Interaction of extracellular factors derived from *Pseudomonas aeruginosa* on Keratinocytes

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Abstract:

Pseudomonas aeruginosa is a Gram negative pathogenic bacterium that has the notable ability to inhabit a broad range of environments, including humans as an opportunistic pathogen. *P aeruginosa* secrets outer membrane vesicles (OMVs) which contain virulence factors such as: pro-elastase, hemolysin, phoshpolipase C, protease, alkaline phosphatase and B-lactamase that can damage the cells of host and also other bacteria. *P aeruginosa* commonly colonises wound beds and this can result in the development of a chronic wound which is an important cause of major pathology.

Two strains of *P aeruginosa* PS3 (hospital strain), which was isolated from the dressing of an established chronic wound and a reference laboratory strain (L) that had no known pathogenic factors were cultured in different media including a simulated wound fluid. The secreted extracellular products were isolated and used to test their virulence on a cultured human keratinocyte cell line (HaCaT) and primary human keratinocytes (NHK). Vesicles derived from the outer membrane of the bacteria were isolated and the protein expression in these outer membrane vesicles (OMV) were compared between the two strains of *P aeruginosa* and with the outer membranes isolated from both strains of bacteria. The virulence of these vesicles and the outer membrane was tested on the keratinocytes.

The initial part of the study demonstrated that HaCaT cells grown for either 4 or 10 days expressed a range of TLRs required for the recognition and response to a wide range of bacterial antigens TLR1, TLR2, TLR4, and both aged cells. In contrast TLR5, TLR9 were found just in10 days cells. The expression of a range of TLRs was investigated and the most expression at the mRNA level was found for TLR2 and TLR4 when the cells were grown for either 4 or 10 days.

Both strains of *P aeruginosa* produced OMVs that appeared to contain a similar protein profile and this was similar to that of the isolated outer membrane. The yield OMVs from PS3 and the lab strain was generally similar but was higher when the bacteria were

treated with gentamycin before isolation and also when the bacteria were grown in the presence of ethanol.

Cultured keratinocyte cells secreted of IL-8 in response to exposure to cell free extracellular material from both strains *P aeruginosa* with little effect on the cell biomass. HaCaT cells produced a much higher concentration of IL-8 in response to OMVs than did NHK or an adapted more proliferative derivative of HaCaT (HaCaTa) with OMVs from the hospital strain stimulating slightly more IL-8 secretion compared to lab strain. NHK also produced significant amounts of IL-8 in response to challenge and with OM of PS3 or the lab strain. *P. aeruginosa* grown in SWF produced OMVs that stimulated a greater production of IL-8 in keratinocytes comparted to bacteria grown in normal bacterial broth (TSB).

These data indicate that *P aeruginosa* which are a common bacterium isolated from the bed of chronic wounds secrete virulence factors that stimulate an inflammatory phenotype in cultured keratinocytes. This pathogenic response is largely driven by the secretion of vesicles derived from the outer membrane and the hospital strain has a slightly greater pathogenicity than the lab strain this has implications for the treatment of wounds.

Acknowledgements:

First of all, I would like to thank my supervisor, Dr. Andrew Collett, for his guidance, advice, encouragement and support throughout the project period, it is a great pleasure to have had the opportunity to work with him. He has been patient, kind and helped me develop a great deal as a researcher. Also, a big ‘thank you’ to my co-supervisor Professor Paul Humphreys who has not only supplied bacterial strains and other lab materials used in this project, but has been also an enduring source of knowledge and advice in all matters bacterial.

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A final massive ‘thank you’ to my amazing daughters and son (Sarah, Lara, Tarah, Razan and Hasanain), who have remained a permanent source of encouragement. I honestly would not have been able to have completed it without them. I would also like to thank them for bringing all the laughter and tears for the duration of my research at the University, I never knew the strength of their patient until the past few years.

Dedication:

I would like to dedicate my work to my parents who I miss and in memory of my father.
Also, to my wonderful daughters and son.

Abbreviations:

CF cystic fibrosis.

EPS extracellular poly saccharides.

LPS lipopolysaccharide.

Pp periplasm.

PG peptidoglycan layer.

IM inner membrane, plasma membrane.

OM outer membrane.

VM vesicle membrane.

Cyt cytosol.

RBC red blood cell.

PRRS pattern recognition receptors.

PAMP pathogen-associated molecular patterns.

TLR toll-like receptor.

ds RNA double-stranded RNA.

ss RNA single-stranded RNA.

TSA tryptone soy agar.

TSB tryptone soy broth.

SWF simulated wound fluid.

FBS fetal bovine serum.

DMEM Dulbecco´s modified eagle medium.

MRD maximum recovery diluent.

CIS cell line services.

EDTA ethylenediaamintetera-acitiacid.

DMSO dimethylsulphoxide.

MEM membrane desalting buffer.

cDNA complementary DNA**.**

PCR poly chain reaction.

qRT-PCR real time –polymerase chain reaction.

PMPS pathogen-associated molecular patterns.

DAMA damage (host)-associated molecular patterns.

PGN peptidoglycan.

CD14 cluster of differentiation.

ELISA enzyme-linked immunosorbent assay.

CXCL8 Cxc Chemokine Ligand 8, “Interleukin 8”.

IL-8 interleukin-8.

L. laboratory strain.

10421 laboratory strain.

PS3 hospital strain.

OPrI lipoprotein I.

OPrL lipoprotein L.

OPrL lipoprotein F.

SDS Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis.

TNF-α Tumour Necrosis Factor Alpha.

NFκB Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells.

NHKs Normal Human Keratinocytes.

HaCaT an immortalized cell line.

HaCaTa cell line adapted from HaCaT.

LOR loricrin.

FLG filaggrin.

SC stratum corneum.

TNF- α Tumor Necrosis factor α.

RPE Rentinal pigment epithelium.

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Chapter 1

Introduction

1.1 *Pseudomonas aeruginosa*

P. aeruginosa initially isolated by Gessard in 1882 was first characterised as a pathogen by Charrin in 1980 (Bodey et al., 1983) it is now recognized as a common nosocomial pathogen which can cause disease in a variety of hosts (Feinbaum et al., 2012) as it has a number of virulence factors, that are important in both animal and plant diseases (Deep et al., 2011). *P. aeruginosa* is a Gram-negative, rod shaped bacterium about 1-5 µm long and 0.5-1.0 µm wide (Bhawsar and Singh, 2014b) it is motile by means of one single polar, monotrichous flagellum and a type IV pili (TFP) (Sadikot et al., 2005) these gives the bacterium the ability to move on surfaces (Semmler et al., 1999). The majority of bacteria require either flagella or a TFP to move, but *P. aeruginosa* is one of the few bacteria that requires both of these (Figure1.1), the surface-associated so-called twitching motility is powered by extension and retraction of type IV pili (Skerker and Berg, 2001). In pond water samples it is one of the fastest moving bacteria.

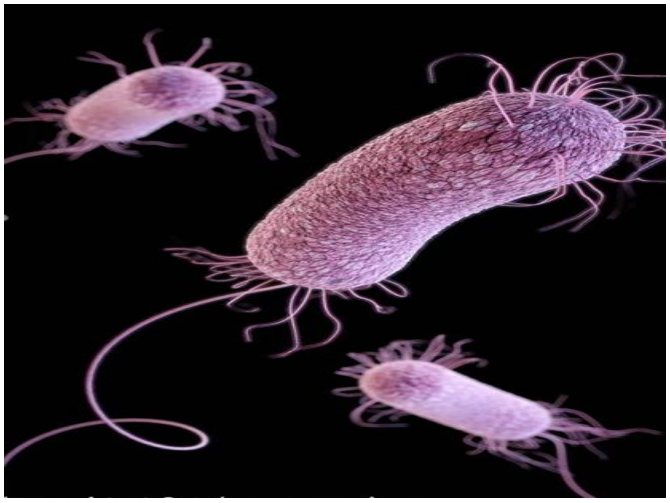

Figure 1. 1: A Scanning electromicrograph of *Pseudomonas aeruginosa* shoeing the flagellum and pilus.

(Taken from www.news-medical.net/whitepaper/20150526/Type-IV-pili-influence-swarming-of-*Pseudomonas-aeruginosa*-an-overview.aspx).

P. aeruginosa is ubiquitous in moist habitats such as soil, marshes, water, sewage plants and various foods such as leafy vegetables, fresh fruit juice, saline solutions, utensils and also cosmetics (Bonten et al., 1999). Its metabolism is respiratory not fermentative it can be anaerobic or aerobic it can use a variety of carbon sources and grows optimally at 37°C but it can grow in temperatures up to 42°C. *P aeruginosa* is tolerant of a wide variety of environmental stresses such as salts, dyes, weak antiseptics, antibiotics and moderate concentrations of disinfectants (Todar, 2006) (Andonova and Urumova, 2013b).

P aeruginosa produces characteristic fruity sweet odour and some strains produce the soluble fluorescent blue pigments, pyoverdin and pyocyanin the latter is produced by bacteria in some infections and this can result in characteristic blue pus (Todar, 2006). *P aeruginosa* is an opportunistic human pathogen that seldom infects healthy individuals but often colonizes susceptible people, such as those suffering from: pneumonia, acute leukaemia, organ transplants, intravenous-drug addiction (Choi et al., 2011) cancer or AIDS (Botzenhart and Döring, 1993) (Venza et al., 2009). *P aeruginosa* infection in the lungs of individuals with cystic fibrosis is a particular problem as the specific mutation which causes this disease, increases the general venerability to lung infections but in addition seems to increase the susceptibility of colonisation by this bacterium specifically which results in a subsequent serious increase in morbidity and mortality. *P.aeruginosa* also regularly colonizes the sites of burns and wounds.

P.aeruginosa is becoming increasingly prevalent pathogen and most studies indicate that antibiotic resistance is increasing in clinical strains (Todar, 2008). The majority of *P. aeruginosa* infections occur in hospitals (Mathee et al., 2008) and is responsible for about 10% of hospital infections in immunocompromised subjects, patients with cancer or diabetes and it is second most frequent agent causing skin infections in burn patients (Andonova and Urumova, 2013a). It can cause both acute and chronic infections (Howe and Iglewski, 1984). In common with most bacteria no new antibiotics effective against *P. aeruginosa* have been developed recently (Feinbaum et al., 2012). The high survivability and pathogenicity of *P. aeruginosa* can be a attributed to its structural characteristics (Carter et al., 2010) (Anwar et al., 1989). In addition it can readily form

biofilms (see below) in many environments including *in vivo* and this makes treatment with antibiotics less effective and therefore promotes chronic infections (Anwar et al., 1989, Rasamiravaka et al., 2015). All this makes it one of the pathogens most frequently isolated from intensive care units (Vincent, 2000).

Some species of *Pseudomonas* such as, *P putida* have been used beneficially as a bio-scrubber to aid in the biodegradation of a diverse range of organic compounds in waste water and polluted air (Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist)(Samuel et al., 2014, Siegrist) (Siegrist, Samuel et al., 2014, Siegrist). Furthermore, *P aeruginosa* is a very important soil bacterium that is capable of breaking down polycyclic aromatic hydrocarbons and synthesising rhamnolipids, quinolones, hydrogen cyanide, phenazines, and lectins (Bhawsar and Singh, 2014a).

1.1.2 Biofilms and *Pseudomonas aeruginosa*

The growth of bacteria in pure cultures has been the mainstay of microbiological since the time of Pasteur (Costerton et al., 1987). However, most bacteria can grow into organized communities known as biofilms. Evidence for biofilm formation appears early in the fossil record (nearly 3.25 billion years ago) (Hall-Stoodley et al., 2004). Biofilms are defined as a group of bacteria cells that stick to each other on a surface or without a surface and encase themselves in a secreted polymeric substances, such biofilms are more tolerant of most antimicrobials and host defences (Bjarnsholt, 2013) and thus can help produce persistent infections (Costerton, 1999) (Costerton and Stewart, 2000). The polymeric substance that the bacteria are embedded into can represent up to 85% of total biofilm biomass particularly in the *in vitro* situation (Rasamiravaka et al., 2015).

Rogovska et al. were the first to use the term biofilm in 1961 (Bjarnsholt, 2013). The clinical importance was first recognized in 1977 when Høiby described a collection of *P*

aeruginosa in an infected patient with cystic fibrosis (CF) (Hoiby et al., 1977). Despite long-term antibiotic treatment, pulmonary infections caused by *P aeruginosa* biofilms usually become chronic in patients with cystic fibrosis (Nickel et al., 1985). The importance of biofilms in human health has become increasingly apparent over the last 15 years and it is now recognised that they are an important factor in 65-80% of all microbial infections (Rasamiravaka et al., 2015).

The polymeric substance that biofilms are encased in are composed of biomolecules derived from bacteria including: polysaccharides, DNA, lipids, protein, flagella, pili and sometimes products from the host such as surfactants and red blood cells (RBC) (Rasamiravaka et al., 2015). Most biofilms have water channels which play important role as the distribution system for gas and nutrients.

Exopolysaccharides (EPS) play a central role in the establishment of biofilms they also help to protect the bacteria from antibiotics and phagocytes (Bjarnsholt, 2013). In addition the EPS matrix protects the individual bacteria from environment stresses, scavenges nutrients from the environment and provides shelter for the unique heterogeneous micro-niches inside the biofilm (Stoodley et al., 2002). *P aeruginosa* can make at least three types of polysaccharides molecules (alginate, Psl and Pel) which determine the stability of the biofilm structure (Sutherland, 2001) (Ghafoor et al., 2011). Alginate is a linear non-divaricate polymer that consists of homopolymeric units of 1-4 β D-mannuronic acid and L-guluronic residues (Ertesvåg and Valla, 1998) plays a fundamental role in the structural stability of biofilms and the preservation of water and other molecules as nutrients (Sutherland, 2001). The Pel polysaccharide is rich in glucose but its exact composition is unknown (Friedman and Kolter, 2004). Psl consists of a recurring pentasaccharides such as D-mannose and L-rhamnose (Byrd et al., 2009).

Most acute infections with have *P. aeruginosa* develop rapidly but are simple to treat with antibiotics because the bacteria exist as planktonic cells (single or as a small groups) by contrast the chronicity of the pathogenic bacteria is normally due to formation of the biofilms as outlined above (Costerton et al., 2007). Biofilms and can result in non-healing wounds (Davis and James, 2008). The most common bacterial

found in wounds is *Staphylococcus aureus* but *P. aeruginosa* has been reported to colonise more than 50% of the chronic wounds in clinical studies (Gjødsbøl et al., 2006), moreover *P. aeruginosa* can reduce the rate healing or even prevent it altogether (Høgsberg et al., 2011) and this is made worse if the bacteria form biofilms in which the bacteria have a distinct phenotype compared to planktonic cells (Harrison-Balestra et al., 2003).

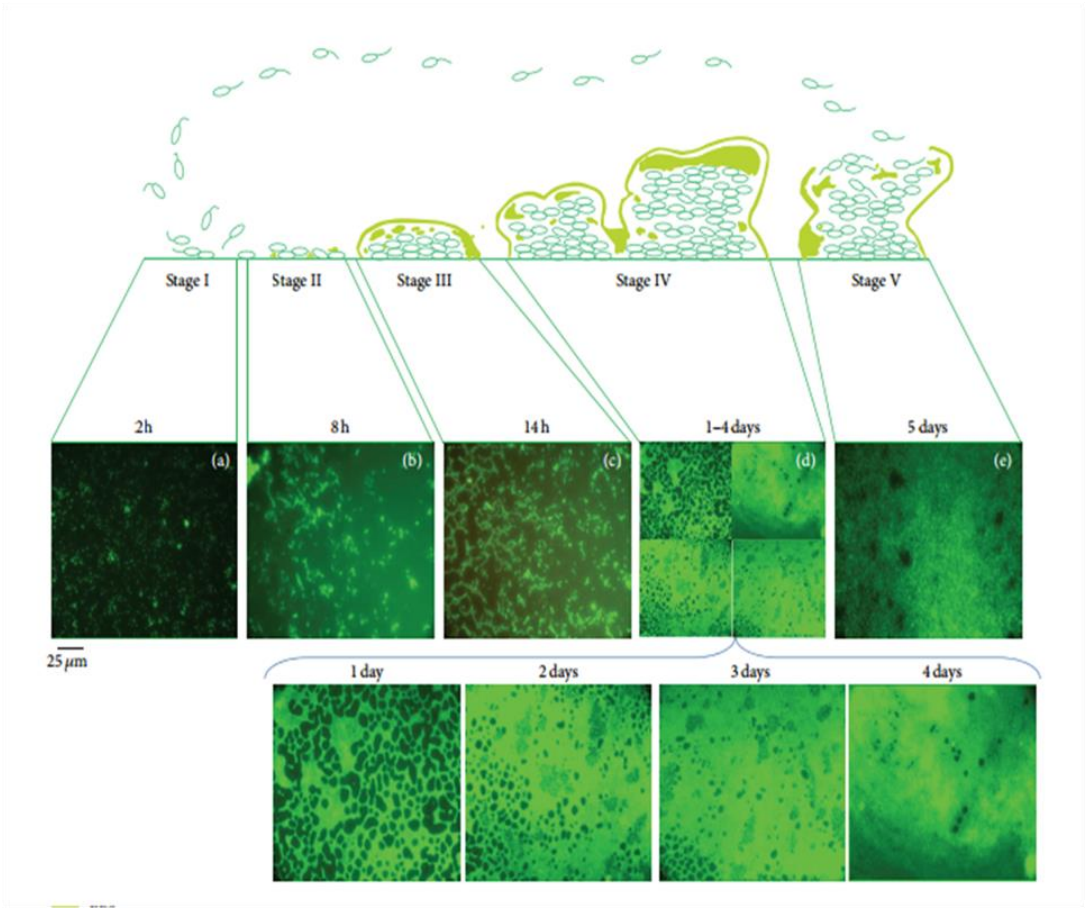

Figure 1. 2: Biofilm life cycle of *Pseudomonas aeruginosa.*

In stage I, planktonic bacteria initiate attachment to an abiotic surface, which become irreversible in stage II. Stage III micro colony formation. Stage IV biofilm maturation and growth of the three-dimensional community. The images were obtained using fluorescence microscope with 400x magnification each image representing the different stages were obtained at the time indicated, after the initiation of the culture at 2 h. (stage I), 8 h (stage II), 14 h (stage III), 1 to 4

days (stage IV), and 5 days (stage V). Images represent a 250x250µm field. Taken from (Rasamiravaka, 2015).

The process of biofilm development can be divided into 3 distinct phases these are: attachment, maturation and dispersion (figure 1.2) (Sauer et al., 2002 and Kalusen et al. 2004). Biofilms of *P aeruginosa* are highly differentiated in structure and this is dependent on the nutrient availability (Kalusen et al. 2004) and specific isolates and thus variation in the overall biofilm phenotypes may develop (Rasamiravaka et al., 2015). Biofilm infections can persist for months, years, or even a lifetime but are rarely fatal (Stewart and Costerton, 2001).

The study of biofilms in relation to the wounds was not considered until the 90s but it is now thought that 80% of human infectious disease are caused by biofilms (National Institutes of Health, 1997).

1.2.1 Treatment of Biofilms

As outlined above the resistance that biofilms provide for individual bacteria is multifactorial (Nickel et al., 1985). But this is dependent on accumulation of bacteria into communities, therefore one method to improve treatment is to breakdown the organization of the biofilm, allowing the host defences a greater opportunity to eliminate the infection (Stewart and Costerton, 2001) potentially such approaches could involve the use of enzymes which breakdown the matrix polymers of biofilm (Nemoto et al., 2000). (Molobela et al., 2010) tested the activity of proteases such as savinase, everlase and polarzyme and amylases including amyloglucosidase and the bacterial amylase novo, on both biofilm removal and the digestion of EPS. In this study everlase and savinase were found to be the most efficient treatments to eliminate biofilms.

It has been shown that some substances, such as penicillic acid and patulin as well two secondary metabolites from *Penicillium* species can prevent biofilm structural synthesis (Yasuda et al., 1993) interestingly these were shown to impact quorum sensing (QS) by

controlling the expression of specific genes in *P aeruginosa* which led to a reduction in the formation of biofilms (Rasmussen et al., 2005) (Irie and Parsek, 2008).

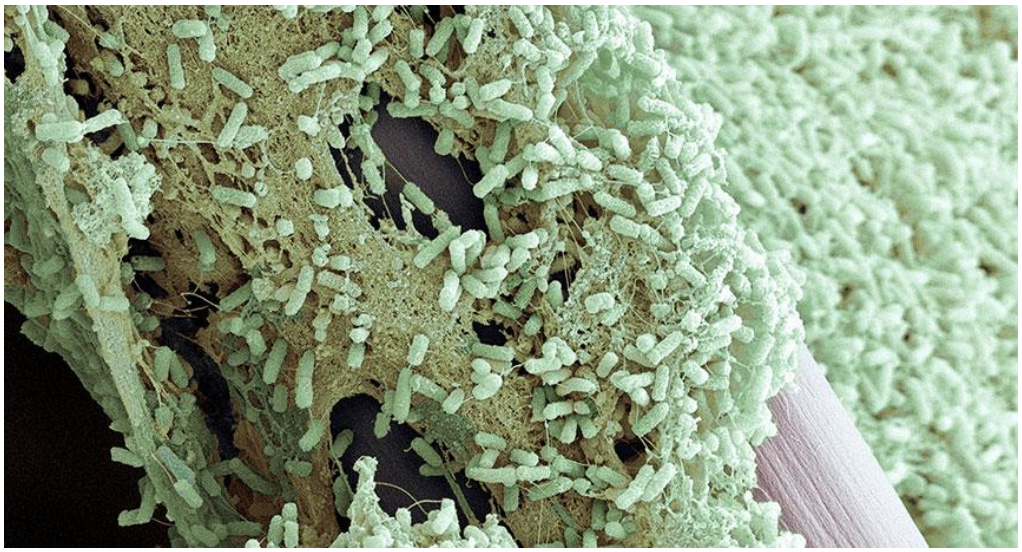

Figure 1. 3: Biofilm of *Pseudomonas aeruginosa*

Biofilms are communities of bacteria (light green cells) encased in a coating primarily of matrix polymers (dark green), taken from https://www.sciencenews.org/article/scientists-find-way-break-through-bad-bacteria%E2%80%99s-defenses.

1.3 The human skin

The skin is the largest organ of the body consisting of about 20m². It provides a protective barrier against mechanical, thermal and physical elements, hazardous substances and it also transmits information about surroundings and temperature regulation to the brain. The skin consists of three layers (different in function, thickness

and strength) the epidermis, dermis and hypodermis (figure 1.4) (McGrath and Mellerio, 2010).

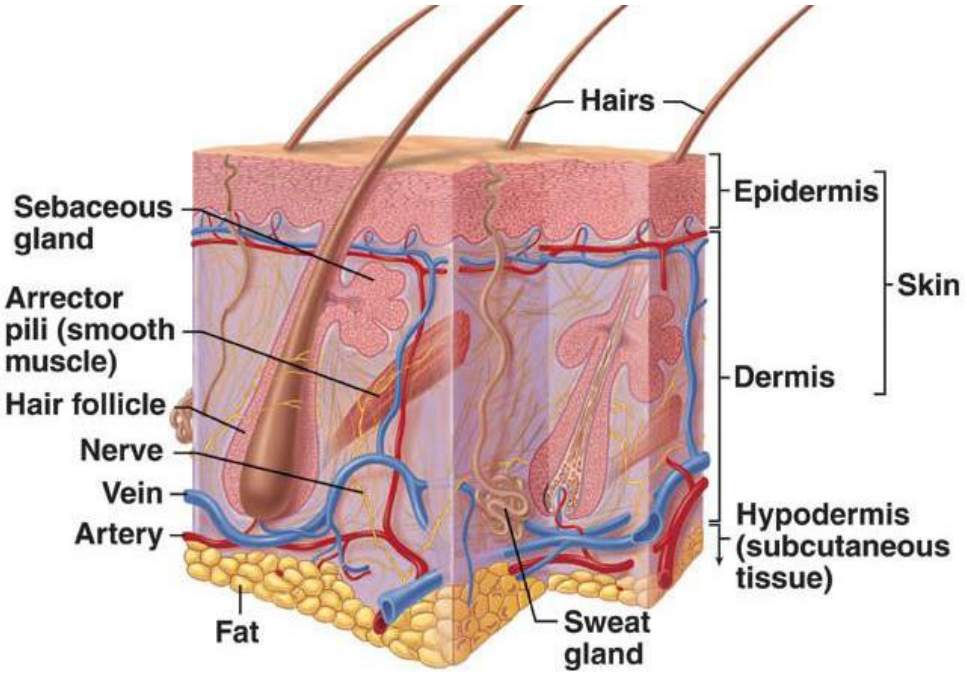

Figure 1. 4: Longitudinal section of the layers of the skin shows: epidermis, dermis and hypodermis.

(Taken from Picture of the Skin, Human Anatomy, By Matthew Hoffman, MD, 2014 WebMD, and LLC.)

The epidermis protects the skin from pathogens and injuries from the environment, its thickness varies in different types of skin, 95% of it is composed of keratinocytes but it also contains melanocytes (which produce the protein melanin a pigment that gives skin a darker colour) Langerhans's cells (epidermal dendritic cells) and tactile epithelial cells or Merkel cells, these are neuroendocrine cells that resemble nerve cells but also have some features of endocrine cells.

The epidermis is made up of five sub layers that work together to continually rebuild the surface of the skin these are:

a) Stratum corneum

b) Stratum lucidum (only in palm and the soles of feet)

c) Stratum granulosum

d) Stratum spinosum

e) Stratum germinativum (also known as stratum Basale)

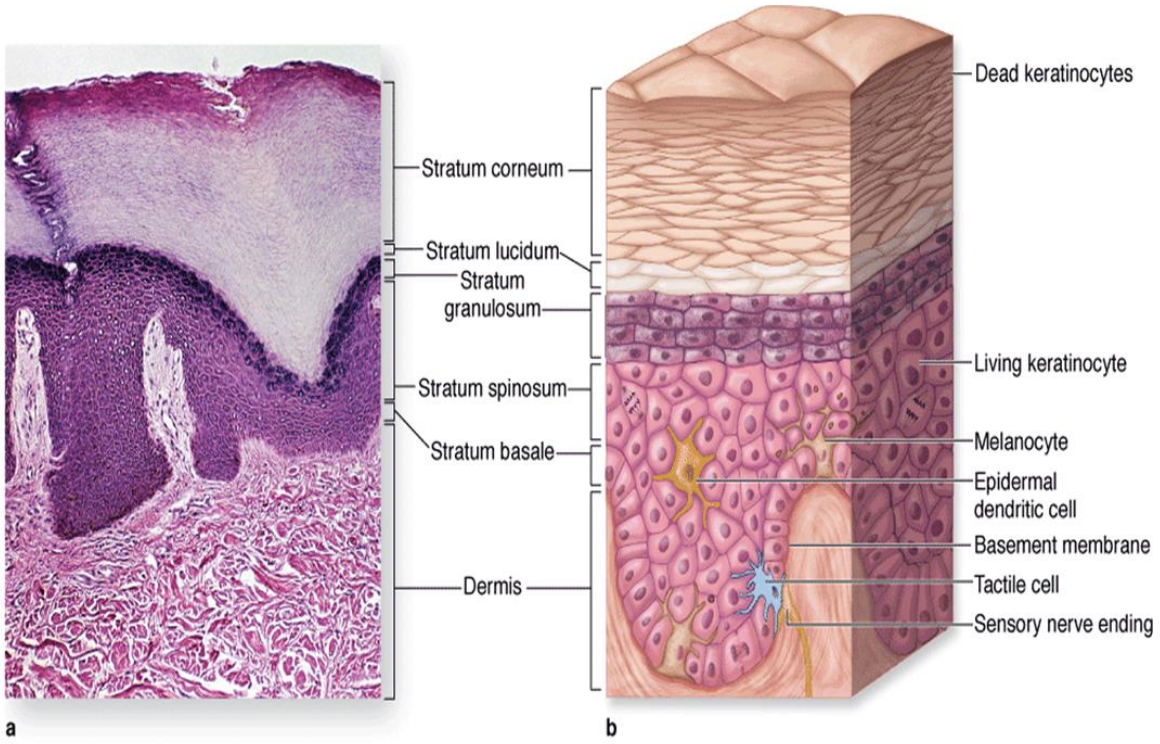

Figure 1. 5: Section of the layers of the human skin (layers of epidermis).

(a): Micrograph showing the epidermal layers in skin.

(b): Diagram illustrating the sequence of the epidermal layers also indicates the normal locations of three important non-keratinocyte cells in the epidermis: melanocytes, a dendritic (Langerhans) cell, and a tactile epithelial cell or Merkel cells. **Taken from: (Elder, 2014)**

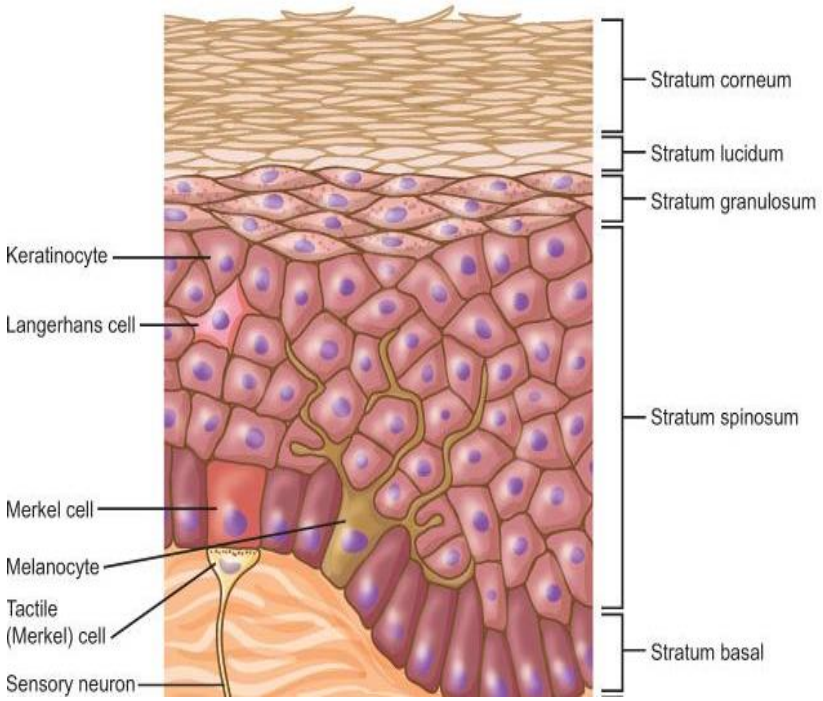

Figure 1. 6: The layers of Epidermis: Stratum corneum, Stratum lucidum, Stratum granulosum, Stratum spinosum, Stratum basal.

Taken from: ***http://headandneckcancerguide.org/adults/introduction-to-head-and-neck-cancer/skin-cancer/anatomy/.***

In the stratum basal, keratinocytes proliferate by the process of mitosis and as a result the cells rise through the layers of skin and undergoes changes in both shape and structure during differentiation until ultimately they become enucleated. The essential role of keratinocytes is to act as a barrier against agents such as pathogens (bacteria, viruses and parasites), heat, and radiation it also reduces losing of water, this is facilitated by the formation of tight junctions but between keratinocytes and with other cells such as the nerve cells. In addition within the keratinocytes are Langerhans cells which are located in the epidermis and lymphocytes cells in the dermis. Keratinocytes also produce recreate proteins such as keratin and lipids that form extracellular units that provides strength to the skin. Keratinocytes continue to migrate through the stratum corneum until they are eventually shed from the surface of the skin in a process known as desquamation.

The dermis is located beneath the epidermis its main function is to regulate temperature. This layer includes blood and a lymph vessels in addition to hair follicles (Gawkrodger and Ardern-Jones, 2016). The hypodermis the last layer and is made up of adipocytes, which contain fat and are used for both energy storage and thermal insulation and in addition to connective tissue (Gawkrodger and Ardern-Jones, 2016).

Not only is the skin a mechanical barrier between internal organs and the outer environment, it is also a fundamental part of the innate immune response (Janeway Jr and Medzhitov, 2002)and in addition can elicit an adaptive immune response against antigens (Nakajima et al., 2014). Keratinocytes are pivotal to both the skin's immunological system (Schloegl et al., 2012), participating in both the innate and acquired immunity (Nickoloff et al., 1995) The innate immune response is triggered through TOL-like receptors (TLRs) (see section 1.8) that are expressed by keratinocytes cells and this activation stimulates acquired immunity via, for example Langerhans cells (LCs) and antigen-specific T cells (Sugita et al., 2007).

1.3.1 Calcium Signalling in the Skin

Calcium signalling is an important intracellular pathway in many types of cell (Cordeiro and Strauss, 2011) and an excess concentration of intracellular calcium can lead to cell impairment and death (Dong et al., 2006). Calcium is the main regulator of growth and differentiation in keratinocytes and stimulates the formulation of desmosome, adherents and tight junctions (Kobashi et al., 2017b) (Pillai et al., 1993). Abnormal keratinocyte calcium concentration can cause disease such as Darier disease which was the first genetic skin disease caused by a loss of cell-to-cell adhesion and abnormal keratinisation described (Savignac et al., 2011).

The mobilization of intracellular calcium activates IL-8 production and secretion by keratinocytes (Yang et al., 2015). NF-kB is the major transcription element participate in the metabolic of intracellular calcium which is mediated by IL-8 gene activation in retinal pigment epithelial cells of human (Yang et al., 2015) Inactivated blood neutrophil cells

released less concentration of IL-8, the calcium ionophore, A23187, released high amount of IL-8 comparing to concentration which is recognized in exudative neutrophil removed from an infected part in human (Kuhns et al., 1998). Calcium controls activation and production of IL-8 gene from intracellular supplies in both colonic and airway epithelial cells in human (Yu et al., 2001, Carmona et al., 2010).

An epidermal Ca^{2+} gradient was first described in 1983 when analysis of skin samples indicated high calcium levels in stratum corneum which is the upper part of epidermis (Malmqvist et al., 1982). Further research confirmed that the superficial part of epidermis in man contains the highest concentration of calcium (Leinonen et al., 2009). It is now known generally in the mammalian epidermis there is a Ca^{2+} gradient, with a lower concentration in both basal and spinous layers and high levels in the stratum granulosum (Elias et al., 2002). The calcium concentration of the basal keratinocyte layer is higher than that of the lowest spinous cell layer in normal epidermis (Leinonen et al., 2009). A low Ca^{2+} concentration decreases the cell density needed to initiate growth (McGrath and Soule, 1984). If keratinocytes are exposed to high Ca^{2+} environments this can disrupt normal skin barrier function due to abnormal/premature differentiation of keratinocytes (Sah et al., 2017a). In addition high calcium concentrations stimulates the expression of serine protease inhibitors such as; lymphoepithelial kazal type related inhibitor (LEKTI), secretory leucocyte peptidase inhibitor (SLPI) and elafin in epidermal keratinocytes (Kobashi et al., 2017b). Interestingly the human keratinocyte cell line (HaCaT) responds to differentiation promoting stimuli, such as contact inhibition and high calcium concentrations in the culture medium by adopting a more differentiated phenotype (Berning et al., 2015).

1.3.2 The Immune Response and the Skin

Innate immunity is an essential part of the stimulation of acquired immunity. Innate immune cells include macrophages, dendritic cells and nonprofessional cells such as epithelial cells, endothelial cells, and fibroblasts (Honda, 2006). The cells that make up the skin immune system are distributed on both sides of the basement membrane. The

epidermal components are (keratinocytes and Langerhans cells). The dermal component contains dendritic cells, mast cells and resident perivascular T cells which are all professional immune cells. (Bos, 1997).

1.3.2.1 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are be defined as non-clonal germ line-encoded sensory molecules and represent an important element of the innate immune system (Akira et al., 2006). The innate immune system uses different PRRs which can be either extra or intracellular (Medzhitov et al., 1997). They recognise bacteria and other microorganisms via specific components which are called microbial-associated molecular patterns (MAMP) (Alexopoulou et al., 2001). Once stimulated PRRs initiate downstream signalling pathways that activate cellular responses that facilitate the elimination of the microbe (Yujie and Jianping, 2017) Because specific MAMPs are central to normal microbial physiology it is hard for microbes to evolve alterations in their structure in order to avoid activating the hosts immune response (Akira et al., 2006).

The principal functions of pattern recognition receptors are: opsonisation, stimulating phagocytosis, stimulation of complement, activation of proinflammatory signalling process, and induction of apoptosis {Gewurz, 1981 #168; Schwalbe, 1992 #169; Fraser, 1998 #170. PRRs also recognize endogenous elements which are released from damaged cells, these are named damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010) Different classes of PRRs have been identified including: transmembrane proteins, Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and intracellular proteins such as NOD-like receptors (NLRs) (Takeuchi and Akira, 2010) AIM2-like receptors (ALRs) and intracellular DNA sensors such as cyclic GMP-AMP synthase (cGAS) which is a cytosolic DNA sensor that stimulates the type 1 interferon signalling pathway (MacCallum et al., 2014) (see figure 8). TLRs are the most fundamental and essential of these immune receptors (Beutler, 2004) and appear to be the first line of host defence against microorganisms in many systems (Carstens and Akiyama, 2014).

1.3.2.2 Toll-like receptors (TLRs)

TLRs are evolutionarily conserved from the worm *Caenorhabditis elegans* to mammals (Beutler, 2004, Akira and Takeda, 2004, Hoffmann, 2003). The identification of mammalian Toll-like receptors (TLRs) by Medzhitov and Janeway was considered to represent a new chapter in our understanding of viral recognition and the innate immune system more generally (Medzhitov et al., 1997). The Toll signalling pathway was first discovered in Drosophila where it was identified for its role the control of dorsal-ventral polarity (Anderson et al., 1985)

At least ten human (TLR1-TLR10) and twelve murine (TLR1-TLR9, TLR11-TLR13) TLRs have been identified (MacCallum et al., 2014), and many researches have focused on both the detection the specific component recognized by each TLR, and the role of those interactions on microbial pathogenesis (Akira et al., 2006). TLRs are expressed on several skin cells including: keratinocytes, melanocyte and Langerhans cells (Ellis et al., 2010, Akira et al., 2001) also cells associated with the skin such as macrophages and fibroblast cells (Kawasaki and Kawai, 2014). TLRs are also found in a variety of other cells including those in the central nervous system (CNS) and the peripheral nervous system (PNS) including: microglia, astrocytes, neurons and Schwann cells (Okun et al., 2011 {Buchanan, 2010 #1788) (Lehnardt, 2010).

TLRs consists of an N-terminal ectodomain with repeats rich in leucine (LRRs), which mediate recognition of PAMPs, a transmembrane domain and another molecule of the cytoplasmic Toll/IL-1 receptor (TIR) domain which initiates downstream signalling {Barbalat, 2009 #2342}; (Cai et al., 2014). The ectodomain has a horseshoe-like structure and binds the specific MAMP or DAMP as a homo- or heterodimer along with a co-receptor or accessory molecule such as CD14 (Botos et al., 2011) (figure1.7).

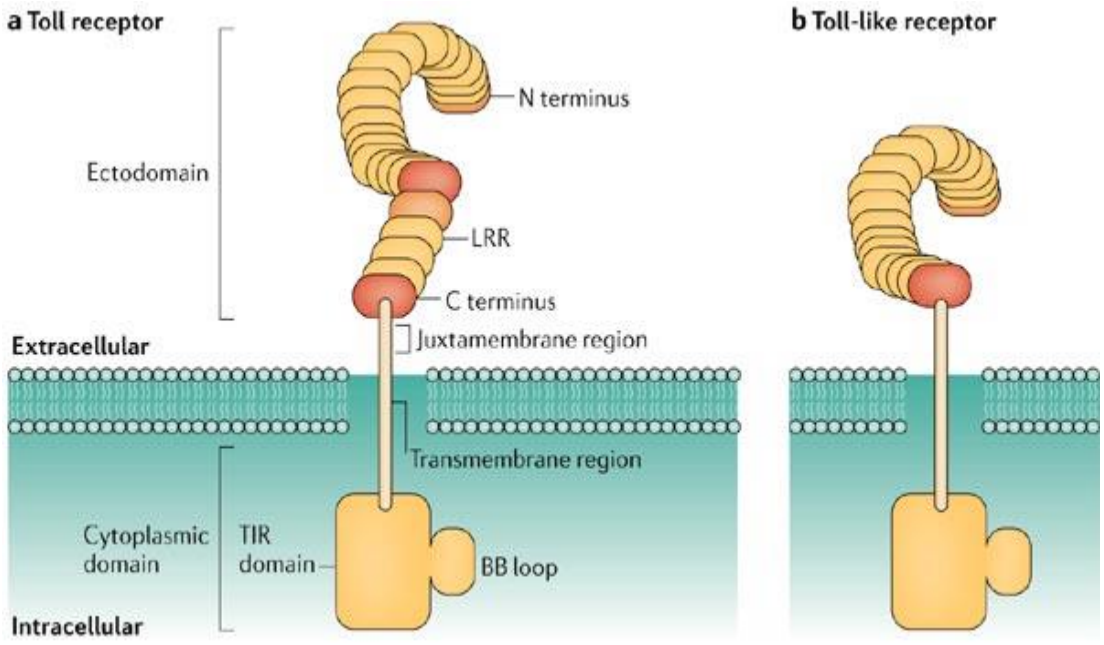

Figure 1. 7: Schematic diagram of the basic architecture of Toll and Toll-like receptors:

Toll receptors (a) and Toll-like receptors (b), extracellular, transmembrane and cytoplasmic are shown. Toll receptors contain two regions of leucine-rich repeats labelled (LRRs). C is the carboxyl terminus; N the amino terminus and TIR domain is the Toll/interleukin-1–receptor-containing domain. (Taken from Nicholas, 2006).

TLRs detect a variety of PAMPs leading to a transcriptional up regulation of distinct genes in a TLR and cell type dependent manner (Takeuchi and Akira, 2010). MAMPs include: bacterial lipopolysaccharide (TLR4), double-stranded (DS) RNA, small interfering RNA, and self–RNA derived from damage cells (TLR3) (Takemura et al., 2014), and single-stranded (ss) RNA from viruses (TLR7) (Takeda et al., 2003). TLR7 is mostly expressed in plasmacytoid DCs (pDCs) (Mancuso et al., 2009). TLR8 recognizes viral and bacterial RNA (Guiducci et al., 2013). TLR13 responds to bacterial 23S rRNA (Hidmark et al., 2012). TLR2, TLR1 and TLR6 recognize a variety of PAMPs, in a species dependent manner, including peptidoglycans, lipoproteins, lipotechoic acids mannan, zymosan and tGPI- mucin (Kawai and Akira, 2010). TLR5 recognizes bacterial flagellin (Akira et al., 2006). Human TLR10 with the co-receptor TLR2 recognize ligands from listeria (Regan et al., 2013), and influenza (Son et al., 2014). TLR9 responds to

bacterial and viral DNA due to the high levels of unmethylated CpG-DNA motifs (Coban et al., 2010). TLR11 recognizes flagellin (Mathur et al., 2012) and responds to a profilin-like protein from the parasite *Toxoplasma gondii* (Yarovinsky et al., 2005). TLR12 is very similar to TLR11, it can form a heterodimer with it (TLR11 also forms a homodimer) and is also stimulated by the same protein from *Toxoplasma gondii* (Koblansky et al., 2013, Andrade et al., 2013, Broz and Monack, 2013) table 1.

TLRs are all synthesized in the endoplasmic reticulum (ER), passing to the Golgi (Kawasaki and Kawai, 2014). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface, whereas other TLRs including TLR3, TLR7, TLR8, TLR9, TLR11, TLR12 and TLR13 are expressed on endocytic vesicle membranes or other intercellular compartments (Akira, 2003) eg the ER, or lysosomes, (Cai et al., 2014, Celhar et al., 2012).

Cell surface TLRs predominantly respond to microbial membrane components such as lipids, proteins, and lipoproteins for example TLR4 recognize bacterial lipopolysaccharide (LPS) (Kawai and Akira, 2010), and some of the intercellular TLRs recognize nucleic acids derived from bacteria and viruses in addition they can respond to self-nucleic acids in conditions such as autoimmune disease (Blasius and Beutler, 2010). Several TLRs respond to the same stimuli (Cai et al., 2014) (figure1.3.2).

After the detection of a pathogen TLRs mediate the activation of both the innate and adaptive immune systems via the modulation of gene expression (Akira et al., 2001). This includes a variety of host defence genes such as inflammatory cytokines, chemokines, antimicrobial peptides and the major histocompatibility complex (MHC) (Kopp and Medzhitov, 1999).

TLR signalling is divided into two distinct pathways depending on the specific adaptor molecules, myeloid differentiation factor 88 (MyD88) and Toll-receptor-associated activator of interferon (TRIF), that is incorporated into the signal (Akira et al., 2006). MyD88 consists of a death domain (DD) in addition to a TIR (Toll/interleukin-1 receptor) domain and is central to signalling in various TLRs, but not (TLR3). MyD88 is also an essential component of how most vaccines produce an immunostimulatory effect (Schnare et al., 2001)This pathway is also important in natural immunity and as such

individuals with MyD88 deficiency suffer from recurrent pyogenic bacterial infections (Kawagoe et al., 2008).

A variety of mechanisms can be used by pathogens to evade TLR signalling pathways these include: inhibition of mitogen activated protein kinases (MAPKs) cascade reactions, inhibition of NF-ΚB activation and by the production Toll/interleukin-1 receptor (TIR)-containing proteins that interact directly with TLRs or adaptor proteins in the signalling pathway and prevent signal transduction (Yujie and Jianping, 2017) (figure1.8).

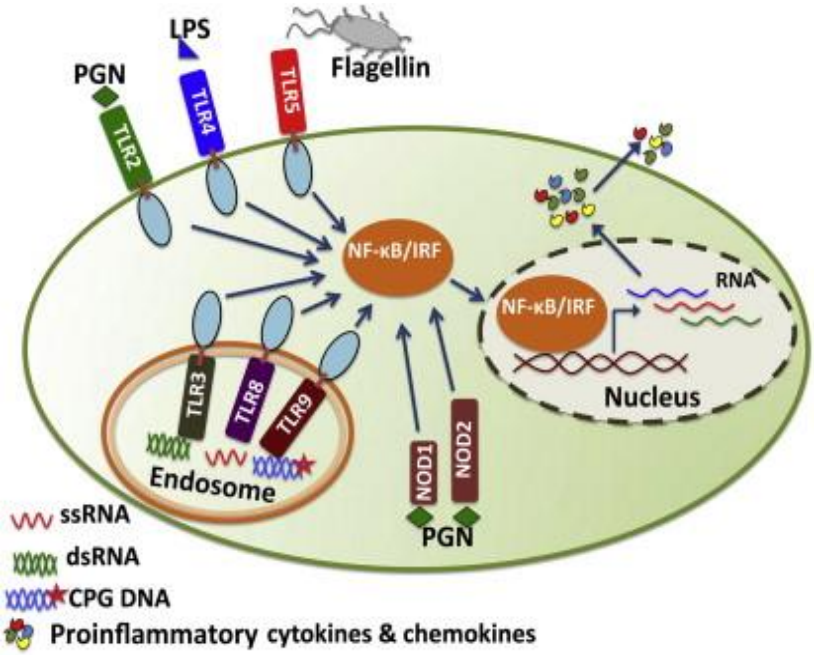

Figure 1. 8: Toll like receptor and cystolic proteins Nod1 and Nod2 are pattern recognition receptors PRRs.

Activation of TLRs by pathogen-derived molecules including LPS, flagellin, and PGN. Signalling through most TLRs induces the activation of nuclear factor-kB (NF-kB), which leads to activated NF-kB leading to induce transcription of gene for proinfammatory cytokines and chemokines (antimicrobial peptides) which are synthesized and realised. Cytokines also travel throughout the body and produce systemic effects such as vasodilation, fever and alteration of metabolism. (Taken from H.C. Lai 2014).

