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Research Article

ANTIBIOTIC SUSCEPTIBILITIES OF BIOFILM PRODUCING BACTERIA ISOLATED FROM HORSE WOUNDS

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ABSTRACT: The occurrence of wounds in horses is very high due to their flight instinct and the nature of their environment, however, the high risk of infection by environmental pathogens complicate the healing process with most wound becoming chronic. Biofilm formation has been identified as a major consequence of infected wound, implicated in chronic non-healing wound. In this study, we demonstrated biofilm forming bacteria in horses wound and described their antibiotics susceptibility. Swab samples from wound of 30 horses were cultured and the biofilm forming potential of the bacteria isolate was assessed. The susceptibility of the biofilm state to ciprofloxacin (CIP), gentamycin (CN) and tetracycline (TE) were also determined. A total of 65 bacterial isolates were identified from the wound, of which 48 prominent bacteria isolates were tested for BFP with 8.3% being strong biofilm formers, 6.3% moderate, 68.7% weak and 16.7% non-biofilm formers. There was significantly (P<0.05) higher minimum biofilm eradication concentration (MBEC) for the three antibiotics tested against the biofilm formers than the minimum inhibition concentration (MIC) required to inhibit the bacteria growth in their planktonic state. The MBEC was highest for the strong biofilm formers, follow by moderate and weak biofilm formers. CIP has the least MBEC for all the isolates tested. In conclusion, there is presence of bacteria biofilm in equine wound and irrespective of the type of biofilm formers, susceptibility to antibiotic is low as higher antibiotics concentrations is required to eradicate the bacteria in biofilm state.

Key words: Bacterial biofilms, Horse, Wound, Antibiotic susceptibility.

INTRODUCTION

The occurrence of wounds in equine species is quite high due to their flight instinct and the nature of their environment (Sole *et al.* 2015, Theoret *et al.* 2016). The wound can either be due to traumatic injuries or following surgical intervention (Knubben *et al*. 2008). However, traumatic injury is the most common causes of wound in horses, and most often occur in the lower limbs due to laceration, abrasion or puncture from foreign objects. As such managing wound has become an integral part of equine veterinary practice and a major challenge to the equine veterinarians (Carter *et al.* 2003).

Wound in horses has a high risk of becoming infected either by direct introduction of bacteria from the foreign material causing the injury or penetration and colonization of the wound by the normal microbiota of the skin or from the environment (Westgate *et al*. 2011). The infection of the wound complicate healing with most wound becoming chronic non-healing, thus making the affected horses losing its ability to perform, retire from athletic activity or even euthanasia (Owen *et al*. 2012, Sànchez-Casanova *et al*. 2014).

The difficulty of managing wound in horses, particularly those in the lower limbs, with most becoming chronic non-healing wound have been attributed to poor blood circulation, frequent joint movement and minimal soft tissue between skin and bone (Quinn 2010). However, there are now evidences of bacteria biofilm formation in chronic wound development. Importantly, biofilm formation has been identified as a major consequence of

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infected wound, be it surgical or traumatic caused, implicated in chronic non-healing wound in human (Percival 2017). In veterinary medicine, the development of biofilms has been related to difficulty in managing intravenous jugular catheters and post-surgical infections, canine pyoderma and bovine mastitis (Lloyd *et al.* 1999, Vaneechoutte *et al*. 2000). However, the possibility of biofilm responsible for non-healing of equine wound is now been reported (Jørgensen *et al.* 2017, König *et al*. 2015, Westgate *et al.* 2011).

Bacterial biofilms are communities of complex microbes that adhere to a surface or each other and survive within a self-synthetized matrix of extracellular polymeric substances (Costerton *et al*. 2003, Flemming and Wingender 2010). The structural arrangement of bacteria biofilm, particularly the extracellular polymer matrix, provides protection for the bacteria against any detrimental conditions within its immediate surrounding including antimicrobial agents (Stewart and Costerton 2001, Percival *et al*. 2010). Consequently, with biofilm formation the wound contaminants (microorganism) that are readily susceptible to antimicrobial agents become protected within the biofilm extracellular polymer matrix, leading to chronic non-healing wound. Furthermore, bacterial biofilm in equine wound causes great distress in terms of wound management and burden to the equine practitioners (Westgate *et al*. 2010). Although, evidence of biofilm from equine wound infection has been documented, information on biofilm susceptibility to antibiotics is still lacking for equine chronic wound. Therefore, the determination of biofilm susceptibility level to common antimicrobial agents will proffer guide on choice of clinically effective antibiotics. The objectives of this study were, therefore, to demonstrate biofilm forming bacteria in horses wound and determine their antibiotics susceptibility.

