Biofilm and latent bacteria in equine uterus

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Abstract

Subfertility in the mare can be frustrating to the clinician. Recent data suggested that bacteria in either a biofilm or latent state may be responsible for certain cases of subfertility. Recent work has identified the pathophysiology for these types of chronic infections in the mare and best methods for therapy. The goal is to elucidate the pathophysiology of biofilm and latent infections and the diagnosis, and treatment of these types of infections in clinical practice.

Keywords: Mare, endometritis, biofilm, latent bacteria

Introduction

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

Mare's uterine defense mechanisms to bacterial infection are well understood and consist of physical, immunological, and mechanical barriers.[1] Bacteria utilize several 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 remain undetected by the host immune system, prevent exposure to antibiotics, and allow for exchange of genetic material leading to antibiotic resistance.[2]

Pathophysiology

Presence of bacteria within the uterine lumen results in a rapid influx of neutrophils, immunoglobulins, and serum proteins. Neutrophils from susceptible mares have reduced in vitro ability to phagocytize bacteria compared to resistant mares. Inflammation associated with the innate immune system results in fluid production into uterine lumen.[3-5]

Final defense mechanism against bacterial endometritis is mechanical uterine clearance of bacteria and inflammatory products. Mares susceptible to uterine infections have decreased clearance of uterine fluid compared to resistant mares. After intrauterine inoculation with bacteria, susceptible and resistant mares had similar uterine myometrial contractions for 6 - 8 hours postinoculation; however, the contractile activity decreased in susceptible mares after 8 hours.[6-8] 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.

Bacterial lifestyle-planktonic versus biofilm

Bacteria are capable of living in 2 lifestyles (planktonic or biofilm state). 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 (e.g., desiccation, lack of nutrients, and others), and sensitive to antibiotics. However, the planktonic cell paradigm does not accurately reflect the growth of bacteria in nature associated with a biofilm.[2]

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

First step in biofilm formation is migration and adherence to a surface. 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 (EPS) matrix forms the scaffold for the biofilm community.

Clinically, biofilms can cause substantial difficulty for clinicians to eliminate once these chronic infections are 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 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 that also makes them more resistant to antimicrobial agents. Ultimately, biofilms are associated with development and maintenance of subpopulations of 'persister cells.'[9-14]

As antimicrobial agents come in contact with biofilm, agents must traverse through a thick layer of 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 concentrations of antibiotics reaching the inner layer bacteria contributes to the formation of a nidus for chronic infection.

Thick layer of EPS in biofilms not only prevents antibiotics from penetrating, but limits diffusion of oxygen and nutrients. Oxygen and nutrient deprivation consequently result in a decrease in metabolic rate 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.[10,14-16]

It has been proposed that biofilms have an important role in chronic uterine infections resistant to antimicrobials due to biofilm production. Additionally, acute and chronic nonhealing wounds on the distal equine limb contained a significantly greater incidence of biofilm producing bacteria compared to a skin sample near the wound.[17]

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 (E.coli)*, *Pseudomonas aeruginosa (P. Aeruginosa)*, and *Klebsiella pneumonia* are capable of producing a biofilm in vitro.[18] In mares, using a model of infectious endometritis, a biofilm involved infection has been clearly identified. The adherent biofilm material is multi-focal with the greatest adherence occurring between the tissue folds and in uterine horns. Bacteria are at greater numbers deep in the endometrial glands compared to the luminal surface. Treatment options may need 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 compared to areas free of bacteria. Unfortunately, no clinical diagnostic tests are available for detection of a biofilm-related infection.[19] In human medicine, a biofilm is suspected if appropriate antibiotic therapy is given and the infection is unable to be eliminated.

Treatment options for biofilms

Bacteria residing in a biofilm can be up to 1,000 times more resistant to treatment with antibiotics compared to free-living (i.e., planktonic) bacteria. Simple treatment 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.

A series of in vitro and in vivo studies were conducted to assess biofilm dispersal and/or bacterial killing for antibiotics and nonantibiotic agents alone or in combination against gram-negative bacteria.[19] Data indicate that antibiotics and nonantibiotic agents are more effective against biofilm if treated concurrently (i.e., in the same syringe). Uterine infusions (based on the in vitro data) are provided (Table). Amount of either antibiotic or nonantibiotic agent for each infusion is the minimum effective concentrations against *E. coli, K. Pneumoniae*, and *P. aeruginosa*. Duration of treatment must be at least 72 hours, repeated 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 nonantibiotic agents and antibiotics should <u>not</u> be combined in the same syringe. For example, in vitro data indicated that mixing acetylcysteine with antibiotics in the same syringe reduced antibiotics activity.

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 nonantibiotic agent.

Latent bacteria or persister cells and infections

Persister cells, representing ~ 1% of all bacteria in a free-floating state, are characterized by tolerance to antibiotics with no change in genetic expression. It is often believed 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.[20] Although this work was conducted before genetic sequencing was available, 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

It has been clearly identified that some mares can have a population of dormant *Streptococcus equi* subsp. *zooepidemicus* deep in the uterine glands.[21] This population of bacteria would not be identified on routine culture (not actively dividing bacteria) or cause substantial inflammation or infection. However, if these bacteria were to leave this dormant stage after the establishment of pregnancy, the resulting bacterial growth will induce inflammation and infection leading to pregnancy loss.

Table. Antibiotic and nonantibiotic 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). Two x concentration of Tris-EDTA solution will be further diluted by the antibiotics below to the proper final concentration.

Antibiotic	Drug amount	Tris EDTA	Expansion volume	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 2 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	Expansion Volume	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	Expansion Volume	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	

Treatment for latent bacteria

Goal for treating mares with latent or dormant bacteria is to force the bacteria to move from the dormant state into a metabolically active state in which identification and treatment can be performed. Dormant *Streptococcus zooepidemicus* can be activated by infusing a proprietary medium (bActivate) into uterus.[22] At 24 hours after infusion, 64% (15/25) mares were positive for *Streptococcus zooepidemicus* compared to 8% (1/12) mares infused with PBS. Proprietary medium forced bacteria to move from the dormant state to a metabolically active state to initiate treatment.[22]

Interestingly, breeding may also activate dormant state bacteria, as 55% (16 of 29) of mares with a negative culture prior to breeding that retained fluid postbreeding were positive for *Streptococcus zooepidemicus*.[23] Authors concluded that it was more likely to be dormant *Streptococcus zooepidemicus* that was reactivated compared to introduction at breeding.[23] Development of post-mating fluid in barren mares could be due to inflammation from breeding and reactivation of dormant bacteria.

Conclusion

Overall, the incidence of biofilm or latent bacteria is unknown in the broodmare population. With relatively higher per cycle overall pregnancy rates in broodmares, one can suspect that the incidence rate of biofilm or latent bacteria is low. Biofilm and latent bacteria must be considered as a cause of subfertility in individual mares failing to become pregnant. Research has helped to understand the development of infections, improve diagnostic techniques, and provide effective treatment strategies for biofilm or latent bacterial endometritis.

Conflict of interest

None to declare.

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