**Original article**

# Characterisation and Drug susceptibility testing of Rapidly growing mycobacteria in extrapulmonary tuberculosis

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**Abstract**

**Introduction:** There has been an increasing awareness of the rapidly growing mycobacteria (RGM), of which numerous species and phylogenetic groups are clearly established human pathogens. It is important to appropriately distinguish RGM from other mycobacteria as first line antituberculous drugs are ineffective for their treatment. Variability in susceptibility of RGM is seen in relation to species, different geographical areas and time. Therefore we conducted a study to speciate the isolates of RGM and perform drug susceptibility testing (DST) from cases of extrapulmonary tuberculosis (EPTB).

**Methods:** Forty isolates of rapidly growing mycobacteria were speciated by phenotypic methods and DST was done by disk diffusion method.

**Observation and Results:** Of the 40 isolates, 55% were *M. fortuitum* group, 35% were *M .smegmatis* group and 10% were *M. chelonae–abscessus* group. Disk diffusion method showed that all isolates of RGM were sensitive to amikacin, clarithromycin and linezolid. All *M. fortuitum* group isolates showed sensitivity to tobramycin. All isolates of *M. chelonae abscessus* were susceptible to tobramycin and gatifloxacin. High degree of resistance was seen to cephalosporins, minocycline, amoxicillin clavulanic acid and cotrimoxazole.

**Conclusion:** Variability in sensitivity to different antimicrobials exists in RGM. Therefore, each isolate must be individually evaluated, and it is advisable to perform DST before commencement of therapy. Disk diffusion can help in tentative identification of the commonly encountered RGM and help to decide empiric antibiotic therapy.

**Keywords:** *M.fortuitum group, M. smegmatis group, M. chelonae abscessus group*

## Introduction

Rapidly growing mycobacteria (RGM) cause a wide variety of infections involving the lungs, skin, lymphnodes, soft tissue, and also disseminated infections. There are currently 5 groups or complexes of RGM based on pigmentation and genetic relatedness. Non pigmented pathogenic species now include 12 species within the *M. fortuitum* group, 5 species in the *M. chelonae abscessus* group, and 3 species in the *M. mucogenicum* group. The pigmenting groups are the late pigmenters *M. smegmatis* group and the 5th group of early pigmenting RGM.[1]

A recent study in India showed that 36% of all NTM infections were RGM. [2]It is important to distinguish RGM from others as conventional antituberculous drugs are ineffective for their treatment.



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Therefore we conducted a study on characterisation and drug susceptibility testing(DST) of RGM from clinically suspected cases of extrapulmonary tuberculosis (EPTB) by attempting the more economical disk diffusion method.

**Aims and Objectives:**

To speciate the isolates of RGM isolated from clinically suspected cases of extrapulmonary tuberculosis (EPTB)and perform drug susceptibility testing (DST) bydisk diffusion method.

**Material and methods**

The study was carried out in the department of Microbiology of a tertiary care hospital in Mumbai after approval from the institutional ethics committee. This study included 40 isolates of RGM obtained from clinical specimens from suspected cases of EPTB over a period of one year from April 2010 to March 2011. Aseptic collection of body fluids, pus and lymph nodes were done by needle aspiration and that of tissue specimens by surgical procedures. The urine specimens included in the study were from patients who had undergone cystoscopy and developed dysuria. They had submitted early morning midstream samples collected by the patients in sterile containers on three consecutive days after periurethral cleaning. Only if the same RGM had been isolated from at least two of the urine specimens were they included in our study. The specimens were decontaminated using NALC NaOH (N acetyl L cysteine sodium hydroxide) method and were inoculated on Lowenstein Jensen (LJ) media. Any growth appearing within one week were confirmed to be acid fast bacilli by Ziehl Neelsen stain and were further characterized by 3-Day Arylsulfatase test, growth on Mac Conkey's agar without crystal violet, 5% NaCl tolerance test and nitrate reduction test.[3, 4]

