Pferdeheilkunde 32 (2016) 1 (January/February) 46-48

Short communication

Equine endometritis and biofilm forming Escherichia coli

David P. Beehan, Dale Paccamonti and Sarah K. Lyle

Department Veterinary Clinical Sciences, Louisiana State University, Baton Rouge, Louisiana, USA

Summary: A common cause of chronic equine endometritis is Escherichia coli (E. coli), and its ability to form a biofilm within the uterus is a potential cause of persistent endometrial infections. Bacterial biofilm is defined as a community of microorganisms irreversibly attached to a surface, producing extracellular polymeric substance (EPS), exhibiting an altered phenotype and having increased resistance to innate immune responses and antibiotics. Our study objectives were to screen uterine E. coli for their biofilm forming potential (BFP) and to investigate antibiotic resistance of any identified BFP-E.coli isolates against commonly used equine intra-uterine antibiotics. The BFP of uterine E. coli isolates (n = 52) was evaluated using the Crystal Violet (CV) assay. E. coli were grown in Luria enrichment broth (LB) for 24 h at 37 °C, bacterial suspensions were diluted 1:100 with fresh LB, and 100- L aliquots were incubated in 96-well plates for 24 h. Plates were washed to remove free bacteria, air dried, 125 µL 0.5% CV stain was added to each well for 10 min, followed by washing to remove excess stain. Acetic acid (30%) was used to solubilize any remaining stain and the optical density OD570 of each well was measured. Based on the OD value, isolates are categorized as strong (OD > 0.4) moderate (0.4 ≥ OD ≥ 0.2) or weak (OD < 0.2) BFP isolates. The minimum inhibitory concentrations of strong BFP isolates (n = 9) for timentin, ceftiofur, gentamicin and amikacin (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL) in their planktonic state (PlankMIC) and in their biofilm state (BioMIC) was measured using the resazurin assay. Resazurin is a blue reagent that fluoresces red when reduced by metabolically active viable cells. The PlankMIC was measured by adding antibiotics and diluted bacterial suspensions to 96-well plates, incubating for 24 h at 37 °C, followed by the addition of 10 µl of resazurin, incubation at 37 °C for an additional 90 min, and the absorbance of each well was read at 590 nm. The BioMIC was determined by first allowing biofilm formation within wells for 24 h, followed by washing to remove free bacteria. Antibiotics and fresh LB was added to the biofilm-coated wells and the BioMIC evaluated as described for PlankMIC using the resazurin assay. Evaluated E. coli were classified as having strong (31%; 16/52), moderate (8%; 4/52), or weak (61.5%; 32/52) BFP. The combined median fold increase of BioMIC over PlankMIC for timentin, ceftiofur, gentamicin and amikacin was 8, 10, 8 and 7 respectively. These results show that 31% of uterine E. coli isolates demonstrate strong BFP in vitro and that strong biofilm-forming isolates in a biofilm state exhibit a large increase in antibiotic resistance in comparison to the isolate's planktonic state. In conclusion, by combining clinical history and evaluating E. coli BFP, we believe treatment strategies can be better optimized for mares with chronic endometritis.

Keywords: mare / reproduction / uterus / biofilm / equine / endometritis / Escherichia coli

