Current understanding of bacterial biofilms and latent infections
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Introduction
Most encounters between bacteria and the equine endometrium lead to an acute period of subclinical infection and occasionally clinical signs. 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. 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.

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. 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.

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 following inoculation, but contractions are depressed in susceptible mares after eight hours. Failure to clear bacteria and inflammatory products from the uterus, results in continued activation of the innate immune system and results 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.

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.

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 organized 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. 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. Ultimately, biofilms are associated with development and maintenance of ‘persister cells’.

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.

A popular theory currently is that growth of bacteria in biofilms produces ‘persister cells’. These cells are unique in that they do not appear to grow and are highly multi-drug resistant to a wide variety antimicrobials. Further work is warranted to understand the role of ‘persister cells’ in chronic infections and biofilms.

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.

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.

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 pneumoniae* are capable of producing a biofilm in vitro. However, to date in vivo biofilm production and identification has not occurred in the endometrium of the mare. 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.

**Persistor cells and infections**

Persistor cells represent about 1% of all bacteria in a free-floating state, 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.

In the mare it has been clearly identified that some mares 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.

**Conclusion**

Development of chronic infections is dependent upon a decrease in host susceptibility and the pathogenicity of causative bacteria. The idea of persistent bacteria and bacteria living within a biofilm are becoming solidified in human medicine and recent research is suggesting that these bacterial states play a significant role in chronic infections in equine reproduction.

**Selected references**

Ferris RA, McCue PM, Borlee GI, et al: In vitro efficacy of nonantibiotic treatments on biofilm disruption of gram-negative