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RESEARCH ARTICLE

Drug susceptibility of *Mycobacterium avium* and *Mycobacterium simiae* isolated in a rural hospital in central India.

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Abstract

Drug susceptibility testing of nontuberculous mycobacterial species has gained importance after their increased isolation from AIDS patients and the fact that their treatment not only differs from Mycobacterium tuberculosis but also among themselves. Drug susceptibility testing was performed for 3 clinical and 4 environmental isolates of Mycobacterium avium and 4 clinical isolates of Mycobacterium simiae by macrodilution method using BACTEC 460TB system and microtitre broth dilution method using Middlebrook 7H9 medium. Six drugs including Streptomycin, Isoniazid, Rifampicin, Ethambutol, Ofloxacin and Kanamycin were tested. In addition, Azithromycin was tested against Mycobacterium avium only by BACTEC 460TB system. All clinical isolates of Mycobacterium avium and Mycobacterium simiae were resistant to all the drugs tested while environmental isolates were relatively sensitive. All Mycobacterium avium isolates whether clinical or environmental were sensitive to azithromycin. Total concordance between macro and microdilution methods was observed. To the best of our knowledge, this is the first report of drug susceptibility of Mycobacterium simiae in AIDS patients from India.

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INTRODUCTION

In the era of HIV/AIDS, it has become important to isolate and speciate mycobacteria from clinical samples, as in such patients' nontuberculous mycobacteria (NTM) can also cause disease in addition to the usual *Mycobacterium tuberculosis*.[1] The susceptibility to antimicrobial drugs is different among the mycobacterial species and hence for their proper treatment both speciation and drug susceptibility testing (DST) are mandatory[2]. To put up DST for NTM – both slowly and rapidly growing, CLSI brought out approved guidelines[3] (NCCLS M24-A, Vol. 3, No.18 April 2003) based on various studies. For slowly growing NTM various methods have been proposed for drug susceptibility testing including broth based macrodilution (BACTEC 460TB system, Becton Dickinson, Sparks, MD, USA)[4, 5] and microdilution (Middlebrook 7H9 broth supplemented with OADC or OAD) methods and agar based modified proportion method [6]. In India, there have been two reports on DST of *M. avium*[7, 8] in which agar dilution method was compared with broth dilution method [7] and Resazurin microtitre assay[8] respectively. The present study on DST of *M. avium* and *M. simiae* isolated from AIDS patients and their environment was undertaken due to paucity of data on this aspect, to know the antibiogram of clinical isolates for recommending treatment and to compare antibiogram of clinical and environmental isolates for typing purpose. The two CLSI recommended methods viz. macrodilution and microdilution MIC were also compared.

METHODOLOGY

Three *M. avium* (2 from blood and 1 from stool) and four *M. simiae* (3 from blood and 1 from stool) were isolated from five AIDS patients in an ongoing approved study[9]. Two patients in whom the isolates were recovered from stool samples, their blood samples also showed growth of the same species. In addition, four *M. avium* isolates were recovered from samples of soil obtained from the house hold and work area of the above patients. These eleven isolates were identified by sequencing of a part of 16S rRNA gene region nt- 28-341 of *E. coli* [10] and tested for DST using standard guidelines recommended by CLSI [3]. The methods included macrodilution testing using BACTEC 460TB system and microdilution testing using minimum inhibitory concentration (MIC) in Middlebrook 7H9 medium (BBL-Difco). Standard strains of *M. avium* (NCTC 8551) and *M. simiae* (TMC 1226/ATCC 25275) obtained from Mycobacterial Repository, National JALMA Institute, Agra were also tested along with the test strains. *Enterococcus faecalis* ATCC® 29212 (rifampicin MIC 1µg/mL) was used as a quality control strain for *M. simiae* as recommended in the CLSI guidelines.[3]

i) Antimicrobial agents:

Pure powders of seven drugs, Streptomycin, Isoniazid, Rifampicin, Ethambutol, Ofloxacin, Kanamycin and Azithromycin, were purchased from Sigma Chemical Co., Mumbai, India. Stock solutions were prepared in distilled water for all these drugs except rifampicin, which was prepared in dimethylformamide. These solutions were kept at -20ºC until used.

ii) Macrodilution testing using BACTEC 460 TB system:

Commercially available BACTEC 12B medium with a pH of 6.8 was used in this test. The concentrations for stock solution were 10,000µg/mL for all drugs except Azithromycin for which it was 20,480 µg/mL. From these stock solutions, working solutions were made in distilled water to be incorporated into 12B medium. Following were the final concentrations of different drugs which were used: Streptomycin 10 µg/mL, Isoniazid 5 µg/mL, Rifampicin 2 µg/mL, Ethambutol 5 µg/mL, Ofloxacin 2 µg/mL, Kanamycin 32 µg/mL and Azithromycin128, 258, 512 µg/mL. To obtain these final concentrations, 40 times concentrated working solutions were prepared for each drug. Working solutions of the drugs were added in a volume of 0.1 mL to vials containing 4mL BACTEC 12B medium to achieve the desired final concentrations.

