UNIVERSITI PUTRA MALAYSIA

ANTIBIOTIC RESISTANCE AND BIOFILM-FORMING PROPERTIES OF BACTERIA FROM HORSES' WOUND

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By

NUR ADILAH BINTI AHMAD NADZIR

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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DEDICATION

This thesis I dedicated specially to my beloved parents, Haji Ahmad Nadzir bin Mansor and Hajjah Rokiah binti Yaacob, who raised me to the person I am today. To my siblings, Kak Nor, Kak Nurul, Kak Ain, Izzati and Nazirul, who always picked me up on time and encouraged me to go on every adventure, especially this one. To my brother-in-laws, nieces, and nephews, who inspired me along the way. Thank you for everything.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

ANTIBIOTIC RESISTANCE AND BIOFILM-FORMING PROPERTIES OF BACTERIA FROM HORSES' WOUND

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Chairman : Zunita binti Zakaria, PhD
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Wounds occur commonly in horses, whether surgical or accidental. The moisture and warmness of the wound provides essential environment for the growth of microorganism. It has been suggested that chronic, non healing wounds may results from biofilm where there is increased antimicrobial resistance of the bacteria residing in biofilms. Therefore presence of resistant organism in chronic wounds further complicates the problem. This research was conducted to identify bacteria on wounds, ability to form biofilm and antibiotic susceptibility of planktonic and biofilm state of bacteria. A total of 122 bacterial isolates were obtained from 30 horses' wound between January 2015 to June 2015. Eighty-one were Gram-negative (66.4%) and 41 were Gram-positive (33.6%). The predominant organisms isolates were *Escherichia coli* (26.2%), *Enterobacter* sp (20.0%), *Staphylococcus* sp (16.9%), *Streptococcus* sp (6.2%) and *Acinetobacter* sp (4.6%). The isolates were then tested for antibiotic susceptibility test, biofilm forming potential (BFP), minimum inhibitory concentration (MIC) and minimum biofilm eradication concentration (MBEC). Gentamycin and Levofloxacin were found to be effective in inhibiting both Gram-negative and Gram-positive bacteria. Thirteen (27.1%) of 48 isolates that constitute of the predominant isolates were found to be multidrug resistant (MDR) with MDR *E. coli* (4.2%), MDR *Enterobacter* (10.4%), MDR *Staphylococcus* (10.4%), MDR *Streptococcus* (2.1%). 96-well microtiter plates biofilm production revealed that 83.3% (40/48) were biofilm forming. While the antibiotic sensitivities of biofilm organisms tested by MBEC assays were lower than the antibiotic sensitivities of the same organisms in their planktonic state, as tested by MIC assays. These data suggest the prevalence of biofilm in wounds may contribute to prolonged healing and antibiotic tolerance thus plays a role in wound management in horses.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

KERINTANGAN ANTIBIOTIK DAN PEMBENTUKAN SIFAT-SIFAT BAKTERIA BIOFILEM DARI LUKA KUDA

Oleh

NUR ADILAH BINTI AHMAD NADZIR

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Luka kerap berlaku pada kuda, sama ada disebabkan surgikal atau kemalangan. Kelembapan dan kehangatan luka menyediakan persekitaran yang sesuai untuk pertumbuhan mikroorganisma. Oleh yang demikian, luka kronik yang tidak sembuh mungkin disebabkan oleh biofilem dimana terdapat peningkatan rintangan bakteria antimikrob yang tinggal di dalam biofilem. Oleh itu, kehadiran organisma di dalam luka kronik merumitkan masalah ini. Kajian ini dijalankan untuk mengenal pasti bakteria pada luka, keupayaan bakteria untuk membentuk biofilem dan kerentanan bakteria bersifat plankton dan biofilem terhadap antibiotik. Sebanyak 122 pencilan bakteria diperolehi daripada 30 luka kuda yang di ambil di antara bulan Januari 2015 hingga Jun 2015. Lapan puluh satu adalah Gram-negatif (66.4%) dan 41 adalah Gram-positif (33.6%). Organisma utama yang dipencilkan adalah *Escherichia coli* (26.2%), *Enterobacter* sp (20.0%), *Staphylococcus* sp (16.9%), *Streptococcus* sp (6.2%) dan *Acinetobacter* sp (4.6%). Organisma tersebut kemudiannya diuji untuk kerentanan antibiotik, kepekatan perencatan minimum (MIC), minimum kepekatan pembasmian biofilem (MBEC) dan potensi membentuk biofilem (BFP). Gentamicin dan levofloxacin didapati berkesan dalam membasmi kedua-dua bakteria Gram-negatif dan Gram-positif. Tiga belas (27.1%) daripada 48 pencilan yang terdiri daripada pencilan utama dikenal pasti rintang sebagai pelbagai rintang antibiotik (MDR) iaitu MDR *E. coli* (4.2%), MDR *Enterobacter* (10.4%), MDR *Staphylococcus* (10.4%), MDR *Streptococcus* (2.1%). 96-lubang plat mikrotiter biofilem mendedahkan bahawa 83.3% (40/48) pencilan menghasilkan biofilem. Sementara itu sensitiviti antibiotik bakteria yang menghasilkan biofilem diuji oleh MBEC adalah lebih rendah berbanding bakteria yang sama dalam keadaan plankton yang diuji oleh MIC. Data yang diperoleh ini mencadangkan bahawa prevalen biofilem pada luka boleh menyumbang kepada penangguhan dalam penyembuhan di mana toleransi terhadap antibiotik memainkan peranan dalam pengurusan luka pada kuda.

