# *Pseudomonas aeruginosa* Bacterial Biofilms

**By**

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## ABSTRACT

### *Pseudomonas aeruginosa* Bacterial Biofilms



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This thesis is an investigation of *Pseudomonas aeruginosa* bacterial biofilms. The objective of the first study was to evaluate the biofilm-forming capacity of canine otitis isolates of *P. aeruginosa* and to compare the minimum inhibitory concentrations (MICs) of antimicrobials for planktonic versus biofilm-embedded bacteria. Biofilm forming ability was assessed using a microtitre plate assay. Broth microdilution was used to assess the MICs of neomycin, polymyxin B, enrofloxacin and gentamicin for the planktonic and biofilm-embedded bacteria of eighty-three isolates. Thirty-three (40%) isolates were biofilm producers and MICs for biofilm-embedded bacteria were significantly higher than their planktonic counterparts for all antimicrobials (all $P<0.05$).

The objective of the second study was to evaluate the impact of Tromethamine edetate disodium dihydrate (Triz-EDTA®) in combination with antimicrobials on antimicrobial susceptibility of *P. aeruginosa* biofilm-embedded bacteria. MICs of the four antimicrobials for the biofilm embedded bacteria and biofilm-embedded bacteria with added Triz-EDTA® were assessed with broth microdilution for thirty-one biofilm-producing isolates. Addition of Triz-EDTA® significantly reduced MICs for neomycin ($P < 0.008$) and gentamicin ($P < 0.04$) but not enrofloxacin ($P = 0.7$), or polymyxin B ($P = 0.5$).

The objective of the third study was to determine the presence of biofilm-associated genes

in biofilm forming and non-biofilm forming isolates. Four genes involved with carbohydrate matrix production (*pelA*), irreversible attachment (*sadB*) and quorum sensing (*lasB*, *rhlA*) were selected. DNA was extracted and polymerase chain reaction (PCR) was performed for all isolates**.** All isolates possessed *lasB* and *sadB,* 74 (90%) possessed *pelA* and 74 (90%) possessed *rhlA*. All thirty-two (100%) isolates that were classified as biofilm producers contained all genes. There was an association between the presence of *pelA* and *rhlA* and biofilm production ($P < 0.017$) and between the presence of *rhlA* and *pelA* and the quantity of biofilm produced (both $P < 0.001$).

These results highlight that biofilm formation of *Pseudomonas aeruginosa* otic isolates does occur and can impact antimicrobial therapy. Certain compounds can also influence antimicrobial susceptibility of biofilm-embedded bacteria. Genetics may also play a role in biofilm formation.

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## DECLARATION OF WORK PERFORMED

I declare that, with the exception of the items below, all work reported in this thesis was performed by me.

Original isolate collection was performed by the Animal Health Laboratory (Guelph, Ontario, Canada) and IDEXX (Markham, Ontario, Canada). Dr Scott Weese assisted with statistical analysis of the results of this study. Ms. Joyce Rousseau submitted samples for sequencing, which was performed by Macrogen Inc.

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## LIST OF ABBREVIATIONS USED IN THE TEST

**AHL** N-acyl homoserine lactones

**c-di-GMP** bis-(3'-5')-cyclic dimeric guanosine monophosphate

**C2** N-decanoyl-L-homoserine benzyl ester

**CBD** Calgary Biofilm Device

**CF** Cystic fibrosis

**Cup** Chaperone usher pathways

**DGC** Diguanylate cyclase

**eDNA** Extracellular DNA

**EPS** Extrapolymeric Substance

**ESBL** Extended-spectrum ß-lactamases

**IFN-gamma** Interferon gamma

**LPS** Lipopolysaccharide

**MBC** Minimum Bactericidal Concentration

**MBEC** Minimum Biofilm Eradication Concentration

**Mbp** Mega base pairs

**MCP** Methyl-accepting chemotaxis protein

**MDR** Multidrug resistant

**MIC** Minimum Inhibitory Concentration

**NO** Nitrogen oxide

**OD** Optical Density

**OE** Otitis externa

**PAMPs** Pathogen associated molecular patterns

**PCR** Polymerase chain reaction

**PI3K** Phosphatidylinositol-3-kinase

**$PIP_3$** Phosphatidylinositol (3,4,5)-triphosphate

**PQS** *Pseudomonas* quinolone signal

**PRRs** Pattern recognition receptors

**QS** Quorum sensing

**RSCVs** Rugose small-colony variants

**SCV** Small colony variants

**TLRs** Toll-like receptors

**TpbA** Tyrosine phosphatase related to biofilm formation A

**TPS** Two-partner-secretion

**Triz-EDTA** Tromethamine Edetate Disodium Dihydrate

# CHAPTER ONE: Literature Review and Objectives

## 1.1 Introduction

Otitis externa (OE) is a common condition in dogs and can account for anywhere between 4.5-20% of all cases presented to a small animal veterinarian (Hill *et al* 2006, Miller *et al* 2013). Otitis externa is due to primary, predisposing and perpetuating factors. Primary factors account for the underlying etiology that triggers the changes within the ear canal, such as allergic skin disease. Predisposing factors are present prior to the development of the otitis, including anatomic variation and moisture within the ear canal. Perpetuating factors occur as a result of the inflammation and include excessive debris (Miller *et al* 2013). Other perpetuating factors include fungal and bacterial infections. The most commonly isolated pathogens in cases of infectious canine OE are *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa* and *Malassezia pachydermatis* (Miller *et al* 2013).

In cases of bacterial otitis, *Pseudomonas aeruginosa* is often implicated (Cole *et al* 1998, Nuttall *et al* 2007). *P. aeruginosa* has not been isolated from healthy canine ears and, when present, can result in inflammation and ulceration within the external ear canal (Tater *et al* 2003, Miller *et al* 2013). Treatment may be difficult as *P. aeruginosa* is intrinsically resistant to multiple antimicrobial classes and can also acquire resistance (Nuttall *et al* 2007). In order to resolve the otitis, treatment must involve a regimen for the underlying primary and predisposing factors (Miller *et al* 2013). Due to this multi-modal approach, intrinsic resistance and the chronic nature of the disease, treatment may be of a long duration and can become costly. In refractory cases, surgical treatment of OE, which consists of total ear canal ablation, may be required (Miller *et al* 2013).

The vast majority of study of bacterial ecology and infection involves the free-living planktonic form. Yet, some bacteria are able to produce biofilm. A bacterial biofilm is a community of sessile bacteria that form layers of planktonic bacterial cells. As the biofilm matures, the cells become irreversibly attached to a surface and produce a matrix (extrapolymeric substance, EPS) made of carbohydrates, proteins and DNA (Donlan 2001). This matrix will provide the bacteria with protection from dessication, the host immune response and antimicrobials. Biofilm-embedded bacteria have an altered metabolism and enhanced cell-cell communication (Costerton 1999, Clutterbuck *et al* 2007). This will serve to increase the resistance to antimicrobials and evasion of the host immune system (Clutterbuck *et al* 2007). Biofilm formation is thought to be an important virulence factor for some bacteria and some infection types.

The role of planktonic *P. aeruginosa* in cases of otitis externa has been well described in the veterinary literature. However, there has been limited study of *P.* aeruginosa biofilms in canine otitis despite the fact that this is an infection that could be strongly associated with biofilm production (Clutterbuck *et al* 2007). *Pseudomonas* spp. have been noted to form biofilms in any environment whereas other bacterial species often require a specific temperature or pH (Clutterbuck *et al* 2007).

In human medicine, bacterial biofilms have been studied extensively and have been noted to form on indwelling catheters, tooth enamel, surgical implants, wounds, burns and in the lungs of patients with cystic fibrosis (Darouiche 2004, Kirketerp-Moller *et al* 2008, Moreau-Marquis *et al* 2008). Biofilms have also been studied, to a lesser degree, in the veterinary literature, and have been found to form within the middle ear cavity, in cases of mastitis, on surgical implants and on wounds (Donlan 2001, Ehrlich *et al* 2002, Melchior *et al* 2006, Watters *et al* 2012, Singh *et al* 2013). However, studies regarding *Pseudomonas* biofilm formation are limited.

Previous studies have documented that cells within a biofilm are less susceptible to antimicrobials *in vitro* than their planktonic counterparts (Hoyle *et al* 1992, Allesen-Holm *et al* 2006). This may be due to the physical protection provided by the EPS, which prevents antimicrobials from reaching the bacteria. In addition, resistance could also be due to the metabolic changes within the biofilm that can affect the target of the antimicrobial (Caiazza *et al* 2004, Khan *et al* 2010). As the biofilm-embedded bacteria may be more resistant to antimicrobials, understanding the ability of clinical otic isolates of *Pseudomonas aeruginosa* to form biofilms, and their susceptibility to these antimicrobials is paramount to allow treatment plans to be formulated. It is also important to investigate other treatment modalities that may help increase penetration of antimicrobials through the biofilm, or disrupt the biofilm. Tromethamine (Tris), edetate disodium dihydrate (EDTA) buffered to pH8 with Tromethamine HCL and deionized water, (Triz-EDTA® Aqueous flush, Dechra Veterinary Products, Kansas, US) is used as an adjunct therapy for dogs with *Pseudomonas* otitis (Nuttall *et al* 2007). The tris buffer enhances the effect of the EDTA, which damages the cell surface of gram-negative bacteria such as *Pseudomonas* (Wooley *et al* 1983). Studies have shown that Triz-EDTA® used in combination with an antimicrobial, will reduce the minimum inhibitory concentration and minimum bactericidal concentration for certain antibiotics for both planktonic bacteria and those within biofilms (Wooley *et al* 1983, Sparks *et al* 1994, Buckley *et al* 2012).

*Pseudomonas aeruginosa* has a genome containing 6.26 Mega base pairs (Mbp) that encode for 5567 genes (Lambert 2002). Studies have highlighted the potential role certain genes may have in biofilm formation (O'Toole *et al* 1998, Musken *et al* 2010). *SadB* (surface attachment deficient) has been shown to contribute to the transition from reversible to irreversible attachment leading to initial biofilm formation (Lambert 2002, Caiazza *et al* 2004). *Pel* codes for a specific type of carbohydrate expression within the biofilm matrix. Mutants will develop a different

morphology and may not form attachment structures needed for biofilm formation (Friedman *et al* 2004). Cell-to-cell signalling systems (quorum sensing systems) are important for communication between cells in the biofilm. It has been noted that the *lasR-lasI* and *rhlR-rhl* systems are two of the main systems operating in *P. aeruginosa* (Davies *et al* 2007). Previous studies have found that mutations in genes coding for these quorum sensing signals can lead to defective biofilm production, which could lead to decreased protection from the external environment and antimicrobials (Sauer *et al* 2002). Identifying genes that are present during biofilm formation or those that may play a role in biofilm formation will help us further understand the biofilm production of *Pseudomonas* otic isolates.

**1.2 Anatomy of the canine external ear**

The canine external ear includes the external pinna and both the vertical and horizontal canals (Appendix 1). The pinna and vertical canal are formed from auricular cartilage, which becomes funnel shaped at the base of the pinna and forms the beginning of the vertical canal (Heine 2004). The tragus and anthelix, protrusions of the auricular cartilage, are found at the base of the pinna and mark the entrance to the vertical canal (Cole 2004, Heine 2004). This vertical canal measures approximately two and a half centimetres, extending ventrally and rostrally before turning medially into the horizontal canal (Cole 2004). Annular cartilage and auricular cartilage of the osseous external auditory meatus comprise the horizontal canal (Heine 2004). The external canal stops at the tympanic membrane where there is a separate band of the annular cartilage. This represents the junction between the external ear canal and the middle ear, formed by the osseous bulla (Cole 2004).

The skin lining the external ear canal is an extension of the skin covering the rest of the body. This skin has a thin epidermis and a dermis containing adnexa (hair follicles, sebaceous glands,

ceruminous glands) (Miller *et al* 2013). There are breed specific variations in the size and shape of the ear pinnae, the number of sebaceous glands and ceruminous glands within the external ear canal (Heine 2004, Cole 2009). Cerumen, made from desquamated keratinized epithelial cells and secretions from the ceruminous and sebaceous glands, coats the canal (Heine 2004). Neutral lipids make up the majority of the lipid content of cerumen in normal canine ears (Huang *et al* 1994). There is a regular turnover of the cerumen and epithelium within the ear canal, which will remove debris within the external ear (Heine 2004).

A healthy canine ear canal will still contain low numbers of bacteria and yeast. In one study by Tater *et al*, they documented that 96% of normal canine ears contained yeast and the highest number noted was 2.1 per high power field (Tater *et al* 2003). The most commonly isolated bacteria from the canine ear are *Staphylococcus* spp. and *Streptococcus* spp. Rod shaped bacteria, other than *Corynebacterium*, are not usually found in normal canine ear canals (Angus 2005).

The function of the external ear canal is to collect sound waves at the ear pinnae, funnel them into the external canal and then transmit them to the tympanic membrane through to the inner ear (Miller *et al* 2013, Cole 2009, Cole 2012). In a non-inflamed canine external ear, the tympanic membrane can usually be visualized via otoscopy and the pH of the canal is 6.1-6.2 (Grono 1970).

### 1.3 Otitis externa

Otitis externa (OE) is defined as inflammation of the external ear canal as a result of multiple underlying causes and etiologies (Miller *et al* 2013). In response to the inflammatory triggers, the blood vessels in the dermis dilate leading to increased permeability and edema. Stenosis of the external canal may then occur due to edema present within the tissue surrounding the canal. The subsequent inflammation leads to a highly permeable epithelium causing loss of epidermal

barrier function. As epidermal barrier integrity is lost, bacterial and toxin penetration is increased through the skin, which further exacerbates the inflammatory process (Angus 2005). The external ear canal becomes filled with cerumen, as production increases in times of inflammation (Angus 2005). Cerumen blocking the external ear canal, combined with the stenosis, will provide a warm, moist environment that favours growth of microorganisms (Angus 2005). There are also changes in the cerumen composition including decreased lipid content which changes the microenvironment within the ear and may further facilitate proliferation of microorganisms (Angus 2005, Cole 2009).

In dogs with acute OE, the pH of the external ear canal becomes more acidic and can drop to approximately 5.9; which maybe a more appropriate pH for bacterial populations to flourish (Grono 1970). As the disease process becomes more chronic, the continued inflammation leads to hyperplastic epithelium, increased epidermal turnover and hyperkeratosis (Angus 2005). Normal epithelial migration within the external ear canal is disrupted and therefore, more cellular debris accumulates blocking the ability of debris to exit the canal exacerbating accumulation of excess cerumen. Neutrophils will migrate into the ear canal in any acute inflammatory process but this will be exacerbated during a bacterial infection. A purulent exudate will begin to form and in certain cases can be seen draining from the ear canal (Angus 2005). Bacterial exotoxins, in combination with neutrophil proteases, can further compromise the epithelium causing erosion and ulceration. This is especially the case if the bacterium *Pseudomonas aeruginosa* is present (Angus 2005). Chronic changes to the ear canal also include mineralization of the cartilage surrounding the ear canal, glandular hyperplasia and further stenosis (Angus 2005).

Otitis externa is a common condition of dogs. The prevalence of canine OE varies from 4.5% to 20% or above (Hill *et al* 2006, Miller *et al* 2013). In 2011, OE was the most common pet health insurance claim in North America and has been for years prior to this survey (Veterinary

Pet Insurance Co. 2012). OE is a frustrating problem for pet owners and veterinarians. Treatment can be difficult as certain bacteria are intrinsically resistant to multiple antimicrobial classes and may also acquire resistance (Nuttall *et al* 2007). This will lead to increased treatment costs, the potential for further antimicrobial resistance to develop and the need for multi-modal therapy. If medical treatment fails, patients may need surgical treatment such as a total ear canal ablation or lateral bulla osteotomy which both have the potential for increased morbidity and complications (Miller *et al* 2013). The inflammation and pain suffered by canine OE patients is also of great concern to owners and veterinarians.

Otitis externa is due to primary, predisposing and perpetuating factors. Primary factors include underlying etiologies that trigger the changes within the ear canal; such as allergic skin disease and endocrine disease. Predisposing factors are present prior to the development of the otitis, and include anatomic variation in pinnal size and shape, and excess moisture within the ear canal. Perpetuating factors include middle ear disease or excessive production of debris and occur as a result of the inflammation (Miller *et al* 2013). Other perpetuating factors, sometimes known as secondary causes of OE, include fungal and bacterial infections (Miller *et al* 2013). The dominant pathogens in cases of infectious canine OE are *Staphylococcus pseudintermedius*, *Pseudomonas aeruginosa* and *Malassezia pachydermatis* (Miller *et al* 2013).

Otitis externa occurs at any age but certain breeds are predisposed, including Cocker spaniels, Brittany spaniels, Jura des Alpes, Golden retrievers, Poodles, West Highland White terriers, German shepherds, Pyrenean Shepherds and Labrador retrievers (Saridomichelakis *et al* 2007, Miller *et al* 2013). Clinical signs associated with OE include head shaking, aural pruritus, pain, alopecia of the pinnae, discharge and odour from the ear canal (Miller *et al* 2013). Dogs will often have clinical signs associated with the underlying primary factor causing the otitis, such as interdigital erythema (allergic skin disease) or symmetric alopecia (endocrine disorders) (Rosser

2004). Cases of OE can be both unilateral or bilateral (Miller *et al* 2013). A previous study documented that cases of bilateral otitis externa were slightly more prevalent at 53% of total otitis cases (Pugh *et al* 1974).

Otitis externa can be acute or chronic in nature. There is currently no accepted definition for what constitutes chronicity in cases of OE; clinically a patient with chronic disease will present with glandular hyperplasia, stenosis of the external ear canal and mineralization of the ear canal. The infection present can also move into the middle ear to cause otitis media (Miller *et al* 2013).

Performing a thorough dermatological examination is extremely important in cases of suspected OE, as there may also be alopecia, excoriations, erythema on the side of the face, back of the ear pinna and aural hematomas (Cole 2012, Miller *et al* 2013). Palpation of the external ear canal may reveal thickening of the pinna or mineralization of the external ear canal and hyperesthesia. In cases of ear canal mineralization due to chronic otitis, medical treatment is rarely successful and surgical management is usually required (Miller *et al* 2013).

### 1.4 *Pseudomonas aeruginosa* otitis externa

*Pseudomonas aeruginosa* and other gram-negative rod shaped bacteria are not commensal bacteria of the external ear canal (Cole *et al* 1998, Yoshida *et al* 2002, Tater *et al* 2003, Sturgeon *et al* 2012). *P. aeruginosa* is one of the most common bacterial species isolated from the ear canal of dogs with OE and is isolated in up to 35% of cases of otitis externa and media (Cole 2012, Nuttall *et al* 2007). In one study, *P. aeruginosa* was isolated from 27.8% of canine ear samples submitted over a 6-year period and the frequency of isolation per year did not change over this same period (Petersen *et al* 2002).

Aural examination will reveal erythema, possibly alopecia around the ear, crusting and usually pruritus. There may be a yellow/green malodorous discharge emanating from the external

ear canal containing bacteria, white blood cells and debris (Shaw 2012, Miller *et al* 2013). Infections of the external ear canal due to *Pseudomonas aeruginosa* also present with pain and ulceration (Nuttall *et al* 2007). If the otitis externa is left untreated, it can progress to otitis media after rupture of the tympanic membrane due to inflammation. Otitis media can result in neurological signs (Miller *et al* 2013).

### 1.5 *Pseudomonas aeruginosa* microbiology and infections

*Pseudomonas* spp. are gram-negative bacteria that are not part of the enterobacteriaceae family. They are found ubiquitously within the environment in soil, water, decaying vegetation and on animals. The most clinically significant member of this family is *Pseudomonas aeruginosa* (Koenig 2012). *Pseudomonas aeruginosa* is an opportunistic pathogen and will lead to infection in individuals that are immunocompromised or if skin epithelium is damaged from trauma. In human hospitals, *Pseudomonas* is a leading cause of nosocomial infections via colonization of catheters, skin wounds, ventilator-associated pneumonia and it is also a cause of respiratory infections in individuals with cystic fibrosis (CF) (Pier 1998, Kierbel *et al* 2007). Colonization by *Pseudomonas* spp. occurs when the fibronectin coat surrounding host cells is destroyed due to trauma or infection (Koenig 2012). The same is true in animals: those that are immunocompromised or have impaired immune function are more likely to succumb to infection with *P. aeruginosa*. The bacteria are normally found in the gastrointestinal tract, genital regions and upper respiratory tract but rarely lead to clinical disease as numbers are kept low by normal bacterial flora in these areas. If there is injury to these sites, inflammation or chronic antibiotic therapy that destroys normal flora, numbers can increase and lead to infection (Koenig 2012).

*Pseudomonas aeruginosa* and other gram-negative bacteria have an outer membrane plus the inner cytoplasmic membrane and intermediate peptidoglycan layer (Koenig 2012). This outer

membrane is not present in gram-positive bacteria. The polysaccharide capsule (also known as the glycocalyx) surrounds the outside of the bacteria. This capsule is a virulence factor and acts to protect the outer membrane from attack by components of the complement membrane complex and inhibits the attachment by phagocytic cells (Koenig 2012). The outer layer of the membrane is composed of mainly lipopolysaccharides (LPS), another important virulence factor for *Pseudomonas*. The LPS consists of a lipid portion embedded in the membrane, (lipid A, which is the component of endotoxin) and polysaccharide (also known as the 'O' antigen) that extends from the bacterial surface (Pier 2007, Koenig 2012). The 'O' antigen is what determines the serogroup of the bacteria. There are approximately 11 variants of this 'O' antigen for *Pseudomonas* spp. The serogroup O14 and O15 lack detectable 'O' antigens but are still able to cause infection, indicating that there may be other components within the LPS, such as lipid A, acting as virulence factors (Pier 2007). Pattern-recognition receptors (PRRs) are expressed on cells of the innate immune system. Their function is to bind to pathogen-associated molecular patterns (PAMPs) entering the body. The lipid A portion of LPS acts as a PAMP that binds specifically to Toll-like receptor 4 (TLR-4), a PRR found on monocytes and at lower levels on B cells. The lipid A is thus a trigger of the innate immune response to infection by *Pseudomonas* (Clutterbuck *et al* 2007, Pier 2007). If TLR-4 recognizes the LPS and the innate immune response is activated there may be resistance to infection. However, if the LPS is protected from binding to TLR-4, the innate immune response will not be triggered (Pier 2007). LPS also stimulates cytokines to be produced and released and also triggers the complement cascade (Pier 2007). *Pseudomonas* can produce collagenase, lecithinase, lipase, protease, hemolysin, fibrinolysin, leukocidin and enterotoxin (Koenig 2012). These enzymes play a role in inflammation and the damage done to the host tissue by *Pseudomonas* spp. Complement is then activated and opsonization of the bacteria will occur. Bacterial cells will also be phagocytized

and the adaptive immune system will be activated (Clutterbuck *et al* 2007).

*Pseudomonas* spp. can survive on a wide range of substrates and adapt to changes in the environment (Lambert 2002). *Pseudomonas* causes infections ranging from ear disease to sepsis and the pathogenicity of the bacteria depends upon the presence of virulence factors, such as the LPS, toxins and adhesins (Klemm *et al* 2000, Pier 2007). For an infection to occur, the bacteria must first adhere to an epithelium via the presence of adhesins on the bacterial cell binding to lectins on the host cell surface. Adhesins are structures located on the bacterial outer membrane that are part of the bacterial fimbriae (an appendage on the outer surface of the bacterium). These adhesins are relatively sensitive and will only bind to certain molecules/proteins (Klemm *et al* 2000, Koenig 2012).

*Pseudomonas aeruginosa* will preferentially bind and enter cells via the basolateral surface (Koenig 2012). Organs, including skin, are usually lined with a multicellular epithelium containing cells that have both apical and basolateral surfaces. Each surface has specific proteins and lipids and are separated by tight junctions. The apical surface is a barrier to the outer environment and helps to exchange materials between the lumen of the cell and the external environment; whereas the basolateral surface is used for interactions with other cells and exchange with blood (Kierbel *et al* 2007). *Pseudomonas* will bind near a cell-to-cell junction and activate phosphatidylinositol-3-kinase (PI3K) to move to the apical surface. Protrusions from the membrane that are enriched with phosphatidylinositol (3,4,5)-triphosphate ($PIP_3$) and actin will accumulate at the apical surface as the site of binding. The protrusions lack apical membrane markers and are made of constituents of the basolateral membrane. Through this mechanism, epithelial damage makes it easier for *Pseudomonas* to enter the cell (Kierbel *et al* 2007).

*Pseudomonas* has few nutritional requirements and thrives in many different environments (Siegrist 2007). Moist environments provide the best resources for the bacteria; such as drains,

bath tubs etc. Open wounds, catheters or inflamed epithelium are susceptible to contamination from moist environments and hence infection by *Pseudomonas* (Koenig 2012).

### 1.6 Diagnosis of *Pseudomonas aeruginosa* otitis externa

Diagnosis of otitis externa is based on history, clinical signs, dermatologic examination, otoscopic examination, otic cytology and possibly cultures from the external ear canal. History taking should include questions regarding underlying disease and previous episodes of OE to determine what the primary factor is and whether this is a new episode of acute OE or whether OE is recurrent and chronic (Miller *et al* 2013). A thorough dermatologic exam should take place to document whether there are any primary or secondary lesions in other regions of the body, such as erythema, epidermal collarettes, pustules or excoriations, that would provide evidence for the primary factor underlying the development of OE (Miller *et al* 2013).