Pattern recognition receptor (PRR)	Location(s)	Ligand(s)	Source(s)
TLR1	Plasma membrane	Triacyl lipopeptides Soluble factors	Bacteria & mycobacteria. Neisseria meningitides.
TLR2	Plasma membrane	Peptidoglycan Lipoprotein/ lipopeptides Phospholipomannan Haemagglutinin and protein	Bacteria Fungi Viruses
TLR2-TLR1	Plasma membrane	Triacyl lipopeptides	Gram-negative bacteria
TLR3	Endosomal	Double-stranded RNA	Bacteria
TLR4	Plasma membrane	Lipopolysaccharide manna	Gram-negative bacteria Fungi
TLR5-TLR11	Plasma membrane	Flagellin	Bacteria
TLR8	Endosomal	Single-stranded RNA	Viruses
TLR9	Plasma membrane and endosomal	CPG-DNA	Bacteria Viruses Protozoa
TLR10-TLR2	Plasma membrane	Listeria	Bacteria
TLR10	Plasma membrane	Influenza	Viruses
TLR11-TLR12	Endosomal	Profiling-like protein	Parasite
TLR13	Endosomal	23S rRNA	Bacteria

Table 1.1: Toll-like receptors (TLRs), Location, their known agonists and the origin of the ligands.

1.4 Gram Positive and Negative Bacteria

Bacteria can be classifying into two groups: Gram positive and Gram negative this is based on two fundamental structural differences Gram positive bacteria have a relatively thick bacterial cell wall (30 to 100nm) outside the bacterial membrane by contrast Gram negative bacteria have a thinner cell wall (10 to 50 nm) with an additional outer membrane outside this cell wall (figure 1.9).

1.4.1 Bacterial Cell wall

The bacterial cell wall is a complex dynamic structure that plays a variety of protective and adaptive roles. (Silhavy et al., 2010), in addition there are fundamental differences in the structure of the Gram positive and negative cell wall. Specifically there are differences in the structure of the peptidoglycan which is the major conserved component of all bacterial cell walls and is essential for stabilizing cell membranes against high internal osmotic pressures. The Gram negative cell wall contains many layers, of peptidoglycan which are long anionic polymers composed of teichoic and lipoteichoic acids (Silhavy et al., 2010) (Neuhaus and Baddiley, 2003). Collectively, these polymers can constitute over 60% of the mass of the Gram positive cell wall (Dramsi et al., 2008). The Gram negative cell wall has few layers of peptidoglycan but contains a large number of proteins and is the location for several important functions such as nutrient acquisition, adherence, secretion, signalling, and protection from the environment (Thompson et al., 1985, Ciofu et al., 2000).

1.4.2 The Gram negative outer membrane

The second defining feature of Gram negative bacteria is the outer membrane (OM), which is located outside the cell wall (figure 1.9) and is abundant in lipids and proteins which can be divided into two classes: lipoproteins and proteins (Nikaido, 2003).

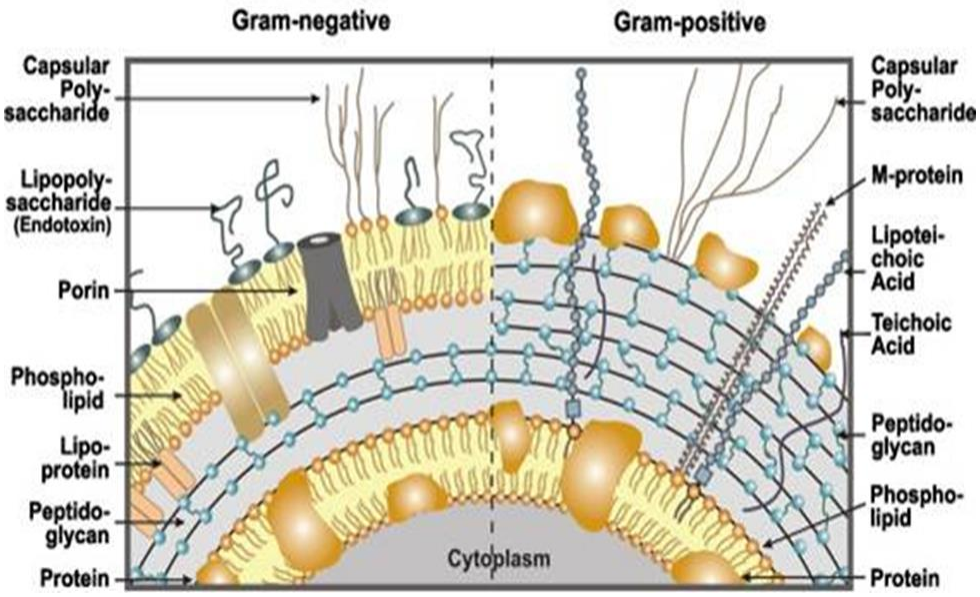

Figure 1. 9: Illustrates the structure of cell wall in Gram-negative and Gram-positive bacteria.

The main difference between Gram-negative and positive is the thickness of peptidoglycan cell wall and the outer membrane in Gram-negative bacteria (Funaharat & Nikaido, 1980).

The outer membrane (OM) also has a trilamellar structure with an asymmetric distribution of lipids. The outer leaflet is composed primarily of lipopolysaccharide (LPS) and the inner leaflet phospholipids and lipoproteins (Avila-Flores and Medellin, 2004). In addition the outer membrane has a very large number of transmembrane pore forming beta-barrel proteins known as porins {Cusumano, 1997 #2343; Smit, 1978 #2344} allowing the passage of a range of substances.

1.4.3 Gram Negative Lipopolysaccharide

The LPS that is present in the outer membrane of Gram negative bacteria can be important in its pathogenicity and is composed of three different parts:

i) Lipid-A

ii) The core polysaccharide comprising the inner and outer cores

iii) The O-specific polysaccharide chains

The O-specific polysaccharide chains consist of repetitive sub-units which extend outward from the bacteria and are in direct contact with a host immune system and thus can act as one mechanism by which the immune response can be triggered. In addition to this these polysaccharide chains can protect the pathogen from the effect of many antibiotics (Caroff and Karibian, 2003) (figure 1.10). The lipid portion of LPS serves as the lipid centre and commonly consists of fatty acids, sugars, and phosphate groups depending on the type of the bacteria.

The space between the outer and inner membrane of Gram negative bacteria, as well as containing the cell wall also has an aqueous compartment called the periplasm which is densely filled with proteins and peptidoglycan (PG) which results in it being more viscous than the cytoplasm (Mullineaux et al., 2006). The distance between the two membranes is approximately 13 nm and makes up between 7 and 40% of the total cell volume. It is connected to both membranes through different protein-protein interactions and it contains destructive proteins such as RNase which contributes to the greater virulence of Gram-negative bacteria compared to Gram positive (van den Berg, 2010).

The multilayered outer membrane explains why Gram negative bacteria are more resistant to severe environmental conditions including toxins and antibiotics compard to Gram positive bacteria (Martorana et al., 2014). For example the outer membrane of the Gram negative bacteria *P aeruginosa* has three (lipoproteins: I, L, F), OPrI, OPrL, and OPrF, which connect the OM to the PG cell wall (Deatherage et al., 2009, Collins et al., 2007, Stock et al., 1977) each of these has different virulent properties and different effects on the host defences (Bodey et al., 1983).

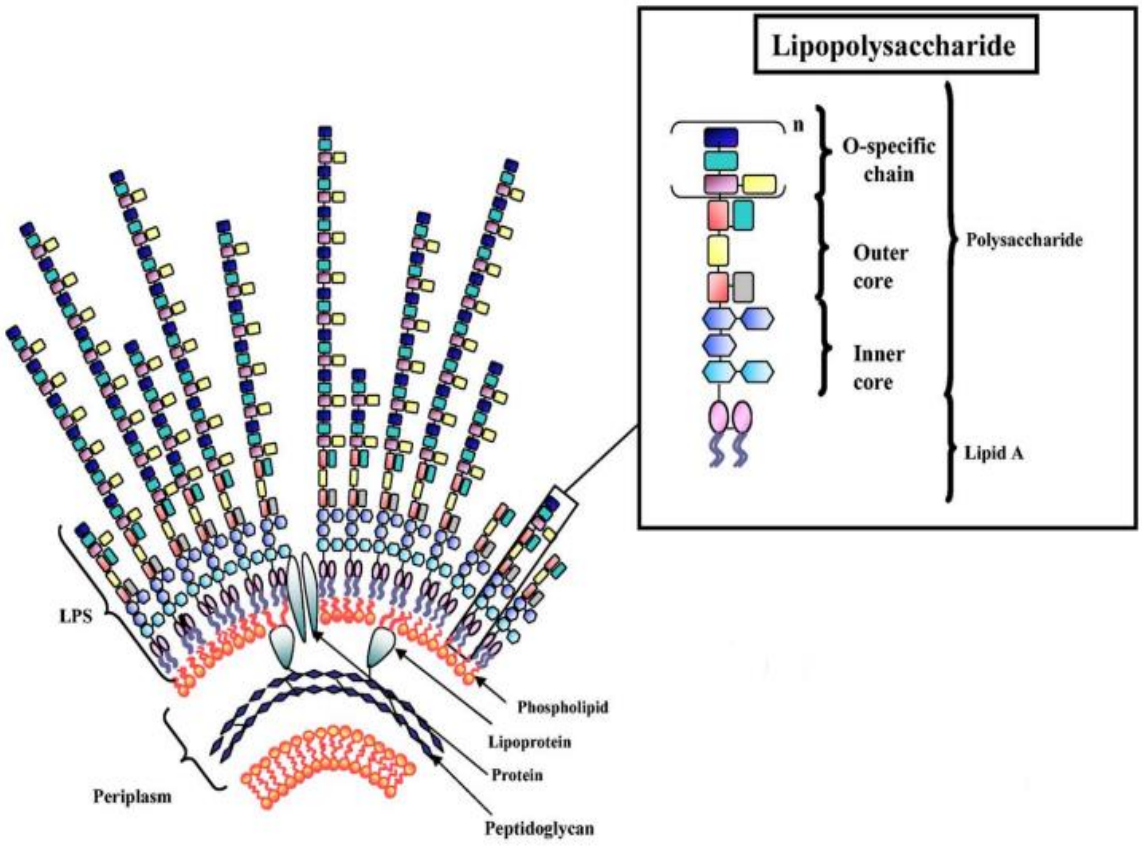

Figure 1. 10: Schematic representations of the Gram-negative cell wall (left) and the structure of lipopolysaccharide (right).

Taken from: (Caroff and Karibian, 2003).

1.5 Outer membranes vesicles

In recent years that there has been increasing interest in vesicles produced by Gram negative bacteria. The vesicles are derived from the outer membrane in a process that is conserved among all Gram-negative bacteria so far studied (Kuehn and Kesty, 2005), including many pathogenic strains (Necchi et al., 2007) (Marsollier et al., 2007), it is now assumed that outer membranes vesicles (OMVs) have an important role in their physiology. OMVs were first detected when they were observed in the supernatant of *Escherichia coli* cultures grown under growth limiting conditions and were found to contained soluble LPS (Bishop and Work, 1965). It was hypothesised that the vesicles

were produced because the growth limiting conditions used inhibited peptidoglycan synthesis which affected the synthesis of the bacterial cell wall but did not alter outer membrane synthesis and thus an unstable membrane was produced and this resulted in vesicles being produced. However, subsequent studies showed that similar structures were also formed under normal growth conditions in vitro as well as in infected host tissues and serum (Hellman et al., 2000). It is now thought that OMV production is ubiquitous in Gram negative bacteria and these vesicles have a range of functions and in the case of pathogenic bacteria will contain pathogenic factors which could cause damage to the host cells and other bacteria (Choi et al., 2011).

The OMVs are complex spherical, bilayer membranous structures which contain a subset of envelope and protein components they are on average 50 to 250 nm in diameter depending on the strain (Ellis et al., 2010). OVMs are produce by growing cells and are not products of cell lysis or death (McBroom and Kuehn, 2007) they are produced at all stages of bacterial growth in vitro and in vivo (Kuehn and Kesty, 2005) (Beveridge, 1999, Keenan et al., 2000, Ismail et al., 2003). When bacteria are grown in culture the maximum rate of vesicles production occurs during the end of log phase (Hoekstra et al., 1976). OMVs production can be increased by a variety of conditions such as limited nutrition, changes in temperature, exposure to antibiotics and oxidative stress (Klimentová and Stulík, 2015) it is of note that conditions will often be encountered by bacteria during an infection (Chutkan et al., 2013).

The components of OMVs will be similar to the outer membrane itself and as such will include lipopolysaccharide, proteins and phospholipids (Klimentová & Stulík, 2015). In addition the will also include products derived from the periplasm or even the contents of the cell such as nucleic acids. In common with other Gram negative bacteria *P aeruginosa*, produce outer membrane vesicles (OMVs). In general, vesicles from *E. coli* contain 0.2-%-0.5% of outer membrane and periplasmic proteins (Mug-Opstelten and Witholt, 1978, Kesty and Kuehn, 2004, Hoekstra *et al*., 1976). It is thought that OMVs have an important role in stimulating the innate immune system due to their small size, which will allow them to easily penetrate infected tissues and potentially further into the

body and the high concentration of MAMPs that are associated with them (Ellis *et al*., 2010).

As it is unlikely that bacteria produce OMVs without purpose or function they presumably have functions that are beneficial to the bacteria that produced them (figure 1.11). It is thought that OMVs have roles that could be beneficial to bacteria both in the context of infecting a host and also within its own environment. These include the ability to transfer many biological molecules to the host cells including virulence factors that enhances bacterial survival (Klimentová and Stulík, 2015). In both pathogenic and non-pathogenic bacteria, OMV can have a protective by absorbing or internalising toxic molecules or factors produced by the hosts immune system such as antibodies which reduces the concentration that the bacteria are exposed to (Loeb, 1974, Loeb and Kilner, 1978). Sometimes OMVs production is a bacterial stress response and can aid nutrient procuration, biofilm formation and growth, quorum signalling, horizontal gene transfer and pathogenesis (Kulp and Kuehn, 2010). For example they can allow bacterial enzymes to spread within the environment which can change its chemical composition (Kulp and Kuehn, 2010). In addition Gram negative bacteria can use OMVs for bacterial communication and even to cause toxicity in other strains of bacteria in a crowded environment (Kulp and Kuehn, 2010).

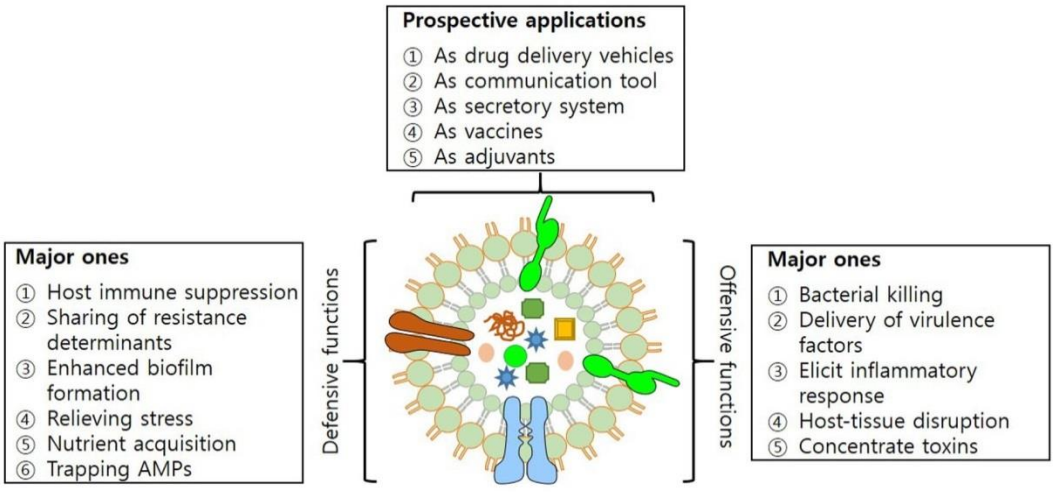

Figure 1.11: Composition of OMV.

Figure illustrating aggressive and protective roles of OMVs utilized in bacteria-bacteria and bacteria-host interactions; and their potential applications. (Tasleem, 2017).

Many *P aeruginosa* virulence factors affect the growth, and immune evasion of this bacterium and can combine to cause inflammation and tissue damage in the host. *P. aeruginosa* lung infections is a main reason for mobidity and mortality in individuals with cystic fibrosis (CF) (Sadikot et al., 2005).

P aeruginosa has two extracellular phospholipase C (PLCs) homologous, a haemolytic PLC (PlcHR) and a non- haemolytic PLC (PlcN) (Ostroff et al., 1990). While PlcN has no pathogenic activity, PleHR may be an important virulence factor during *P aeruginosa* infection in mammals (Ostroff et al., 1989), plants (Jander et al., 2000), yeast (Hogan and Kolter, 2002), and insects (Jander et al., 2000). Purified PleHR is toxic to living cells and by interfering with signalling pathways of eukaryotic cells (Terada et al., 1999). Specifically, a rise int the concentration of the haemolytic PLC (PlcHR) has been linked to the decline in lung function in individuals with CF (Lanotte et al., 2003), the second messengers normally involved in PleH activity are phosphatidylcholine (PC) and sphingomyelin (López et al., 2011) These lipid derived molecules are precursors of pulmonary surfactant. Numerous studies have detailed the potentially deleterious effect of PlcH pc-phospholipase C/sphingomyelinase (pc-plc/s Mase) activity during *P aeruginosa* infection (Wiener-Kronish et al., 1993). For example PlcH may impact on disease course due to its influence on pulmonary surfactant fluid which is largely composed of dipalmitoylphosphatidylcholine (Wargo et al., 2011).

1.5.1 Formation and Pathogenicity of Outer Membrane Vesicles

Outer membrane vesicles are formed by a process of bulging out and pinching off a portion of the bacterial outer membrane {Chatterjee, 1967 #2352} thus OMVs originate from the bacterial surface (figure 1.12) and contain outer membrane proteins OMPs, phospholipids, and LPS {Wai, 2003 #2355; Kuehn, 2005 #2354; Horstman, 2000 #2353}. As OMVs bleb from the outer membrane, periplasm fills their lumen and is retained there {Schooling, 2006 #2356} which means, they entrap some of the underlying periplasmic component that probably varies dependent on the specific

bacterial growth conditions and species. Generally, the periplasm will include toxins, enzymes, DNA, adhesions molecules and other virulence factors (Ellis et al., 2010). Vesicles have been identified in different host tissues, revealing the ability of vesicles to access a variety of environments within the host.

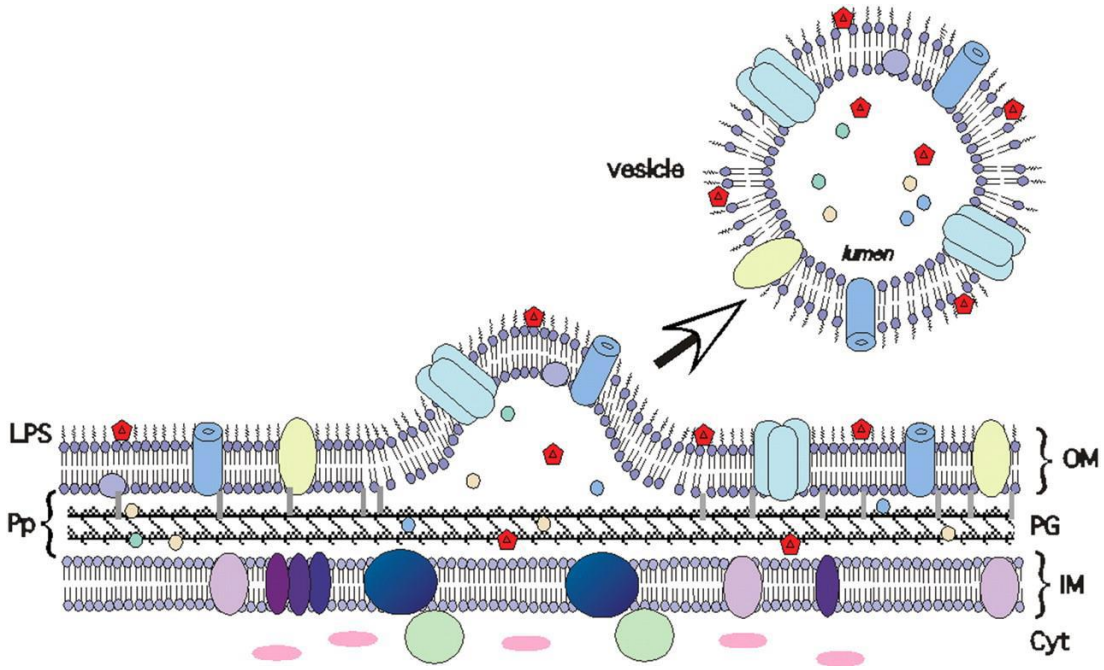

Figure 1. 12: Formation of vesicle membrane.

OMVs are proteoliposomes made of OM phospholipids, lipopolysaccharides, a raft of OM proteins, and proteins from periplasmic space (luminal). (LPS) Lipopolysaccharide; (Pp) periplasm; (OM) outer membrane; (PG) peptidoglycan; (IM) inner membrane; (Cyt) cytosol (red symbol) outer membrane protein (Kuehn and Kesty, 2005).

Vesicles are thought to bud out from sites where contacts between the peptidoglycan and OM are infrequent, missing, or knocked out. It has been shown that almost all OMVs preparations are enriched in envelope components (Kuehn and Kesty, 2005). Some of the preparation were also found to contain a small amount of cytosolic and inner membrane proteins, the basis of which remains unclear or rather controversial (Scorza et al., 2008, Ellis and Kuehn, 2010).

1.5.2 Virulence Factors of OMVs

Virulence factors have been discovered to be associated with vesicles produced by pathogenic bacteria (Kuehn and Kesty, 2005) such as *P aeruginosa* vesicles from which can possess a wide spectrum of virulence factors that can mediate the killing of host cells or other bacteria. These virulence factors include: pro-elastase, hemolysin, phospholipase C, protease and alkaline phosphatase, in addition to the penicillin-degrading enzyme B-lactamase (Kadurugamuwa and Beveridge, 1995, Kadurugamuwa and Beveridge, 1997) and antibacterial factors such as hydrolases (Choi et al., 2011). In addition OMVs released from pathogenic bacteria, contain a wide range of immunomodulatory molecules {Allan, 2003a #160; Allan, 2003b #161} and produce more outer membrane vesicle than non-pathogenic bacteria (Lai et al., 1981, Wai et al., 1995). Proteolytic analysis of the cell-free supernatants of mid-log phase cultures derived from an enterotoxigenic *E. coli* produced about 10-fold more outer membrane vesicles than non-pathogenic *E. coli* (Horstman and Kuehn, 2002). OMVs have the ability to combine with the membranes of host cells such as epithelial cells and thus realise their virulence factors into the cells where they degrade their cellular structure which can be an important factor that mediates the pathogenicity caused an infection by the pathogenic bacteria (Kadurugamuwa and Beveridge, 1997).

Vesicles have been detected in different host tissues, which indicates that vesicles have the ability to penetrate different milieu of the host also they have been identified in the fluids distant from the infected tissues of the host, which indicates the ability of OMVs to spread to sites far from the infected area (Kuehn and Kesty, 2005).

The structure of vesicles of a laboratory *P aeruginosa* strain was reported to be different to the vesicles secreted by pathogenic hospital strains of isolated from an individual with cystic fibrosis (CF), the former one lack LPS with O-antigen which is essential for the pathogenicity of the *P aeruginosa* hospital strains (Pier, 2000) and the pathogenic vesicles were enriched with an amino peptidase that facilities their ability to fuse to lung epithelial cells when compared to vesicles derived from laboratory strains (Kuehn and

Kesty, 2005). It has also been reported that there is a dynamic change in the structure of OMVs that is related to different phases of bacterial growth and this impacts on their biological function (Tasleem, 2017).

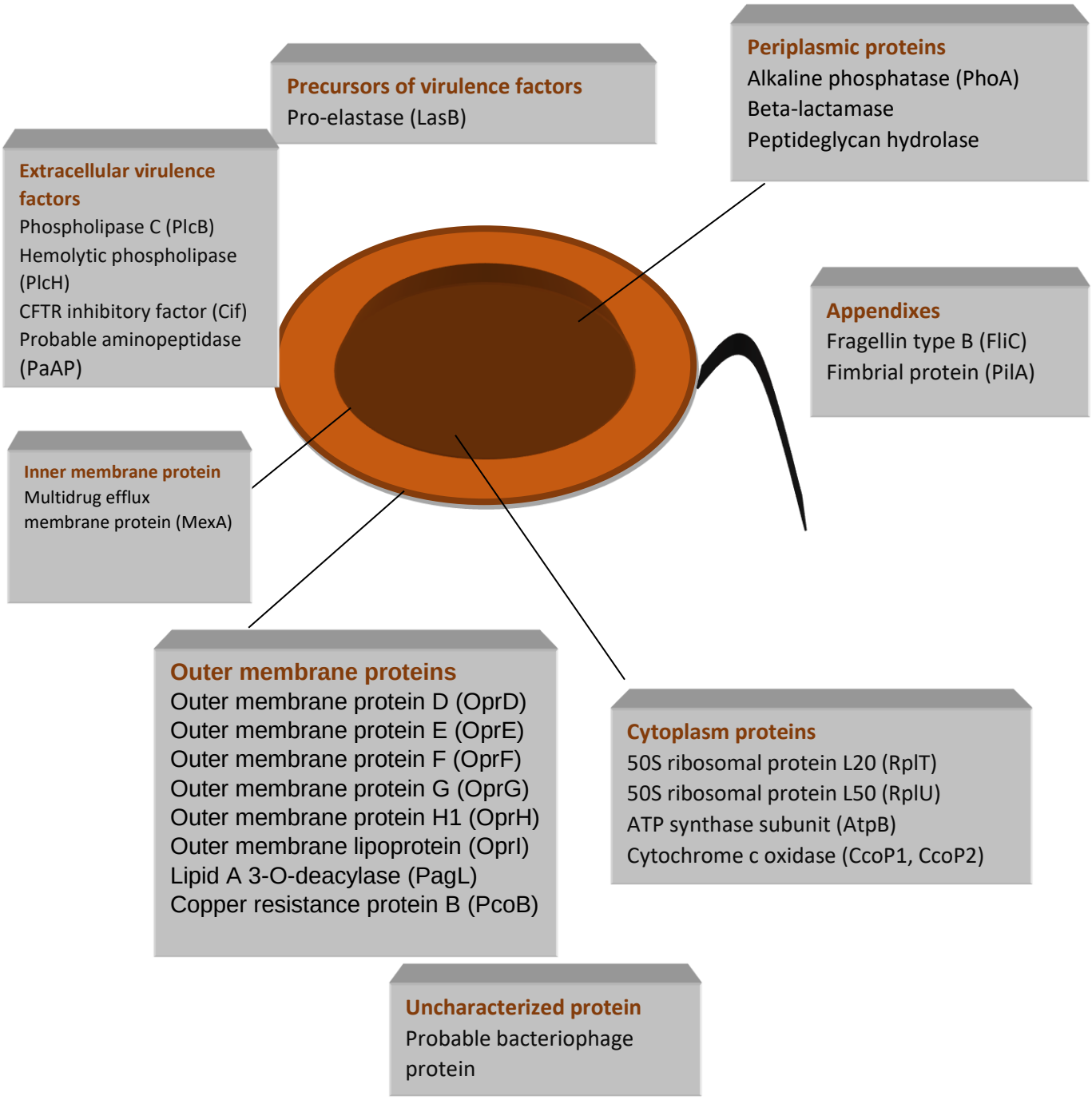

Figure 1. 13: Proteins associated with Pseudomonas aeruginosa OMVs.

The outer membrane of Gram-negative bacteria contains extracellular virulence agents (Tashiro et al., 2012) such as phospholipase C (Sabharwal et al., 2014), that cleaves host cell phospholipids which induces a number of cell responses including the stimulation of the cystic fibrosis transmembrane conductance controlling inhibitory factor (cif) (Ballok and O'Toole, 2013), β-lactamase, that can destroy β-lactam based antibiotics and thus aid bacterial survival within a host (Tashiro et al., 2012) and on occasions cell necrosis (Assis et al., 2014).

1.6 Chronic Wounds

Chronic wounds often cause increasing loss of the functional ability of the skin and is occupied by increasing pain and reduction in the quality of life. In addition due to their persistence they are a significant burden for patients carers and health care resources {Rizzi, 2010 #1986

1.6.1 Pathology of Wounds and Chronic Wounds

The fundamental role of the skin is to act as a barrier against different external factors. Loss of the integrity of specific parts of the skin because of injury or illness can cause disability or death {Kondo, 2010 #1987}. A wound is defined as a disruption of the normal anatomical composition and function of the skin (Atiyeh et al., 2002).

Wound healing is a complex physiology process and has been simplified by separating it into distinct programmed stages: homeostasis, inflammation, proliferation and maturation (Stojadinovic et al., 2008), any change in this processes can result in pathological conditions of a variety medical relevance. Wound healing involves a well-organized interaction between various tissues and cells (Branski et al., 2009) in distinct phases which have different physiology functions which must take place in the proper sequence, at a particular time and continue for a specific period of time and at an

optimal intensity as regulated by the activity of distinct cells types that are associated with the skin including: keratinocytes, fibroblasts, endothelial cells, immune cells in addition to blood cells and platelets {Marfia, 2015 #1991}.

The management of infected wounds is a worldwide health care issue (Akbar et al., 2015) and has been further complicated by the rise of antimicrobial resistance (Gottrup et al., 2013).

A wound infection is present when bacteria have invaded the wound and/or the surrounding tissue and are replicating to numbers that result in significantly impaired local healing, overt wound infection and/or a systemic toxicity (Stojadinovic et al., 2008). A wound with adequate blood supply and $<10^5$ bacteria per gram of wound tissue should heal or accept grafting well {Sibbald, 2000 #1992}.

There is an essential difference between acute wounds which proceed to uncomplicated healing (these progress through the normal stages of wound healing and show definite signs of healing within four weeks) (Demidova-Rice et al., 2012) and those that become chronic wounds (Stojadinovic et al., 2008). Chronic wounds or non-healing wounds are wounds that do not progress through the normal wound healing process resulting in an open laceration of varying levels of severity (Silva et al., 2015). All wounds are contaminated, in that there are co-habitating, non-replicating bacteria, in addition many wounds are colonized with replicating bacteria, but without a host response to their presence thus healing is not normally impaired in contaminated or colonized wounds (Stojadinovic et al., 2008). However if wounds are heavily infected and this extends to the wound bed a chronic wound can develop. The clinical management of chronic wounds attempts to accelerate endogenous healing or facilitate the effectiveness of other therapeutic interventions and this can be followed with systemic antibiotics with an appropriate Staphylococcal, Streptococcal, Coliform and anaerobic coverage (Sibbald et al., 2000). However, chronic wound infections often do not respond to traditional antimicrobial therapies (Percival, 2004) for this reason new therapeutic modalities may be required (Percival et al., 2008). Recently regenerative medicine and in particular the use of mesenchymal stem cells is being developed a potential new treatment (Marfia et al., 2015).

A wound is considered to be infected when bacteria have invaded either the wound its self and/or the tissue that surrounds it and are replicating to such numbers that local healing is significantly impaired this can then go on to produce systemic toxicity (Stojadinovic et al., 2008). In otherwise healthy individuals if a wound has a good blood supply it should be able to tolerate over 10^5 bacteria per gram of wound tissue and still heal or accept a skin graft {Sibbald, 2000 #1992}.

Tissue repair is a complex process and is dependent on the tissue's capacity to regenerate and also particularly if the wound is infected the inflammatory response (Eming et al., 2017), which is governed by a balance of cytokines, chemokines, growth factors and effects of immune cells which collect at the wound site (Chanson et al., 2005). These factors also effect the rate of healing via stimulation of cell proliferation and matrix synthesis (Chanson et al., 2005).

Non-healing wounds can develop as a complication of a range of different conditions such as trauma, surgery, acute illness, or various chronic disease and worldwide affects millions of individuals every year. Ultimately it is the result of the defective regulation of the processes required for the normal healthy repair of wounds and includes inflammation, angiogenesis, and cell recruitment. The defective functioning of one or more of these cellular mechanisms and is normally triggered by specific chronic diseases, including vascular disease, diabetics or aging. Research aimed at improving clinical methods to overcome chronic wounds will require an understanding of the essential biological methods of repair and regeneration (Chanson et al., 2005). Globally chronic and poorly healing wounds represent a significant health, social and economic burden. (Bird and Emery, 2009 {Brem, 2007 #2017).

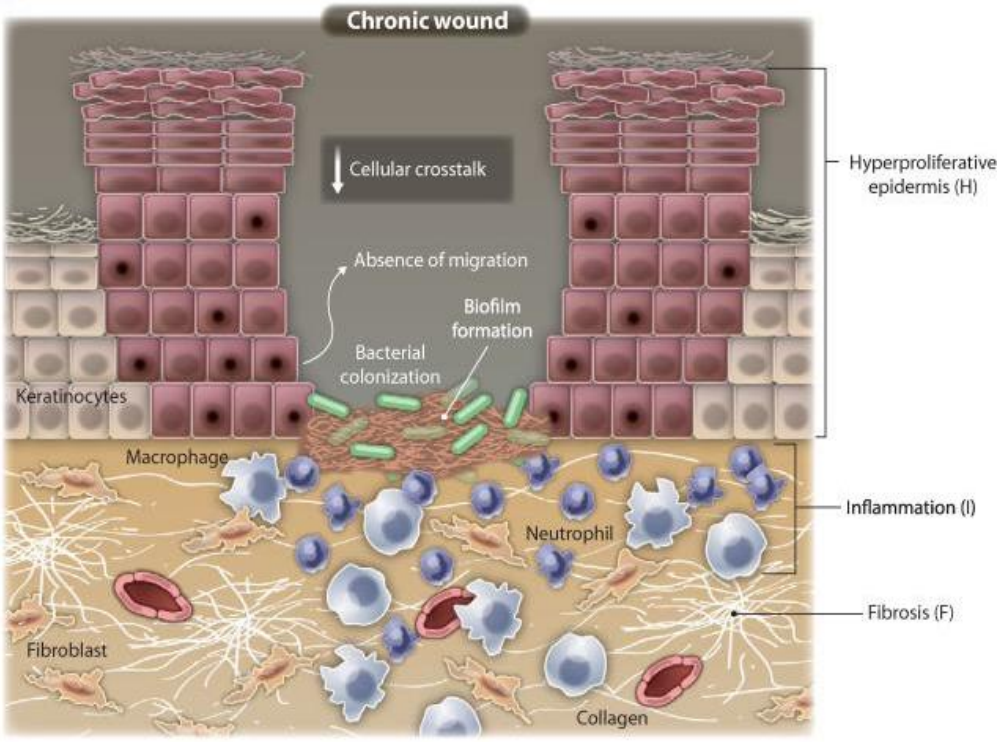

Figure 1. 14: Molecular pathology of chronic wounds.

Chronic wounds show rapid proliferative and nonmigratory epidermis, unresolved inflammation, existence of infection, and formation of biofilm. There is an accumulation of inflammatory cells (neutrophils and macrophages) and uncontrolled protease interference with fundamental repair mechanisms. Some fibroblasts become senescent and there is low level angiogenesis, stem cell recruitment and activation, and a redesigning of the extracellular matrix. (From Eming et al. 2017).

Chapter 2

Materials & Methods

2.1 Materials

All standard chemicals were obtained from Sigma-Aldrich or Fisher Scientific unless otherwise stated. Other consumables were obtained from the manufactory stated at their first mention in the text. Table 2.1 shows the use and supplier of the basic cell bacterial culture media.

Culture medium and Solutions	Application	Supplier
Dulbecco's Modified Eagle Medium (DMEM) (high glucose) with sodium bicarbonate, without L-Glutamine	Growth medium	Sigma-Aldrich
Foetal bovine serum (FBS) 500ml	Supplements	Sigma-Aldrich
L-Glutamine 200mM solution	Supplements	Sigma-Aldrich
Keratinocyte-SFM Medium	Growth medium	Fisher scientific
Supplements for keratinocyte-SFM	Supplements	Fisher scientific
DPBS (10x concentrated) liquid 500ml	Cell washing	Invitrogen
Trypsin-EDTA solution 0.25%	Cell detachment	Sigma-Aldrich
Simulated Wound Fluid (SWF)	Bacterial growth	Sigma-Aldrich
Tryptone Soy Broth (TSB)	Bacterial growth	Fisher scientific
Tryptone Soy Agar (TSA)	Bacterial growth	Fisher scientific

Table 2.1: Growth medium, supplements and basic bacterial media.

2.2 Bacterial Culture

Two strains of *Pseudomonas aeruginosa* was used in this study these were a hospital strain PS3 which had been previously isolated from the dressing of a chronic wound and a laboratory strain 10421 which has no known pathogenic markers. In addition an enteric bacteria *E.coli* C25 was also used all 3 strains were kindly provided by Prof. P.

Humphreys (Department of Biological, University of Huddersfield). Routinely the bacteria were grown on tryptone soy agar (TSA) plates and in tryptone soy broth (TSB) at 37°C in aerobic conditions. The broth and agar were made up using the manufactures instructions. In addition to this and for selected experiments both strains of *Pseudomonas aeruginosa* were also grown in simulated wound fluid or DMEM which was prepared as described below.

2.2.1 Simulated Wound Fluid (SWF)

Simulated wound fluid (SWF) was prepared using 1:1 v:v mix of fetal bovine serum (FBS) and maximum recovery diluent (MRD). MRD is a peptone saline solution routinely used as an isotonic diluent for maintaining the viability of micro-organisms during dilution procedures in a laboratory setting, it contains a low concentration (0.1% w:v) of peptone to reduce organism multiplication in addition it contains nitrogen, carbon, vitamins, and minerals with a pH of 7.0 ± 0.2 at 25°C. (Said et al., 2014).

2.2.2 Dulbecco's Modified Eagle Medium (DMEM) and *P. aeruginosa*

DMEM powder medium was prepared as per the manufacture's instruction (13.4 grams of powder per litre of ultra-pure water) of and filtered sterilised with 0.22 µm pore size sterile filters (Millipore). pH was adjusted to 7.4 using HEPES buffer.

2.2.3 Establishing *P. aeruginosa* strains

To establish the initial bacterial cutlers one bead of both strains of previously bio-banked *P aeruginosa* was removed in aseptic conditions and grown in 30 ml of TSB at 37°C in an orbital incubator (S1500, Stuart Scientific) at 120 rpm or until the solution became cloudy. This solution was then streaked on 4 TSA plates and grown overnight at 37°C it was visually established that all the colonies had the same appearance.

These plates were stored at 4°C for up to 6 months. To establish a bacterial culture 1 colony was removed from the plate and added to 30mls of TSB and incubated overnight at 37°C in an orbital shaker set at 120 rpm these conditions had previously been shown to provide a plateau phase culture.

2.2.4 Obtaining a growth curve for the strains of *P. aeruginosa*

Two sterilized flasks with 50ml of SWF or DMEM were inoculated with both strains of *P. aeruginosa* and the absorbency at 600 nM was adjusted to 0.255 au for Laboratory strain (L.) and 0.270 au for Hospital strain (PS3) at 270 nm respectively which had been determine to give a suspension of approximately 10^8 cfu/ml. 5ml of the suspension was added to 45ml of growth media in a flask and incubated in an orbital shaker at 37°C and 120 rpm absorbance at 600 nM was measured each hour for 7 hours and then at various time point up to 24, 72, 96 hours.

In addition to absorbance bacterial growth curves were established by sampling the bacteria and producing a serial dilution to give a final dilution of 10^2,10^4,10^6 these were then added to TSA plates using an automatic spiral plater, (Don Whitley Scientific, UK) in duplicate for each dilution after which the plates are incubated at 37°C overnight and then the colonises were counted using aCOLyte plate reader and accompanying software.

2.2.5 *E coli* C25

E coli C25 was obtained from bio-banked stores described for *P. aeruginosa* the bacteria was grown in TSB or on TSA plates.

2.2.6 Total carbohydrate analysis by the phenol-sulphuric acid assay

The phenol-sulphuric acid method is a simple and rapid colorimetric method to determine total carbohydrates in a sample (Dubois et al., 1956). To quantify the concentration of glucose a calibration curve was constructed using (0, 50, 100, 150, 200, 250, 300, 350, 400) mg/l. Samples were taken from the bacterial solution at the time intervals indicated in the results and then centrifuged at (4000 rpm) for 10 min, then 100µl of the supernatant was added to 500µl of 50% phenol and 2.5ml of concentrated 99.9% (v:v) sulphuric acid vortexed and incubated at 70°C for 20 min, the samples were then cooled in a water bath at 10°C for 20 min. Absorbance was measured at 490 nm and the glucose concentration calculated.

2.2.7 Determination of glucose concentration by pico-trace analysis method

To detect the glucose consumption during *Pseudomonas aeruginosa* growth a pico-trace was used (Pico-trace Analytics, Richard-Wagner-Strab, Braunschweing, Germany). 10µl samples were first centrifuged at 4000 rpm and then added to Pico-trace buffer solution and vortexed and the glucose concentration was measured.

2.2.8 Isolation of bacterial supernatant solutions

A loopful of Laboratory strain (L.) and Hospital strain (PS3) of *P. aeruginosa* were taken and streaked onto separate TSA plates. In aerobic conditions the plates were incubated in incubator (Sanyo MIR 262) at 37ºC. By using a sterile inoculation loop, colonies were taken from the TSA plates to inoculate 50ml of sterile media which was incubated overnight in a shaking incubator at 37ºC, 120rpm to grow to stationary phase. The bacteria were centrifuged at 10000 x g for 10 minutes and the supernatant was retained and then filtered through a 0.45µm syringe-driven filter and stored at -30 ºC until required.

2.2.9 Sonicated bacteria

Bacteria were grown as described in section 2.2.4 and 10 ml aliquots of stationary phase bacterial cultures were sonicated on ice, using a Vibracell VCX 130 (Sonics and Materials Inc., Newtown, CT, USA) at 85% amplitude for a 5 x 6 sec pulses with a 25 sec cooling between pulses, to prevent an excessive increase in temperature. The resulting solutions were filtered through a 0.45µm syringe-driven filter and stored at -30 ºC until required.

2.2.10 Whole Cell Extract Preparation

The 10ml of a plateau phase of bacteria were centrifuged at 10000 x g for 10 minutes the supernatant was removed and the pellet re-suspended in water. This extract is then sonicated following the method described above and stored in aliquots at -30 ºC.

2.2.11 Isolation and quantification of outer membrane vesicles isolated from *P. aeruginosa* and *E coli*

The method used for the isolation of outer membrane vesicles (OMVs) was based on that described by Kadurugamuwa & Beveridge (1995). A colony of *P. aeruginosa* both PS3 and lab strain and *E. coli* (C25) from tryptone soy agar plate was used to inoculated 30 ml aliquots of tryptone soy broth (TSB) and incubated at 37°C overnight in an orbital shaker at 120 rpm to achieve the plateau phase of the growth curve. These cultures were then added to 270ml of TSB and again grown overnight in an orbital shaker or until the plateau phase was achieved. 1ml of the bacteria culture just before the isolation of OMV was taken to determine the CFU/ml for each culture following the procedure described in section 2.1.4. The remaining culture was centrifuged at 10,000 x g (Beckman Coulter Avanti J-26 XPI centrifuge, F250 rotor) for 20 minutes to pellet the bacteria. The supernatant was collected and filtered through 0.45µm pore filter, to

remove any remaining bacteria, the filtrate was centrifuged at 40,000 x g (Beckman Coulter Optima L-100K, 50Ti rotor) at 4°C for 2 hours to pellet out the OMVs. The small pellets that were distributed in 8 tubes were suspended using 1ml of HEPES buffer per tube and pooled in 1 tube the volume was then made up to 28mls and centrifuged again at 40,000 x g at 4°C for 1.5 hours to re-pellet the OMVs. The supernatant was removed and the pellet was re-suspended in specific volumes for each strain in HEPES buffer solutions at pH 6.8, this was filtered aseptically through 0.45µm pore filter to sterilise and stored at -20°C.

2.2.12 Isolation of bacterial outer membranes

The isolation of bacterial outer membranes was carried out using a method previously described (Zhou et al, 1998). A plateau phase culture of either one of the two strains of *P. aeruginosa* or *E. coli* (C25) grown in TSB was centrifuged at 10000g for 10 mins and the pellet resuspended in 10ml phosphate buffer saline (PBS) the centrifugation step was repeated twice after which the bacterial pellet was resuspended in 10ml PBS containing 0.01M ethylenediaminetetraacetic acid (EDTA) and incubated at room temperature for 30 mins, the suspension was then placed on ice and sonicated for 10 seconds at 85% amplitude and then centrifuged at 10000g for 10 mins at 4°C. The resultant supernatant was again centrifuged at 80000g (using the Beckman Ti rotor) for 2 hours at 4°C, this produced a translucent yellow pellet that was resuspended in sterile water the two previous centrifugation steps were repeated and the pellets, which represents the outer membranes, are pooled and resuspended in a final volume of 0.5ml of sterile water the samples were stored at -20°C.

2.2.13 Lowry protein assay

A BioRad Lowry assay kit was used in a 96 well plate format using the manufactures instructions with BSA used as standards. The supplied 2X (2N) Folin-Ciocalteu reagent was diluted1:1 with ultrapure water to prepare 1X (1N) reagent and used on the same

day. 40µL of all standards and protein samples was added into a 96 well plate to which was added 200µL of the modified Lowry reagent using a multi-channel pipette. Immediately the contents of the wells were mixed on a plate mixer for 30 seconds. The microplate was covered with sealing tape and incubated at room temperature (RT) for 10 minutes. The absorbance at 750nm was then measured using a plate reader. The average absorbance value of the blanks, standards and protein samples were calculated.

2.2.14 SDS Polyacrylamide gel electrophoresis analysis of outer membrane vesicles

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is the most commonly used laboratory technique to separate proteins and to estimate their molecular weight. This was used to compare the proteins expressed in the OMVs from the bacteria grown in various culture conditions and to those obtained from the outer membrane of the bacteria. In order to run the SDS-PAGE 2ml of the OMVs or OM suspensions from the different bacteria grown in different conditions were centrifuged at 150000 x g for 1.5 hours at 5°C. The resultant pellets were resuspended in 85µl of NuPAGE® LDS sample buffer (4X) and 10µl DTT. Loading buffer was added to the purified sample at a 1:1 v: v ratio. 500µl NuPAGE LDS 4X sample buffer, 300µl water and 200µl DTT resulting in a total of 1ml the samples were incubated at 70°C for 10 minutes.

10µl of the sample along with 10µl of the Novex pre-stained protein standards were pipetted into individual wells. 1x MES buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.0) was added to the chambers of a one-dimensional, 4-12% NuPAGE Novex Bis-Tris precast protein gel in a vertical gel container system. The gel was run at 200V, 250mA for 45 minutes after which the gel was stained in Coomassie blue stain on an IKA Labortechnik KS250 basic flat shaker bed for approximately an hour. The gel was destained overnight in destain (10% acetic acid, 30% methanol, 60%

water) and viewed using the InGenius Syngene gel viewing cabinet and images were taken with the GeneSnap software.

As there were no clear bands visible in for the OMV samples, the method was modified and 30µl of sample was pipetted into individual wells along with 30µl of the Novex pre-stained protein standard. The gel was stained for approximately 1 hour and destained for 3 days.

