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Molecular characterization of clinical isolates of *Rhodococcus equi* with PCR assay based on virulence plasmid marker

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ABSTRACT

Rhodococcus equi is one of the most important pathogens of foals, in which it causes a disease manifesting in pyogranulomatous bronchopneumonia, abscesses, lymphadenitis or ulcerative enterocolitis. *R. equi* can be pathogenic to other domestic and wild animals and humans as well. Although, *R. equi* is prevalent in India, the work carried out in our country has not gone much beyond isolation of organism from clinical cases of foal pneumonia. Therefore, the present study was carried out for characterization of *R. equi* strains isolated from clinical cases based on plasmid markers (*tra*A, *vap*A and *vap*B genes) and antibiotic sensitivity. In the present study, 298 samples (nasal swab, 136; fecal sample, 130; soil, 28; tissue, 4) were collected and processed for isolation, identification, and characterization of *R. equi* via biochemical test, antimicrobial susceptibility test and PCR. A total of 28 *R. equi* isolates could be recovered from clinical samples. All the 28 isolates were found sensitive to chloramphenicol, erythromycin, oxytetracycline, ciprofloxacine, neomycin and rifampin while resistant to ampicillin, trimethoprim, sulphadiazine, cloxacin, amikacin, cephalexin, and kanamycin in *in vitro* antimicrobial assay. PCR typing based on plasmid gene markers: *traA, vapA,* and *vapB* revealed that vapA plasmid was present in 26 isolates whereas it was absent in 2 isolates. Periodic monitoring of horse farm before and after foaling season is recommended for diagnosis of *R. equi* and initiating requisite bio-security and therapeutic measures.

Key words: Antimicrobial susceptibility, PCR, Plasmid, *Rhodococcus equi*, *vapA* gene

Rhodococcus equi is a soil actinomycete responsible for severe respiratory disease in foals between 1–4 months of age, however, other domestic animals like cattle, pigs, goat, sheep, and camels are also affected (Weinstock and Brown 2002, Cohen 2014). The classical form of *R. equi* infection in foals is characterized by suppurative bronchopneumonia; other clinical manifestations of *R. equi* in foals include ulcerative enterocolitis, colonic or mesenteric lymphadenopathy, osteomyelitis, septic arthritis, synovitis, and uveitis (Cohen 2014). *R. equi* is considered as one of the most economically important pathogen of horse breeding industry worldwide due to high mortality rate, non availability of suitable diagnostics in early stage, prolonged and expensive treatment regimen. *R. equi* infection was also observed in immuno-compromised human patients (Dias *et al.* 2013).

The specific and timely diagnosis of *R. equi* infection is of paramount importance for clinical management of the infected foals, including timely therapeutic and biosecurity intervention. Efforts for early diagnosis using serologic methods have failed because of its inherent limitations like questionable specificity and a considerable time lag (Sellon *et al.* 2001). A number of polymerase chain reaction (PCR) assays have been optimized for detection and characterization of *R. equi* (Arriaga *et al.* 2002, Ladro´n *et al.* 2003, Pusterla *et al.* 2007, Monego *et al.* 2009). The virulence of *R. equi* is associated with the presence of large plasmids about 85–90 kb in size which encode virulence-associated protein A (VapA) or protein B (VapB) (Takai *et al.* 1991, Letek *et al.* 2008). However, the work carried out in our country has not gone much beyond isolation of organism from clinical cases of foal pneumonia (Saxena and Narwal 2009, Khurana *et al.* 2009). Therefore, the present study was carried out for characterization of *R. equi* strains isolated from clinical cases based on plasmid markers (*tra*A, *vap*A and *vap*B genes).

MATERIALS AND METHODS

Samples (298: (nasal swab, 136; fecal sample, 130; soil, 28; tissue, 4) used for the isolation of *R. equi* were collected from foals housed at different premises spread across

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Haryana state during the period between 2005 and 2010. These premises had a history of foal mortality. At the time of sample collection, 108 foals were showing respiratory symptoms (cough, nasal discharge, abnormal auscultation, fever, and/or rapid respiratory rate) and 28 were apparently healthy in-contact foals. Age of the foals ranged between 1–6 months. Nasal swabs were obtained from the distal nares using a sterile culturette swab with PDC-300 Amies modified medium. All the samples were transported to laboratory at 4°C. Nasal swabs and tissue swabs were plated directly on nutrient agar (NA) media and incubated at 37°C for 48 h. Fecal and soil samples were processed by suspending in brain heart infusion (BHI) broth for 12 h at 30°C and then plated on NA media. Mucoid colonies showing salmon pink pigmentation after 48 h were Gram stained and observed under microscope.

