OCCURRENCE OF *Rhodococcus equi* IN SOIL AND FAECES IN SELECTED STUD FARMS IN MALAYSIA

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ABSTRACT. The world widely distributed infection by Rhodococcus equi usually leads to pneumonia and associated respiratory signs. This study is aimed at detecting the occurrence of this pathogen in selected horse farms. A total of 12 R. equi isolates from few samples (13.89%) were successfully obtained from soil and faeces collected from two selected farms. However, based on the *vap*A gene classification, only one virulent *R. equi* isolate was identified.

Keywords: Rhodococcus equi; Multiplex PCR

INTRODUCTION

Rhodococcus equi is an important opportunistic pathogen in animals and humans especially in foals and immunocompromised horses. It mainly causes pneumonia in foals; however its existence as one of major factor of respiratory illness is always overshadowed by other more renowned aetiologies of equine pneumonia (http://www.merckvetmanual.com). Infection by this bacterium leads to significant economic losses due to mortality, prolonged treatment, surveillance programmes for early detection and relatively expensive prophylactic strategies (Buckley, 2007). The virulence factor associated to *R. equi* infection in horses is the thermoregulated virulence associated antigen (VapA) encoded by *vapA* gene which is located in the 85–90 Kb virulence plasmid (Krewer *et al*., 2008).

In humans, it causes a lung disease reminiscent of pulmonary tuberculosis, and ulcerative lymphangitis in cattle (Ladron *et al*., 2003). Infection in cats has been reported in Japan (Takai *et al*., 2003) and Malaysia (Alimah *et al*., 2008).

The Malaysian climate invariably favours the propagation of *R. equi* as evidenced by unreported cases of pneumonia in foals suggestive of *R. equi* infection. However, the causative agent of the disease has never been confirmed. This study is aimed at detecting the occurrence of *R. equi* in horses and its environment in selected stud farm and the presence of the virulence factor (*vap*A gene).

MATERIALS AND METHODS

Sampling

Two horse breeding farms in Peninsular Malaysia were selected for this study. One farm is located in Perak while the other is in Selangor. Soil samples were collected by scraping from the ground surface for not more than 30 cm depth using a clean auger and placed in clean containers. The fresh faeces samples were collected directly from the stable floor among apparently healthy horses. Samples were transported to the Bacteriology Laboratory, Faculty of Veterinary Medicine, UPM to be processed. One gram of each sample was enriched in Trypticase Soy Broth (TSB) and incubated at 37°C for 24 hours. The enriched samples were then inoculated onto M-CAZ medium and incubated at 37°C for three days.

Isolation and Identification of *R. equi* from soil and faeces

Presumptive *R. equi* colonies were subjected to Gram staining. Gram positive pleomorphic cells were selected and subcultured to obtain pure culture. Pure cultures were tested for catalase production. Positive catalase cultures were subjected to three different biochemical tests; urease, glucose and nitrate.

Multiplex PCR to confirm R. equi identity and detection of *vap*A gene

Four to five colonies of presumptive *R. equi* culture isolates were then transferred into 100 µl of sterile distilled water in a microfuge tube and heated on dry bath at 96°C for 10 minutes. Then, the tubes were centrifuged for 3 minutes at 13 000 rpm. The supernatant were transferred into clean microfuge tubes and used as DNA template for multiplex PCR. Primers used in this study were RG – Forward (5'–CGT CTA ATA CCG GAT ATG AGC TCC TGT C–3') ; RG–Reverse (5'–CGC AAG CTT GGG GTT GAG CCC CAA–3') and *vap*A–Forward (5'–GAC TCT TCA CAA GAC GGT–3') ; *vap*A–Reverse (5'–TAG GCG TTG TGC CAG CTA–3') which amplify the 16S rRNA and *vap*A gene fragments respectively (Krewer *et al.*, 2008). Multiplex PCR was performed in 50 µl reaction volumes containing template DNA (3 µl); 1 × TopTaq PCR Buffer (Qiagen); 0.25 mMdNTP (mix) (Qiagen); 1 × Coral Load (Qiagen), 16S rRNA forward and reverse (0.2 µM each); *vap*A forward and reverse (0.2 µM each); TopTaq DNA Polymerase (Qiagen) (1 unit) and sterile distilled water or RNAse free water (Qiagen). Reactions were carried out in a thermal cycler (Bio-Rad) under the following conditions: initial denaturation at 96°C for 3 minutes; 35 cycles of 96°C for 30 seconds, 59°C for 30 seconds and 65°C for 1 minute; and final extension at 65°C for 10 minutes. The PCR products (7 µl) were applied to 1.5% agarose gel containing 0.1

µl/ml of gel red and electrophoresed for 1 hour 30 minutes at 80 V. *Rhodococcus equi* ATCC 6939 were used as the positive control. Upon completion, the gel were visualized under ultraviolet source and documented, using Alpha Imager (Alpha Innotech).