MATERIALS AND METHODS

Sample collection

Thirty horses with traumatic wound from Kelantan and Terengganu, East coast of Peninsular Malaysia were included in this study. The wounds were classified into acute and chronic. Acute wounds are those that follow normal healing process without any signs infection while chronic wounds are those that do not follow normal healing progress and are more than a month old without evidence of healing. None of the horses were treated with either antibiotics or antiseptics in the last one week prior to sample collection. For each horse, two swab samples were collected: one from the wound and another from intact skin around the anatomical location of the wound. The wound surface and the intact skin were initially irrigated with sterile saline before the swabbing. The swabbing of the wound was done using Levine technique by sterile swabs stick (Amies, Italy). The location of the wound on the horses includes the lower limbs, abdominal region, neck and the face regions. The horses comprise of different breed, namely thoroughbred, warmblood, Arabian and polo pony, and their age range from 2 to 15 years. The study was consented to by horse owners and approved by Institutional Animal Care and Use Committee (IACUC) Universiti Putra Malaysia, (R037/2014).

Bacterial isolation and identification

The swab samples were enriched in Tryptone Soya Broth (Oxoid, UK) for 24 hours at 37°C prior to culturing on blood (7% horse blood, Oxoid, UK) and MacConkey (Oxoid, UK) agar. All the agar plates were incubated aerobically at 37°C for 18-24 hours. Colonies of isolates were sub-cultured onto Trypticase Soy Agar (Oxoid, UK) to obtained pure cultures. Identification of pure bacteria colony was done based on cellular morphology, gram staining, and biochemical tests.

Biofilm formation assay

The potential of the cultured bacteria in forming biofilm were tested on the predominant bacteria isolates: *Escherichia coli*, *Enterobacter* spp., *Acinetobacter* spp., *Staphylococcus* spp. and *Streptococcus* spp. from the wound culture, using previously described method with few modifications (Wakimoto *et al*. 2004). Briefly, 150µL freshly grown bacteria isolate(s) (10^8 cfu mL^{-1}) in Muller Hinton broth were dispensed into sterile 96 well plates and incubated in an orbital incubator (80 rpm) at 37°C for 24hours. After incubation, the wells were empty and rinsed three times with sterile saline to remove the non-adherent bacteria. The wells were then filled with 175 µL 96% ethanol to fix the attached biofilm and then stained with 2% crystal violet for 10 minutes at room temperature. The wells were then washed three times with sterile distilled water. Presence of visible ring lined inside the wall of wells was considered positive for biofilm formation. After drying the stained biofilm for 1 to 2 hours by placing the plate in inverted position, the wells were filled with 175 µL of 33% acetic acid and optical density (OD) of the solubilised stained biofilm was measured at 550 nm using ELISA microtiter plate reader (Sunrise Tecan, Switzerland). All isolates and controls were tested in triplicate. The isolates were then classified into non biofilm former if OD_{550} is less than 0, weak biofilm former (OD_{550} = 0.2), moderate biofilm former (0.2 < OD_{550} =

Table1. Number and percentages of bacteria isolates from equine wound and intact skin swab samples.

	Bacteria	Wound samples		Intact Skin samples		
		Acute	Chronic	% isolates	No. of isolates	% isolates
Gram-negative	*Escherichia coli*	5	12	26.2	5	8.8
	Enterobacter spp.	8	5	20.0	12	21.1
	Acinetobacter spp.	1	2	4.6	7	12.3
	Aeromonas spp.	1	1	3.1	1	1.8
	Providencia spp.	1	1	3.1	2	3.5
	Klebsiella spp.	1	1	3.1	2	3.5
	Pseudomonas spp.	0	2	3.1	2	3.5
	Citrobacter spp.	0	1	1.5	2	3.5
	Stenotrophomonas spp.	0	0	0	2	3.5
	Serratia spp.	1	0	1.5	0	0
	Chromobacterium spp.	0	1	1.5	0	0
	Yersinia spp.	0	1	1.5	0	0
	Vibrio spp.	1	0	1.5	0	0
Gram-positive	*Staphylococcus* spp.	6	5	16.9	14	24.6
	Streptococcus spp.	0	4	6.2	2	3.5
	Corynebacterium spp.	2	0	3.1	3	5.2
	Bacillus spp.	2	0	3.1	3	5.2
TOTAL		29	36	100	57	100

0.4) and strong biofilm former (OD_{550} = 0.4) (Stepanovic *et al*. 2004).