In any case of otitis externa, cytology must be performed to diagnose any secondary infections such as *Pseudomonas*. Visual appearance of otic exudate is often misleading and a veterinarian cannot make a definitive diagnosis of an infection due to a specific colour or texture of the exudate (Miller *et al* 2013). A cotton swab is inserted into the external ear canal to the level of the junction of the horizontal and vertical canals. This swab is then removed and rolled onto a slide (Shaw 2012). The sample is heat fixed and stained using a Romanowsky-type stain (modified Wright's stain or DiffQuik®) (Cole 2012). There is discrepancy between studies as to how many organisms constitute an active infection versus normal flora for Staphylococci. However, for *Pseudomonas*, even small numbers of the organism can constitute an infection, as gram-negative rods are not usually seen within the ear canal of unaffected dogs (Tater *et al* 2003, Cole 2012). When inflammatory cells are noted on cytology, this is a significant finding and the number of organisms is irrelevant; especially if the organisms are located within the cells (Miller

*et al* 2013). Although cytology can identify the shape and number of microorganisms within the external ear canal, it cannot definitively determine the species. A bacterial culture is required to speciate bacteria and allow for antimicrobial susceptibility testing.

Cytology results will serve as a guide as to whether aerobic bacterial culture and susceptibility testing is required. In most cases where gram-negative rods are visualized, a bacterial culture and susceptibility testing is recommended as well as in cases where previous topical antibiotics have been ineffective (Shaw 2012, Cole 2012). Susceptibility testing indicates the minimum inhibitory concentration (MIC) of systemic doses of antibiotics. If an oral antibiotic is selected, especially for cases of otitis media, these culture results will guide the choice of oral antibiotic. For most cases of otitis externa, topical therapy will be used. Response to topical medication does not often correlate with susceptibility testing results and the choice of topical antimicrobial may not always be based on the culture results (Morris 2004). In one study 10/16 cases were reported to be resistant to a certain antimicrobial based on bacterial culture and susceptibility results. Clinically and cytologically 90% of these cases responded to topical therapy with this same antimicrobial (Robson *et al* 2010). There is concern as to whether bacterial culture and susceptibilities are reproducible and their validity. A study by Graham-Mize *et al* found that parallel cultures submitted to the same laboratory for *Pseudomonas aeruginosa* differed in their susceptibility patterns in 11% of cases and the cytology findings were in agreement with the culture findings only 68% of the time (Graham-Mize 2004). Another study found that some diagnostic laboratories inconsistently isolated *Pseudomonas* from bacterial cultures with rods noted on cytology, and there was also variation between susceptibility patterns (Schick *et al* 2007). This variation could be due to small numbers of organisms being present or differences in sample preparation and processing. However, these results are concerning as

*Pseudomonas* otitis can be challenging to treat and with antibiotic resistance always a concern, using antibiotics that *Pseudomonas* is not susceptible to, could lead to further resistance.

Otoscopic examination will detect whether foreign bodies are present within the ear canal, the magnitude of stenosis of the ear canal and whether exudate is present in the canal (Miller *et al* 2013). Both ears should be examined and cytology must be taken from both ear canals to compare the difference between the two in cases of bilateral OE and to compare abnormal to normal in cases of unilateral OE. The unaffected ear, or less affected, should be examined first to determine the baseline for that individual and to prevent the patient from becoming painful early in the examination, necessitating sedation or anesthesia (Miller *et al* 2013). In some cases of chronic or painful ear disease, sedation or anesthesia maybe required and even then this may not allow adequate visualization if the canal is stenotic or filled with debris/exudate. In these cases, anti-inflammatory therapy maybe needed for 7-14 days before otoscopic examination is successful (Miller *et al* 2013). In these latter cases where debris is present, flushing of the ear canal with sterile saline is required to remove exudate (Gortel 2004). When examining both ears, the otoscopic cone must be changed between the ears, as previous studies have documented that *Pseudomonas* and other organisms can be transmitted from one ear to the other via an otoscope cone (Newton *et al* 2006).

### 1.7 Treatment of *Pseudomonas aeruginosa* otitis externa

When treating *Pseudomonas aeruginosa* otitis there are two factors that must be addressed: keeping the car canal clean and treatment with appropriate antimicrobials. Ear cleaners can be prescribed for patients with instructions for owners to properly clean the external ear canal. There are many products available including those with ceruminolytics, anti-inflammatories and anti-microbials. Cleaners routinely used in cases of OE show varying degrees of antimicrobial activity

as well as their "cleansing" activity (Cole *et al* 2003, Swinney *et al* 2008, Steen *et al* 2012). Ear flushing, under anesthesia or sedation, maybe important for treatment success if excess exudate is preventing administration of topical compounds or owner ear cleaning is not thorough enough. Flushing acts to manually remove excess cerumen and purulent exudate within the ear canal to allow increased contact of the topical antimicrobial with the ear canal epithelium (Gotthelf 2005). Ear flushing is also necessary if treatment fails to resolve the otitis. It is often recommended to flush ears infected with *Pseudomonas* prior to any treatment, as the exudate is usually purulent and can be very thick (Gortel 2004).

Topical therapy is most commonly used to treat canine otitis externa because antibiotic concentrations of 100-1000 times that of systemically delivered antimicrobials can be achieved in order to overcome resistant populations of *P. aeruginosa* (Morris 2004, Cole 2012). If medications are to be given topically, they must be able to reach the surface of the skin within the canal. This may mean, as stated above, that there is indication for using ear cleaners, measuring out the specific volume of the topical otic medication or using adjunctive therapy such as Tromethamine (Tris), edetate disodium dihydrate (EDTA) buffered to pH8 with Tromethamine HCL and deionized water, (Triz-EDTA® Aqueous flush, Dechra Veterinary Products, Kansas, US) to help antibiotics reach their site of action (Shaw 2012). Triz-EDTA® is commonly used as an adjunct therapy for dogs with *Pseudomonas* otitis (Nuttall *et al* 2007). It has been documented that flushing the canal with Triz-EDTA® 15 minutes prior to the application of a topical antimicrobial agent is beneficial in resolving the infection (Cole 2012). The tris buffer within the compound is thought to enhance the effect of the EDTA. EDTA acts to damage the cell surface of gram-negative bacteria such as *Pseudomonas* (Wooley *et al* 1983). EDTA treatment leads to chelation of cations from the outer membrane and hence disruption of the membrane (Hancock *et al* 1984). Previous studies have shown that Triz-EDTA® used in combination with an

antimicrobial can reduce the MIC and minimum bactericidal concentration (MBC) for certain antibiotics against planktonic bacteria (Wooley *et al* 1983, Sparks *et al* 1994). In a study by Farca *et al*, cases of canine otitis externa were treated successfully with topical Triz-EDTA® followed by topical enrofloxacin. Every dog in this study had an otic bacterial culture performed and *Pseudomonas aeruginosa* isolated. For every isolate, the bacterial population was noted to be resistant to enrofloxacin administered at systemic doses. All of these canines had been non-responsive to topical and systemic enrofloxacin previously (Farca *et al 1997*).

There are many otic medications available and care should be taken when selecting the appropriate medication for topical therapy. All medications have different antibiotics included, which have different mechanisms of action and spectrum of activity. The majority of otic medications contain not only an antibacterial agent but also a topical steroid and antifungal along with a specific vehicle or surfactant (Morris 2004). When rods are noted on cytology, otic medications including gentamicin, polymyxin B and enrofloxacin can be considered for treatment (Shaw 2012). Most otic medications come with label directions to instill a specific number of drops into the ear canal. Some clinicians prefer for a volume of fluid to be instilled as opposed to a number of drops i.e. 1ml in a large dog. This is due to growing concern that a small number of drops will not fully coat the external ear canal and potentially lead to subtherapeutic concentrations of antimicrobials being used therefore extending treatment and potentiating resistance (Morris 2004). Ticarcillin combined with potassium clavulanate, hypromellose, benzalkonium chloride and disodium edetate was found to successfully treat *Pseudomonas* otitis in a small numbers of cases and was also found to be stable for a prolonged period of time. Combining the ticarcillin with the clavulanic acid prevents resistance to the ticarcillin from developing, as the clavulanic acid would inactivate ß-lactamases produced by the *Pseudomonas* (Bateman *et al* 2012).

Antibiotic therapy success depends on selecting the appropriate antibiotic, dose and duration of therapy (Shaw 2012). Any antibiotic selected, whether given systemically or topically, must be effective against gram-negative bacteria. With OE, there is debate as to whether systemically administered antibiotics will reach the desired concentration within the external ear canal. Unless the ear canal epithelium is ulcerated, which may be the case with *P. aeruginosa*, systemic antimicrobials are unlikely to reach therapeutic concentrations within the canal (Morris 2004). The fluoroquinolones are the only family of antibiotics with activity against *Pseudomonas* that can be given orally. All other systemic antibiotics must be given by injection or topical medications must be used (Guardabassi *et al* 2008). Previous studies show that levels of antibiotics given intravenously do not reach the required MIC within tissue for *Pseudomonas* (Cole *et al* 2009).

Antimicrobials for canine OE are most often selected empirically based on otic cytology, unless a bacterial culture and susceptibility has been performed (Morris 2004). There is currently no standard of care treatment for *Pseudomonas* OE due to limited data available and low numbers of studies performed. From a review by Nuttall *et al*, it was noted that the treatments with the highest success rates were Triz-EDTA® plus topical enrofloxacin, specific ear cleaners with antibacterial action, an otic gel containing norfloxacin and ketoconazole and both topical and injectable ticarcillin (Nuttall *et al* 2007). Susceptibility patterns of *Pseudomonas aeruginosa* isolates taken from cases of chronic canine OE have been studied and the most common antibiotics that *Pseudomonas* are susceptible to are aminoglycosides (gentamicin, tobramycin, neomycin and amikacin), polymyxin B, fluoroquinolones (marbofloxacin, enrofloxacin), ceftazidime, ticarcillin and imipenem (Barrassa *et al* 2000, Guardabassi *et al* 2008). Neomycin and gentamicin may not always be successful in clearing OE as they are inactivated in purulent material, so maybe best selected when there is scarce pus within the ear canal (Guardabassi

2008).

Monitoring of otitis externa is important to determine when there is resolution of the otitis. Resolution should not be judged on improvement of clinical signs solely but rather based on resolution of clinical signs and negative cytological findings (Miller *et al* 2013).

For acute *Pseudomonas* OE a first-line antimicrobial should be selected based on cytologic findings, for example polymyxin B. Therapy for at least 7-14 days is warranted and then the patient should be re-evaluated (Morris 2004). For chronic or recurrent OE, treatment maybe needed for a longer period of time and oral anti-inflammatories, such as glucocorticoids, may also be required. Topical treatment may require second or third line antibiotics, such as fluoroquinolones or aminoglycosides (Morris 2004). Oral antibiotics may also be needed if there is ulceration and pain (Morris 2004). Regular ear cleaning may be performed to maintain a clean microenvironment within the ear and to prevent recurrence of otitis (Nuttall *et al* 2004).

Treatment of the underlying condition is most important for resolution of the OE (Nuttall 2007, Miller *et al* 2013, Cole 2012). Each underlying cause or factor should be addressed i.e. primary, predisposing, perpetuating and secondary factors (Miller *et al* 2013). Depending on the severity of the otitis externa, oral anti-inflammatory therapy may be required to increase the patency of the ear canal (Gortel 2004).

### 1.8 *Pseudomonas aeruginosa* resistance to antimicrobials

Treatment of *Pseudomonas aeruginosa* otitis can be difficult, as these bacteria are intrinsically resistant to multiple antimicrobial classes and may also acquire resistance during treatment (Nuttall *et al* 2007). *P. aeruginosa* is frequently resistant to fluoroquinolones, first and second generation cephalosporins and penicillin derived ß lactam antibiotics (Bateman *et al* 2012, Nuttall *et al* 2007). A study by *Petersen et al* performed over a six-year period noted that all otic

isolates of *P. aeruginosa* were resistant to ampicillin, cephalothin, trimethoprim sulfadiazine and tetracycline but most were susceptible to aminoglycosides, ticarcillin, piperacillin and ciprofloxacin. This is in contrast to other studies that found the bacteria was resistant to other fluoroquinolones (Cole *et al* 1998, Petersen *et al* 2002). Previous studies have documented that high levels of fluoroquinolones are detected in ear tissue after systemic administration, which would prevent resistance from occurring readily if the correct dosages are being utilized (Cole *et al* 2008). It is interesting to note that in this same study by Cole *et al*, when otic samples of *Pseudomonas* were compared to skin samples, fewer otic isolates were susceptible to gentamicin and amikacin. This could be due to the common usage of aminoglycosides in topical ear medications (Petersen *et al* 2002). Resistance leads to decreased treatment options and increased morbidity.

Emergence of antimicrobial resistance is steadily increasing which could be due to transmission between patients or due to exposure to multiple antimicrobials over the lifetime of a patient. Resistance after antimicrobial exposure can result in multidrug resistant *Pseudomonas aeruginosa* strains. In humans, Multi Drug Resistant (MDR) *Pseudomonas* previously occurred rarely in patients without cystic fibrosis (CF) (Aloush *et al* 2006). However, overall resistance is prevalent in isolates from hospitalized patients and resistance to certain antimicrobials is rising (Livermore 2002).

The overall intrinsic resistance noted for *P. aeruginosa* is due to low permeability of its cell wall (Lambert 2002). Any antimicrobials used for *P. aeruginosa* infections must cross the cell wall to reach the target. Aminoglycosides inhibit protein synthesis by binding to the 30s subunit of the ribosome, fluoroquinolones bind to the A subunit of DNA gyrase, ß-lactams inhibit transpeptides on the outer face of the membrane that assemble peptidoglycan and polymyxin B will bind to phospholipids in the outer membrane destroying barrier function (Lambert 2002). If

these antimicrobials are unable to reach their target molecules, they cannot exert their antimicrobial action.

There are three basic mechanisms of resistance in any organism including failure of the antibiotic to accumulate in the cell, changes in targets of the antimicrobial or inactivation of the drug (Lambert 2002, Stewart 1994). Failure of antimicrobials to accumulate within an organism is often due to either restricted permeability, as is seen with *Pseudomonas*, or efficient removal of antibiotic molecules from the cell by action of efflux pumps (Lambert 2002). Efflux systems are comprised of energy dependent pumps in cytoplasmic membranes, the outer membrane porin and linker proteins that join these other two molecules together (Lambert 2002). All antibiotics, except polymyxins, are susceptible to extrusion via efflux systems (Lambert 2002). The Opr family are a group of outer membrane porins in *Pseudomonas* species and other gram-negative bacteria. *Pseudomonas* will produce different proteins within the Opr family and a loss of one of these proteins is not always the cause of antibiotic resistance (Lambert 2002). However, loss of *oprD* is associated with resistance to imipenem and reduced susceptibility to meropenem, as these antibiotics require a porin to cross the outer membrane (Livermore 2002, Quale *et al* 2006).

The Mex family of proteins are part of the efflux pump system found in many bacterial species. *OprD* is regulated with *MexEF-oprN*. *MexAB-oprM* is another pump system, combined with the porin protein, that is responsible for extrusion of ß lactams, chloramphenicol, fluoroquinolones, macrolides, tetracyclines, sulfonamides, trimethoprim and a range of disinfectants. If this system is upregulated there will be resistance to those antibiotics that can be pumped via this system. Other efflux pump systems have also been documented and shown to increase resistance to antimicrobials when upregulated. *MexXY-oprM* extrudes aminoglycosides and *MexEF-oprN* extrudes carbapenems and fluoroquinolones (Lambert 2002, Livermore 2002). *MexT* mutants are strains with mutations in both the pump system and the porin protein leading to

upregulation of the *MexEF-oprN* and reduced *oprD* function, the result of which is resistance to both fluoroquinolones and imipenem and reduced susceptibility to meropenem (Livermore 2002). If all efflux pumps systems are upregulated this means that individual isolates can become resistant to greater numbers of antibiotics. Resistance to aminoglycosides has been noted in laboratory strains due to overexpression of *oprH* leading to decreased permeability (Livermore 2002).

Changes in the target molecule can include mutations in target enzymes. For example the mutation in *gyrA* gene (encodes A subunit of DNA gyrase) and *parC* gene (encodes topoisomerase IV) have been documented to lead to resistance to fluoroquinolones (Tejedor *et al* 2003). Although in Tejedor's study, they found that the *parC* gene was not mutated in any of the fluoroquinolone resistant *P. aeruginosa* isolates. This suggests this gene is not solely responsible for resistance to this family of antimicrobials. In this same study the use of an efflux pump inhibitor lead to decreased MICs for the fluoroquinolones so it is possible that a combination of decreased efflux and genetic mutation are responsible for the resistance documented for the fluoroquinolones (Tejedor *et al* 2003). Changes in the 30s subunit of the ribosome influence sensitivity to streptomycin and other aminoglycosides and resistance to ß lactams stems from alteration of penicillin-binding proteins (Lambert 2002).

Inactivation and modification of antibiotics occurs via multiple routes. All *Pseudomonas* strains possess the *ampC* gene for inducible ß lactamases (Lambert 2002, Livermore 2002). ß lactamases will break down the ß lactam ring forming part of ß lactam antibiotics rendering the antibiotics ineffective. The efflux pump system previously mentioned is thought to contribute to antimicrobial resistance in combination with *ampC* (chromosomal ß lactamases) (Lambert 2002). Over-expression of this enzyme results from spontaneous mutation in the regulatory gene *ampR*. Over production of the *ampC* ß lactamase poses a threat to cephalosporins and other ß lactam

antibiotics (Lambert 2002). Other ß lactamases produced include extended-spectrum ß-lactamases (ESBLs), which are plasmid-mediated enzymes active against penicillins and cephalosporins (Lambert 2002). An example of an ESBL is PER-1, which is a ß-lactamase that confers resistance to ceftazidime. Use of ß lactamase inhibitors, such as clavulanic acid, confers protection of the antibiotic against the plasmid-mediated enzyme but not that produced via induction of the *ampC* enzyme. Inactivation of aminoglycosides occurs through production of enzymes which transfer acetyl, phosphate or adenylyl groups to amino and hydroxyl substituents on antibiotics (Lambert 2002). Modifying these enzymes uses cytoplasmic cofactors (acetyl co-enzyme A or ATP) to supply molecules added to the aminoglycoside antibiotics. Acquisition of the genes for the modifying enzymes would require transfer from bacterial strains bearing plasmids (Lambert 2002). If cells mutate during antibiotic treatment, this does not lead to over expression of these enzymes, as is the case with chromosomal ß lactamases (Lambert 2002).

Trying to prevent resistance from developing is challenging with many different routes to consider. This necessitates the need to select appropriate antimicrobials and use appropriate doses over the treatment period to prevent subtherapeutic concentrations from occurring.

### 1.9 Bacterial Biofilms

In nature bacteria can exist in a planktonic and biofilm embedded state (Clutterbuck 2007). Biofilm growth is the most predominant mode of growth for bacteria within the environment and is likely a survival mechanism (Boles *et al* 2005). A biofilm is a bacterial population that is adherent to a biological or non-biological surface and is enclosed by an extra-polymeric substance (EPS) (Costerton *et al* 1995). Microorganisms form biofilms that exist in the environment, whether upon inanimate objects or living animals. The bacteria attach to these surfaces, including soil and aquatic systems, and have been documented in the human literature

developing on medical devices, within the middle ear, external ear, lungs, heart valves, surgical implants and tooth enamel (Donlan 2001). Biofilms have been noted in the veterinary literature associated with chronic mastitis in cows, within the middle ear cavity, on surgical implants and wounds and as a potential virulence factor in *Staphylococcus pseudintermedius* which is the most common cause of skin and surgical site infections in dogs (Ehrlich *et al* 2003, Melchior *et al* 2006, Singh *et al* 2013).

In order for biofilms to form there must be specific environmental cues, such as nutrient availability, presence of iron and oxygen limitation that trigger this lifestyle switch (Costerton *et al* 1995, O'Toole *et al* 1998, O'Toole *et al* 2000, Clutterbuck 2007). Increased levels of acylhomoserine lactone produced by individual cells will also lead to specific gene expression that contributes to biofilm formation (Costerton *et al* 1999). Other signals come from quorum sensing (QS) molecules. These molecules aid in cell-to-cell signaling and genes such as *las* and *rhl* are responsible for the expression of some of these molecules in *Pseudomonas* spp. These molecules will also regulate expression of virulence determinants (Parsek *et al* 2000).

Biofilm formation occurs during a five-stage cycle: initial attachment, irreversible attachment, two maturation stages and dispersal (see Appendix 2). This cycle begins when planktonic cells attach to a surface (O'Toole 2003, Ma *et al* 2009). Biofilm formation in gram-negative bacteria occurs when bacterial cells first swim along a surface, using flagellar-mediated motility, until attachment occurs at a specific site (O'Toole *et al* 2000). These cells then form a monolayer where their attachment is initially reversible. Reversible attachment involves cell pole mediated interactions where the cells make contact with a surface via the cell pole (Clutterbuck 2007). Van der Waals forces and electrostatic charge play a role in this initial attachment (van Loosdrecht *et al* 1989). Once contact with a surface is made and reversible attachment has occurred, bacteria use type IV pili to move slowly within the monolayer. Movement occurs when

the pili extend and then retract to propel the bacterium forward (Stoodley *et al* 2002). When surface contact occurs the bacteria are still motile. Initial growth of bacteria is the production of microcolonies (clusters of cells), which form when cells aggregate together. With further movement of the cells using type IV pili, known as twitching motility, the attachment becomes irreversible. This will develop once surface interactions are stable (Stoodley *et al* 2002, Clutterbuck 2007). Twitching motility is also thought to be involved in further microcolony production (O'Toole *et al* 1998). Remaining bacteria will move between the colonies that have formed (Klausen *et al* 2003).

Irreversible attachment occurs when the cells cannot be removed by gentle rinsing. At this time the individual cells reorient to the longitudinal cell axis (Jackson *et al* 2004). It is thought that the twitching motility enables bacteria to overcome electrostatic repulsion present on the surface and adhere to this same surface (van Loosdrecht *et al* 1989). The bacteria undergo cell division, form larger microcolonies and produce extrapolymeric matrix (EPS) (Donlan 2001). Previous studies document that the EPS of a biofilm is visible within 5 hours after inoculation and characteristics associated with a mature biofilm can be seen after 10 hours (Harrison-Balestra *et al* 2003). The *lasR-lasI* QS system in *Pseudomonas aeruginosa* will also be activated when attachment becomes irreversible and *rhlR-rhlI* will be activated during the first maturation stage. Both of these are QS systems that activate genes that contribute to biofilm formation (Clutterbuck 2007).

The EPS forms around the cells and is composed of polysaccharides, nucleic acid and proteins. This self-produced barrier protects the cells from environmental conditions, shear stress, antimicrobials and the host immune response (Clutterbuck 2007). The EPS is highly hydrated with a composition of 98% water (Donlan 2001). This hydrated matrix surrounding the bacterial cells will protect against dessication from the external environment (Clutterbuck 2007). All

bacterial cells produce glycocalyx but in biofilm, cells are cemented together and immobilized by this glycocalyx *(*Davies et *al* 1998, Clutterbuck 2007). Due to the increased number of bacterial cells in a biofilm and the glycocalyx surrounding them, making them immobile, diffusion of nutrients and oxygen to individual cells is minimal. Water channels form within the biofilm allowing for transport of nutrients and oxygen to the cells. Water channels are maintained by rhamnolipid surfactants, whose production is controlled by quorum sensing molecules (Davey *et al* 2003). This limited supply of nutrients and oxygen means that cells grow slower within a biofilm (Donlan 2001). *RpoS*, a transcription factor, can be activated in *Pseudomonas* biofilms when nutrients become limited. *RpoS* is a stationary-phase sigma factor that can also regulate resistance for gram-negative bacteria. The activation of this gene is a self-protective mechanism to facilitate survival of the bacteria by initiating release of cells from the biofilm due to nutrient depletion (Clutterbuck 2007).

The other components of the EPS depend on environmental conditions, age of the biofilm and the strain forming the biofilm (Harmsen *et al* 2010). Three genes *psl*, *pel* and one for alginate, contribute to biofilm formation in *Pseudomonas aeruginosa* by encoding for specific exopolysaccharides that make up part of the EPS (Friedman *et al* 2004, Leid *et al* 2005). *Pel* is involved in the production of glucose and is involved in pellicle formation. *Psl* (polysaccharide synthesis locus) is involved in mannose production (Clutterbuck 2007). The study by Ma *et al* looked at the distribution of *psl* exopolysaccharide and found that, during the attachment stage, this polysaccharide was noted primarily on the bacterial cell surface in a helical pattern (Ma *et al* 2009). It is hypothesized that this helical nature may contribute to cell-to-cell interactions with adjacent bacteria, which will begin to establish a matrix between the two individual cells (Ma *et al* 2009). Another theory is that other proteins or lipids may have a similar shape and so insertion of cells is easier (Ma *et al* 2009). Cells treated with cellulase showed dissociation of the *psl* from

the surface of the bacteria. This could indicate that this polysaccharide is attached to the cell surface via the target of cellulase ß-1,3 or ß-1,4-linked glucose (Ma *et al* 2009). Further studies showed that cellulase treatment reduced biofilm development, indicating that *psl* expression is likely essential for biofilm development (Ma *et al* 2009). This finding is further substantiated by studies documenting that *pslAB* mutants are unable to initiate biofilm formation; the A and B are different genes within the *psl* cluster of genes (Jackson *et al* 2004). Levels of *psl* were detected in regions with no bacterial cells but it is unknown whether the genetic material dispersed from a cell or whether it remains attached to a surface after the bacteria have dispersed. This expression could facilitate recruitment of cells to the surface and connecting them together (Ma *et al* 2009).

Depending on environmental conditions and nutrient cues, biofilms can form flat structures or microcolonies with a three-dimensional arrangement. Conditions that are high in nutrients favour production of flat biofilms whereas in low nutrient environments, the "classical" three-dimensional mushroom shaped biofilm will form (Clutterbuck 2007). A pellicle may form which is a part of the biofilm that forms at the air-liquid interface (Sakuragi *et al* 2007). Distribution of polysaccharides within each of these microcolonies is different (Ma *et al* 2009). In the "classical" mushroom shaped colonies there is less of *psl* expressed in the lower centre (Ma *et al* 2009). Studies have also shown that if colonies form mushroom structures, the stalks are formed from growth of non-motile bacteria and the caps are aggregations of motile bacteria. There will also be cavities within these microcolonies (Klausen et al 2003).

