

Research Article

Characterization of *Rhodococcus equi* Isolates from Foals with Respiratory Problems using a Multiplex PCR for the *vap* Genes

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Abstract | *Rhodococcus equi* is widely associated with subacute or chronic suppurative bronchopneumonia, ulcerative lymphangitis, lymphadenitis and enteritis in foals aged 1-4 months. Identification of the virulence plasmids associated with *R. equi* is of great importance in ascertaining pathogenicity in equines. Virulence factor is associated with presence of 85-90kb plasmid which encodes various virulence associated proteins (VAP -A,-B,-C,-D,-E,-F,-G,-H). VAP A is essential for virulence in foals. This study was aimed at the evaluation of molecular characteristics of *R. equi* isolates from foals with respiratory problems by multiplex PCR assay that amplifies the *vap* gene family (*vap*-A,-B,-C,-D,-E,-F,-G,-H). Twenty-eight isolates of *R. equi* from foals with respiratory problems were used in this study. The PCR amplification reaction was performed using specific primers for *vap* gene family viz. *vap*-A,-B,-C,-D,-E,-F,-G,-H which yielded the bands of 350, 400, 450, 500, 600, 650 and 700 bp, respectively. All the 28 isolates were positive for *vap* A,C,D,E,F,G and H and negative for *vap* B gene which was characteristic of virulent *R. equi* isolates of equine origin. The multiplex PCR assay for the *vap* gene family allowed identification and characterization of virulent isolates in a simple one-step reaction. This is a significant advantage in comparison with the conventional diagnostic methodologies of *R. equi* strains and simultaneously evaluated the presence of the *vap* A gene which is responsible for virulence in foals.

Keywords | *Rhodococcus equi*, Equine, *vap* gene, Virulence- associated protein

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INTRODUCTION

R. equi is Gram positive and facultative intracellular pathogen belonging to order *Actinomycetales*. These are coccobacillary, aerobic and non-motile organisms formerly known as *Corynebacterium equi*. Infection causes subacute or chronic abscessating or suppurative bronchopneumonia, ulcerative lymphangitis and enteritis (Meijer and Prescott, 2004) in foals aged 1-4 months (Tkachuksaad and Prescott, 1991; Yager et al., 1991; Khurana et al., 2009; Von Bargen and Haas, 2009; Khurana, 2015a, 2015b; Khurana et al., 2015) months old. *R. equi* has been reported to infect number of domestic animals including pig, sheep, camel and cattle and has been also recognized as opportunistic pathogen in both immunocompetent and immunocom-

promised humans and readily cause infection in HIV positive patients (Takai et al., 1995; Mizuno et al., 2005; Napoleao et al., 2005; Khurana, 2014; Khurana et al., 2014). It is commonly isolated from soil, feces and gut from healthy and sick animals. Identification of the virulence plasmids associated with *R. equi* is of great importance in characterizing pathogenicity of disease in equines (Monego et al., 2009). The virulence is associated with the ability of the bacteria to prevent phagosome-lysosome fusion and replication within macrophages resisting clearance by organism defence (Kanaly et al., 1993; Krewer et al., 2008). Virulence factor is associated with presence of 85-90kb plasmid which encodes various virulence associated antigen (Byrne et al., 2001). According to many researchers virulent and intermittently virulent *R. equi* is presented with virulence

associated antigen Vap A (15-17 kDa) and Vap B (20 kDa) respectively. Gene for the *vapA* antigen is virtually found in all clinical *R. equi* isolated from foals (Costa et al., 2006; Takai et al., 1996) and *vap B* gene on isolates from pigs and humans. Earlier studies identified pathogenicity island within the plasmid which contains seven *vap* genes and discovery of more *vap* proteins has taken this number to nine *vap* genes *vap-A*, -B, -C, -D, -E, -F, -G, H and I. Thus the presence or absence of the *vapA* gene can be used as a marker to show presence or absence of the *vapA* encoding virulence plasmid. Virulence plasmid negative strains of *R. equi* do not express *vapA* gene and are therefore incapable of causing disease (Jain et al., 2003). The multiplex PCR assay for the *vap* gene family allowed identification and characterization of virulent isolates in a simple one-step reaction. This is a significant advantage in comparison with the diagnostic methodologies presented by Halbert et al. 2005 which allowed identification of *R. equi* strains and simultaneously evaluated the presence of the *vapA* gene. The PCR technique has the potential to identify virulent *R. equi* rapidly by amplification of gene sequences that are unique to the virulence plasmids. This approach is useful not only for epidemiological investigations but also for early diagnosis programs. This study was aimed at the evaluation of molecular characteristics of *R. equi* isolates obtained from foals and adult horses from different geographical locations in India by Multiplex PCR assay that amplifies the *vap* gene family (*vap-A*, -B, -C, -D, -E, -F, -G, -H).

with respiratory problems from Haryana and Rajasthan previously identified as *R. equi* by biochemical tests.