2.2.15 Transmission electron microscopy of outer membrane vesicles

Transmission electron microscopy (TEM) of the OMVs was carried out in collaboration with Dr Dan Patten at the University of Birmingham UK. OMVs were isolated as described in section 2.1.11. The OMVs from a 300ml overnight plateau phase bacterial growth in TSB was resuspended in 1ml of 50mM HEPES buffer (pH 6.8). The vesicles were put on carbon films that were placed on a 400 copper mesh grid (Agar Scientific) and left for 1 minute at room temperature. The grids were then negatively stained with 1% aqueous uranyl acetate for 1 min at room temperature. The grids were then mounted on the viewing platform of on a 1200EX transmission electron microscope (LoJeol) and images of the OMVs were taken.

2.3 Cell culture

The culture of the mammalian keratinocytes used in this study was performed aseptically within a HEPA filtration microbiological safety class II cabinet (CellGarda manufactured by NUAIRE, Triple Red Technologies). To avoid contamination, working areas of the cabinet were cleaned before and after use with 70% ethanol (w/v). For a monthly routine sterilisation, the hood was disinfected by using Mikrozid® (Gompel Healthcare). Contaminated solutions and unwanted cells were treated with 10% (w/v) virkon for at least 30 mins before being disposed of via the normal waste drain. For

centrifugation of the cells Hettich Zentrifugen Universal 320 bench top centrifuge for pelleting of cells they were routinely spun for 5 mins at 1200rpm.

2.3.1 HaCaT Cells

The immortalized human skin keratinocyte cell line HaCaT was obtained from Cell Line Services (CIS) and used between passages 49-61. These cells were in grown in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (4500 mg/l) supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine at 37 C in 5% CO_2 and 100% humidity.

2.3.2 Sub Culturing Cells

Cells were sub cultured when they reached 80-95% confluence. The media was poured off the cells and they were washed with 10 ml of EDTA 0.1% (w:v) solution in HBSS without of calcium and magnesium to remove any remaining serum from the cells and dissociated the tight junction between the cells, allowing the trypsin to access the cell adherence sites and break the bonds with the surface of the flasks. After the cells were washed, 2 ml of 0.05% trypsin/ EDTA solution were added to the flasks. The cells were then incubated with trypsin solution at 37 C for 5-10 minutes until all the cells were detached, fresh media was added to inactivate the trypsin. The HaCaT suspension produced was pipetted a number of times to disperse the cells. The cell suspension was transferred to a new T75 culture flask containing 12 ml fresh medium a split ratio 1:4 was used routinely for continuation cultures. The cells were also seeded on specific plastic-ware for experiments as indicated.

2.3.3 HaCaT adaptation to serum-free medium

A sequential adaptation methodology was followed to switch the culture conditions of HaCaT cells from a serum-supplemented to a serum-free, low calcium medium (KSFM). This involved culture and passaging whilst gradually reducing the proportion of standard culture medium DMEM/10% FBS (DMEM complete, DMEMc) and replacing it with KSFM/EGF/BPE (KSFM complete, KSFMc) over a period of six passages. To do this the following media ratios changes were used passage 1 (P1) from DMEMc to 3:1 (v/v) DMEMc: KSFMc, (P2) change to 1:1 (v/v) DMEMc: KSFMc (P3) change to 1:3 (v/v) DMEMc: KSFMc (P4) medium-change to 1:9 (v/v) DMEMc: KSFMc (P4) and (P5), final medium-change to KSFMc and subsequent passage (P6). After this, HaCaT cells had fully adapted to the new culture medium and were named HaCaTa.

2.3.4 HaCaTa cells

HaCaT cell were adapted to culture conditions designed for NHKs (Keratinocyte serum free medium KSFM) (see section 2.2.3) in order to render them more representative of normal cells, this newly adapted cell line was named HaCaTa. HaCaTa were kindly provided by Dr Nikolaos T. Georgopoulos (Department of Biological Sciences, University of Huddersfield).

HaCaTa cell were cultured in Keratinocyte serum free medium (KSFM) supplemented with epidermal growth factor (EGF) and bovine pituitary extract (BPE) as recommended by the manufacturer.

2.3.5 Normal Human keratinocytes (NHKs)

Normal Human keratinocytes (NHKs) were kindly provided by Dr Nikolaos T. Georgopoulos (Department of Biological Sciences, University of Huddersfield) (and were originally isolated from human obtained from various surgical procedures). NHKs were cultured in keratinocyte serum free medium (KSFM) supplemented with epidermal

growth factor (EGF) and bovine pituitary extract (BPE) as recommended by the manufacturer. NHKs were used at passages 1–6 to ensure maximal proliferative capacity. NHKs were seeded into 96-well tissue culture plates at a density of 7.5×10^3 cells per well. For routine maintenance and experiments, NHKs were cultured in Primaria™ (Scientific Laboratory Supplies) or Cell Plus (Sarstedt) plasticware, whereas original and adapted HaCaT cells were maintained in standard plasticware (Sarstedt).

2.3.6 Effects of extracellular calcium on the growth and differentiation of normal human keratinocytes

NHK's were cultured in keratinocyte serum free media (KSFM) already containing 0.09 mM calcium, supplemented with 25 µg / ml Bovine Pituitary Extract (BPE) and 1.5 $ng.ml^{-1}$ of recombinant epidermal growth factor (EGF). Cultures were maintained in a humidified 5% CO_2 atmosphere at 37°C. For all experiments, cells were seeded at a density of $3x\ 10^4$ $cells.cm^2$. Cells were allowed to adhere for 24 h in LC KSFM (0.09 mM calcium) prior to exposure to high calcium (HC) KSFM supplemented with 1.2 mM calcium, for 24 hours.

2.3.7 Seeding of cells for bacterial challenged experiments

HaCaT or HaCaTa were seeded into 96-well tissue culture plates at a density of 5×10^3 cells per well, NHKs (both low and high calcium) were seeded at 7.5×10^3 cells per well optimal density for each cell type was determined by pre-titration experiments. All 3 types of keratinocytes were grown in standard tissue culture conditions before the experiments.

For IL-8 analysis HaCaT and HaCaTa were seeded at 1.5×10^5 cell/well and NHKs (both low and high calcium) were seeded at 7.5×10^3 cells per well in 6 well plates and grown in standard tissue culture conditions for 3 days until they became confluent during this

time the media was changed every once. After this the cells were challenged with various bacterial products as described below.

2.3.8 Cryopreservation of HaCaT, HaCaTa and NHK cells

For cryopreservation of the cells they were first trypsinized as described in section 2.2.2 above and then centrifuged at 1200 rpm for 5 minutes the supernatant removed and the cell pellet resuspended in freeze media (80% normal complete media (for the specific cell line) supplemented with 10% FBS and 10% dimethylsulphoxide (DMSO). Cells were aliquoted into 1ml storage cryovials and transferred to a 'Mr. Frosty'™ supplied by Thermo Scientific this contains ethanol and provides controlled and gradually cooling when placed at -80°C overnight after this the vials were transferred to a liquid nitrogen at -176 °C for long term storage.

2.3.9 RNA isolation

RNA was isolated using an isolate ll RNA mini kit (Bioline) following the manufactures instructions. Each kit contains: filter columns, collection tubes (2ml and 1.5ml), lysis buffer, wash buffer, membrane desalting buffer MEM (to reduce DNase I activity), and reaction buffer for DNase I, DNase, RNase-free water. The cells were cultured in a T75 flask the supernatant was removed and the cells were lysed with the addition of 350µl lysis buffer with 3.5µl of β-mercaptolethanol the cell lysates were stored at -80°C until required.

After defrosting the lysates were loaded on the filter which was placed inside a 2ml collection tube and spun at 11000xg for 1 min. 350µl of 70% ethanol was added to the filter and mixed by pipetting up and down several times. The homogenized cell lysate was loaded on the filter column and centrifuged at 11000xg for 30s. 350µl of membrane desalting buffer (MEM) was added to the column and centrifuge at 11000xg for 1min. 10µl of DNase I was added to 90µl of reaction buffer and 95µl of this DNase I reaction

mixture was added to the centre of the silica membrane and incubated at room temperature for 15 min. After which 200µl of wash buffer was added to the column and centrifuged at 11000xg for 30s 2 further wash steps were carried out and then 60µl of RNase-free water was added to the column and centrifuged at 11000xg for 1 min. The filtrate, containing RNA was then collected and the UV absorbance at 260nm was measured using a Nanodrop (manufactory etc) to determine the concentration of RNA which was stored in aliquots at -80°C until required.

2.3.10 cDNA production by reverse transcription

mRNA was converted to cDNA by reverse transcription (RT) reaction using the SensiFAST cDNA synthesis kit from (Bioline,UK) following the manufactures instruction. Up to 1µg of RNA was added to 4µl of 5x trans amplification buffer and 1µl reverse transcriptase the volume was made up to 20µl with DNase/RNase free water. The solution was incubated in a Techne TC-3000 Thermocycle, using the following protocol: 25°C for 10 min, 42°C for 15 min, 85°C for 5 min and the reaction was held at 4°C. The cDNA was stored at -80°C.

2.3.11 Quantification of gene expression

Quantitative PCR (qRT-PCR) was used to quantify the relative abundance of TLR-1, TLR-2, TLR-4, TLR-5, TLR-9 and actin. qRT-PCR was performed on the 7500 Fast Real time PCR system (Life Technologies). Probes and primers were designed using the Roche ProbeFinder software (Roche Diagnostics Ltd). Table 2.1 shows the sequences of the primers and the Universal probe number used to quantify the expression of the genes in this study. The probes were obtained from Roche Products Limited and primers from MWG Eurofins. qRT-PCR reaction was carried out in final volume of 20µl with the following reaction mix: 0.5µl of 20µM primers, 0.5µl universal probelibrary probe, and 4µl of 5 x lightcycler® Taqman®Master mix, 0.5µl of 8.95µg/ml cDNA and 13 µLPCR-grade water. All PCR assay were performed in triplicate using the following incubation steps: activation at 95C° for 10 min, followed by cycles of 95°C for

10s, 60°C for 30s and 72°C for 1s this was repeated for 44 cycles after which the reaction was stopped with a final cooling step at 40°C for 30 s.

Gene	Forward Primer sequence	Reverse Primer sequence	Universal Probe No
Actin	ccaaccgcgagaagatga	tccatcacgatgccagtg	64
TLR1	aaacaacattgaaacaacttggaa	cacgtttgaaattgagaaatacca	64
TLR2	ctctcggtgtcggaatgtc	aggatcagcaggaacagagc	56
TLR4	gaaggttcccagaaaagaatgtt	cctgattgtccttttcttgaatg	75
TLR5	ctccacagtcaccaaaccag	cctgtgtattgatgggcaaa	72
TLR9	tgtgaagcatccttccctgta	gagagacagcgggtgcag	56

Table 2.2: Primers and Universal probes used for the genes indicated in qRT-PCR.

2.3 Bacterial products challenge experiments

2.3.1 Keratinocytes challenge cell free bacterial conditioned media

In order to challenge HaCaT cell with the soluble factors secreted from the bacterial strains they were grown for 24 hours in 30ml of growth media either DMEM or TSB after this the solution was centrifuged at 1200 RPM for 5 minutes to remove the bacteria. The supernatant was then filtered using 0.4µm pore size aseptically the solutions were aliquoted and stored at -20°C before being used to challenge of the cells.

HaCaT cells were grown in 6 well plates until became confluent, the media was removed and replace with 1 ml of bacteria conditioned TSB or DMEM diluted in complete tissue culture media as indicated or the corresponding non-conditioned bacterial growth media. The cells were incubated 4 hours at 37°C after this the challenge solution was removed and then fresh tissue culture media was added and the

cells were returned to the cell culture incubator for 24 hours after which the supernatant was removed and stored at -20°C for subsequent IL-8 analysis. After the supernatant was removed the biomass of the cells was routinely measured using the CellTiter 96® AQueous One Solution as described in section 2.2.7.

2.3.2 Cell challenge with OMVs and OM

Cells were seeded at $1.5x10^5$ cells/well in 6 well plates and grown in standard tissue culture conditions until they became confluent during this time the media was changed every 2nd day. After this the cells were challenged with 1ml of several concentrations of OMV and OM in tissue culture media derived from both strains of *P. aeruginosa* for 4 hours. The solution was then removed and 1ml of tissue culture media was added to each well and incubated over night at 37°C. The culture media was then collected into several Eppendorf tubes and stored at (-20°C) for subsequent IL-8 analysis. Micrographs of the cells were obtained to allow a visual representation of their viability cell viability was also quantified using CellTiter 96® AQueous One Solution as described in section 2.3.3.

2.3.3. Quantification of cell biomass

Quantification of cell viability was performed via the determination of cell biomass using the CellTiter 96® AQ_{ueous} One Solution Cell proliferation assay (Promega UK). This assay involves the use of the MTS tetrazolium ion which is yellow and is reduced to a brown formazan derivative by cellular respiration and this colour change is proportional to the number of viable cells. To perform the cells were plated into 96 well plates at a density of 5×10^3 cells per well incubated overnight and then challenged with various bacterial culture supernatants or outer membrane vesicles (OMV's) as indicated in the results. After this 20µl of CellTiter 96® AQ_{ueous} One Solution was added to each well and the plate was incubated at 37°C in 5% CO_2 for 4 hours. The concentration of formazan formation/cell viability was quantified by measuring absorbance at 492nm on

a FLUOstar OPTIMA plate reader (BMG Labtech) the data was processed using MARS software (BMG Labtech). Cell viability was calculated as percentage viability in comparison to controls using equation 1:

$$\text{Cell viability (\%)} = T/C \times 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

T= treated cells and C= controls cells.

In addition to Cell Titer assay, cell viability was also monitored by inverted contrast microscopy and phase contrast images were obtained using an EVOS XL core microscope (life Technologies) at 100x magnification.

2.3.4 IL-8 Quantification

IL-8 quantification was carried out using the DuoSet® ELISA development system Human CXCL8/IL-8 kit (DY008) provided by R&D Systems, following the protocol supplied. The capture antibody was diluted to the working concentration in PBS only and 100µl per well was added to a 96-wellplate which was then sealed with sealing film and incubated overnight at room temperature. The wells were then washed with 310µl of diluted wash buffer this was then removed by inverting the plate and blotting it against clean paper towels this step was repeated 3 times. 300µl of blocking buffer per well was added and the plate was incubated for one hour after this the wash step as reported above was repeated. The standards were prepared by serial dilution of the IL-8 stock solution in reagent diluent to give the following concentrations: 1000, 500, 250, 125, 62.5 and 31.3 pg/ml. 100µl of these were added to the well in duplicate. Samples (which were diluted if required) were added to the remaining wells. The plate was then covered and incubated for 2 hours at room temperature after which the wash step was repeated again. The detection antibody was prepared by dilution in the regent diluent and 100µl of this was added per well. The plate was covered with a new adhesive strip and incubated for 2 hours at room temperature. After this the wash step was repeated

as before and 100µl of a 1:40 dilution of streptavidin-HRP solution was added to each well the plate was covered and incubated for 20 minutes away from light. The wash was repeated and then 100µl of the substrate solution was added to each well and the plate incubated at room temperature away from direct light for 20 minutes. 50µl of stop solution was added to each well, the plate was taped gently to mix the contents and a 96 well plate reader (FULOstar OPTIMA) (BMG labtech) was used to measure the optical density at 540nm.

Statistical Analysis

By using Microsoft Excel the results were collected and analysed. All data were presented as mean values ± SEM. Statistical analysis was performed by using statistical software (Minitab 17). P-value was generated by using two tailed paired student t-test.

Chapter 3

The Results

Section 1

3.1.1 HaCaT, HaCaTa and NHK cell lines

Three cells lines were used to study the interactions of bacterial products with keratinocytes. Micrograph images were routinly taken to check that they grow with a consitent phenotype and repersientiative images are presented here.

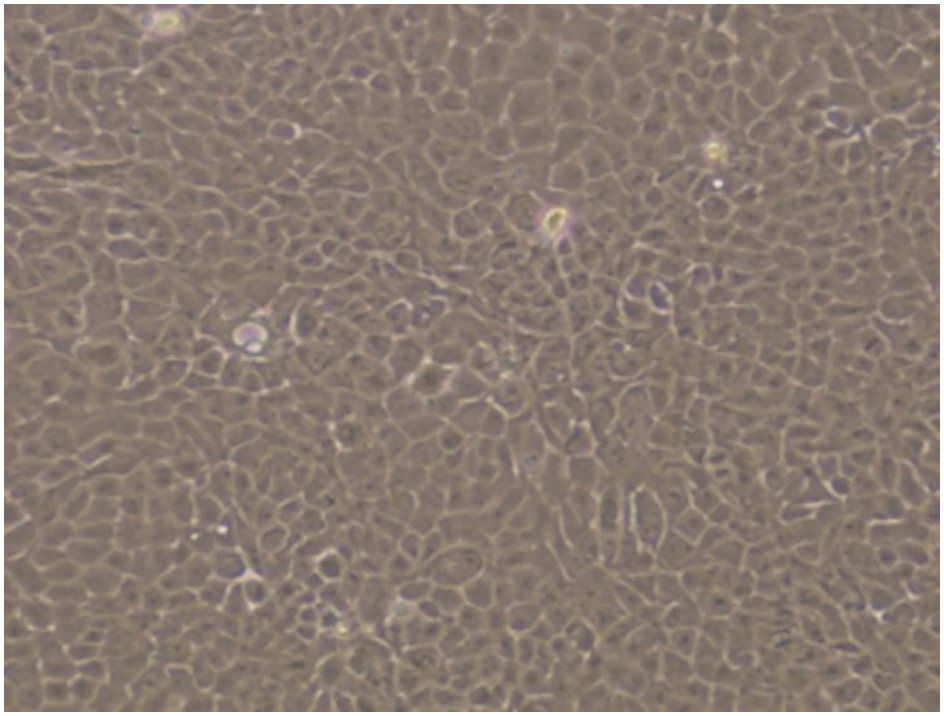

Figure 3.1.1: HaCaT cells cultured in complete media DMEM to demonstrate their normal growth pattern

Classic morphology of HaCaT cells can be seen. Image is representive phase contrast micrographs (taken at 100x magnification.

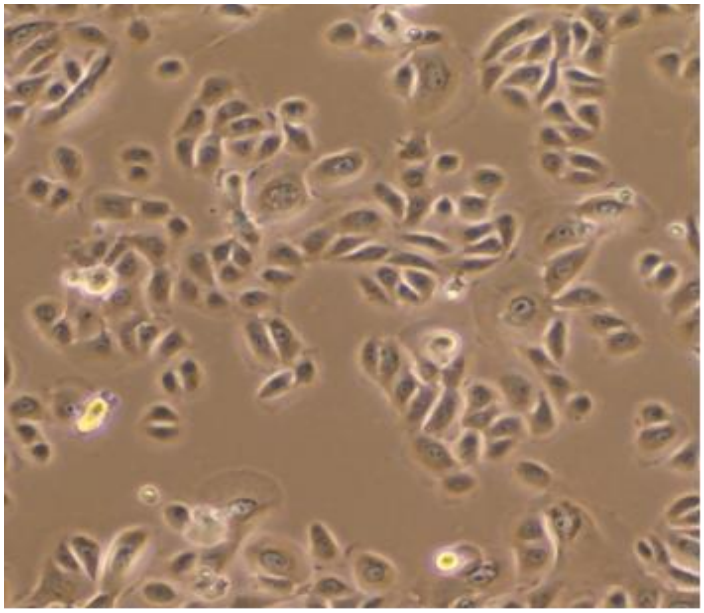

Figure 3.1.2: A representative image of Normal Human Keratinocyte

Image is representative phase contrast micrograph (taken at 100x magnification).

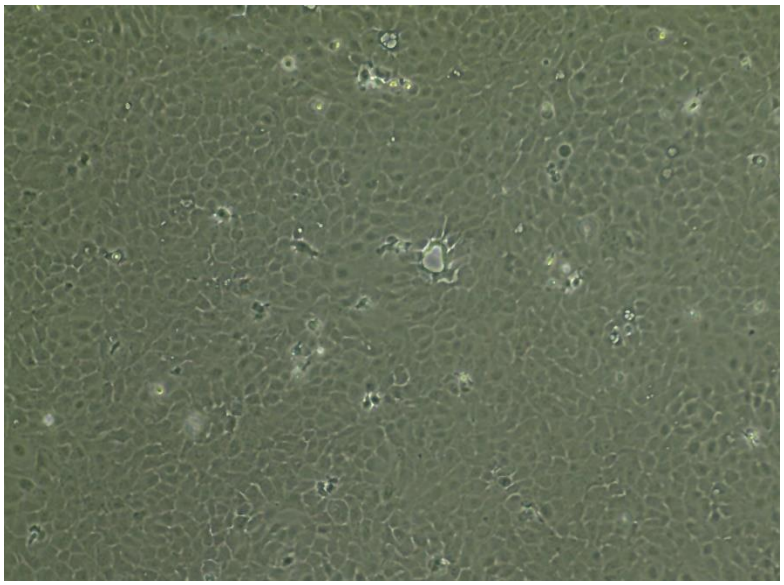

Figure 3.1.3: A representative image of HaCaTa.

Image is a representative phase contrast micrograph (taken at 100x magnification).

3.1.2 NHK in low and high Calcium

In order to study the effect of differentiation of the NHK cells on their response to bacterial secreted products they were grown in different concentrations of calcium the effect of this on their basic morphology are presented here.

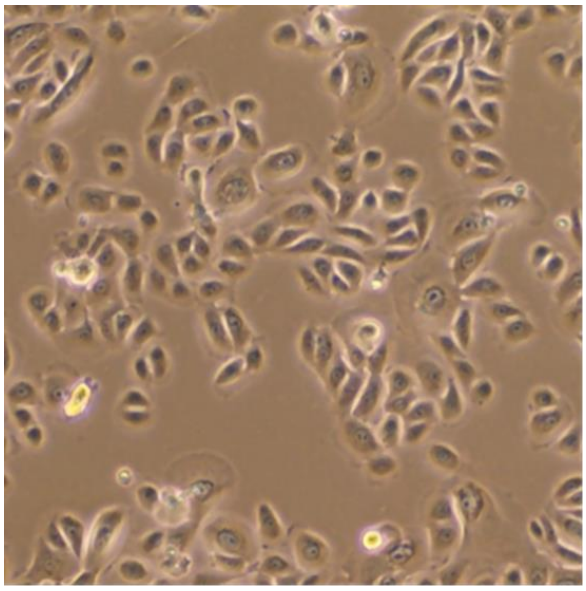

Figure 3.1.4: Human Keratinocyte control calcium.

Image is represented phase contrast micrographs (taken at 100x magnification).

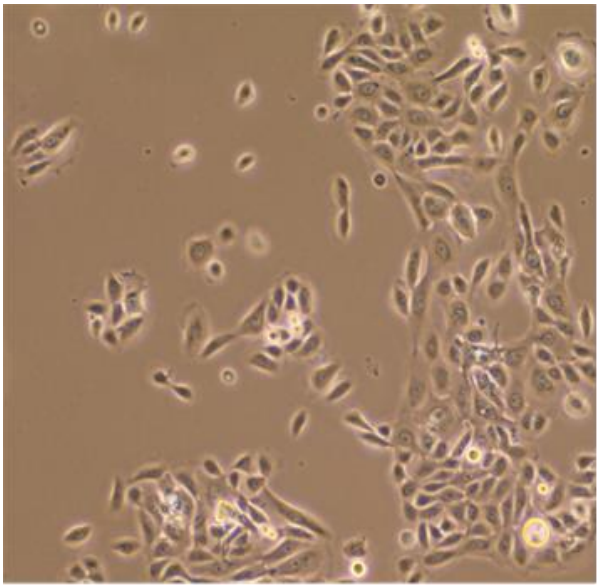

Figure 3.1.5: A representative image of Normal Human Keratinocyte after exposure to low concentration of calcium medium (LC) after three days.

Morphological changed can be seen as a result of calcium addition, cells became flat and spread out, and a steady increase in cell growth was observed. Image is representative phase contrast micrographs (taken at 100x magnification).

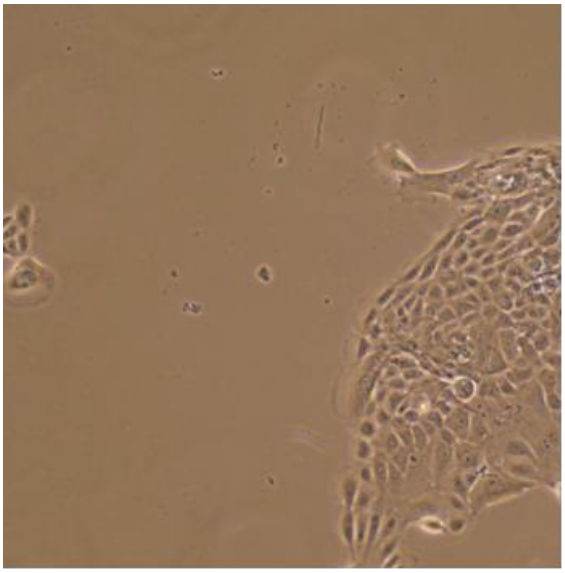

Figure 3.1.6: A representative image of Normal Human Keratinocyte after exposure to high concentration of calcium (HC) after three days.

Morphological changed can be seen as a result of calcium addition, cells became small and cobble-stoned and cell growth was slower than LC medium. Image is representive phase contrast micrographs (taken at 100x magnification).

3.1.4 Expression Pattern Recognition Receptors in HaCaT cells

In order to understand if the HaCaT cells express a range of Pattern Recognition Receptors when grown in culture a series of quantitative PCR experiments were performed. These are the mammalian receptors that respond to a series of microbial-associated molecular pattern molecules. HaCaT cells were grown for either 4 or 10 days in standard culture conditions before the RNA was extracted.

	10 Days		**4 Days**	
Gene	CT1	CT2	CT1	CT2
Actin	18.862	-	18.7644	-
TLR1	29.824	10.9617	27.928	9.1637
TLR2	24.853	5.9909	25.8853	7.1209
TLR4	28.012	9.1498	24.8197	6.0553
TLR5	24.852	5.9898	25.3899	6.6255
TLR9	28.062	9.2005	32.3793	13.6149

Table 3.1. 1: Gene expression HaCaT cells grown for 4 days, 10 days in culture.

CT: (Threshold cycle) the cycle number at which the fluorescence signal crosses threshold. Also called cp (cross point cycle) for light cycle terminology. Gene amplification represented relative amount of mRNA for that gene. ΔCT: value of CT of the Gene - value of CT for housekeeping (Actin) Gene. Representative standard amplification curves are shown in the appendix

The expression of the house keeping gene actin is the same in both age of cells and TLR9 has more expression in day 4 however, TLR1 and TLR4 had greater expression in days 10 cells.

Overall, with the exception of TLR9, there was generally high expression in the older cell. Expression, at the mRNA level, was observed for all the TLRs although relatively little TLR9 mRNA seems to be present.

Section 2

3.2.1 TEM image of outer membrane vesicles (OMVs) isolated from bacteria

Outer membrane vesicles were obtained from both strains of *Pseudomonas aeruginosa* using standard isolation techniques as described in the methods. Following this samples were processed for TEM and the following images where obtained.

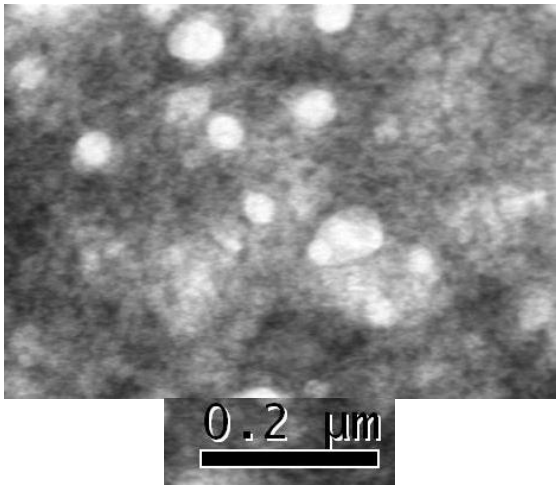

Figure 3.2.1: TEM image of outer membrane vesicles (OMVs) isolated from Pseudomonas aeruginosa Hospital strain (PS3).

The vesicles can be seen as white 30 -50 nm sized spheres**.**

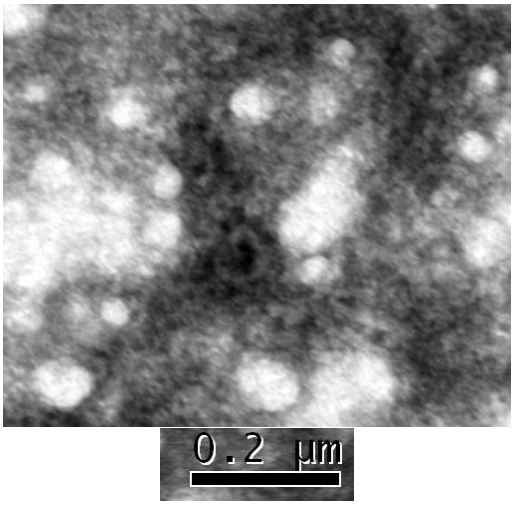

Figure 3.2.2: TEM image of outer membrane vesicles (OMVs) isolated from Pseudomonas aeruginosa Laboratory strain (L.).

The vesicles can be seen as white 30 -50 nm sized spheres.

As a comparison and to verify the isolation of OMVs from *Pseudomonas aeruginosa* a similar isolation was performed on *E coli* C25 and TEM images were obtained although isolated at a lower density similar images of vesicles were obtained.

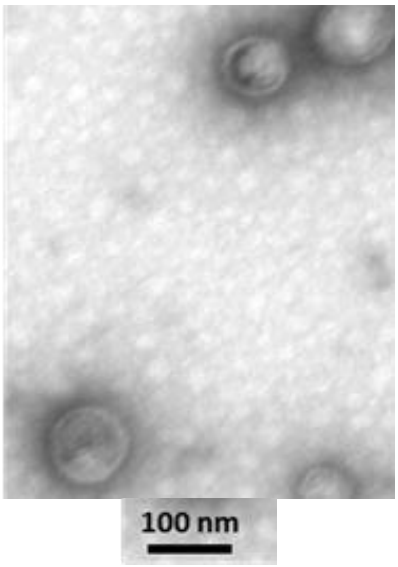

Figure 3.2.3: Image illustrates Outer Membrane Vesicles OMVs of E. Coli (C25).

The vesicles can be seen as white 30 -70 nm sized spheres.

3.2.2 Protein assay for OMVs isolated from *Pseudomonas* bacterial strains

In order to quantify the OMV obtained from each preparation a Lowry assay was performed to measure the concentration of protein associated with the OMVs obtained following each preparation performed on the 2 strains of *Pseudomonas* and different growth conditions used in this study.

Samples	Protein concentration (µg/ml)	Total Protein (µg)
OMVs of Hospital strain (PS3) without gentamicin in TSB	7.95	3.62
OMVs of Hospital strain (PS3) with gentamicin in TSB	8.10	6.68
OMVs of Laboratory strain (L.) without gentamicin in TSB	8.00	0.289
OMVs of Laboratory strain (L.) with gentamicin in TSB	18.00	0.318

Table 3.2. 1: Protein concentration for OMVs isolated from *P. aeruginosa* strains in TSB using a Lowry assay (Preparation 1).

In order to calculate the relative yield of OMVs from the 2 strains of *Pseudomonas* and different growth conditions were used in this study, the number of bacteria in the overnight culture used to isolate the vesicles from was calculated. This was then used to normalise the protein content of the OMVs in µg per 10^8 bacteria from the culture that the OMV were isolated from the results for preparation 1 is shown below.

Samples	CFU/ml× Vol. per ml centrifuged at first high speed rotation = overall CFU in bacteria sample	Total protein in OMV (µg/ml)	Protein (µg) in CFU bacteria10^8
OMVs of Hospital strain (PS3) without gentamicin in TSB	$(1.01 \times 10^7) \times 50 = 5.05 \times 10^8$	7.95 x 2.3 =18.28	18.282/5.05=3.62
OMVs of Hospital strain (PS3) with gentamicin in TSB	$(6.3 \times 10^6) \times 50 = 3.15 \times 10^8$	8.1 x 2.6 =21.06	21.06/3.15=6.68
OMVs of Laboratory strain (L.) without gentamicin in TSB	$(1.16 \times 10^7) \times 100 = 11.6 \times 10^8$	8.0 x 0.42 =3.36	3.36/11.6=0.29
OMVs of Laboratory strain (L.) with gentamicin in TSB	$(1.58 \times 10^7) \times 100 = 15.8 \times 10^8$	18.0 x 0.28 =5.04	5.04/15.8=0.32

Table 3.2. 2: OMVs purified from *P. aeruginosa* strains in TSB and the overall protein concentration in each sample (preparation 1).

In addition, the CFU/ml of bacteria that was used to isolate the OMVs is indicated.

The Hospital strain PS3 produced over 10 fold more OMVs compared to the lab strain, as assessed by protein concentration (table 3.2.2). It also showed that addition of gentamicin 30 minutes before isolation of OMV increased the amount of vesicles produced in both strains but this increase was much greater in the Hospital strain.

Samples	Protein concentration (µg/ml)	Total protein (µg)
OMVs of Hospital strain (PS3) in TSB	49.6 (4ml)	24.5
OMVs of Laboratory strain (L.) without gentamicin in TSB	37.0 (5ml)	19.6
Laboratory strain (L.) with gentamicin in TSB	56.0 (2ml)	298.7

Table 3.2. 3: Protein concentration for OMVs isolated from P. aeruginosa strains in TSB using a Lowry assay (Preparation 2).

In order to compare the total yield of OMVs from the different strains of bacteria the total amount of protein in the OMVs from the Hospital PS3 and Laboratory L. strains of *P. aeruginosa* was normalised to the CFU of the bacteria in the culture that the vesicles were isolated from using the calculation outlined below in (Table 3).

In addition, the effect on the yield of OMVs of adding gentamycin 30 mins before purification in the laboratory strain L. was assessed.

The total volume for the OMVs concentration was calculated as it stated in the table below (Table 3). First of all, the concentration of OMVs for Hospital strain (OMVs PS3) was (24.5µg per 10^{11} of bacteria) where the total volume of protein for laboratory strain (OMVs L.) was (19.6µg per 10^{11} of bacteria), the protein concentration in the laboratory strain with the presence of Gentamicin (gOMVs L.) was (298.7µg per 10^{11} of bacteria), and that was the highest concentration from all *P. aeruginosa* strains.

Samples	CFU/ml× Vol. per ml centrifuged at first high speed rotation=overall CFU in bacteria sample	Total protein in OMV (µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Hospital strain (PS3) in TSB	$(2.7 \times 10^9) \times 300 = 8.1 \times 10^{11}$	49.6×4 =198.4	198.4 /8.1= 24.49
OMVs of Laboratory strain (L.) without gentamicin in TSB	$(2.7 \times 10^9) \times 350 = 9.45 \times 10^{11}$	37 ×5= 185	185/9.45=19.58
gOMVs of Laboratory strain (L.) with gentamicin in TSB	$(1.5 \times 10^8) \times 250 = 0.375 \times 10^{11}$	56×2 = 112	112/0.375=298.66

Table 3.2. 4: OMVs purified from P. aeruginosa strains in TSB and the overall protein concentration in each sample (preparation 2).

The data above represents that the concentration of protein was different in the Hospital (PS3) and Laboratory strains (L.) and Laboratory strain (L.) treated with gentamicin had the highest in protein concentration followed by Hospital strain (PS3) and untreated Laboratory strain (L.). This was taken as an indication of a greater production of OMVs. The preparation was repeated and the data is indicated in the table.

Samples	Protein concentration (µg/ml)	Total protein (µg)
OMVs of Hospital strain (PS3) in TSB	35.2 (2ml)	70.4
OMVs of Laboratory strain (L.) in TSB	49.0 (2ml)	98.0

Table 3.2. 5: Protein concentration for OMVs isolated from *P. aeruginosa* strain in TSB using a Lowry assay (Preparation 3).

Samples	CFU/mlx Vol. Per ml centrifuged at first high speed rotation=overall CFU in bacterial sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV (µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Hospital (PS3) Strain in TSB	(2.79x 10^{9}) x300 =8.37x10^{11}	35.2	70.4	8.4
OMVs of Laboratory (L.)Strain in TSB	(2.65x10^{9}) x300 =7.95x10^{11}	49.0	98.0	12.3

Table 3.2. 6: The data of the OMVs purified from *P. aeruginosa* strains in TSB and overall protein concentration in each sample has been stated with CFU/ml of both strains which used for (preparation 3) OMVs Isolation.

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg)
OMVs of Hospital (PS3)strain in TSB	32.3 (3.5ml)	113.1
OMVs of Laboratory strain (L.) in TSB	22.4 (3.5ml)	78.4

Table 3.2. 7: Protein concentration for OMVs isolated from *P. aeruginosa* strains in TSB using a Lowry assay (Preparation 4).

Samples	CFU/ml x Vol. Per ml centrifuged at first high speed rotation=overall CFU in bacterial sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV (µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Hospital (PS3) Strain in TSB	$(2.38x\ 10^{9})$ x300 $=7.14x10^{11}$	32.3	113.1	15.8
OMVs of Laboratory Strain(L.) in TSB	$(2.06x10^{9})$ x300 $=6.18x10^{11}=$	22.4	78.4	12.7

Table 3.2. 8: Shows the data of the OMVs purified from *P. aeruginosa* strains in TSB and overall protein concentration in each sample has been stated with CFU/ml of both strains which used for (preparation 4) OMVs Isolation.

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg)
OMVs of Laboratory strain (L.) strain in SWF	535	1605
OMVs of Laboratory strain (L.) in SWF+Ethanol	650	1950

Table 3.2. 9: Protein concentration for OMVs isolated from *P. aeruginosa* strains in TSB using a Lowry assay (Preparation 5).

Samples	CFU/ml x Vol. Per ml centrifuged at first high speed rotation=overall CFU in bacterial sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV(µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Laboratory strain (L.) in SWF	(3.17 x 10^9) x300 =9.5x10^{11}	535	535x3=1605	1605/9.5=169
OMVs of Laboratory strain in SWF+Ethanol	(2.18 x10^9) x300 =6.54x10^{11}=	650	650x3=1950	1950/6.54=298

Table 3.2.10: The data of the OMVs purified from *P. aeruginosa* Laboratory strain (L.) in SWF and SWF + Ethanol overall protein concentration in each sample has been stated with CFU/ml of Laboratory strain (L.) which used for OMVs Isolation (Preparation 5).

The overall of protein concentration in OMVs samples µg/ml can be used to calculate the total OMVs protein per 10^{11} CFU. In the table above the colony count data (CFU/ml) was obtained during the OMVs isolation process as shown in in the first column of (Table 3.2.10). This number of bacteria was multiplied by the total volume of the culture

media used and gives the total CFU/in 300ml culture. The total protein in OMVs (µg/ml) was the amount of HEPES buffer that was added to the OMVs pellet when isolated from *P. aeruginosa* multiplied by the protein concentration (µg/ml) of the total protein. Concentration was obtained by multiplying the total protein in OMV (µg/ml) by the CFU/ml of total bacteria. Finally, the results show that the bacteria which were grown in SWF with ethanol produced OMVs more than the bacteria grown in only SWF.

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg).
OMVs of Laboratory strain (L.) in TSB	191.66	1916.6
OMVs of Laboratory strain (L.) in SWF	543.75	5437.5

Table 3.2.20: Protein concentration for OMVs isolated from *P. aeruginosa* strain in TSB using a Lowry assay (Preparation 6).

Samples	CFU/ml x Vol. Per ml centrifuged at first high speed rotation=overall CFU in bacterial sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV(µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Laboratory strain (L.) in TSB	$(3.5 \times 10^9) \times 300 = 10.5 \times 10^{11}$	1916.6	1916.6 x 0.2 = 383.32	36.506
OMVs of Laboratory strain (L.) in SWF	(2.9×10^9) X 300 = 8.7×10^{11}	5437.5	5437.5x 0.5=	312.5

Table 3.2.21: The data of the OMVs purified from *P. aeruginosa* Laboratory strain (L.) in TSB and SWF overall protein concentration in each sample has been stated with CFU/ml of Laboratory strain (L.) which used for OMVs Isolation (Preparation 6).

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg)
OMVs of Laboratory strain (L.) in TSB	160	1600
OMVs of Laboratory strain (L.) in SWF	382	76400

Table 3.2.22: Protein concentration for OMVs isolated from *P. aeruginosa* Laboratory strain (L.) in TSB and SWF using a Lowry assay (Preparation 7)

Samples	CFU/ml x Vol. per ml centrifuged at 1st high speed rotation- Overall CFU in bacteria sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV(µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Laboratory strain (L.) in TSB	$(3.3 \times 10^9) \times 300 = 9.9 \times 10^{11}$	1600	1600 x0.2 =320	320/9.9=32.3
OMVs of Laboratory strain (L.) in SWF	$(8.6 \times 10^9) \times 300 = 25.8 \times 10^{11}$	3820	76400 x 0.5=	305600/2.58=11844.9

Table 3.2.23: Shows the data of the OMVs purified from *P. aeruginosa* Laboratory strain (L.) in TSB and SWF overall protein concentration in each sample has been stated with CFU/ml of Laboratory strain (L.) which used for OMVs Isolation (Preparation 7).

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg)
OMVs of Hospital strain (PS3) in SWF	44.2	884
OMVs of Laboratory strain (L.) in SWF	49.4	988

Table 3.2. 24: Protein concentration for OMVs isolated from *P. aeruginosa* Hospital strain (PS3) and Laboratory strain (L.) in SWF using a Lowry assay (Preparation 8).

Samples	CFU/ml x Vol. per ml centrifuged at 1st high speed rotation-Overall CFU in bacteria sample	Protein concentration (µg/ml) in OMV samples	Total protein in OMV(µg/ml)	Protein (µg) in CFU bacteria10^{11}
OMVs of Hospital strain (PS3) in SWF	$(2.59 \times 10^{9}) \times 300 = 7.8 \times 10^{11}$	44.2	44.2x20=884	884/7.8=113.33
OMVs of Laboratory strain (L.) in SWF	$(2.7 \times 10^{9}) \times 300 = 8.1 \times 10^{11}$	49.4	49.4x20=998	998/8.1=121.97

Table 3.2. 25: The OMVs purified from *P. aeruginosa* Hospital strain (PS3) and Laboratory strain (L.) in SWF overall protein concentration in each sample has been stated with CFU/ml of Laboratory strain (L.) which used for OMVs Isolation (Preparation 8).

3.2.3 Preparation of Outer Membrane (OM)

Samples	Protein concentration (µg/ml) in samples	Total protein in samples (µg)
OM of Hospital strain (PS3) in SWF	14.3	71.5
OM of Laboratory strain (L.) in SWF	11.4	57

Table 3.2.26: Protein concentration for OM isolated from *P. aeruginosa* Laboratory strain (L.) and Hospital strain (PS3) in SWF using a Lowry assay (Preparation 1).

Samples	CFU/ml x Vol. per ml centrifuged at 1st high speed rotation-Overall CFU in bacteria sample	Protein concentration (µg/ml) in OM samples	Total protein in OM (µg/ml)	Protein (µg) in CFU bacteria10^{11}
OM of Hospital strain (PS3) in SWF	$(2.32 \times 10^9) \times 300 = 7.0 \times 10^{11}$	14.3	71.5	71.5/7.0=10.21
OM of Laboratory strain (L.) in SWF	$(2.10 \times 10^9) \times 300 = 6.3 \times 10^{11}$	11.4	57	57/6.3=9.04

Table 3.2.27: The OM purified from *P. aeruginosa* Laboratory strain (L.) and Hospital strain (PS3) in SWF overall protein concentration in each sample has been stated with CFU/ml of both strains which used for OM Isolation (Preparation 1).

3.2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis results

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate and determine the molecular weight of proteins expressed in the samples of *P. aeruginosa*.

Gel image of OMV proteins derived from *P. aeruginosa* (Preparation 5):

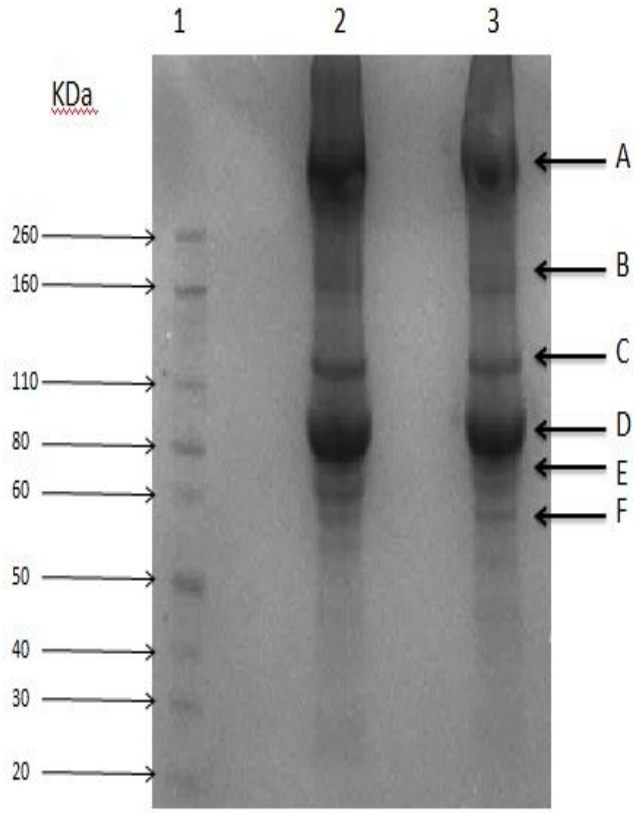

Figure 3.2.4: SDS-PAGE of OMV determination of an unknown protein isolated from *P.aeuginosa* Laboratory strain (L.) (Preparation 5).

Lane 1 contains 10 µl of Protein standard standards; lane 2 has 30 µl of OMVs of *P.aeuginosa* grown in SWF and lane 3 has 30 µl of OMVs of *P.aeuginosa* grown in SWF with ethanol. Proteins were separated by SDS-PAGE in a Criterion 4–20% precast gel and stained with Coomassie blue stain. Molecular weights markers are indicated.

Figure 3.2.4 shows the bands in the lane 2 and lane 3 are identical to each other which refer to that the OMVs of *P. aeruginosa* grown in SWF contain the same protein in the OMVs of *P. aeruginosa* grown in SWF with ethanol. Estimation of molecular weight of unknown proteins in OMVs were based on the values of the bands on the standard where the graph was plotted by using the logarithm of the molecular weight of standard protein.

Molecular weight of protein standard (KDa)	log of standard molecular weight	migration distance (mm)
260	2.41	22
160	2.20	29
110	2.04	40
80	1.90	48
60	1.78	54
50	1.61	64
40	1.60	73
30	1.51	79
20	1.30	88

Table 3.2.19: The values of molecular weight of protein standard (KDa) and its log, the migration distance (mm).

unknown protein bands	Molecular weight (KDa)	Protein name
A	295	unknown
B	182	unknown
C	126	unknown
D	91	FpvA
E	72	OprC
F	62	XcpQ

Table 3.2.20: Possible identification of the unknown protein in OMVs isolated from *P. aeruginosa* grown in Simulated Wound Fluid (SWF) and SWF with ethanol.

The approximate molecular weights (MW) of each unknown protein was used to provide to identify a potential protein based on the similar size of MW which can be found on data bases of outer membrane proteins of *Pseudomouns aeruginosa.* In this study three proteins were identified in OMVs (FpvA, OprC and XcpQ) whereas, the other three bands have high MW could not be determined.

