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Bacterial Biofilm In Veterinary Medicine: Mechanisms And Its Clinical Implications

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Abstract: Bacteria can exist in natural ecosystem in two forms: (1) free-floating 'planktonic organisms' and (2) in biofilms which is recently recognized and the predominant form of microbial growth attached to surfaces. Biofilms are clinically important in both human and veterinary medicine, having the ability to form on both medical devices and living tissue. Biofilm formation is a multistage process that starts with microbial adhesion with a subsequent production of extracellular matrix, involving proliferation, maturation and latter detachment of biofilm. Biofilm associated infections are generally persistent and respond poorly to commonly used antibiotics, disinfectants. Moreover, such types of infections also have the potential to evade the defense mechanisms of the host immune system. Biofilm formation by pathogenic bacteria has deleterious, sometimes fatal consequences, and leads to severe contamination problems in medicine, dentistry, food processing, water treatment and other areas that directly affect human health and life. The inability to treat many bacterial infections like chronic non-healing wounds and mastitis is related to the capacity of bacteria to form a biofilm. Although less research exists about biofilms in animals, they are believed to be involved in many diseases, such as pneumonia, liver abscesses, enteritis, wound infections and mastitis. A greater understanding of bacterial biofilm is required for the development of novel, effective control strategies thus resulting improvement in patient management. Therefore, this review attempts to compile scientific information regarding the mechanisms of the formation of bacterial biofilm with regard to the clinical importance in veterinary medicine and also public health. Emphasis will be given to the areas of diagnosis, prevention and treatment.

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1. Introduction

Bacteria can be found in natural ecosystem in two forms: (1) planktonic cells characterized by better freedom for migration, more prone to mutation, sensitivity to environment, more active metabolically, sensitive to antimicrobial agents and (2) biofilm form characterized by better protection, less subjects to mutation, high resistance to antibiotics and disinfectants and also less active metabolically (Clutterbuck *et al.,* 2007). By definition, a biofilm is a community of microorganisms adhering to a biotic or abiotic surface and surrounded by a complex matrix of extra-polymeric substances which can adapt to sudden shifts in nutrient availability as well as the host immune defenses. Such characteristics are achieved through systematized gene expression with an ability to grow as part of a sessile (Jefferson, 2004). Nearly 80% of bacteria in natural environment can form biofilm. For example, recent studies indicated biofilms can grow in the most extreme environments and found on various surfaces, including, natural aquatic or potable water system, living tissues, medical devices that causes health problems for the patients with indwelling medical devices via attachment of cells to the surface matrix (Rampelotto, 2013). Four potential incentives behind the formation of biofilms by bacteria during infection are considered: defense, colonization, utilization of cooperative benefits and biofilms normally grow as biofilms and planktonic cultures are an in vitro artifact (Jefferson, 2004). Bacterial biofilm has increased antibiotic resistance and involved in many persistent diseases (Singh *et al.,* 2017).

The problem associated bacterial biofilms are common in many branches of industry, human and veterinary medicine. In the food industry, bacterial biofilms are considered a main problem especially in dairy, fresh products, poultry and meat processing plants. Outbreaks of foodborne disease caused by various species of *Listeria*, *Salmonella*, and *Staphylococcus* have been linked to biofilm production. Biofilms formed by pathogens in food processing plants are a major culprit in the spread of foodborne diseases, which claim thousands of lives and amount to losses of about $78 billion/year in the

United States alone, for example (Scharff, 2012). Biofilms are recognized as being of major medical importance, as they have the ability to form on medical devices and also on living tissue (Costerton *et al.,* 1999). Biofilms are thought to be responsible for 65% of all human bacterial infections (Rowson and Townsend, 2016). They have been found to be involved in a wide variety of microbial infections in humans, including urinary tract infections, endocarditis, periodontitis, pneumonia in cystic fibrosis, chronic bacterial prostatitis and otitis media (Bjarnsholt, 2013; Donlan and Costerton, 2002; Hall-Stoodley *et al.,* 2006; Singh *et al.,* 2002).

The same is true in veterinary medicine in which biofilms are recognized as being clinically important (Gardner *et al.,* 2011). Biofilms affect both livestock and companion animals and have been implicated in animal diseases as diverse as bovine mastitis (Aslantaş and Demir, 2016; Gomes *et al.,* 2016; Melchior *et al.,* 2006a, 2006b), equine wounds (Cochrane *et al.,* 2009; Westgate *et al.,* 2011, 2010); and in canine and feline urinary tract disease (Shimizu and Harada, 2017), gastrointestinal disease (Reis *et al.,* 2014; Silva *et al.,* 2014) periodontal disease (Holcombe *et al.,* 2014; Oliveira *et al.,* 2016), otitis (Moreira *et al.,* 2012; Pye *et al.,* 2013), dermatitis (Bumroongthai *et al.,* 2016; Proietti *et al.,* 2015) and wounds (Bayne, 2014; Swanson *et al.,* 2014) and implant infections (Gallagher and Mertens, 2012; Nicoll *et al.,* 2014; Savicky *et al.,* 2013; Thompson *et al.,* 2011).

Although microorganisms predominantly exist as multi-cellular communities within biofilms in most environments, scientists are still exploring these complex systems in order to understand the complexity of the interactions within the biofilms, the processes involved in their formation and marked resistance to antimicrobial agent is of crucial importance for their control. Significant advances have been made to reveal new insights into biofilms and their constituents (Bridier *et al.,* 2011). The aim of this paper is to review the scientific information regarding the mechanisms of the formation of bacterial biofilm with regard to the clinical importance in veterinary medicine and also public health. Emphasis will be given to the areas of diagnosis, prevention and treatment.

