Biofilms in Veterinary Medicine "Impact and Consequences of Food Quality and the Treatment of Infectious Disease"

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Biofilms consist of microorganisms that are attached to any surface. These organisms are wrapped in a layer of polysaccharide that is located in a microenvironment that is favorable for their survival. This chapter, titled Biofilms in Veterinary Medicine, aims to address the issue of biofilms, with emphasis on the consequences and problems in food quality and the treatment of infectious diseases of animals. This theme has not been well documented in some microbiology books despite being an actual topic of research, and there are few reports that address this subject in food microbiology and animal diseases. Due to the limited data in this area, it was suggested that this chapter address this topic in a comprehensive, updated and specific manner, which would allow the reader a better understanding of this subject. This chapter studies the role of biofilms in veterinary medical research and also addresses the subtopic of food quality. The purpose of this book chapter is to provide a general definition of biofilms, laboratory identification methods and methods of control and prevention of the formation. We provide information that will be useful for researchers in microbiology, particularly those studying food microbiology and infectious diseases of animals.

Keywords biofilms; food quality; infectious disease; veterinary medicine.

1. Biofilms: General information and definition

A biofilm is a microbial community where microorganisms are attached to any surface and to each other. These microorganisms are surrounded by polysaccharides that contain a diverse set of proteins, lipids and carbohydrates, which are the most important component.

The first step in the formation of biofilms is the adhesion of the microorganisms to a surface that can be biotic or abiotic. The process of biofilm formation can be divided into five stages: conditioning the surface by adsorption of organic material, transportation of cells and nutrients to the site of adhesion, processing of bacterial adhesion by reversible electrostatic attraction, cell multiplication and colonization and irreversible adhesion [1] cited by [2].

It is now recognized that biofilm formation is an important aspect of many, if not most, bacterial diseases. Established biofilms can tolerate antimicrobial agents at concentrations of 10-1000 times that which is needed to kill genetically equivalent planktonic bacteria. Biofilm microorganisms are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts [3, 4].

The serious and pervasive clinical impact of bacterial biofilms has inspired many researchers to investigate the regulatory mechanisms behind their formation and dissolution, with the ultimate goal of pinpointing specific targets for chemotherapeutic agents [4, 5].

The formation of a biofilm alters the microenvironment of its own inhabitants, which then leads to additional alterations in gene expression and further maturation of the biofilm. The study of biofilms is further complicated by the heterogeneous composition of the bacteria. Disease-related biofilms can be multi-species or multi-kingdom, such as the biofilms involved in mastitis, or single-species, such as those involved in other microbial diseases. However, even bacteria within single-species biofilms are heterogeneous with respect to gene expression [6].

Many biofilm infections share clinical characteristics. Biofilms develop preferentially on inert surfaces, or on dead tissue, and occur commonly on medical devices or fragments of dead tissue, such as sequestra of dead bone [6]. They can also form on living tissues, as in the case of endocarditis. Biofilms grow slowly and can grow in one or more locations. Therefore, biofilm infections are often slow to produce overt symptoms [7]. Sessile bacterial cells release antigens and stimulate the production of antibodies, but the antibodies are not effective at killing bacteria within biofilms and may cause immune complex damage to the surrounding tissues [8]. Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms [9]. Antibiotic therapy typically reverses the symptoms caused by planktonic cells that are released from the biofilm but fails to kill the biofilm [10]. For this reason, biofilm infections typically recur after cycles of antibiotic therapy until the sessile population is surgically removed from the body. Planktonic bacterial cells are released from biofilms, and evidence supports the hypothesis that there is a natural pattern of programmed detachment.

2. Laboratory methods of identification

There are several methods for detecting the production of biofilms. These methods can be divided into phenotypic and genotypic strategies. The standard method used to identify biofilm formation is the measurement of colony forming units (CFU). This method aims to enumerate the colonies that are present (living microorganisms in the biofilm). This

method is related to the use of a microplate for the measurement of biofilm formation: once formed, the biofilm is scraped from the surface and the cells are sonicated, diluted and counted in an appropriate culture medium for each strain (genus and species specific).

Freeman et al. (1989) proposed Congo Red Agar (CRA) as an alternative method for the detection of slime (biofilm) production by coagulase-negative *Staphylococcus*. Using Congo Red agar, biofilm production was detected in 77.78% of the strains tested. This culture medium can be used for verifying the phenotypic changes within colonies of coagulase-negative *Staphylococcus*. Colonies can be categorized by color: the slime-producing colonies are black and those that do not produce slime are red [11].

