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INVITRO DRUG SENSITIVITY OF *Rhodococcus equi* ISOLATED FROM NORMAL MICRO-FLORA OF SKIN AND UPPER RESPIRATORY TRACT OF EQUINES

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AUTHOR'S CONTRIBUTION
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ABSTRACT

Rhodococcus equi (*R. equi*) is asignificant pathogen with equines being the most susceptible host. This facultative intracellular pathogen is quite stable in nature and contaminates equine environments. In this paper aimed to antibiogram study of *R. equi*. Out of 220 samples processed 37 isolates were found to be R. equiin out of 75 *Rhodococcus* sp. All the 37 isolates of *R. equi* were subjected to resistotyping with 16 different antibiotics. All isolates of *R. equi* showed 100 percent sensitivity to Gentamicin followed by ciprofloxacin and streptomycin (sensitivity-97.30%) while higher resistance i.e. 97.30 percent to furazolidone and 59.46 percent to sulphadiazine was observed.

Keywords: *Rhodococcus equi;* antibiogram; sensitivity; gentamicin.

1. INTRODUCTION

R. equi is facultative intracellular pathogen mostly causing pyogranulomatous pneumonia, ulcerative typhlocolitis, mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis in foals [1].This Gram positive organism is responsible for approximately 3% ofglobal foal mortality [2]. *R. equi* can infect many other farm animals (cats, goats, pigs, cattle and camels) and human beings also.The infections caused by *R. equi* are mostly sub-clinical for a long period of time. When the symptoms are expressed the foals become untreatable. *R. equi* is an intracellular pathogen surviving and replicating in macrophages and causing granulomatous lesions with thick caseous material. Acquired resistance among *R. equi* isolates has been reported with multiple antibiotics, including doxycycline, penicillin, erythromycin, vancomycin, co-trimoxazole and rifampin [3]. Therefore, prolonged treatment with judicious choice of antibiotics, probably in combination is required for effective treatment and to avoid relapse of infection [4]. It has been seen that with appropriate use of antibiotics survival rate of sick foals can be increased from 20% to nearly 90%. Total 17 isolates were identified, including *Bacillus, Rhodococcus, Staphylococcus, Micrococcus, Streptococcus, Aerococcus, Diplococcus, Enterobacter etc.* [5]. The present study was conducted to studyantibiogram of *R. equi* in apparently healthy animals in the arid region of Bikaner.

2. MATERIALS AND METHODS

A total of 220 samples from skin and upper respiratory tract of apparently healthy horses were collected using sterilized swabs and placed in test

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tubes containing nutrient broth.Out of 220 samples processed for the present investigation 132 were from the non-organized farms and 88 were from animals of organized farm (Table 1).

The samples collected were subjected to aerobic cultivation. Each swab was streaked on nutrient agar in order to obtain isolated colonies of bacteria. The cultures were incubated for 24 hours at 37°C. The growth was examined for the colonial morphology and pigmentation and different types of colonies were sub cultured on separate plates in order to obtain pure cultures. To check the purity of cultures, smears were prepared from each of the sub cultured colony, fixed by gentle heat and stained by Gram's Method. On observing the cultures for their purity, Gram's properties and rhodococcocal morphology (cocobacilli) the bacterial isolates were allocated code numbers accordingly and the colonies were transferred to paired nutrient slants. One of the slants was kept under refrigeration at 4°C after proper sealing with paraffin wax and the other was used for different tests. The organisms were isolated and identified as described by Quinn et al. [6].

2.1 Primary Tests for Identification of *R. equi*

Gram's staining: The Gram's staining was carried out on 18-24 hr old cultures according to the [7] method. A loop full of an overnight culture was air-dried and heat fixed on a glass slide. Crystal violet stain (0.3% w/v) was added and allowed to stand for one minute. Excess stain was washed off with a gentle stream of water. Then Gram's iodine (0.4% w/v) was added and allowed to stand for 30 sec before being rinsed off. The smear was destained with ethanol (95% v/v) and then stained with the secondary stain, safranin (0.4% v/v), for one min. This was then washed with water for 5 sec. Gram-negative bacteria, appeared pink under the microscope whereas Gram-positive cultures appeared purple under the microscope.

Catalase test: A loop full of young culture of bacterial isolate was mixed with a drop of 3 per cent hydrogen peroxide over a clean glass slide. The production of gas bubbles or any effervescence within a few seconds was considered as catalase positive and absence of gas bubbles as catalase negative.

Oxidase test: Filter paper was impregnated with a 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine dihydrochloride. Bacterial cultures were smeared across the filter paper with a glass rod. Appearance of dark purple colourwithin 5-10 sec indicated positive oxidase test.

