

## *Mycobacterium kansasii*: antibiotic susceptibility and PCR-restriction analysis of clinical isolates

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*Mycobacterium kansasii* is the second most common cause of non-tuberculosis mycobacterial diseases in Sao Paulo, Brazil. An important component of the management of infections caused by this organism is antibiotic susceptibility testing. The objective of this study was to determine the drug susceptibility profiles and genotypes of clinical isolates of *M. kansasii* obtained from patients with or without an infection that met the American Thoracic Society's case definition criteria of *M. kansasii* disease. One hundred and sixty-nine clinical isolates of *M. kansasii* collected between 1993 and 1998 in Sao Paulo, Brazil, were tested consecutively. The isolates were genotyped by PCR restriction-enzyme pattern analysis (PRA). Most of the *M. kansasii* strains were susceptible to isoniazid, streptomycin, rifabutin, rifampicin, clarithromycin, ethionamide, amikacin, clofazimine and cycloserine, and resistant to ethambutol, ciprofloxacin and doxycycline. Of 169 isolates, 167 belonged to the type I PRA genotype and one each belonged to type II and III genotypes. There was no correlation between PRA subtype and *M. kansasii* disease according to the American Thoracic Society case definition. Clinical trials may be needed to better correlate MIC values with treatment outcomes to identify appropriate parameters for drug-resistance testing of *M. kansasii*.

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## INTRODUCTION

*Mycobacterium kansasii* causes both pulmonary and extra-pulmonary infections (Bolivar *et al.*, 1980; Wolinsky, 1979; Woods & Washington, 1987). In addition to chronic pulmonary disease resembling tuberculosis, which is the most frequent clinical presentation, *M. kansasii* can cause cervical lymphadenitis, dermatitis, osteomyelitis and arthritis. Disseminated infection occurs most commonly in immunocompromised patients (Scherer *et al.*, 1986; Valainis *et al.*, 1991). Prior to the human immunodeficiency virus (HIV) epidemic, *M. kansasii* pulmonary diseases were commonly reported from the USA and western Europe, particularly from patients with pre-existing lung pathology (Schraufnagel *et al.*, 1986). In the early period of the AIDS epidemic and before the advent of highly active anti-retroviral therapy (HAART), *M. kansasii* ranked second behind *Mycobacterium avium* complex as the most common non-tuberculosis mycobacteria (NTM) species isolated from AIDS-associated opportunistic NTM infections (Valainis

*et al.*, 1991). Recent studies suggest an increasing incidence of *M. kansasii* infections in both HIV-positive (Shafer & Sierra, 1992; Witzig *et al.*, 1995) and HIV-negative patients (Bittner *et al.*, 1996). Currently, in many areas of the world, *M. kansasii* is the most frequent NTM isolated (Bloch *et al.*, 1998; Chobot *et al.*, 1997; Taillard *et al.*, 2003).

One complicating feature of *M. kansasii* infections is in the assessment of the clinical significance of the infection. The problem of contamination of clinical specimens with environmental strains is well recognized, and has led some authors to develop a strict set of criteria for *M. kansasii* infection (Ahn *et al.*, 1982). The American Thoracic Society (ATS) guidelines from 1990 required at least two positive respiratory samples for the diagnosis of pulmonary infection (American Thoracic Society, 1990). However, the isolation of *M. kansasii* from respiratory sites appears to correlate well with pulmonary disease in those with HIV infection (Bamberger *et al.*, 1994; Wolinsky, 1981), which led the ATS to modify its recommendation in 1997 (American Thoracic Society, 1997).

Another approach to assess the clinical significance of *M. kansasii* infection suggested by Alcaide *et al.* (1997, 1999) involves genotyping *M. kansasii* by PCR restriction-fragment analysis of the *hsp65* gene (PRA). These authors reported that types I and II are frequently clinical isolates,

Abbreviations: AMK, amikacin; ATS, American Thoracic Society; CIP, ciprofloxacin; CLO, clofazimine; CLR, clarithromycin; CS, cycloserine; DOX, doxycycline; EMB, ethambutol; ETH, ethionamide; HIV, human immunodeficiency virus; INH, isoniazid; NTM, non-tuberculosis mycobacteria; PRA, PCR restriction-enzyme pattern analysis; RFB, rifabutin; RIF, rifampicin; SPT, streptomycin.

while the other types are likely to be from environmental samples (Alcaide *et al.*, 1997a). They suggested that genotyping *M. kansasii* may help to differentiate clinically important infections from contamination.

