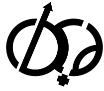 Rev. Bras. Reprod. Anim., Belo Horizonte, v.41, n.1, p.74-80, jan./mar. 2017. Disponível em www.cbra.org.br

Current understanding of bacterial biofilms and latent infections: A clinical perspective

Conceitos atuais sobre biofilmes e bactérias latentes em casos clínicos de endometrite

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Abstract

Subfertility in the mare can be frustrating to the clinician. Recent data has suggested that bacteria in either a biofilm or latent state may be responsible for explaining many cases of subfertility. Recent work has identified how the pathophysiology for these type of chronic infections in the horse and identified the best methods for therapy. The goal of this discussion is to explain the pathophysiology of biofilm and latent infections and how the clinician can diagnose and treat these type of infections in clinical practice.

Keywords: bacteria, biofilm, latent, chronic, endometritis.

Introduction

Most encounters between bacteria and the equine endometrium lead to an acute period of subclinical infection and occasionally clinical symptoms. Following an acute infection in the majority of mares the invading bacteria will be eliminated and the infection resolved. However, in a minority of cases, small numbers of bacteria survive and cause persistent infections that can be difficult to eliminate. The development of acute and chronic cases of endometritis is the result of deficiencies in the mare's ability to eliminate an infection and the causative bacteria's unique pathogenic properties.

The mare's uterine defense mechanisms to bacterial infection are well understood and consist of physical, immunological, and mechanical barriers (LeBlanc and Causey 2009). Bacteria utilize numerous methods to survive degradation by the host immune system and antibiotic therapy. One survival tool utilized by bacteria is the production of a biofilm. Biofilms allow bacteria to be unrecognized by the host immune system, prevent exposure to antibiotics, and allow for exchange of genetic material leading to antibiotic resistance (Donlan and Costerton 2002).

The purpose of this review is to describe how alterations to host defenses in combination with the pathogenicity of bacteria result in chronic cases of bacterial endometritis.

Pathophysiology

Host defense mechanisms

The mare has three main defense mechanisms to prevent bacterial infections in the uterus, physical barriers of the reproductive tract, the innate immune system, and mechanical uterine clearance. The physical barriers include the vulva, vagino-vestibular sphincter, and cervix. These barriers prevent feces, air and environmental pathogens from reaching the uterus. A reduction in the pathogenicity and quantity of bacteria occurs from the vulva to the cervix. Any disturbance in conformation of the reproductive tract will increase the likelihood of bacteria entering the uterus (Ricketts and Barrelet, 2001). Consequently, this results in a decrease in pregnancy rates. Once bacteria have reached the uterus the mare's innate immune system is activated.

The presence of bacteria within the uterine lumen results in a rapid influx of neutrophils, immunoglobulins, and serum proteins. This binding of complement and opsonins to bacteria greatly increase the ability and rate at which neutrophils phagocytize bacteria. Neutrophils from susceptible mares have reduced in vitro ability to phagocytize bacteria as compared to resistant mares. The inflammation associated the innate immune system results in fluid production into the uterine lumen (Troedsson et al., 1993a; Ohman et al., 2011; Troedsson et al., 1993b).

The final defense mechanism against bacterial endometritis is mechanical uterine clearance of bacteria and inflammatory products. Several studies have shown that mares susceptible to uterine infections have decreased clearance of uterine fluid as compared to resistant mares. After intrauterine inoculation with bacteria susceptible and resistant mares have similar uterine myometrial contractions for 6-8 hours post inoculation, but depresses in susceptible mares after 8 hours (LeBlanc et al., 1989; Troedsson et al., 1993; Troedsson et al., 1993c). Failure to clear bacteria and inflammatory products from the uterus, results in continued activation of the innate immune system. Resulting in a further increase in inflammatory cells, immunoglobulins, and serum proteins reaching the uterus that continue to activate the innate immune system.

A single alteration to any of the defense mechanisms of a mare may allow for colonization of the uterus with a bacterial pathogen leading to a chronic infection.

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Bacterial lifestyle

Bacterial are capable of living in two different lifestyles planktonic or biofilm states. Planktonic bacteria are single bacterial cells free flowing in suspension. Bacteria in this lifestyle are utilizing available nutrients for procreation. These individual cells are relatively susceptible to recognition and degradation by the host immune system, susceptible to changes in environment (desiccation, lack of nutrients, etc), and sensitivity to antibiotics. However, the planktonic cell paradigm does not accurately reflect the growth of bacteria in nature associated with a biofilm (Donlan and Costerton 2002).

In the last several decades the biofilm state has been considered to be the more prevalent lifestyle with ~99% of the overall world bacterial biomass living in a biofilm. In natural environments these biofilms are invariably a multispecies microbial community harboring bacteria that stay and leave with purpose, share their genetic material at high rates and fill distinct niches within the biofilm.