The inoculum was made from an initial BACTEC 12B broth culture after it had reached the maximum growth (growth index [GI] 999) detected radiometrically. This culture was diluted 1:100 using BACTEC diluting fluid, and 0.1 mL of this dilution (A) was inoculated into each vial, providing an initial concentration of 10^4 to 10^5 CFU per mL.[11] Two drug-free controls were used: one BACTEC 12 B vial was inoculated with 0.1mL of the above mentioned dilution (A) to match the drug-containing test vials (C1), and the second was inoculated with 0.1mL of a further 1:100 dilution of the diluted inoculum (A) to represent 1% (C2) of the bacterial population (10^2 to 10^3 CFU/mL). The vials were incubated at 37°C, and the growth index (G.I.) readings were recorded daily in the BACTEC-460TB instrument. The final results were recorded once the 1:100 control (B) gave G.I. of >10 for 3 consecutive days.[11]

Organism which produced daily G.I. increases but final G.I. reading lower than those in the 1: 100 control (C2), were considered to have been inhibited more than 99% in that drug concentration and were therefore considered as sensitive. Organisms were defined as resistant if daily increases and final GI reading in drug containing vials was more than that of control (C2).

Since 3 different concentrations for Azithromycin were used, MIC could be calculated and following criteria were used to define the strains –Susceptible: $\leq$128 µg/mL; Intermediate: =256 µg/mL; Resistant: $\geq$ 512 µg/mL.[3]

iii) Minimum inhibitory concentration testing:

a) Preparation of the inoculum:

Once there was sufficient growth on the Lowenstein Jensen slant, the isolates were subcultured in Middlebrook 7H9 broth (BBL-Difco) supplemented with OADC (BBL-Difco) and incubated at 37ºC for one week. Before use, cultures were vortexed with glass beads for 20-30seconds and then left for 3-5 minutes to settle the heavy particles. A standard bacterial suspension equivalent in turbidity to that of a no. 1 McFarland's standard was prepared and diluted 1:20 in 7H9 broth. A 10µL inoculum was used for each well.[12]

b) Preparation of drug solutions: The concentration of stock solution of antimicrobial agents to be tested was 10,240µg/mL for different drugs. For each test the drug was dissolved twofold in 7H9 broth (pH 6.8), and 100µL volumes were dispensed into wells of sterile 96-well U bottomed microtitre plates (Tarsons, India) to put up the test.[13] Following antimicrobial agents with their range of concentration in µg/mL were used: Streptomycin 0.5 – 64, Isoniazid 0.125-16, Rifampicin 0.125-16, Ethambutol 0.5 – 64, Kanamycin 0.5 – 64, and Ofloxacin0.5 – 64. Azithromycin was not tested by MIC.

c) Preparation of broth micro dilution plates:

As mentioned above, 100µL of the different concentrations of drugs were dispensed in the microtitre plate columns 3-10. Column 2 was used as a drug free control well and column 11 was used as a control well with only 7H9 medium. Perimeter wells of the plate were filled with sterile water to avoid dehydration of the medium during incubation. The 7H9 containing columns (with and without drug) were inoculated with 100µL of the inoculum. Plates were sealed with paraffin film and put in plastic bags and incubated at 37ºC in ambient air for at least 7 days. If good growth was observed in the control well, MIC was determined for drugs on that day only. Otherwise, the plates were incubated up to 14 days and then read.

d) Reading of plates:

Plates were read using inverted mirror. A bead up to 2mm in the control well indicated good growth. MIC was defined as the concentration that totally inhibited the growth of NTM in the well. Since cut off titres for drugs other than azithromycin have not been mentioned for *M. avium* in the CLSI guidelines, to decide sensitivity or resistance cut off, titre given by Heifets[11] were used as given in Table-1. For *M. simiae* the cut off titres have been mentioned in CLSI guidelines as Streptomycin 10 µg/mL, Isoniazid 5 µg/mL, Rifampicin 2 µg/mL, Ethambutol 5 µg/mL, Ciprofloxacin 2 µg/mL (used for Ofloxacin), and Amikacin 32 µg/mL (used for Kanamycin).