Stepanovic et al. (2000) noted that for quantification of biofilm formation by *Staphylococcus* sp, the microtiter adhesion test is one of the most frequently used methods. This test will also function as an indicator of pathogenic micro-organisms [12].

Jain & Agarwal (2009) evaluated whether phenotypic Congo Red Agar or microplate test would more efficiently identify *Staphylococcus* sp isolated from biofilms that were found on swabs of nasal mucosa from venous catheters. They concluded that both tests demonstrated good sensitivity and specificity in the detection of microorganisms that produced biofilms [13].

Microbiological tests for evaluating the number of cells in the biofilms are primarily used to test substances that act on biofilms. There are difficulties in the execution of this test because much care is needed to remove the biofilms from the polystyrene plates and to transfer them to agar plates for counting. However, when this test is performed well, it is considered the gold standard for reporting the actual number of microorganisms in biofilms [14].

Electron microscopy is used for evaluating the interactions within the microbial biofilm. This method preserves many of the associated structures that are found in a hydrated and viable state. Sample fixation is used for the assessment of microbial interactions in the biofilm matrix [2]. In one of the first studies that investigated bacterial adhesion to surfaces that come into contact with food, [15] used a scanning electron microscope to show the adherence of *Staphylococcus aureus* and *Pseudomonas fragi* to stainless steel surfaces and glass. According to Pizzolitto (1997), the scanning electron microscope image shows a three-dimensional interaction, and the surface topography of the sample is clearly revealed [16, 17].

3. Methods of control, removal and prevention of biofilm formation

Silver-containing nanomaterials are now considered to be one of the most promising strategies to combat bacterial infections related to indwelling medical devices, such as intravenous catheters. Nanoscale materials have recently appeared as new antimicrobial agents due to their high surface area to volume ratio and unique chemical and physical properties [18].

Different types of nanomaterials, such as copper, zinc, titanium, magnesium, gold, alginate and silver, have been developed in recent years. Nevertheless, silver nanoparticles (NPs) have demonstrated more effectiveness and good antimicrobial activity against bacteria, viruses and eukaryotic microorganisms [19].

Furthermore, silver NPs have not been shown to cause bacterial resistance, which is presumably because, unlike antibiotics, silver NPs exert their antibacterial effects at several sites, such as the bacterial wall, proteosynthesis and DNA [20]. The considerable surface-to-volume ratio of NPs enables a constant local supply of silver (Ag) ions to be maintained at the coating tissue interface and allows improved and increased contact with microorganisms [18]. NPs also protect the outer and inner surfaces of devices [21].

Although some studies have raised concerns regarding the biosafety of silver NPs [22], they are emerging as the next-generation of antibacterial agents and there are currently reports demonstrating their efficacy.

There are studies that demonstrate the ability of NPs to reduce or prevent biofilm formation on catheter materials, both in vitro [23] and in animal models [24]. There are still few studies that have been done in patients.

Bacteriophages are viruses that infect prokaryotes. They can be used to control biofilms when the bacteria are inoculated with compatible viruses. Some of these viruses can induce bacteria to enter the lysogenic cycle and integrate their genetic material in bacteria, or the lytic cycle, which inactivates it. The study of phages has fundamental importance for the control of biofilms because it does not present a risk to human and animal health [25].

Phages are widely distributed in the world, and it is estimated that there are 10 phages for each bacterial cell. Some phages have a wide scope, while others are extremely specific. The use of phage lytic therapy is being investigated in the search for new, more efficient alternative treatments for infections. The Committee on the Taxonomy of Viruses (ICTV) recognizes 13 families and 31 genera of phages [26].

When incorporated phages are in the lytic cycle, the host bacteria are directed to express the genes and proteins responsible for the assembly of new phage particles. The use of phages would be an interesting option because they affect only specific bacteria and are not toxic to plants and animals [26].

Antimicrobial peptides (AMPs) are small cationic peptides and conserved components of the immune response that are involved in the defense mechanisms of a wide range of organisms [27]. Members of the AMP family are widely distributed in nature; there are more than 1500 AMPs that have been reported in organisms such as bacteria, fungi,

insects, plants and humans [28]. Some classes of AMPs, such as b-defensins, indolicidin, cecropin A, and magainins, have demonstrated effectiveness in killing bacteria, fungi, parasites and even viruses [29]. Importantly, AMPs have also been found to be effective against superbugs that have developed resistance to antibiotics such as MRSA and quinolone-resistant *Enterobacteriaceae* [30].