Pure cultures of clinical isolates were assayed for biochemical fermentation properties and antibiotic susceptibility. Biochemical assays like CAMP test and other sugar fermentation tests were performed as described earlier (Anzai *et al.* 1997). Suspected colonies were identified as *R. equi* on the basis of biochemical and morphological characteristics (Sellon *et al*. 2001). Antimicrobial susceptibility testing was performed by disc-diffusion method (Giguère *et al.* 2010) using antimicrobial discs impregnated with 17 different antimicrobial agents which included amoxycillin, gentamycin, ampicillin, trimethoprim, chloramphenicol, sulphadiazine, cloxacillin, oxytetracycline, amikacin, streptomycin, cotrimoxazole, cephalexin, kanamycin, erythromycin, ciprofloxacin, neomycin, and rifampin. Antimicrobial resistant zone was read by antibiotic zone reader. The interpretation of antibiotic susceptibility/ resistance was done as per the manufacturer's recommendations.

Genomic DNA was isolated from overnight cultures of *R. equi* isolates grown in brain-heart infusion (BHI) broth using a cetyltrimethylammonium bromide (CTAB)-based protocol (Sambrook and Russell 2001). The DNA quality was assessed visually on agarose gels and concentration was determined spectrophotometrically using Nanodrop spectrophotometer. The identity of prospective *R. equi* clinical isolates was confirmed by species-specific and cholesterol oxidase (*choE*) PCR using genomic DNA as template. The analytical sensitivity (detection limit) of PCR assays was determined on 10-fold serial dilutions of genomic DNA. For plasmid characterization, whole DNA (genomic and plasmid DNA) of *R. equi* was isolated by boiling and chilling method. Briefly, overnight cultures of *R. equi* (approximately 1 ml) were centrifuged at 6,000 rpm for 10 min. The recovered pellet was resuspended in 100 µl of nuclease-free water. The suspensions were boiled for 10 min and then snap chilled in crushed ice. The obtained lysate was briefly centrifuged and supernatant was collected in a fresh tube. The 5µl of whole DNA was used as template in PCR reaction for plasmid characterization using *traA, vapA* and *vapB* primers.

The oligonucleotide primers used in the study are shown in Table 1; first 2 sets of primer were used for specific detection of *R. equi* and rest of three primer sets were used for plasmid characterization. The reaction mixture for PCR amplification consisted of 2 µl of DNA template, 1 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 2.5 µl of 10X PCR amplification buffer, 10 pmol of each primer, 200 µM each dNTPs, and deionized water to a final volume of 25 µl. The DNA was first denatured at 95°C for 5 min and was then subjected to 30 cycles comprising of denaturation at 95°C for 1 min, annealing at defined temperature as indicated in table 2 for respective primer for 1 min, and extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were analyzed in 1.2 % agarose gel and results were documented using gel documentation system.

RESULTS AND DISCUSSION

R. equi was recovered from 28 clinical specimen (nasal swab, 26; soil,1; tissue,1). It is pertinent to mention that 14 isolates previously reported by this group (Khurana *et al.* 2009) were also taken into count and used for molecular characterization in the present study. However, no *R. equi* could be isolated from the fecal samples. One time collection of faecal sample from a foal – as is the case in the present study- might have less diagnostic value because of individual animal variation as well as farm-to-farm variation in the number of *R equi* in the faecal shedding

Table 1. Oligonucleotide primers used in polymerase chain reactions (PCR)