All non pigmenting isolates which exhibited aryl sulphatse activity at 3 days were considered to be *M. fortuitum* group or *M. chelonae abscessus* group or *M.mucogenicum* group and were further characterized by nitrate reduction and iron uptake, both the tests are positive only in *M. fortuitum* group. None of the RGM isolated in our study belonged to *M. mucogenicum* which gives variable nitrate reduction, distinct tan appearance on iron uptake and is negative on 5% NaCl.[3, 4]Also polymixin B (300 units) was used to differentiate between *M. fortuitum* group and *M. chelonae abscessus* group as the former gave an inhibition zone of > 10 mm while the latter group showed no zone of inhibition.

Drug susceptibility test (DST) was performed by disk diffusion method using the following antibiotics: linezolid(10µg), clarithromycin(15µg), amikacin(30µg), cefoxitin(30µg), imipenem(10µg), minocycline(30 µg), tobramycin(10 µg), ciprofloxacin (5µg), gatifloxacin(10µg), ceftriaxone(30 µg), amoxicillin/clavulanic acid (20/10 µg) and trimethoprim/sulfamethoxazole(1.25/23.75µg) supplied by Hi media Laboratories.[5,6,7] Briefly colonies were taken from the LJ media and transferred to sterile distilled water. Turbidity was then adjusted to 0.5 Mac Farland. This was inoculated as a lawn culture on blood agar plates and 6 disks were applied per plate. The plates were incubated at 30 $^0$C for 72 hours and the zones of inhibition were measured.



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**Observation and Results**

A total 40 isolates of rapidly growing mycobacteria from clinically suspected cases of extrapulmonary tuberculosis were obtained from pus (50%), lymphnode aspirates (22.5%), fluids (12.5%), tissue (7.5%) and urine (7.5%). All isolates were nonpigmented. Of these, 22 isolates (55%) were *M.fortuitum* group, 14 (35%) were *M.smegmatis* group and 4 (10%) were *M.chelonae – abscessus* group. Samplewise distribution of various groups of RGM is shown in **Table 1.** Drug susceptibility of *M.fortuitum* group , *M.chelonae-abscessus* group and *M. smegmatis* groupby disk diffusion method is depicted in **tables 2,3,4** respectively. All isolates of *M.fortuitum* group were sensitive to amikacin, clarithromycin, linezolid and tobramycin.None of the isolates was sensitive to cefoxitin.

All isolates of *M.chelonae -abscessus* group were sensitive to amikacin, clarithromycin, linezolid, tobramycin, gatifloxacin and were resistant to cefoxitin, ceftriaxone, minocycline, amoxycillin-clavulanic acid and trimethoprim-sulfamethoxazole. Three isolates (75%) were susceptible to ciprofloxacin, imipenem.

The isolates were mostly *M. chelonae* as it is known to show resistance to cefoxitin but susceptibility to tobramycin, linezolid and gatifloxacin.

*M.wolinskyi* are known to be resistant to tobramycin (disk diffusion zone ≤10mm and hence 3 of the isolates in *M.smegmatis* group were *M.wolinskyi.* The other 11 isolates may have been *M. goodii* or *M.smegmatis).*[4]

Amikacin, clarithromycin and linezolid were active against all *M.smegmatis* group isolates by disk diffusion method while all isolates were resistant to cefoxitin, ceftriaxone, minocycline and cotrimoxazole.

