



**UNIVERSITI PUTRA MALAYSIA**

***PREVALENCE, ANTIBIOTIC SUSCEPTIBILITY AND  
PATHOGENICITY OF RHODOCOCCLUS EQUI IN HORSE  
FAECES AND SOILS FROM SELECTED STUD FARMS IN  
PENINSULAR MALAYSIA***

***MOHAMMAD FHITRI BIN SHARI***

**FPV 2014 37**



**UPM**  
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BERILMU BERBAKTI

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**MASTER OF VETERINARY SCIENCE  
UNIVERSITI PUTRA MALAYSIA**

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By

**MOHAMMAD FHITRI BIN SHARI**

**This thesis submitted to School of Graduate Studies, Universiti Putra Malaysia, in  
fulfilment of the requirements for the Master of Veterinary Science**

**NOVEMBER 2014**

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## DEDICATION

I dedicate this thesis especially to:

**My lovely mother,**

SARIDAH BT CHE EMBI

And

**My beloved brothers and sisters,**

MOHAMAD HAFIZ BIN SHARI, ASMIDA BT MAN AND THEIR CHILD,  
MOHAMMAD HAZIQ AZHFAR BIN MOHAMAD HAFIZ  
NOOR SARFINA BT SHARI  
MUHAMMAD HIDZIR BIN SHARI  
SITI AISHAH BT SHARI  
ABDUL LATIFF BIN SHARI

For their eternal love, continual support and immortal inspiration throughout  
my life

Who gave me the strength and faith to accomplish my goals and reach my  
dreams

Only Allah knows how grateful I am destined to live together with them

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment for the award of Master of Veterinary Science

**PREVALENCE, ANTIBIOTIC SUSCEPTIBILITY AND PATHOGENICITY OF *RHODOCOCCLUS EQUI* IN HORSE FAECES AND SOILS FROM SELECTED STUD FARMS IN PENINSULAR MALAYSIA**

By

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**Chairman: Associate Professor Zunita Zakaria, PhD  
Faculty: Veterinary Medicine**

*Rhodococcus equi* is considered as a major bacterial veterinary pathogen that is difficult to treat amongst the important diseases in the equine industry. It has been isolated from clinical specimens from human, various species of animals as well as environmental samples such as soil, air, bedding materials and others in many countries but reports are scarce in South East Asian countries including Malaysia. This study was conducted to determine the appropriate selective medium for the isolation of *R. equi*, to determine its prevalence in horse's faeces and soil in selected farms, to determine the antimicrobial susceptibility patterns and to evaluate the pathogenicity of the virulent *R. equi* isolates.

Two types of selective medium; Nalidixic Acid-Novobiocin-Actidione-Tellurite medium (NANAT) and modified Ceftazidime-Novobiocin medium (m-CAZ) were evaluated to isolate *R. equi* from clinical and environmental samples. Samples were cultured on both medium and presumptive isolates were identified using conventional biochemical test and confirmed using species specific polymerase chain reaction (PCR). The m-CAZ medium was shown to be the better selective medium with 36/81 (44.44%) successful isolations compared to none (0%) on the NANAT. Prevalence of *R. equi* in selected farms were conducted in four farms (A, B, C and D) comprising of 103 healthy animal faeces (mares, n=59; foals, n=44) and 139 soil samples. The prevalence of *R. equi* from farms A, B, C and D was recorded as 14.29% (6/42), 38.60% (22/57), 52.81% (47/89) and 42.59% (23/54) respectively. Of 98 *R. equi* isolates collected, 53.06% (52/98) were isolated from soil while the remaining was derived from faeces. From these number, 3.85% (2/52) of soil isolates and 6.52% (3/46) of faecal isolates were virulent detected through multiplex PCR. All five virulent isolates were from farm C.

The isolates were subjected to antibiotic sensitivity test using disc diffusion technique. All were tested against 12 different antibiotics namely Ampicillin, Azithromycin, Ceftiofur, Cephalexin, Doxycycline, Enrofloxacin, Erythromycin, Gentamicin, Levofloxacin, Oxytetracycline, Penicillin and Streptomycin. Six of 98 isolates (6.12%) were susceptible to all antibiotics, 92 of 98 isolates (93.88%) were resistant to at least one antibiotic, 47.96% (47/98) showing mono-resistant and 45.92% (45/98) were multidrug resistant. The isolates showed the highest susceptibility rate against four antibiotics which were Doxycycline, Levofloxacin, Enrofloxacin and Gentamicin. Besides that, 93.88% (92/98) isolates were intermediately resistant to Streptomycin and 2.04% (2/98) were found to resistant to Erythromycin although others not.