Sl.No.	Target gene	Oligonucleotide sequence(5´- 3´)	Annealing temperature (°C)	Amplicon size (bp)	Reference
1	Species specific	Forward–tccagaagcgggatgaggattc Reverse-tggtgtgatggcggaagatc	46	700	(Arriaga *et al.* 2002)
2	*choE*	Forward-gtcaacaacatcgaccaggcg Reverse- cgagccgtccacgacgtacag	56	959	(Ladron *et al.* 2003)
3	*vap*A	Forward-gagcaagcgataccgccgg Reverse- ctggatatggccgaggaagc	53	286	(Ocampo-Sosa *et al.* 2007)
4	*vapB*	Forward- tgcgatagccacagccgct Reverse- ctggatatggccgaggaagc	53	477	
5	*traA*	Forward- agagttcatgcgtgacaacg Reverse- gtccacaggtcaccgttctt	53	959	

(Pusterla *et al.* 2007, Takai *et al.* 1986). On the basis of cultural characteristics and biochemical tests, all these 28 isolates were characterized as *R. equi*. All the isolates showed positive reaction in hydrolysis of urea, hydrogen sulphide production, nitrate reduction and were catalase positive. All the isolates showed negative reaction in methyl red and Voges-Proskauer test and were unable to ferment citrate, arabinose, rhamnose, dextrose, sucrose, maltose, lactose, sorbitol, and salicin.

In the antimicrobial susceptibility assay, all of the 28 isolates were susceptible to chloramphenicol, erythromycin, oxytetracycline, ciprofloxacine, neomycin and rifampin and resistant to ampicillin, trimethoprim, sulphadiazine, cloxacin, amikacin, cephalexin and kanamycin. However, the isolates showed differential susceptibility to amoxycillin, gentamycin, streptomycin and cotrimoxazole. Fifteen isolates were resistant and 13 isolates were sensitive to amoxicillin but most of the *R. equi* isolates were resistant to gentamycin (24), streptomycin (23), and cotrimoxazole (21). Erythromycin and other macrolides preferably coupled with rifampicin are considered to be the antibiotics of choice for treatment against rhodococcal infections (Giguère *et al.* 2011). However, frequent use of rifampicin may lead to the emergence of rifampin resistance *R. equi* (Giguère *et al.* 2010, Buckley *et al.* 2007). Therefore, this drug should be combined with other antimicrobials such as erythromycin, ciprofloxacin and/or newer and safer macrolides like azithromycin and clarithromycin (Cisek *et al*. 2014). Here, in this study, all the isolates being susceptible to these antimicrobial agents, treatment with suitable combinations of these drugs may be appropriate for effective treatment of rhodococcal infection in foals.

Besides classical methods of identifying *R. equi* isolates by Gram-staining and biochemical assay, PCR technique with two different sets of primer were also used for further confirmation of the isolates. PCR assay with species-specific and *choE* primer resulted in the amplification of the expected 700 bp and 959 bp amplicons (Figs 1, 2) which further authenticates the identity of the clinical isolates as *R. equi*. The assessment of *in vitro* sensitivity of the PCR assays revealed that it could detect as less as 10 pg genomic DNA which is equivalent to 3 *R. equi* colony forming units (CFUs) (Fig. 3).

The virulence of *R. equi* is associated with the presence of extra-chromosomal plasmid DNA. In horse isolates, these plasmids are 85–90 kb in size and encode virulence-associated protein A (VapA), a 15–17-kDa surface lipoprotein antigen important for intramacrophage survival, cytotoxicity, and horse pathogenicity (Takai *et al.* 1991, Benoit *et al.* 2001). In contrast, non-equine (pig, camel etc.) *R. equi* isolates often contined a larger (20-kDa) variant of surface lipoprotein antigen known as VapB (Letek *et al.* 2008, Ribeiro *et al.* 2011). In this study, we focus to characterize the isolates on the basis of plasmid markers (*tra*A, *vap*A and *vap*B).The gene *tra*A is required for conjugal transfer of plasmids between strains and used for identifying plasmid positive strain. Out of the 28 isolates, 26 showed 959 bp fragment of the *traA* gene (Fig. 4) indicating that they contained a plasmid. Two isolates did not have plasmid. Further, *vap*A and *vap*B PCR revealed that all the plasmid- positive *R. equi* isolates were harboring virulent *vap*A gene as evident by amplification of 286 bp *vap*A fragment (Fig. 4). Exclusive presence of *vap*A plasmid in 26 *R. equi* isolates is in agreement with the previous observations that *vap*A and *vap*B gene are mutually exclusive and are allelic variants of one locus that has divergently evolved in two different plasmid subpopulations