Minimum inhibitory concentration (MIC) and Minimum biofilm eradication concentration (MBEC) assay

Antimicrobials susceptibility of the biofilm bacteria and their planktonic state to ciprofloxacin (CIP), gentamicin (CN) and tetracycline (TE) was determined by minimum biofilm eradication concentration (MBEC) and minimum inhibitory concentration (MIC) assay respectively. The MIC assay was done using the Clinical Laboratory Standard Institutes (CLSI 2017) broth microdilution method. For MBEC assay, the bacteria biofilm was first grown in sterile 96 well plates as previously described. The bacteria biofilm was then challenged with about 175 µL of two-fold dilution of each antibiotic (concentration tested range between 0.015 ìg/mL and 2048 ìg/mL) and incubated at 37°C for 24 hours. Following incubation, antibiotic solutions were discarded, and the plates washed 3 times with sterile saline. Each well was then filled with 175 µL MHB for biofilm recovery and then sonicated for 1 minute (Ultrasonic sonicator, J.P Selecta, Spain), before the OD each plate was read at550 nm using ELISA microtiter plate reader (Sunrise Tecan, Switzerland).

Statistical analysis

Descriptive statistic was used to express the distribution of cultured isolates. The possible BFP difference between the gram negative and positive bacteria isolates was assessed using Chi-square test while Student t-test was used to compare MIC and MBEC of each antibiotic. One-way ANOVA was used to test the susceptibility difference among the strong, moderate and weak biofilm formers. Statistical analyses were performed using Graph Pad Prism version 8 with $P < 0.05$ considered as significant.

RESULTS AND DISCUSSION

Bacteria isolation and biofilm formation

A total of 122 bacteria isolates comprises of 65 isolates from wound swab samples and 57 isolates from intact skin swab samples were cultured (Table 1). Of the 65 isolates from the wound samples, 29(44.6%) are from acute wound and 36(55.4%) from chronic wound.

Table 2. Biofilm ability of bacterial isolates from equine wounds in microtiter plate assay.

Organism	No of Isolates	Number (%) isolate from acute wound				No of Isolates	Number (%) isolate from chronic wound			
		Strong	Moderate	Weak	Non		Strong	Moderate	Weak	Non
Escherichia coli	5	0	0	3 (15.0)	2 (10.0)	12	1(3.6)	1 (3.6)	7 (25.0)	3 (10.7)
Enterobacter sp.	8	0	0	6 (30.0)	2 (10.0)	5	3 (10.7)	1 (3.6)	1 (3.6)	0
Acinetobacter sp.	1	0	0	1 (5.0)	0	2	0	0	2 (7.1)	0
Staphylococcus sp.	6	0	0	6 (30.0)	0	5	0	1 (3.6)	4 (14.3)	0
Streptococcus sp.	0	0	0	0	0	4	0	0	4 (14.3)	0
TOTAL	20	0	0	15 (80.0)	4 (20.0)	28	4 (14.3)	3 (10.8)	18 (64.3)	3 (10.7)

However, there was no significant difference in the occurrence of the isolates in acute and chronic wound as well as between wound and skin isolates. Similar to our findings, previous study in dogs, goats and sheep also reported no significant differences between the proportions of bacteria isolated from wound and intact skin (Bukar-Kolo *et al*. 2016).

The biofilm forming potential (BFP) of the five prominent bacteria: *Escherichia coli, Enterobacter* spp., *Acinetobacter* spp., *Staphylococcus* spp., *Streptococcus* spp. (48 isolates) cultured from the wound samples were assessed using microtiter plate assay (Table 2). Of all the 48 isolates tested, 68.7% displayed weak biofilm forming potential, 6.3% moderate biofilm former, 8.3% strong biofilm former, and 16.7% non-biofilm former. When compared isolates from acute and chronic wound, 80% of isolates from acute wound are weak biofilm formers. However, for the 28 isolates from chronic wound, 14.3% are strong biofilm formers, 10.8% moderate biofilm former and 64.3% weak biofilm formers. Only an isolate (3.6%) of *Escherichia coli* and 3 (10.7%) isolates of *Enterobacter* spp. displayed stronger biofilm forming potential, while an isolate each (2.1%) for *Escherichia coli, Enterobacter* spp. and *Staphylococcus* spp. showed moderate biofilm forming potential. The display of different degree of biofilm forming potential among larger proportion 40 (83.3%) of the bacteria isolates is in agreement with earlier studies (Freeman *et al*. 2009, Westgate *et al.* 2011). Although most of the isolates were not characterized to species level, the varying biofilm forming ability exhibited by isolates of the same genus suggest that bacteria species difference does exist in the ability to form biofilm. This knowledge of species difference in biofilm formation is important as it will improve veterinarians and equine researchers understanding of the common equine wound bacteria isolates with high biofilm forming potential.

