence of chemical agents.

1.2.4.1Adherence factors

The ability of the organisms to adhere to surfaces as well as the rate of adherence will influence biofilm formation (Shanks *et al,* 2007; Lasaro *et al*, 2009). The twitching motility of biofilm organisms which has been associated with type IV pili determines the rate of adherence which consequently influences the formation of biofilm (O'Toole and Kolter,1998). Flagella and pili therefore play significant roles at the initial stage of biofilm formation since they are required for adherence (O'Toole and Kolter, 1998).

1.2.4.2 Nutritional Factors

Biofilm formation depends on availability of nutrients and also varies amongst species (Mcdougald *et al,* 2006). It has been demonstrated that increased amount of nutrients enhanced the production of quorum sensing molecules, enzymes and other essential amino acids necessary for the formation and growth of biofilm (Rice *et al,* 2005; Heydorn *et al*, 2002).

1.2.5 Effect of chemical and physical agents on biofilm

1.2.5.1 Indole

Indole, secreted by some gram negative organisms, is one of such substances which have been shown to affect biofilm formation. It was observed that indole reduced biofilm formation in organisms such as *Escherichia coli* that synthesize it whereas it increased biofilm formation by some other organisms such as *P. aeruginosa* that do not synthesize it (Lee *et al,* 2007).

1.2.5.2 Lactoferrin

The presence of certain substances in biofilm culture has a substantial effect on biofilm formation and sustenance. Iron in low concentration favours the growth of biofilm but reduces the rate of release of deoxyribonucleic acid (DNA) in cells at higher concentrations thereby reducing the rate of biofilm formation (Yang *et al,* 2007; Banin, *et al,* 2005; Banin *et al,* 2006; Dinty *et al,* 2005). Lactoferrin commonly found in human secretions such as tears, airways secretions and milk also prevents biofilm formation by chelating iron thereby initiating twitching motility of the organisms which causes the organisms to move around and prevents them from attaching to surfaces to form biofilm (Singh *et al,* 2002). However once the biofilm has formed biofilm becomes resistant to lactoferrin (Singh *et al,* 2002).

1.2.5.3 Electric current

Application of electric currents to biofilms has shown reduction of biofilms with time (van der Borden *et al*, 2004) hence its application in improving the efficacy of antimicrobial killing on biofilm was explored (Caubet *et al*, 2004). Bioelectric application in addition to exposure to antimicrobial provides synergistic killing effect on

biofilm cells especially the organisms that are susceptible to the antimicrobials in the planktonic forms (Jass and Lappin-Scott, 1996).

1.2.5.4 Water current (Turbulent or laminar flow)

The flow of water or liquid either in the natural environment such as the ecosystem that has high shear forces (turbulent) seems to enhance bacterial adhesion and biofilm formation (Donlan and Costerton, 2002). In the laboratory the rate of flow has been observed as a factor that affects biofilm formation, but the rate of flow can be controlled, either a turbulent or the low flow rate (laminar) to suit the purpose of the study. The effect of fluid flow has been shown to influence the tensile strength of biofilms and the sloughing of organisms within biofilm, the tensile strength of biofilm is higher with turbulent flow and the sloughing is reduced (Donlan and Costerton, 2002). The impact of flow rate on QS was investigated by Kirisits and colleagues (2007) using laminar flows (0.04 ml/min and 4.0 ml/min) and turbulent (380ml/min) and found that biofilm growth was achieved in both laminar and turbulent flows. However it was observed that the biomass required for full QS induction for the laminar flow rate was at bacterial population of about $6x10^7$ cfu/ml whereas full QS induction of biofilm population at the turbulent flow rate was not observed. The detection of QS is therefore dependent on the rate of fluid flow and may not be fully operative at high flow rate.

1.2.6 Resistance of biofilm to immune system and antimicrobial agents

Biofilm organisms are known to cause persistent infections due to their inherent resistance to body immune mechanism and antimicrobials (Leid *et al*, 2002 and 2005; Webster *et al,* 2006; Chandra *et al*, 2001; Hentzer *et al*, 2001; Costerton *et al,* 1999; Gilbert *et al*, 1997).

1.2.6.1 Mechanism of biofilm resistance to body immune systems

Antibiotic resistance in pathogenic organisms such as *P. aeruginosa* has been linked to biofilm formation and phenotypic variation which arise at high frequency in cultures (Drenkard and Ausubel, 2002). The entire biofilm architecture as well as the chemical substances produced by the organisms within the biofilm aid resistance to body immune mechanisms and antimicrobials. Biofilm organisms also grow at a slow rate (Adams and McLean, 1999) which is a factor that enhances their ability to resist host immune mechanisms and antimicrobial interventions The components of extracellular polysaccharides (EPS) of biofilm usually prevent phagocytosis of biofilm organisms (Leid *et al*, 2005; Jeff *et al*, 2005; Jesaitis *et al*, 2003). The older biofilm are more resistant to phagocytic actions of polymorphonuclear neutrophils (PMNs) than the younger biofilm (Günther *et al,* 2009). Some chemicals and enzymes produced by *Pseudomonas aeruginosa* biofilm phenotypes such as proteases are some virulence factors that damage host tissues and interfere with host antibacterial defence mechanisms (Ołdak and Trafny, 2005) although human leucocyte was able to penetrate and responded to the biofilm of *Staphylococcus aureus* (Leid *et al*, 2002) but generally phagocytic and immune resistances of organisms are high in biofilm and have been linked to the large molecules which pose problems to immune recognition and signal molecules control (Jensen *et al,*, 2006; Bjarnsholt *et al,*, 2005) and protection by the EPS (Leid *et al*, 2005).

1.2.6.2 Mechanism of biofilm resistance to antimicrobials

Organisms within the biofilm are able to resist antimicrobials through various mechanisms due to the architecture and composition of biofilm. The mechanisms of resistance are either innate due to the physiology and the architecture of biofilm and may be due to genetic acquisition of resistant genes (Anderson and O'Toole, 2008; Fux

et al, 2005). The resistance of biofilm organisms to antimicrobial agents starts from the attachment phase and increases with the development of the biofilm (Patel, 2005).

The mechanisms involved in biofilm resistance include the following

a) The extracellular matrices and their contents act as barrier which tends to physically restrict the diffusion of antimicrobial agents into the biofilm niche thus reducing the availability of antimicrobial inside the biofilm niche and protecting the organisms from the effect of the antimicrobials.

(b) Nutrient and oxygen depletion within the biofilm environments with consequent slow rate of growth of the organisms within the biofilm hence the organisms are less susceptible to growth-dependent antimicrobial killing (Borriello *et al,* 2004).

(c) The heterogeneous environment within biofilm such as the pH, oxygen tension and other chemical substances have been shown to reduce the activities of antimicrobials hence the effect of antimicrobials will vary with the location within the biofilm (Borriello *et al,* 2004)

(d) The development of biofilm/attachment specific phenotype which might be due to the stress response by the organisms to the biofilm environment resulting in the selection of subpopulations (Costerton *et al,* 1999)

(e) Transfer and acquisition of antimicrobial resistance genes amongst the organisms within the biofilms (Gilbert *et al,* 1997). The ability of biofilm organisms to rapidly mutate has been shown to influence the rate of antimicrobial resistance (Driffield *et al*, 2008).

(f) The regulation of genes by quorum sensing molecules including antimicrobial resistance genes (Schertzer *et al,* 2009). Although QS deficient strains of *P. aeruginosa* that caused infections have shown reduced susceptible to antimicrobials (Karatuna and Yagci, 2010; Schaber *et al*, 2007).

(h) The age of biofilms tends to affect the resistance of the biofilm to antimicrobials. It has been shown that older biofilms are more resistant to antibiotics (Donlan, 2001).

(i) Synergistic effects were shown to enhance biofilm biomass, resistance of the biofilm organisms to antimicrobial agents and bacterial invasion in multispecies biofilms (Burmolle *et al*, 2006; Mette *et al*, 2006).

1.2.7 Biofilm detection methods

Various methods have been used to determine the presence of biofilm in cultures *in vitro* and tissues. These procedures have been used singly or in combinations to detect biofilm in wounds and they include:

(1) *Light microscopic examination of stained cultures and tissue samples*: Light microscopic examination of stained cultures will provide information about the intact physiology of the biofilm. Stains such as Congo red in Tween 80 can be used to stain biofilm EPS which often appear pinkish-orange while the organisms appear purplish-red when stained with Ziehl carbol fuchsin. The stains reveal the structures of the organisms within the biofilm matrix which usually provide information regarding the arrangement of the organisms as well as the size of biofilm and quantity of EPS (Harrison- Balestra *et al*, 2003). Basic information about the organisms such as shape and size can be obtained and can be used for pure and mixed cultures. Examination of tissue samples may show the location of the biofilm within the tissue and host components such as the PMNs (Dongari-Bagtzoglou *et al*, 2009).

(2) *Confocal laser scanning electron microscopic examination of biofilm*: Confocal laser scanning electron microscopic (CLSM) examination provides the opportunity for live visualisation of biofilm to observe viable and non-viable organisms on continuous basis. Activities such as multiplication of organisms, production of EPS or interaction amongst the organisms within the biofilm can be observed. Organisms can also be

stained with dyes that will differentiate between dead or living organisms or reveal movement of organisms from one location to another within the biofilm (Tolker-Nielsen *et al*, 2000).

(3) *Atomic force microscopy (AFM) of biofilm*: Atomic force microscopy (AFM) can be used to observe biofilm at high magnification and can provide measurement of each organism within the biofilm (Surman *et al*, 1996).

(4) *Scanning electron microscopic examination of tissue samples (SEM)*: Scanning electron microscopic examination of tissue samples (SEM) reveals detailed structures of the biofilm components especially the arrangement of the organisms within the biofilm as well as the adherence of the organisms to the matrix (Davis *et al*, 2008).

(5) *Epifluorescent microscopy*: Fluorescent microscopy is employed when the organisms are stained with fluorophores which absorb light emitted at specific wavelengths to emit lights of longer wavelength (Davis *et al*, 2008) and the stained preparations are examined with an epifluorescent microscope.

(6) *Fluorescent in situ hybridization (FISH) or (Peptide nucleic acid-based fluorescence)*: Fluorescent in situ hybridization (FISH) (Peptide nucleic acid-based fluorescence) is the utilisation of fluorescence technique to stain specific components of the cell such as the deoxyribonucleic acid (DNA). The fluorescent probes bind to the specific component with genetic similarity for species identification of organisms within a biofilm (Kirketerp-Moller *et al*, 2008; Lefmann *et al*, 2006).

(7) *Sonication and/or vortex mixing and estimation of colony forming units (cfu)*: These procedures are often used to estimate the extent of a biofilm. The numbers of organisms provide indirect information about the size of biofilm relative to time or an indication of the effect of substances such as antimicrobial on biofilm (Sun et al, 2008). The method does not provide information about the morphology of the biofilm or the interaction amongst the organisms within the biofilm.

(8) *Detection of quorum sensing molecules*: Biofilm markers including quorum sensing molecules has been used for the detection of biofilm formation by a particular class of organisms (like Gram negative organisms such as *P. aeruginosa* that produces AHL). This was applied to catheters (Stickler *et al*, 1998); sputum (Singh *et al*, 2000) and biopsy specimens from rats (Nakagami *et al*, 2008). In polymicrobial infections, other tests are required to identify each of the organisms in such group.

(9) *Detection of EPS components*: EPS components such as alginate can be used to determine the presence of biofilm in cultures, or materials, medical devices such as catheters. The detection can be achieved by means of SEM examination (Stickler *et al*, 1998), staining and examination with light microscope (Harrison – Balestra *et al*, 2003) or chemical methods (Kives *et al*, 2006). EPS can also be detected using antibody (Roper *et al*, 2007) or by other chemical reactions.

(10) *Biofilm adherence assay (in microtitre plate or on slides)*: This method is mostly used for screening organisms for biofilm *in vitro*. The microtitre plate provides the medium for biofilm forming organisms to adhere and form biofilm in a batch culture (Christensen *et al*, 1985). Although the morphology of the biofilm can not be seen but the ability of organisms to form biofilm can easily be determined in a simple and reproducible analysis. Biofilm culture on slide can be achieved in a batch or continuous culture and biofilm can be stained and examined with light microscope or by determination of viable counts after releasing the organisms by sonication or vortex (Sun *et al*, 2008).

1.2.7 Medical importance of biofilm

The physiological nature of biofilm particularly the protection of the organisms from immune (Leid *et al*, 2002) and antimicrobial strategies by the EPS (Shih and Huang, 2002) and the slow growth rate of organisms arising from distribution gradient of air and nutrients greatly enhanced the ability of biofilm organisms to further resist

antimicrobials thus resulting in chronic infections (Borriello *et al,* 2004). The effect of quorum sensing molecules on the up- regulation of virulent factors (Goldsworthy 2008; Schembri *et al*, 2003; Sauer *et al,* 2002) and the cumulative effects of the expression of these virulence factors by the large numbers of organisms within biofilm could aggravate pathogenic consequences (Asad and Opal, 2008; Dowd *et al*, 2008; Rhoads *et al,* 2008). The proximity of the organisms within biofilm enhances the transfer and acquisition of virulence genes such as antimicrobial resistance, toxin and enzyme production amongst biofilms of mixed organisms (Burmølle *et al*, 2008; Weigel *et al,* 2006; Molin and Tolker-Nielsen, 2003; Donlan and Costerton 2002) which tends to increase significantly the virulence of the organisms (Goldsworthy, 2008; Jacobsen, 2008). The cumulative factors of the resistant strategies employed by biofilm organisms has resulted in huge resistance to antimicrobials which could range between 10 to 1000 times that of the planktonic counterparts (Lewis, 2001; Evans and Holmes, 1987; Nickel *et al,* 1985). The cells from biofilm detach to initiate infections at other sites within a host including the bloodstream and the internal organs (Wood *et al*, 2007; Rice *et al,* 2005). In certain circumstances the biofilm slough could be transported through the veins and can lead to embolism which could be life threatening (Wenzel, 2007).

Biofilm organisms may colonise medical devices and equipment (Fux *et al*, 2006; Donlan 2001), such as heart valves catheters, joint prostheses and the central venous system devices (CNS) (Jacobsen *et al*, 2008; Donlan Costerton, 2002; Donlan, 2001) where they serve as reservoirs for the organisms and sources of nosocomial infections particularly in immunocompromised patients. Many species of bacteria implicated in diseases form biofilms (Dowd *et al,* 2008; Donlan, 2001); including *Streptococcus* species (Lembke *et al,* 2006), *Staphylococcus* species (O'Gara and Humphreys, 2001; Donlan, 2001), *Enterococcus* species (Mohamed and Huang, 2007), *Pseudomonas*

aeruginosa (Hassett, 2009; Wagner and Iglewski, 2008), enteric bacteria (Gaetti-Jardim *et al,* 2008) and *Candida albicans* (Douglas 2003; Chadra *et al*, 2001). Most biofilms are usually polymicrobial and the synergistic effect of the virulence factors of the organisms, consequently results in complications and chronicity of infections (Bjarnsholt *et al*, 2008; Dowd *et al*, 2008).

In natural environments biofilm organisms can influence each other in a synergistic or antagonistic manner depending on species-specific physical interactions between cells or on extracellularly secreted factors which are less specific interactions. Studies have shown that *Candida* species produce certain chemical substances which are inhibitory to quorum sensing in *P. aeruginosa* (Rasmussen *et al,* 2005). In humans, it has been shown that biofilms are responsible for over 60 - 80% of chronic infections (Costerton *et al*, 1995; National Institutes of Health (USA), 1997) as well as 65% of nosocomial infections (Mah and O'Toole, 2001; Potera, 1999) with associated high cost of treatments (Wilson, 2001; Habash and Reid, 1999; Costerton *et al*, 1995). In the USA over 14 million infections are caused by biofilm every year and over 350,000 cases result in death (Wolcott *et al,* 2010). In the developed countries such as USA about 2% of the population have from chronic wounds with the attendant complications (Gottrup *et al*, 2004). Biofilms are implicated in a wide variety of microbial infections including urinary tract (Jacobsen *et al*, 2008; Trautner and Darouiche 2004**;** Anderson *et al*, 2003) and catheter infections (Fux *et al*, 2004; Stickler *et al*, 1998), otitis media (Hong *et al,* 2007) and other ear infections, dental infections (Thomas and Nakaishi, 2006**;** Robert *et al,* 2003), endocarditis (Manetti *et al,* 2007; Nallapareddy *et al,* 2006), cystic fibrosis (Hassett *et al*, 2009; Costerton *et al,* 1999) and human wounds (Malic *et al*, 2009; Wolcott and Rhoads, 2008; James *et al*, 2008; Dowd *et al,* 2008; Ngo *et al*, 2007).

1.3 Wound

1.3.1 Skin and wound formation

The skin is a complex tissue that consists of various components that effectively protect the body against the external environment due to its multi-layered and cornified anatomical barrier and keeps other organs in place (Figure 1.3) (Thibodeau and Patton, 2007). Its role includes homeostatic preservation of body fluid (Thibodeau and Patton, 2007; Guyton and Hall, 2006), thermoregulation (Charkoudian, 2003) and body protection against infection (Fuchs and Horsley, 2008; Fuchs, 2008). The acidity and dryness of the skin, and the secretion of antibodies and antimicrobial inhibitory substances as well as the microbial normal flora on the skin prevent infection (Drake *et al,* 2008; Gariboldi *et al,* 2008).

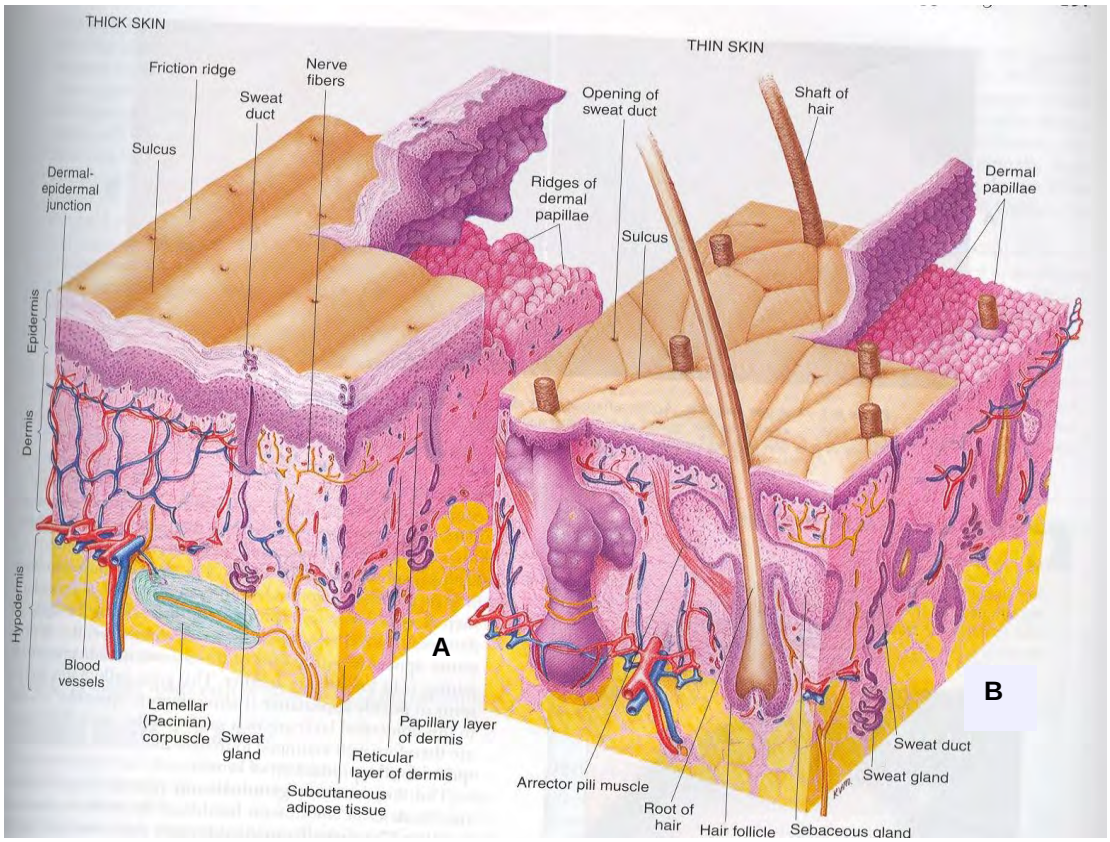

Figure 1.3: Diagrams of skin structure. (A) Thick skin found on surfaces of the palms and souls of the feet and (B) thin skin on most areas of the body. Raised epidermis at a corner reveals the papillae of the dermis. (Thibodeau and Patton, 2007; with permission - Appendix 1).

A wound is a tissue damage which could be caused by mechanical force or chemical substances and it could involve more than one tissue or organ. The cause of injury could be accidental, for example penetrating plants, animal bites (Bygott *et al,* 2008; Goldstein *et al,* 1978) or deliberate cases such as surgical interventions and gun shots (Fallouh, 2009; Ogston, 1898). The discontinuity of the skin at such sites allows organisms and other foreign bodies to gain access into the tissues (Dombrádi *et al*, 2009; Davis *et al,* 2008; Nasser *et al,* 2003) or the internal organs. Proper management of wounds is therefore vital for timely healing.

1.3.2 Types of wounds

(1) Abrasions: These are superficial injuries that damage the epidermis but do not usually result in bleeding. Abrasions could occur on other organs of the body such as the cornea due to the effect of surgical procedures (Batra and Bali, 1977).

*(2) Bruises***:** These are blunt trauma or injuries to tissues, which often lead to blood vessel damage resulting in blood leakage into surrounding tissues from damaged capillaries, veins and arterioles. They may be superficial or deep seated within tissues or organs. Bruises are more common with infants (Labbé and Caouette, 2001; Sugar *et al*, 1999) and the elderly (Schwendimann *et al,* 2006).

(3) Lacerations: They occur when the full depth of the skin is torn due to blunt trauma which usually results in profuse bleeding (Milroy and Rutty, 1997; Kelly, 1911).

(4) Incised wounds: They are caused by sharp objects such as knives or broken glass (Banasr *et al,* 2003; Milroy and Rutty, 1997; Kelly, 1911). The wound edges are determined by the nature of the cutting edge of the object; razor will leave regular margins, whereas a broken glass would create irregular margins. Incised wounds

include slash and stab wounds. Slash wounds have greater length than the depth and can be life threatening when wound involves major blood vessels. Stab wounds are those that the depth of injury is greater than the length due to deep penetration which usually affect vital organs particularly in the chest and stomach (Banasr *et al,* 2003).

1.3.3 Wound Healing Process

This is a process by which the body repairs the damaged tissue which involves series of stages including haemostatic, inflammatory, proliferative and remodelling phases but these processes overlap in time.

1.3.3.1 Haemostatic phase

The injury to the skin or any tissue could cause damage to blood vessels as well as other tissue components which would cause release of blood components. The immediate response of the body to wound is homeostasis through blood clotting cascade to limit blood loss as well as the restoration of the integrity of the damaged tissue (Guyton and Hall, 2006). Homeostasis comprises three main stages: vasoconstriction, platelet plug, and formation of a clot that seals the gap until the tissue is repaired thus providing the temporary matrix for cellular migration (Grinnell *et al,* 1981). Some scientists do not consider haemostasis as one of the healing phases but an immediate response towards the restoration of homeostasis (Bentley, 2004; Singer and Clark, 1999). However, the involvement of fibrin clot in the migration of fibroblast has been highlighted (Geer and Andreadis, 2003).

1.3.3.2 Inflammatory phase

Wounds are usually contaminated with microorganisms from various sources and

sometimes debris hence the role of polymorphonuclear neutrophils (PMNs) in their removal is vital to wound healing (Park and Babul, 2004). At this phase, the white cells remove the foreign bodies while fibroblast cells produce collagen and the exracellular matrix. Activation of complement, platelets and initiation of molecular cascade of processes usually result in infiltration of the wound with granulocytes or PMNs within 24 to 48 hours of injury to eliminate any microorganisms from the wound (Kim *et al,* 2008; Enoch and Price, 2004). The PMNs usually reach their maximal numbers in 24 - 48 hours and the number begins to reduce in the wound from 72 hours as the PMNs clear the bacteria and cellular debris from wound within three to four days of wound formation (Mercandetti and Cohen, 2008). Induction of proliferative signals on human keratinocytes and fibroblasts occurs (Iyer *et al,* 1999) with the involvement of neutrophils and macrophages (Sylvia, 2003). Other substances or proteins which influence wound healing process such as transforming growth factors (TGFs), TGF-β1, cytokines and interleukin-1 (IL-1), interleukin-8 (IL-8), tumour necrosis factor (TNF), are also secreted by macrophages at this time. Inflammatory substances such as p38 mitogen-activated kinase (MAPkinase) are released towards repairing the defect in barrier within 6-8 hours of wounding (Kobayashi *et al,* 2003). Studies have shown that wound contraction was significantly diminished by topical IL-8 (Blumenfeld *et al*, 2000; Rennekampff *et al,* 2000) indicating the function of endogenous IL-8 in all phases of human wound healing. At the late inflammatory phase the monocytes change to tissue macrophages and they further release more cytokines and growth factors into the wound which recruit fibroblasts, keratinocytes and endothelial cells to repair of damaged blood vessels. In normal healing process, a late inflammatory phase is attained in three to four days after wounding, while the granulation tissue formation follows four to five days later (Singer and Clark, 1999). Dead cells are cleared by macrophages through phagocytosis or as sloughs of tissue layers (Schultz *et al,* 2005).

1.3.3.3 Proliferative phase

This phase is comprised of fibroplasia, matrix deposition, angiogenesis and re-epithelialisation and may last up to 4 weeks in an uninfected wound (Mercandetti and Cohen, 2008). Granulation also takes place when new blood vessels and collagen are formed (Gabriel *et al*, 2009). In about five to seven days, fibroblasts migrate into the wound with new collagen being put in place and the wound is covered with matrix and fibronectin as they are being deposited (Schultz *et al,* 2005). Some authors believe that the fibroblasts are the most significant cells in this phase and they attain the maximum number about the seventh day after injury and are responsible for initiating angiogenesis, epithelialisation, and collagen formation (Gabriel *et al*, 2009). Angiogenesis is modulated by vascular endothelial growth factor while the formation of new extracellular matrix as well as the migration, mitosis, and maturation of endothelial cells are modulated by epidermal growth factors (EGF) and tissue growth factors (TGF) (Gray *et al,* 1993). Re-epithelialisation of wounds has been shown to begin within hours after injury on removal of clotted blood and damaged stroma from the wound (Singer and Clark, 1999). Granulation tissue usually appears during this phase even as early as the 4^{th} day after injury (Gabriel *et al*, 2009). A study in mice showed that the reorganization of keratin filaments in wound edge corresponds with the onset of epithelisation (Paladini *et al,* 1996) suggesting the role of keratinocyte migration in wound healing. At this stage of epidermal cell migration the granular tissue starts to contract for re-arrangement of cells into appropriate pattern within the tissue (Gabbiani *et al*, 1978). Basement-membrane proteins reappear from the margin of the wound inward and the stimulation of fibrinogen continues to promote the proliferation of fibroblasts (Gray *et al,* 1993).

1.3.3.4 Remodelling

This is the final (maturation) stage of wound repair process and the peak is attained by the third week after the wound is created but contraction of the wound is an ongoing process involving the proliferation of myofibroblasts (Mercandetti and Cohen, 2008), collagen and scar formation (Harding *et al*, 2002). Formation of granulation tissue begins in the wound within four days after injury. Growth factors stimulate fibroblasts within the tissue to proliferate while the fibroblasts synthesise, deposit, and remodel the extracellular matrix. Collagen is degraded and deposited in the wound for contraction of the wound and this can take years after the initial injury occurred. Contraction takes place during the second week in primary healing when the newly formed matrix are linked together and the surrounding tissue are drawn inwards towards achieving the maximal tensile strength but it is usually around the 12th week during secondary healing (Mercandetti and Cohen, 2008). The scar from a wound can only attain 80% tensile strength of an uninjured tissue (Gabriel *et al*, 2009; Mercandetti and Cohen, 2008; Bentley, 2004). Chemokines are vital at all the stages of wound healing because chemokines uniquely activate and selectively guide various leukocyte subsets to specific micro-anatomical sites of wounds (Gillitzer and Goebeler, 2001).

The temporal and spatial expression of macrophage chemokines at the surgical wound site of a mice model wound was examined and there were differences in appearance and level of expression over time suggesting distinctive functions for each chemokine and their influences on macrophage recruitment during wound healing (Jackman *et al*, 2000). In a similar study in mice, the dynamics of inflammatory cytokines such as interleukin-1α (IL-lα, interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα) in wound healing was examined by Kondo and Ohshima (1996). The results indicate that TNFα and IL-1β play an important role at the commencement of inflammation and at the proliferative stage. In humans Engelhardt and colleagues

(1998) studied wound healing with 14 healthy adult volunteers (eight women and six men) on whom incisions five mm deep and five mm long were made on the ulnar forearm confirmed the involvement of battery of inflammatory reactions when dynamic set of chemokines were secreted with sequential participation and close regulation of neutrophils, macrophages, and lymphocytes associated with skin wound healing. During the study they also noticed that wound closure was completed after 2 and 4 days indicating how rapid acute wounds can heal.

The clearance of organisms and cellular debris from cutaneous wounds was confirmed in granulocyte macrophage colony-stimulating factor (GM-CSF) treated-mice or *S. aureus*-infected mice and the clearance of organisms from the wound correlated with an increase in the number of neutrophils infiltrating the wound unlike the results obtained from saline injected mice (Kim *et al*, 2008). The PMNs recognise and engulf microorganisms in the wound during wound repair, although the process requires a lot of signal transduction event but they are mainly confined to the site of infection which limits pro-inflammatory and tissue-destructive processes (Brown, 1995).

The process of healing for all wounds are similar but the duration of each of the stages differs from acute to chronic wounds (Bentley, 2004). A wound that heals by primary intention has minimal tissue loss but when there is a moderate to large amount of tissue loss, wounds heal by secondary intention with formation of granulation tissue at the proliferation stage and development of obvious scar (Gabriel *et al*, 2009; Bentley, 2004).

1.3.4 Classification of Wounds

Wounds are classified into 2 main groups; acute and chronic.

1.3.4.1 Acute wounds

An acute wound is the one that shows obvious signs of healing without any complication. In most individuals with competent immune systems, acute wounds heal within a reasonable time frame, usually within a month (Enoch et *al,* 2006).

1.3.4.2 Chronic wounds

A chronic wound is a wound that does not heal in an orderly set of stages and/ or within a predictable time frame as most wounds do (Enoch and Price, 2004; Lazarus *et al*, 1994). Such wound usually fails to heal despite all efforts to aid healing and becomes persistent or reoccurs after a period of time when the wound has healed (Schultz *et al*, 2003; Gottrup, 2004). In general any wound that fails to heal within three months is often considered a chronic wound.

Failure of keratinocyte migration during wound healing process has been established in chronic wound (Usui *et al,* 2008; Martin 1997). Non-healing wounds are sometimes associated with underlying conditions such as diabetes (Marston, 2006; Phillips *et al*, 1994), obesity (Wilson and Clark, 2003; Armstrong, 1998), age particularly from 60 years (Harding *et al*, 2002) and poor nutrition (Graue *et al* ,2008) (Table 1.1). Chronic wounds include pressure ulcers, diabetic foot ulcers (Phillips *et al,* 1994) and venous leg ulcers (Marston, 2006).

Table 1.1 Some predisposing factors to chronic wounds.

Local factors	**Systemic factors**
Inadequate blood supply	Advancing age
Increased skin tension	Obesity
Poor surgical apposition	Malnutrition
Wound dehiscence	Systemic malignancy
Poor venous drainage	Chemotherapy/radiotherapy
Presence of foreign body	Neutrophil disorders
Presence of microorganisms	Leucocyte adhesion deficiency
Excessive local mobility	Immunosuppressant drugs
	Impaired macrophage activity
	Use of anticoagulants

1.3.5 Microbial burden of Wound

Wound formation exposes the subcutaneous tissue and microbial contamination becomes inevitable (Dombrádi *et al*, 2009; Davis *et al,* 2008; Robson, 1997).

1.3.5.1 Wound contamination

The presence of microorganisms within the wound without any host reaction is referred to as contamination (Ayton, 1985). The source of contamination may be endogenous or exogenous. Endogenous sources include the surrounding tissue and the normal body flora of the patient depending on wound location. The exogenous sources are diverse and depend on the causes of the wounds and the environment; and may include sand, bullets, water, medical devices, surgical instruments, hospital staff and airborne contaminants (Gardner, 2008; Hollenbeak *et al*, 2006; Cooper *et al*, 2002; Bowler *et al*, 2001). In immunocompetent individuals the body immune system eliminates the

organisms. However contamination can lead to colonisation and sometimes wound infection due to various factors.

1.3.5.2 Wound colonization

Colonisation is often referred to as the presence of microorganisms within the wound that are either multiplying or associated with host reactions (Goyal *et al*, 2010; Oluwatosin, 2005; Ayton, 1985). The wound environment provides favourable conditions of warmth, moisture and nutrition for easy colonization of various species of organisms hence colonisation of wounds are mostly polymicrobial (Bowler and Davies, 1999**;** Mousa, 1997). Protective colonization could occur in wounds whereby some organisms within the wound could produce substances that will reduce or inhibit the growth of others (Kingsley *et al*, 2001). Colonisation could become critical even without any obvious host reaction and it may lead to infection particularly when microbial proliferation rate is high (White and Cutting, 2006; Cutting and Harding, 1994).

1.3.5.3 Wound infection

Wound infection occurs when the microbial load and virulence factors overwhelm the host‟s immune system (Landis, 2008; Cooper, 2005; Kingsley, 2001). In individuals with compromised immunity, microbial growth may increase optimally within a short period especially in devitalised tissues (Bowler *et al,* 2001). The microbial density (Gardner, 2008), types and virulence of microorganisms (Cooper *et al*, 2003; Bowler *et al*, 2001), the host immune response (Kaya *et al*, 2007) and the condition of the tissue (Hollenbeak *et al*, 2006) are the main factors that determine wound infection. A wound that has microbial burden of more than 10^5 organisms per gram of tissue is considered to be infected (Robson, 1997) but Levine and colleagues (1976) suggested a quantitative swab culture method for viable bacteria count of 10^6 or more bacteria. Most often,

practitioners rely on clinical symptoms to confirm wound infection especially increasing pain and wound breakdown (Gardener *et al,* 2001). The major signs of infection are redness, swelling, heat and pain while others may include increased exudates, odour, contact bleeding, delayed healing, and abnormal granulation of tissue (Cutting and White, 2004). Wound infections are mostly polymicrobial (Landis 2008; Dowd *et al*, 2008 a and b; Bowler *et al*, 2001) and usually associated with the synergistic effect of the microbial virulence (Kingsley, 2001) and could lead to septicaemia (Ceilley, 1977) and death (Everett *et al*, 1994). Proper management of wound infections is therefore crucial to wound healing and general wellbeing of patient.

1.3.5.4 Common pathogens in wounds

Few of the pathogenic organisms commonly found in wounds are shown in Table 1.2 below.

Table 1.2: **Potential wound pathogens**

Group	**Organisms**
Gram-positive cocci	*Streptococcus pyogenes* *Enterococcus faecalis* *Staphylococcus aureus*
Gram-negative rods	*Pseudomonas aeruginosa* *Escherichia coli* *Klebsiella* species *Proteus* species
Gram-positive rods	*Bacteroides* species *Clostridium* species
Fungi	*Candida albicans* / *Candida* species other Yeasts

1.3.5.5 Surgical site infection (SSI)

SSIs could be a major problem (Chalidis *et al*, 2009; Benger *et al*, 1998) and the risk of such infections is sometimes influenced by technical problems with the operation (Singhal *et al,* 2009), medical equipment or hospital staff team (Kaminski *et al*, 2007). Contamination from the surrounding tissues of the patient (Shukla *et al*, 2009; Sanderson *et al,* 1979), underlying disease and the immune competence of the patient also have impact on infection rate (Chalidis *et al*, 2009; McKinnon *et al,* 2001). Technical problems such as bleeding, the size of devitalized tissue produced, and drains within the wound with underlying disease such as obesity and diabetes could complicate infection (Singhal *et al*, 2009; Rubin, 2006).

According to the guideline on surgical site infection: prevention and treatment of surgical site infection by the national institute for clinical excellence (UK); the National Prevalence Survey of Infections in Hospitals (1996) conducted in 157 hospitals in England, Scotland, Wales and Ireland involving data from 37,111 patients reported an overall prevalence of hospital acquired infection of 9.0% and prevalence of SSI was 10.7% amongst the identified groups of infections. In another survey, between 1997 and 2001 an incidence of SSIs of 4.2% was reported from 152 English hospitals. In 2006, the prevalence survey suggested that approximately 8% of patients in hospital in the UK have a health-care associated infection (HCAI) and SSIs accounted for 14% of these infections and nearly 5% of patients who had undergone a surgical procedure were found to have developed an SSI. Recently, a post-discharge surveillance to identify colorectal surgical site infection rates and related costs; colorectal SSI, 29 (27%) out of 105 patients developed SSI with additional treatment costs of £10,523 per patient (Tanner *et al*, 2009). In the USA, a similar study of the SSI in a 415-bed community hospital in the 1990s, Kirkland and his group (1999) found that patients who developed SSI had longer and costlier hospitalizations than patients who did not develop such

infections. They are twice as likely to die, 60% more likely to spend time in an intensive care unit (ICU) and more than five times more likely to be readmitted to the hospital. In another study Engemann and his group (2003) observed that methicillin resistance is independently associated with increased mortality and hospital charges among patients with *S. aureus* SSI. Another survey in the USA showed that orthopaedic surgery patients with SSI required more rehospitalisations which obviously increase overall cost of treatment and the quality of life was adversely affected for patients with SSI (Whitehouse *et al,* 2002). In a study involving patients undergoing abdominal surgeries in Nigeria 17.4% of the 144 patients studied developed surgical site infections (Mofikoya *et al*, 2009).

A series of procedures are usually put in place to prevent SSIs (Tanner *et al*, 2008) although they vary from place to place they aim towards minimising SSIs. The surgical team tries to eliminate or reduce the risk of infections as much as possible (Budnar *et al*, 2009) through infection prevention control procedures. Preoperative antibiotic prophylaxis is sometimes administered to further decrease the incidence of infections. Although research shows poor compliance to guidelines (Parulekar *et al*, 2009; Fennessy *et al*, 2006) but a recent review of the factors influencing antibiotic prophylaxis administration for prevention of surgical site infection in general surgery between 1996 and 2007 (Gagliardi *et al*, 2009) favours implementation of multidisciplinary pathways, individualized performance data and written or computerized order sets as quality-improvement strategies.

1.3.6 Diagnosis of wound infections

Although, diagnosis of wound infection mostly relies on clinical symptoms the shortcoming of this approach has been observed. It was shown in a study that the microbial load of 6 wound samples were higher than the predicable value of 1×10^5 organisms /g of tissue yet no clinical signs of infection were perceived in the patients,

hence the need to reassess the guidelines of diagnosing wound infections (Cooper *et al*, 2008). Some wounds fail to show the clinical symptoms and it is still not uncommon to see chronic wound infection progressing to septicaemia and death (Castillo *et al*, 1981). Traditionally swab samples are obtained from wounds for aerobic and anaerobic cultures to isolate pathogens and for selection of appropriate antibiotics for treatment of infections. Recently, it is believed that available growth media for isolating and identifying organisms in wounds would only isolate and identify about 5% of bacterial species in wounds especially some fastidious organisms that are uncultivable (Amann *et al*., 2001; Moter & Goebel, 2000) hence biopsy samples are being advocated as a preferred option. The cultural methods can not detect biofilm hence biopsy samples become inevitable when biofilm infections are suspected and the presence of biofilm in human has been revealed (Malic *et al*, 2009; James *et al*, 2008; Ngo *et al,* 2007).

1.3.7 Complications and problems associated with chronic wounds

The complications of chronic wounds are numerous, some of which include formation of sinus or fistula, unrecognised malignant tumour, Marjolin's ulcer, osteomyelitis, contractures and deformity in surrounding joints, systemic amyloidosis, and heterotopic calcification, colonisation by multi-drug resistant pathogens (Gardner and Frantz, 2008; Paavola *et al*, 2000), anaemia and septicaemia and sometimes amputation (Armstrong *et al,* 1997; Pecoraro *et al*, 1990). The effect of wound complications manifest mostly in people with compromised immunity especially individuals with underlying health conditions such as diabetes mellitus, the elderly, those that have prolonged hospital stay or are bedridden. In diabetics the infections may progress rapidly from localized infection to spreading infection and severe infection as often experienced in diabetes foot ulcers (Edmonds, 2009; Pecoraro *et al*, 1990). It has been predicted that 15% of diabetics will develop foot ulcers (Reiber *et al*, 2001). According to a study of the

causes of diabetic limb amputation (Pecoraro *et al*, 1990), 23 unique causal pathways to diabetic limb amputation were identified of which minor trauma, cutaneous ulceration, and wound-healing failure applied to 72% of the amputations due to association of infection and gangrene. Cumulative proportion of incident showed that 46% of the amputations were attributed to ischemia, 59% to infection, 61% to neuropathy, 81% to faulty wound healing, 84% to ulceration, 55% to gangrene, and 81% to initial minor trauma. This staggering revelation shows the importance of management of infections in the diabetics" wounds. In the world it has been estimated that limb amputation occurs every 30 seconds (Reiber, 2001). The complications associated with infections in diabetic wounds have led to the guidelines on the treatment of diabetic wounds by Infectious Diseases Society of America (IDSA) (Lipsky, 2004). In a longitudinal study of 1666 persons with diabetes, an increase risk towards amputation of limbs was observed (Lavery *et al,* 2007).

Human activities and the advancement in medical and health treatments to prolong lives including surgical interventions often create wounds in some individuals with compromised immune system (Chalidis *et al,* 2009). In such patients, complications might occur with the healing process and wounds might not heal in a timely manner. Bacterial infections of wounds usually complicate wound management which often result in extra days of hospitalization and associated costs (Marston, 2006; Plowman, 2000). Surgical wounds commonly acquire hospital infections with high morbidity and increased stay in the hospital due to delay in recovery consequently doubles hospital costs incurred by such patients and occasionally results in death (Plowman, 2000). The Nosocomial Infection National Surveillance Service (NINSS) survey report between October 1997 and September 2001, showed that the incidence rate of hospital acquired infection (HAI) related to surgical wounds was 10% with the attendant problem costing the National Health Service (NHS) almost £1 billion pounds per annum (NINSS,2002).

The problems of delayed wound healing can be inconvenient ranging from pain (Boutoille *et al*, 2008; Gardner *et al*, 2001) to partial or complete immobility (Boutoille *et al*, 2008) infection and in extreme circumstances amputation especially in diabetic foot ulcers (Boutoille *et al*, 2008; Harish *et al,* 2007; Wu and Armstrong, 2005) and sometimes death (Hellinger *et al*, 2009; Horvath *et al*, 2007). Other related problems of wound infections or chronicity include inability to work/ reduction in income, absenteeism, reduced social interactions. Wound chronicity has also been associated with other psychological problems (Cole-King and Harding, 2001).

1.3.8 Management and treatment of wounds

The process of wound healing involves the entire body‟s systems and the problems associated with delayed healing can affect the quality of life enormously (Ruvo *et al*, 2005; Ragnarson and Apelqvist, 2000). The main purpose of treating wounds is essentially to ensure that the wound heals within a reasonable time frame and the management strategies depend on the condition of the wound as well as the patient‟s circumstance. Wound healing process requires essential nutrients for the metabolic activities of the body as well as the anabolic processes associated with wound healing. The nutritional status of the patient plays a vital role in wound healing and poor and imbalanced nutrition has been associated with delayed healing (Demling, 2009; Church *et al*, 2006).

1.3.9 Treatment of infected and chronic wounds

Infected and chronic wounds often heal slowly or occasionally not healing at all due to some predisposing factors such as underlying diseases (Prompers *et al,* 2008; Yasuhara *et al,* 2008) and microbial infection especially with biofilm forming organisms (Dowd *et al*, 2008 a and b; James *at al*, 2008; Ngo *et al*, 2007; Gjødsbøl *et al,* 2006). Various

strategies targeted towards enhancing the healing of such chronic wounds are usually employed in their treatment depending on the factors responsible for the delayed healing. Once clinical signs of infection are observed in wounds, efforts should be made towards the diagnosis of infection by microbial identification and appropriate antimicrobial treatments should be given as soon as possible to prevent complications (Neil and Munro, 1997). In complicated and more difficult cases, other treatment strategies are often explored including use of hyperbaric oxygen, negative pressure therapy, electric stimulation and electromagnetic therapy, growth factors, ultrasound, bone marrow derived cells, debridement and in severe conditions that are life threatening surgical interventions for removal of tissues or amputation.

1.3.9.1 Wound cleansing and debridement

The presence of foreign bodies in a wound prevents healing (Grey *et al,* 2006; Granick *et al,* 2006; Stadelmann *et al,* 1998) as the body tends to react to such objects thus hampering healing processes (Ng *et al,* 1997). Similarly the presence of devitalised tissue in wound hinders wound contraction and also provides an enabling environment for microbial proliferation thereby encouraging infection (Leaper, 2002), which consequently might delay or prevent healing. In order for wounds to heal foreign bodies and devitalised tissues have to be removed from them. The process of removal of microorganisms, foreign materials and slough from the wound is termed cleansing and removal of devitalised tissue is debridement.

The first approach to wound management is usually cleansing to remove any impediment to healing such as foreign substances, microbes and devitalised tissues. There are various methods of cleansing the wound which may be mechanical, chemical or biological. They include rinsing, irrigation and usage of agents that aid the removal of debris such as aggregators and detergents (Anglen, 2001; Cooper *et al*, 2002) and debridement (Steenvoorde and Oskam, 2006; Armstrong *et al*, 2005). Cleansing of

wounds can be done with water (Gannon 2007; Riyat and Quinton, 1997), saline (Gannon 2007) by mechanical washing or irrigation (Luedtke-Hoffmann and Schafer, 2000). Antiseptic solutions are sometimes employed in the cleansing of acute wounds to further reduce the bioburden and to minimise or prevent the proliferation of microorganisms in the wounds (Ferguson *et al*, 2003; Anglen, 2001). Moore and Cowman in their study of cleansing solutions in 2008 discovered that pressure ulcers that were cleaned with saline spray containing aloe vera, silver chloride and decyl glucoside had significant improvement in healing unlike those cleaned with saline alone.

1.3.9.2 Autolytic debridement

Autolytic debridement occurs naturally in wounds, although when the wound is not healing properly this process might be hampered. The process is influenced by the moisture level of the wound hence autolytic debridement occurs better in moist wound and it might be relatively impossible in a dry wound. It utilises the patients own enzymes to debride the wound of eschar and slough under moist environment (Hofman, 2007; König *et al*, 2005).

1.3.9.3 Maggot (larval) Debridement

Maggots have been successfully employed in the debridement of wounds (Chan *et al,* 2007; Church and Courtenay, 2002). The larvae of the green bottle fly are mostly used for wound debridement. Its effectiveness as wound debridement agent is linked to the ability of the maggots to feed on the dead tissues and the larvae also secrete bactericidal metabolic products that reduce the bioburden of the wounds (Steenvoorde and Oskam, 2006; Armstrong *et al*, 2005; Church and Courtenay, 2002). The side effect of maggot debridement is the pain that is sometimes associated with the therapy. It has been

shown that *P. aeruginosa* QS molecules caused the death of debridement larva (Andersen *et al,* 2010).

1.3.9.4 Enzymatic debridement

This is achieved with the use of enzymatic preparations from microorganisms such as collagenase, fibrinolysin, streptokinase and those from plants, urea and papain or the combination of urea and papain to digest the extracellular proteins of the slough or eschar of a wound (Marazzi *et al*, 2006). Enzymatic debridement is more selective since the enzyme will only digest the particular component of the tissue such as the digestion of collagen from the slough of a wound with collagenase (Marazzi *et al*, 2006; Moore and Jensen, 2004).

1.3.9.5 Surgical debridement

This is often used for removal of large amount of necrotic tissues and it is usually done under anaesthesia in the operating theatres. It provides effective and fast form of debridement for chronic wounds especially for patients with underlying conditions such as diabetes (Werner *et al*, 2008; Wang *et al*, 2008; Armstrong *et al*, 2002). Armstrong and his team (2002) described simple techniques by which surgical debridements of diabetic neuropathic ulcers which otherwise could result in complications and probably amputation can be achieved. Surgical debridement of chronic wounds in general often reduces the chances of complications which might lead to amputations (Faglia *et al*, 2006).

1.3.9.6 Topical Oxygen Therapy

Oxygen therapy can be applied to a wound in the form of supersaturated oxygen which will be delivered to a wound over a period of time (Davis *et al,* 2007; Gottrup, 2004). This will increase the energy supply to the cells and improve protein production, cell homeostasis and angiogenesis.

1.3.9.7 Hyperbaric oxygen therapy

Wound healing requires adequate tissue oxygen tension but this is sometimes lacking in devitalized chronic wounds particularly in individuals with underlying conditions such as diabetes (Kalani *et al*, 2002). Adequate oxygen tension can be achieved by hyperbaric oxygenation (Flanagan *et al,* 2006; Roeckl-Wiedmann *et al*, 2005).

1.3.9.8 Negative pressure wound therapy (NPWT) via vacuum-assisted closure

Negative pressure therapy has been used in wound treatment to improve wound closure and healing (Armstrong *et al*, 2007; Steenvoorde and Oskam, 2006) but there is still the need for additional clinical evidence (Gregor *et al*, 2008).

1.3.9.9 Antimicrobials

Topical and systemic antibiotic therapies are employed for infected and chronic wounds (Howell-Jones *et al*, 2005) but topical therapies are employed more often for peripheral wounds because of the metabolism of the antibiotics and their reduced concentrations at the arterioles and the skin. In order to prevent wound infection in immune deficient individuals, prophylactic uses of antimicrobials for surgical wounds are more frequently employed especially at sites such as the abdominal region with high microbial flora (Bratzler *et al,* 2005; Classen *et al,* 1992) but such regimens provide beneficial results when administered at the appropriate time (Silver *et al,* 1996). Prophylactic treatments

of minor injuries have been shown to yield positive results in terms of reduction of rates of infection especially in children (Langford *et al,* 1997) and burn wounds (Church *et al*, 2006).

Wound assessment to determine infection is important before the decision to use antimicrobials (Wahie, and Lawrence**,** 2007; Grey *et al*, 2006; Bowler *et al*, 2001) and to determine the route of antimicrobial administration (Nathwani, 2006). Broad-spectrum antimicrobials can be used for prophylaxis but when infection has been confirmed it is best practice to culture the wound sample to identify the organisms and determine the appropriate antimicrobials before the treatment commences (Church *et al*, 2006). This will minimise the development of mutants and resistance strains of microorganisms (Silver, 2003; Bridges *et al*, 1979; McHugh *et al*, 1975). Antimicrobials could be applied as solutions for cleaning the wounds, or topically as ointments or dressings, and they could be administered orally, injected subcutaneously or intravenously when systemic application is required.

1.3.9.9.1 Antiseptics/ topical applications

Antiseptics and topical antibiotics are commonly used in the treatment of wounds to reduce the microbial load of wounds and in some circumstances completely remove the microorganisms. The antimicrobial could be used directly on the wound as a cleaning agent (Moore and Cowman, 2008; Smith, 2005; Cooper and Lawrence, 1996) or as dressing material (Ip *et al,* 2006; Church *et al,* 2006). Topic antiseptic applications can be employed as a prophylaxis in preventing or reducing the risk of wound infections (Heal *et al*, 2009; White *et al*, 2001) but development of resistant strains of microorganisms to the prophylactic drug might occur (Cheng *et al*, 2008).

(a) Povidone iodine

It can be used alone or in combination with other antimicrobials (Ferguson *et al*, 2003; Sherlock, 1984) for better antimicrobial activities. Although some research on the use of povidone-iodine solution for treating wounds as an antiseptic suggested that it did not reduce significantly the bioburden of wounds with time hence the limitation of its efficacy; but iodine continues to be effective against microorganisms (Giacometti *et al,* 2002). In a recent review of the use of iodine as antimicrobial (Cooper, 2007) appropriate use of iodine has been suggested as a potential source for wound management being an effective, broad-spectrum antimicrobial agent without known record of microbial resistance. Povidone iodine-containing formulations are less toxic than most of the antimicrobials examined in a study done by Muller and Kramer (2008) which makes iodine a better tolerated topical antimicrobial for wound management.

(b) Silver dressings

Treatment of wounds with topical antimicrobial dressings has proved successful. Such antimicrobials include silver compounds (Parsons *et al,* 2005). There are various silver impregnated or coated dressings available for the treatment of wounds and their efficacies have been determined *in vitro* and *in vivo* (Ip *et al,* 2006; Church *et al,* 2006) as silver sulphadiazine and silver-impregnated activated charcoal dressing. X-ray microanalysis of silver treated bacteria cells revealed the presence of within the cells which suggests that silver may cause loss of cellular ions that are vital for the maintenance of cells (Hobot *et al,* 2008). Silver has lethal a effect on biofilm organisms but usually at high concentrations which might be up to 10-100 times the concentration required to kill the planktonic organisms (Knight *et al*, 2009; Bjarnsholt *et al,* 2007). This has been demonstrated on single and mixed bacterial biofilms using silver dressing which showed lethal effects on the biofilms from 3 hours of exposure and by

24 hours over 90% of organisms were killed (Percival *et al*, 2008) unlike the planktonic organisms that were killed within 3 hours as observed with confocal laser scanning microscope (Newman *et al,* 2006). Resistance of wound isolates to silver is still low (Percival *et al*, 2008 b). The rate at which the silver is released from the dressings determines the effectiveness of the antimicrobial activity of the silver dressing (Thomas and McCubbin, 2003) and this factor should be considered when choosing the dressing. Combinations of topical antimicrobials are often used for treating wounds because of higher degree of efficacy which is achieved due to their synergistic effects. Such preparations include 1% silver-zinc allantoinate cream (Margraf and Covey, 1977), Povidone iodine and alcohol (Mertz *et al*, 1984), bacitracin-polymyxin ointment (Palmieri and Greenhalgh, 2002). In some cases these preparations may not be efficient probably due to heavy microbial burden (Mertz *et al*, 1984) or the presence of biofilm (Percival and Bowler, 2004) particularly when the host competence is much lower than the pathogenic effect of the microorganisms.

1.3.9.10 Surgical interventions

Non - healing wounds sometime need surgical interventions to forestall systemic infections and complications that may lead to loss of vital organs and death. Necrotic tissues usually reduce or prevent blood circulation to the affected site thus affecting oxygen and nutritional supply to the tissues which consequently affect wound healing. Surgical interventions include removal of dead tissues, skin replacement, skin flap reconstruction and amputation of a toe, foot or the entire limb.

1.4 Effect of biofilm on wound healing

Pathogenicity of most organisms is mainly determined by the virulent capability of the organisms within the wound such as production of tissue damaging toxins and enzymes

(Cooper *et al,* 2002) and biofilm formation and immunological status of the patient. Biofilms have been associated with chronic infections in wounds (James *et al,* 2008; Ngo *et al*, 2007), because these organisms often resist host mechanisms and antimicrobial interventions (Figure 1.4) (Bjarnsholt, 2008; Patel, 2005; Anderson and O'Toole, 2008; Fux *et al,* 2005; Leid *et al*, 2002).

Despite the efficiency of the body immune mechanism in eliminating microorganisms, a single macrophage can clear up to 100 bacteria (Guyton and Hall, 2006) coupled with the action of the neutrophils and the walling - off process that produces fibrinogen clot to inhibit the spread of organisms, biofilms have been particularly recalcitrant. Biofilm mostly exist as multispecies community of organisms so the microbial virulence synergy often results in wound complications (Bjarmsholt *et al,* 2008; Rhoads *et al*, 2008). Colonisation of wound by virulent organisms such as *S. aureus* in biofilm has been demonstrated *in vivo* in pigs and the biofilms were less susceptible to antimicrobials than the planktonic counterparts (Davis *et al,* 2008).

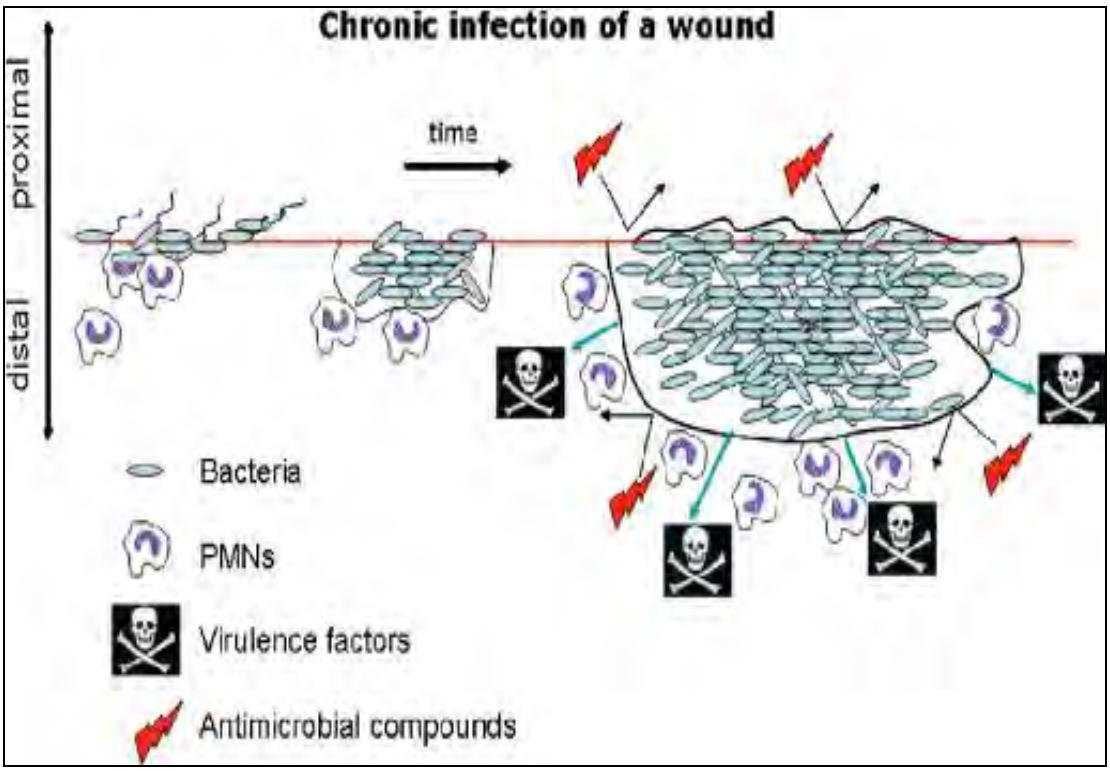

Figure 1.4 Development of a biofilm in a chronic infected wound: barrier to body immune and antimicrobial strategies (Bjarnsholt *et al*, 2008). Planktonic bacteria develop over time into biofilms in the wound. The biofilm organisms are encased in extracellular polymeric matrix and are able to impede the action of host immune system and antimicrobials. The cell-to-cell signalling molecules produced by the organisms during biofilm formation influence the production of virulence factors, which often shield phagocytic activities by PMNs.

The ability of biofilm to resist phagocytic action (Günther, *et al,* 2009; Leid *et al*, 2005) as well as prevention of opsonisation by macrophages especially due to protection by extracellular polymeric substances (EPS) (Conway *et al*, 2004) and presence of high level of antigens within a biofilm (Cerca *et al,* 2006) have grave consequences on wound healing. Biofilms are often polymicrobial (Malic *et al*, 2009; Dowd *et al,* 2008 a and b; Ngo *et al*, 2007) and the synergistic effect of the virulence factors produced by individual organism would exert enormous effect within the wound space and in the host at large (Bjarnsholt *et al*, 2008; Chen *et al*, 2009).

Quorum sensing molecules (QSMs) are required for biofilm formation and regulation of virulent factors in pathogenic organisms (Parsek and Greenberg, 2005; Rice *et al*, 2005). Nakagami and team (2008) in an experimental study demonstrated the presence of quorum sensing signals from *P. aeruginosa* infected ischaemic wound in rats. Increased production of virulence factors such as cytotoxic enzymes would frequently increase the breakdown of wounds. Higher production of toxins by the organisms would further drain the immune mechanism of the patient and further reduce the healing process. Loryman and Mansbridge (2007) observed the inhibition of keratinocyte migration by lipopolysaccharide (LPS) derived from Gram negative bacteria such as *P. aeruginosa* or *E. coli* due to the effect of QSMs. All these factors explain the inability of wounds with biofilms from healing.

Biofilms innately resist immune system and when established in infections antimicrobial interventions have limited effect on embedded organisms and eradication of biofilm with conventional antimicrobials has been extremely difficult. It has even been demonstrated that sub - lethal concentrations of imipenem enhanced biofilm formation in *P aeruginosa* (Bagge *et al*, 2004) while sub-inhibitory concentrations of aminoglycoside antibiotics induced biofilm formation in *P. aeruginosa* and *E. coli* (Hoffman *et al*, 2005) the phenomenon that is likely to occur mostly within biofilm due to slow penetration of antibiotics offered by biofilm EPS. Hoffman and colleagues (2005) in their study found in *P. aeruginosa,* that a gene aminoglycoside response regulator (*arr*) was essential for this induction and contributed to biofilm-specific aminoglycoside resistance. Intriguingly, exposure of *P. aeruginosa* biofilms to high levels of tobramycin antibiotic caused differential expression of 20 genes, the response that was proposed as being critical for the development of biofilm resistance to the antibiotic (Whitely *et al*, 2001).

A study found that resistant mutants readily developed against some antimicrobials such as azithromycin that are bactericidal to *P. aeruginosa* biofilms, furthermore, they frequently show cross-resistance to other unrelated antipseudomonal agents such as ciprofloxacin and hypersusceptibility to others such as imipenem or tobramycin (Mulet *et al*, 2009). These resistance strategies by biofilms have contributed to chronic infections and development of mutants (Hoffman *et al*, 2005) hence eradication of biofilm is paramount in medical care and in particular wound management.