---

Equine Endometritis und Biofilm formierende Escherichia coli

Eine häufige Ursache für die chronische equine Endometritis stellt Escherichia coli (E. coli) und seiner Fähigkeit, einen Biofilm in der Gebärmutter zu bilden, dar. Dies ist eine mögliche Ursache für eine persistierende endometriale Infektion. Bakterielle Biofilme sind definiert als Lebensgemeinschaft von Mikroorganismen, die irreversibel an eine Oberfläche gebunden sind, extrazelluläre polymere Substanzen (EPS) produzieren und die einen veränderten Phänotyp mit erhöhter Resistenz gegen angeborene Immunreaktionen und Antibiotika haben. Die Ziele unserer Studie waren es, uterine E. coli auf ihr Potential einer Biofilmbildung (BFP) zu überprüfen und die Antibiotikaresistenz von jedem identifizierten BFP-E.coli-Isolat gegen beim Pferd im Endometrium häufig verwendete Antibiotika zu untersuchen. Der BFP von uterinen E. coli-Isolaten (n = 52) wurde unter Verwendung des Kristallviolettassays (CV) ausgewertet. E. coli wurden in Luria Anreicherungsbrühe (LB) für 24 h bei 37 °C gezüchtet, die Bakteriensuspensionen 1:100 mit frischem LB verdünnt und 100 µl-Aliquots in 96-Well-Platten für 24 h inkubiert. Die Platten wurden gewaschen, um freien Bakterien zu entfernen, luftgetrocknet, 125 µl 0,5%iger CV Lösung in jedes Well für 10 Minuten zugegeben, gefolgt von einer weiteren Spülung, um überschüssigen Farbstoff zu entfernen. Essigsäure (30%) wurde verwendet, um jegliche verbleibende Färbung aufzulösen und die optische Dichte OD570 jedes Wells gemessen. Auf der Grundlage des OD-Wertes, wurden die Isolate als starke (OD > 0,4) moderate (0,4 ≥ OD ≥ 0,2) oder schwache BFP-Isolate (OD < 0,2) eingestuft. Die minimalen Hemmkonzentrationen starker BFP Isolate (n = 9) für Timentin, Ceftiofur, Gentamicin und Amikacin (0,25, 0,5, 1, 2, 4, 8, 16, 32, 64, 128, 256 und 512 µg/ml) in ihrem Planktonzustand (PlankMIC) und in ihrem Biofilm Zustand (BioMIC) wurde mit dem Resazurin-Test gemessen. Resazurin ist ein blaues Reagenz, das rot fluoresziert, wenn es von metabolisch aktiven lebenden Zellen reduziert wird. Die PlankMIC wurde durch Zugabe von Antibiotika und verdünnten Bakteriensuspensionen auf Platten mit 96 Wells, einer Inkubation für 24 h bei 37 °C, gefolgt von der Zugabe von 10 µ Resazurin, einer weiteren Inkubation bei 37 °C für 90 Minuten ermittelt, wobei die Extinktion jedes Wells bei 590 nm erfasst wurde. Die BIOMIC wurde bestimmt, indem sich erst die Biofilme im Well für 24 h bilden konnten, gefolgt von einer Spülung, um freie Bakterien zu entfernen. Antibiotika und frische LB wurden den mit Biofilm beschichteten Wells zugefügt. Die BioMIC-Auswertung erfolgte unter Verwendung des Resazurin-Assays, wie er für den PlankMIC beschrieben wurde. Die untersuchten E. coli wurden klassifiziert als starke (31%; 16/52), moderate (8%; 4/52) oder schwache (61,5%; 32/52) BFP. Der kombinierte mittlere Anstieg von BIOMIC gegenüber PlankMIC für Timentin, Ceftiofur, Gentamicin und Amikacin betrug 8, 10, 8 und 7. Diese Ergebnisse zeigen, dass 31% der equinen E. coli-Isolate starke BFP in vitro aufweisen und dass starke Biofilmbildende Isolate im Biofilmzustand eine erhebliche Zunahme einer Antibiotika-Resistenz gegenüber dem entsprechenden Isolat im Planktonzustand zeigen. Es wird der Schluss gezogen, dass die Behandlungsstrategien bei Stuten mit chronischer Endometritis optimiert werden können, indem die klinische Anamnese und die Auswertung der E. coli BFP kombiniert werden.

Schlüsselwörter: Stute / Reproduktion / Uterus / Biofilm / Pferd / Endometritis / Escherichia coli

---

Citation: Beehan D. P., Paccamonti D., Lyle S. (2016) Equine endometritis and biofilm forming Escherichia coli. Pferdeheilkunde 32, 46-48

Correspondence: David P. Beehan MVB (Hons), MS, DipACT, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana 70803, USA; Email: dbeehan@lsu.edu

Aim of the study

A common bacterial pathogen of the equine uterus is Escherichia coli (E. coli). Despite seemingly appropriate veterinary intervention E. coli diagnosed endometrial infections persist in a small proportion of mares, causing long-term infertility. The presence of bacterial biofilm within the uterus is now believed to be a cause of these unresponsive endometrial infections in the mare. In human medicine bacterial biofilm formation is a well-accepted cause of persistent unresponsive infection in a wide range of body systems, but to date limited studies have confirmed biofilm-forming bacteria as a cause of equine endometrial disease. Its role in equine uterine disease is suggested based on the strong clinical similarities between chronic endometritis cases and human biofilm-associated diseases (chronicity, recurrence and poor response to antibiotics). In addition uterine biofilms have occasionally been visualized and described by clinicians during equine hysteroscopic and transrectal-ultrasonographic, lending support to the theory of a biofilm involvement.