**Table 1. Samplewise distribution of various groups of RGM**

| Samples | Isolates | | |
|---|---|---|---|
| | *M.fortuitum* group (n=22) | *M.chelonae-abscessus* group (n=4) | *M.smegmatis* group (n=14) |
| Pus | 12 (54.54%) | 1 (25%) | 7 (50%) |
| FNAC | 6 (27.27%) | 0 | 3 (22%) |
| Fluids | 3 (13.63%) | 1 (25%) | 1 (7%) |
| Tissue | 0 | 1 (25%) | 2 (14%) |
| Urine | 1 (4.54%) | 1 (25%) | 1 (7%) |
| Total | 22 | 4 | 14 |



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**Table 2. Drug susceptibility of *M.fortuitum* group by disk diffusion method**

| | No. of isolates of *M.fortuitum* group (n=22) | | |
|---|---|---|---|
| Antimicrobial agents | Susceptible | Intermediate | Resistant |
| Amikacin | 22 (100%) | - | - |
| Cefoxitin | - | - | 22 (100%) |
| Ceftriaxone | 2 (9.09%) | - | 20 (90.90%) |
| Ciprofloxacin | 17 (77.27%) | - | 5 (22.72%) |
| Clarithromycin | 22 (100%) | - | - |
| Minocycline | 2 (9.09%) | - | 20 (90.90%) |
| Imipenem | 18 (81.81%) | - | 4 (18.18%) |
| Linezolid | 22 (100%) | - | - |
| Tobramycin | 22 (100%) | - | - |
| Gatifloxacin | 17 (77.27%) | - | 5 (22.72%) |
| Amoxycillin-clavulanic acid | 3 (13.63%) | - | 19 (86.36%) |
| Trimethoprim-sulfamethoxazole | 1 (4.54%) | - | 21 (95.45%) |

**Table 3. Drug susceptibility of *M.chelonae-abscessus* group by disk diffusion method**

| | No. of isolates of *M.chelonae-abscessus* group (n=4) | | |
|---|---|---|---|
| Antimicrobial agents | Susceptible | Intermediate | Resistant |
| Amikacin | 4 (100%) | - | - |
| Cefoxitin | - | - | 4 (100%) |
| Ceftriaxone | - | - | 4 (100%) |
| Ciprofloxacin | 3 (75%) | - | 1 (25%) |
| Clarithromycin | 4 (100%) | - | - |
| Minocycline | - | - | 4 (100%) |
| Imipenem | 3 (75%) | - | 1 (25%) |
| Linezolid | 4 (100%) | - | - |
| Tobramycin | 4 (100%) | - | - |
| Gatifloxacin | 4(100%) | - | - |
| Amoxycillin-clavulanic acid | - | - | 4 (100%) |
| Trimethoprim-sulfamethoxazole | - | - | 4 (100%) |



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**Table 4. Drug susceptibility of *M. smegmatis* group by diskdiffusion method**

| | No. of isolates of *M.smegmatis* group(n=14) | | |
|---|---|---|---|
| Antimicrobial agents | Susceptible | Intermediate | Resistant |
| Amikacin | 14 (100%) | - | - |
| Cefoxitin | - | - | 14(100%) |
| Ceftriaxone | - | - | 14 (100%) |
| Ciprofloxacin | 7 (50%) | - | 7 (50%) |
| Clarithromycin | 14 (100%) | - | - |
| Minocycline | - | - | 14 (100%) |
| Imipenem | 11(78.57%) | - | 3 (21.42%) |
| Linezolid | 14 (100%) | - | - |
| Tobramycin | 11 (78.57%) | - | 3 (21.42%) |
| Gatifloxacin | 9 (64.28%) | - | 5 (35.71%) |
| Amoxycillin-clavulanic acid | 1 (7.14%) | - | 13 (92.85%) |
| Trimethoprim-sulfamethoxazole | - | - | 14 (100%) |

**Discussion**

During the past two decades there has been an increasing awareness of NTM, including the RGM, of which numerous species and phylogenetic groups are clearly established human pathogens. These include primarily the *M. fortuitum* group and the *M.chelonae- M.abscessus* group, previously known collectively as the *M.fortuitum* complex; and to a lesser extent the *M.smegmatis* group. The present study was undertaken to speciate the isolates of RGM and perform drug susceptibility test (DST). We processed 40 isolates of RGM of which *M.fortuitum* group was commonest comprising 55% of the RGM isolates, 35% belonged to *M.smegmatis* group and 10% were *M.chelonae-abscessus* group. The last group is mostly responsible for pulmonary infections. Hence the numbers were less in our study as we had not included pulmonary specimens.