The first step in biofilm formation is migration and adherence to a surface. This is typically performed through the use of flagella and type IV pili in *E. coli, P. aeruginosa, and K. pneumonia. Strep. equi* subsp. *zooepidemicus* bacteria are non-motile and rely on movement from environmental or host factors. Individual bacteria will migrate (if capable) until other bacteria (same species or other) are encountered and micro-colonies start to form. At this point planktonic and biofilm lifestyles start to diverge, genes associated with flagella are down regulated and genes associated with polysaccharide production increase. This exopolysaccaride matrix forms the scaffold for the biofilm community.

As the community of bacteria grows in size the environment within the biofilm because heterogeneous with higher concentrations of oxygen and a more neutral pH on the outside of the biofilm as compared to the core which is relatively low in available oxygen with a slightly acidic pH. Bacteria are not randomly distributed within a biofilm but rather organized to best meet the needs of individual and the group.

Intercellular communication or quorum sensing is carried out through the production of bacterial products that are able to diffuse away from one cells and enter another cell. Signaling between cells is critical in the development of a viable biofilm and in reacting to outside environmental stress.

One of the biggest advantages of biofilm living is the ability to acquire transmissible, genetic elements at accelerated rates. Conjugation occurs naturally among bacteria but appears to be accelerated when bacteria are in a biofilm lifestyle. This allows for the rapid horizontal transfer of genetic material making a biofilm a perfect milieu for emergence of new pathogens by acquisition of antibiotic resistance, virulence factors and environmental survival capabilities.

Clinically biofilms can cause significant difficulty for clinician to eliminate these chronic infections once established. Bacteria within a biofilm are protected from the host immune system as white blood cells have reduced ability for movement and function, and the thick layer of exopolysaccaride (EPS) prevents antibodies from reaching bacteria deep within the biofilm (Jefferson et al., 2005; Brown et al., 1988; Anwar et al., 1992; Stewart and Costerton 2001; Borriello et al. 2004; Shah et al. 2006). Biofilms protect bacteria from antibiotics by providing a diffusion barrier that decreases the amount of antibiotics that reach the protected bacterial colonies and creates a microenvironment that slows down the metabolism and therefore the replication rate of bacteria, which also makes them more resistant to antimicrobial agents (Stewart and Costerton 2001; Anderl et al., 2000; Costerton et al., 1999; Donlan, 2011; Mah and O'Toole 2001).

As antimicrobial agents come in contact with the biofilm, the agents must traverse through a layer of thick EPS, DNA, RNA, lipids and proteins in order to reach bacteria buried deep within this protective barrier. Bacteria in the outer region may be killed, but a decrease in the level of antibiotics reaching the inner layer bacteria contributes to the formation of a nidus for chronic infection.

The thick layer of EPS found in biofilms not only prevents antibiotics from penetrating, but limits the diffusion of oxygen and nutrients. Oxygen and nutrient deprivation consequently results in a decrease in metabolic rate as compared to planktonic or free individual bacteria. This reduction in metabolic rate provides additional antimicrobial resistance as antibiotics typically only act upon rapidly multiplying bacteria (Brown et al., 1988; Chiang et al., 2012; Williamson et al. 2012; Walters et al. 2003).

The innate factors of antimicrobial resistance in bacterial biofilms have led to significant challenges in human medicine. It is estimated that 65% of nosocomial infections are associated with biofilms, and that treatments for biofilm based infections cost >$1 billion annually. In equine medicine, we have just started investigating the role of biofilms in chronic infections (Licking 1999).

It has been proposed that biofilms play an important role in chronic infections in the horse including chronic uterine infections resistant to antimicrobials may be due to biofilm production. Acute and chronic non-healing wounds on the distal equine limb contained a significantly greater incidence of biofilm producing bacteria as compared to a skin sample near the wound.

Biofilms in the horse

Evaluation of bacteria isolated from the equine uterus suggests that the majority of isolates of *Streptococcus equi* subsp. *zooepidemicus, Escherichia coli, Pseudomonas aeruginosa,* and *Klebsiella pneumonia* are capable of producing a biofilm *in vitro* (Ferris et al., 2016; Ferris 2014; Ferris et al., 2014; Loncar et al., 2015; Beehan et al., 2015). In the horse using a model of infectious endometritis a biofilm involved infection has been clearly identified. The adherent biofilm material is multi-focal with the greatest adherent occurring between the tissue folds and in the uterine horns. The bacteria are at greater numbers deep in the endometrial glands as compared to the luminal surface. Treatment options may need to be able to penetrate deeper into the glands and tissue to effectively clear these infections. There is an alteration in the host immune response with reduced PMNs surrounding areas of adherent biofilm as compared to areas free of bacteria (Ferris et al. 2016). Unfortunately, no clinical diagnostic tests are available for the detection of a biofilm related infection. In human medicine a biofilm is suspected if appropriate antibiotic therapy is administered and the infection is unable to be eliminated.