RESULTS AND DISCUSSION

A total of 31 isolates were confirmed as *R. equi* by isolation. Sixteen isolates were isolated from soil and 15 from the faeces samples. Twenty-seven isolates were found to be positive for the *R. equi* 16SrRNA gene confirming the identity (Table 1, Figure 1). The remaining four isolates (9.6%) were misidentified using the conventional tests. Only one isolate (3.7%) among the 27 *R. equi* isolates were found to possess the *vap*A gene. The multiplex PCR was found to be very useful in confirming the identity of the isolates. In studies conducted by Monego *et al.* (2009) and Halbert *et al.* (2005), the researchers reported the usage of multiplex PCR technique to identify virulent *R. equi* rapidly by amplication of gene sequences that are unique to the virulence plasmids. Detection of *R. equi* using conventional methods was found to inaccurate and time consuming (Halbert *et al.*, 2005). Multiplex PCR assay also provide an efficient and accurate method for epidemiologic screening of soil and tissue or fluid samples from non equine mammalian and environmental sources even when the prevalence of *vap*A strains of the bacterium is generally low (Halbert *et al.*, 2005). Barreto (2000) suggests that conventional biochemical tests are not preferred as it does not guarantee accurate identification because of the difficulties such as lack of adequate reproducibility and the variability of phenotypes that may lead to ambiguous or erroneous results. In addition, PCR also enable differentiation of virulent (Figure 1) from avirulent strains (Figure 2) of *R. equi* via detection of the v*ap*A gene fragments in the isolates.

Table 1: Biochemical Test and Multiplex PCR results of isolates obtained from 2 selected horse breeding farms

Farm	Number of Samples	Type of Samples	Biochemical Test (%)	Multiplex PCR	
				R. equi (%)	*Vap*A (%)
A	33	Faeces (13)	3	3/10 (30%)	0
		Soil (20)	7	3/10 (30%)	0
Total			10 (30.30%)	6/10 (60%)	0
B	39	Faeces (24)	12	12/21 (57.14%)	1/21 (4.76%)
		Soil (15)	9	9/21 (42.86%)	0
Total			21 (53.85%)	21/21 (100%)	1/21 (4.76%)

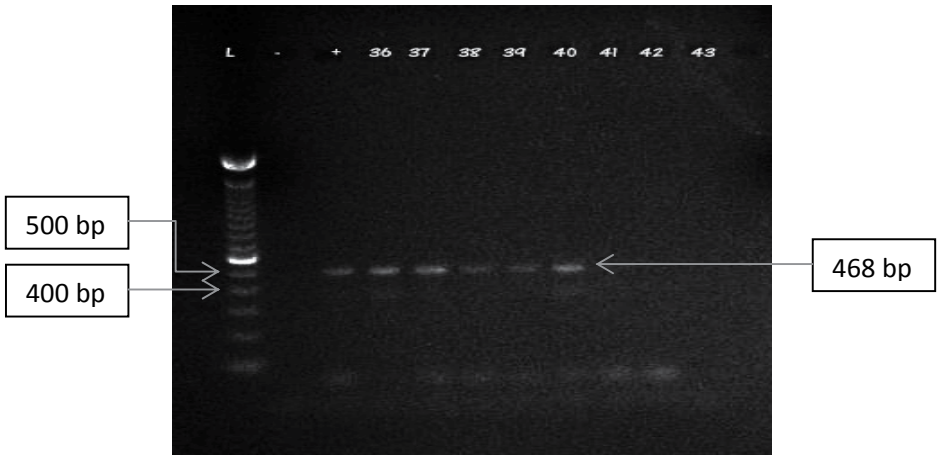

Figure 1 a): Multiplex PCR amplification of 16S rRNA gene fragments on samples at Farm A. L: Molecular Ladder (Ladder 100 bp Invitrogen); 36,37,38,39,40,41,42 & 43: Samples, -: Negative Control & +: Positive Control (*Rhodococcus equi* ATCC 6939)