1.5 Control and potential treatment of biofilm in wounds

The best control for biofilm infections should be preventive; however the treatment intervention is aimed at reducing the quantity of organisms and the virulent expressions of the organisms for easy clearance by the immune mechanism.

1.5.1 Interference with biofilm formation

One of the commonly isolated pathogens from wound has been shown to form biofilm *in vitro* within 10 hours of culture (Harrison – Balestra *et al,* 2003) and biofilm formation has also been demonstrated *in vivo* in animals within 48-72 hours of bacterial colonisation (Davis *et al*, 2008; Serralta *et al*, 2001). It is therefore relevant to prevent biofilm formation soon after a wound is formed particularly in patients that are predisposed to infection. Studies have shown that some agents prevent biofilm formation; application of such agents in biofilm preventive control would alleviate the agonizing effect of biofilm in wounds.

1.5.2 Attenuation of quorum sensing

Because of the role of QS in the regulation of virulence factors including biofilm formation, blockage of quorum sensing in pathogenic organisms has therefore been

suggested as a novel treatment strategy especially in the control of biofilm infections (Bjarnsholt and Givskov, 2007). Attenuation of *P. aeruginosa* QS by garlic has resulted in rapid clearing of *P. aeruginosa* from the lungs of mice models (Bjarnsholt *et al,* 2005a) and susceptibility of *P. aeruginosa* biofilms to antimicrobials (Bjarnsholt *et al,* 2005b; Rasmussen *et al,* 2005a) and reduction of the killing of *Caenorhabditis elegans* cells by the organisms was also observed (Rasmussen *et al,* 2005b). The synergistic activity of tobramycin and bismuth against quorum sensing, virulence factors and biofilms of *P. aeruginosa* was investigated (Alipour *et al*, 2010). The effectiveness of both tobramycin and bismuth was superior when they were administered together as a liposomal formulation. Honey has been found to reduce biofilm formed overtime *in vitro* (Okhiria *et al*, 2009 and 2004; Merckoll *et al*, 2009) and the effect of quorum sensing has also been demonstrated (Truchado *et al*, 2009; Okhiria *et al*, 2007). The *in vitro* broad spectrum antimicrobial properties of honey, prophylactic use and topical application in wound management have been extensively reviewed (Cooper *et al,* 2008, Molan, 2006).

1.5.3 Interference with iron metabolism in biofilm

Lactoferrin, an iron-binding antimicrobial protein present in saliva and gingival crevicular fluids, has been found to prevent biofilm formation *in vitro* (Wakabayashi *et al*, 2009; Singh *et al*, 2002). Lactoferrin was used in combination with 4 antibiotics against biofilms and was found that the amounts of a preformed biofilm reduced were higher when compared with the level of reduction when the antibiotics were used alone (Wakabayashi *et al*, 2009). Another agent that inhibits biofilm by chelating iron in the medium is gallium and has been found to inhibit biofilm formation and kill biofilm organisms *in vitro* (Kaneko *et al*, 2007).

Other synthetic iron chelators that have successfully inhibited biofilm include 2,2'-dipyridyl (2DP), diethylenetriaminepentacetic acid (DTPA), EDTA, deferoxamine mesylate (DM) and ethylenediamine-*N*,*N*'-diacetic acid (EDDA) (O‟May *et al,* 2009).

1.5.4 Enhancement of immune mechanism

Enhancement of regenerative process of the skin has been achieved using naturally occurring substances that are capable of stimulating cellular growth such as growth factors (Brown *et al,* 1989). Treatment with bone marrow- derived cells (Badiavas and Falanga, 2003), gene therapies (Asai *et al*, 2006) and stem cells (Branski *et al,* 2009) have also been applied as topical treatment strategies. Garlic was found to enhance the clearance of *Pseudomonas* in mice (Bjarnsholt *et al*, 2005). The quantity of garlic that an adult will use to achieve this preventive measure is very high but research into the extraction of the component involved in the inhibition will usher in the solution. Shuford and colleagues (2005) also demonstrated the antibiofilm effect of fresh garlic extract on biofilms of *Candida* species. Reduction of the killing of *Caenorhabditis elegans* cells by the organisms was achieved on attenuation of QS (Rasmussen *et al,* 2005).

1.5.5 Application of infective agent

The use of infective agents such as phage to prevent biofilm formation on catheters has been documented *in vitro* (Donlan, 2009; Curtin and Donlan, 2006) as well as disrupting already formed biofilms (Sillankorva *et al,* 2008). Younger biofilms were found to be more susceptible to the phage than the older biofilm (Sillankorva *et al,* 2008).

1.5.6 Other therapeutic agents used for biofilm treatment

Other treatment strategies include the use of ultrasound (Uhlemann *et al*, 2003), electrical stimulation (Petrofsky *et al,* 2009; Liebano *et al,* 2008) and electromagnetic therapy for soft tissue injuries and chronic wounds.

1.6 Summary / Statement of intent

Wound healing is a complex process by which the tissue restores its integrity after injury at the earliest possible time. It is inevitable that wounds will be colonised with microorganisms but the innate immune system of the body usually eliminate the microbial load in the course of wound healing. However, when the immune system is compromised it might be difficult to cope with the microbial burden and infection might occur. Under certain circumstances even when the individual is immune competent, the microbial load may be high or the virulence propensity of the colonising microbes might overwhelm the immune system of the host. These problems often lead from wound colonisation to infection and the healing processes will be adversely affected. Adequate treatment strategies will resolve the problems associated with such circumstances that may prolong or prevent healing. Biofilm formation by certain pathogens such as *P. aeruginosa* can sometimes be rapid (Harrison Balestra *et al*, 2003) and the presence of such organisms in wounds could lead to the development of biofilms in such wounds even within a period of 24 to 48 hours after colonisation. Biofilm formation is associated with quorum sensing molecules (Xu *et al*, 2006; Rice *et al*, 2005; Davies *et al*, 1998) which also regulate other virulence factors (Sakuragi and Kolter, 2007; Xu *et al,* 2000) and EPS formation (Hentzer *et al*, 2001; Costerton *et al*, 1987). Biofilm EPS and the large number of organisms within it make phagocytic engulfment and opsonisation impossible (Günther *et al,* 2009; Leid *et al,* 2005). The fast rate of genetic transfer within biofilm provides higher potential for biofilm

organisms to resist antimicrobials (Anderson and O'Toole, 2008; Fux *et al,* 2005). Quorum sensing molecules and biofilm formation are therefore a great burden in terms of virulence expressions to the host environment. Biofilm infections in wounds have resulted in chronicity and complications leading to amputations, high morbidity and mortality. Treatment strategies are geared towards prevention or reduction of microbial burden and control of any underlying conditions. Whatever the treatment intervention, efforts should be focused on prevention of biofilm formation in wounds to enhance wound healing and reduce the problem of wound chronicity and the attendant complications.

1.7 Aims of the research

Presently, biopsy samples are used for diagnosis of biofilm in wounds; the procedures for the collection of the samples are invasive and discomfort patients. Uneven distribution of organisms within the wound space might prevent the full opportunity for detection of biofilm in wounds. Furthermore the method of diagnosis is expensive and not available for routine diagnosis. There is therefore the need for simple and less expensive procedure for detecting the presence of biofilm in wounds. Also a less cumbersome but convenient procedure for sampling might be necessary.

The problem of antibiotic resistance in biofilm has informed various suggestions such as the use of compounds that target quorum sensing communication system in bacteria to forestall biofilm formation (Bjarnsholt and Givskov, 2007; Hentzer and Givskov, 2003). This will further reduce the problems of resistance and selection of mutants across various species because it is generic and will forestall virulence expressions. This study aims to focus on the role of biofilm in wounds by investigating the potential of organisms in wounds to form biofilm and development of non - invasive procedures for investigating the presence of biofilm in chronic wounds. The antibacterial effect of

honeys on biofilm would also be explored and in particular the mode of action of honey on biofilm organisms. The aims are

- To investigate the characteristics of *P. aeruginosa* and other organisms isolated from wounds *in vitro*.
- To explore novel ways to detect the presence of biofilm in chronic wounds
- To study the effect of honey on biofilm

Objectives

- To determine the antibiotic sensitivity pattern of organisms isolated from wounds.
- To develop an assay method for screening wound isolates for biofilm forming potential (biofilm index)
- To screen organisms isolated from wounds for ability to form biofilm *in vitro*
- To investigate the relationship between quorum sensing molecules and biofilm formation.
- To develop a wound model for biofilms
- To analyse phenotypic changes that occur during biofilm formation.
- To develop assay methods for screening wounds for the presence of biofilm.
- To examine the antimicrobial effect of honey on biofilm
- To determine the effect of honey on quorum sensing

Chapter 2

Characterisation of *Pseudomonas aeruginosa* isolated from wounds

2.1 Introduction

Wound colonisation following contamination by microorganisms from the cause of an injury, the patient‟s flora or the environment (Kühme *et al*, 2007; Lowbury *et al,* 1970) is inevitable hence a wound is not usually sterile. During the process of wound healing, particularly at the inflammatory stage, the body eliminates the colonising organisms (Mercandetti and Cohen, 2008; Enoch and Price, 2004). However, few colonising organisms may have the ability to overcome the immune system and establish infection (Olson *et al*, 2002; Costerton *et al*, 2009). Biofilm formation is one of the evasive strategies of pathogenic organisms (Donlan and Costerton, 2002). It has been documented that microorganisms in biofilm flourish within the extra-polymeric substances produced by them (Sutherland, 2001; Costerton *et al,* 1999). Series of physiological and metabolic activities occur during biofilm formation including the production of certain chemical substances known as quorum sensing signal molecules utilised for the formation and maintenance of biofilm. Signal molecules can be species specific (Fuqua *et al*, 1994) and are population dependent (Wilson, 2001). In Gram negative bacteria such as *P. aeruginosa*, the most commonly utilised quorum sensing signalling molecules during biofilm formation and maintenance of metabolic activities within the biofilm are the acylhomoserine lactones (AHL) (Parsek and Greenberg, 2000). The association of signalling molecules with biofilm has been established (Labbate *et al*, 2004; Fuqua *et al*, 2001; Davies *et al*, 1998) and this enhances production of virulence factors (Camilli and Bassler, 2006; Zhu *et al*, 2002; Whitely *et al*, 2001) which tends to potentiate the pathogenic effect of the organisms. The influence of signalling molecules such as AHL on the inhibition of phagocytic activity by biofilm has been demonstrated (Alhede *et al*, 2009; Bjarnsholt *et al*, 2005). These signalling molecules are often used as biofilm markers to diagnose biofilm infections. Stickler and colleagues (1998) established the presence of biofilm in urinary catheters

by screening for Gram negative quorum sensing molecules (AHL). Singh and colleagues (2000) confirmed the presence of biofilm in cystic fibrotic lung through detection of signalling molecules in patient‟s sputum. Production of signalling molecules by pathogenic bacteria can be site dependent (Soto *et al*, 2007; Choy *et al*, 2008; Hamood *et al*, 1996) as they play a vital role in the regulation of virulence factors in such organisms (Choy *et al*, 2008; Soto *et al*, 2007; Favre-Bonté *et al,* 2007; Hamood *et al*, 1996).

In an animal model study devised by Serralta and colleagues (2001), partial thickness wounds made on pigs challenged with *P. aeruginosa* and covered with plastic coverslips or polyurethane dressings produced biofilm within 72 hours which were dislodged from the wounds by flushing with surfactant solution. The dislodged biomass was cultured and results showed two distinct populations of bacteria in the wounds, the planktonic and the biofilm phenotypes. Microscopic examination of the wound curetting after staining revealed the presence of EPS matrix surrounding the bacteria which was an indication of biofilm formation. In 2008, Davis and his colleagues also demonstrated the evidence of biofilm associated wound colonisation in six pigs using light microscopy (LM), epifluorescence microscopy (EpiM) and scanning electron microscopy (SEM) in 2 sets of experiments. Partial thickness wounds were created on the flanks and the back of each animal. Wounds were challenged with *S. aureus* and covered with polyurethane wound dressing for 48 hours to allow colonisation. At 48 hours, a set of three wounds were cultured using flush and scrub technique for establishment of biofilm baseline count. Biopsies were taken for LM, EpiM and SEM examinations. Results of the LM examination of stained biopsy sample showed aggregates of *S. aureus* surrounded by biofilm EPS matrix. SEM examination demonstrated cocci embedded in amorphous matrix at 24 hours and after 48 hours the aggregates were larger within the embedded matrix. The images of the growth of *S.*

aurues within the wound showed evidence of biofilm matrix unlike the agar culture which showed the organism in planktonic form devoid of EPS matrix.

The ability of bacteria such as *P. aeruginosa* isolated from wounds to produce biofilm *in vitro* within 10 hours has been demonstrated (Harrisson – Balestra *et al*, 2003) which suggests evidence that biofilm can form shortly after wound contamination especially in patients with compromised immunity. Gristina and colleagues (1985) observed the presence of biofilm in surgical debridement material from osteomyelitic bone through scanning electron microscopy but it was over two decades later that their finding was confirmed by various evidences through scanning of wound materials in humans. Examinations of wound biopsies and debridement materials have provided evidence for the presence of biofilm in human wounds (Malic *et al*, 2009; James *et al*, 2008, Davis *et al*, 2008; Ngo *et al*, 2007).

2.1.2 Choice of organism for this study

P. aeruginosa is one of the opportunistic pathogens commonly found in wound infections (Lyczak, 2000) and has been known to form biofilm easily and quickly (Harrison- Balestra, 2003). It produces high levels of quorum sensing molecules (Fuqua, 2006; Zhu *et al*, 2005) and alginate which potentiates its ability to evade immune mechanisms (Leid *et al*, 2002 and 2005). The quorum sensing signalling system of *P. aeruginosa* has been widely investigated and some detection methods are simple, easy and less expensive. *P. aeruginosa* has been used extensively as a model organism for studying physiological properties of Gram negative organisms due to its ability to utilise various organic compounds as an energy source and requires minimal nutrients for growth.

2.1.3 Pseudomonas aeruginosa

2.1.3.1 Introduction and general characteristics of Pseudomonas aeruginosa

Pseudomonas aeruginosa is a Gram-negative, aerobic rod shaped bacterium that belongs to the Pseudomonadaceae family. It measures about 0.5 to 0.8 µm by 1.5 to 3.0 µm and it is motile by means of a single polar flagellum. *P. aeruginosa* can thrive with little oxygen especially in biofilm but can grow under anaerobic conditions (O'May, 2006) and can thrive within a wide range of temperature up to 42^{o}C (Wolska, 2008) with the optimum temperature of 37^{o}C. It is a free-living organism, commonly found in soil, vegetation and water, marshes, and coastal marine habitats (Van Delden and Iglewski, 1998) and occasionally animals and human. About 4-12 % of the human population harbour *P. aeruginosa* in the gastrointestinal tract (GIT) (Bodey *et al* 1983).

P. aeruginosa produces variety of pigments such as pyoverdin (also known as fluorescein); a yellow-green fluorescent pigment is produced abundantly in media of low-iron content, pyorubin (red-brown) and the blue/green pigment pyocyanin (Figure 2.1).

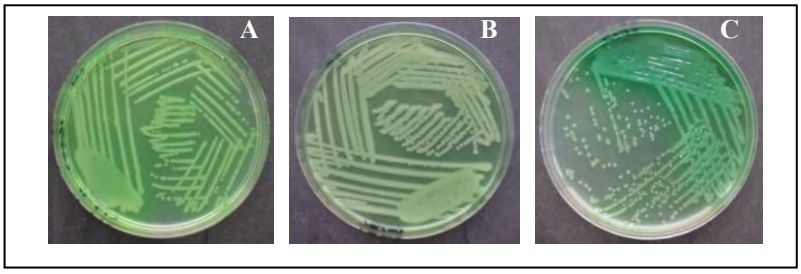

Figure 2.1: *Pseudomonas aeruginosa* pigmentation in cultures (A and B) *Pseudomonas* selective medium with gentamicin supplement agar and (C) nutrient agar.

Pyocyanin is characteristic of suppurative infections caused by *Pseudomonas aeruginosa*; "pyocyaneus" is known as the blue pus. The pigments particularly pyocyanin plays a role in the pathogenicity of *P. aeruginosa* by inhibiting cellular respiration thus accelerating neutrophil apoptosis (Allen *et al*, 2005; Usher *et al*, 2002)

P. aeruginosa isolates from environment sources such as soil or water usually produce small, rough colonies while those from clinical samples may appear as fried-egg shaped, large, smooth with flat edges or mucoid in appearance due to production of alginate, which plays a significant role in colonization and virulence (Pritt *et al* 2008; Li *et al,* 2005; Mathee *et al* 1999).

2.1.3.2 Pathogenicity and virulence factors of P. aeruginosa infections

P. aeruginosa is a pathogen of plants, animals and humans with colonies as diverse as the site of isolation and is able to cause devastating infections because of the strong attachment potential with pili (Lyczak *et al*, 2000; Van Delden and Iglewski 1998) and production of high level of virulence factors such as elastase (Blackwood *et al*, 1983), phospholipase C (Lyczak *et al*, 2000), exotoxin A (Van Delden and Iglewski, 1998), and exoenzyme S (Jia *et al*, 2006; Woods *et al* 1997; Hamood *et al*, 1996). Others include proteases, lipopolysaccharide (Woods *et al* 1997) and alginate (Potvin *et al*, 2003). Some of the virulence factors act locally while some are remote in their activities but the overall effect results in overcoming the host‟s immune system mainly due to tissue damage, dissemination, systemic infection, multiple organ failure and consequently death if infection is not checked (Tang *et al*, 1996).

Elastase produced by *P. aeruginosa* breaks down host elastin, and other connective tissue proteins such as laminin and collagen types III and IV (Yanagihara *et al*, 2003; Bejarano *et al,* 1989); it impairs the structure of the arteries causing haemorrhage (Komori *et al*, 2001) and degradation of surfactant proteins A (SP-A) and D (SP-D) (Mariencheck *et al*, 2003). These destructive events enhance the invasiveness of *P aeruginosa* during infection. In the lungs *P. aeruginosa* protease IV contributes to lung injury due to degradation of surfactant function (Malloy *et al,* 2005) and investigation has shown the production of protease IV by *P. aeruginosa* isolates from the cystic fibrotic (CF) lung as evidence of its important role in the pathogenesis of CF patients

(Smith *et al,* 2006). *P aeruginosa* secretes exotoxin S to induce apoptosis pathways in the host and inhibits the anti-apoptosis pathway, which can shut down host survival signal pathways (Jia *et al*, 2006). The damaging effect of *P. aeruginosa* toxins on the tissues and organs of the host coupled with initiation of host immune responses often leads to septic shock and determines the mortality and morbidity of infections associated with *P aeruginosa* (Ciornei *et al*, 2009; Vitkauskiene *et al*, 2005). In a reported case of some patients in critical health conditions in a Brazilian hospital, *P aeruginosa* infections resulted in the death of over 50% of the patients (Pinheiro *et al*, 2008). The smooth LPS O-antigen is known to initiate the host immune response (Vitkauskiene *et al*, 2005) which results in high level of host‟s immune responses but the rough strain LPS is easily destroyed by serum complement (Hancock *et al*, 1983). Studies have shown that the level of virulence factors produced by *P. aeruginosa* was site dependent; although elastase and phospholipase were produced at all infection sites but toxin A and exoenzyme S production were found to be higher in wound infections (Runbaugh *et al* 1999; Hamood *et al*, 1996) suggesting the importance of *P. aeruginosa* virulence in wounds. The presence of exotoxins in wound has been found to prevent healing (Engel and Balachandran, 2009).

2.1.3.3 P. aeruginosa opportunistic and nosocomial infections

The clinical relevance of *P. aeruginosa* in causing opportunistic infections in man and nosocomial infections (Srinivasan *et al*, 2003; Moolenaar *et al*, 2000; Morrison and Wenzel, 1984) has been on the increase in the last few decades, especially with the advancement of medical interventions geared towards prolonging lives of people with diseases. *P. aeruginosa* can survive on basic minimal nutritional resources particularly on moist surfaces and has been found on medical devices, instruments and worktops in the hospital environments, which sometimes serve as sources of infection (Kolar *et al*,

2009; Srinivasan *et al*, 2003). Infections can also be acquired by patients through the hospital staff (Moolenaar *et al*, 2000). In recent times *P. aeruginosa* infections in healthy individuals have been reported (Hatchette *et al*, 2000). *P. aeruginosa* has been implicated in various infections including the urinary tract (Marcus *et al*, 2008; Gupta *et al*, 2005), respiratory system (Runbaugh *et al*, 1999), endocarditic (Bodey *et al* 1983), wounds (Cooper *et al*, 2009; Fazli *et al*, 2009), severe burns (Estahbanati *et al*, 2002; Lyczak *et al*, 2000) and surgical infections (Efem and Iwara 1992). *P. aeruginosa* is frequently isolated as opportunistic pathogen in systemic infections, particularly in patients with underlying health problems or compromised health conditions such as cystic fibrosis (Santucci *et al*, 2003; Pier, 2000; Wine, 1999), diabetes (Dowd *et al*, 2008), cancer (Kolar *et al*, 2009; Varaiya *et al*, 2008) and autoimmune deficiency syndrome (AIDS) patients (Mendelson *et al*, 1994), as well as patients with *in situ* medical devices (Hardalo & Edberg, 1997). According to a survey carried out in a cardiovascular intensive unit in Ohio, USA, *P aeruginosa* was responsible for 10% of the blood stream infections amongst 40,207 patients between January 1986 and December 1997 (Gordon *et al*, 1998). *Staphylococcus aureus* seems to be the most commonly isolated pathogen from wounds, however some studies have found *P. aeruginosa* as the most prevalent of the isolated organisms (Agnihotri *et al*, 2004; Singh *et al*, 2003) while some studies found *P. aeruginosa* as one of the most commonly isolated bacteria in wounds (Akinjogunla *et al*, 2009; Percival *et al*, 2004; Pirnay *et al*, 2000).

P. aeruginosa is also prevalent in the wounds of patient with underlying health problems especially in diabetic foot and leg ulcers (Ako-Nai *et al*, 2006), burns (Bielecki *et al,* 2008; Estahbanati *et al,* 2002) and surgical wound infections (Hani *et al*, 2009; Masaadeh and Jaran, 2009). According to the 2006 England and Wales survey, the number of reported cases of bacteraemia attributable to *Pseudomonas* species

increased by 41% from 2605 in 2002 to 3663 in 2006; of the *Pseudomonas* species isolated from bacteraemia 92% were *P. aeruginosa*. The increased rate for *P. aeruginosa* bacteraemia alone rose from 2063 in 2002 to 2881 in 2006, an increase of 40%. In another survey, *P. aeruginosa* bacteraemia including susceptibility tests for at least one antimicrobial agent has increased from 77% in 2002 to 90% in 2006 in the UK (HPA, 2007). In the United State, according to the American Thoracic Association, Infectious Diseases Society of America: Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia (2005) *P. aeruginosa* is second most common of the bacteria that cause nosocomial infections and the major cause of death (Zavascki *et al*, 2006) especially due to multidrug resistance (Obritsch *et al,* 2005). It is the second most prevalent organism in surgical site infections (Richards *et al*, 1999). The prevalence and mortality rates have been on the increase in the last few decades (Lautenbach *et al,* 2006) particularly in patients with longer duration of hospital stay (Onguru *et al*, 2008) and associated high economic impact especially antibiotic treatment (Inan *et al*, 2005).

2.1.3.4 Antibiotic resistance by Pseudomonas aeruginosa

P. aeruginosa can tolerate a wide variety of physiologic conditions such as high concentrations of salts and dyes; and commonly used antimicrobials which have influenced its pathogenicity in causing chronic infections. The microbial resistance of *P. aeruginosa* has been on the increase (Kouda *et al*, 2009; Wang *et al*, 2005; Gür *et al*, 1995) and has been linked to exposure to antimicrobials (Onguru *et al*, 2008; Akinci *et al*, 2005; Harris *et al*, 2002). In England and Wales, resistance of *P. aeruginosa* to some antimicrobials increased from 8% to 11% for imipenem and from 6% to 10% for meropenem from 2002 to 2006 (HPA, 2007). Increase in resistance to gentamicin was observed between 2001 and 2002 from 5% to 8% while resistance to ciprofloxacin was

from 10% to 12% during the same period according to the Department of Healthcare-Associated Infection and Antimicrobial Surveillance of the Health Protection Agency (HPA), UK report (2005). In 2008, 26% of the 230 *P. aeruginosa* isolates from diabetic and cancer patients were resistant to carbapenem (Varaiya *et al*, 2008).

2.1.3.5 Mechanism of antimicrobial resistance by *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is able to resist antimicrobials through various means, some of which are intrinsic while others are acquired. The mechanisms of resistance include the following:

(a) Reduced permeability of cell wall to antimicrobials: All strains of *P. aeruginosa* intrinsically resist some antibiotics due to reduced permeability of antimicrobials through the cell wall (Lambert, 2002; Brinkman *et al,* 2000). The antibiotics mainly employed in the treatment of *Pseudomonas* infection have to cross the cell wall into the cytoplasm to get to the targets. Reduced penetration does not allow adequate amount of antibiotic to get through to the cytoplasm for effective interference with the mechanism of protein synthesis of the organism. Antibiotics such as the aminoglycosides (gentamicin or tobramycin) will not be able to effectively inhibit protein synthesis by binding to the 30S subunit of the ribosome while the action of quinolones such as ciprofloxacin binding to the A subunit of DNA gyrase will be reduced (Lambert, 2002). The effect of permeability does not affect low molecule hydrophilic antibiotics like β-lactams (piperacillin) that inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the cytoplasmic membrane. Such antibiotics pass through the aqueous porin proteins channels however loss of ability to produce a porin protein (oprD) has been associated with resistance of *P. aeruginosa* to imipenem (Livermore, 2001). Antibiotics such as the aminoglycosides bind to the lipopolysaccharides (LPS) on the outer surface of the cell wall and are transported actively into the bacteria where

they interfere with ribosomal protein synthesis (Brodersen *et al*, 2000). Over - expression of the outer membrane proteins prevents LPS from binding to such antibiotics thus resisting the effect of the antibiotics.

(b) Efflux pump system: Another effective resistance mechanism is the efflux pump system of *P. aeruginosa* (Laohavaleeson *et al,* 2008; Hocquet *et al,* 2003; Zhang *et al*, 2001; Nikaido, 1994) which eliminates microbial agents that penetrate through the cell wall into the cytoplasm. *P. aeruginosa* has 4 types of efflux systems - mexAB-oprM, mexXY-oprM, mexCD-oprJ and mexEF-oprN10 which are effective in the removal of various groups of antibiotics (Lambert, 2002).

(c) β-lactamase production: All strains of *P. aeruginosa* possess the ampC gene for the induction of β-lactamase production which destroys penicillins and cephalosporins (Nicasio *et al,* 2008; Bradford, 2001).

(d) Production of extracellular substances: P. aeruginosa produces extracellular substances such as mucus and capsule and alginate when in biofilm, which influence the penetration of antimicrobials through the cell wall. Alginate in *P. aeruginosa* biofilm prevents antimicrobial penetration (Hentzer *et al*, 2001). Some strains of *P. aeruginosa* with alginate have been found to be sensitive to antimicrobials (Hoffmann *et al*, 2007) due to the suppression of the virulence factors that are regulated via quorum sensing (Skindersoe *et al*, 2008), which is indicative of the modulating effect of quorum sensing signalling molecules on antibiotic resistance of bacteria.

(e) Mutational changes of target enzymes: Some antimicrobial agents target metabolic enzymes and compete with substrates required for proteins and amino acids that are required for metabolism of micro-organisms; but *P. aeruginosa* has the capability for mutational changes of target enzymes and often render such antimicrobials ineffective (Martinez and Baquero, 2000). Quinolones such as ciprofloxacin binds to the A subunit of DNA gyrase. When the mutations in quinolone-resistance-determining regions

(QRDR) of gyrA, gyrB, parC, and parE genes were examined in some clinical isolates, mutations in parC or parE which lead to amino acid changes were found to confer resistance to quinolones (Akasaka *et al*, 2001).

(f) Change of metabolic processes: Certain antimicrobials are targeted towards the aerobic metabolic processes of bacteria but *P. aeruginosa* can grow anaerobically to circumvent the activity of such antimicrobial. It has been demonstrated that *P. aeruginosa* in the lungs of the cystic fibrosis patients has the ability to grow anaerobically which might result in resistance or inefficacy of the antimicrobials that are targeted towards the aerobic metabolic processes of the bacteria (Hassett *et al,* 2002).

(g) Transfer of genetic materials: P. aeruginosa is able to acquire antibiotic resistance through genetic transfer (Enabulele *et al*, 2006; Bremner, 1979) and able to form biofilm easily which has an implication on the antibiotic resistance potential. The high rate of transfer of genetic materials within biofilm including antimicrobial resistance gene (Ghigo, 2001) suggests that resistance to antimicrobials increase when in biofilm.

2.1.3.6 Biofilm formation and quorum sensing by *P. aeruginosa*

P. aeruginosa has the ability to grow in a wide range of conditions and forms biofilm easily with high production of quorum sensing molecules (Kirisits and Parsek, 2006; Stickler *et al*, 1998). Biofilm formation and high production of signalling molecules have a profound influence on *P. aeruginosa* pathogenicity (Høiby *et al*, 2001; Rumbaugh *et al*, 2000) especially in the lungs of cystic fibrotic patients (Hassett *et al*, 2009; Singh *et al,* 2000), and those with medical devices such as urinary catheters (Stickler *et al,* 1998).

P. aeruginosa is a model species for studying group-related behaviour in bacteria including biofilm formation. Production of biofilm by *P. aeruginosa* wound isolate has been demonstrated within 10 hours of culture *in vitro* (Harrison-Balestra *et al,* 2003).

Production of high levels of extracellular polymeric substances (EPS), which usually circumvent antimicrobial action, has been associated with the bacteria (Prasad *et al,* 2009; Pritt *et al,* 2008; Li *et al,* 2005). Quorum sensing molecules are an essential communication mechanism which biofilm forming organisms are known to produce and utilise during biofilm formation and control of metabolic activities within the biofilm. Acyl-homoserine lactones are mostly utilised by Gram negative organisms whereas Gram positive organisms use peptides. *P. aeruginosa* as a Gram negative organism utilises two quorum sensing systems: LasR-LasI and the RhlR-RhlI systems (Schuster *et al*, 2003; Fuqua *et al,* 2001; Latifi *et al,* 1995; Gambello and Iglewski, 1991) for the production of AHL quorum sensing molecules. The LasR is also the transcriptional regulator for elastase (Gambello and Iglewski, 1991). Schuster *et al*, in 2003 identified 315 quorum-induced and 38 quorum-repressed genes, which represent almost 6% of the *P. aeruginosa* genome. AHL quorum sensing modulates the expression of several genes which were induced at different stages of the culture. According to Schuster and his team some genes were expressed early in growth, most genes at the transition from the logarithmic phase to the stationary phase while some were induced during the stationary phase. It was observed that genes were up-regulated more than 5 times by AHL modulation and 85 genes which were repressed by furanone were QS-controlled (Hentzer *et al,* 2003). *P. aeruginosa* major virulence factors such as *lasB* gene encoding elastase, *lasA* encoding *LasA* protease, the *rhlAB* operon for rhamnolipid production, the *phzA-G* operon encoding phenazine biosynthesis, the *hcnABC* operon for hydrogen cyanide production and the *chiC* gene encoding chitinase activity and 38 of such genes were repressed (Schuster *et al*, 2003). It was observed in another study that 222 genes were repressed by AHL (Wagner *et al*, 2003). According to the review summary by Smith and Iglewski (2003), 97 genes were common to the 3 studies that were done about the same period (Wagner *et al*, 2003; Schuster *et al*, 2003

and Hentzer *et al*, 2003) although media composition and oxygen concentration had significant effect on the expression (down or up-regulation) of the genes. AHL remains an important factor in biofilm formation as well as virulence expression of *P. aeruginosa*. There are three quorum-sensing systems, two of which are linked to *R* and *I* genes as discussed previously but the third *LuxR* homologue which is the furanone system is yet to be fully understood. The evidence of quorum sensing molecule production *in vivo* by *P. aeruginosa* has been demonstrated in cystic fibrosis sputum (Middleton *et al*, 2002; Singh *et al*, 2000), medical devices (catheter) *in vivo* and *in vitro* (Stickler *et al*, 1998) and debridement samples from wounds (Rickard *et al,* 2010). Dissemination of *P. aeruginosa* infections from biofilm to other locations is aided through the production of alginate lysate, an enzyme that cleaves alginate in biofilm and causes detachment of organisms within the biofilm. The process causes dispersion of organisms from biofilm causing the spread of *Pseudomonas* infection (Boyd and Chakrabarty, 1995) which further complicates a patient‟s condition. Although some strains of *P. aeruginosa* are deficient in production of quorum sensing molecules such strains are still able to cause infections and form biofilm (Favre-Bonte *et al*, 2007; Schaber *et al*, 2007 and 2004).

2.1.3.7 *P. aeruginosa* in wound infections

P. aeruginosa has been found known to cause opportunistic and chronic infections in wounds where it produces series of virulence factors such as enzymes and toxins which breakdown the host‟s tissues (Yanagihara *et al*, 2003; Bejarano *et al,* 1989). Mucus and extracellular polymeric substances including alginate are often produced by the organism to prevent the action of phagocytes and other immune mechanisms (Leid *et al*, 2002 and 2005). The ability to multiply quickly and produce biofilm within a short period (Harrison-Balestra *et al*, 2003) has enhanced its pathogenicity. *P. aeruginosa* is

one of the organisms that are predominant in wound infections (Akinjogunla *et al*, 2009; Cooper *et al*, 2009; Fazli *et al,* 2009) and has been associated with large ulcers and linked to biofilm formation in wounds (Malic *et al,* 2009; James *et al*, 2008; Ngo *et al*, 2007).

The ability to form biofilm enhances the resistance to the body‟s immune system and antimicrobials which further increases the virulence capability and this phenomenon has been linked to the impediment of wound healing and cause of chronic wounds (Bjarnsholt *et al*, 2008; Wolcott and Rhoads, 2008). According to the investigation conducted by James and his colleagues (2008), in which wound debridement materials and biopsies of chronic wounds in which biofilms were implicated, *P. aeruginosa* was isolated from 35% of the 50 chronic wounds and 20% of the 16 acute wounds. *P. aeruginosa* has been identified as the most predominant in multispecies biofilm of chronic wounds (Malic *et al*, 2009) particularly embedded in the innermost niche of wound biofilm (Kirketerp-Møller *et al*, 2008).

In this chapter, the ability of *P. aeruginosa* clinical isolates from infected and chronic wounds to form biofilm and produce AHL was examined. Using polymerase chain reaction (PCR) to screen the isolates, the possibility of the similarity in the genetic diversity of the isolates to one another were explored and the results obtained analysed in relation to biofilm forming potential and antibiotic susceptibility of the isolates. The correlation between biofilm forming capability of the isolates and AHL production was examined and compared with *P. aeruginosa* cultures from a non- clinical source (water pipes) to assess virulence characteristics based on site of isolation.

The growth curves of a reference culture of *P. aeruginosa* NCIB 8626, a biofilm and non-biofilm forming wound isolates were determined to serve as a guide for the growth phases for subsequent assays. The cultures will also provide the opportunity to

determine the exponential phase when the organism is most viable and metabolically active as well as the period of stress (the stationary / decline phases) when the waste and toxic products are high in the medium and the nutrients are depleted with the resultant reduction in viability of the bacteria. The growth curves were also to serve as a guide on the estimate of number of organisms in a culture at any given time during the experiment. The growth rate was monitored by estimation of optical density and total viable count of bacteria at pre-determined intervals during the period of culture.

2.1.4 Aims

- To determine the biofilm phenotypic expressions of a cohort of *P. aeruginosa* isolated from infected and chronic wounds *in vitro.*
- To explore the link between biofilm phenotypic expressions, antimicrobial susceptibility and similarities in genetic fingerprints of the isolates.

Objectives

- To determine the sensitivity pattern of *P. aeruginosa* isolated from wounds to some commonly used antibiotics.
- To determine the biofilm forming capability of *P. aeruginosa* wound isolates.
- To determine the ability of *P. aeruginosa* wound isolates to produce quorum sensing molecules in cultures as a marker for biofilm formation.
- To characterise *P. aeruginosa* isolates using RAPD - PCR to determine the genotypic similarities
- To investigate any associations between the above characteristics.
- To compare phenotypic characteristics of *P. aeruginosa* strains from wounds with the strains from water pipes as a study of relationship between site and virulence expressions

2.2 Materials and Methods

2.2.1 Organisms

2.2.1.1 Sources of organisms

Cultures of reference organisms: *P. aeruginosa* NCTC 27853, NCIB 8626 and PAO1; *Chromobacterium violaceum* ATCC 12472 and 31532 were purchased from Health Protection Agency (HPA), UK. Prof. Clay Fuqua of Indiana University kindly provided the reporter bacteria *A. tumefaciens* NTL4 (pCF218) (pCF372) and *A. tumefaciens* KYC6 (pCF218). Eighty *P. aeruginosa* wound isolates were obtained from the Department of Medical Microbiology of the University Hospital of Wales from infected wound swabs being routinely processed in the laboratory for diagnosis and seventeen were provided from a collection made by Prof. Rose Cooper, who had isolated them from chronic venous leg ulcers (cvlu) of out - patients attending the Wound Healing Research Unit, Cardiff University (Table 2.1). Nine of biofilm forming *P. aeruginosa* isolated from water pipes were selected from the stock culture collections of Dr Hugh Griffiths of the Cardiff School of Health Sciences, University of Wales Institute Cardiff.

2.2.1.2 Maintenance of cultures

All cultures used for this study (reference organisms and clinical isolates) were maintained at -80^{o}C in broth with beads (PROTECT Bacterial Preserver System, UK). *A. tumefaciens* NTL4 traR plasmid (218) codes for traR protein and (272) codes for traI-lacZ for detection of acyl-homoserine lactones (AHLs) and KYC6 (pCF218) traR over-expression plasmid (pCF218) producing high levels of autoinducers. To maintain *A. tumefaciens* strains plasmids, the bacterium was cultured on LB with 4.5µg/ml tetracycline and 100 µg/ml kanamycin antibiotics while NTL4 was maintained with 4.5µg/ml tetracycline and 50 µg/ml spectinomycin. Each organism except *A. tumefaciens* was cultured on nutrient agar (Oxoid, Basingstoke, UK) and incubated at 37^{o}C for 24 hours prior to each assay.

Table 2.1: Sources of *Pseudomonas aeruginosa* clinical isolates

ID No	Organism	Wound type	ID No	Organism	Wound type
P.1	NCTC8626	Control	P.56	53343	IW
P.2	GW16	cvlu	P.57	53646	IW
P.4	MY20	cvlu	P.58	53499	IW
P.5	LEO8	cvlu	P.59	50568	IW
P.6	CR01	cvlu	P.60	53453	IW
P.7	62816	IW	P.61	71569	IW
P.8	74196	IW	P.62	72401	IW
P.9	64055	IW	P.63	71331	IW
P.10	62852	IW	P.64	72081	IW
P.11	74545	IW	P.65	63875	IW
P.12	62413	IW	P.66	71523	IW
P.13	79595	IW	P.67	71083	IW
P.14	62731	IW	P.68	71668	IW
P.15	62989	IW	P.69	71327	IW
P.16	64594	IW	P.70	71324	IW
P.17	62844	IW	P.71	72793	IW
P.18	74344	IW	P.72	71127	IW
P.19	62993	IW	P.73	65942	IW
P.20	64521	IW	P.74	72057	IW
P.21	62974	IW	P.75	63551	IW
P.22	74618	IW	P.76	58261	IW
P.23	74443	IW	P.77	63256	IW
P.24	62808	IW	P.78	58267	IW
P.25	WD3105	cvlu	P.79	50993	IW
P.28	BH2443	cvlu	P.80	63080	IW
P.29	JH1704	cvlu	P.81	50996	IW
P.30	PP15	cvlu	P.82	71698	IW
P.31	GW16	cvlu	P.83	63380	IW
P.33	OS3103	cvlu	P.84	71575	IW
P.34	PB18	cvlu	P.85	71657	IW
P.35	NJ19	cvlu	P.86	71692	IW
P.36	RC03	cvlu	P.87	59008	IW
P.37	AN3103	cvlu	P.88	50785	IW
P.39	JS13	cvlu	P.89	71042	IW
P.40	JT17	cvlu	P.90	50822	IW
P.41	53708	IW	P.91	50870	IW
P.42	53844	IW	P.92	53940	IW
P.44	50688	IW	P.93	59074	IW
P.45	50618	IW	P.94	63642	IW
P.46	50552	IW	P.95	71037	IW
P.47	50613	IW	P.96	71196	IW
P.48	53810	IW	P.97	59371	IW
P.49	50547	IW	P.98	71330	IW
P.50	50650	IW	P.99	71323	IW
P.51	53491	IW	P.100	71190	IW
P.52	50510	IW	P.101	71219	IW
P.53	53436	IW	P.102	63979	IW
P.54	53707	IW	P.103	63998	IW
P.55	50428	IW	P.104	TE1261	cvlu

Key: cvlu = chronic venous leg ulcer; IW = Infected wound

2.2.2 Media and reagent Preparation

2.2.2.1 Preparation of solid media

The range of solid media used in this study included nutrient agar (NA) for inoculating organisms from -80^{o}C, for propagation of organisms for total viable count and for growing organisms for stock cultures. Luria broth (LB) agar for isolating the reporter strains on addition of appropriate antibiotics and for AHL detection assays. Blood agar (BA), chocolate agar, cysteine lactose electrolyte deficient (CLED) agar, fastidious anaerobic agar, streptococcus and staphylococcus selective agar and Sabouraud agar were utilised for the isolation of various organisms from the wound dressings (details discussed in chapter 4). Appropriate quantity of each culture media was weighed into the media preparator (AES Laboratoire, Combourg, France) bucket and the appropriate volume of deionised water was added. The bucket was placed in the media preparator and set at the required cycle programme for sterilisation of the media at 121^{o}C for 15 minutes. On completion of the sterilising cycle, using the automatic media dispenser (AES Laboratoire, Combourg, France), 25 mls of each media was dispensed into each of the transparent 90 mm diameter triple vent Petri dishes (Fisher, UK). The media were allowed to solidify and the plates were removed, labelled appropriately and kept in the cold room at 4^{o}C. The plates were dried in the oven (Gallenkamp, UK) at 45^{o}C for 20 - 30 minutes prior to use. Media with supplements or blood were prepared in 1 litre reagent bottles and sterilised at 121^{o}C for 15 minutes in the autoclave (LTE Scientific, UK) and cooled to 50^{o}C before the addition of supplements or blood (10% v/v). Twenty ml of each media was dispensed aseptically into each Petri dish and Petri dishes were covered while the media were allowed to set. The plates were packed and labelled appropriately. All media in Petri dishes were kept in the cold room at 4^{o}C prior to use.

2.2.2.2 Preparation of liquid media and buffers

The liquid media include nutrient broth, Luria broth (LB), ½, ¼ and $^{1}/_{10}$ strengths of LB for continuous cultures of reference organisms and wound isolates. Tryptone soy broth (TSB) for biofilm culture in microtitre plates and ¼ strength Ringer‟s solution for dilution of cultures and recovery of organisms from wound dressings before plating on to the isolation media. The phosphate buffered saline (PBS) was used for washing biofilm cultures in microtitre plates to remove the planktonic organisms. Appropriate quantities of each of the culture media (Nutrient or Luria broth (LB) (Oxoid) were weighed into a litre conical flask and the appropriate amount of deionised water was added. The broth was mixed until the solution became homogenous by placing magnetic stir bars inside the flask containing the broth and placing the flask on the magnetic stirrer (Stuart Scientific, UK). Ten millilitres of each broth was dispensed into universal containers which were sterilised at 121^{o}C for 15 minutes.

Phosphate buffered saline (PBS) (Oxoid, Cambridge, UK) and ¼ strength Ringers solution (Oxoid, UK) were prepared in 1 litre reagent bottles according to Manufacturer‟s instructions. The PBS was sterilised at 110^{o}C for 10 minutes while the ¼ strength Ringers solution was sterilised at 121^{o}C for 15 minutes. The broths, PBS and the Ringers solution were appropriately labelled and stored at room temperature in the laboratory prior to use.

2.2.2.3 Preparation of TBE buffer

Tris/ boric acid /EDTA (TBE) 90 mM buffer was prepared by weighing on a balance (Fisherbrand) and dissolving 10.89 g of Tris acetate (BDH, UK) (90 mM), 5.56 g of Boric acid (Sigma, UK) (90 mM) and 0.74 g of Ethylene-diamine-tetra-acetic acid (EDTA) (Fisher Scientific, UK) (2 mM) in 1 litre of deionised water. The pH was adjusted to pH 8.5 with hydrochloric acid (10 M) (BDH, UK).

2.2.2.4 Preparation of Chelex Solution

One gram of iminodiacetid acid ionic resin (chelex) was weighed into a reagent bottle and 10ml of sterile deionised water was added. The chelex was dissolved in the sterile water while stirring at 60°C and the solution was allowed to cool.

2.2.2.5 Preparation of stock antibiotic solutions for maintenance of A. tumefaciens

Stock solutions of tetracycline (Sigma, UK) (0.045gm/10ml) and kanamycin (Sigma, UK) (1gm/10ml) were prepared by weighing the appropriate quantity of each antibiotic into a sterile universal bottle and ten ml of sterile de- ionised water was added to each bottle according to the manufacturer‟s instructions. Stock solution of spectinomycin (Sigma, UK) (0.5gm/5ml) was prepared accordingly and all the antibiotic solutions were kept in the freezer at -20^{o}C. Appropriate quantity of each antibiotic solution was added into the media at 50^{o}C during preparation to obtain the required final concentration for the maintenance of the plasmids of the *Agrobacterium tumefaciens* strains. *A. tumefaciens* NTL 4 was maintained with 4.5µg/ml tetracycline and 50µg/ml spectinomycin while *A. tumefaciens* KYC6 was maintained with 4.5 µg/ml tetracycline and 100µg/ml kanamycin.

2.2.2.6 Preparation of stock X-gal solution

X-gal solution 20 mg/ml (w/v) (Sigma-Aldrich, UK) was prepared in dimethyl-formamide (BDH, UK) and sterilised with Millipore filters (Fisher Scientific, UK) in a fume cupboard (AIRONE FC-640, carbon filter c-100). This solution must be prepared prior to use to prevent deterioration.

2.2.3 Growth Curve of *Pseudomonas aeruginosa*

A colony from the overnight NA plate culture of *P. aeruginosa* NCIB 8626, a biofilm forming and non- biofilm forming wound isolate was each inoculated into 10 ml LB(Oxoid, UK) in a universal container and incubated at 37^{o}C for 18 hours in a shaking waterbath (Grant OLS200, UK) at 100 cycles per minute. The culture was diluted 10^{-3} with Luria broth (LB) and 5 ml of the diluted culture was aseptically pipetted into 95 ml LB in a conical flask. The flask was incubated at 37^{o}C in a shaking waterbath for 24 hours at 100 cycles per minute. Samples of the culture were taken every hour up to 12 hours and at 24 hrs to determine the optical density at 550 nm with the spectrophotometer (Cecil CE2010, UK) and total viable count (TVC) using spread plate method.

2.2.3.1 Estimation of bacteria in culture (Total Viable count)

Ten fold serial dilutions were made from the culture samples with ¼ strength Ringers solution (Oxoid, UK) and 20 µl of each diluted culture was dropped on to a dry nutrient agar (NA) plate using sterile pipette tips. The culture was spread with the aid of a plastic Lazy-L spreader (Fisher Scientific, UK) and plates were incubated at 37^{o}C for 18 - 24 hours. The colonies were enumerated with a colony counter (Stuart, UK) and the total viable count was calculated from the dilution factor and expressed as the colony forming units per ml (cfu/ml) of culture. The TVC for each dilution was estimated in duplicate to obtain a mean value.

2.2.3.2 Turbidimetric estimation of bacteria in culture (optical density)

0.5 ml of the culture was removed aseptically from the flask and poured into a microcuvette (Fisher Scientific, UK) and the optical density was read at 550nm with a spectrophotometer (Cecil CE2010, UK).

2.2.4 Determination of antibiotic susceptibility of *P. aeruginosa* wound isolates

The test was performed according to the guideline of the British Society for Antimicrobial Chemotherapy (BSAC) standardized disc diffusion susceptibility testing method (version 5) of January, 2006. Two to three colonies of the reference *P. aeruginosa* NCIB 8626 or each of the clinical isolates were touched with wire loop and the organisms were emulsified into 2 ml of sterile distilled water in a test tube and the suspension was matched with 0.5 McFarland standard to ensure it was the right turbidity. The suspension was diluted to10^{-2} and it was streaked on the sensitivity agar with the aid of a sterile cotton - wool swab, covering the entire surface of the plate. The antibiotics discs (ciprofloxacin 1 µg, cefuroxime 30 µg, amoxicillin 10 µg, gentamicin 10 µg, imipenem 10 µg, trimethoprim 2.5 µg and piperacillin 75 µg) (all discs were from Oxoid, UK) were placed on the plates on which the organism had been streaked. The plates were incubated for 18 hours at 37^{o}C and observed for zones of inhibition. The diameters of the zones of inhibition were measured with callipers. The bacterial growth inhibition zones due to effect of antibiotics (Fig. 2.3) were interpreted for determination of susceptibility based on the guidelines provided by BSAC (version 5) (2006) (Table 2.2). Pictures of some of the plates were taken with a Nikon digital camera.

Table 2.2: Guidelines for interpretation of antibiotic susceptibility (BSAC, 2006)

Antibiotics	R ≤ (mm)	I (mm)	S ≥ (mm)	**Acceptable zone for control organism (mm)**
Ciprofloxacin1 µg	12	13 - 22	23	24 - 30
Cefuroxime 30 µg	24	-	25	25 - 32
Amoxicillin 10 µg	11	12 - 14	15	24 - 30
Gentamicin 10 µg	17	-	18	22 - 28
Imipenem 10 µg	21	-	22	23 - 28
Trimethoprim 2.5 µg	14	15 - 19	20	30 – 37
Piperacillin 75 µg	23	-	24	27 - 34

Key: R = Resistant; I = Indeterminate and S = Susceptible

2.2.5 Biofilm forming potential of *Pseudomonas aeruginosa* isolated from wounds

2. 2.5.1 Biofilm formation by P. aeruginosa wounds isolates in microtitre plate

The protocol described by Christensen and colleagues in 1985 was employed in this assay. A colony from each of the 24 hour cultures of *P. aeruginosa* NCIB 8626 and 97 *P. aeruginosa* clinical isolates from wounds was picked into Tryptone soy broth (TSB) (Oxoid, UK) and incubated at 37^{o}C for 18 hours in a shaking waterbath (Grant OLS200, UK) at 100 cycles per minute (rpm). The overnight culture was diluted with TSB to 10^{-2} and 200 µl was pipetted into each microtitre plate well (8 replicates for each isolate). The first column of wells in the microtitre plate was inoculated with TSB only as blank control and the last with the reference organism *P. aeruginosa* NCIB 8626. The plates were incubated in an incubator (Gallenkamp, UK) at 37^{o}C for 24 hours. All the diluted cultures were inoculated on nutrient agar (NA) (Oxoid, UK) and incubated at 37^{o}C overnight to check the purity of the cultures. The total viable count of the diluted cultures of the reference organism and some selected clinical isolates were determined as previously described in section 2.2.4 to confirm the inoculum size (≥10^{8} cfu/ml). The

microtitre plate cultures were removed from the incubator at 24 hours and the broth was aseptically removed from each well with sterile tips (Sigma-Aldrich, UK) and discarded. The wells were washed 4 times with sterile phosphate buffered saline (PBS) (Oxoid, UK) using automatic plate washer (Mikura Ltd, UK) to release planktonic bacteria from the culture. The biofilm was fixed with 200 µl of 2.5% (w/v) glutaraldehyde for 5 minutes and the wells were washed 2 times with sterile PBS. Biofilm in each well was stained with 200 µl of 0.25% (w/v) crystal violet stain for 5 minutes and the wells were washed 8 times with sterile PBS. The wells were drained off by inverting the wells and wells were allowed to air dry. The crystal violet dye was solubilised from the stained biofilm with 200 µl of acetone/ethanol (1:1) mixture. The optical densities of the dye released from the biofilms were read using the Elisa plate reader (Dynex technologies, UK; MRX revelation software) at 570 nm.

2.2.5.2 Microtitre plate biofilm reproducibility assay

The microtitre plate biofilm cultures of the reference organism *P. aeruginosa* NCIB 8626 and 21 selected clinical isolates were determined on 3 different occasions as described above in section 2. 2.6.1 to determine the reproducibility of the method.

2.2.6 Examination of *P. aeruginosa* wound isolates for AHL production

2.2.6.1 AHL detection by cross feeding assay.

The protocol described by Stickler and his colleagues in 1998 was utilised for this assay with modifications. Cultures of *P. aeruginosa* were inoculated on nutrient agar (Oxoid) and incubated at 37^{o}C; and those of A. *tumefaciens* on LB agar (Oxoid) supplemented with spectinomycin and tetracycline (strain NTL4); tetracycline and kanamycin for KYC6 and incubated at 30°C. Luria Bertani (LB) agar plates were dried at 45^{o}C and 0.4µl of 20mg X-gal (w/v) (Sigma-Aldrich, UK) in dimethyl- formamide (BDH, UK)

was dropped on each plate and spread over it with the aid of a sterile plastic Lazy-L spreader (Fisher Scientific, UK). The plates were allowed to dry at room temperature for about 1-2 hours. A colony from the 24 hour culture of the control organism *P. aeruginosa* (NCIB 8626) or *P. aeruginosa* clinical isolate was streaked across each of the appropriately labelled plate of X-gal covered LB agar. *A. tumefaciens* NTL4 was streaked across the plate, parallel to the control organism or isolate, at 1cm distance. The plates were incubated at 30°C for 48 hours. Cultures were observed at 24, 36 and 48 hours for evidence of quorum sensing molecule production by the test organism which should result in change in the colour of *A. tumefaciens* NTL4 colonies from cream to blue. Positive control (*A. tumefaciens* KYC6 and A. *tumefaciens* NTL4) and negative control (*A. tumefaciens* KYC6 against itself) were included in the assay.

2.2.6.2 Detection of AHL in P. aeruginosa wound isolates by indirect method – (Quorum sensing inhibition (QSI) assay)

A method used by McLean and colleagues (2004) was modified for this study. The reference *P. aeruginosa* (NCIB 8626) and PAO1, and the clinical isolates were cultured from -80°C on NA plates at 37°C for 24 hours. A colony from each of the 24 hours culture of the reference organisms (*P. aeruginosa* NCIB 8626 and PAO1), and each of the clinical isolates was suspended into ¼ strength Ringers solution (Oxoid, UK) and 5µl of the suspension was dropped on to nutrient agar plate at the centre and incubated at 37°C for 24 hours. Two colonies of *C. violaceum* ATCC 12472 were cultured in 10 ml of Luria broth at 30°C overnight in a waterbath. Ten ml of full strength LB with 0.6% agar (w/v) was melted and placed in a waterbath to cool to 45°C. The molten LB agar was inoculated with 20µl of the overnight broth culture of *C. violaceum* and was mixed gently for even distribution of the organisms within the molten agar. The *C. violaceum* inoculated semi-solid LB agar was layered on top of the nutrient agar on

which the colony of *P. aeruginosa* control organism or clinical isolate from wound was grown overnight by pouring the semisolid LB agar gently on top of the LB agar. Each plate was incubated at 30^{o}C for 24 hours and examined for quorum sensing inhibition of *C. violaceum* due to AHL production by *P. aeruginosa* control organism or the clinical isolates. An AHL producer *P. aeruginosa* NCIB 8626 was used as positive control and a non-AHL producer *P. aeruginosa* PAO1 as negative control bacteria. The inhibition zones were measured and photographs were taken with Olympus plate reader (ProtoCol, UK).

2.2.7 Phenotypic characteristics of *P. aeruginosa* isolated from tap water

The purpose of this assay was to compare the physiological and phenotypic characteristics of the biofilm forming *P. aeruginosa* isolated from wounds with those obtained from tap water. Nine *P. aeruginosa* biofilm forming cultures from water pipes were cultured on NA plates for 24 hours at 37 ^{o}C from stock culture for 24 hours. Each of the cultures was tested for formation of biofilm in a microtitre plate, production of AHL molecules and QSI as described in 2.2.5.1, 2.2.6.1 and 2.2.6.2, respectively.

2.2.8 Polymerase chain reaction (PCR)

The random amplified polymorphic deoxyribonucleic acid (RAPD) analysis of the *P. aeruginosa* isolates from wounds was done using the protocol described by Babalola and his colleagues (2002). RAPD analysis will provide the opportunity to examine the genetic diversity of the isolates which will not be possible if specific genetic probes are used for particular phenotypic characteristics.

2.2.8.1 Preparation of DNA Solution and DNA Quantification

Eppendorf tubes (Sigma-Aldrich, UK) were labelled appropriately and 500µl solution of 10% chelex (w/v) solution was pipetted into each Eppendorf tube and 2 colonies of each isolate were added to the chelex solution. The mixture was placed inside a water bath (Grant, UK) at 95^{o}C for 10 minutes to lyse the cell walls of the bacteria to release the DNA. The solution was centrifuged (Amazon centrifuge, UK) at 13,000 g for 5 minutes. The supernatant (DNA solution) was aseptically transferred into a sterile Eppendorf tube. Into an Eppendorf tube 68 µl of DNA free sterile deionised water was pipetted and 2 µl of the DNA solution was added and mixed. The DNA in the solution was estimated spectrophotometrically with the DNA reader (Gene-quant probe, Amershan Biosciences, UK) using DNA free water as blank.

2.2.8.2 PCR Master Mix solution

150 µl sterile deionised water, 21µl PCR buffer, 1.3µl Deoxinucleotide master mix (DNTP), 1.0µl Taq polymerase enzyme and 1.3µl Primer (OPA-09: 5‟GGGTAACGCC3‟) (Sigma - Aldrich, UK) were pipetted into an Eppendorf tube and mixed with the aid of vortex mixer (Stuart Scientific, UK).

2.2.8.3 PCR amplification procedures and PCR optimisation reaction

35µl of the PCR master mix solution was pipetted into each PCR tube (Fisher Scientific, UK) and the appropriate Eppendorf tubes and various volumes (10, 7, 5, 3, 2 and 1 µl) of the DNA solution of the reference *P. aeruginosa* NCTC 27853 were appropriately pipetted into the labelled PCR tubes containing the PCR solution. The marker and the reagent blank were included in the assay. The tubes were placed in a thermal cycler (Applied BioSystems, GeneAmp PCR system 9700, UK). The thermal cycler was programmed for the amplification and annealing of the DNA (initial denaturing time of 5 minutes at 94^{o}C, denaturing cycles for 1minute at 94^{o}C, annealing

for 1 minute at 40°C and at 2 minutes at 72°C, final extension time for 10 minutes at 72°C in 35 cycles and final holding time at 4°C till removal of PCR products). The amplification products were analysed by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide and examined under ultra violet (UV) light with BioRad UV Transilluminator 2000. Gel pictures (Fig. 2 .1A) were taken using Gel Doc™ 2000 and Quantity One™ 4.0.3 software (BioRad Laboratories, Hercules, CA).

2.2.8.4 Agarose gel bed preparation

Agarose powder (0.45g) (Sigma - Aldrich, UK) was weighed into a flask and 30 ml of TRIS buffer (0.09M) were added to obtain 1.5% (w/v) agarose concentration. The mixture was placed in the boiling water bath to dissolve the agarose. The molten agarose solution was cooled to 55 - 60°C and was poured on to the bed receptacle inside the electrophoresis tank and allowed to set.

2.2.8.5 Gel Electrophoresis and Staining

The electrophoresis tank (Life Technologies, USA; model 250) was filled with 150 mls of the TRIS buffer. Drops of 8 µl of the loading buffer (0.25% Bromophenol blue) were placed onto a sterile Petri dish, 7 µl of the PCR products of the reference organism *P. aeruginosa* NCTC 27853 from each tube was added to each drop of loading buffer, mixed together and the wells of the agarose gel were appropriately charged. The 1kb marker (Sigma - Aldrich, UK) was loaded on the last lane (1.3 µl was mixed with 8µl of the loading buffer) and the reagent blank (PCR reagents and loading buffer) was loaded on the first lane wells respectively. The tank was closed, plugged into the socket and the electricity (250 Amps and 1.5volts) was switched on to run for 90 minutes. The gel was immersed in 100mls of ethidium bromide solution (0.02mg/100mls w/v) for 30 minutes to stain the DNA and the gel was washed under running tap water for 30 - 60

minutes. The gel was later examined with the transluminator and photographs were taken. The optimisation reaction showed prominent bands but the lines with 2 µl, 3µl and 5 µl DNA solutions were better than the 1 µl, 7 µl and 10 µl volumes. The 5 µl volume gave the most distinct bands and was chosen for subsequent PCR assays.

2.2.8.6 PCR of P. aeruginosa wound isolates

Based on the results of the optimization (section 2.2.8.3 – 2.2.8.5), 5 µl of the DNA solution of each isolate was used for the PCR run as in 2.2.8.3 and 2.2.8.5 above.

2.2.9 Data analysis

2.2.9.1 Statistical analysis

The sets of data obtained from the growth curve and microtitre plate biofilm cultures were analysed with Excel 2003 and mean values and SD were obtained as well as calibration curves were represented in graphical forms. Tests of association between biofilm formation and AHL production and antibiotic susceptibility were carried out using Minitab (version 15) Pearson's chi-square tests. *P* values <0.05 were considered significant and 95 % confidence intervals (CIs) were used to determine the strength of the associations. The similarity linkage was analysed with Minitab using Jaccard similarity coefficient and probability graphs were plotted.