When focusing on motile bacteria, two factors contribute universally to biofilm formation. These are extracellular polysaccharides and swarming motility (O'Toole 2008). There is an inverse relationship between biofilm formation and swarming motility (O'Toole 2008). This stands to reason as bacteria within the core of the biofilm that are embedded are non motile and

the more the bacteria move once irreversibly attached, the more difficult biofilm formation becomes.

Extracellular DNA (eDNA) is a third important component of the biofilm matrix. The eDNA is derived from chromosomal DNA and functions in cell-to-cell connections within the biofilm. It is released in the largest amounts during the late phase of growth (Allensen-Holm *et al* 2006). This release is not noted in QS mutants, suggesting that QS plays a role in DNA release (Allensen-Holm *et al* 2006). This eDNA is intertwined between cells in large clumps (Allensen-Holm *et al* 2006). A *fliMpilA Pseudomonas aeruginosa* mutant that does not have the cellular appendages of flagella and pili did not produce eDNA. eDNA is found primarily within the stalks of mushroom shaped biofilms. Pili will attach to DNA, which explains why the caps of the mushrooms are formed from motile bacteria (Allensen-Holm *et al* 2006). Cell autolysis occurs in microcolonies and it is thought to mediate eDNA release (Webb *et al* 2003). Previous studies indicate that treatment with DNase leads to disruption of biofilm integrity (Webb *et al* 2003).

As the cells cycle through two maturation stages, certain genes are upregulated and there is marked phenotypic variation between the planktonic cells and the biofilm embedded cells (Clutterbuck 2007). *Arc* proteins are involved with anaerobic processes and play a role in amino acid metabolism and these are also upregulated during the first maturation phase. This highlights that there is likely oxygen limitation in regions of the biofilm (Sauer *et al* 2002, Clutterbuck 2007). During the second maturation phase, where optimal thickness is achieved, over 70 genes will undergo alterations in expression with the result of proteins being significantly different to those in the first maturation stage (Whiteley *et al* 2001).

Biofilm embedded bacteria have a decreased ability to evade stresses as they are confined to the biofilm and their motility is repressed (Whiteley *et al* 2001, Sauer *et al* 2002). When nutrients are depleted as a result of biofilm growth, it is necessary for bacteria to be able to separate from

the biofilm. This process is known as detachment (Boles *et al* 2005). Once the biofilm reaches its maximum thickness, detachment, or dispersion, will occur. This increase in thickness occurs after activation of *las* and *rhl* quorum sensing systems. During this time, proteins within the biofilm are more similar to those in planktonic cells (Clutterbuck 2007). Biofilm reaches maximum thickness during the second maturation stage, which is the point where biofilm bacteria are phenotypically different from planktonic bacteria. At least 50% of the proteins undergo changes in regulation between the planktonic stage and this second maturation stage (Sauer *et al* 2002). Detachment can be continual (erosion) or can be rapid with a larger number of cells being dispersed (sloughing). Detachment of individual or clumps of cells creates motile bacteria that can cause infection (Boles *et al* 2005). Cells that are lost can retain characteristics such as antimicrobial resistance (Clutterbuck 2007). Cells detach from the biofilm due to either growth and division or removal of biofilm aggregates that contain masses of cells (Donlan 2001). At the dispersion stage, there are numerous bacteria within the centre of the microcolonies. Swarming is facilitated by biosurfactant and rhamnolipid (Kohler *et al* 2000). Rhamnolipids are glycolipids with many functions including facilitating swarming, altering surface polarity of bacteria and they have surface-active properties that decrease adhesive interactions and therefore can function as detachment factors (Al-Tahhan *et al* 2000, Kohler *et al* 2000, Boles *et al* 2005). They also have antimicrobial activity against other bacteria (Haba *et al* 2003). Rhamnolipids can cause release of lipopolysaccharides from *P.aeruginosa,* which will enhance the hydrophobicity of the bacteria leading to increased adhesiveness, which can contribute to initial biofilm formation (Al-Tahhan *et al* 2000, Harmsen *et al* 2010). In previous studies, inactivation of the rhamnolipid genes eliminated the accelerated detachment phenotype. We can presume that rhamnolipids are needed for detachment to occur (Boles *et al* 2005). Dispersion of biofilm embedded cells can also

be induced by the addition of alternate carbon sources such as glutamate or citrate and ammonium chloride (Sauer *et al* 2004).

Most wild-type biofilms release a small number of cells continuously and will also spontaneously detach after prolonged growth (Boles *et al* 2005). An *rhlAB*, *Pseudomonas aeruginosa* mutant shows the following growth pattern for detachment: First biofilm slackens and individual bacteria will begin to move within the structure. Cavities then form within the biofilm where bacteria can be seen swimming around. The cavity size increases over time and then ruptures (Boles *et al* 2005). The remaining biofilm will then detach leaving a monolayer of cells behind (Boles *et al* 2005). Growth in wild type bacterial populations is similar but occurs after longer periods of time (Boles *et al* 2005). The presence of bacteria within cavities highlights that some motility is retained, whereas previous work has shown that swimming motility is suppressed during biofilm growth (Whiteley *et al* 2001). Cells detach slowly from the biofilm and can show intermediate resistance to certain antimicrobials when compared to those within biofilm (Boles *et al* 2005). This suggests that detachment may restore certain phenotypes including antibiotic sensitivity and motility. In mixed biofilms with both wild type and mutant bacteria, the wild type may localize to the centre of biofilm and the mutant to the exterior. Pure wild type bacterial biofilms do not undergo detachment until later whereas when mixed genotypes are present, detachment occurs earlier (Boles *et al* 2005).

The surface the biofilm develops on can be smooth or rough and ranges from hydrophobic material such as Teflon, to hydrophilic material such as metal or glass (Donlan 2001). Biofilms have been found to grow more rapidly on surfaces that are rough and hydrophobic (Donlan 2001). The presence of flagella, pili and the glycocalyx capsule will impact the rate of growth of bacterial biofilms (Donlan 2001). A study by Hoyle *et al* documented that calcium treatment of biofilms stopped piperacillin from diffusing across the biofilm, indicating that calcium may

change the structure of the EPS or interact with efflux pumps or genes controlling entry of the antimicrobial into the cell (Hoyle *et al* 1992).

Once the bacteria are embedded within the biofilm they avoid being cleared by both the innate and adaptive immune response. Host defences are often not effective against biofilms. Indwelling devices, if colonized by a biofilm, must be removed as they cannot be adequately treated with antimicrobials (Jesaitis *et al* 2003). This extra protection from the EPS also means that traditional concentrations of antimicrobials, that would be used to kill the planktonic form, will be ineffective in killing cells within a biofilm (Clutterbuck 2007).

In the human literature, it has been noted that biofilm embedded bacteria are less susceptible to antimicrobials than their planktonic counterparts (Drenkard *et al* 2002). Attack by antimicrobials can change the phenotype of the bacteria within the biofilm in many ways, which may be yet another reason why biofilm embedded bacteria are less susceptible to antimicrobials. Levels of alginate are actually increased in response to imipenem by as much as a twenty fold increase in the level of alginate in biofilms exposed to imipenem, as opposed to control biofilms (Bagge *et al* 2004). Certain antimicrobials have also been found to alter biofilm formation; such as azithromycin delaying biofilm formation *in vitro* (Gillis *et al* 2004). It has been noted that azithromycin retards biofilm growth and formation by blocking QS controlled *lasB*, by suppressing the virulence factors in mucoid strains of the bacteria and inhibits alginate production. Azithromycin also increases the sensitivity to hydrogen peroxide for biofilm embedded cells. However, in this same study it was noted that azithromycin has no effect on mature biofilms (Hoffman *et al* 2007).

Bacteria embedded within biofilms also have other methods of resistance. First the EPS provides a barrier between the external environment and the bacteria preventing penetration by the host immune response and antimicrobials (Lambert 2002, Clutterbuck 2007, Donlan *et al*

2011). For example small hydrophilic molecules such as ß lactam antibiotics can only pass through channels created by porin proteins and certain mutations in genes coding for these proteins can occur within biofilms (Lambert 2002). The nutrient limitations within the biofilm mean that the cellular growth rate is decreased. As many antimicrobials function on actively growing cells this means that the antimicrobial function maybe decreased. The efficacy of certain antibiotics is also reduced due to the lower oxygen requirements within the biofilm e.g. tobramycin and ciprofloxacin (Clutterbuck 2007). This decreased rate of growth will also decrease the rate that antimicrobial agents are taken into the cell. If no antimicrobial is present then there will be no antimicrobial action (Donlan *et al* 2011). Due to the close proximity within biofilms, plasmids may also be exchanged between individual cells, which can confer resistance from one cell to another (Donlan *et al* 2011). These factors along with altered gene expression and quorum sensing lead to increased resistance to antibiotics once bacteria are embedded within the biofilm (Clutterbuck 2007).

## 1.10 *Pseudomonas aeruginosa* biofilm

### 1.10.1 *Pseudomonas aeruginosa* biofilm microbiology

*Pseudomonas* can readily form biofilms in any environment conducive to growth, compared to other bacteria that require specific conditions such as temperature and pH (Clutterbuck 2007). *Pseudomonas aeruginosa* biofilms are commonly noted in humans with cystic fibrosis and diabetics with foot ulcers (Moreau-Marquis et *al* 2008, Watters *et al* 2012). Most of the human research targeting *Pseudomonas* biofilm production stems from these two diseases. For isolates obtained from the lungs of CF patients, there is variation in their ability to form biofilm (Head *et al* 2004). In the lungs of CF patients, the mucus produced is hyper viscous and hence there is reduced clearance of inhaled particles. *Pseudomonas* can attach to the mucin and form biofilms.

The low oxygen environment facilitates biofilm formation and the calcium imbalance documented further allows bacterial adherence (Clutterbuck 2007). The presence of QS molecules in CF sputum has been documented as evidence that Pseudomonas forms biofilms in the lungs of CF patients (Moreau-Marquis *et al* 2008). The ratio of these molecules noted in sputum from CF patients is similar to that seen *in vitro* with biofilm growth, as opposed to planktonic growth (Moreau-Marquis *et al* 2008). Recent studies performed show that treatment with azithromycin *in vitro*, which interferes with QS signaling, as well as alginate production, helps clear *P. aeruginosa* from a mouse model of chronic lung infection (Hoffman *et al* 2007).

Some *Pseudomonas* isolates do form biofilms and others do not. Reasons why some isolates form biofilms have been hypothesized. It has been documented that nutrient depleted environments favour biofilm formation and high concentrations of nutrients suppress biofilm formation (Costerton *et al* 1995). Hydrophobicity of a surface and minor temperature changes do not appear to impact biofilm formation for *Pseudomonas* (Head *et al* 2004). However, other studies have found that transcription of certain genes that play a role in biofilm formation are temperature dependent such as *pel (*Sakuragi *et al* 2007).

Biofilms can also form in anaerobic conditions and are thicker than those formed under aerobic conditions (Yoon *et al* 2002). It is unknown why this is the case but is likely linked to the fact that *Pseudomonas* biofilms grow better in low oxygen environments (Walter *et al* 2003, Clutterbuck 2007). This could potentially be an adaptive mechanism due to the fact that oxygen transport within biofilms is limited (Clutterbuck 2007). *Pseudomonas* can grow anerobically via denitrification, where nitrate or nitrite is used as an alternate substrate to oxygen. *In vitro* nitrate supplementation decreased killing of biofilm embedded cells by ciprofloxacin and tobramycin and diminished the activity of other antibiotics tested (Borriello *et al* 2004). This fact suggests that oxygen limitation within a biofilm plays a large role in antibiotic tolerance for *Pseudomonas*.

*PilA* mutants, those with defective pili, are unable to form biofilms under anaerobic conditions suggesting that pili are needed for anaerobic biofilm production, potentially to begin the attachment process (Yoon *et al* 2002).

*P. aeruginosa* biofilms have a dense cell mass at the base of the biofilm with 27% of the biomass at the attachment surface (Costerton *et al* 1995). Planktonic cells will interact with a surface dependent on many factors including nutritional status of the environment (Costerton *et al* 1995). *P. aeruginosa* exhibits different behaviours when it encounters solid or semisolid surfaces. The cells can then form biofilms, swarm and begin pili-mediated twitching (O'Toole 2008). Cells within biofilms are thought to be different than their planktonic counterparts (Sauer *et al* 2002). For example, *P. aeruginosa* growing on a surface has increased expression of *algC*, a gene required for synthesis of extracellular polysaccharide (Stapper *et al* 2004). Other differences include changes in expression of pili, fimbriae and proteins (O'Toole *et al* 1998). Strains of *Pseudomonas* that are known as "mucoid types", commonly found in the lungs of CF patients, over-produce alginate (Hentzer *et al* 2001). These mucoid types develop highly differentiated biofilms that are more heterogeneous in their cellular structure (Hentzer *et al* 2001). Mucoid biofilms over producing alginate have also been found to be more resistant to certain antimicrobials and form thicker, rougher biofilms with enhanced microcolony formation (Hentzer *et al* 2001). Other studies document that alginate is not critical for biofilm formation and alginate over production is not responsible for decreased levels of biofilm biomass, but it does change the architecture of the biofilm; when alginate is produced the biofilm bacteria will stay together and form compact colonies and then grow vertically and horizontally (Stapper *et al* 2004). *Pseudomonas* bacteria that lack alginate are killed by human leukocytes in the presence of IFN-gamma, whereas bacteria from wild-type biofilms are not killed by human leukocytes (Leid *et al* 2005). This suggests that alginate also plays a role in defense against the host immune response.

The main family of cells that kill alginate negative bacteria are mononuclear cells (Leid *et al* 2005). Alginate can act as a barrier in biofilms by surrounding cells and binding them together. It can also bind cationic antibiotics such as aminoglycosides and therefore restrict their diffusion into the cell (Lambert 2002).

Neutrophils are likely the most significant component of the host defence against *Pseudomonas* biofilms (Jesaitis *et al* 2003). When bacteria in a biofilm are exposed to neutrophils *in vitro*, the bacteria exhibit increased oxygen consumption and a cloud of bacteria is released that then surrounds the neutrophils. The neutrophils become surrounded by the biofilm but still appear able to engage in phagocytosis, degranulate and mount a respiratory burst. Neutrophil contact stimulates individual cells to move away from this contact (Jesaitis *et al* 2003). Previous studies have documented that the presence of neutrophils will lead to a reduction in the number of planktonic *P. aeruginosa* bacteria present but once the neutrophils have died and lysed, their remaining parts, specifically actin and DNA, lead to an increase in biofilm development (Walker *et al* 2005).

Biofilm formation in half of a population of defective Pseudomonas fluorescens cells in minimal media was restored in one study by supplementing the media with iron or by using either citrate or glutamate as the sole energy source. This suggests that multiple, convergent pathways are involved in biofilm formation and that the presence of iron and appropriate polysaccharides contributes to biofilm formation (O'Toole *et al* 1998). Lactoferrin has activity as an iron chelator. Lactoferrin is one component of the innate immune system and can inhibit bacterial growth at high concentrations (Singh *et al* 2002). Lactoferrin may also be bactericidal by binding LPS and disrupting membranes so bacteria do not differentiate into biofilm structures (Singh *et al* 2002). The absence of lactoferrin will lead to normal biofilm development (Banin *et al* 2005). Lactoferrin has also been noted to alter cell movement and in the presence of lactoferrin,

daughter cells move away from the point of cell division (Singh *et al* 2002). Lactoferrin also prevents biofilm formation by stimulating twitching motility (Singh *et al* 2002). If increased lactoferrin prevents biofilm formation, there is a possibility that iron may play a role in biofilm development as well. Banin *et al* studied the effects of iron on biofilm formation and found that, in the absence of lactoferrin, if no functional iron uptake system was present, biofilms would still form flat, thin colonies (Banin *et al* 2005). *Pseudomonas aeruginosa* cannot acquire sufficient iron for biofilm development via passive diffusion but can obtain iron and support biofilm formation using endogenous pyoverdine and pyochelin (siderophores; iron chelating compounds secreted by microorganisms) or ferric dicitrate or desferrioxamine (Banin *et al* 2005). *Fur* (ferric uptake regulator) is an intracellular iron regulator found in *Pseudomonas* and suppresses transcription dependent on iron levels. However*, Fur* mutants can form biofilms even in the presence of lactoferrin (Banin *et al* 2005).

*Pseudomonas* can induce the synthesis of EPS matrix components in response to signals that are sensed by sensor kinase response regulators such as *LadS, RetS* and *GacS* (Goodman *et al* 2004). *RetS* and *GacS* operate in an opposing manner. *RetS* will suppress genes that encode for polysaccharide components of the biofilm, including *pel*, which plays a role in polysaccharide production in biofilms. *GacS* is needed to activate genes involved in chronic persistence including those involved in biofilm formation; *gacS* mutants did not proceed past the irreversible attachment phase and the biofilms formed were flat and lacked a layered structure typically noted in mature biofilms (Goodman *et al* 2004 Davies *et al* 2007). *GacS* mutants are found to be hyper motile and hence poor biofilm producers (Davies *et al* 2007). However, some *gacS* mutants also give rise to small colony variants (SCV). These variants display a hyper-biofilm forming phenotype and are less motile and more tolerant to antimicrobial agents than the mutant strains (Davies *et al* 2007). This suggests that *gacS* may have a role in regulating the conversion of

variants back to a normal morphology (Davies *et al* 2007). Previous studies have documented that, for CF isolates, low concentrations of antibiotics may select for hyper-biofilm-forming SCVs from biofilms of the mutant *gacS*. This indicates that if subtherapeutic concentrations of antibiotic are used for the treatment of biofilms, this may increase the biofilm-forming ability of the bacteria leading to further difficulties with treatment (Drenkard *et al* 2002, Davies *et al* 2007). This highlights the need to develop a screening test for biofilm antibiotic susceptibility and to accurately document when biofilms may form. The study by Davies *et al* also showed that silver ions might trigger these hyper-biofilm-forming SCVs (Davies *et al* 2007).

It has been shown previously that eDNA in the matrix can induce antibiotic resistance (Mulcahy *et al* 2008). The eDNA within the extracellular matrix creates a cation-limited environment that is detected by *Pseudomonas*. LPS modification genes are induced leading to antimicrobial resistance, especially to aminoglycosides (Mulcahy *et al* 2008). The DNA also disrupts the integrity of the cell envelope leading to cell lysis by chelating cations (Mulcahy *et al* 2008).

Specifically for *Pseudomonas aeruginosa*, changing bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) concentrations also affect biofilm formation and swarming motility. They do this by production of specific polysaccharides within the biofilm matrix and by control of flagellar function (O'Toole 2008). C-di-GMP is a second messenger found in bacteria that will regulate processes such as the change from planktonic motile cells to an adhesive structure such as a biofilm. A study by Head *et al* documented that, among a group of flagellar mutants and a wild-type strain of *Pseudomonas aeruginosa*, the isolates forming the most biofilm were the isolates with mutations in the flagellar gene. This same study also documented isolates with twitching motility formed the smallest amount of biofilm (Head *et al* 2004). This suggests that, although twitching motility is important in the initial stages of biofilm attachment, continued

motility has a negative impact on biofilm formation.

Along with the *las* and *rhl* QS systems, *Pseudomonas* has its own third cascade known as the *Pseudomonas* quinolone signal (PQS). This is synthesized from anthranilic acid (Musk *et al* 2006). Links between this cascade and biofilm formation have yet to be precisely documented.

N-decanoyl-L-homoserine benzyl ester (C2) is a newly discovered QS inhibitor. This C2 will decrease rhamnolipid production to 50% compared to control populations (Yang *et al* 2012). Swarming motility is dependent on rhamnolipids acting as a biosurfactant and these are dependent on QS. Changes in colony morphology and size have been documented with the addition of C2 (Yang *et al* 2012). Certain antibiotics such as gentamicin, meropenem and tobramycin also show synergistic activity when combined with C2 (Yang *et al* 2012). C2 had been noted to inhibit virulence of *Pseudomonas aeruginosa* by inhibiting expression of *lasR, lasI, rhlR* and *rhlI*, all QS molecules with a role in expression of virulence factors (Yang *et al* 2012).

Cell death and lysis occur in microcolonies of *Pseudomonas aeruginosa*; after 12 days of growth, up to 50% of the microcolonies can be lysed (Webb *et al* 2003). No cell death is noted in *rpoN* mutants, which are deficient in both type IV pili and flagella. *Las* mutants and *rhl* mutants showed wild-type death in biofilms, suggesting that both of these QS molecule may contribute to longevity in non-mutant strains (Webb *et al* 2003). Detachment in *Pseudomonas* biofilms is similar to other bacteria and involves either the discharge of individual bacteria, separation of cell clusters or the mass detachment of whole colonies (Stoodley *et al* 2001, Boles *et al* 2005).

### 1.10.2 Treatment of *Pseudomonas aeruginosa* biofilm

We know from previous research that bacteria within biofilms may be up to one thousand times more resistant to antimicrobials (Mah *et al* 2003). Bacteria within biofilms may also be more resistant to disinfectants and other compounds. *P. aeruginosa* biofilms are resistant to toxic

oxygen products such as hydrogen peroxide. The bacteria can convert this product back to oxygen with catalase and superoxide dismutase activity rendering it ineffective (Hassett *et al* 1998, Lu *et al* 1998). This indicates that selecting the appropriate antimicrobial is even more important if bacterial biofilm growth is suspected. The same principals of selecting antimicrobials without intrinsic resistance to *P. aeruginosa,* apply to biofilm embedded bacteria as for those noted for the planktonic bacteria.

It has also been documented that levels of alginate are actually increased in response to imipenem by as much as a 20-fold increase in the level of alginate in biofilms exposed to imipenem as opposed to control biofilms (Bagge *et al* 2004). This fact is concerning as it suggests that exposure to antimicrobials may actually promote biofilm development. This same mechanism has also been documented for aminoglycosides (Hoffman *et al* 2005). As with the planktonic form of the bacteria, topical therapy maybe more effective for *Pseudomonas aeruginosa* biofilms as increased antimicrobial concentrations can be reached.

Research into compounds that may affect the EPS and barriers to antimicrobial penetration are underway. As with planktonic cells, studies have shown that Triz-EDTA® used in combination with an antimicrobial, can reduce the MIC and minimum bactericidal concentration (MBC) for certain antibiotics against biofilm embedded bacteria (Wooley *et al* 1983, Buckley *et al* 2012). Gram-negative bacteria exposed to Triz-EDTA® have increased permeability to extracellular solutes and leakage of intracellular solutes and are also sensitized to lysozyme, bactericides and antibiotics. The bacterial cells release periplasmic enzymes, cell membrane associated proteins, lipopolysaccharides, protein, phospholipid and divalent cations from the cell wall (Wooley *et al* 1983). EDTA enhances the loss of *P. aeruginosa* biofilm cells and is more effective when combined with gentamicin as opposed to being used as the sole agent (Banin *et al* 2006). A combination of EDTA and gentamicin in a Tris buffer eliminated biofilm-associated

cells in a previous study (Banin *et al* 2006). EDTA was also noted to kill bacterial cells within the mushroom structure of the biofilm and will also induce dispersal of cells from a biofilm (Banin *et al* 2006). A study by Wooley *et al* documented that *P. aeruginosa* did not grow in media in the presence of EDTA and was lysed in the presence of EDTA-Tris in sterile water. This is in contrast to other studies that documented that Triz-EDTA® alone does not have any anti-microbial activity (Wooley *et al* 1983, Buckley *et al* 2012). Other compounds being researched to disrupt biofilm formation include: iron salts, plant extracts and vegetable extracts. Iron salts have been documented to decrease the formation of biofilms by decreasing expression of virulence factors and genes, which express proteins essential for scavenging iron (Musk *et al* 2005). Extracts from marine plants, such as red algae *Delisea pulchra*, have also shown promise in preventing growth of bacterial biofilms via inhibiting N-acyl homoserine lactones (AHL), which are part of the QS signalling system in gram-negative bacteria that can start the QS cascade (Bauer *et al* 2002, Musk *et al* 2006). Certain vegetables can secrete compounds similar in composition to the AHL will can also interfere with biofilm synthesis (Bauer *et al* 2002). AHL derivatives are also being manufactured to attempt to disrupt bacterial biofilms (Musk *et al* 2006). The protein *GroEL1* is produced by Mycobacteria and has been found to have some anti-biofilm activity (Musk *et al* 2006). Further work will be needed to determine if these compounds will have an application in the treatment of biofilms.

### 1.10.3 Biofilm MIC testing

Multiple methods are currently available for determining and measuring biofilm growth. Biofilm formation in *Pseudomonas aeruginosa* is noted to be the best on minimal arginine medium or LB medium (Caiazza *et al* 2004). Current methods to identify biofilm production include; scanning electron microscopy, transfocal electron microscopy, confocal laser

microscopy and detection of genes associated with the ability of bacteria to form biofilms (Gotz 2002, Davies *et al* 2007). Many of these methods are costly and time consuming, so cheaper and more convenient *in vitro* tests have also been developed.

The method outlined by Stepanovic *et al* documents biofilm growth for *Staphylococcus* within a microtitre plate, staining with crystal violet and measurement of optical density (Stepanovic *et al* 2000). In previous studies, a positive correlation has been noted between biofilm colony forming units and optical density, which suggests optical density is an appropriate method for measurement of bacterial ability to form biofilms (Head *et al* 2004). This method has been changed slightly by many researchers depending on the bacterial species being studied, but is commonly used (Bendouah *et al* 2006).