Six isolates (virulent, n=5; avirulent, n=1) were subjected to pathogenicity test in mice. The results revealed that three of virulent isolates caused death in mice while others did not after being inoculated intraperitoneally. The avirulent isolate and the other two virulent isolates did not cause death in tested mice. Post-mortem on the dead mice showed that the major visceral organ affected were lung, liver and spleen while others showed non significant lesion. All affected lung were haemorrhagic while all affected liver and spleen were congested. Histological examination proved that all of these visceral organs were severely damaged with the lesion score of three instead of one or two. In conclusion, this study showed low prevalence of virulent *R. equi* in all selected stud farms in Peninsular Malaysia.



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

**PREVALENS, KEPEKAAN ANTIBIOTIK DAN PATOGENISITI  
*RHODOCOCCUS EQUI* YANG DIPENCILKAN DARIPADA TINJA KUDA DAN  
TANAH DI LADANG KUDA TERPILIH DI SEMENANJUNG MALAYSIA**

Oleh

**MOHAMMAD FHITRI BIN SHARI  
NOVEMBER 2014**

**Pengerusi: Profesor Madya Zunita Zakaria, PhD  
Fakulti: Perubatan Veterinar**

*Rhodococcus equi* dianggap kuman penyebab penyakit veterinar utama yang sukar untuk dirawat diantara penyakit terpenting dalam industri kuda. Ia telah dipencilkan daripada specimen-spesimen klinikal daripada manusia, pelbagai spesis haiwan serta daripada sampel persekitaran seperti tanah, udara, perkakas tempat tinggal dan sebagainya di banyak negara. Walaubagaimanapun, laporan tentangnya sangat sedikit di negara-negara Asia Tenggara termasuk Malaysia. Kajian ini dilakukan untuk menentukan media pemilih yang sesuai untuk pemencilan *R. equi*, untuk menentukan prevalens *R. equi* dalam kuda dan tanah di ladang terpilih, menentukan corak kepekaan antibiotik dan untuk menilai patogenisiti pencilan *R. equi* virulen.

Dua jenis media pemilih; Nalidixic Acid-Novobiocin-Actidione-Tellurite (NANAT) dan Ceftazidime-Novobiocin yang diubahsuai (m-CAZ) telah dinilai untuk memencilkan *R. equi* daripada sampel tinja dan tanah. Sampel telah dikultur pada kedua-dua media dan pencilan ramalan telah dikenalpasti menggunakan ujian biokimia konvensional serta disahkan menggunakan tindak balas rantaian polymerase (PCR) spesis spesifik. Media m-CAZ telah dibuktikan media pemilih lebih baik dengan 36/81 (44.44%) pencilan berjaya diasingkan berbanding tiada (0%) pada NANAT. Prevalens *R. equi* di ladang terpilih telah dilakukan di empat buah ladang (A, B, C dan D) yang terdiri daripada 103 sampel tinja (kuda betina, n=59; anak kuda, n=44) dan 139 sampel tanah. Prevalens *R. equi* daripada ladang A, B, C dan D ialah 14.29% (6/42), 38.60% (22/57), 52.81% (47/89) dan 42.59% (23/54). Daripada 98 pencilan *R. equi* yang diambil, 53.06% (52/98) dipencilkan daripada tanah manakala selebihnya diperoleh daripada tinja. Daripada jumlah ini, 3.85% (2/52) pencilan tanah dan 6.52% (3/46) pencilan tinja adalah virulen yang dikesan melalui multiplex PCR. Kelima-lima pencilan virulen diperoleh daripada ladang C.

Pencilan-pencilan didedahkan kepada ujian kepekaan antibiotik menggunakan teknik penyebaran cakera. Semua pencilan diuji dengan 12 antibiotik yang berbeza iaitu Ampicillin, Azithromycin, Ceftiofur, Cephalexin, Doxycycline, Enrofloxacin, Erythromycin, Gentamicin, Levofloxacin, Oxytetracycline, Penicillin dan Streptomycin. Enam daripada 98 pencilan (6.12%) sensitif terhadap semua antibiotik, 92 daripada 98 pencilan (93.88%) rintang pada sekurang-kurangnya satu antibiotik, 47.96% (47/98) menunjukkan kerintang tunggal dan 45.92% (45/98) adalah rintang pada pelbagai antibiotik. Pencilan-pencilan menunjukkan kadar kepekaan tertinggi terhadap empat antibiotik seperti Doxycycline, Levofloxacin, Enrofloxacin dan Gentamicin. Selain itu, 93.88% (92/98) pencilan didapati separa rintang pada Streptomycin dan 2.04% (2/98) pula rintang terhadap Erythromycin walaupun yang selebihnya tidak.