Overall, there was significant ($P < 0.05$) association between the gram positive and negative isolates and ability to form biofilm, with gram positive being higher than gram negative isolates. The higher biofilm forming potential seen with the gram-negative isolates, particularly *E. coli* and *Enterobacter* sp. is similar with previous studies and it is attributed to various extracellular components such as flagella, fibrils, fimbriae and outer membrane proteins possesses by these bacteria (Kim *et al.* 2012, Nair *et al.* 2013, Tadepalli *et al.* 2016). Bacteria flagella and fimbriae for instance mediate attachment and invasion of bacteria to variety of host proteins, thus the host-cell adhesion could be responsible for their higher biofilm formation abilities (Olsen *et al.* 1993, Sjobring *et al.* 1994, Nair *et al.* 2013). Alteration of genes by at least two-fold has also been reported with *E. coli* biofilm when compared with its planktonic state (Prigent-Combaret *et al.* 1999). Apart from the cellular components like flagella shared by both gram positive and negative bacteria, the *ica* gene of *Staphylococcus* sp. and *Streptococcus* sp. has also been acknowledged as a contributor to intracellular adhesion of these bacteria and has been identified to play role in biofilm formation (Cramton *et al.* 1999, Brady *et al*. 2017, Nasr *et al.* 2012). It was also suggested that the bacteria environmental conditions such pH level, ionic strength, temperature and substrata may contribute to adhesion mechanism of the bacteria responsible for biofilm formation (Bakker *et al*. 2004).

Antibiotic susceptibility of biofilm and planktonic state

The determination of susceptibility of equine chronic wound biofilm isolates to common antibiotics used among equine veterinarians in the study area is justifiable in this study considering the fact that bacteria in biofilm state have been acknowledged to possesses some resistance

Table 3. The comparison between MIC and MBEC of antibiotics tested on the biofilm forming isolates from equine chronic wound.

Antibiotics (µg/mL)	Gram-negative isolates		Gram-positive isolates	
	MIC	MBEC	MIC	MBEC
Ciprofloxacin	0.27 ± 0.04	54.00 ± 12.44*	0.40 ± 0.06	25.60 ± 10.55*
Gentamycin	0.88 ± 0.18	576.0 ± 141.0*	1.30 ± 0.30	332.80 ± 76.80*
Tetracycline	2.25 ± 0.41	736.0 ± 112.8*	0.90 ± 0.29	268.80 ± 71.27*

MIC: Minimum Inhibitory Concentration; MBEC: Minimum Biofilm Eradication Concentration; Values are expressed as mean ± SEM. *Values with significantly different between MIC and MBEC

mechanisms against host defense systems as well as antimicrobial agents (Hall and Mah 2017). Biofilm susceptibility test was conducted for 13 biofilm producing isolates comprising of strong, moderate and weak biofilm formers. The three antibiotics studied are among the common antibiotics with wide spectrum of activity used to treat bacterial infection in horses in the study area. As anticipated, there was significantly ($P < 0.05$) higher MBEC for the three antibiotics tested against the biofilm formers than the MIC required to inhibit the bacteria growth in their planktonic state (Table 3). The MIC of the planktonic state of all tested isolates shows susceptibility to CIP, CN and TE, however, higher concentrations of the antibiotics were required to eradicate the bacteria in their biofilm state. When compared biofilm susceptibility between gram negative and positive bacteria isolates, higher MBEC was required to eradicate the gram-negative bacteria isolates. This finding is not surprising as previous studies have documented that antibiotics susceptibility is less in biofilm when compare with their planktonic cells (Fayaz *et al.* 2014). Nevertheless, of the antibiotics tested CIP shown to be the most potent against the biofilm state.

The antibiotic susceptibility of each isolate in their planktonic and biofilm state to CIP, CN and TE is shown in Table 4. All isolates in their planktonic state were

Table 4. The comparison of antibiotic inhibitory and eradication concentration of planktonic and biofilm isolates from equine chronic wound.