The Calgary biofilm device (CBD) uses the innate ability of biofilms to form on any surface and grows a biofilm on plastic pegs inserted into a microtitre plate with the appropriate media. The CBD was developed due to enhanced antimicrobial resistance within biofilms (Olsen *et al* 2002). After incubation and biofilm formation, sonication is used to remove the cells from the peg and determine the extent of biofilm formation via counts on plates. Susceptibility to antimicrobials is noted by growing biofilms on these pegs with stock solutions of antimicrobial dilutions within the wells of a microtitre plate (Ceri *et al* 1999). This method has also been changed slightly based on individual researchers, including breaking the pegs with biofilm on and isolating in culture (Davie*s et al* 2007, Sandoe *et al* 2006).

The Modified Robbins Device measures tubular flow through membranes. These membranes can have antimicrobial discs attached, which are then removed and susceptibility is tested (Hoiby *et al* 2001).

There is the obvious question as to which of these methods is most appropriate to evaluate biofilm growth and that question is unanswered at this time. Currently there is no approved

method for evaluating the effectiveness of antimicrobial agents against biofilm-associated bacteria outlined by the National Committee for Clinical Laboratory Standards (Curtin *et al* 2003, Moreau-Marquis *et al* 2008).

### 1.10.4 Minimum Biofilm Eradication Concentration (MBEC)

Minimum inhibitory concentration (MIC) is the conventional way to determine susceptibility of planktonic bacterial cells to antimicrobials (Curtin *et al* 2003). The MIC is defined as the lowest concentration of an antimicrobial agent that prevents visible growth of the microorganism and is measured by exposing a growing bacterial population to different concentrations of antimicrobial agents (Curtin *et al* 2003). The MIC can be altered based on the numbers of cells present, the medium used or a longer incubation period; therefore standardized methodology is documented by the National Committee for Clinical Laboratory Standards (Curtin *et al* 2003). The minimum bactericidal concentration is the lowest concentration of an antimicrobial that lead to a 99.9% reduction of the bacterial population (Curtin *et al* 2003).

The Minimum Biofilm Eradication/eliminating Concentration (MBEC) is the concentration of an antimicrobial agent required to kill a bacterial biofilm (Olsen *et al* 2002). Determination of this may help with selecting the most appropriate antibiotic that results in disruption of the biofilm and success of antimicrobial therapy (Olsen *et al* 2002). Recently, one commercial laboratory has developed an *in vitro* test to determine MBEC of a bacterial population and this is the becSCREEN® (Spectrum laboratories®, Phoenix, Arizona) (Spectrum Laboratories Inc. 2013). This test uses a modification of the Calgary Biofilm device to determine MBEC. Previous studies have documented difference between the MIC and MBEC for *Pseudomonas aeruginosa* with the MBEC being much higher than the MIC (Sepandj *et al* 2004). Other than the becScreen®, there are no commercially available tests to measure MBEC.

MBEC requires growth of a biofilm first prior to susceptibility testing. Finding the MBEC, if a bacterial biofilm has formed or is suspected, is important due to the fact that bacteria within biofilms are less susceptible to antimicrobials than their planktonic counterparts (Drenkard *et al* 2002, Clutterbuck 2007). This will mean that a more appropriate antimicrobial and dose of antimicrobial will be used to treat a bacterial biofilm infection.

### 1.10.5 *Pseudomonas aeruginosa* genetics noted in human literature and how these impact biofilm formation

*Pseudomonas aeruginosa* has a large genome that contains 6.26 Mbp (encoding 5567 genes) (Lambert 2002). In an effort to determine whether certain genes play a role in biofilm formation, multiple studies within the human literature denote the presence of genes within the *Pseudomonas* genome that may or may not contribute to bacterial biofilm formation

One study uncovered 15 *Pseudomonas* mutant strains known as surface attachment deficient (*sad*) mutants. In these strains there was a cluster of genes named *sad* genes (O'Toole *et al* 1998). In a full screen identifying 394 and 285 genetic determinants contributing to the biofilm phenotype, only the gene *sadB* was identified from this family of *sad* mutants (Musken *et al* 2010). The *sadB* gene codes for a protein with unknown function and *sad* genes play a role in attachment of the bacteria within the biofilm. *P. aeruginosa* PA14 *sad* mutants were found to be non-motile and it has also been documented that *sadB* is required for the transition from the reversible to the irreversible stage of biofilm formation (Caiazza *et al* 2004, O' Toole *et al* 1998). If an isolate has a mutated form of the *sadB* gene, biofilm will not be produced even if there is good twitching motility (Clutterbuck 2007, Musken *et al* 2010). At 24 hours, *sadB* mutants have very low numbers of irreversibly attached cells (90% of the cells are reversibly attached in the mutant strain whereas only 25% are reversibly attached in the wild-type strain) which maybe due

to an unstable interaction between the mutant and the surface (Caiazza *et al* 2004). It has also been shown that *sadB* levels correlate with the transition to irreversible attachment. This suggests that biofilm formation is positively correlated with *sadB* levels (Caiazza *et al* 2004). The highest levels of *sadB* proteins are present when *P. aeruginosa* is grown on LB medium or when arginine is provided as a carbon source. Lower levels of *sadB* protein were observed in cells grown in glucose compared to those grown on arginine (Caiazza *et al* 2004). Other studies have noted that bacteria with the *sadB* mutation also had defects in swimming motility and produced denser biofilm than the wild-type strains (Musken *et al* 2010). As mentioned earlier, evidence exists that flagellar driven motility for biofilm establishment is important and can vary depending on environmental conditions. Mutations in *sadB* increases flagellar reversal rates but has a minimal effect on the linear swim speed (O'Toole 2008). These effects are observed only under conditions with high viscosity. The *cheIV* chemotaxis-like cluster also works with *sadB* and *sadC* to regulate flagellar function. Mutations in *cheIV* make cells deficient in type IV pili-mediated twitching motility (O'Toole 2008). S*adB* mutants are also unable to form biofilm on surfaces other than PVC (O' Toole *et al* 1998). *SadC* mutants, another gene located in the *sad* gene cluster, are defective in forming biofilms and have increased swarming motility. The *sadC* gene produces a protein containing a GGDEF domain, which is common among proteins with a diguanylate cyclase (DGC) activity. DGC is required for synthesizing bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP), an intracellular signalling molecule. Levels of c-di-GMP in bacterial cells correlate with the transition from the sessile form to motile form. High levels of this molecule promotes biofilm formation (O'Toole 2008). Elevated c-di-GMP leads to induction of specific gene transcription, including those belonging to *pel* and *psl* clusters (Starkey *et al* 2009). *P. aeruginosa* has over 60 genes involved in making and binding c-di-GMP (O'Toole 2008).

Two cell-to-cell signalling systems (QS) in P. aeruginosa are *lasR-lasI* and *rhlR-rhlI*. The *lasI* gene product directs synthesis of an extracellular signal; N-(3-oxododecanoyl)-L-homoserine lactone. The *lasR* product is a transcriptional regulator that requires N-(3-oxododecanoyl)-L-homoserine lactone to activate virulence genes *(*Davies *et al* 1998). L*asI* mutants will form flat colonies similar to those strains with a mutation in *pelA* (Sakuragi *et al* 2007). Development and study of the wild type versus mutant strain showed that the *Las* quorum-sensing system is involved in the development of cell clusters (Davies *et al* 1998). Studies have documented that *Pseudomonas* strains defective in *Las* quorum sensing produce similar quantities of EPS as wild-type bacteria but the mutants were unable to form normal biofilms (Davies *et al* 1998). It was observed that these *Las* mutant biofilms had greater space between individual cells that was not filled with EPS (Sauer *et al* 2002). A *lasI* mutant formed only a thin layer of biofilm that was easily broken, indicating that the *las* mutant is defective in matrix production. Overall *las* mutants had a 60% reduction in adhered biofilm formation indicating that the *las* QS mutants are defective in biofilm formation (Sakuragi *et al* 2007). The *Rhl* system is highly expressed within *Pseudomonas* biofilms (Singh *et al* 2000). R*hlI* products direct the synthesis of the extracellular signal N-buytryl-L-homoserine lactone, which is needed for activation of virulence genes and expression of stationary-phase factor *RpoS* by the *rhlR* gene product. Quorum sensing requires a specific density of bacteria to function *(*Davies *et al* 1998).

Along with the *las* and *rhl* QS systems, another cell-to-cell signal has also been identified as the *Pseudomonas* quinolone signal (PQS). In the study by Allensen-Holm *et al*, it was found that isolates deficient in the *pqs*A gene, which produces PQS, generated low amounts of eDNA and *pqs* mutants, which overproduce PQS, generated increased mounts of eDNA. PQS has also been noted to be involved in the lysis of *P. aeruginosa* cells (Allensen-Holm *et al* 2006). In nature, an active *lasR* (QS) protein is required for PQS production. This indicates that a gene

required for PQS synthesis is controlled through the *las* quorum sensing system *(Pesci* et al *1999).* PQS has also been shown to induce *rhlI* in *Pseudomonas aeruginosa,* which suggests that the quorum sensing signals may be intertwined (McKnight *et al* 2002).

*Pel* and *psl* are two genes that code for the different carbohydrate expression within the biofilm, as outlined previously. *Pel* genes are thought to play a role in manufacturing glucose-rich components of the biofilm matrix and *P. aeruginosa* strain *PA14* relies on the *pel* locus, that contains seven genes, to produce a glucose-rich extracellular polysaccharide, which then becomes part of the pellicle (Friedman *et al* 2004, Ryder *et al* 2007). *Pel* levels are affected by c-di-GMP (O'Toole 2008). A role for both *pel* and type IV pili in the initial attachment process of bacteria has also been suggested (Ryder *et al* 2007). *Pel* mutants exhibit differences in colony morphology appearing smoother that other colonies and will not form a pellicle/stalk (Friedman *et al* 2004). With glucose as a carbon source, the bacteria within the biofilm will differentiate into nonmotile and motile subpopulations. The nonmotile cells form microcolonies that become the "stalk" of the mushroom shape and the motile cells become the "cap" of the mushroom (Klausen *et al* 2003). If citrate is the carbon source, the biofilm will be flat, as the entire cell population is motile at the initial phase of biofilm development (Klausen *et al* 2003). Transcription of *pel* genes is activated by the *las* QS system (Sakuragi *et al* 2007). *Psl* genes contribute to a mannose-rich biofilm (Friedman *et al* 2004). The *psl* locus contains approximately 11 genes (Friedman *et al* 2004). Loss of the *pel* gene does not lower resistance to most antimicrobials, but will lead to enhanced biofilm resistance for aminoglycoside antibiotics (Khan *et al* 2010). This result is interesting as it suggests that even with decreased EPS formation, biofilm-embedded bacteria continue to be more resistant to antimicrobials than their planktonic counterparts.

Tyrosine phosphatase related to biofilm formation A (TpbA), is a tyrosine phosphatase that will suppress biofilm formation via the *pel* locus. Mutants of this phosphatase will exhibit hyper-

biofilm-formation, enhanced production of EPS, altered colony morphology, elevated c-di-GMP and little swarming (Ueda *et al* 2009). TpbA, if inactivated, leads to increased biofilm formation and when activated, leads to decreased swarming. TpbA reduces the amount of cellular c-di-GMP present and therefore affects biofilm formation, as c-di-GMP is a positive regulator of biofilm formation, EPS production and swarming motility (Ueda *et al* 2009).

Chaperone usher pathways (cup) contain genes belonging to a specific cluster numbered A1-A5. CupA2 was previously identified as the *ladN66* gene. The gene product belongs to the FGS family, which are chaperones that assemble fimbriae (Vallet *et al* 2001). Not much is known about these genes other than *cupA3* is likely involved in biofilm formation, as *cupA3* mutants are deficient in biofilm formation (Vallet *et al* 2001).

Rugose small-colony variants (RSCVs) are variants of *Pseudomonas* that produce wrinkled, small colonies able to form a biofilm. These RSCVs have elevated levels of c-di-GMP compared to wild-type strains (Starkey *et al* 2009). Overexpression of a phosphodiesterase that degraded c-di-GMP eliminated the formation of hyper-biofilm production in these RSCV phenotypes (Starkey *et al* 2009). Within the RSCVs, EPS formation and motility appear to be controlled by c-di-GMP, as is the case in wild type biofilms (Starkey *et al* 2009). C-di-GMP promotes biofilm formation by downregulating motility and upregulating production of secreted polysaccharides (Borlee *et al* 2010).

Other genes that play a role in biofilm have also been documented. The *pilA* gene is required for synthesis of type IV pili and *filC* is required for flagellar synthesis (Sauer 2004). *PilA* mutants do not grow normally and form a biofilm with incomplete surface coverage and irregularly shaped colonies (Klausen *et al* 2003). *FleR* or *rpoN* are also genes that control the expression of pili and flagella. When mutant strains of these genes were grown, levels of *sadB* were elevated leading to increased biofilm production (Caiazza *et al* 2004). If proteins leading to

pili or flagella expression are not made, the bacteria will be non-motile and biofilm formation can occur. Two-partner-secretion system (TPS) encoded by *cdrA* and *cdrB,* encode a large adhesin and its transporter and contribute to biofilm formation (Starkey *et al* 2009). *CdrA* expression is elevated with high levels of c-di-GMP which, in turn, leads to biofilm formation (Borlee *et al* 2010). *UreB* encodes a urease and is one of the most extensively differentiated regulated genes identified in *Pseudomonas aeruginosa* (Whiteley *et al* 2001). The enzyme is thought to be involved in the maintenance of pH homeostasis in biofilm cultures (Musken *et al* 2010). This enzyme leads to alkalinzation of the environment; urea is hydrolyzed to ammonia and carbon dioxide (Musken *et al* 2010). *UreB* mutants produced low levels of biofilm (Musken *et al* 2010).

The lungs of CF patients are initially colonized with a nonmucoid *P.aeruginosa* strain. Over time the mucoid variant can emerge. This mucoid phenotype is usually due to overproduction of alginate, which is known to be a capsular polysaccharide virulence factor (Ryder *et al* 2007). Mutations most often occur in the regulator gene *mucA*, which negatively regulates the expression of alginate (Ryder *et al* 2007). Alginate appears to protect *Pseudomonas* from inflammation by scavenging free radicals released by macrophages and from clearance via phagocytosis (Pier 1998). The cells are also more resistant to IFN-gamma mediated killing by cells of the innate immune system (Leid *et al* 2005). Alginate production is not required for biofilm formation but, through mutations in *mucA*, does affect resistance to antimicrobials (Ryder *et al* 2007). In planktonic cells, the *ampC* gene is induced upon exposure to imipenem (Bagge *et al* 2004). Biofilm cells exposed to imipenem increased in average thickness and many genes involved in alginate synthesis are expressed at slightly higher levels (Bagge *et al* 2004).

Anaerobic biofilms of *Pseudomonas aeruginosa* require *rhl* quorum sensing to survive (Yoon *et al* 2002). *Rhl* mutants exhibit high levels of toxic nitrogen oxide (NO), which may account for premature cell death (Yoon *et al* 2002). Research into the porin proteins, members of

the *Opr* family, identified an *oprF* mutant that was a poor biofilm producer (Musken *et al* 2010). It has been suggested that *OprF* maybe important for NO detoxification and the establishment of biofilms in anaerobic conditions (Yoon *et al* 2002).

Death and cell lysis can occur based on the orientation of the biofilm, which suggests *Pseudomonas* maybe capable of programmed cell death (Ma *et al* 2009). Two genetic loci have been identified in *Pseudomonas*, *cidAB* and *lrgAB*, which are similar to proteins found in *Staphylococcus aureus* that contribute to cell death and cell lysis (Rice *et al* 2008). C*idAB* is a holin and *lrgAB* is an anti-holin. Holins are small proteins located in the cell membrane encoded by phages. They control hydrolases and the timing of cell lysis during bacteriophage infection. Holins are antagonized by anti-holins (Rice *et al* 2008). Deletion of either of these loci led to accumulation of more dead cells and a high Optical Density (OD) (Ma *et al* 2009). *Pseudomonas lrgAB* and *cidAB* mutants resulted in more cell death but normal and reduced cell lysis respectively within biofilms (Ma *et al* 2009). This resulted in a larger cavity within microcolonies and premature dispersal of cells. This suggests that cell death and lysis contribute to the formation of the *psl* matrix cavity and dispersal.

As biofilm embedded bacteria generally have an increased MIC for antimicrobials when compared to their planktonic counterparts, and we know that genetics may play a role in this resistance; it stands to reason that some *Pseudomonas* mutants may have decreased resistance to antimicrobials when in a biofilm. At the gene locus 45E7 in *Pseudomonas aeruginosa* there was no difference between the MBC for planktonic cells for the wild type and mutant strains for certain antimicrobials. However, when comparing the MBC for the same antimicrobials for biofilm embedded versus mutant strains, the MBC was lower for those within the biofilm (Mah *et al* 2003). The *ndv*B gene codes for glucosyltransferase, which is required for the synthesis of cyclic-ß-(1,3)-glucans. Glucans play a role in adaptation to low osmotic media and flagella-

mediated motility. The *ndvB* mutant was more sensitive to antimicrobials in a biofilm but not planktonic (when compared to the wild-type) (Mah *et al* 2003).

## 1.11 Objectives

The objectives of this research were as follows:

A. To evaluate the biofilm-forming capacity of canine otic isolates of *Pseudomonas aeruginosa.*

B. To compare the minimum inhibitory concentration (MIC) for gentamicin, neomycin, polymyxin B and enrofloxacin for the planktonic versus biofilm-embedded bacteria *in vitro*.

C. To evaluate the impact of Tromethamine Edetate Disodium Dihydrate (Triz-EDTA®) use on *in vitro* antimicrobial susceptibility of biofilm-embedded *Pseudomonas aeruginosa*.

D. To determine the prevalence of selected biofilm-associated genes in *Pseudomonas aeruginosa* and to evaluate the association of these genes with *in vitro* biofilm production.

## 1.12 References


Allensen-Holm M, Barken KB, Yang L, *et al*. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molec. Microbiol.* 2006; 59: 1114-1128.

Aloush V, Navon-Venezia, Seigman-Igra Y, *et al*. Multidrug-Resistant Pseudomonas aeruginosa: Risk Factors and Clinical Impact. *Antimicrob. Agents Chemother.* 2006; 50: 43-48.

Al-Tahhan RA, Sandrin TR, Bodour AA, *et al*. Rhamnolipid-induced removal of lipopolysaccharides from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates. *Appl. Environ. Microbiol.* 2000; 66: 3262–3268.

Angus JC. Cytology and Histopathology of the Ear in Health and Disease. In: Gotthelf LN. *Small Animal Ear Diseases: An Illustrated Guide 2nd Ed*. 2005; 41-75.

Bagge N, Schuster M, Hentzer M, *et al*. Pseudomonas aeruginosa Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and ß-Lactamase and Alginate Production. *Antimicrob. Agents Chemother.* 2004; 48: 1175-1187.

Banin E, Vasil ML, Greenberg EP. Iron and *Pseudomonas aeruginosa* biofilm formation. *PNAS* 2005; 102: 11076-11081.

Banin E, Brady KM, Greenberg EP. Chelator-Induced Dispersal and Killing of Pseudomonas aeruginosa Cells in a Biofilm. *Appl. Environ. Microbiol.* 2006; 72: 2064-2069.

Barrassa JLM, Gomiez PL, Lama ZG, *et al*. Antibacterial Susceptibility Patterns of *Pseudomonas* Strains Isolated from Chronic Canine Otitis Externa. *J. Vet. Med. B* 2000; 47: 191-196.

Bateman FL, Moss SM, Trott DJ, *et al*. Biological efficacy and stability of diluted ticarcillin-clavulanic acid in the topical treatment of *Pseudomonas aeruginosa* infections. *Vet Derm* 2012; 23: 97-102.

Bauer WD, Robinson JB. Disruption of bacterial quorum sensing by other organisms. *Curr. Opin. Biotech.* 2002; 13: 234-237.

Bendouah Z, Barbeau J, Hamad WA, *et al*. Use of an *in vitro* assay for determination of biofilms-forming capacity of bacteria in chronic rhinosinusitis. *Am. J. Rhinol.* 2006; 20: 434-438.

Borlee BR, Goldman AD, Murakami K, *et al*. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. *Molec. Microbiol.* 2010; 75: 827-842.

Boles BR, Thoendel M, Singh PK. Rhamnolipids mediate detachment of *Pseudomonas aeruginosa* from biofilms. *Molec. Microbiol.* 2005; 57: 1210-1223.

Borriello G, Werner E, Roe F, *et al*. Oxygen Limitation Contribute to Antibiotic Tolerance of Pseudomonas aeruginosa in Biofilms. *Antimicrob. Agents Chemother.* 2004; 48: 2659-2664.

Buckley LM, McEwan NA, Graham P, et al. TrisEDTA significantly potentiates the bactericidal activity of silver sulfadiazine against multi-drug resistant *Pseudomonas aeruginosa*. In 7th World Congress of Veterinary Dermatology 2012; 23: 30.

Caiazza NC, O'Toole GA. SadB is required for the Transition from Reversible to Irreversible Attachment during Biofilm formation by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 2004; 186: 4476-4485.

Clutterbuck AL, Woods EJ, Knottenbelt, *et al*. Biofilms and their relevance to veterinary medicine. *Vet. Microbiol.* 2007; 121: 1-17.

Cole LK, Kwochka KW, Kowalski JJ, *et al*. Microbial flora and antimicrobial susceptibility patterns of isolated pathogens from the horizontal canal and middle ear in dogs with otitis media. *J Am. Vet. Med. Assoc.* 1998; 212: 534-538.

Cole LK, Kwochka KW, Kowalski JJ, *et al*. Evaluation of an ear cleanser for the treatment of infectious otitis externa in dogs. *Vet. Ther.* 2003; 4: 12-23.

Cole LK. Otoscopic evaluation of the ear canal. *Vet Clin Small Anim* 2004; 34: 397-410.

Cole LK, Papich MG, Kwochka KW, *et al*. Plasma and ear tissue concentrations of enrofloxacin and its metabolite ciprofloxacin in dogs with chronic end-stage otitis externa after intravenous administration of enrofloxacin. *Vet Derm* 2008; 20: 51–59.

Cole LK. Anatomy and physiology of the canine ear. *Vet Derm* 2009; 20: 412-421.

Cole LK. Otitis Externa. In: Greene CE. *Infectious Diseases of the Dog and Cat 4th Ed.* St Louis: Elsevier Saunders, 2012; 885-892.

Costerton JW, Lewandowski Z, Caldwell D, *et al*. Microbial Biofilms. *Annu. Rev. Microbiol.* 1995; 49: 711-745.

Costerton JW. Introduction to biofilm. *Int J Antimicrob Agents* 1999; 11: 217-221.

Costerton JW, Stewart PS, Greenberg EP. Bacterial Biofilms: A common cause of persistent infections. *Science* 1999; 284: 1318-1322.

Curtin J, Cormican M. Measuring antimicrobial activity against biofilm bacteria. *Rev. Environ. Sci. Biotech.* 2003; 2: 285-291.

Darouiche RO. Treatment of Infections Associated with Surgical Implants. *N. Engl. J. Med.* 2004; 350: 1422-1429.

Davey ME, O'Toole GA. Microbial Biofilms: from Ecology to Molecular Genetics. *Microbiol. Mol. Biol. Rev.* 2000; 64: 847-867.

Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosa PA01. *J. Bacteriol.* 2003; 185: 1027–1036.

Davies DG Parsek MR, Pearson JP. The Involvement of Cell-to-Cell Signals in the Development of a Bacterial Biofilm. *Science* 1998; 280: 295-298.

Davies JA, Harrison JJ, Marques LLR, *et al*. The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. *FEMS Microbiol. Ecol.* 2007; 59: 32-46.

Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin. Infect. Dis.* 2001; 33: 1387-1392.

Drenkard E, Ausubel FM. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* 2002; 416: 740-743.

Ehrlich GD, Veeh R, Wang X, *et al*. Mucosal Biofilm Formation on Middle-Ear Mucosa in the Chinchilla Model of Otitis Media. *J. Anim. Med. Assoc.* 2002; 287: 1710-1715.

Ellison RT. The effects of lactoferrin on Gram-negative bacteria. *Adv. Exp. Med. Biol*. 1994; 357: 71–90.

Farca AM, Piromallo G, Maffei F, *et al.* Potentiating effect of EDTA-Tris on the activity of antibiotics against resistant bacteria associated with otitis, dermatitis and cystitis. *J. Sm. An. Prac.* 1997; 38: 243-245.

Friedman L, Kolter R. Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the *Pseudomonas aeruginosa* Biofilm Matrix. *J. Bacteriol.* 2004; 186: 4457-4465.

Gillis RJ, Iglewski BH. Azithromycin Retards Pseudomonas aeruginosa Biofilm formation. *J. Clin. Microbiol.* 2004; 42: 5842-5845.

Goodman Al, Kulasekara B, Rietsch A, *et al*. A Signaling network reciprocally regulates genes associated with acute infection and chronic persistence in *Pseudomonas aeruginosa. Dev Cell* 2004; 7: 745-754.

Gortel K. Otic Flushing. *Vet Clin Small Anim* 2004; 34: 557-565.

Gotthelf LN. Perpetuating Factors and Treatment of Otitis Externa. In: Gotthelf LN. *Small Animal Ear Diseases: An Illustrated Guide 2nd Ed*. 2005; 174-185.

Gotz F. *Staphylococcus* and biofilms. *Molec. Microbiol.* 2002; 43: 1367-1378.

Graham-Mize CA, Rosser EJ. Comparison of microbial isolates and susceptibility patterns from the external ear canal of dogs with otitis externa. *J. Am. Anim. Hosp. Assoc.* 2004; 40: 102–108.

Grono LR. Studies of the microclimate of the external auditory canal in the dog. I. Aural Temperature. *Res. Vet. Sci.* 1970; 11: 307-311.

Guardabassi L, Houser GA, Frank LA, *et al*. Guidelines for antimicrobial use in dogs and cat. In: Guardabassi L, Jensen LB, Kruse H. *Guide to Antimicrobial Use in Animals*. Ames: Blackwell Publishing Ltd 2008; 183-207.