Gel image of OMV proteins derived from *P. aeruginosa* in TSB (Preparation 6):

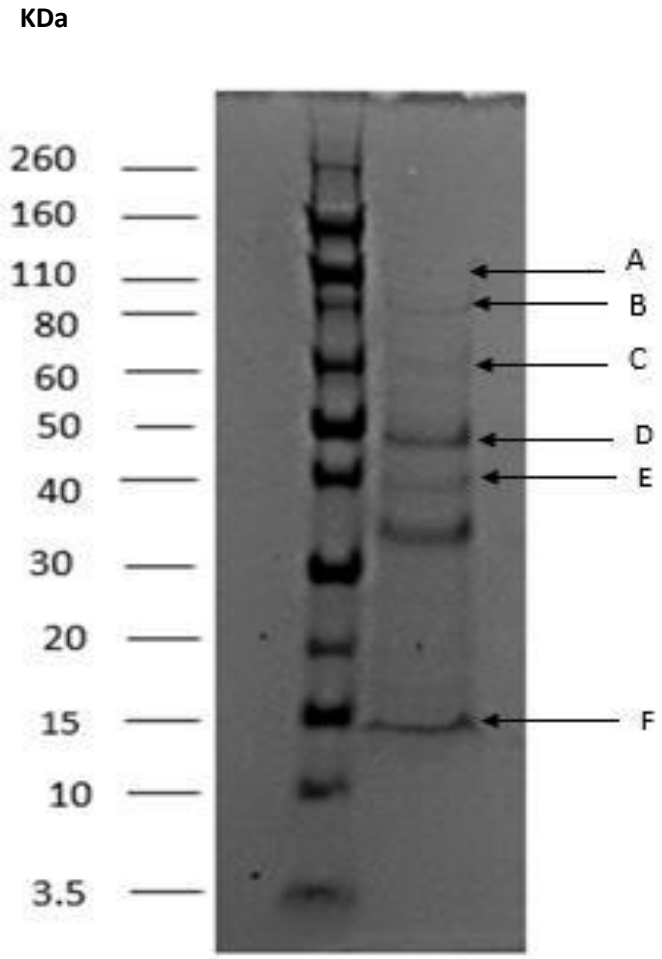

Figure 3.2. 5: showing SDS-PAGE of OMV determination of an unknown protein isolated from *P. aeuginosa* Laboratory strain (L.) in TSB (Preparation 6).

Lane 1 contains 10 µl of Protein standard standards; lane 2 has 40 µl of OMVs of *P. aeuginosa* grown in only TSB Proteins were separated by SDS-PAGE in a Criterion 4–20% precast gel stained with Coomassie Blue. Molecular weights are indicated. The unknown OMV protein are labelled with letters.

Unknown protein bands	Molecular weight (KDa)	Protein name
A	110	unknown
B	106	unknown
C	80	PiuA
D	50	OprM
E	39	OprF
F	13	unknown

Table 3.2.21: Possible identification of the unknown protein from OMVs isolated from *P. aeruginosa* grown in TSB.

Gel image of OMVs proteins derived from *P. aeruginosa* in Simulated Wound Fluid (SWF) (Preparation 6):

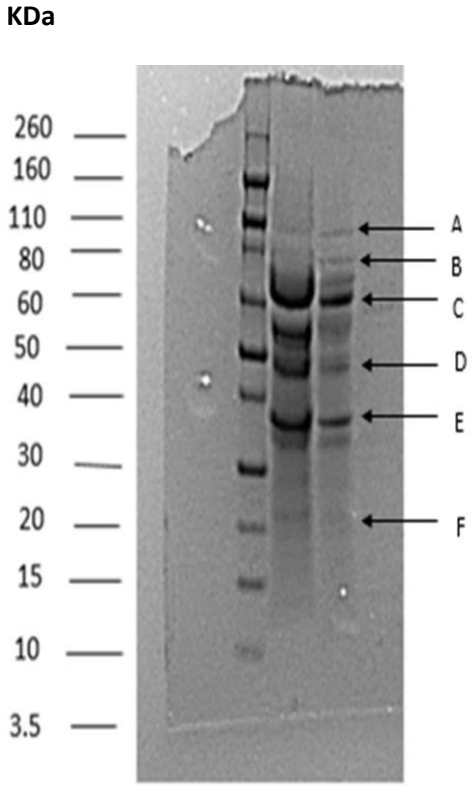

Figure 3.2 6: showing SDS-PAGE of OMV determination of an unknown protein isolated from *P. aeruginosa* Laboratory strain (L.) in SWF (Belong table 11 and 12) (Preparation 6).

Lane 1 contains 10µL of protein standard, lane 2 has 30µL and lane 3 has 10µL. Proteins were separated by SDS-PAGE stained with Coomassie Blue. Molecular weights are indicated.

Unknown Protein bands in SWF	Molecular weight (KDa)	Protein name
A	122	Unknown
B	100	Unknown
C	78	PfuA
D	51	OprM
E	40	OprF
F	16	OprX

Table 3.2.22: Possible identification of the unknown protein in OMVs isolated from *P. aeruginosa* grown in SWF. (Preparation 6).

Image of OMVs proteins taken from *P. aeruginosa* Laboratory strain in TSB (Preparation 7):

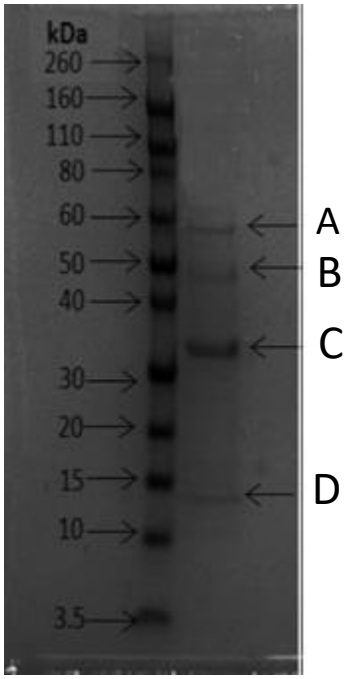

Figure 3.2.7; SDS-PAGE of OMV determination of an unknown protein isolated from *P. aeruginosa* Laboratory strain (L.) in tryptone soy broth media TSB.

(Belong table 12 and 13) (Preparation 6). Lane 1 contains 10µL of protein standard, lane 2 has 40µL, of of P. aeruginosa Laboratory strain in TSB. Proteins were separated by SDS-PAGE and stained with Coomassie Blue. Molecular weights are labelled. The unknown OMV protein bands indicated.

Unknown protein bands in TSB	Molecular weight (kDa)	protein name
A	97720	unknown
B	51280	OprM
C	28180	PilD
D	12580	unknown

Table 3.2.23: Estimation of the unknown protein in OMVs isolated from *P. aeruginosa* Laboratory strain (L.) grown in TSB (Preparation 7).

Gel image of OMVs proteins derives from *Pseudomonas aeruginosa* in SWF (Preparation 7):

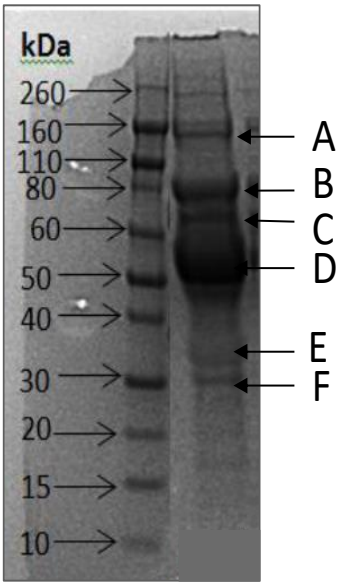

Figure 3.2.8: Showing SDS-PAGE of OMV determination of an unknown protein isolated from *P. aeruginosa* Laboratory strain (L.) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of *P. aeruginosa* Laboratory strain in TSB. Proteins were separated by SDS-PAGE gel stained with Coomassie Blue. Molecular weights are indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	194	unknown
B	158	unknown
C	117	unknown
D	97	unknown
E	68	XcpQ
F	15	unknown
G	16	OprH

Table 3.2.24: Possible identification of the unknown protein in OMVs isolated from *P. aeruginosa* Laboratory strain (L.) grown in TSB (Preparation 7*).*

There was a lower protein concentration in OMVs isolated from *Pseudomonas aeruginosa* in TSB compared to the same bacteria cultured in SWF which indicates that *Pseudomonas aeruginosa* produce higher amounts of OMVs in SWF.

OMVs have variety of sizes and proteins, the protein expression seems to vary a little depending on their media the bacteria were grown in *P. aeruginosa* cell-free supernatants contain a high concentration of vesicles, but could also contain other secreted elements including pili, flagella, and R-type pyocins that increase the protein content (Bauman and Kuehn, 2006).

Gel image of OM proteins derives from *Pseudomonas aeruginosa* Laboratory strain (L.) in SWF:

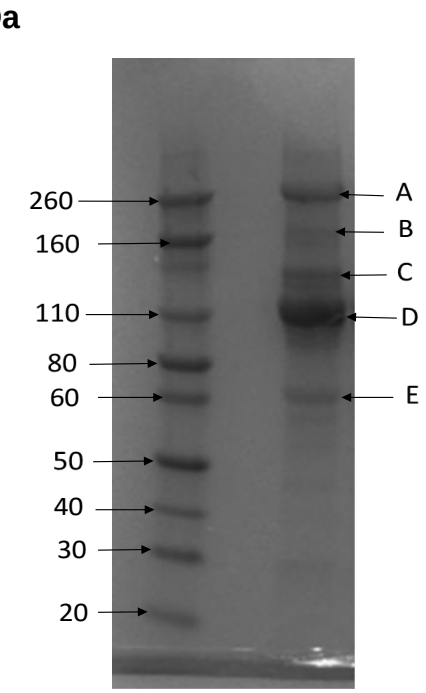

Figure 3.2.9: SDS-PAGE of OM determination of an unknown protein isolated from *P. aeruginosa* laboratory strain (L.) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of *P. aeruginosa* Laboratory strain in SWF. Proteins were separated by SDS-PAGE in a Criterion 4–20% precast gel stained with Coomassie Blue. Molecular weights (in kilodaltons) are indicated on the left. The unknown OM protein bands indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	154.43	rPOB
B	112.42	NrdA
C	88.61	Cation-Transporting p-type ATPase
D	69.84	XcpQ
E	41.06	OprD

Table 3.2.25: Possible identification of the unknown protein in OM isolated from *P. aeruginosa* Laboratory strain (L.) grown in SWF.

Gel image of OMV proteins derives from *Pseudomonas aeruginosa* Laboratory strain (L.) in SWF

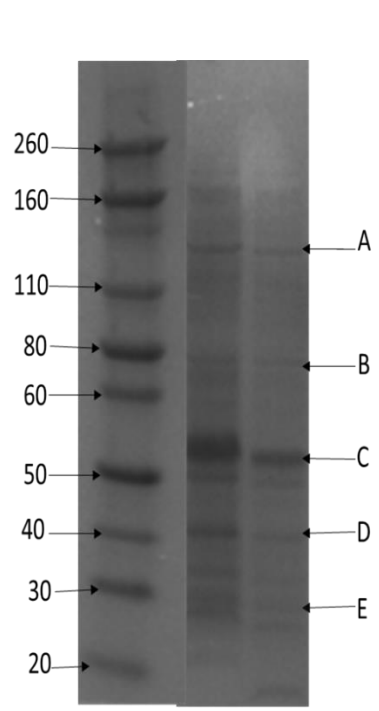

Figure 3.2.10: SDS-PAGE of OMV determination of unknown proteins derived from *P. aeruginosa* laboratory strain (L.) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of *P. aeruginosa* Laboratory strain in SWF. Proteins were separated by SDS-PAGE gel stained with Coomassie Blue. Molecular weights markers are indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	88.61	Cation-Transporting p-type ATPase
B	51280	OprM
C	30.35	Unknown
D	18.12	OPrH
E	14.86	PilG

Table 3.2. 26: Possible identification of the unknown protein in OMV isolated from *P. aeruginosa* Laboratory strain (L.) grown in SWF (Preparation 8).

Image of OM proteins derives from *Pseudomonas aeruginosa* Hospital strain (PS3) in SWF:

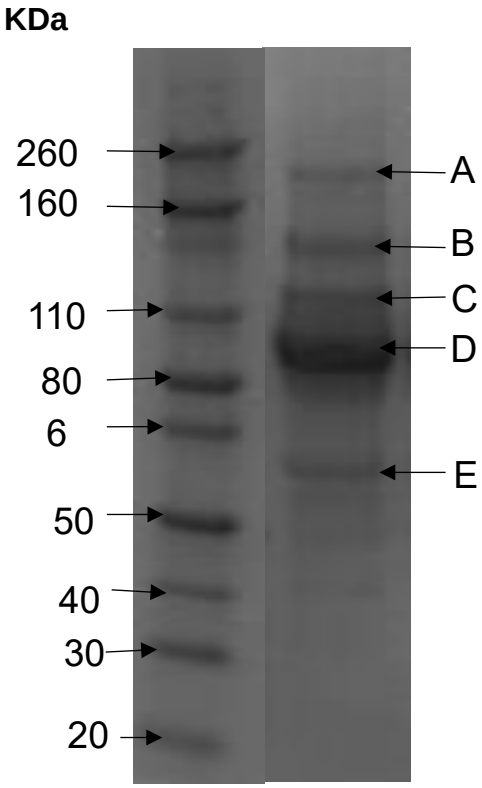

Figure 3.2.11: SDS-PAGE of OM determination of an unknown protein isolated from *P. aeruginosa* Hospital strain (PS3) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of *P. aeruginosa* Laboratory strain in SWF. Proteins were separated by SDS-PAGE gel stained with Coomassie Blue. Molecular weights marked are indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	154.4	rPOB
B	117.0	unknown
C	88.6	Cation-Transporting p-type ATPase
D	69.8	XcpQ
E	41.1	OprD

Table 3.2. 27: Possible identification of the unknown protein in OM isolated from *P. aeruginosa* Hospital strain (PS3) grown in SWF.

Gel image of OMV proteins derives from *Pseudomonas aeruginosa* Hospital strain (PS3) in SWF:

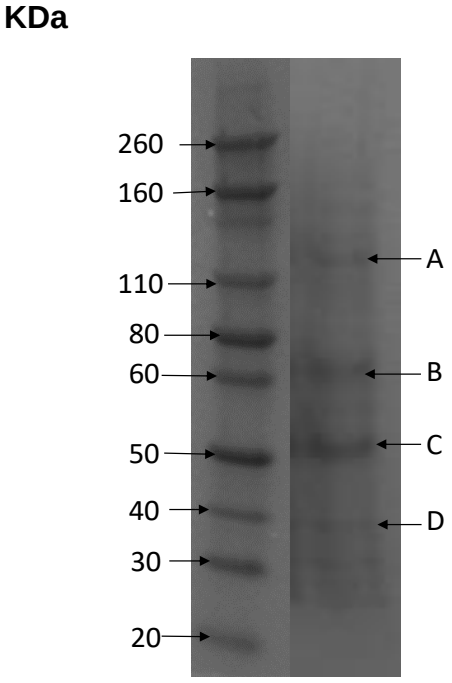

Figure 3.2.12: SDS-PAGE of OMV determination of unknown proteins derived from *P. aeruginosa* Hospital strain (PS3) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of *P. aeruginosa* Laboratory strain in SWF. Proteins were separated by SDS-PAGE gel stained with Coomassie Blue. Molecular weights markers are indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	Protein name
A	88.6	Cation-Transporting p-type ATPase
B	51.3	OprM
C	30.4	unknown
D	18.1	OPrH

Table 3.2. 28: Possible identification of the unknown protein in OMVs isolated from *P. aeruginosa* Hospital strain (PS3) grown in SWF (Preparation 8).

Gel image of OM proteins derives from *E. coli* strain (C25) in SWF:

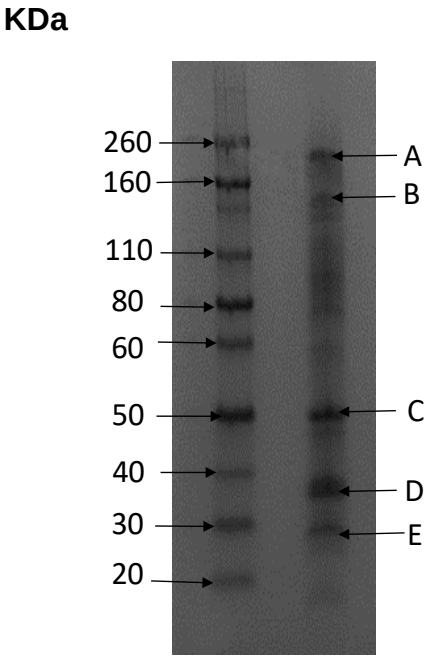

Figure 3.2.13: SDS-PAGE of OM determination of unknown proteins derived from *E. coli* strain (C25) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of strain *E. coli* in SWF. Proteins were separated by SDS-PAGE gel stained with Coomassie Blue. Molecular weights of the markers are indicated.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	78.86	
B	55.04	
C	17.52	
D	13.21	
E	11.81	

Table 3.2. 29: Estimation of the unknown protein in OM isolated from *E. coli* strain (C25) grown in simulated wound fluid SWF.

Gel image of OMV proteins derives from *E. coli* strain (C25) in SWF:

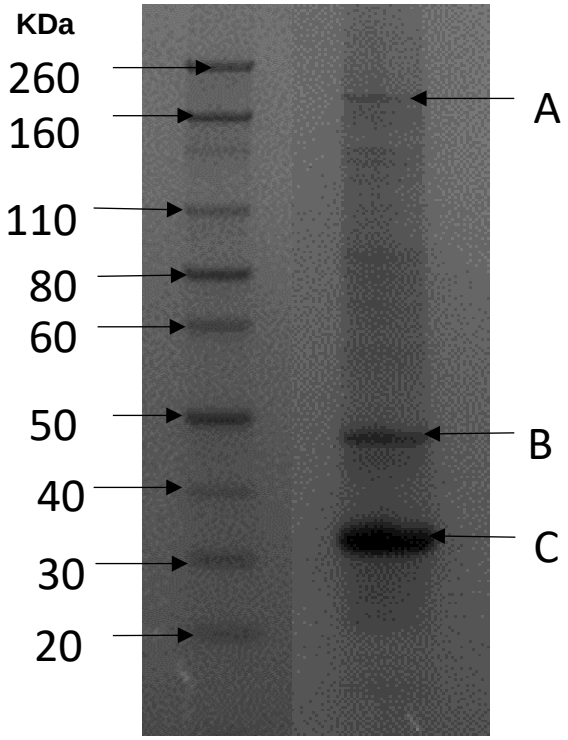

Figure 3.2.14: SDS-PAGE of OMV determination of unknown proteins derived from *E. coli* strain (C25) grown in simulated wound fluid SWF.

Lane 1 contains 10µL of protein standard, lane 2 has 30µL, of sample all containing OM proteins of strain *E. coli* in SWF. Proteins were separated by SDS-PAGE in a Criterion 4–20% precast gel stained with Coomassie Blue. Molecular weights markers are indicated on the left.

Unknown protein bands in SWF	Molecular weight (KDa)	protein name
A	79	
B	18	
C	13	

Table 3.2.30: Molecular weight of the unknown protein in OMVs isolated from *E. coli* strain (C25) grown in simulated wound fluid SWF.

Section 3

Bacterial Growth curves

Pseudomonas aeruginosa laboratory strain (L.) 10421 was grown in tryptone soy broth (TSB) and Simulated wound fluid (SWF) for 7 hours and >2.5 au after 24hours.

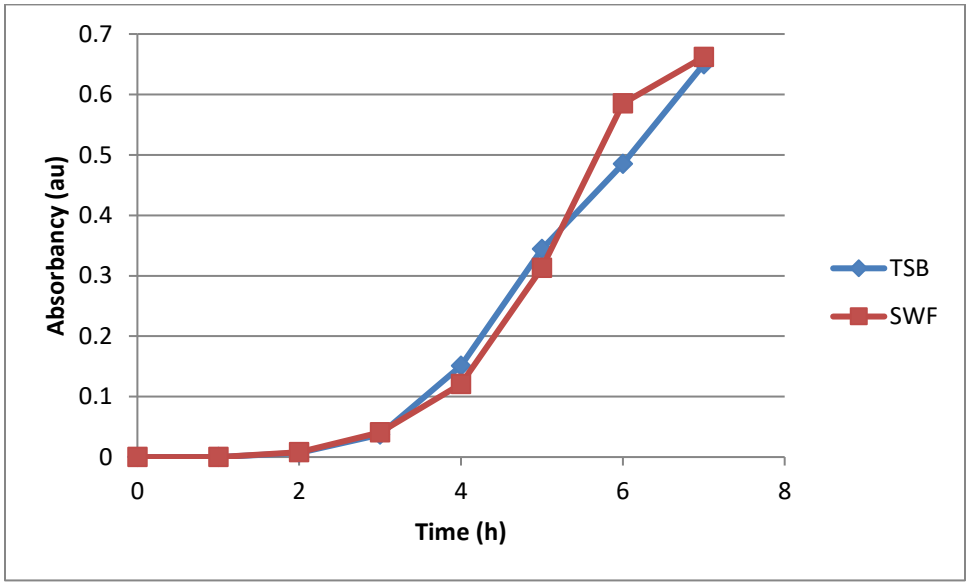

Figure 3.3.1: Measurement of absorbency for *Pseudomonas aeruginosa* laboratory strain (L.) 10421 In TSB and SWF for 7 hours.

Three independent biological experiments, each consisting of 6-8 technical replicates.

Pseudomonas aeruginosa laboratory strain (L.) 10421 was grown in either TSB (the basic media for the bacteria) or simulated wound fluid (SWF) (which is much closer to the conditions at the wound bed). The absorbance at 620nm was monitored. The absorbance data indicated that both strains had very similar growth characteristics in both culture conditions.

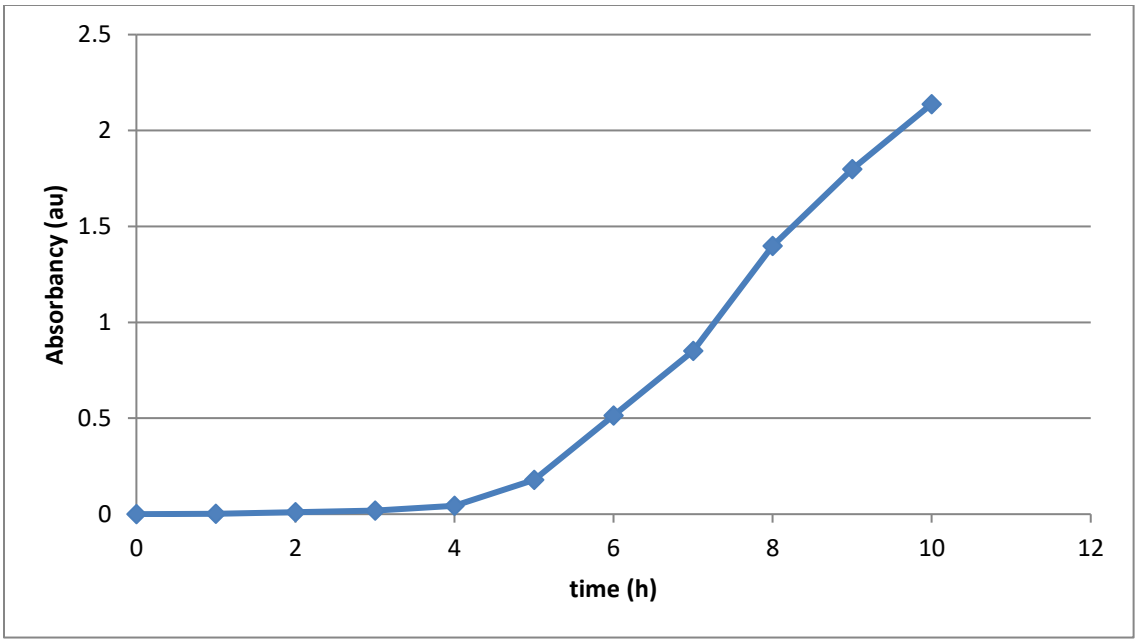

Figure 3.3.2: Measurement of absorbency for *Pseudomonas aeruginosa* Hospital strain (PS3) in TSB for 7 hours.

Three independent biological experiments, each consisting of 6-8 technical replicates.

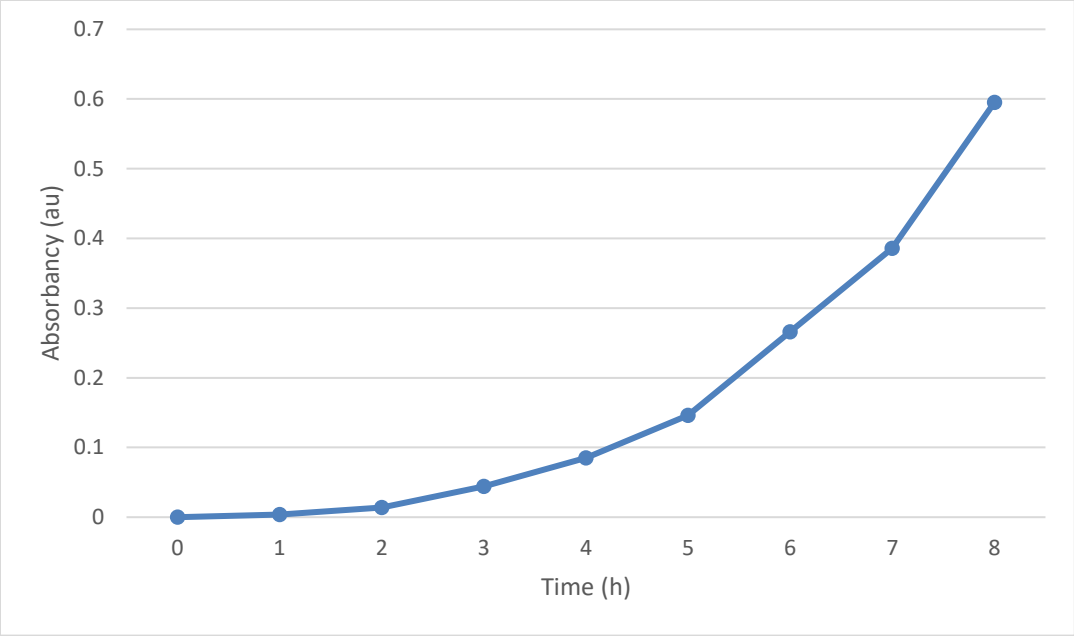

Figure 3.3.3: Measurement of absorbency for *Pseudomonas aeruginosa* Hospital strain (PS3) in SWF for 7 hours.

Three independent biological experiments, each consisting of 6-8 technical replicates.

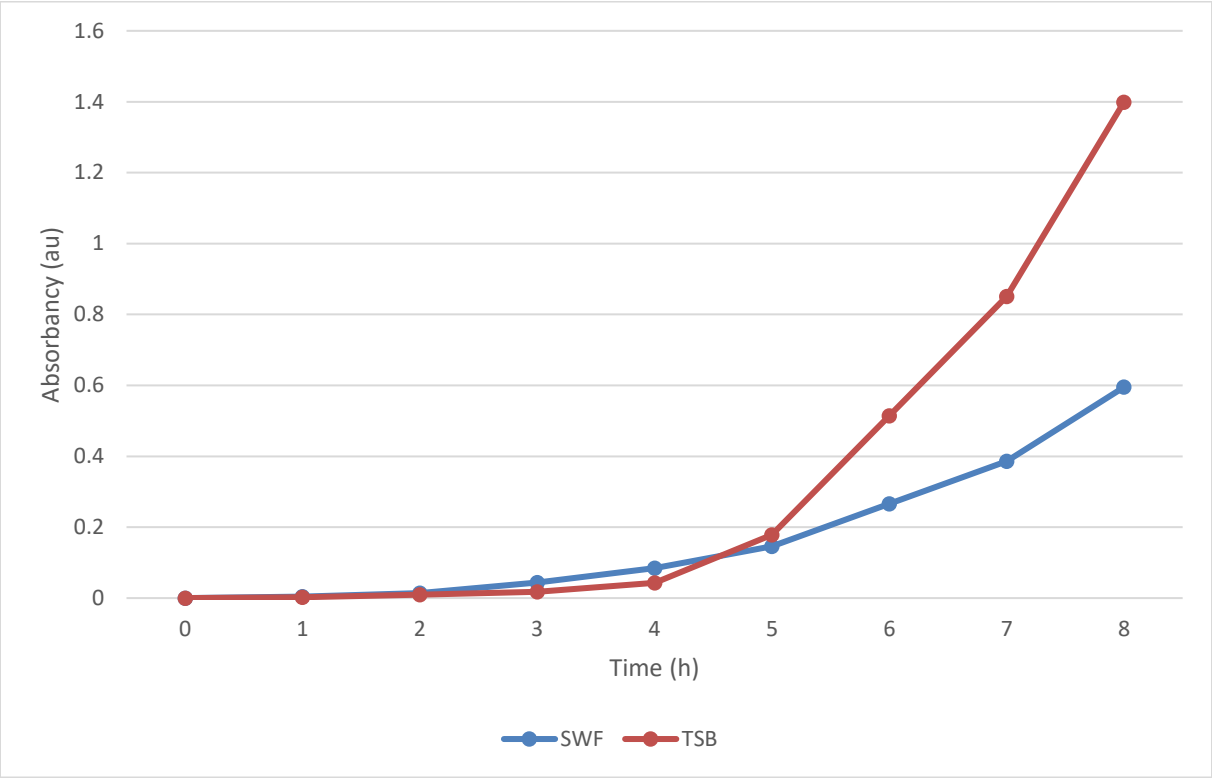

Figure 3.3.4: Measurement of absorbency at 620nm of *Pseudomonas aeruginosa* Hospital strain type 3 (PS3) in SWF and TSB for 8 hours in 24hours, note change of scale from previous graph.

Three independent biological experiments, each consisting of 6-8 technical replicates.

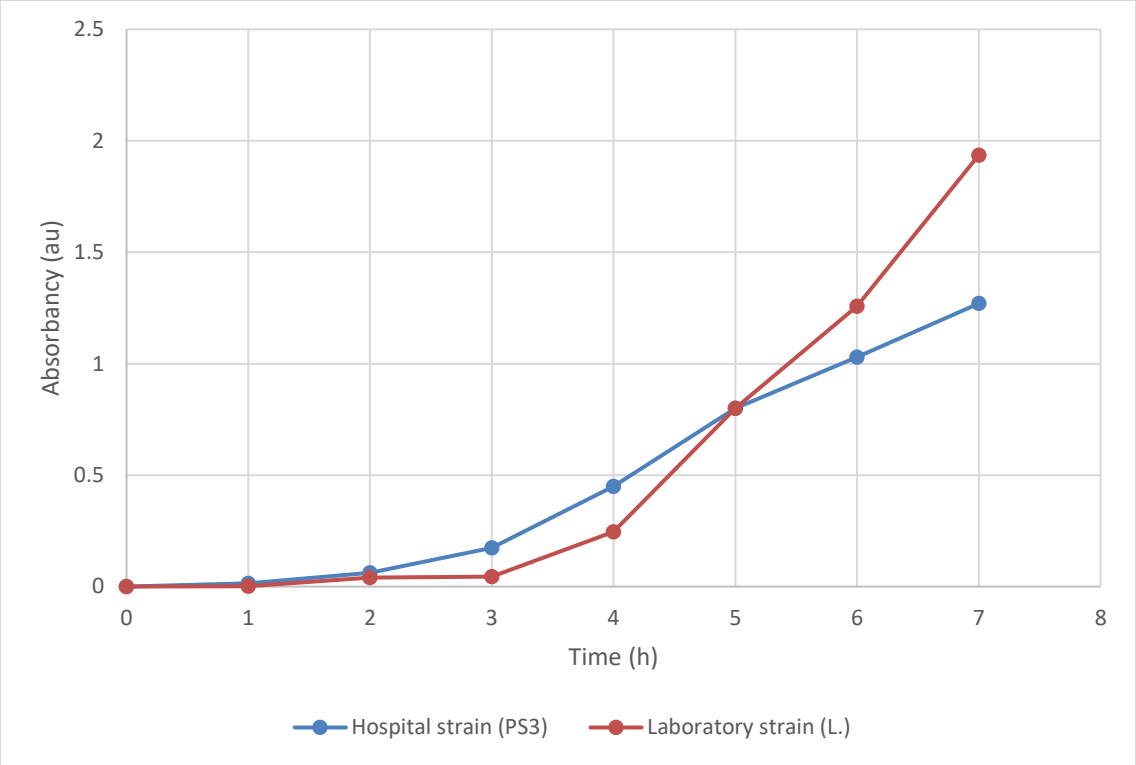

Figure 3.3.5: Measurement of absorbency at 620nm of *Pseudomonas aeruginosa* both Laboratory strain (L.) 10421 and Hospital strain type 3 (PS3) in DMEM for 7 hours in 24hours, note change of scale from previous graph.

Three independent biological experiments, each consisting of 6-8 technical replicates.

Both strains (Hospital and lab.) grown in DMEM and reached the stationary phase after about 7 hours. The absorbance data indicates that greater bacteria numbers grow in DMEM compared to TSB or SWF.

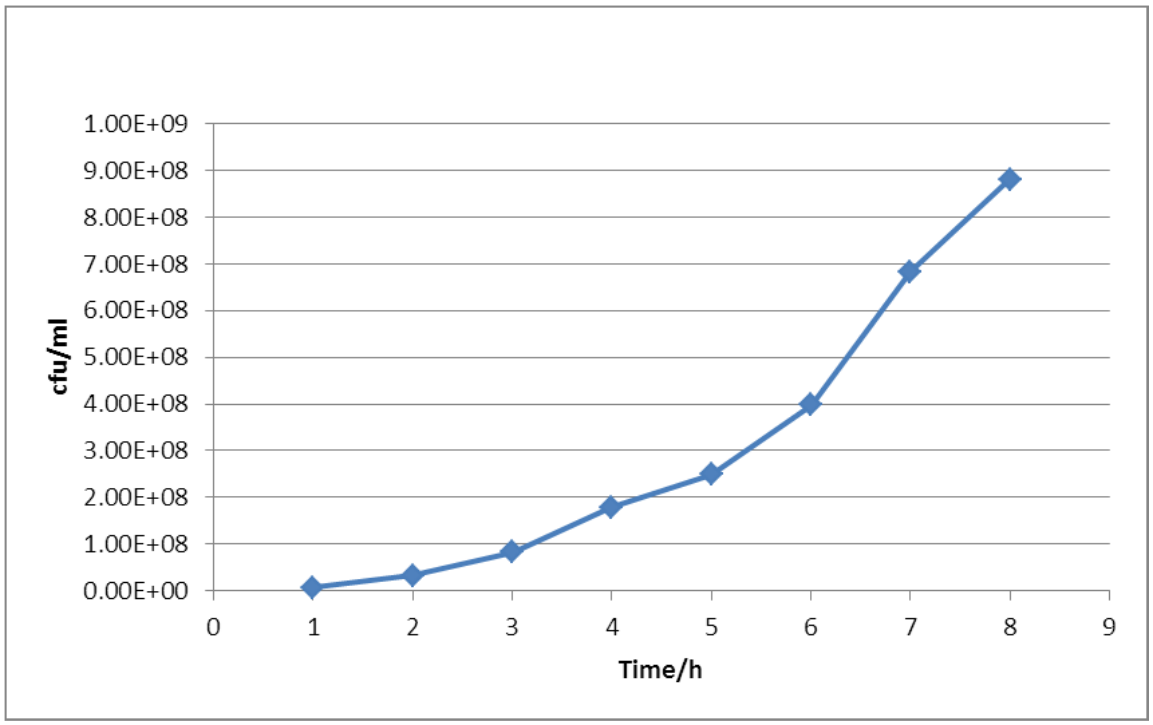

Figure 3.3.6: Number of bacteria *Pseudomonas aeruginosa* Hospital strain type 3 (PS3) in DMEM for 8 hours.

Three independent biological experiments, each consisting of 6-8 technical replicates.

There was also rapid increase in the number of bacteria *Pseudomonas aeruginosa* in DMEM. When bacteria numbers were directly quantified by plating on agar plates as the rapid growth as indicated by change in absorbency was confirmed.

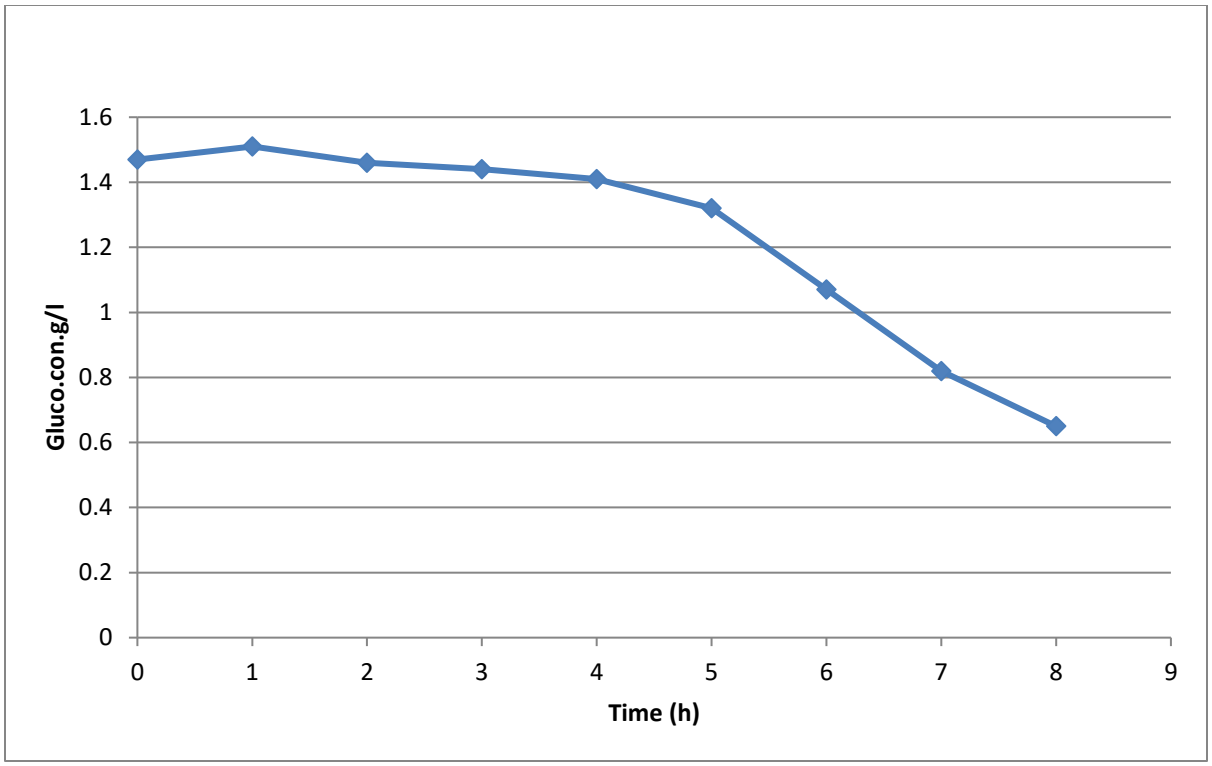

Figure 3.3.7: Glucose concentrations in DMEM whilst growing of *Pseudomonas aeruginosa* Hospital strain (PS3).

Three independent biological experiments, each consisting of 6-8 technical replicates.

Figure 3.3.7 shows the consumption of glucose, decline slowly for 4 hours after that the rate of glucose consumption increased.

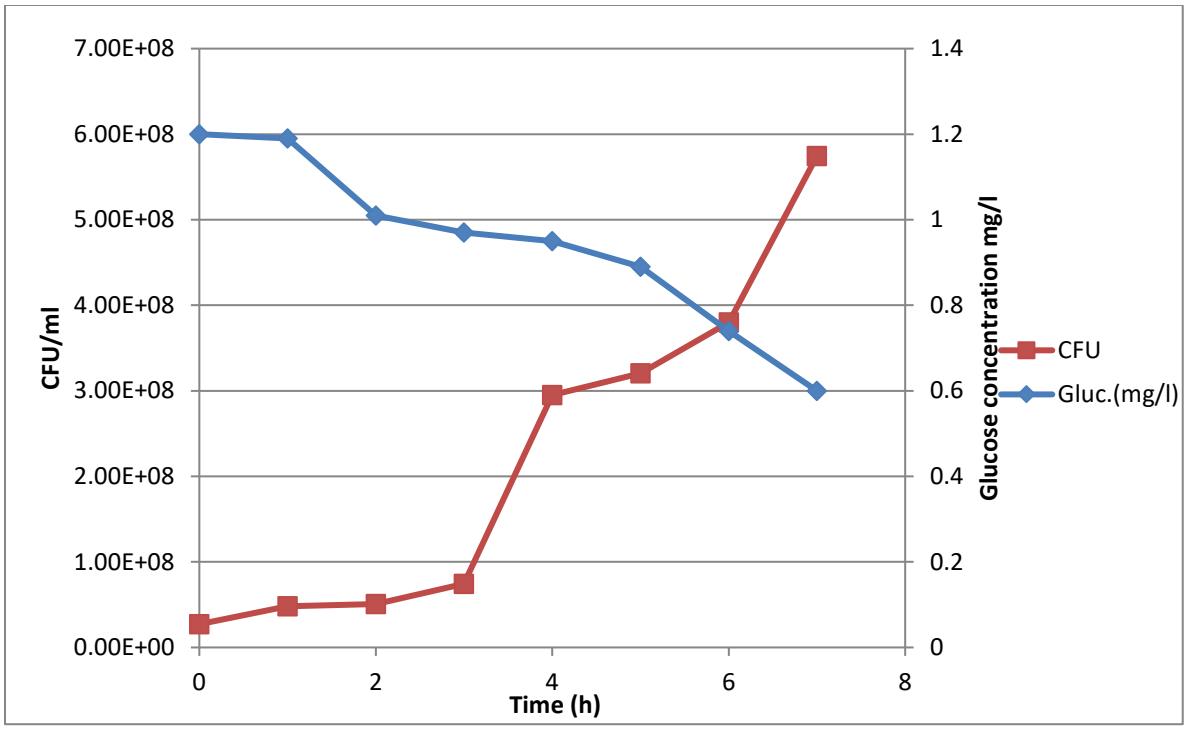

Figure 3.3.8: Glucose concentration (mg/l) and growth of *Pseudomonas aeruginosa* (ml) in DMEM in first day (8 hours).

Three independent biological experiments, each consisting of 6-8 technical replicates.

As shown in figure 3.3.8 there is a rapid increase in the number of bacteria that is associated with consumption of glucose.

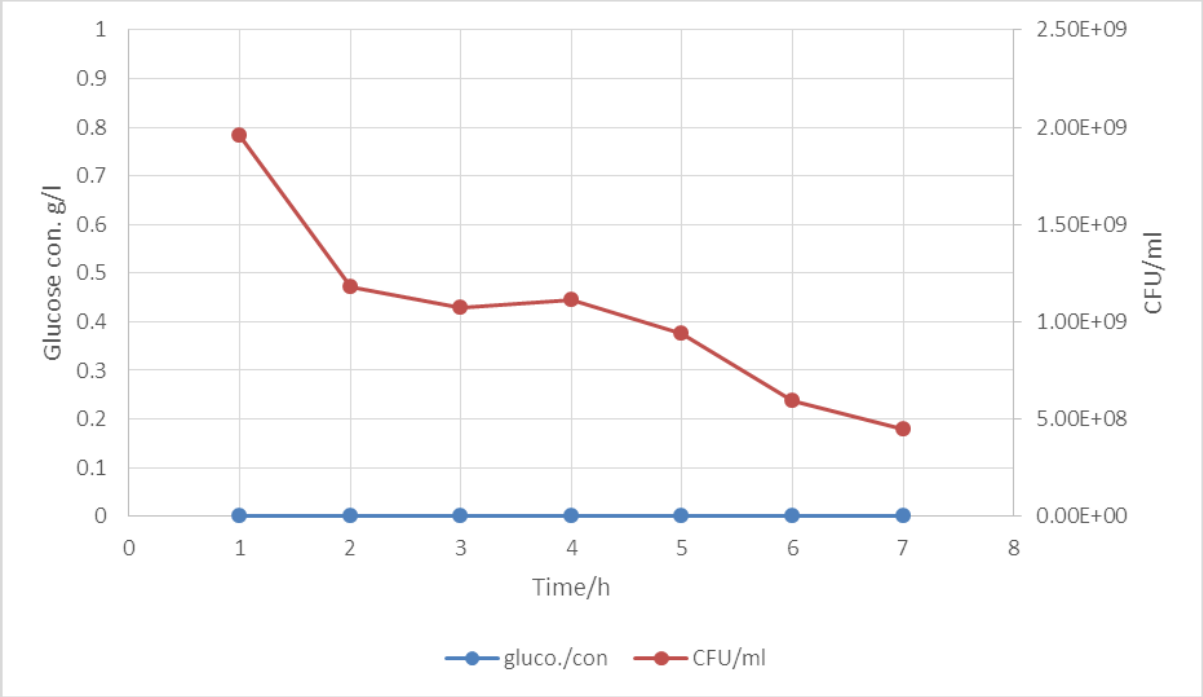

Figure 3.3.9: Glucose concentration (g/l) and growth of *Pseudomonas aeruginosa* (m/l) in DMEM on the second day (7 hours).

Three independent biological experiments, each consisting of 6-8 technical replicates.

After overnight growth all glucose was used and the bacterial numbers declined over the next 7 hours in DMEM. Showing that the decline phase of the growth curve had been reached this, at least in part, this is due to the fact that all the glucose had been used.

Section 4

Interaction of Outer membrane vesicles and outer membranes isolated from *Pseudomonas aeruginosa* on keratinocyte cells

3.4.1 Cytotoxicity of OMV isolated from *Pseudomonas* grown in TSB

A cytotoxicity assay was determined to assess the toxicity of the OMVs isolated from the two strains of *Pseudomonas* grown in TSB on the HaCaT cells at different concentrations the average biomass of the treated cells was compared to control to give an average % biomass relative to control.

Mean	**5.71 µg/ml OMV (PS3)**	**28.55µg/ml OMV (PS3)**	**14.28 µg/ml OMV (L.)**	**71.40µg/ml OMV (L.)**
Biomass%	105.05	82.01	130.62	98.11
S.E.M	10.02	0.50	4.99	16.49

Table 3.4.1: The means of the biomass for all concentration for OMVs of *Pseudomonas aeruginosa* strains (preparation 1).

HaCaT cells were exposed to the indicated concentrations of OMVs for four hours. The data is from for three independent biological experiments, each consisting of 6-8 technical replicates.

There was a lower biomass of HaCaT cells on exposure to OMVs from PS3 compared to Laboratory Strain L. and this was the case even when the cells were exposed to a greater concentration of Laboratory Strain L. OMV (figure 3.4.1).

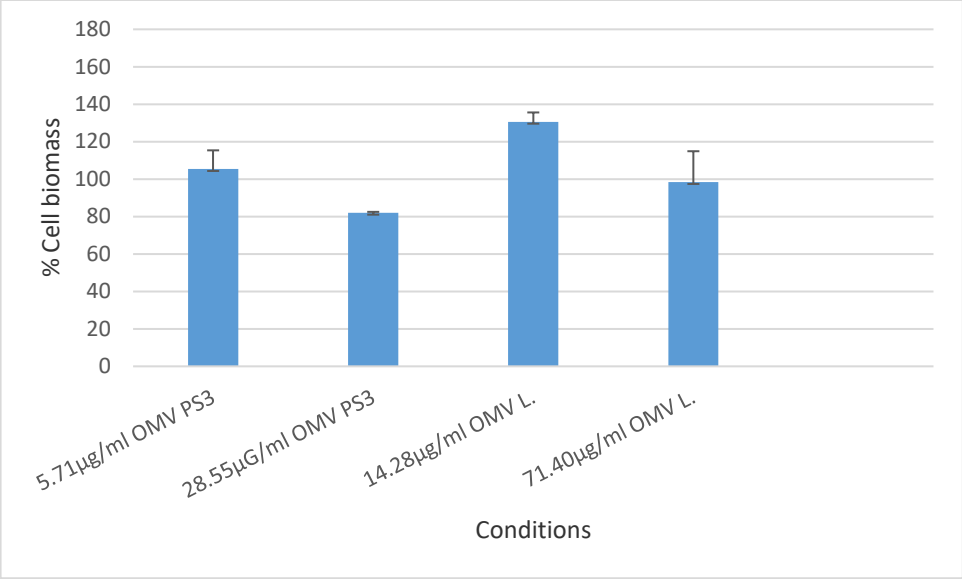

Figure 3.4.1: The average cell biomass for HaCaT cells challenged with OMVs of Hospital strain (PS3) and Laboratory Strain (L.) in two different concentrations. (For preparation 1).