Bacteria	BFP	CIP			CN			TE		
		MIC (µg/ml)	MBEC (µg/ml)	Ratio	MIC (µg/ml)	MBEC (µg/ml)	Ratio	MIC (µg/ml)	MBEC (µg/ml)	Ratio
Escherichia coli	S	0.25	128	512	2	=1024	512	1	1024	1024
	M	0.25	32	128	1	512	512	2	512	256
	W	0.125	32	256	0.5	128	256	4	512	128
Enterobacter sp.	S	0.25	64	256	1	= 1024	1024	2	1024	512
	S	0.25	64	256	0.5	512	1024	2	1024	512
	S	0.25	64	256	1	1024	1024	1	1024	1024
	M	0.25	32	128	0.5	256	512	2	512	256
	W	0.50	16	32	0.5	128	256	4	256	64
Staphylococcus sp.	M	0.25	64	256	1	512	512	1	512	512
	W	0.50	32	64	1	512	512	0.5	256	512
	W	0.25	8	32	0.5	256	512	0.5	256	512
Streptococcus sp.	W	0.50	16	32	2	256	128	0.5	64	128
	W	0.50	8	16	2	128	64	2	256	128

BFP: Biofilm forming potential; S; Strong; M: Moderate; W: Weak; CIP: Ciprofloxacin; CN: Gentamycin; TE: Tetracycline; MIC: Minimum Inhibitory Concentration; MBEC: Minimum Biofilm Eradication Concentration; Ratio: MBEC/MIC

susceptible to all the tested antibiotics (CLSI, 2017). The concentration of CIP in order of potency against the isolates tested were $MBEC_{Streptococcus} > MBEC_{Enterobacter} > MBEC_{E.\ coli} > MBEC_{Staphylococcus}$. Similar trend was observed for TE and CN for *Streptococcus* spp. and *E. coli* were $MBEC_{Streptococcus}$ was greater than the $MBEC_{E.\ coli}$. However, $MBEC_{Staphylococcus}$ was greater than the $MBEC_{Enterobacter}$ for CN while $MBEC_{Enterobacter} > MBEC_{Staphylococcus}$ for TE. The higher MBEC observed for the isolates tested is similar with earlier studies (Machado *et al.* 2013, Masadeh *et al.* 2019, Shrestha *et al.* 2019). Abdallah *et al.* (2011) reported higher MBEC of ciprofloxacin for *Staphylococcus aureus*, *E. coli*, *Klebsiella* spp. isolated from urinary tract (catheter) infections. Biofilm susceptibility of different isolates of *E. coli* to CIP, CN showed significantly higher MBEC than the MIC (Sepandi *et al.* 2004).

There was significant ($p < 0.05$) difference in antibiotics susceptibilities among the three classification of the biofilm formers with susceptibility higher in the weak biofilm formers. That is, the MBEC was highest for the strong biofilm formers, followed in order by moderate and weak biofilm former. The MBEC of CIP for the strong biofilm formers was more than 300x higher than its planktonic MIC while TE and CN were 700x and about 800x higher, respectively. Similarly, CIP has the least MBEC (170-fold higher than MIC) for moderate biofilm formers when compared with TE (300x) and CN (500x). The susceptibility to CIP was however higher in weak biofilm formers with lower antibiotics concentrations (72-fold higher than MIC). The MBEC of TE is more than 200-fold higher and CN is about 400-fold higher than their respective planktonic states.

Furthermore, the MBEC/MIC ratio for three antibiotics was highest for the strong biofilm forming isolates when compared with the moderate and weak biofilm formers. The variation in the susceptibility of different degree of biofilm formers with strong biofilm former having the highest MBEC/MIC could be attributed to slow diffusion of antibiotics into the multiple layer of the bacteria biofilm, thus the gradual exposure of the bacteria to low antibiotics concentrations could promote development of resistance among the bacteria (Antunes *et al.* 2011). In addition to low diffusion of antibiotics, the adaptation to low nutrient requirement with slow growth of bacteria in biofilm state make them tolerance to antibiotics (Lewis 2001, Macia *et al.* 2014). This is because the action of antibiotics is target at rapidly dividing cells. Therefore, the higher antibiotic concentrations required to eradicate bacteria in biofilm state could be among the factors responsible for the treatment challenges affecting equine wound management, as achieving this concentration systemically may not be possible through parenteral therapy. It is therefore imperative to recognize these when formulating antibiotic treatment for wounds management in horses so as not encourage emergence of antibiotic resistant bacterial strains.

CONCLUSION

The equine wound bacteria isolates vary in their ability to form biofilm with majority being weak biofilm formers. Irrespective of the degree of the biofilm formers, antibiotic susceptibility is low as higher concentrations of antibiotics is required to eradicate the bacteria in biofilm state than their planktonic cells.

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