Haba E, Pinazo A, Jauregui O, *et al*. Physicochemical characterization and antimicrobial properties of rhamnolipids produced by Pseudomonas aeruginosa 47T2 NCBIM 40044. *Biotechnol. Bioeng.* 2003; 81: 316–322.

Hancock REW, Wong PGW. Compounds which increase the Permeability of *Pseudomonas aeruginosa* Outer Membrane. *Antimicrob. Agents Chemother.* 1984; 26: 48-52.

Harmsen M, Yang L, Pamp SJ, *et al*. An update on *Pseudomonas aeruginosa* biofilm formation, tolerance and dispersal. *FEMS Immunol. Med. Microbiol.* 2010; 59: 253-268.

Harrison-Balestra C, Cazzaniga AL, Davis SC *et al*. A Wound-Isolated *Pseudomonas aeruginosa* Grows a Biofilm In Vitro Within 10 Hours and Is Visualized by Light Microscopy. *Dermatol. Surg.* 2003; 29: 631-635.

Hassett DJ, Schweizer HP, Ohman DE. Pseudomonas aeruginosa sodA and sodB mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. *J. Bacteriol.* 1998; 175: 7658-7665.

Head NE, Yu H. Cross-sectional Analysis of Clinical and environmental isolates of Pseudomonas aeruginosa: Biofilm formation, virulence and Genome Diversity. *Infect. Immun.* 2004;72: 133-144.

Heine PA. Anatomy of the ear. *Vet Clin Small Anim* 2004; 34: 379-395.

Hentzer M, Teitzel GM, Balzer GJ, *et al*. Alginate Overproduction Affects Pseudomonas aeruginosa Biofilm Structure and Function. *J. Bacteriol.* 2001; 183: 5395-5401.

Hill PB, Lo A, Eden CA, *et al*. Survey of the prevalence, diagnosis and treatment of dermatological conditions in small animals in general practice. *Vet. Rec.* 2006; 158: 533-539.

Hoffman LR, D'Argenio DA, MacCoss MJ, *et al*. Aminoglycoside antibiotics induce bacterial biofilm. *Nature* 2005; 436: 1171-1175.

Hoffmann N, Lee B, Hentzer M, *et al*. Azithromycin Blocks Quorum Sensing and Alginate Polymer Formation and Increases the Sensitivity to Serum and Stationary-Growth-Phase Killing of Pseudomonas aeruginosa and Attenuates Chronic P. aeruginosa Lung infection in Cftr-/- Mice. *Antimicrob. Agents Chemother.* 2007; 51: 3677-3687.

Hoyle BD, Alcantara J, Costerton JW. Pseudomonas aeruginosa biofilms as a diffusion barrier to piperacillin. *Antimicrob. Agents Chemother.*1992; 36: 2054-2056.

Hoyle BD, Wong CKW, Costerton JW. Disparate efficacy of tobramycin on Ca+2-, Mg+2-, and HEPES-treated *Pseudomonas aeruginosa* biofilms. *Can. J. Microbiol.* 1992; 38: 1214-1218.

Huang HP, Fixter LM, Little CJL. Lipid content of cerumen from normal dogs and otitic canine ears. *Vet. Rec.*1994; 134: 380-381.

Huang CT, Xu KD, McFeters GA, *et al*. Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. *Appl. Environ. Microbiol.* 1998*;* 64: 1526–1531.

Jackson KD, Starkey M, Kremer S, *et al*. Identification of *psl*, a Locus Encoding a Potential Exopolysaccharide that is Essential for *Pseudomonas aeruginosa* PAO1 Biofilm Formation. *Am. Soc. Microbiol.* 2004; 186: 4466-4475

Jesaitis AJ, Franklin MJ, Berglund D, *et al*. Compromised Host Defense of Pseudomonas aeruginosa Biofilms: Characterization of Neutrophil and Biofilm Interactions. *J. Immunol.* 2003; 171: 4329-4339.

Khan W, Bernier SP, Kuchman SL, *et al*. Antibiotic resistance of *Pseudomonas aeruginosa* biofilms modulated by extracellular polysaccharide. *Int. Microbiol.* 2010; 13: 207-212.

Kierbel A, Gassama-Diagne A, Rocha C*, et al*. *Pseudomonas aeruginosa* exploits a PIP3-dependent pathway to transform apical into basolateral membrane. *J. Cell Biol.* 2007; 177: 21-27.

Kirketerp-Moller K, Jensen PO, Fazli M, et al. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *J Clin Microbiol* 2008; 46: 2717-2722.

Klausen M, Aaes-Jergensen A, Molin S, *et al*. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Molec. Microbiol.* 2003; 50: 61-68.

Klemm P, Schembri MA. Bacterial adhesins: function and structure. *Int. J. Med. Microbiol.* 2000; 290: 27-35.

Koenig A. Gram-negative Bacterial Infections. In: Greene CE. *Infectious Diseases of the Dog and Cat 4th Ed.* St Louis: Elsevier Saunders, 2012; 349-359.

Kohler T, Curty L K, Barja F, *et al*. Swarming of Pseudomonas aeruginosa is dependent on cell-to-cell signaling and requires flagella and pili. *J. Bacteriol.* 2000; 182: 5990–5996.

Lambert PA. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J. R. Soc. Med.* 2002; 95: 22-26.

Leid JG, Willson CJ, Shirtliff ME, *et al*. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from IFN-{gamma}-mediated macrophage killing. *J. Immunol.* 2005; 175: 7512–7518.

Livermore DM. Multiple Mechanisms of Antimicrobial Resistance in *Pseudomonas aeruginosa*: Our Worst Nightmare. *Clin. Inf. Dis.* 2002; 34: 634-640.

Lu XF, Roe A. Jesaitis Z, *et al*. Resistance of biofilms to the catalase inhibitor 3-amino-1,2,4-triazole. *Biotechnol. Bioeng* 1998; 20: 59:156.

Ma L, Conover M, Lu H, *et al*. Assembly and Development of the *Pseudomonas aeruginosa* Biofilm Matrix. *PLoS Pathog.* 2009; 5: 1-11.

Mah TF, Pitts B, Pollock B, *et al*. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 2003; 426: 306-310.

McKnight SL, Iglewski BH, Pasci EC. The Pseudomonas *Quinolone Signal regulates* rhl *Quorum sensing in* Pseudomonas aeruginosa*. J. Bacteriol.* 2000; 182: 2702-2708.

Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: A role in recurrent mastitis infections? *Vet. J.* 2006; 171: 398-407.

Miller WH, Griffin CE, Campbell KL. Diseases of eyelids, Claws, Anal Sacs, and Ears. In: Miller WH, Griffin CE, Campbell KL. *Small Animal Dermatology 7th Ed*. St Louis: Elsevier Mosby, 2013; 741-767.

Moreau-Marquis S, Stanton BA, O'Toole GA. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. A short review. *Pulm. Pharmacol. Ther.* 2008; 21: 595-599.

Morris DO. Medical therapy of otitis externa and otitis media. *Vet Clin Small Anim*. 2004; 34: 541-555.

Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA Chelates Cations and Induces Antibiotic Resistance in *Pseudomonas aeruginosa* Biofilms. *PLoS Pathog.* 2008; 4: 1-12.

Musk DJ, Banko DA, Hergenrother PJ. Iron salts perturb Biofilm formation and Disrupt existing Biofilms of *Pseudomonas aeruginosa*. *Chem. Bio.* 2005; 12: 789-796.

Musk DJ, Hergenrother PJ. Chemical Countermeasures for the Control of Bacterial Biofilms: Effective Compounds and Promising targets. *Curr. Medicinal. Chem.* 2006; 13: 2163-2177.

Musken M, Di Fiore SD, Dotsch A. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. *Microbiol.* 2010; 156: 431-441.

Newton HM, Rosenkratz WS, Muse R, *et al*. Evaluation of otoscope cone cleaning and disinfection procedures commonly used in veterinary medical practices: a pilot study. *Vet Derm* 2006; 17: 147-150.

Nuttall TJ, Cole LK. Ear cleaning: the UK and US perspective. *Vet Derm* 2004; 15: 127–136.

Nuttall T, Cole LK. Evidence-based veterinary dermatology: a systematic review of interventions for treatment of *Pseudomonas* otitis in dogs. *Vet Derm* 2007; 19: 69-77.

Olsen ME, Ceri H, Morck DW, *et al*. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can. J. Vet. Res.* 2002; 66: 86-92.

O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molec. Microbiol.* 1998; 30: 295-304.

O'Toole GA, Kaplan HB, Kolter R. Biofilm Formation as Microbial Development. *Annu. Rev Microbiol.* 2000; 54: 49-79.

O'Toole GA. To Build a Biofilm. *J. Bacteriol*. 2003; 185: 2687-2689

O'Toole GA. How *Pseudomonas aeruginosa* Regulates Surface Behaviours. *Microbe* 2008; 3: 65-71.

Parsek MR, Greenberg EP. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria: a signaling mechanism involved in associations with higher organisms. *Proc. Natl. Acad. Sci.* 2000; 97: 8789–8793.

Pesci EC, Milbank JBJ, Pearson HP, *et al*. Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 1999; 96: 11229-11234.

Petersen AD, Walker RD, Bowman MM, *et al*. Frequency of Isolation and Antimicrobial Susceptibility Patterns of Staphylococcus intermedius and Pseudomonas aeruginosa Isolates From Canine Skin and Ear Samples Over a 6-Year Period (1992-1997). *J. Am Anim. Hosp. Assoc*. 2002; 38: 407-413.

Pier GB. Pseudomonas aeruginosa: a key problem in cystic fibrosis. *ASM News* 1998; 6: 339–347.

Pier GB. Pseudomonas aeruginosa lipopolysaccharide: a major virulence factor, initiator of inflammation and target for effective immunity. *Int. J. Med. Microbiol.* 2007; 297: 277-295.

Pugh KE, Evans JM, Hendy PG. Otitis externa in the dog and cat – an evaluation of a new treatment. *J. Small Anim. Pract.* 1974; 15: 387-400.

Quale J, Bratu S, Gupta J, *et al*. Interplay of Efflux System, ampC and oprD Expression in Carbapenem Resistance of Pseudomonas aeruginosa Clinical Isolates. *Antimicrob. Agents Chemother.* 2006; 50: 1633-1641.

Rice KC, Bayles KW. Molecular control of bacterial death and lysis. *Microbiol. Molec. Biol. Rev.* 2008; 72: 85–109.

Robson D, Burton G, Bassett R. Correlation between topical antibiotic selection, in vitro bacterial antibiotic sensitivity and clinical response in 16 cases of canine otitis externa complicated by *Pseudomonas aeruginosa*. In: *Proceedings of the North American Veterinary Dermatology Forum*, Portland, Oregon, 2010.

Rosser EJ. Causes of otitis externa. *Vet Clin Small Anim* 2004; 34: 459-468.

Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 2007; 10: 644-648.

Sakuragi Y, Kolter R. Quorum-sensing Regulation of the Biofilm Matrix Genes (pel) of Pseudomonas aeruginosa. *J. Bacteriol.* 2007; 189: 5383-5386.

Sandoe JAT, Wysome J, West AP, *et al*. Measurement of ampicillin, vancomycin, linezolid and gentamicin activity against enterococcal biofilms. *J. Antimicr. Chemother.* 2006; 57: 767-770.

Saridomichelakis MN, Farmaki R, Leontides LS, *et al.* Aetiology of canine otitis externa: a retrospective study of 100 cases. *Vet Derm* 2007; 18: 341-347.

Sauer K, Camper AK, Ehrlich GD, *et al*. *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. *J. Bacteriol.* 2002; 184: 1140-1154.

Sauer K, Cullen MC, Rickard AH, *et al*. Characterization of Nutrient-Induced Dispersion in Pseudomonas aeruginosa PAO1 Biofilm. *J. Bacteriol*. 2004; 186: 7312-7326.

Schick AE, Angus JC, Coyner KS. Variability of laboratory identification and antibiotic susceptibility reporting of *Pseudomonas* spp. isolates from dogs with chronic otitis externa. *Vet Derm* 2007; 18: 120-126.

Shaw S. The use of antibiotics to treat bacterial infections in dogs and cats. *Companion Animal* 2012; 17: 37-41.

Siegrist J. (2007). Pseudomonas Media and Tests. Retrieved June 23, 2013, from http://www.sigmaaldrich.com/technical-documents/articles/analytix/pseudomonas-media.html.

Singh A, Walker M, Rousseau J *et al*. Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. *BMC Vet. Res.* 2013; 9: 93-98.

Singh PK, Parsek MR, Greenberg EP, *et al*. A component of innate immunity prevents bacterial biofilm development. *Nature* 2002; 417: 552-555.

Sparks TA, Kemp DT, Wooley RE, et al. Antimicrobial effect of combinations of EDTA-Tris and amikacin or neomycin on the microorganisms associated with otitis externa in dogs. *Vet. Res. Commum.* 1994; 18: 241-249.

Spectrum Laboratories Inc. (2013, June). Introduction to becSCREEN. Retrieved June 23, 20130, from http://www.becscreen.com/about/biofilm-eradication-concentration.

Stapper AP, Narasimhan G, Ohman DE, *et al*. Alginate production affects *Pseudomonas aeruginosa* biofilm development and architecture but is not essential for biofilm formation. *J. Med. Microbiol.* 2004; 53: 679-690.

Steen SI, Paterson S. The susceptibility of *Pseudomonas spp.* isolated from dogs with otitis to topical ear cleaners. *J. Sm. Anim. Prac.* 2012; 53: 599-603.

Stepanovic S, Vukovic D, Dakic I, *et al*. A modified microtitre-plate test for quantification of staphylococcal biofilm formation. *J. Microbiol. Methods*. 2000; 40: 175-179.

Stewart PS. Biofilm accumulation model that predicts antibiotic resistance of Pseudomonas aeruginosa biofilms. *Antimicrob. Agents Chemother.* 1994; 38: 1052-1058.

Stoodley P, Wilson S, Hall-Stoodley L, *et al*. Growth and detachment of cell clusters from mature mixed-species biofilms. *Appl. Environ. Microbiol.* 2001; 67: 5608–5613.

Stoodley P, Sauer K, Davies DG, *et al*. Biofilms as Complex Differentiated Communities. *Annu. Rev. Microbiol.* 2002; 56: 187-209.

Sturgeon A, Costa M, Weese JS. Preliminary evaluation of the bacterial microbiome of the skin and ear in dogs. In 7th World Congress of Veterinary Dermatology 2012; 23: 63.

Swinney A, Fazakerley J, McEwan, *et al*. Comparative *in vitro* antimicrobial efficacy of commercial ear cleaners. *Vet Derm* 2008; 19: 373-379.

Tater KC, Scott DW, Miller WH, *et al*. The cytology of the external ear canal in the normal dog and cat. *J. Vet. Med. Series A – Physiology Pathology Clinical Medicine* 2003; 50: 370–374.

Tejedor MT, Martin JL, Navia M, *et al*. Mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from canine infections. *Vet. Microbiol.* 2003; 94: 295-301.

Ueda A, Wood TK. Connecting Quorum sensing, c-di-GMP, Pel polysaccharide, and Biofilm formation in *Pseudomonas aeruginosa* through Tyrosine Phosphatase TpbA (PA3885). *PLoS Pathogens* 2009; 5: 1-15.

Vallet I, Olson JW, Lory S, *et al*. The chaperone/usher pathways of *Pseudomonas aeruginosa*: Identification of fimbrial gene clusters (*cup*) and their involvement in biofilm formation. *PNAS* 2001; 98: 6911-6916.

Van Loosdrecht MCM, Lyklema J, Norde W *et al*. Bacterial Adhesion: A Physiochemical Approach. *Microb. Ecol.* 1989;17: 1-15.

Veterinary Pet Insurance Co. (2012, May 1). VPI names top 10 canine, feline medical conditions in 2011. Retrieved June 11, 2013, from http://veterinarynews.dvm360.com/dvm/Veterinary+news/VPI-names-top-10-canine-feline-medical-conditions-/ArticleStandard/Article/detail/773988.

Walker TS, Tomlin KL, Worthen GS, *et al*. Enhanced Pseudomonas aeruginosa Biofilm Development Mediated by Human Neutrophils. *Infect. Immun.* 2005; 73: 3693-3701.

Watters C, DeLeon K, Trivedi U, *et al*. *Pseudomonas aeruginosa* biofilms perturb wound resolution and antibiotic tolerance in diabetic mice. *Med. Microbiol. Immunol.* 2012; 1-11.

Webb JS, Thompson LS, James S, *et al*. Cell death in Pseudomonas aeruginosa biofilm development. *J. Bacteriol.* 2003; 185: 4585–4592.

Whiteley M, Greenberg EP. Promoter Specificity Elements in *Pseudomonas aeruginosa* Quorum-Sensing-Controlled Genes. *J. Bacteriol.* 2001; 183: 5529-5534.

Wooley RE, Jones MS. Action of EDTA-Tris and antimicrobial agent combinations on selected pathogenic bacteria. *Vet. Microbiol.* 1983; 8: 271-280.

Yang YX, Xu ZH, Zhang YQ, *et al*. A New Quorum –sensing Inhibitor Attenuates Virulence and decreases Antibiotic Resistance in *Pseudomonas aeruginosa*. *J. Microbiol*. 2012; 50: 987-993.

Yoon SS, Hennigen RF, Hilliard GM, *et al*. *Pseudomonas aeruginosa* Anaerobic Respiration in Biofilms: Relationships to Cystic Fibrosis Pathogenesis. *Dev. Cell* 2002; 3: 593-603.

Yoshida N, Naito F, Fukata T. Studies of certain factors affecting the microenvironment and microflora of the external ear of the dog in health and disease. *J Vet Med Sci* 2002; 64: 1145–1147.

# CHAPTER 2: Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro*

## Manuscript

**Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro***

The following manuscript was submitted to Veterinary Dermatology on January 14, 2013. The manuscript was accepted for publication on April 23, 2013. The article is included in this thesis in the final edited version.

**Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro***

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**Conflict of interest:** None declared.

**Running title:** Pseudomonas biofilm antibiotic susceptibility

**2.1 Abstract:**

**Background:** *Pseudomonas aeruginosa* is a common cause of canine otitis. *P. aeruginosa* biofilm formation has been documented in human medicine, but the role of biofilms in canine disease is not well documented. Bacteria within biofilms can be more resistant to antibiotics compared with their planktonic form; therefore understanding the biofilm-forming capacity of isolates and their susceptibility to antimicrobials is important when developing treatment regimens.

**Objectives:** To evaluate the biofilm-forming capacity of canine otic isolates of *P. aeruginosa* and to compare the minimum inhibitory concentration (MIC) of the planktonic versus biofilm-embedded bacteria.

**Methods:** Biofilm-forming ability was assessed using a microtitre plate assay. Broth microdilution was used to assess the MIC of neomycin, polymyxin B, enrofloxacin and gentamicin for the planktonic and biofilm embedded bacteria.

**Results:** 83 isolates from dogs with otitis were tested. 33 (40%) were classified as biofilm producers. Biofilm MICs for polymyxin B, neomycin, gentamicin and enrofloxacin were significantly higher than for the planktonic form ($P<0.05$).

**Conclusions and clinical importance:** Biofilm production by otitis isolates of *P. aeruginosa* is common and may play a role in the pathogenesis of disease. The MICs for biofilm-embedded bacteria differ from their planktonic counterparts, potentially leading to a lack of response to treatment. If polymyxin B, gentamicin, neomycin or enrofloxacin is to be used for topical treatment of a *Pseudomonas* otitis, the concentration of the medication should be increased, in particular if addressing chronic otitis because biofilms may have developed.

## 2.2 Introduction

Bacterial otitis is a common problem in dogs and *Pseudomonas aeruginosa* is often implicated.[1-2] This bacterium is not considered part of the normal otic microflora.[3-4] When present, *P. aeruginosa* infection can lead to ulceration and inflammation within the ear canal. Otitis is believed to be multifactorial, and treatment can be difficult in some cases. Although poorly investigated, the presence of bacterial biofilm may be an important factor in the pathophysiology of disease and resistance to treatment.

A bacterial biofilm is a sessile community of bacteria that develops as monolayers of planktonic bacterial cells irreversibly attach to a surface that produce a matrix (extrapolymeric substance, EPS) comprised of carbohydrates, proteins and DNA.[5] Once the exopolysaccharide matrix has been formed, it provides extra protection for the sessile bacterial cells inside the biofilm from external factors, such as dessication, the host immune response and antimicrobials.[6,7] Bacteria within a biofilm also have markedly altered metabolism and enhanced cell-cell communication; factors which further assist evasion of the immune system and antimicrobials.[6]

In human medicine, formation of biofilms and their role in many types of infections have been extensively evaluated. Biofilms may form in the lungs of patients with cystic fibrosis, within wounds, on surgical implants and within the middle ear. [8-10] Previous studies from the human literature have confirmed that bacterial cells within biofilms are less susceptible to antimicrobials *in vitro* than their planktonic counterparts.[5,11-12] This decreased susceptibility may be partly due to the extracellular polysaccharide components present within the biofilm, to the biofilm providing physical protection to prevent antimicrobials reaching the bacteria, or to metabolic alterations that affect the antibiotic target.[13-14]

Although the role of the planktonic *P. aeruginosa* has been well described, the role of biofilms in canine otitis is not well documented.[1] As bacteria embedded within a biofilm may be more resistant to antimicrobials, understanding the biofilm-forming ability of clinical isolates and their susceptibility, in comparison to their counterpart planktonic form, is important for developing appropriate treatment regimens for canine patients.

The objectives of this study were to evaluate the biofilm forming ability of canine otic isolates of *P. aeruginosa* and to evaluate the impact of biofilm formation on *in vitro* antimicrobial susceptibility.

## 2.3 Materials and Methods

### Bacterial Isolates

Eighty-three epidemiologically unrelated, multi-drug resistant *Pseudomonas aeruginosa* isolates from dogs with otitis externa were collected from the Animal Health Laboratory (Guelph, Ontario, Canada) and IDEXX (Markham, Ontario, Canada) between the years of 2010-2011. Multidrug resistance was defined as resistance to two or more antimicrobial classes. Isolates were plated and then stored in cryostore vials (Innovatek Medical Inc., Vancouver, Canada).

### Planktonic minimum inhibitory concentrations (MICs)

Antimicrobial susceptibility was tested using agar dilution as per Clinical and Laboratory Standards Institute (CLSI) guidelines.[15] Enrofloxacin, neomycin, gentamicin and polymyxin B, antimicrobials commonly included in topical otic medications, were tested at 2-fold concentrations between 1 and 256 μg/mL.

### Biofilm Growth

*In vitro* biofilm production was assessed using a microtitre plate assay.[16] Pure, 18-24 h growth of *P. aeruginosa* isolates were inoculated to a 0.5 McFarland suspension in enrichment broth containing 10 g/L tryptone (Oxoid, Nepean, Canada), 5 g/L yeast (Oxoid, Nepean, Canada) and 5 g/L sodium chloride (Fisher Science, Ottawa, Canada). Aliquots of 200 μl were inoculated in triplicate into a flat-bottomed 96 well microtitre plate (Sarstedt Inc., Montreal, Canada) and incubated for 24 hours at 35-37$^0$C. A negative control consisting of uninoculated broth was included in triplicate on each plate. Following incubation, the contents of the wells were discarded and each well was washed three times with phosphate buffered saline (PBS) using micropipetting to ensure maintenance of biofilm integrity. Microtitre plates were then heat fixed

at $60^0$C for 60 min. Adhered cells were stained using 150 µl of 0.1% crystal violet for 15 min at room temperature. The crystal violet was then aspirated with a pipette and excess stain was rinsed off with running water. The microtitre plates were then dried at $35^0$C for 15-30 min and crystal violet was re-solubilized with 150 µl of 95% ethyl alcohol. The optical density of each well was measured at 570nm ($OD_{570}$). The triplicate negative control mean $OD_{570}$ was subtracted from the triplicate mean $OD_{570}$ of the study samples. Isolates were classified as biofilm producers if the net $OD_{570}$ was greater than 0.135. They were further classified as low biofilm producers if the net $OD_{570}$ was > 0.135 but ≤ 0.27, moderate biofilm producers if $OD_{570}$ was > 0.27 but ≤ 0.54 and high biofilm producers if $OD_{570}$ was > 0.54 in accordance with a previous study.[16]

**Biofilm bacteria susceptibility testing**

A subset of thirty-one isolates was tested. Biofilm was produced on microtitre plates as described above, but after initial rinsing with PBS, wells were not stained. Instead, 100 µl of LB broth was added to each well along with 100 µl of enrofloxacin, gentamicin, polymyxin B or neomycin at two-fold dilutions from 0.5-2048 µg/ml. Testing was performed in triplicate. A positive control of 200 µl of LB broth was also tested in triplicate. Growth or no growth was visually assessed after 24 h incubation at $35\text{-}37^0$ and the lowest concentration that inhibited visible growth was considered the MIC.

**Statistical analysis**

Results of the susceptibility testing were analyzed with SPSS using a paired t-test. Significance was set as $P < 0.05$.

## 2.4 Results

Thirty-three (33/83; 40%) of the isolates were classified as biofilm producers. Eleven isolates (11/83; 13%) were classified as high biofilm producers, fifteen (15/83; 18%) as moderate biofilm producers and seven (7/83; 8%) as low biofilm producers. Biofilm bacteria susceptibility testing revealed that MICs were significantly higher in biofilm-embedded versus planktonic bacteria for all antimicrobials; enrofloxacin ($P < 0.028$), gentamicin ($p < 0.049$), polymyxin B ($P < 0.0003$) and neomycin ($P < 0.009$) (Table 1 and Figure 1).