2.2.9.2 Gel Analysis

The fingerprint data analysis of the bands was done with GelCompar II version *4* (Applied Maths, Belgium) (Fig. 2 .3 A and B) for the percentage similarities using Jaccard similarity coefficient and the dendrogram (Fig. 2 .4).

2.3 Results and Interpretations

2.3.1 Growth Curve for *Pseudomonas aeruginosa* NCIB 8626

Growth curves of the reference strain were obtained by plotting graphs of either the relationship between the optical densities (OD) against time of growth (Fig. 2.2A) or the number of viable organisms (TVC) against time of incubation (Fig. 2.2B). The relationship between OD and TVC was also determined Fig. 2.2C. The OD was readable when the number of bacteria in the culture increased to 10^7 organisms/ml. The results gave the information about the growth phases of the species as a guide for the expected number of bacteria in the culture at any particular time of an assay. This also served as a guide in diluting cultures in order to obtain the inoculum size for an assay. The growth patterns of the wound isolates (data not presented) were similar to that of the reference organism

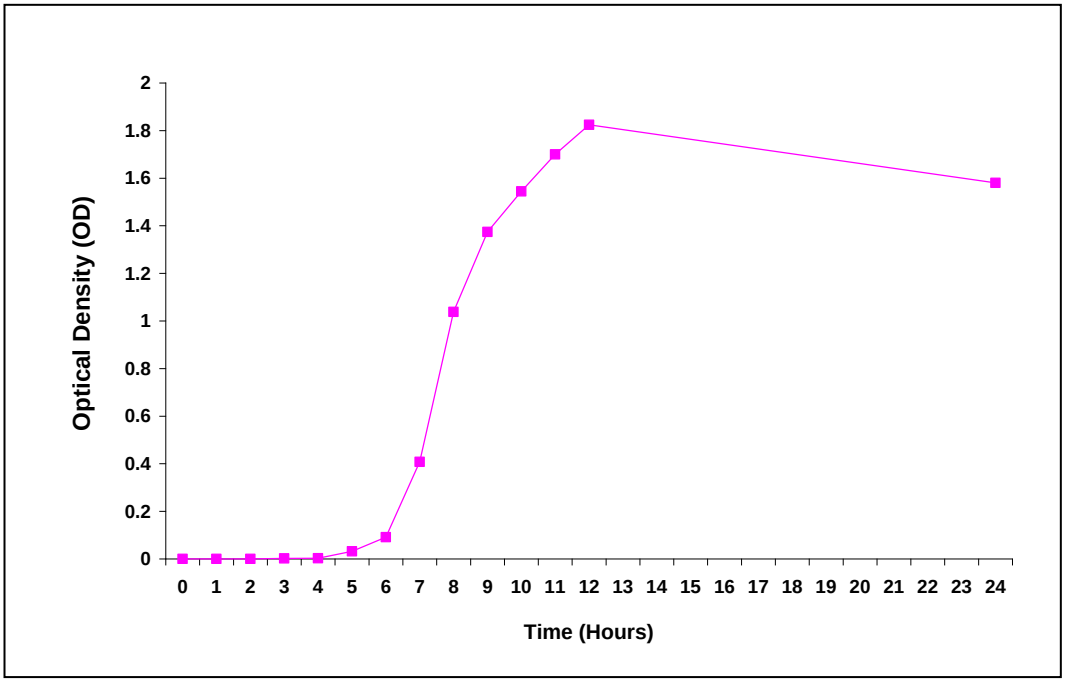

Figure 2.2A The growth pattern of *P. aeruginosa* NCIB 8626 in a 24 hour Luria broth culture. The optical densities of the cultures were determined every hour to monitor the growth pattern of the bacteria.

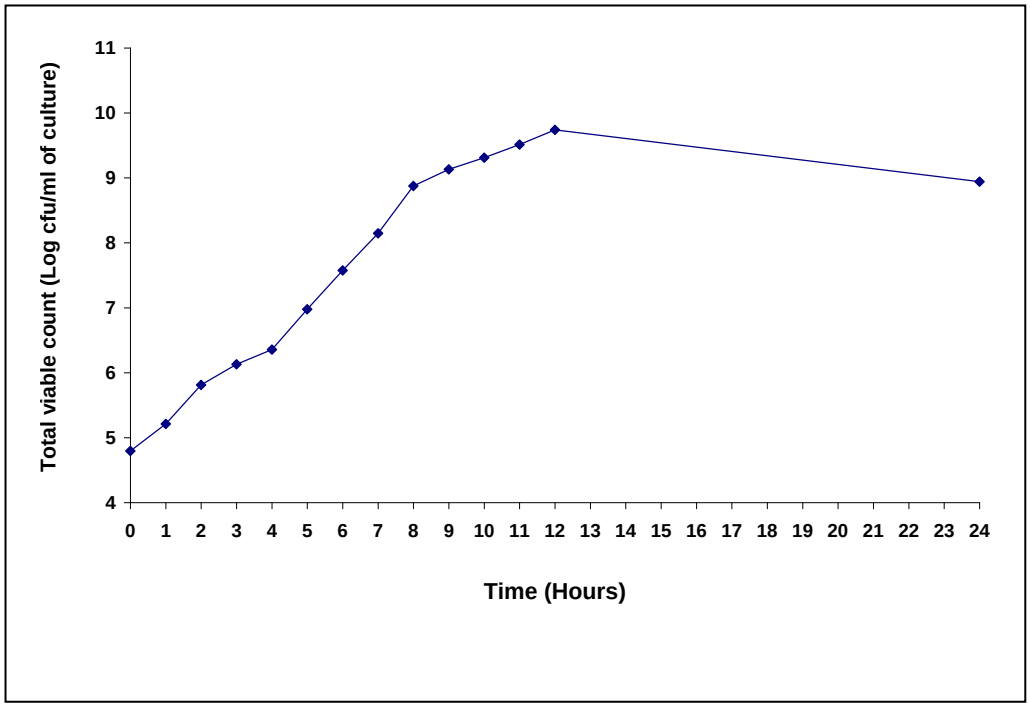

Figure 2.2B: The growth pattern of *P. aeruginosa* NCIB 8626 in a 24 hour broth culture. The growth was monitored by determining the total viable count.

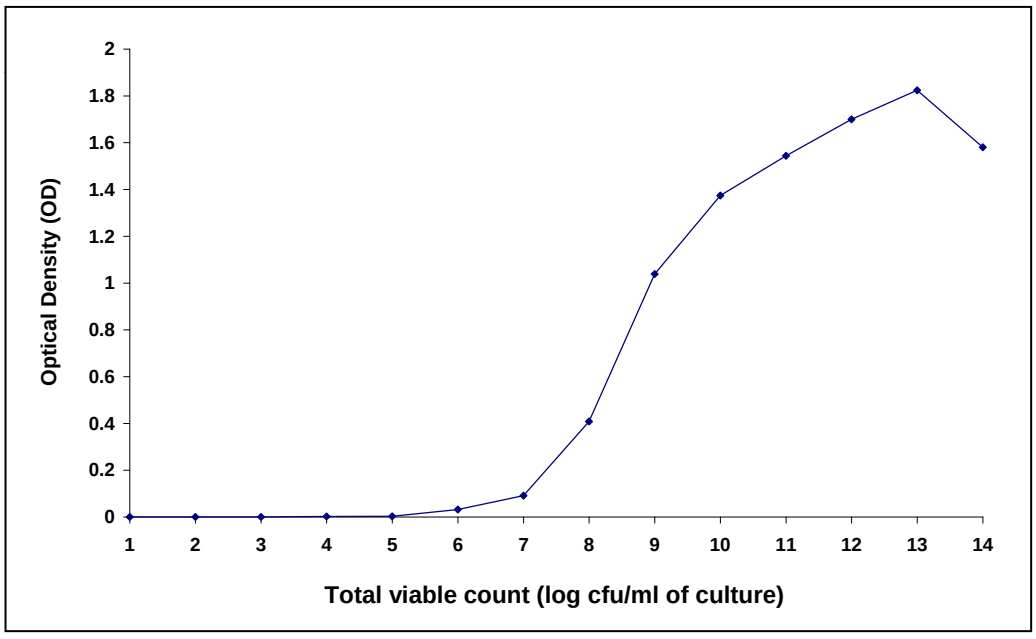

Figure 2.2C: Relationship between optical density and growth of *P. aeruginosa* NCIB 8626 in 24 hour LB culture.

2.3.2 Antibiotic susceptibility assay of *P. aeruginosa* clinical isolates

The results of the antibiotic susceptibility for the 97 isolates (Fig. 2.3) as discussed in section 2.2.4 above were summarised in Table 2.3.

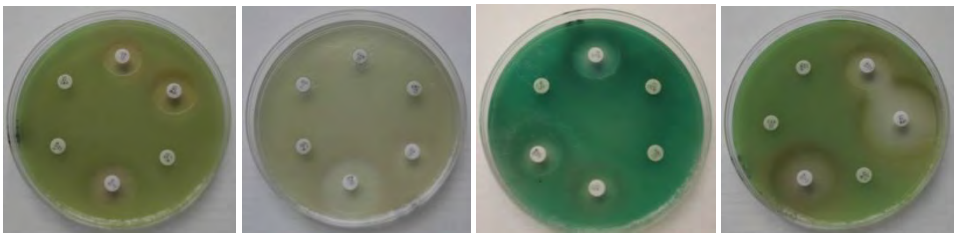

Figure 2.3: Cultures of *P. aeruginosa* wound isolates on sensitivity agar plates with antibiotic discs.

Table 2.3: Summary of the susceptibility results of the isolates to each antibiotic.

Antibiotics	**susceptible isolates**	**Resistant isolates**
Ciprofloxacin (CIP)1µg	77 (78.38 %)	20 (20.62 %)
Cefuroxime (CXM) 30µg	1 (1.03 %)	96 (98.97 %)
Amoxicillin (AML) 10 µg	3 (3.1 %)	94 (96.9 %)
Gentamicin (CN) 10µg	79 (81.44 %)	18 (18.56%)
Imipenem (IPM) 10µg	89 (91.75 %)	8 (8.25 %)
Trimethoprim (W) 2.5 µg	1 (1.03 %)	96 (98.97 %)
Piperacillin (PRL) 75µg	92 (94.85 %)	5 (5.15 %)

2.3.3. Biofilm formation by *P. aeruginosa* wound isolates

2.3.3.1 Interpretations of results

The maximum optical density (OD) measurement that can be obtained from the Dynex reader that was used for the measurement of the OD of the solubilised stained microtitre plate biofilm cultures (Fig. 2.4 b) was 3.5. Some of the cultures had optical densities of over 3.5 but for the purpose of the experiment any value above the maximum limit of the calibration of the reader was recorded as OD of 3.5. The interpretation of results

was done according to Christensen *et al,* 1985. The mean of all the blanks (global mean) and the standard deviation (SD) were calculated. The SD was multiplied by 3 and the result was added to the global mean of blank OD, any value below it was scored as non adherent (Non-adherent value (NAV). The NAV was multiplied by 2; the value between NAV and this value (2 NAV) was a weak biofilm formation value (WB). Any value above the WB was scored as strong biofilm formation value (SB). The biofilm index NAV was a negative result, WB was weak biofilm formation and SB was strong biofilm formation.

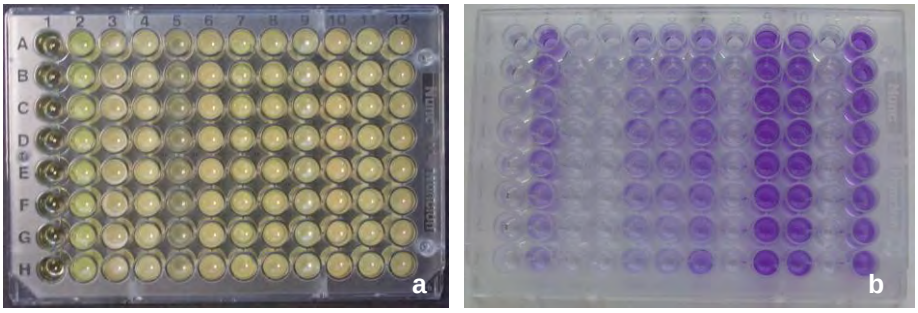

Figure 2.4: 24 hour biofilm cultures of *P. aeruginosa* wound isolates in microtitre plates. (**a**) Unstained and (**b**) Crystal violet stained biofilm cultures, in column 1 was TSB as blank; columns 2 to 11 contained the clinical isolates and column 12 the reference organism *P. aeruginosa* NCIB 8626 as a positive control.

2.3.3.2 Microtitre plate biofilm formation reproducibility assays

Biofilm assays of selected *P. aeruginosa* clinical isolates were performed at 3 different times and each biofilm index score based on the optical density as explained in section 2.3.3.1 was determined. The isolates were classified into 3 categories as either strong, weak or non-biofilm producing isolates according to the mean of the 3 assays. Reagent blank and reference *P. aeruginosa* NCIB 8626 were included as controls. The reproducibility assays for biofilm formation as detailed in Table 2.4 showed that the majority of the assay results (90.86%) were consistent and the assay is reproducible ($p < 0.05$).

Table 2.4: The summary of microtitre plate biofilm reproducibility assays.

Wound isolates/ controls	Assay 1 Biofilm Index	Assay 2 Biofilm Index	Assay 3 Biofilm Index	Mean Biofilm Index	Category Biofilm classification
Blank	NAV	NAV	NAV	NAV	Blank control
1	SB	SB	SB	SB	Strong biofilm former
2	SB	SB	SB	SB	Strong biofilm former
4	NAV	WB	WB	WB	Weak biofilm former
5	SB	SB	SB	SB	Strong biofilm former
6	WB	WB	WB	WB	Weak biofilm former
7	SB	SB	SB	SB	Strong biofilm former
8	SB	SB	SB	SB	Strong biofilm former
9	WB	NAV	WB	WB	Weak biofilm former
10	SB	SB	SB	SB	Strong biofilm former
11	SB	WB	WB	WB	Weak biofilm former
12	SB	SB	WB	SB	Strong biofilm former
13	SB	SB	SB	SB	Strong biofilm former
14	NAV	NAV	NAV	NAV	Non - biofilm former
15	NAV	NAV	NAV	NAV	Non - biofilm former
16	NAV	NAV	NAV	NAV	Non - biofilm former
17	SB	SB	SB	SB	Strong biofilm former
18	NAV	WB	NAV	NAV	Non - biofilm former
19	SB	SB	WB	SB	Strong biofilm former
20	SB	WB	WB	WB	Weak biofilm former
21	WB	WB	WB	WB	Weak biofilm former
22	SB	SB	WB	SB	Strong biofilm former
NCIB8626	SB	SB	SB	SB	Strong biofilm former

2.3.3.3 Biofilm formation results for all P. aeruginosa wound isolates

In total, 88 (90.7%) out of the 97 *P. aeruginosa* isolates that were tested formed biofilm in microtitre plates; of which 68 (72.3 %) were strong biofilm formers and 20 (22.7 %) formed biofilm weakly while 9 (8.3%) did not form biofilm (Table 2.5).

Table 2.5: Data summary: Biofilm formation by *P. aeruginosa* wound isolates

Category	Number	Percentage
Strong biofilm former	68	70.1%
Weak biofilm former	20	20.6%
All biofilm formers	88	90.7%
Non - biofilm former	9	8.3%

2.3.4 Production of AHL by *P. aeruginosa* wound isolates

2.3.4.1 AHL production by cross feeding assay

Some bacteria such as *A. tumefaciens* and *Escherichia coli* have a competent system of metabolising lactose by conserving energy source and only switch on the gene for production of lac operon protein when lactose is present in the medium. Three proteins are involved in the metabolism of lactose and they are encoded in sequence on a DNA called the lac operon. *Lac* operon, an inducible system is repressed in the absence of lactose because it is bound by the repressor protein but when lactose is present in the medium, the repressor protein unbinds from the *Lac* operator (Hensel and Xiao, 2009). This process allows the RNA polymerase to transcribe the 3 genes of the *lac* operon; *lac* Y, *lac* Z and *lac* A that work in cooperation (Oehler *et al*, 1990). This system was observed to be expressed at higher levels in *A. tumefaciens* than *E coli* (Chen and Winans, 1991).

The quorum sensing molecule, produced by *A. tumefaciens* KYC6 or *P. aeruginosa* cultures diffused through the agar and resulted in the activation of the transcription of traI-*lac*Z (*lac* operon) in *A. tumefaciens* (NTL4). The process leads to the production of β-galactosidase enzyme which metabolises 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) releasing galactose (colourless) and an insoluble blue 4-chloro-3-brom-indigo. The 4-chloro-3-brom-indigo forms precipitate that changes the colonies of the reporter organism *A. tumefaciens* NTL4 from cream to blue (Figure 2.5a and b). The negative control (NTL4 against itself) (Fig. 2.5 c) or lack of AHL production by the non - AHL producing *P. aeruginosa* clinical isolates (Fig. 2.5 d) did not result in any change of colour. Out of the 97 clinical isolates that were tested, 72 (74.2%) produced AHL while 25 (25.8%) gave negative results for AHL production in this assay (Table 2.6).

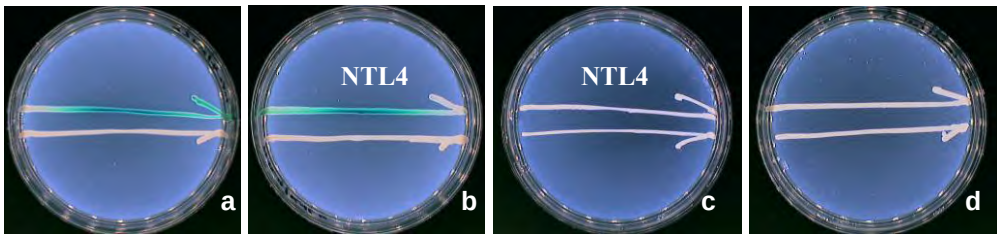

Figure 2.5 Detection of acylhomoserine lactone (AHL) by cross feeding assay. (**a**) AHL producing organism *Agrobacterium tumefaciens* KYC6 and the reporter strain *A. tumefaciens* NTL4 showing bluish colonies as positive control; (**b**) an AHL producing *P. aeruginosa* isolate from wound; (**c**) *A. tumefaciens* KYC6 against itself as negative control showing cream colonies and (**d**) a non- AHL producing *P. aeruginosa* isolate from wound.

Table 2.6: Data summary: AHL detection in *P. aeruginosa* wound isolates (cross-feeding assay)

Category	Number	Percentage
AHL producers	72	74.2 %
Non-AHL producers	25	25.8 %
Total no of isolates	97	100 %

2.3.4.2 AHL detection by quorum sensing inhibition (QSI) assay

Production of violacein pigment by *Chromobacterium violaceum* is regulated by N-hexanoyl-homoserine lactone (C6-HSL) quorum sensing molecule, which can be inhibited by antagonists such as antibiotics, furanones or AHLs produced by other bacteria. Gram negative quorum sensing molecules (AHL) produced by *P. aeruginosa* interferes with the production of violacein by *C. violaceum* ATCC 12475 around the vicinity of the AHL producer thereby resulting in whitish colonies of *C. violaceum* ATCC 12475 instead of purple colonies; there was complete growth inhibition around the colonies, an indication of complete inhibition of growth (Fig. 2.6 a and b). There was no change in the colour of the reporter organism around the colonies of non producer of AHL (Fig. 2.6 c and d). A total of 78 of the 97 (80.4%) *P. aeruginosa* wound isolates tested positive while 19.6% showed negative results (Table 2. 7).

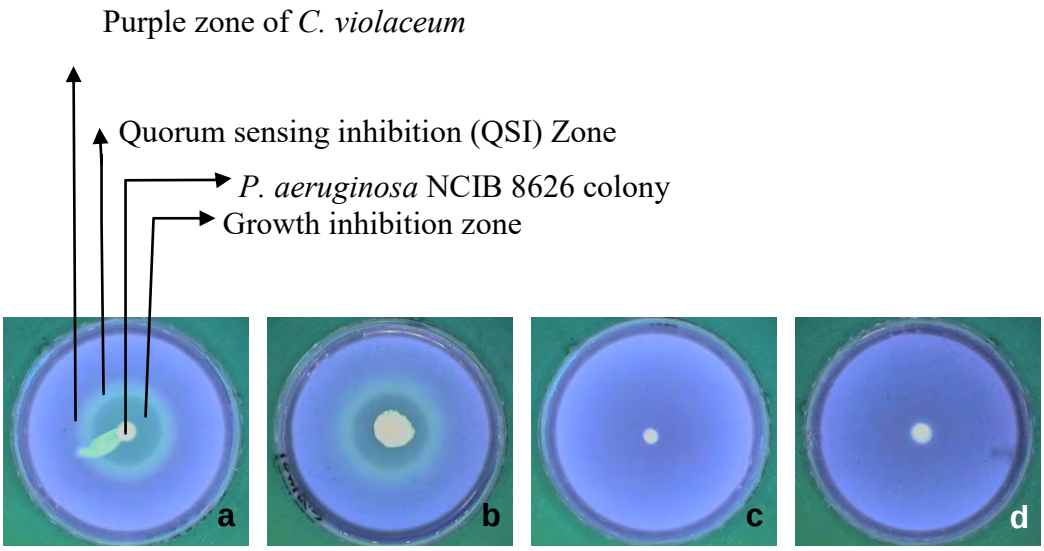

Figure 2.6: AHL detection by quorum sensing inhibition assay.

(**a**) AHL producing organism *P. aeruginosa* NCIB 8626 colony at the centre of the plate showing clear zone of inhibition and whitish quorum sensing inhibition zone next to the purple coloured reporter organism *C. tumefaciens* ATCC 12475 as positive control; arrows indicating quorum sensing inhibition (QSI) zone, growth inhibition zone and *P. aeruginosa* NCIB 8626 colony; (**b**) a Gram negative AHL producing *P. aeruginosa* isolate from wound; (**c**) a non-AHL producing reference organism *P. aeruginosa* PAO1 as negative control showing no inhibition zone and (**d**) a non - AHL producing *P. aeruginosa* isolated from a wound. High level production of AHL also resulted in complete inhibition of growth of the reporter organism at the zone closest to the colonies of test organisms as shown above (Fig. 2.6 a and b).

Table 2.7 Data summary: AHL detection in *P. aeruginosa* wound isolates (QSI assay)

Category	**Number**	**Percentage**
QSI positive	78	80.4 %
QSI Negative	19	19.6 %
Total no of isolates	97	100 %

2.3.5 Summary of biofilm markers

In summary, of the 97 *P. aeruginosa* wound isolates 88 (90.7 %) formed biofilm *in vitro,* 72 (74.2 %) tested positive for AHL production by cross feeding assay while 78 (80.4 %) were positive for AHL production by quorum sensing inhibition method as shown in Figure 2.7.

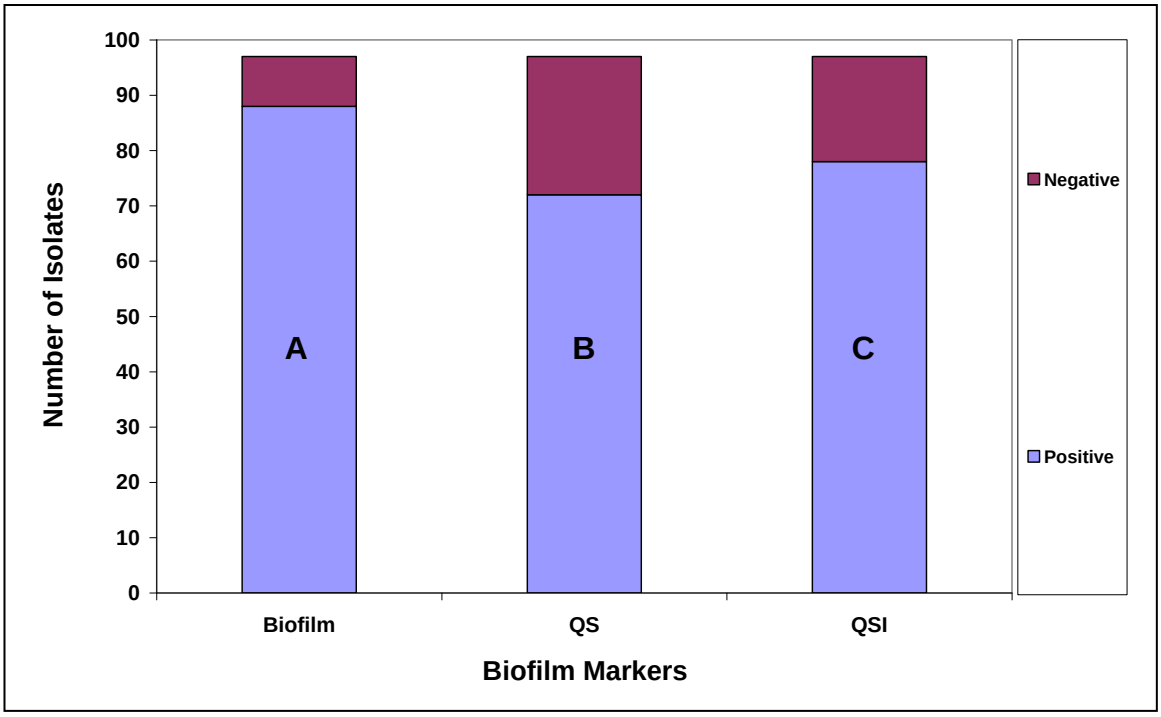

Figure 2.7: Histogram of biofilm markers of the *P. aeruginosa* clinical isolates from wounds. Columns A and B showing AHL detection in cultures by cross feeding assay (QS) and by inhibition assay (QSI) respectively, and column C biofilm formation in microtitre plate. The positive groups are shown as purple sections while the maroon columns are the negatives.

2.3.6 Biofilm phenotypic expressions of *P. aeruginosa* isolated from tap water

Of the biofilm forming *P. aeruginosa* recovered from water 7 (77.8%) produced biofilm strongly while 2 (22.2%) were weak biofilm formers. Five (55.6%) of the samples produced quorum sensing molecules with either of both (cross feeding or inhibitory method); 3 were positive with cross feeding assay and 2 for the inhibitory method but none was positive for both assays together (Table 2.8).

Table 2.8 Results of Biofilm, QS and QSI for *P. aeruginosa* from water pipes.

Organisms	**Biofilm OD**	**Biofilm Index**	**QS - AHL (direct assay)**	**QSI - AHL (indirect assay)**
P. aeruginosa NCIB 8626	2.65	2	**Positive**	**Positive**
101	0.575	2	Negative	Negative
105	0.84	2	Negative	Negative
140	0.525	2	**Positive**	Negative
149	0.685	2	**Positive**	Negative
184	3.08	2	Negative	Negative
190	0.51	2	**Positive**	Negative
200	2.975	2	Negative	**Positive**
212	0.55	2	Negative	Negative
265	1.285	2	Negative	**Positive**

2.3.7 PCR characterisation of *P. aeruginosa* wound isolates

The randomly amplified polymorphic DNA (RAPD)-PCR analysis characterisation of the 97 isolates with OPA-09 (5‟GGGTAACGCC3‟) primer yielded numerous products which were separated with gel electrophoresis. Each band pattern represents a genetic fingerprint which can be employed in the characterisation of specific strains of organisms (Fig. 2 .8). The bands for the isolates ranged mostly from 6 to 10 with identical nucleic acid sequence of the same molecular weight having similar bands.

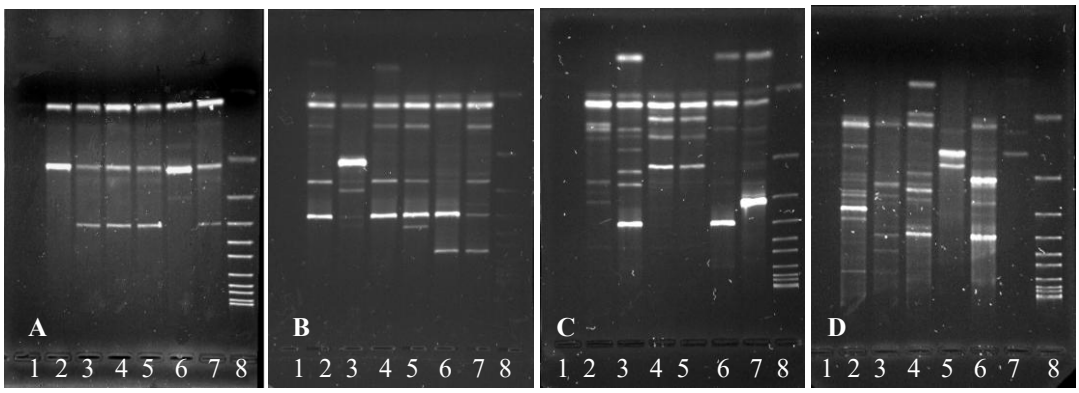

Figure 2.8: Gel electrophoresis of PCR products of *P. aeruginosa* type culture and wound isolates. (Gel **A**) PCR optimization of reference *P. aeruginosa* NCTC 27853: lanes 1(blank), 2 -7(1µl, 2 µl, 3µl, 5 µl, 7 µl and 10 µl of *P. aeruginosa* NCTC 27853 DNA solution respectively and lane 8 (marker). Gels **B, C** and **D** are for some *P. aeruginosa* wound isolates; lane 1 (blank); lanes 2 -7 wound isolates and lane 8 (marker).

Analysis of the bands of the marker for assays performed at different times shows the consistency and reproducibility of the assay (Fig. 2.9). The fingerprint data analysis of the bands for the isolates (Fig. 2.10) using similarity coefficient based on Jaccard; linking all the isolates showed that 82 (84.53%) of all the isolates have 100% similarity linkage to the cohort, 3 (3.09%) have 75 - 99% while 12 (12.37%) are of 50 - 75% linkage (Fig. 2 .11). The similarity coefficient is based on the collective analysis of the genetic diversity of the cohort and not on individual genetic or phenotypic characteristic. The phylogenetic tree also indicated the subgroups within the cohort.

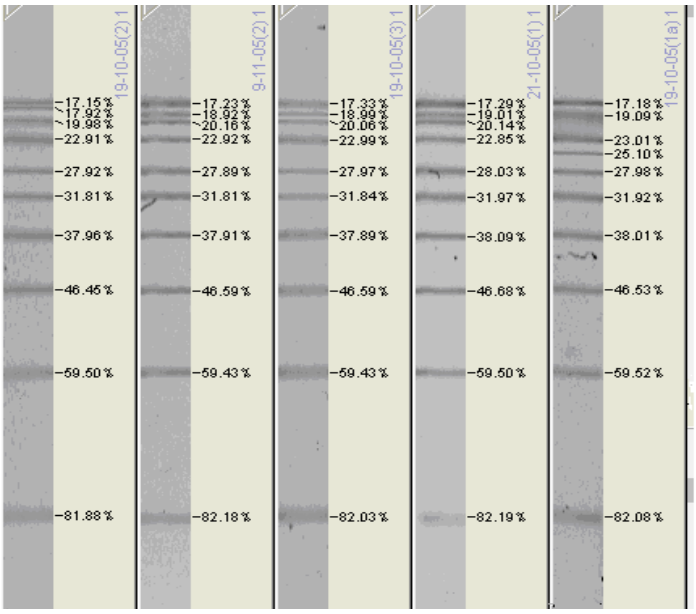

Figure 2.9: Analysis of bands of marker obtained from PCR at different times GelCompar II (version 4) software was used for band analysis and results obtained from the analysis confirmed the reproducibility of the assays.

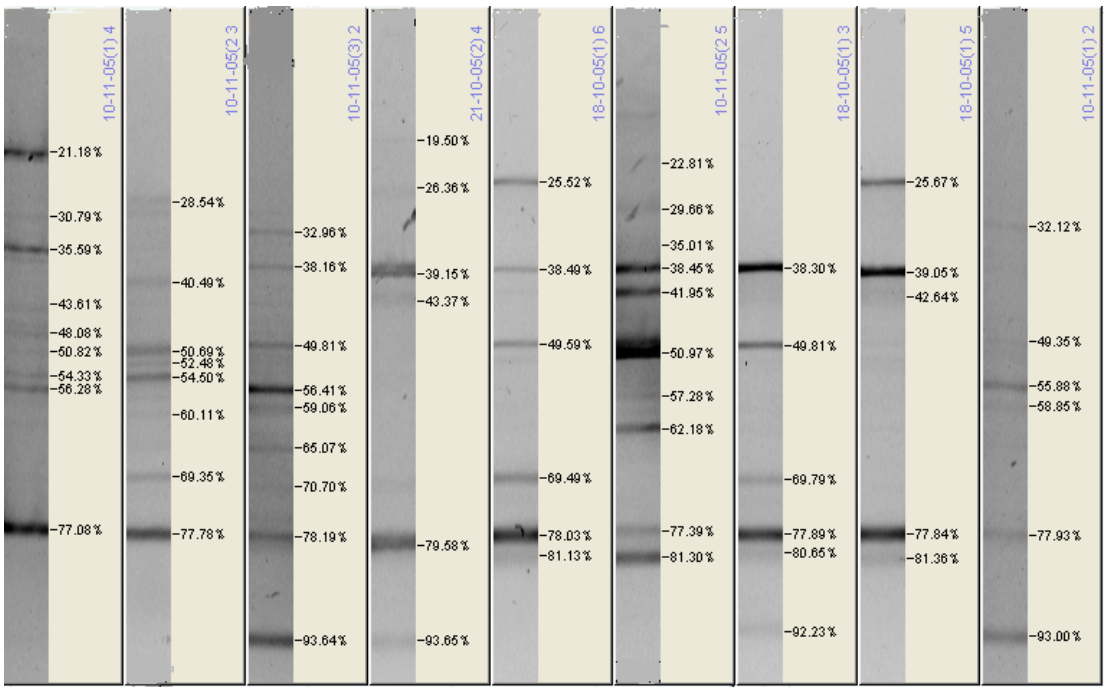

Figure 2.10: Fingerprint analysis of PCR products of *P. aeruginosa* wound isolates using *GelCompar* II version 4

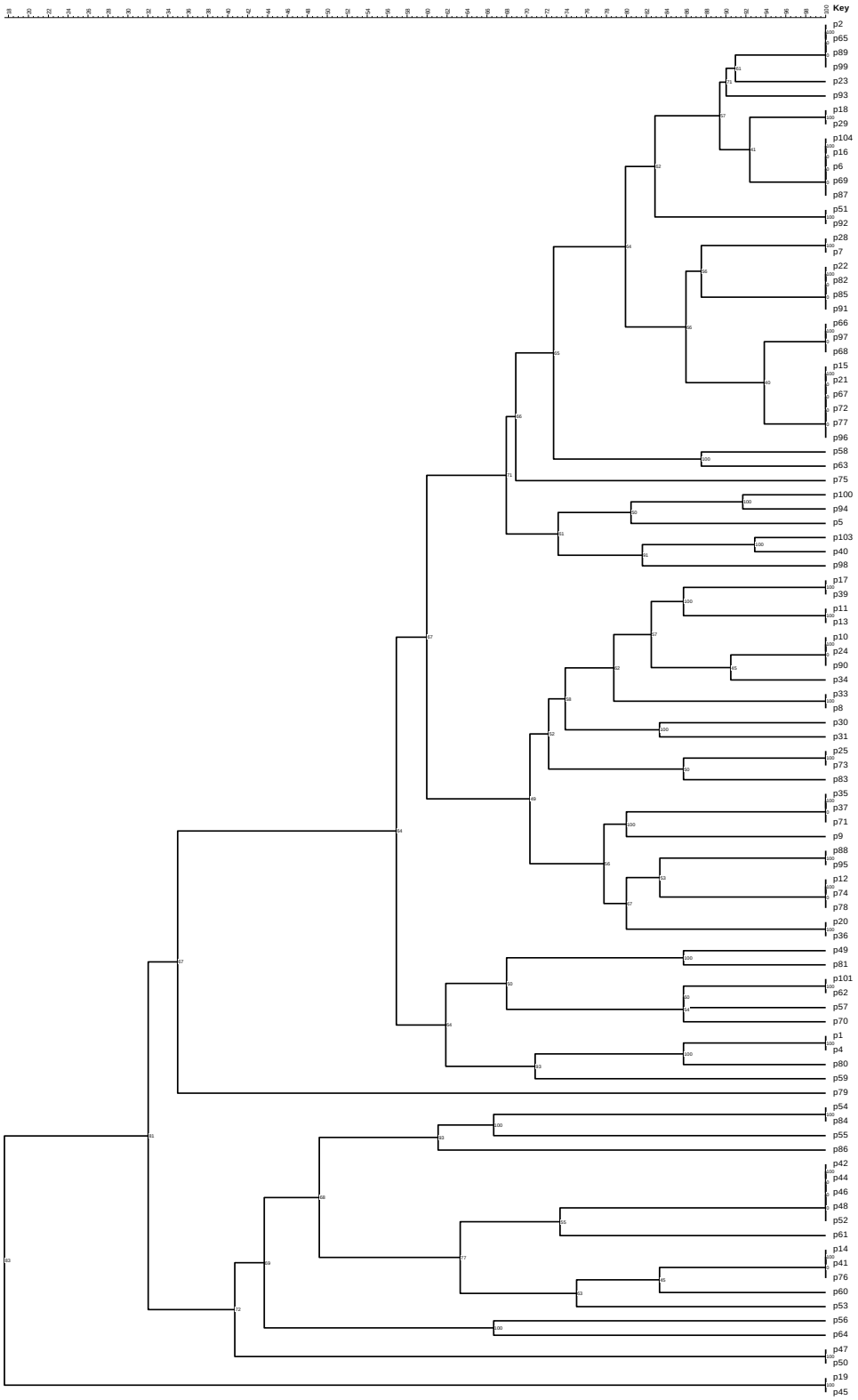

Figure 2.11: Dendrogram of the complete linkage between the 97 *P. aeruginosa* wound isolates based on Jaccard similarity coefficient (Jaccard index).

2.3.8 Statistical Analysis

The statistical analysis of data was performed with Minitab (version 15); Jaccard index showed that the genetic diversity of the isolates was significantly similar ($p < 0.005$) and the probability plot indicates that the majority have 100% similarity (Figure 2.12). There was correlation between QS and QSI (AHL production assays), $p < 0.005$. There was no correlation between similarity index, biofilm formation and AHL production but there was an association between the number of isolates that were positive for AHL (QS, QSI) and biofilm formation using Pearson's chi-square test ($p<0.006$). The biofilm forming potentials of the isolates were also significant ($p<0.005$), the distribution pattern showing that the majority of the wounds‟ isolates formed biofilm (Figure 2.13). Statistically there was no correlation between the biofilm forming potentials of the isolates and the various antibiotics that were investigated in this study; however there were significant correlations between AHL production and a few of the antibiotic susceptibility patterns as shown in Table 2.9.

Table 2.9 Correlation between AHL production by isolates and antibiotic susceptibility.

Parameters for Pearson Correlation	P values
Correlations: AHL production and amoxicillin	P-Value = 0.0001
Correlations: AHL production and gentamicin	P-Value = 0.002
Correlations: AHL production and piperacillin	P-Value = 0.017
Correlations: AHL production and imipenem	P-Value = 0.097

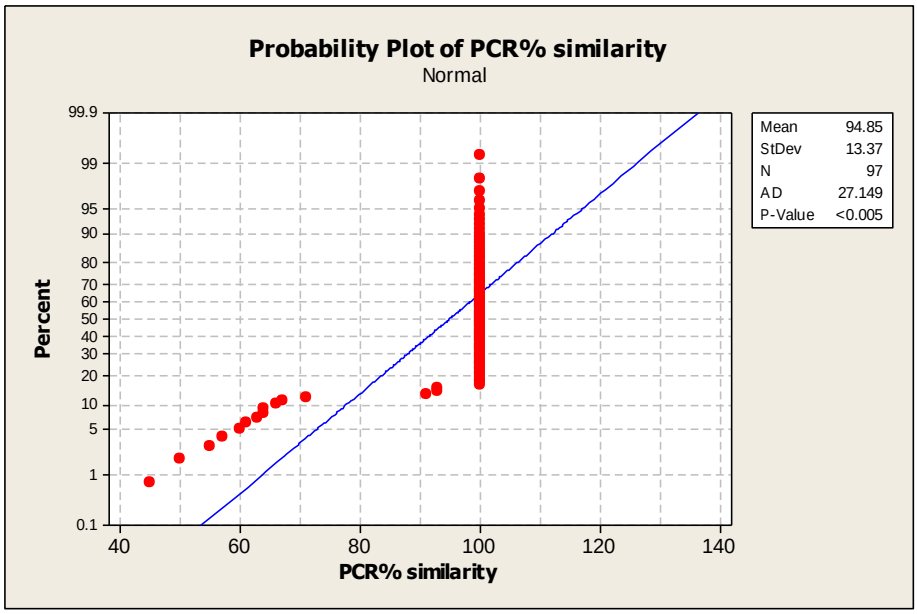

Figure 2.12: Probability graph of similarity index of *P. aeruginosa* cohort isolated from wounds. The majority of the wound isolates have 100% genetic similarity linkage, $p < 0.005$.

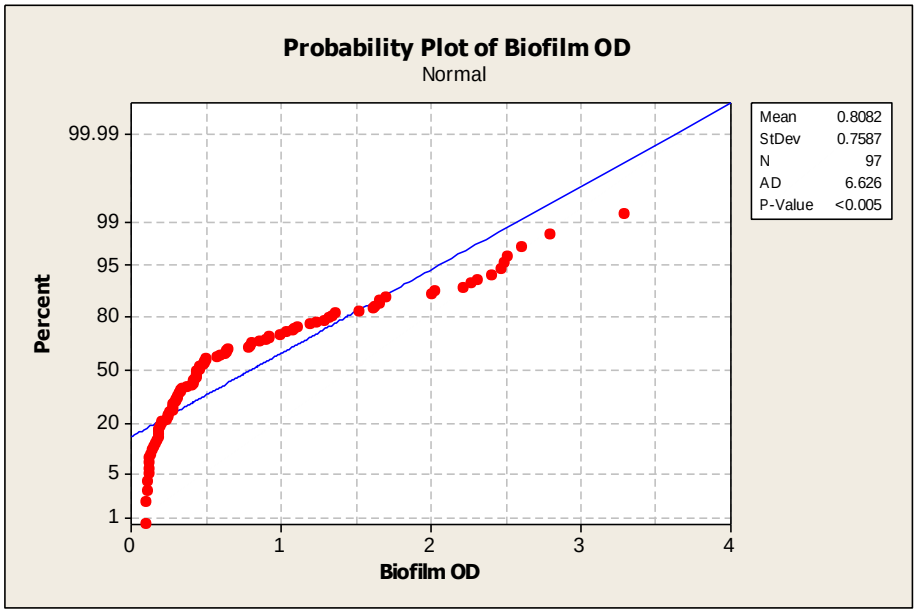

Figure 2.13: A chart representation of the distribution pattern of optical densities of biofilm formed by *P. aeruginosa* isolates from infected and chronic wounds. The significance of the biofilm formation capability of the isolates is shown, $p< 0.005$.

2.4 Discussion

The ability of microorganisms to cause infections is dependent on possession of virulent factors. Pathogenic organisms overcome the host defence mechanisms by means of virulence factors to initiate infection and for sustenance within the host‟s tissues. These often result in extensive damages to the host and in some cases the infections may become persistent particularly those involving biofilms (Soto *et al*, 2007; Choy *et al*, 2008). Signalling molecules are involved in the regulation of various physiological processes of microorganisms (Schertzer *et al,* 2009; Williams *et al,* 2007; Parsek and Greenberg, 2005) such as production of more or higher degrees of virulence factors including biofilm formation (Choy *et al*, 2008; Soto *et al*, 2007) which consequently increases the degree of pathogenicity (Bjarnsholt and Givskov, 2007). Gram negative bacteria such as *P. aeruginosa* utilise acyl-homoserine lactones (AHL) for quorum sensing (Kirisits *et al,* 2006; Favre-Bonté *et al,* 2003; Stickler *et al,*1998) which have been found to influence the production of several virulence factors such as lipase (Sauer *et al,* 2002; Pessi *et al,* 2001; Passador *et al,*1993;) exotoxin A, quinolones, hyaluronidase, ß- lactamase and biofilm formation and interferes with the immune system of the host such as reduction of cytokine and prevention of antibody production (Telford *et al,* 1998). AHL deficient strains of *P. aeruginosa* were not able to cause infection whereas the AHL producing strains caused severe keratitis of the cornea (Zhu *et al,* 2005) and *P. aeruginosa* has been implicated in various biofilm related and persistent infections (Costerton, 2001; Bowler *et al,* 1999). *P. aeruginosa* biofilm and quorum sensing system have been widely studied and there are 3 quorum-sensing systems, 2 of which are linked to *R* and *I* genes (Latifi *et al,* 1995; Fuqua *et al,* 2001) and the third are the quinolones.

The results of the antibiotic susceptibility assays showed large numbers of the isolates were susceptible to ciprofloxacin (80.4%), gentamicin (92.78%), imipenem (91.75%) and piperacillin (96.9%) (Fig. 2.3 and Table 2.3). This results were similar to the report on the United States antimicrobial susceptibility surveillance of clinical isolates in 65 laboratories (1998 to 2001), over 90% of *P. aeruginosa* clinical isolates were susceptible to piperacillin, 80 to 90% to imipenem while 70 to 80% of isolates were susceptible to ciprofloxacin and gentamicin (Karlowsky *et al,* 2003) with the higher susceptibility observed in gentamicin. In another survey of susceptibility rates *P. aeruginosa* to some antibiotics were found to be susceptible to piperacillin-tazobactam (86%), ceftazidime (80%), ciprofloxacin (68%), and levofloxacin (67%), but gentamicin 72.3% and imipenem 78.8% between 2001 and 2003 (Karlowsky *et al*, 2005). In the United Kingdom, a survey of resistance of *P. aeruginosa* to six commonly prescribed antimicrobial agents to patients with cystic fibrosis in 17 hospitals reported a high incidence of resistance (Pitt *et al,* 2003). Amongst the 417 *P. aeruginosa* isolates the resistant rates were gentamicin about 50%, ceftazidime (39%), piperacillin (32%), ciprofloxacin (30%), tobramycin (10%), and colistin (3%) while the multi resistant rate was almost 40%.

The results obtained from this study gave lower resistance rates for ciprofloxacin, gentamicin, and piperacillin than those obtained from some studies (Karlowsky *et al*, 2005; Pitt *et al,* 2005). This could have been due to the site of isolation of the organisms, since *P. aeruginosa* has been found to express virulence due to site of isolation (Favre-Bonte *et al*, 2007; Hamood *et al,* 1996) and this may be linked to the antibiotic sensitivity. This could also be due to prolonged exposure of the strain to antibiotics especially those isolated from cystic fibrosis patients who often suffer chronic infections. The isolates were resistant to amoxicillin (94.9%), cefuroxime (99%), and trimethoprim (99 %). This was similar to the findings of Gad and colleagues

(2008) on the study of the antimicrobial resistance of *P. aeruginosa* clinical and environmental isolates in Egypt; and *P. aeruginosa* isolates from the skin infection showed 100% resistant to ampicillin and 89% to cefuroxime amongst others. According to past studies from different parts of the world, the resistance patterns of the organisms vary from place to place which might probably be due to the exposure of the strains to various antibiotics in use at such places (pattern of drug administration) (Rogues *et al*, 2007) . Nonetheless, the increasing rate of antimicrobial resistance is a common factor to all which might be the cumulative result of the effect of innate antimicrobial resistant mechanisms, selection of mutants and acquisition of resistant genes.

The results of the antimicrobial sensitivity pattern of the isolates as observed in this study are similar to those obtained from other studies; ciprofloxacin (Karlowsky *et al,* 2003 and 2005); gentamicin (Karlowsky *et al,* 2003 and 2005; Dohar *et al,* 1995); imipenem (Karlowsky *et al,* 2003); piperacillin (Karlowsky *et al,* 2003; Bouza *et al*,1999). Although the resistance pattern was contrary to the ones obtained by some studies particularly resistance to gentamicin (Gad *et al,* 2008; Strateva *et al,* 2007) ciprofloxacin (Strateva *et al,* 2007; Pitt *et al,* 2005) and imipenem (Gad *et al,* 2008; Strateva *et al,* 2007), this would be the cumulative result of the dose administration, exposure of organism to the particular antibiotic, the sight of isolation as well as the bacterial phenotype. The biofilm forming and AHL producing phenotypes would be more resistant to antimicrobial interventions because of the role of biofilm such as extracellular matrices barrier (Chandra *et al*, 2001; Costerton *et al,* 1999) and acquisition of resistance gene (Anderson and O'Toole, 2008; Fux *et al,* 2005; Gilbert *et al,* 1997) in antimicrobial resistance as well as that of AHL in the upregulation of genes including antimicrobial resistance genes (Goldsworthy 2008; Sauer *et al*, 2002; de Kievit and Iglewski 2000). However, some studies revealed that biofilm matrix

polymers do not play any significant role in antibiotic resistance (Baillie and Douglas, 2000). All the isolates except one were resistant to cefuroxime which was a confirmation of its inactivity against most *Pseudomonas* strains (Greenwood *et al,* 1976) although its efficacy is usually enhanced in combination with other agents (Zhang and Li, 2001). While most of the isolates were susceptible to four of the antibiotics tested, on the contrary almost all the isolates were resistant to the remaining three, the *P. aeruginosa* wound isolates had similar antibiotic susceptibility pattern which was an indication of close species identification. The antibiotic susceptibility patterns confirmed that the isolates are *P. aeruginosa* and also suggested that the resistance to some antibiotics such as ciprofloxacin, gentamicin and imipenem are on the increase.

Out of the 97 isolates, 88 (90.7 %) formed biofilm which supports other findings (Tunney *et al,* 2007; Harrison-Balestra *et al,* 1998) while 9 (9.3 %) were not able to form biofilm (Figure 2.4 and Table 2. 5). Out of the 88 isolates that tested positive for biofilm; 68 (70.1%) formed strong biofilm and 20 (20.6 %) formed biofilm weakly (Table 2. 5). It has been shown that *P. aeruginosa* formed biofilm in animal model (Serralta *et al*, 2001); 35% of organisms isolated from chronic wound biofilms were *P. aeruginosa* (James *et al*, 2008), and the implication is that the organisms might form biofilm in wound.

The AHL production was determined by direct estimation of AHL through cross feeding assay of *A. tumefaciens* NTL4 and indirectly by inhibition of quorum sensing production by *C. violaceum.* A positive result for either of the 2 assays is indicative of positive AHL production by the isolates, 72 (74.2 %) were positive by the direct assay (Figure 2.5; Table 2. 6) while 78 (80.4 %) were positive for the inhibitory assay (Figure 2.6; Tables 2. 7). It was observed from this study that all the isolates that tested positive for AHL cross feeding assay also showed positive result for AHL by inhibition

assay. In total 78 (80.4 %) were positive for AHL production according to the combined results of the two assays (Table 2. 10), of which 75 (77.3 %) formed biofilms *in vitro* and 6 (6.2 %) did not produce biofilm (Table 2. 10). Although there were 19 (19.6%) AHL negative isolates, 16 (84.2 %) of these AHL negative isolates formed biofilm in microtitre plates but 3 (15.8 %) were biofilm negative (Table 2. 10).

Table 2.10: Relationship between biofilm formation and AHL production amongst *P. aeruginosa* clinical isolates

Biofilm markers	**BIOFILM Positive**	**BIOFILM Negative**	**Total**
AHL Positive	72	6	**78**
AHL negative	16	3	**19**
Total	**88**	**9**	**97**

It was evident that majority of the isolates 78 (80.4%) produced AHL, a signalling molecule that is involved in biofilm formation in Gram negative bacteria (Favre-Bonté *et al*, 2003; de Kievit *et al*, 2001; Davies *et al*, 1998) which has been found to significantly influence the virulence of *P. aeruginosa* in animals (Wu *et al*, 2001; Rumbaugh 1999) and persistence of biofilm infections (Christensen *et al*, 2007). The formation of biofilm by the AHL producing *P. aeruginosa* clinical isolates from wounds as observed in this study was in agreement with other scientific evidence (Rice *et al,* 2005; Labbate *et al,* 2004; Sabine Favre-Bonté *et al,* 2003).

Interestingly few of the isolates produced biofilm but tested negative for AHL production. The isolates that formed biofilm but were deficient in AHL production could have produced the pseudomonas quinolone signalling (PQS) molecules (2-heptyl-3-hydroxy-4-quinolone), a non - AHL signalling (Deziel *et al*, 2004). The test for detection of PQS was not investigated in this study. Scientific findings have shown that

AHL might not be necessary for biofilm formation (Schaber *et al*, 2004 & 2007). One of the AHL deficient strains that were investigated by Schaber and his colleagues in 2004 produced biofilm of up to 82% that of PAO1 wild type control organism. AHL plays vital role in the control of virulence factors and consequently the pathogenesis of organisms; however studies have shown that AHL deficient *P. aeruginosa* are able to cause infections especially where there are underlying health conditions (Schaber *et al*, 2007 and 2004; Boşgelmez-Tınaz *et al*, 2005). Boşgelmez-Tınaz and colleagues (2005) isolated some AHL deficient *P. aeruginosa* from intensive care unit patients and the ability of *P. aeruginosa* to cause acute infection in burn mice was demonstrated by Schaber and his colleagues in 2007. In another study, Boşgelmez-Tınaz and Ulusoy (2008) characterised 50 *P. aeruginosa* clinical isolates and found that 4 out of the isolates were QS deficient which confirmed that QS deficient *P. aeruginosa* are capable of causing infections.

Some of the wound isolates produced AHL but did not form biofilm in the laboratory. However, studies have shown that other factors such as flagella and twitching motility (O'Toole and Kolter, 1998), catabolite repression control (Crc) protein (O'Toole *et al*, 2000); GacA (Parkins *et al*, 2001) and pili (Klausen *et al*, 2003) amongst others are also relevant for biofilm formation. Six of the isolates (6.18 %) produced AHL but failed to form biofilm *in vitro* which might be due to defect in other genetic materials or structures that aid biofilm formation. It is also possible that the organisms do not produce biofilm since AHL modulates series of genetic activities.

In order to survive in a host an organism has to overcome environmental stresses (Davey and O‟Toole, 2000) which are due to various factors such as opsonisation, phagocytosis, antibody effect and other host defence system activities (Kumar *et al,* 2009; Medzhitov 2007; Bascones-Martínez *et al,* 2006). In natural environments the degree of stress encountered by the organisms would be significantly less than in the

human host environment. Therefore the likelihood for the organisms that infect human or animal to develop some degree of resistance mechanism to the environmental stress for sustenance might be higher. Pathogenicity, the ability of organisms to cause infection is therefore dependent on the ability to overpower the host defence system which is dependent on the expression of virulence of the organism. Virulence is determined by the number of virulence factors possessed by an organism and the extent of production of such virulence factors. Biofilm formation, one of the virulence factors employed by pathogenic organisms in causing persistent infections is associated with production of quorum sensing molecules (Waters *et al,* 2008; Parsek and Greenberg, 2005; De Kievit *et al,* 2001; Costerton *et al,* 1999). In Gram negative bacteria including *P. aeruginosa* the most common signalling molecules are the AHLs (Fuqua, 2006; Lequette *et al*, 2006; Fuqua *et al*, 1994). The role of quorum sensing including the Gram negative quorum sensing signalling molecules (AHLs) in pathogenesis has been extensively investigated and studies have shown that they play dual role in the pathogenicity of the bacteria by modulating the production of virulence factors (Hooi *et al,* 2004; Erickson *et al,* 2002; de Kievit *et al,* 2000) as well as inducing immune responses in the host (Hooi *et al,* 2004; Smith *et al,* 2002 (a and b); Smith *et al,* 2001). AHL involvement in the activation of regulatory systems for production of virulence factors in *P. aeruginosa* has been observed previously (Hooi *et al,* 2004; Erickson *et al,* 2002; de Kievit *et al,* 2000; Rumbaugh *et al,* 2000; Whiteley *et al,* 1999). AHL induction of inflammatory processes in the hosts (Smith *et al,* 2002 and 2001) has been suggested as one of the major factors causing the damage in the lungs of the cystic fibrosis patients.

Studies have also shown that production of quorum sensing molecules by biofilm organisms may be site dependent (Favre-Bonté *et al,* 2007; Schuhmacher and Klose, 1999). In a recent study Prasad and his group (2009) investigated the ability of

P. aeruginosa strains from different infection sites to produce virulence markers such as slime and observed that the isolates from wounds produced higher quantities of slime than the lung isolates suggesting that the wound isolates might be more virulent.

In order to confirm that *P. aeruginosa* strains exhibit virulence characteristics depending on the site of colonisation, biofilm forming strains that were isolated from water taps were investigated for biofilm characteristics in order to compare the virulence potentials of both cohorts of *P. aeruginosa* strains. A great majority (90.7%) of the 97 *P. aeruginosa* wound isolates that were tested formed biofilm in microtitre plates. Out of these 88 biofilm positive isolates 78 of them produced AHL (88.6%). However of 5 out of 9 (55.6 %) of the water isolated biofilm forming *P. aeruginosa* also produced AHL in the laboratory. The greater percentage of the biofilm forming wound isolates produced AHL unlike the biofilm forming tap water isolates according to the results of this study which supports previous findings that virulence factors are site dependent (Choy *et al*, 2008; Favre-Bonté *et al,* 2007; Schuhmacher and Klose, 1999). Schuhmacher and Klose (1999) observed that bile activates the production of regulatory protein ToxT, an activator of virulence factors in *Vibrio cholera*; which enhances the pathogenicity of the bacteria in the gut. Favre-Bonté and his colleagues in 2007 observed that AHL production by *P aeruginosa* strains in the lungs were site dependent. The results of their study showed that the type and degree of production of virulence factors in the intubation devices differ from those obtained from the host‟s trachea particularly the production of elastase, an enzyme that the bacteria utilise for proteolytic damage of the host‟s tissue. *Pseudomonas* autoinducer has been suggested as causing the production of a chemokine IL-8, which often result in the damage of the lungs of the cystic fibrosis patients (Smith *et al,* 2001). *P. aeruginosa* isolated from wounds have been found to produce higher degrees of virulence factors than those isolated from other sites (Prasad *et al,* 2009). According to the study of the functional characterization of

P. aeruginosa by Wiehlmann and his colleagues (2007), 15 proteins play highly diverse roles in the cell and became habitat-specific virulence factors on exposure to specific hosts and/or on exposure to specific stress conditions or host defence mechanisms. It was expected that clinical isolates from wounds would express certain virulence factors different from environmental strains and this has been demonstrated by this study.

The genetic similarity index showed that majority of all the isolates have 100% similarity linkage (Figure 2.11) which may suggest that most of the organisms might have some similar virulence characteristics. Fifteen (88.2%) of the isolates from chronic venous leg ulcers (CVLU) have 100% similarity linkage to the member(s) of the cohort while 83.8% of the strains from the infected wounds showed such similarity in genetic diversity. The results showed that the greater percentages of the isolates exhibited biofilm phenotypic characteristics including biofilm formation (Figures 2.4 and 2.13), AHL production (Figures 2.5 and 2.6), and antibiotic resistance (Figure 2.3); the degree of which were similar to those of the genetic similarities results (Figures 2.11 and 2.12; Tables 2.11 and 2.12). Of the 17 chronic wound isolates, 16 (94.12 %) formed biofilm *in vitro* (Table 2.10) while 72 (90%) out of 80 infected wound isolates tested positive for biofilm (Table 2.11). Six of isolates from chronic wounds (35.3%) did not produce AHL, five of which (83.3%) formed biofilm while 11 (84.62%) of the 13 AHL negative isolates from infected wounds formed biofilm.