2. Literature Review

2.1. Biofilm Structure and Its Formation

Biofilm formation is a dynamic process that takes place through a series of different phases. The initial phase is attachment of the microorganisms to a surface. After attachment, cell division starts and the microorganisms start to form micro-colonies. Bacterial cells then produce a protective, slimy extracellular polymeric substance (EPS) that provides a structural scaffold for the biofilm and binds it to the underlying surface (Donlan and Costerton, 2002). As the biofilm matures, multiple layers of cells build up and are incorporated into the matrix. The final structure is a complex three dimensional construction, in which the matrix is interspersed with open water channels that provide nutrients and oxygen to developing micro-colonies that remain protected against antibiotics, toxic chemicals and where necessary the body's immune system, within their EPS (Donlan, 2002). The final phase of biofilm development is when bacterial cells detach and disperse to colonize new sites. Microbes form a biofilm in response to various different factors which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional scarcity, or exposure of planktonic cells to sub-inhibitory concentrations of antibiotics or disinfectants (Lebeaux *et al.,* 2014).

EPS produced by microorganisms are a complex mixture of biopolymers primarily consisting of polysaccharides, as well as proteins, nucleic acids, lipids and humic substances. EPS make up the intercellular space of microbial aggregates and form the structure and architecture of the biofilm matrix. The key functions of EPS comprise the mediation of the initial attachment of cells to different substrata and protection against environmental stress and dehydration (Vu *et al.,* 2009). Attachment itself is governed by specific protein-protein interactions of bacterial surface with human matrix proteins. After attachment to tissue or matrix-covered devices is accomplished, infectious bacterial biofilms grow by proliferation and production of an extracellular matrix. The function of the matrix is to provide adhesion between bacterial cells, thereby enabling the formation of a multilayered biofilm (Joo and Otto, 2013; Vuong *et al.,* 2004).

Biofilm matrix involve proliferation, embedding in an extracellular matrix, and maturation. The latter depends on cell-cell disruptive factors, recently identified to be primarily surfactants. Strong production of surfactants, which are controlled by quoreum sensing (QS), leads to biofilm detachment (dispersal) (Joo and Otto, 2013). Therefore, detachment is not only just important for promoting genetic diversity but also for escaping unfavorable habitats aiding in the development of new niches. Once these planktonic cells are shed from the biofilm during dispersion they can go on to colonize new areas and the process of forming a classic biofilm structure begins again (Clutterbuck *et al.,* 2007).

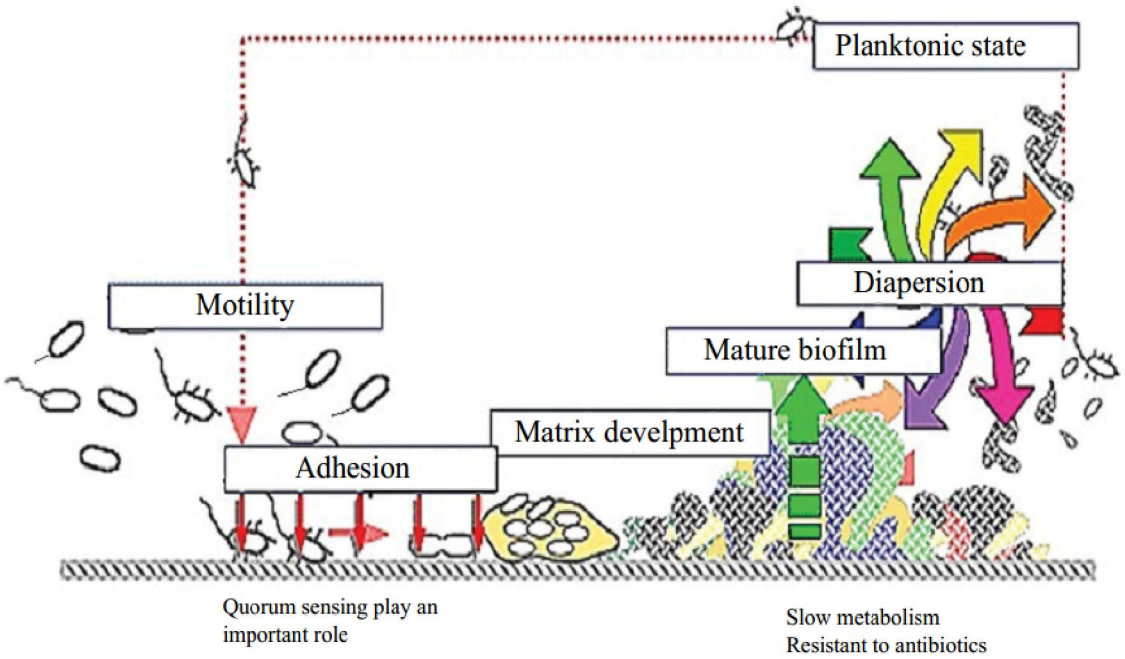

Figure 1: Steps in biofilm formation, The step of biofilm formation include; adhesion, matrix development, mature biofilm and dispersion. **Source:** (Shakibaie, 2018)**.**

2.2. Molecular Regulation of Biofilm Formation

In general, biofilm formation is a two-stage process that begins with the adherence of bacteria to a substrate surface (Adhesion Stage), and continues by proliferation and differentiation of the attached cells (Maturation Stage). From a molecular biology point of view, these two stages are mainly controlled by surface adhesins and quoreum sensing respectively (Chen and Wen, 2011).

2.2.1. Adhesins are key regulators of the adhesion stage

Adhesins are substances which confer virulence in bacteria by allowing them to adhere to epithelial surfaces. Pathogens colonize different sites in the host body because they express multiple adhesins. These are usually proteins that recognize specific receptors expressed at various sites of the host. Bacteria also produce different types of polysaccharides that are specifically designed to form the structural components of the biofilm. The expression of adhesins seems to be regulated by a variety of inputs. For example, for *S. epidermidis*, initial adhesion to the naked or coated polymer surface is mediated by polysaccharide adhesion (PS/A) (Tojo *et al.,* 1988; Zhang *et al.,* 2003). The expression of PS/A is controlled by the intercellular adhesion operon (Ica). In *S. mutans*, the adhesin is critical for *S. mutans* adhesion to tooth surfaces and the process is further enhanced by sucrose or pre-existing biofilms. Although *S. mutans* is normally known as an oral bacterium, and the etiological agent for dental caries (Nobbs *et al.,* 2009).