## 2.5 Discussion

This study illustrates that biofilm production by canine otic isolates of *P. aeruginosa* is common, with variability in the degree of *in vitro* biofilm production between isolates. The ability to produce biofilm could be an important virulence factor by facilitating establishment of resistant infections within the ear canal and middle ear and by hampering elimination of infection by the immune system and antimicrobials. Although many bacterial species require specific pH or temperature to grow and produce biofilm, *Pseudomonas* has been reported to produce EPS wherever conditions are appropriate for bacterial colonization.[6] This ability might be a reason that *Pseudomonas* is a leading cause of bacterial canine otitis.

While many *Pseudomonas* isolates produced biofilm *in vitro* in our study, 60% either produced no biofilm or a very small amount. Factors affecting biofilm formation by these isolates were not evaluated in this study. Time, *in vivo* factors and a variety of genes may play a role in biofilm formation. Future studies may investigate the genetic components of canine isolates of *P. aeruginosa* that may contribute to biofilm formation as has been highlighted with human isolates, because investigation of these genes in canine *Pseudomonas* is currently lacking.[17-18] Better understanding of the mechanisms involved in biofilm formation is required in order to understand the pathophysiology of biofilm-associated infections and to develop preventive and treatment measures.

As anticipated, these data indicate that MICs for biofilm-embedded bacteria are significantly higher than their planktonic counterparts, as is expected based on previous studies of *Pseudomonas* of human origin.[19] This also suggests that biofilm formation may be an important survival characteristic in canine otitis. Biofilm can physically protect bacteria from antimicrobial exposure, particularly with large polar molecules (e.g. aminoglycosides) as well as antimicrobial peptides.[20] Both the aminoglycoside antibiotics and enrofloxacin bind to structures within the

bacterial cell.[21-22] With the physical protection provided by the biofilm matrix, these antibiotics may not be able to penetrate the cell and exert their effects, hence contributing to increased antimicrobial resistance. Polymyxin B binds to the lipopolysaccharides of the cell membrane to alter structure and make the cell more permeable.[23] If it is also not able to penetrate the polysaccharide matrix it cannot bind to the LPS and lead to cellular death. The population of cells within a biofilm is heterogeneous with both fast and slow growing cells. Downregulation of metabolism and growth may contribute to biofilm resistance, particularly for antibiotics that act on actively growing cells such as enrofloxacin and aminoglycosides.[24] An additional factor is the potential for antimicrobials such as aminoglycosides to induce biofilm formation.[25] While not studied here, it is possible that this phenomenon could have played a role in significantly higher MIC for biofilm-embedded bacteria versus the planktonic form.

One concern with this study is whether *in vitro* susceptibility testing can approximate an *in vivo* biofilm. There is disagreement within the literature regarding the appropriate test to determine antibiotic MIC values for bacterial biofilms.[26] Multiple tests exist, however, there is a lack of standardization between the testing modalities and a lack of consensus over which test is the most appropriate for biofilm MIC determination.[26,27] While the microtitre plate assay is widely used, it is clearly an artificial system that cannot mimic the complexity of the microenvironment of an inflamed ear. However, biofilm formation within the middle ear cavity of chinchillas has been documented *in vivo,* and other studies have demonstrated biofilm formation on other mucosal surfaces such as within sinuses of rabbits.[28-29] These studies support the notion that biofilms form readily on mucosal surfaces and therefore potentially form within the external ear canal. Although conditions may be appropriate for biofilm formation *in vitro*, in a diseased state the epithelium of the external ear canal may have an increased turnover rate and

cerumen production.[30] This may serve as an efficient defense mechanism as it will prevent adherence of larger numbers of micro-organisms and disrupt biofilm formation.

Topical therapy is most commonly used to treat canine otitis externa. Even culture-resistant planktonic populations of *P. aeruginosa* can be overcome, because topically applied antibiotic concentrations reach 100-1000 times that of systemically delivered antimicrobials at the site of infection.[31,32] This study has highlighted the potential role of biofilm formation in canine otitis and the possibility that biofilm could impact the success of antimicrobial therapy. Biofilm formation may be an important virulence factor in *P. aeruginosa* and an important step in the development of otitis externa. Further study of the role of biofilm in disease, the impact of biofilm formation on clinical treatment failure and factors that regulate biofilm production are indicated to help elucidate the pathophysiology of otitis and to develop better treatment approaches.

## 2.6 Tables

| | $MIC_{50}$ (μg/ml) | | | $MIC_{90}$ (μg/ml) | | | MIC Range (μg/ml) | |
|---|---|---|---|---|---|---|---|---|
| Drug | Planktonic | Biofilm | Fold diff | Planktonic | Biofilm | Fold diff | Planktonic | Biofilm |
| Enrofloxacin | 1 | 8 | 8 | 8 | 16 | 2 | 1-16 | 1-256 |
| Gentamicin | 4 | 16 | 4 | 8 | 128 | 16 | 1-64 | 4-1024 |
| Polymyxin B | 4 | 64 | 16 | 64 | 256 | 4 | 1-256 | 2-256 |
| Neomycin | 64 | 128 | 2 | 128 | 256 | 2 | 8-256 | 8-1024 |

**Table 1: Comparison of mean $MIC_{50}$ and $MIC_{90}$ for planktonic bacteria and biofilm-embedded bacteria of 31 isolates.**

## 2.7 Figures

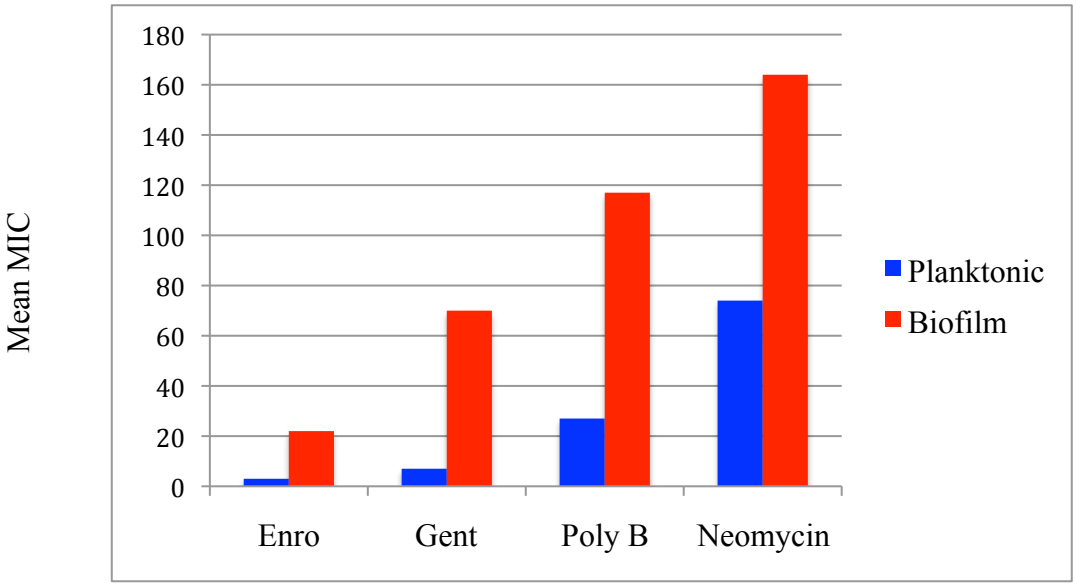


**Figure 1: Comparison of mean MIC of enrofloxacin, gentamicin, polymyxin B and neomycin for planktonic bacteria versus biofilm-embedded for 31 *P. aeruginosa* isolates.**

## 2.8 References


1. Nuttall T, Cole AK. Evidence-based veterinary dermatology: a systematic review of interventions for treatment of *Pseudomonas* otitis in dogs. *Vet Derm* 2007; 18: 69-77.
2. Cole LK, Kwochka KW, Kowalski JJ, et al. Microbial flora and antimicrobial susceptibility patterns of isolated pathogens from the horizontal ear canal and middle ear in dogs with otitis media. *JAVMA* 1998; 212: 534–538.
3. Tater KC, Scott DW, Miller WH, et al. The cytology of the external ear canal in the normal dog and cat. Journal of Veterinary Medicine Series A – Physiology. *Path Clin Med* 2003; 50: 370–374.
4. Weese JS. The canine and feline skin microbiome in health and disease. *Vet Derm* 2013; 24: 137–145. e31.
5. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin Infect Dis* 2001; 33: 1387-1392.
6. Clutterbuck AL, Woods EJ, Knottenbelt DC, et al. Biofilms and their relevance to veterinary medicine. *Vet Micro* 2007; 121: 1-17.
7. Costerton JW. Introduction to biofilm. *Int J Antimicrob Agents* 1999; 11: 217-221.
8. Moreau-Marquis S, Stanton BA, O'Toole G. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. A short review. *Pulm Pharmacol Ther* 2008; 21: 595-599.
9. Kirketerp-Moller K, Jensen PO, Fazli M, et al. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *J Clin Microbiol* 2008; 46: 2717-2722.
10. Darouiche RO. Treatment of Infections Associated with Surgical Implants. *N Engl J Med* 2004; 350: 1422-1429.

11. Hoyle BD, Wong CKW, Costerton JW. Disparate efficacy of tobramycin on Ca+2-, Mg+2-, and HEPES-treated *Pseudomonas aeruginosa* biofilms. *Can J Microbiol* 1992; 38: 1214-1218.

12. Allesen-Holm M, Barken KB, Yang L, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molec Micro* 2006; 59: 1114-1128.

13. Khan W, Bernier SP, Kuchma SL, et al. Aminoglycoside resistance of *Pseudomonas aeruginosa* biofilms modulated by extracellular polysaccharide. *Int Micro* 2010; 13: 207-212.

14. Caiazza N, O'Toole GA. SadB Is Required for the Transition from Reversible to Irreversible Attachment furing Biofilm Formation of *Pseudomonas aeruginosa* PA14. *J Bact* 2004; 186: 4476-4485.

15. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved standard-Seventh edition. Clinical and laboratory Standards Institute document M7-A7 2006; 26: 1-49.

16. Stepanovic S, Vukovic D, Dakic I, et al. A modified microtitre-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 2000; 40: 175-179.

17. McKnight S, Iglewski BH, Pesci EC. The *Pseudomonas* Quinolone Signal Regulates *rhl* Quorum Sensing in *Pseudomonas aeruginosa*. *J Bacteriol* 2000; 182: 2702-2708.

18. Klausen M, Heydorn A, Ragas P, et al. Biofilm formation by *Pseudomonas aeruginosa* wild type, flagella and type IV pili mutants. *Molec Micro* 2003; 48: 1511-1524.

19. Drenkard E. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes Inf* 2003; 5: 1213-1219.

20. Lewis K. Riddle of Biofilm Resistance. *Antimicrob Agents Chemother* 2001; 45: 999-1007.

21. Howland RD, Mycek MJ. Protein synthesis inhibitors. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 367-380.

22. Howland RD, Mycek MJ. Quinolones, Folic Acid Antagonists, and Urinary Tract Antiseptics. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 381-386.

23. Zavascki AP, Goldani LZ, Li J, et al. Polymyxin B for the Treatment of multi-drug resistant pathogens: a critical review. *J Antimicrob Chemother* 2007; 60: 1206-1215.

24. Lambert PA. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J Royal Society of Med* 2002; 95: 22-26.

25. Hoffman LR, D'Argenio DA, MacCoss MJ, et al. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 2005; 436: 1171-1175.

26. Curtin J, Cormican M. Measuring antimicrobial activity against biofilm bacteria. *Rev. Environ. Sci. Biotech.* 2003; 2: 285-291.

27. Ceri H, Olson ME, Stremick C et al. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* 1999; 37: 1771–1776.

28. Byrd M, Pang B, Hong W, et al. Direct Evaluation of *Pseudomonas aeruginosa* Biofilm Mediators in a Chronic Infection Model. *Inf Imm* 2011; 79: 3087-3095.

29. Perloff JR, Palmer JN. Evidence of Bacterial Biofilms in a Rabbit Model of Sinusitis. *Am J Rhin* 2005; 19: 1-6.

30. Angus JC. Cytology and Histopathology of the Ear in Health and Disease. In: Gotthelf L. *Small Animal Ear Diseases: An Illustrated Guide*. 2nd ed. St Louis: Elsevier Saunders, 2005; 41-75.

31. Hnilica KA. Otitis Externa. In: Hnilica KA. *Small Animal Dermatology: A Colour Atlas and Therapeutic Guide.* 3rd ed. St Louis: Elsevier Saunders. 2011; 395-409.

32. Morris DO. Medical therapy of otitis externa and otitis media. *Vet Clin North Small Anim Pract*. 2004; 34: 541-555.

## CHAPTER 3: Evaluation of the impact of Tromethamine edetate disodium dihydrate on antimicrobial susceptibility of *Pseudomonas aeruginosa* in biofilm *in vitro*

**Manuscript**

**Evaluation of the impact of Tromethamine edetate disodium dihydrate on antimicrobial susceptibility of *Pseudomonas aeruginosa* in biofilm *in vitro***

The following manuscript was submitted to Veterinary Dermatology on June 6, 2013. The article is included in this thesis in the submitted version.

**Evaluation of the impact of Tromethamine edetate disodium dihydrate on antimicrobial susceptibility of *Pseudomonas aeruginosa* in biofilm *in vitro***

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**Funding:** This study was self-funded.

**Conflict of interest:** None declared.

**Running title:** Biofilm susceptibility with Triz-EDTA®

**3.1 Abstract:**

**Background:** Biofilm formation by *Pseudomonas aeruginosa* has been documented in canine otic isolates. An increase in minimum inhibitory concentration (MIC) for specific antibiotics has been noted for biofilm embedded bacteria. Tromethamine edetate disodium dihydrate buffered to pH8 with Tromethamine HCL and deionized water (Triz-EDTA®) has been documented to potentiate bactericidal activity when used in combination with topical antibiotics but the impact on biofilm-embedded bacteria is unknown.

**Objective:** The objective of this study was to evaluate the impact of Triz-EDTA® use on *in vitro* antimicrobial susceptibility of biofilm-embedded *Pseudomonas aeruginosa*.

**Methods:** Biofilm formation was documented using a microtitre plate assay. Broth microdilution was used to assess the MIC of neomycin, polymyxin B, enrofloxacin and gentamicin for the biofilm embedded bacteria and biofilm-embedded bacteria with added Triz-EDTA®.

**Results:** 31 isolates from dogs with otitis were tested. Addition of Triz-EDTA® significantly reduced MICs for neomycin ($P < 0.008$) and gentamicin ($p < 0.04$) but not enrofloxacin ($P = 0.7$), or polymyxin B ($P = 0.5$).

**Conclusions and clinical importance:** Triz-EDTA® may be a useful adjunctive treatment for chronic cases of *Pseudomonas* otitis where biofilms may have developed, if gentamicin or neomycin are to be used as a topical treatment. *In vivo* study is required to confirm this effect.

## 3.2 Introduction

Bacterial otitis externa is a common condition seen in dogs and the bacterium *Pseudomonas aeruginosa* is often implicated in the pathogenesis of the disease.[1] *Pseudomonas aeruginosa* is a bacterium that is not a commensal organism of the external ear canal and has not been identified on cultures obtained from healthy canine ears.[2-5] *Pseudomonas* can cause infection when inflammation is present within the ear canal due to an underlying etiology such as allergic skin disease, endocrinopathy or immune mediated disease.[6] *Pseudomonas* otitis externa can be a frustrating disorder to treat with an unpredictable response to antimicrobial therapy. Various factors may account for this, including an underlying disorder and the degree of aural inflammation present. Another possible reason is the presence of bacterial biofilm. A bacterial biofilm develops from monolayers of planktonic bacterial cells that first irreversibly attach to a surface, produce a matrix of carbohydrates, proteins and DNA (extrapolymeric substance, EPS) and then become irreversibly attached to that surface.[7] Bacteria within the biofilm can evade the host immune response, dessication and antimicrobial therapy by physical protection provided by EPS, altered metabolism and various other changes in the individual bacteria and the community as a whole.[8]

Biofilm formation has been documented extensively in human medicine, with multiple studies demonstrating biofilm production by clinical isolates of *P. aeruginosa* and reduced *in vitro* susceptibility of biofilm embedded bacteria, including otic isolates.[9-13] Recently, biofilm formation was identified in canine otic isolates of *Pseudomonas aeruginosa,* with 40% of the isolates forming a quantifiable biofilm.[14] The role of biofilm formation in cases of canine otitis externa has not been well described, but biofilm production could play an important role in development and maintenance of otitis externa despite antimicrobial treatment. Investigating

treatment modalities or compounds that may increase penetration of antimicrobials through the biofilm, or biofilm disruption, is important.

Tromethamine (Tris), edetate disodium dihydrate (EDTA) buffered to pH8 with Tromethamine HCL and deionized water, (Triz-EDTA® Aqueous flush, Dechra Veterinary Products, Kansas, US) is commonly used as an adjunct therapy for dogs with *Pseudomonas* otitis.[1] The tris buffer within the compound is thought to enhance the effect of the EDTA, which acts to damage the cell surface of gram-negative bacteria such as *Pseudomonas*.[15] Previous studies have shown that Triz-EDTA® used in combination with an antimicrobial, can reduce the MIC and minimum bactericidal concentration (MBC) for certain antibiotics against planktonic bacteria and those within biofilms.[15-17] The objective of this study was to evaluate the impact of Triz-EDTA® use on *in vitro* antimicrobial susceptibility of preformed *Pseudomonas aeruginosa* biofilms.

### 3.3 Materials and Methods

#### Isolates

Thirty-one multi-drug resistant *P. aeruginosa* isolates recovered from the ears of dogs with otitis externa that were previously demonstrated to produce biofilm in vitro were studied. Each isolate was obtained from a different canine.[14]

#### Biofilm Growth and biofilm bacteria susceptibility testing

Biofilm growth and susceptibility testing was carried out as documented in a previous study.[14] *In vitro* biofilm production was assessed using a microtitre plate assay.[18] Biofilm forming capacity of each isolate had previously been documented by measuring the optical density of each well at 570nm ($OD_{570}$), after overnight incubation. Isolates were inoculated in triplicate along with a triplicate negative control. The triplicate negative control mean $OD_{570}$ was subtracted from the triplicate mean $OD_{570}$ of the study samples. Isolates were classified as biofilm producers if the net $OD_{570}$ was greater than 0.135. They were further classified as low biofilm producers if the net $OD_{570}$ was > 0.135 but ≤ 0.27, moderate biofilm producers if $OD_{570}$ was > 0.27 but ≤ 0.54 and high biofilm producers if $OD_{570}$ was > 0.54 in accordance with a previous study.[14,18] For susceptibility testing, pure, 18-24 h growth of *P. aeruginosa* isolates were inoculated to a 0.5 McFarland suspension in enrichment broth containing 10 g/L tryptone (Oxoid, Nepean, Canada), 5 g/L yeast (Oxoid, Nepean, Canada) and 5 g/L sodium chloride (Fisher Science, Ottawa, Canada). Aliquots of 200 µl were inoculated in triplicate into a flat-bottomed 96 well microtitre plate (Sarstedt Inc., Montreal, Canada) and incubated for 24 hours at 35-37$^0$C. A negative control consisting of uninoculated broth was included in triplicate on each plate. Following incubation, the contents of the wells were discarded and each well was washed three times with phosphate buffered saline (PBS) using micropipetting to ensure maintenance of

biofilm integrity. 100 µl of LB broth was then added to each well along with 100 µl of enrofloxacin, gentamicin, polymyxin B or neomycin at two-fold dilutions from 0.5-2048 µg/ml. Testing was performed in triplicate. A positive control of 200 µl of LB broth was also tested in triplicate. Growth or no growth was visually assessed after 24 h incubation at 35-37$^0$ and the lowest concentration that inhibited visible growth was considered the MIC.

**Triz-EDTA® susceptibility testing**

LB broth containing 10 g/L tryptone (Oxoid, Nepean, Canada), 5 g/L yeast (Oxoid, Nepean, Canada) and 5 g/L sodium chloride (Fisher Science, Ottawa, Canada) was produced to include 24% Triz-EDTA® (Dechra Veterinary Products, Kansas, US). This broth contained 0.53 mg/ml Tris and 0.14 mg/ml EDTA. Biofilm was produced on microtitre plates as described above. After initial rinsing with PBS, 100µl aliquots of this LB broth with added Triz-EDTA® were inoculated into a flat-bottomed 96 well microtitre plate (Sarstedt Inc., Montreal, Canada). 100 µl of enrofloxacin, gentamicin, polymyxin B or neomycin at two-fold dilutions from 1.0-512 µg/ml was then added to each well. Testing was performed in triplicate. A positive control of 200µl of LB broth was also tested in triplicate along with a second control of 200µl of LB broth containing Triz-EDTA®. Growth or no growth was visually assessed after 24 h incubation at 35-37$^0$ as per Clinical and Laboratory Standards Institute (CLSI) guidelines for planktonic bacteria and MIC testing.[19] The lowest concentration that inhibited visible growth was considered the MIC.

**Statistical analysis**

The impact of Triz-EDTA® on MIC was evaluated for each antimicrobial with SPSS using a paired t-test. The impact of biofilm category (high/moderate/low) was assessed using ANOVA with Tukey's test. Significance was set as $P < 0.05$.

### 3.4 Results

Addition of Triz-EDTA® resulted in significantly lower MICs for biofilm-embedded bacteria treated with neomycin ($P < 0.003$) and gentamicin ($p < 0.02$), but not for enrofloxacin ($P = 0.5$), or polymyxin B ($P = 0.3$) (Figure 1 and Table 1). For enrofloxacin, the MIC actually increased in the presence of Triz-EDTA® for 20 (64.5%) isolates. For 12 (38.7%) isolates, the increase in MIC was 4-fold or higher. In contrast, a 4-fold or higher increase in MIC was only noted for 4 (12.9%) isolates for polymyxin B, 1 (3.2%) isolate for neomycin and no isolates for gentamicin. 10 (32.3%) isolates were high biofilm producers, 13 (41.9%) were moderate and 8 (25.8%) were low.[14] There was no impact on biofilm categorization (high/moderate/low) on the impact of Triz-EDTA®, as indicated by the fold difference in MIC (all $P > 0.12$). There was no inhibitory effect of Triz-EDTA® without antibiotic for any isolate.

### 3.5 Discussion

This study indicates that the addition of Triz-EDTA® can have a significant impact on the susceptibility of biofilm-embedded *P. aeruginosa* to certain antimicrobials. This is consistent with previous studies that documented a decrease in the minimum inhibitory concentration of specific antibiotics for *Pseudomonas aeruginosa* with the addition of Triz-EDTA®.[16-17,20]

As evidenced by the lack of an effect of Triz-EDTA® alone on bacterial growth, the effect on MICs is not simply an additive effect of another antibacterial substance. Rather this is a synergistic effect that could be mediated by Triz-EDTA® opening pores in the bacterial cell wall to facilitate drug penetration.[21] When exposed to Triz-EDTA® , the cell surfaces of gram negative bacteria can become damaged, leading to increased leakage of intracellular components and increased penetration of antimicrobials.[15] This finding is consistent with previous studies.[22]

The difference in the impact of Triz-EDTA® on the efficacy of different antimicrobials bears consideration. A significant effect was only noted for neomycin and gentamicin, two aminoglycosides, which could indicate that the mechanism of activity is specific to these two drugs or this drug class. However, both aminoglycoside and fluoroquinolones (enrofloxacin) bind to structures within the bacterial cell.[23-24] It stands to reason that if Triz-EDTA® facilitates penetration of the antimicrobials into the bacterial cell, these antibiotics could exert their effects more readily. The lack of an impact of enrofloxacin and polymyxin B was therefore surprising. It is possible that this is related to statistical power; however, of additional interest (and concern) was the increase in biofilm MIC with added Triz-EDTA®, in 64.5% of isolates in the presence of enrofloxacin. While a one-dilution (2-fold) difference in MIC could simply represent test-to-test variation, the 4-fold or higher difference that was identified for 39% of isolates suggests that this was a true increase in MIC in response to Triz-EDTA®. Potential reasons for this are unclear and require further investigation. These data are also in contrast with a previous study that showed

that addition of Triz-EDTA® into the treatment regimen of otitis externa, previously unresponsive to enrofloxacin alone and noted to be resistant to enrofloxacin on a bacterial culture, lead to resolution of the otitis.[20] However, that study looked at low numbers of clinical cases of *P. aeruginosa* otitis externa where the bacteria were not defined as being planktonic or biofilm-embedded, as opposed to this study of only biofilm-forming *P. aeruginosa*. Therefore, the results may not truly be contradictory and rather indicate differences in effect with different study populations. Another limitation with this study is whether an *in vitro* susceptibility test can be compared to *in vivo* biofilm formation. Biofilm formation within the middle ear cavity of chinchillas has been documented *in vivo* and other studies have demonstrated biofilm formation on other mucosal surfaces such as within sinuses of rabbits.[25-26] These studies support that biofilm formation on mucosal surfaces is possible. *Pseudomonas aeruginosa* biofilm formation has been consistently documented in the lungs of patients with cystic fibrosis where mucus production is altered and greatly increased.[9] Both cerumen and the mucus produced by cystic fibrosis patients, contain high levels of glycolipids, and have many other similarities, which could potentially facilitate biofilm formation in both of these anatomical locations.[27-28] Conditions and host defense within the external ear canal should also be taken into account. Local production of antibodies and increased cerumen production in diseased states may contribute to changes in biofilm formation or resistance.[6] Multiple tests exist to determine antibiotic MIC values for bacterial biofilms. Currently, there is a lack of standardization as to which test is most appropriate.[29-30] Another consideration is the isolate collection that was used. Selection of biofilm producers was required for the isolates; however, up to 60% of canine otic *P. aeruginosa* do not produce biofilm *in vitro*.[14] Therefore, the results must be taken as an indication of the impact of Triz-EDTA® on MIC for those isolates that do produce biofilm, not *P. aeruginosa* as a whole.