Persister cells and Infections

Persister cells represent about 1% of all bacteria in a free-floating state, an are characterized by being tolerant to antibiotics with no change in genetic expression. It is often thought that these bacteria are potentially dormant and metabolically inactive. This phenomenon was originally described in the 1940’s in that cultures of *Staphylococcus aureus* exposed to lethal doses of penicillin resulted in <1% of the original CFUs surviving penicillin exposure. While this work was conducted before genetic sequencing was available the authors did not feel the acquired antibiotic resistance was due to a mutation in the bacteria as subsequent culturing and exposure to antibiotics resulted in continued susceptibility of these previous tolerant colonies.

Latent bacteria in the horse

In the mare it has been clearly identified that some mare can have a population of dormant *Streptococcus equi* subsp *zooepidemicus* deep in the uterine glands. This population of bacteria would not be identified on routine culture (not actively dividing bacteria) or cause significant inflammation or infection. However, if these bacteria were to leave this dormant stage the resulting bacterial growth will result in inflammation and infection leading to pregnancy loss.

Treatment For Latent Bacteria

The goal for treating mares with latent or dormant bacteria is to get the bacteria to move from the dormant state into a metabolically active state in which identification and treatment can be performed. Recent work by Petersen et al. (2015) has shown that dormant *Streptococcus zooepidemicus* can be activated by infusing a proprietary media (bActivate) into the uterus. After the infusion (24 hours) 64% (15/25) mares were culture positive for *Streptococcus zooepidemicus* as compared to 8% (1/12) mares infused with PBS (Petersen et al., 2015). The proprietary media is capable is able to get bacteria to convert from the dormant state to a metabolically active state and treatment can be initiated.

Interestingly, it should be noted that breeding also may result in bacteria being activated from a dormant state. Recent work by Christoffersen et al. (2015) showed that 55% (16 of 29) of mares with a negative culture prior to breeding but retained fluid post breeding were positive for growth of *Streptococcus zooepidemicus*. The authors conclude that it was more likely to be dormant *Streptococcus zooepidemicus* that was reactivated as compared to introduction at the time of breeding (Christoffersen et al., 2015). The development of post mating fluid in barren mares could be due to both inflammation from breeding but also reactivation of dormant bacteria.

Treatment Options For Biofilms

Bacteria residing in a biofilm can be up 1000 times more resistant to treatment with antibiotics as compared to free-living (planktonic) bacteria. The simple administration of antibiotics has been unable to eliminate chronic infections suspected of involving a biofilm in both human and veterinary medicine. The goal in treating a biofilm associated infection is to remove the biofilm material and kill the bacteria residing within the biofilm.

We have tested various products for efficacy to disrupt a biofilm or kill the bacteria within a preformed biofilm. This is an *in vitro* assay looking at the effects of these agents specifically on bacteria residing in a biofilm and the effects observed.

N-acetylcysteine

Treatment with 3.3% NAC significantly ($P < 0.05$) reduced the number of CFUs in all *P. aeruginosa* isolates evaluated, and no significant reduction in biofilm biomass was observed. The *E. coli* and *S. zooepidemicus* isolates had a significant reduction in CFUs and biofilm biomass as compared to the untreated control. The MIC for N-acetylcysteine was found to be 3.3% dilution to 1.6% did not disrupt preformed biofilms or kill the bacteria within the biofilm. Treatment with NAC did not reduce biofilm biomass or reduced the number of CFUs for *K. pneumoniae*.

Hydrogen Peroxide

Challenge with 1% hydrogen peroxide significantly reduced the number of CFUs for 9 of the 10 *K. pneumoniae* isolates, but did not reduce the biofilm biomass. *E. coli* isolates were significantly reduced in biofilm biomass and had a reduction in CFUs following challenge. *P. aeruginosa* isolates had reduced biofilm biomass in five isolates but no reduction in CFUs following treatment with 1% hydrogen peroxide (Table 1). *S. zooepidemicus* isolates had significantly reduced biofilm biomass and CFUs as compared to untreated controls. Dilution to 0.5% was found to be just as effective as a 1% concentration.

Chelating Agents. No significant difference in biofilm biomass or CFUs was observed following a 6h challenge with either Tris-EDTA (50 mM and 3.5 mM, respectively) or THAM-EDTA (20 mM and 8 mM, respectively) for isolates of *P. aeruginosa*, or *K. pneumoniae*. *E. coli* had a significant reduction in biofilm biomass following challenge with Tris-EDTA or THAM-EDTA. *S. zooepidemicus* isolates had significantly reduced biofilm biomass and CFUs as compared to untreated controls. Dilution from recommended concentrations failed to disrupt a preformed biofilm or kill the bacteria within the biofilm.