2.2.2. Quorum sensing systems control biofilm maturation stage

During biofilm formation many species of bacteria are able to communicate with one another through a mechanism called quoreum sensing. QS is a cell-density-dependent chemical signaling system that allows individual cells to release small signal molecules to the surroundings to make their presence known. Using a QS system, individual cells can produce and release small QS signaling molecules called auto-inducers. Auto-inducers is used to stimulate bacteria within the biofilm to modify gene expression to regulate such factors as surface attachment, maturation of the biofilm and dispersal of cells from the biofilm. A breakdown of the cell-to-cell communication may keep cells in the planktonic state (Naves *et al.,* 2010).

2.3. Biofilm and Antibiotic Resistance

Biofilm are 1000-1500 times more resistant to antibiotics than planktonic state. Treatment of infections with biofilm forming bacteria is extremely difficult, requires higher doses or combination of antibiotics, and removal of foreign bodies when implicated in device related infections (Modarresi *et al.,* 2015). In biofilms characterized by poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multilayered defense (Lebeaux *et al.,* 2014).

Firstly, the protective EPS matrix is thought to limit antibiotic penetration. It has been suggested that if the antimicrobial agent is deactivated in the outer layer of the biofilm faster that it can diffuse into it then a penetration barrier is created. EPS also can act as an ion exchanger and is able to sequester hydrophobic and positively charged antibiotics such as aminoglycosides. This limited penetration is thought to account for resistance to a single application of antibiotics but not during long-term chemotherapy. It is important to note that, the EPS that cover biofilm bacteria have been found to be less immunogenic, thus hiding the proteins and lipopolysaccharides on bacteria surfaces (Shakibaie, 2018).

Due to slow growth rate there is limited availability of oxygen and nutrients inside biofilms, so biofilm cells, especially those in the deep layers have a slow metabolic rate, as well as growth rate and division rate. These features make biofilm bacteria insensitive to antibiotic that target dividing cells (Pena *et al.,* 2011; Sutherland, 2001). The changes in the bacterial growth cycle influenced the level of enzyme synthesis in proportional to cell mass. In stationary phase or slow growing bacteria cellular enzyme synthesis is arrested. Biocides kill the metabolically active bacteria, whereas at the dormant growth phase, bacteria are less susceptible to the antimicrobial agents and protect them from the antimicrobial action. The metabolic activities are controlled by oxygen availability within biofilms. Bacterial biofilm also increases the level of resistance against antibiotics through expressing specific genes under the anaerobic conditions (Gilbert *et al.,* 2002).

Bacterial biofilm contains resistant persister cells that exhibit multidrug and bactericidal agent tolerance and are responsible for the severe chronic infectious disease (Lewis, 2005). Persisters cells formation controlled by the growth stages of bacterial communities, which are rapidly propagated and survive in the presence of lethal doses of antimicrobial agents (Brooun *et al.,* 2000). Stationary phase bacteria produced a high level of persister cells and correlated with the increasing resistance inside biofilm (Del Pozo and Patel, 2007). Persister variants are thought to survive by having a defective programmed cell death (PCD). This theory states that antimicrobials indirectly kill cells by causing cell damage and thereby triggering PCD. Therefore, persister cells with disabled PCD can ensure the survival of a population even if every cell has been exposed to antimicrobial activity (Lewis, 2001).

Efflux pumps are protein structures, either express constitutively or intermittently which facilitates bacterial survival under extreme conditions. It has substrate specificity and found inside the bacteria in the periplasmic area which is involved in antagonized accumulation of antibiotics. They show resistant to multiple antibiotics. Efflux pumps are also expressed in planktonic cells, but some efflux pump genes are up regulated in biofilm, indicating that they contribute to antibiotic resistance (De Kievit *et al.,* 2001; O'Toole *et al.,* 2000).

Nutrient limitation is experienced by some of the cells within a biofilm causing them to exist in a state of slow growth or even no growth. A decrease in growth has been shown to be synonymous with an increase in resistance to antibiotics. Oxygen limitation is also thought to contribute to the antibiotic resistance of biofilms since the efficacy of some antibiotics is reduced in its absence (Zhang and Mah, 2008).

Phenotypic or phase variation is another mechanism that is proposed to contribute to antibiotic resistance. It is a control mechanism that adapts a bacterium to many environments. The variations are commonly reversible and occur randomly at high frequencies resulting in a phenotypically heterogeneous population. This mixed population increases the probability of the survival of some bacterial cells in the event of sudden environmental change. The transition between the planktonic and biofilm mode of growth was regulated by phase variation (Déziel *et al.,* 2001). Therefore, phase variation would ensure the presence of bacterial cells which were able to initiate biofilm formation once conditions became favorable. Altered gene expression by organisms within the biofilm can result in a phenotype with reduced susceptibility to an antimicrobial agent. It is thought that a biofilm-specific phenotype is induced in a subpopulation of the community that results in the expression of active mechanisms to combat the detrimental effects of antimicrobial agents (Cochran *et al.,* 2000).

Antibiotics resistance in biofilm also occurred due to the presence of neutralizing enzymes which degrade or inactivate antibiotics. These enzymes are proteins which confer resistance by mechanisms such as hydrolysis, modification of antimicrobials by different biochemical reactions. Accumulations of these enzymes occur in the glycocalyx from the biofilm surface by the action of antibiotics. Neutralization by enzymes is enhanced by slow penetration of antibiotics and also antibiotics degradation in the biofilm. In cystic fibrosis which is caused by *P. aeruginosa*, overproduction of cephalosporinase enzymes is responsible for resistance to different antibiotics. This enzyme confers resistance to β-lactam in the presence of even much more concentration of carbapenems (Rojas and Del Valle, 2009).

Lastly, QS is thought to play a role in the antibiotic resistance of biofilms. This method of bacterial cell communication is known to control the

expression of extracellular virulence factors but its role in biofilm resistance is unknown. Resistance is a problem because it means diseases are difficult to cure causing increased health costs and serious welfare implications (Drenkard, 2003). Multiple mechanisms are thought to contribute to the antibiotic resistance of biofilms (Table 1).