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I certify that a Thesis Examination Committee has met on 6 June 2017 to conduct the final examination of Nur Adilah binti Ahmad Nadzir on her thesis entitled "Antibiotic Resistance and Biofilm-Forming Properties of Bacteria From Horses' Wound" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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The thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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LIST OF ABBREVIATIONS/ SYMBOLS

µg/mL	Microgram per mililitre
µL	Microlitre
AST	Antibiotic susceptibility test
BA	Blood agar
cfu	Colony-forming unit
CLSI	Clinical Laboratory Standard Institutes
EPS	Extracellular polymeric substance
h	Hour(s)
MBEC	Minimum Biofilm Eradication Concentration
MDR	Multidrug resistance
mg/mL	Miligram per mililitre
MHA	Muller-Hinton Agar
MHB	Muller-Hinton Broth
MIC	Minimum Inhibition Concentration
min	Minute(s)
MRSA	Methicillin-resistant *Staphylococcus aureus*
nm	Nanometer
OD	Optical density
OIE	Office International des Epizooties (World Organization of Animal Health)
pH	Potential of hydrogen
rpm	Revolutions per minutes
s	Second(s)
SD	Standard deviation
SEM	Standard error of mean
sp	Species
TSA	Tryptic Soy Agar

Abbreviations for Antibiotics

AMK	Amikacin
AMC	Amoxicillin/clavunic acid
AMP	Ampicillin
C	Chloramphenicol
CIP	Ciprofloxacin
CN	Gentamycin
DO	Doxycycline
IPM	Imipenem
LEV	Levofloxacin
MEM	Meropenem
SAM	Ampicillin/sulbactam
SXT	Trimethoprim/sulphamethaxole
TE	Tetracycline
TIM	Ticarcillin clavunate

CHAPTER 1

INTRODUCTION

A wound is a type of physical injury involving damage in the skin. The exposed subcutaneous tissues provide a favourable substratum for a wide variety of microorganisms to contaminate and colonize. If the involved tissue is devitalized and the host immune response is compromised, the conditions become optimal for microbial growth. This is because the host immune response plays a critical role in determining whether wound infection will arise (Esebelehie *et al.*, 2013). Wound infection refers to the deposition and multiplication of bacteria in the tissue with an associated host reaction (Ayton, 1998). This may be characterized by the classic signs of redness, pain, swelling and fever (Calvin, 1998). Due to horse's nature and the environment in which it lives, wound are quite common in horse patients and it frequently involves a significant amount of tissue trauma. The most frequent wound occurrences are legs caught in fences, panels, wire or gates. While laceration originated from steel siding, trailer accidents and riding accidents. Horse wounds have a high risk of becoming infected due to their environment in which they live in.

Infected wounds harbour diverse populations of microorganisms, however in some cases these microorganisms can be difficult to identify and fail to respond to antibiotic treatment, resulting in chronic non-healing wounds (Westgate *et al.*, 2011). Wounds on the distal limbs of horses can be difficult to manage because of poor circulation, joint movement and minimal soft tissue between skin and bone. There is also always the risk of contamination from the environment. Majority of the cases handled by University Veterinary Hospital (UVH), Universiti Putra Malaysia (UPM) and Department of Veterinary Services (DVS) Malaysia involve treatment of wounds, both acute and chronic. Chronic wounds are highly prevalent in horses and it is a challenge for treatment.

More often nowadays, the veterinarians experience handling wounds that display delayed healing and recurrent infection. The wounds are particularly prone to infection as it is always being exposed to environmental microorganisms. In human, chronic non-healing wounds has been proven to be caused by the ability of bacteria to survive in a biofilm phenotypic state. Biofilms are known to delay wound healing, principally due to their recalcitrance towards antimicrobial therapies and components of the innate immune response.

Maintaining the wound microenvironment helps suppress the microbial proliferation, especially at the problematic anatomical sites that has poor blood supply and reduced tissue oxygenation (Westgate *et al.*, 2010). Apart from applying appropriate wound management strategies, if the wound is at increased risk of infection or is known to be infected, suitable antibiotic regime must be employed to promote healing. In horses, the following organisms are commonly associated with wound infections result from commensal flora acting as opportunistic pathogens: *Streptococcus zooepidemicus*, *Staphylococcus aureus*, alpha *Streptococcus sp*, *Escherichia coli*, and *Pseudomonas*

aeruginosa. Among of the pathogens, *S. aureus* is the major pathogen present in wounds (Hussain *et al*., 2005 and Ahmed, 2012).

Therefore the presence of pathogens in wound cause great distress in terms of associated mortality and morbidity, increased length of hospital stay, delayed wound healing, profound discomfort and increasing of healthcare cost (Esebelahie *et al.*, 2013). It has been suggested that chronic, non-healing wounds may results from biofilm (Broomfield *et al*., 2009). Several researchers have shown that there is increased antimicrobial resistance of the bacteria residing in biofilms (Leid *et al*., 2000). Most of the research on biofilm formation on horse wounds has been carried out elsewhere (Cochrane *et al.*, 2009; Westgate *et al.*, 2011), however there is no data available in Malaysia.

This study is carried out to unveil preliminary evidence of the presence of bacterial biofilm and multidrug resistant (MDR) bacteria in horses' wound. This present study may also provide the clinicians or veterinarians with the necessary information to select appropriate drugs and doses for treatment as well as increase the effectiveness of preliminary screening of diagnosis, prevention and control of wound infection cause by biofilm. Therefore, it is necessary to investigate the presence of bacteria and composition of biofilms on horses' wound. It is hypothesized that the biofilms in the wound can lead to delay wound healing due to recalcitrance towards antimicrobial therapies and innate immune response.

The objectives of this study were to:

1) determine the distribution and antibiotic resistance properties of microorganisms present on acute and chronic horses' wound
2) determine the biofilm forming ability among the five dominant species of isolates

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