Hypochlorous acid

No significant reduction in biofilm biomass or CFUs was observed following a 6 hour challenge with either Vetricyn® (Vetricyn, Riverside Ca) or Omniphase® (Integrated Healing Technologies, Franklin TN) for *P. aeruginosa, E. coli, or K. pneumonia.* Isolates of *S. zooepidemicus* had a significant reduction in biofilm biomass and CFUs following challenge with Vetricyn® or Omniphase®.

Antimicrobial Peptide Mimic

A significant reduction in biofilm biomass was observed for isolates of *E. coli and K. pneumoniae* but no change in CFUs was detected following challenge. In *P. aeruginosa* 50% of isolates had reduced biofilm biomass, but none of these isolates had decreased CFUs. Ceragyn® (Ceragyn, Spanish Fork UT) effectively reduced the biofilm biomass and reduced the number of CFUs for isolates of *S. zooepidemicus*. For *K. pneumoniae* and *E. coli* isolates we were able to dilute this product to 1.5%, and 0.5%, respectively and still maintain equal disruption and killing to the recommended concentration.

Combination Antibiotic and non-antibiotic therapy

No significant changes in biofilm biomass or CFUs was observed following a 6 hour challenge with ozone for *E. coli, K. pneumonia, P. aeruginosa,* and *S. zooepidemicus.*

A series of *in vitro* studies were conducted to assess biofilm dispersal and/or bacterial killing for antibiotics and non-antibiotic agents alone or in combination against Gram-negative bacteria. Our data indicates that antibiotics and non-antibiotic agents are more effective against biofilm if administered concurrently (i.e. in the same syringe). The attached table (Tab.1) explains how to make up clinical treatments for local infusion into the uterus based on the *in vitro* data. The amount of either antibiotic or non-antibiotic agent for each infusion are the minimum effective concentrations against *E. coli, K. pneumoniae* and *P. aeruginosa*. The treatment period should be at least 72 hours in duration, with repeated treatments every 24 hours (i.e. a uterine infusion of the selected combination once every 24 hours for 3 consecutive days). This treatment protocol resulted in complete biofilm dispersal and bacterial killing *in vitro*.

It is important to note that some non-antibiotic agents and antibiotics should not be combined in the same syringe. For example, the *in vitro* data indicated that mixing acetylcysteine with antibiotics in the same syringe resulted in reduced activity of the antibiotics.

We recommend antibiotic sensitivity testing for all Gram-negative organisms. Bacteria inherently resistant to an antibiotic will still be resistant when that antibiotic is used in combination with a non-antibiotic agent.

Table 1. Antibiotic and non-antibiotic combinations for the treatment of biofilm associated bacterial endometritis in mares.

Tris EDTA- final concentration in the syringe should be 50 mM Tris and 3.5 mM EDTA
Note: Tris-EDTA and Tricide are similar; however Tricide is not equivalent to Tris-EDTA in regards to bacterial killing
To make Tris-EDTA: 16oz bottle of Dechra Triz-EDTA crystals; add 8 oz of sterile water (this is different than the bottle instructions). The 2x concentration of Tris-EDTA solution will be further diluted by the antibiotics below to the proper final concentration.

Antibiotic	**Drug Amount**	**Tris EDTA**	**QS**	**Final volume**	**Notes:**
Amikacin (250 mg/ml)	4 mls (1 gram)	30 mls	16 mls sterile fluid (Saline, LRS, Sterile H_2O)	60 mls	10 mls of 8.4% sodium bicarbonate should be added to the amikacin
Ceftiofur (1 gram reconstituted in 20 mls)	20 mls (1 gram)	30 mls	10 mls sterile fluid (Sterile H_2O)	60 mls	
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	40 mls	0	80 mls	Split between two syringes

H_2O_2- 1% final concentration in the syringe
A 3% stock solution is available at many drug stores and veterinary distributors

Antibiotic	**Drug Amount**	**H_2O_2**	**QS**	**Final volume**	**Notes:**
Amikacin (250 mg/ml)	4 mls (1 gram)	20 mls	26 mls sterile fluid (Saline, LRS, Sterile H_2O)	60 mls	10 mls of 8.4% sodium bicarbonate should be added to the amikacin
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	20 mls	0	60 mls	

DMSO- 30% final concentration in the syringe
99% stock solution is used for calculations below

Antibiotic	**Drug Amount**	**DMSO**	**QS**	**Final volume**	**Notes:**
Ceftiofur (1 gram reconstituted in 20 mls)	20 mls (1 gram)	20 mls	20 mls sterile fluid (Sterile H_2O)	60 mls	
Ciprofloxacin (10 mg/ml)	40 mls (400 mg)	20 mls	0	60 mls	

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