Given that the killing mechanism of AMPs involves targeting the fundamental structures of bacteria, such as the membrane, the emergence of resistant mutants is unlikely to occur because of the essential functions of the membrane in maintaining microbial homeostasis, metabolism and viability [31].

Therefore, there are many reasons that AMPs are attractive as potential antimicrobial agents. For example, they have a broad spectrum of activity, relative selectivity toward their targets (microbial membranes), a rapid mechanism of action and, above all, a low frequency in selecting resistant strains [32]. However, the work of Perron et al. (2006) has demonstrated the development of some level of resistance to AMPs in vitro [41].

The cationic peptides have a net positive charge at neutral pH because of multiple arginines and/or lysines in their sequences [28]. The surface of several synthetic materials that are used as biomaterials, such as silicone and polyesters, which are normally subjected to microbial colonization and biofilm formation, are negatively charged at pH 7. This property permits the binding of cationic molecules, such as AMPs [33]. Taking into account that biofilm tolerance to antibiotics is generally due to the slow growth rate and low metabolic activity of bacteria, the use of AMPs to inhibit biofilm formation could be a promising strategy.

Because the AMPs' primary mechanism of action is the ability to permeabilize and/or to form pores within cytoplasmic membranes, they also have a high potential to be effective on slow growing or inactive bacteria [32]. The cathelicidin peptide BMAP-28 was reported by Cirioni et al. [34] to have good antimicrobial activity against *S. aureus* biofilms. It also had a tendency to attach to the biomaterial surface, making pre-treatment with BMAP-28 an attractive choice to control device-related infections caused by staphylococci.

4. Biofilms in the food industry and veterinary infectious diseases

The growth of microorganisms on the surface of food is a major cause of deterioration and loss of fresh and processed products. Thus, to prolong the life of products, antimicrobial agents are added directly to the feed or sprayed on the surface to control the growth of microbes. Active packaging is adopted to interact with the food product in order to eliminate or inhibit microorganisms. Nitrates, lactate, nisin, natamycin, sodium benzoate, propionate, and more recently, the compound triclosan have been used [35].

Both microbial adhesion and biofilms are of great importance to the food industry and occur on a variety of food contact surfaces. Both spoilage and pathogenic microorganisms contribute to the intensity of adhesion processes and biofilm formation. *Pseudomonas aeruginosa, Pseudomonas fragi, Micrococcus* sp and *Enterococcus faecium* [36, 37, 38] are some of the microorganisms associated with spoilage, while *Listeria monocytogenes, Yersinia enterocolitica, Salmonella thyphimurium, Escherichia coli* O157:H7*, Staphylococcus aureus* and *Bacillus cereus* [36, 39, 40] belong to the pathogenic group.

Stainless steel, glass, rubber, and polypropylene surfaces can be contaminated either by spoilage or pathogenic microorganisms. Under certain conditions, these microorganisms are deposited, adhere to, and interact with the surface, initiating cellular growth and consequently leading to biofilm formation [39, 40, 42, 43, 44].

The surfaces of conveyor belts on the food industry premises, which the products may come into the direct contact with, are frequently contaminated even after sanitation according to valid procedures. The contact surface of the conveyor belt in a dairy plant was contaminated with 10^5 to 10^6 CFU/100 cm^2 (CFU = Colony Forming Units) of *Staphylococcus* spp., *Pseudomonas* spp., and other bacteria, even after cleaning [45].

Bioaerosols may become a source of contamination on these open surfaces because the microorganisms are stuck to the liquid particles of aerosol. In the environment of the industry, a bioaerosol is formed during water and air flow and by the release of bacteria from the biofilms present in the waste or on the floors of the production plants with coarse or otherwise damaged surfaces. Such surfaces may be contaminated with bacteria (up to 10^8 CFU/100 cm^2). Dangerous strains of *Pseudomonas* and *Staphylococci* were most often isolated from meat processing plants and dairies [46]. *L. monocytogenes* was also isolated [47].

