Rhodococcus equi isolation from sputum of patients with suspected tuberculosis

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Rhodococcus equi *has emerged as an opportunistic pathogen associated with pulmonary, invasive or systemic infections in immunocompromised patients. We report the identification of 51* R. equi *isolates found in sputum samples of 546 individuals suspected to have pulmonary tuberculosis in two Public Health Hospital Units in Brazil. The epidemiology of* R. equi *infection as well as the phenotypic identification and drug susceptibility profile of isolates are described in this paper.*

Key words: *Rhodococcus equi* - identification - tuberculosis - antimicrobial profile - epidemiology

The genus *Rhodococcus* belongs to the group of nocardioform, Gram-positive rods containing mycolic acids in the cell wall; this group also includes the genera *Mycobacterium*, *Nocardia*, *Corynebacterium, Dietzia*, *Gordonia*, *Millisia*, *Segniliparus, Skermania*, *Tsukamurella* and *Williamsia* (Soddell et al. 2006a, b, Tsitko et al. 2006). The genus *Rhodococcus* was discovered by Zopf in 1891 (Goodfellow & Alderson 1977) and comprises 30 species, of which *Rhodococcus equi* is considered the most opportunistic pathogen in mammals, including humans (Meijer & Prescott 2004). The first case of human infection was reported in 1967 in a patient presenting with a pulmonary abscess. The two first cases that occurred in Brazil were reported by Severo et al. (2001). However, with the AIDS epidemic, the number of patients infected by *R. equi* has grown (Roda et al. 2009). *R. equi* also causes infection in patients with lymphoma, chronic renal failure, alcoholism, lung cancer, leukaemia, diabetes mellitus and other immunodeficient syndromes. Some cases have been reported in which the infection can also occur in immunocompetent hosts (von Bargen & Haas 2009).

When incubated aerobically at 37°C, *R. equi* grows efficiently in the majority of nonselective culture media, including media used in mycobacteria isolation and produces irregular, smooth and mucoid colonies that turn a shade of salmon pink to yellow after a week of growth (Prescott 1991). *R. equi* appears coccoid on stained smears of clinical specimens, especially purulent material and tissue (obtained by biopsy, during surgery and upon autopsy). However, long rods have been reported in clinical specimens isolated from blood, sputum and bronchial lavage fluid (von Bargen & Haas 2009). Colony morphology and acid-fast staining are characteristic features in the initial identification of different nocardioform genera (Christopher & Bruno 2002). In general, *R. equi* is biochemically non-reactive, has no proteolytic activity and fails to oxidize or ferment carbohydrates, with the exception of glucose, which is oxidized by *R. equi* in 14 days. *R. equi* is strictly aerobic, catalase positive, oxidase negative and mostly urease positive. *R. equi* produces soluble "*equi* factors" that are associated with phospholipase and cholesterol oxidase activity and interact with phospholipase D of *Listeria ivanovii* to induce complete haemolysis of sheep erythrocytes (Prescott 1991).

The Laboratory of Mycobacteriology at the Adolfo Lutz Institute in Ribeirão Preto (SP, Brazil) has frequently isolated partial acid-fast bacteria from sputum of patients suspected to have pulmonary tuberculosis. Considering the emerging clinical importance of *R. equi*, our goal was to identify the 51 partial acid-fast bacteria isolated from sputum, to determine the antimicrobial profiles of these isolates and to analyse the epidemiological characteristics of patients with *R. equi* infection.

PATIENTS, MATERIALS AND METHODS

Clinical samples - Sputum samples (duplicates/triplicates) were obtained from 546 patients suspected to have pulmonary tuberculosis in Public Health Hospital Units in Ribeirão Preto and the surrounding region. The samples were analysed by the Laboratory of Mycobacteriology at the Adolfo Lutz Institute in Ribeirão Preto.

R. equi isolation and identification - After testing for acid-fast bacilli (AFB), samples from 296 patients showed the presence of AFB; in 60 patients, partial AFB or coccobacilli, suggestive of *Rhodococcus* spp, were isolated. The AFB samples were cultured using the automated MB/BacT system (bioMérieux) and subjected to mycobacteria identification by phenotypic methods and Accuprobe® (GenProbe). The 60 AFPB were cultured on Müeller Hinton agar (MH) plates and using the automated MB/BacT system (bioMérieux) to verify the absence of *Mycobacterium* spp. Isolated colonies were transferred to MH agar slants in tubes. The identifica-

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tion of *R. equi* was based on microscopic observation of bacteria subjected to Gram's and Ziehl-Neelsen staining (Ballows et al. 1991) and phenotypic biochemical tests described in the Manual of Systematic and Clinic Microbiology (Ballows et al. 1991, Holt et al. 1994, Murray et al. 1999, Koneman et al. 2000, Mac Faddin 2000). Gordon's base culture medium was used for acid production in carbohydrate tests (McNeil & Brown 1994). The CAMP test was performed as described by Bille and Doyle (1991), using *L. ivanovii* ATCC 19119 and *R. equi* ATCC 6939 as standard strains.