Bacterial biofilm is defined as a community of microorganisms irreversibly attached to a surface, producing extracellular polymeric substance (EPS) and exhibiting an altered phenotype (*Fux* et al. 2005). When a biofilm associated infection is present in a body location, the bacteria are protected by the EPS layer from innate immune responses and display a high resistance to antibiotics. In addition the biofilm bacteria display lower metabolic and growth rates (referred to as the biofilm state) and this allows bacteria to survive long periods in unsuitable stressful environments. When environmental conditions improve the biofilm cells can revert back to their free (planktonic) state and be released from the biofilm so as to colonize new surfaces and cause recurring disease episodes, e.g. recurring episodes of endometritis. To date, equine studies examining biofilm-associated disease have been limited. Bacteria from equine chronic wounds have been shown to possess a higher biofilm forming potential (BFP) than skin microbiota (*Westgate* et al. 2011). Also anti-biofilm therapies have been investigated in a limited number of equine reproductive pathogens (*Ferris* et al. 2014).

Table 1 Biofilm classification table | *Klassifikation der Biofilme*

Optical Density	Interpretation
≤ 0.2	Weakly adherent (Weak BFP)
0.2 - 0.4	Moderately adherent (Moderate BFP)
> 0.4	Strongly adherent (Strong BFP)

Our study objectives were 1) to screen a large number of uterine E. coli for their BFP and 2) to investigate antibiotic resistance of any identified biofilm-forming E. coli isolates against commonly used equine intra-uterine antibiotics.

Materials and methods

E. coli isolates (n = 52) were collected from mare uterine samples. The BFP of each isolate was evaluated using the crystal violet (CV) assay. The CV assay was performed by inoculating Luria enrichment broth (LB) with colonies from a single isolate of E. coli and incubating for 24 h at 37 °C in an orbital incubator (70 rpm). After incubation, bacterial suspensions were diluted 1:100 with fresh LB. After dilution, 100 µL was transferred into eight wells of a flat-bottom 96-well plate and incubated for 24 h at 37 °C in an orbiting incubator. At the end of incubation, plates were washed three times with sterile distilled water and allowed to air dry. After drying, 125 µL of 0.5% CV was added to each well and allowed to incubate at room temperature for 10 min. The plates were then rinsed three times with sterile distilled water, or until all excess stain was removed, and allowed to air-dry. Any remaining stain in each well was solubilized by adding 125 µL of 30% acetic acid and the optical density (OD570) of each well was used to quantify the biofilm biomass in each well. Based on the OD570 value, isolates were categorized as strong, moderate or weak BFP isolates (Table 1). An E. coli K-12 biofilm-forming strain was used as a positive control.

Nine isolates with strong BFP were selected for determination of planktonic (PlankMIC) and biofilm (BioMIC) minimum inhibitory concentrations using the resazurin assay. Resazurin is a blue reagent that is reduced in the presence of metabolically active viable cells to a fluorescent red color. Antibiotics evaluated were timentin, ceftiofur, gentamicin and amikacin at concentrations of 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL. The PlankMIC was measured by adding a 10-µl aliquot of a 10 × antibiotic dilution and 90 µl of 1:100 diluted bacteria into each well of a 96-well microtiter plate, resulting in the final desired antibiotic concentration. Following incubation for 24 h at 37 °C, 10 µl of resazurin solution was added to each well and then incubated at 37 °C for an additional 90 min. The absorbance of each well was read at 590 nm using an automated plate reader. The BioMIC was measured by adding 100 µl of 1:100 bacterial dilutions to each well of a 96-well plate and incubated for 24 h at 37 °C to allow biofilm formation to occur within the wells. At the end of incubation the wells were washed 3 times with sterile distilled water to remove any non-attached cells, followed by adding a 10-µl aliquot of a 10× antibiotic dilution and 90 µl of fresh LB broth being added to each well, and plates were incubated for a further 24 h at 37 °C. The BioMIC plates