Table 2.11: Relationship between antibiotic susceptibility and genetic index of isolates

ID No	CIP	CXM	AML	CN	IPM	W	PRL	Genetic linkage to member(s)	ID No	CIP	CXM	AML	CN	IPM	W	PRL	Genetic linkage to member(s)
P2	S	R	R	S	S	R	S	100%	P57	R	R	R	R	S	R	S	54%
P 4	R	R	R	S	S	R	S	100%	P58	S	R	R	S	S	R	S	100%
P 5	S	R	R	S	S	R	S	60%	P59	S	R	R	S	S	R	S	93%
P 6	S	S	R	S	S	S	S	100%	P60	S	R	R	S	S	R	S	45%
P7	S	R	R	S	S	R	S	100%	P61	S	R	R	S	S	R	S	55%
P8	R	R	R	S	S	R	R	100%	P62	S	R	R	S	S	R	S	100%
P9	S	R	R	S	S	R	S	100%	P63	S	R	R	S	S	R	S	100%
P10	S	R	R	S	S	R	S	100%	P64	R	R	R	R	R	R	R	100%
P11	S	R	R	S	S	R	S	100%	P65	S	R	R	R	S	R	S	100%
P12	S	R	R	R	S	R	S	100%	P66	S	R	R	S	S	R	S	100%
P13	S	R	R	S	S	R	S	100%	P67	S	R	R	S	S	R	S	100%
P14	S	R	R	S	S	R	R	100%	P68	S	R	R	S	S	R	S	100%
P15	S	R	R	S	S	R	S	100%	P69	R	R	R	S	S	R	S	100%
P16	S	R	R	S	S	R	S	100%	P70	S	R	R	S	S	R	S	54%
P17	R	R	R	R	S	R	S	100%	P71	S	R	R	R	S	R	S	100%
P18	S	R	R	S	S	R	S	100%	P72	S	R	R	S	S	R	S	100%
P19	S	R	R	R	S	R	S	100%	P73	S	R	R	S	S	R	S	100%
P20	S	R	R	S	S	R	S	100%	P74	S	R	R	S	S	R	S	100%
P21	S	R	R	S	S	R	S	100%	P75	S	R	S	S	S	R	S	66%
P22	S	R	R	S	S	R	S	100%	P76	S	R	R	S	S	R	S	100%
P23	S	R	R	S	S	R	S	61%	P77	S	R	R	S	S	R	S	100%
P24	S	R	R	S	S	R	S	100%	P78	S	R	R	S	S	R	S	100%
P25	S	R	R	S	S	R	S	100%	P79	S	R	R	S	S	R	S	67%
P28	S	R	R	S	S	R	S	100%	P80	R	R	R	R	R	R	R	100%
P29	R	R	R	S	R	R	S	100%	P81	S	R	R	R	S	R	S	100%
P30	S	R	R	S	S	R	S	100%	P82	S	R	R	S	S	R	S	100%
P31	S	R	R	S	S	R	S	100%	P83	S	R	R	S	S	R	S	50%
P33	R	R	R	R	S	R	S	100%	P84	S	R	R	S	S	R	S	100%
P34	S	R	R	S	S	R	S	45%	P85	S	R	R	S	S	R	S	100%
P35	S	R	R	S	S	R	S	100%	P86	S	R	R	S	S	R	S	93%
P36	S	R	R	S	S	R	S	100%	P87	S	R	S	S	R	R	S	100%
P37	R	R	R	S	S	R	S	100%	P88	S	R	R	S	S	R	S	100%
P39	S	R	R	S	S	R	S	100%	P89	R	R	S	S	S	R	S	100%
P40	S	R	R	S	S	R	S	100%	P90	S	R	R	S	R	R	R	100%
P41	R	R	R	R	S	R	S	100%	P91	R	R	R	R	R	R	S	100%
P42	S	R	R	S	S	R	S	100%	P92	S	R	R	S	S	R	S	100%
P44	S	R	R	S	S	R	S	100%	P93	S	R	R	S	S	R	S	71%
P45	S	R	R	S	S	R	S	100%	P94	S	R	R	S	S	R	S	100%
P46	S	R	R	S	S	R	S	100%	P95	S	R	R	S	R	R	S	100%
P47	R	R	R	S	S	R	S	100%	P96	S	R	R	S	S	R	S	100%
P48	S	R	R	S	S	R	S	100%	P97	S	R	R	S	S	R	S	100%
P49	S	R	R	R	S	R	S	100%	P98	R	R	R	S	S	R	S	91%
P50	S	R	R	S	S	R	S	100%	P99	S	R	R	R	S	R	S	100%
P51	S	R	R	S	S	R	S	100%	P100	R	R	R	R	S	R	S	100%
P52	R	R	R	R	S	R	S	100%	P101	S	R	R	S	S	R	S	100%
P53	S	R	R	R	S	R	S	63%	P102	R	R	R	S	R	R	S	100%
P54	S	R	R	S	S	R	S	100%	P103	R	R	R	S	S	R	S	100%
P55	S	R	R	R	S	R	S	100%	P104	R	R	R	S	S	R	S	100%
P56	S	R	R	S	S	R	S	100%									

Key: CIP = ciprofloxacin 1 µg; CXM = cefuroxime 30 µg; AML = amoxicillin10 µg; CN = gentamicin10 µg; IPM = imipenem 10 µg; W = trimethoprim 2.5 µg; PRL = piperacillin 75 µg; S = susceptible and R = resistant.

Table 2.12: Biofilm characteristics and genetic similarity index of *P. aeruginosa* isolated from wounds

ID No	Biofilm	QS	QSI	Genetic linkage to cohort	ID No	Biofilm	QS	QSI	Genetic linkage to cohort
P2	+	+	+	100%	P57	+	+	+	54%
P 4	+	+	+	100%	P58	-	-	-	100%
P 5	+	+	+	60%	P59	+	+	+	93%
P 6	+	+	+	100%	P60	+	+	+	45%
P7	+	-	-	100%	P61	+	+	+	55%
P8	+	+	+	100%	P62	+	+	+	100%
P9	+	+	+	100%	P63	+	+	+	100%
P10	+	+	+	100%	P64	+	-	-	100%
P11	+	+	+	100%	P65	+	-	+	100%
P12	+	+	+	100%	P66	+	+	+	100%
P13	+	+	+	100%	P67	+	+	+	100%
P14	-	+	+	100%	P68	+	+	+	100%
P15	-	+	+	100%	P69	+	+	+	100%
P16	-	+	+	100%	P70	+	+	+	54%
P17	+	+	+	100%	P71	+	+	+	100%
P18	-	+	+	100%	P72	+	+	+	100%
P19	+	+	+	100%	P73	+	+	+	100%
P20	+	+	+	100%	P74	+	-	-	100%
P21	+	+	+	100%	P75	+	-	-	66%
P22	+	+	+	100%	P76	+	-	-	100%
P23	+	+	+	61%	P77	+	-	-	100%
P24	-	+	+	100%	P78	+	+	+	100%
P 25	+	-	+	100%	P79	+	+	+	67%
P28	+	+	+	100%	P80	+	-	-	100%
P29	+	+	+	100%	P81	+	+	+	100%
P30	+	+	+	100%	P82	+	+	+	100%
P31	+	+	+	100%	P83	+	+	+	50%
P33	+	-	-	100%	P84	+	+	+	100%
P34	+	-	-	45%	P85	+	+	+	100%
P35	+	+	+	100%	P86	+	+	+	93%
P36	+	+	+	100%	P87	+	-	-	100%
P37	-	-	-	100%	P88	+	+	+	100%
P39	+	-	-	100%	P89	+	-	+	100%
P40	+	-	-	100%	P90	+	+	+	100%
P41	+	-	-	100%	P91	+	-	-	100%
P42	+	+	+	100%	P92	+	+	+	100%
P44	+	+	+	100%	P93	+	+	+	71%
P45	+	+	+	100%	P94	+	+	+	100%
P46	+	-	+	100%	P95	+	+	+	100%
P47	+	+	+	100%	P96	+	-	+	100%
P48	+	+	+	100%	P97	-	+	+	100%
P49	+	+	+	100%	P98	+	+	+	91%
P50	+	+	+	100%	P99	+	+	+	100%
P51	-	-	-	100%	P100	+	+	+	100%
P52	+	-	+	100%	P101	+	+	+	100%
P53	+	+	+	63%	P102	+	-	-	100%
P54	+	+	+	100%	P103	+	-	-	100%
P55	+	+	+	100%	P104	+	+	+	100%
P56	+	+	+	100%					

Key: + = Positive result; - = Negative result

Environmental factors often play a vital role in the virulence of an organism especially in clinical infections (Prasad *et al,* 2009; Choy *et al,* 2008; Wu *et al,* 2003; Feltman *et al,* 2001); the findings confirm that the wound isolates have high virulence potentials to be able to cause infections in the host that the water isolates might not possess because of less exposure to environmental stress. The percentage of the biofilm forming tap water isolates that produced AHL was much lower than those of the wound isolates, an indication that the wound isolates might be more virulent than the water tap isolates. Although biofilm organisms usually show high resistance to antimicrobials (Hentzer *et al,* 2001) the sensitivity pattern observed in these studies was not performed on the biofilm but the planktonic cultures of the isolates. Bacterial resistance to antimicrobial compounds sometimes depends on persister cells amongst the bacterial population (Lewis, 2001; Spoering and Lewis, 2001) and not biofilm formation.

The *P. aeruginosa* wound isolates that were examined in this study have strong potential for biofilm formation. Since wound colonisation is inevitable (Cooper *et al,* 2002; Bowler *et al,* 2001) the tendency for such bacteria to form biofilm in wounds might be relatively high.

Conclusion

Most of the wound isolates formed biofilm and produced AHL, which are important to pathogenicity. The findings suggest that the wound isolates have the potential to exhibit virulence determinants that allow them to cause infections whereas water isolates did not. There is the possibility for the organisms to form biofilm in wounds. In this study an observation of a strong association between the genetic similarity index and biofilm phenotypic characteristics of the *P. aeruginosa* wound isolates was found. The wound isolates were largely sensitive to the most commonly used antimicrobials for *P. aeruginosa* infections.

Chapter 3

The study of physiological and phenotypic characteristics of *Pseudomonas aeruginosa* in a wound model

3.1 Introduction

Generally, the growth of microorganisms depends on various factors such as nutrient availability (Candy *et al,* 2009; Rochex and Lebeault, 2007) and temperature (Alves *et al*, 2008). In a closed system or batch culture, depletion of essential growth requirements as a result of an increase in bacterial population, reduces the growth rate and the toxicity of metabolic wastes that are being released by the organisms further contribute to the reduction of the bacterial population. In such cultures, apart from the stress that these conditions pose to the organisms, culture periods are often limited since they are dependent on the nutrients within the system at the onset of the culture. The population size of organisms remains constant during the period of nutritional depletion and accumulation of toxic waste (usually the end of exponential phase and the beginning of the stationary phase) because the rate of multiplication has reduced and the death of the organisms in the culture has commenced hence there is a state of equilibrium as the new cells are replacing the dead ones (as demonstrated in section 2.2.3 of chapter 2). At this phase, phenotypic changes involving the expression of various genes usually occur (Nakamura *et al,* 2003; Thompson *et al,* 2003*)* for the survival of the organisms. Nakamura and colleagues studied 2 strains of *Bordetella pertussis* and observed that during this transition from exponential phase to stationary phase 144 array elements showed an increase in transcription, while 177 elements showed a decrease in transcription rate. It was observed that there was a significant switch in virulence gene expressions of *Helicobacter pylori* during the transition from late exponential phase to stationary phase (Thompson *et al,* 2003). In the host environment stress arising from the immune mechanism often induces the production of virulence factors as a means of circumventing the circumstance. In an *in vitro* study the acquisition and retention of the ability of *Salmonella typhimurium* to induce the death of macrophages was observed only during the transition from late exponential and

stationary growth phases (Lundberg *et al,* 1999). Cowell‟s group (1999) also observed in their study that *Pseudomonas aeruginosa* cells that were carbon limited showed a greater increase in adhesion to artificial tear coated contact lenses; and that lipopolysccharide may have been involved in the constitutive adhesion to the lenses that occurred during carbon limitation which was an indication of biofilm formation. The reduction in metabolic activities and switching on of virulence genes such as spore formation, biofilm formation, production of cytotoxic enzymes and toxins have been extensively reviewed (Siegele and Kolter, 1992; Brown *et al*, 1988). These phenomena imply that pathogenic microorganisms are able to sense the conditions within the environments including that of the host and adjust to such conditions through growth control and virulent expressions. Studies have shown that nutrient depletion encourages production of signalling molecules (Kay *et al*, 2006) and development of biofilm in cultures of microorganisms *in vitro* (López *et al,* 2009; Haase *et al,* 2006). These phenomena are often utilised by organisms within biofilms (Fux *et al,* 2005) to enhance the ability to cause diverse infections and resist antimicrobials (Hill *et al,* 2005; Borriello *et al,* 2004). Biofilm forming organisms such as *Pseudomonas aeruginosa* therefore require minimal nutrients for growth and can thrive well in both aerobic and anaerobic conditions (Waite and Curtis, 2009; O'May *et al,* 2006). An increase in nutritional supply was observed to aid the dispersion of *P. aeruginosa* biofilm (Sauer *et al*, 2004) as this tends to increase the expression of the flagella while reducing the expression of pili thus promoting the detachment of the organisms from the biofilm. Evidences of increased yield in biofilm culture *in vitro* with availability of nutrients have been reported (Yang *et al*, 2008; Hawser and Douglas, 1994).

Once the nutrients become totally depleted, the organisms cease to multiply and eventually die and this further limits the application of batch culture in studies that require long period of microbial culture. In a host environment such as a wound

nutrients are usually available but the general conditions within the host due to host defence against the organisms (Hu *et al*, 2009) and products released by colonising organisms (Chen *et al*, 2009) would induce stress in the organisms. Zaborina and colleagues (2007) in their study observed that *P. aeruginosa* can be alerted to the presence of a physiological disturbance in its host by dynorphin released by humans during severe stress to produce harmful toxins and to suppress the growth of probiotic bacteria in the gut.

3.1.1 Continuous culture

Continuous culture is a means by which the culture of microorganisms is maintained under constant conditions for a long period to enable for continuous growth. In this culture procedure, the medium is constantly being replaced thus the metabolic products are being eliminated while the nutrients are being maintained (Herbert *et al,* 1956). Continuous cultures prevent cultural deterioration or mutant strain development (Williams and. Wimpenny, 1978; Detrois, 1959) even for a long period. Continuous culture methods have been recommended for the maintenance of phenotypic characteristic of the organisms for a long period (Herbert *et al,* 1956) because of the difficulty in cultivating organisms that will retain their phenotypic characteristics in a static culture. Continuous cultures have been successfully utilised to determine production of metabolic products (Guerra-Santos *et al*, 1984) and phenotypic characteristic of biofilm organisms such as *P. aeruginosa* (Robinson *et al*, 2004; Hentzer *et al*, 2001; Davies and Geesey, 1995; Davies *et al*, 1993).

3.1.2 Phenotypic expressions of *Pseudomonas aeruginosa* in biofilm

In the course of biofilm formation, quorum sensing signalling molecules are released for the modulation of genetic expressions of biofilm forming organisms (Fuqua *et al,* 2001; Whiteley *et al,* 1999) as well as other substances. The sensing and response to these signal molecules or autoinducers transforms to coordinated group activities when the quorum population density is attained (Bjarnsholt and Givskov, 2007; Parsek and Greenberg, 2005; Waters and Bassler, 2005; Fuqua *et al,* 1994). In *P. aeruginosa* these signalling molecules have been identified as mainly 2 systems of AHLs (Fuqua *et al*, 2001; Parsek Greenberg, 2000; Whiteley *et al,* 1999) and quinolones (Diggle *et al,* 2006; Pesci *et al,* 1999). Organisms within biofilm typically produce extrapolymeric substances for encasement and for attachment (Watnick and Kolter, 1999; McKenny *et al,* 1998). These phenotypic changes have been used as biomarkers in biofilm infections (Rickard *et al*, 2010; Sing *et al*, 2000; Stickler *et al*, 1998).

3.1.3 Models /wound models for biofilm study

3.1.3.1 In vitro models

In vitro models are often employed for the study of agents such as antimicrobials and healing enhancement agents on cells in the laboratory. The system is mostly cost effective and the ability to test multiple agents and series of concentrations of agents at the same time offers an advantage over the use of *in vivo* models. A variety of cell lines and tissue cultures are usually employed as *in-vitro* wound models (Oberringer *et al*, 2007). The effect of cellular hypoxia on treatment in wound healing was demonstrated *in vitro* using a cell line wound model (Oberringer *et al*, 2007). The proliferative and cellular wound fill was shown to be more rapid with a particular cell (gingival fibroblasts) than periodontal ligament cells (Oates *et al,* 2001). The study was able to show an indication that cell specific attributes play a significant role in healing.

The main purpose of a study usually determines the type and the form of a model, as well as the material used in the construction of the model. Various models have been used in the study of biofilm cultures in the laboratory. *P. aeruginosa* biofilm architecture and susceptibility of the biofilm to tobramycin antibiotics have been studied in a continuous culture reactor system using polycarbonate chips as the stratum on which the biofilm attached (Hentzer *et al,* 2001), whereas the effect of sucrose and silver on biofilm of *S. aureus* was successfully studied using biofilm model grown on microscope coverslips in rabbit plasma in tissue culture dishes for 24 hours (Akiyama *et al*, 1998). Sriramulu and colleagues (2005) simulated the cystic fibrotic environment as a biofilm model by growing *P. aeruginosa* in artificial sputum in a conical flask, while Walker and Sedlacek (2007) developed a biofilm model with ceramic calcium hydroxyapatite disks. Thorn and his colleagues (2009) studied the effects of iodine and silver dressings on biofilms of *S aureus* and *P. aeruginosa* grown within cellulose matrices using flat- bed perfusion biofilm model by inoculating the organisms and then perfusing the culture for 24 hours. The 24 hour biofilms were removed and placed on pre-incubated hydrogel polymer test-bed assay plates and dressings were cut (2.5 x 2.5 cm) and placed on top of the biofilms. Test samples were removed at intervals and biofilm assessed by sonication and TVC determination. Both iodine and silver were found to exert significant antimicrobial effects on the two biofilms, although iodine was more potent than silver in biofilm control of the two organisms after 8 hours of exposure. Evidence of biofilm growth was found under silver dressings after 24 hours. The antimicrobial activity of silver on biofilms of *P. aeruginosa, Candida albicans, S. aureus* and other organisms in a poloxamer gel was investigated (Percival *et al,* 2007). Of the two dressings tested, the silver-containing hydrofibre (SCH) dressings showed higher antimicrobial activity against biofilms than the nanocrystalline silver containing (NSC) dressings. Although the SCH dressings were supposed to contain 10

times the concentration of silver than the NSC it was found that the concentration did not correlate to the level of activity.

Multispecies biofilms have been mostly recognised in wounds. An *in vitro* validation of this polymicrobial biofilms was demonstrated in Lubbock chronic wound biofilm (LCWB) model to study cooperation growth amongst the commonly isolated wound pathogens (Sun *et al*, 2008). The 24 hour polymicrobial biofilm grown in the model was tested against various concentrations of sodium hypochlorite (bleach) solution. The (LCWB) model was also used to propagate the polymicrobial biofilm comprising of aerobic and anaerobic bacteria commonly isolated from chronic wounds to simulate the microbiota within the wound environment (Sun *et al*, 2009). The organisms included 5 anaerobes and 3 aerobes and were cultured with Bolton broth, heparinised plasma and laked blood media to represent the available nutritions in the wound environment. Electron microscopic examination showed the association of the bacteria in biofilm with the fibrous network of EPS. PCR analysis also confirmed the identification of the various organisms within the biofilm and the results of the population monitoring showed the dominance of *P. aeruginosa* at 24 and 48 hours over the other bacteria.

Although the environment within the *in vitro* model can be controlled the inability to simulate an environment that is akin to the natural one in the human system poses a great limitation, hence the use of animal model to simulate a wound environment closer to that of human.

3.1.3.2 In vivo wound models

These are used in the study of wound healing processes or the effect of agents on wound healing in animals or humans. Many models have been used to study various

mechanisms and reactions as well as those involved in the pathophysiology of cutaneous wounds. The most commonly used in wound studies are the animal models because the opportunities to carry out controlled, clinical trials on human wounds are limited (Sullivan *et al,* 2001). The animal models also reduce the variability often encountered with human subjects. Although an animal model will not provide the full concept due to the differences in the physiological and genetic make up, immunological response and tissue architecture the information obtained usually provides relevant information that can be useful in the treatment of human subjects.

Issues of costs, availability of a particular animal and maintenance of the animal during the period of experiment also inform the decision on the type of model to use. The wound inflicted depends on the experiment and could range from incisional, partial or full thickness, contraction, blister and burn wounds of various degrees depending on the study requirement. In some countries (in the European Union; EU regulation 76/768 of Feb. 2003) the use of animal models is restricted for humane treatment of animals. Wound models that are commonly used include porcine (pig) wound models (Davis *et al*, 2008; Serralta *et al*, 2001) because the pig skin is physiologically close to the human skin. Other animal models often used are rat models (Patil *et al*, 2009; Manjunatha *et al*, 2006) and mice (Manjunatha *et al,* 2006). A continuous biofilm model was designed using murine models (Kadurugamuwa *et al,* 2003) to study formation of biofilm on catheters. The study of biofilm formation and detection of AHL by clinical and environmental isolates was studied in mice wound model (Kumar *et al*, 2009).

Although none of the *in vitro* and *in vivo* animal wound models can provide the ideal situation in human wound they will provide necessary information and guidelines to improve wound management.

3.1.4 Study hypothesis

The development of a model is dependent on various factors including the type of study, sample size, the convenience / ease of maintenance, cost, size of model and availability of material. The majority of the *P. aeruginosa* cultures from infected and chronic wounds were able to produce biofilm and AHL in the batch cultures. Nutrient limitation and accumulations of toxic wastes are stress factors that initiate phenotypic changes in batch cultures (Nakagura *et al,* 2003; Thompson *et al,* 2003). Such stress factors are not common in the wound continuum where nutritional factors are constantly available for the colonising organism.

In this chapter, the phenotypic diversity of the *P. aeruginosa* wound isolates was determined using continuous culture method in a wound model. The study of the phenotypic expressions of the biofilm forming and the non- biofilm forming isolates allowed more information about the diversity in the expression of the 2 phenotypes of the bacteria which are often found in the patient during *P. aeruginosa* infections. The results of the assay provided judgemental evidence regarding the potential of the isolates to form biofilm in wound. Furthermore, detection of these markers in the wound model would suggest that the markers can be detected in the wound by screening the dressings that have been in contact with wounds for biofilm markers. The period for the development of biofilm within the wound model and detection of biomarkers would throw better light on the timescale for biofilm development in wound contaminated with biofilm forming bacteria such as *P. aeruginosa*.

Aim of this chapter

- To study the phenotypic characteristics of *Pseudomonas aeruginosa* wound isolates in a continuous culture wound model.

Objectives

- To monitor the growth of *P. aeruginosa* and AHL production on a wound dressing in a batch culture
- To develop a wound model for continuous biofilm culture
- To study the growth pattern and AHL production of *P. aeruginosa* wound isolates in a wound model.
- To monitor the stages of biofilm formation of *P. aeruginosa* wound isolates in a wound model.

3. 2 Materials and Methods

3.2.1 Developing a wound model

In this study, a wound model was devised for continuous cultures in order to study the phenotypic characteristics of selected isolates without mutant strain development and to prevent the accumulation of toxic waste which might affect the analysis. However, the development of the wound model was done in two stages, first with a batch culture and secondly with continuous culture system. Gauze, a conventional dressing material was chosen as the support material for the wound model (the substratum on which organisms were grown), since it is a traditional wound dressing. It was tested for its ability to support the growth of the test organisms and to retain the Gram negative quorum sensing molecules, such as the acyl- homoserine lactones (AHL).

3.2.1.1 Batch Cultures

Selected biofilm forming and non biofilm forming wound isolates and a reference culture *P. aeruginosa* NCIB 8626 were investigated in batch cultures to determine the phenotypic expression. AHL production was determined through the gauze that had been incorporated in the medium to establish the phenotypic expressions of the selected cultures.

3.2.1.1.1 Batch cultures in flasks for AHL detection at 1 cm distance.

Overnight culture of *P. aeruginosa* was diluted 10^{-3} with Luria broth (LB); 5 mls of the diluted inoculum was pipetted into 95 ml of LB or LB and foetal calf serum (FCS) (1:1) (Fisher – UK) in which some pieces of sterile gauze (8 layers of 2.5 x 2 cm per piece) have been included (Fig. 3.1). The addition of FCS was to simulate wound environment and to examine the effect of serum on biofilm formation. Blank controls flasks containing LB with gauze or LB and FCS mixture with gauze pieces without test

organism were included in the assay. The flasks were incubated at 37^{o}C in the static waterbath for 48 hours. The gauze materials were taken out of each flask into a sterile universal container at 24 and 48 hours and the universal containers were sterilized at 121 ^{o}C for 15 minutes in an autoclave (LTE Scientific, UK). The AHL detection (cross feeding assay) was determined as previously described in chapter 2 with *Agrobacterium tumefaciens* (NTL4) and the gauze placed 1 cm away from reporter strain. The controls for the reporter strain, the reference organism *P. aeruginosa* NCTC 8626 and the blank were included in the assay.

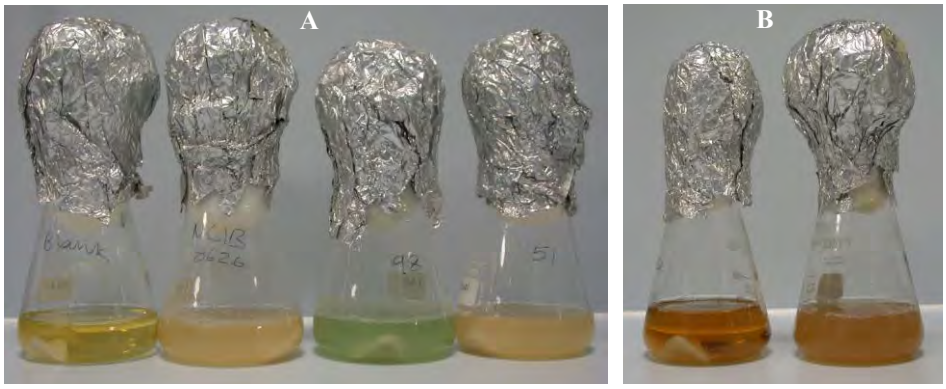

Figure 3.1: Batch culture wound model (A) Flasks containing LB and pieces of gauze (the blank control), 48 hour LB cultures of *P. aeruginosa* NCIB 8626, a biofilm forming AHL producing *P. aeruginosa* and non- biofilm forming and non-AHL producing *P. aeruginosa* isolated from wounds respectively. **(B)** Foetal calf serum (blank control) and *P. aeruginosa* NCIB 8626 LB and foetal calf serum 48 hour culture respectively. All the flasks contained pieces of gauze for AHL detection.

3.2.1.1.2 Batch culture of P. aeruginosa for AHL detection at 1 mm distance.

The experiment was repeated as described in 3.2.1.1.1 above but the gauze from the each culture was placed beside the NTL4 streak, 1-2 mm apart. This was to evaluate the influence of distance on AHL detection.

3.2.1.1.3 Determination of ideal distance for detection of AHL in gauze.

The assay was to determine the farthest distance between the reporter strain and the gauze at which the weak/ average AHL positive results could be detected. A reference organism *P. aeruginosa* NCIB 8626 and a strongly positive AHL producing *P. aeruginosa* clinical isolate (P.98) were investigated. The gauze materials from the cultures were placed 1 - 6 mm away from the inoculation streak of the reporter organism *Agrobacterium tumefaciens* NTL4; the cultures were incubated at 30^{o}C and the cultures were observed at 24, 36 and 48 hours and photographs were taken with Nikon digital camera.

3.2.1.2 Wound model (Continuous culture)

3.2.1.2.1 Design of the wound model

The idea to design a wound model was to culture the isolates in a system that will provide adequate nutrients at a steady state without flooding the culture and to allow the removal of bacterial metabolic wastes. The wound model (Fig. 3.2) was prepared with 2 gauze rolls, (each with 8 layers of 2.5 by 2 cm gauze) inside a Leur lock glass syringe (Fisher Scientific) (held in place with the aid of the stand and clamp) with tubing passed from the media reservoir to a syringe (anti - grow back trap) from where the tubing was connected to a Leur lock stainless steel needle (BD, USA) to continuously feed the culture. The culture was fed with the drips of nutrients with the aid of a Watson Marlow 505U peristaltic pump (Watson Marlow Ltd., England, UK), the effluent drips from the syringe to the waste receptacle which collects the culture. The volume of the broth contained in the gauze was determined by pumping the broth through the model and counting the number of drops that completely soaked the piece of gauze and multiplying by the volume of each drop which had been earlier determined. The 2 rolled pieces of gauze used for each culture in the model, were found to retain between 1.41 ml and 1.54 ml of broth (an average of about 1.475 mls) was calculated. All

components parts excluding the pump were sterilised at 121°C for 15 minutes and dried in the oven at 60°C prior to each culture.

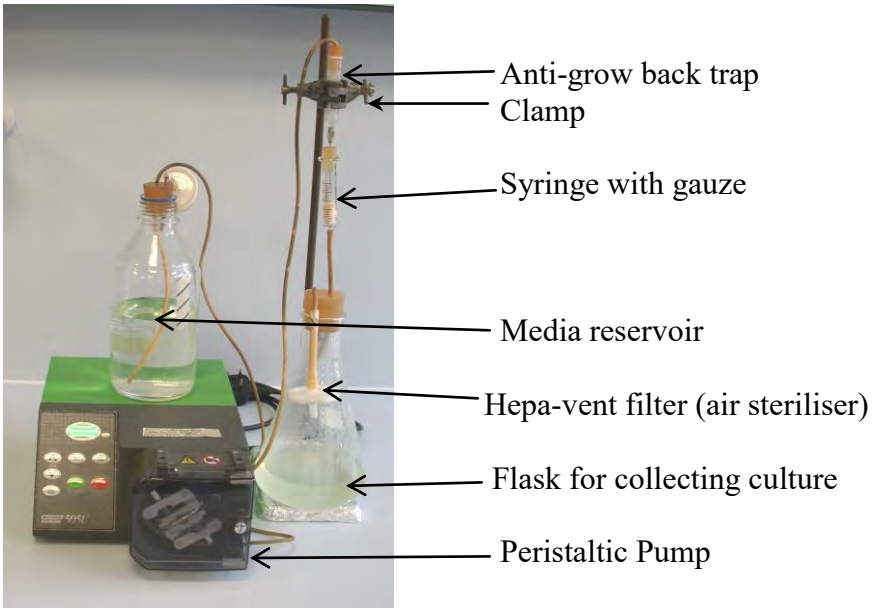

Figure 3.2: The Continuous Culture Wound Model: A system designed and assembled for the study of the growth pattern and phenotypic characteristics of biofilm and non- biofilm forming *P. aeruginosa* in continuous culture. The system was sterile and closed to the outside environment but equilibrium with atmospheric pressure was maintained by fitting to each bottle a Whatman® Hepa-vent air-permeable filter.

3.2.1.2.2 Preliminary study of phenotypic diversity of P. aeruginosa strains in continuous culture wound model with full strength Luria broth.

The flow rate of the LB through the model was calculated from the amount of exudate produced by wounds, especially external wounds or wounds associated with virulent bacterial mechanisms that often cause vasodilation and extravasation (Wilson *et al,* 2002). High production of exudates had been estimated to be more than 10 ml every 24 hours (Mulder, 1994). This volume could not be achieved with the pump available for this study and the minimum volume that could be delivered with the pump was 8.4 ml per hour. This was achieved by using tubing with the lowest diameter (0.5mm) for the pump and selecting the appropriate speed according to the calibration of the pump.

This was utilised for most of the experiments except for the high flow rate of 84 ml per hour. The wound model was placed in the incubator which was maintained at 37^{o}C throughout the period of the study. An overnight culture of the reference or test organism was diluted 10^{-5} with Luria broth. The model was inoculated for 1 hour with the 10^{-5} diluted overnight culture of the reference organism *P. aeruginosa* NCIB 8626 in Luria broth (LB), an AHL positive and AHL negative clinical isolate (a sample at a time) via the tubing and the broth run through the model for 24 hours with the aid of the peristaltic pump. Samples were collected from the model culture at the distal end close to the waste receptacle by removing the tubing at predetermined intervals into sterile Bijou bottles (Fig. 3.2). Total viable count (TVC) of each sample was determined and the remaining of the culture sample was sterilised with a syringe filter (Millipore Millex Durapore 33mm 0.22µm pore filter - Fisher – Scientific, UK) for acylhomoserine lactone (AHL) detection by dropping the sterilized effluent on the streaked culture of *C. violaceum* before incubation (modification of previously described method in chapter 2, section 2.2.7.1). At the end of each experiment, the 2 gauze rolls were removed, one was put in a sterile universal container with 10 mls of sterile deionised water for TVC after releasing the organisms by vortex mixing for 15 or 30 seconds (for optimisation of release of organisms) while the second gauze was sterilised at 121 ^{o}C for 15 minutes in an autoclave (LTE Scientific, UK) and tested for the presence of AHL as described previously in section 3.2.1.1.2.

3.2.1.2.3 Selection of media for wound model cultures (Effect of nutrient)

In order to determine the effect of nutrients on biofilm formation, and to select a suitable concentration of broth that will support the growth of biofilm in the model without the EPS from biofilm clogging the outlet tubing for the culture effluent. Each of an AHL producing biofilm forming *P. aeruginosa* wound isolate (P98) and a non - AHL

producing and non biofilm forming *P. aeruginosa* wound isolate (P51) was cultured in the wound model for 48 hours with ½, ¼ and $^{1}/_{10}$ strength LB. Samples were taken from the model culture at intervals for total viable count (TVC) and acylhomoserine lactone (AHL) detection using *A. tumefaciens* NTL 4 as the reporter organism.

3.2.1.2.4 Examining the effect of flow rates of broth on the wound model culture

A biofilm forming (P.98) and a non biofilm forming (P.51) wound isolates were each cultured in the wound model using $^{1}/_{10}$ LB at a high flow rate of 84 mls per hour. This was to ensure that there was no trapping of the bacteria within the gauze and to observe effect of the hydrodynamic flow on the culture in particular if the non biofilm former will be washed out of the model. The model was inoculated for 1 hour at the flow rate of 8.4 mls per hour and the rate of the media was thereafter increased to 84 mls per hour for the 48 hour duration of the experiment. Samples of the effluent were collected at intervals and the AHL was determined and smears were made on glass slides which were stained were stained and examined for biofilm formation.

3.2.1.2.5 Determination of reproducibility of assays for wound model culture

A biofilm forming and AHL producing *P. aeruginosa* was cultured in the wound model on 3 different occasions with $^{1}/_{10}$ LB to determine the reproducibility of the model using TVC and AHL assays. Viable counts of the culture samples were determined to establish the growth pattern of the organism and the AHL assay to determine the period of AHL production. Slide smears of the culture effluent samples were also made, fixed and stained for EPS to study biofilm morphological structure. Slides were examined with Olympus microscope with digital camera (Volocity software) for EPS and biofilm morphology.

3.2.1.2.7 Examining the effect of serum on biofilm formation

A biofilm forming and AHL producing *P. aeruginosa* (P.98) was cultured in the wound model using FCS and full strength LB (1:1) and FCS in $^{1}/_{8}$ LB (1:4) (giving final concentration of $^{1}/_{5}$ FCS and $^{1}/_{10}$ LB) to determine growth pattern and biofilm formation as previously described section 3.2.1.2.2. Viable count of the culture samples and production of quorum sensing molecules (AHL) were determined as above using culture effluent samples.

3.2.2 Staining procedure for EPS detection and biofilm morphology

The slide smears were fixed with 10 mM cetylpyridium chloride (Sigma, UK) for 5 minutes and air dried for 30 minutes. Slides were fixed with gentle heat, cooled and stained with 2:1 mixture (v/v) of saturated aqueous Congo red (BDH, UK)and 10% Tween 80 (Sigma –Aldrich, UK) for 15 minutes. Slides were rinsed with water and were counterstained with 10% Ziehl‟s Carbol Fuschin (BDH, UK) for 5 minutes. Slides were rinsed with water and air dried (Harrison – Balestra *et al,* 2003).

3.2.3 Scanning electron microscopic (SEM) examination of biofilm on the gauze from the wound model culture.

It was necessary to examine the gauze from the wound model to determine the presence of biofilm. A biofilm forming *P. aeruginosa* isolate (P.98) and a non biofilm forming *P. aeruginosa* isolate (P.51) were each cultured in the wound model for a period of 30 hours as described previously (section 3.2.1.2) using $^{1}/_{10}$ strength Luria broth. Samples of the culture effluent were collected at determined intervals for TVC and AHL. Effluent slide smears were prepared, stained and examined to establish biofilm formation. After terminating the culture at 30 hours, the gauze was fixed with 2.5% (w/v) glutaraldehyde (Fluka, UK), dehydrated through ascending concentrations of

ethyl alcohol (30%, 50%, 70%, 80% and 90%) for 10 minutes each. The gauze was finally dehydrated in three changes of 100% ethyl alcohol. It was dried in CPD030 (Bullzer‟s clinical drier) with carbon - dioxide to flush out the alcohol and saturate the material completely at 10^{o}C and 90 mmHg pressure for an hour. The pressure was released and the temperature was increased to 40^{o}C for 3 hours for critical point drying (CPD). Sputter coating of the gauze was done by spraying the gauze with gold (Emscope Gold Sputtering Coater AE 1231 (Emscope, Kent, UK) for 10 minutes and was examined with a scanning electron microscope (Philips XL 20 SEM, Eindhoven, Netherlands). This was done at the Electron Microscopy (EM) unit, Cardiff University.

3.3 Results

3.3.1 Detection of AHL from gauze incubated in batch culture

Gram negative quorum sensing (QS) molecules AHLs present in the gauze which was produced by *A. tumefaciens* KYC6 (an AHL producer as positive control) and the *P. aeruginosa* wound isolates (P.98 and P100), diffused through the medium changing NTL4 colonies from cream to blue (Fig. 3.3A) as discussed in chapter 2 section 2.3.5. Negative controls (Fig. 3.3B); the blank control (Fig. 3.3C) and the wound isolates (P.51 and P.58) that did not produce QS molecules (Fig. 3.3E) remained unchanged after 48 hours incubation at 30^{o}C.

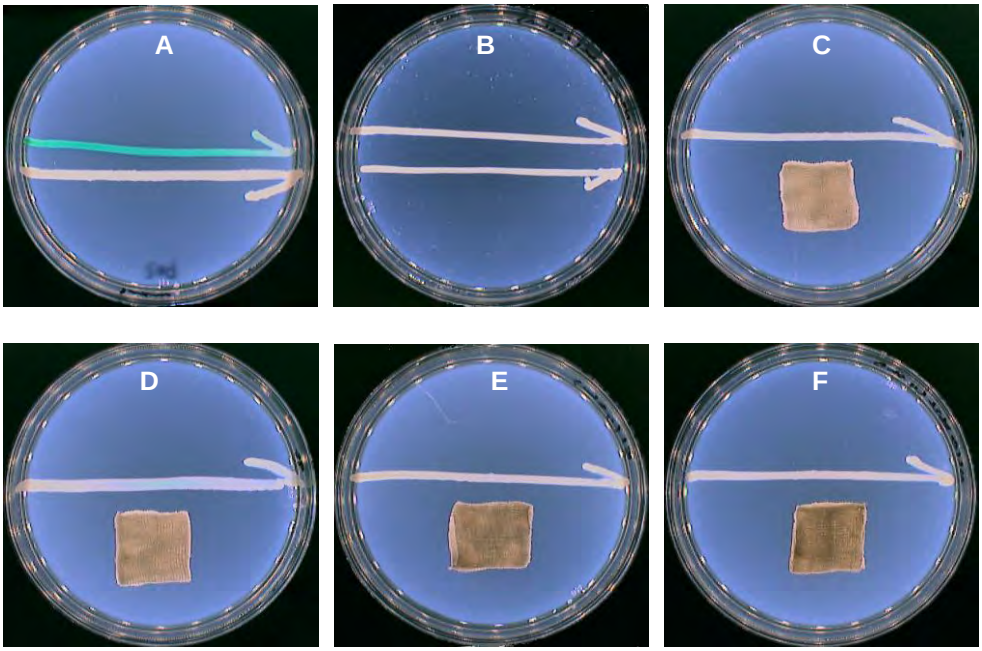

Figure 3.3: Detection of AHL in gauze from 48 hours batch cultures showing **(A)** the reporter bacteria *Agrobacterium tumefaciens* NTL4 with A. tumefaciens KYC6 (an AHL producing biofilm forming bacteria) as positive control; **(B)** KYC6 against itself as negative control; **(C)** LB without inoculation of organism as blank control; **(D)** an AHL producing biofilm forming *P. aeruginosa* isolate from wound; **(E)** a non-AHL producing non- biofilm forming *P. aeruginosa* isolate from wound; **(F)** an AHL producing biofilm forming *P. aeruginosa* NCIB8626 as positive culture control.

However the colour change from cream to blue that was observed between 36 - 48 hours for the strong AHL producing wound isolate (P.98) (Figure 3.3D) was faint and not as obvious as had been observed with the solid agar culture in the cross feeding assay as discussed in chapter 2, (section 2.3.5). AHL could not be detected in the gauze from the reference organism NCIB 8626 as there was no visible change of colour on plate (Fig. 3.3F) even though the organism had tested positive in the cross feeding assay. In the cross feeding assay on solid agar plate, the AHLs being produced by the bacteria diffused through the agar within the 1cm distance to initiate the effect that was observed in the reporter bacteria. In the batch culture, the AHLs were being diluted by the culture medium as were being produced and the amount in the gauze might not have been high enough to initiate the expected reaction when it diffused over the distance of 1cm. Although when the level of AHLs produced by the bacteria is high due the numbers of organisms or the ability of organisms to produce high level of AHL, the detection might be possible at this distance but when the concentrations of the AHLs produced in the culture medium are low, detection from such a distance might be difficult. In order to confirm that the gauze sustained the growth of the organisms and the AHLs produced by the organisms the gauze was placed one millimetre away from the NTL4 in the plate. The results showed positive results with pronounced colour intensity for the positive control (Fig. 3.4A), an AHL producing biofilm forming *P. aeruginosa* isolate from wound (Fig. 3.4B) and an AHL producing biofilm forming *P. aeruginosa* NCIB 8626 (Fig. 3.4C) as positive reference organism control. KYC6 against itself as negative control (Fig. 3.4D), LB without inoculation of organism as blank control (Fig. 3.4E), and a non-AHL producing non- biofilm forming *P. aeruginosa* isolate from wound (Fig. 3.4F) did not show any colour change.

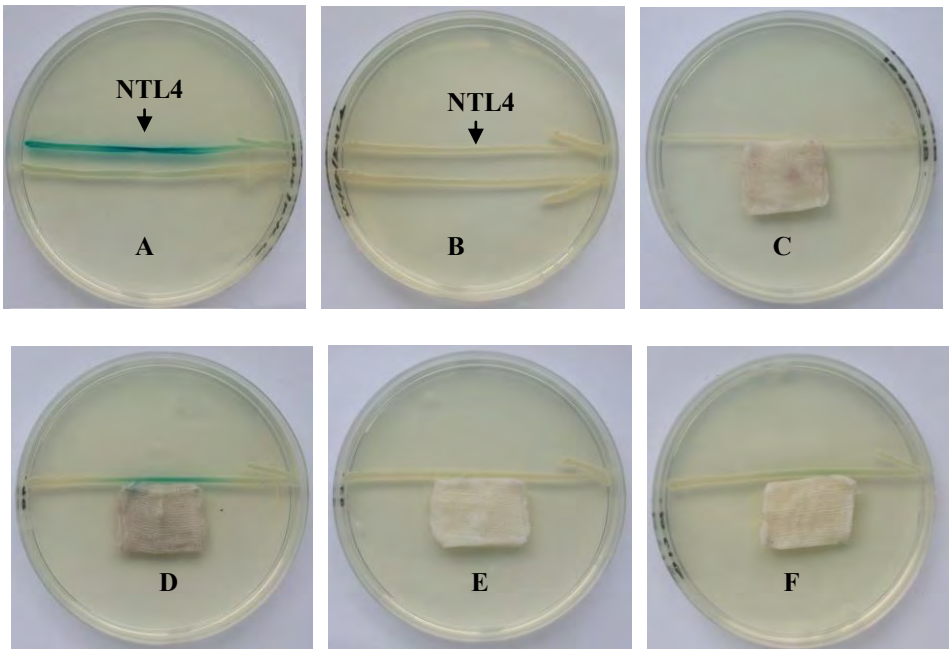

Figure 3.4: Detection of AHL in gauze from batch culture placed 1mm from reporter bacteria. (**A**) reporter bacteria *A. tumefaciens* NTL4 with A. *tumefaciens* KYC6 (AHL producing biofilm forming bacteria) as positive control; (**B**) KYC6 against itself as negative control; (**C**) LB without inoculation of organism as blank control; (**D**) AHL producing biofilm forming *P. aeruginosa* isolate from wound (P.98); (**E**) a non-AHL producing non-biofilm forming *P. aeruginosa* isolate from wound (P.51) and (**F**) **AHL** producing biofilm forming *P. aeruginosa* NCIB 8626 as positive reference culture.

3.3.2 The Ideal distance for AHL detection in gauze

In order to determine the ideal distance between the reporter strain and lowest AHL concentrations in samples of gauze removed from wound model, various distances between the reporter strain and the sterilised culture material (gauze) were investigated. This was to serve as a guide for the greatest distance between the reporter strain and the gauze at which the weak and average positive results can be determined. A type culture *P. aeruginosa* NCIB 8626 and a strong AHL molecule producing wound isolate (P.98) were investigated in an assay in which the sterilised gauze materials from the cultures were placed between 1 and 6 mm (at 1mm interval) away from the NTL4 streak before

incubation. Optimal colour development was observed at 36 hours which remained stable at 48 hours. The strong QS molecule producer showed strong positive results for the 1- 6 mm distance from NTL4 (Figure 3.5a ; Table3.1) while NCIB 8626 was positive up to 3 mm with reduced colour intensity (Figure 3.5b ; Table3.1). These results signified that the gauze should be placed as close as possible (about 1-2mm) to the reporter strain to detect low AHL production.

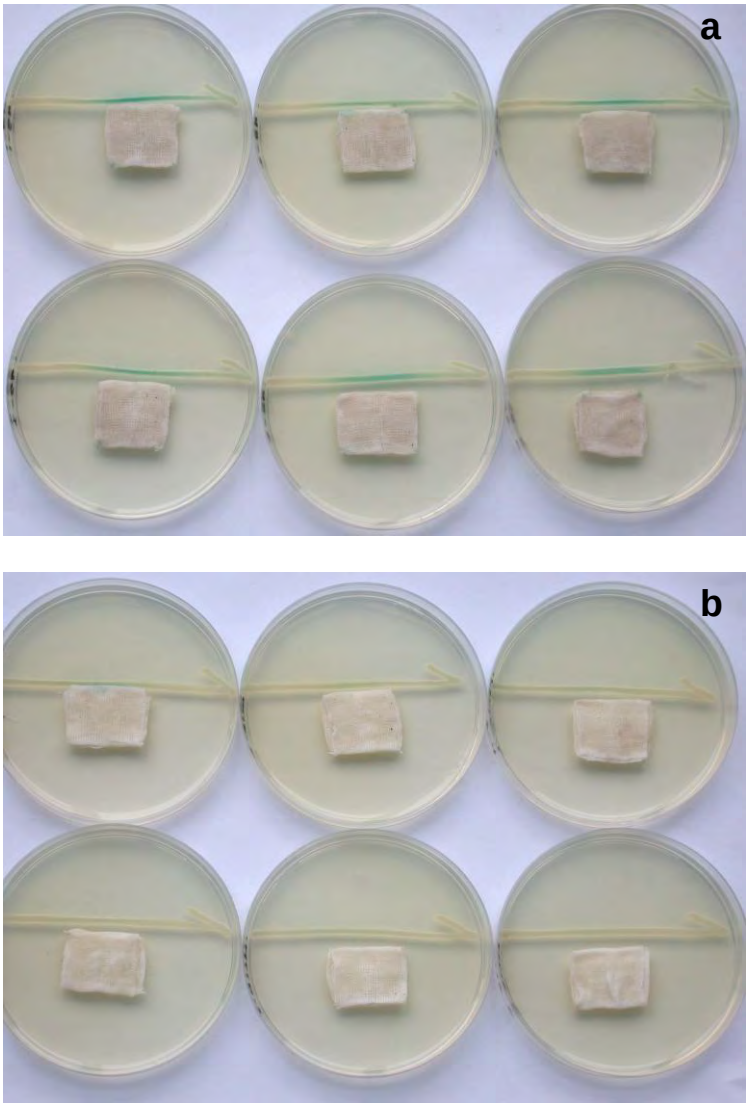

Figure 3.5: AHL detection in gauze samples removed from *P. aeruginosa* 48 hour batch cultures placed between 1 and 6 mm distance from the reporter organism. (**a**) a strong biofilm and high AHL producing *P. aeruginosa* wound isolate and (**b**) a reference *P. aeruginosa* NCIB 8626 a strong biofilm former and low AHL producer.

Table 3.1: Colour intensity index of optimisation of AHL detection assay

Distance between gauze and *A. tumefaciens*	**Colour intensity index score**	
	P. aeruginosa NCIB 8626	*P. aeruginosa* wound isolate (P.98)
1mm	+	+++
2mm	+	+++
3mm	+/-	+++
4mm	-	+++
5mm	-	+++
6mm	-	+++

Key: (±) slightly positive; (+) positive; (++) strong positive; (+++) very strong positive

The results of the batch cultures showed that gauze provided support for bacterial growth and AHL detection. The AHL produced by biofilm forming organisms were detectable from the gauze samples which were sterilized after removal from the culture. The wound model batch cultures provided information to distinguish between biofilm forming and the non- biofilm forming *P. aeruginosa* cultures isolated from wounds. The ideal distance for detection of AHL was also established as 1 mm especially for detection of low concentration of AHL.

3.3.3 Effect of nutrients on biofilm development in continuous culture

The effect of nutrients on bacterial growth and in particular on biofilm formation, was determined using various concentrations of LB to culture each of the biofilm forming and non- biofilm forming wound isolates in the wound model continuous culture.

3.3.3.1 Preliminary study of phenotypic diversity of P. aeruginosa strains in continuous culture wound model

In this study, the growth pattern for the 3 organisms tested with full strength Luria broth appeared similar, however AHL was first detected in isolate P.98 culture samples at 12 hours, when the number of organisms attained 9.6 x 10^9 cfu/ml of culture (Fig. 3.6; 3.7 C and D) while the reference organism *P. aeruginosa* NCIB 8626 tested positive for AHL only after 22 hours with the bacterial population of 6.46 x 10^{11} cfu/ml of culture whereas isolate P.51 did not produce AHL within the period studied (Figures 3.6 and 3.7 (E and F).

The results of the TVC for each effluent sample are presented in a graph for the LB cultures of the *P. aeruginosa* wound isolates, a biofilm former (isolate P. 98), a non biofilm former (isolate P.51) and a reference organism *P. aeruginosa* NCIB 8626 (Fig. 3.6). The organisms trapped within the gauze that were released at 15 seconds and 30 seconds showed higher numbers of organisms for the biofilm formers than the non biofilm former. Larger numbers of *P. aeruginosa* wound isolate (P.98) were recovered from the gauze than for the reference culture *P. aeruginosa* NCIB 8626 (Table 3.2) while those for isolate P.51 were the lowest. AHL detection results for P.98 were more pronounced than those for NCIB 8626 (Fig. 3. 4 D and F; Table 3.1). The stronger biofilm former isolate P. 98 had more organisms trapped within the biofilm matrix indicating that the larger the biofilm the greater the number of organisms within the matrix. The 2 periods of sonication were used in the preliminary study for the purpose of optimisation to determine the appropriate time for the release of organisms from the gauze throughout the period of the study.

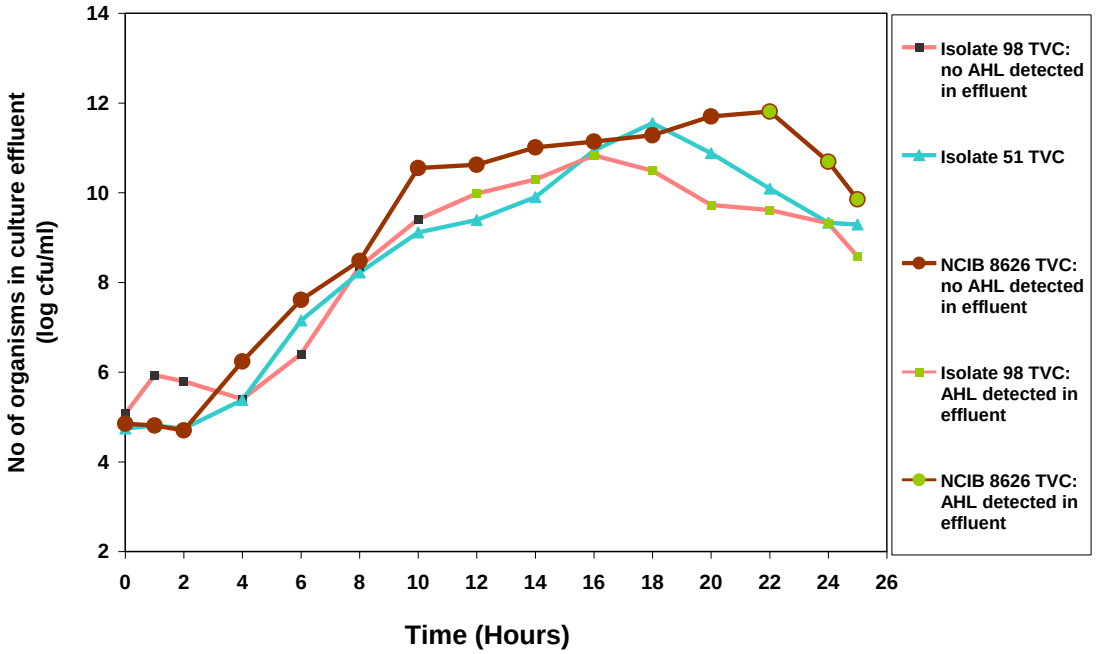

Figure 3.6: Phenotypic characteristics of *Pseudomonas aeruginosa* strains shown by growth curve and AHL production in wound model continuous cultures. The growth curves of *P. aeruginosa* wound isolates (biofilm forming P.98 and a non - biofilm former P.51) and a reference organism *P. aeruginosa* NCIB 8626 in wound model continuous culture using full strength LB at the flow rate of 8.4 ml/hr for a period of 25 hours including one hour inoculation time are represented graphically.

Table 3.2 Organisms released from gauze after 24 hour culture of each *P. aeruginosa* strain in a continuous culture wound model using full strength Luria broth at 37°C

Organisms	**15 sec vortex (Log cfu/ml)**	**30 sec vortex (Log cfu/ml)**
P. aeruginosa NCIB 8626	9.5×10^{10}	3.28×10^{11}
Wound isolate (P.98)	2×10^{9}	1.63×10^{10}
Wound isolate (P.51)	2.25×10^{9}	5.5×10^{9}

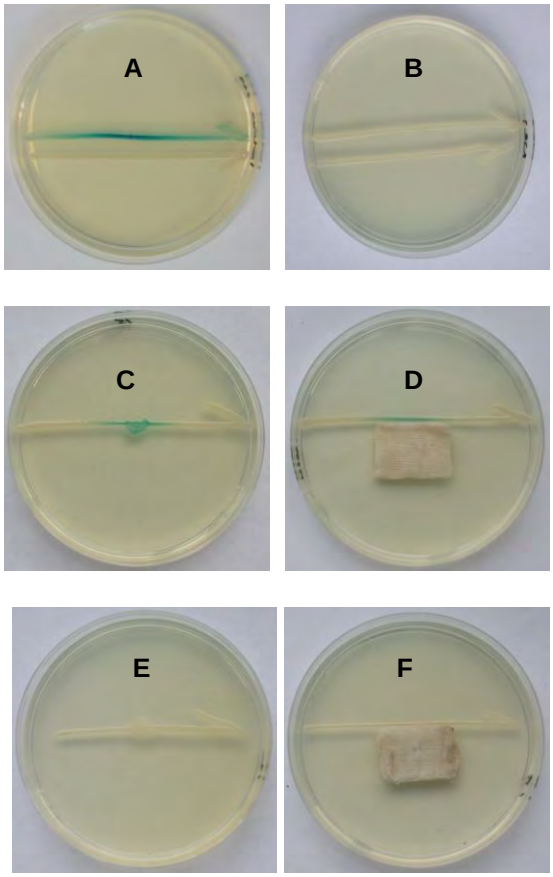

Figure 3.7: AHL detection in culture effluent and sterilized gauze. (**A**) *A. tumefaciens* KYC6 with *A. tumefaciens* NTL 4 as positive control; (**B**) KYC 6 with KYC6 as negative AHL control; (**C**) a drop of sterilised culture effluent sample from a biofilm forming *P. aeruginosa* wound isolate (P.98) taken during the continuous culture in a wound model (**D**) the gauze from the wound model after the culture (P.98) with NTL4 tested positive for AHL; (**E**) a drop of the sterilised culture effluent sample of a non-biofilm forming *P. aeruginosa* wound isolate (P.51) taken during the continuous culture in a wound model and (**F**) the gauze from the wound model at the end of the culture (P.51) showing negative result for AHL.

3.3.3.2 Effect of nutrient concentration on biofilm

The growth pattern and AHL production for the biofilm forming isolate P.98 for the various concentrations of Luria broth (Full strength, 1/2, 1/4 and 1/10) appeared similar over 24 and 48 hours (Fig. 3.8 and 3.9) (Table 3.3). The AHL was detectable at 12 hours for the full strength LB culture when the bacterial population was above log 9 per ml and the 1/2, 1/4 and 1/10 strength LB cultures when the bacterial populations were above log 7 per ml of culture. Although the non - biofilm forming isolate showed similar growth pattern over 24 - hour and 48 - hour incubation periods (Fig. 3.10 and 3.11), AHL was not detected in any of the culture samples throughout the period of experiment. It was apparent that the growth yield reduced with the decrease in

concentration of broth (Fig. 3.8, 3.9, 3.10 and 3.11). The continuous wound model culture for the diluted LB (1/2, 1/4 and 1/10 strength LB) showed increase in TVC of P.98 up till 36 hours whereas with full strength LB cultures numbers started declining from 21 hours (Fig. 3.8, 3.9, 3.10 and 3.11). For the purpose of this study, it was important to have a steady growth that would not be too rapid in order to study the organism for a reasonable period of time. Excessive growth would cause blockage of the tubing to the effluent collection flask with bacteria and EPS that are being produced during biofilm formation which might cause the wound model to collapse. The broth that sustained steady growth with the least growth rate, 1/10 Luria broth was chosen as the ideal concentration of broth for the wound model cultures.

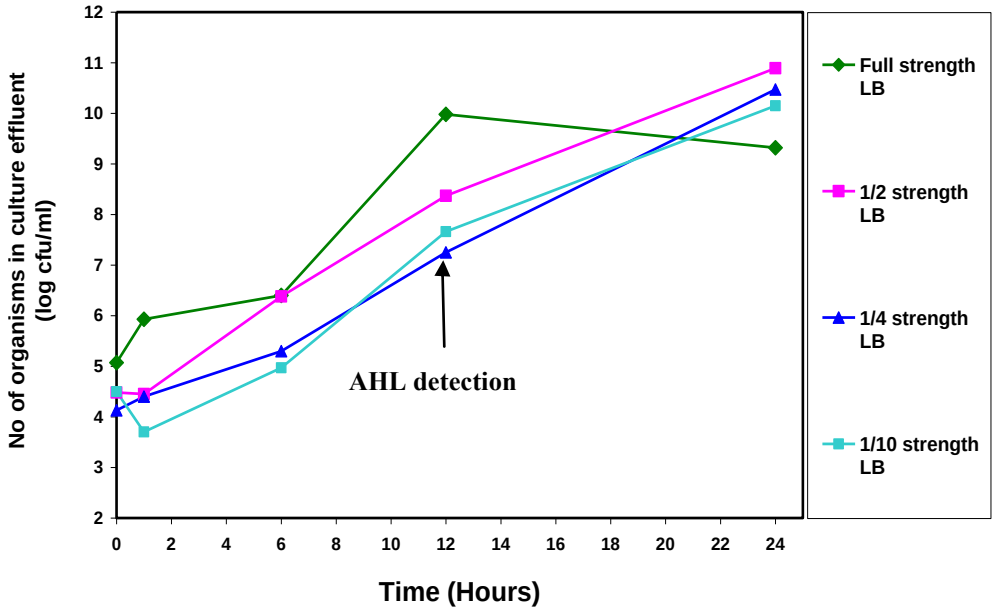

Figure 3.8 The 24 hour continuous cultures of a biofilm forming *P. aeruginosa* wound isolate (P. 98) in the wound model with various Luria broth concentrations. The wound model continuous culture of the biofilm forming isolate (P. 98) was run at 8.4 mls per hour flow rate with each of the media for a period of 24 hours at different times. TVC and AHL production were determined at intervals of 2 or 3 hours during the period of culture.

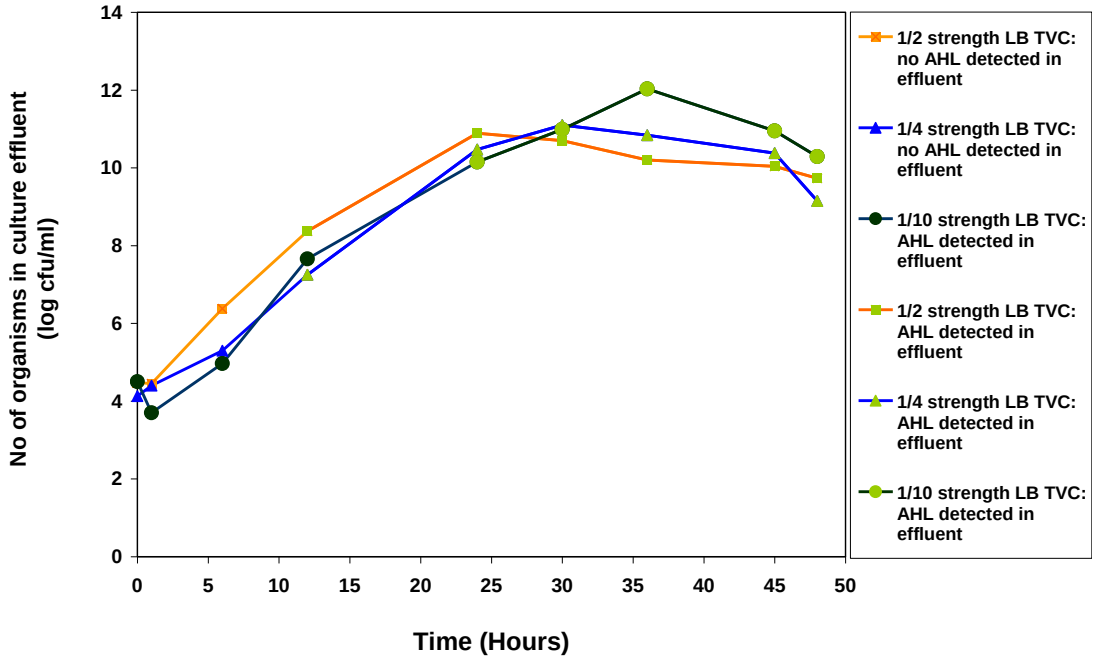

Figure 3.9 Growth curves of 48 hour continuous cultures of a biofilm forming *P. aeruginosa* wound isolate (P. 98) in the wound model using various strengths of Luria broth. AHL was detected from culture effluent samples from 12 hours of culture

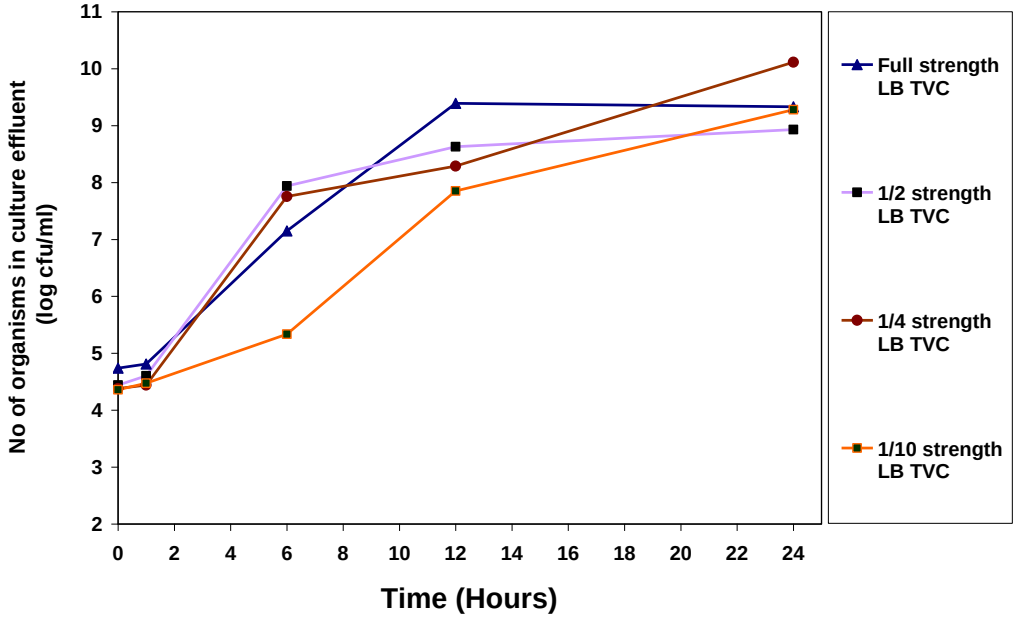

Figure 3.10 The wound model continuous cultures of a non- biofilm forming *P. aeruginosa* wound isolate (P.51) with various concentrations of Luria broth. AHL was not detected in the culture samples during the period of experiment.