For most NTM, *in vitro* susceptibility tests are not standardized. However, with *M. kansasii*, the interpretative criteria used for *Mycobacterium tuberculosis* susceptibility tests appear to correlate with clinical response (Inderlied & Pfyffer, 2003). In this study, we attempted to characterize the susceptibility profiles of the clinical isolates of *M. kansasii* by the MIC method and compare the results to the types of infection as defined by ATS and the *M. kansasii* PRA genotypes.

## METHODS

**Isolates.** All 169 clinical isolates of *M. kansasii* consecutively referred to the reference laboratory Instituto Adolfo Lutz (IAL) in Sao Paulo, Brazil, from 1993 to 1998 were analysed. They were cultured from sputa (141 samples), abscesses from unknown sites (8), bronchial washings (5), urine (3), skin biopsies (2), gastric washes (2), blood (2), bone marrow (2), cerebrospinal fluid (1), pericardial fluid (1) and synovial fluid (2). These isolates were identified as *M. kansasii* by standard biochemical tests at IAL. *M. kansasii* strain ATCC 12478 was used as a reference strain for MIC testing.

The clinical isolates were obtained from 106 patients, of whom 26 (24 %) met the American Thoracic Society (1997) case definition criteria for *M. kansasii* disease, that is, they had at least three isolates recovered from non-sterile sites or one isolate from a sterile site identified as *M. kansasii*. Those patients who had less than three isolates from non-sterile sites were defined as suspected cases. Among the 26 patients who met the ATS case definition criteria, 19 (73 %) were HIV positive, one (4 %) was HIV negative and six (23 %) had no information concerning HIV status. These isolates were sent to the reference laboratory without any clinical information. Most of them were the first isolate from the patient but some of the patients had more than one isolate analysed. For PRA analysis, only one isolate (the first isolate) from each patient was studied ( $n = 106$ ).

**MIC determination.** MIC determination was performed in 96-well microtitre plates with U-shaped wells. All wells were filled with 0.1 ml Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC) enrichment (Difco) (Telles & Yates, 1994; Wallace *et al.*, 1996; Witzig & Franzblau, 1993).

The antimicrobial agents and the concentrations tested were as follows: streptomycin (SPT), 0.5–32 µg ml<sup>-1</sup>; isoniazid (INH), 0.25–16 µg ml<sup>-1</sup>; ethambutol (EMB), 0.25–16 µg ml<sup>-1</sup>; rifampicin (RIF), 0.12–8 µg ml<sup>-1</sup>; rifabutin (RFB), 0.06–4 µg ml<sup>-1</sup>; amikacin (AMK), 0.5–32 µg ml<sup>-1</sup>; ciprofloxacin (CIP), 0.25–16 µg ml<sup>-1</sup>; clofazimine (CLO), 0.12–8 µg ml<sup>-1</sup>; clarithromycin (CLR), 0.5–32 µg ml<sup>-1</sup>; ethionamide (ETH), 0.5–32 µg ml<sup>-1</sup>; cycloserine (CS), 2–128 µg ml<sup>-1</sup>; doxycycline (DOX), 1–64 µg ml<sup>-1</sup>. All antimicrobial agents were kept as a 1 % stock solution in distilled water except for RIF, which was dissolved in methanol, and RFB, CLR and ETH, which were dissolved in dimethyl sulfoxide. The drugs were stored at –20 °C. The stock solutions of the drugs were diluted in Middlebrook 7H9 medium (Difco) and 0.1 ml of each drug was added to each of the 12 columns. Two-fold dilutions were then made across each row by a multi-channel pipette with the transfer of 0.1 ml aliquots. The last row (H) included medium only, as a drug-free control for bacterial growth.