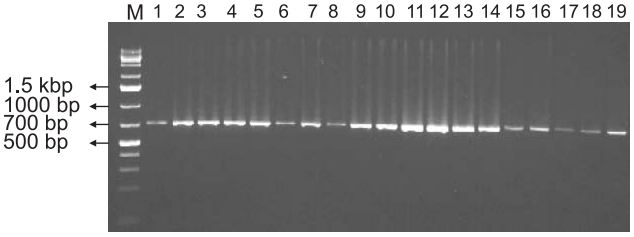

Fig. 1. Representative species-specific PCR amplification products (700 bp) obtained from genomic DNA samples of *R. equi*. Lanes: M, 1 kb DNA ladder plus; 1, reference strain; 2 to 19, *R. equi* clinical isolates.

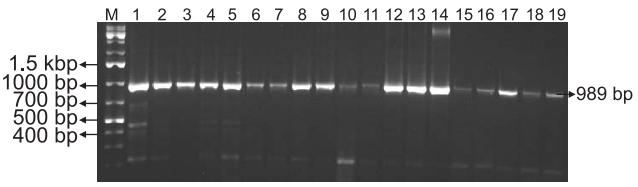

Fig. 2. Representative *choE* PCR amplification products (959 bp) obtained from genomic DNA samples of *R. equi*. Lanes: M, 1 kb DNA ladder plus; 1, reference strain; 2 to 19 *R. equi* clinical isolates.

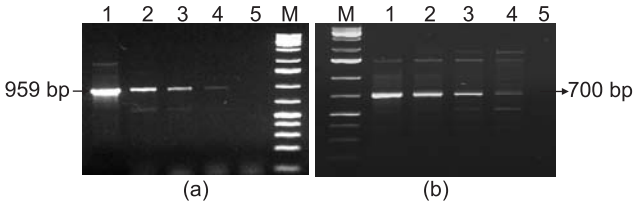

Fig. 3. Sensitivity of the *choE* PCR (a) and species-specific primer PCR (b). Purified genomic DNA obtained from *R. equi* isolate was diluted (10- fold serial dilution) and detection limit of the *choE* PCR was observed with various amounts of genomic DNA template. Lanes 1 to 5 (10 ng, 1 ng, 100 pg, 10 pg and 1 pg genomic DNA respectively). Lane M, 1 kb DNA Ladder plus.

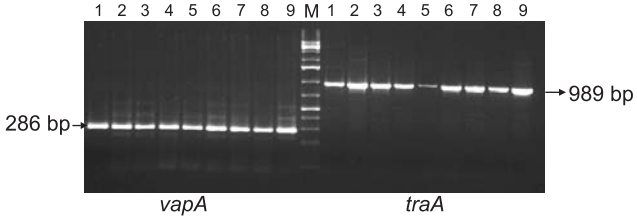

Fig. 4. PCR amplification of *vapA* and *traA* gene. Lanes: 1, reference strain ; 2 to 9 *R. equi* clinical isolates; M, 1 kb DNA ladder plus.

(Ocampo-Sosa *et al.* 2007; Letek *et al.* 2008). Although, it is generally believed that isolates causing disease in foals are *vapA*-plasmid positive, the plasmid less *R. equi* isolates (n=2) recovered from diseased foals are exceptional findings in this study. However, similar observation was made by Morton *et al.* (2001) where a number of plasmid less *R. equi* isolates were found to be associated with clinical cases of foal pneumonia.Therefore, it may be anticipated that occurrence of virulent plasmid may not entail the disease causing ability of *R. equi* rather it intensify the pathogenic behavior of the organism and aggravate the disease prognosis.

R. equi infection is prevalent in equine breeding farm in India and, as such, extensive studies with clinical samples from *R. equi*-infected and non-infected foals, adult equines, and environmental samples from various geographical location are required for the molecular epidemiological analysis of *R. equi.* Periodic monitoring of horse premises before and after foaling season would be of immense help for diagnosis of *R. equi* and initiating requisite bio-security and therapeutic measures.

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