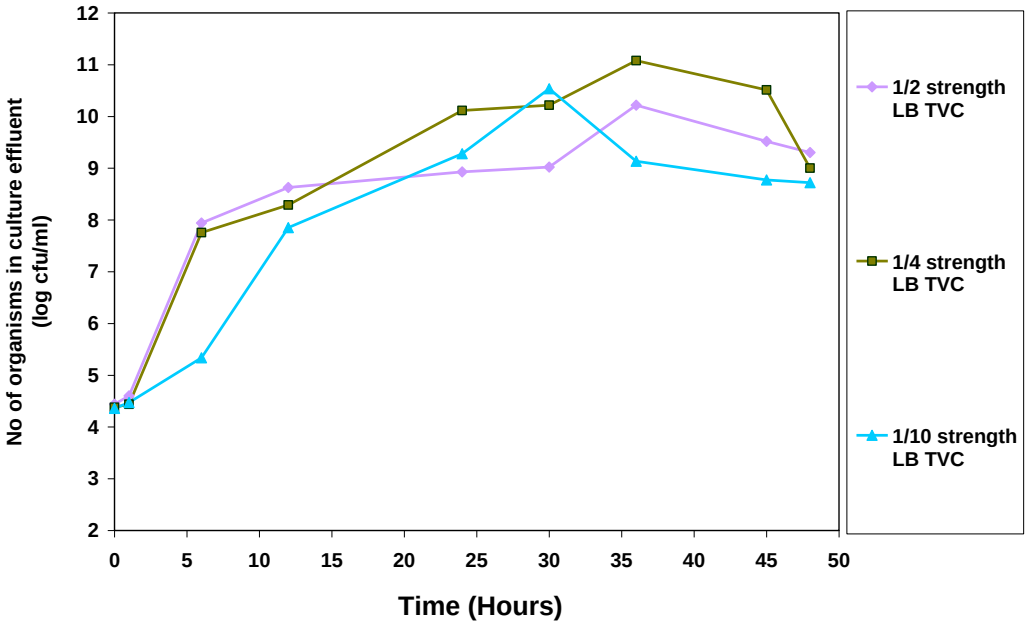

Figure 3.11 The graphical representation of the continuous cultures of a non-biofilm forming *P. aeruginosa* wound isolate (P. 51) in the wound model using various strengths of Luria broth for a period of 48 hours. AHL was not detected in any of the culture samples throughout the experiment. Higher concentration of broth produced higher numbers of organisms.

Table 3.3 Detection of AHL during the development of *Pseudomonas aeruginosa* biofilm in wound model continuous cultures.

Variables (Media and Flow rate)	**Organism**	**Time of AHL detection**	**TVC (log cfu/ml)**
Full strength LB	(isolate P. 98)	12 hours	9.6×10^{9}
½ strength LB	(isolate P. 98)	12 hours	2.36×10^{8}
¼ strength LB	(isolate P. 98)	12 hours	1.78×10^{7}
$^{1}/_{10}$ strength LB	(isolate P. 98)	12 - 21 hours	1×10^{9}
Full strength LB + FCS (1:1)	(isolate P. 98)	16 hours	3.3×10^{10}
FCS +12.5%LB (20 % FCS in 1/10 LB)	(isolate P. 98)	24 hours	1.99×10^{9}
1/10 LB (High flow rate)	(isolate P. 98)	30 hours	1.02×10^{10}
Full strength LB	(NCIB 8626)	22 hours	6.46×10^{11}

3.3.4 Effect of broth flow rate

Ten fold increase in the rate of broth flow to the culture provided increase availability of nutrient for bacterial growth which increased the rate of discharge of culture effluent from the wound model which had effect on the organisms‟ growth rate. Both the biofilm forming and non-biofilm forming *P. aeruginosa* wound isolates for low and high flow rates (Fig. 3.12) showed similar growth curves in the wound model. Whereas the biofilm forming isolate (P. 98) grew at a slower rate during the lag phase of the culture (prolonged lag phase), the non-biofilm forming isolate (P. 51) grew rapidly at the onset of the culture. The growth of P.51 increased more rapidly with a longer log phase at the higher 84 mls/hour flow rate than that of the 8.4 mls/hr flow rate. Whereas P. 98 had a longer lag phase and decreased growth output during the log phase with the 84 mls/hour flow rate although the growth curve for each isolate remained similar for the two flow rates. The number of dislodged organisms from the gauze after culture was significantly higher when 8.4 ml/hour flow rate was applied; log cfu/ml was 1.03×10^{10} whereas that for the 84 mls/hr flow rate was 7.2×10^{8} (Table 3.4).

AHL detection with the 84 mls/hour flow rate was from 30 hours at the bacterial count of 1.02×10^{10} cfu/ml of culture whereas AHL was detected at 21 hours when 8.4ml/hour flow rate was applied with population of 1×10^{9} cfu/ml of culture (Table 3.3 and fig. 3.13). The non - biofilm former tested negative for AHL production throughout the duration of the culture.

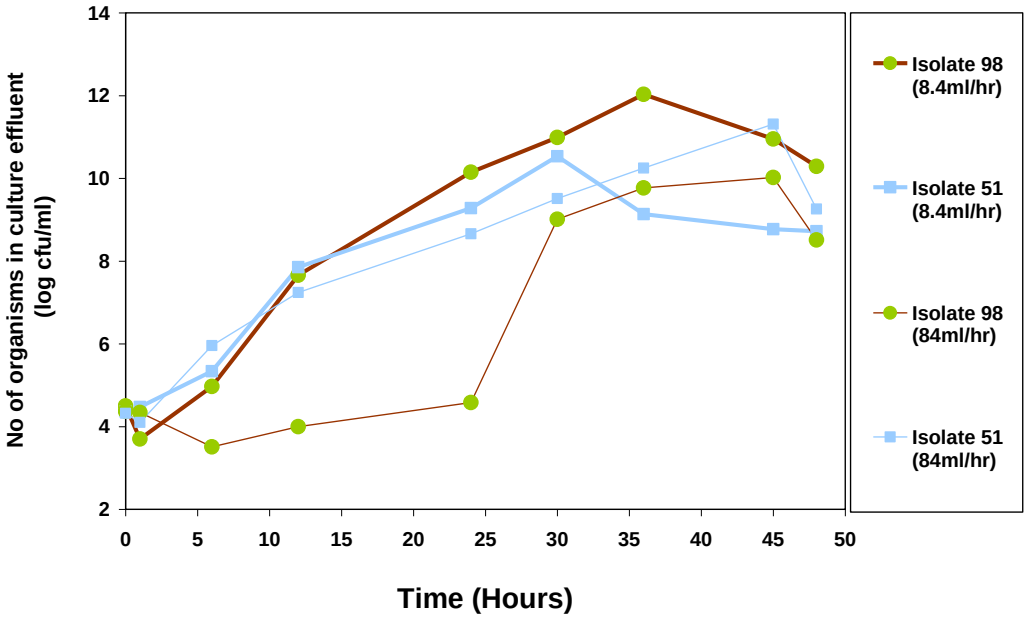

Figure 3.12 Effect of broth flow rate on phenotypic expressions of *P. aeruginosa* strains in continuous culture wound model. The growth curves of a biofilm forming (P.98) and non-biofilm forming (P51) wound isolates using 2 different broth flow rates.

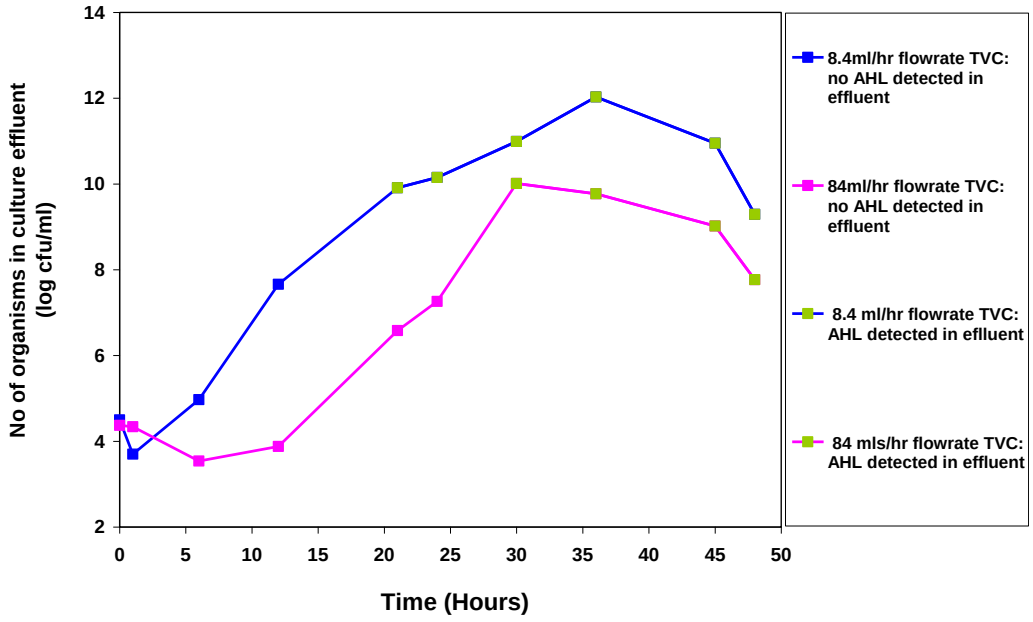

Figure 3.13 Effect of flow rate on *P. aeruginosa* wound isolate biofilm formation in continuous culture wound model. The growth curves the isolate (P.98) using 2 different broth flow rates are shown. AHL production was detected at 21 hours at the lower flow rate whereas at 30 hours at the higher flow rate.

Table 3.4 Organisms released from *P. aeruginosa* (P.98) wound isolate biofilm culture at different broth flow rates in a continuous culture wound model using $^{1}/_{10}$ LB.

Broth flow rate (mls/hr) Using $^{1}/_{10}$ LB	**Organisms released from gauze after 48 hours culture at 37°C (Log cfu/ml)**
8.4	1.03×10^{10}
84	7.2×10^{8}

3.3.5 Reproducibility of assays

The reproducibility of the analysis was examined by culturing a strong biofilm forming *P. aeruginosa* wound isolate (P. 98) in the wound model using 1/10 strength Luria broth on 3 different occasions. The TVCs were determined for each effluent culture sample; the mean of the 3 assays including the standard deviations (SD) were calculated and presented in Fig. 3.14. AHL was detectable in the wound model culture effluent samples from 21 hours when the bacterial population was above 1×10^{9} per ml of culture effluent and in the gauze at the end of each culture (Figure 3.13 and Table3.2). Statistical analysis of variance using Minitab (version 15) one-way ANOVA (unstacked), $p < 0.001$ (95% CIs for mean based on pooled SD).

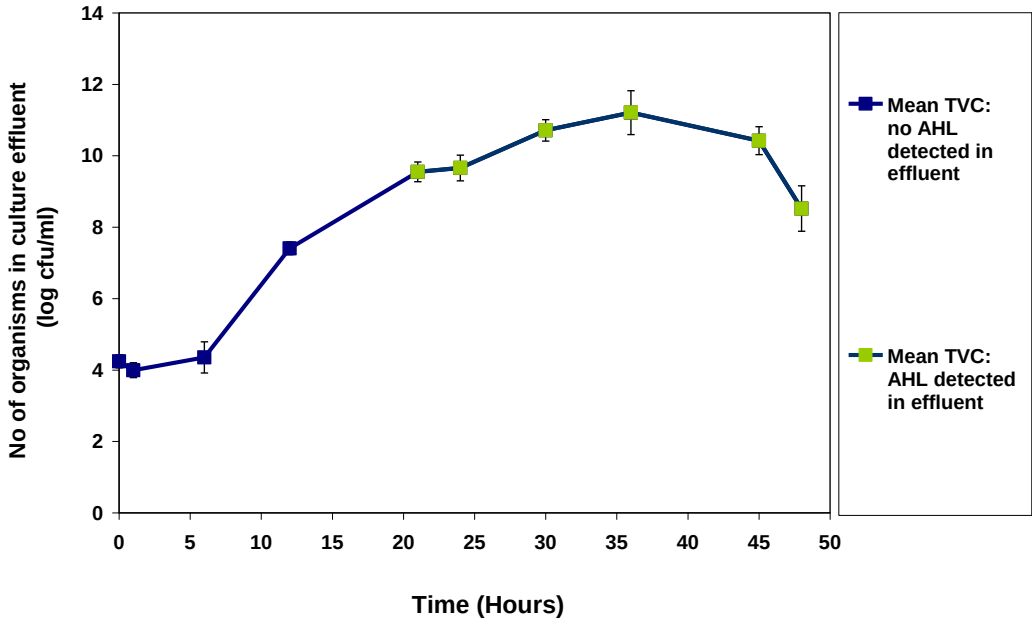

Figure 3.14 Graph of reproducibility assay of a biofilm forming *P. aeruginosa* wound isolate in wound model continuous cultures. The error bars indicate the standard deviations between the sets of bacterial populations for the 3 separate experiments. AHL was detected from 21 hours for the 3 cultures at TVC of over log10 ^{9} cfu/ml of culture effluent.

3.3.6 Effect of serum on biofilm formation and AHL production

In this study it was intended to simulate the circumstances that exist in wound as closely as possible. In the wound there is blood and the components of blood which are contained in the serum particularly the immune components such as complement, interferon or antibodies which might interfere with the metabolic activities of the organisms. The presence of such agents might have an effect on the development of biofilm structure and or production of quorum sensing molecules.

When P. 98 was cultured in the continuous wound model with full strength LB, AHL was detected at 12 hours when TVC was 9.6 x10^{9} cfu/ml of culture sample but when FCS was added to the LB AHL was detected at 16 hours with TVC of 3.3x10^{10} cfu/ ml of culture sample (Fig. 3.15; Table 3.3). Similarly using 1/10 LB and P.98 AHL was first detected between 12 and 21 hours with TVC of between 4.6 x10^{7} and 1x10^{9} cfu/ml

of culture but when FCS (12.5% v/v) was added to LB at LB concentration of $^{1}/_{10}$ AHL was detected at 24 hours when the bacterial population was up to $1.99x10^{9}$ cfu/ml of culure (Fig. 3.16; Table 3.3). Similarly the organisms released from gauze after each culture showed higher number for LB (1.63×10^{10}) and $^{1}/_{10}$ LB(1.03×10^{10}cfu/ml) than FCS and LB (1:1) and FCS and $^{1}/_{8}$ LB (1:4) which were (7.7×10^{9}) and (2.45×10^{9}) respectively (Table 3.5).

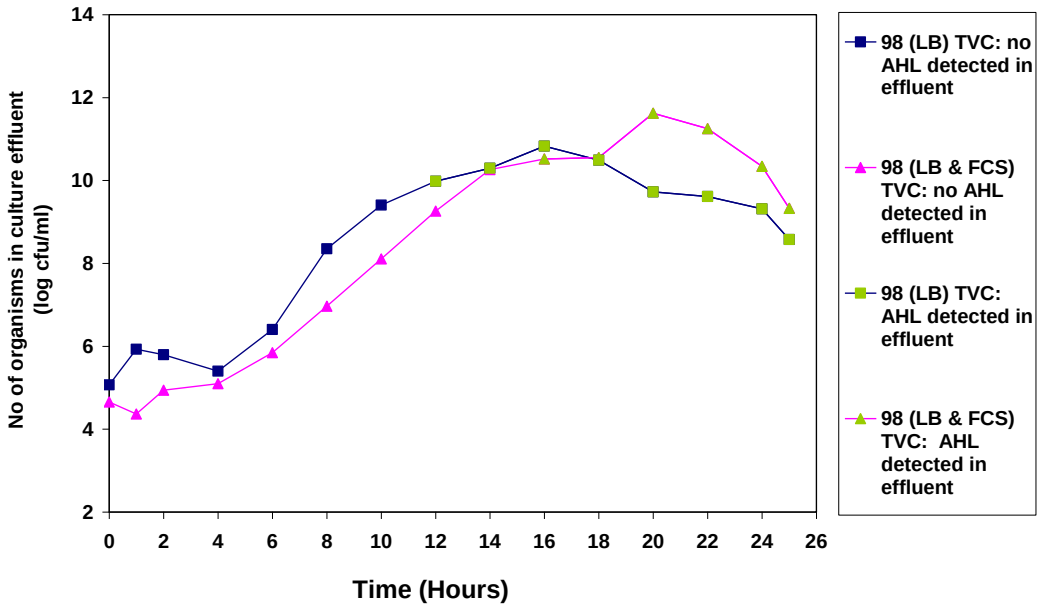

Figure 3.15: The effect of serum on growth of P. 98 and AHL production in the continuous culture wound model. AHL production by the isolate was detected at 12 hours in full strength LB, while AHL was detected 16 hours in LB/FCS (1:1).

Table 3.5 Populations of bacteria released from gauze after each culture of P.98 biofilm using LB, or LB and FCS in wound model.

Culture media	**30 sec vortex (Log cfu/ml)**
LB	1.63×10^{10}
$^{1}/_{10}$ LB	1.03×10^{10}
FCS and LB (1:1)	7.7×10^{9}
FCS and LB (1:4)	2.45×10^{9}

It was found in this study that the addition of FCS to the culture medium did not prevent growth and AHL production but the time of detection of AHL were affected by the concentration of the FCS.

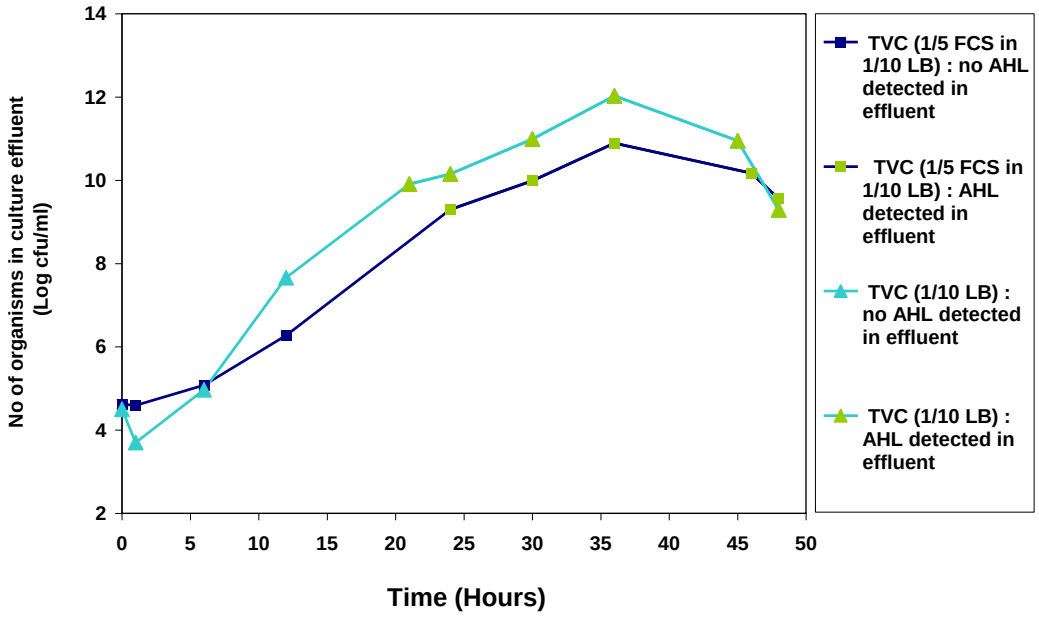

Figure 3.16 Comparison of the growth curve and AHL production of a biofilm forming *P aeruginosa* wound isolate P. 98, in a wound model continuous culture using LB, FCS and LB (1:1) and $^{1}/_{5}$ faecal calf serum in $^{1}/_{10}$ Luria broth.

3.3.7 Biofilm structure

3.3.7.1 Light microscopic examination of biofilm culture from the wound model

Stained slide smears of the culture effluent samples from the wound model showed the progressive stages of biofilm formation, some of which are presented in fig. 3.17. There is a relationship between AHL production and biofilm formation with time. The detection of AHL in the culture effluents at 12 or 21 hours coincided with detection of biofilm microcolonies and EPS in model effluent (Fig. 3.17) when 8.4 ml/hr flow rate was used for the wound model continuous culture. AHL was detected at 30 hours in the cultures with 84 ml/hr flow rate when biofilm was visible. The details of the time of AHL detection in the corresponding cultures of the biofilm forming *P. aeruginosa* isolate and reference *P. aeruginosa* NCIB 8626 are detailed in Table 3.3. Biofilm

microcolonies became larger and EPS formation increased with time in the culture effluent of the biofilm forming isolate (P. 98). There was evidence of mature biofilm from 36 hours and high rate of dispersion of organisms from the biofilm with the 8.4 ml/hr flow rate cultures. The culture usually entered the decline phase shortly at this period (Fig. 3.8; 3.9; 3.12; 3.13; 3.14; 3.15 and 3.16). The non- biofilm forming isolate (P. 51) did not produce AHL or biofilm throughout the period of experiment.

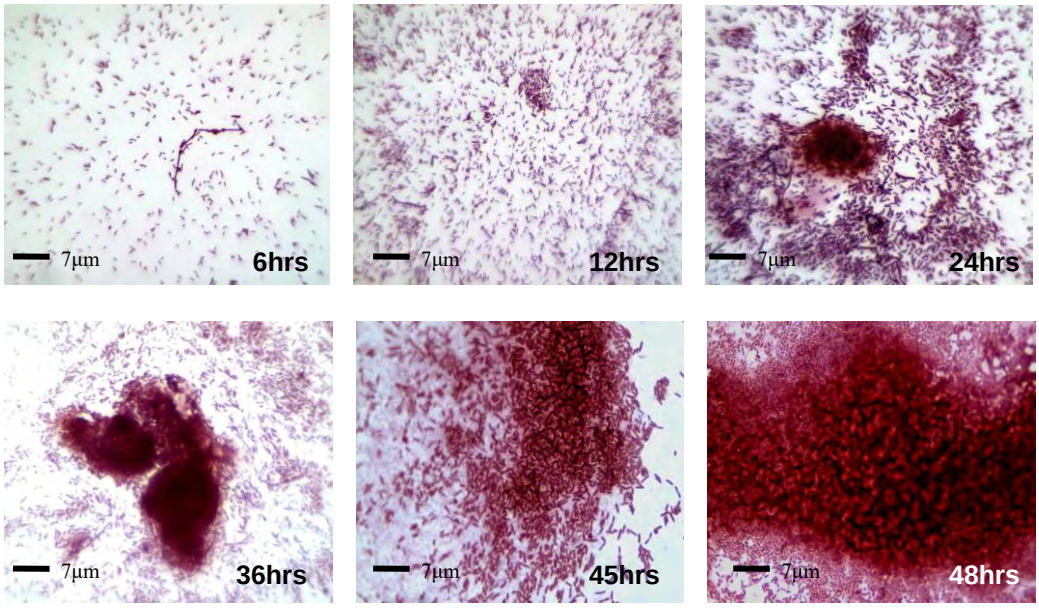

Figure 3.17: Biofilm of *P. aeruginosa* wound isolate (P.98) in a wound model continuous culture over a period of 48 hours. Microscopic examination of stained slide smears taken every 3 to 6 hours during culture showing progressive development of biofilm micro-colonies and increase EPS production.

3.3.7.2 SEM examination of biofilm culture materials from the wound model

The scanning electron microscopy of the gauze removed from the wound model at 30 hour with biofilm forming *P. aeruginosa* wound isolate (P. 98) revealed the presence of biofilm in the form of microcolonies that were attached to the gauze fibres, as well as what appeared to be extracellular polymeric substances (EPS) (Figure 3. 18 A to C). The gauze from the 30 hour culture of a non- biofilm forming *P aeruginosa* wound isolate (P. 51) did not show any micro - colony or alginate (Figure 3.18 D to F).

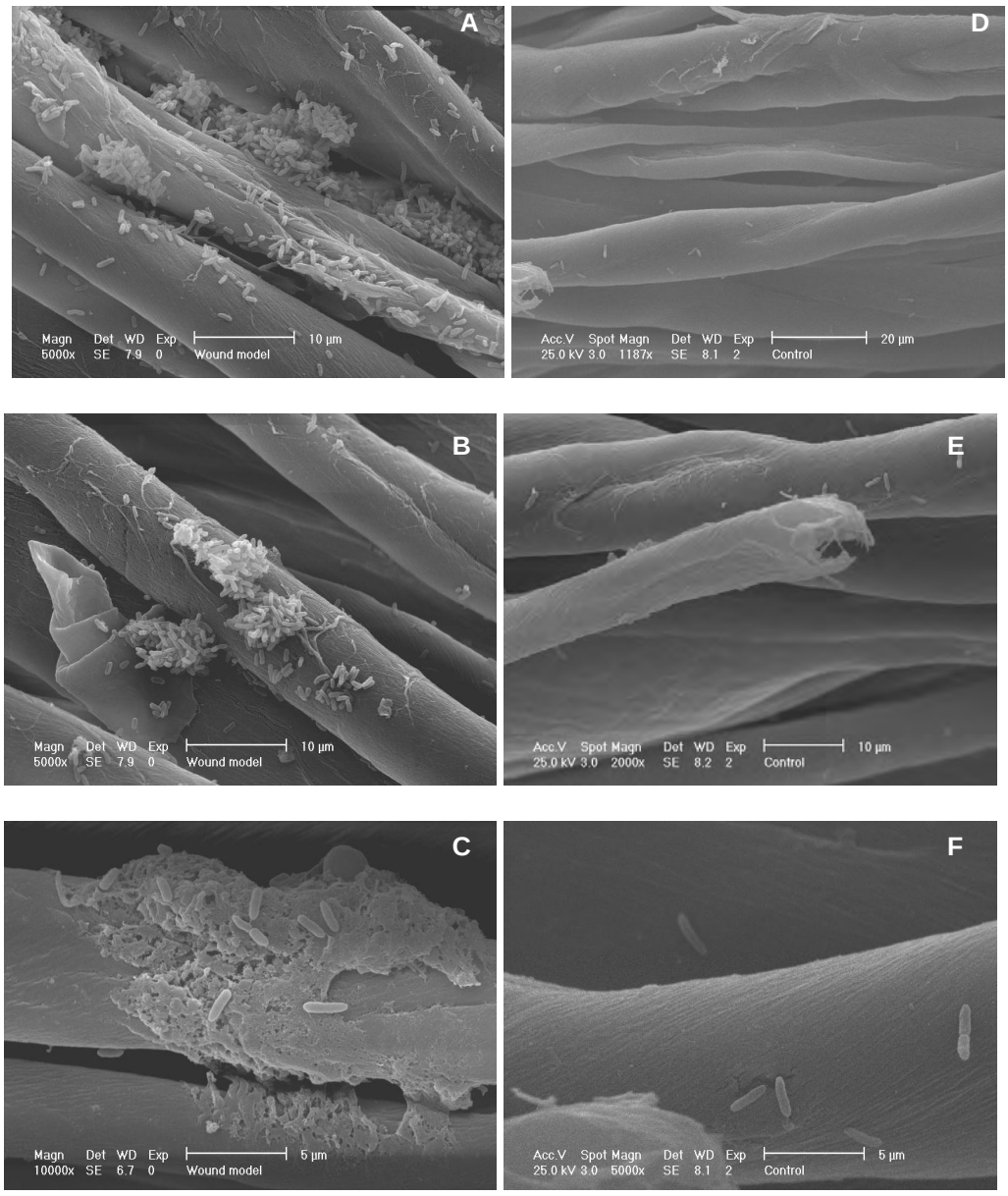

Figure 3.18 Scanning electron micrographs of gauze from 30 hour wound model continuous cultures of *Pseudomonas aeruginosa* biofilm and non – biofilm forming wound isolates. Micrographs (A and B) showing biofilm microcolonies of the biofilm forming isolate culture (P. 98) attached to the gauze fibres and (C) some bacteria that were attached to EPS on gauze fibre. Micrographs (D to F) are showing the non – biofilm forming wound isolate (P. 51) with few organisms attached to the strands of the gauze, with no evidence of microcolony of extra-polymeric substances (EPS).

3.4 Discussion

Biofilms have been of particular interest in medicine because they have been implicated in 65% of human infections (Potera, 1999) and about 80% of persistent infections (Lewis, 2001). In fact the problems of failure to heal often encountered in chronic wounds have been attributed to the presence of biofilms in such wounds (Bjarnsholt, 2008). Recently, the presence of biofilm in wounds was unequivocally established (Malic *et al,* 2009; James *et al,* 2008; Ngo *et al*, 2007). Wound associated biofilms have been found to be polymicrobial and the cumulative effect of the destructive processes encountered from the release of enzymes and toxins by the organisms often exert more devastating effect on the wound which further prevent healing. Although the location of wounds (superficial or deep seated) and the viability of the tissues can sometimes influence production of exudates the effect of bacterial virulence and proteolytic activities mostly result in inflammation and production of higher exudates (Wilson *et al*, 2002). Some organisms such as *Pseudomonas aeruginosa* that are commonly isolated from wounds have been found to stimulate the release of heparin binding protein (HBP) from neutrophils thereby aggravating chronic inflammation by increasing endothelial hyper-permeability (Lundqvist *et al,* 2004). The amount of exudates produced by any type of wound would therefore depend on various factors. Quorum sensing is an important factor in the regulation of microbial phenotypic expressions and has been found to play a significant role in the pathogenicity of microorganisms of medical importance. The ability of most of these pathogenic organisms to form biofilm has been associated with the production and response to quorum sensing molecules (Parsek and Greenberg, 2005; De Kievit *et al,* 2001) and detection of quorum sensing in biofilm has been established *in vivo* and *in vitro* (Singh *et al*, 2000; Stickler *et al,* 1998).

The purpose for this chapter was to explore the possibility of growing isolated *P. aeruginosa* from wounds in a continuous culture in which nutrients will be supplied

to simulate the condition in the wound. The wound model was to provide the environment to study the ability of the isolates to form biofilm *in vitro* and produce quorum sensing molecules. The deductions from the experiment would be a pointer to the pathogenic role of the organisms within the wound environment particularly biofilm formation and signalling molecules production.

The batch cultures of the biofilm forming *P. aeruginosa* NCIB 8626 and the wound isolate (P. 98) in flasks containing pieces of gauze indicated that AHL could be detected while the cultures of the non- biofilm forming *P. aeruginosa* wound isolate (P. 51) tested negative for AHL. The detection of the AHL produced by P. 98 was weakly detected when the gauze was placed 1 cm away from the reporter organism *A. tumefaciens* (Fig. 3.3D) and reference organism *P. aeruginosa* NCIB 8626 showed a negative result (Fig. 3.3 F. When the gauze from the cultures were placed close to *A. tumefaciens* (1-2 mm distance) both isolate P. 98 and *P. aeruginosa* NCIB 8626 tested positive (Fig. 3.4 D and F) and isolate P. 98 showing stronger reaction than *P. aeruginosa* NCIB 8626. In solid culture where the method is normally used the population of organisms is high and the production of the AHL is easily detectable 1 cm away from the reporter organism whereas in broth the AHL secreted by organisms becomes diluted as it is being produced. It was imperative to modify the procedure to suit the material being used in this study. The second factor that affected the result was the concentration gradient as a substance diffused through a medium. AHL from the gauze would have to diffuse through the agar to reach the reporter organism to initiate the expression of lac operon gene in the reporter organism, a process that further diluted the concentration of the AHL. The resultant effect is that the concentration of AHL reduced as it diffused through the agar farther away from the gauze and detection

becomes weaker and more difficult. In order to detect AHL in the gauze it was vital to place the gauze close to the reporter bacterium.

In order to have the adequate amount of AHL within the agar on diffusion to initiate a gene switch in *A. tumefaciens* that will be visibly detectable it became necessary to determine the ideal distance between the reporter organism and the gauze for detecting AHL. The distance between the gauze and the reporter organism within which AHL could be detected was determined for a strong AHL producing biofilm forming *P. aeruginosa* (isolate P. 98) and a low AHL producing biofilm forming *P. aeruginosa* NCIB 8626. The detection assays for AHL in gauze removed from the biofilm cultures showed that it was ideal to place the gauze as close as possible to the reporter organism *A. tumefaciens*, (Fig. 3. 4 D and F; Fig. 3. 5 A and B) otherwise low concentrations of AHL might not be detectable (Fig. 3.3F and 3.5 B). It was apparent that the biofilm forming isolate P. 98 and the reference organism *P. aeruginosa* NCIB 8626 produced AHL in the batch culture and the AHL was detectable when the gauze was placed beside the reporter organism *A. tumefaciens.* However when the gauze was placed further away from reporter organism, the detection was weak or impossible particularly when AHL concentrations are low. The gauze from the culture of non- biofilm forming and non AHL producing *P. aeruginosa* wound isolate tested negative (Fig. 3.3E and 3.4 E) regardless of the distance.

Biofilm and non- biofilm *P. aeruginosa* wound isolates and the reference organism were cultivated in the continuous culture wound model and the phenotypic diversity of the strains were observed. Detection of AHL by the biofilm forming *P. aeruginosa* wound isolate (P. 98) was dependent on media and flow rate of broth. AHL was detected in wound model continuous cultures at the period when the populations of the bacteria were higher than $1x10^7$ cfu/ml with broths of ¼ strength to full strength broth

and mostly around the late log phase and the beginning of stationary phase (Fig. 3.8; 3.9; 3.12 and 3.13, and Table 3.3). At those periods when AHL was detected, microscopic examination of the stained culture effluent slide smears showed the presence of biofilm structures (Fig. 3.17) and the scanning electron microscopic examination of the culture material (gauze) confirmed its presence (Fig. 3.18). These observations concur with other studies that production of virulence factors such as AHL production, biofilm formation, production of cytotoxic enzymes and toxins by pathogenic microorganisms occur during the late exponential and beginning of stationary phases (Mari *et al*, 2006; Thompson *et al*, 2003; Lundberg *et al*, 1999). It has also been observed that some bacteria usually develop resistance to antimicrobials during this period (Siegele and Kolter, 1992).

Detection of AHL and biofilm formation became obvious when the nutrients in the medium were becoming limited as the population of the bacteria within the wound model increased as observed in this study. Evidence from studies has shown that the higher the depletion of nutrient the greater the increase in adhesion of *P. aeruginosa* to surfaces due to the production of EPS and biofilm formation (Soo *et al*, 2009; Ryu *et al,* 2004). EPS production was observed with the features of biofilm at population of over$1x10^7$ cfu/ml of culture effluent when quorum sensing communication was detected in the cultures. This coincidence between the detection of AHL in cultures and the detection of biofilm features like EPS and microcolonies was in line with others that indicated that AHL is responded to by biofilm forming organisms such as *P. aeruginosa* to form biofilm (De Kievit *et al,* 2001; Davies *et al*, 1998) indicating the importance of signalling molecules in the regulation of genes in biofilm organisms. Kiristis and colleagues (2007) also observed full QS induction of biofilm cultures at bacterial population of $6x10^7$ CFU/ml of cultures in laminar flow rates of 0.04ml/min and 0.4mlm/min biofilm cultures as observed in this study. At the stationary phase, though

the growth of the organisms was low and the population was reducing but biofilm growth was increasing particularly the size of EPS (Fig. 3.17) because the nutrient was diverted into EPS production which was in line with the study done by Tempest and colleagues (1981).

The findings from this study suggests that AHL can be detected in gauze and if dressings which are made of similar materials are screened for biofilm markers such as AHL the presence of biofilm in such wounds could be determined. Other scientific findings have utilised the presence of biofilm markers to confirm the presence of biofilm in urine catheters (Stickler *et al,* 1998) and in cystic fibrosis patient‟s lungs by examining the sputum (Singh *et al*, 2000).

It was evident that the continuous wound model cultures supported the growth of all the bacteria studied and the results were consistent. The reliability of the model was demonstrated through the phenotypic characteristics of the biofilm forming *P. aeruginosa* under study which was shown by the reproducibility assay (Fig. 3.14).

The biofilm - forming phenotypes *P. aeruginosa* isolate P. 98 and the reference organism *P. aeruginosa* NCIB 8626 produced AHL (Fig. 3.4; 3.5; 3.6; 3.7; 3.8; 3.9; 3.12 and 3.14) and formed biofilm (Fig. 3.17 and 3.18 A to C) while the non- biofilm forming phenotype *P. aeruginosa* isolate P. 51 did not produce AHL (Fig. 3.4; 3.5; 3.10 and 3.11) or form biofilm (Fig. 3.18 D to F). The confirmation of biofilm structures such as the biofilm microcolonies and extracellular polymeric substances (EPS) from the stained slides (Fig. 3.17) and with electron microscopic scanning of the gauze from wound model culture (Fig. 3.18) provided the evidence of this study. This finding has been supported by other studies that demonstrated the presence of biofilm by staining the EPS in biofilm matrix (Harrison - Balestra *et al*, 2003) and electron scanning microscopy (Chandra *et al*, 2008; Stickler *et al*, 1998).

This continuous culture wound model can be used to culture biofilm forming bacteria in order to study phenotypic characteristics of various strains.

P. aeruginosa biofilm requires minimal nutrients for growth (Waite and Curtis, 2009) and the $^{1}/_{10}$ LB provided the ideal medium for the continuous cultures of *P. aeruginosa* in the wound model. The growth patterns of the biofilm forming isolate and the reference organisms during the continuous biofilm cultures showed similar growth patterns but the time of AHL detection and biofilm formation differed with the nutritional supply (Fig.3.13). Biofilm formation was observed when the population of the bacteria had increased to a point that the nutrient was becoming limited to all the organisms usually from $1.78x10^{7}$ cfu/ml of culture effluent. It has been shown that nutritional depletion enhances biofilm formation (Cowell *et al,* 1999) which was observed in this study that biofilm structures was detected when the population was high relative to the supply of nutrient (Fig. 3.6; 3.8; 3.9; 3.13 and 3.14). Cowell and others observed that when the nutrient (nitrogen source) was reduced the attachment of cells and biofilm formation became higher and lipopolysccharide increased the attachment of carbon limited cells to surfaces confirming the role of EPS on biofilm formation. In this study, EPS was observed at the stages when the population was overwhelming for the nutrition and the biofilm grew bigger even at the stationary phase due to more production of EPS.

At the mid / end of log phase when the bacterial population was high relative to nutrient availability, AHL production was detected in the cultures and this coincided with the appearance of biofilm structures in the culture effluent stained smears. When the nutritional supply was increased by increasing the rate at which the broth was being delivered to the culture, the period at which the biofilm structures appeared was longer (from 12 hours to 21 hours) for biofilm structures and AHL detection to 18 - 30 hours

for biofilm structures and AHL detection. The results show that biofilm formation is dependent on nutritional stress.

The variation of concentration of LB reduced the nutrients available to culture but did not prevent biofilm formation. However, population of organisms in biofilm reduced with concentration of LB. Various stages of biofilm formation were observed with varying strengths of LB and AHL production was observed at 12 hours when full strength LB, ½ strength and ¼ strength LB were supplied to the culture whereas AHL production was observed at 21 hours with $^{1}/_{10}$ LB. No samples were taken between 13 and 20 hours for the detection of AHL. In a similar study of biofilm formation in a flow-through system, it was observed that difference in media composition did not markedly affect biofilm formation (De Kievit *et al*, 2001).

The increase in nutritional supply due to increased broth flow rate caused reduction in the growth of the biofilm relative to time when compared with the lower flow rate. The nutrients available to the organisms at that period were in abundance which would reduce nutritional stress and probably increased the expression of certain genes. Increase in nutritional supply was observed to aid the dispersion of *P. aeruginosa* biofilm (Sauer *et al*, 2004) as this tends to increase the expression of the flagella activities while reducing the expression of pili activities. This was observed in this study when the flow rate of the broth was increased ten fold thus increasing the supply of nutrients to the organism.

The intensity of the AHL detection was reduced in the FCS and LB (1:1) culture with a final LB concentration of ½ in the batch culture; this may not be due to dilution of the nutrient since the intensity in the $^{1}/_{10}$ LB had visible level of AHL in the continuous culture but the effect of the serum on the organism‟s biofilm formation. The reduction in the rate of biofilm development of the *P. aeruginosa* isolate P. 98 and longer time

before the AHL was detected in the wound model continuous culture was possibly due to the inhibitory properties of serum constituents which might have delayed biofilm formation by the bacteria. Although foetal calf serum that was used in this study delayed biofilm formation it did not inhibit biofilm formation. This was contrary to other findings that observed inhibition of biofim by human serum. Hammond and her colleagues (2008) found that serum was inhibitory to *P. aeruginosa* biofilm formation on plastics surfaces and intravenous catheters and bovine serum caused a significant decrease in biofilm development and reduced an already-developed PAO1 biofilm. The fact that clinical isolates are usually more virulent than environmental or wild type strains might be the reason for the major factor why the biofilm was not inhibited by the serum as observed in this study.

3.5 Conclusion

In this chapter it has been shown that gauze can be used as a substratum in a wound model for the detection of biofilm markers such as AHL and EPS. It is therefore possible that dressings obtained from wounds infected with biofilm might contain biofilm markers which can be detected in the laboratory.

Biofilm structures were demonstrated in the culture effluents and on the gauze strands microscopically. Wound dressings can also be examined for biofilm markers such as AHL, microcolonies and EPS.

The wound model system was a simple and convenient method for studying biofilm and could be used for testing efficacy of antimicrobial agents against microorganisms in biofilm cultures.

Chapter 4

Detection of biofilm markers in chronic wound dressings

4.1 Introduction

Bacteria exist in many different phenotypes, planktonic (free floating) and the sessile forms but much attention has been given to the planktonic ones and the sessile forms which are associated with biofilm formation are yet to be fully understood. Biofilms have been mostly studied in human infections and colonisation of medical devices including dental caries, endocarditis, urinary tract infections and chronic bronchitis particularly in cystic fibrosis while recent studies are focusing on the elucidation of the pathogenesis of biofilm in wounds. Application of biofilm science to the study bacterial infections has been suggested as an important factor in the control of chronic infections (Costerton *et al*, 2003). Characteristic features of biofilm such as extracellular polymeric substances (EPS), signalling molecules and microcolonies have been used to identify the presence of biofilm *in vitro* and *in vivo*.

The presence of bacterial biofilms in catheters that have been indwelling from three to eighty-three days was established using electron scanning microscopy (Ganderton *et al*, 1992). There was evidence of biofilm microcolonies in 44 of the 50 catheters and bacterial composition ranged from single to multispecies. There was no correlation between biofilm formation and the number of days the catheter had been in situ indicating that biofilm can be formed within a short period of time. Another study examined the ability of Gram negative bacilli isolated from catheters to produce quorum sensing molecules such as AHL. The isolated *P. aeruginosa* gave strong positive reactions through cross feeing assay while other isolates showed weak or negative results (Stickler *et al,* 1998). Cultures of *P. aeruginosa* isolated from patient catheter were cultivated in artificial urine in the laboratory in which catheters were incorporated. The sections of the catheters both cleaned and the sections not cleaned of biofilm showed positive reactions to AHL detection assay. Production of AHL was determined in nine catheters colonised by biofilm and freshly removed from patients, of which four

tested positive for AHL. The results of the catheters removed from patients then confirmed that the AHLs that were detected were produced by the organisms *in situ* in the bladder (Stickler *et al*, 1998). Singh and colleagues (2000) examined the sputum of cystic fibrosis patients and detected QS molecules and *P. aeruginosa*. When the isolates were later grown in the laboratory, they produced quorum-sensing signal to indicate the presence of biofilm in the lungs of cystic fibrosis patients and that quorum-sensing signal has been claimed to serve as a biofilm marker. Using samples of the lung tissues *P. aeruginosa* infection was confirmed by AHL detection (Favre – Bonte *et al*, 2002). In that study, it was also found the level of AHL *in vivo* was considerably lower than detected *in vitro*. In another study, Ramseier and colleagues (2009) examined 100 human subjects and used host and microbially derived biomarkers to identify periodontal disease status from whole saliva and plaque biofilm. Salivary biomarker data were correlated to comprehensive clinical, radiographic, and microbial plaque biofilm levels.

Traditionally, diagnosis of wound infections has mostly relied on clinical symptoms and recalcitrant infections have been investigated with swabs cultures. However, the use of biopsies has been advocated because of the opinion that swab samples do not provide opportunity to fully isolate the microorganisms usually associated with wound infections. It has also been shown that some organisms are not cultivable in the laboratory due to the fastidious nature of such organisms (Frankel *et al*, 2009). Furthermore, it is known that currently available growth media for isolating and identifying organisms in wounds would isolate and identify about 5% of bacterial species in wounds (Amann *et al*, 2001; Moter and Goebel, 2000).

The distribution and ecology of bacteria in chronic wounds using fluorescent in situ hybridization (FISH) was studied and no good correlation was found between bacteria detected by culturing methods and FISH (Kirketerp – Moller *et al*, 2008). Patients for

the study were selected based on suspicion of *P. aeruginosa* infection due to colour, odour and suspicion from an experience surgeon. Results showed that standard culturing techniques showed 86% (19) of the 22 wounds were colonised with bacteria; 12 (60%) of which were colonised with *S. aureus* and less than 30% (5) were colonised wit *P. aeruginosa*. Contrarily, FISH revealed 60% (13) of the wounds as being colonised of which 15% (2) were *S. aureus* and 70% (9) were of *P. aeruginosa*. Examination of the slides with PNA-FISH technique showed that *S. aureus* were located on the surface while *P. aeruginosa* were situated inside the wound beds. The importance of non - uniform distribution of microorganisms within the wounds was highlighted. The spatial organization of *Pseudomonas aeruginosa* and *S. aureus* in chronic wounds was investigated by obtaining wound biopsies and using peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH) and confocal laser scanning microscopy (CLSM) to examine the sections (Fazli *et al*, 2009). The distance of the bacterial aggregates to the wound surface were measured and the distance of *P. aeruginosa* aggregates to the wound surface was found to be significantly higher than that of *S. aureus* aggregates indicating that the distribution of organisms in chronic wounds was non-random thus swab culturing techniques may underestimate the presence of organisms that are embedded in tissues. The study thus suggests the inadequacy of swab samples in diagnosis of wound infections.

However, some practitioners are still of the opinion that cultural methods and use of swab samples are still valuable in diagnosis of infection. Hence studies have been performed to compare biopsy samples with swab samples in evaluating the microbial burden of wounds. A comprehensive study of the microbiology of chronic venous leg ulcers to evaluate the clinical predictive value of tissue biopsies and swabs was done (Davies *et al*, 2007). The study compared the usefulness of surface swabs against tissue biopsies. The microbiota of 70 chronic venous leg ulcers was quantified with swab

samples and biopsies. Regression analysis provided no additional prognostic information when compared with analysis of surface microbiota since both sampling methods gave almost the same microbial yield. Patients‟ response to colonisation was considered more important than the number of bacteria within the wound. The predominant organisms isolated were *P. aeruginosa*, (34.8% from swabs and 31.8% from biopsy), *S. aureus* (71.2 % from both tissues and swab samples) and *Candida albicans* (10.6%). Isolation of organisms suggested the role of the organisms in impeding wound healing. It was concluded that the use of biopsies might not contribute significantly to the patient management hence their use should be discouraged in clinically uninfected wounds.

A pilot study in which the assessment of the microbiota of locally infected venous leg ulcers were investigated using three sampling methods to compare the distribution of microbial flora within the wounds (Cooper *et al*, 2009) showed a contrary view. The swab gave the highest number of isolated organism followed by the polyvinyl acetate (PVA) foam disc while the biopsy provided the lowest recovery of organisms. The lowest agreement of colonisation and counts of microorganisms in the study was particularly obvious between the distribution of anaerobes, corynebacteria, *P. aeruginosa* and *S. aureus.* The study further increased the awareness that microorganisms are not evenly distributed within the wound and the recovery of organisms is dependent on the area of the wound sampled.

Despite these arguments about cultural methods, a vital issue in the diagnosis of wound infection is the inability to cultivate biofilm in the laboratory using the conventional cultural methods which has necessitated the use of other diagnostic methods which are not simple and direct and they are often expensive to diagnose biofilm in the laboratory. Examination of samples by microscopic examinations has been used mainly to confirm

the presence of biofilm in cultures, medical devices or implants such as catheters, tissue or biopsy samples from wounds. Other methods in use recently include fluorescent in situ hybridisation (FISH) and molecular methods such as bacterial tag encoded FLX amplicon pyrosequencing (bTEFAP) analysis. As sophisticated as these methods are, the common factor that affects the cultural methods still remains. The samples for these methods are the tissue biopsies taken from specific locations in the wounds which are still affected by the uneven distribution of organisms within the wound space, hence full isolation of all the microbial pathogens in a wound at any given time might not be possible. In this study therefore, wound dressings removed from chronic wounds were investigated for biofilm markers to determine the presence of biofilms in the wounds. Although, *in vitro* investigations provide the required information about the physiologic and virulence expressions of pathogenic organisms, the environment and factors that come to play in the host are usually different from those available *in vitro*. In order to further reveal host - pathogen effect in biofilm study in wounds, animal models have been used to shed more light into this relationship.

In rats, the presence of *P. aeruginosa* biofilm *in vivo* was confirmed by detection of AHL in the infected rats (Nakagami *et al*, 2008). Pressure induced wounds were created on 12 rats and then infected with *P. aeruginosa*. Quorum sensing signals were then quantified for each rat sample on days three and seven. Microscopic examination of tissue samples also revealed the presence of biofilm. The study suggested that QS contributes to establishment of chronic wound infections and detection of auto-inducers in chronic wounds could be a novel technique for diagnosing chronic wound infections. In the study AHL was not detectable in rats with low bacterial count ($<2 \times 10^6$ CFU/g) or high ($> 1.1 \times 10^8$) bacterial counts which could have affected the sensitivity of the assay. To further expand the knowledge on biofilm in wounds, partial thickness wounds were made on pigs with a dermatome and challenged with *P. aeruginosa* and

covered for 72 hours. Examination of Congo red stained curettage from the wounds revealed the organisms surrounded by EPS (Serralta *et al*, 2001). The wounds were flushed with sterile saline solution to dislodge the non-adherent bacteria and later scrubbed with sterile spatula. The flushed and the scrubbed wound materials were cultured and 2 phenotypes of bacterial populations were revealed, the biofilm phenotype from the scrubbed material were less in number than the organisms not in biofilm in the flushed samples which showed that two populations of organisms live in the wounds. The study also demonstrated *in vivo* the presence of biofilm in wounds. In fact this phenotypic variation in biofilm organisms has been linked to antibiotic resistance in pathogenic biofilm forming organisms such as *P. aeruginosa* (Drenkard and Ausubel, 2002). In 2008, Davis and his colleagues created a biofilm associated wound using a porcine model. *S. aureus* strain was inoculated into the partial thickness wounds. Wound biopsies were taken immediately after inoculating the wounds with the organism and 48 hours after inoculation. Biofilm-like structures in form of attached microcolonies and bacterial colonies encased in extracellular matrix attached to wound beds were observed after 48 hours on examination of the wounds biopsies with scanning electron microscopy and epifluorescent microscopy. The structural and physiological findings supported the hypothesis of the role of biofilm in wound colonisation and infection (Davis *et al*, 2008). The stained microcolonies with calcoflour white revealed bacterial colonies within the matrix when examined with light microscope. The study also compared the colony of bacteria grown on agar plate which had no amorphous matrix with the microcolony from the surface of the wound bed which were surrounded with amorphous matrix. The study has also shown that biofilm can be established in acute wounds soon after formation. In the same study the formed biofilm was found to be more resistant to antimicrobials compared to the planktonic counterparts.

To establish the ability of clinical isolates to form biofilm *in vitro*, Harrison – Balestra and her colleagues (2003) cultured *Pseudomonas aeruginosa* isolated from a human wound in the laboratory and formed biofilm within 10 hours of culture. Biofilm features were visualised by light microscopy after staining with saturated Congo red solution in Tween 80 and Ziehl carbol fuschin. The rapidity of biofilm formation by the bacteria suggested the ability of wound pathogens to form biofilm in wounds soon after colonisation to shield immune mechanisms and subsequently antimicrobial intervention.

Laboratory studies have mostly utilised monospecies biofilm concept whereas in natural environments and in the hosts mono-species biofilms are relatively rare but exist as a range of species. This polymicrobial existence of biofilms composed of mixtures of micro-organisms has been observed in wounds (Malic *et al*, 2009; James *et al*, 2008; Dowd *et al*, 2008; Ngo *et al,* 2007). The communal existence amongst the microorganisms aids the interspecies and intraspecies interactions and the molecular complexity within biofilm.

In humans, the presence of biofilm in chronic wounds has been established on microscopic examination of biopsy samples from chronic wounds which revealed the colonial aggregation of microorganisms surrounded by EPS matrix (Malic *et al*, 2009; James *et al*, 2008; Ngo *et al*, 2007). The investigators utilised multiple approach to the investigations using light microscope, scanning electron microscope (SEM), confocal laser microscope (CSLM) to visualise the microorganisms embedded within the biofilm matrix. A revelation about the presence of biofilm in human chronic wounds was first revealed when biopsy samples from 12 chronic wounds were observed with electron microscope and 7 (58.3%) of the 12 wounds had biofilms (Ngo *et al*, 2007). The study highlighted the inability of biopsy sampling to fully reveal the expected outcome of the investigation because microorganisms are usually not distributed evenly within the

wounds hence some positive results could have been missed out. The invasiveness of biopsy collection and discomfort to patient was discussed in the report of that study.

Fifty chronic wounds and 16 acute wounds biopsy specimens were examined using scanning electron microscopic technique for the presence of biofilm (James *et al*, 2008). Of the 50 chronic specimens, 30 (60%) revealed the presence of biofilms which appeared as large aggregates whereas biofilm was observed in only 1 of the 16 (6.25%) acute wound specimens. The samples were stained and examined with light microscopes and the chronic wound samples showed large aggregates of organisms while the acute wounds except 1 were mostly seen as individual cells. The biofilm aggregates were seen comprising of mixed species of organisms corroborating the polymicrobial nature of biofilm in wounds as well as the predominance of biofilm in chronic wounds. Malic and his colleagues (2009) examined biopsies of tissues from chronic wounds for identification of specific bacteria in the biofilm and also to characterise the species distribution of *in vitro* biofilms to demonstrate the applicability of the technique to tissues from chronic wounds. Characterization of bacterial populations and communities within the wound biofilm was done using fluorescent in situ hybridization (FISH) which allows individual bacteria in human disease states to be visualized and identified *in situ* and slides were examined with confocal laser microscopy (CSLM). The culture of multispecies biofilm in a biofilm model of constant depth film fermentor and biopsies from patients with non-infected CVLU wounds were examined. Examination of the multispecies biofilm revealed the predominance of *P. aeruginosa* (49±15.75%) which was distributed throughout the biofilm followed by *S. aureus* 5±7.86% that concentrated towards the surface of the biofilm and the cocci were the least in numbers. The finding demonstrated the presence of biofilm in both the *in vitro* cultures and the tissue biopsies. *P. aeruginosa* were seen in the deeper regions of the tissues. It was also observed that once the integrity of epithelial barrier of the skin

has been disrupted, the bacteria can invade the tissue. Uneven distribution of wound microbiota was also found in the study.

Efforts to utilise the various markers associated with biofilm to diagnose biofilm infection has led to a recent study in which quorum sensing molecules was used in the detection of biofilm in debridement materials from chronic wounds (Rickard *et al*, 2010). In the study, AHL and/or AI-2 signalling molecules were detected in 21 (70%) of 30 debridement samples, although the microbial contents of the debridement samples were not characterised. The ability of isolated organisms from other chronic wounds to produce quorum sensing signals (AHL and autoinducer-2 (AI-2) cell–cell signalling molecules were determined of which 69·6% were inferred to produce AI-2, while 19·6% were inferred to produce AHL molecules. It was suggested that the presence of AHLs and AI-2 could be used as a predictor of wound severity.

The electron microscope, the most frequently utilised approach in diagnostic procedures for biofilm detection is not available routinely and it is relatively expensive. Hence there is need for a simple and inexpensive method of screening wounds for the presence of biofilm. Collection of biopsy samples for microscopic examinations have been found to be invasive and associated with inconveniences for the patients. The time spent on collection of biopsies by medical and health practitioners is often longer unlike other samples such as swabs. Uneven distribution of organisms within the wound space could result in failure to identify biofilm.

During wound healing process exudates containing vital molecules and cells to support the healing are normally produced in wounds (Baker and Leaper, 2000). However the presence of virulent organisms within wound space especially in infected and chronic wounds often increases the amount of exudates (Falanga, 2000), the composition of which will include the organisms, degradative enzymes and toxins and other metabolic

products from the organisms as well as host‟s immune substances (White and Cutting, 2006).

It was therefore decided to investigate the presence of biofilm in wounds using an approach that would address the disadvantages identified above by examining dressings removed from chronic wounds for biofilm markers. By screening the organisms isolated from the dressings for their ability to express biofilm phenotypic characteristics and produce biofilm markers *in vitro*, it was hoped to provide evidence to confirm *in vivo* observations.

Aim

To explore novel ways of detecting biofilms in wounds

Objectives

- To screen dressings collected from patients with chronic wounds for the presence of biofilm markers.
- To isolate and identify organisms associated with dressings removed from patients with chronic wounds.
- To investigate the potential of isolated organisms to exhibit biofilm markers *in vitro*.

4.2 Materials and Methods

4.2.1 Research ethics approval

Approval for this project was obtained from the Research Committee of the University of Wales Institute Cardiff (UWIC) and the research and development (Research Risk Review Committee) of Gwent Healthcare NHS Trust, Newport, South Wales, United Kingdom (Appendices 2 and 3). Patients‟ consent was not required because the used wound dressings were normally discarded when removed from the wounds at dressing changes and their collection for this study did not influence their treatment regimes.

4.2.2 Collection of chronic wound dressings

Thirty five wound dressings were kindly collected by a Tissue Viability Nurse (Tracy Morgan) from either a chronic wound of patients attending an out-patient wound healing clinic at Royal Gwent hospital or a patient receiving treatment at home. No patient was used on more than one occasion. The dressing from each chronic wound was removed during normal treatment and dressing change by the Nurse and was placed immediately into a sterile stomacher bag (Fisher, UK) and labelled appropriately. The stomacher bag was placed into another bag to prevent cross contamination. Each dressing was transported immediately after collection to the laboratory in biohazard transport box (Electrolux, UK) for processing in accordance with the British standard operating procedures (BSOP 11) (Health Protection Agency (HPA), 2008).

4.2.3 Taking samples from each chronic wound dressing

Three samples were taken from each dressing by cutting 2 by 2.5 cm pieces from the portion that had been in direct contact with the wound with sterile scissors inside the safety cabinet (HERA safe, Thermo-Fisher Scientific, Basingstoke, UK). Each of the samples was placed into an appropriately labelled sterile universal container. The

remaining part of each dressing was then discarded into the clinical waste bin in the laboratory immediately.

4.2.4 Investigations of chronic wound dressings for biofilm markers

4.2.4.1 Detection of quorum sensing molecules (AHL) in wound dressings

One of the 2 x 2.5 mm pieces of each dressing contained in a universal container was autoclaved at 121^{o}C for 15 min. Then it was tested for the presence of AHL as previously described in chapter 3, section 3.2.1.1.2(adapted from Stickler *et al*, 1998).

4.2.4.2 Detection of extrapolysaccharides (EPS) in wound dressings

The second sample from each dressing was smeared on to two microscope glass slides and were fixed with 10 mM cetylpyridium chloride (CPC) and stained for EPS as previously described in chapter 3, section 3.2.1.2.6 (Harrison - Balestra *et al,* 2003).