HaCaT cells were exposed for four hours and the MTS assay was performed after further 24 hour incubation in cell cutler media. Data are represented as mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

The micrographs below demonstrate HaCaT cells exposed to OMVs for both strains of *P. aeruginosa*.

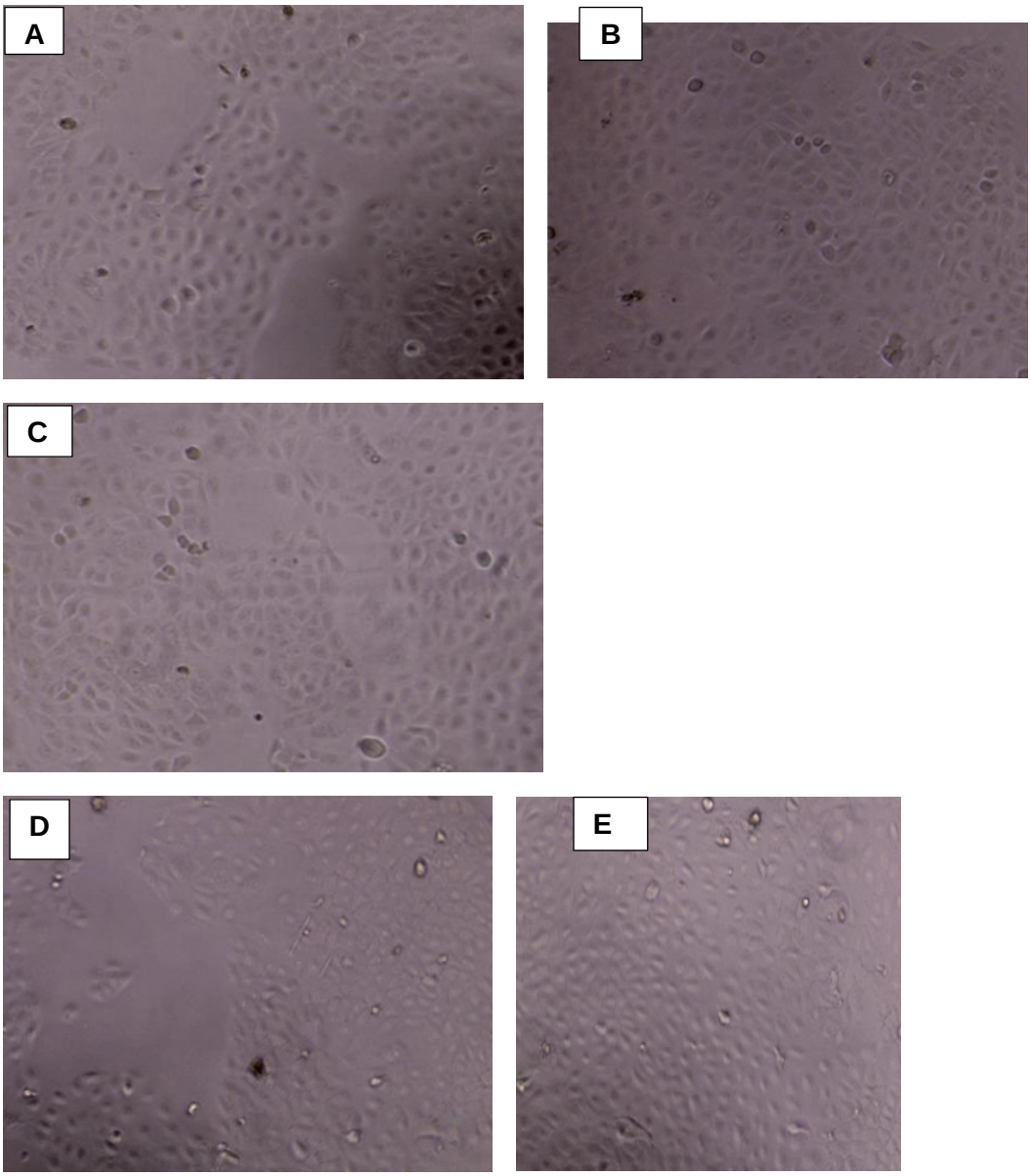

Figure 3.4.2. Photomicrographs of keratinocytes cells exposed to OMVs of *P. aeruginosa*.

(A) Untreated cells**. (B)** Challenged with OMVs of the laboratory strain **(L.) at** 14.28µg/ml, the cells appear not be affect at this concentration of OMVs**. (C)** Challenged with 71.4µg/ml of laboratory strain (L.) OMV showing many dead cells. **(D)** Treated with 5.714µg/ml of OMVs form the Hospital strain (PS3), has less effect on the cells. **(E)** 28.55 µg/ml of Hospital strain OMVs (PS3) shows death of some cell.

A second preparation of OMVs was produced and the MTS biomass assay was used to determine the toxicity of the OMVs on the HaCaT cells at different concentration of OMV from the 2 strains of Pseudomonas grown in TSB

Mean	6.67µg/ml OMV (PS3)	33.3µg/ml OMV(PS3)	6.67µg/ml OMV (L.)	33.3µg/ml OMV (L.)
Biomass%	117.12	153.12	123.55	133.93
S.E.M	11.08	12.52	18.10	14.54

Table 3.4.2 the means for the % change in biomass for OMVs derived from *Pseudomonas aeruginosa* strains taken from preparation 2.

HaCaT cells were exposed for four hours and the MTS assay was performed after further 24 hour incubation in cell cutler media. Data are represented as mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

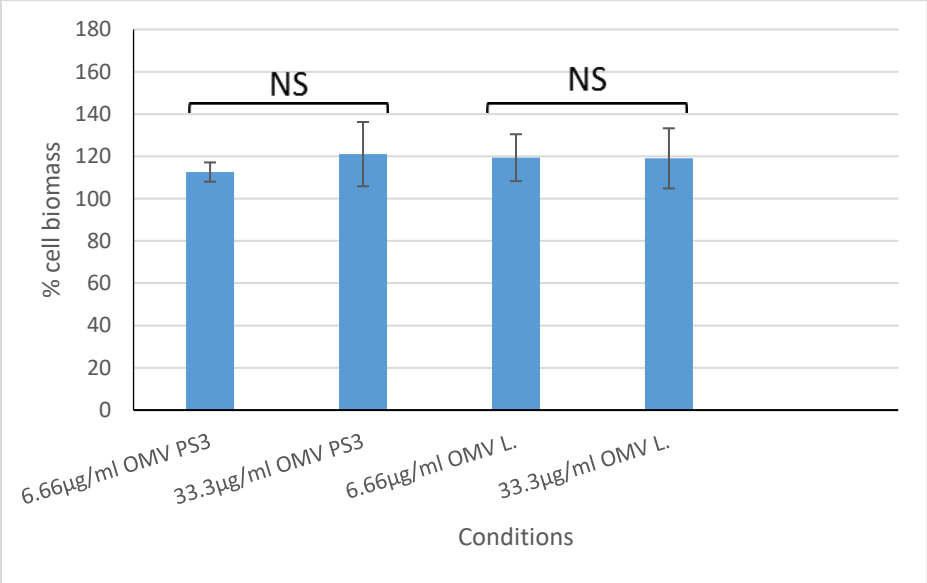

Figure 3.4.3: The average cell biomass for HaCaT cells challenged with OMVs of Hospital (PS3) strain and laboratory strain (L.) in two different concentrations. (For preparation 2).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, paired Student T-test Cell biomass of HaCaT cells compared for challenging this cells with different concentration of (OMV) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

A 3rd OMVs isolation from *Pseudomonas* grown in TSB was performed and their effect on the biomass of HaCaT keratinocytes was tested.

Mean	11.67µg/ml OMV (PS3)	58.33µg/ml OMV (PS3)	11.67µg/ml OMV (L.)	58.33µg/ml OMV (L.)
Biomass%	125.55	127.96	126.67	126.29
S.D.	2.10	4.24	2.15	7.34

Table 4.3: The means of the biomass and absorbance for all concentration for OMVs of *Pseudomonas aeruginosa* strains (preparation 3).

HaCaT cells were exposed for four hours and the MTS assay was performed after further 24 hour incubation in cell cutler media. Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates

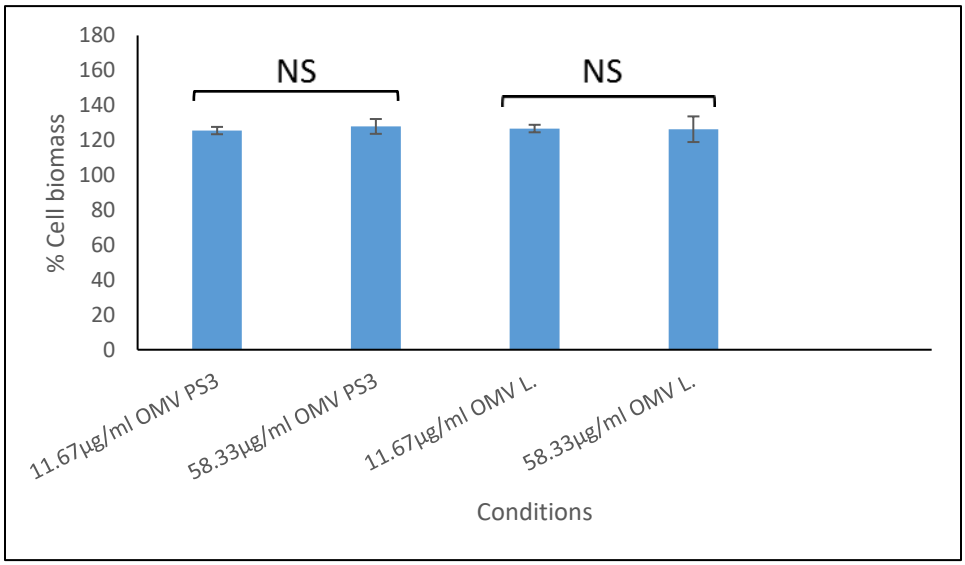

Figure 3.4.4: The average cell biomass for HaCaT cells challenged with OMVs of Hospital (PS3) strain and laboratory strain (L.) in two different concentrations. (For preparation 3).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, paired Student T-test Cell biomass of HaCaT cells compared for challenging this cells with different concentration of (OMV) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

Overall the 3 preparation of OMV's from both strains of *Pseudomonas aeruginosa* grown in TSB had little effect on HaCaT cells biomass and there was no difference between the Hospital and Laboratory strains.

To confirm the data from the cytotoxicity assays photomicrographs were taken to give a visual representation of the cells.

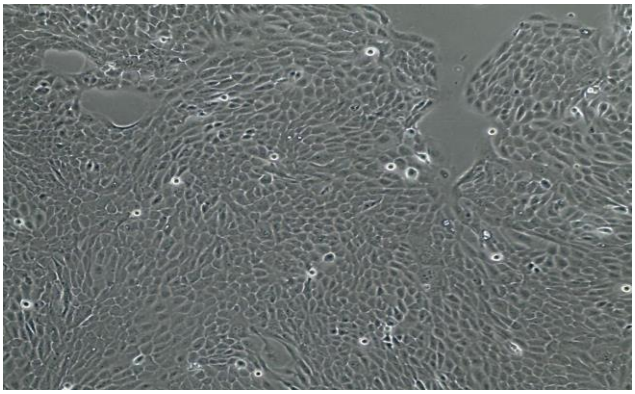

Figure 3.4.5: HaCaT cells growing in DMEM as control sample to demonstrate their normal growth pattern.

(Classic morphology of HaCaT cells can be seen in this figure). Image is representative phase contrast micrographs (taken at 100x magnification).

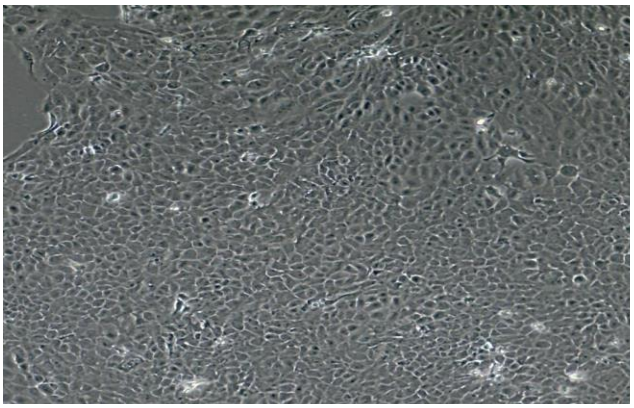

Figure 3.4.6: HaCaT cells exposed to the OMVs of Laboratory strain 6.66µg/ml of preparation 2.

The effect of OMVs is clear compared with non-treated cells as control. (In agreement with cytotoxicity assay). Image is representative phase contrast micrographs (taken at 100x magnification).

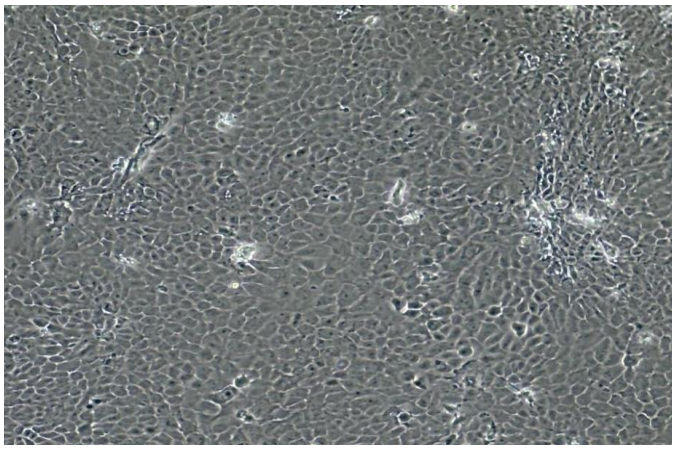

Figure 3.4.7: HaCaT cells exposed to the OMVs of Hospital strain (PS3) with concentration 6.66µg/ml preparation 2.

The effect of OMVs on the shape of the cells is clear comparing with the control. Image is representative phase contrast micrographs (taken at 100x magnification).

3.4.2 IL-8 secretion from HaCaT cells in response to OMV isolated from *Pseudomonas* grown in TSB

HaCaTa cells were exposed for 4 hours to OMV taken from the 3 OMV preparations from both strains *Pseudomonas* grown in TSB after this the cells were returned to normal culture conditions for a further 24 hours after which the culture media was tested for the concentration of IL-8.

Samples	IL-8 Concentration (pg/ml)			
	Preparation 1	Preparation 2	Preparation 3	Mean
Control	9.3	18.3	4.4	10.7
OMVs Laboratory strain (L.)	42.8	17.4	13.4	24.5
OMVs Hospital strain (PS3)	44.4	71.5	20.3	30.6

Table 4.4: IL-8 Concentration form HaCaT cells treated with 11.7µg/ml of OMV obtained from preparation 1, 2, 3.

For each preparation the data are mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. An overall mean is also provided.

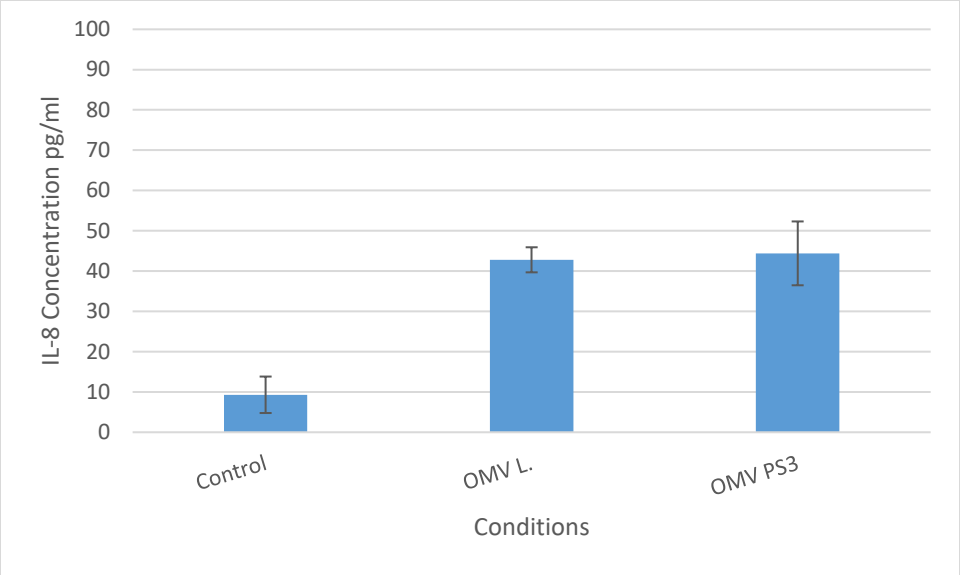

Figure 3.4.8: The concentration of IL-8 (pg/ml) of HaCaT cells treated with outer membrane vesicles (OMVs) for *P. aeruginosa* in TSB for both strains (preparation 1).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

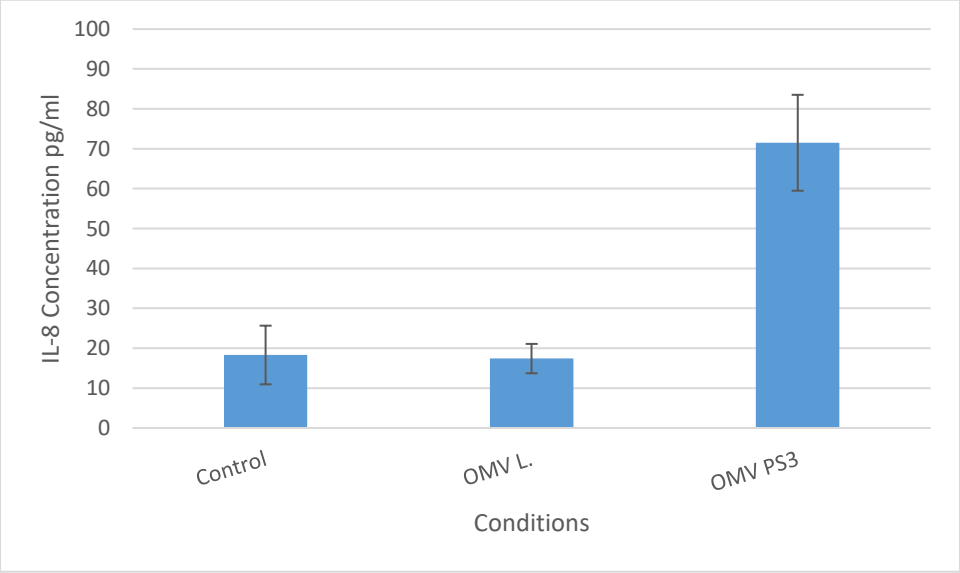

Figure 3.4.9: The concentration of IL-8 (pg/ml) of HaCaT cells treated with outer membrane vesicles (OMVs) for *P. aeruginosa* in TSB for both strains (preparation 2).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

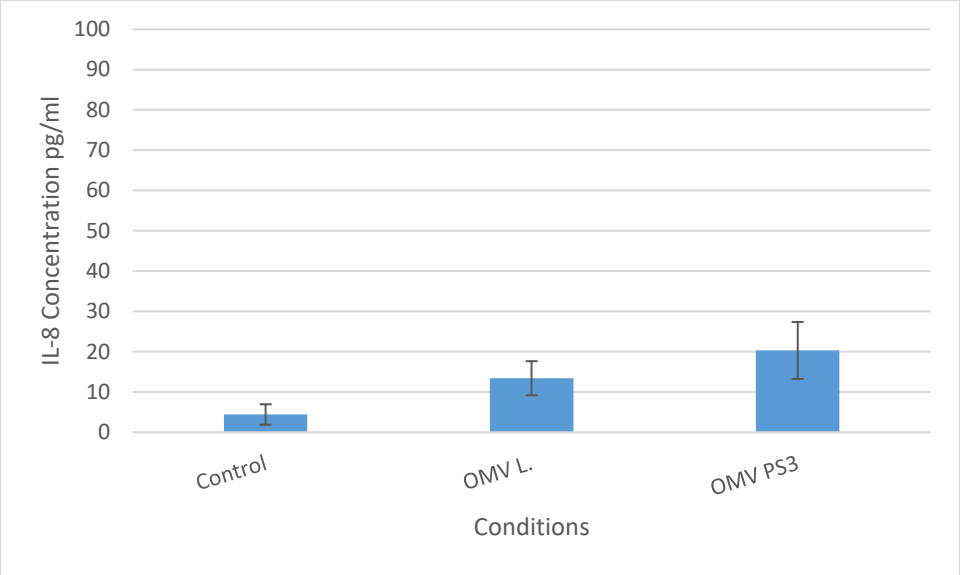

Figure 3.4.10: The concentration of IL-8 (pg/ml) of HaCaT cells treated with outer membrane vesicles (OMVs) for *P. aeruginosa* in TSB for both strains (preparation 3).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

Overall the data indicates that OMVs derived from both strains *P. aeruginosa* grown in TSB stimulates an increase in IL-8 from HaCaT cells however, PS3 resulted in a greater secretion of IL-8

3.4.3 HaCaT viability in response to OMVs isolated from *Pseudomonas* grown in simulated wound fluid with glucose or ethanol as the carbon source

HaCaT cells were challenged with OMVs from *P.aeruginosa* grown in SWF with either glucose or ethanol as the carbon source using the same protocol as described previously

3.4.3.1 Images of HaCaT cells challenged with OMVs from simulated wound fluid with glucose or ethanol as the carbon source

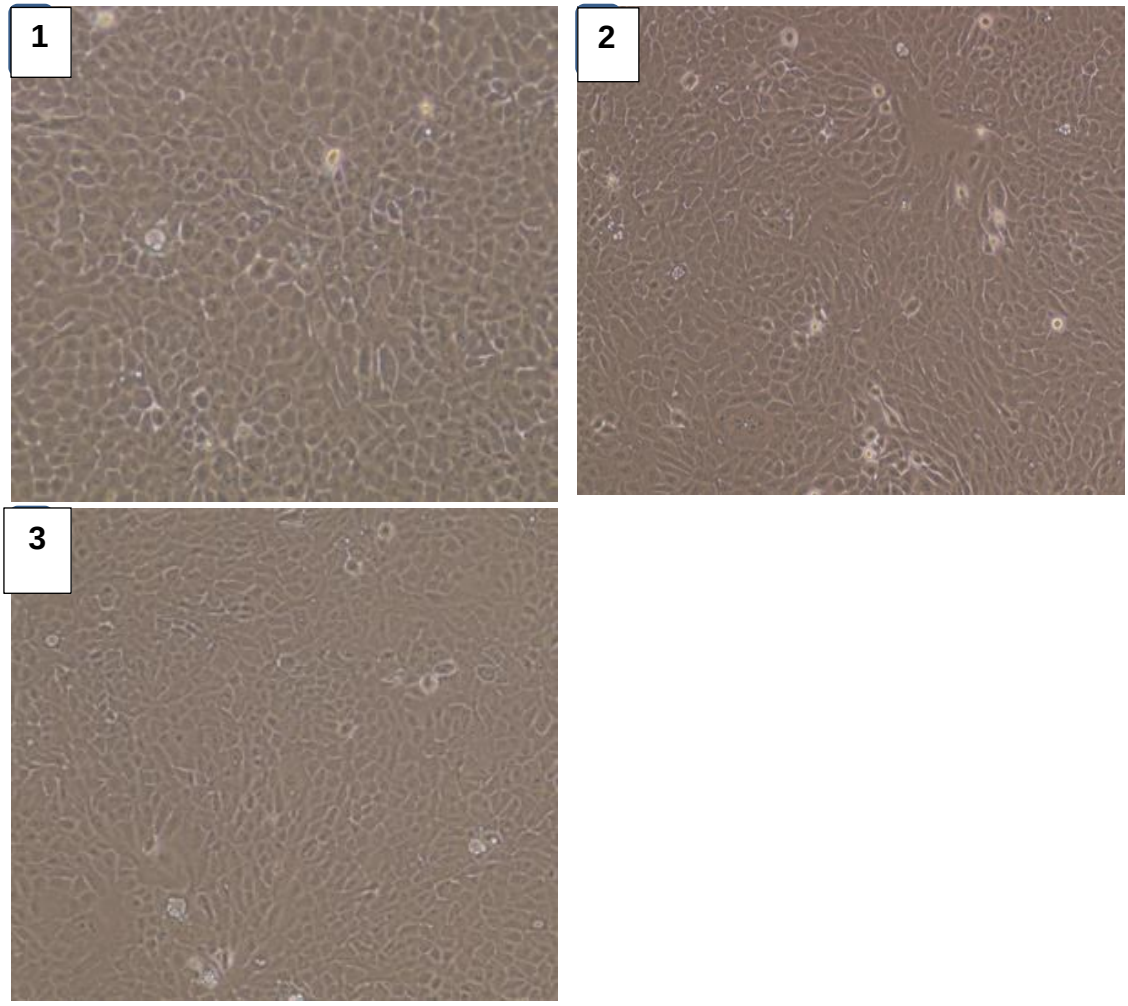

Figure 3.4.11: Photomicrographs for HaCaT cells treated with OMVs.

Picture (1) shows HaCaT cells without OMVs challenge, picture (2) shows cells challenged with OMVs derived from *P. aeruganosa* Laboratory strain (L.) grown in Simulated Wound fluid (SWF with ethanol and picture (3) challenged OMVs from the *P*. *aeruganosa* Laboratory strain (L.) These images show little effect on the size and shape of the cells however, there is no significant change in the numbers of the cells after treated with OMVs compared with the control in the image (1). Image is representative phase contrast micrographs (taken at 100x magnification).

3.4.3.2 HaCaT IL-8 secretion in response to OMVs isolated from *Pseudomonas* grown in simulated wound fluid with glucose or ethanol

Following the protocol described above OMVs isolated from *P. aeruginosa* lab strain (L.) grown in SWF with either glucose or EtOH as the main carbon source was used to challenge HaCaT cells and the concentration of IL-8 secreted was quantified by ELISA.

Samples	IL-8 concentration (pg/ml)
Control	2.0
OMVs of *P.aeruginosa* (L.) in SWF	21.7
OMVs of *P.aeruginosa* (L.) in SWF+Ethanol	20.9

Table 4.5: The concentration of IL-8 (pg/ml) of HaCaT cells treated with outer membrane vesicles (OMVs) for *P. aeruginosa* lab strain in SWF with glucose or ethanol as the carbon source.

Data are represented as mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

The data above shows the concentrations of IL-8 produced by HaCaT cells after challenging with culture media in different conditions, where the control sample represents concentration of IL-8 produced by HaCaT in culture media without OMVs whereas, the other samples represent the concentrations of IL-8 which secreted from HaCaT after challenged with OMVs of *P. aeruginosa* Laboratory strain (L.) growth in Simulated Wound Fluid (SWF) and with ethanol. These data were similar to OMVs from bacteria grown in TSB

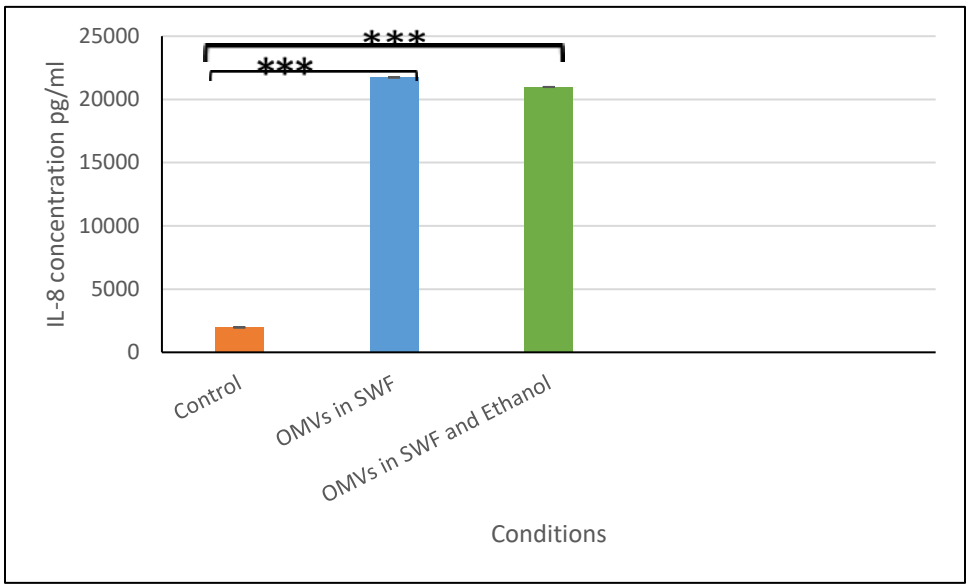

Figure 3.4.12: The concentration of IL-8 (pg/ml) of HaCaT cells treated with outer membrane vesicles (OMVs) for *P. aeruginosa* lab strain in Simulated Wound Fluid with and without ethanol.

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats:***, p<0.001 paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by HaCaT cells challenge with different concentration of (OMV) of *Pseudomonas aeruginosa* Laboratory (L.) strain.

3.4.3.3 HaCaT Biomass in response to OMVs isolated from *Pseudomonas* grown in simulated wound fluid

HaCaT were challenged with OMVs derived from PS3 and lab strain grown in SWF. For this series of experiments the quantification of OMVs was based on fold concentration of the OMVs from the culture solution they were originally isolated from ie if the OMVs were used at their original concentration this would be a 1:1 if it was a 10 fold concentration this would be 1:10.

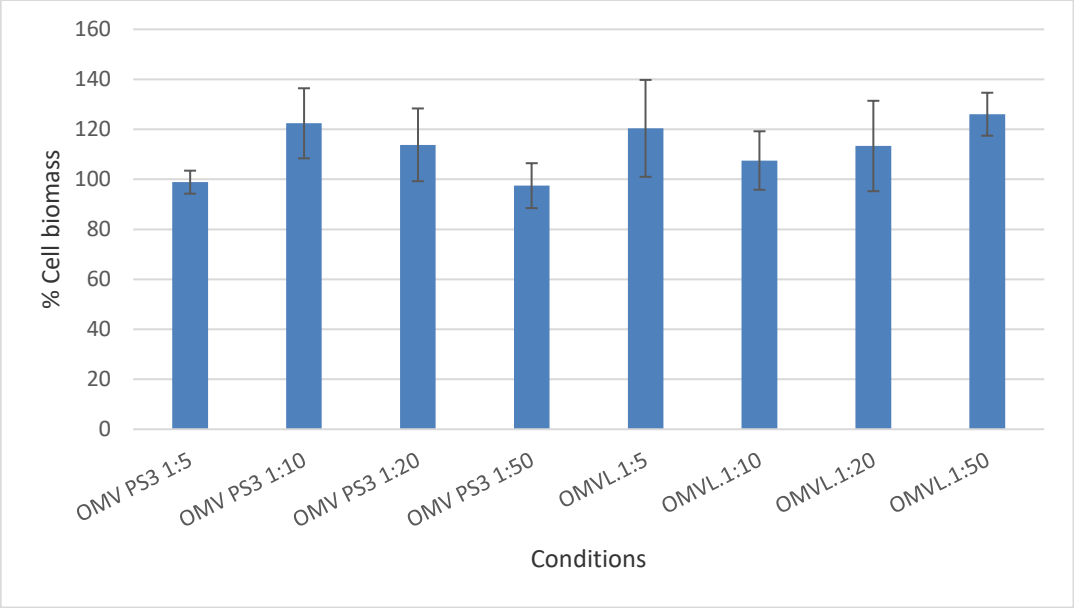

Figure 3.4.13: The average cell biomass of HaCaT cells challenged with outer membrane vesicles OMV of *Pseudomonas aeruginosa* Laboratory (L.) and Hospital strains (PS3) in SWF.

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

Although there is some small variation the OMVs from either strain of *Pseudomonas* produces little variation in biomass.

3.4.3.4 NHK Biomass in response to outer membrane isolated from both *Pseudomonas* grown in simulated wound fluid

The effect of outer membrane (OM) from *Pseudomonas aeruginosa* PS3 grown in SWF on the biomass of NHK cells was investigated.

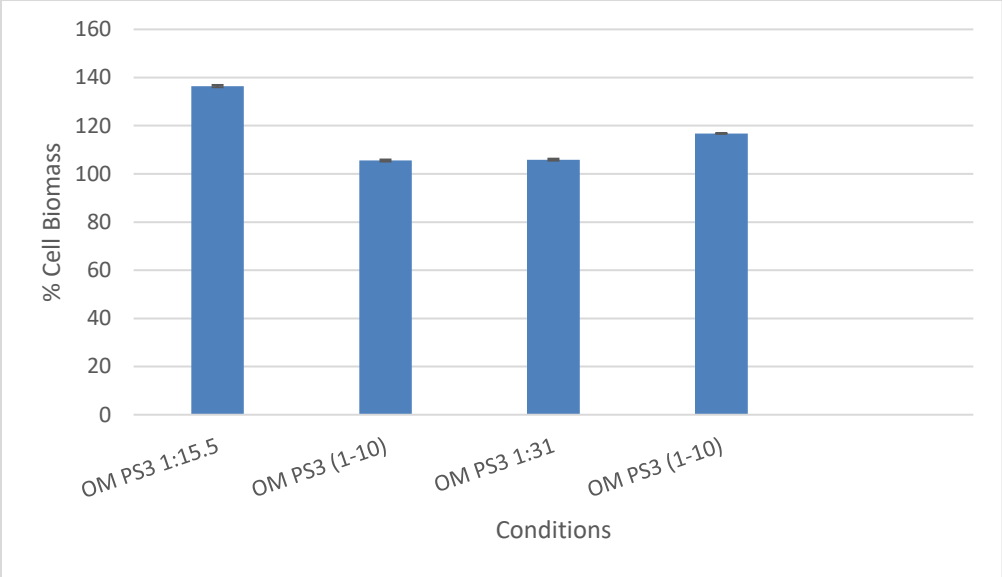

Figure 3.4.14: The average cell biomass of NHK cells challenged with outer membrane OM of *Pseudomonas aeruginosa* PS3 in SWF.

Data are represented as mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

The bar chart shows that the NHK treated cells with and 1-10 of OM Hospital strain (PS3) showed a small stimulation of biomass for 1:15.5 concentration of OM but little effect overall.

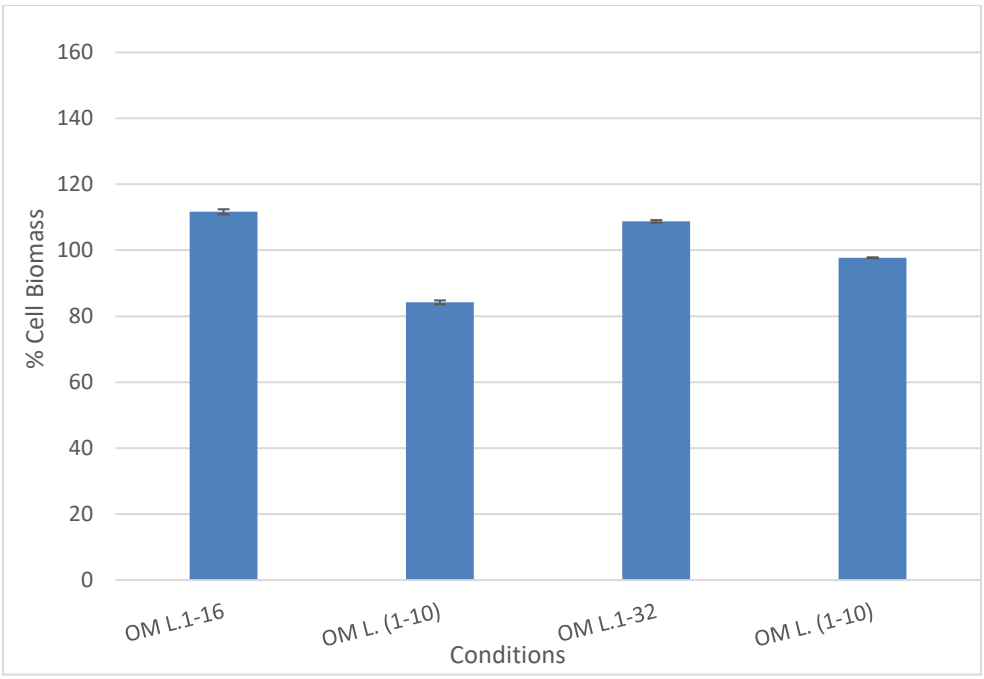

Figure 3.4.15: The average cell biomass of NHKs challenged by outer membrane OM of *Pseudomonas aeruginosa* laboratory (L.) in SWF.

Data are represented as mean values ± S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

Overall the data showed that NHKs treated with OM from *Pseudomonas aeruginosa* laboratory strain showed little overall effect on biomass.

3.4.3.5 IL-8 secretion of HaCaT cells, HaCaTa cells, NHK cells in response to outer membrane isolated from both *Pseudomonas* grown in simulated wound fluid

The effect of the OM isolated from *Pseudomonas* grown in simulated wound fluid on IL-8 secretion of the 3 cultured keratinocyte cell lines was tested.

	HaCaT cells	**HaCaTa cells**	**NHK cells**
Control	455	86.3	250
Outer membrane of *Pseudomonas aeruginosa* (L.) (1:16)	3690	395	310
Outer membrane of *Pseudomonas aeruginosa* (L.) (1:32)	3600	340	300
Outer membrane of *Pseudomonas aeruginosa* (PS3) (1:15.5)	3700	350	370
Outer membrane of *Pseudomonas aeruginosa* (PS3) (1:31)	2600	317	345

Table 4. 6: Concentration of IL-8 pg/ml for HaCaT, HaCaTa, NHK cells after expose to OM of *Pseudomonas aeruginosa* Laboratory (L.) and Hospital (PS3) strains.

There is variation in the concentration of IL-8 secreted from the keratinocytes in response to the control samples, which only contained DMEM or KSFM.

HaCaT cells were much more sensitive to OM derived from both strains of *Pseudomonas aeruginosa* than with NHK or HaCaTa cells but all three cell types responded similarly to the both PS3 and lab strain.

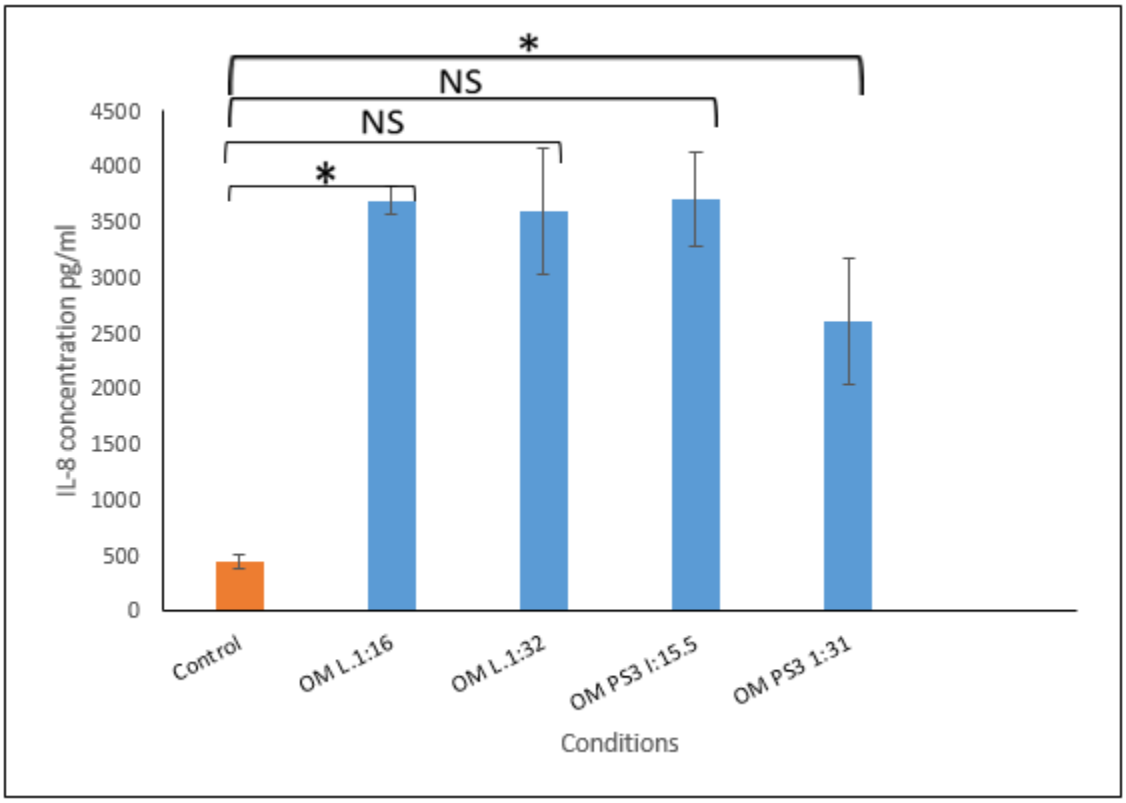

Figure 3.4.16: Concentration of IL-8 of HaCaT cells after expose them to outer membrane (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital strains (PS3).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, and *, $p<0.05$ paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by HaCaT cells challenge with different concentration of (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

There was an increase in IL-8 concentration in the solution surrounding the keratinocytes that have been challenged with outer membrane (OM) of *Pseudomonas aeruginosa* the highest concentration of IL-8 with 1:15.5 µg/ml of (OM) Hospital strain (PS3).

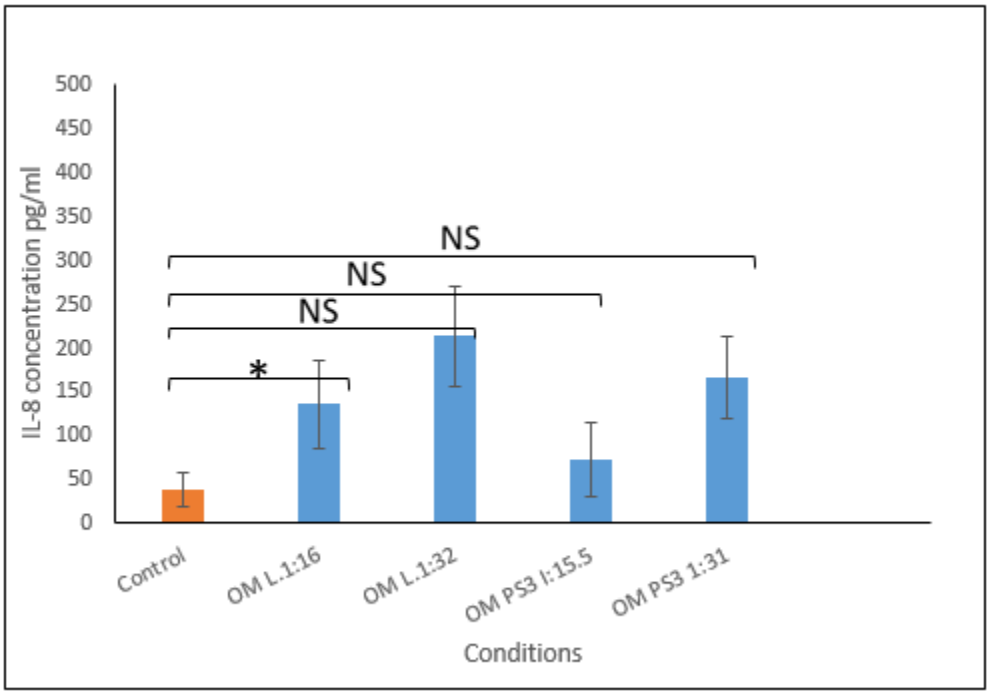

Figure 3.4.17: Concentration of IL-8 of HaCaTa cells after exposure to the outer membrane (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital strains (PS3).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, and *, $p<0.05$ paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by HaCaTa cells challenge with different concentration of (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

There was an increase in IL-8 concentration in the solution surrounding HaCaTa that have been challenged with outer membrane (OM) of *Pseudomonas aeruginosa* the highest concentration of IL-8 with 1:32 of (OM) Hospital strain (PS3).

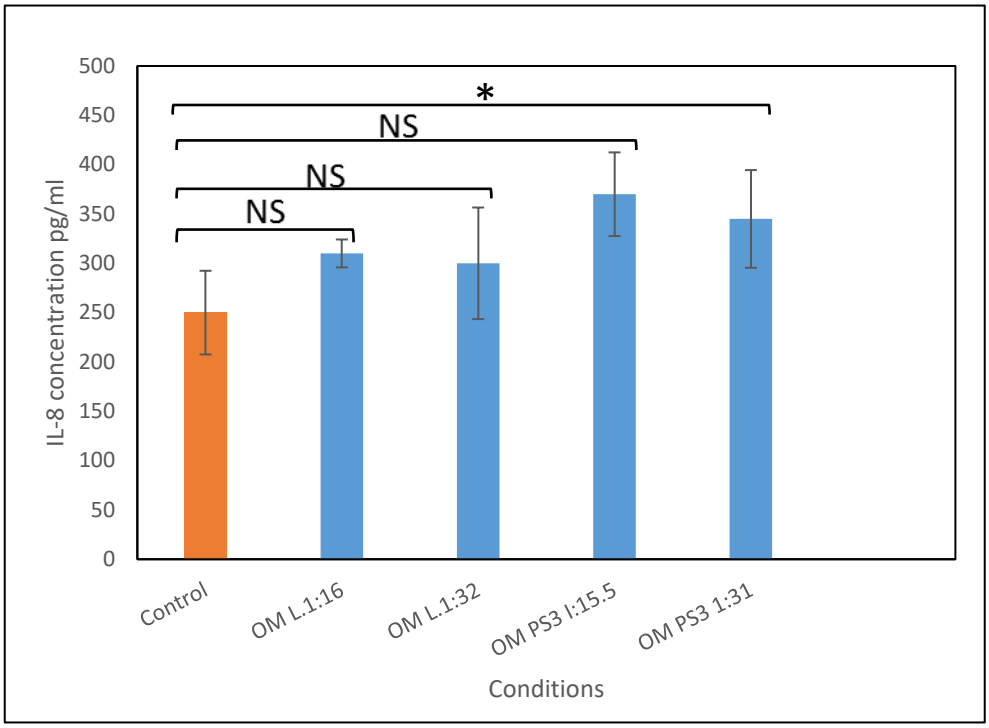

Figure 3.4.18: Concentration of IL-8 of NHK cells after expose them to outer membrane (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital strains (PS3).

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, and *, $p<0.05$ paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by NHK cells challenge with different concentration of (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

3.4.3.6 IL-8 secretion of HaCaT cells, HaCaTa cells, NHK cells in response to outer membrane vesicles isolated from both *Pseudomonas* grown in simulated wound fluid

The effect of the OMVs isolated from *Pseudomonas* grown in simulated wound fluid on IL-8 secretion of the 3 cultured keratinocyte cell lines was tested.

	HaCaT cells	HaCaTa cells	NHK cells
Control	**219**	**133**	**259**
Outer membrane vesicles of *Pseudomonas aeruginosa* (L.) (1-5)	**5621**	**286**	**218**
Outer membrane vesicles of *Pseudomonas aeruginosa* (L.) (1-10)	**3437**	**253**	**137**
Outer membrane vesicles of *Pseudomonas aeruginosa* (PS3) (1-5)	**7390**	**389**	**254**
Outer membrane vesicles of *Pseudomonas aeruginosa* (PS3) (1-10)	**6006**	**226**	**172**

Table 4.7: Concentration of IL-8 pg/ml for HaCaT, HaCaTa, NHK cells. After expose HaCaT, HaCaTa, NHK cells to OMVs of *Pseudomonas aeruginosa* Laboratory (L.) and Hospital (PS3) strains.