Table 1: Examples of antibiotic resistance bacteria in veterinary pathogens.

Bacterial organism	Animal species	Disease/infection	Antibiotic resistance	Reference
Acinetobacter baumannii	Horse	Jugular catheter infection	Amoxicillin/clavulanic acid	Vaneechoutte *et al.* (2000)
Actinobacillu sspp.	Horse	Post-operative wound infection	Penicillin	Smith and Ross (2002)
Klebsiella sspp	Horse	Musculoskeletal infection	Ampicillin, amoxicillin/ clavulanic acid	Moore *et al.* (1992)
Pseudomonas aeruginosa	Dog	Otitis	Amoxicillin/clavulanic acid	Hariharan *et al.* (1995)
Staphylococcus aureus	Cow	Mastitis	Amoxicillin, ampicillin, lincomycin, penicillin	San Martín *et al.* (2002)
Staphylococcus epidermis	Horse	Post-operative wound infection	Methicillin	Trostle *et al.* (2001)
Staphylococcus intermedius	Dog	Pyoderma	Ciprofloxacin	Lloyd *et al.* (1999)

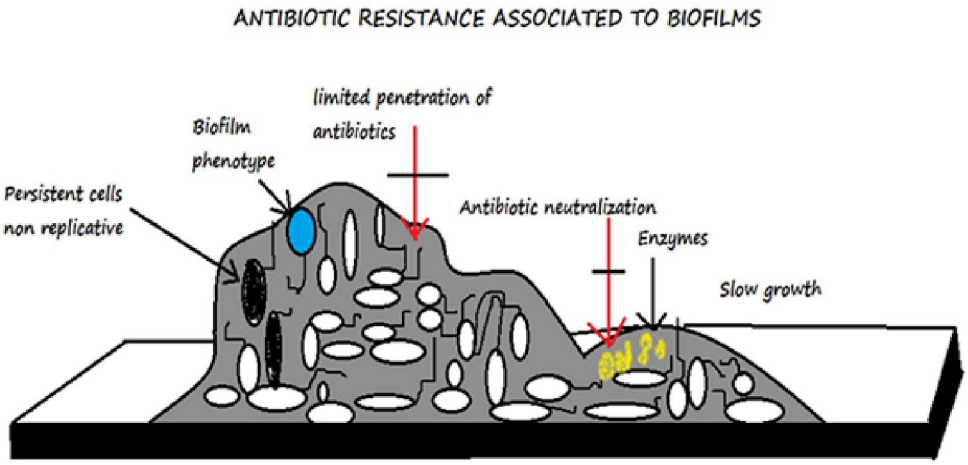

Figure 2. Mechanism involved in antibiotic resistance in the biofilm. Description of the key mechanisms involved in antibiotic resistance such as enzyme causing neutralizations, presence of persistent (non- dividing) cells and biofilm phenotype. **Source:** (Jamal *et al.,* 2015)**.**

2.4. Biofilms in the Healthcare Setting

In human medicine, biofilms have been shown to form on central venous catheters, prosthetic heart valves, urinary catheters, prosthetic joints and endotracheal tubes (Donlan and Costerton, 2002). Biofilm associated infections are generally persistent and respond poorly to commonly used antibiotics and disinfectants. Biofilms protect the cells from assaults like UV radiation, pH stress, chemical exposure, phagocytosis, dehydration and antibiotics (Gupta *et al.,* 2016). A significant disadvantage of biofilm formation in such instances is that there is a potential link between biofilm formation and the spread of resistant genes through plasmid exchange via conjugation, or gene uptake via transformation. This naturally leads to increased difficulties in treating biofilm related infections, increasing the cost to healthcare organizations and increases patient suffering due to prolonged infection. Biofilm producing bacteria have the potential to evade the defense mechanisms of the host immune system (Pour *et al.,* 2011).

Bacterial biofilm contamination of surfaces in clinical workspaces is likely ubiquitous, and serves as a potential source of infection. One of the first clinical infections associated with biofilm formation was medical device related infections. Pacemakers, electrical dialysers, joint prosthetics, intravenous catheters, urinary catheter are indispensable for the patients as there has not been any other alternative against these devices. These devices also come up with a heightened risk of biofilm associated infection. Mostly, *Staphylococci* and *Pseudomonas species* opportunistically infect a medically intervening device and get entry to the host (Gupta *et al.,* 2016).

For example, central venous catheters are used for fluid administration, medication, administering nutrients and monitoring hemodynamic activities. Biofilm-forming organisms have been reported to be found dwelling on the surface of these catheters. The

colonizing microorganisms in such cases are *S. epidermis*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae,* etc. These biofilms may be present either on the lumen or on the outer surface of the catheter. It has also been reported that microbial colonization on catheters may occur within 10 days of catheterization. In cases where the catheter is administered for long time period, biofilms occur in the lumen of the catheter (Kokare *et al.,* 2009).

Nowadays, mechanical valves along with bio-prostheses are used as prosthetic heart valves. The implantation of such prosthetics is susceptible to microbial colonization and subsequent biofilm formation. During surgical procedure, tissue damage may occur that leads to platelet and fibrin accumulation at the site of suture and also on the device. Microbes colonize to these surfaces with higher affinity as a result of which biofilms develop on the surrounding tissues of the prosthesis (Kokare *et al.,* 2009).

Urinary catheters are inserted through the urethra to the bladder where the device measures urine during surgical procedures. Catheters can have either open or closed systems. In case of an open system catheter, the urine is drained in an open collection center. This type of system is susceptible to contamination and may also lead to the development of urinary tract infection (UTI) within a matter of few days. In a closed system, the catheter is emptied in a tightly fastened plastic bag. This type of a closed system is less susceptible to opportunistic infections in comparison with the open system ones. Prolonged use of catheters leads to a higher chance of acquiring UTI. The organisms contaminating such devices are *S. epidermis*, *E. coli*, *P. mirabilis*, *P. aeruginosa*, *K. pneumoniae*, *E. faecalis* and some gram-negative bacteria. Intra-uterine devices (IUD) and contact lenses also harbor biofilm causing infections. The tail of the IUDs is very susceptible to contamination (Kokare *et al.,* 2009).