4.2.5 Isolation and identification of microorganisms in wound dressings

4.2.5.1Culturing of wound dressings

British standard operating procedure (BSOP) 11 from the Health Protection Agency (HPA), UK which is usually used for processing wound swabs and soft tissues was used as the basis for the wound dressings samples, because there was no SOP for wound dressings. The third piece of each wound dressing sample was aseptically transferred into a universal bottle containing 10 ml maximum recovery diluent (MRD) (Oxoid, Cambridge, UK) and mixed using a vortex mixer (Stuart, UK) at 3000 g for 30 seconds to release the organisms from the dressings into the diluent. 10 µl of the resulting suspension was inoculated by streaking on to each of the isolation media: blood agar, chocolate agar, staphylococcus and streptococcus selective media, fastidious anaerobic

agar supplemented with neomycin (Oxoid, UK), Sabouraud dextrose agar and cystine lactose electrolyte deficient (CLED) (Oxoid, Basingstoke, UK). All plates were incubated at 37^{o}C for 24 - 48 hours; all media were incubated aerobically except chocolate agar which was incubated in 5% carbon dioxide in candle jars while the anaerobic agar was incubated anaerobically in an anaerobic cabinet (Don Whitey Scientific Ltd, Yorkshire, UK). The blood agar served as an enriched medium for the isolation of most organisms including fastidious organisms and differential medium for detecting haemolysis. Staphylococcus and streptococcus selective served as the medium for isolating staphylococci and streptococci. CLED agar was used as a selective medium for the isolation of coliforms especially to prevent *Proteus* species from swarming. It also provided means of preliminary identification between lactose fermenters and non-fermenters. Chocolate agar was for isolating *Haemophilus* species while the fastidious anaerobic agar with neomycin served as enriched and selective medium for the isolation of anaerobes and Sabouraud agar to isolate fungi.

4.2.5.2 Preliminary identification of isolated organisms

The isolated organisms were identified by colonial appearance and Gram staining.

4.2.5.3 Maintenance of cultures (isolated organisms' stock cultures)

Each of the different colonies recovered from dressings was cultured on nutrient agar (Oxoid, Basingstoke, UK) to obtain pure colonies for further identification tests and stock cultures. The stock cultures were prepared with the Protect bacterial preservation system media and stored in the freezer at -80^{o}C.

4.2.5.4 Biochemical Tests

A catalase test was performed on all the isolated Gram positive cocci using 3% v/v hydrogen peroxide (Fisher Scientific, UK) while *Staphylococcus aureus* isolates were confirmed with Staphylococcus agglutination test kit (Remel, USA). All *S. aureus* were screened for methicillin susceptibility using the BSAC method. All the isolated Gram positive bacteria except *S. aureus* were identified to species level with rapid diagnostic kits (BBL Crystal; BD, USA) according to the manufacturers‟ instructions. All the streptococcus species were further examined for Lancefield group using the antigen/antibody agglutination rapid diagnostic kit (Remel, USA). All the isolated Gram negative bacteria were tested for oxidase and indole (BBL Crystal; BD, USA) and other biochemical tests such as substrate utilisation using rapid diagnostic kit (BBL Crystal; BD, USA). All the anaerobic bacteria were tested for catalase and indole production and further identification up to species level was done with BBL crystal anaerobic rapid diagnostic kit in accordance with the manufacturer‟s guidelines. The cultures of yeast (identified during Gram staining) were tested for germ tube production and negative results were confirmed after 24 hours incubation. All germ tube negative yeasts were later identified with rapid diagnostic kit (API 20 C AUX, bioMérieux, France) according to the manufacturer‟s instructions.

4.2.6 Examination of phenotypic characteristics of organisms isolated from chronic wound dressings

4.2.6.1 Screening Gram negative wound dressing isolates for AHL production

The isolated Gram negative bacteria were cultured at 37^{o}C from the frozen stock culture and tested for the production of AHL using cross feeding assay as previously described in chapter 2, section 2.2.7.1 (Stickler *et al*, 1998).

4.2.6.2 Screening wound dressings isolates for biofilm formation

The potential of the isolated organisms to form biofilm at 37^{o}C in microtitre plates was investigated using the assay described previously in chapter 2, section 2.2.6.2 (Christensen *et al,* 1985).

4.2.6.3 Examination of phenotypic characteristics of Gram negative isolates from chronic wound dressings in continuous culture wound model

Two of the chronic wound dressing isolates that formed biofilm in microtitre plate assay (M3 (1) and M16 (5) were cultured in the continuous culture wound model to observe their phenotypic characteristics. The culture samples were collected at intervals to determine the growth pattern, AHL production and biofilm detection as described in chapter 3, section 3.2.1.2.3.

4.2.7 Data analysis

Regression analysis of the data was done using Excel 2003 and graphs of the growth curve of the organisms were plotted. Minitab version 15 was used to determine the correlation between EPS detection in wound dressings and *in vitro* formation of biofilm by the isolated organisms. AHL detection in dressings and isolation of Gram negative bacteria was analysed. The association between the detection of AHL in dressings and isolation of *P. aeruginosa* from the dressings was determined using Pearson correlation.

4.3 Results

The summary of this study is presented in Table 4.1, but each of the parameters is discussed in detail below.

Table 4.1: Summary of biofilm markers detected in 35 wounds dressings removed from patients with chronic wounds.

Biofilm Markers	Positive	Negative	Not determined
AHL	11 (31.4%)	22 (62.8%)	2 (5.7%)
EPS	28 (80%)	3 (8.6%)	4 (11.4%)
Dressings that contained organisms that formed biofilm	32 (91.4%)	3 (8.6%)	0
Dressings that contained Gram negative bacteria	24 (68.6%)	11 (31.4%)	0
Dressings that contained AHL producing Gram negative bacteria	13 (37.1%)	22 (62.9%)	0

4.3.1 Detection of AHL in wound dressings

Out of the 35 wound dressings investigated, 11 dressings (31%) were positive for AHL (Fig. 4.1 a and b; Table 4.1), 22 (62.8%) were negative (Fig. 4.1 c and d; Table 4.1) and in two dressings AHL production could not be determined due to the inhibition of growth of the indicator organism (*Agrobacterium tumefaciens*) by the antimicrobial components in the wound dressings.

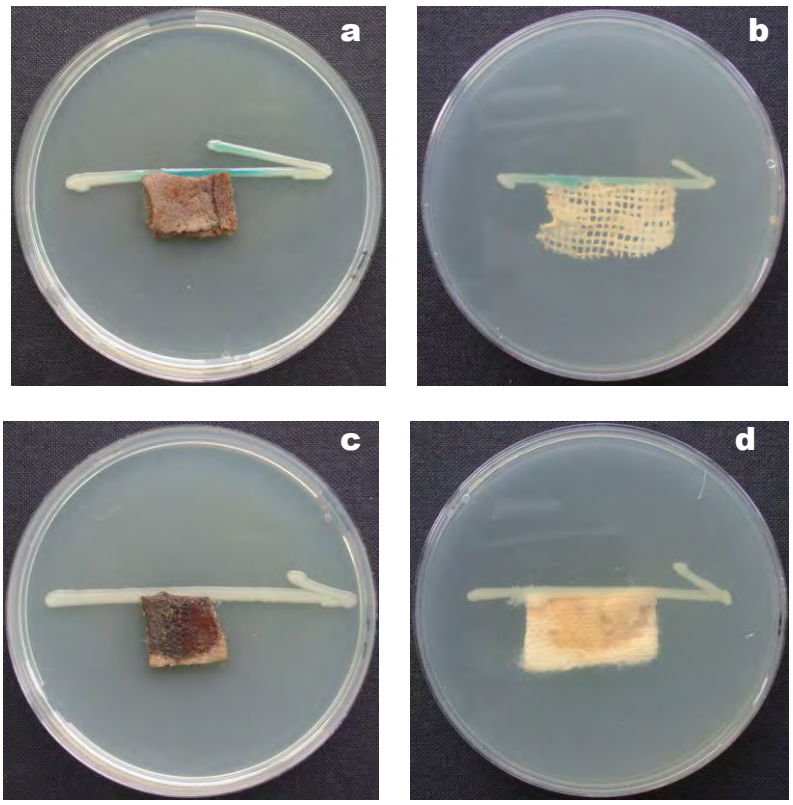

Figure 4.1: Samples of chronic wound dressings tested for AHL. Examples of sterilised wound dressings‟ samples (a and b) in which AHL was indicated by the reporter strain *A. tumefaciens* NTL4, (c and d) wound dressings in which AHL was not detected.

4.3.2 Detection of extracellular polymeric substances (EPS) in wound dressings

EPS were detected in 28 (80%) of the dressings (Table 4.1; Fig. 4.2a), 3 (8.6%) dressings tested negative for EPS (Table 4.1, Fig 4.2b) and 4 dressings could not be tested because the dressings were too dry to make suitable smears for EPS examination.

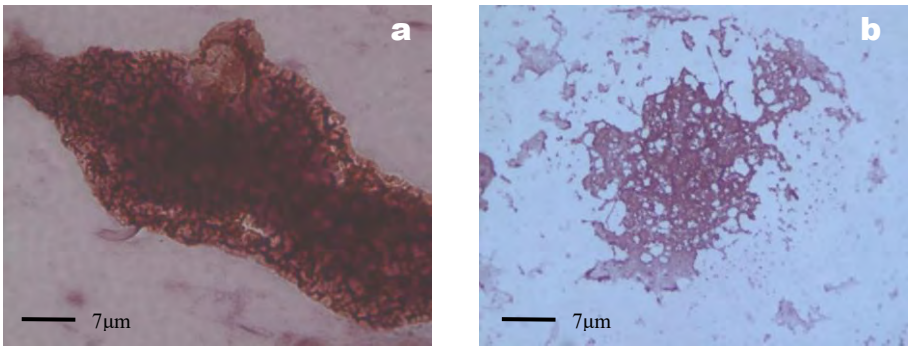

Figure 4.2 EPS positive and EPS negative smears derived from chronic wound dressings. Stained slide smears of chronic wound exudates observed under light microscope (x100 objective): (a) chronic wound dressing that tested positive for EPS showing embedded organisms and (b) a wound dressing that did not contain EPS.

4.3.3 Microorganisms isolated from chronic wound dressings

4.3.3.1 Species and frequency distribution of isolated organisms

The organisms recovered from the dressings that were characterised included aerobic and anaerobic bacteria and yeasts (Fig. 4.4). In total 108 isolates were recovered from the dressings ranging from one to six organisms per dressing with a mean of three isolates per dressing (Fig. 4.5). Twenty two *Staphylococcus aureus* were isolated, of which eight were methicillin resistant *S. aureus* (MRSA) (Fig. 4.3). They were obtained from 22.9 % of the dressings while *P. aeruginosa* was isolated from 14 dressings (40%) and *Corynebacterium striatum* from 10 dressings (28.6%) (Fig. 4.4 and Appendices 4 and 5).

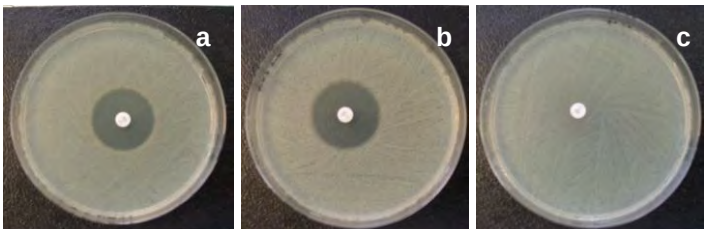

Figure 4.3: Cultures of *Staphylococcus aureus* tested for methicillin susceptibility. (a) Reference *S. aureus* NCTC 6571, (b) a methicillin sensitive *S. aureus* (MSSA) and (c) methicillin resistant *S. aureus* (MRSA) isolated from chronic wound dressings.

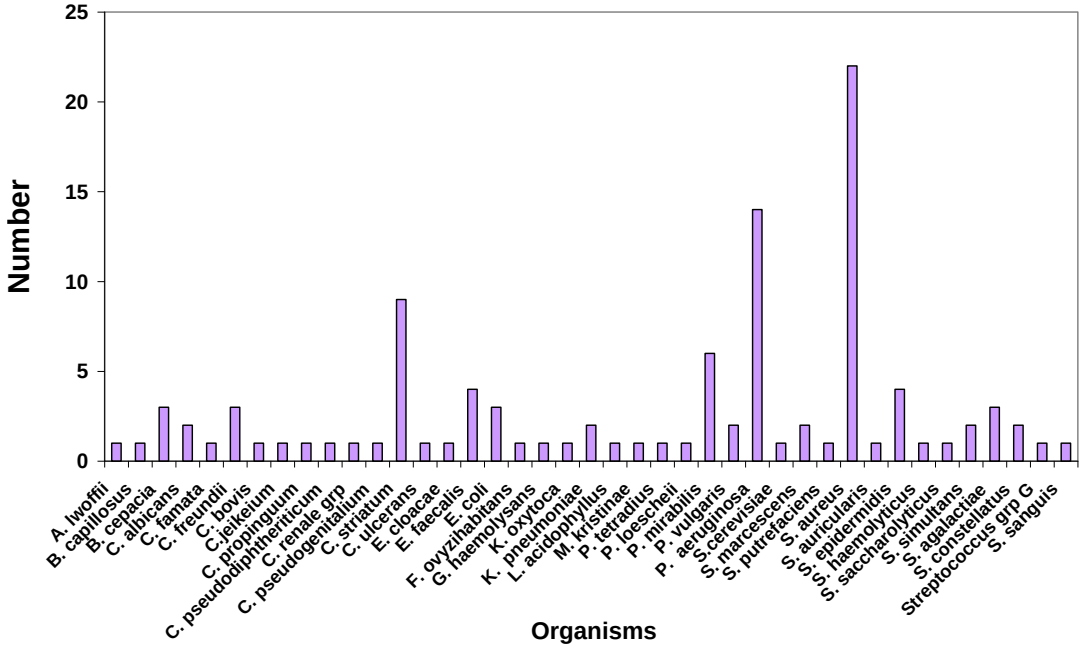

Figure 4.4: Frequency of isolated organisms from chronic wound dressings

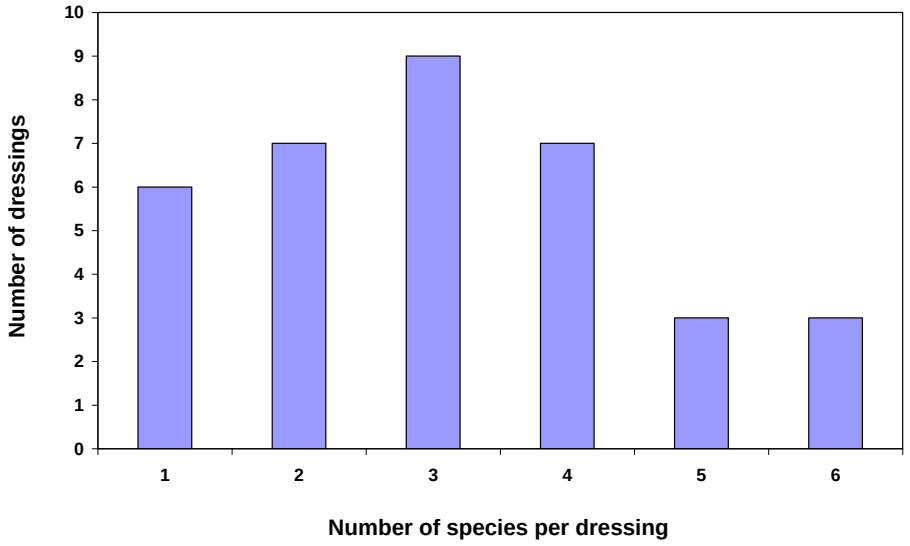

Figure 4.5: Distribution of species of organisms isolated from each chronic wound dressing. The number of species of organisms isolated from each dressing ranges from 1 to 6 with a mean of 3 species per dressing.

4.3.3.2 Biofilm phenotypic expressions of isolated organisms from chronic wound dressings

4.3.3.2.1 AHL production by Gram negative bacteria isolated from the wound dressings

In total Gram negative bacteria were isolated from 23 of the 35 wound dressings but Gram negative bacteria that produced AHL *in vitro* (Table 4.1; Fig. 4.6 a) were isolated from 13 dressings (Tables 4.1 and 4.2) Gram negative bacteria from 10 dressings tested negative for AHL production (Figure 4.6 b).

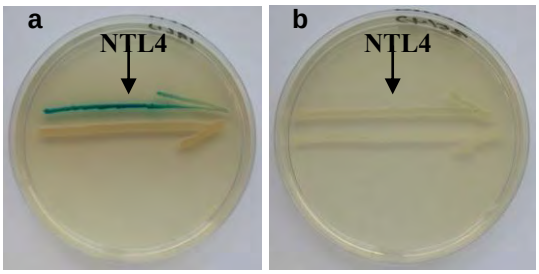

Figure 4.6 AHL positive and AHL negative Gram negative bacteria recovered from chronic wound dressings. Pictures (a) *A. tumefaciens* NTL4 streaked beside an AHL producing Gram negative bacteria isolated (b) a non- AHL producing Gram negative isolate.

Table 4.2 Biofilm markers detected in chronic wound dressings.

Dressings	AHL detection	EPS detection	Biofilm forming isolate(s)	Gram neg. bacteria isolated	Gram neg. isolate(s) produced AHL	No of orgs isolated
1	-	+	+	-	N/A	1
2	+	+	+	+	+	3
3	-	Not tested	+	+	-	4
4	+	+	+	+	+	3
5	-	+	+	+	-	2
6	-	Not tested	+	+	-	4
7	-	Not tested	+	+	-	3
8	-	-	-	-	N/A	1
9	-	-	-	-	N/A	1
10	+	Not tested	+	-	N/A	1
11	-	+	+	-	N/A	2
12	-	+	+	+	-	5
13	-	+	+	-	N/A	4
14	-	+	+	+	-	3
15	-	+	+	-	N/A	2
16	+	+	+	+	+	6
17	+	+	+	+	+	4
18	R.I.	+	+	+	+	6
19	+	+	+	+	+	3
20	-	+	+	+	-	1
21	R.I.	+	+	+	+	2
22	-	+	+	+	-	4
23	-	+	+	-	N/A	2
24	-	+	+	+	-	3
25	-	+	+	-	N/A	3
26	-	+	+	+	+	5
27	-	-	-	-	N/A	1
28	+	+	+	+	+	2
29	+	+	+	+	+	5
30	+	+	+	+	+	4
31	-	+	+	+	N/A	3
32	+	+	+	+	+	6
33	+	+	+	+	+	3
34	-	+	+	-	N/A	2
35	-	+	+	+	-	4

Key: Positive (+); Negative (-); Reporter inhibited (R.I.); Not applicable (NA)

4.3.3.2.2 Biofilm forming potential of organisms isolated from chronic wound dressings

Out of these 108 isolates, 63 including Gram negative and Gram positive bacteria as well as yeasts from 32 dressings (91.43%) formed biofilm in a microtitre plate assay (Tables 4.1 and 4.2). A positive result for a wound dressing in Table 4.2 (biofilm forming isolate) is indicative of one or more isolates from the dressing forming biofilm.

4.3.3.2.3 Biofilm phenotypic characteristics of Gram negative isolates from chronic wound dressings in wound model continuous culture.

Two of the Gram negative isolates from the chronic wound dressings *P. aeruginosa* M16 (5) and *B. cepacia* M3 (1) were cultured in the wound model to study their phenotypic characteristics. The effluent from the wound model cultures of each of the 2 isolates showed evidence of progressive biofilm formation including increase in bacterial population and AHL production (Fig. 4.7a and 4.8a) as well as EPS production and biofilm morphology (Fig. 4.7 a and c ; 4.8 b and c) during the period of culture.

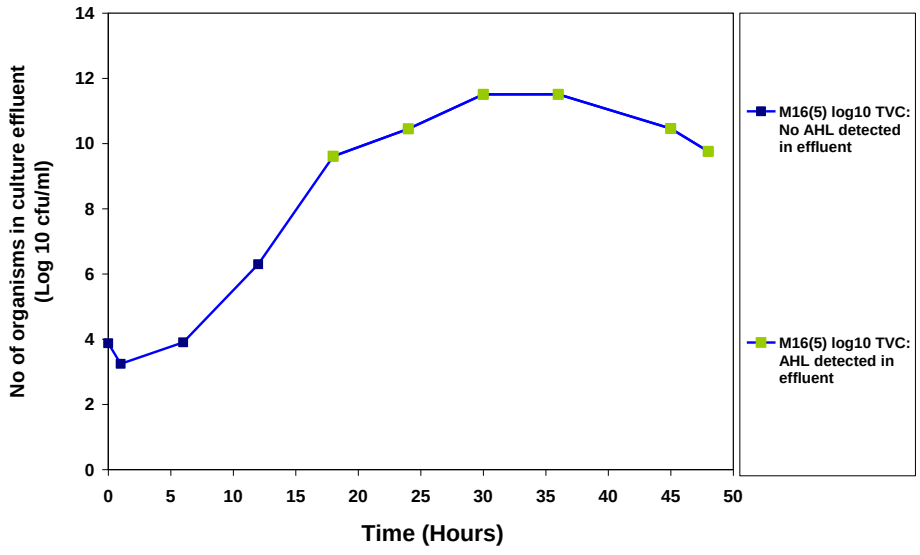

Figure 4.7a Growth pattern of a Gram negative isolate *P. aeruginosa M* 16(5) from a chronic wound dressing in continuous culture wound model. AHL was detected from 18 hours when culture effluent bacterial count was 4.04 x 10^9 cfu/ ml till the end of culture and in the gauze.

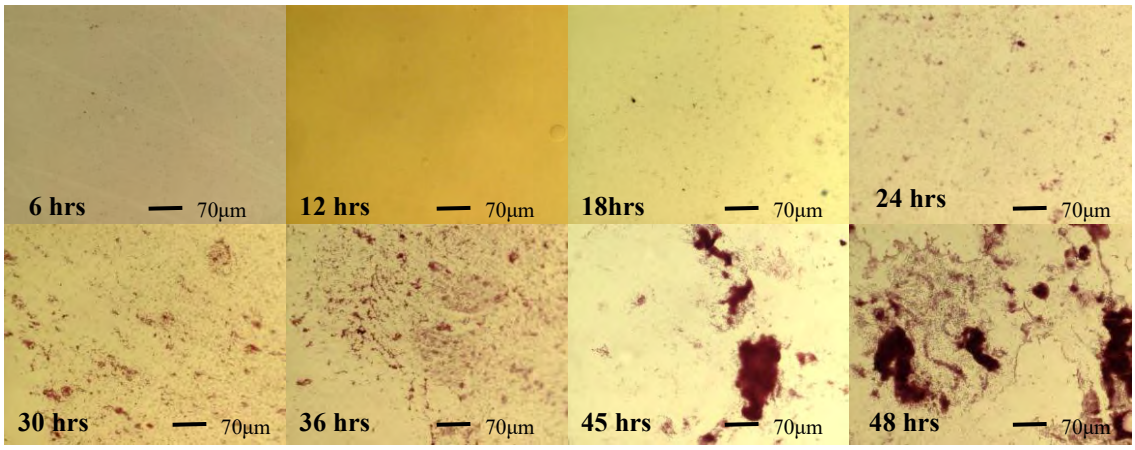

Figure 4.7b Biofilm developments of a wound dressing isolate *P. aeruginosa* M16 (5) in wound model continuous culture. Microcolonies became bigger with increase in number of organisms and EPS production relative to time as shown on microscopic examination (x10 objectives).

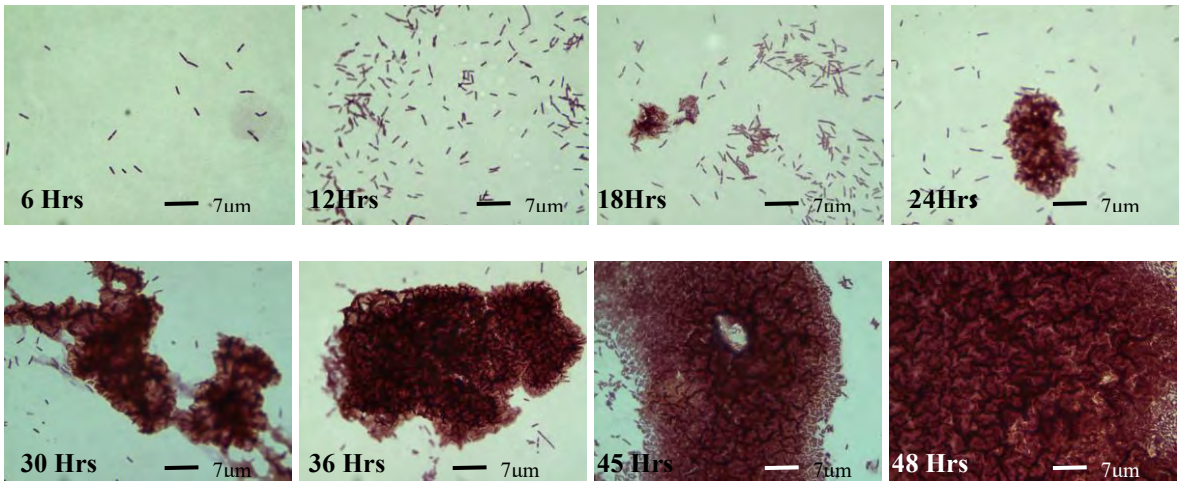

Figure 4.7c Biofilm development of a *P. aeruginosa* M16 (5) isolated from a wound dressing in wound model continuous culture. Microcolonies became bigger with increase in bacterial population and EPS production with time as shown on microscopic examination (x100 objectives).

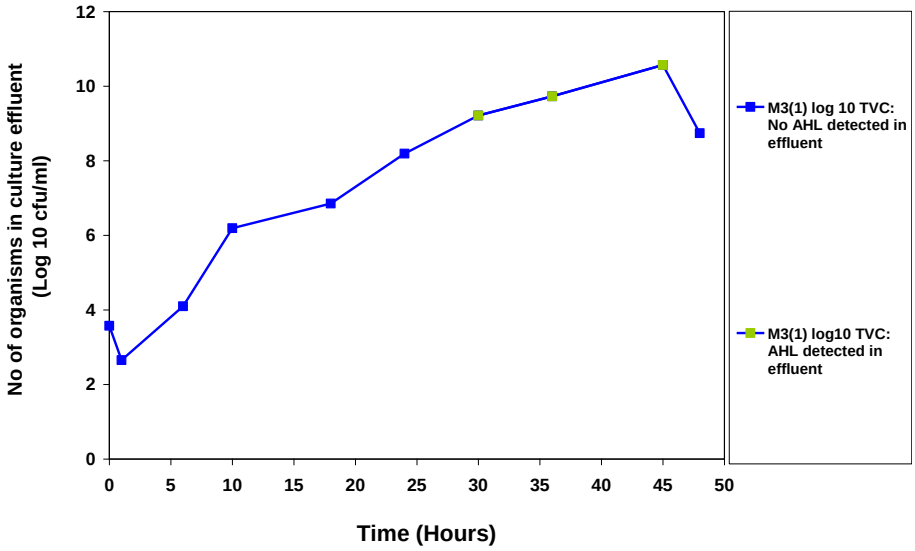

Figure 4.8a Growth pattern of a Gram negative isolate, *B. cepacia* M 3(1), from a chronic wound dressing in a wound model culture. Biofilm formation was evident from 12 hours and AHL was detected from 30 hours when the culture effluent bacterial viable count was 1.63 x 10^9 cfu/ ml up to 45 hours but no AHL was detected at 48 hours and in the gauze.

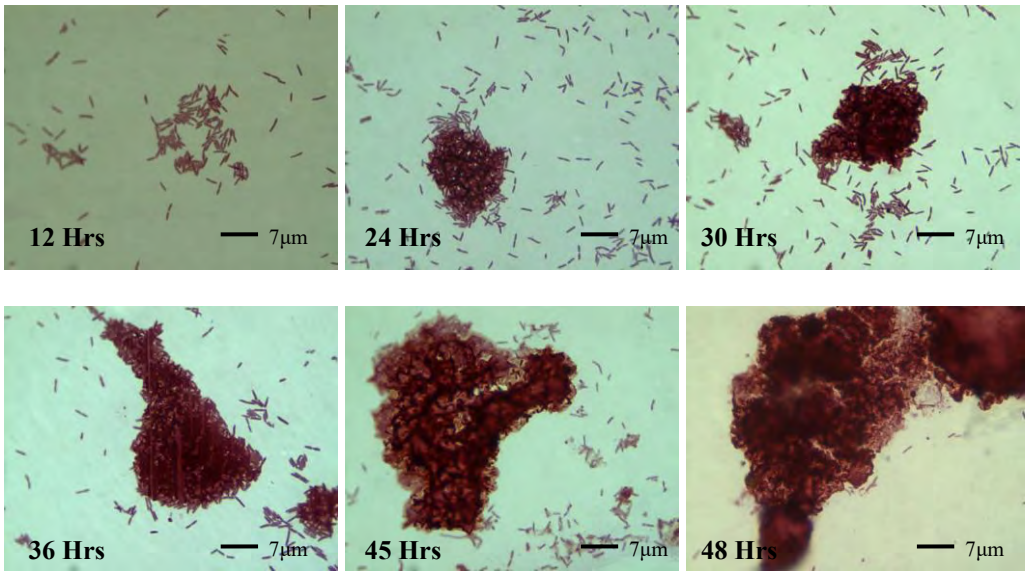

Figure 4.8b Biofilm developments of a *Burkholderia cepacia* M3 (1) isolated from wound dressing in wound model continuous culture. Microcolonies became bigger with increase in number of organisms and EPS production with time as shown on microscopic examination (x100 objectives).

4.3.4 Statistical analysis and correlation

The data were analysed with Minitab version 15. Pearson correlation shows a strong association between the detection of AHL in dressings and isolation of *P. aeruginosa* from the dressings (P< 0.005) (Fig. 4.9). There was also a correlation between EPS detection in the dressings and *in vitro* formation of biofilm by the organisms isolated from the dressings (P< 0.0005) (Fig. 4.10). AHL detection in dressings and isolation of Gram negative bacteria was also significant, P< 0.034.

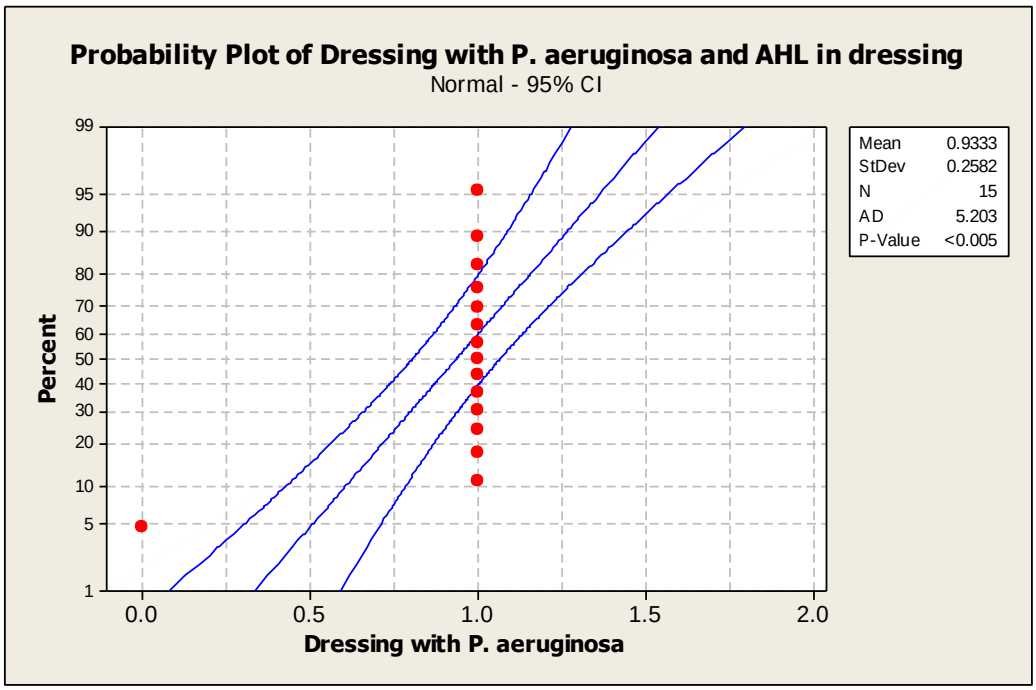

Figure 4.9 Graphical representation of the relationship between AHL detection and isolation of *Pseudomonas aeruginosa* from chronic wound dressings

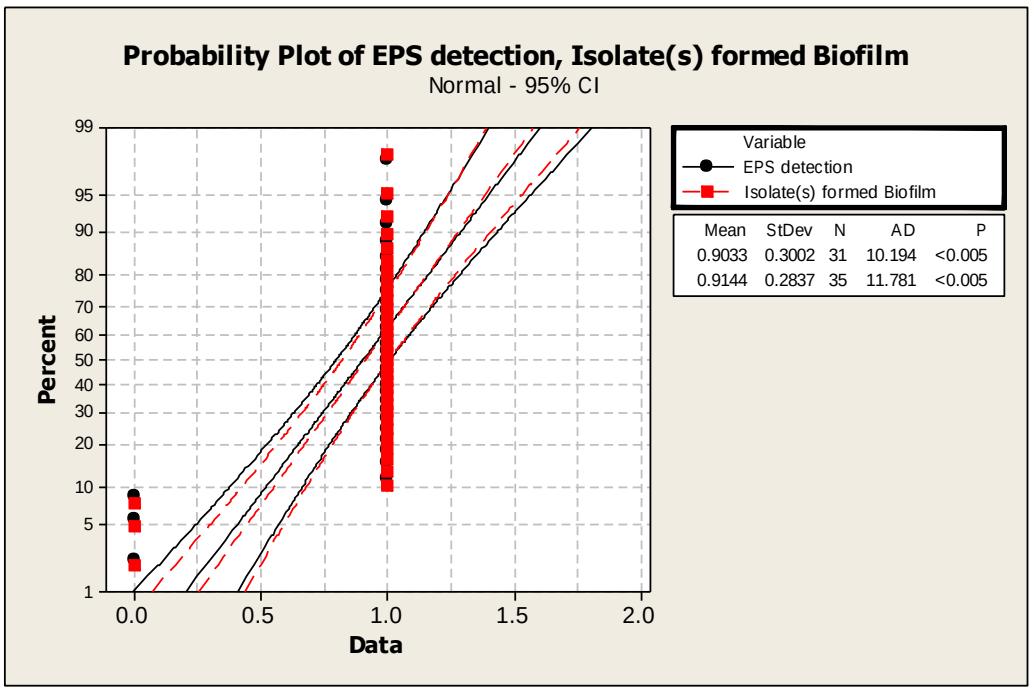

Figure 4.10 Graphical representation of the relationship between detection of EPS in chronic wound dressings and formation of biofilm by the isolated organisms from the dressings.

4.4 Discussion

According to the US National Institute of Health about 80% of chronic infections are due to biofilm (NIH, USA, 1997) which has been associated with the incessant resistance to antimicrobial agents (Davies, 2003). It has been suggested that the presence of biofilm in wounds impedes wound healing processes (Bjarnsholt, 2008). In the past, various studies have been done to elucidate the growth and physiology of biofilm as well as the resistance mechanisms to immune and antimicrobial interventions *in vitro* and *in vivo* using animal models. To date investigations designed to determine the presence of biofilm in wounds have relied on wound biopsies which might be inconvenient to patients because the procedure is invasive, although recently wound debridement samples have been used. It would be a novel idea to screen for biofilm in wounds without any discomfort to the patient by examining used wound dressings. It has also been observed that non- cultural methods are able to detect wound pathogens more extensively than cultural methods, screening of wounds for biofilm using non-cultural methods such as detection of biofilm markers in dressings removed from chronic wounds might increase awareness in the diagnosis of biofilm in wounds and contribute to wound management.

In infections involving biofilms, quorum sensing molecules are the main pivot of biofilm formation (Huang *et al,* 2009; Costerton *et al*, 1999) and detection of these molecules is therefore suggestive of biofilm colonisation of the wound.

In this study, there was evidence of biofilm markers including AHL and EPS in the dressings removed from 35 chronic wounds, and the organisms isolated from the dressings expressed biofilm phenotypic characteristics such as biofilm formation and production of AHL and EPS *in vitro*.

AHL was detected in 11 dressings (Table 4. 1, Fig. 4.1 a and b) 10 (90.9%) of which contained Gram negative bacteria that produced AHL *in vitro* (Table 4. 1, Fig. 4.6a) ($p < 0.005$). However no Gram negative bacteria were isolated from the remaining wound and any organisms in this dressing might have been inhibited by a component present in the wound dressing. The inability of the culture method to isolate the organism is one of the factors that usually necessitate the use of alternative means of identification of organisms such as PCR which relies on the bacterial DNA (Rayner *et al,* 1998). All of the Gram negative bacteria that were isolated from the AHL positive dressings were strong biofilm formers in the microtitre assay and this suggests the organisms have the potential to form biofilm in a wound.

Detection of AHL in wound dressings could provide a useful means of diagnosis of biofilm in wounds. In the past, the detection of AHL in urinary catheters (Stickler *et al,* 1998) and in the sputum of cystic fibrosis patients (Singh *et al,* 2000) has been used to determine the presence of biofilm. Wu and colleagues (2000) confirmed the pathogenicity of *P. aeruginosa* by detecting AHL in the lungs of *P. aeruginosa* intratracheally infected mice. This finding corroborates previous studies of AHL detection as biofilm marker and recently in chronic wound debridement samples (Rickard *et al,* 2010).

In all, Gram negative bacteria were isolated from 23 dressings but Gram negative bacteria that tested positive for AHL were isolated from only 13 dressings (56.5%). Out of the 13 dressings, 10 dressings (43.5%) tested positive for AHL screening while 2 (8.7%) dressings results could not be determined due to inhibition of the growth of the reporter strain. Gram negative bacteria that tested negative for AHL (Table 4.1; Fig. 4.6 b) were isolated from 10 dressings (43.5%) and the dressings with non-AHL producing Gram negative bacteria tested negative for AHL. The detection of AHL in used wound

dressings seemed to be a sensitive assay and easily executed. AHL producing bacteria were isolated from all the dressings in which AHL was detected during screening.

All of the dressings that tested positive for AHL were polymicrobial and in particular contained biofilm forming *P. aeruginosa.* This confirms the importance of *P. aeruginosa* biofilm formation in wounds and the involvement with multispecies biofilms, which has been demonstrated by other studies (Malic *et al,* 2009). Malic and colleagues in their study observed that *P. aeruginosa* was the most predominant organism within multi-species biofilms. The detection of AHL in almost all of the dressings in which *P aeruginosa* was isolated reveals the vital role of signalling molecules in the formation of *P. aeruginosa* biofilm (Van Delden and Iglewski, 1998). In particular the association of virulence expression by *P. aeruginosa* during infection with increase in AHL production (Van Delden and Iglewski, 1998) has been shown and usually coincides with pathogenicity of *P. aeruginosa* (Wu *et al*, 2000). Rickard and colleagues (2010) found that although most of the resident organisms within the wound produced AI-2 molecules but aggressive species such as *P. aeruginosa* produce AHLs which was revealed in this study. A strong association between the presence of *P. aeruginosa* and detection of AHL in chronic wounds has been observed in this investigation.

In this study, one wound dressing tested positive for AHL but the culture of the dressing did not yield the growth of any Gram negative organisms. It has been extensively reviewed that the use of cultural methods often miss out some pathogens and lack of identification of such organisms has a great impact on treatment strategies hence the need to utilise non - cultural methods (Rogers *et al,* 2009). In a study of the 40 diabetic foot ulcers by Dowd and his colleagues (2008), it was observed that traditional cultural methods select for easily cultured organisms against the difficult to culture organisms.

In a similar study of the microbial microbiota of chronic wounds, Kirketerp-Moller and his colleagues (2008) also support the development of new diagnostic strategies for chronic wounds because they observed that more organisms were identified by non-cultural methods than from the cultures. The detection of *P. aeruginosa* was higher in non-cultural methods than the cultural method in the study. In another study the non-cultural method revealed 4 times the number of organisms from the wounds than the cultural methods (Price *et al,* 2009) and high prevalence of *P. aeruginosa* was observed amongst patients treated with antibiotics. These might explain why a wound dressing tested positive for AHL but no Gram negative organism was isolated from the dressing. Although in this circumstance, the possibility of antibiotic resistance is suggested as the main reason for inability to isolate the organism.

Investigation of the biofilm phenotypic characteristics of the selected Gram negative wound dressing isolates showed that *Burkholderia cepacia* M3 (1) produced AHL in the continuous culture investigation between 30 hours and 45 hours, after which the AHL became undetectable (Figure 4. 8a), whereas the organism had tested negative for the AHL in the cross feeding assay (1cm away from reporter strain). It has been observed that some strains of biofilm forming *B. cepacia* show dynamic changes in cultures and the production of AHL decreases rapidly during the stationary phase (Soo *et al,* 2009; Chen *et al,* 2005) and similar response was observed in this study. The findings of the analysis of QS and QSI in chapter 2 showed that the closer the reporter organism was to the test organism, the higher the chance of detecting the AHL production. This low production of AHL by some of the organisms could have resulted in false negatives such as the case with M3 (1) that tested negative for cross- feeding assay but produced AHL in wound model continuous culture from 30 hours of culture up till 45 hours. It also tested negative for AHL at 48 hours (Figure 4. 8a).

Some studies have discovered that certain strains of bacteria such as *P. aeruginosa* form biofilm independently of signalling molecules (Schaber *et al*, 2007; Favre - Bonte *et al*, 2007). It is possible to miss out the diagnosis of such biofilm organisms when established in wounds by using signalling molecules as a lone means of diagnosis. This study investigated AHL signalling molecule alone and not other signalling molecules. Hence a limitation of this study is not detecting other signalling molecules, which would have provided more information about the relevance of detecting signalling molecules in wound dressings.

The results obtained from this study showed that most of the wound dressings (28 (80%) of the 35 tested) contained EPS, three tested negative while four could not be tested due to the nature of the dressings because they were dry. Organisms that formed biofilm in a microtitre plate assay were isolated from 32 dressings inclusive of all the 28 EPS positive dressings. There was a significant correlation between the detection of EPS in dressings and biofilm formation of the organisms isolated from the dressings; $p< 0.005$. EPS serves as the housing for the biofilm community preventing the organisms from the environmental stress such as body immune system and antibiotics (Leid *et al*, 2005; Ryu and Beuchat, 2005) consequently resulting in the persistence of infection therefore detection of EPS in the chronic wound dressings suggests the presence of biofilm in such wounds. Many biofilm detection studies have utilised methods that detect EPS (Harrison- Balestra *et al*, 2003), even for the determination of biofilm age (Ahimou *et al*, 2007).

Of the wound dressing isolates tested here, two [M16 (5) and M3 (1)] demonstrated progressive biofilm formation in continuous cultures with high production of EPS and AHL. The presence of EPS in chronic wound dressings is another indication of the presence of biofilms in the wounds from which the dressings were obtained, and the

organisms that were isolated from the dressings also produced biofilm in the laboratory (Table 4.1). The three findings provide evidence of the presence of biofilm in the chronic wounds where the dressings were removed.

A total of 108 organisms were isolated and characterised from the chronic wound dressings, 63 (58.3%) which were isolated from 32 dressings (91.4%) formed biofilm *in vitro* while 46 did not form biofilm suggesting the potential of the organisms to form biofilm in wounds. Out of the 32 dressings from which biofilm forming organisms were isolated, 28 (87.5%) tested positive for EPS but 4 could not be screened. There is a strong correlation between EPS detection in the wound dressings and biofilm formation by the isolated organisms ($P< 0.005$).

In a study the *Pseudomonas aeruginosa* strains biofilms were cultivated in a reactor to compare the phenotypic expressions of type culture and clinical isolates. A type culture PAO 1 and four clinical isolates, derived from CF patients were investigated for phenotypic expressions of the organisms in the laboratory. The clinical variants displayed many of the same characteristics as the laboratory variants, suggesting a link between laboratory and cystic fibrosis biofilms (Kirisits *et al*, 2005). Applying the same concept, detection of AHL in catheters removed from patients confirmed the presence of biofilm in the bladder from which the catheters were obtained (Stickler *et al*, 1998).

The principle was also demonstrated by Malic and his colleagues (2009) using species distribution of chronic wound biofilms bacteria *in vitro* to demonstrate biofilm organism distribution in tissues from chronic wounds.

Most of the dressings were polymicrobial (82.9%) which was in agreement with other studies (Dowd *et al,* 2008; Kennedy *et al,* 2009) especially the involvement of biofilms in such wounds (Kennedy *et al,* 2009; Kathju *et al,* 2009; Dowd *et al,* 2008). The

abundance of *P. aeruginosa* in wounds as observed in this study has been confirmed by previous studies (Price *et al,* 2009; Fazli *et al,* 2009; Oguntibeju and Nwobu, 2004).
In all 40 Gram negative bacteria were isolated from 23 dressings, 17 isolates (42.5%) from 13 dressings produced AHL *in vitro*. Thirty four of the Gram negative bacteria from 24 dressings formed biofilm in the laboratory. AHL detection can only be utilised as a diagnostic tool for biofilms of AHL producing organisms hence the limitation of this method alone in biofilm detection. AHL detection was highly sensitive and detected most of the AHL producing bacteria, therefore it will provide a good diagnosis for investigation of biofilm involving AHL producers in wounds.

Two of the isolates were cultured in a continuous culture system and evidence of AHL (Fig. 4.7a and Fig. 4.8a) and EPS (Fig. 4.7b and c and Fig. 4.8b) production were observed which confirmed that the AHL and EPS that were detected in the wound dressings were produced by the biofilm forming organisms that were isolated from the same dressings.

It was observed from this study that the most prevalent organisms isolated from the dressings were *Staphylococcus aureus* (13%) or including MRSA (20.4%); *P. aeruginosa* (13%), *Corynebacterium striatum* (9.3%) and MRSA (7.4%) (Fig. 4.4) which was in agreement with other studies of wound flora (Malic *et al,* 2009; Kirketerp-Møller *et al,* 2008).

The detection of biofilm markers such as AHL and EPS in dressings is a good indicator of the presence of biofilm in wounds, which in turn is of clinical significance, in view of the findings that the presence of biofilms in wounds is linked to failure to heal (Bjarnsholt *et al*, 2008; Percival and Bowler, 2004). Detecting bacterial infections through culture independent approaches has been suggested as the solution to inability of cultural methods to isolate some pathogenic organisms (Rogers *et al,* 2009). In this

study, AHL was detected in particular in almost all of the dressings that biofilm forming *Ps. aeruginosa* strains were isolated confirming the importance of quorum sensing in the pathogenicity of *Ps. aeruginosa* infections. These findings also corroborate the role of AHL in modulating the virulence of pathogenic organisms such as *P. aeruginosa* (Jensen *et al,* 2006; Bjarnsholt *et al*, 2005; Sauer *et al*, 2002). The dressings from which *P. aeruginosa* cultures were isolated were multi-microbial and most of the organisms formed biofilm *in vitro* which also shows the vital role of quorum sensing to biofilm community. Regrettably, other signalling molecules were not examined in this study hence the precise number of dressings containing signalling molecules could not be determined, to corroborate the biofilm forming potential of the isolated organisms *in vitro* and the EPS detection in dressings.

EPS was detected in the majority of the dressings. EPS is known to protect biofilm against body immune system and antibiotics (Leid *et al,* 2005; Ryu and Beuchat, 2005); the implication is that the organisms will be able to resist treatment interventions and persistently cause damage to the tissues and breakdown of wounds.

The presence of biofilms in wounds has been shown to interfere with the healing process leading to chronicity of such wounds and recently the presence of biofilm in wound have been reported (Malic *et al*, 2009; Dowd *et al,*, 2008; James *et al*, 2008; Ngo *et al,* 2007). In previous studies that the presence of biofilm had been investigated using tissue biopsy samples from wounds, out of 12 chronic wounds, 7 (58.3%) tested positive (Ngo *et al*, 2007), of 50 chronic wounds 30 (60%) tested positive while 21 (70%) out of 30 tested positive when debridement materials were used. From the results of the investigations, the fact that wound pathogens are not evenly distributed within the wound plays a vital role in the ability to isolate or detect the biofilm. This was discussed by one of the groups in their report as a factor that

probably affected the result because they were of the opinion that the positive results ought to have been higher (Ngo *et al*, 2007). The trends of the result are increasing and the debridement materials might likely have larger sampling area depending on whether the whole wound surface was debrided and how deep since it has been found that some organisms such as *P. aeruginosa* are found in the deeper regions of the wound. Detection of AHL in 70% wound debridement samples has not only shed more light into the diagnosis of biofilm in wounds through biofilm markers but also the high prevalence of biofilm in wounds.

However, the results obtained in this study showed higher trend because wound dressings cover a larger sampling areas than biopsies. Since the signalling molecules would diffuse though the dressing while EPS from the organisms within the wound surface would be picked as well as those of the organisms in the deeper region due to the pressure of the dressing. In this study, of the 35 chronic wound dressings that were investigated, only three were negative for all the biofilm markers. 32 of the dressings contained organisms that formed biofilm *in vitro*, 28 of which contained EPS. Although not all the dressings contained Gram negative organisms or AHL positive Gram negative organisms but AHL positive bacteria were isolated from the dressings that tested positive for AHL and the bacteria formed biofilm in microtitre assay, and produced AHL in culture and EPS in continuous cultures to confirm biofilm phenotype. Using the 2 parameters (EPS and biofilm formation) 80% of the chronic wounds showed evidence of biofilm colonisation. But the 3 biomarkers: EPS, AHL and biofilm formation by isolates, gave the result of 32 (91%) of the chronic wounds were inferred to have been colonised by biofilm.

Conclusion

A high prevalence of biofilm markers in dressings removed from chronic wounds was found in this study. The polymicrobial nature of wounds was also confirmed, especially in wounds thought to be colonised with biofilm. *S. aureus, P. aeruginosa* and *Corynebacterium striatum* were found to be the most frequently isolated organisms. There was a strong association between the detection of AHL and the presence of *P. aeruginosa* in wound. Examination of dressings removed from wounds for microbially derived biomarkers may be used to detect biofilm in wounds. This study has supported other studies that biofilm is present in chronic wounds.

Application of the techniques as used in this study as a diagnostic tool for detecting biofilm could simplify the process of identification of infections associated with biofilm in wounds. It could also be employed in assessing biofilm management strategies and monitoring treatment s designed to eradicate biofilm from wounds.

AHL was the only signalling molecule tested here; therefore the ability to detect the presence of other signalling molecules in wounds could not be fully accomplished. There is a need to utilise other assay methods to detect all the various signalling molecules of both Gram positive and Gram negative organisms, including the intra and intercellular signalling molecules in future studies. A larger cohort might be needed to determine whether detection of signalling molecules can be used as a lone indicator of biofilm in wounds. In future scanning electron microscopic examination of the wound dressings could be done to reveal biofilm architecture. Further studies are needed to link the presence of biofilm in a wound *in vivo* to the presence of biofilm markers in dressings.

Chapter 5

Antimicrobial effect of honey on biofilm and quorum sensing

5.1 Introduction

Treatments of pathogenic infections with antimicrobials have evolved over the years particularly from the discovery of penicillin, the first antibiotic derived from *Penicillium notatum* fungi by Sir Alexander Fleming, Scottish biologist and pharmacologist in 1929. Numerous synthetic antimicrobials have since been produced for killing or rendering ineffective several microorganisms. The major effects of antimicrobials on microorganisms are interference with nucleic acid, protein or cell wall synthesis and inhibition of metabolic pathways (Tenover, 2006) or by increasing the membrane permeability of the organisms (Mangoni *et al*, 2004).

The limitation of the magnificent achievements of antimicrobial therapy is the ability of microorganisms to develop ways of circumventing the lethal effect of antimicrobials. The resistance of pathogenic organisms to antimicrobial agents has been on the increase due to various resistance mechanisms acquired by the pathogens as well as over- usage of antimicrobials. The major factors are the innate ability of microorganisms to resist specific antimicrobials and to acquire resistance genes from other organisms within the environment by conjugation, transduction or transformation. Selection of mutants could occur as a result of antibiotic treatment regimes which often kill susceptible bacteria, allowing resistant strains to dominate (Tegnell et al, 2003). These genotypic changes sometimes lead to the selection of mutants and consequent resistance to specific or multiple antimicrobials which often result in treatment failures and other complications that may be life threatening. Prolonged use of antibiotic could also lead to selection of antibiotic resistant strains that may spread within the community or the hospital (Pedersen *et al*, 1986). The economic implication of antibiotic resistance to healthcare is enormous (Cosgrove, 2006). The available funds for developing new antimicrobials are fast depleting and the lack of development of new antimicrobials is being envisaged as a serious potential threat to public health (Norrby *et al,* 2005). The impact on treatment

of infections has therefore reawakened the awareness on the use of natural products such as honey for treatment.

5.1.1 Honey and medical history

Honey is a nutritional product produced by bees from nectar which they collect from plants as source of food. The composition of honey usually depends on the floral sources particularly the phytochemical components of the honey (Molan, 2001). The major components of honey are the sugars, fructose (about 38.5%) and glucose (about 31.0%), (White and Doner, 1980). Other components include the enzymes produced by bees, sucrose between 1.0% and 12%, water 17.0% other sugars (such as maltose, melezitose) 9.0%, ash 0.17% to 0.77 respectively according to the analysis of a typical honeys (Kamal *et al*, 2002) and other components. The enzymes commonly found in honeys are diastase (amylase), invertase (α-glucosidase), glucose oxidase, catalase and acid which contribute to their overall activity. Honeys also contain phenols; the amount of which influences the antimicrobial activity of the honey (Aljadi and Yusoff, 2003). Other substances that have been identified in honeys are minerals such as K, Na, Ca, Mg, Fe, Zn, Cu, Ni and Co, with K (Saif-ur-Rehman *et al,* 2008). The composition of honey remains constant with storage at temperature of 20^{o}C or below (Rybak-Chmielewska and Szczesna, 1995) hence temperature is of high importance in the storage of honey for the maintenance of potency when honey is to be used as a medicament. Individual honeys vary from their components due to the type of bees that produced them, the plants from where they are produced and the conditions of storage. Higher storage temperatures tend to increase the hydroxymethylfurfuraldehyde (HMF) of honey which could be up to 3 times within 12 months storage at high temperatures (Rybak-Chmielewska and Szczesna, 1995) hence it is advisable to store honey below 4^{o}C.

5.1.2 Physico-chemical properties of honey

The constituents of honey determine the various properties including the osmolarity, pH and other chemical properties. The chemical properties are due mainly to the presence of hydrogen peroxide, acids and enzymes such as catalase, glucose oxidase and sugars.

5.1.2.1 pH of honey

Although the pH of honey varies from honey to honey but generally they have an acidic pH; ranging between 3.2 and 4.5 (White and Doner, 1980) and could be as low as 2. 19 (Ghazali, 2009) and this pH range is highly unfavourable for most microorganisms to thrive. Other substances present in honey such as caffeic acid and ferulic acid have also been found as contributory agents to the pH and antimicrobial effect of honey (Wahdan, 1998).

5.1.2.2 Osmolarity of honey

Honey has high osmolarity because it is mainly a highly saturated mixture of monosaccharides with low water content, the molecules of which are mostly associated with the sugars. The resultant osmotic effect prevents microbial growth but the antimicrobial activity of honey is not exclusively due to the osmotic effect but other substances (Molan, 2006; French *et al*, 2005). These were observed when diluted honey inhibited microorganisms unlike concentrated sugar solution (French *et al*, 2005).

5.1.2.3Hydrogen peroxide activity of Honey

Hydrogen peroxide is produced in honey when glucose is broken down by glucose oxidase to produce gluconic acid and hydrogen peroxide (White Jr. *et al*, 1963). The

hydrogen peroxide is broken down by the catalase enzyme which is also present in the honey.

Glucose + H_2O + O_2 → gluconic acid + H_2O_2

In diluted honey, the effect of glucose oxidase enzyme lowers the pH which further reduces the activity of the enzyme and consequently the production of peroxide (Molan, 1992). The slow release of the hydrogen peroxide prevents toxicity to the tissues but acts as antiseptic without causing damage to the tissues when honey is applied to wound (Molan, 2006). The oxygen free radicals released by the hydrogen is oxidised by the iron present in honey which neutralises the effect on the tissue. The release of the hydrogen peroxide has been particularly linked to the antimicrobial activity of some honeys (Weston *et al*, 2000). Some honeys like manuka honey are non-peroxide honey but the antimicrobial properties have been linked to other components especially the phytochemical components (Atrott and Henle, 2009; Mavric *et al*, 2008; Adams *et al*, 2008).

5.1.2.4 Phytochemical components of honey

The phytochemical components have major effect on the antibacterial properties of honeys which determines the potency of various honeys (Mboto *et al,* 2009; Molan, 2006) and they are dependent on the type of plants from which the bees derived the nectar. In manuka honey, it has been shown that the antimicrobial properties are not dependent on peroxide activity (Molan, 1992) and methylglyoxal (MGO) has been identified as one of the major active antimicrobial components responsible for the effectiveness against microorganisms (Atrott and Henle, 2009; Mavric *et al,* 2008; Adams *et al,* 2008). In their studies Atrott and Henle (2009) analysed 61 manuka honey samples and the results showed that the level of methylglyoxal in the honey samples ranged from 189 to 835 mg/kg honey with corresponding antibacterial activities of 12.4

to 30.9 % equivalent phenol concentration. Mavric and colleagues (2008) evaluated the MGO in six manuka honeys and found the concentrations of the MGO to be 1000 times higher than the conventional honeys and that 15 -30% w/v manuka honey showed antimicrobial effect whereas other honeys were only inhibitory to organisms at 80% concentration. The study also linked the 1.1 -1.8 mM concentrations of MGO to 15 - 30% manuka honey concentration and to the unique manuka factor (UMF) values of medical grade manuka honeys. Adams and colleagues (2008) characterized the components of 49 manuka honeys alongside 39 other honeys from New Zealand; they confirmed the non peroxide nature of manuka honey using an agar well diffusion assay with *S. aureus*. Analysis with HPLC detection assay revealed the effective antimicrobial component of manuka honey as methylglyoxal (Adams *et al*, 2008). Adams and colleagues in 2009 explored the origin of methylglyoxal in manuka honey by studying the chemical components of nectar from manuka shrubs (*Leptospernum scoparium*) and other plants and linking the chemical composition to the methylglyoxal in manuka honey. The activities of enzymes in honey are responsible for the conversion of dihydroxyacetone to methylglyoxal (Adams *et al,* 2008). The concentration of methylglyoxal in manuka honey increased with temperature according to the study indicating the effect of temperature on the conversion of the chemical components of honey (Adams *et al,* 2008). Another chemical component, defensin 1, has been identified in honey recently (Kwakman *et al*, 2010).

5.1.2.5 Antioxidants properties of honey

Honey has antioxidant properties (Oddo *et al*, 2008; Henriques *et al,* 2006) which are capable of neutralizing biologically destructive chemicals in the body. The antioxidant contents of honey diminish with time particularly from six months of storage notwithstanding the temperature and storage containers (Jiménez *et al,* 2006) hence

fresh honeys might be more beneficial than the older ones when used as antioxidant. Total phenolic content have been shown to influence the antioxidant activity of honeys although it is not solely responsible for it (Gheldof *et al,* 2002). Antioxidant activities vary with the floral content and source of honey (Hegazi and El-Hady, 2007; Gheldof *et al,* 2002 and 2003). The variations in the antioxidant property of honeys were demonstrated by Henriques and colleagues (2006) with three types of honey. Manuka honey (a medical grade honey) was found to have completed quenching of the generated free radical by peroxide in 5 minutes whereas Pasture honey (another medical grade honey) quenched the free radicals completely in 1 hour. The beneficial effect of antioxidant property of honey can not be overemphasized in wound management particularly in the removal of free radicals which are often produced by some pathogenic organisms like *Pseudomonas aeruginosa* to invade tissues during infection.

5.1.3 Sterility of honey

Although there are numerous benefits of honey, honey sometimes contain microorganisms (Cooper and Jenkins, 2009; Olaitan *et al,* 2007; Malika *et al,* 2005) especially yeasts and spores of some organisms such as the *Bacillus* species (Cooper and Jenkins, 2009) and *Clostridium botulinum* (Midura *et al*, 1979) which could be from the bees, the environment or from the human handlers (Snowdon and Cliver, 1996). A similar range of microorganisms have been found in honeys obtained from the same plant source suggesting that the source of the organisms might be from the plants (Malika *et al,* 2005). Although due to the antimicrobial nature of honey, the organisms might not thrive and be destroyed overtime but the ability of the spores of some organisms to thrive in honey could jeopardize the beneficial effect especially in children and the immuno-compromised. It is therefore pertinent to sterilise honeys that are to be used for treatment of infections hence medically prepared honeys which are

usually sterilized to avoid the risk associated with the organisms should always be used for treatment purposes. According to the study by Cooper and Jenkins (2009), while the medical grade honey (MGH) examined was found to be sterile the non- MGHs (table honeys) contained a range of microorganisms.

5.1.4 Medical importance of Honey

5.1.4.1 Antibacterial properties of honey

The antibacterial activities of honey have been extensively studied (Williams *et al,* 2009; George and Cutting, 2007; French *et al,* 2005; Cooper *et al,* 2002). Honey was found to be many times more potent against coagulase-negative staphylococci than if bacterial inhibition were due to their osmolarity alone when compared with artificial honey or sugar solution (French *et al,* 2005; Efem and. Iwara, 1992) even when diluted up to 7 to 15 fold honey still inhibited bacterial growth (Cooper *et al,* 2002). Antimicrobial effects of honey have been demonstrated on common wound isolates such as *Pseudomonas* (Cooper *et al*, 2002 b), *Staphylococcus* (French *et al,* 2005; Cooper *et al,* 1999), fungi (Koc *et al,* 2009; Basson and Grobler, 2008), MRSA (Cooper *et al,* 2002), VRE (Cooper *et al,* 2002a) *Streptococcus* species (Efem and Iwara,1992) and recently on biofilms *in vitro* (Alandejani *et al,* 2009; Okhiria *et al,* 2009; Merckoll *et al*, 2009). Although honey was antifungal *Candida* species were found to be more resistant to honey than bacteria (Basson and Grobler, 2008). Koc and colleagues (2009) tested the effect of various honeys on 40 yeast strains (*Candida albicans, C. krusei, C. glabrata* and *Trichosoporon* species) and all the organisms were inhibited by the honeys. According to a review of the laboratory and clinical evidence of antimicrobial properties of honey by Cooper (2008) the evidence of broad antimicrobial effect of honey against bacteria, fungi, viruses and protozoa has been recognised but much of the evidence is anecdotal and further research is needed to appreciate the mechanism of

inhibition. Basson and Grobler‟s study also confirmed variation in honey‟s antimicrobial effect as a result of the phytochemical properties which are dependent on the plant from which the bees derive the nectar.