Sejumlah enam pencilan (virulen, n=5; tidak virulen, n=1) disaring untuk ujian patogenisiti dalam tikus. Keputusan menunjukkan tiga daripada pencilan virulen menyebabkan kematian pada tikus sedangkan yang lain tidak selepas disuntik secara intraperitoneal. Pencilan tidak virulen dan dua pencilan virulen lain tidak menyebabkan kematian pada tikus yang diuji. Post-mortem terhadap tikus yang mati menunjukkan bahawa organ dalaman utama yang dijangkiti adalah paru-paru, hati dan limpa manakala selainnya tidak menunjukkan kesan sampingan. Semua paru-paru yang dijangkiti mengalami pendarahan manakala semua hati dan limpa mengalami kongesi. Pemeriksaan histologi membuktikan semua organ dalaman ini rosak teruk sehingga diberikan skor luka tiga berbanding satu atau dua. Kesimpulannya, kajian ini menunjukkan prevalens *R. equi* virulen adalah rendah di semua ladang kuda terpilih di Semenanjung Malaysia.

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**MOHAMMAD FHITRI BIN SHARI**



I certify that a Thesis Examination Committee has met on 3 November 2014 to conduct the final examination of Mohammad Fhitri Bin Shari on his thesis entitled “Prevalence, Antibiotic Susceptibility and Pathogenicity of *Rhodococcus equi* In Horse Faeces and Soils From Selected Stud Farms In Peninsular Malaysia” in accordance with the Universities and University College 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 Veterinary Science.

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## LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion
AIDS	Acquired Immune Deficiency Syndrome
AMP	Ampicillin
ATCC	American Type Culture Collection
AZM	Azithromycin
BA	Blood Agar
bp	Base Pair
β	Beta
CFU	Colony Forming Unit
CL	Cephalexin
CLSI	Clinical Laboratory Standard Institute
cm	Centimeter
CN	Gentamicin
°C	Degree Celcius
DH <sub>2</sub> O	Distilled Water
DNA	Deoxyribonucleic Acid
DO	Doxycycline
E	Erythromycin
EFT	Ceftiofur
EHV	Equine Herpes Virus
ELISA	Enzyme-Linked Immunosorbent Assay

ENR	Enrofloxacin
G	Gauge
g	Gram
HI	Hyper Immune
HIP	Hyper Immune Plasma
HIV	Human Immunodeficiency Virus
IACUC	Institutional Animal Care and Use Committees
IL	Interleukin
IU	International Unit
kb	Kilobase
kDA	Kilodalton
LEV	Levofloxacin
MAA	<i>Mycobacterium avium subsp avium</i>
Mb	Mega base
MHA	Mueller Hinton Agar
min	Minute
ml	Milliliter
mM	Milimolar
mm	Millimeter
μg	Microgram
μl	Microliter
μM	Micromolar
nm	Nanometer



NO <sub>2</sub>	Nitrite
NO <sub>3</sub>	Nitrate
OT	Oxytetracycline
P	Penicillin
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT-PCR	Real Time Polymerase Chain Reaction
S	Streptomycin
s	Second
TBA	Trachea Broncheol Aspirate
TBE	Tris-Borate-EDTA
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
V	Volt
vapA	Virulence Associated Plasmid A
vapB	Virulence Associated Plasmid B

## CHAPTER 1

### INTRODUCTION

*Rhodococcus equi* is an opportunistic pathogen in mammals including humans (Meijer and Prescott, 2004) causing potentially life threatening infections in severely immunocompromised people (Ladron *et al.* 2003) and cause major disease in foals worldwide (Haites *et al.* 1997). It has been ranked as among the four most important diseases of the horse industry in many Australian Thoroughbred stud farms (Muscatello *et al.* 2006A) and listed as a major bacterial veterinary pathogen (Vazquez-Boland *et al.* 2010). The genus *Rhodococcus* was first discovered by Zopf in 1891 and comprises 30 species (Meijer and Prescott, 2004) which belong to 'mycolic acid containing group' of actinomycetes (Letek *et al.* 2010). Two species in the genus *Rhodococcus* are known to have parasitic lifestyles, the phytopathogen *Rhodococcus fascians* and the animal pathogen *Rhodococcus equi* (Vazquez-Boland *et al.* 2010). *Rhodococcus equi* parasitizes macrophages and like *Mycobacterium tuberculosis*, replicates within a membrane bound vacuole (Letek *et al.* 2010) thus belonging to the group of organisms called facultative intracellular parasites (Vazquez-Boland *et al.* 2010).

