UNIVERSITI PUTRA MALAYSIA

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

MOHAMMAD FHITRI BIN SHARI

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MASTER OF VETERINARY SCIENCE
UNIVERSITI PUTRA MALAYSIA

2014
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By

MOHAMMAD FHITRI BIN SHARI

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

By

MOHAMMAD FHITRI BIN SHARI

NOVEMBER 2014

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, Levofoxacin, 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, Levofoxacin, 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 \textit{R. equi} in all selected stud farms in Peninsular Malaysia.
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. Walau bagaimanapun, 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 kerintangan 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.

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In the name of Allah the most compassionate and the most merciful.

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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.

Members of the Thesis Examination Committee were as follows:

**Abdul Rani Bin Bahaman, PhD**
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

**Abdul Rahim Bin Abdul Mutalib, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Siti Khairani Binti Bejo, PhD**
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

**Sharifah Binti Syed Hassan, PhD**
Associate Professor
Monash University Malaysia
Malaysia
(External Examiner)

**ZULKARNAIN ZAINAL, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 26 February 2015
This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the award of Master of Veterinary Science. The members of the Supervisory Committee were as follows:

Zunita Binti Zakaria, PhD  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Chairman)

Noordin Bin Mohamed Mustapha, PhD  
Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
(Member)

Latiffah Binti Hassan, PhD  
Associate Professor  
Faculty of Veterinary Medicine  
Universiti Putra Malaysia  
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<td>AGID</td>
<td>Agar Gel Immunodiffusion</td>
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<td>bp</td>
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<td>CFU</td>
<td>Colony Forming Unit</td>
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<td>EHV</td>
<td>Equine Herpes Virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td>ENR</td>
<td>Enrofloxacin</td>
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<tr>
<td>G</td>
<td>Gauge</td>
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<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>HI</td>
<td>Hyper Immune</td>
</tr>
<tr>
<td>HIP</td>
<td>Hyper Immune Plasma</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committees</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>IU</td>
<td>International Unit</td>
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<td>Kilodalton</td>
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<tr>
<td>Mb</td>
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<td>MHA</td>
<td>Mueller Hinton Agar</td>
</tr>
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<td>min</td>
<td>Minute</td>
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<td>nm</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<td>PFGE</td>
<td>Pulsed Field Gel Electrophoresis</td>
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<tr>
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<td>Ribonucleic Acid</td>
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<td>Real Time Polymerase Chain Reaction</td>
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<td>Second</td>
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<td>TBE</td>
<td>Tris-Borate-EDTA</td>
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<tr>
<td>V</td>
<td>Volt</td>
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<td>vapA</td>
<td>Virulence Associated Plasmid A</td>
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<td>vapB</td>
<td>Virulence Associated Plasmid B</td>
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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). 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

Doreen Phee, Veterinary Officer of National Stud Farm, Tanjung Rambutan, Perak, pers. comm. 18 October 2010.

Web Citations