5.1.4.2 Enhancement of immune function

Scientific research with extracts from honey showed chemotactic activity for neutrophils and antitumour characteristic in mice (Fukuda *et al,* 2009; Hamzaoglu *et al,* 2000). Various components in honey other than the sugars are responsible for the potency of honey. Tonks and her group (2003) observed the stimulation of cytokines by three honeys (jelly bush, manuka and pasture) and later (2007) discovered the 5.8-kDa component of manuka honey stimulated cytokine production whereas the sugar solution did not; and honey increased cellular viability which might explain the reason for the effectiveness of the healing properties of honey particularly in wounds. When honey extract were injected into mice, stimulation of the immune function and anti-tumour activity were observed in the test mice unlike the control mice (Fukuda *et al*, 2009). This enhancement of immune function has a great impact on the healing potential of honey.

5.1.4.3 Application of Honey in wound treatment

The application of honey in the treatment of wounds dates back to the history of mankind (Majno, 1975). There are unique properties of honey that enhance wound healing potential which make honey an all encompassing remedy for wound treatment. These factors include the antibacterial activity which prevents mal-odour and inflammation, provides moist environment within the wound which aids debridement; and stimulation of wound healing processes. The use of sugars in the honey by bacteria instead of the amino acids in the dead tissues of the wounds further reduces the

unpleasant odour of the wounds (Molan, 2001). The uses of honey in treatment of infected wounds and topical infections have been shown through several studies (Robson *et al* 2009; Cooper and Molan, 1999; Subrahmanyam, 2005; Efem, 1988). Honey has been used successfully in dressing infected wounds (Ingle *et al*, 2006; Dunford *et al*, 2000).

It has been shown that honey keeps wound sterile even when such wounds have been colonised and it debrides wounds easily (Efem, 1988). An acidic medium aids the healing of wounds and the pH of honey reduces the pH and maintains lower pH when applied to a wound. This reduction of wound pH on application of honey reduces microbial load which aids wound healing (Molan, 2001). Gethin and her colleagues (2008) observed that chronic wounds with pH of less than 7 which was achieved on the application of honey healed faster than those at higher pH before honey was applied. Honey is hygroscopic so when applied to wounds it keeps the wound moist and prevents formation of scalds. The moisture also prevents the dressings from getting stuck to the wound surface therefore reducing the discomfort to patients when dressings are being removed. In a randomised control trial by Ingle and colleagues (2006) honey treatment showed no significant advantage as effective healing agent over other agents but treatment with honey offered other advantages of being cost-effective safe and satisfying. Molan (2006) in a review article highlighted various positive evidence on the application of honey in the treatment of wounds; including 17 randomized controlled trials involving 1,965 participants, 5 clinical trials of other forms involving 97 participants and the effectiveness of honey in assisting wound healing demonstrated in 16 trials on 533 wounds. Lotfi (2008) discussed extensively the use of honey in animal and human wounds. Simon and his colleagues (2009) exemplified the success rate of wounds treated with honey in Germany especially the medical grade honey. Cooper (2008) also reviewed the application of honey in the treatment of wounds and inhibition

of pathogens. The importance and benefits of honey as a modern medicine in the treatment of wounds as well as the ways by which honey effectively heals wounds were highlighted. Evidence of reduction of inflammation in wounds by honey has also been reported (Dunford, 2000) so the effect of tissue damage and delayed healing through excessive inflammation could be avoided which might be due to the antioxidant in honey which removes the free radicals (Phillips *et al,* 2009; Henriques *et al,* 2006) which often reduces inflammation (Molan, 2002).

Some researchers are of the opinion that honey has no better healing potential than other topical agents that are being used as wound dressings because such dressings have similar effect on wound healing as honey (Jull *et al*, 2008; Moore, 2008). The use of honey in health care was recently reviewed by Bardy and others (2008) which include 43 studies in relation to burns, skin, cancer and other wounds. The results of the review showed that many of the studies were not replicable because of poor description, incomplete methodologies or inconsistencies between the groups studied while some studies did not include standard deviations in their statistical data. Some studies did not show the inclusion or exclusion criteria and sample size and lack of proper documentation of informed consent in some studies particularly of note were those involving minors. Meta-analysis of the systematic review was not possible due to the heterogeneity amongst the studies (study designs, data presentations, variety of conditions, different populations). Other factors that render the analysis inconclusive include factors such as age, varying wound sizes and sites, presence of infection and other conditions that may delay wound healing. They found that there is scope for usage of honey for cancer patients. In conclusion, the analysis found honey to be particularly effective when standard therapy failed and irrespective of the outcome of the review, it was concluded that honey seems to provide an alternative treatment option according to the overwhelming evidence. The application of honey in wounds particularly in

reduction of bioburden, minimising scarring and pain reduction was highlighted. The use of honey in the management of other skin conditions such as dandruff, seborrheic dermatitis, psoriasis and other fungal infections as well as chemotherapy or radiation induced skin reactions was analysed. Evidence of reduction of oral mucosis in cancer patients (Biswal *et al,* 2003) was shown to be comprehensive, well designed, controlled and reliable. A major problem identified in the use of honey is the varying degrees of potencies of various honeys which suggest that the antimicrobial effect of honeys might vary significantly. The use of honey in oncology and medicine in general was diverse and the significant role of honey in healthcare can not be overemphasized. Future research was suggested to further unravel and elucidate the healing potentials of honey.

In another meta-analysis; the application of honey in wound treatment was reviewed (Jull *et al*, 2008) which include acute and chronic wounds of all types. 19 trials were included in the review including minor acute wounds, burns, acute and chronic wounds such as pressure ulcers, diabetic ulcers, varicose ulcers and tropic ulcers. According to the review, there does not seem to be enough evidence to support the use of honey in minor uncomplicated wounds. In burn wounds treatment, honey was found to be more effective than conventional dressings but there appears to be delayed healing in mixed depth burns treated with honey. The reviewers are of the opinion that the rationale for conducting the trials of the treatment of acute and chronic wounds except venous leg ulcers is unclear and most of the trials are small therefore the findings might not influence clinical practice. In summary, it was admitted that honey improves healing over conventional dressing in mild to moderate superficial and partial thickness burns but not chronic venous ulcer. Appropriate procedure for reporting clinical trials has been recommended.

Simon and colleagues (2009) in a commentary of a brief review on honey titled „‟Medical honey for wound care - still the latest resort?““opined that honey did not meet

the criteria for an ideal wound antiseptic because honey has no fast onset of activity. Honey does not reduce bacteria and fungi by more than five log reductions in 1-10 minutes as an antiseptic. However unlike conventional antimicrobials such as iodine and silver products that are involved with problems of metabolism honey is safe to use even for paediatric patients. Raised liver enzymes has been reported in patients using silver coated dressings (Trop *et al,* 2006) while the effect of iodine on thyroid functions has been an issue to be considered when applying the product on infants. The uses of honey as treatment interventions were highlighted in the review including recalcitrant wounds (Simon *et al*, 2006;Blaster *et al,* 2007;Cooper *et al,* 2001), oral mucositis and gingivalis in cancer patients (Biswal *et al,* 2003), wound care in neonates (Vardi *et al,* 1998), viral treatment (Al-Waili, 2004), ocular treatment (Albietz *et al*, 2006). Honey treatment is less expensive than conventional antimicrobial agents (Ingle *et al,* 2006) an additional benefit to encourage health professionals to use honey in wound care. However certified honey with standardised antibacterial activity should be used as this will eliminate the problem of the non- uniformity of efficacy of various honeys.

5.1.5 Biofilm resistance and justification for alternative antimicrobials

The problem of biofilm infections in particular resistance to body immune system and commonly used antimicrobials have been highlighted and discussed extensively and these phenomena often result in chronic infections. The formation of biofilm in wounds by pathogenic organisms has been associated with impairment of healing processes due to the combined effects of the virulence of the organisms within the biofilm (Bjarnsholt *et al*, 2008). Biofilm formation involves a series of physiological and phenotypic activities in which several genes are regulated in response to quorum sensing signalling molecules produced by biofilm organisms (Sauer *et al*, 2002).

Inhibition of quorum sensing by some conventional antimicrobials such as azithromycin (AZM), ceftazidime (CFT) and ciprofloxacin (CPR) were investigated by using DNA micro array analysis and reverse transcription polymerase chain reaction (rt-PCR) (Skindersoe *et al,* 2008). The results showed that the drugs decreased the expression of a range of quorum sensing regulated virulence factors in *P. aeruginosa.* The effect of attenuation of the organism through inhibition of quorum sensing signalling molecules will allow the body defence system of the host (patient) to easily eliminate the organism from the site of infection. A study in which *P. aeruginosa* quorum sensing was inhibited resulted in the attenuation of the organism and the clearance from the mice lungs was more effective (Hentzer *et al*, 2003).

Honey as an alternative antimicrobial has been explored in the treatment of infections and as a result the antimicrobial effects of honey have been widely investigated on various pathogenic organisms in the last two decades. Although many studies have been done on the effect of honey on microorganisms (French *et al,* 2005; Cooper *et al,* 2002) including biofilm (Alandejani *et al,* 2009; Okhiria *et al,* 2009 and 2004; Merckoll *et al*, 2009) the mode of action of honey on pathogenic organisms and biofilm in particular is yet to be fully elucidated. More investigations will increase the understanding of the mode of action of honey relevant to treatment of infections caused by virulent pathogens in wounds and topical infections.

5.1.6 Recent studies on the antibacterial activities of various honeys

In a study done by Rasmussen and his colleagues (2005), various natural products (54) were screened for ability to inhibit quorum sensing, of which 11 showed quorum sensing inhibitory activity. The effect of garlic, one of the agents that inhibited QS, was further tested on biofilm of PAO 1 and also in combination with tobramicin antibiotic. It was observed that most of the bacteria in the biofilm treated with both garlic and

tobramicin died, while the cells at the top layer of the tobramycin treated biofilm died but garlic extracts alone had no effect on biofilm. The control biofilm culture exhibited the classical mushroom-like biofilm structure. The study showed evidence that natural products alone or in combination with conventional antimicrobial might exert some effect on biofilm by interfering with the quorum sensing systems.

The antimicrobial effects of honey on planktonic and biofilm embedded organisms in chronic wounds were examined (Merckoll *et al*, 2009). In the study, bacteria were grown as biofilm individually and as mixed cultures in broth and later exposed to honey. The bacteria were also grown in various concentrations of irradiated and commercially prepared honeys in broth with glucose. Difference in the susceptibility of the individual organism was observed and the planktonic counterparts of the individual organisms were susceptible to the honeys at low concentrations unlike the biofilms. ESBL *Klebsiella* was least susceptible of the individual cultures as well as in the mixed cultures. Medihoney® inhibited the growth of biofilm at 1.6% concentration but not at 0.8% and completely killed *Pseudomonas* at 6% (w/v) concentration whereas a Norwegian honey was inhibitory to MRSA at 25% over the 20 hour period of experiment. The study showed the variations in the resistance of various planktonic and biofilm organisms to different honeys and that honey can support the growth of microorganisms. Manuka honey was found to be of higher antimicrobial activity than the Norwegian honey. The researchers highlighted the advantages of the multiple modes of actions of honey on microorganisms. The interference of fructose content of honey with fuctose, which is involved in binding of *P. aeruginosa* to surfaces, might further prevent biofilm formation by *P. aeruginosa* (Lerrer *et al,* 2007).

In a study, 24 hour biofilms of 6 *P. aeruginosa* cultures (5 wound isolates and 1 reference culture) were exposed to 20% and 40% manuka honey (Okhiria *et al,* 2009). Biofilms exposed to the 40% honey reduced in quantity overtime but increased growth

was observed after 48 hours whereas the 20% honey solution biofilm growth was evident throughout the culture period for all the isolates and from 6 hours for the reference organism. The study shows the lethal effect of manuka honey on biofilm at 40% concentration but the tendency of the sugars contained in honey to enhance growth at sub-lethal concentration was revealed in the study.

Alandejani and his colleagues (2009) in a preliminary study examined the effects of Sidr and manuka honeys on 11 cultures of each of methicillin susceptible *S. aureus* (MSSA), methicillin resistant *S. aureus* (MRSA) and *P. aeruginosa* in both planktonic and biofilm forms. The honeys were able to kill all the planktonic cultures but Sidr and manuka honeys were effective against the biofilms of MSSA 63% -82%, MRSA 73% - 63% and *P. aeruginosa* 91% - 91% respectively at 1 in 2 dilutions of honeys showing the increase resistance of biofilm than the planktonic counterparts. The strong antimicrobial effects of the 2 honeys were demonstrated in their finding. Although the initial study started with 4 honeys but 2 were dropped because of lack of cidal effect on biofilm by the particular honeys, an indication that not all honeys should be used on wounds that have biofilms since such honeys may enhance the growth of the biofilm organisms (Okhiria *et al,* 2009).

In one of the recent studies, Ansari and Alexander (2009) tested the antimicrobial effect of a honey produced by *African sculata* obtained from Guyana against *Escherichia coli*, *Pseudomonas* species, *S. aureus* and *Candida albicans* using disc diffusion method. It was observed that the honey was effective against all the bacteria but *C. albicans* was not affected by the honey. The study showed that all honeys are not effective against all pathogenic organisms that may be found in wound.

Another examination of the mode of inhibition of manuka honey on *S. aureus* NCTC 10017 (Henriques *et al,* 2010), scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of the organism before and after exposure to honey showed

that the inhibition of *S. aurues* 10017 by manuka honey was irreversible (MIC and MBC values of 2.9 and 4.5% (w/v) respectively). The effect of 10% (w/v) manuka honey on *S. aureus* revealed that manuka honey interfered with cell division of the organism. There was an increase in the number of cells with crosswalls in the culture treated with 10% (w/v) manuka honey unlike the cells in the control cultures indicating that the cells could not divide.

Recently, the antimicrobial activity of 30 honeys on quorum sensing inhibition of *C. violaceum* was investigated (Truchado *et al*, 2009). Inhibition of quorum sensing of *C violaceum* by the honeys investigated was shown using agar diffusion method, and estimation of violacein production by the reporter organism in broth culture. During the investigation, the relationship between the peroxide activities and phenolic composition of the various honeys were determined. The 15 unifloral honeys of different geographical origins showed different QSI activities in dose dependent manner from 0.2g/ml. Reduced production of violacein pigment was found in broth cultures of *C. violaceum* in the presence of honey samples and increase in honey concentration resulted in significant increase in QSI activities of the honeys. The study also found that QSI activity of individual honey is not solely dependent on the peroxide activity of the honeys although there was reduction in QSI activities on addition of catalase to the culture. There was no correlation between the phenolic content of the honeys and the QSI activity of the honeys. The study demonstrated that QSI activity of honey which was demonstrated by lack of violacein production by the reporter organism was not due to inhibition of growth but inhibition of quorum sensing of the organism.

5.1.7 Treatment of biofilm embedded in wounds with honey

In the biofilm niche, signalling molecules mainly control the genetic and physiologic activities which sequentially influence the pathogenicity of the organism (Zhu *et al*,

2002; de Kievit and Iglewski 2000). Biofilms have been found to be mostly polymicrobial particularly in wounds (Malic *et al*, 2009; Dowd *et al*, 2008; James *et al*, 2008) and communication amongst organisms within biofilm could be via the intra or interspecies QS molecules (Donlan and Costerton, 2002; Williams *et al*, 2007). Although the Gram positive organisms utilise short chain peptides as QS, the Gram negative organisms utilise the homoserine lactones such as AHL which are different but interestingly the organisms are able to communicate with the global QS system across the species and genera of microorganisms.

Organisms embedded in biofilms have been shown to be highly resistant to microorganisms even up to 10 - 1000 times more resistant than their planktonic counterparts (Costerton *et al*, 1995). This has been associated with the phenotypic changes that occur when organisms are in biofilm state (Sauer *et al*, 2002) in response to quorum sensing signalling molecules as well as the EPS secreted by the biofilm organisms themselves (Hentzer *et al*, 2002). A study of the effect of silver sulfadiazine (SSD) antimicrobial against biofilm in a continuous culture was published (Bjarnsholt *et al*, 2007). Biofilms of *P. aeruginosa* were cultivated for 4 days in flow cells and various concentrations silver sulfadiazine solution were tested against the cultures over a period of 24 hours. Tobramycin antibiotic was tested in a similar manner. Results showed that silver sulfadiazine concentrations that will kill biofilm were 10 – 100 times higher than the lethal concentration for the planktonic counterpart, whereas tobramycin had little or no effect on the mature biofilm even at concentration as high as 340 µg/ml. In the same study the effect of QS on the sensitivity of SSD and tobramycin were tested. The biofilms of *P. aeruginosa* were rendered sensitive to tobramycin when the QS was inhibited whereas SSD‟s activity was not QS dependent having similar result as seen in the wild type *P. aeruginosa* biofilm.

In biofilm infections various treatment interventions have been suggested and in particular the disruption of the mode of interaction of biofilm organisms such as the inhibition of quorum sensing molecules (Bjarnsholt and Givskov, 2007; Hentzer and Givskov, 2003) and has been shown to reduce intercellular communication even across bacterial species and biofilm formation (Bjarnsholt *et al* , 2005), and enhanced the clearance of organisms by the host immune system (Wu *et al,* 2004; Balaban *et al,* 2003). Studies have confirmed that quorum-sensing inhibition of pathogens such as *P. aeruginosa* by natural and synthetic compounds such as garlic and plants consequently reduced the *P. aeruginosa* tolerance to tobramycin (Rasmussen *et al,* 2004). In similar study antibiotics like AZM, CFT, and CPR have been found to decrease the expression of a range of QS-regulated virulence factors (Skindersoe *et al,* 2008) and it has been suggested that attenuation of virulent organisms such as *P. aeruginosa* by using quorum-sensing inhibitors like salicylic acid, nifuroxazide, and chlorzoxazone would provide antipathogenic effect (Yang and colleagues, 2009).

McLean and his colleagues (1997) devised a method to determine the inhibition of AHL signalling molecules by synthetic analogues. In their study, a Gram negative bacterium *C. violaceum* with characteristic of purple pigment (violacein) production was used as a biosensor. *C. violaceum* produces purple pigmentation due to production of quorum sensing molecules N-hexanoyl-L-homoserine lactones (HHL), inhibition of which results in the lack of purple colouration but whitish colonies as shown in (MacLean *et al*, 1997). This assay has been designed to suit a wide variety of assays involving AHL and signalling molecules using *C violaceum* as a biosensor.

In this chapter the effect of honey as antimicrobial against biofilm in continuous culture was determined. The mode of action of honey on quorum sensing of biofilm organism was investigated using *C. violaceum* as an indicator organism.

5.1.8 Aim of the chapter

The aim of this study was to investigate the effect of honey on biofilm.

Objectives

- To determine the effect of honey on *P. aeruginosa* biofilm in a continuous culture wound model.
- To study the effect of honey on quorum sensing in a biofilm forming organism.
- To develop a screening method for evaluating the potency of honeys as antimicrobial agent.
- To determine the inhibitory effects of some honeys against biofilm forming organism as a measure of potency.

5. 2 Materials and Methods

5.2.1 Organisms and reagents

5.2.1.1 Preparation and maintenance of cultures

Chromobacterium violaceum ATCC 31532 was obtained from the American type culture collection, USA and cultured on Luria broth agar at 30^{o}C for 48 hours. Colonies were harvested into the bacterial preserve media with beads (PROTECT Bacterial Preserver System, UK) and stored at -80^{o}C. The bacteria were cultured on LB agar for 24 hours prior to use. *Pseudomonas aeruginosa* P.98 clinical isolate (source and preservation have been discussed in chapter 2, section 2.1).

5.2.1.2 Preparation of reagents

Preparation of 0.3 % (w/v) Luria broth agar

Luria broth was prepared according to the manufacturer‟s specification and Technical agar powder (Oxoid, UK) was added to obtain 0.3% (w/v) concentration. The contents of the flask were boiled to dissolve the agar. Five ml aliquots of the media was dispensed at 60 ^{o}C into each universal container and sterilised at 121 ^{o}C for 15 minutes in an autoclave. The media was kept at room temperature prior to use.

Preparation of X-Gal solution

20 mg X-gal stock solution was prepared as described in chapter 2, section (2.2.2.6) prior to use.

Preparation of Chloramphenicol antibiotic solution

Five ml of 50 µg/ml stock solution of Chloramphenicol antibiotic (Sigma, UK) was prepared using sterile deionised water into a sterile Bijou bottle and the solution was kept in a freezer at -20 ^{o}C prior to use.

5.2.1.3 Honeys used in this study

The details of the honeys used in this study are provided in Table 5.1 below.

Table 5.1: Sources and antibacterial activities of the honeys tested

Honey	**Grade**	**Source**	**Total activity***	**Non-peroxide Activity****
Activon® (UMF 12)	Medical grade	New Zealand	18.9	18.9
Pasture honey	Table honey	New Zealand	14.8	0
M109 (UMF 18)	Table honey	New Zealand	18.3	18.3
Trinidad honey	Table honey	Trinidad	6.8	0
Kent honey	Table honey	United Kingdom	0	0
Manuka Active UMF20+	Table honey	New Zealand	14.6	15.1
Manuka Active UMF10+	Table honey	New Zealand	13.0	13.2
Manuka Active UMF5+	Table honey	New Zealand	6.4	7.1
Carmarthenshire honey	Table honey	United Kingdom	12.3	0
Mel de Saule	Table honey	France	Not tested	Not tested
Comvita® (UMF18)	Medical grade	New Zealand	18.8	18.6

All honeys were kept in the refrigerator at 4 ^{o}C prior to use.

* Antimicrobial activity of honey in relation to phenol activity

** The antimicrobial activity of honey after the addition of catalase to remove hydrogen peroxide

5.2.2 Effect of manuka honey on *P. aeruginosa* biofilm in a wound model continuous culture.

An overnight broth culture of the strong biofilm forming *P. aeruginosa* wound isolate P.98 was diluted 10^{-5} with Luria broth to obtain an inoculum of approximately $\log_{10} 4$ to 4.5 cfu/ml and TVC was determined. The wound model was set up as described in chapter 3, section 3.2.1.2.2 and inoculated for 1 hour with the diluted culture. The continuous wound model culture was supplied with $^{1}/_{10}$ LB for 30 hours and was replaced with 40% w/v honey (Comvita medical-woundcare, active UMF 18+) in LB for 18 hours. Culture effluent samples were taken for TVC and AHL detection at known

time intervals throughout the period of culture. Slide smears were made from each sample for biofilm detection as described previously in chapter 3 (section 3.2.1.2.2 and 3.2.2). At the end of the experiment the gauze from the wound model was removed aseptically and sterilised at 121^{o}C for 15 minutes in an autoclave (LTE Scientific, UK) and tested for AHL. The minimum detection level of the organism was determined by preparing serial dilution of the culture and determining the total viable count as previously described in chapter 2, section 2.2.3.1.

5.2.3 Effect of honey on growth and quorum sensing of *C. violaceum*

5.2.3.1 Determining growth and quorum sensing inhibitory properties of honey

To determine the effect of honey on quorum sensing, a reporter organism, *C. violaceum* was tested with various samples of honey in LB semi-solid agar at 30 ^{o}C for 24 hours according to the method of McLean and colleagues (1997). Five millilitres of 0.3% (w/v) sterile LB semi-solid agar was inoculated with 5 µl of the 24 hour *C. violaceum* ATCC 31532 broth culture and poured onto a pre-warmed LB agar. The inoculated agar was allowed to gel and form a thin layer before a well was bored into the centre of the plate with a sterile 6 mm stainless steel cork borer. 50% (w/v) concentration of M109 honey solution was prepared with sterile deionised water immediately before use; either 50 µl or 100 µl of diluted honey sample was pipetted into the well (2 plates were used for each volume of honey solution). The blank control included in the assay was 50 µl or 100 µl of sterile deionised water. The plates were incubated at 30 ^{o}C for 24 hours and cultures were observed with plate reader (ProtoCol, UK) for growth and quorum sensing inhibitions. Pictures of the plates were taken with the digital camera which was incorporated into the plate reader (ProtoCol, UK).

5.2.3.2 Interpretation of results of QSI culture

The reporter bacteria *C. violaceum* 31532 regulates pigment production by *N*-hexanoyl-HSL (C6-HSL) QS. Inhibition of QS molecules would be indicated by the lack of pigment production by the reporter strain. Growth inhibition of the reporter organism indicated an antibiotic effect of the honey sample (Fig.5.1). The diameter of the well was subtracted from the diameter of growth inhibition (GI) zone to obtain the actual GI value while the GI value was subtracted from the QSI zone to obtain the QSI value.

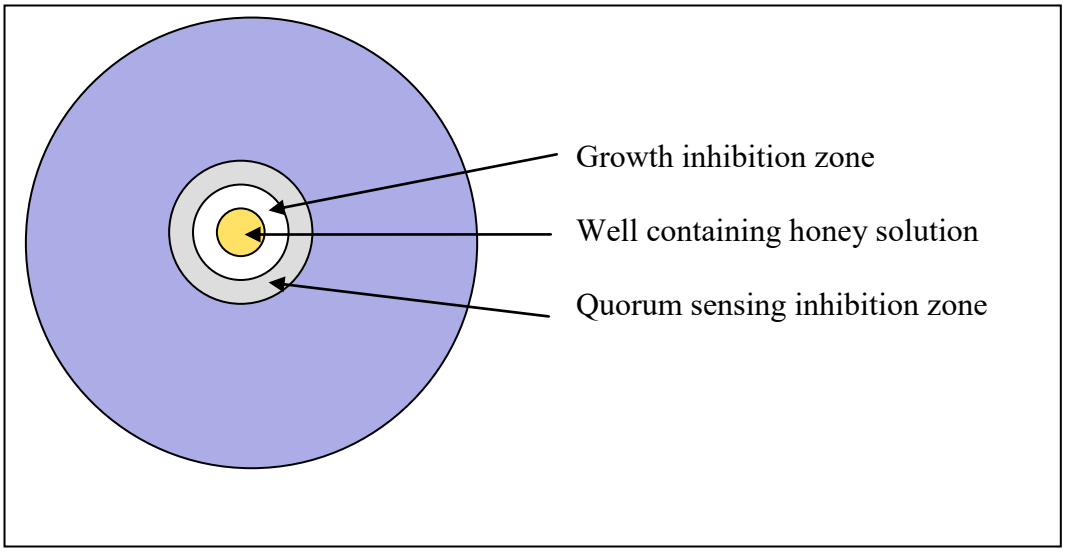

Figure 5.1 Diagram of honey QSI assay. *C. violaceum* ATCC 31532 culture showing the well containing honey solution (golden yellow) at the centre of the plate, the white zone representing growth inhibition and the grey zone showing the growth of *C. violaceum* without colour production due to inhibition of quorum sensing of the organism by honey while the growth of the organism with production of pigmentation due to quorum sensing is shown in the purple area.

5.2.4 Developing a screening method for evaluating the growth and quorum sensing inhibition potential of honeys (QSI assay)

5.2.4.1 Determination of inoculum size and volume of honey for QSI assay

In order to determine the effect of inoculum size on the quorum sensing assay, honey solutions were tested using various inocula of *C. violaceum* 31532 in the overlays. In each well either 100 µl or 50 µl of 50% (w/v) M 109 honey solutions was used and each set was tested in duplicate. The population size of *C. violaceum* in the overlay was estimated by TVC of each inoculum.

5.2.4.2 Effect of honey concentration on growth and QSI of C. violaceum

Concentration is an important factor in the efficacy of antimicrobial agents. It was therefore necessary to study the effect of honey concentration on the growth and quorum sensing of the reporter organism *C. violaceum* ATCC 31532. Various (10% to 50% (w/v) concentrations of M109 honey solutions were prepared with sterile deionised water immediately prior to use. Based on the result of the preliminary assays in section (5.4.2.1), 50 µl of each diluted honey solutions was pipetted into each well (each test was done in duplicate). The quorum sensing inhibition assay was performed by using various concentrations of honey solutions against the reporter bacterium, *C. violaceum* ATCC 31532. The cultures were examined for the inhibition of growth or quorum sensing after 24 hours incubation at 30 ^{o}C.

5.2.4.3 Reproducibility test for QSI assay

The consistency and reliability of the assay method was ascertained by testing (Activon®, manuka UMF 5+ and manuka UMF 10+) for ability to inhibit the growth and quorum sensing of *C. violaceum* ATCC 31532. The tests were performed on three different occasions using various concentrations (10%, 20%, 30%, 40% and 50%) of

each honey on each occasion and each test was performed in duplicate with 50 µl of each honey solution in each well.

5.2.5 Evaluating the potency of various honeys with QS assay

The growth and quorum sensing inhibition potentials of 10 honeys were determined once conditions for the QS assay were optimised. Various concentrations of each honey were tested against *C. violaceum* ATCC 31532 (in duplicates) using 50 µl of each honey solution per well. Chloramphenicol was used as a reference antibiotic at various concentrations (50, 25, 12.5, 6.25 and 3.125 µg/ml.) and sterile deionised water as the blank control.

5.2.6 Regression and statistical analysis

Regression analysis was done with Excel 2003 on each set of data obtained from the estimation of the viable organisms to determine the calibration curves of the continuous cultures, the histogram for the growth inhibition and the quorum sensing inhibition zones to determine the effect of inocula on growth and quorum sensing inhibitory properties of honey as well as the effect of honey concentration on the growth and quorum inhibitory properties of honey. Standard deviations were included in the data plot for the reproducibility assay data histogram plotted. Minitab (version15) was used to determine the correlation (Pearson correlation) between honey concentration and inhibitory effect as well as the UMF of honey and the inhibitory properties with 95% confidence limit.

5.3 Results

5.3.1 Effect of manuka honey on *P. aeruginosa* biofilm in a continuous culture wound model

The effect of 40% (w/v) comvita (medical grade – UMF 18+) honey on the 30 hour - biofilm of a strong biofilm forming *Pseudomonas aeruginosa* isolated from wound (isolate 98) in a continuous culture wound model showed evidence of an inhibitory effect. The biofilm was cultured with 1/10 strength Luria broth for 30 hours so that once the biofilm was established 40% (w/v) manuka honey (Comvita®) (UMF18) was added for 18 hours. The growth curve was found to be similar to those observed for the isolate previously (Fig. 5.2a) up to 30 hours and production of AHL by the bacteria was detected from the effluent samples between 24 hours and 30 hours (Figure 5.2). The bacterial growth was inhibited by the introduction of the honey to the model; from 36 hours (6 hours after the addition of honey) until the termination of the continuous culture at 48 hours. During the 18 hour period of exposure of the biofilm culture to 40% (w/v) manuka honey solution, no viable organisms were recovered in the effluent culture samples and AHL was not detected (Fig. 5.2). Minimum detection level for the wound isolate *P. aeruginosa* P.98 used in this study was 2.4×10^{1} cfu/ml of culture.

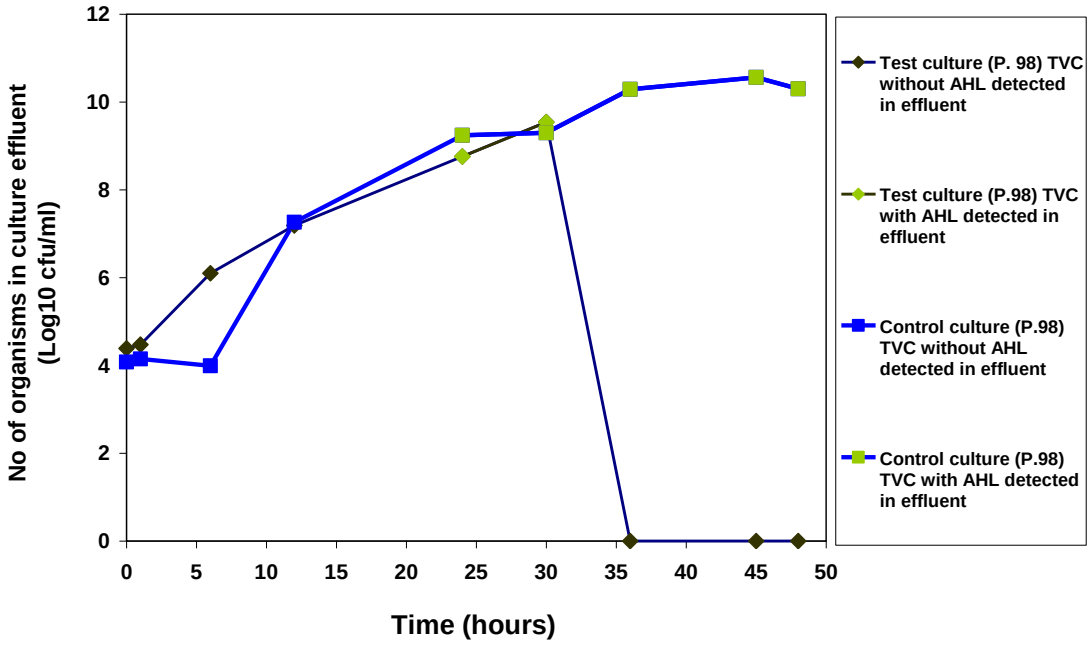

Figure 5.2: A chart showing effect of honey on biofilm in a wound model continuous culture. The 48-hour growth curve of a strong biofilm forming *P. aeruginosa* wound isolate (P.98) in a wound model continuous culture using 1/10 LB for 48 hours is shown with the culture of the 30-hour biofilm that was exposed to 40% (w/v) manuka honey in the wound model 18 hours. Biofilm growth increased and AHL was detected in culture effluents between 24 and 30 hours prior to exposure to honey. A sudden decline in growth was observed after the introduction of the honey sample; no viable organism was recovered from the culture after 6 hours.

5.3.2 Growth and quorum sensing inhibitory properties of honey

A 50% (w/v) M109 honey solution inhibited the growth of the *C. violaceum* in the area closest to the well containing the honey solution (clear zone). The honey also inhibited quorum sensing in *C violaceum* which resulted in the reduction of the purplish colouration of *C. violaceum* to greyish white due to honey solution interfering with secretion of signalling molecule by *C. violaceum*. The colour of the *C. violaceum* was restored from the zone where the effect of the honey was ineffective due to reduction in concentration of honey (dilution effect) as it diffused through the agar (Fig. 5.3a) while the blank control in which the well contained sterile deionised water showed

homogenous culture of *C. violaceum* without any growth or quorum sensing inhibition (Fig. 5.3b).

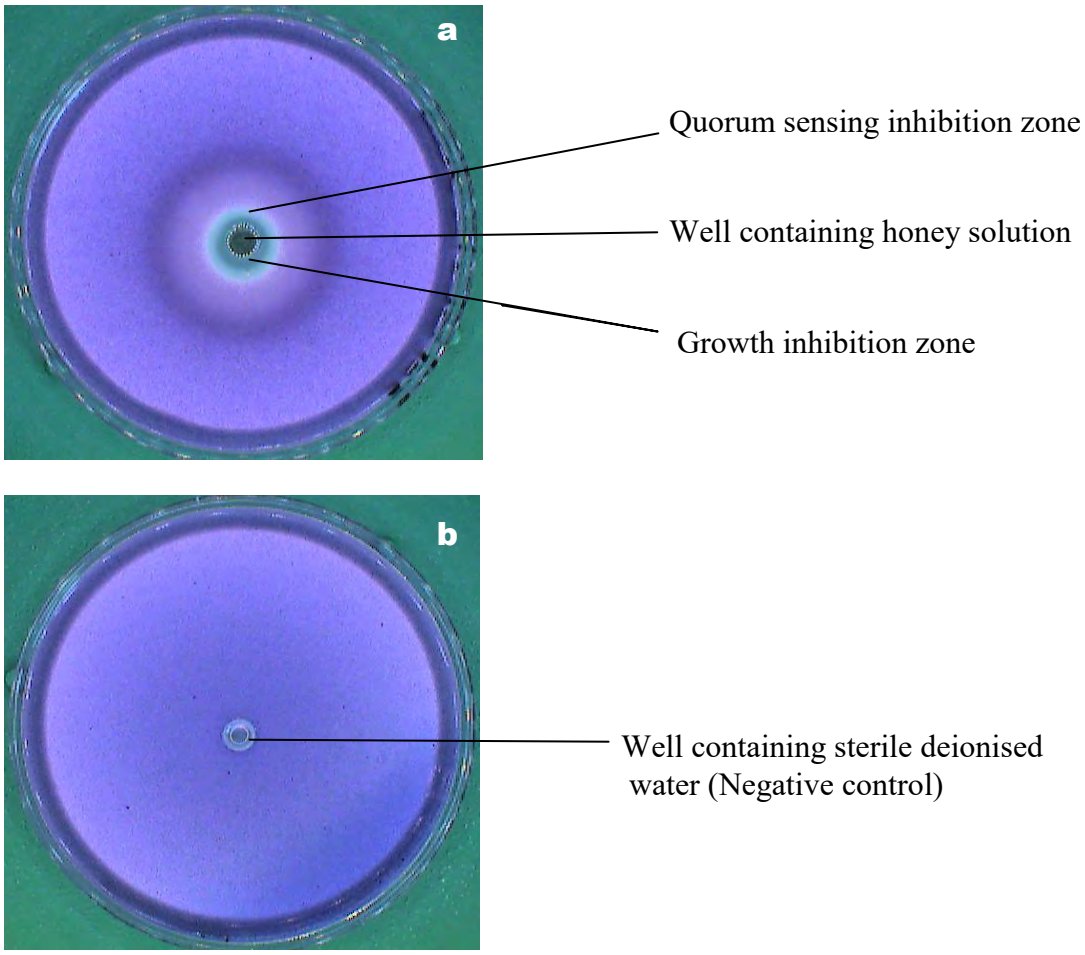

Figure 5.3: Quorum sensing inhibition effect of honey on *C. violaceum* ATCC 31532 in LB agar. Pictures: (a) 50% M109 honey solution in a 6mm diameter well at the centre showing culture of *C. violaceum* with growth and quorum sensing inhibition (QSI) zones due to antimicrobial effect of honey (b) blank control well containing sterile deionised water, *C. violaceum* culture showing no growth or QSI.

5.3.3 Effect of inoculum size on QSI assay

An inoculum below log10^7 cfu/ml of *C. violaceum* was found to give maximum GI when 50 µl of 50% (w/v) honey solution was introduced to the well above which the GI zones were reduced and remained almost constant (Fig. 5.4a). The QSI was higher when inoculum was about log 10^7 cfu/ml (9.5x10^6 and 1.9x10^7) of culture but reduced

with higher inocula ($4.75x10^7$ and $9.5x10^7$) cfu/ml (Fig. 5.4a). However when the 100 µl of 50% (w/v) honey solution was introduced, the GI zones were double those observed with 50 µl volume while the QSI zones of *C. violaceum* were half of the values observed with the 50 µl volume (Figure 5.4b). The lower inocula showed bigger zones of QSI than the higher inocula when the 100 µl honey sample was introduced which was the reverse of the effect of the 50 µl volumes. Interestingly the log $4.75x10^7$ cfu/ml inoculum has lower QSI values for both the 50 µl and 100 µl of honey than $9.5x10^7$cfu/ml inoculum (Fig. 5.4a and 5.4b).

The cumulative effect of the 50% (w/v) honey solution on the inoculum of *C. violaceum* as shown in table 5.2 indicates that the combined effect of the growth and quorum sensing inhibition activities of honey decreases with higher inoculum size (Figure 5.4a; Figure 5.4b) in relation to the volume of honey applied, $p<0.0005$. The purpose of this study was to examine the effect of honey on quorum sensing hence it was vital to prevent complete inhibition of growth of the organism therefore the 50µl volume of honey samples were found to be more useful than the 100µl volume.

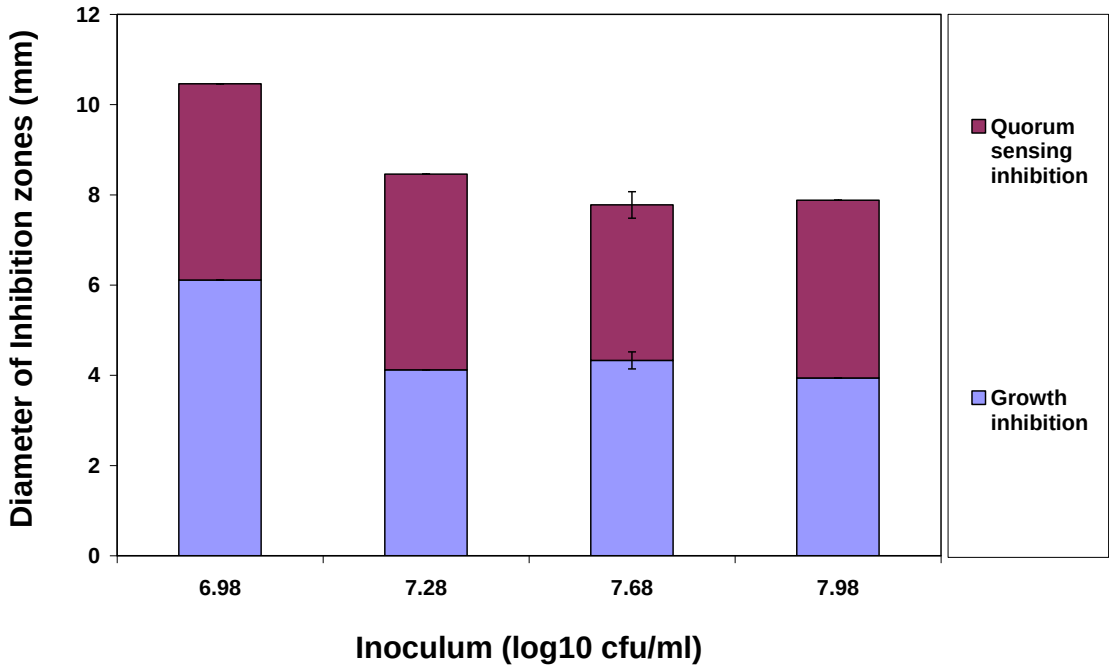

Figure 5.4a: Effect of inoculum size of *C. violaceum* on honey inhibitory properties using 50 µl of 50% (w/v) M109 honey solution in each well. The effect of honey against the growth and quorum sensing of reporter organism *C. violaceum* using various inocula against 50 µl of 50% (w/v) honey solution in each well revealed varying degrees of growth and quorum sensing inhibition zones. The effect on the growth of the reporter organism was higher when the inoculum was less than 10^7 organisms per ml of culture but lower when the inoculum was greater than 10^7 organisms per ml of culture.

Key: log 6.98 = 9.5 x 10^6; log 7.28 = 1.9 x 10^7; log7.68 = 4.75 x 10^7 and log 7.98 = 9.5 x 10^7.

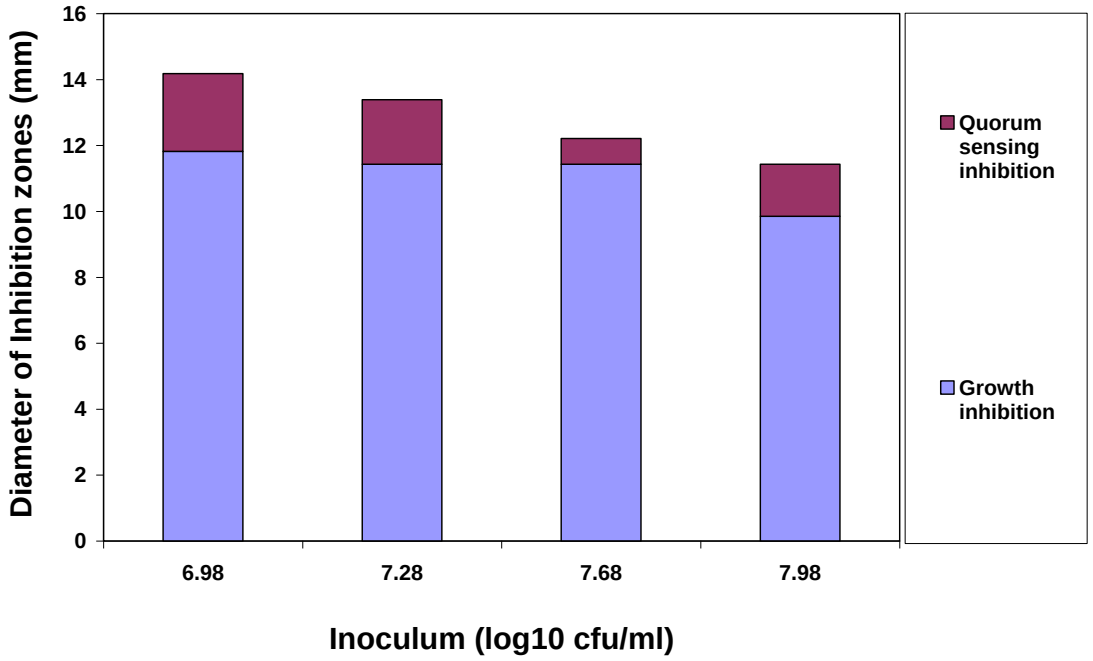

Figure 5.4b: Effect of inoculum size of *C. violaceum* on honey inhibitory properties 100 µl of 50% (w/v) M109 honey solution in each well. The chart shows the effect of inoculum of *C. violaceum* on honey concentration; the various inocula were tested against 100 µl of 50% (w/v) honey solution in each well. The effect showed marked growth inhibition zones and relatively reduced quorum sensing inhibition zones.

Key: log 6.98 = 9.5 x 10^6; log 7.28 = 1.9 x 10^7; log7.68 = 4.75 x 10^7 and log 7.98 = 9.5 x 10^7.

5.3.3.1 The ideal inoculum size for QSI assay

Based on the results of the preliminary assays, the 1.9x10^7 inoculum was chosen for subsequent assays while 50 µl volumes of honey samples was used for each well.

5.3.4 Effect of honey concentration on QSI assay

The effect of concentration on the inhibition of growth and quorum sensing was examined in 2 sets of assays by applying 50 µl of series of honey dilutions (10% - 50% (w/v) to the well in each culture plate showed increase in honey activity with higher concentration, $p<0.025$. Quorum sensing of *C. violaceum* was inhibited by 100 µl honey solution from 10% (w/v) concentration and it increases up to 30% (w/v) when the quorum sensing inhibition remained almost constant (Fig. 5.5) but the growth inhibition

of *C. violaceum* culture increases with increase in honey concentration (Fig. 5.5). The total effect of antimicrobial activities of honey was higher when 100 µl honey solution was applied to the culture than the 50 µl of honey solution.

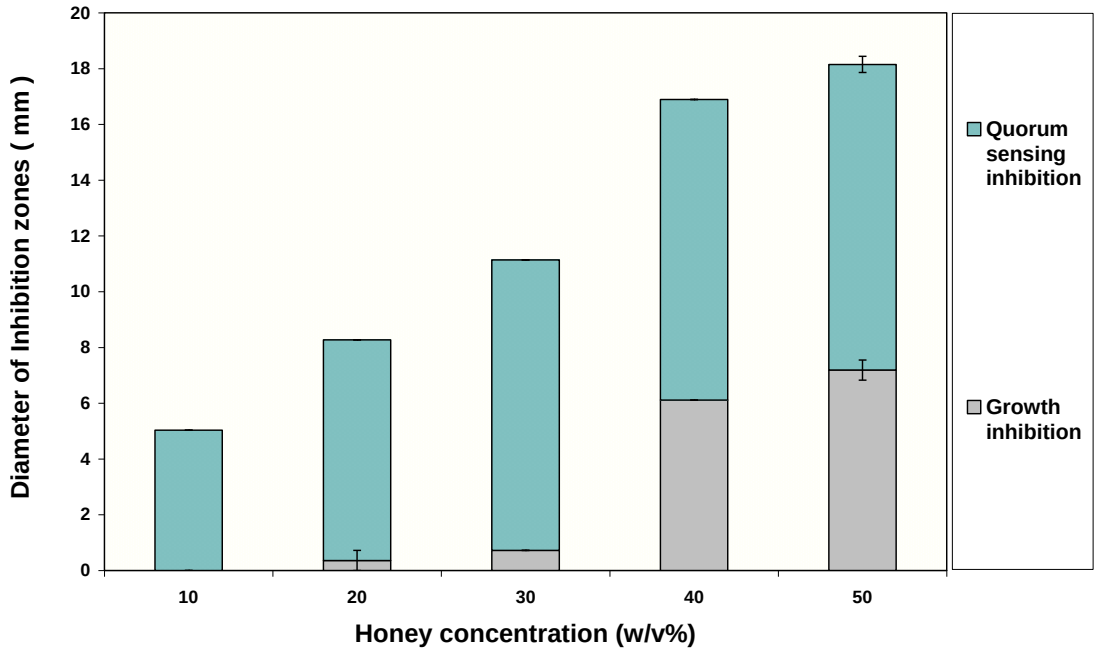

Figure 5.5: Graphical representation of the effect of honey concentration on growth and quorum sensing inhibitions of *C. violaceum*. Antibacterial activities of various concentrations of M 109 manuka honey were tested against *C. violaceum* in QSI assay. The growth and quorum sensing inhibitions of *C. violaceum* using 50 µl of honey solution in 6mm diameter well in each culture showed increase in honey activities with higher concentrations of honey solution.

The inhibition of *C. violaceum* was dependent on the concentration of honey. The cumulative (total) effect of the antimicrobial activities of honey on *C. violaceum* (combined growth and quorum sensing inhibition zones) also increased with concentration (Fig. 5.5) indicating the significance of honey concentration on the activity of honey, $p < 0.025$ using one – way ANOVA; Minitab (version 15) software.

5.3.5 Reproducibility of QSI assay

Reproducibility of QSI assay was determined by testing three honeys on three separate occasions in duplicate. Serial dilutions of each honey showed that each of the honeys tested inhibited the growth and quorum sensing of the reporter bacterium in a dose dependent manner thus demonstrating the effect of concentration on the activity of the honey samples (Fig. 5.6a, 5.6b, 5.6c and 5.6d).

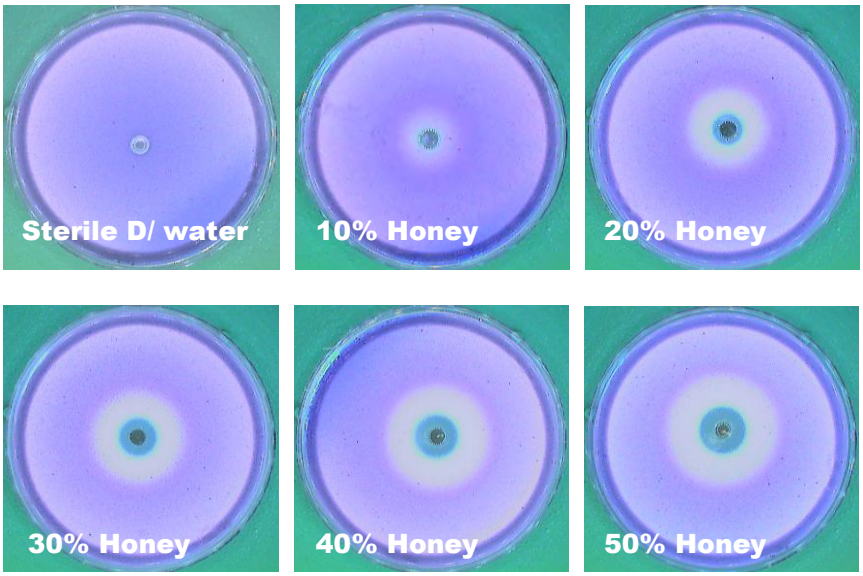

Figure 5.6a: Cultures showing the effect of honey concentrations on the growth and quorum sensing of *C. violaceum*.

The results were analysed with Excel statistical data analysis software and the charts of the means and standard deviations of the analysis were plotted (Fig. 5.6b, 5.6c and 5.6d). The results obtained from the 3 honeys were reproducible and there was significant correlation between honey concentrations and inhibition of growth and quorum sensing of *C. violaceum* ($p < 0.025$), using one-way ANOVA, Minitab (version 15). The honey with higher UMF factor 10 + (Fig. 5.6d) had greater activity than the honey with UMF factor 5+ (Fig. 5.6c) which is also indicative of the reliability of the assay.

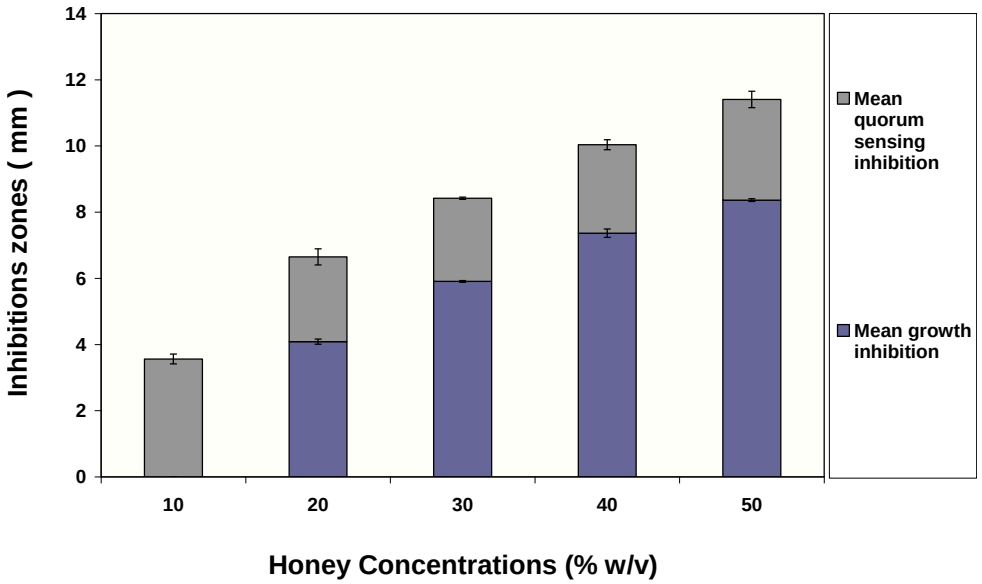

Figure 5.6b: **The effect of various concentrations of a medical grade honey (Activon® - UMF 12) on the growth and quorum sensing of *C. violaceum*.**

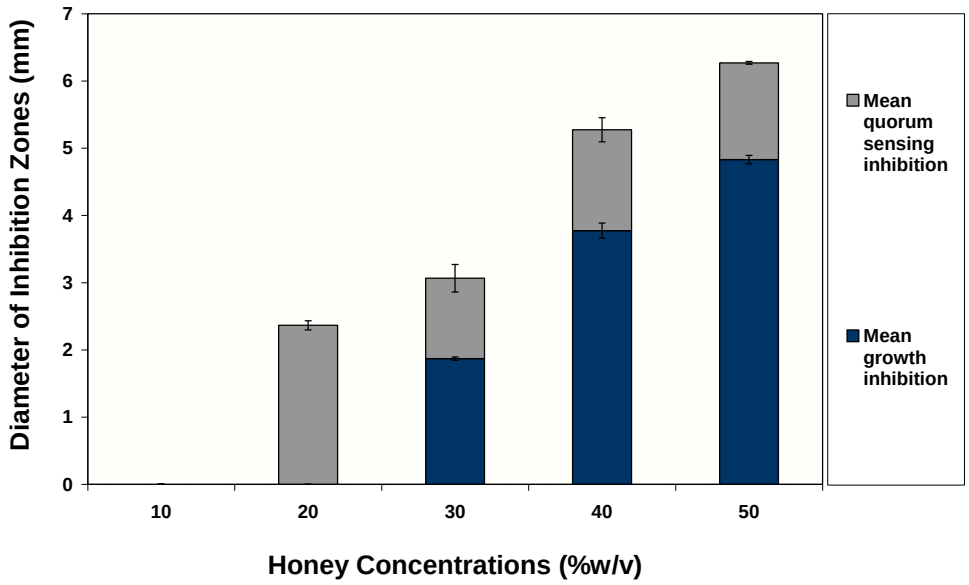

Figure 5.6c: **The effect of various concentrations of a manuka honey UMF 10+ on the growth and quorum sensing of *C. violaceum*.**

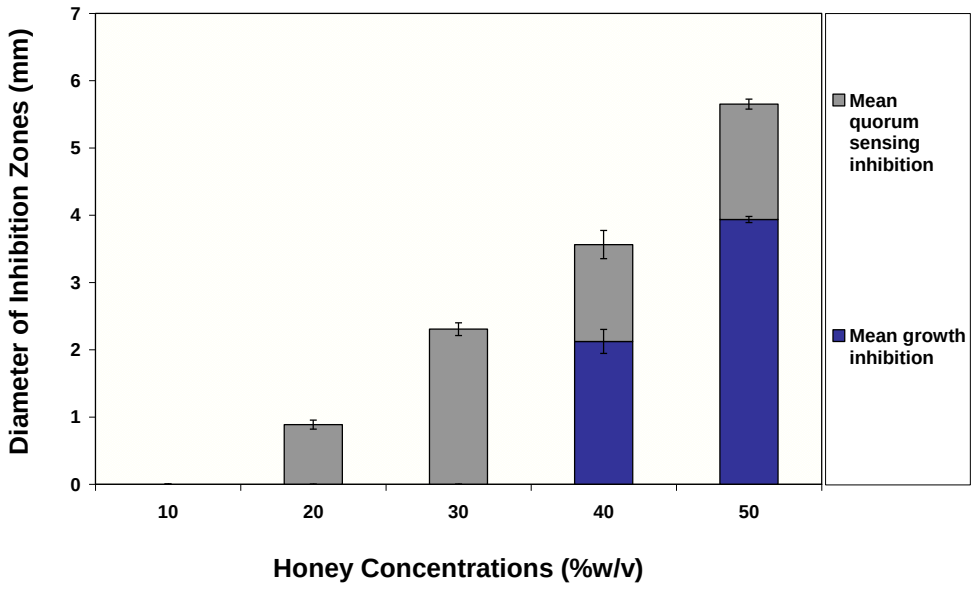

Figure 5.6d: **The effect of various concentrations of a manuka honey UMF 5+ on the growth and quorum sensing of *C. violaceum*.**

4.3.6 Growth and quorum sensing inhibitory properties of 10 honeys.

Out of the 10 honeys that were screened, 8 inhibited growth and quorum sensing of *C. violaceum*. A table honey (Kent honey) exhibited no growth or quorum sensing inhibitory effect on *C. violaceum* while another table honey (Miel de Saule) had no growth inhibitory effect but exhibited quorum sensing inhibitory effect on *C. violaceum*. A reference antibiotic (chloramphenicol) only inhibited the growth of the reporter bacterium at concentrations of 50 µg/ml and quorum sensing inhibition at 25 and 50 µg/ml (Fig. 5.7a). The different honeys revealed varying degrees of inhibitory activities against the reporter bacterium (Fig. 5.7b and 5.7c). Increase in both growth and quorum sensing inhibitory activities of honeys were directly related to increase in honey concentrations (Fig. 5.7b and 5.7c) $p < 0.025$. The activities of the manuka honeys with known unique manuka factors (UMF) showed activities relative to the UMF factors (Fig. 5.7d). It was found that the higher the UMF the stronger the inhibitory effect $p<0.005$, (Pearson correlation- Minitab version 15).

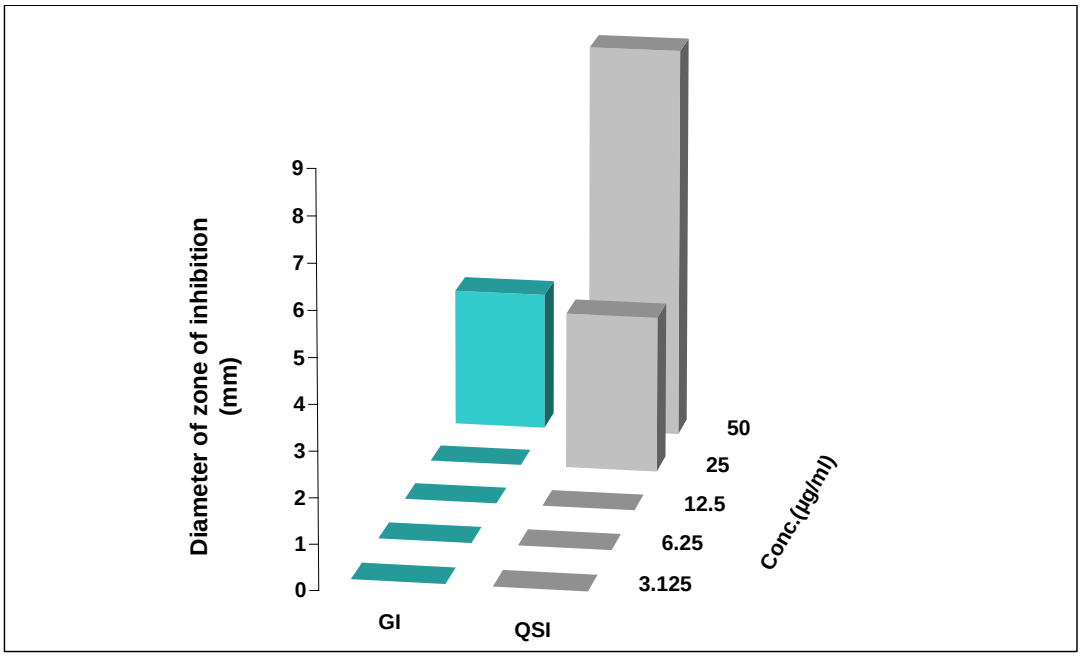

Figure 5.7a: Growth and quorum sensing inhibition of *C violaceum* by chloramphenicol.