Table 2 MIC results | *Ergebnisse der MIC (minimale Hemmkonzentration)*

	Planktonic MIC		Biofilm MIC		
Antibiotic	Range (µg/mL)	Median (µg/mL)	MIC range (µg/mL)	Median (µg/mL)	Fold Increase
Timentin	4 - 512	4	64 - ≥512	≥512	8
Ceftiofur	0.5 - 8	1	128 - ≥512	≥512	10
Gentamicin	2 - 256	4	≥512	≥512	8
Amikacin	4 - 16	8	≥512	≥512	7

were the evaluated using the resazurin assay as described for PlankMIC. Positive controls (100% reduced resazurin reagent), negative controls (bacteria free LB broth), and a positive quality control E. coli isolate (ATCC® 25922™) were included. The resazurin assay MIC was defined as the lowest antimicrobial concentration that resulted in a 90% reduction of resazurin stain when compared to the positive control value. All evaluations were performed in triplicate.

Results

Of the uterine E. coli isolates evaluated, 31% (16/52), 8% (4/52) and 61.5% (32/52) were classified as having strong, moderate and weak BFP, respectively, using the CV assay. The combined median fold increase of BioMIC over PlankMIC of the tested isolates for timentin, ceftiofur, gentamicin and amikacin was at least 8, 10, 8 and 7 respectively (Table 2).

Conclusion

The results show that 31% of E. coli isolates collected from the equine uterus demonstrate strong BFP in vitro and provide support for the theory that biofilm-forming bacteria could be a cause of chronic endometritis. A disadvantage of our study was that information on the reproductive status of the mare (e.g. routine pre-breeding culture or problem mare) from which each E. coli isolate collected was not available; therefore, establishment of an association between clinical status and in vitro biofilm formation was not possible. In people with clinically symptomatic E. coli-associated cystitis and prostatitis, 53% to 63% of isolates demonstrated in vitro biofilm formation using the CV assay (*Soto* et al. 2006, *Soto* et al. 2007).

The strong BFP isolates evaluated in this study showed a substantial increase in antibiotic resistance. Biofilm antibiotic resistance is due to a number of factors, including: physical and chemical barriers to antibiotic diffusion, slow turnover and metabolism of biofilm cells, activation of a general stress response, and a biofilm specific phenotype (*Mah* and *O'Toole* 2001). Current standard clinical microbiology laboratories determine the MIC of an antibiotic against planktonic bacteria in the exponential phase of growth and therefore predict antibiotic efficacy against rapidly dividing cells. Bacteria within a biofilm exist in a sessile state, with low rates of metabolism and cell division, making planktonic-based assays for antibiotic sensitivity evaluation unsuitable if biofilm is suspected to be present in the patient.

These findings delineate the importance of determining the BFP of E. coli isolated from problem mares to help optimize treatment strategies and effectively treat intra-uterine biofilm. In view of the high BioMIC of the isolates evaluated, it is imperative to consider non-antibiotic alternatives for the treatment of chronic bacterial endometritis. Combining clinical history and evaluation of E. coli BFP should optimize endometritis treatment strategies.

Acknowledgments

The authors would like to acknowledge Dr. William Doerrler for his advice and technical support. The authors would like to gratefully acknowledge the Equine Health Studies Program, School of Veterinary Medicine, Louisiana State University for project funding.

References

Ferris R. A., Wittstock S. M., McCue P. M., Borlee B. R. (2014). Evaluation of biofilms in gram-negative bacteria isolated from the equine uterus. Equine Vet. Sci. 34, 121

Fux C. A., Costerton J. W., Stewart P. S., Stoodley P. (2005). Survival strategies of infectious biofilms. Trends Microbiol. 13, 34-40

Mah T. F., O'Toole G. A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9, 34-39

Soto S. M., Smithson A., Horcajada J. P., Martinez J. A., Mensa J. P., Vila J. (2006) Implication of biofilm formation in the persistence of urinary tract infection caused by uropathogenic Escherichia coli. Clin. Microbiol. Inf. 12, 1034-1036

Soto S. M., Smithson A., Horcajada J. P., Martinez J. A., Mensa J. P., Vila J. (2007). Biofilm formation in uropathogenic Escherichia coli strains: relationship with prostatitis, urovirulence factors and antimicrobial resistance. Urology 177, 365-368

Westgate S. J., Percival S. L., Knottenbelt D. C., Clegg P. D., Cochrane C. A. (2011). Microbiology of equine wounds and evidence of bacterial biofilms. Vet. Microbiol. 150, 152-159