Dangerous biofilms were also detected in closed systems. Pathogenic microorganisms (from the genera *Bacillus, Streptococcus, Staphylococcus, Shigella, Escherichia*, and *Enterobacter aerogenes*) participated in biofilm formation on the surfaces of a post-pasteurization unit in a dairy plant.

Haun and Cristianini (2004) investigated *Pseudomonas fluorescens* biofilm formation on stainless steel plates, using milk as a substrate, using a static or dynamic system and by simulating heat exchangers and storage tanks. Through the static model, it was possible to obtain counts above 10^5 CFU per stainless steel plate, even after the plates were washed three times in sterile water with stirring [48].

The adherence and biofilm formation by *Pseudomonas fluorescens* on surfaces of marble, granite and stainless steel were studied as a function of time and temperature. The high number of this microorganism is disturbing in certain areas because it is capable of growing at low temperatures and is frequently the cause of deterioration of dairy products due to the production of thermostable proteases and lipases [49].

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, ducts, 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 [50].

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. 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 [51].

The bacteria growing in a biofilm on a stainless steel surface of a heat exchanger in a pasteurization unit of a dairy still contained contaminated milk at a concentration of 10^6 CFU/ml after pasteurization [52]. It was previously discovered that the production of a thermoresistant toxin by the *S. aureus* bacterium surviving in a conduit tap in the dairy was the cause of an extensive crisis in Japan in 2000, which affected over 13,000 people [53].

To prevent the formation of biofilms in the food industry, it is essential that adequate hygiene and sanitation procedures are established [54]. According to Lemos (2002), it takes two to four weeks for a biofilm to form, so formation would only occur in systems where cleanliness and sanitation are deficient [55].

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 [56, 57, 58]. 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 microflora.

Through a combination of endogenous and exogenous factors, these generally harmless commensals may become pathogenic. For example, *S. aureus*, which makes up part of the normal flora of the equine skin and hoof [59], is a well-documented source of post-operative wound infections [60] and mastitis [57, 58,14].

Biofilms are involved in many veterinary diseases, and wound infections are a particular problem in the treatment of hospitalized animals. The natural bacterial flora of the skin is a major source of contamination [60].The process of biofilm formation in animal wounds may follow the same pattern as that documented in human wounds [61, 62].

Furthermore, lower leg injuries are fairly common in horses, and bacterial contamination can occur from environmental pathogens in the mud, feces and bedding. Biofilms have been demonstrated to form in animal wounds by Serralta et al. (2001), who infected pig wounds with *P. aeruginosa*. Biofilms have also been linked to bovine mastitis. Baselga et al. (1993) have suggested that biofilm producing strains of *S. aureus* showed a superior ability to attach to mammary mucosal surfaces and to cause infection than non-biofilm producing strains [63, 64].

Recently, Cucarella et al. (2004) reported that the presence of biofilm associated proteins (Bap) in the bovine intramammary gland may facilitate persistent *S. aureus*-associated biofilm formation in chronic mastitis infections [65]. In fact, *S. aureus* isolates were found [66] to be significantly more likely to produce a biofilm when obtained from intramammary sources, i.e., milk, than when isolated from external sources, such as the milking machines, suggesting that biofilm production is definitely a risk factor during intramammary infection.

S. aureus is one of the most important causes of subclinical, clinical, recurrent and chronic mastitis in dairy cattle. Epidemiological studies have shown that the correlation between the in vitro antimicrobial susceptibility of *S. aureus* isolates from each case of mastitis and the actual bacteriological cure rate after antimicrobial treatment is only moderate [67]. Various hypotheses have been presented to explain these differences. The duration of the infection and the susceptibility of a strain to antibiotics, e.g., penicillin, seem to play a role in the bacteriological cure rate [67, 68, 69, 14]. Epidemiological studies also revealed that the treatment of older cows, or cows with high somatic cell counts (SCC) in general, is less successful than the treatment of cows with a high SCC due to a chronic infection with *S. aureus*. Sometimes, treatment of the latter fails entirely [70, 71].