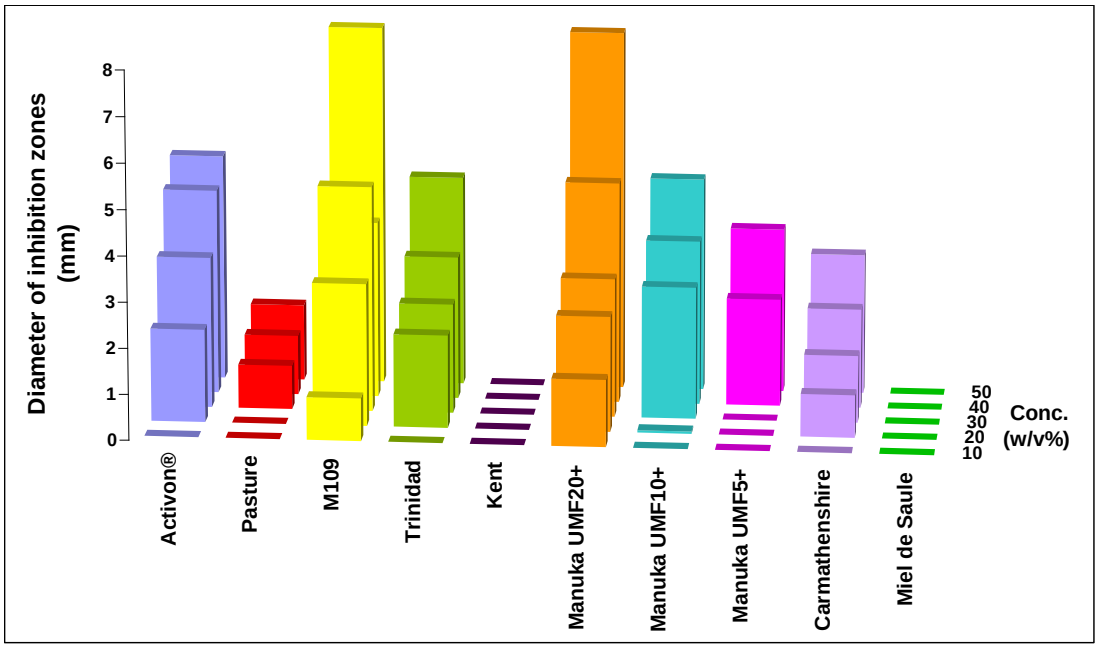

Figure 5.7b: Assessment of potency of honeys using growth inhibition of *C. violaceum*. The chart shows the growth inhibitory activities of 10 honeys at various concentrations against *C. violaceum* culture in LB semi solid agar using 50 µl of each honey solution in each well.

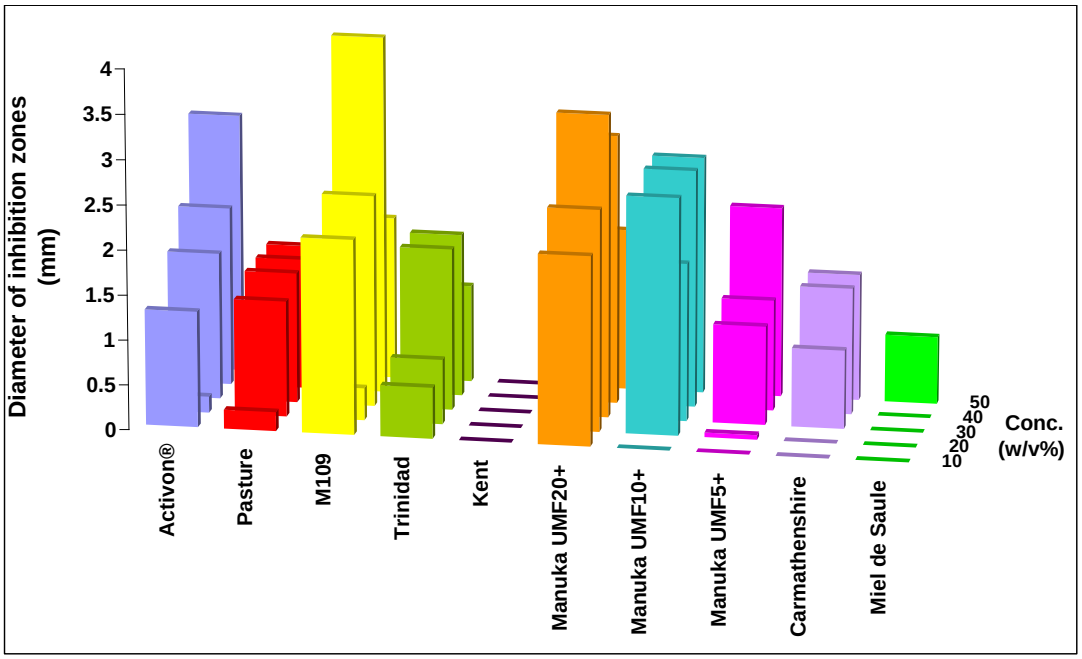

Figure 5.7c: Assessment of potency of honeys by QSI assay. The chart representation of quorum sensing inhibitory properties of various concentrations of 10 honeys against *C violaceum* in LB semisolid agar culture using 50 µl of each honey solution in 6mm diameter well.

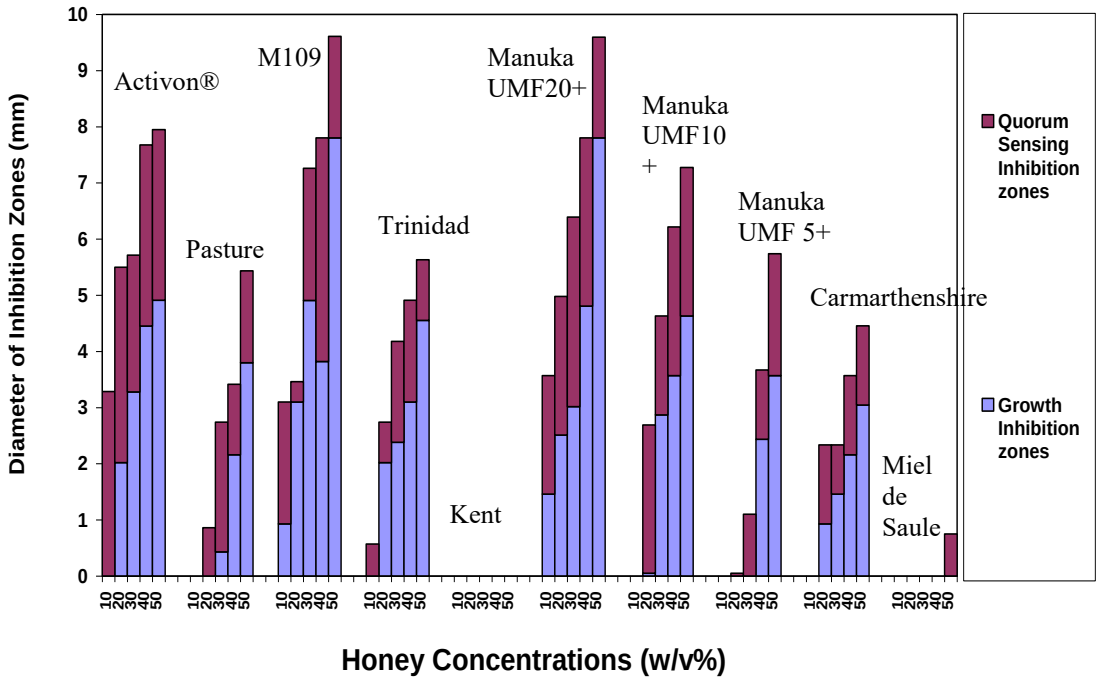

Figure 5.7d: Combined effect of each honey on growth and quorum sensing of *C. violaceum* relative to concentrations. The honeys showed various degrees of growth or QS inhibition of the reporter organism.

5.4 Discussion

The effect of honey on growth and quorum sensing inhibition of a medical grade honey (Comvita®) was examined in a continuous culture wound model. The exposure of 30 hour biofilm of *P. aeruginosa* wound isolate (P.98) to 40% (w/v) solution of a medical grade honey (Comvita medical-woundcare, active UMF 18+) in a wound model culture resulted in the complete inhibition of the *P. aeruginosa* within 6 hours. The AHL signalling molecule which was earlier detected in the culture samples between 24 and 30 hours prior to exposure of the culture to honey was not detected after exposing the culture to honey until the termination of the culture (Fig. 5.2b). This is an indication that the 40% (w/v) honey solution had penetrated the biofilm, and inhibited the organisms embedded within the biofilm. The consequence of the lethal effect was the sudden drop in the viable count of the culture from $3.5x10^9$ cfu/ml bacteria of culture to undetectable level of bacteria. The ability of the continuous culture wound model to grow biofilm had been previously determined in chapter 3, section 3.2.1.2, the reproducibility assay was found to be sensitive, and according statistical analysis using one-way ANOVA (unstacked), $p< 0.001$ (95% confidence limits for mean based on pooled SD). This finding was in line with the previous findings of inhibition of *P. aeruginosa* biofilm cultures in microtitre plates (Okhiria *et al*, 2004 and 2009) and the susceptibility of 91% of 11 *P. aeruginosa* biofilms to honeys (Alandejani *et al,* 2009). In the microtitre plate assay, the dissolved biofilm components as a result of the action of honey remained in the culture because they were not washed out. Biofilm EPS components were quantified by the measurement of absorbed dye in microtitre plate culture. Adherent biofilm biomass retained in the microtitre plate could still contribute to the overall value of biofilm estimation whether it was viable or not, whereas dissolved biofilm components were eliminated in the continuous culture as the medium was being replaced at a constant rate. This suggests that the effect of honey on biofilm would be better

appreciated in the wound model which is a continuous culture in comparison with the microtitre plate biofilm culture assay. Biofilm viability would be better assessed by using fluorescent dyes such as LIVE/DEAD BacLight bacterial viability kit that can stain the nucleic acids of both living and dead bacteria and the second dye that will not be able to penetrate living bacteria with intact cells except the dead cells. There are some of such dyes that are not toxic to the organisms and the cultures can still be used for further assay when there is need to keep the culture for longer period during the experiment. This study therefore reveals the full potential of the action of honey on biofilm suggesting the *in vivo* effectiveness of honey in wound management. Manuka honey has been shown to exert a bactericidal effect on planktonic *P. aeruginosa* cultures (Cooper *et al,* 2002) even though biofilm can resist antimicrobial up to 500 to 1000 times that of the planktonic counterpart (Amorena *et al,* 1999) the antibacterial effect of honey on biofilm at 40% (w/v) concentration shows that honey can inhibit biofilm and confirms previous findings (Okhiria *et al,* 2004 and 2009). Despite an observation that nutritional starvation and high cell density enhance the resistance of biofilm organisms to antimicrobial tolerance (Fux *et al*, 2005) *P. aeruginosa* biofilm was susceptible to the 40% (w/v) manuka honey. This study has confirmed the high antimicrobial effect of manuka honey on biofilm.

In this study, inhibition of growth and quorum sensing of *C. violaceum* by various concentrations (10 - 50%) (w/v) of M109 (manuka honey) was observed (Fig. 5.3 and 5.6a). Inhibition of quorum sensing of biofilm organisms by honey will prevent biofilm formation and expression of certain genes that are vital to the virulence of the organisms. This suggests that honey can be used alone or in combination with other antimicrobials to effectively treat biofilm infections.

After establishing the mode of action of honey on biofilm, an assay method was modified for assessing the growth and quorum sensing inhibitory properties of honey. The method was optimised by assessing the effect of inoculum and honey concentrations on the activity of honey and the reproducibility of the assay. The effect of various inocula on the activity of M109 honey was examined and 50% (w/v) concentration found to inhibit the growth and quorum sensing of *C. violaceum* at the various inocula (Fig. 5. 4 a and b).

Inhibition of growth and quorum sensing by honey was dependent on concentration. Inhibition of quorum sensing was observed on honey concentrations as low as 10% (w/v) honey was bactericidal to planktonic cultures of *P. aeruginosa* (Cooper *et al,* 2002) at 10% concentration (v/v) but at this concentration M109 honey showed some antimicrobial properties against *P. aeruginosa* biofilm by preventing quorum sensing.

The larger proportion of biofilm constituents are made up of water, Zhang and his colleagues in their study discovered that 97% of biofilm is water while another study estimated the water contents of biofilm to be 70% to 80% water (Sandt *et al*, 2008). The high osmolarity of honey would have a dehydrating effect on the biofilm because of the high water content of biofilm while the acids in the honey would reduce the pH of the biofilm culture environment which would have further increased the lethal effect of honey on the organisms within the biofilm. The antimicrobial properties of honey are multifactorial with all the factors working synergistically to produce a strong lethal effect on biofilm microorganisms; the chances of biofilm organisms developing resistance to honey might be remote.

Three samples of manuka honey (Activon®, manuka honey UMF 10+ and manuka honey UMF 5+) were tested to ascertain the reproducibility of the QSI assay. The assays showed evidence of reproducibility, with low standard deviations. A higher

activity for the manuka UMF 10+ honey over that of manuka honey UMF 5+ was observed in the analysis which confirmed the effect of concentration on the potency of the honeys. This also shows the importance of grading in honey and the relationship of grading to the effectiveness or potency of the honeys.

Ten honeys were screened for their potential to inhibit the growth and quorum sensing of *C. violaceum* as an assessment of their potency. Eight honeys showed varying degrees of growth and quorum sensing inhibitions to *C. violaceum* whereas two of the table honeys had little or no effect on the reporter organism. One of the honeys only inhibited the quorum sensing of *C. violaceum* at 50% (w/v) concentration while the second honey had no inhibitory effect on the growth and quorum sensing of the organism. The effect of the investigated honeys on QS has confirmed the recent findings by Truchado and her colleagues (2009).

The activities of the honeys relative to phenolic concentrations were also found to be correlated with the quorum sensing inhibition of *C. violaceum* as well as the growth inhibition. Using Minitab version 15, one way Anova unstacked, there were correlations between the total activity of the honeys and quorum sensing inhibition $p \leq 0.0001$, as well as total activity of the honeys and growth inhibition, $p \leq 0.002$. There was significant correlation between QSI and UMF factor of the manuka honeys, $p \leq 0.001$ while UMF and QSI showed significant correlation, $p \leq 0.0001$. The varying potencies of different honeys as indicated by the UMF values means that care should be taken in selecting honey for topical and wound treatment. The higher potency of medical grade honeys over the potency of some table honeys as observed in this study was in agreement with the study done by Cooper and Jenkins (2009).

Five manuka honeys were included in this study and all of the honeys showed effective growth and quorum sensing inhibitory effects relative to concentrations and UMF

factors confirming the high potency of medical grade honeys and manuka honey in particular. Although manuka honey is a non-peroxide honey the phytochemical content which has been identified as methylglyoxal (Adams *et al,* 2008; Mavric *et al*, 2008) has given manuka the antimicrobial effect. The potencies of these honeys as observed in this study was similar to the bactericidal activities of New Zealand manuka honey and Yemen honey on biofilm demonstrated by Alandejani"s group (2009).

The use of honey in modern medicine is gaining increased awareness, especially in wound treatment (Cooper, 2008; Molan, 2006; Cooper & Molan, 1999). Recently, biofilms have been implicated in chronic wounds (James *et al,* 2008; Ngo *et al,* 2007) but any honey to be used on biofilm infected wounds ought to be able to effect antimicrobial action on the biofilm. Exposure of microorganisms to sub-lethal concentrations could lead to development of resistance or reduced susceptibility (Moore *et al*, 2008) which could occur if organisms are constantly exposed to honeys that are ineffective. However, Blair and her colleagues (2009) found that *S. aureus* and *P. aeruginosa* developed resistance to conventional antibiotics such as tetracycline, oxacillin or ciprofloxacin but not against Leptospermum (manuka) honey and peroxide producing honeys even when each of the known components were eliminated during the assay. It was also found that there was no cross resistance to honey as observed in the organisms against other antimicrobials via decreased cell wall permeability or drug accumulation or increased efflux of drugs.

It has been reported that quorum sensing molecules regulate genes involved in relieving oxidative stress in biofilm organisms, a process that increases the tolerance of the organisms to hydrogen peroxide (Bjarnsholt *et al,* 2005; Hassett *et al,* 1999). Inhibition of quorum sensing by honey will therefore interfere with the tolerance of biofilm organisms and render them susceptible to hydrogen peroxide released from honey via

glucose oxidase activity on glucose. Honey would therefore provide a unique multifaceted treatment strategy for biofilm in wounds.

It is important to investigate and identify possible active components of other honeys that are used for wound treatment. It has been shown that honey can enhance the growth of biofilm (Okhiria *et al,* 2009) especially at low concentrations and at high concentration the dilution effect and metabolic activities of the microorganisms could reduce the effect of honey to sub-inhibitory level. However, in order to standardize the use of honey as an antimicrobial agent and prevent development of mutants and resistant strains to honey, it is pertinent that honeys being used for treatment are potent enough to kill pathogenic organisms. Therefore determining the potency of honey before being used as topical agent on infected wounds might be necessary.

5.5 Conclusion

In this study, the ability of manuka honey to disrupt biofilm and inhibit organisms embedded in biofilm within 6 hours in a continuous wound model has been confirmed. Inhibition of quorum sensing of biofilm organism by manuka and other honeys has also been demonstrated. Varying antibacterial activities of the honeys were also observed although a table honey did not show any effect on the reporter bacterium. Further studies are necessary to reconcile the *in vitro* effect of honey on biofilm to the *in vivo* activity of honey in wounds with biofilm.

5.6 Recommendation for future studies

There is need to link the quorum sensing inhibitory effect of honey *in vitro* to the effect of honey in wound. Molecular techniques could be used to investigate the effect of honey on biofilm. Determination of minimum inhibitory concentration (MIC) and

minimum bactericidal concentration (MBC) of honeys on *P. aeruginosa* cultures in the microtitre and the continuous culture assays relative to time will provide more information about the effect of honey on biofilm.

Chapter 6

Synopsis and conclusions

6.1 Summary

In this thesis the role of biofilms in wounds has been investigated by studying the antibiotic susceptibility pattern and the ability of *Pseudomonas aeruginosa* isolated from infected and chronic wounds to form biofilm and produce quorum sensing signalling molecules *in vitro* in batch and continuous cultures. The genetic fingerprint patterns of the isolates were determined by PCR and gel electrophoresis and the similarity between each isolate‟s fingerprints to the cohort was studied in relation to antibiotic susceptibility, biofilm formation and acyl-homoserine lactones (AHL) production. The ability of gauze to support the growth of biofilm in batch cultures and detection of AHL in the gauze after sterilization was established. A wound model was then developed for biofilm continuous culture using gauze as the substratum and phenotypic expressions of the isolates were studied including biofilm development and structure, AHL and extracellular polymeric substances (EPS) production. The presence of biofilm in chronic wounds was investigated using dressings that were removed from chronic wounds for the presence of biofilm markers including quorum sensing molecules and EPS. Furthermore organisms from the wound dressings were isolated and examined for biofilm phenotypic expressions such as biofilm formation in batch culture and continuous cultures. All isolated Gram negative bacteria were examined for production of quorum sensing signalling molecules. The results of biofilm markers obtained from the wound dressings were linked with those of the isolated organisms from each of the dressings to draw conclusions for the presence of biofilm in chronic wounds. The effect of honey on biofilm was investigated in continuous culture wound model; biofilm was disrupted within six hours of exposure to 50% (w/v) honey. Ten honeys were investigated for quorum sensing inhibitory activity and all but one exhibited quorum sensing inhibitory activity against biofilm forming organism, *C. violaceum* in a dose dependent manner.

6. 2 Synoptic discussion

In this study the susceptibility of the isolates to the commonly employed antibiotics was determined using the British Society for Antimicrobial Chemotherapy (BSAC) guidelines (version 4) (Andrews, 2005). Interestingly, almost all the organisms were resistant to cefuroxime, amoxicillin and trimethoprim while about one fifth of the cohort of organisms showed resistance to ciprofloxacin. On the other hand less than 10% of the entire isolates were resistant to imipenem, gentamicin and piperacillin. This finding is in support of other studies regarding the resistance of biofilm organisms to antibiotics (Anderson and O'Toole, 2008; Fux *et al,* 2005; Donlan, 2000). This result reveals more information about the role of biofilm forming organisms with respect to antimicrobial resistance. The resistance of biofilm organisms has been shown to be due to various factors including the protection by the extracellular polymeric substances (EPS) in which they are encased acting as barrier against the penetration of chemical substances such as antibiotics and the acquisition of resistant genes within biofilm. Some strains of *P. aeruginosa*, particularly the biofilm forming phenotypes are known to produce high level of alginate which usually enhances the resistant potential of the organism (Hentzer *et al*, 2001). In their study, Hentzer and colleagues found that the non-mucoid biofilm was almost entirely susceptible to tobramycin, while the mucoid biofilm strains remained viable. The antibiotic assay was done on solid agar and the organisms were therefore in a quasi sessile form, which may stimulate the production of alginate.

Biofilm formation by *P. aeruginosa* isolated from infected and chronic wounds was determined in microtitre plate (Christensen *et al*, 1985) and 88 (90.7%) of the 97 isolates were able to produce biofilm in cultures within 24 hours. The majority of the isolates produced biofilm strongly; an indication of high probability to produce biofilm in the wound by the organisms and this can happen as soon as the wound is

contaminated by such organisms. It has been shown that a wound isolated *P. aeruginosa* produced biofilm within 10 hours *in vitro* (Harrison – Balestra *et al*, 2003). In animal models, biofilms were formed in wounds within 48 – 72 hours after challenging the wounds with *S. aureus* or *P. aeruginosa* (Davis *et al*, 2008; Serralta *et al*, 2001).

Production of AHL was examined using cross feeding (Stickler *et al,* 1998) and quorum sensing inhibition (QSI) (MacLean *et al,* 2004) assays. The results showed 78 (80.4%) of the isolates produced AHL in the laboratory. There was a significant correlation between the number of isolates that formed biofilm and produced AHL $p< 0.005$. It was found that the QSI assay was more sensitive than the cross-feeding assay in detecting AHL produced by the isolates tested here.

Several authors have linked signalling molecules such as AHL to biofilm formation (Williams *et al*, 2007; Singh *et al* 2000; Rumbaugh *et al*, 2000; Chandra *et al*, 2001; Costerton *et al*, 1999). Recently, AHL has been detected in chronic wound isolates and debridement samples (Rickard *et al*, 2010). An inference is that the marked production of virulence factors (such as cytotoxic enzymes, toxins and biofilm formation), will further promote wound breakdown and impairment of healing processes. *Pseudomonas aeruginosa* has been associated with delayed wound healing (Dowd *et al,* 2008; Howell- Jones *et al*, 2005; Bowler *et al*, 2001) and particularly with large ulcers (Halbert *et al,* 1992).

Interestingly, it was found that 16 (84.2%) of the 19 AHL negative isolates formed biofilm *in vitro*. Studies have shown that some pathogenic *P. aeruginosa* formed biofilm independent of AHL signalling molecules (Schaber *et al*, 2007 and 2004; Favre – Bonte, 2007). *P. aeruginosa* have signalling molecules other than AHL such as the quinolones (Deziel, 2004) which could have been used by the AHL negative but biofilm positive isolates. Other signalling molecules were not investigated in this study; there is

a need for further study to determine other signalling molecules, such as quinolones or autoinducer-2 which are used by *P. aeruginosa* for regulation of virulence factors including biofilm formation in the AHL negative strains that produced biofilm.

Although the majority of the *P. aeruginosa* wound isolates that formed biofilm produced AHL, six (7.7%) of the 78 AHL positive wound isolates did not form biofilm *in vitro*. Signalling molecules such as AHL are involved in the regulation of virulence by pathogenic organisms (Bassler and Losick, 2006; de Kievit and Iglewski, 2000) even across species (Atkinson and Williams, 2009; Williams *et al,* 2007; Williams, 2007). The production of AHL by the biofilm negative isolates could be for the regulation of virulence factors other than biofilm formation and more investigations need to be done to unravel this finding. It has been observed that virulence expression could be site dependent (Favre Bonte, 2007; Schuhmacher and Klose, 1999); it is possible for the isolates to form biofilm in wounds even though the laboratory assay showed a negative result. The regulatory protein ToxT directly activates the transcription of virulence factors in *Vibrio cholerae*, including cholera toxin (CT) is modulated by environmental signals with little or no expression outside the host (Schuhmacher and Klose, 1999).

The implication for the wound is that a biofilm forming organism that contaminates a wound could form biofilm within 24 hours, making it especially difficult for the body‟s immune system to eliminate the organisms consequently delaying wound healing.

The relationship between virulence and site of isolation of organism was studied by using biofilm forming *P. aeruginosa* isolated from water pipes. Biofilm formation (Christensen *et al*, 1985) and AHL production by cross-feeding (Stickler *et al,* 1998) and QSI (McLean *et al,* 1997) of the water isolates were determined and the results were compared with those of the wound isolates. Seventy two (74.2%) of the 97 wound isolates tested positive by cross -feeding assay and 78 were positive by QSI assay,

whereas of the 9 biofilm forming *P. aeruginosa* water isolates 5 (55.6%) were positive for AHL production by either of the 2 assays (3 by cross-feeding and 2 by QSI assay) but none showed positive for both methods. This shows that the strains from wound expressed higher virulence propensity than the water isolates. Studies have shown that virulence expressions are site dependent, particularly outside the host environment (Schuhmacher and Klose, 1999). Series of phenotypic changes which characterise biofilm formation (Sauer *et al,* 2002) are modulated by quorum sensing signalling molecules (Parsek, *et al*, 2005; Williams *et al*, 2007; Parsek and Greenberg, 2000); the presence of which has been demonstrated *in vivo* in animal (rat) wounds (Nakagami *et al*, 2008).

The genetic relationships of the wound isolates were then determined by polymerase chain reaction (PCR) and analysis of the complete linkage of the amino acid bands (*GelCompar* II version 4) with Jacquard similarity index revealed that the majority of the isolates (84.5%) had 100% genetic similarity, 3% were 75 - 99% linked while (12.4%) were of about 50 - 75% similarity. The high genetic similarity therefore might be linked to the biofilm phenotypic expressions such as biofilm formation, antibiotic susceptibility and AHL production observed in this study. Further study is needed to link the specific gene that codes for each factor with the expression of the factors in the wound isolates. The results obtained from the biofilm phenotypic characterisation of the isolates (antibiotic sensitivity, biofilm formation, AHL production and PCR genetic fingerprint) was intriguing and particularly the similarity of the trends which strongly suggest that the isolates might have genetic similarities.

Gauze has been in use as a primary or secondary wound dressing for centuries because it is absorptive and allows moisture to escape. Gauze dressings have been utilised in diverse ways as dry or moist; alone (Ubbink *et al,* 2008; Motta *et al,* 2004) or

impregnated with agents such as petrolatum, antiseptics (Motta *et al,* 2004; Cazzaniga *et al,* 2002) or other healing agents. The ability of gauze to support the growth of biofilm and retain AHL secreted by biofilm organisms was determined in batch cultures. The results of the cultures showed that gauze satisfactorily supported the growth of biofilm and was able to retain the AHL which was detected after sterilising the gauze at 121^{o}C for 15 minutes with reporter organism *C. violaceum*.

Growing microorganisms in batch cultures normally provides abundant nutrients at the onset of the culture but nutrients reduce with time and microbial wastes accumulate in the culture medium. Such cultures would not be appropriate for studies that require long period of culture of organisms since the accumulation of wastes and nutrient depletion usually leads to the death of the organisms and affects the phenotypic expression of the organisms. The continuous system offers a long period of study for the organisms without change in phenotypic expressions because there is constant replenishment of the media as well as the continuous elimination of the metabolic wastes from the culture.

In order to elucidate the phenotypic characteristics in biofilm, a wound model was developed for biofilm in continuous culture using gauze as the substratum for biofilm. In this study the phenotypic expressions of selected biofilm and non- biofilm forming wound isolates were investigated in a wound model to simulate what happens *in vivo.* The isolates were examined for biofilm formation and AHL production over a period of 48 hours. The microscopic examination of the cultures of biofilm forming isolates revealed progressive formation of biofilm microcolonies and increase in EPS throughout the duration of the experiment as well as production of AHL which was detectable when the bacterial population attained 10^{7} cfu/ml of culture and also coincided with the appearance of biofilm structures while a non-biofilm forming isolates did not produce biofilm or AHL. The detection of AHL at the bacterial

population of 10^7 cfu/ml of culture using the low flow rate was similar to the finding of Kirisits and colleagues (2007). In their study QS induction for the laminar flow rate (2.4 and 24 ml per minute) was at bacterial population of about $6x10^7$ cfu/ml. The growth patterns of a biofilm and a non - biofilm forming *P. aeruginosa* wound isolates were similar but the biofilm forming isolate formed biofilm and produced AHL which was detected in culture effluent samples (Fig. 3.6, 3.7 B and C, 3.8, 3.9) and the microscopic examination of stained slide smears of culture effluents (Fig. 3.17) and electron microscopic scanning of gauze after 30 hours of culture (Fig.3.18) whereas the non- biofilm forming isolate was not able to produce biofilm (Chapter 3; Fig. 3.19) or AHL (Fig. 3.6, 3.7 E and F,3.10 and 3.11) in wound model cultures. In the wound model, the development of biofilm microcolonies were evident and increase in EPS formation and biofilm features at all stages up to maturation were observed at determined intervals during the period of culture. The demonstration of biofilm features was in agreement with other studies that biofilms have been cultured and demonstrated *in vitro* (Harrison – Balestra *et al*, 2003).

The findings from these investigations of the *P. aeruginosa* isolates from wounds have revealed the biofilm forming potential of most of these isolates in the laboratory which suggested an ability to form biofilm *in vivo*. The link between the ability of the organisms to form biofilm in the laboratory with formation of biofilm in the wound was therefore explored. This hypothesis was investigated in wounds by examining wound dressings for the presence of biofilm although previous studies had used biopsies and debridement samples.

Clinical assessment of wounds for infection is not straightforward since it involves the consideration of various parameters such as pain, bleeding, production of purulent exudates or discharge (Grey *et al*, 2006; Petherick *et al*, 2006) as well as localised

swelling, redness or heat (Petherick *et al*, 2006). Once infection is established, it is best practice for wound infection to be confirmed by isolation of organisms from wound swab or wound biopsy (Petherick *et al*, 2006) for appropriate antibiotic treatment because of the problem of resistance (Ciofu *et al,* 2009; Neu, 1999). In practice infected wounds are treated on the basis of clinical signs and symptoms before identifying causative agents and this might encourage antimicrobial resistance and development of mutants. It has been shown that the use of antimicrobials induces the alteration of community structure by reducing some bacteria while selecting for others (Price *et al,* 2009).

Isolation of organisms by cultural methods provides opportunity to determine the susceptibility of the organisms to antimicrobials for the selection of the most appropriate antimicrobial for treatment. But the inadequacy of cultural methods have been revealed recently, particularly the inability to isolate some fastidious organisms (Price *et al*, 2009; Singh *et al,* 2009; James *et al,* 2008; Dowd *et al,* 2008 a and b; Kirketerp-Møller *et al*, 2008). The studies have compared cultural and non- cultural methods and confirmed that more organisms were identified when non - cultural methods were used (Price *et al*, 2009; Sing *et al,* 2009; Dowd *et al,* 2008 a and b). Price and colleagues observed in their study that microbial burden of the investigated wounds were 4 times more than estimated culture-based method.

According to the study by James and colleagues (2008) using 77 chronic wounds and 16 acute wounds specimens, 50 of the chronic wounds were analysed with light and scanning electron microscopy techniques and 60 % had biofilm whereas just 1 (6%) of the 16 acute wounds was positive for biofilm while molecular analyses of the remaining 27 chronic wounds specimens showed diverse polymicrobial communities which was not observed by cultures. Kirketerp-Møller and colleagues confirmed that the organisms

within wounds are aggregated in microcolonies. The presence of biofilm has therefore been confirmed in human wounds (Price *et al*, 2009; Singh *et al,* 2009; James *et al,* 2008; Dowd *et al,* 2008 a and b; Kirketerp-Møller *et al*,2008; Ngo *et al,* 2007) but cultural methods would not usually reveal the presence of biofilm. The inadequacies of cultural methods have led to the use of culture independent approach to studying microorganisms and have been suggested (Rogers *et al*, 2009; Davies *et al*, 2001).

The present procedure for the assessment of wounds for biofilm involve collection of wound biopsies (which is an invasive procedure) and the available procedures for processing the samples such as scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) or fluorescent in –situ hybridisation (FISH) are quite expensive and usually not available for routine use. Wounds normally produce exudates which would increase when the wounds are infected because the microorganisms metabolic activities and toxins continually breakdown the tissues within the wound space (Kingsley, 2001). The processes stimulate the body immune system to increase the production of inflammatory cytokines and proteases such as TNF-alpha, the interleukins and heparin-binding protein (HBP) (Lundqvist *et al*, 2004) and infiltration of neutrophils (Kim *et al,* 2008) to eliminate the organisms from the wound. All these activities increase the blood flow to the wound area consequently increasing the amount of exudates (Ovington, 2003; Kingsley, 2001).

Since biofilms are often polymicrobial (Malic *et al*, 2009; Dowd *et al,* 2008 a and b; James *et al,* 2008), the presence of biofilm in wound would result in the combined and synergistic effect of all the organisms‟ virulent expressions, such as the production of proteolytic enzymes and toxins within the biofilm which further aggravates the breakdown of tissues, the consequence of which usually contribute to the inability of such wounds to heal (Bjarnsholt *et al*, 2008). Problems associated with biofilm such as

the prevention of phagocytic activities by PMNs, the effects of signalling molecules on the expression of virulence factors and inhibition of antimicrobials by biofilm organisms give rise to overwhelming numbers of organisms; the phenomena that further complicate the healing process of wounds with biofilm (Bjarnsholt *et al,* 2008). Wound exudates will ooze out into the dressings and usually contain the products released due to proteolytic activities of the organisms in addition to the body fluid.

In this study wound dressings were obtained to provide some insight into the presence of biofilm in wounds by examining the exudates for biofilm markers such as EPS and AHL and isolating organisms from the dressings. The isolated organisms were then examined for biofilm phenotypic expressions *in vitro*.

In wounds, exudate production varies according to the state of the wound (White and Cutting, 2006), the acute wounds exudates are usually low but higher in infected wounds due to microbial virulence expressions (Wilson *et al,* 2002) and internal wounds. The diverse range of exudate production in wounds was taken into consideration in the construction of the wound model for continuous cultures regarding the quantities of media that were delivered into the culture during the period of each culture.

The presence of biofilm in human wounds has been established with the examination of wound biopsies using histological methods, fluorescence in situ hybridization (FISH), confocal laser scanning microscopy (CSLM) and scanning electron microscopy (SEM) (Malic *et al*, 2009; Kirketerp-Møller *et al,* 2008; James *et al,* 2008; Ngo *et al,* 2007).

In the past detection of biofilm markers such as AHL signalling molecules have been utilised to confirm the presence of biofilm in urinary catheters (Stickler *et al,* 1998) and cystic fibrosis lung by examining the sputum (Singh *et al,* 2000). Recently, debridement

materials have been examined for biofilm markers such as signalling molecules (AHL and autoinducer-2) (Rickard *et al*, 2010).

Here the examination of wound dressings that were removed from patients with chronic wounds was a novel idea in the determination of the presence of biofilm in wounds. The method was expected to provide a quick and less expensive screening for the detection of biofilm in wounds.

The dressings from 35 patients with chronic wounds were screened for biofilm markers such as AHL signalling molecule, EPS and the ability of the isolated organisms from the dressings to express biofilm characteristics. Since microorganisms within the wound space would form part of the constituents of wound exudates (White and Cutting, 2006) and the organisms to a large extent would present the microbial flora of such wound, detection of biofilm markers would give an indication of the possible presence of biofilm in the wounds.

The investigation of chronic wound dressings revealed that the majority (80%) of the 35 dressings contained EPS although 11.4% could not be tested. Interestingly, organisms that produced biofilm *in vitro* were isolated from 32 out of the 35 chronic wound dressings (91.4%) inclusive of the 80% in which EPS was detected. Since EPS produced by biofilm organisms during biofilm formation serves as the means by which biofilm adheres to abiotic and biotic surfaces (Flemming *et al,* 2007; Costerton *et al,* 1995) the detection of EPS in the wounds dressings suggests that it was produced by organisms present in the wounds. Some constituents of certain wound dressings include alginate but to prevent false positive results, examination of the stained smear of the exudates were carefully monitored and only evidence of microcolonies in which organisms were embedded were taken as a positive result (Fig. 4.2a).

AHL was detected in 11 dressings, 10 (77%) of which had AHL producing bacteria (P. *aeruginosa*); although in total AHL producing bacteria (Chapter 4; Fig. 4.1 a) were isolated from 13 dressings but the rate of detection of AHL in the dressings was high since 10 were detected. Statistical analysis using one way ANOVA (Minitab version 15), the relationship between AHL detection in the wound dressings and production of AHL by the isolates *in vitro* was significant $p< 0.005$. All of the isolates that produced AHL also formed biofilm in the microtitre plate assay. These results suggest that the AHLs that were detected in the dressings were produced by the isolated organisms, as these organisms were able to produce the AHLs *in vitro.* One dressing tested positive for AHL but no Gram negative organism was isolated from it, which might be due to the inhibitory effect of the antimicrobial agent in the dressing. This situation supports the fact that cultural methods often fail to isolate some organisms in wounds which non - cultural methods are able to detect (Price *et al*, 2009; Sing *et al,* 2009; James *et al,* 2008; Dowd *et al,* 2008; Kirketerp-Møller *et al*,2008).

It was observed in this study that the detection of AHL in wound dressings was associated with the presence of *P. aeruginosa*, $p < 0.005$. A similar study in which AHL was detected in wound debridement materials found an association between AHL detection and the presence of certain Gram negative bacteria including *P. aeruginosa* (Rickard *et al*, 2010).

Gram negative bacteria were isolated from 23 dressings out of which 13 produced AHL, therefore AHL detection method is only useful for AHL producing biofilm organisms and may not be applicable for general biofilm screening involving Gram positive and non-AHL producing Gram negative organisms. This study has supported previous findings that *P. aeruginosa* usually produces high level of AHL during biofilm formation (Christensen *et al,* 2007; Kirisits and Parsek, 2006).

P. aeruginosa were isolated from 14 dressings but 1 did not produce biofilm in the laboratory and AHL was not detected in the dressing from which the organism was isolated, even though it has been shown that some bacteria such as *P. aeruginosa* can cause infection and form biofilm without producing AHL (Favre-Bonte *et al*, 2007; Schaber *et al*, 2007 and 2004).

Most of the chronic wound dressings that were investigated were polymicrobial with a mean of 3 organisms per dressing, a situation that has been found in wound infections and in particular chronic wounds (Price *et al,* 2009; Dowd *et al,* 2008; Dowd *et al*, 2008). The most prevalent organisms were *Staphylococcus aureus* (20.4%) (MSSA: 13%) and MRSA: 7.41%), *P. aeruginosa* (13%) and *Corynebacterium striatum* (9.3%). Some studies have shown that *S. aureus* and *P. aeruginosa* were prevalent in chronic wounds (Price *et al,* 2009; Dowd *et al,* 2008) especially antibiotic resistant strains (Lipsky, 2008) whereas *Corynebacterium striatum* is mainly prevalent in chronic diabetic ulcers (Dowd *et al,* 2008; Citron *et al,* 2007). The results obtained from this chronic leg ulcers study seem to follow a similar pattern to previous studies.

The wounds in which *P. aeruginosa* were isolated were also polymicrobial which confirms the association of *P. aeruginosa* with polymicrobial wound infections (Price *et al*, 2009; Sing *et al,* 2009; James *et al,* 2008; Dowd *et al,* 2008 a and b; Kirketerp-Møller *et al*, 2008).

Infected wounds often lead to complications and delay in healing but treatments of wounds have evolved over a long period of time especially the management of chronic wounds (Forrest, 1982; Ayton, 1985).

A series of problems are associated with wounds including pain, malodour and mobility constraints to patients (White and Cutting, 2006; Percival and Bowler, 2004) with the attendant socio-economic problems (Church *et al,* 2006; Wysocki, 2002; Philips *et al,* 1994). The development of resistance strains of pathogenic organisms to conventional antimicrobials has aggravated the problems associated with chronic wounds particularly health related and life threatening complications such as septicaemia, damage to organs (Church *et al*, 2006), amputations (Ramsey *et al,* 1999) and in extreme circumstances death (Ramsey *et al,* 1999).

Various studies have shown that resistance to antimicrobials by microorganisms is higher in biofilm organisms than the planktonic counterparts (Lewis, 2001; Nickel *et al,* 1985) and biofilm organisms have been associated with chronic wounds (Bjarnsholt *et al,* 2008; Costerton *et al*, 2003 and 1999). Biofilm infected wounds are often polymicrobial and the damaging impact on the wounds is much higher due to the combined pathogenic effect of the organisms within the biofilm (Dowd *et al*, 2008; Rhoads *et al*, 2008; Bjarnsholt *et al,* 2008). In recent times, because of the problem of antimicrobial resistance (Khorasani *et al,* 2008; Zavascki *et al*, 2006; McDonald *et al,* 2004), the use of honey in the treatment of wounds has been revisited (Cooper *et al,* 2008; Molan, 2006, Cooper *et al* 2001; Cooper and Molan, 1999).

The effectiveness of honey against virulent organisms such as *S. aureus* and opportunistic pathogen like *P. aeruginosa* has been linked to the composition and the physio-chemical properties of honey. These properties include the acidic pH, high osmolarity due to sugar content, release of hydrogen peroxide which increases with the dilution of honey and the phyto-chemical properties. The phyto-chemical property of honey is determined by the plants from which the honey is formed and it influences the potency of honey (Mboto *et al,* 2009; Molan, 2006) hence the variation in the

antimicrobial properties of different honeys (Cooper and Jenkins, 2009). Several studies have demonstrated the antimicrobial effect of honey against many microorganisms (Williams *et al,* 2009; Amghalia *et al*, 2009; George *et al,* 2007; French *et al,* 2005; Cooper *et al,* 2002). The application of honey in wound treatment has been extensively evaluated (Majtán 2009; Cooper, 2008; Molan, 2006; Cooper & Molan, 1999); in particular the effect of honey on microorganisms with respect to the low pH (Simon *et al,* 2009; Lotfi, 2008), acid content (Wahdan, 1998), osmotic effect (Cooper *et al,* 2002; Molan, 2006), peroxidase activity (Molan, 2006 and 1992; White Jr. *et al*, 1963) antioxidant effect on free radical (Henriques *et al,* 2006; Frankel, 1998) and phytochemical effect (Mboto *et al,* 2009; Molan, 2006). The antimicrobial effects of honey have been demonstrated in the past on planktonic microorganisms (French *et al,* 2005; Mulu, 2004; Cooper *et al*, 2002; Efem and Iwara, 1992) and biofilm organisms (Alandejani *et al,* 2009; Okhiria *et al,* 2009; Merckoll *et al*, 2009). However, there is need for more information about the antimicrobial effect of honey on pathogenic organisms.

In this study the effect of honey on 30 hours biofilm of a strong biofilm forming *P. aeruginosa* wound isolate in a continuous wound model culture was examined. The biofilm was penetrated and the EPS were dissolved (dispersed) within 6 hours of exposure of the biofilm to the 40% (w/v) manuka honey solution. The organisms could not be recovered from wound model effluent when cultured on nutrient agar after exposure to honey. This result indicates that 40% (w/v) manuka honey had a bactericidal effect on *P. aeruginosa* biofilm. This finding has supported previous findings on the effect of honey on biofilm (Alandejani *et al,* 2009; Okhiria *et al,* 2009; Merckoll *et al*, 2009).

Signalling molecules are vital to the formation of biofilm and virulence of pathogenic organisms particularly regulation of various genetic activities involved with biofilm formation as well as the population density of biofilm community. The need to understand the mode of action of honey on biofilm phenotypic expression is important to the application of honey in wound treatment especially the effect of honey on signalling molecules which are the global gene regulator of biofilm organisms.

In this study, the effect of honey on the action of Gram negative signalling molecules, acyl- homoserine lactones (AHL) was investigated. The study revealed the inhibitory effect of honey against quorum sensing of a biofilm organism, *Chromobacterium violaceum,* in a dose dependent manner (Fig. 5.5; and 5.6 a – d).

A screening method was developed for the evaluation of the potency of honey as a topical and wound dressing antimicrobial (MacLean *et al,* 1997) and 10 honeys were examined. The growth and quorum sensing inhibitory effects of the various concentrations of each honey against *C. violaceum* were determined and the potencies of various honeys were then evaluated based on the obtained results. The investigation revealed the various antimicrobial effects of the different honeys on biofilm and the effects were dose dependent. The majority (8) of the 10 honeys examined in this study showed both growth and quorum sensing inhibitory effects on biofilm forming organisms whereas one honey did not exhibit such properties (Fig. 5.7 b - d). The inhibition of quorum sensing signalling of the organisms within biofilm would probably have prevented the formation and secretion of more EPS and penetration of honey through the EPS would have led to dissolution of the biofilm which consequently gave room for the honey to act directly on individual cell. The cumulative effect has resulted in the death of the organisms as evident from the result of this study. The potency of manuka honey in eradicating pathogenic organisms within biofilm is further restated.

Previous studies have shown the reliability of the potency of medical grade honey on pathogenic organisms (Cooper and Jenkins, 2009) and *P. aeruginosa* biofilm in batch culture (Okhiria *et al,* 2009). Inhibition of quorum sensing of *C. violaceum* by some honeys has recently been demonstrated (Truchado *et al*, 2009).

This study has shown that one of the antibacterial effects of honey on biofilm- forming organisms is inhibition of quorum sensing. Quorum sensing inhibition will stall most of the genetic expressions that are vital to proteolytic activities of the organisms, which has been suggested as treatment strategy for pathogenic infections. The effect of honey on the regulator of metabolic activities that are vital to the existence and sustenance of biofilm consequently lead to the sudden death of the organisms. The signalling molecule being the modulator of the metabolic and other relevant genetic activities within the biofilm (Passador *et al***,** 1993; Xu *et al*, 2000 and 2006; Patel, 2005) including virulence factors (Xu *et al*, 2000 and 2006; Patel, 2005; de Kievit and Iglewski 2000) and production of EPS matrix that houses and protect the biofilm organisms (Flemming *et al,* 2007; Costerton *et al,* 1995) from environmental stress. Quorum sensing inhibition of pathogens such as *P. aeruginosa* has been shown to reduce the tolerance to antimicrobials (Hoffmann *et al,* 2007) which would further increase the chances of the elimination of such organisms from the wound. It has also been shown that inhibition of *P. aeruginosa* signalling molecules reduced or completely suppressed the production of some virulence factors (Hentzer *et al*, 2002). Communication amongst bacterial species with AHL signalling molecules have been demonstrated (Rasmussen *et al,* 2000; Riedel *et al*, 2001) indicating that a particular species of bacteria can utilise a signalling molecule from another species for modulation of virulence factors. Inhibition of quorum sensing signalling molecules might be the solution to the problem of biofilm infections and in particular development of mutants

by biofilm organism. Application of honey as an inhibitor of quorum sensing is a potential benefit that needs to be utilised.

The importance of inhibition of quorum sensing by honey can not be overemphasized as one of the key factors that determine the effectiveness of honey as a treatment intervention in the management of wounds with biofilm. The ability of honey to dissolve the EPS of biofilm rapidly shows the organisms within the biofilm would be exposed to other antibacterial effects of honey thereby allowing the killing of the organisms and enhancing wound healing. The inhibitory effect of honey on quorum sensing signalling molecules would further reduce the organisms‟ virulence expressions or totally render the organisms ineffective thus providing an enabling environment for wound healing.

6.3 Conclusions

In this thesis, the ability of wound isolates to form biofilm and produce signalling molecules such as AHL *in vitro* both in batch and continuous cultures has been demonstrated. Production of EPS concordant with biofilm development was observed in cultures during these investigations. Examination of wound dressings revealed evidence of biofilm markers such as AHL and EPS an evidence of biofilm formation *in vivo* and the organisms isolated from the dressings demonstrated biofilm phenotypic characteristics *in vitro*.

- This thesis has presented evidence of the potential of *P. aeruginosa* isolated from wounds to form biofilm in the laboratory. The majority of the isolates formed strong biofilm within the 24 - hour period of investigation.

- This study showed evidence of AHL production by *P. aeruginosa* wound isolates and most of the AHL positive isolates also produced biofilm.

- The evidence of antibiotic resistance by *P. aeruginosa* wound isolate was demonstrated in this study.

- The genetic diversity of *P. aeruginosa* wound isolates was investigated and showed that most of the isolates have 100% genetic similarity linkage during the fingerprint analysis using GelCompar II software.

- In this study a wound model was designed for *in vitro* study of biofilm in continuous cultures. Biofilm phenotypic expression of *P. aeruginosa* wound isolates including biofilm formation; AHL and EPS production were observed in continuous cultures in the wound model even within 12 hours of culture. This assay could be used to predict biofilm formation capability in contaminated wound.

- This study established the use of wound dressing to determine the presence of biofilm in wounds by screening the dressings for biofilm markers such as signalling molecules and extracellular polymeric substances (EPS).

- The ability of the various species of organisms that had been isolated from wound dressings removed from patients with chronic wounds to form biofilm in the laboratory was demonstrated. Isolated Gram negative bacteria produced AHL while selected Gram negative isolates (*Pseudomonas aeruginosa* and

Burkholderia cepacia) displayed biofilm phenotypic expressions in continuous cultures in the wound model.

- It was revealed that the presence of *P. aeruginosa* was associated with polymicrobial wounds.

- A screening method for honey was developed in this work as a tool for establishing the potency of honey as a topical antimicrobial agent and in particular for biofilm eradication in wounds.

- In this study some honeys were screened for growth and quorum sensing inhibitory effect on biofilm forming organism, *Chromobacterium violaceum*

- This work has established the inhibitory effect of manuka and other honeys on the quorum sensing of a biofilm forming organism. The phenomenon that might prevent the formation of biofilm in a wound colonised by pathogenic biofilm forming organisms.

- The effect of manuka honey in disrupting biofilm in continuous culture in a wound model within 6 hours of exposure is also presented in this study. This demonstrates that manuka honey is highly effective against biofilm *in vitro*.

In conclusion, this study has added to the knowledge of evidence of biofilm in chronic wounds and provided another way to determine the presence of biofilm in wounds and the microbial population of the wound through examination of wound dressings. The formation of biofilm was evident within 12 hours of culture in the continuous cultures

and the implication is that biofilm can be formed in a wound within 24 hours of colonisation by pathogenic organisms. The presence of biofilm in wounds may delay healing; prevention of biofilm formation in wound at the earliest possible time will play a vital role in wound management and enhancement of wound healing.

The resistance of planktonic forms of *P. aeruginosa* biofilm forming isolates to conventional antimicrobials was apparent but curiously the biofilm counterpart of the organism was susceptible to 40% (w/v) manuka honey. The inhibitory effect of honey on quorum sensing of biofilm organism might be the key factor in the ability of honey to kill biofilm organisms. The ability of honey to prevent quorum sensing and consequently biofilm formation or preventing the maintenance of biofilm activities by quorum sensing molecules when biofilm already exists would play a major role in the management of infected wounds. Application of honey as topical antimicrobial agent in wound treatment might resolve the problem of resistance and prevent the development of mutants in wound infections.

6.4 Limitations of study

- The constraints in terms of equipment have not allowed the wound model biofilm culture to be examined under confocal scanning laser microscope.

- The screening of the wound isolate with biofilm forming gene array to determine the ability of the isolates to form biofilm via gene analysis.

- The use of fluorescence in situ hybridization (FISH) to examine the biofilm in wound model cultures and wound dressings would have made the organisms

within the biofilms more visible and distinctively reveal the organisms from the matrix.

6.5 Recommendations

- Further studies are necessary to connect the presence of biofilm markers in wound dressings to the presence of biofilm in wounds; it will be necessary to examine wound biopsies alongside wound dressings in a single study.

- This study investigated Gram negative quorum sensing molecules (acyl-homoserine lactones), future work should investigate other signalling molecules employed by Gram negative and Gram positive organisms. This will give more chances for detection of signalling molecules in the wound dressings and higher correlation between signalling molecules and biofilm for more accurate diagnosis.

- The possibility of detecting virulence factors in biofilm forming organisms such as *P. aeruginosa* should be explored and the link between these virulence factors and biofilm formation could be investigated.

- It is also pertinent to study the bactericidal activity of honey against the biofilms of commonly isolated wound pathogens in the continuous culture in particular the determination of MIC and MBC. The wound model used in this study will provide a useful tool for such study.

- Since wounds are mostly polymicrobial, the possibility of the effect of honey on polymicrobial biofilm culture should also be explored in order to simulate the

condition that exists in most wounds. The wound model can easily be adapted to such analysis.

- There is need to connect the effect of honey in disrupting formed biofilm *in vitro* with *in vivo* activity in the wound.

- The effect of honey on quorum sensing of biofilm organisms *in vitro* should be linked with *in vivo* effect of honey in wounds. This will further boost the credence of the acclaimed role of honey in wound management.

Appendices

(Appendix 1)

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(Appendix 2)

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24th July 2008
cshs/als/ethics/app

Okhira, Olusola, A
PhD
Cardiff School of Health Sciences
Llandaf Campus
Western Avenue
Cardiff CF5 2YB

Dear Applicant

Re: Application for Ethical Approval

Detection of biofilm and quorum sensing molecules in wound dressings

Your research project proposal, as shown above, was amongst those considered at the meeting of the Applied Life Sciences Ethics Panel on 24th July 2008

I am pleased to inform you that your application for ethical approval was **APPROVED** subject to the conditions listed below – please read carefully.

Conditions of approval

That any changes in connection to the proposal as approved, are referred to the Panel.

That any untoward incident which occurs in connection with this proposal should be reported back to the Panel **without delay**.

Yours sincerely

Prof K Jones
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Cc: Cooper, Rose

PLEASE RETAIN THIS LETTER FOR REFERENCE

(Appendix 3)

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Research & Development
Research Risk Review Committee
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Professor Rose Cooper
Professor of Microbiology
Centre for Biomedical Sciences
University of Wales Cardiff
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Ref: RRR.117.08
26th June 2008

Dear Professor Cooper
Re: Used Wound Dressing

Thank you for your email dated 20th June advising the committee of your project.

The committee noted this at its meeting on 25th June 2008 and approves the collection of dressings for this purpose.

Yours sincerely

Dr A Anstey
Chairman
Research Risk Review Committee

(Appendix 4)

Frequency of isolates recovered from chronic wound dressings

Species	No	Species	No
Acinetobacter lwoffii	1	*Lactobacillus acidophyllus*	1
Bacteroides capillosus	1	*Micrococcus kristinae*	1
Burkholderia cepacia	3	*Peptostreptococcus tetradius*	1
Candida albicans	2	*Prevotella loescheii*	1
Candida famata	1	*Proteus mirabilis*	6
Citrobacter freundii	3	*Proteus vulgaris*	2
Corynebacterium bovis	1	*Pseudomonas aeruginosa*	14
Corynebacterium jeikeium	1	*Saccharomyces cerevisiae*	1
Corynebacterium propinguum	1	*Serratia marcescens*	2
Corynebacterium pseudodiphtheriticum	1	*Shewanella putrefaciens*	1
Corynebacterium renale grp	1	*Staphylococcus aureus* (8 MRSA)	22
Corynebacterium pseudogenitalium	1	*Staphylococcus auricularis*	1
Corynebacterium striatum	9	*Staphylococcus epidermidis*	4
Corynebacterium ulcerans	1	*Staphylococcus haemolyticus*	1
Enterobacter cloacae	1	*Staphylococcus saccharolyticus*	1
Enterococcus faecalis	4	*Staphylococcus simultans*	2
Escherichia coli	3	*Streptococcus agalactiae*	3
Flavimonas ovyzihabitans	1	*Streptococcus constellatus*	2
Gemella haemolysans	1	*Streptococcus grp G*	1
Klebsiella oxytoca	1	*Streptococcus sanguis*	1
Klebsiella pneumoniae	2		

(Appendix 5)

Organisms isolated from dressings removed from chronic wounds

S/No	Dressing	ID	Organisms
1	1	M1(1)	*Staphylococcus aureus (MRSA)*
2	2	M2(2)	*Pseudomonas aeruginosa*
3		M2(3)	*Staphylococcus aureus (MRSA)*
4		M2(4)	*Enterococcus faecalis*
5	3	M3(1)	*Burkholderia cepacia*
6		M3(2)	*Corynebacterium striatum*
7		M3(3)	*Staphylococcus aureus*
8		M3(9)	*Escherichia coli*
9	4	M4(1)	*Pseudomonas aeruginosa*
10		M4(2)	*Staphylococcus aureus*
11		M4(4)	*Candida albicans*
12	5	M5(1)	*Pseudomonas aeruginosa*
13		M5(2)	*Corynebacterium striatum*
14	6	M6(1)	*Proteus mirabilis*
15		M6(4)	*Corynebacterium striatum*
16		M6(5)	*Gemella haemolysans*
17		M6(9)	*Candida albicans*
18	7	M7(1)	*Klebsiella pneumoniae*
19		M7(2)	*Corynebacterium bovis*
20		M7(8)	*Staphylococcus epidermidis*
21	8	M8(1)	*Corynebacterium renale grp*
22	9	M9(1)	*Corynebacterium propinguum*
23	10	M10(1)	*Micrococcus kristinae*
24	11	M11(1)	*Staphylococcus aureus (MRSA)*
25		M11(3)	*Corynebacterium striatum*
26	12	M12(2)	*Staphylococcus aureus (MRSA)*
27		M12(3)	*Streptococcus agalactiae*
28		M12(4)	*Enterobacter cloacae*
29		M12(7)	*Bacteroides capillosus*
30		M12(8)	*Staphylococcus saccharolyticus*
31	13	M13(1)	*Staphylococcus aureus (MRSA)*
32		M13(2)	*Streptococcus agalactiae*
33		M13(4)	*Staphylococcus haemolyticus*
34		M13(5)	*Prevotella loescheii*
35	14	M14(1)	*Klebsiella pneumoniae*
36		M14(2)	*Staphylococcus aureus*
37		M14(3)	*Staphylococcus epidermidis*
38	15	M15(1)	*Staphylococcus aureus*
39		M15(2)	*Streptococcus grp G*
40	16	M16(1)	*Klebsiella oxytoca*
41		M16(2)	*Citrobacter freundii*
42		M16(3)	*Corynebacterium striatum*
43		M16(4)	*Proteus mirabilis*
44		M16(5)	*Pseudomonas aeruginosa*
45		M16(6)	*Staphylococcus aureus (MRSA)*
46	17	M17(1)	*Flavimonas oryzihabitans*
47		M17(2)	*Pseudomonas aeruginosa*

S/No	Dressing	ID	Organisms
48		M17(3)	*Corynebacterium ulcerans*
49		M17(4)	*Citrobacter freundii*
50	18	M18(1)	*Pseudomonas aeruginosa*
51		M18(3)	*Enterococcus faecalis*
52		M18(4)	*Staphylococcus simulans*
53		M18(8)	*Lactobacillus acidophyllus*
54		M18(9)	*Peptostreptococcus tetradius*
55		M18(10)	*Staphlococcus aureus*
56	19	M19(1)	*Pseudomonas aeruginosa*
57		M19(2)	*Staphylococcus aureus (MRSA)*
58		M19(3)	*Corynebacterium striatum*
59	20	M20(1)	*Escherichia coli*
60	21	M21(1)	Saccharomyces cerevisiae
61		M21(2)	*Proteus mirabilis*
62	22	M22(2)	*Pseudomonas aeruginosa*
63		M22(3)	*Streptococcus agalactiae*
64		M22(4)	*Staphylococcus aureus*
65		M22(6)	*Proteus mirabilis*
66	23	M23(1)	*Staphylococcus aureus*
67		M23(4)	*Staphylococcus simultans*
68	24	M24(1)	*Staphylococcus aureus*
69		M24(2)	*Staphylococcus epidermidis*
70		M24(3)	*Corynebacterium jeikeium*
71	25	M25(1)	*Staphylococcus aureus*
72		M25(2)	*Streptococcus saiguis*
73		M25(3)	*Corynebacterium striatum*
74	26	M26(1)	*Serratia marcescens*
75		M26(2)	*Streptococcus constellatus*
76		M26(3)	*Burkholderia cepacia*
77		M26(4)	*Pseudomonas aeruginosa*
78		M26(5)	*Proteus vulgaris*
79	27	M27(1)	*Staphylococcus aureus*
80	28	M28(1)	*Burkholderia cepacia*
81		M28(2)	*Pseudomonas aeruginosa*
82	29	M29(1)	*Streptococcus constellatus*
83		M29(2)	*Staphylococcus aureus*
84		M29(3)	Proteus mirabilis
85		M29(4)	*Pseudomonas aeruginosa*
86		M29(5)	*Corynebacterium pseudodiphtheriticum*
87	30	M30(1)	*Citrobacter freundii*
88		M30(2)	*Pseudomonas aeruginosa*
89		M30(4)	*Staphylococcus aureus (MRSA)*
90		M30(5)	*Proteus mirabilis*
91	31	M31(1)	*Escherichia coli*
92		M31(2)	*Corynebacterium striatum*
93		M31(3)	*Enterococcus faecalis*
94	32	M32(1)	*Pseudomonas aeruginosa*
95		M32(2)	*Shewanella putrefaciens*
96		M32(3)	*Corynebacterium pseudogenitalium*
97		M32(5)	*Serratia marcescens*
98		M32(6)	*Staphylococcus aureus*

S/No	Dressing	ID	Organisms
99		M32(7)	*Proteus vulgaris*
100	33	M33(1)	*Pseudomonas aeruginosa*
101		M33(2)	*Staphylococcus epidermidis*
102		M33(3)	*Staphylococcus auricularis*
103	34	M34(1)	*Staphylococcus aureus*
104		M34(3)	*Enterococcus faecalis*
105	35	M35(1)	*Staphylococcus aureus*
106		M35(2)	*Corynebacterium striatum*
107		M35(4)	*Acinetobacter lwoffii*
108		M35(5)	*Candida famata*

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