DNA EXTRACTION

All twenty eight isolates were cultured 5% sheep blood agar and then transferred to nutrient agar and incubated for 24 hours at 37°C. Then the colonies of all 28 isolates were inoculated in BHI broth and incubated at 37°C for 24 hours. The genomic DNA of all isolates from overnight BHI broth culture was isolated by snap chilling. One ml of overnight BHI broth culture of all the isolates was taken in eppendorf tube and centrifuged at 7000 rpm for 5 min. at 4°C and supernatant was discarded. Again one ml of BHI is added to same tube and above step of centrifugation was repeated and supernatant was discarded. 100-200 µl of nuclease free water (NFW) was added to the precipitate in the eppendorf tube and kept in water bath for 5 min. at 95°C and immediately kept in ice for 5 min. The suspension obtained was centrifuged at 7000 rpm for 5 min. at 4°C. The supernatant was stored in fresh eppendorf tube and used as DNA template for Multiplex PCR. The purity of DNA was evaluated by taking the ratio of optical densities (OD) at 260 nm to that of 280 nm, by spectrophotometer (Biorad Smart Spec™ Plus). The samples having OD ratio between 1.7-1.9 were considered having acceptable purity and used in future experiments. For computing the concentration of plasmid DNA, it is known that one OD value at 260 nm corresponds to 50 µg/ml of double stranded DNA. Hence, the concentration of DNA was calculated using the following formula:

$$DNA\ concentration\ (\mu g\ DNA/ml) = OD\ 260 \times 50 \times dilution\ factor$$

MATERIALS AND METHODS

BACTERIAL ISOLATES

The study was conducted on 28 bacterial isolates from foals

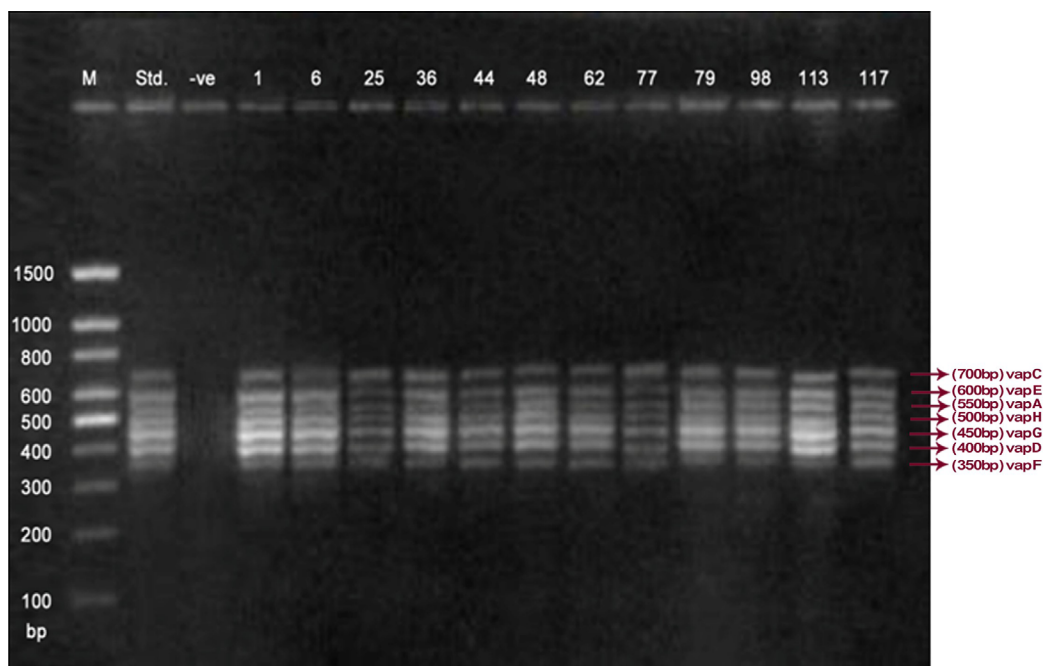


Figure 1: Multiplex PCR amplification of *vap* gene family.