Susceptibility tests - The disk-diffusion method was performed as recommended in the Clinical and Laboratory Standard Institute/National Committee and Clinical Laboratory Standards guidelines. In this method, 28 antimicrobial agents were used (results of *Staphylococcus* sp. were used as the basis for data interpretation) (CLSI/NCCLS 2004) and *R. equi* ATCC 6939, *Enterococcus fecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923 served as the reference strains.

Epidemiological investigation - The available patient data, such as gender, age and immunosuppressive condition, were utilized for epidemiological investigation.

RESULTS

Sixty patient samples out of a total of 546 patients had evidence of *Rhodococcus* spp isolates and among these, 51 were identified as *R. equi* based on the results of the phenotypic tests presented in Table I. All of the 51 isolates were catalase positive, oxidase negative and failed to oxidize or to ferment carbohydrates or alcohols. These isolates produced *equi* factors (CAMP test) and lipase but not gelatinase, esculinase, H_2S or indole and they did not use citrate or malonate. For nitrate reductase, urease and hippurate reduction, the 51 isolates showed variable results. There was 100% similarity between the 51 isolates and *R. equi* ATCC 6939, the standard strain. Nine isolates could not be identified as *R. equi* because the CAMP test was negative, despite the similarity of results in all other tests and in comparison to the standard strain.

TABLE I

Phenotypic identification of *Rhodococcus equi*

Phenotypic characteristics of *R. equi*	Phenotypic characteristics of 51 isolates		
	Reaction[a] %	Positives n	Result %
Catalase	100 (+)	51	100 (+)
Oxidase	1-5 (+)	0	100 (–)
Motility	100 (–)	0	100 (–)
Obligate aerobe	100 (+)	51	100 (+)
Glucose fermentation	100 (–)	0	100 (–)
Glucose oxidation	100 (+)	51	100 (+)
Sole carbon source[b]	100 (–)	0	100 (–)
equi factor (CAMP test)	100 (+)	51	100 (+)
Gelatinase	100 (–)	0	100 (–)
Indol	100 (–)	0	100 (–)
H_2S	32 (+)	0	100 (–)
Urease	95 (+)	45	88 (+)
Nitrate reduction	88 (+)	50	98 (+)
Lipase	100 (+)	51	100 (+)
DNase	100 (–)	0	100 (–)
Lecithinase	100 (–)	0	100 (–)
Hippurate hydrolysis	1 (+)	06	88 (–)
Esculin hydrolysis	4 (+)	0	100 (–)
Adenine hydrolysis	100 (+)	51	100 (+)
Casein hydrolysis	100 (–)	0	100 (–)
Hypoxanthine hydrolysis	100 (–)	0	100 (–)
Tyrosine hydrolysis	100 (–)	0	100 (–)
Xanthine hydrolysis	100 (–)	0	100 (–)

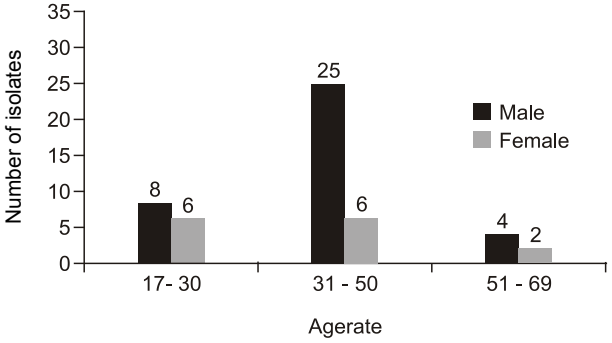
a: adapted from Prescott 1991, McNeil and Brown 1994 and Christopher and Bruno 2002; *b*: all isolates failed to use acetate, citrate or malonate as sole carbon source, or produce acid from adonitol, arabinose, cellobiose, erythritol, fructose, galactose, glycerol, inositol, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, sucrose, salicin, sorbitol, starch and xylose; +: positive reaction; -: negative reaction.