2.5. Biofilms and Their Relevance in the Veterinary Medicine and Food Industry

Although less research exists about biofilms in animals, they are believed to be involved in many diseases, such as pneumonia, liver abscesses, enteritis, wound infections and mastitis infections (Melchior *et al.,* 2006b, 2006a; Olson *et al.,* 2002). These infections can be caused by environmental organisms, such as *P. aeruginosa*, which are commonly found in wound infections, or by species of bacteria that constitute part of the normal mammalian micro flora (Galuppo *et al.,* 1999).

Implant‐associated infection rates have been reported to be 5% and 4.7–12% in human and veterinary medicine, respectively (von Eiff *et al.,* 2001). An implant‐associated infection is a major complication that in general requires device removal for resolution, because of formation of a biofilm on the implant. Biofilm development begins with bacterial adherence via host‐tissue ligands present on the implant such as fibronectin and fibrinogen. Once microbes attach to an implant, host cells have difficulty displacing them (Hoyle and Costerton, 1991).

For example there is increased use of orthopaedic implants and prosthetic devices in veterinary surgical techniques (Allen, 2012). This has led to similar post-operative risks in companion animals to humans. One retrospective study, involving 902 dogs undergoing surgery for cranial cruciate rupture, reported an infection rate post-surgery in the 406 dogs undergoing tibial plateau levelling osteotomy (TPLO) of 8.4% (Frey *et al.,* 2010). Another study to determine the implant removal rate due to infection after TPLO, reported 7.4% of dogs needed removal of the implant, with approximately 70% of the infections being caused by *Staphylococcus* spp. *Staphylococcus* spp. are capable of forming biofilms (Gallagher and Mertens, 2012).

The hip replacement in dogs, retrospective studies suggest a lower implant infection rate. In one study, implant failure leading to its removal due to infection was seen in only a single dog in a series of 60 animals (Guerrero and Montavon, 2009). This dog had concurrent aspiration pneumonia that may have been the source of the infection. The factors that increase the risk of biofilm formation and implant associated infections includes the presence of extensive soft tissue trauma, the location of the implant, immunosuppression due to concurrent disease or medication (chemotherapy and corticosteroids) are important (Rowson and Townsend, 2016). Several pathogens have been implicated, including most commonly *E. coli* and *S. pseudintermedius*, *P. aeruginosa*, *K. pneumonia* and *P. mirabilis* (Shimizu and Harada, 2017). Periodontal disease is a significant problem in dogs, affecting 44–63.6% of the population. It is known to be caused by plaque, which a microbial biofilm that colonizes teeth and causes inflammation in the gingiva (Holcombe *et al.,* 2014).

Biofilms are involved in many veterinary diseases, and wound infections are a particular problem in the treatment of hospitalized animals (Percival, 2004; Rhoads *et al.,* 2008). The inability to treat many bacterial infections like chronic non-healing wounds and mastitis is related to the capacity of bacteria to form a biofilm. Unlike planktonic bacterial infections which are typically rapid and acute in onset, in biofilm-related disease there is a temporal delay in the clinical appearance. Wounds, whether of surgical or traumatic origin, are a frequent occurrence in veterinary clinics and are common sites for biofilm formation. Bacterial infection of wounds is an

important aspect of patient care in veterinary practice (Orsini *et al.,* 2004). Open wounds can provide an environment conducive to the formation of a biofilm (Xu *et al.,* 2000).

Once a biofilm has colonized the wound bed, the repair process is interrupted and fails to progress through the sequential stages of wound healing. Interruption of the process due to the presence of a biofilm is primarily identified by a prolonged inflammatory response that results in a chronic non-healing wound. Horses are particularly at risk from chronic non-healing wounds of the lower limb. Once the biofilm has become established within the wound bed, the wound becomes difficult to manage with traditional antibiotic treatments (Theoret, 2004)

Mastitis is another significant health issue involving biofilm infection in animals. It affects many species but is most commonly isolated in the cow where the importance placed on the commercial value of dairy products is the highest. Adhesion of bacterial cells (e.g. *S. aureus*) to the mammary gland epithelium has been considered the primary step in the pathogenesis of mastitis. Research has confirmed that bacterial strains growing as a biofilm in mastitis are less susceptible to current mastitis therapies (Melchior *et al.,* 2007, 2006a, 2006b).

The ability of strains isolated from mastitis causing pathogens to adhere to stainless steel, glass, rubber and polypropylene surfaces has been widely studied. In dairy farms, a recent investigation showed that 42% and 39% of 31 *S. aureus* strains isolated from milking parlor environments were biofilm producers on stainless steel and rubber, respectively, indicating a possible persistence of this pathogen in the milking environment. These findings are of major concern in dairy farms, taking into account the association between the occurrence of biofilms and bovine mastitis. Moreover, *S. aureus* strains with phenotypically active genes encoding biofilm components may have the ability to start biofilm production, causing persistent intra-mammary infections (Melchior *et al.,* 2007).

In veterinary medicine, the relationship between the presence of biofilm and diverse diseases in different animal species has been reported, such as:

Table 2: The relationship between bacteria biofilm and their respective infection.