HaCaT produced more IL-8 in response to OMV derived from both strains of *Pseudomonas aeruginosa* than NHK or HaCaTa. In addition OMVs from PS3 stimulated a greater secretion of IL-8 than the lab strain in HaCaT.

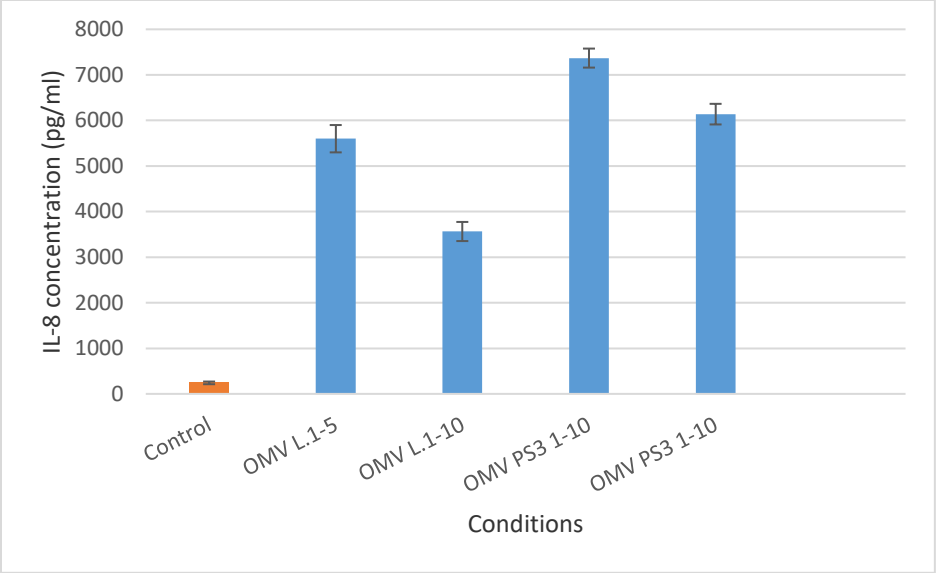

Figure 3.4.19: Concentration of IL-8 of HaCaT cells after expose them to outer membrane vesicles (OMV) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates.

A greater secretion of IL-8 from HaCaT cells occurred on challenge from PS3 OMVs

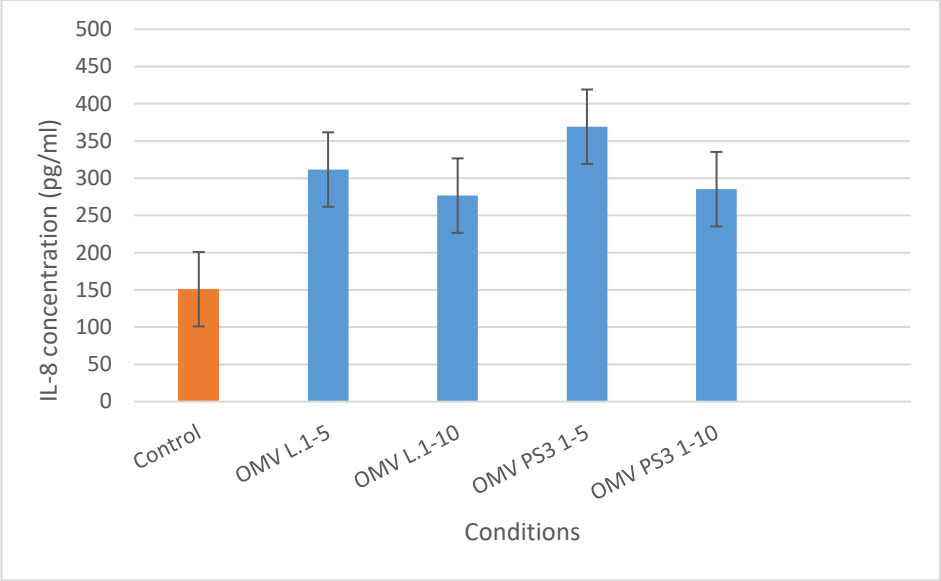

Figure 3.4.20: Concentration of IL-8 of HaCaTa cells after expose them to outer membrane vesicles of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

Data are represented as mean values ±S.E.M for three independent biological experiments, each consisting of 6-8 technical replicates.

A similar secretion of IL-8 from HaCaTa cells occurred on challenge from PS3 OMVs

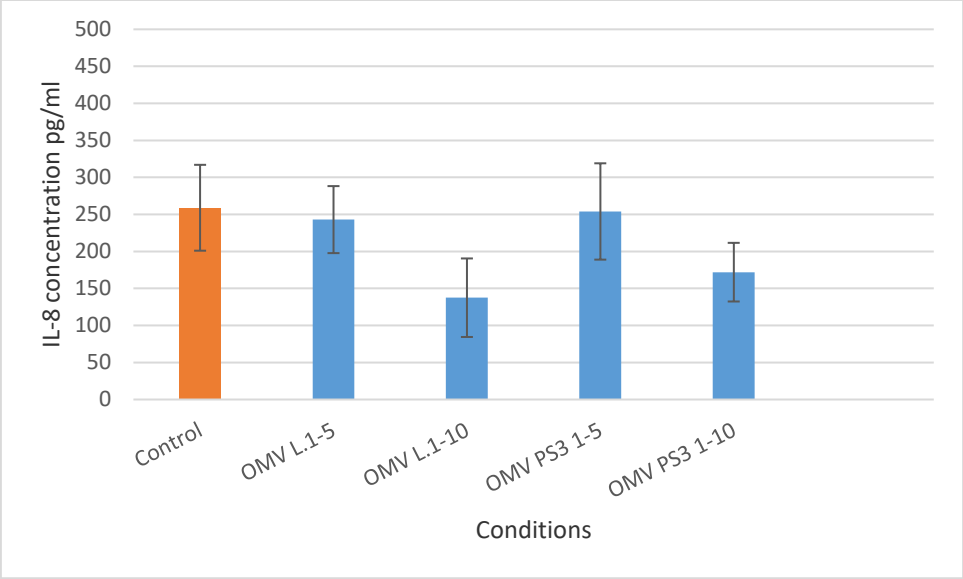

Figure 3.4.21: Concentration of IL-8 of NHK cells after expose them to outer membrane vesicles of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

Data are represented as mean values ±S.E.M for three independent biological experiments, each consisting of 6-8 technical replicates.

A similar secretion of IL-8 from NHKs occurred on challenge from PS3 OMVs

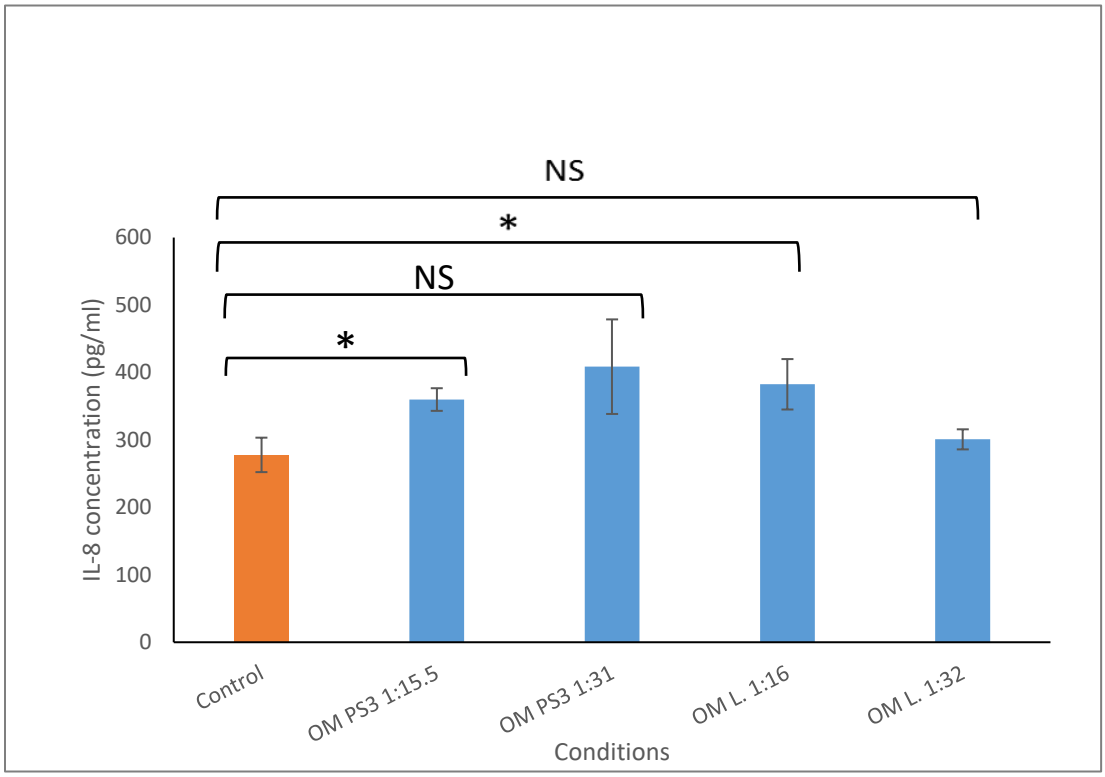

Figure 3.4.22: Concentration of IL-8 of NHK cells after expose them to outer membrane (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains. (In low concentration of Calcium). Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, and *, p<0.05 paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by NHK grown in high concentration of Calcium following challenge with different concentration of (OMVs) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

Bar graph to show the concentration of IL-8 the concentration (pg/ml) of IL-8 present in solution surrounding NHK following challenge with different concentration of (OM) or control KSFM. The results on the graph above are the average concentration of IL-8 for each sample type and the highest concentration of IL-8 with 1:31 µg/ml of (OM) Hospital strain (PS3). In low concentration of calcium.

3.4.3.6 IL-8 secretion of NHK grown in high calcium in response to outer membrane isolated from both *Pseudomonas* strains grown in simulated wound fluid

The effect of the OM isolated from *Pseudomonas* grown in simulated wound fluid on IL-8 secretion of NHK cells grown in high calcium solutions.

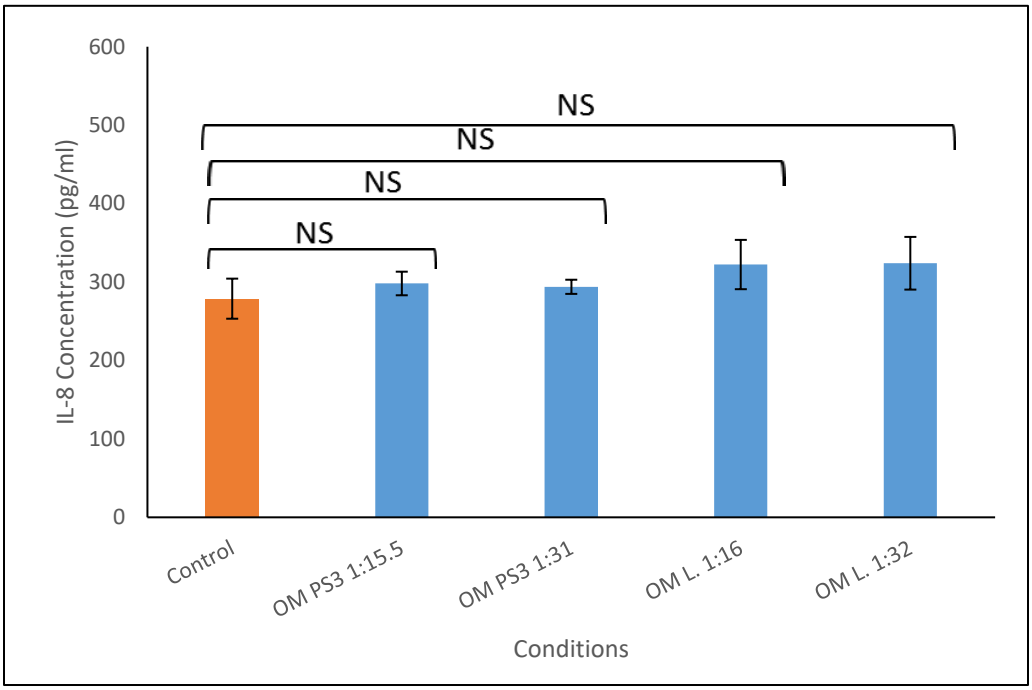

Figure 3.4.23: Concentration of IL-8 of NHK cells after expose them to outer membrane (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains. (In high concentration of Calcium). Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, paired Student T-test Control was statically compared with the concentration (pg/ml) of IL-8 released by NHK grown in high concentration of Calcium following challenge with different concentration of (OM) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

The results on the graph above are the average concentration of IL-8 for each sample type and the highest concentration of IL-8 with 1:32 µg/ml of (OM) Hospital strain (PS3). In high concentration of Calcium.

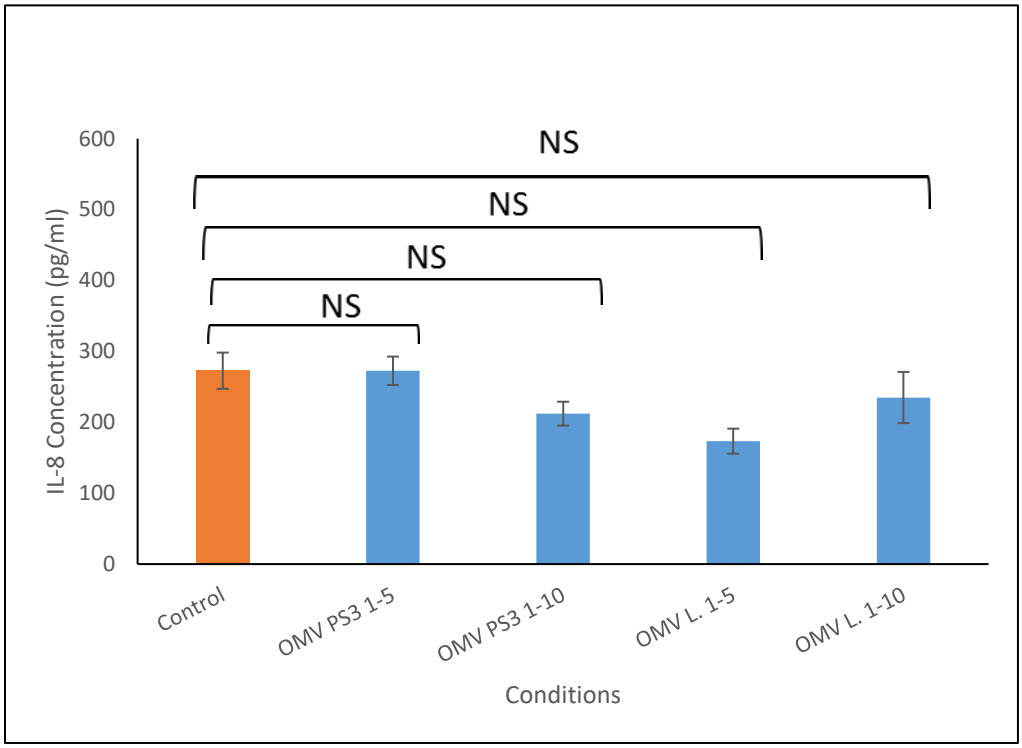

Figure 3.4.24: Concentration of IL-8 of NHK cells after expose them to outer membrane vesicles (OMV) of Pseudomonas aeruginosa Laboratory (L.) & Hospital (PS3) strains. (In Low concentration of Calcium). Data are represented as mean values ±S.E.M. for three independent biological experiments, each consisting of 6-8 technical replicates. Stats: NS. Non-significant, paired Student T-test Control was statically compared with the concentration (pg/ml) of IL-8 released by NHK grown in low concentration of Calcium following challenge with different concentration of (OMVs) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

The bar chart shows that NHK produced the highest concentration (pg/ml) of IL-8 when exposed to 1-5 µg/ml of OMV Hospital strain (PS3). In low concentration of calcium).

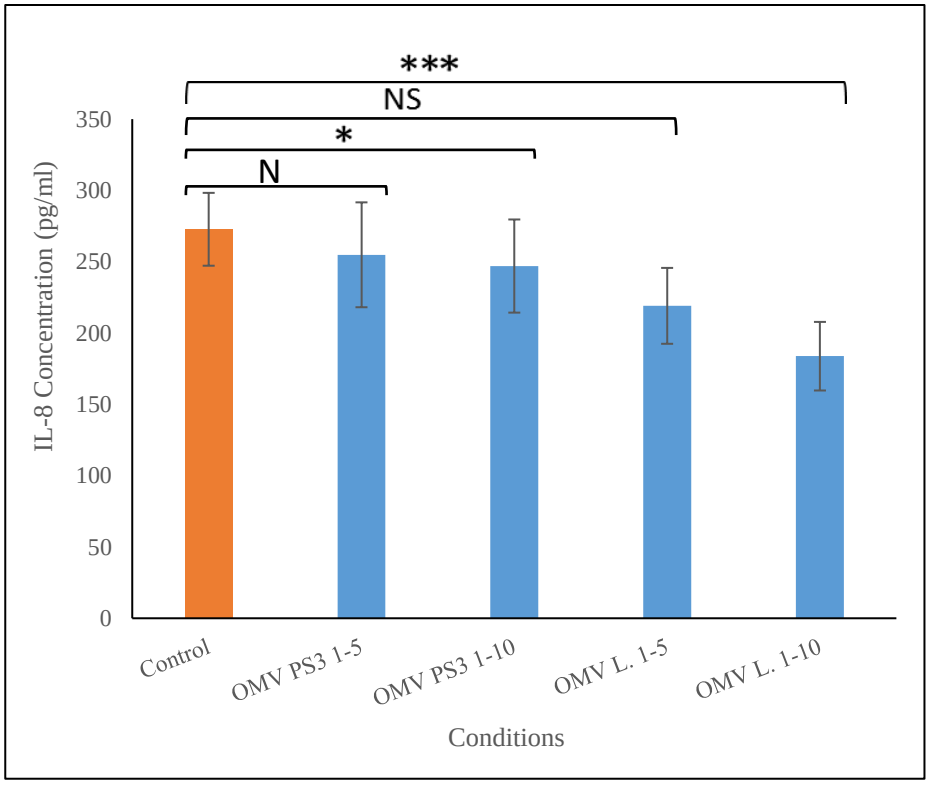

Figure 3.4.25: Concentration of IL-8 of NHK cells after expose them to outer membrane vesicles (OMVs) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains. (In high concentration of Calcium).

Data are represented as mean values ±S.E.M. for three independent biological experiments, **each consisting of 6-8 technical replicates.** Stats: NS. Non-significant, *, p<0.05 and ***, p<0.001paired Student T-test Control compared with the concentration (pg/ml) of IL-8 released by NHK grown in high concentration of Calcium following challenge with different concentration of (OMVs) of *Pseudomonas aeruginosa* Laboratory (L.) & Hospital (PS3) strains.

The bar chart shows the concentration (pg/ml) of IL-8 present in solution surrounding NHK grown in high concentration of Calcium following challenge with different concentration of (OMVs) or control KSFM. The results show that culturing NHK in high calcium had little effect on their response to OMVs

Section 5

IL-8 of supernatant HaCaT challenge with cell free *Pseudomonas aeruginosa* culture media

5.1 HaCaT challenge with cell free *Pseudomonas aeruginosa* culture media

The HaCaT cells were challenged with the cell free growth media Dulbecco's Modified Eagle Medium (DMEM) or Tryptone Soy Broth (TSB) after 24 hours of bacterial growth the controls were cells left in culture media of DMEM or TSB that had been placed in an incubator at 37°C for 24 hours.

Sample	Concentration of IL-8 (pg/ml) in Control	Concentration of IL-8 (pg/ml) in (with *P.aeruginosa*)
Laboratory Strain (L.) in TSB	794	7795
Laboratory Strain (L.) in DMEM	997	842
Hospital Strain (PS3) in TSB	1022	8732
Hospital Strain (PS3) in DMEM	845	8451

Table 3.5. 1: The average concentrations of Interlukine-8 (IL-8) after expose the HaCaT cells to supernatant of *Pseudomonas aeruginosa* Laboratory (L.) and Hospital strains (PS3).

The averages include all results across the sample type. Control samples are those collected from the challenge of keratinocytes with Dulbecco's Eagles Medium alone. Samples "with *P. aeruginosa*" are those that were obtained from the solution surrounding the cultured keratinocytes challenged with supernatant from the growth of *Pseudomonas aeruginosa* in the same growth media.

Keratinocytes HaCaT cells challenged with the supernatant from the growth of laboratory strain in Dulbecco's Modified Eagles Medium (DMEM) show little difference in the concentration the proinflammatory cytokine IL-8 secreted when compared to control sample. The control, which followed the challenge with DMEM only, show that

there is an increase in contained a higher concentration of IL-8, containing 997pg/ml, than the sample that challenged with bacterial growth media, which contained 842pg/ml (table 3.5.1).

All other samples assayed contained a higher level in IL-8 concentration in the solution surrounding the keratinocytes that have been challenged with supernatant from growth media which contained *Pseudomonas aeruginosa (*figure 3.5.1and 3.5.2).

Samples of cultured HaCaT cells that exposed to the supernatant from the growth of Hospital strain (PS3) in (DMEM) showed the highest concentration of IL-8. Also, both control samples from this challenge are higher than the other samples in comparison.

When the bacteria were grown in tryptone soy broth (TSB) there was a higher concentration of IL-8 present in the samples following the challenge with supernatant from laboratory strain growth media when compared to Hospital strain (PS3) in TSB. When looking at results (table 1) it is clear that there is variation in the level of IL-8 secretion following the challenge of the keratinocytes with DMEM. The highest and lowest concentrations are both from samples alongside the samples from the challenge with Hospital strain (PS3).

The data indicates a relatively high spontaneous release of IL-8 but a significant increase when exposed to cell free bacterial supernatant in all conditions apart from the laboratory strain when grown in DMEM. In addition, there was a greater secretion of the proinflammatory cytokine IL-8 on exposure to the PS3 strain and this was particularly evident when grown in TSB.

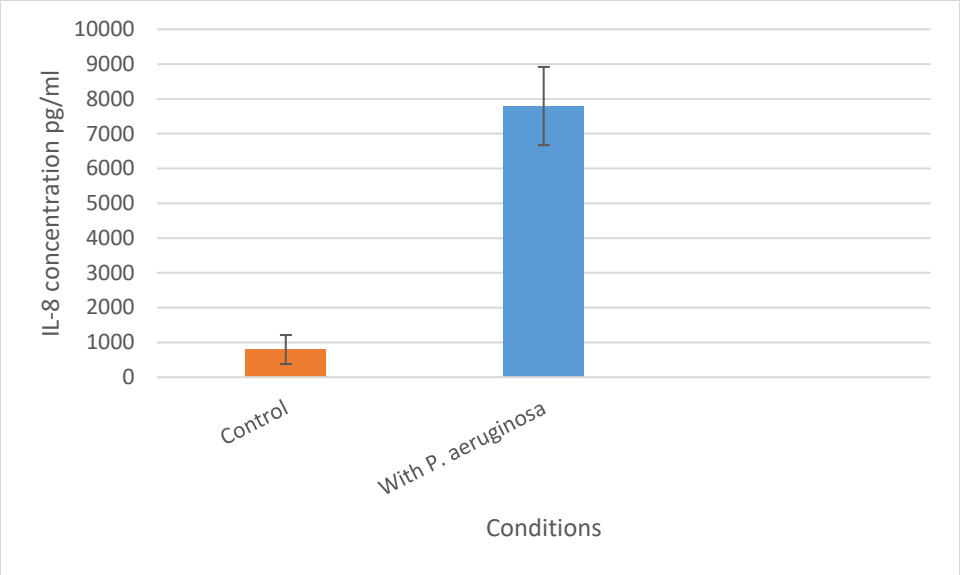

Figure 3.5.1: Concentration of IL-8 (pg/ml) in supernatant of Laboratory strain (L.) in TSB.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control DMEM. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ± S.E.M.for three independent biological experiments.

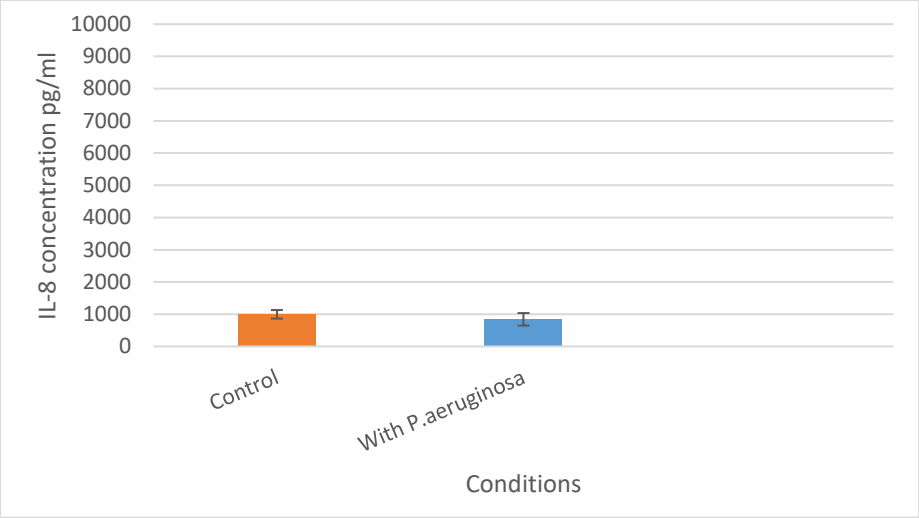

Figure 3.5.2: Concentration of IL-8 (pg/ml) in supernatant of Laboratory strain (L.) in DMEM.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control DMEM. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ±S.E.M.for three independent biological experiments.

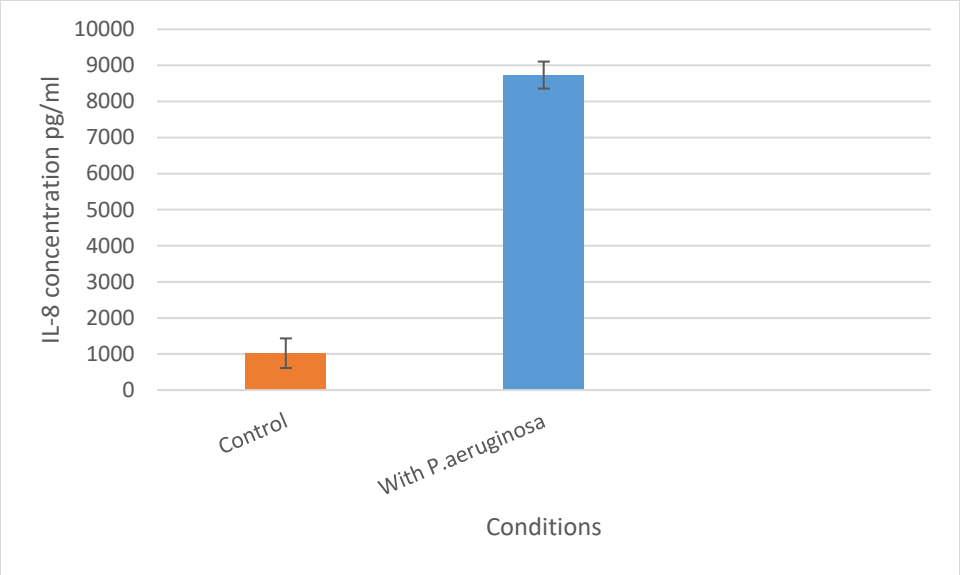

Figure 3.5.3: Concentration of IL-8 (pg/ml) in supernatant of Hospital strain (PS3 strain) in TSB.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control DMEM. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ±S.E.M.for three independent biological experiments.

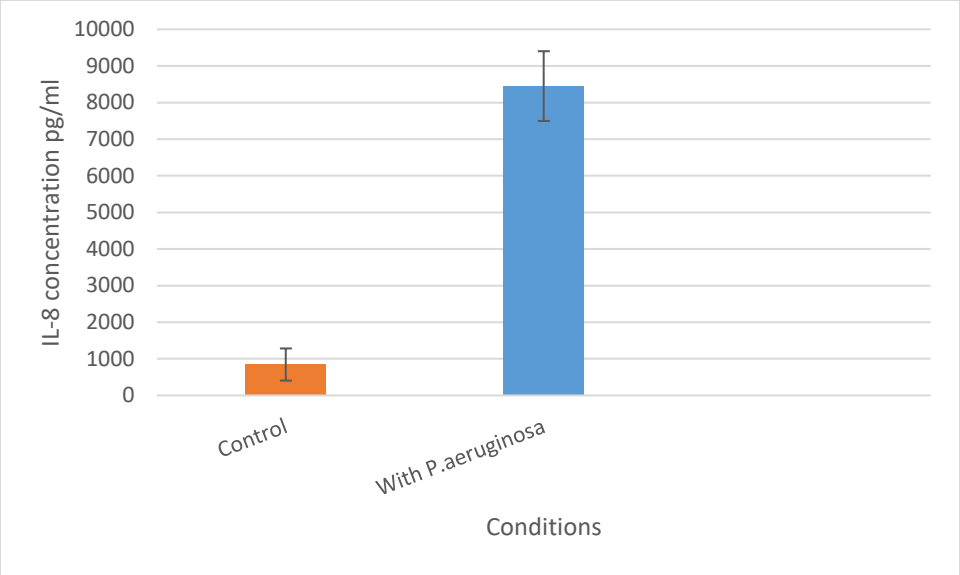

Figure 3.5.4: Concentration of IL-8 (pg/ml) in supernatant of Hospital strain (PS3) in DMEM.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control DMEM. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ±S.E.M.for three independent biological experiments.

Sample	Concentration of IL-8 (pg/ml) in Control	Concentration of IL-8 (pg/ml) in (with *P.aeruginosa*)
Hospital Strain(PS3) in DMEM	**1548.3**	**8925.5**
Hospital Strain(PS3) in TSB	**577.1**	**7022.3**
Hospital Strain(PS3) in TSB	**577.1**	**10130.77**

Table 3.5.2: The average concentrations of Interlukine-8 (IL-8) after expose the HaCaT cells to supernatant of *Pseudomonas aeruginosa* Hospital strains (PS3).

The averages include all results across the sample type. Control samples are those collected from the challenge of keratinocytes with Dulbecco's Eagles Medium (DMEM) and (TSB). Samples "with P. aeruginosa" are those that were obtained from the solution surrounding the cultured keratinocytes challenged with supernatant from the growth of *Pseudomonas aeruginosa* Hospital strains (PS3) in the same growth media.

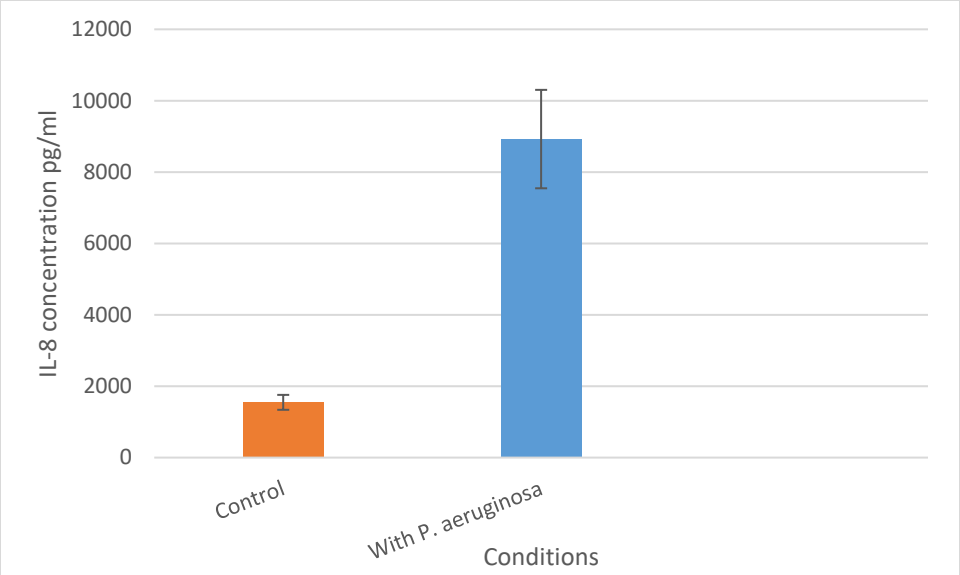

Figure 3.5.5: Concentration of IL-8 (pg/ml) in supernatant of Hospital strain (PS3) in DMEM.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control DMEM. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ±S.E.M.for three independent biological experiments.

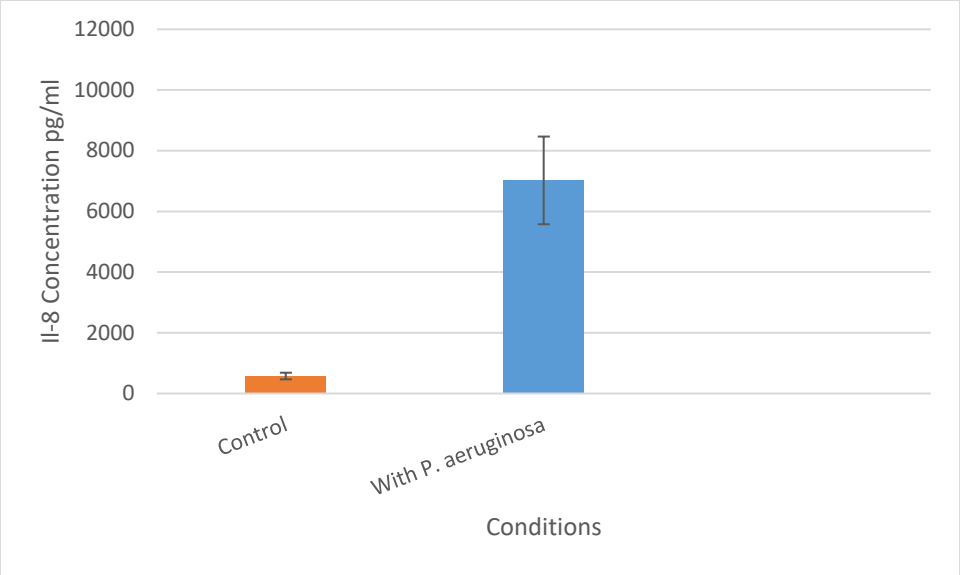

Figure 3.5.6: Concentration of IL-8 (pg/ml) in supernatant of Hospital strain (PS3) in TSB.

A graph showing the concentration (pg/ml) of IL-8 present in solution surrounding keratinocytes following challenge with growth media or control TSB. The results on the graph above are the average concentration of IL-8 for each sample type. Data are represented as mean values ±S.E.M.for three independent biological experiments.

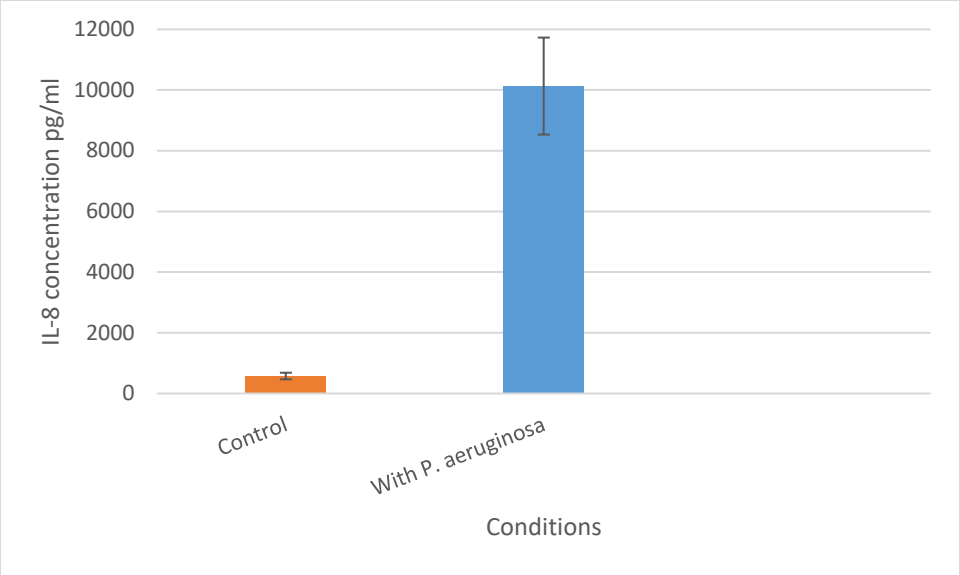

Figure 3.5.7: Concentration of IL-8 (pg/ml) in supernatant of Hospital strain (PS3) in TSB.

Bar chart showing the concentration of IL-8 present in samples of keratinocytes exposed to medium only (Control) and keratinocytes in cell culture medium: bacterial solution of TSB-grown *P. aeruginosa* solution. 1:10 dilutions were used to find the actual concentrations. (1:50) Data are represented as mean values ± S.E.M.for three independent biological experiments.

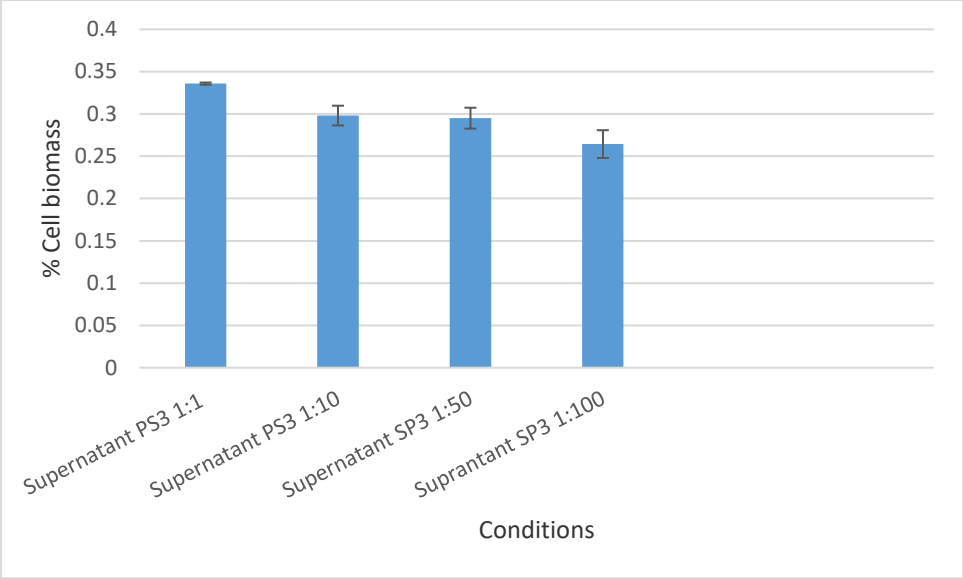

Figure 3.5.8: Biomass of HaCaT cells, exposé to supernatant of *P. aeruginosa* Hospital (PS3) strains.

The bar chart shows that the treated cells with supernatant 1:100 of Hospital strain (PS3) showed a loss of mass where the other concentration showed less dead cells. Data are represented as mean values ± S.E.M. for three independent biological experiments.

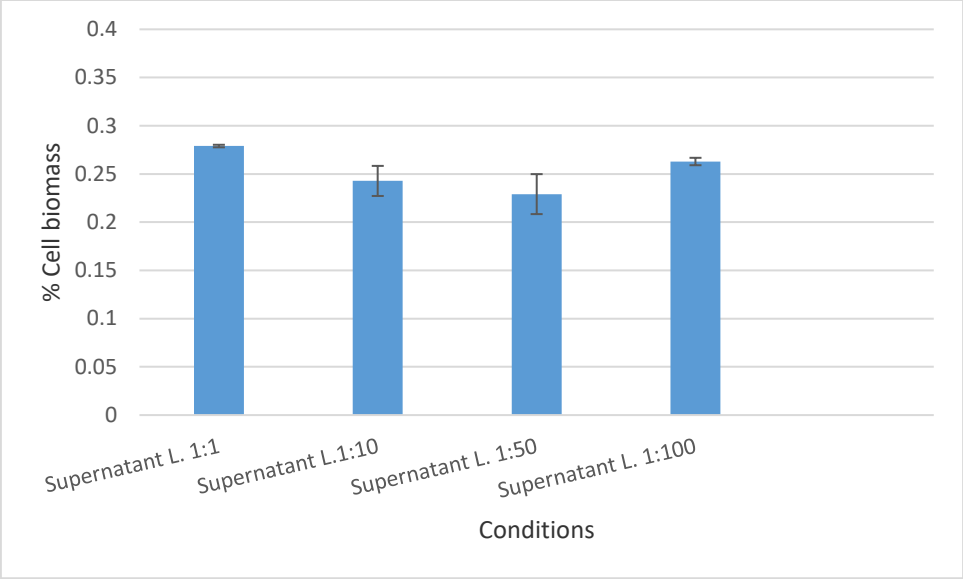

Figure 3.5.9: Biomass of HaCaT cells, exposé to supernatant of *P. aeruginosa* Laboratory (L.) strains.

A graph shows that the treated cells with supernatant 1:50 of Laboratory strain (L.) showed a loss of mass where the other concentration showed less dead cells. Data are represented as mean values ± S.E.M. for three independent biological experiments.

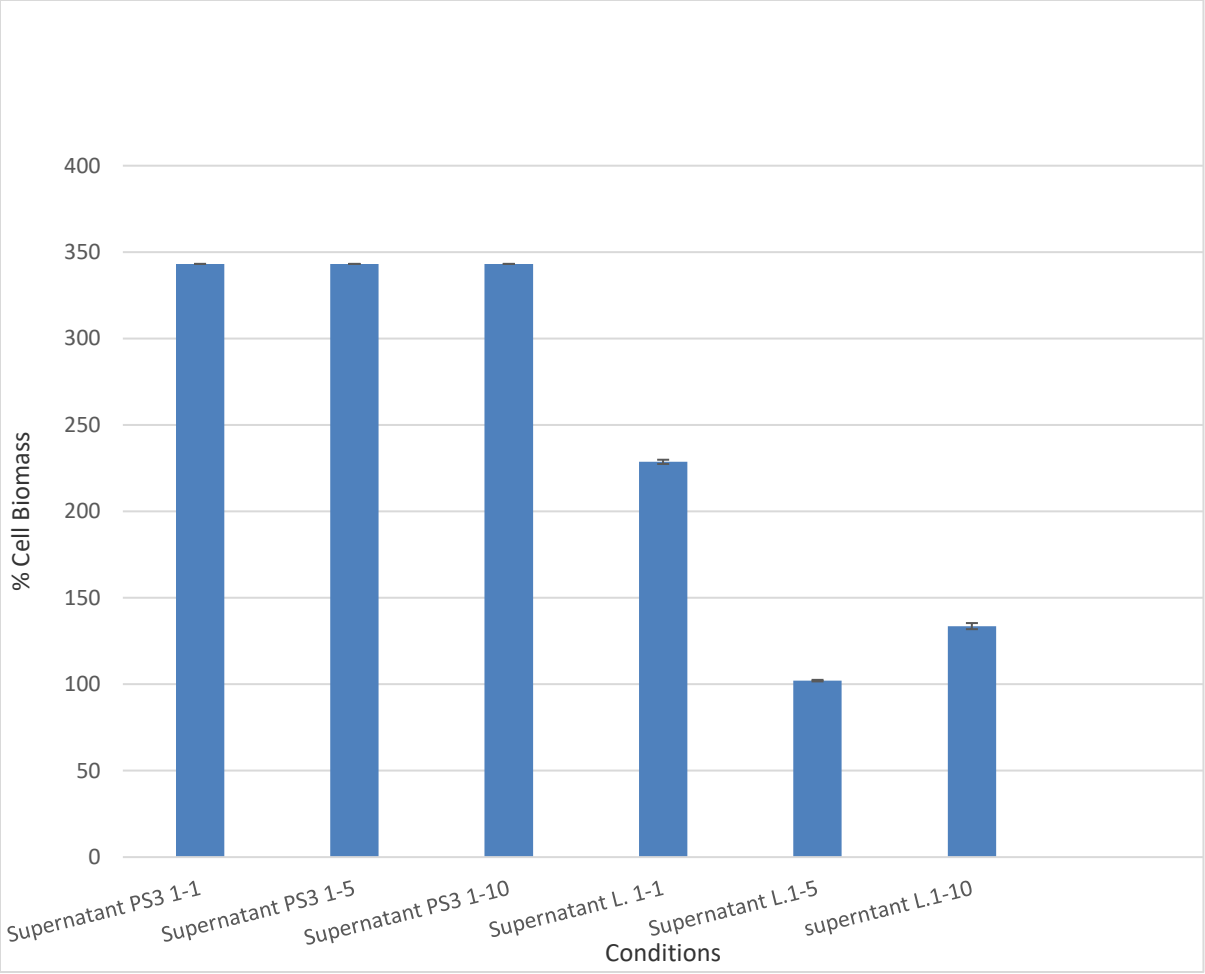

Figure 3.5.10: Biomass of HaCaTa cells, exposé to supernatant of *P. aeruginosa* Laboratory (L.) and Hospital (PS3) strains.

The bar chart shows that the treated cells with supernatant 1:5 of Laboratory strain (L.) showed a loss of mass where the other concentration showed less dead cells. Data are represented as mean values ± S.E.M. for three independent biological experiments.

Chapter 4

Discussion

Section 1

4.1 HaCaT, HaCaTa and NHK cells:

HaCaT, HaCaTa and NHK cells have been shown to possess different capacities for IL-8 release according to the factors to which they are exposed (various concentration of OMVs and supernatant of *P. aeruginosa* and the outer membrane OM of this bacteria for both clinical or laboratory strains). This *in vitro* study used HaCaT cells which are immortal non-tumorigenic keratinocytes (Wilson, 2013). HaCaT is abridgement for (Ha = human adult, Ca = calcium, T = temperature) to appoint its provenance and the original culture milieu (Boukamp, 1988). The HaCaT cell line is mainly used as a keratinocyte model because of its ability to proliferate and differentiate forming an epidermal community *in vitro* (Schoop et al., 1999). Keratinocyte differentiation requires a complicated group of processes characterized by a normal and equal level of growth and differentiation. The HaCaT cell line became spontaneously immortalised (Micallef et al., 2009).

The HaCaTa cells used in this study were adapt from HaCaT by the use of a stepwise change of the culture media of HaCaT cells from a serum-supplemented media with a high calcium concentration to a serum-free low calcium media ie keratinocyte serum free media (KSFM), when HaCaT cells had totally adapted to the new culture medium and they were named HaCaTa (Al-Tameemi et al., 2014).

Normal Human Keratinocyte (NHK) being a primary cells are somewhat more complicated to culture and have a much shorter period where they are viable in culture than the 2 cell lines (Micallef et al., 2009).

The epidermis is an important location of microorganism interactions with keratinocytes and host defences (Wingens et al., 1998). Epidermis keratinocytes do not compose a passive barrier, it mediates a controlled cutaneous inflammation and functions as part and innate immune system and can also activate an adaptive immune response (Frohm et al., 1997). In order to model these interactions it was proposed that the human keratinocyte cell line HaCaT was used. However, for these cells to represent a viable model of bacterial interactions with the skin they, as a minimum, must express a similar range of receptors that recognise bacterial products, as normal keratinocytes. In

order to establish that the HaCaT cells cultured here maintain these receptors it was determined if the cells expressed mRNA for a range of TLRs.

The initial study demonstrated that both that HaCaT cells grown for either 4 or 10 days expressed a range of TLRs needed for the identification and response to a vast range of bacterial antigens. The receptors expressed were TLR1, TLR2, TLR4, and in both aged cells. In contrast TLR5, TLR9 were found just in 10 days cells. The expression of a range of TLRs has previously been reported in HaCaT cells (Köllisch et al., 2005). Most expression at the mRNA level was found for TLR2 and TLR4 when the cells were grown for either 4 or 10 days.

