RESISTOTYPES OF *Rhodococcus equi* ISOLATED FROM FOALS WITH RESPIRATORY PROBLEMS

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KEYWORDS

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Marker

ABSTRACT

Rhodococcus equi has been recognized primarily as a respiratory pathogen of equines particularly of foals between one and four months of age. *R. equi* is ranked among the most important disease problems in equines especially because of its high prevalence and mortality rate. *R. equi* being an intracellular pathogen is very fastidious and requires prolonged specific antibiotic combination therapy lasting up to three months for successful treatment. This assumes further importance as no effective vaccination is available for prevention. It has zoonotic potential and may be responsible for infection in immunocompromised humans. This study is aimed at analyzing the distribution pattern of the resistotypes (R-types) of various isolates of *R. equi* in different areas of Haryana and Rajasthan, India. Antimicrobial susceptibility pattern of *R. equi* isolates was determined by Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines. A total of twenty eight clinical isolates of *R. equi* from foals from different parts of Haryana and Rajasthan were used in this study. Antibigram of *R. equi* isolates with 33 antibiotics revealed ten distinct R-types: R-type 1 to R-type 10, on the basis of variable results of four antibiotics i.e. amoxycillin, gentamycin, colistin and streptomycin. Out of ten resistotypes obtained the relative frequencies of R-1 resistotype and R-4 resistotype were found to be high i.e. 28.57% and 25%, respectively. Differentiation of *R. equi* strains into R-types is an important tool for therapeutics. In addition to direct foal management, it may have implication in identifying the source and spread of infection, and as epidemiological marker to correlate various isolates from various places, ultimately helping in therapeutics for timely control.

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1 Introduction

Rhodococcus equi is aerobic, non-sporulating, non-motile, gram-positive bacteria, found in soil, herbivore dung and the intestinal tract of cattle, horse, sheep, pig and some other animals. It is primarily a respiratory pathogen mainly affecting the foals between 1-4 months of age (Khurana et al., 2015). The disease is direct anthroponosis since the animals are primary reservoirs of the etiological agent (Khurana, 2014). It can be responsible for infection in humans compromised by immunosuppressive drug therapy, lymphoma or AIDS (Takai et al., 1995; Mizuno et al., 2005; Napoleao et al., 2005). *R. equi* has been ranked one of the most important disease problems in the horse industry especially because of its high prevalence and mortality rate (Blood-Horse, 2009; Muscatello, 2009; Elissalde et al., 1980; Mir et al., 2015).

The symptoms in foals include pyrexia, respiratory distress, pulmonary lesions and chronic lung abscesses, which when left untreated lead to death due to asphyxiation (Wichtel et al., 1991; Lavoie et al., 1994). The infection can spread from the lungs to other organs and joints when granulomatous foci in the lung open up (Prescott, 1991; Giguere, et al., 2011). It has been reported that only a small proportion of all *R. equi* in soil are able to cause the infection and only *R. equi* carrying virulence plasmids can cause disease in foals (Muscatello et al., 2006). Foal survival requires successful antimicrobial therapy (Sweeney et al., 1987).

Most of the conventional antibiotics are not effective against *R. equi* because it is an intracellular pathogen. *R. equi* is usually resistant to beta-lactam antibiotics such as penicillin G, oxacillin, ampicillin, carbenicillin and cefazolin (Kedlaya et al., 2001). Current treatment comprises the use of a combination of erythromycin and rifampin (Jacks, 2003).

This combination facilitates the drugs to penetrate the lung abscesses, macrophages or neutrophils where the bacteria survive and multiply (Prescott, 1991). During the last decade the minimum inhibitory concentrations (MIC) of erythromycin and rifampin for *R. equi* are rising and there are reports of resistance to these antibiotics (von Bargaen & Haas, 2009). Therapy with orally administered macrolides has greatly improved survival rates for foals with *R. equi* pneumonia (Sweeney et al., 1987). *R. equi* isolates resistant to the commonly used macrolides (azithromycin, clarithromycin and erythromycin) as well as rifampin are emerging these days. The overall prevalence of *R. equi* isolates resistant to macrolides or rifampin has been reported as 4% (Giguere et al., 2010). Keeping in view, the emerging variable resistance to various important antibiotics and difficulty in identification of *R. equi* isolates due to inconsistent biochemical tests, the present study was undertaken to study the resistance pattern of various isolates of *R. equi* and to find out various resistotypes.