Bacterial organism	Disease	Reference
Acinetobacter baumannii	Intravenous jugular catheters infection	Vaneechoutte *et al.* (2000)
Haemophilus parasuis	pneumonias	Jin *et al.* (2006)
Mycoplasma mycoides subspecies mycoides SC	Bovine pleuropneumonia	McAuliffe *et al.* 2008)
Actinobacillus pleuropneumoniae and *Staphylococcus epidermidis*	Postoperative infections	Kaplan and Mulks (2005); Orsini *et al.* (2005); Smith and Ross (2002)
Pseudomonas aeruginosa	Otitis	Aguilar-Romero *et al.* (2010)
Klebsiella spp	Skeletal muscle infections	Moore *et al.* (1992)
Staphylococcus intermedius	Pyoderma	Lloyd *et al.* (1999)
Staphylococcus aureus and *Staphylococcus epidermidis*	Mastitis	Fox *et al.* (2005); Oliveira *et al.* (2006); Vasudevan *et al.* (2003)

The prevalence of biofilms is a significant problem in food and the food industry, major foodborne pathogens such as *E. coli, L. monocytogenes, Salmonella* spp., and *C. jejuni* can form a biofilm and remain a significant food safety challenge for the food industry (Brandl, 2006; Gandhi and Chikindas, 2007; Murphy *et al.,* 2006). In food processing environments, a variety of microorganisms colonize food and food contact surfaces, survive, grow, and sometimes form multispecies biofilm communities. Once developed, biofilms are a significant potential source of contamination of food products; biofilms may lead to spoilage of food and/or substantial risks for consumer health after consumption. Many outbreaks that are associated with the consumption of fresh produce, such as lettuce, onions, spinach, milk, and tomatoes, have been linked to surface colonization by a biofilm-forming pathogen (Brandl, 2006; Zhang *et al.,* 2008)

Another problem faced by industry is called biocorrosion, defined as a complex of materials that is being deteriorated by microorganisms and causing damage to structures (cooling systems, tanks, etc.). Such damage not only leads to high economic losses but also leads to health and safety issues. Several microorganisms are involved in this process, and SRB (sulfate reducing bacteria) have been identified as the group responsible for the most serious cases of biocorrosion (Melo, 2013).

Additionally, biofilms in industry can have a beneficial effect. For example, biofilms are needed for the production and degradation of organic matter, the degradation of pollutants or the recycling of nitrogen, sulfur and various metals. Most of these processes require the collective effort of organisms with different metabolic capabilities (de Macêdo, 2000). Thus, biofilms are used in the aerobic and anaerobic treatment of domestic and industrial effluents, sewage

and contaminated metabolites. The treatment of drinking water requires the removal of nitrogen, carbon and biodegradable precursors of trihalomethanes, which can be performed by submerged microbial biofilms. Another example is the existing biofilm reactors that produce fermented products (de Macêdo, 2000). To prevent the formation of biofilms in the food industry, it is essential that adequate hygiene and sanitation procedures are established.

2.6. Diagnosis of Biofilm-Associated Infections (BAI)

As a rule of thumb, effective treatment of any disease requires accurate diagnosis of the disease.

However, due to the complex nature of biofilm, achieving accurate diagnosis through the conventional culture and isolation diagnostic method is quite difficult (Rowson and Townsend, 2016). At present, diagnostic techniques such as; serology, fluorescent *in situ* hybridization (FISH), conventional radiographic approaches (computed tomography, magnetic resonance imaging and radioinuclide scans), polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and other molecular technique has shown promising result in effectively diagnosing biofilm diseases (Abdullahi *et al.,* 2015). Reliance on culture as the 'gold standard' of medical microbiology exclusively for the identification of bacterial pathogens as a diagnostic criterion in clinical laboratories is not clear-cut with biofilm-associated infections (BAI). In culture identifies a pathogen around 25–30% of the time, while culture-independent methods such as PCR and/or FISH identify pathogens 80–100% of the time (Hall-Stoodley *et al.,* 2006).

However, culture of the heart valve tissue itself was not necessarily more effective, whereas molecular methods were more successful at identifying a causative microorganism. The identification by broad range PCR and subsequent sequencing of heart valve material could be confirmed by FISH analysis showing extensive biofilms in culture-negative endocarditis (Mallmann *et al.,* 2010). As FISH targets ribosomal RNA, this molecular method also indicates recent metabolic activity of the bacteria. For sub-acute bacterial endocarditis, which may be present for weeks or even months before being diagnosed, an antibody response may be helpful. Diagnosis of prosthetic joint infection in orthopedics is another example where culture is suspected of producing a high rate of false negative results and suggests that infection might be commonly misdiagnosed as 'aseptic loosening'. Even in cases when the surface is sampled directly by swabbing, it has been shown that bacteria may be extremely hard to detach (Bjerkan *et al.,* 2009; Kobayashi *et al.,* 2009, 2007).

2.6.1. The importance of molecular diagnostic approaches

The development of molecular based diagnostic approaches to BAI is central to improving the detection and identification of microorganisms and establishing their role in pathogenesis. This is consistent with molecular diagnostics increasingly being applied to microbial detection and identification in the microbiology laboratory for many putative infections that are either not able to be cultured (viruses) or are fastidious or slow-growing. Several molecular techniques are now used routinely to either augment existing culture results (for bacteria) or to detect and identify pathogens in the absence of culture (primarily for virus detection). The most widespread molecular methods are nucleic acid (NA) amplification techniques such as the PCR. Advantages of PCR include: high sensitivity that may detect very few microorganisms, availability of primer/probe sets for most common pathogens, routine extraction protocols for nucleic acid extraction, and the development of automated systems and readouts for higher throughput of samples. Quantitative PCR can also provide quantitative data on the relative abundance of microorganisms that are present. Thus, PCR is a powerful approach that needs to be interpreted in the context of other diagnostic approaches and clinical data (Larsen *et al.,* 2008; Rudkjøbing *et al.,* 2011; Wolff *et al.,* 2011).

FISH is another sensitive and specific approach, which is particularly well suited to the study of complex tissue samples and evaluation of the presence of microbial aggregates. FISH relies on hybridization of a fluorescently labeled probe to the 16S or 23S ribosomal RNA (huose keeping gene) in bacteria or the 18S or 26S ribosomal subunits in eukaryotic microorganisms. These molecular regions are specific to species level in microorganisms, and with careful optimization and use of controls, this approach can give robust in situ evidence of pathogens in a sample. Disadvantages include: the dependence on laboratory expertise, requirement for fluorescence microscopy or confocal laser scanning microscopy (CLSM) for research purposes, the need for fixation and permeabilization of the sample, few commercially available probes for diagnostic use coupled with the need for testing and of validating new probes, and cost (Heydorn *et al.,* 2000).