The incidence of pneumonia due to *R. equi* infection appears to be increasing in all breeds of animals (Haites *et al.* 1997) since firstly isolated from pulmonary lesions of foals in Sweden (Hondalus, 1997). Since then, researchers have identified *R. equi* in a variety of land and water animals including cats, dogs, cattles, goats, swine, buffaloes, sheeps, crocodiles, wild birds, deers, seals, marmosets and koala bears (Taouji *et al.* 2008; Takai *et al.* 2003; Weinstock and Brown, 2002). The first case of human infection was reported in 1967 (Silva *et al.* 2010) in a patient presented with fever and cavitary pneumonia. Thereafter the incidence of *R. equi* infection in human has increased markedly which coincide with the increase in HIV infection and advances in organ transplantation and cancer treatment (Weinstock and Brown, 2002).

*Rhodococcus equi* causes chronic bronchopneumonia in young foals along with other clinical conditions such as intestinal disease, non-specific synovitis and sporadic abscesses (Buckley *et al.* 2007). It is recognized in many countries as the leading cause of mortality in foals and is a cause of serious concern to the equine industry as there is no effective vaccine for its prevention and it can become endemic in stud farms (Rodriguez-Lazaro *et al.* 2006). The lack of sensitive diagnostic techniques for identifying the early stages of the infection in foals, the extent of *R. equi* subclinical carriage, its intrinsic resistance to a number of antibiotics such as Penicillins, Cephalosporins, Sulphonamides, Quinolones, Tetracyclines, Clindamycin and Chloramphenicol and the

intracellular localization of this pathogen complicates the treatment in the farm (Vazquez-Boland *et al.* 2010).

Muscatello *et al.* (2006A) reported that the Australian equine industry bears and estimated \$2-4 million annually due to 1-10% of foals affected every year even though mortalities are usually maintained below 1% by early aggressive therapy. However mortality can reach 20% or higher as reported in a few stud farms (Muscatello *et al.* 2006A). The Malaysian equine industry is small compared to Australia's. Malaysia is estimated to have over 5000 stabled equines without taking into consideration the numbers of horses and ponies in Kelantan and Sabah ([www.equinemalaysia.com.my](http://www.equinemalaysia.com.my)). However, little is known about the significance of *R. equi* infection in Malaysian horses which may be due to absence of infection or under/misdiagnosis of the infection. *Rhodococcus equi* can be acquired by inhalation from the contaminated soil or infectious aerosols, inoculation into wound or mucous membrane or via ingestion (Weinstock and Brown, 2002). However, studies conducted by Muscatello *et al.* (2006B) and Vazquez-Boland *et al.* (2010) proved that inhalation of virulent *R. equi* is the main route of transmission.

Laboratory diagnosis of rhodococcal infections currently relies on classical bacteriological methods involving the isolation of the organism from clinical samples or postmortem materials (Rodriguez-Lazaro *et al.* 2006) followed by routine biochemical identifications. The polymerase chain reaction (PCR) has also been used to support or replace the traditional methods because it is more rapid, sensitive and highly specific (Arriaga *et al.* 2002). Two different primers which were 16S rRNA which is species specific and *vapA* which amplifies a gene 85 to 90 kb of the virulence plasmid carried by this bacterium are available in the market. Horse isolates of *R. equi* typically harbor an 85 to 90 kb virulence plasmid that encodes virulence associated protein A or *VapA* which is responsible for the virulence of the organism but is less frequently found in non-horse isolates (Rodriguez-Lazaro *et al.* 2006).

The occurrence of *R. equi* in human and animals from various geographical regions has been reported in countries such as Japan (Takai *et al.* 1991; Takai and Tsubaki, 1985), Unites States of America (USA) (Takai *et al.* 2006; Takai *et al.* 2001), Thailand (Poolkhet *et al.* 2009; Takai *et al.* 2002), China (Takai *et al.* 2006), Netherland (Komijn *et al.* 2007), Brazil (Silva *et al.* 2010; Krewer *et al.* 2008). In Malaysia, Liew, (2009) and Puthucheary *et al.* (2006) did some work on the organism but the scope of the study is limited.

It is hypothesized that *R. equi* is widely distributed in horses and stud farms in Malaysia and its occurrence in the equine environment may pose serious

veterinary health threats to the susceptible or immunosuppressed individuals and foals. The objectives of this study were:

- i. to determine the prevalence of *R. equi* in horses and soil in the farms using two selective media such as NANAT and m-CAZ
- ii. to determine the antibiotic resistance patterns of *R. equi* isolates
- iii. to determine the pathogenicity of virulent *R. equi* isolates in laboratory animals.



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## Personal Communications

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