Lane 1: Molecular Ladder; **Lane 2:** *R. equi* Standard; **Lane 3:** Negative control; **Other Lane:** Respective *R. equi* isolate number

Table 1: Primers utilized with multiplex PCR for *vap* gene family amplification

Identification	GeneBank access	Sequence	Size (bp)
VAPA-F	AF116907	ACAAGACGGTTTCTAAGGCG	550
VAPA-R	AF116907	TTGTGCCAGCTACCAGAGCC	
VAPB-F	RER20KDAVA	GAATTTCGAAAGCGCAAAGGT	650
VAPB-R	RER20KDAVA	TTCCGTGAACATCGTACTGC	
VAPC-F	AF116907	GGGTCGTCCATCCAAATCGA	700
VAPC-R	AF116907	GGTCAGGCCTATCACCCTTG	
VAPD-F	AF118814	GGTGGTGCATGTCAGAATG	400
VAPD-R	AF118814	TGGAACGTCTTGCCCTTCTT	
VAPE-F	AF116907	ATATGACGACCGTTCACAAG	600
VAPE-R	AF116907	CTCCGATGCCACCAAATA	
VAPF-F	AF116907	AGAATATGCCTGGTATGGGC	350
VAPF-R	AF116907	TCGTCGTATAGCTGCTGCAG	
VAPG-F	AF116907	TCATTGCCACCCTCCGGTTC	450
VAPG-R	AF116907	GCGAACGCGGAAACTTCAAT	
VAPH-F	AF116907	AATTCCTATCAAGGACAGC	500
VAPH-R	AF116907	ATACCGATTACGGAGCTCAC	

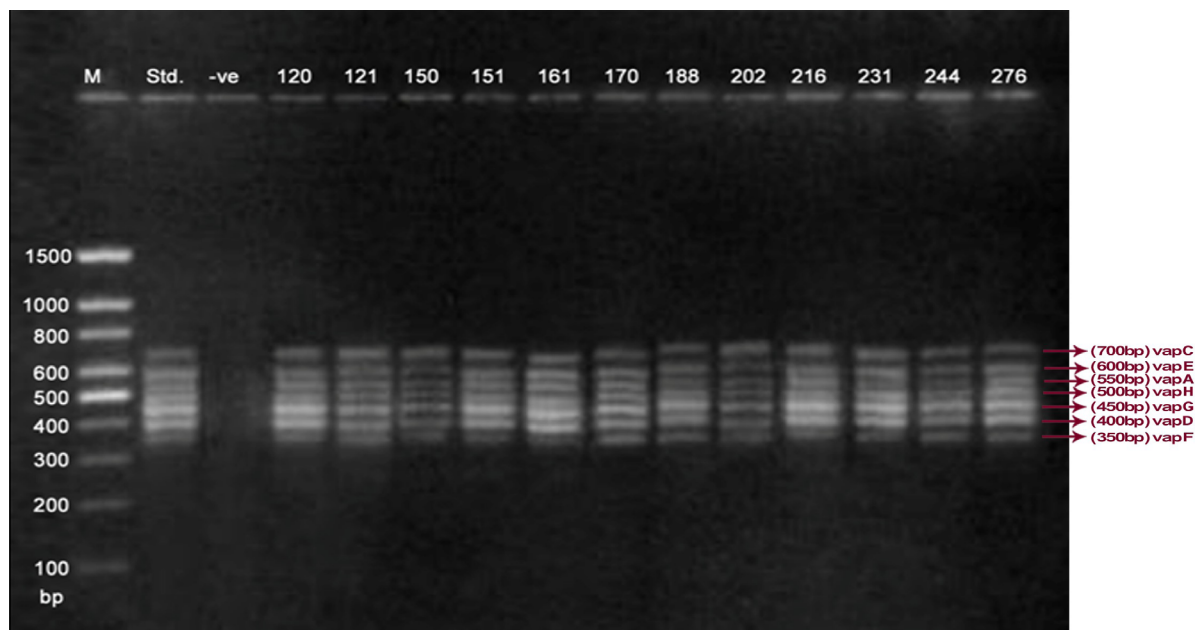


Figure 2: Multiplex PCR amplification of *vap* gene family.

Lane 1: Molecular Ladder; Lane 2: *R. equi* Standard; Lane 3: Negative control; Other Lane: Respective *R. equi* isolate number

MULTIPLEX PCR ASSAY FOR VAP GENE FAMILY

The PCR reaction was carried out as described by Monego et al. (2009) with some modifications. The virulence associated protein (vap) family genes viz. vap-A, -C, -D, -E, -F, -G and -H were amplified using gene specific primers. The details of the primers were mentioned in Table 1. The multiplex PCR cycling conditions consisted of initial denaturation for 5min. at 95°C, denaturation for 45sec. at 95°C, primer annealing for 1 min. at 54°C and extension for 1.5 min. at 72°C following final extension which lasted 10 minutes at 72°C. The samples were subjected to 34 cycles of amplification in thermal cycler (XP gradient cycler Bioer Technology PR, China). For each reaction we

used 3.0 µl of DNA preparation in 25 µl reaction mixture containing 10.2 µl NFW, 2.5µl of 10X PCR buffer with 15mM MgCl₂ (Fermentas), 1.0µl 10mM dNTP mixture (Fermentas), 0.5µl of 10µM of each forward primers, 0.5µl of 10µM of each reverse primers, Taq DNA Polymerase 0.3 µl (5U/ µl stock)(Fermentas). The amplification products (10µl) were submitted to 1.5% agarose gel electrophoresis at 75 volts for 1 hour 25 minutes.