2 Materials and Methods

A total of twenty eight *R. equi* isolates from foals with respiratory problems from various parts of Haryana and Rajasthan were used. Nasal swabs from each case were collected in Cary-Blair transport medium and processed in the laboratory for isolation. All the isolates of *R. equi* were maintained on nutrient agar slants. All the twenty eight isolates of *R. equi* were identified by cultural characteristics, gram staining and biochemical tests. After 48 hrs incubation of nutrient agar plates characteristic 1-2 mm irregularly round smooth, mucoid, glistening, semi transparent, salmon pink to yellow coloured and coalescing colonies on nutrient agar were observed. The antibiotic sensitivity test was conducted by Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines (Bauer et al., 1966).

Table 1 Antibiotics used and their concentration.

S. No	Antibiotics	Concentration	S. No	Antibiotics	Concentration
1	Azithromycin	30 mcg	18	Ampicillin	25 mcg
2	Ciprofloxacin	10 mcg	19	Cloxacillin	30 mcg
3	Chloramphenicol	25 mcg	20	Kanamycin	05 mcg
4	Ceftriaxone	30 mcg	21	Nalidixic acid	30 mcg
5	Cefapazone	75 mcg	22	Oxacillin	05 mcg
6	Enrofloxacin	10 mcg	23	Penicillin G	10 mcg
7	Erythromycin	10 mcg	24	Sulphadiazine	100 mcg
8	Methicillin	10 mcg	25	Trimethoprim	25 mcg
9	Norfloxacin	10 mcg	26	Amoxycillin	10 mcg
10	Neomycin	03 mcg	27	Colistin	25 mcg
11	Ofloxacin	02 mcg	28	Gentamycin	30 mcg
12	Oxytetracycline	30 mcg	29	Streptomycin	25 mcg
13	Rifampicin	30 mcg	30	Tetracycline	10 mcg
14	Roxithromycin	30 mcg	31	Lincomycin	15 mcg
15	Tobramycin	30 mcg	32	Pefloxacin	05 mcg
16	Vancomycin	05 mcg	33	Clindamycin	25 mcg
17	Amikacin	30 mcg			

mcg = Micrograms

A few colonies of the *R. equi* were picked with a wire loop from the nutrient agar slant and inoculated into a test tube containing brain heart infusion broth (BHI). These tubes were incubated at 37°C for 16-18 hours to produce a bacterial suspension of moderate cloudiness. The bacterial broth suspension was spread evenly onto the surface of Mueller Hinton Agar (MHA) plates covering whole of the plate with a sterile cotton swab. After the inoculums had dried, the disks were placed on the agar. The discs of thirty three antibiotics were used (Table 1).

Plates were then incubated immediately at 37°C for 24 hrs. After incubation, the diameter of zone of inhibition was measured with antibiotic zone reader and interpreted as per manufacturer interpretation values. The results of antibiotic sensitivity were analyzed to find out antibiotics which are sensitive or resistant to all isolates. Antibiotics which were showing variable results i.e. some of isolates were sensitive and some were resistant to antibiotics were identified. On the basis of antibiotic sensitivity/ resistance twenty eight *R. equi* isolates were classified into various resistotypes.

3 Results

All the isolates were sensitive to 20 antibiotics (zone range with mean in mm): azithromycin (30-34, 32), ciprofloxacin (31-38, 34.5), chloramphenicol (20-30, 25), ceftriaxone (28-34, 31), clindamycin (20-28, 24), cefapazone

(22-30, 26), enrofloxacin (28-34, 31), erythromycin (32-38, 35), lincomycin (30-34, 32), methicillin (20-28, 24), norfloxacin (21-30, 25.5), neomycin (20-32, 26), ofloxacin (24-32, 28), oxytetracycline (10-19, 14.5), pefloxacin (25-31, 28), rifampicin (31-36, 33.5), roxithromycin (31-37, 34), tetracycline (25-33, 29), tobramycin (20-34, 27), vancomycin (22-28, 24). All the isolates were resistant showing no inhibition zone or zone of inhibition within resistant range to 9 antibiotics viz. amikacin, ampicillin, cloxacillin, kanamycin, nalidixic acid, oxacillin, penicillin-G, sulphadiazine, trimethoprim. However, 4 antibiotics viz., amoxycillin, colistin, gentamycin, streptomycin showed variable results (Table 2) which formed the basis to formulate resistotypes of *R. equi*. Ten resistotypes could be detected amongst 28 isolates of *R. equi* (Table 3). The resistotype R-1 represented eight isolates (NS-1, NS-44, NS-98, NS-117, NS-120, NS-151, NS-244, NS-290) which showed 28.50 % relative frequency and found to be resistant to amoxycillin, gentamycin, streptomycin and sensitive to colistin. Resistotype R-2 represented only one isolate (NS-6) which showed 03.57% relative frequency and found to be sensitive with all the four antibiotics, Resistotype R-3 represented two isolates (NS-25, NS-276) which showed 07.14 % relative frequency and found to be resistant to all four antibiotics, Resistotype R-4 represented seven isolates (NS-36, NS-79, NS-113, NS-150, NS-231, FNS-4, FNS-5) which showed 25 % relative frequency and found to be resistant to gentamycin, streptomycin and sensitive to amoxycillin, colistin.

