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

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Received: 2 February 2015; Accepted: 10 June 2015

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 (*traA*, *vapA* and *vapB* 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, oxetetracycline, ciprofloxacine, neomycin and rifampin while resistant to ampicillin, trimethoprim, sulphadiazine, cloxacin, amikacin, cephalixin, 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) (Takei 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 (*traA*, *vapA* and *vapB* 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...
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, cephalixin, 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 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.

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>
<thead>
<tr>
<th>Sl.No.</th>
<th>Target gene</th>
<th>Oligonucleotide sequence(5´- 3´)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>choE</td>
<td>Forward- ctgcagacagcagccggcggc</td>
<td>56</td>
<td>959</td>
<td>(Ladron et al. 2003)</td>
</tr>
<tr>
<td>2</td>
<td>vapA</td>
<td>Forward- gataggtgacagtgacagcg</td>
<td>53</td>
<td>286</td>
<td>(Ocampo-Sosa et al. 2007)</td>
</tr>
<tr>
<td>3</td>
<td>vapB</td>
<td>Reverse- cgcgagccggcggcgacagc</td>
<td>53</td>
<td>477</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>traA</td>
<td>Reverse- agtcagctgaagcgcggc</td>
<td>53</td>
<td>959</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotide primers used in polymerase chain reactions (PCR)
MOLECULAR CHARACTERIZATION OF RHODOCOCCUS EQUI

(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, ciprofloxacin, neomycin and rifampin and resistant to ampicillin, trimethoprim, sulphadiazine, cloxacitin, amikacin, cephalexin and kanamycin. However, the isolates showed differential susceptibility to amoxicillin, 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 like azithromycin and clarithromycin (Cisek 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 contain 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 (traA, vapA and vapB). The gene traA 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, vapA and vapB PCR revealed that all the plasmid-positive R. equi isolates were harboring virulent vapA gene as evident by amplification of 286 bp vapA fragment (Fig. 4). Exclusive presence of vapA plasmid in 26 R. equi isolates is in agreement with the previous observations that vapA and vapB 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.](image1)

![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.](image2)

![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.](image3)

![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.](image4)
(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.

ACKNOWLEDGEMENT

We thank the Indian Council of Agricultural Research, New Delhi for providing necessary financial assistance. The authors also acknowledge the assistance provided by various state animal husbandry departments and stakeholders during sample collection and the technical assistance of Mr Sita Ram and Mr Guru Dutt, National Research Centre on Equines, Hisar, India.

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