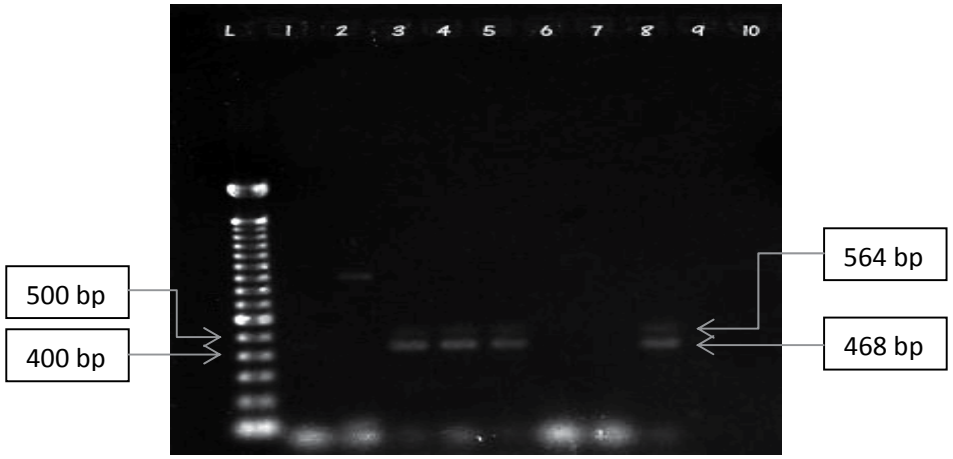

Figure 1 b): Multiplex PCR amplification of both 16S rRNA gene fragments &VapA gene fragments on samples at Farm B. L: Molecular Ladder (Ladder 100 bp Invitrogen); 1,2,3,4,5,6,7 & 10: Samples; 8: Positive Control (*Rhodococcus equi* ATCC 6939) & 9: Negative Control

REFERENCES

1. Barreto M.M., Chacon C.A.V., Rivero C.B., Blanco P.A., Jost K.C.J., Balandrano S. and Diaz H.O. (2000). Comparison among three methods for mycobacteria identification. *Salud Publica Mex*, **42**; 484-489.
2. Buckley T., McManamon E. and Stanbridge S. (2007). Resistance studies of erythromycin and rifampin for *Rhodococcus equi* over a 10-year period. Irish Veterinary Journal, **60**; 728-731.
3. Halbert N.D., Reitzel R.A., Martens R.J. and Cohen N.D. (2005). Evaluation of a multiplex polymerase chain reaction assay for simultaneous detection of *Rhodococcus equi* and the *vap*A gene. *AJVR Journal*, **66**; 1380 – 1385.

4. Krewer C.D.C., Spricigo D.A., Botton S.D.A., Costa M.M.D. Schrank I. and Vargas A.C.D. (2008). Molecular characterisation of *Rhodococcus equi* isolates of horse breeding farms from an endemic region in south of Brazil by multiplex PCR. *Brazilian Journal Of Microbiology*, **39**; 188-193.
5. Ladron, N., Fernandez, M., Aguera, J., Zorn, B.G., Boland, J.A.V & Navas, J. (2003). Rapid identification of *Rhodococcus equi* by a PCR assay targeting the choE gene. *Journal of Clinical Microbiology*, **41**; 3241-3245.
6. Monego F., Maboni F., Krewer C., Vargas A., Costa M. and Loreto E. (2009). Molecular characterisation of *Rhodococcus equi* from horse breeding farms by means of multiplex PCR for the *vap* gene family. *Curr Microbiology,* **58**; 399-403.
7. Nor-Alimah R., Cheng N.A.B.Y., Hardany Primarizky and Zunita Z. (2008). *Rhodococcus equi* from a discharging, cavitating cutaneous mass of domestic cat. Preceedings of 20th VAM Congress. pp 71.
8. Takai S., Martens R.J., Julian A., Ribeiro M.G., Farias M.R.D., Sasaki Y., Inuzuka K., Kakuda T., Tsubaki S. and Prescott J.F. (2003). Virulence of *Rhodococcus equi* isolated from cats and dogs. *Journal of Clinical Microbiology*, **41**; 4468-4470.
9. http://www.merckvetmanual.com. Accessed on 27. 07. 11 At 11.30 p.m.

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