PNA FISH (FISH using peptide nucleic acid probes) probes abide by Watson/Crick pairing but possess unique hybridization characteristics because of their uncharged chemical backbone, including rapid and stronger binding to complementary targets compared with traditional DNA probes. PNA probes can also be used with unfixed biological samples; however, only a limited number of probes are

currently available, restricting the use of PNA FISH for the present. CLSM and FISH emphasize that demonstrating biofilm spatial organization is extremely important to: (1) identify whether the bacteria present are aggregated, (2) indicate a polymicrobial nature of a biofilm, (3) indicate the extent of biofilm on a surface that CFU (colony forming unity) may vastly underestimate, and (4) to show biofilm EPS that may comprise a greater part of the biofilm than cells alone. On non-biological, flat surfaces, biofilm spatial organization can best be measured by various parameters using image analysis software (Heydorn *et al.,* 2000)

Chronic or recurrent infection itself has been suggested as a diagnostic criterion along with recalcitrance of the infection to antibiotic treatment (Høiby *et al.,* 2010a). For example, the BAI in CF (cystic fibrosis) is characterized by progressive chronic lung infection in response to multiple respiratory pathogens, which are eventually dominated by *P. aeruginosa*. This organism then may adopt a mucoid phenotype that is highly resistant to clearance by antibiotic or host immune responses. CF illustrates several aspects of biofilm associated disease (Høiby et al., 2010b) and contrasts with acute pneumonias that are resolved with antibiotic therapy (Høiby *et al.,* 2010b).

Loop-Mediated Isothermal Amplification (LAMP) is innovative gene amplification technique, amplifies nucleic acid at a very rapid pace, maintaining high sensitivity, specificity and efficiency. The cheapness and user-friendliness of LAMP amongst other advantages made it an ideal diagnostic tool that provides solution to the odds of PCR (Abdullahi *et al.,* 2015).

2.7. Therapy and Prevention of Biofilm Infections

There are range of different strategies that can be utilized to manage biofilm infection. Because of the difficulty in dealing with such infections the most important initial step should be, especially in surgical procedures where implants (catheter) are used, to prevent infection. Strategies to prevent initial microbial contamination of implants include the institution of strict hygiene levels for device insertion; and the use of systemic, prophylactic, perioperative antibiotics (Aiken *et al.,* 2015; Nazarali *et al.,* 2014; Weese and Halling, 2006; Whittem *et al.,* 1999).

Once biofilm infection has been diagnosed then a range of different treatment options may be explored. Often, combinations of therapy that include antimicrobial drugs with other anti-biofilm agents prove the most successful. Possible treatment options include; antimicrobial therapy, mechanical removal of biofilms, quorum sensing inhibitors, anti-adhesive agents, bacteriophages, and bacteriocin (Paterson, 2017).

2.7.1. Antimicrobial therapy

Where biofilm infection is present, topical administration of antibiotics provides high local concentrations of drug at the site of the infection. By achieving high concentrations of drug locally, the mean inhibitory concentration (MIC) of the bacteria is exceeded several fold. Therefore bacteria are killed, reducing both the risk of biofilm formation and the development of antimicrobial resistance. Topical therapy is also helpful in avoiding systemic side effects (Paterson, 2017). Nebulized antibiotics are the treatment of choice for cystic fibrosis, where chronic biofilm infections of lung are common. Likewise, antibiotic impregnated materials with gentamicin, tobramycin and vancomycin have been shown to reduce the incidence of prosthetic joint infections associated with biofilms in man (Ciofu *et al.,* 2017).

Systemic antibiotics are often employed to treat canine otitis. Although studies in both man (Belfield *et al.,* 2015) and dogs (Cole *et al.,* 2009) have shown good levels of antibiotics can be achieved in the middle ear (Belfield *et al.,* 2015) and external ear canal (Cole *et al.,* 2009). Depending on the type of device, systemic antibiotic prophylaxis can be proposed in order to reduce the risk of microbial contamination. In that case, antibiotics are injected a few minutes before skin incision and are dedicated to eradicating any microorganisms that are not removed by skin disinfection. This approach is recommended in the case of surgically implanted devices, such as orthopedic and cardiac devices (Baddour *et al.,* 2010).

Some antibiotics have been shown to have the ability to penetrate the extracellular matrix and therefore achieve high localized bactericidal levels of drug. Prolonged courses and high dose of systemic antibiotics are always needed for biofilm associated infections (Lebeaux *et al.,* 2014). Some antimicrobials, such as colistin, sodium dodecyl sulphate (SDS), ethylenediaminetetraacetic acid (EDTA) and chlorhexidine, preferentially kill the non-growing bacteria located in the inner part of the biofilm, which means that such products have the potential to be used with topical antibiotic therapy to target all of the physiological stages of bacterial growth within the biofilm (Ciofu *et al.,* 2017).

2.7.2. Antibiotic coating of implanted devices

The principle of antibiotic coating of implanted devices is to deliver a locally high concentration of antimicrobials at the site of potential colonization. Depending on the type of device and the length of implantation, these antibiotic coated materials can efficiently reduce the rate of colonization (Hetrick and Schoenfisch, 2006). Silver is an antimicrobial

nontoxic metal. The anti-biofilm effectiveness of silver nanoparticles against *P. aeruginosa* and *S. epidermidis* strains. Silver-impregnated dressings have been shown to reduce the viability of biofilm bacteria in wounds and increase their susceptibility to antibiotics (Kostenko *et al.,* 2010; Percival *et al.,* 2007). Silver-impregnated coatings on orthopedic implants and catheter has been shown to reduce the risk of biofilm infections (Azab *et al.,* 2016; Mala *et al.,* 2017; Secinti *et al.,* 2011). Colloidal silver has also been used topically to treat biofilms infections (Richter *et al.,* 2017).