The analysis of epidemiological data showed that, from a total of 51 patients infected by *R. equi*, 37 (72.5%) were male and 14 (27.4%) were female. The age range most affected was between 31-50 years, comprising 60.8% of patients. There were no cases of childhood infection. The youngest patient was 17 years old and the oldest was 69 years old (Figure). All patients showed some degree of immunosuppression, such as through infection with HIV, chronic alcoholism, drug use or transplant surgery.

Twenty-eight antimicrobial agents were tested against the 51 *R. equi* isolates by the disk-diffusion method. The sensitivity profile can be seen in Table II. The aminoglycosides (amikacin and gentamicin), the tetracycline (minocycline) and the glycopeptides (teicoplamin and vancomycin) were 100% effective. The macrolides (azithromycin and clarithromycin) were also 100% effective, with the exception of erythromycin, for which one isolate showed intermediate sensitivity (98.04% effective). The β-lactam agents alone showed poor activity (49.02% for cefoxitin, 45.10% for cefepime, 23.53% for cefotaxime, 19.60% for cephalothin, 13.73% for cefazolin, 7.84% for ampicillin, 5.88% for penicillin and 1.96% for oxacillin) but when associated with a β-lactamase inhibitor, the activity rose significantly (98.04% for amoxicillin and clavulanate). Imipenem (98.04%) and ceftriaxone (80.39%) were the most effective β-lactam agents. Other antimicrobial agents that showed low activity were clindamycin and sulfamethoxazole + thrimethoprim, in which 90.20% and 58.84% of the strains were resistant, respectively. The quinolones, levofloxacin (96.08%), ciprofloxacin (92.16%) and norfloxacin (90.20%), were very effective, as were rifampin (98.04%), doxycycline (96%), chloramphenicol (84.31%) and tetracycline (78.43%).

DISCUSSION

Among the 546 sputum samples from individuals suspected to have pulmonary tuberculosis, it was found that 406 patient samples effectively gave acid-fast or partial acid-fast positive results. *Mycobacterium tuberculosis* and nontuberculous mycobacteria were isolated



Age and sex distribution of 51 isolates phenotypically characterized as *Rhodococcus equi* from sputum.

from 67.5% (274/406) and 17.7% (72/406) of the patients, respectively. *R. equi* was identified as the single agent in 12.6% (51/406) of patients. Another nine isolates (2.2%) gave negative CAMP test results and were considered only to be *Rhodococcus* spp, although these isolates showed similar results as the standard strain in all other biochemical tests. According to Bille and Doyle (1991) and Prescott (1991), *R. equi* isolates with a negative *equi* factor have been described. In spite of the general consensus that human infections with *R. equi* are rare, our results indicated that infection is not so infrequent. Corti et al. (2009) and Martin et al. (2007) suggested that in patients with *M. tuberculosis*/HIV co-infection, respiratory pathogens such as *R. equi* should also be considered. Due to the similarity of the clinical symptoms of rhodococcosis and tuberculosis (Tsitko et al. 2006), as well as the high prevalence of tuberculosis in the population and the partial acid-fastness of *R. equi*, a mistaken diagnosis can easily be made in the laboratory and in medical practice.

The epidemiological data analysis showed that *R. equi* was isolated four times more often in male patients, whose predominant (60.8%) age range was from 31-50 years. A male predominance of 3:1 for pulmonary rhodococcosis was also observed by Kedlaya et al. (2001). HIV infection is one of the main factors predisposing patients to *R. equi* infection (Roda et al. 2009) and normally, the incidence of HIV is higher among males, which explains the predominance of *R. equi* strains in males. In our study, all 51 patients infected with *R. equi* were also co-infected with HIV. Torres-Tortosa et al. (2003) assessed *R. equi* infection in 67 patients co-infected with HIV. Fifty-five patients were male with ages varying between 25-37 years. These authors also found that most (52.2%) of these bacterial isolates came from sputum.

Treatment of *R. equi* infection must be based on the results of antimicrobial susceptibility tests because there is no standard drug regimen for *R. equi* infection in humans (Roda et al. 2009) and the emergence of some strains resistant to various antibiotics has been reported within the last 10 years (Buckley et al. 2007). *R. equi* isolates in our study showed resistance to β-lactam agents, in agreement with the results described by Roda et al. (2009). When β-lactam agents were combined with a

TABLE II

Susceptibility test results in disk-diffusion test with 28 antimicrobial agents against 51 *Rhodococcus equi* isolates[a]