RESULTS

The PCR methodology was applied to analyse 28 isolates. The PCR amplification reaction was performed using the

specific primers for vap gene family viz. *vap* A,-B,-C,-D,-E,-F,-G,-H which yielded the bands of 350, 400, 450, 500, 600, 650 and 700 bp (Figure 1, 2 and 3). The length of DNA fragments amplified by multiplex PCR was in accordance with previous results of Monego et al. (2009). The accuracy of PCR amplification of the *vap* gene family in our control strains was verified by matching the results with that of standard and negative control used in PCR reaction. All the 28 isolates isolated from foals and adult horses with respiratory problems were positive for *vap* -A,-C,-D,-E,-F,-G and-H. The amplicon specific for *vap*-B was absent in all isolates of *R. equi*.

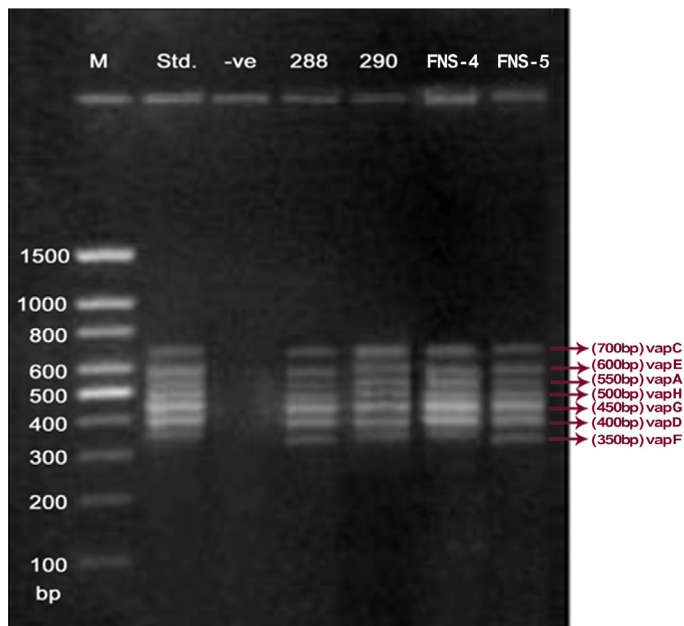


Figure 3: Multiplex PCR amplification of *vap* gene family. Lane 1: Molecular Ladder; Lane 2: *R. equi* Standard; Lane 3: Negative control; Other Lane: Respective *R. equi* isolate number

DISCUSSION

The virulence associated antigens (Vap-A) and plasmids are used as epidemiological markers for *R. equi* virulence in foals (Cohen et al., 2005; Takai et al., 1999). Two categories of plasmids: VapA and VapB family are mainly responsible for pathogenesis and host tropism of *R. equi* (Takai, 1991a, 1991b; Oldfield et al., 2004; Ocampo-Sosa et al., 2007). *R. equi* isolated from clinical cases express only one of these two plasmids and carries the gene(s) encoded either in *vap*A or *vap*B families (Takai et al., 1995). The VapA+ B- type plasmid is associated with the infection in horse (Takai et al., 1995; Ocampo-Sosa et al., 2007). This unique plasmid-determined host-specificity has been found only in Rhodococci, however the precise role and mechanism of the *vap* antigens remain enigmatic. Monego et al. (2009) identified that none of the 32 *vap*A-positive isolates showed the presence of vap B gene. The presence of *vap*-A gene in *R. equi* clinical isolates and its relationship with lethality of *R. equi* to susceptible foal was reported by

many researchers (Takai et al., 1995; Takai, 1997; Wada et al., 1997). In present study we analyzed 28 *R. equi* isolates from different geographical regions of India for the presence of *vap* genes (*vap*-A,-B,-C,-D,-E,-F,-G,-H) using Multiplex PCR. The molecular profile of the *vap* gene family found in our study presented *vap*A, and *vap*C to *vap*H genes. The profile all the isolates showed *vap*A, *vap*D, and *vap*G, which in agreement with result data of Jacks et al. (2007), which indicate that these *vap* genes are the most biologically relevant as they are preferentially induced during infection in the natural host. None of the 28 *vap*-A positive isolates showed the presence of *vap*-B gene. The results obtained agreed with that of Takai et al., (1995) reported that *R. equi* isolates can express either *vap*-A or *vap*-B but not both. In our study all isolates were found positive for *vap*-A gene and negative for *vap*-B gene, thus providing a *vap* A+B- profile, which is specific for virulent *R. equi* isolates of equine origin.

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