4.1.1 Function of TLR:

TLR1 and TLR2 recognise various bacterial cell wall elements for example peptidoglycan (PGN) is recognized by TLR2 (Schwandner et al., 1999). The first human homologue of Toll to be discovered was TLR4 (Medzhitov et al., 1997) and was thereafter considered as a receptor for LPS signalling pathway (Poltorak et al., 1998). It is now known that TLR4 consists of a family that can recognize many ligands for example the fusion molecule of a protein that is synthesized by a respiratory syncytial virus and taxol subunits in the mouse. It has been reported that LPS activity is mediated by TLR4 in primary human keratinocyte cells. However this is a controversial data because other groups have failed to find such expression (Smith et al., 2003, Kawai, 2003). Besides pathogen-associated molecular patterns (PMPS) TLR4 has been reported to also identified host damage associated molecular patterns (DAMPS) (Akira et al., 2001) an example of which is serum amyloid A.

In order to function some TLR such as TLR4 require the binding of a co-receptor known as CD14. Cluster of differentiation 14 (CD14) is a human gene that was first recognized on the surface of both monocytes and macrophages (Griffin et al., 1981). It is now known that CD14 acts as a pattern recognition receptor in innate immune system and recognizes different ligands, from apoptotic cells and fungi to the products of bacteria such as components of microbial cell walls and whole bacteria (Savill et al., 1990, Devitt

et al., 1998). CD14 considered as homing receptor for the LPS component endotoxin of Gram-negative bacteria (Wright et al., 1990) #2041; Ziegler-Heitbrock, 1995 #2042}.

It has been reported that TLR4 and CD14 are expressed by both HaCaT and primary human keratinocytes and are activated by LPS (Song et al., 2002, Pivarcsi et al., 2003, Pivarcsi et al., 2004) Our result showed that TLR4 mRNA is found in HaCaT cells, however, by contrast Kawai, 2003 showed no TLR4 expression in these cells. Interestingly TLR4 expression has been found to be correlated to keratinocyte differentiation (Pivarcsi et al., 2004) so this might explain these discrepancies.

TLR5 is an independent subfamily of the mammalian group of Toll homologues that recognizes the flagellin protein of bacteria through a specific part on the flagellin protein that is needed for motility of the bacteria (Smith et al., 2003); (Hayashi et al., 2001). Specifically it was reported that TLR5 is expressed in HaCaT cells grown for 10 days.

It has been elucidated that for TLR9 a TLR9-CPG DNA interaction often take place after TLR9 recruitment from the endoplasmic reticulum to a tubular lysosome structure (Latz et al., 2004).

Interestingly (Köllisch et al., 2005) has suggested that there are differences in the functional expression of some TLRs and TLR cofactor components between HaCaT cells and primary keratinocytes these data illustrate the caution required when extrapolating data from immortalized cell lines and primary cells into tissue. Despite this it is clear that the variety of TLR expression in keratinocytes indicates that these cells could act as sentinels of skin homeostasis.

4.1.2 Role of TLR in skin Inflammation:

In addition to their role in wounds PAMPs could play important in skin inflammation conditions. Inflammatory cutaneous diseases such as atopic dermatitis and psoriasis that disturbed the physical skin barrier and may affect the state of the immune barrier (Hatano et al., 2013). TLR participate in the stimulation of immune activity in infection and inflammation of the skin (Kupper and Fuhlbrigge, 2004, Leung, 2000). According to

Panzer et al., 2014 TLR2 was expressed in the suprabasal layers in diseased skin, but its expression is restricted to the basal epidermis in normal skin. This change in the TLR expression may be due to disturbed skin barrier function and need to boost the immune defence because of the skins increased vulnerability to invading of pathogens.

Calcium is a main regulator of the differentiation of normal keratinocyte, and stimulated the formation of desmosome, adherens junctions and tight junctions. In part this is by the stimulation of calcium receptors that stimulate cellular signalling pathways which are necessary for differentiation (Kobashi et al., 2017a). In connective tissues, intercellular adhesion is essential for tissue morphogenesis, development and wound healing (Ko et al., 2001).

It is established that Ca^{2+} ions are an essential part to a complex intracellular messenger that mediates vast sets of biological processes including: muscle contraction, secretion, glycolysis and gluconeogenesis, ion transport, division and growth of the cells (Forsen and Kordel, 1994).

The importance of extracellular calcium in epidermal differentiation has been recognized for many years (Savignac et al., 2011). An increase in extracellular calcium concentration enhances the intracellular levels of free calcium which promote the differentiation of keratinocyte (Pillai and Bikle, 1991).

NHK cells are the principle cell culture model to study the physiological mechanisms of human keratinocyte differentiation mechanisms that can be activated by supplements the in the media including calcium (Garach-Jehoshua et al., 1998). The explanation of the process of normal keratinocyte growth and differentiation in vitro is still insufficient understood in part because they have slow growth rates and are relatively complicated to handle. To aid in vitro keratinocyte research a spontaneously immortalized human keratinocyte cell line is often used as a keratinocyte model because their capacity for proliferating and differentiating (Boukamp et al., 1988). These HaCaT cells are capable of maintaining a balance between differentiation and growth, in part, because cells they are immortalized.

When normal keratinocyte cells were cultured in low calcium (LC) medium (0.09 mM), cells became more proliferative and only had a few cell-cell contacts without any desmosomes formation (Watt et al., 1984).

Data in this study that was supported by morphological observations by contrast phase microscopy (Figure 1.8) indicated that in LC medium there was a steady increase in cell growth in NHK cells and they became flattened and spread-out. NHK proliferation decreased with high calcium (HC) KSFM (1.2mM), specifically cell growth was slowed by about 30% at day 3 and this congruent with on-going process of differentiation as observed by the small and cobble-stoned appearance of the cells in HC medium. This is in agreement with other studies that have shown that NHK proliferation can be prevented and terminal differentiation initiated by increasing the calcium concentration (Kolly et al., 2005a). It has been confirmed that when NHK are incubated in physiological concentrations of calcium (1-1.5 mM) this results in an intracellular calcium concentration that can slow cell proliferation (Sakaguchi et al., 2003). Similar results obtained (Dr. Christopher Dunnill, 2017- personal communication). The influence of calcium on intercellular adhesion and cell morphology such as cell-cell contacts has also been shown by Micallef *et al* (Micallef et al., 2009)

Discussion

Section 2

4.2 Isolation and purification and yield of outer membrane vesicles

Outer membrane vesicles were successfully isolated from both strains of the Gram negative bacteria *P. aeruginosa* using a standard centrifugation technique. To help verify this the OMVs were visualised using transmission electron microscopy. As a comparison and to check the isolation method could isolate Gram negative OMVs vesicles from an enteric bacteria *E coli* C25 was also isolated. This bacterium had previously been reported to secrete abundant OMVs (Patten ***et al*** *'*17)

The yield of outer membrane vesicles has an inverse relationship with purification process where the amount of OMVs reduces as the purity increasing, based on this the volume of OMVs isolated from *P. aeruginosa* was relatively small due to the levels of purification (Chutkan et al., 2013). The production of sufficient vesicles of a high purity in a reproducible manner remains a critical challenge for their analysis in most bacteria (Klimentová and Stulík, 2015) the precise purification protocol and subsequent analysis the OMVs production might determine the resulting data. It has previously been shown that, there are many factors that control the production of outer membrane vesicles such as the temperature, the number of bacteria and the type of media used grow the bacteria.

Choi *et al* stated that the size of the filter used in filtration step plays a profound role in the yield of OMVs (Choi et al., 2011). The pore size of filters used in this study was 0.22µm and the diameter of outer membrane vesicles are approximately 10-250nm, as result: some of these OMVs are blocked by the filter which will affect the yield of OMVs of both strains (Hospital and Laboratory) of *P. aeruginosa.* Also temperature plays a fundamental role in the production of vesicles, higher temperatures leads to an increase in the secretion of vesicles because temperature affects the cell membrane proteins and makes them less stable which activates various signalling pathways within the membrane, which in turn affects the production of vesicles. In addition, higher temperatures make the membrane more fluid which may lead to a greater release OMVs from outer membrane (Kulp and Kuehn, 2010). In this research *P. aeruginosa* was grown at 37°C which is considered to be the optimal temperature for growth however, it is not known if this temperature has impact yield of OMVs.

Here we used the protein composition of the OMVs to estimate the concentration of the OMVs isolated. This has the advantage of being quick and reliable, however, it has to be acknowledged that if vesicles from different strains of bacteria or when grown in different conditions had a higher content of protein than this method could not distinguish between this or an increase in the production of vesicles. When the vesicles are quantified on the basis of protein it is was clear that the yield of OMVs derived from *P. aeruginosa* laboratory strain (L.) was increased when cultured with the antibiotic gentamycin which is considered to be a stress factor and this is in agreement with Ellis and Kuehn (Ellis and Kuehn, 2010). In addition the increase in yield stimulated by some antibiotics could at least in part be explained by the fact that they target the outer membrane (Hancock et al., 1981), however, this is not the case with gentamycin, and its target is ribosomal.

Here the production of OMVs from Hospital isolate (PS3) in control conditions was greater than the production of OMVs from the laboratory strain by contrast, there was a greater production from the Laboratory strain (L.) on treatment with gentamicin compared to the Hospital strain (PS3) without treatment. It has been suggested that the production of OMVs is an important mechanism by which bacteria can save themselves from the effect of antibiotics. Kukavica-Ibrulj et al. stated that the clinical isolate (PS3) is more toxic than the Laboratory strain (10421) (Kukavica-Ibrulj et al., 2008) and the greater secretion of OMVs compared to the Laboratory strain (L.) 10421. The greater production of OMVs could increase the virulence of PS3 because OMVs are known to deliver virulence factors to other cells this will be discussed further below. Poole, 2005 also suggested that the OMVs increase resistance to antibiotics, particularly aminoglycosides, by the protein OprH. The same report showed that bacteria with OprH knockout mutations have compromised resistance to aminoglycoside because of its impact on the expression of the phoQP genes, which leads to a change the lipid A of lipopolysaccharide (LPS), and then the modification of the LPS component of the outer membrane which is involved in resistance to aminoglycoside (Poole, 2005).

The CFU of Hospital strain (PS3) was slightly lower than the unchallenged Laboratory stain (L.). The production of OMVs depends on many factors of stress including: the

CFU of the bacteria, temperature, the nutrition factors in the media and other stress inducers of vesiculation such as the biosynthesis of the cell wall inhibitor D-cycloserine and the OM targeted antimicrobial peptide polymyxin B raises the generated OMV in *P. aeruginosa* (Klimentová and Stulík, 2015).

OMVs derived from *P. aeruginosa* Laboratory isolate (L.) which are grown in SWF with ethanol was higher than the protein concentration that produced from OMVs isolated from *P. aeruginosa* Laboratory isolate (L.) which grown in SWF without ethanol (Table 2.9), this provides evidence that the yield of vesicles was higher in the presence of ethanol in culture media in comparison to *P. aeruginosa* grown in SWF without ethanol which suggests that the ethanol increased the yield of OMVs. It was shown that *P. aeruginosa* PS3 also produced more OMV when it was grown in the culture media contains ethanol and in this case these would be potentially pathogenic.

4.2.1 SDS-PAGE Analysis Outer Membrane Vesicle Proteins

In order to compare the proteins in the OMVs isolated from the 2 strains of *P. aeruginosa* grown in different culture conditions SDS-PAGE was used to compare proteins based on their molecular weight (MW). Here it was showen that OMVs derived from *P. aeruginosa* grown in SWF without ethanol have similar proteins to OMVs derived from *P. aeruginosa* grown in SWF with ethanol. The resultant of gel image has six protein bands which were tentativly identified based on their molecular weight. Three outer membrane proteins were identified by comparing their molecular weight to the known *P*. *aruginosa* outer membrane proteins on the proteomic database for *P*. *aruginosa* proteins that have been identified include FpvA, OprC and XcpQ.

4.2.1.1 Ferripyoverdine receptor FpvA

Many species of *Pseudomonas* produce pyoverdine siderophores. Pyoverdine is a 6, 7-dihydroxyyquinolone containing a fluorescent compound which is linked to a partly cyclic octapeptide and has an affinity to Fe^{3+}. It is the main siderophore that traffics iron from transferrin or serum to bacteria *in vivo*. Siderophores are chemically heterogenic and each bacterium tends to use its own siderophore and is less able to utilize others. This specificity is mediated at the level of the outer membrane receptors and gated porins (Hoffmann et al., 2002b).

FpvA is a receptor for pyoverdine which co-purifies with iron-free pyoverdine in *P. aeruginosa* and has no role in active transport. Iron-free pyoverdine is dislodge by Ferric-pyoverdine with the internal kinetics and as result the complexes PpvA-pyoverdine-Fe3C were formed in this reaction. Final reaction is TonB dependent*. P. aeruginosa* has two homologous of TonB (TonB1 and TonB2) and it has been noted that TonB2 is heavily involved in this displacement. This process causes the ferric-pyoverdine to move across the membrane (Hoffmann et al., 2002b).

4.2.1.2 Outer membrane copper receptor (OprC)

OprC was first identified as a nonselective porin that produces slightly anion selective small diffusion pores that seems to have no role in the uptake of antibiotics. It was found to have 65% homolog with *P. stuzeri* NosA which is an outer membrane protein required for insertion of copper into nitrate reductase (Hoffmann et al., 2002a). NosA its self contains copper and was only when the bacteria are maintained in anaerobic conditions (Lee et al., 1989). Purified NosA can form channels in black lipid bilayers.

Production of OprC is an anaerobic process which is inhibited by high copper concentrations. It is homologous to PfeA a ferric enterobactin receptor making it a member of large TonB-dependent family of proteins the expression of which is correlated with complexed iron uptake. BtuB is another less common member of TonB

family of outer membrane receptor which is also a vitamin B12 receptor in *P. aeruginosa and E. coli* and is identical to BtuB (Hoffmann et al., 2002b).

4.2.1.3 XcpQ (type II secretion system protein D)

(Bleves et al., 2010) outlines the numerous secretion systems *P. aeruginosa* expresses. These systems allow the secretion and utilization of virulence factors that promote infection as well as increase bacterial adaptability and survival. Five different pathways of protein secretion have been described in *Pseudomonas aeruginosa* including the type II secretion system (T2SS) that is employed by bacteria in order to secrete toxic factors into cells. T2SS is responsible for exporting most of the exoproteins (Florez et al., 2017). The T2SS encoded by Xcp gegns contains 12 different oligomeric protein molecules (Van der Meeren et al., 2012). *P. aeruginosa* have 12 different proteins that comprise this secretion machinery. Generally, the Xcp secretion machinery requires very large pores to transport folded exoproteins across the outer membrane. XcpQ is protein contains approximately 659 amino acids. (Akrim, 1993 #1681). It is an element of type II secretion pathways in *Pseudomonas aeruginosa*, specifically it is the outer membrane component of this machinery and also has been shown to form large channels. XcpQ is a member of the protein family specific for the secretions. The other members of this family are involved in type III protein secretion which results in the formation of filamentous type IV pili. Hence secretin makes up an essential group of transporters in the outer membrane of Gram negative bacteria (Florez et al., 2017). *P.aeruginosa* produces only a small amount of XcpQ that is around 50-100 copies per bacterium, the high expression of this protein is considered to be toxic to the cells (Brok, 1999 #1682).

Interestingly it has been shown that type III secretion system (T3SS) expression results in worsened disease outcome. For example in mice inoculated with *P. aeruginosa* isolates genetically adapt to have only T3SS, there was a 100% fatality rate by contrast there was an 80% rate, in strains exhibiting a Type II secretion system only (Lavoie et al., 2011, Fito-Boncompte et al., 2011).

4.2.1.4 PiuA: (a Ton-B-dependent receptor).

PiuA is one of 35 different in Ton-B-dependent receptors (TTBDRs) in *P. aeruginosa* (Luscher et al., 2018). PiuA is a siderophore receptor, the crystal structure of which has been obtained from the protein in *P. aeruginosa.* Some strains of this bacteria have no PiuA gene, but contain other TBDR such as PfuA, OptE and pyochelin which can transport siderophore-drug conjugates in an iron-limited environment (Luscher et al., 2018). PiuA can also be involved in uptake of xenosiderophore (Luscher et al., 2018).

PfuA is also a TBDR, the natural substrate is unknown. Luscher *et al*. identified PfuA with the other Ton-B-dependent receptor (TBDRs) of *P. aeruginosa* isolate namely OptE and OptJ. (Luscher et al., 2018).

4.2.1.5 Outer membrane protein M OprM:

In *P. aeruginosa* lipoprotein OprM plays a fundamental role in the antibiotic resistance and works by trafficking molecules through the cell membrane (Koch et al., 2012). It is is a member of the Mex-OprM xenobiotic antibiotic transporter family (Akama et al., 2004) and as such can actively export antibiotic thus reducing their concentration in the cell. The OprM monomer consist of 468 amino acid residues and a N-terminal fatty acid modifcation. (Akama et al., 2004).

4.2.1.6 Outer membrane porin F (OprF)

OprF is the main surface protein in wild type isolates of *P. aeruginosa* (Yu et al., 2016). OprF is likely to be important in the modification of *P. aeruginosa* to the host immune defence system. As part of this process it binds IFNγ and can thus sense the activation of an immune response and in response produce a more virulent phenotype (Sharma et al., 2011). OprF protein is the major OM component participating in the production of non-specific channels for the diffusion of ions, small polar molecules and also antibiotics (Nestorovich et al., 2006). The pathogenicity of *P. aeruginosa* is affect by absence of

OprF protein (Fito-Boncompte et al., 2011). Interestingly the lack of OprF increases OMV production via increased *Pseudomonas* quinolone signal (PQS) production (Wessel et al., 2012).

4.2.1.7 OprX

OmpX, is a lipoprotein the over expression which is known to increase the production of OMVs in *Salmonella enterica*.

4.2.1.8 Type 4 prepilin-like proteins leader peptide-processing enzyme (PilD):

In *P. aeruginosa*, PilD is prepilin peptidase and represent one of the integral membrane aspartic acid proteases (IMAAPs) (Aly et al., 2013). This enzyme is required to change many proteins of the type IV pilin subunits to other structures such as filaments (Pepe and Lory, 1998). PilD cleaves the unique leader peptides of type-IV pilin system that is found in a variety of Gram-negative bacteria (Lory, 1997 #1757).

4.2.1.9 Outer membrane protein H1 (OprH)

OprH Is an outer membrane protein which increases the stability of the outer membrane and its components by interacting with LPS (Lee et al., 2017). In the early stages of infection OprH is highly expressed and is also part of the *P. aeruginosa* in stress response (Lee et al., 2017).

4.2.1.10 DNA-directed RNA polymerase subunit beta (rpoB)

rpoB is an RNA polymerase beta subunit (Mollet et al., 1997). One copy of the rpoB gene is present in all bacteria because of it is important role in cellular metabolism as such rpoB is a highly conserved housekeeping gene (Qi et al., 2001). It has been used to study the phylogenetics of the genus *Pseudomonas* and for the identification of clinic isolates (Tayeb et al., 2005).

Mollet, suggested that the rpoB gene is a suitable target on which to base identification of enteric bacteria (Mollet et al., 1997), it could also be used for S*pirochetes* (Renesto et al., 2000), *Bartonellas* (Renesto et al., 2001) and *Rickettsias* (Drancourt and Raoult, 1999).

4.2.1.11 Pilus response regulator (pilG)

The pilus response regulator **(**pilG) is a type IV pilin protein (T4P) (Bertrand et al., 2010). These are long, thin (5-8 nm diameter) hair like annexations located on the bacterial and surface (Pelicic, 2008). The appendages play essential roles in surface attachment, cell-cell aggregation, formation of biofilms and motility of bacteria (Burrows, 2005, Pelicic, 2008 #1691, Pelicic, 2008 #1691). Type IV pilin are also important for virulence. They are divided in two major subfamilies, type IVa (T4aP) and type IVb (T4bP) pili (Burrows, 2012) (Darzins, 1993).

4.2.1.12 Cation-transporting P-type ATPase

P-type ATPases are found in bacteria and in a number of eukaryotic plasma membranes and organelles (Axelsen and Palmgren, 1998). P-type ATPases include proteins that participate in the active pumping of charged molecules through the cell membrane (Møller et al., 1996).

In many types of organisms P-type ATPase have been found to be involved in the transport of copper. The P-type ATPases constitute a large class of homologous ATP-dependent cation transporters (Lutsenko and Kaplan, 1995). All P-type ATPases have a conserved aspartate residue that is phosphorylated by ATP during the catalytic cycle (Fan and Rosen, 2002).

Spillman *et al.*, 2013 provided evidence of the cation ATPase pfATP4 pump being a Na^+ efflux pump, maintaining copper homeostasis in *E coli* (Spillman et al., 2013). The mutation of copA gene led to less copper being secreted and an increase copper toxicity. (Rensing et al., 2000).

4.3 Comparison of Outer Membrane vesicles and Outer Membrane proteins

The results reported here indicated that most proteins of OMV are the same to OM which support the hypothesis that the OMV isolated here are indeed derived from OM. Wurpel, 2015 stated that 70-80% OM-associated proteins are trafficked to OMVs (Wurpel et al., 2015). It has been proposed that the proteomic analysing of the outer membrane is a fundamental process to identify the pathogen that is causing the disease (Tiwari 2016). It is intriguing to think that these could be obtained coulters or blood samples via the isolation of OMVs.

Discussion

Section 3

4.3 Growth curve of bacteria

This study examined and assessed the effect of different coulter conditions on the growth of *Pseudomonas aeruginosa* bacteria, a growth curve was produced to establish the growth rate of the bacteria in tryptone soy broth (TSB) and simulated wound fluid (SWF) for *Pseudomonas aeruginosa.* The same procedure was performed for both Laboratory (L.) 10421 and Hospital (PS3) strains (PS3). To establish how the bacteria would grow in cell culture media growth curves were also performed in Dulbecco's Modified Eagles Medium (DMEM). Growth was assessed using both absorbency at 620nm and CFU/ml to provide a more accurate enumeration of bacterial numbers. The growth of *Pseudomonas aeruginosa* was found to follow a standard pattern of growth, displaying logarithmic, exponential and stationary phases.

Pseudomonas aeruginosa was grown in either TSB (as basic medium for the bacteria) or simulated wound fluid (SWF) which is much closer to the condition in wound for both strains Laboratory strain (L.) 10421 and Hospital strain (PS3) the bacteria were grown for 3 days and absorbance at 620nm was monitored. The absorbance data indicated that both strains had very similar growth characteristics in both culture conditions. By contrast *Pseudomonas aeruginosa* (both strains) grown in high glucose DMEM resulted in a much greater maximum absorbance (2 times) after 7 hours compared to either TSB or SWF it is likely that this is mainly because it contains 4.5g/l of glucose while TSB contains 2.5g/l. However, there was little effect on the immediate growth of the bacteria in DMEM, there may be problems using the media for bacterial growth, as its intended purpose is for use as a constituent of tissue culture media at least in part because of the effects of bacteria on the pH of the media. In an attempt to avoid this measures were taken to ensure that the pH of the media was at 6.9, the optimum pH for the bacterial growth, by using HEPES buffer and 1M HCl. In cell culture using a 5% CO2 incubator is commonly used to maintain the pH of the media (Kim et al., 2013) because the bacteria were not cultured in a CO_2 incubator HEPES was required to act as a buffer to maintain the pH.

Mathee et al. (2008) showed that different strains of *P. aeruginosa* have genomic variation and demonstrated that these variations have effects on bacterial survivability in different environments. *P. aeruginosa* present in hospitals may have more opportunity to be exposed to more harsh environmental conditions for example the routine use of antimicrobial products and this could selected for a bacterial strains that can thrive in less-optimal environments (Mathee et al., 2008). This may be through direct resistance mechanisms to condition of alkaline pH, or developed mechanisms that have a positive effect with regard to pH resistance.

Despite the finding of reduced *P. aeruginosa* growth in different pH, clinical implications related to wound treatment may be insubstantial. Gethin (2007) suggested the ability of wounds to heal in alkaline pH environments was reduced and thus promoted the idea that wounds heal better within more neutral and acidic conditions (Gethin, 2007). This information leads to practical considerations for the effect of pH on *P. aeruginosa* when looking at direct wound treatment. *P. aeruginosa* remains a concern in hospital environments due to its ability to adapt and grow on a range of surfaces, including medical equipment which can for example result in surgical wound infections (Secher et al., 2005). A possible implication for the effect of pH on the bacteria is in the treatment and storage of equipment, such as catheters, prior to use. Storage within an acidic condition may inhibit the growth of *P. aeruginosa* on the surfaces of equipment and then reduce the incidence of infection. There are some considerations when exploring this, including the link between biofilm formation and the effectiveness of pH treatment. *P. aeruginosa* can exist as a biofilm when colonizing medical equipment, this can be a particular issue in a medical context because biofilm formation can result increased resistance to a number of antibiotics (Breidenstein et al., 2011). Some researchers have assessed the use of liquid-infused silicone as a strategy to control the infection. It was found that treatment of equipment with silicone oil, a slippery polymer, resulted in reduced bacterial surface adhesion and reduced biofilm formation (MacCallum et al., 2014). Recently, carbon monoxide releasing molecules (EBOR-CORM-1) have been proposed as a new synthetic type of antimicrobials to treat bacterial infections by reducing the growth of a range of clinical *P. aeruginosa* strains (Flanagan et al., 2018) (Sônego et al., 2018).

Glucose consumption could not be determined in the media because the high level of glucose in this cell culture media saturated the glucose assay for this reason the media was diluted (3.325g/l instead of 13.3g/l) which brings the glucose concentration down to a point where the changes caused by the bacteria could be detected. In this way it was confirmed that the bacteria consumed glucose during the first 7 hours in culture (figure 3.7). After an overnight incubation all the glucose was used up and the bacteria reached the stationary phase of the growth curve.

Simulated Wound Fliud (SWF) consisted of fetal bovine serum (FBS) and Maximum Recovery Diluent (MRD). Important molecules for bacterial present in FBS include; different proteins, polypeptides, fat, carbohydrates, growth factor, vitamins and minerals. MRD contains low level of peptone with a pH 7.0 and it reduces replication of the bacteria for at least one hour (Yang & Xiong, 2012). The use of Simulated Wound Fliud (SWF) as a bacterial growth media produced a growth curve with a long lag phase which supports the fact that MRD in SWF reduced the replication of *P. aeruginosa*, however 6 hours after inoculation, the bacterial population entered exponential growth phase of growth.

The result obtained from growing *Pseudomonas aeruginosa* in different conditions with optimal temperature at 37°Ċ indicates the impact of culture media on the growth rate of *Pseudomonas aeruginosa* laboratory strain (L.). The growth of *P. aeruginosa* in SWF without ethanol showed that the CFU was higher than the bacteria grown in combination of SWF with ethanol. This indicated that ethanol has a negative effect on the growth of bacteria, as the high levels of the ethanol may cause a complete inhabitation of the growth rate of bacteria. The amount of the ethanol added was 4.02ml into 300ml of simulated wound fluid, which is identical to adding 0.0134ml of ethanol per ml of culture media as reported by Kretzschmar *et al*, this concentration is low enough when added to bacterial culture media to act as a carbon source for bacterial growth as was shown here. Interestingly, the addition of ethanol reduced the growth of *P. aeruginosa* compared to the sample of SWF without ethanol. Koshiro and Olie, reported that when the concentration of ethanol reaches 30% and the *P. aeruginosa* are grown at 20°C this resulted in the killing of approximately 10^6 CFU/ml in 30 min and when the concentration

of ethanol was increased to 40% it killed approximately the same number of *P. aeruginosa* within 20 second (Koshiro and Oie, 1984).

Discussion

Section 4

4.4 The cytotoxicity of OMVs

The effect of a variety of OMVs at different concentrations on the viability of the 2 keratinocyte cell lines and on NHKs was determined using an assay of biomass which compares the metabolic activity of live cells to that of the controls.

For HaCaT cells initially OMV form both laboratory (L.) and Hospital strains (PS3) were added at a concentration of 14.28 µg/ml and 5.71 µg/ml and this had little impact on the cells. However, when 71.4 µg/ml of the laboratory strain (L.) OMV was added there was a slight reduction in cell viability. The most toxic concentration for OMV was the second experiment in which a concentration 28.55 µg/ml from the Hospital strain (PS3) was used and this produced a lower biomass. This indicates some variability between the different preparation of the vesicles but supports the theory proposed by Kukavica-Ibrulj that *P. aeruginosa* Hospital strain (PS3) is more toxic than the laboratory strain (L.) (Kukavica-Ibrulj et al., 2008) and this, at least in part, could be due to the different properties of vesicles that they produce. However, HaCaT showed no response to low amounts of OMV s for laboratory (L.) and clinical strains (PS3). Importantly, these findings demonstrated that at low concentrations of OMVs the cell growth remain unaffected and show rapid growth compared to control and this was particularly clear for the lower concentrations of the laboratory strain (L.) where there appeared to be a stimulation in the rate of cell growth. This increase in biomass in response to moderate cell stress has been reported previously by (Al-Tameemi et al., 2014) and others who have shown that that a low concentrations of the cytotoxic drugs doxorubicin and 4-OH-CP increases cell growth in comparison to untreated cells. This response of increase cell proliferation at sub-lethal doses of toxic chemotherapy compounds has been observed in other cell types (Kayamba et al., 2013, Paus et al., 2013).

The images collected 24hrs after exposing the cells to OMV showed that the most affected cells with an apparent loss of viability was in those exposed to the Hospital strain (PS3) and the laboratory strain (L.) OMVs, had less effect on the cells. This was the same in both groups of images. Specifically, for preparation 2, the cells were treated with 6.67µg/ml of OMVs from the clinical strain (PS3) and this produced a loss of

biomass whereas lower concentration of vesicles produced a smaller affect, thus the most toxic concentration for OMVs was 6.67µg/ml for the Hospital strain (PS3). In addition, in these conditions the shape of the HaCaT cells did not represent classical morphology indicting that the OMVs are a stress factor and again these results confirm the idea that clinical strain (PS3) for *P. aeruginosa* is more toxic than the laboratory strain (L.) (Kukavica-Ibrulj et al., 2008).

A diversity of environmental factors effects the proteins in OMVs and alteration of the protein content in response to environmental cues could have important implications on how OMVs impact host cell function and immune response (Ballok et al., 2014). The fact that both strains of *Pseudomonas aeruginosa* produce OMV is an indication of the central role that these must have in the function of this bacteria and shows that mechanisms exist to promote the formation of OMV in both lab-adapted and clinical strains of *Pseudomonas aeruginosa* (Florez et al., 2017). Previous results have shown that *P. aeruginosa* OMVs are damaging to epithelia, being both inflammatory and cytotoxic (Bomberger et al., 2009).

The potential of OMVs to stimulate an inflammatory phenotype in the keratinocytes was investigated here by quantifying the secretion of IL-8 (CXCL8) from the cells. CXCL8 or Interleukin-8 (IL-8) was the first chemokine to be described (Koch et al., 1992). IL-8 is a member of CXC chemokine family, which are a small inducible proteins (6-15 kDa) that possess diverse biological activities (Zaja-Milatovic and Richmond, 2008), they are characterized by their capacity to attract subsets of leukocytes. More than 20 chemokines have been isolated in humans (Liebler et al., 1994). CXC's are secreted by cells such as: macrophages and epithelial cells in response to environmental stress including the presence of bacteria, components of bacteria and/or bacteria virulence factors (Lotti and Maggi, 2013) and as such they play a key role in the initiation of the innate immune system in both health and disease (Daig et al., 1996). Specifically, IL-8 plays a major role in the recruitment of neutrophils to inflammatory sites (Kuhns et al., 1998) and thus evokes a neutrophil-mediated inflammation which can lead to tissue destruction (De Boer et al., 1993), IL-8 expression is also found after mechanical injury in the retinal pigment epithelium (RPE) during the wound healing process (Yoshida et

al., 2001). IL-8 is one of the defences against tissue damage and end-pathogen invasion (Dinarello, 2009).

Recently, non-thermal atmospheric pressure plasma (NTAPP) has emerged as a novel medical therapy for skin wounds. NTAPP irradiation has been reported to promote production of IL-8 (Hotta et al., 2018). In this context IL-8 is thought to play an important role in wound healing by initiating an immune response.

HaCaT, HaCaTa and NHK cells have been shown here to possess different capacities for IL-8 release according to the factors to which they are exposed including various concentration of outer membrane vesicles OMVs and the outer membrane OM of *P. aeruginosa* isolated from either clinical PS3 or laboratory strains (L.). The response of HaCaT, HaCaTa and NHK cells was determined by measuring the amount of interleukin 8 (IL-8) secreted using an enzyme linked immunosorbant assay (ELISA). The production and secretion of IL-8 is known to be related to environmental stress upon the cell (Lotti & Maggi, 2013), such as bacterial infection which in this research simulated by OMVs or OM isolated from *P. aeruginosa.*

Our results indicated that OMVs have similar proteins to those find in OM, which they are derived from. Some studies show that 70-80% of OM associated proteins make up the protein content of OMVs (Wurpel et al., 2015)The quantitative proteomic differences between OMVs and the OM vesicles they are derived from shows that the OMVs were enriched with proteins and trace metal uptake and transport proteins (Lappann et al., 2013). If this occurred here it be expected that the effect of OM on HaCaT, HaCaTa and NHK would be like exposing them to OMVs. Both laboratory and clinical strains of bacteria produce OMVs, their protein content showed high similarity to the OM of the bacteria, from where the originate and this is in agreement with previous studies (Jan, 2017)OMVs from pathogenic bacteria are considered as a stress factor. It has been shown that a diversity of environmental factors affects the proteins that are expression in OMVs (Ballok et al., 2014) and presumable this is a result of changes in the protein expressed in the OM of the bacteria.

The highest concentration of IL-8 secretion from HaCaT cells occurred after challenging with OM from the Hospital strain (PS3) thus again these results support the hypothesis

that the clinical strain PS3 of *P. aeruginosa* is more toxic than the laboratory strain (L.) (Kukavica-Ibrulj et al., 2008).

HaCaT cells adapted to grow in low calcium (0.09mM) and serum-free conditions the to produce a new cell line (HaCaTa cells) which was cultured in Keratinocyte Serum Free Medium (KSFM) which contains low calcium concentration (~0.09mM) – in comparison to physiological (~2mM) calcium (Georgopoulos et al., 2010). When HaCaTa cells were challenged with OMV again the highest concentration of IL-8 was produced in response to the hospital strain and this was the same for the OM.

The concentration of extracellular calcium has a central role as a switch between a differentiation and growth phase of epithelial keratinocytes, accordingly calcium stimulates terminal differentiation producing specific changes in cell structure and cell cycle withdrawal and stimulation of a group of terminal differentiation-related genes (Boelsma et al., 1999).

Deyrieux and Wilson, 2007 stated that reducing the amounts of calcium in the media of HaCaT cells resulting in a high growth and low differentiation phenotype which was confirmed by decrease of specific molecular markers including K1and involucrin (Deyrieux and Wilson, 2007).

Normal Human Keratinocyte NHK that are cultured in Keratinocyte Serum Free Medium (KSFM), respond to different concentration of OM by producing IL-8, again the highest level of IL-8 was stimulated by the Hospital strain (PS3) it was also more toxic more toxic than the laboratory strain which is similar to the other keratinocytes studied here and confirms previous work (Kukavica-Ibrulj et al., 2008). When normal keratinocytes are challenged with *S. aureus* and increase in the secretion of interleukin IL-6 and significantly attenuated expression of terminal differentiation markers keratin, loricrin and filaggrin in the stratum corneum (SC) of the skin *in vivo* has been reported. In addition, *S. aureus* inhibits the terminal differentiation of keratinocytes by stimulating IL-6 secretion. (Son et al., 2014)

Cultured HaCaT keratinocyte cells secreted a higher concentration of IL-8 in response to exposure to outer membrane vesicles (OMV) Hospital strain (PS3) of *P. aeruginosa*

and the Laboratory isolate than either HaCaTa or NHK both of which are grown in a lower concentration of calcium (0.09mM) and serum free conditions and exhibited higher proliferation rates cells and are less differentiated than HaCaT (Al-Tameemi et al., 2014). This could be due to the fact that in normal skin such undifferentiated and proliferating cells would, because of their location be less exposed to bacteria or their products. In this regard it is interestingly HaCaT cells and NHKs have the different Th cell cytokine-dependent transcriptional profiles and expression of epidermal differentiation markers such as: filaggrin, loricrin, involucrin, and KRT10, which is essential in skin permeability barrier formation (Seo et al., 2012).

Venza et al. (2009) reported that heat-killed *P. aeruginosa* bacteria had a stimulatory effect on IL-8 secretion from human conjunctiva. This suggests that stressed or heat-killed bacteria may produce an upregulation of secreted factors and therefore have an increased effect on IL-8 production (Venza et al., 2009).

Calcium as well as being the main regulator of keratinocyte differentiation is also responsible for the stimulating the formation of desmosome, adherens junctions and tight junctions (Kobashi et al., 2017a). In connective tissues, intercellular adhesion is essential for tissue morphogenesis, development and wound healing (Ko et al., 2001).

NHK cells have been shown to possess different capacities for IL-8 release according to the factors to which they are exposed various concentration of outer membrane vesicles OMVs and the outer membrane OM of *P. aeruginosa* for both clinical (PS3) or laboratory strains (L.) in low and high levels of calcium. Differentiation of Normal Human Keratinocyte can be activated by supplementing calcium in the media (Hennings et al., 1980, Micallef et al., 2009, Tu et al., 2004, Tu et al., 2007). Indeed, calcium induces terminal differentiation, therefore the concentration of extracellular calcium has a profound role as a switch between epithelial growth and differentiation of keratinocytes (Boelsma et al., 1999).

In vivo high concentration of $Ca2^{+}$ in keratinocytes can affect normal skin barrier due to abnormal differentiation of cells (Sah et al., 2017b, Sah et al., 2017a). High calcium concentrations also increased the expression of serine protease inhibitors such as: lympho epithelial kazal type related inhibitor (LEKTI), secretory leucocyte peptidase

inhibitor (SLPI) and elafin in epidermal keratinocytes (Kobashi et al., 2017a). High concentrations of calcium can disrupt the normal skin barrier due to abnormal differentiation of keratinocytes (Sah et al., 2017a). The abnormality of differentiation and proliferation can lead to many diseases such as: dermatitis, angioedema, macropapular rashes, psoriasis and skin cancer (Sah et al., 2017a).

Here it was shown that for NHKs an increase in the concentration of calcium in the media produced a reduction in the secretion of IL-8 when the cells are challenged with OM or OMVs.

The results showed a significant increase of IL-8 the highest concentration of IL-8 with 1:31 µg/ml of (OM) Hospital strain (PS3) and the lower concentration with 1:32 µg/ml of (OM) Laboratory strain 12041. In low concentration of calcium (~0.09 mM) comparing to physiological calcium (~2 mM) (Georgopoulos et al., 2010) (figure 4.19). It has been stated that reducing the concentration of calcium of keratinocyte HaCaT cells resulted in a high level of proliferation and less differentiation (Deyrieux and Wilson, 2007). A low Ca^{2+} concentration decreases the cell density needed to initiate growth (McGrath and Soule, 1984). When Normal Human Keratinocyte (NHK) were maintained in L.C. levels in the culture medium, cells were described to proliferate and grew as monolayers and have less cell-cell contact without desmosome formation (Watt et al., 1984).

In vivo a high calcium environment around keratinocytes can disrupt normal skin barrier function due to abnormal and/or premature differentiation of keratinocytes (Sah et al., 2017a). Changes in the concentration of extracellular calcium affect the balance between proliferation and differentiation in epidermal keratinocytes {(Menon et al., 1992)#1957}. In our results morphological changes were observed by contrast phase microscopy. NHK growth can be stopped and terminal differentiation activated by raising the concentration of calcium (Kolly et al., 2005b). Thus, the type of cultured medium used is fundamental {Papp, 2003 #53

Discussion

Section 5

The effect of *P. aeruginosa* secreted factors on keratinocytes

The supernatant of *P. aeruginosa* PS3 was found to be cytotoxic to keratinocytes in a dose dependent manner to a dilution of 1:100. Other dilutions produced an increase in the biomass of the keratinocyte cells which could be due to to a sub-lethal stress mediated effect on the cells as described previously for the OMV and previously reported for sub-lethal concentrations of cytoxic drugs (Al-Tameemi et al., 2014). For the Laboratory isolate a 1:50 dilution of the cell free supernatant produced a loss of biomass resulting from the treatment of the cells, while other concentrations resulted in no loss of biomass.

HaCaTa cells were less affected by the supernatant of *P. aeruginosa* Hospital (PS3) and this could be due to the fact that the growth rate of HaCaTa cells were higher than that of HaCaT. Al-Tameemi, 2014 state that (the proliferation rate of the HaCaTa in comparison to the HaCaT cell line, with HaCaTa having about a 50% higher proliferation rate (Al-Tameemi et al., 2014).

4.5 HaCaT IL-8 secretion in response to *P. aeruginosa* secreted factors

It has been previously reported that when HaCaT cells are exposed to bacteria and/or bacteria virulence factors (Olaru and Jensen, 2010) they secrete IL-8. Here the keratinocyte cells secreted the highest concentration of IL-8 when exposed to the supernatant from Hospital strain (PS3) grown in TSB, and the lowest value of IL-8 in response to laboratory isolate grown in DMEM. This indicates that the conditions in which bacteria are grown affects their phenotype there is issue with using DMEM for bacterial growth, as it is not the normal growth media for bacteria, it was used here because when the cells were challenged with this media because it was similar to the media used for the cells in this study in contrast to the bacterial broth. When it was evident that coulter conditions changed the phenotype of the bacteria it was decided that it was also important to grow bacteria in conditions that could be found at a wound

bed and thus the simulated wound fluid (SWF) used to more closely mimic the situation found in an infected wound. The relatively low concentration of IL-8 in control samples increased on challenge with bacterial products this is in agreement with (DiMango et al., 1995), (Sar et al., 1999), Denning et al., 1998) as they showed that IL-8 expression was low in a normal environment but its secretion form epithelial cells could be stimulated by conditions such as infection by P*. aeruginosa* and this results in a significant recruitment of neutrophils to infected area. It has been stated that IL-8 expression is activated by *P. aeruginosa* associated molecules such as pilin, flagellin, pyocyanin, and phospholipase C (Kipnis et al., 2006). These finding suggest that at least some of these factors are secreted into the extracellular fluid of wounds and that as described previously in this study could be in the form of OMVs.

The production and secretion of IL-8 in response to factors from *Pseudomonas aeruginosa* is related to environmental stress on the cells (Lotti and Maggi, 2013). In addition to IL-8 *P. aeruginosa* infection potently induces other proinflammatory mediators, such as: IL-1, IL-6 and IL-10 (Epelman et al., 2000, Schultz et al., 2002, Kube et al., 2001). Also, *P. aeruginosa* can affect the host cells, Shao (2010) states that infections with *P. aeruginosa* can be lethal to individuals with impaired respiratory and immune systems *P. aeruginosa* produces cyanide which can kill the nematode *C. elegans* within hours (Shao, 2010).

Keratinocytes play a fundamental role in coordinating immune responses by releasing chemokines following TLR engagement (Lebre et al., 2007). Keratinocytes express a range of Toll-like receptors (TLRs) which are a group of receptors that can detect microbial derived factors (MAMPs) which are produced by pathogenic and non-pathogenic bacteria (Takeda and Akira, 2005). Both keratinocytes and HaCaT cells expressed TLR2, 3 and 5 while TLR4 was only observed in HaCaT cells and this was confirmed here. TLRs are normally highly expressed in keratinocytes (Köllisch *et al*., 2005) which is indicative of the function of skin as the first line of defence to pathogenic components via detection of microbial associated molecular patterns (MAMPs) (Andonova and Urumova, 2013). It is likely that TLRs respond to OMVs because they are enriched in, not only outer membrane components (such as lipoproteins), but also

because of the method of their synthesis which results in other structures from the bacterial envelope such as peptidoglycan being incorporated into the vesicles (Kuehn and Kesty, 2005) and these are known to stimulate TLRs.

It has reported that there are differences in the basic structure and function of some TLRs and TLR cofactor molecules between primary keratinocytes and the HaCaT cell line and this provides evidence for why it is important to use a range of models in addition to the immortalised non-malignant human keratinocyte line HaCaT, when investigating epithelial TLR expression. However, HaCaT cells have been shown to demonstrate some features and behaviour that are identical to normal keratinocytes in some molecular studies (Boukamp et al., 1988 and Deyrieux and Wilson, 2007), and both of them have been used in 2D organotypic cultures (Margulis et al., 2005) and they have also been used to investigate drug-interactions (Luanpitpong et al., 2011).

The keratinocytes secreted the highest concentration of IL-8 after exposure to PS3 grown in TSB and the lowest concentration when exposed to the supernatant from PS3 grown in DMEM. This could be due to the reduced growth of the bacteria in this media however, the same environmental stress may have provoked an increased secretion of outer membrane vesicles (Ellis & Kuehn, 2010) or an increase in pathogenic factors in the vesicles of the hospital strain (PS3), compared to the bacterial grown in TSB this could be due to changes in pH in DMEM when used to cutler bacteria. Acute inflammation is known as an important strategy that the body uses to combat infection. However, over stimulation of innate immunity can result in a worsening of the disease outcome (De Lima, 2012) and in the case of the skin the development of a chronic wound.

The IL-8 data presented here provides more information related to various factors which are released by *P. aeruginosa* and result in a further understanding of the impact these have on the innate immune response. Future studies may lead to the direct control of pro-inflammatory molecules and thus reduce the over stimulation of the immune response which can be responsible for the development of chronic wounds. For example it is already understood that elafin plays a vital role in preventing tissue damage Meyer-Hoffart et al. (2003) in addition it also has antimicrobial effect against *P.*

aeruginosa (Gales et al. 2000) but is also an example of an immuno-modulating molecule that produces an anti-inflammatory effect reducing the neutrophil-caused cell damage. With future research, both pro-inflammatory and anti-inflammatory molecules may be shown to have the ability to be used to control the immune response at a molecular level and thus reduce the possible clinical complications that may occur.

Future work

Determine the effect of bacterial cell free supernatant that has had the OMVs removed on the IL-8 secretion of keratinocytes.

Determine the TLR expression in NHK and HaCaTa cells and verify this using protein expression studies such as western blot.

Determine TLR expression in keratinocytes after exposure to OMVs to help confirm which pathways are being stimulated.

Use protein expression to determine the intracellular pathways stimulated by the *Pseudomonas* strains.

Use a proteomic approach to investigate the differences in protein expression in the OMVs of *Pseudomonas* grown in SWF and TSB.