Antimicrobial agents (concentration)	Isolates/isolates tested n (%) Interpretation categories		
	Sensitive	Intermediate	Resistant
Aminoglycosides			
AMI (30 µg)	51 (100)	-	-
GEN (10 µg)	51 (100)	-	-
β-lactams			
AMC (30 µg)	50 (98)	-	01 (20)
AMP (10 µg)	04 (7.8)	-	47 (92.2)
CFL (30 µg)	10 (19.6)	01 (2)	40 (78.4)
CFZ (30 µg)	07 (13.7)	01 (2)	43 (84.3)
CRO (30 µg)	41 (80.4)	03 (5.9)	07 (13.7)
CFO (30 µg)	25 (49)	11 (21.6)	15 (29.4)
CTX (30 µg)	12 (23.5)	01 (2)	38 (74.5)
CPM (30 µg)	23 (45.1)	06 (11.8)	22 (43.1)
IPM (10 µg)	50 (98)	-	01 (2)
OXA (5 µg)	01 (2)	-	50 (98)
PEN (10UI)	03 (5.9)	-	48 (94.1)
Chloramphenicol			
CLO (30 µg)	43 (84.3)	02 (4)	06 (11.7)
Glycopeptides	-	-	-
TEC (30 µg)	51 (100)	-	-
VAN (30 µg)	51 (100)	-	-
Lincosamide			
CLI (2 µg)	01 (2)	04 (7.8)	46 (90.2)
Macrolides	-	-	-
AZI (15 µg)	51 (100)	-	-
CLA (15 µg)	51 (100)	-	-
ERI (15 µg)	50 (98)	01 (2)	-
Quinolones			
CIP (5 µg)	47 (92.1)	03 (5.9)	01 (20)
LVX (5 µg)	49 (96)	01 (2)	01 (2)
NOR (10 µg)	46 (90.2)	01 (2)	04 (7.8)
Rifampin	-	-	-
RIF (5 µg)	50 (98)	-	01 (2)
Sulfa + trimethoprim			
SMT (25 µg)	21 (41.2)	-	30 (58.8)
Tetracyclines	-	-	-
TET (30 µg)	40 (78.4)	08 (15.7)	03 (5.9)
DOX (30 µg)	49 (96)	01 (2)	01 (2)
MIN (30 µg)	51 (100)	-	-

a: the Interpretative Patterns of Halo Inhibition Diameters were those determined for *Staphylococcus* sp., in agreement with Clinical and Laboratory Standards Institute, Performance Standards for Antimicrobial Susceptibility Testing, Fifteenth Informational Supplement, document M100-S15 (ISBN 1-56238-556-9. 2005); AMC: amoxicillin + clavulanate; AMI: amikacin. AMP: ampicillin; AZI: azithromycin; CFL: cephalothin; CFO: cefoxitin; CFZ: cefazolin; CIP: ciprofloxacin; CLA: clarithromycin; CLI: clindamycin; CLO: chloramphenicol; CPM: cefepime; CRO: ceftriaxone; CTX: cefotaxime; DOX: doxycycline; ERI: erythromycin; GEN: gentamicin; IPM: imipenem; LVX: levofloxacin; MIN: minocycline; NOR: norfloxacin; OXA: oxacillin; PEN: penicillin G; RIF: rifampin; SMT: sulfamethoxazole + Trimethoprim; TEC: teicoplanin; TET: tetracycline; VAN: vancomycin.

beta-lactamase inhibitor, the activity was significantly increased (98.04% for amoxicillin-clavulanate), confirming the correlation of β-lactamase enzyme production and resistance.

Because *R. equi* is an intracellular bacteria, the infection can be treated with macrolides, such as erythromycin and rifampicin (Heidmann et al. 2006, Buckley et al. 2007). The macrolides tested in our study, azithromycin and clarithromycin, were 100% effective, while erythromycin was 98% effective against the *R. equi* isolates. The quinolones were very effective against *R. equi*, with sensitivity similar to that against *M. tuberculosis* clinical isolates (Ginsburg et al. 2003). Rifampin, a first-line drug for tuberculosis control, was also effective in 98.04% of isolates. Torres-Tortosa et al. (2003) reported that in 55 patients infected with *R. equi,* the most effective antibiotics were vancomycin, amikacin, rifampin, imipenem, ciprofloxacin and erythromycin. The effectiveness of these antibiotics was confirmed in our 51 isolates.

R. equi infection in humans is an underidentified disease and can be confused with tuberculosis or other granulomatous pathologies. Our intention is to alert health professionals about the importance of investigating suspected pulmonary pathologies for potential *R. equi* infection, especially in immunocompromised patients with cavitary lesions.

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