2.7.3. Mechanical removal of biofilm

Mechanical removal of biofilm is most common in periodontal disease. The microbial biofilm or plaque that colonizes teeth and causes inflammation in the gingiva (Holcombe *et al.,* 2014). Where wounds have biofilm infection, debridement forms an important part of the treatment process. Therefore successful treatment of chronic non healing wounds should ideally involve a combined treatment effort using surgical debridement of the biofilm and necrotic tissue, thorough lavage with physiologic saline and topical and/or systemic administration of antibiotics. Where standard medical treatments fail, for example in chronic cases of endocarditis or osteomyelitis, debridement or excision of infected tissue may be the only successful option for therapy (Paterson, 2017).

2.7.4. Bacteriocin

Bacteriocins are ribosomally synthesized antimicrobial peptides that are produced by all prokaryotic lineages and are generally active against closely related species. Due to their anti-biofilm properties, bacteriocins have been well studied. Treatment with a bacteriocin is a promising method for the reduction of bacterial attachment and biofilm formation (Mahdavi and Jalali, 2007). Despite powerful antimicrobial and anti-biofilm properties, application of bacteriocins to the biomedical and food industries has been hampered by the slow development of a reliable bacteriocin delivery system (Yamakami *et al.,* 2013).

2.7.5. Anti-adhesive agents

Given the fact that without initial adhesion a biofilm cannot develop, the objective of inhibiting microbial adhesion is to impede the initial steps in biofilm formation. Anti-adhesive agents should specifically interact with the adhesins of the pathogen, to prevent the union between the pathogen and the eukaryotic cell. Anti-adhesive agents include mannosides, curlicides and pilicides (Soto, 2014). Mannosides, which are molecules containing mannose sugar groups, are utilized in veterinary medicine in urinary supplements to help treat and maintain animals prone to urinary tract disease. Another major strategy for reducing bacterial adhesion is to modify the surface so it is protected by grafting anti-adhesive molecules (Größner‐Schreiber *et al.,* 2009).

2.7.6. Quorum sensing inhibitors

Quorum sensing inhibitors (QSIs) and antagonists are currently one of the most promising areas for new therapeutic options for therapy of biofilm infections. QSIs have been suggested as novel anti-biofilm agents (Brackman and Coenye, 2015). There are several established quorum-quenching strategies through which a QS mechanism can be interrupted such as inhibition of signal synthesis or direct degradation of a signaling molecule, inhibition of binding of the signaling molecule to its receptor, and/or inhibition of binding of the signal transduction cascade. The quorum-quenching approach leads to the dissociation of the biofilm architecture but not to killing of the biofilm microorganism. Nonetheless, QSIs have the potential to increase the sensitivity of biofilm-forming bacteria to antibiotics. As a consequence, many researchers have combined quorum quenching with antibiotic treatments and demonstrated in animal studies that these methods work well. The goal of vaccination is to induce the production of antibodies against bacterial biofilm antigens, such as structures involved in adhesion or biofilm maturation (Lebeaux *et al.,* 2014).

2.7.7. Bacteriophages

Bacteriophages are viruses that specifically infect bacteria. Among them, lytic phages are able to disrupt the normal bacterial metabolism, favoring viral replication. Phages are currently considered a potential alternative or adjunct to antibiotics for bacterial infections, especially for biofilm inhibition or disruption. Phages have been tested as potential anti-biofilm agents. For example, T4 phage can effectively infect and replicate within *E. coli* biofilms and can disrupt the biofilm matrix by destroying bacterial cells. So phages have the ability to control biofilms by being able to multiply at the site of the infection and produce enzymes (polysaccharide depolymerase) that degrade the EPS of the biofilm matrix (Donlan, 2009). Bacteriophages have shown some promise in veterinary medicine, with some preliminary studies showing they may have potential to treat *Pseudomonas* spp. infections in otitis (Furusawa *et al.,* 2016; Soothill, 2013).

2.8. Advancements in Biofilm Research

Biofilm formation of infectious agents is mostly found on “implant devices”. Recently another saprophytic organism, the incidence of which is increased in nosocomial (hospital acquired) infections, is a risk factor for medical device-carrying patients (Buommino *et al.,* 2014). *P. aeruginosa* is the 2nd most common reason for ventilator-associated pneumonia (VAP) and catheter-associated urinary tract infections (CAUTI). *P. aeruginosa* forms biofilms on

endocardial tubes and catheters in CAUTI and VAP patients. Another recent advancement in biofilm research has been applied to control energy crises. This approach is using microbial fuel cells (MFCs). MFCs produce electricity by utilizing chemical energy found in organic and some in-organic compounds. Electrogenic microbes play a role in this process by accepting or donating electrons to an external object (electrode), while some non-electrogenic microbes are also involved as part of a synergistic electrogenic biofilm (Zhou *et al.,* 2013).

3. Conclusion And Recommendations

Bacterial biofilms are highly organized structures composed of communities of bacteria covered within extracellular matrices. They are a major cause of infection in man and animals. Biofilm formation enables bacterial pathogens to colonize a wide variety of host niches and persist in harsh environments, making their eradication particularly difficult. They are an important reason for orthopedic implant failure and contribute to a wide range of veterinary diseases. In medicine, biofilm forming bacteria are responsible for chronic and persistence infections. It is important that veterinary surgeons are aware of the presence of biofilms in disease, and the need to modify their therapeutic approach to deal with them. The development of effective strategies to combat biofilms infection is a challenging task. Numerous innovative anti-biofilm approaches have been published. Some of the emerging novel approaches, such as bacteriophages, quorum quenching, bacteriocin, and anti-adhesive agents, are promising and may help to find the therapy of biofilm infection. This paper has highlighted the scientific information regarding the mechanisms of the formation of bacterial biofilm with regard to the clinical importance in veterinary medicine and also public health.

Therefore, based on the above facts the following recommendations are forwarded:

- A combination of these novel techniques with conventional methods (antibiotics, disinfectants, and physical methods) is expected to solve the "biofilm problem" in the near future.
- Molecular technique has shown promising result in effectively diagnosing biofilm diseases.
- The combination of QSI and antibiotics to handle biofilm has been suggested.

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