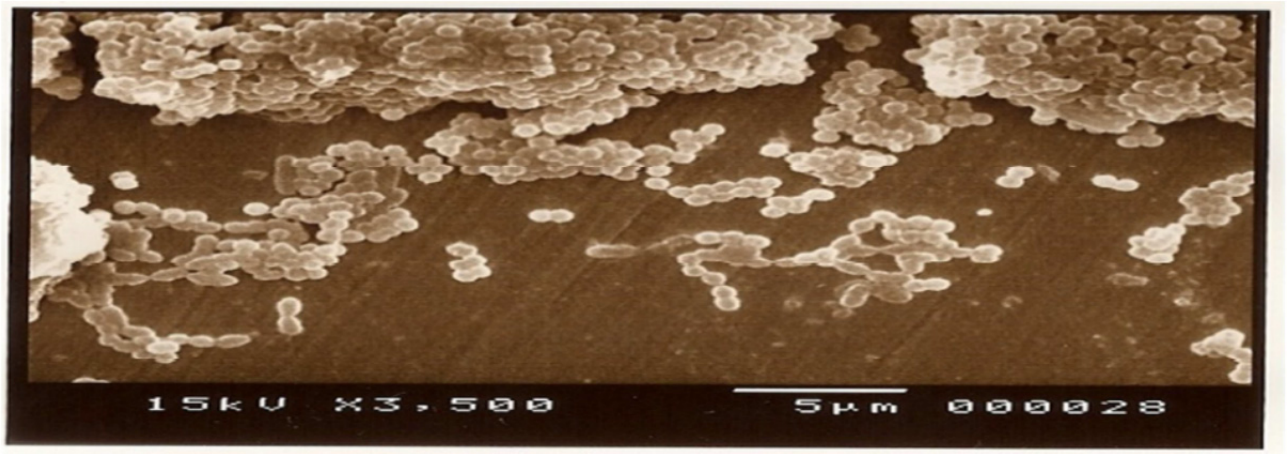

Fig. 1 Biofilms of *S. aureus* derived from milk conductive rubbers viewed by scanning electron microscopy (SEM) on steel coupons, [14].

5. Resistance of microorganisms in biofilms to disinfectants and antimicrobials

Surface-attached microorganisms have an increased resistance to the action of disinfectants. Based on different research, it was shown that there was between 150 and 3,000 times more resistance to the action of hypochlorous acid than that shown by non-adherent microorganisms. Surface-associated microorganisms were 2-100 times more resistant to the action of monoclarinas. Similarly, cells of *L. monocytogenes* that were not adhered were eliminated after 30 seconds of contact with the sanitizing agent benzalkonium chloride. In contrast, adhered cells resisted the same sanitizing treatment for 10 to 20 minutes [51].

Melo (2011) investigated the effectiveness of sodium hypochlorite (NaOCl) against strains of *S. aureus* that were isolated from the raw milk of cows with subclinical mastitis and *Staphylococcus aureus* isolated from the milking environment (blowers and milk conducting tubes). They found that after a contact period of five minutes with NaOCl (150 ppm), four strains (two strains from the milk, one from the blowers and one from a conductive rubber) were still able to grow. Increasing the contact time between the bacteria and the NaOCl (150 ppm) inhibited the growth of all of the strains. With regard to the efficiency of NaOCl on total biofilm biomass formation by each *S. aureus* strain, a decrease was observed when these strains were in contact with 150 ppm NaOCl for a total period of 10 minutes. This study highlights the importance of an appropriate sanitation protocol for all of the milk processing units. Proper sanitation can significantly reduce the presence of microorganisms, leading to a decrease in mastitis occurrence and milk contamination [14].

The adsorption of biosurfactants to solid surfaces can be a new and effective way to combat the adhesion of pathogenic microorganisms in food processing plants. These biosurfactants consist of metabolic by-products of bacteria, fungi and yeasts that exhibit surfactant properties, i.e., lower the surface tension or have a high emulsifying capacity. Applications in the industrial environment are promising because they show structural diversity, a possibility for large-scale production from renewable sources, are biodegradable and have a low toxicity. In food areas, the biosurfactant can be used for conditioning surfaces and as an antimicrobial agent and emulsifier or as a multi-functional additive [54, 72].

The effectiveness of cleaning is primarily preconditioned by four factors: (1) chemical agents, (2) mechanical power, (3) temperature, and (4) duration of the procedure, which together form the Sinner circle [73]. The Sinner circle is described as an economically ideal cleaning process used for the optimization of interactions between these basic characteristics. The reduction of one of them must be compensated by the strengthening of another factor. However, the compensation cannot be applied without the knowledge of the specific causes and of the microorganisms that form the biofilm. For example, the combination of the effects of an EDTA chelating agent with ultrasound had an unambiguous synergistic effect on the release of a model biofilm formed by *E. coli.*

The effectiveness of the cleaning agents is of primary concern in the food industry because these remove the biofilm deposits and protect the surfaces by the effect of disinfectants [74]. During the cleaning stage, up to 99.8% of bacteria present on a stainless steel surface can be removed [75].

