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Bacterial endometritis: a focus on biofilms
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Abstract
Treatment of chronic bacterial endometritis with antimicrobials is often unsuccessful. Bacteria have developed multiple mechanisms of antimicrobial resistance, including the production of biofilm. Biofilms are an extracellular matrix produced by a community of bacteria that provide antimicrobial resistance through prevention of antibiotic diffusion into the community of bacteria, a decrease in the metabolism of bacteria that increase their resistance to antimicrobial agents, and ultimately cultivate a population of ‘persister cells’ that are multi-drug resistant. Additionally biofilms prevent recognition of the infection by the host immune system by altering the movement and function of white blood cells, and preventing antibodies from binding to bacteria. Treatment with buffered chelators or hydrogen peroxide is unable to routinely disrupt in vitro preformed biofilms.

Keywords: Equine endometritis, chronic, biofilm

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 causitive bacteria’s unique pathogenic properties.

The mare’s uterine defense mechanisms to bacterial 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 six to eight hours after inoculation, but depresses 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. 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.

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 utilize 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 that are typically 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. pneumoniae*. *Strep. equi* subsp. *zooepidemicus* 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 heterogenous 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 the 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 subpopulations 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.

LeBlanc and Causey have suggested that chronic uterine infections resistant to antimicrobials may be due to biofilm production. 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 (Ferris 2014, unpublished). 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.

Treatment options

Bacteria residing in a biofilm can be up 1000 times more resistant to treatment with antibiotics as compared to free-living (planktonic) bacteria. The administration of antibiotics has been unable to eliminate chronic infections suspected of involving a biofilm in both human and veterinary medicine.

Work in other species has shown that buffered chelating agents (tris-EDTA) may potentiate antimicrobials and break up biofilms. Gray et al showed that EDTA disrupted the lipopolysaccharide membrane of Pseudomonas aeruginosa through the binding of heavy metals. A concentration dependent effect was observed on solubilizing of the carbohydrates and phosphorus present in the cell wall. Essentially the EDTA is able to ‘poke holes’ in the bacterial plasma membrane increasing the cell permeability potentially making the organism more susceptible to antibiotic therapy. First generation tris-EDTA (0.5 M tris tromethamine and 3.5 M ethylene-diaminetetra acetic acid) has been shown to kill and/or decrease survivability of Pseudomonas aeruginosa isolated from the equine uterus.

For buffered chelating agents to have an effect they must come in contact with the bacterial cell wall and stay in contact with the bacterial cells wall to maintain effectiveness. It has been recommended to increase the infusion size to 200 to 500 mls to allow for dispersion completely through the uterus. Combination of tris-EDTA and antibiotics (amikacin, ticarcillin with clavulonic acid, and ceftiofur) decreases the minimal inhibitory concentration over treatment with tris-EDTA or antibiotics alone.

Tris-EDTA has been shown to induce dispersal and killing of preformed biofilms in laboratory strains of P. aeruginosa. However tris-EDTA has been unable to consistently disrupt preformed biofilm from clinical strains of P. aeruginosa isolated from the equine uterus using an in vitro model.
Hydrogen peroxide is a common anti-septic utilized in human and veterinary medicine to non-specifically cause lysis of bacteria. In equine reproduction a common concentration of 1% hydrogen peroxide has been proposed to be beneficial in chronic cases of endometritis. Using an in vitro system to evaluate P. aeruginosa preformed biofilms 1% hydrogen peroxide was capable of disrupting the biofilm in 50% of cases. (Ferris 2014, unpublished).

Conclusion
Development of chronic infections is dependent upon a decrease in host susceptibility and the pathogenicity of causative bacteria. If a biofilm is associated with these chronic infections it may be difficult to appropriately diagnose the infection and even if diagnosed treatment failure is common. Unfortunately, when a biofilm is suspected in cases of chronic endometritis classically recommended therapies are not efficacious at disrupting pre-formed biofilms. Further work is needed to investigate the effectiveness of various agents utilized in human medicine to disrupt biofilms produced by the bacteria that cause endometritis in the mare.

References