Table 2 Antibiotic sensitivity of twenty eight isolates of *Rhodococcus equi*.

Antibiotics to which all the isolates were		Antibiotics to which isolates showed variable resistance pattern
Sensitive	Resistant	
Azithromycin	Amikacin	Amoxycillin (12S, 16 R)
Ciprofloxacin	Ampicillin	Colistin (21S, 7R)
Chloramphenicol	Cloxacillin	Gentamycin (4S, 24R)
Ceftriaxone	Kanamycin	Streptomycin (5S, 23 R)
Clindamycin	Nalidixic acid	
Cefapazone	Oxacillin	
Enrofloxacin	Penicillin-G	
Erythromycin	Sulphadiazine	
Lincomycin	Trimethoprim	
Methicillin		
Norfloxacin		
Neomycin		
Ofloxacin		
Oxytetracycline		
Pefloxacin		
Rifampicin		
Roxithromycin		
Tobramycin		
Tetracycline		
Vancomycin		

*Number in parentheses represent sensitive (S) and resistant (R) isolates

Table 3 Resistotypes recognized amongst twenty eight *Rhodococcus equi* isolates.

Resistotype	Sensitive or Resistant to antibiotic				Sample No. (No. of Isolates)	Relative frequency
	A	C	G	Str		
R-1	R	S	R	R	NS-1, NS-44, NS-98, NS-117, NS-120, NS-151, NS-244 NS-290 (8)	28.57%
R-2	S	S	S	S	NS-6 (1)	03.57%
R-3	R	R	R	R	NS-25, NS-276 (2)	07.14%
R-4	S	S	R	R	NS-36, NS-79, NS-113, NS-150, NS-231, FNS-4, FNS-5 (7)	25.00%
R-5	R	S	S	R	NS-48, NS-216 (2)	07.14%
R-6	S	S	R	S	NS-62 (1)	03.57%
R-7	R	R	R	S	NS-77 (1)	03.57%
R-8	S	R	R	R	NS-121, NS-161, NS-288 (3)	10.71%
R-9	R	R	S	R	NS-170 (1)	03.57%
R-10	R	S	R	S	NS-202, NS-188 (2)	07.14%

A = Amoxycillin, C = Colistin, Str = Streptomycin, G = Gentamycin, R = Resistant, S = Sensitive

Resistotype R-5 represented two isolates (NS-48, NS-216) which showed 03.57% relative frequency and found to be resistant to amoxycillin, streptomycin and sensitive to gentamycin, colistin., Resistotype R-6 represented one isolate (NS-62) which showed 03.57% relative frequency and found to be resistant to gentamycin and sensitive to amoxycillin, colistin, streptomycin.

Resistotype R-7 represented one isolate (NS-77) which showed 03.57% relative frequency and found to be resistant to amoxycillin, gentamycin, colistin and sensitive to streptomycin.

Resistotype R-8 represented three isolates (NS-121, NS-161, NS-288) which showed 10.71 % relative frequency and found to be resistant to gentamycin, streptomycin, colistin and sensitive to amoxycillin. Resistotype R-9 represented one isolate (NS-170) which showed 03.57% relative frequency and

found to be resistant to amoxycillin, colistin, streptomycin and sensitive to gentamycin , Resistotype R-10 represented two isolates (NS-202, NS-188) which showed 07.14% relative frequency and found to be resistant to amoxycillin, gentamycin and colistin, streptomycin (Figure 1). Out of ten resistotypes obtained the relative frequencies of R-1 resistotype and R-4 resistotype were higher i.e. 28.57% and 25%, respectively. Resistotype R-1 included eight isolates of *R. equi* which were isolated from nasal swabs of foals belonging to various places i.e. Tohana (NS-1), Sorkhi (NS-44), Gangua (NS-98), Hanumangarh (Rajasthan) (NS-177), Ismaila (NS-120), Muklan (NS-151), Barwala (NS-244) and Kaimri (NS-290). Whereas resistotypes R-4 included isolates of *R. equi* from various places viz. Sorkhi (NS-36), Sachakhera (NS-79), Muklan (NS-113 and 150), Jakhal (NS-231) and Tohana (FNS-4 and FNS 5). Four isolates of *R. equi* of Tohana were characterized into three resistotypes viz. R-1 (NS-1), R-2 (NS-6) and R-4 (FNS-4 and FNS-5).