This study evaluated the MIC, not minimum bactericidal concentration (MBC). Whether this is of relevance is unclear, but research has also shown that the addition of Triz-EDTA® can significantly reduce the MBC of silver sulfadiazene against multi drug resistant *Pseudomonas aeruginosa*.[17] The minimum inhibitory concentration is defined as the lowest concentration of an antimicrobial that will inhibit visible growth of the microorganism after overnight incubation. The minimum bactericidal concentration is defined as the lowest concentration of an antimicrobial that will prevent growth of an organism after subculture onto antimicrobial-free media.[31] Although visible growth was inhibited at the MICs documented in this study, the bacteria within the biofilm may not have been completely eliminated and hence could potentially re-grow after cessation of antimicrobial therapy. The clinical relevance of this is unclear but it may be of importance since persistence of a small percentage of bacteria viable within the biofilm could result in recrudescence of infection. The impact of Triz-EDTA® on MBC of isolates within this study was not documented but this would be an area for future research.

This study has highlighted the potential role for Triz-EDTA® as an adjunctive treatment of *Pseudomonas* otitis. Further study will be needed to investigate the role of biofilm formation in disease and the impact of anti-biofilm approaches for treatment of prevention of disease *in vivo.* Study of other topical compounds that may interact synergistically with Triz-EDTA® is also indicated.

### 3.6 Tables

| | $MIC_{50}$ (µg/ml) | | | $MIC_{90}$ (µg/ml) | | | MIC Range (µg/ml) | |
|---|---|---|---|---|---|---|---|---|
| Drug | Biofilm | Tris Biofilm | Fold diff | Biofilm | Tris Biofilm | Fold diff | Biofilm | Tris Biofilm |
| Enrofloxacin | 8 | 16 | 2 | 16 | 32 | 2 | 1-256 | 1-256 |
| Gentamicin | 16 | 4 | -4 | 128 | 8 | -16 | 4-1024 | 1-32 |
| Polymyxin B | 64 | 32 | -2 | 256 | 256 | 1 | 2-256 | 4-512 |
| Neomycin | 128 | 64 | -2 | 256 | 128 | -2 | 8-1024 | 16-256 |

**Table 1: Comparison of mean minimal inhibitory concentrations MIC50 and MIC90 for biofilm-embedded bacteria and biofilm-embedded bacteria grown in broth inoculated with Triz-EDTA® for 31 *Pseudomonas aeruginosa* isolates.**

### 3.7 Figures

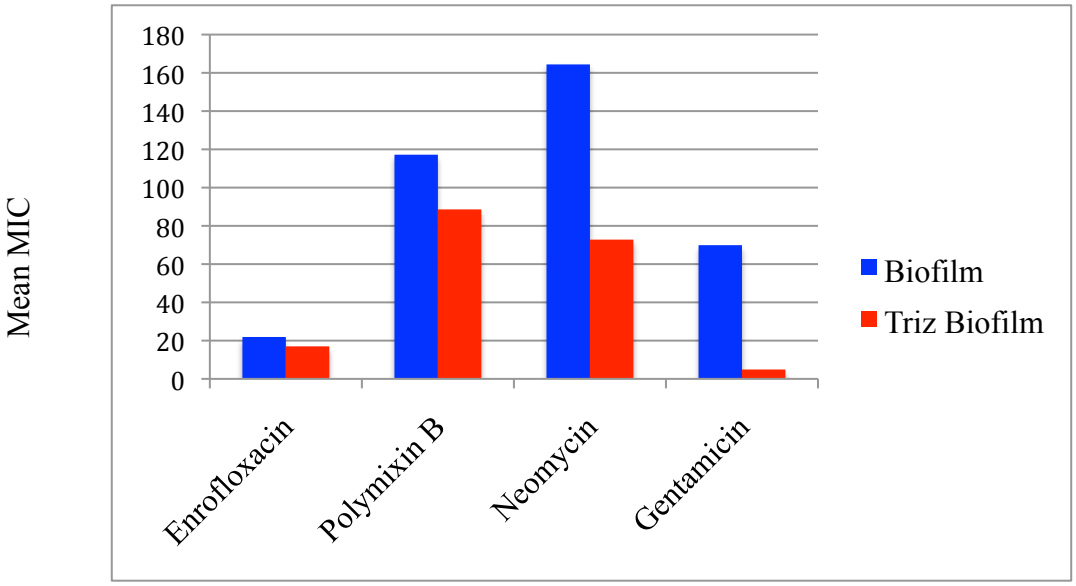


**Figure 1: Comparison of mean MIC of enrofloxacin, polymyxin B, neomycin and gentamicin for biofilm-embedded bacteria versus biofilm-embedded bacteria grown in broth inoculated with Triz-EDTA® for 31 *Pseudomonas aeruginosa* isolates.**

## 3.8 References


1. Nuttall T, Cole AK. Evidence-based veterinary dermatology: a systematic review of interventions for treatment of *Pseudomonas* otitis in dogs. *Vet Derm* 2007; 18: 69-77.
2. Sturgeon A, Costa M, Weese JS. Preliminary evaluation of the bacterial microbiome of the skin and ear in dogs. In 7th World Congress of Veterinary Dermatology 2012; 23: 63.
3. Tejedor MT, Martin JL, Navia M, *et al*. Mechanisms of fluoroquinolone resistance in Pseudomonas aeruginosa isolates from canine infections. *Vet Microbiol* 2003; 94: 295–301.
4. Yoshida N, Naito F, Fukata T. Studies of certain factors affecting the microenvironment and microflora of the external ear of the dog in health and disease. *J Vet Med Sci* 2002; 64: 1145–1147.
5. Tater KC, Scott DW, Miller WH, *et al*. The cytology of the external ear canal in the normal dog and cat. *J Vet Med Series A – Physiology Pathology Clinical Medicine* 2003; 50: 370–374.
6. Miller WH, Griffin CE, Campbell KL. Disease of Eyelids, Claws, Anal Sacs, and Ears. In: Miller WH, Griffin CE, Campbell KL. *Muller and Kirk's Small Animal Dermatology 3rd Ed*. St.Louis: Elsevier Mosby. 2013; 741-767.
7. Clutterbuck AL, Woods EJ, Knottenbelt DC, et al. Biofilms and their relevance to veterinary medicine. *Vet Micro* 2007; 121: 1-17.
8. Costerton JW. Introduction to biofilm. *Int J Antimicrob Agents* 1999; 11: 217-221.
9. Moreau-Marquis S, Stanton BA, O'Toole G. *Pseudomonas aeruginosa* biofilm formation in the cystic fibrosis airway. A short review. *Pulm Pharmacol Ther* 2008; 21: 595-599.
10. Kirketerp-Moller K, Jensen PO, Fazli M, et al. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *J Clin Microbiol* 2008; 46: 2717-2722.

11. Darouiche RO. Treatment of Infections Associated with Surgical Implants. *N Engl J Med* 2004; 350: 1422-1429.

12. Hoyle BD, Wong CKW, Costerton JW. Disparate efficacy of tobramycin on Ca+2-, Mg+2-, and HEPES-treated *Pseudomonas aeruginosa* biofilms. *Can J Microbiol* 1992; 38: 1214-1218.

13. Allesen-Holm M, Barken KB, Yang L, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molec Micro* 2006; 59: 1114-1128.

14. Pye CC. Yu AA, Weese JS. Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro*. *Vet Derm* 2013; 24: 446-e99.

15. Wooley RE, Jones MS. Action of EDTA-Tris and antimicrobial agent combinations on selected pathogenic bacteria. *Vet Microbiol* 1983; 8: 271-280.

16. Sparks TA, Kemp DT, Wooley RE, et al. Antimicrobial effect of combinations of EDTA-Tris and amikacin or neomycin on the microorganisms associated with otitis externa in dogs. *Vet Res Commum* 1994; 18: 241-249.

17. Buckley LM, McEwan NA, Graham P, et al. TrisEDTA significantly potentiates the bactericidal activity of silver sulfadiazine against multi-drug resistant *Pseudomonas aeruginosa*. In 7th World Congress of Veterinary Dermatology 2012; 23: 30.

18. Stepanovic S, Vukovic D, Dakic I, et al. A modified microtitre-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 2000; 40: 175-179.

19. Clinical and Laboratory Standards Institute. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically: Approved standard-Seventh edition. Clinical and laboratory Standards Institute document M7-A7 2006; 26: 1-49.

20. Farca AM, Piromallo G, Maffei F, *et al.* Potentiating effect of EDTA-Tris on the activity of antibiotics against resistant bacteria associated with otitis, dermatitis and cystitis. *J Sm An Prac* 1997; 38: 243-245.

21. Guardabassi L, Houser GA, Frank LA *et al*. Guidelines for antimicrobial use in dogs and cats. In: Guardabassi L, Jensen LB, Kruse H. *Guide to antimicrobial use in animals*. Iowa:Blackwell Publishing Ltd. 2008;183-206.

22. Swinney A, Fazakerley J, McEwan N, *et al*. Comparative *in vitro* antimicrobial efficacy of commercial ear cleaners. *Vet Derm* 2008; 19: 373-379.

23. Howland RD, Mycek MJ. Protein synthesis inhibitors. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 367-380.

24. Howland RD, Mycek MJ. Quinolones, Folic Acid Antagonists, and Urinary Tract Antiseptics. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 381-386.

25. Byrd M, Pang B, Hong W, et al. Direct Evaluation of *Pseudomonas aeruginosa* Biofilm Mediators in a Chronic Infection Model. *Inf Imm* 2011; 79: 3087-3095.

26. Perloff JR, Palmer JN. Evidence of Bacterial Biofilms in a Rabbit Model of Sinusitis. *Am J Rhin* 2005; 19(1): 1-6.

27. Lillehoj EP, Kim KC. Airway Mucus: Its Components and Function. *Arch Pharm Res* 2002; 25: 770-780.

28. Feig MA, Hammer E, Volker U, *et al*. In-depth proteomic analysis of the human cerumen— A potential novel diagnostically relevant biofluid 2013; 83: 119-129.

29. Curtin J, Cormican M. Measuring antimicrobial activity against biofilm bacteria. *Rev Environ Sci Biotechnol.* 2003; 2: 285-291.

30. Ceri H, Olson ME, Stremick C, Read RR et al. The Calgary Biofilm device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *J Clin Micro* 1999; 37: 1771-1776.

31. Andrews JM. Determination of minimum inhibitory concentrations. *J Antimic Chemotherapy* 2001; 48: 5-16.

## CHAPTER 4: Evaluation of genetic markers for biofilm production in canine otic isolates of *Pseudomonas aeruginosa*

**Manuscript**

### Evaluation of genetic markers for biofilm production in canine otic isolates of *Pseudomonas aeruginosa*

This manuscript has yet to be submitted for publication.

**Evaluation of the presence of biofilm-associated genes in canine otic isolates of *Pseudomonas aeruginosa***

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**Conflict of interest:** None declared.

**Running title:** *Pseudomonas* biofilm genetics

**4.1 Abstract:**

**Background:** *Pseudomonas aeruginosa* is an important canine pathogen and there is increasing evidence that biofilm formation by this bacterium is common and that biofilm forming ability may be an important virulence factor. Factors regulating biofilm production in canine *P. aeruginosa* have not been investigated, but a variety of genes associated with attachment (*sadB*), extrapolymeric substance formation (*pel*) and quorum sensing (*rhl, las*) may be important.

**Objective:** To determine the prevalence of selected biofilm-associated genes (*sadB, pelA, lasB* and *rhlA)* in both biofilm forming and non-biofilm forming canine otic isolates of *P. aeruginosa*.

**Methods:** DNA was extracted from eighty-three *Pseudomonas aeruginosa* isolates obtained from canine ears. Sequences for the genes to be evaluated were obtained from National Centre for Biotechnology Information GenBan database and primers selected. Polymerase chain reaction (PCR) was performed for all isolates with primers for the above genes.

**Results:** All isolates possessed *lasB* and *sadB,* 74 (90%) possessed *pelA* and 74 (90%) possessed *rhlA*. All isolates that were classified as biofilm producers contained all four genes. There was an association between the presence of *pelA* and *rhlA* and biofilm production. There was also an association between the presence of *rhlA* and *pelA* and biofilm optical density at 570nm (OD570) ($P<0.001$).

**Conclusions and discussion:** These putative biofilm-associated genes were common in *P. aeruginosa* from canine ears. This study highlights the potential role of specific genes in biofilm formation. Future investigation into how genes are expressed within biofilms, as well as study of other potential biofilm-associated genes, would be of value.

## 4.2 Introduction

*Pseudomonas* aeruginosa is an important cause of infection in dogs, particularly in cases of bacterial otitis externa.[1] Antimicrobial resistance is common with *P. aeruginosa*, complicating treatment of infections.[2] Further complicating *P. aeruginosa* infections is the process of biofilm formation.[3] A bacterial biofilm is a sessile community of bacteria that becomes irreversibly attached to a biological or non-biological surface. Once attachment occurs, bacteria produce an extrapolymeric substance matrix (EPS) composed of proteins, carbohydrates and DNA.[3] Bacterial biofilms have been studied extensively in the human literature as they can form on catheters, surgical implants and chronic wounds.[4-5] Biofilm production by *P. aeruginosa* has received particular attention in cystic fibrosis patients, where biofilms in the airways, along with antimicrobial resistance, can result in significant morbidity and mortality.[6] Biofilms have been studied in the veterinary literature, although studies regarding *Pseudomonas* biofilm formation are limited.[7-9] It was recently determined that *Pseudomonas aeruginosa* from canine otic isolates can form biofilms, and biofilm embedded bacteria have increased minimum inhibitory concentrations (MIC) for certain antimicrobials.[10] This is not surprising based on human data but indicates the potential role of biofilm production in the pathophysiology of *Pseudomonas* infections in animals.

In a study of canine otic *P. aeruginosa*, biofilm formation was not ubiquitous, with 40% of the isolates producing a quantifiable bacterial biofilm *in* vitro.[10] This suggests that there may be specific genes that are expressed that allow for biofilm production. *Pseudomonas aeruginosa* has a large (>6Mbp) genome that encodes over 5500 genes.[2] Previous studies have investigated certain potential biofilm-associated genes in human *Pseudomonas* isolates, but canine study is lacking.[11-13]

A variety of genes might be associated with biofilm formation. Surface attachment

defective (*sad*) mutants, mutants defective in one of the genes in the *sad* cluster, have been investigated in biofilm-forming and biofilm-deficient strains and it has been documented that expression of wild-type *sadB* is required for the transition from the reversible to the irreversible stage of biofilm formation.[14]

Production of the carbohydrate matrix is a key step in biofilm formation, and *pel* is thought to play a role in manufacturing glucose-rich components of the matrix.[15-16] Different carbon sources lead to a biofilm forming a different shape and *pel*-deficient mutants exhibit differences in colony morphology, appearing smoother that other colonies with no pellicle.[15,17] As the carbon source for the biofilm becomes incorporated into the extrapolymeric matrix, this may suggest that *pel*-deficient isolates may not produce functioning EPS leading to abnormal biofilm formation.

Two cell-to-cell signalling systems (quorum sensing systems) previously documented in *Pseudomonas aeruginosa* are the *lasR-lasI* and *rhlR-rhl* systems. Studies have documented that *Pseudomonas* strains defective in *Las* QS produce similar quantities of EPS as wild-type bacteria, but the mutants were unable to form 'normal' biofilms due to increased space between cells.[18-19] *Las* mutants have been documented to have a 60% reduction in adhered biofilm formation and the onset of *lasB* activity has also been shown to correlate with initial development of biofilm cell clusters.[19-20] Along with the *las* and *rhl* QS systems, another cell-to-cell signal has been identified in *Pseudomonas,* the *Pseudomonas* quinolone signal (PQS). In nature, an active *lasR* protein is required for PQS production. This indicates that a gene required for PQS synthesis is controlled through the *las* quorum sensing system.[21] *Pseudomonas aeruginosa* biofilms that grow under anaerobic conditions require *rhl* quorum sensing to survive.[22]

The objective of this study was to determine the prevalence of *sadB, pelA, lasB* and *rhlA in* canine otic isolates of *P. aeruginosa* and to evaluate the relationship between the presence of these genes and *in vitro* biofilm formation.

## 4.3 Materials and Methods

### Isolates

Eighty-two multi-drug resistant *P. aeruginosa* isolates recovered from the ears of dogs with otitis externa were studied. Each isolate was obtained from a different canine. Thirty-two of these isolates had previously been demonstrated to form biofilm *in vitro*. The other fifty had been documented as non-biofilm producing isolates.[10] Biofilm formation was documented using a microtitre plate method.[23] Each isolate was documented to produce a specific quantity of biofilm via measurement of the optical density of each well at 570nm ($OD_{570}$). Isolates were inoculated in triplicate along with a triplicate negative control. The triplicate negative control mean $OD_{570}$ was subtracted from the triplicate mean $OD_{570}$ of the study samples. Isolates were classified as biofilm producers if the net $OD_{570}$ was greater than 0.135. They were further classified as low biofilm producers if the net $OD_{570}$ was > 0.135 but ≤ 0.27, moderate biofilm producers if $OD_{570}$ was > 0.27 but ≤ 0.54 and high biofilm producers if $OD_{570}$ was > 0.54 in accordance with a previous study.[23]

### Sub-culture and DNA extraction

Pure 18-24 h growth of *P. aeruginosa* isolates was inoculated into 1ml of PCR-grade water. This mixture was vortexed and centrifuged at 10,000-12,000 rpm for 1-2 min. The supernatant was removed and 200 ul of InstaGene matrix® (Bio-Rad, Mississauga) was added. This mixture was then vortexed and incubated for 30 min in a $56^0$C water bath. After incubation the mixture was vortexed again at high speed for 10 s and then placed in a heat block for 8 min at $100^0$C. After further vortexing for 10 s and centrifugation at 10,000-12,000 rpm for another 2-3 min the DNA was then stored at $-20^0$C until use.

**Polymerase chain reaction and gel electrophoresis**

Previously extracted DNA was used as the DNA template for the PCR reaction. Stock solutions were made to 10pM/µl of both forward and reverse primers for the four genes in question. These primers are described in table 1. The multiplex PCR reaction contained 12.5 µl of KAPA (KAPABiosystems, Boston), 1.0 µl each of the forward and reverse primer stock solutions, 6.5µl of water and 2µl of the template DNA from each isolate. PCR conditions were as follows: $94^0$C for 30 sec; 30 cycles of $94^0$C for 30 sec, 30 cycles of $59^0$C for 30 sec and 30 cycles of $72^0$C for 1 min.; final extension at $72^0$C for 5 min. For gel electrophoresis, 1.5% agarose gel (Invitrogen, Burlington) with 10µl of Gel-red (Biotium, Hayward) added, was used. Samples were run at 150 volts and 80 microamps for 45 minutes. Gels were then visualized under ultraviolet illumination (Genegenius, Syngene, Frederick). Positive and negative controls were included for each run. Positive controls were developed from the existing isolate collection by taking positive samples from a pilot round of multiplex PCR testing, repeating the PCR reaction using only the single target primer set and confirmation of PCR specificity through sequencing of the PCR product and comparison with sequences from the National Centre for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). These isolates were then used as positive controls. Negative controls consisted of PCR-grade water in place of DNA template.

**Statistical Analysis**

Categorical comparisons were performed using chi-squared test. Continuous data were assessed using one-way analysis of variance (ANOVA). A *P* value of $<0.05$ was considered significant.

## 4.4 Results

All isolates possessed *lasB* and *sadB,* 74 (90%) possessed *pelA* and 74 (90%) possessed *rhlA*. Seventy (84%) isolates possessed all four genes, while nine (11%) possessed three (either *lasB, sadB, pelA* or *lasB, sadB* or *rhlA)* and four (5%) two (*lasB* and *sadB*). All thirty-two (100%) isolates that were classified as biofilm producers contained all four genes. There was an association between the presence of *pelA* and *rhlA* and biofilm production (table 2). There was also an association between the presence of *rhlA* and *pelA* and quantity of biofilm production, as determined by OD570 (both $P<0.001$). Analysis of *lasB* and *sadB* was not performed since these genes were present in all isolates.

## 4.5 Discussion

The four studied biofilm-associated genes were common in this group of *P. aeruginosa* otic isolates and all four genes were present in all isolates documented to produce biofilm. While this does not prove that these genes, either individually or in combination, are critical for biofilm formation, these data and the known functions of these genes suggest that they play at least some role in biofilm formation.[13-16,19] However, all four genes were present in 84% of the total isolates, including those not noted to produce biofilm *in vitro*. These results highlight that factors other than presence of these genes must play a role in determining whether biofilm is produced (at least *in vitro*). This could include variable expression of genes, the presence of non-functional genes and the presence of other genes that initiate or repress biofilm formation.

Both *sadB* and *lasB* were present in all isolates, complicating any assessment of their role in biofilm formation. Clearly, they are not the sole drivers of biofilm formation, since they were present in non-biofilm forming isolates. They may still be of importance (or required) for biofilm formation in combination with other factors. Previous studies have reported that bacteria with a mutation of the *sadB* gene were unable to form a biofilm and also had defects in swimming motility.[12,14] This indicates that the presence of *sadB* may not equal a functioning gene, as this gene was present in non-biofilm forming isolates in this study, or that *sadB* could be a critical initial step in biofilm formation but other steps involved are missing in the non-biofilm forming isolates in our study. This could also be the case for *lasB* as it was present in all isolates studied.

There was a positive association between the presence of both *pelA* and *rhlA* and biofilm production, indicating that biofilm formation was more likely and more abundant when those genes were present. Transcription of *pel* genes is known to be activated by the *las* QS system.[22] In all isolates in this study *lasB* was present: even isolates that were negative for the presence of *pelA*. This suggests that other genes must play a role in *pelA* activation and transcription. *Las*

mutants have been documented to have a 60% reduction in adhered biofilm formation indicating that *las* quorum sensing mutants are defective in biofilm formation.[22] The results of this study support that concept that a *lasB* gene may need to be present for biofilm production to occur. However, gene expression was not studied therefore no comment can be made on whether a fully functioning *lasB* gene is required for biofilm formation. A possible role for *pel* in the initial attachment process of bacteria has also been previously suggested, indicating that this gene must be present for biofilm formation to occur.[17-18] Results from this study would also support this due to the association between the presence of *pelA* and biofilm production. However, as with the other genes, these were found in non-biofilm producers and are not a sole indicator of biofilm formation.

There was also an association between the presence of *rhlA* and *pelA* and biofilm OD570 indicating that biofilm production was greater when these genes were present. An increase in thickness of a biofilm has previously been shown to occur after activation of both the *las* and *rhl* quorum sensing systems.[3] The results of this study also suggest this may be the case for *rhlA*.

As with any study, consideration must be given to the study population. The isolates in this study were obtained from the external ear canal of dogs with clinical signs of otitis externa. Results from this study highlight gene presence in *P. aeruginosa* isolates from canine ears and isolates from other regions of the body could be different. Some of these isolates were obtained after treatment and some prior to treatment. Treatment may have played a role in selecting for antimicrobial resistance, which, in turn, could have affected phenotypic variation within isolates. An *in vivo* study would be needed to determine treatment effect on gene presence or expression. Additionally, PCR-based studies such as this simply look for the presence of a gene, not expression of that gene. It is possible that positive PCR results could have been obtained for isolates that were unable to express the gene or that had alterations in the gene sequence that

prevented expression of the target protein. Another limitation of the study is that the microtitre plate assay is an *in vitro* test and therefore does not fully reflect *in vivo* biofilm formation. Biofilm formation is also undoubtedly multifactorial and complex, and this study only assessed four genes. Presumably, there are other genes that may play important roles that were not assessed.

Regardless, this study highlights the potential role of specific genes in biofilm formation. Future research should include investigation into other genes known to play a role in biofilm development. Investigation into how genes are expressed within biofilms would also be of value. If genes that allow biofilm to form can be identified, this could lead to the development of compounds or medications targeting these genes. Biofilm-embedded bacteria require higher MICs for certain antimicrobials, so developing tests that will determine whether an isolate can form a biofilm is also important for therapy. If gene expression within an isolate can be documented via a test, clinicians will have a better understanding of whether that isolate is capable of biofilm formation and they will be able to select the appropriate dose and antimicrobial for treatment.

**4.6 Tables**

| Gene of Interest | Forward Primer | Reverse Primer | Product Length (bp) |
|---|---|---|---|
| *sadB* | GAAAAAGGTGCTGGAAGCGG | GCCTTTCGGTAGTGCGTTTC | 106 |
| *pelA* | GCCATGTGTGCGCCTAAAAA | TGGGTCGAAGGATGTTACGG | 268 |
| *rhlA* | CAAGGACGACGAGGTGGAAA | TCTTCGCAGGTCAAGGGTTC | 673 |
| *lasB* | AGCCATCACCGAAGTCAAGG | TGCCGCGCATATAGAACTCG | 872 |

**Table 1: Forward and Reverse Primers and product length for four genes of interest used for PCR.**

| Gene | Biofilm producers (n=32) | Non biofilm producers (n=50) | *P* |
|---|---|---|---|
| *sadB* | 32 (100%) | 50 (100%) | ND |
| *rhlA* | 32 (100%) | 42 (84%) | 0.017 |
| *pelA* | 32 (100%) | 42 (84%) | 0.017 |
| *lasB* | 32 (100%) | 50 (100%) | ND |

ND: Not done

**Table 2: Prevalence of the presence of biofilm-associated genes in biofilm forming and non-biofilm forming canine otic isolates of *Pseudomonas aeruginosa***

## 4.7 References


1. Nuttall T, Cole AK. Evidence-based veterinary dermatology: a systematic review of interventions for treatment of *Pseudomonas* otitis in dogs. *Vet Derm* 2007; 18: 69-77.
2. Lambert PA. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J. R. Soc. Med.* 2002; 95: 22-26.
3. Clutterbuck AL, Woods EJ, Knottenbelt, *et al*. Biofilms and their relevance to veterinary medicine. *Vet. Microbiol.* 2007; 121: 1-17.
4. Kirketerp-Moller K, Jensen PO, Fazli M, et al. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *J Clin Microbiol* 2008; 46: 2717-2722.
5. Darouiche RO. Treatment of Infections Associated with Surgical Implants. *N Engl J Med* 2004; 350: 1422-1429.
6. Son MS, Matthews WJJ, Kang Y *et al. In Vivo Evidence of Pseudomonas aeruginosa* Nutrient acquisition and pathogenesis in the Lungs of Cystic Fibrosis Patients. *Inf. Immun.* 2007; 75: 5313-5324.
7. Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: A role in recurrent mastitis infections? *Vet. J.* 2006: 171; 398-407.
8. Byrd M, Pang B, Hong W, et al. Direct Evaluation of *Pseudomonas aeruginosa* Biofilm Mediators in a Chronic Infection Model. *Inf Imm* 2011; 79(8): 3087-3095.
9. Singh A, Walker M, Rousseau J *et al*. Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. *BMC Vet. Res.* 2013;9:93-98.
10. Pye CC. Yu AA, Weese JS. Evaluation of biofilm production by *Pseudomonas aeruginosa* from canine ears and the impact of biofilm on antimicrobial susceptibility *in vitro*. *Vet Derm* 2013; 24: 446-e99.