RESULTS

For *M. avium* and *M. simiae*, DST was performed by macrodilution method in BACTEC 12B medium using BACTEC 460TB system and microdilution method using Middlebrook 7H9 broth in microtitre plate. The *M. avium* and *M. simiae* isolates were classified as sensitive or resistant using MIC cut off values as given in Tables 1 and 2.

All *M. avium* isolates were sensitive to azithromycin at the MIC <128 µg/mL (Table-2). All clinical isolates of *M. avium* and *M. simiae* were resistant to all the drugs tested including four first line antiTB drugs. Among four environmental isolates of *M. avium*, two were fully sensitive, while two showed resistance, one to INH and rifampicin and the other to streptomycin, INH, rifampicin and ethambutol. The results of drug susceptibility testing using cut off titres given by CLSI[3] using BACTEC system and those given by Heifets[11] (Table-1) were concordant.

For two isolates of *M. avium* (isolates number 1 and 2 in Table 2) from the blood and stool samples of the same patient the antibiogram was observed to be the same. Same was the observation with two isolates of *M. simiae* from blood and stool samples of another patient (isolates number 8 and 9 in Table 2). These findings indicate that probably the same strain was present both in the gut and the blood. The antibiograms of clinical and environmental isolates were different indicating them to be different strains.

Table-1: Cut off titres for various drugs used in MIC testing of *M. avium* (Heifets 1988)[4]

Drug	**Susceptible** **MIC µg/ml**	**Moderately Susceptible** **MIC µg/ml**	**Moderately Resistant** **MIC µg/ml**	**Resistant** **MIC µg/ml**
Streptomycin	≤0.2	4.0	8.0	≥16
INH	≤0.1	0.2-1.25	2.5	≥5
Rifampicin	≤0.5	1.0-4.0	8.0	≥16.0
Ethambutol	≤2.0	4.0	8.0	≥16.0
Ofloxacin	≤2.0	4.0	8.0	≥16.0
Kanamycin	≤3.0	6.0	12.0	≥24.0

Table 2: MIC (µg/mL) against six drugs for slowly growing NTM isolates

Isolate & Sample	Strep	INH	Rif	ETB	Kana	Oflox	Pattern by MIC	Pattern by BACTEC
1. *M avium* Blood	>64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
2. *M avium* Stool	>64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
3. *M avium* Blood	64 R	>16 R	8 MR	>64 R	32 R	8 MR	RRRRRR	RRRRRR
4. *M avium* Soil	1 S	<0.125 S	<0.125 S	1 S	>64 R	64 R	SSSSRR	*SSSSRR*
5. *M avium* Soil	1 S	>16 R	8 MR	1 S	16 MR	0.5 S	SRRSRS	SRRSRS
6. *M avium* Soil	64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
7. *M avium* Soil	<0. 5 S	1 MS	<0.125 S	<0.5 S	<0. 5 S	<0. 5 S	SSSSSS	*SSSSSS*
8. *M simiae* Blood	>64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
9. *M simiae* Stool	>64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
10. *M simiae* Blood	8 MR	>16 R	16 R	8 MR	>64 R	>64 R	RRRRRR	RRRRRR
11. *M simiae* Blood	64 R	>16 R	>16 R	>64 R	>64 R	>64 R	RRRRRR	RRRRRR
12. Control *M. avium*	<0. 5 S	8 R	<0.125 S	<0.5 S	<0. 5 S	2 S	SRSSSS	SRSSSS
13. Control *M. simiae*	8 S	>16 R	0.25 S	>64 R	64 R	1 S	SRSRRS	SRSRRS

S: Sensitive; MS: Moderately sensitive; MR: Moderately resistant; R: Resistant

MIC for azithromycin put by BACTEC 460TB system was observed to be <128 µg/ml for all the *M. avium* strains.

DISCUSSION

As per CLSI guidelines[3] published in 2003, the susceptibility tests have been described for slowly growing and rapidly growing mycobacterial species. Amongst the slowly growing species the guidelines have been intended for susceptibility testing of only certain NTM i.e. *M. avium* complex, *M. kansasii* and *M. marinum*. These species have been selected as there is sufficient data for them to base general recommendations. There is little information on the correlation of in-vitro susceptibility testing results and clinical outcome in most of the other slowly growing NTM. The recommendations concerning selection of antimicrobial agents have been taken from that opined by American Thoracic Society.[14] As most of the data is available for MAC, following recommendations have been given for situations where DST should be performed in MAC isolates[14] - i) clinically significant isolates from patients on prior macrolide therapy; ii) isolates from patients who develop bacteremia while on macrolide prophylaxis; iii) isolates from patients who relapse while on macrolide therapy, and iv) isolates from blood or tissue (patients with disseminated disease) or from clinically significant respiratory samples to establish baseline values.