Culture growth was transferred with a 10 µl loop from a Löwenstein–Jensen slant into 2 ml Middlebrook 7H9 medium and incubated at

36 °C for 7 days. These suspensions were then diluted 1 : 10 with fresh media and a 5 µl suspension was inoculated into each well. The plates were then sealed with plastic covers and incubated at 36 °C for 7–14 days.

The MIC was defined as the lowest concentration of the drug that inhibited all bacterial growth. The concentrations used to define resistance were as follows: SPT, 10 µg ml<sup>-1</sup>; INH, 5 µg ml<sup>-1</sup>; EMB, 5 µg ml<sup>-1</sup>; RIF, 1 µg ml<sup>-1</sup>; RFB, 2 µg ml<sup>-1</sup>; AMK, 32 µg ml<sup>-1</sup>; CIP, 2 µg ml<sup>-1</sup>; CLO, 1 µg ml<sup>-1</sup>; CLR, 16 µg ml<sup>-1</sup>; ETH, 4 µg ml<sup>-1</sup>; CS, 32 µg ml<sup>-1</sup>; DOX, 4 µg ml<sup>-1</sup> (Wallace *et al.*, 1996; Witzig & Franzblau, 1993).

**DNA preparation for PCR.** A loopful of a bacterial colony was lifted from a Löwenstein–Jensen medium culture and resuspended in 500 µl of distilled water. The sample was then boiled for 10 min, frozen overnight and about 5 µl of the supernatant was used for PCR amplification.

**PRA identification.** Mycobacterial DNA was added to a 45 µl PCR mixture with primers Tb11 and Tb12 by the procedure described by Telenti *et al.* (1993). The amplification product was subjected to *Bst*EII and *Hae*III enzyme digestion, and the fragments were separated by electrophoresis on a 3 % Gibco agarose gel (Life Technologies). The gel was photographed on a UV transilluminator and restriction patterns were evaluated with the help of an algorithm (outlined on the internet site <http://app.chuv.ch/prasite/index.html>).

## RESULTS AND DISCUSSION

We previously standardized a rapid and inexpensive test to determine the antimicrobial drug MICs for *Mycobacterium* species (Telles & Yates, 1994; Wallace *et al.*, 1996; Witzig & Franzblau, 1993). We applied this procedure to test *M. kansasii*. Table 1 shows the MIC ranges, the MIC at which 90 % of the isolates were inhibited (MIC<sub>90</sub>), the MIC<sub>50</sub> and the susceptibility profiles against 12 antimicrobial agents for 103 isolates from 80 suspected patients and 66 isolates from 26 patients who met the bacteriological ATS case definition criteria for *M. kansasii* disease. The MIC ranges of the 12 drugs varied widely. The resulting MICs were categorized into susceptible and resistant concentrations (Wallace *et al.*, 1996; Witzig & Franzblau, 1993). Most of the isolates of *M. kansasii* were susceptible to CLR (99 % of the strains), AMK (97 %), ETH (95 %), RFB (93 %), INH (92 %), RIF (88 %), SPT (86 %) and CLO (57 %). Most of the isolates showed resistance to DOX (99 %), EMB (94 %), CS (86 %) and CIP (66 %). When the analysis was performed with one isolate per patient source ( $n = 106$ ), the overall susceptibility patterns remained similar.

A comparison of the isolates from patients who did ( $n = 26$ ) and did not ( $n = 80$ ) meet the ATS case definition criteria for disease showed that the MIC<sub>90</sub> for ETH approached significance as it was higher for the isolates from suspected patients (14 % resistant) than for the other group (3 %), while for the other 11 drugs no significant differences were found. The explanation for this observation is not clear. Information about prior treatment history of patients would have been helpful in interpreting the results, which was a limitation of the present study.