In a study by Shenai S, Rodrigues C, Mehta A, of the RGM isolated, 43.75% were *M. fortuitum* and 56.25% were *M. abscessus*.[2] Gayathri *et al* reported that of the RGM included in their study, 44.6% were *M. fortuitum*, 52% were *M. abscessus* and one isolate (0.67%) each were of *M.chelonae* and *M. smegmatis*.[6] A study from Taiwan showed that of the RGM isolated, 34.5% were *M. fortuitum*, 46% were *M. abscessus* and 19.5% were *M.chelonae*.[10]*M.fortuitum* group comprised of 28.7% and *M.smegmatis* group along with the fifth group was 6.6% of RGM isolated in a study done in Texas.[11] However, these studies also included pulmonary specimens unlike our study.

*M. smegmatis* group includes *M. smegmatis, M. goodii, M. wolinskyi* which are now known to cause a number of community acquired and health care associated disease. *M. smegmatis* group has been reported in cases of infection following traumatic injury and surgical or medical



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procedures such as cardiac surgery, breast reduction surgery and face lift plastic surgery. *M.goodii* and *M.wolinskyi* have been associated with osteomyelitis. In the present study, since we had not done any sequence analysis which was the limitation of our study, many of the arylsulfatase negative species from the newly described fifth group like *M. vaccae, M. thermoresistible* and *M. flavescens* got included in the *M. smegmatis* group. The distribution of various groups of RGM according to samples has been shown in **table 1**. Majority of the isolates of *M. fortuitum* group (54.54%) and *M. smegmatis* group (50%) were isolated from pus samples, followed by lymphnode aspirates which yielded 27.27% of *M.fortuitum* group and 22% of *M. smegmatis* group. Community acquired disease due to *M. smegmatis* group is now known to involve post traumatic cellulitis, localized abscesses and osteomyelitis of a wound site. The 3 urine specimens which yielded RGM were from patients who had undergone cystoscopy following which had presented with dysuria. They were included in our analysis only if the same RGM had been isolated from at least two of the urine specimens from the same patient.

In a study by Jesudasan *et al* 42.7% isolates were from biopsies, 14.56% were from sputum, 32.03% were from pus, 2.91% each from CSF and gastric juice, 3.88% from blood and 0.97% from urine. Majority of *M.chelonae* (45.28%) and *M.fortuitum*( 40.42%) were isolated from biopsies, followed by pus from which *M.chelonae* constituted 28.30% and *M.fortuitum* 38.29%. One isolate of *M.smegmatis* was obtained from sputum (50%) and urine (50%) each. [9]

For serious disease with *M.fortuitum*, the aminoglycoside amikacin, combined with a beta lactam (cefoxitin or imipenem) or a quinolone (ciprofloxacin or ofloxacin) has been recommended for initial therapy. Macrolides and clarithromycin are important agents for treatment of pulmonary and cutaneous infections caused by *M.chelonae, M.abscessus*, and majority of *M.fortuitum*. The methods for DST for RGM are broth microdilution, disk diffusion, E test and agar disk elution most of which are not affordable to routine laboratories. Therefore, we attempted the more economical method of disk diffusion for DST of RGM. Although molecular methods are required for the definitive species identification of RGM, antimicrobial susceptibility patterns provide useful taxonomic help for the commonly encountered species, including the isolates of *M.fortuitum* complex, *M. chelonae* and *M.abscessus*.[12]