Figure 1 Proportion of resistotypes of *R. equi* isolates.

Discussion and conclusions

For selection of appropriate agents to treat *R. equi* infections, both the *in vivo* and *in vitro* properties of the antimicrobial agents should be considered. Previous reports have shown that *R. equi* is susceptible to ampicillin/sulbactam, amoxycillin/clavulanic acid, gentamycin, erythromycin, tetracycline, rifampin, TMP-SMZ, imipenem, and vancomycin. The organism is usually less susceptible to penicillin, ampicillin, cephalosporins, or quinolones (Prescott, 1991; McNeil & Brown 1992; Nordmann & Ronco 1992; Giguere et al., 2011).

All the twenty eight isolates of *R. equi* of this study were grouped into ten resistotypes on the basis of four antibiotics which showed variable results described vide supra. A total of sixteen resistotypes ($2^4 = 2 \times 2 \times 2 \times 2 = 16$) could be possible on the basis of four variable antibiotics if larger number of isolates could be studied for resistotyping. These results could not be compared to previous reports because of non availability of such type of study in earlier literature which shows that the present study is probably the first study of resistotypes of *R. equi*. However, in recent review Khurana (2015) reported emergence of antibiotic resistance to various antibiotics. Resistance to rifampicin in *R. equi* attributable to monooxygenase like sequence has been reported (Anderson et al., 1997). Mutations in *rpoB* gene leading to rifampicin resistance have been reported (Asoh et al., 2013; Liu et al., 2014).

These resistotypes could be used as epidemiological marker during outbreak studies due to *R. equi* in equines. In this study Resistotype R-1 represented eight isolates (NS-1, NS-44, NS-98, NS-117, NS-120, NS-151, NS-244, NS-290) which showed highest relative frequency (28.50 %) and found to be resistant to amoxycillin, gentamycin, streptomycin and sensitive to colistin. These isolates were from various places i.e. Tohana (NS-1), Sorkhi (NS-44), Gangwa (NS-98), Hanumangarh, Rajasthan (NS-177), Ismaila (NS-120), Muklan (NS-151), Barwala (NS-244) and Kaimri (NS-290).

All these isolates were from the areas near Hisar city within a radius of 70 km except isolate NS-177 from Hanumangarh, Rajasthan. Resistotype R-4 had second highest relative frequency (25%) represented by seven isolates (NS-36, NS-79, NS-113, NS-150, NS-231, FNS-4, FNS-5) resistant to gentamycin, streptomycin and sensitive to amoxycillin, colistin from various places viz. Sorkhi (NS-36), Sacchakhera (NS-79), Muklan (NS-113 and 150), Jakhal (NS-231) and Tohana (FNS-4 and FNS-5). Four isolates of *R. equi* of Tohana were characterized into three resistotypes viz. R-1 (NS-1), R-2 (NS-6) and R-4 (FNS-4 and FNS-5). This finding is very interesting and showing the use of resistotypes as epidemiological marker and could be useful for therapy in a particular endemic area/farm after identifying the resistotype.

In the present study all isolates of *R. equi* were found sensitive to rifampicin and macrolides antibiotics viz. azithromycin,

erythromycin and roxithromycin. Macrolide resistance in *R. equi* has also been reported (Burton et al., 2013; Liu et al., 2014). A glycopeptides resistance operon *vanO* having potential implications in *R. equi* therapy has been described (Gudeta et al., 2014). Cohen (2014) has warned about the challenges of emergence of resistance to macrolide due to non-availability of effective alternative for *R. equi* therapeutics.

Rifampicin along with macrolide is drug of choice for effective treatment of *R. equi* infections. However the emergence of resistance against these antibiotics poses a serious challenge in therapeutic management. But in this study such problem has not been observed, therefore the cases of foals respiratory diseases can be treated successfully in this area with standard antibiotic combination therapy used to treat *R. equi* infections. It is therefore, recommended that veterinarians must use antibiotics for treatment judiciously.

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Conflict of interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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