Appendix

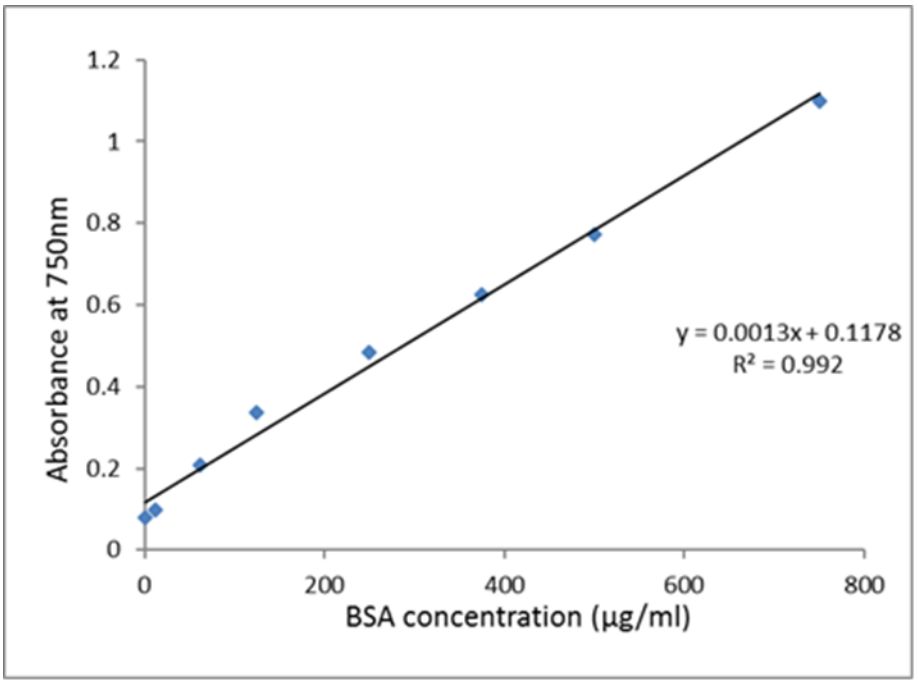

Figure 1: The BSA standard curve which used to find the protein concentration (µg/ml) at 750nm wave length, for the purified OMVs which isolated from *Pseudomonas aeruginosa.*

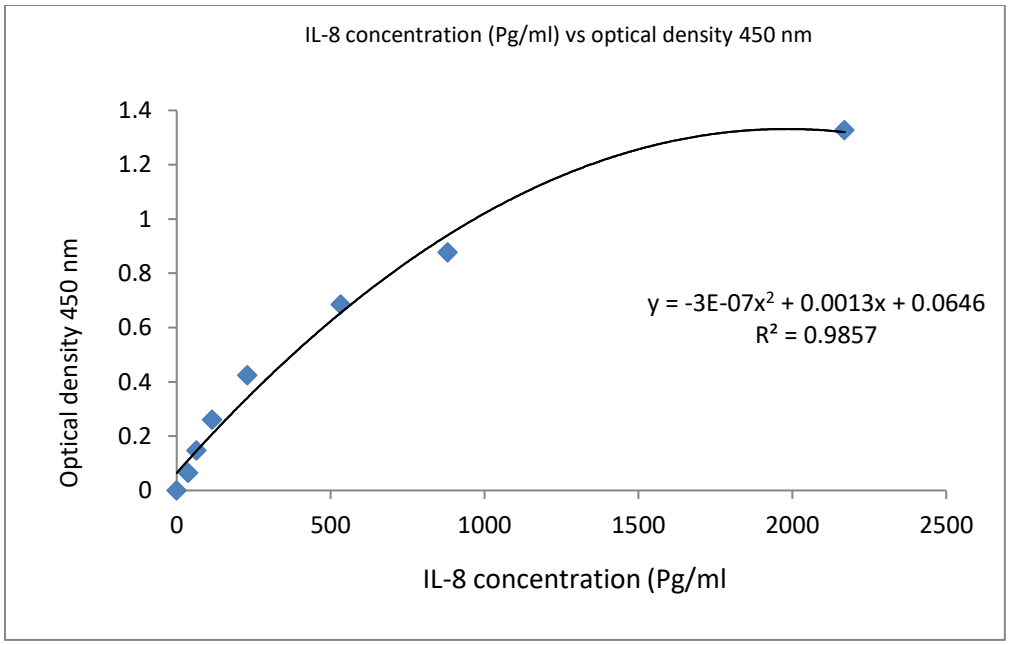

Figure2: Standard curve generated by IL-8 concentration and its optical density 450nm.

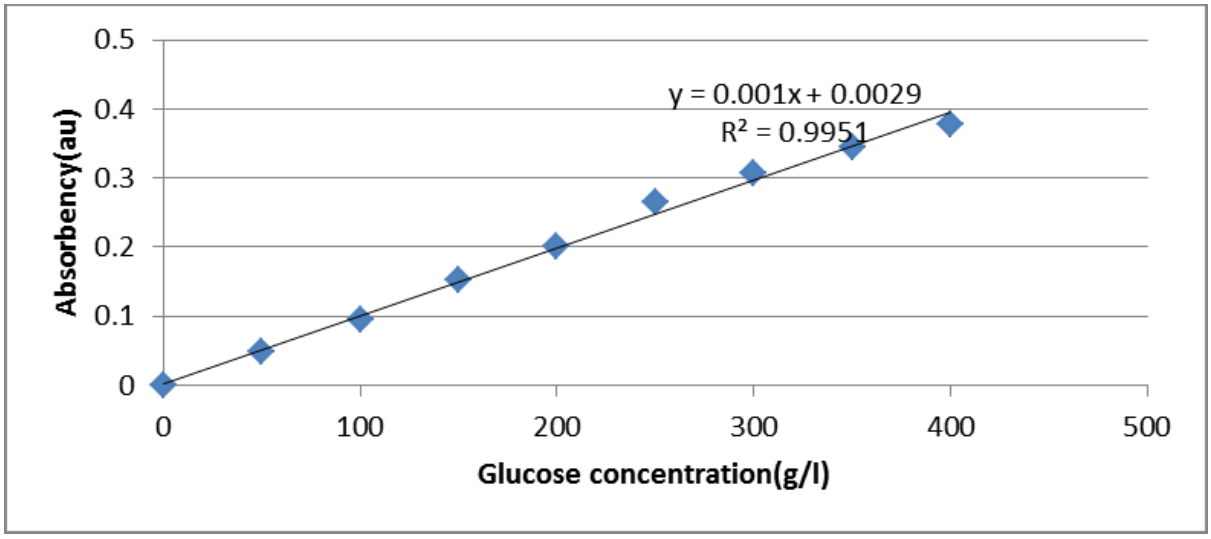

Figure 3: Calibration curve for glucose using the phenol-sulphuric acid assay.

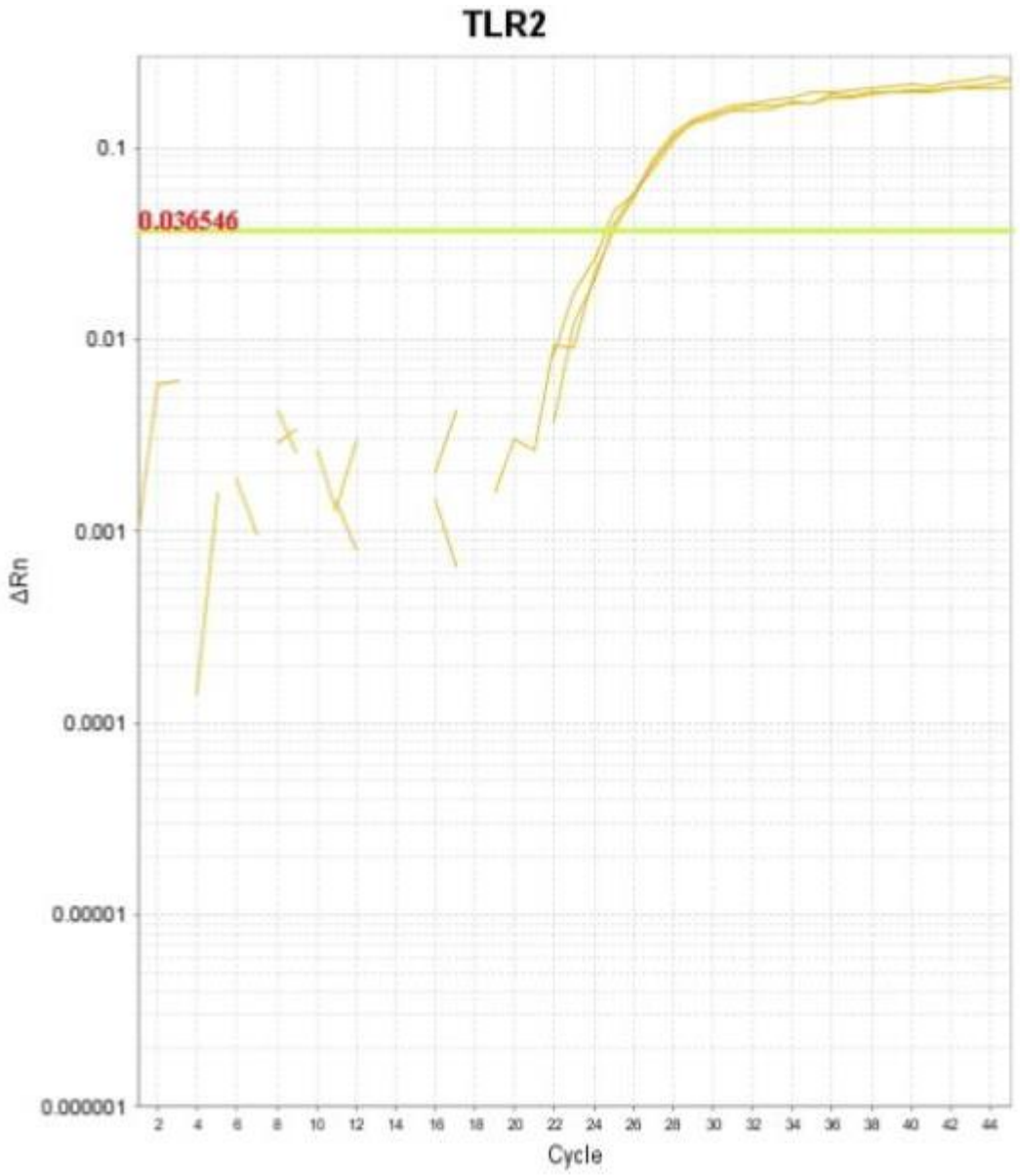

Figure 4: Typical amplification curves of qRT-PCR for TLR2, in 10 days HaCaT cell.

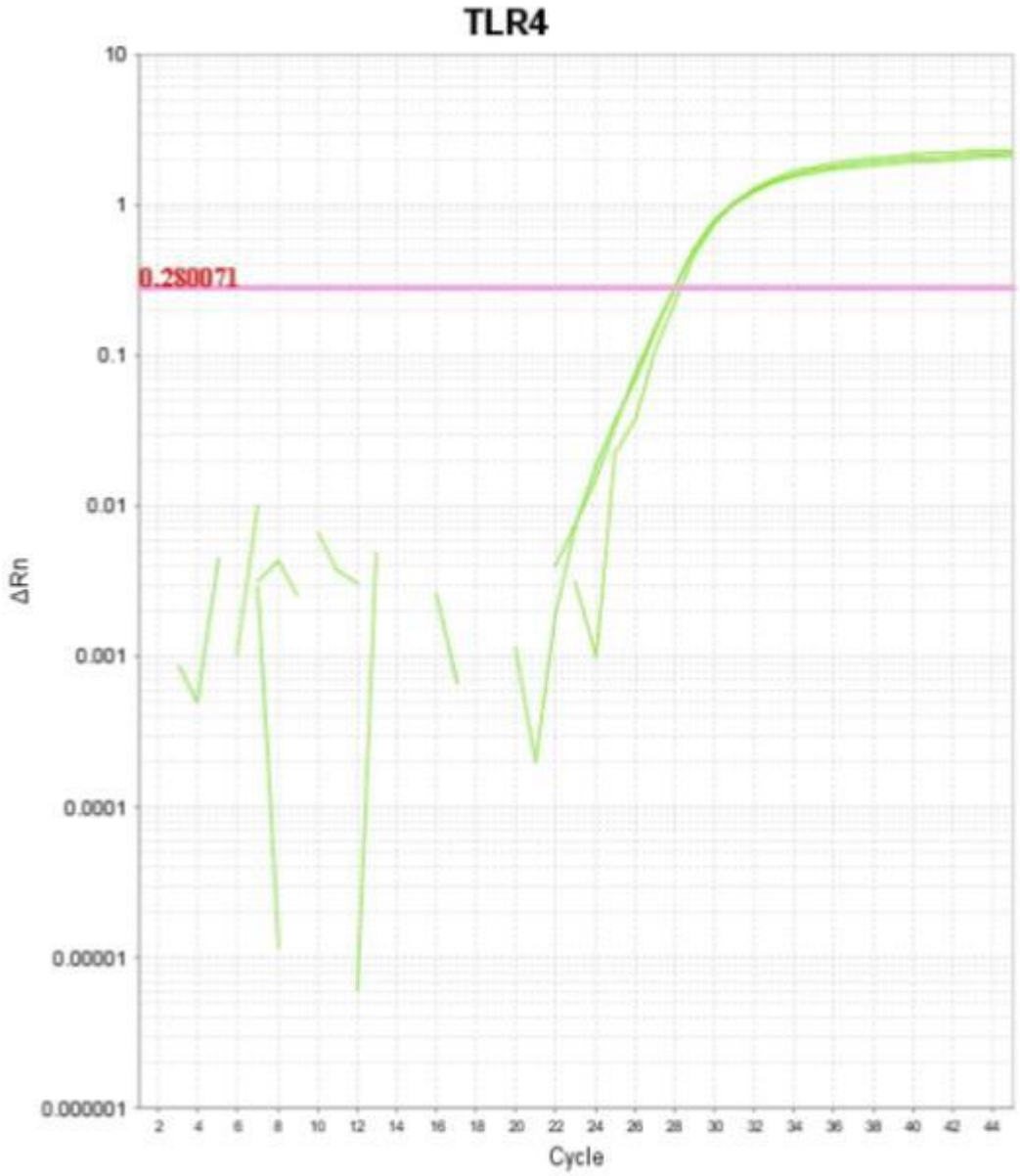

Figure 5: Typical amplification curves of qRT-PCR for TLR4, in 10 days HaCaT cell.

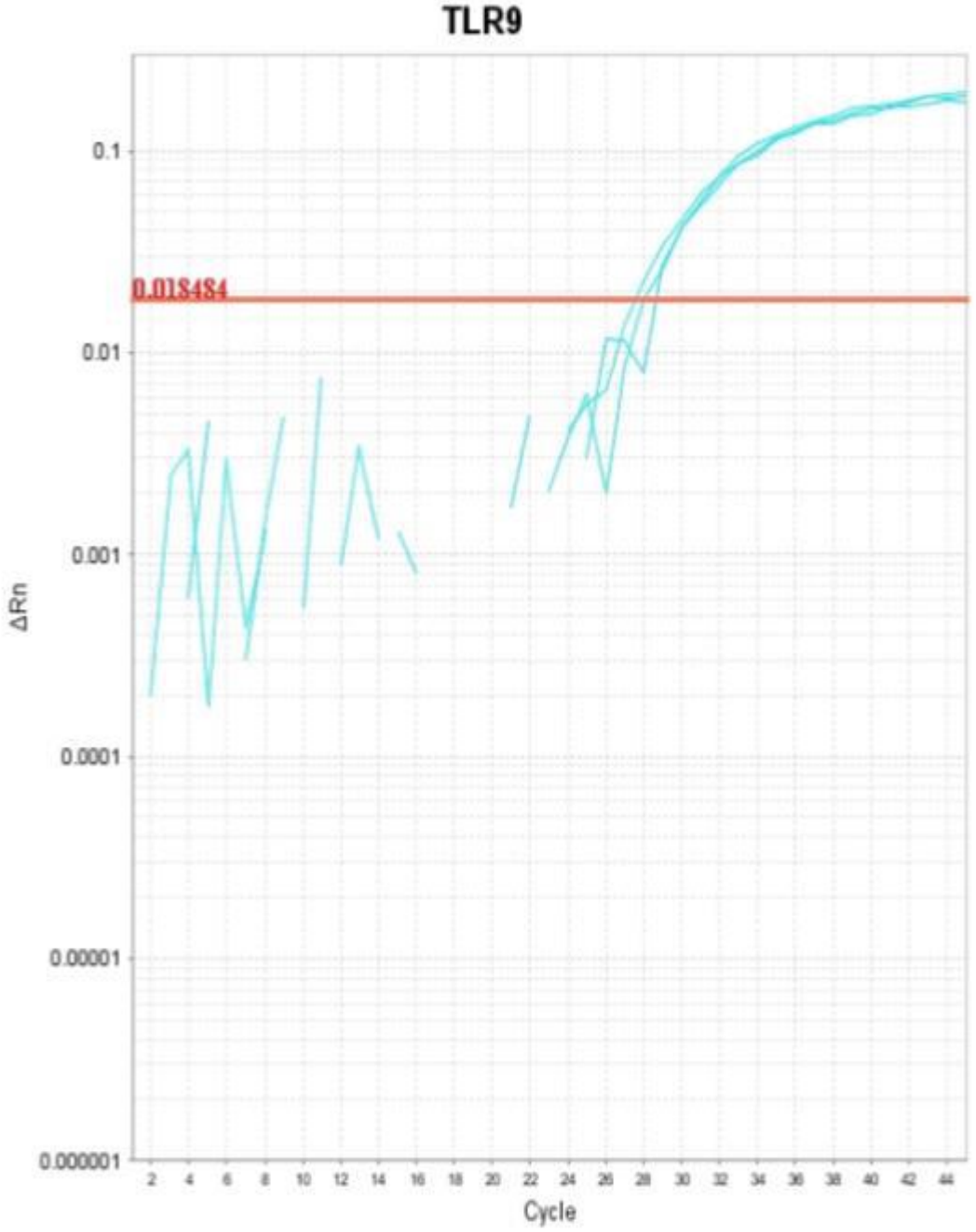

Figure 6: Typical amplification curves of qRT-PCR for TLR5, in (10 days) HaCaT cell.

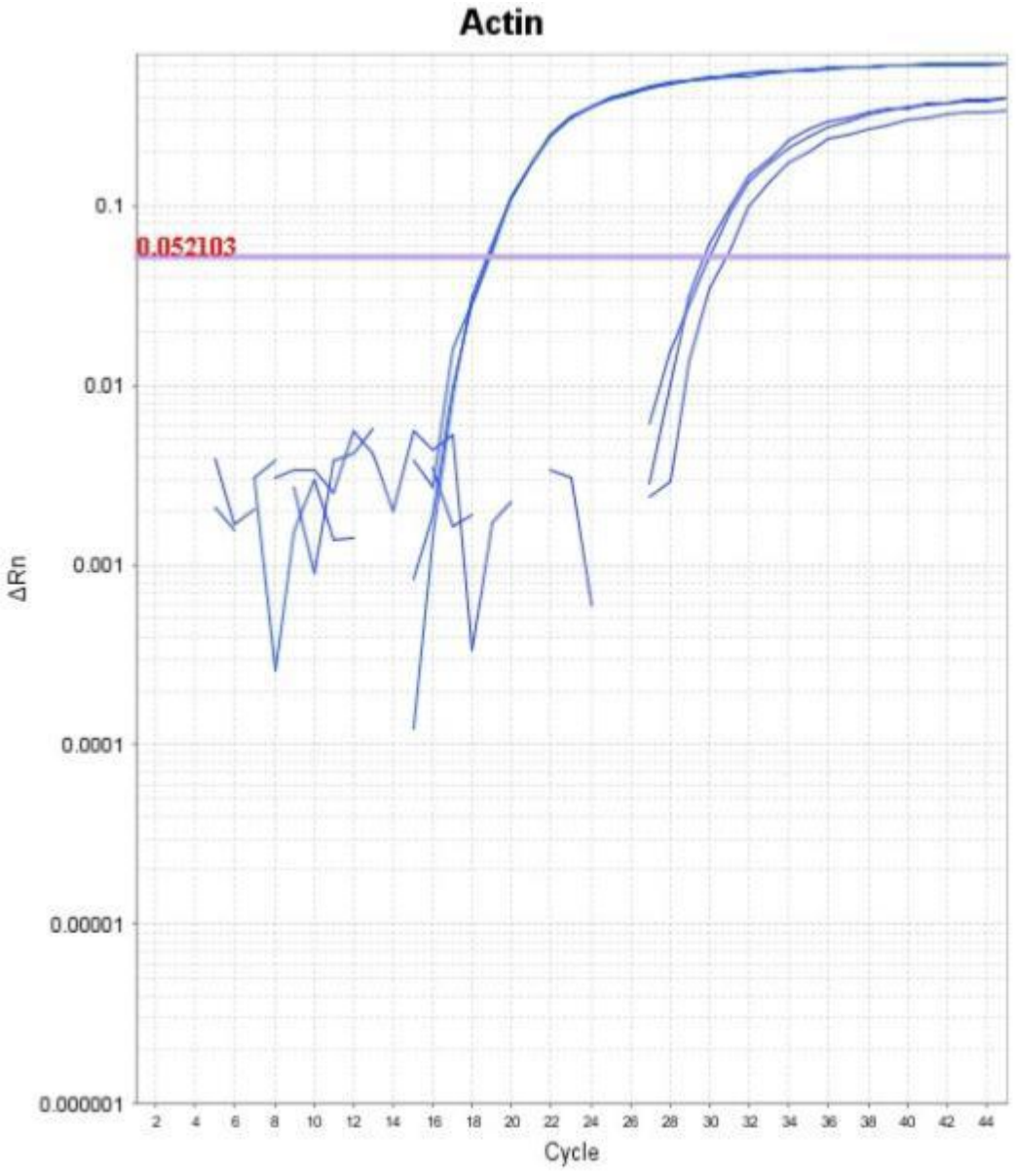

Figure 7: Typical amplification curve of qRT-PSR. For actin gene in 4 days HaCaT cells.

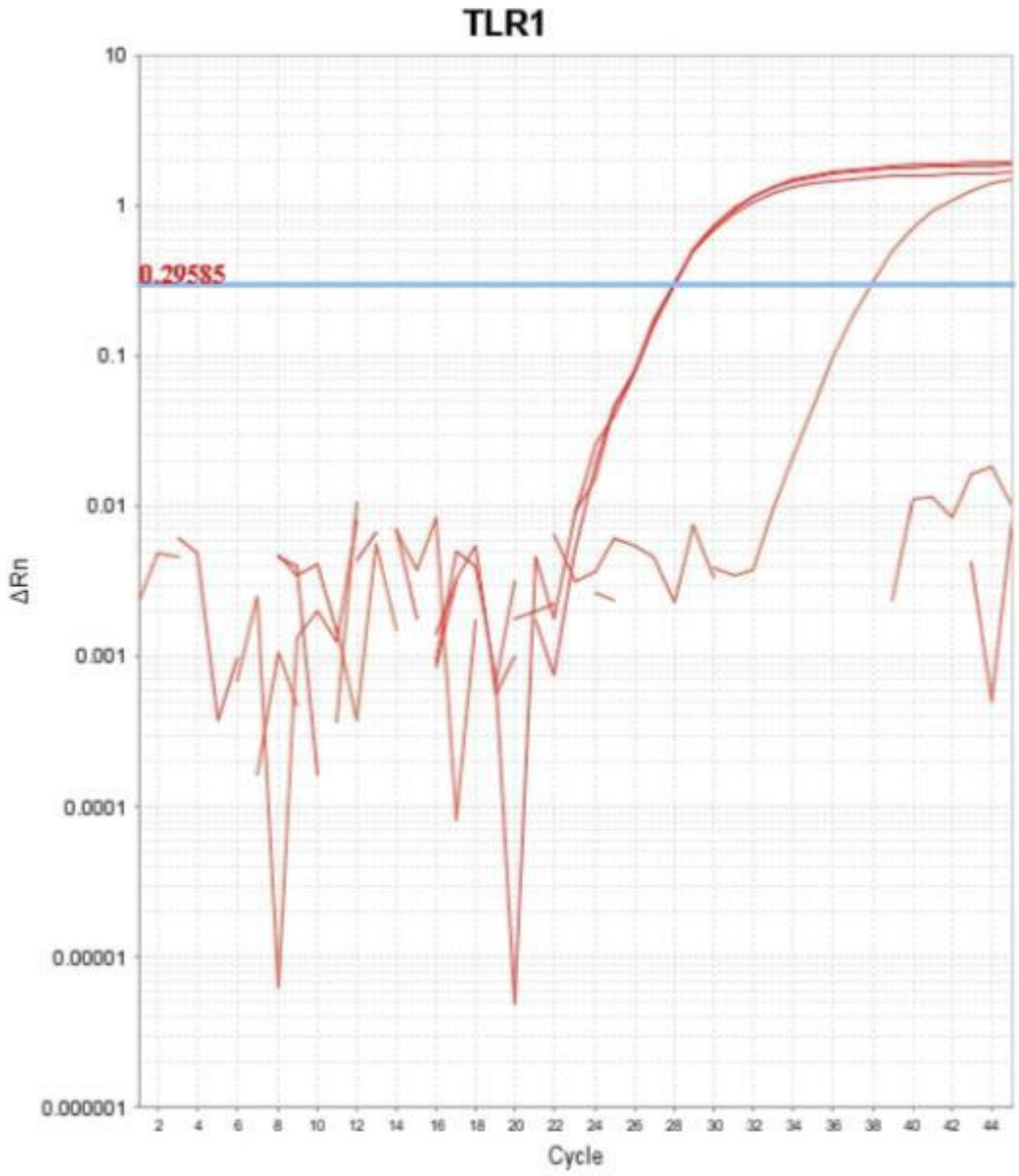

Figure 8: Typical amplification curve of qRT-PSR. For TLR1 gene in 4 days HaCaT cells.

Figure 9: Typical amplification curve of qRT-PSR. For TLR2 gene in 4 days HaCaT cells.

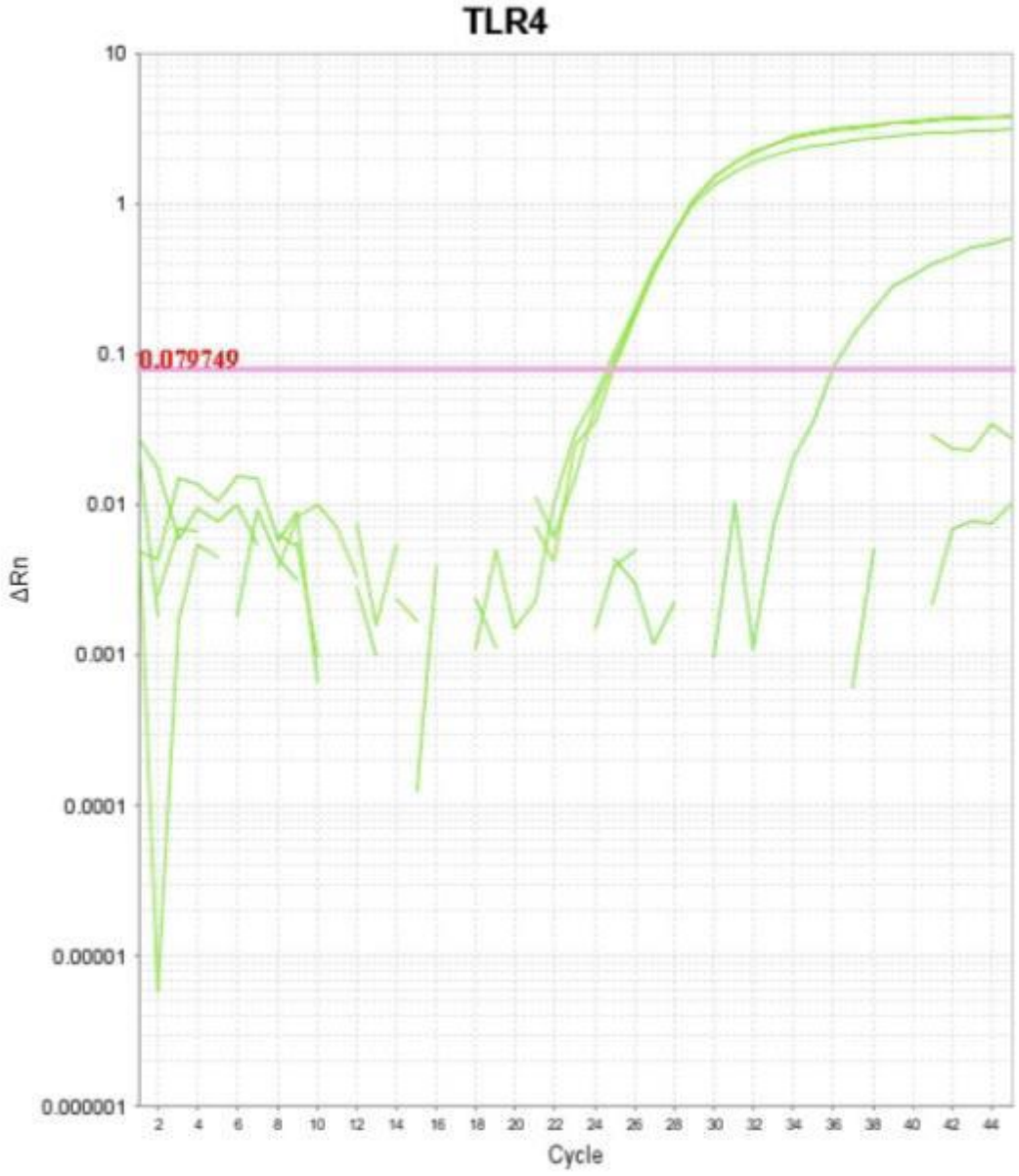

Figure 10:Typical amplification curve of qRT-PSR. For TLR4 gene in 4 days HaCaT cells.

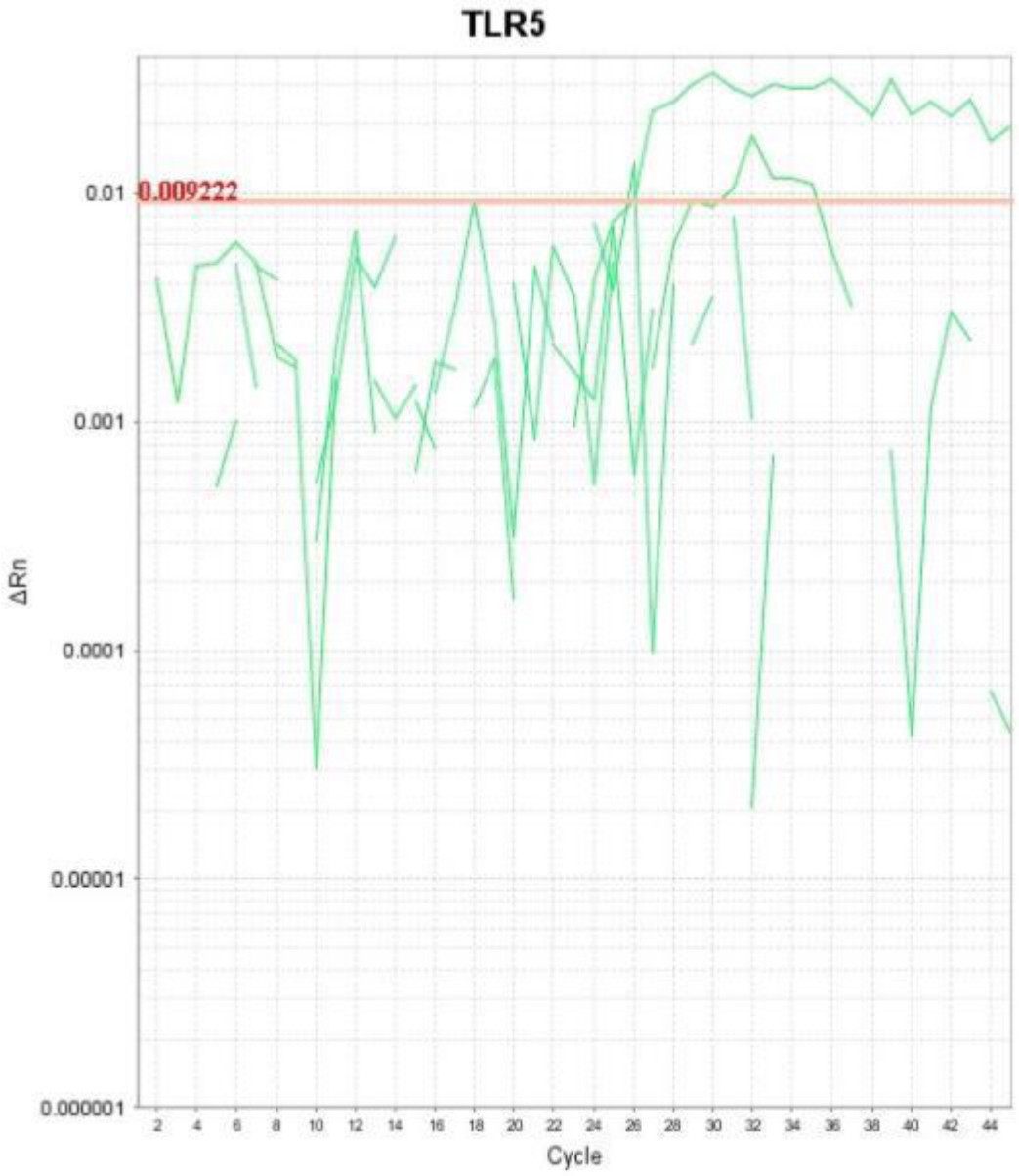

Figure 11: Typical amplification curve of qRT-PSR. For TLR5 gene in 4 days HaCaT cells.

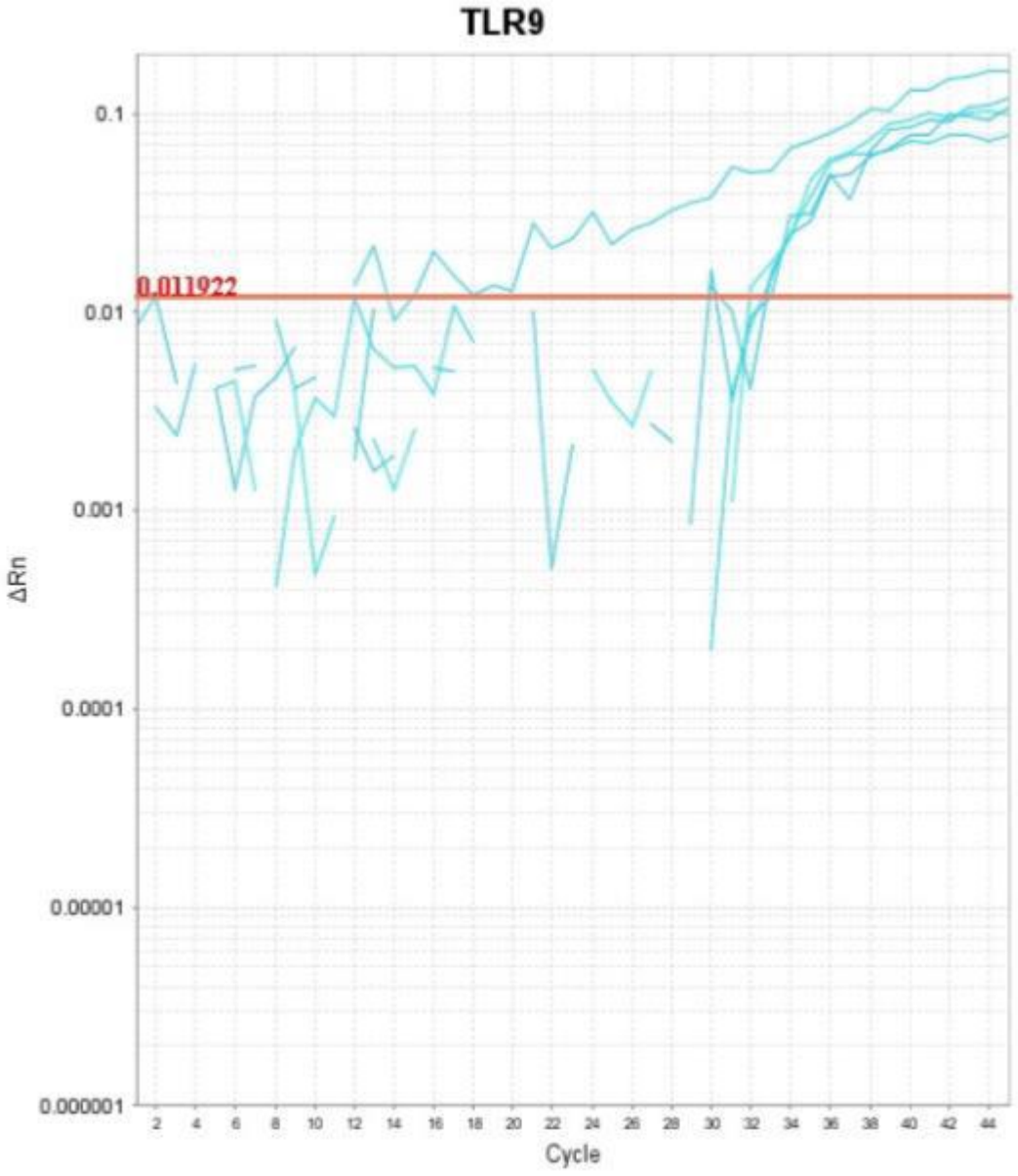

Figure 12: Typical amplification curve of qRT-PSR. For TLR5 gene in 4 days HaCaT cells.

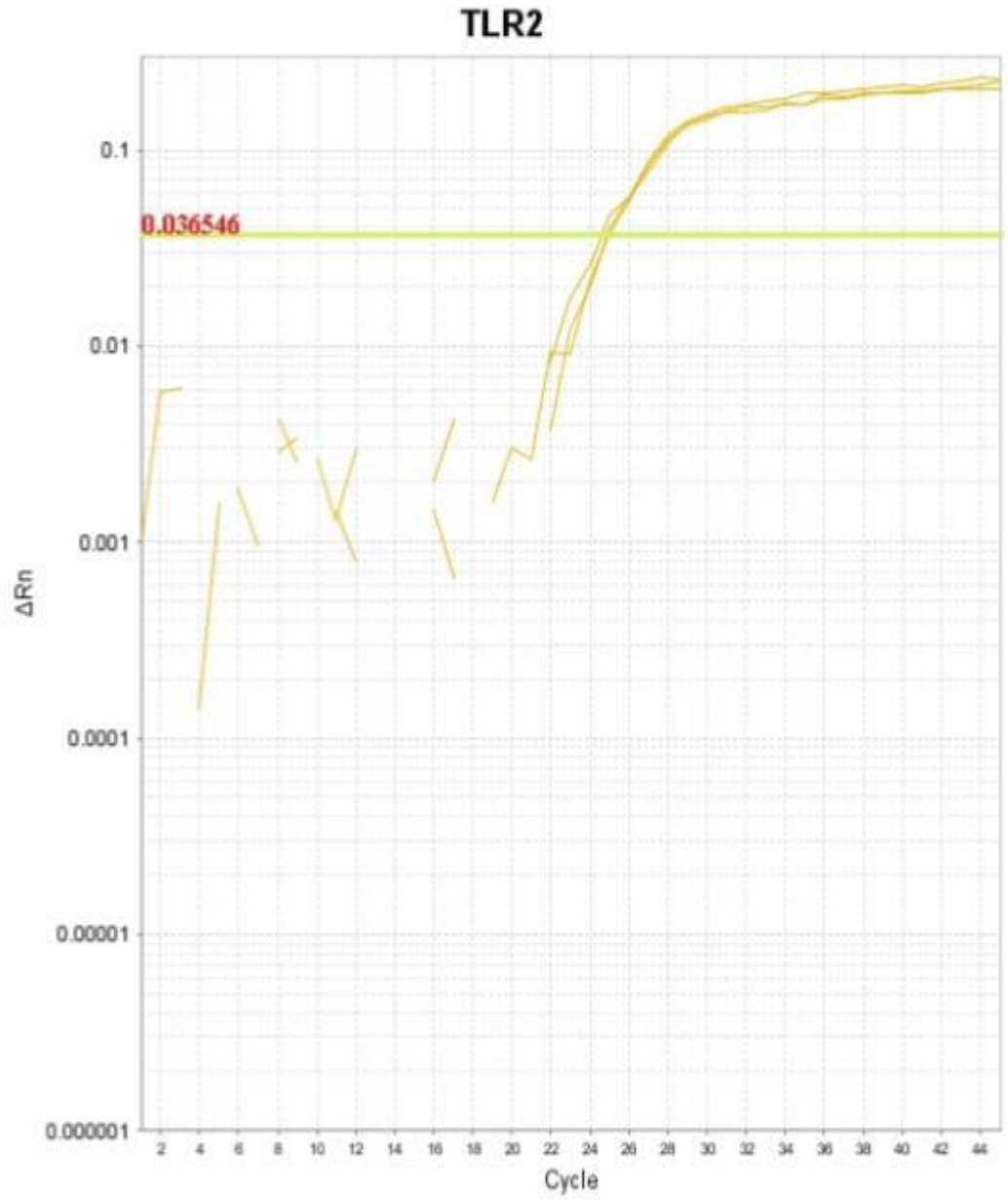

Figure 13: Typical amplification curves of qRT-PCR for TLR2, in 10 days HaCaT cell.

3.2 Gene expression of TLR in HaCaT:

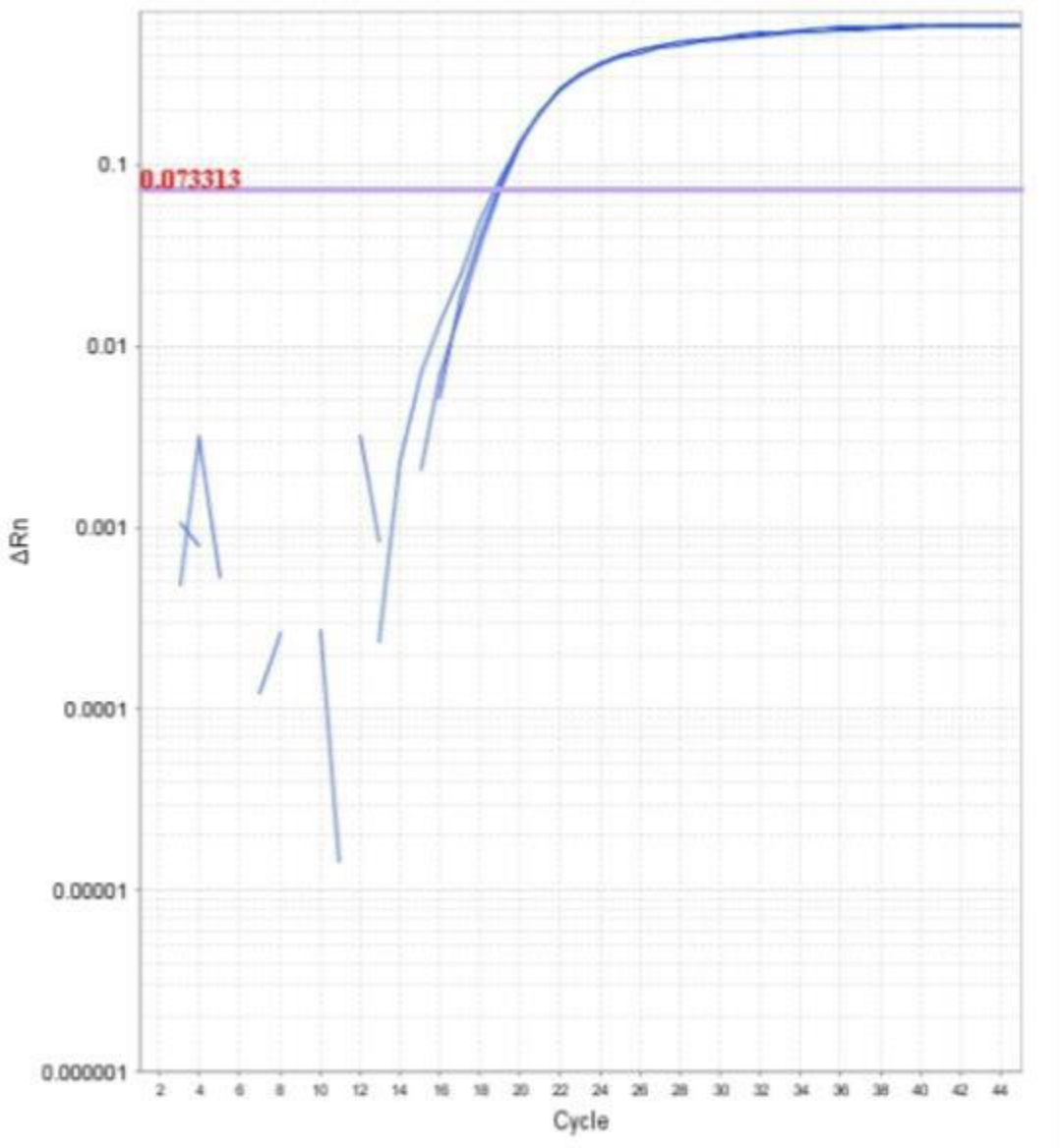

Figure 14: Typical amplification curves of qRT-PCR for Actin, in7 days HaCaT cell.

Δ Rn: Fluorescence signal.

Threshold: (0.073313) at which significant and specific amplification occurs.

Cycle: cycle number of amplification.

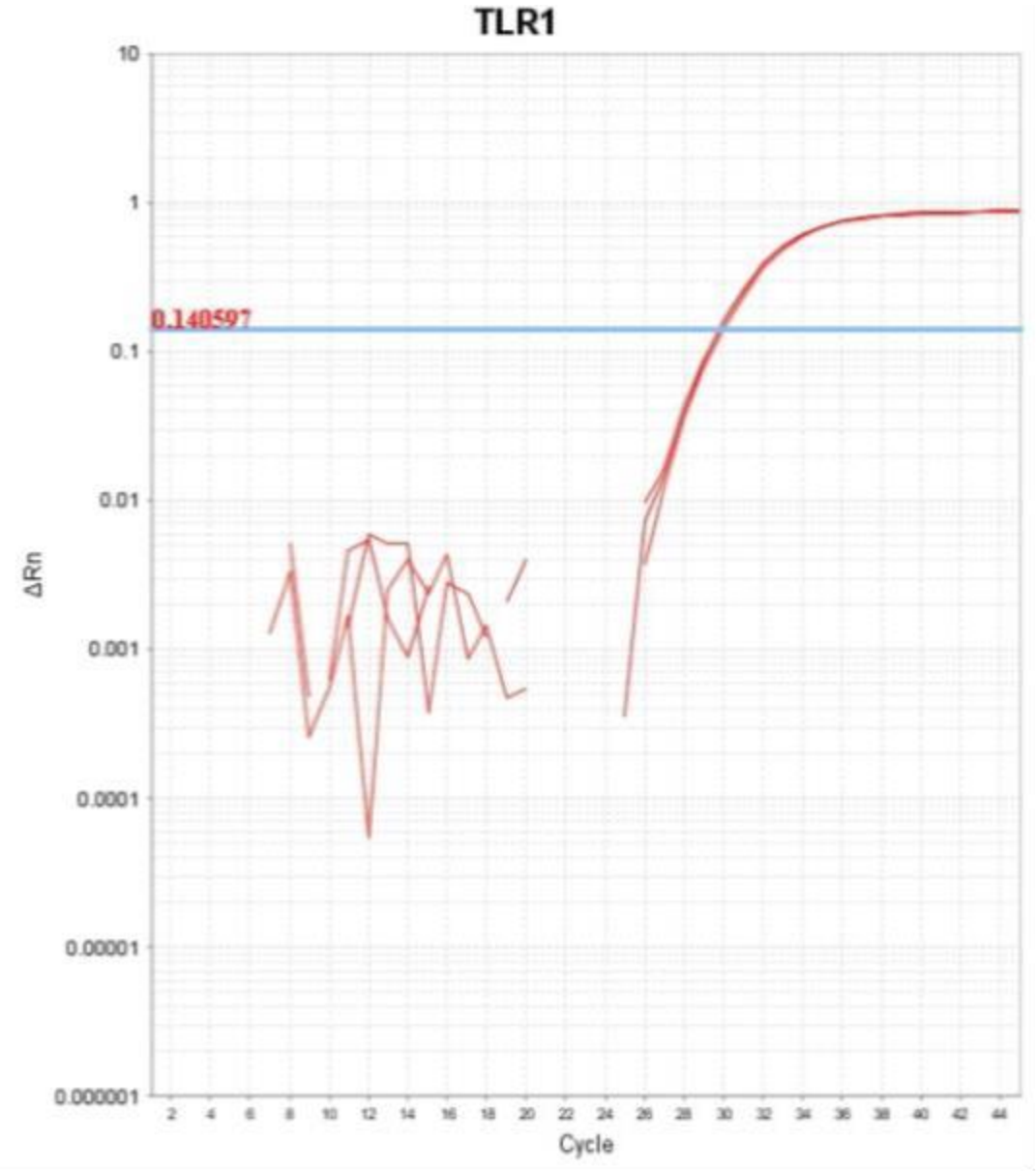

Figure 15: Typical amplification curves of qRT-PCR for TLR1, in10 days HaCaT cell.

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