Naturally, the selection of the disinfection agent, or biocide, is equally important. Before selecting a biocide, the following questions should be answered: (1) how effective is it in the pH range of the sanitized environment, (2) how stable is it in a solution, (3) does it evaporate, (4) is it toxic, irritating, or safe, (5) what is the range of its effectiveness, (6) is its activity related to temperature, (7) does it causes corrosion of the sanitized surfaces, (8) is it surface-active, (9) how stable is it in the reactions with organic materials, and finally, (10) what is its effectiveness relative to the price [76,77].

In human medicine, therapy resistant, recurrent and chronic nosocomial infections caused by Staphylococci have been associated with the growth of these bacteria in biofilms [78].

A low rate of bacterial multiplication with antimicrobial testing was reported [79] in biofilm-associated bacteria that were treated with antimicrobial agents. The effectiveness of the antimicrobial agents is dependent on the multiplication of micro-organisms due to the bactericidal effect of antibiotics. For example, penicillins and cephalosporins have almost no effect on cells that are not multiplying, and the bactericidal effect is proportional to the multiplication of bacterial cells. Several classes of antibiotics, including fluoroquinolones and aminoglycosides, however, are most effective in rapidly dividing cells.

Increasing evidence indicates that biofilm formation by *S. aureus* at the site of an infection also explains the apparent resistance to therapy of *S. aureus* isolates causing bovine mastitis [57, 58, 65, 80]. It has been demonstrated that both *S. aureus* isolates obtained from bovine mastitis and clinical *S. aureus* isolates from humans are 10–1000 times more resistant to antimicrobial agents when growing in a biofilm than the same isolate growing in planktonic (free floating) form [3, 57, 58, 81]. Although there are several tests that have been developed to determine the susceptibility of bacteria growing in biofilms to antimicrobials, including the MBECTM assay (Innovotech Inc.,1 Edmonton, Canada), these assays are not yet considered reliable enough for routine application.

In all cases, the 24 h biofilm susceptibility test resulted in a Minimal Biofilm Eradication Concentration (MBEC) that was higher than the concentration that can be reached in vivo, indicating that all strains were almost identically therapy resistant. This observation, together with the results obtained during several clinical trials, which demonstrated that the chance of a positive therapy outcome increases with a longer duration of the therapy [67, 68, 69] led to the development of the extended MBEC assay.

Table 1 Examples of pathogens and antibiotic resistance in veterinary medicine

Bacterial organism	Animal species	Disease/Infection	Antibiotic resistance
Acinetobacter baumannii	Horse	Jugular catheter infection	Amoxicillin/ clavulanic acid
Actinobacillus spp	Horse	Post-operative infection	Penicillin
Klebsiella sp	Horse	Musculoskeletal infection	Ampicillin/ Amoxacillin/Clavulanic acid
Pseudomonas aeruginosa	Dog	Otitis	Amoxacillin/ acid clavulanic
Staphylococcus aureus	Cow	Mastitis	Amoxacillin/ampicillin/lincomycin/penicillin
Staphylococcus epidermidis	Horse	Post-operative infection	Methicillin
Staphylococcus intermedius	Dog	Pyoderma	Ciprofloxacin

Source: Adapted from Clutterbuck et al. (2007).

6. Conclusion

Biofilms will remain a major challenge in health care in the near future. They are still an important cause of morbidity and mortality. Frequently, the only solution for dealing with biofilms in veterinary medicine is through the development of surfaces and coatings that can eradicate microorganisms in an active way. It is also important to maintain an aseptic environment, and a large number of methods have been developed toward this goal in recent years. Ideally the antimicrobials should be long-lasting or permanent and their mode of action should most likely function throughout multiple pathways, so that the development of resistance, as in the case of antibiotics, ultimately does not occur.

Promising technologies that incorporate novel approaches, such as enzymes, phages and peptides that remove biofilms and enhance antimicrobial activity, also seem to provide useful approaches for the future. The light-activated antimicrobials offer particular promise because they function by generating reactive oxygen species that act on multiple targets within microbes.

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