11. Head NE, Yu H. Cross-sectional Analysis of Clinical and environmental isolates of Pseudomonas aeruginosa: Biofilm formation, virulence and Genome Diversity. *Infect. Immun.* 2004:72; 133-144.
12. O'Toole GA, Kolter R. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Molec. Microbiol.* 1998: 30; 295-304.
13. Musken M, Di Fiore SD, Dotsch A. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. *Microbiol.* 2010: 156; 431-441.
14. Caiazza NC, O'Toole GA. SadB is required for the Transition from Reversible to Irreversible Attachment during Biofilm formation by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 2004; 186: 4476-4485.
15. Friedman L, Kolter R. Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the *Pseudomonas aeruginosa* Biofilm Matrix. *J. Bacteriol.* 2004; 186: 4457-4465.
16. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 2007; 10: 644-648.
17. Klausen M, Aaes-Jergensen A, Molin S, *et al*. Involvement of bacterial migration in the development of complex multicellular structures in *Pseudomonas aeruginosa* biofilms. *Molec. Microbiol.* 2003; 50: 61-68.
18. Davies JA, Harrison JJ, Marques LLR, *et al*. The GacS sensor kinase controls phenotypic reversion of small colony variants isolated from biofilms of *Pseudomonas aeruginosa* PA14. *FEMS Microbiol. Ecol.* 2007; 59: 32-46.
19. Sauer K, Camper AK, Ehrlich GD, *et al*. *Pseudomonas aeruginosa* Displays Multiple Phenotypes during Development as a Biofilm. *J. Bacteriol.* 2002; 184: 1140-1154.
20. Webb JS, Thompson LS, James S, *et al*. Cell death in Pseudomonas aeruginosa biofilm

development. *J. Bacteriol.* 2003; 185: 4585–4592.

21. Yoon SS, Hennigen RF, Hilliard GM, *et al*. *Pseudomonas aeruginosa* Anaerobic Respiration in Biofilms: Relationships to Cystic Fibrosis Pathogenesis. *Dev. Cell* 2002; 3: 593-603.
22. Sakuragi Y, Kolter R. Quorum-sensing Regulation of the Biofilm Matrix Genes (pel) of Pseudomonas aeruginosa. *J. Bacteriol.* 2007; 189: 5383-5386.
23. Stepanovic S, Vukovic D, Dakic I, *et al*. A modified microtitre-plate test for quantification of staphylococcal biofilm formation. *J. Microbiol. Methods*. 2000; 40: 175-179.

# CHAPTER 5: General Discussion and Conclusions

## 5.1 General Discussion and Conclusions

Biofilms are colonies of bacteria that are shielded from environmental stresses, the host immune response and antimicrobial therapy due to the presence of a carbohydrate based EPS matrix.[1] Biofilms have been identified in the human literature as being responsible for chronic infections including those in wounds, on surgical implants and in the lungs of patients with cystic fibrosis.[2-4] Research has showed that production of biofilm is likely an important virulence factor for certain bacterial species as biofilm-embedded bacteria are less susceptible to antimicrobial therapy than their planktonic counterparts.[5-6] In the veterinary literature there is less data regarding biofilm formation and antimicrobial resistance although biofilms have been documented for certain species and conditions.[7-9] Information regarding biofilm formation for *Pseudomonas aeruginosa* is limited and there are currently no reports documenting biofilm forming ability or antimicrobial susceptibility for *P. aeruginosa* isolates obtained from the external ear canal of canines.

The first study illustrates that biofilm production by canine otic isolates of *P. aeruginosa* is possible, although there is variability in the degree of *in vitro* biofilm production. The ability of an isolate to produce biofilm could be an important virulence factor for *P.aeruginosa,* assisting in establishing infections and evading the system and antimicrobials. While many *Pseudomonas* isolates produced biofilm *in vitro*, sixty per cent produced no quantifiable biofilm. Time, *in vivo* factors and genetics could play a role in biofilm formation but these were not evaluated in this study. A more thorough knowledge of biofilm formation is required to understand the pathology of biofilm-associated infections to allow treatment protocols to be designed.

Previous studies have documented that minimum inhibitory concentrations (MICs) for antimicrobials are higher for biofilm-embedded bacteria, as was the case in this study.[10]

Therefore, the ability to form biofilm may be an important virulence factor for some isolates of *P. aeruginosa* and play an important role in the pathogenesis of disease and establishment of chronic, recurrent and refractory infections. This also indicates limitations in interpretation of typical culture and susceptibility results when biofilms may be involved, as testing performed with planktonic bacteria may not accurately represent the likelihood of response to treatment. Previously, certain antimicrobials have been shown to actually induce biofilm formation or induce production of biofilm matrix components.[11-12] This fact was not exclusively studied with this research but may be playing a role in the higher minimum inhibitory concentrations in biofilm-embedded bacteria. Topical therapy is most commonly used to treat canine otitis externa. Populations of *Pseudomonas aeruginosa* that are resistant to a specific antimicrobial i*n vitro* have been shown to be destroyed by this same antimicrobial when used topically. This is due to the much higher concentrations of antimicrobials that can be reached during topical application.[13-14] Further study is needed to determine the role of biofilm in otitis externa and the choice to use topical or systemic therapy.

The second study documents that Triz-EDTA® use can impact the susceptibility of biofilm-embedded *P. aeruginosa* to certain antimicrobials. The clinical impact of this finding was not investigated but the ability to reduce bacterial MICs suggests that Triz-EDTA® could be a useful adjunctive treatment. The mechanism of this effect is unclear but may relate to creation of pores in bacterial membranes which aid in antimicrobial penetration.[15-16] However, a significant effect was only shown for neomycin and gentamicin, and the reason for a lack of impact on enrofloxacin and polymixin B is also unclear. Both aminoglycoside and fluoroquinolones (enrofloxacin) bind to structures within the bacterial cell, therefore it is unlikely that just drug penetration accounted for the difference of the MIC or enrofloxacin's MIC should have also been lowered.[17-18] It could be that this difference is related to statistical power. However, a remarkable

finding was the increase in biofilm MIC with added Triz-EDTA® in 64.5% of isolates with enrofloxacin. Some of these isolates showed a four-fold or higher difference suggesting that this increase may be due to the presence of the Triz-EDTA®, through some unknown but potentially clinically important mechanism.

Results from this study are in disagreement with data previously documented in the literature. Earlier studies show that the addition of Triz-EDTA® for otitis externa that was previously unresponsive to enrofloxacin and found to be resistant to enrofloxacin on bacterial culture, lead to resolution of the otitis.[19] The discrepancy with this study was that the bacteria were never defined as planktonic or biofilm-embedded, whereas the current study was looking at purely biofilm-embedded bacteria. These results may highlight differences pertaining to the study populations.

Minimum inhibitory concentration, not minimum bactericidal concentration was measured in this study. Previous studies have documented a decrease in the minimum bactericidal concentration for specific antimicrobials for *Pseudomonas* with the addition of Triz-EDTA®.[20] How relevant this difference is, is unknown, although this may mean that a small number of bacteria within the biofilm are not completely eradicated after antimicrobial therapy. Whether this could lead to establishment of a new infection or biofilm is unclear and would need further study. Future research could also include the use of other compounds or medications to decrease MICs for antimicrobials against *Pseudomonas* or the use of other topicals that could act synergistically with Triz-EDTA®.

One concern with both these studies is whether this method of *in vitro* susceptibility testing can approximate an *in vivo* biofilm. Several assays are available to determine the antimicrobial minimum inhibitory concentration for bacterial biofilms. There is currently no consensus within the literature as to which test is the most accurate.[21-22] The microtitre plate

assay is widely used but obviously does not reflect the complex microenvironment present within the external ear in cases of otitis externa. Biofilm formation has been documented within the middle ear cavity of chinchillas *in vivo*.[23] This supports the idea that biofilms could potentially form in the external ear canal. However, conditions within the external ear canal during inflammation, including excessive cerumen production, inflammation and host response, will change the microenvironment and potentially change the production of biofilm and its components.[24] In order to determine whether these factors play a role, an *in vivo* study would need to be undertaken.

Results of the third study show that all isolates documented to produce biofilm possessed all four genes being studied. We know that all have been previously documented to contribute to biofilm formation.[25-30] All isolates, those forming biofilms and those non-biofilm forming isolates, possessed *lasB* and *sadB,* 74 (90%) possessed *pelA* and 74 (90%) possessed *rhlA*. The fact that some of the non-biofilm forming isolates still had all four genes present suggests that other factors play a role in biofilm formation independent of the presence of these genes. These results may also suggest that a gene may be responsible for the initial biofilm development, but other factors are required for biofilm production. Future studies looking at expression of these genes would be required to substantiate their role in biofilm production.

There was a positive association between the presence of *pelA* and *rhlA* and biofilm production. *Las* mutants have been documented to have a 60% reduction in adhered biofilm formation indicating that *las* quorum sensing mutants are defective in biofilm formation.[26] The results of this study support the concept that a *lasB* gene must be present for biofilm production to occur. However, gene expression was not studied therefore no comment can be made on whether a fully functioning *lasB* gene is required for biofilm formation. A role for *pel* in the initial attachment of bacteria to a surface has also been suggested.[28,30] Results from this study

would also support this due to the association between the presence of *pelA* and biofilm production.

There was also an association between the presence of *rhlA* and *pelA* and biofilm OD570 indicating that biofilm production was greater when these genes were present. An increase in thickness of a biofilm has previously been shown to occur after activation of both the *las* and *rhlA* quorum sensing systems.[31] Results of the current study support that biofilm thickness may be increased when *lasB* is present.

In summary, we can conclude the following:

1. Biofilm production by otic isolates of *Pseudomonas aeruginosa* is common with variability in the amount of biofilm produced.
2. The minimum inhibitory concentration of biofilm-embedded bacteria is higher for enrofloxacin, gentamicin, neomycin and polymyxin B when compared to their planktonic counterparts *in vitro*.
3. The addition of Triz-EDTA® will lower the minimum inhibitory concentration of biofilm-embedded bacteria for gentamicin and neomycin, *in vitro*.
4. Genetics likely play a role in biofilm development and production and *sadB, pelA, lasB* and *rhlA* maybe some of the important gene contributing to biofilm formation.

These results are highly relevant due to emergence of bacterial biofilms in veterinary medicine and the inherent difficulties in treating such infections.[1,20] Documenting that biofilm formation is common among *Pseudomonas aeruginosa* otic isolates and that biofilm formation increases the MICs of certain antimicrobials, may impact treatment choices in chronic cases of otitis externa where biofilm formation is suspected. This data may also fuel future research into compounds

that can disrupt biofilms to enable lower MICs to be targeted with therapy and bypass the inherent defense mechanisms of biofilms. By evaluating and identifying genes that are present in biofilm forming isolates we can then further research gene expression in biofilms. This research may also enable therapies to be manufactured that target gene expression to prevent biofilm formation. *In vivo* studies are needed to determine the full extent of biofilm formation and appropriate treatment, however, understanding the basis of biofilm formation and susceptibility to antimicrobials is key to determine ways to eradicate bacterial biofilms.

## 5.2 References


1. Donlan RM. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clin Infect Dis* 2001; 33: 1387-1392.
2. Kirketerp-Moller K, Jensen PO, Fazli M, et al. Distribution, Organization, and Ecology of Bacteria in Chronic Wounds. *J Clin Microbiol* 2008; 46: 2717-2722.
3. Darouiche RO. Treatment of Infections Associated with Surgical Implants. *N Engl J Med* 2004; 350: 1422-1429.
4. Son MS, Matthews WJJ, Kang Y *et al. In Vivo Evidence of Pseudomonas aeruginosa* Nutrient acquisition and pathogenesis in the Lungs of Cystic Fibrosis Patients. *Inf. Immun.* 2007; 75: 5313-5324.
5. Hoyle BD, Wong CKW, Costerton JW. Disparate efficacy of tobramycin on Ca+2-, Mg+2-, and HEPES-treated *Pseudomonas aeruginosa* biofilms. *Can J Microbiol* 1992; 38: 1214-1218.
6. Allesen-Holm M, Barken KB, Yang L, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. *Molec Micro* 2006; 59: 1114-1128.
7. Melchior MB, Vaarkamp H, Fink-Gremmels J. Biofilms: A role in recurrent mastitis infections? *Vet. J.* 2006: 171; 398-407.
8. Byrd M, Pang B, Hong W, et al. Direct Evaluation of *Pseudomonas aeruginosa* Biofilm Mediators in a Chronic Infection Model. *Inf Imm* 2011; 79(8): 3087-3095.
9. Singh A, Walker M, Rousseau J *et al*. Characterization of the biofilm forming ability of *Staphylococcus pseudintermedius* from dogs. *BMC Vet. Res.* 2013;9:93-98.
10. Drenkard E. Antimicrobial resistance of *Pseudomonas aeruginosa* biofilms. *Microbes Inf* 2003; 5: 1213-1219.

11. Hoffman LR, D'Argenio DA, MacCoss MJ, et al. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 2005; 436: 1171-1175.

12. Bagge N, Schuster M, Hentzer M, *et al*. Pseudomonas aeruginosa Biofilms Exposed to Imipenem Exhibit Changes in Global Gene Expression and ß-Lactamase and Alginate Production. *Antimicrob. Agents Chemother.* 2004: 48; 1175-1187.

13. Morris DO. Medical therapy of otitis externa and otitis media. *Vet Clin North Small Anim Pract*. 2004; 34: 541-555.

14. Robson D, Burton G, Bassett R. Correlation between topical antibiotic selection, in vitro bacterial antibiotic sensitivity and clinical response in 16 cases of canine otitis externa complicated by *Pseudomonas aeruginosa*. In: *Proceedings of the North American Veterinary Dermatology Forum*, Portland, Oregon, 2010.

15. Wooley RE, Jones MS. Action of EDTA-Tris and antimicrobial agent combinations on selected pathogenic bacteria. *Vet Microbiol* 1983: 8; 271-280.

16. Guardabassi L, Houser GA, Frank LA *et al*. Guidelines for antimicrobial use in dogs and cats. In: Guardabassi L, Jensen LB, Kruse H. *Guide to antimicrobial use in animals*. Iowa:Blackwell Publishing Ltd. 2008;183-206.

17. Howland RD, Mycek MJ. Protein synthesis inhibitors. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 367-380.

18. Howland RD, Mycek MJ. Quinolones, Folic Acid Antagonists, and Urinary Tract Antiseptics. In: Howland RD, Mycek MJ. *Pharmacology.* 3rd ed. Philadelphia: Lippincott Williams and Wilkins. 2006; 381-386.

19. Farca AM, Piromallo G, Maffei F, *et al.* Potentiating effect of EDTA-Tris on the activity of antibiotics against resistant bacteria associated with otitis, dermatitis and cystitis. *J Sm An Prac* 1997: 38; 243-245.

20. Buckley LM, McEwan NA, Graham P, et al. TrisEDTA significantly potentiates the bactericidal activity of silver sulfadiazine against multi-drug resistant *Pseudomonas aeruginosa*. In 7th World Congress of Veterinary Dermatology 2012; 23: 30.

21. Curtin J, Cormican M. Measuring antimicrobial activity against biofilm bacteria. *Rev. Environ. Sci. Biotech.* 2003: 2; 285-291.

22. Ceri H, Olson ME, Stremick C et al. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* 1999; 37: 1771–1776.

23. Byrd M, Pang B, Hong W, et al. Direct Evaluation of *Pseudomonas aeruginosa* Biofilm Mediators in a Chronic Infection Model. *Inf Imm* 2011; 79(8): 3087-3095.

24. Miller WH, Griffin CE, Campbell KL. Disease of Eyelids, Claws, Anal Sacs, and Ears. In: Miller WH, Griffin CE, Campbell KL. *Muller and Kirk's Small Animal Dermatology 3rd Ed*. St.Louis: Elsevier Mosby. 2013; 741-767.

25. Pesci EC, Milbank JBJ, Pearson HP, *et al*. Quinolone signalling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* 1999: 96; 11229-11234.

26. Sakuragi Y, Kolter R. Quorum-sensing Regulation of the Biofilm Matrix Genes (pel) of Pseudomonas aeruginosa. *J. Bacteriol.* 2007; 189: 5383-5386.

27. Caiazza NC, O'Toole GA. SadB is required for the Transition from Reversible to Irreversible Attachment during Biofilm formation by *Pseudomonas aeruginosa* PA14. *J. Bacteriol.* 2004; 186: 4476-4485.

28. Friedman L, Kolter R. Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the *Pseudomonas aeruginosa* Biofilm Matrix. *J. Bacteriol.* 2004; 186: 4457-4465.

29. Musken M, Di Fiore SD, Dotsch A. Genetic determinants of *Pseudomonas aeruginosa* biofilm establishment. *Microbiol.* 2010: 156; 431-441.

30. Ryder C, Byrd M, Wozniak DJ. Role of polysaccharides in *Pseudomonas aeruginosa* biofilm development. *Curr. Opin. Microbiol.* 2007; 10: 644-648.

31. Clutterbuck AL, Woods EJ, Knottenbelt, *et al*. Biofilms and their relevance to veterinary medicine. *Vet. Microbiol.* 2007; 121: 1-17.

**Appendix 1: Diagram of External ear canal**

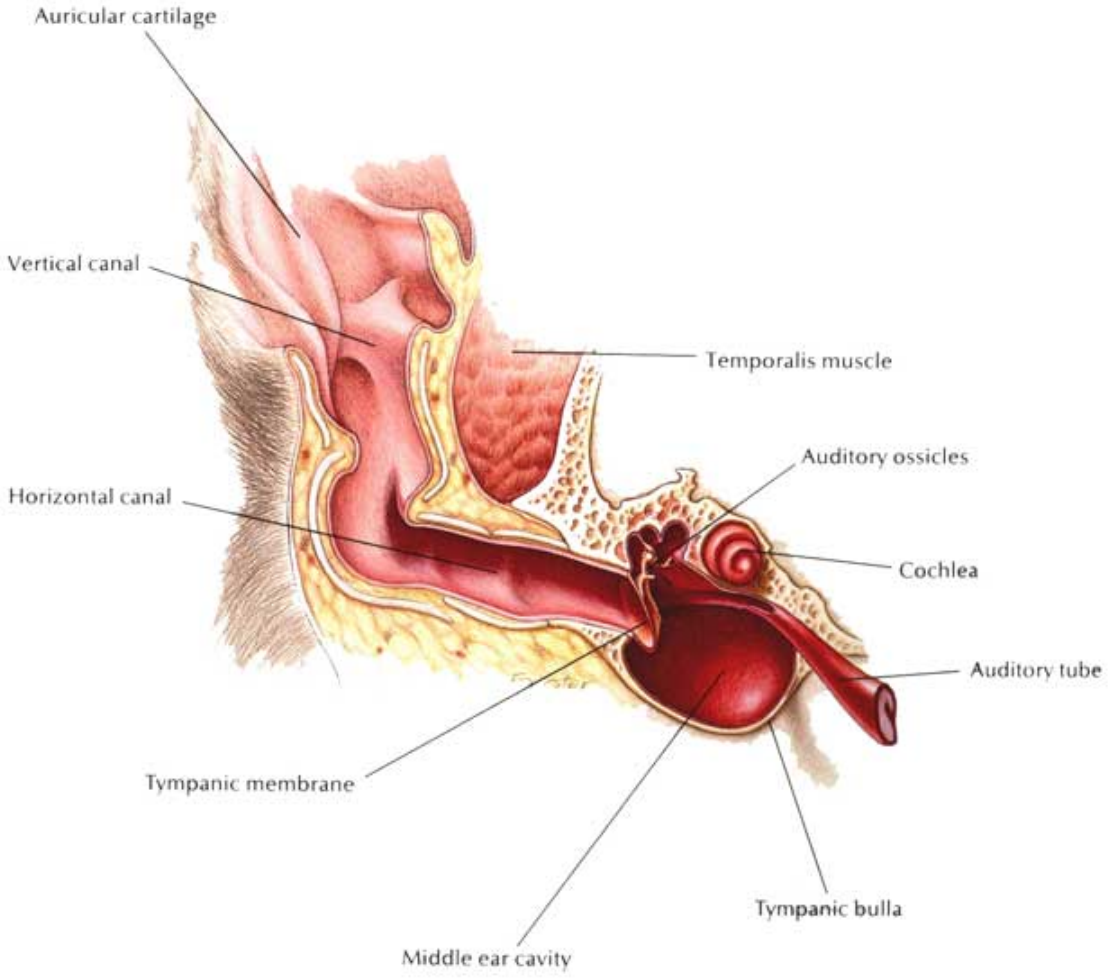


Image courtesy of: Adelaide Animal Hospitals. Obtained from: http://adelaidevet.com.au/pet-library/head-shaking-an-ear-of-an-issue

**Appendix 2: Biofilm Formation**

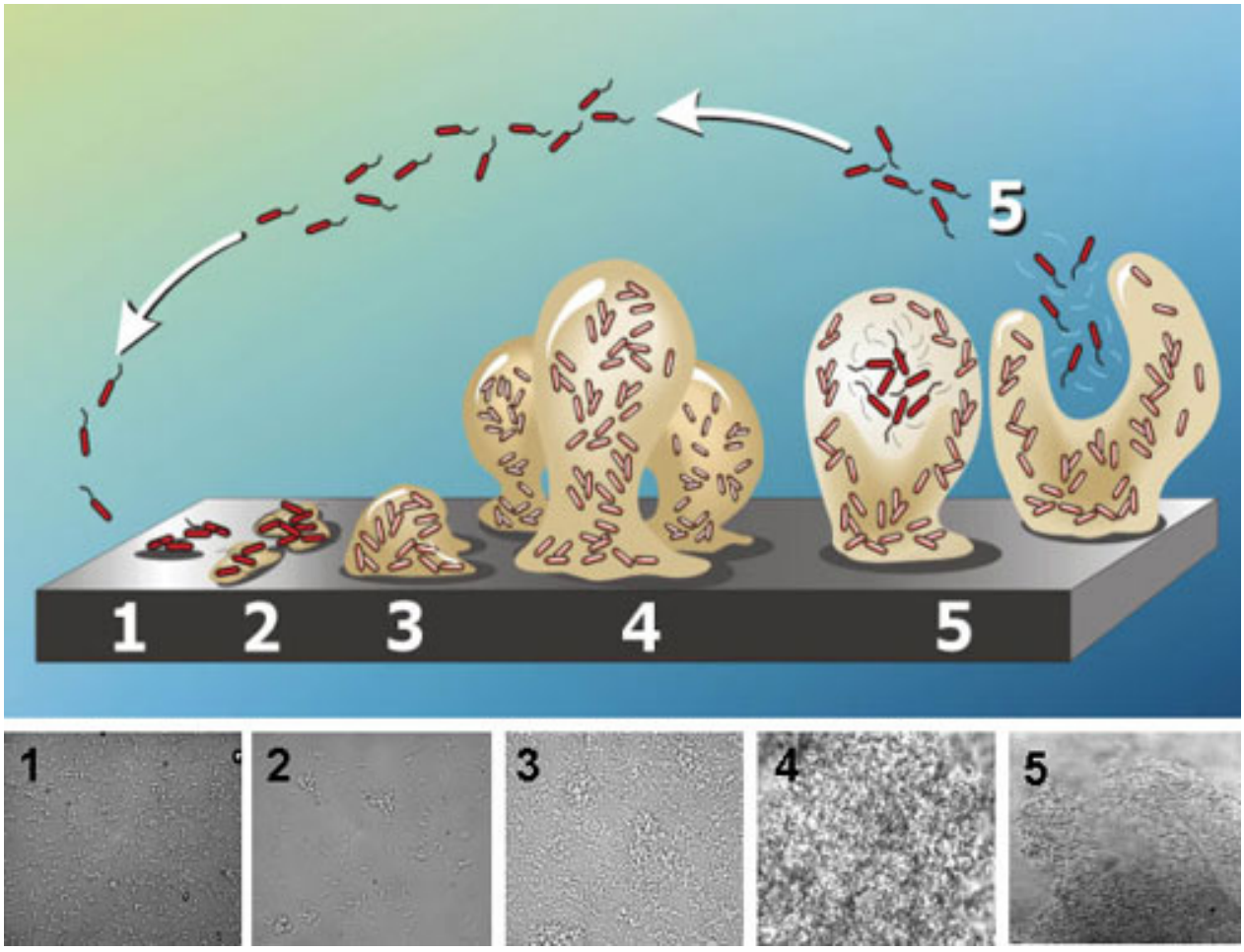


Image courtesy of D. Davis. Obtained from: "Looking for Chinks in the Armor of Bacterial Biofilms" PLoS Biol, Vol 5, issue 11.

**Appendix 3: Examples of PCR**