NTM are important infecting organisms in AIDS patients and are responsible for disseminated disease. However, once isolated they are difficult to treat as their sensitivity to various available drugs differs. None the less there are drugs like macrolides (clarithromycin, azithromycin), ethambutol, clofazimine and rifamycins (especially rifabutin) to which they respond and which are modestly effective in controlling bacteremia.[3] However, MAC responds poorly to available antimycobacterial regimens because of the intrinsic resistance to common anti TB drugs and the fact that many AIDS patients may have polyclonal MAC infection.[15] Antimicrobial agents where correlation between in-vitro susceptibility test and clinical response has been demonstrated against MAC in controlled clinical trials are only macrolides (Azithromycin and Clarithromycin) and based on this data the guidelines suggest that susceptibility of MAC isolates should be evaluated for macrolides only.

The three clinical isolates of MAC in our study were totally resistant to all the drugs tested including first line anti TB drugs and were sensitive only to azithromycin. The environmental isolates of M. avium were also uniformly sensitive to azithromycin but in addition also showed sensitivity to some of the first line antiTB drugs. At least in two other Indian studies drug susceptibility of *M. avium* was tested. In a study from New Delhi, MIC and agar dilution methods were used and a total of 49 isolates of *M. avium* were tested against 9 drugs viz. isoniazid, streptomycin, ethambutol, rifampicin, kanamycin, amikacin, clofazimine, ciprofloxacin, and roxithromycin. More than 40% of MAC isolates were found sensitive to ciprofloxacin, amikacin and roxithromycin, while only 22-37% of the isolates were sensitive to first line antiTB drugs.[7] In another study from North India Jadaun et al[8] tested 20 *M. avium* isolates using Resazurin microtitre plate method and found 80% of the isolates to be resistant to ethambutol considering 3.125mg/L to be cut off point. Considering the resistance levels noted in all the above studies and based on the CLSI guidelines[3], in case of *M. avium*, drug susceptibility testing can be restricted to macrolides only.

M. simiae unlike *M. avium* has been isolated from restricted geographical areas of the world and there are very limited reports on its drug susceptibility testing. In CLSI also no special guidelines have been mentioned for *M. simiae*, in fact it is recommended that the guidelines for *M. kansasii* should be followed for *M. simiae, M. terrae/nonchromogenicum, M. xenopi* and *M. malmoense.*[3] The drugs to be tested are isoniazid, rifampicin, ethambutol, streptomycin, clarithromycin, amikacin, ciprofloxacin, thrimethoprim-sulfamethoxazole or sulfamethoxazole and the methods recommended for testing are BACTEC 460TB system, modified proportion method in 7H10 agar, and broth microdilution.[3] All four isolates of *M. simiae* in the present study were found to be resistant to four first-line anti-TB drugs. In CLSI, it has been recommended that rifampicin should be tested in treatment failure cases or for those who respond poorly to initial treatment with rifampicin, INH and ethambutol. For isolates resistant to the 1µg/mL concentration of rifampicin, susceptibility testing to a total of eight drugs has been recommended.[3] These drugs include rifabutin, ethambutol hydrochloride, isoniazid, streptomycin, clarithromycin, amikacin, ciprofloxacin, thrimethoprim-sulfamethoxazole or sulfamethoxazole. In the present study, sensitivity to rifabutin, thrimethoprim-sulfamethoxazole or sulfamethoxazole could not be tested due to non-availability of these drugs at the time of testing.

Considering the antibiogram for two isolates of *M. avium* from blood and stool sample of the same patient, it was found it matched perfectly but was different between clinical and environmental isolates. For *M. simiae* also antibiogram of two isolates from the same patient was the same. These findings indicate that both these patients had bacteremia and gut infection with the same strain based on antibiogram typing.

To conclude, *M. avium* isolates were found to be uniformly sensitive to azithromycin, the drug of choice for the specie. For other 6 drugs, including four first line antiTB drugs, clinical isolates of both *M. avium* and *M. simiae* were found to be resistant. The antibiogram of different clinical isolates from same patient was also the same, while there was no match either amongst the environmental isolates or between clinical and environmental isolates. The two tested methods also showed concordance with each other.

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