In the early 1980s in Sao Paulo state, Brazil, *M. kansasii* was the most frequently isolated NTM associated with pulmon-

**Table 1.** *In vitro* susceptibility of *M. kansasii* isolates from 26 cases who met the ATS case definition criteria (ATS) and 80 suspected (Sus) patients

AMK, amikacin; CIP, ciprofloxacin; CLO, clofazimine; CLR, clarithromycin; CS, cycloserine; DOX, doxycycline; EMB, ethambutol; ETH, ethionamide; INH, isoniazid; RFB, rifabutin; RIF, rifampicin; SPT, streptomycin.

Drug	Patient	MIC ( $\mu\text{g ml}^{-1}$ )			No. (%) of isolates		
		Range	MIC <sub>50</sub>	MIC <sub>90</sub>	Susceptible	Resistant	Total
INH	ATS	0.25–16.0	0.5	16.0	58 (88)	8 (12)	66
	Sus	0.25–16.0	0.5	4.0	97 (94)	6 (6)	103
RIF	ATS	0.12–0.8	0.12	8.0	55 (85)	10 (15)	65
	Sus	0.12–0.8	0.12	1.0	92 (89)	11 (11)	103
SPT	ATS	0.5–32.0	4.0	16.0	58 (88)	8 (12)	66
	Sus	0.5–32.0	8.0	16.0	87 (85)	16 (15)	103
EMB	ATS	1.0–16.0	16.0	16.0	3 (5)	61 (95)	64
	Sus	2.0–16.0	16.0	16.0	7 (7)	91 (93)	98
RFB	ATS	0.06–2.0	0.06	0.25	63 (97)	2 (3)	65
	Sus	0.06–4.0	0.06	4.0	92 (89)	11 (11)	103
CIP	ATS	0.25–8.0	2.0	4.0	22 (33)	44 (67)	66
	Sus	0.25–16.0	2.0	4.0	35 (34)	68 (66)	103
AMK	ATS	1.0–32.0	4.0	16.0	64 (97)	2 (3)	66
	Sus	0.5–32.0	4.0	16.0	100 (97)	3 (3)	103
CLO	ATS	0.12–8.0	0.5	2.0	36 (54)	30 (46)	66
	Sus	0.12–8.0	0.5	2.0	60 (59)	42 (41)	102
CLR	ATS	0.5–32.0	0.5	1.0	66 (100)	0	66
	Sus	0.5–32.0	0.5	2.0	98 (95)	5 (5)	103
ETH	ATS	0.5–32.0	0.5	1.0	64 (97)	2 (3)	66
	Sus	0.5–32.0	0.5	2.0	89 (86)	14 (14)	103
CS	ATS	8.0–64.0	32.0	64.0	8 (12)	58 (88)	66
	Sus	8.0–128.0	32.0	64.0	15 (15)	88 (85)	103
DOX	ATS	1.0–64.0	16.0	64.0	1 (1)	65 (99)	66
	Sus	4.0–64.0	32.0	64.0	5 (5)	98 (95)	103

any disease (Silva *et al.*, 1987). In another more recent study carried out with isolates from all regions of the state (Ferrazoli *et al.*, 1992), 60 (21 %) of the 289 NTM isolates referred to IAL from 1985 to 1990 were identified as *M. kansasii*. However, only 12 patients were confirmed to have *M. kansasii* disease.

The frequency of *M. kansasii* isolation from clinical specimens varies greatly by region. In Pennsylvania, 1.6 % of all *Mycobacterium* species isolated are *M. kansasii*, while in Cincinnati, *M. kansasii* accounts for 6.3 % of species isolated (Nachamkin *et al.*, 1992). A population-based study of *M. kansasii* infections in San Francisco between 1992 and 1996 found a cumulative incidence of 2.4 cases per 100 000 HIV-infected adults, which was almost five times higher than the national isolation rate in 1980; the cumulative incidence among non-HIV-infected adults (0.75 per 100 000) remained similar to the rate from California in 1980 (0.58 per 100 000) (Bloch *et al.*, 1998). *M. kansasii* appears to have a stable isolation rate in the UK, although it is the most common cause of pulmonary NTM infection in the non-HIV population (Evans *et al.*, 1996). Between 1968 and 1995, *M. kansasii* pulmonary infections steadily increased in a non-

HIV-infected population in north Moravia and Silesia (Chobot *et al.*, 1997). A similar picture has been observed in Sao Paulo, Brazil (Ferrazoli *et al.*, 1992). However, since many more laboratories now perform cultures in Sao Paulo than in the past, part of this apparent increase may reflect an increase in the isolation frequencies of *M. kansasii*.