Oxidation-fermentation test: The bacterial isolates were stab inoculated in a pair of test tubes containing Hugh and Leifson O-F media. One of which was sealed with 1-2 mm thick layer of sterile mineral oil to provide anaerobic condition and the other was left unsealed. The tubes were incubated at 37^{o}C for seven days. If both the inoculated tubes had changed from bluish green to yellow, the bacterial isolate was considered as fermentative and if only unsealed tube turned to yellow, the bacterial isolate was considered as oxidative.

2.2 Other Biochemical and Metabolic Tests Used for Identification

Pigment production: The culture plates incubated at 37ºC for 24-48 hr were observed for any colour production by the isolates.

Urease test: Tubes containing urea broth were inoculated with cultures and incubated at 37ºC. Change in colour of media from yellow to red was observed for 2-3 days. Red colour indicated positive for urease.

Nitrate reduction test: This test was performed in two steps. In step one, nitrate broth was inoculated with culture and incubated at 37ºC for 24 hr. From this an aliquot of 0.5 ml was taken in empty sterile test tube and equal volumes of sulphanilic acid (HiMedia) and α- napthylamine (HiMedia) were added. Appearance of pink colour indicated reduction of nitrate. If no colour change was observed in first step then a pinch of Zinc dust was added to the test tubes and if the colour of the media changed to pink, it indicated negative nitrate reduction in this second step also. But if no change in colour was observed upon addition of Zinc dust, the results were interpreted as positive for nitrate reduction.

Test for production of haemolysin: Sheep blood agar medium was used for testing haemolysin production by bacterial isolates. Blood agar plates were inoculated by streaking across the surface. Plates were incubated at 37ºC for 24 hr and observed for haemolysis*i.e.* α-haemolysis (green zones, cell envelopes intact) or β-haemolysis (Fig. 1) (clear, colourless zone, cell envelopes disrupted) [8].

Test for production of *equi* factor (CAMP test): Sheep blood agar medium was used for testing production of *equi* factor by bacterial isolates. Blood agar plates were inoculated by streaking fresh isolates of beta-hemolytic *Staphylococcus aureus* vertically and test strains were streaked horizontally without touching the vertical streaks across the surface. Plates were incubated at 37ºC for 48 hr. and observed for

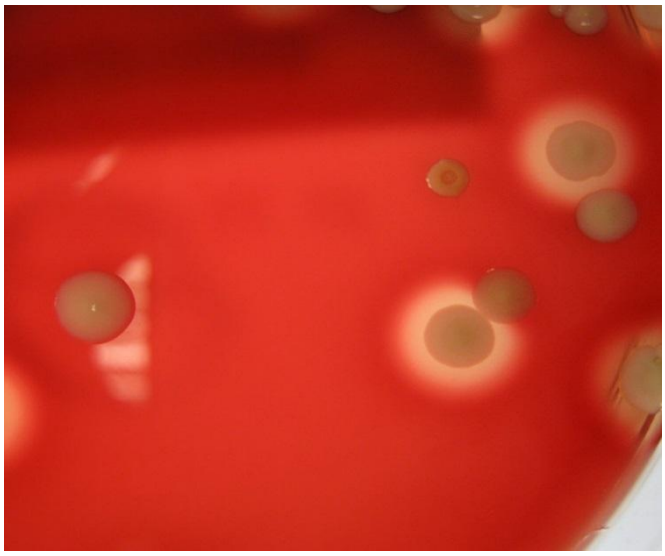


Fig. 1. β-haemolysis of *R. equi*

synergistic haemolysis in the zone of the vertical streaks. Enhancement in haemolysis was observed in the *equi* factor producing isolates.

Congo red dye agar test (CR test): The congo red dye medium used for determination of Congo red binding of the isolates. The colonies were streaked on Congo red agar and incubated at 37ºC for 24 hrs. After 24 hrs incubation, the cultures were left at room temperature for 48 hrs to facilitate annotation of results. The isolates that bind the congo red dye show red colonies and colonies that do not bind the dye give pale colonies.

Test for lipase activity: To detect Lipase activity egg yolk agar was used. Egg yolk agar is used for both lipase and lecithinase activity of bacteria. The test culture was inoculated heavily on the surface of egg yolk agar and incubated at 37ºC for 24 hrs. On solid media containing egg yolk, lipolysis was seen by the formation of a thin, iridescent 'pearly layer' overlying the colonies and a confined opalescence in the medium underlying them.

Phosphatase test: Phosphatase test was used to detect the presence of phosphatase enzyme which causes splitting of phenolphthaleindiphosphate. The test culture was inoculated heavily over the entire slope surface of phosphatase test medium (nutrient agar containing 0.01% phenolphthaleindiphosphate) and incubated at 37ºC for 24 hours. Change in the colour of phenolphthaleindiphosphate indicator from light purple to pink was considered as positive test and no change or yellow colour of the indicator indicated a negative test [9].

2.3 Antibiotic Sensitivity Test

The antibiotic sensitivity pattern of bacterial isolates was determined by disc diffusion method as per technique [10]. The test strain from nutrient agar slant culture was inoculated in to 5 ml of nutrient broth and incubated overnight at 37ºC in shaker incubator. The concentration of these log phase cultures was adjusted to 0.5 McFarland units with the help of nutrient broth. The broth culture was spread over the surface of Mueller-Hinton Agar plate with the help of sterile spreader. The plates were kept as such for 5-10 minutes to allow the inoculum to absorb. The antibiotic discs (HiMedia) were then placed on Mueller-Hinton agar at equal distance with sterilized forceps. These plates were then incubated at 37ºC for 24 hr. The results were recorded by measuring the diameter of clear zone of inhibition around each antibiotic disc and compared with the standard zones to assess the status of the isolate in terms of sensitivity. These samples were subjected to culturing for isolation and 37 *R. equi* isolates were obtained. The organisms were isolated and identified as described by several authors [11,6]. Isolates were confirmed as *R. equi* by 16S rRNA gene based identification carried out as per the method described [12].

3. RESULTS

All the 37 isolates of *R. equi* were subjected to resistotyping with 16 different antibiotic and the results were interpreted according to the instructions of manufacturer. The response of organisms was interpreted as sensitive, intermediate and resistant

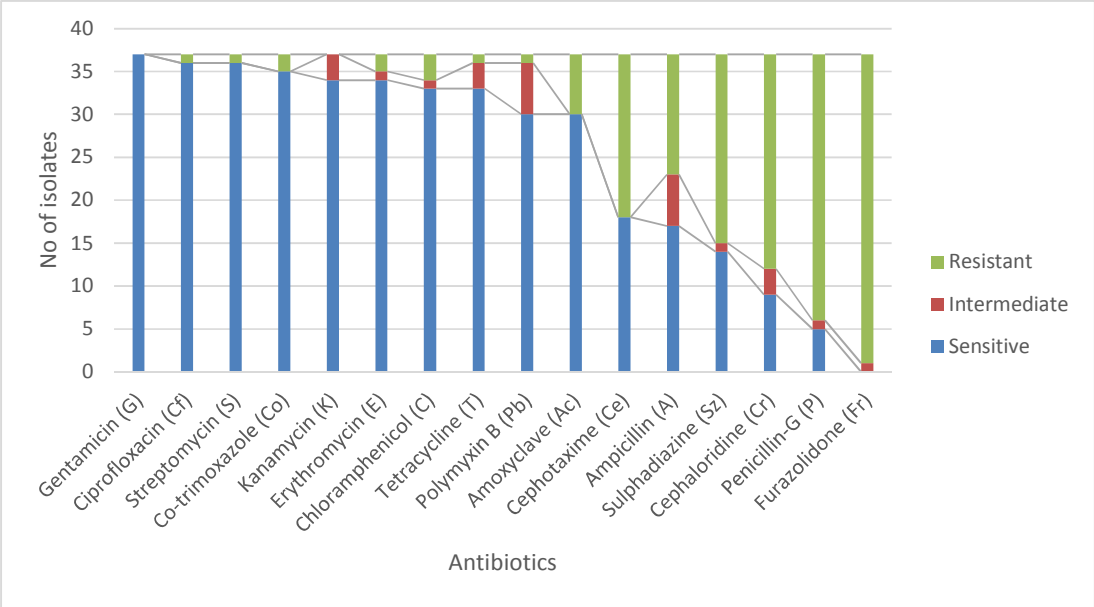
(Table 2). For five antibiotics tested i.e. penicillin-G, Cephaloridine, Sulphadiazine, Furazolidone and Cephotaxime more than 50% isolates were having resistance. All the isolates were sensitive to gentamicin, streptomycin, ciprofloxacin, co-trimoxazole, erythromycin, kanamycin, tetracycline, chloramphenicol, polymyxin B and amoxyclave (Table 2). Among macrolides 91.89% of the *R. equi* isolates were found sensitive to erythromycin. Almost similar sensitivity patterns against erythromycin were obtained by Silva et al. [13] with 98 per cent of efficacy against *R. equi* isolates.

The β-lactam agents alone showed poor sensitivity (48.65% for cephotaxime, 45.94% for ampicillin, 24.32% for cephaloridine and 13.51% for penicillin) but when associated with a β-lactamase inhibitor, the sensitivity rose significantly (81.08%) for amoxycillin and clavulanic acid (amoxyclave). These observation are quite similar with the study of several authors [14,13] in *R. equi* isolates. Among fluoroquinolones, 97.30 per cent of the *R. equi* isolates were found sensitive to ciprofloxacin. Almost similar sensitivity patterns against ciprofloxacin was obtained by Silva et al. [13] with 92.16 per cent of *R. equi* isolates showing sensitivity to ciprofloxacin (Graph 1).