Our strains were resistant to more drugs than the UK strains (Telles & Yates, 1994), which were sensitive to most of the drugs, including INH, STR, EMB, RFB, RIF, CLO and CIP, and were resistant only to AMK. The isolates from the UK and Sao Paulo were tested by the same MIC method, and hence the reason for the high resistance frequency to EMB among the Sao Paulo isolates is not clear. During the same period, the reference laboratory tested hundreds of *M. tuberculosis* clinical isolates by the same method and found a low EMB-resistance frequency. These EMB-resistant *M. kansasii* isolates may represent a clonal strain predominant in the state. A previous study (Sato *et al.*, 1993) of 30 clinical isolates from the state also showed high EMB resistance (90 % were resistant at concentrations of 8 and 16  $\mu\text{g ml}^{-1}$ ). According to the American Thoracic Society (1997), geographic differences are observed in *M. kansasii*

drug-susceptibility patterns. In a study of pulmonary *M. kansasii* infection, Evans *et al.* (1996) reported that of 47 isolates, all were sensitive to RIF and EMB. Another study, in London, studied 10 clinical isolates; all were sensitive to EMB and resistant to INH, nine were sensitive to RIF, and of six isolates tested for CIP, all were fully or partially sensitive (Rooney *et al.*, 1996).

In Brazil, due to the fact that a positive acid-fast smear test is assumed to represent tuberculosis, and the sputum is not often sent for culture at the beginning of treatment, there can be a delay in diagnosing *M. kansasii* disease. Only when there is a treatment failure, the sputum culture is done and eventually species identification is performed. Thus, most of the isolates that did not meet the ATS case definition criteria probably represent environmental strains that were not previously exposed to drugs.

In the absence of good clinical information accompanying the *M. kansasii* isolates, we used the PRA method to genotype *M. kansasii* isolates to see if genotypes suggested to be associated with clinical disease by other investigators were present in our collection. Molecular strain typing analyses have demonstrated that *M. kansasii* consists of a heterogeneous group including several distinct subtypes (Alcaide *et al.*, 1997a; Picardeau *et al.*, 1997; Ross *et al.*, 1992; Zhang *et al.*, 2004). Worldwide, *M. kansasii* genotype I, as defined by PRA, appears to be highly clonal and is the most common genotype associated with human disease (Zhang *et al.*, 2004). It is rarely isolated from the environment. However, HIV-infected patients seem to be particularly susceptible to infection with *M. kansasii* subtype II (Taillard *et al.*, 2003). According to Taillard *et al.* (2003) and Alcaide *et al.* (1997a), the identification of *M. kansasii* at the subtype level may be not only an interesting epidemiological tool but also relevant to the clinical management, as it allows the differentiation of potentially pathogenic subtypes from the non-pathogenic subtypes.

In our study, of 106 isolates typed by PRA, 104 were genotype I and the others were types II and III (one each). All 26 patients who met the ATS disease criteria were infected with genotype I strain. The type II and type III isolates were found in suspected cases that had only one isolate of *M. kansasii*. Type I strains were isolated from both HIV-infected and non-HIV-infected patients who met the ATS case definition criteria. Thus, genotypic characterization of *M. kansasii* isolates in Sao Paulo did not correlate well with clinical disease.

In conclusion, the broad range of MICs represented by the clinical isolates of *M. kansasii* in our study demonstrates that it is difficult to correlate strain susceptibility to treatment outcome, especially since many of the patients from whom *M. kansasii* was isolated did not meet the ATS disease definition criteria. Additional studies are needed to provide recommendations for appropriate and standardized methods to determine the resistance profiles of *M. kansasii* clinical

isolates, especially those isolated from disease. Carefully designed clinical trials may be needed to develop such recommendations.

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