Among remaining antibiotics, higher sensitivity *i.e.* 94.59 per cent to co-trimoxazole, 89.19 per cent to tetracycline and chloramphenicol, and 81.08 to polymyxin B, and higher resistance *i.e.* 97.30 per cent to furazolidone and 59.46 per cent to sulphadiazine was observed in *R. equi* isolates. Several authors [15,16,13] reported almost similar sensitivity to tetracycline and chloramphenicol.

Prevelance of *R. equi* in the arid region: In all, *R. equi* could be isolated from 37 (16.81%) samples out of 220 samples processed. Although no significant difference was found in the isolation rate, higher isolation of *R. equi* was observed from non-organized animals as compared to that of the organized farm animals. 81 bacteria and 5 yeasts were isolated from nasal and wound secretion from equines. In result most of prominent bacteria related to organized and non-organized both like *Bacillus spp., R. equi and Streptococcus spp*. [17] the probable reason for this could be the difference in the hygienic conditions of organized and non-organized sectors.

R. equi multiplies to 10,000 times or more in the dung which is left on the paddock or the pasturing ground. This contaminated soil is one of the important sources for transmission of infection [18]. The percent isolation of *R. equi* was higher in summers i.e. 31.94% and 17.50% than that in winter season i.e. 5% and 13% in non organized and organized farms respectively (Table 1). This finding corroborates with the earlier observations of many workers who have observed increase in isolation rate of *R. equi* in summer months [19,20]. While working on the ecology of *R. equi* observed Takai et al. [21] a sudden increase of up to 80% in isolation rate of *R. equi* during end of March, which remained at that level during April and May as compared to that of winter season.



Graph 1. Sensitivity of *R. equi* on different antibiotics

Table 1. Occurrence of *Rhodococcus equi* isolates in samples obtained from different sources

S. no.	Type of sample collected	Season	Source	Number of sample collected	Number of *R. equi* isolates obtained	Percentage of *R. equi* isolates obtained
1.	From non-organized farm/places	Summer	Skin	36	16	44.44%
			Nasal swab	36	7	19.44%
			Total	72	23	31.94%
		Winter	Skin	30	1	03.33%
			Nasal swab	30	2	06.67%
			Total	60	3	05.00%
		Total		132	26	19.70%
2.	From organized farm/places	Summer	Skin	20	5	25.00%
			Nasal swab	24	2	8.33%
			Total	44	7	15.90%
		Winter	Skin	20	3	15.00%
			Nasal swab	24	1	4.16%
			Total	44	4	9.09%
		Total		88	11	12.50%
	Grand total			220	37	16.81%

Table 2. Sensitivity of *R. equi* isolates to different antibiotics

Antibiotic	Sensitive	Intermediate	Resistant
Ampicillin (A)	17 (45.94%)	6 (16.22%)	14 (37.84%)
Cephotaxime (Ce)	18 (48.65%)	_	19 (51.35%)
Cephaloridine (Cr)	9 (24.32%)	3 (08.11%)	25 (67.57%)
Amoxyclave (Ac)	30 (81.08%)	_	7 (18.92%)
Chloramphenicol (C)	33 (89.19%)	1 (02.70%)	3 (08.11%)
Ciprofloxacin (Cf)	36 (97.30%)	_	1 (02.70%)
Co-trimoxazole (Co)	35 (94.59%)	_	2 (05.41%)
Erythromycin (E)	34 (91.89%)	1 (02.70%)	2 (05.41%)
Furazolidone (Fr)	_	1 (02.70%)	36 (97.30%)
Gentamicin (G)	37 (100%)	_	_
Kanamycin (K)	34 (91.89%)	3 (08.11%)	_
Polymyxin B (Pb)	30 (81.08%)	6 (16.22%)	1 (02.70%)
Tetracycline (T)	33 (89.19%)	3 (08.11%)	1 (02.70%)
Penicillin-G (P)	5 (13.51%)	1 (02.70%)	31 (83.78%)
Streptomycin (S)	36 (97.30%)	_	1 (02.70%)
Sulphadiazine (Sz)	14 (37.84%)	1 (02.70%)	22 (59.46%)

4. CONCLUSION

In the present study out of 220 samples processed 75 (34.09%) had Rhodococcus *species*. Out of these 75 isolates, 44 (58.66%) were from samples of non-organized farm and 31(41.33.00%) from samples of organized farm. As identification for *R. equi* was done a total 37 (49.33%) isolates were found to be *R. equi from* 75 *Rhodococcus sp.* Although species specific identification of non *R. equi* rhodococcus could not be done in the present study but there was an indication of the fact that in addition to *R. equi* other rhodococci can also be found associated with equines.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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