In literature there are studies by Gayathri *et al*, Broda A *et al*, Welch and Kelly and Jesudasan *et al* where DST for RGM has been done by disk diffusion method.[6, 7, 8, 9]The National Mycobacteriology Reference Laboratory (NMRL) in the United Kingdom has conducted DST's for rapidly and slowly growing mycobacteria using disk diffusion method following the BSAC recommendation for many years. Results over time have shown a large proportion of the isolates tested were resistant to a number of drugs. Overall this remained true regardless of the method employed for DST although results did vary for some drugs. Broda A *et a*l in a comparative study got good agreement between broth dilution and disk diffusion for most drugs except ciprofloxacin and tobramycin in *M. chelonae* isolates.[7]

In the present study the drugs most active against RGM were amikacin, clarithromycin and linezolid



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as all RGM isolates were sensitive to these drugs. Susceptibility to amikacin in Gayathri *et al's* study was 97.40% to 100%, [6]Jesudasan *et al* study was 99.2% [9] and 100% in a study by Welch and Kelly.[8] Our findings of amikacin sensitivity are in agreement with these studies. The other aminoglycoside tested was tobramycin to which all isolates of *M. fortuitum* group, *M. chelonae-abscessus* group and 78.57% of *M.smegmatis* group isolates were sensitive. Our study correlates with a study by Welch and Kelly where all isolates were sensitive to tobramycin.[8] However, Gayathri *et al* found 62% of *M.fortuitum* and 100% of *M. abscessus* to be resistant to tobramycin.[6]

Newer macrolides are important antimicrobial agents for treating RGM infections. All RGM isolates in the present study showed sensitivity to clarithromycin. Broda *et al's* susceptibility results showed 83% of *M. chelonae* and 57% of *M. abscessus* to be susceptible.[7]Gayathri *et al* had included azithromycin in their study to which 70% of the RGM were susceptible.[6]The results of our study showed all isolates were susceptible to linezolid. This drug was reported to be moderately active in Broda 's study as majority of the *M. chelonae* and *M. abscessus* isolates were susceptible.[7]

Among the cephalosporins, all RGM isolates were resistant to cefoxitin while 90.90% of *M.fortuitum* group were resistant to ceftriaxone. Gayathri *et al* found89% of *M. fortuitum* and upto 84% of *M. abscessus* to be resistant to ceftriaxone.[6] No cefoxitin activity was seen to *M. chelonae* and 73% susceptibility was seen in *M. abscessus* in Broda A's work.[7]

In the present study, ciprofloxacin was active against 77.27% of *M.fortuitum* group, 75% of *M. chelonae abscessus* group and 50% of *M. smegmatis* group. Gayathri *et al*reported 82% sensitivity of *M.fortuitum* and *M.abscessus* which is similar to our study. [6]

Gatifloxacin was effective against 77.27% of *M.fortuitum* group, 100% of *M.chelonae-abscessus* group and 64.28% of *M.smegmatis* group which is close to Gayathri *et al's* gatifloxacin sensitivity in 92% of *M. fortuitum* and 91% of *M.abscessus.*[6]

Our results showed, 81.81% of *M. fortuitum* group, 75% of *M.chelonae-abscessus* group and 78.57% of *M.smegmatis* group were sensitive to imipenem. Sensitivity to amoxicillin-clavulanic acid was low as it was active against 13.63% of *M.fortuitum* group and 7.14 % of *M.smegmatis* group. Broda A reported all strains of *M. chelonae* and *M. abscessus* to be resistant to imipenem and amoxicillin - clavulanic acid.[7]

Tetracyclines used to be very popular therapeutic drugs for RGM but their use has declined in last 20 years due to development of drug resistance. Our data shows tetracycline like minocycline to be effective against only 9.09% of *M.fortuitum* group. Broda also showed poor activity of minocycline.They also found high resistance to trimethoprim-sulfamethoxazole in their study which was on isolates of *M.abscessus* and *M. chelonae*.[7]In our study only 4.54% of *M.fortuitum* group were sensitive to trimethoprim-sulfamethoxazole.

Thus, in our study, disk diffusion method showed that all isolates of RGM were sensitive to amikacin, clarithromycin, linezolid. All *M. fortuitum* group isolates showed sensitivity to tobramycin. All isolates of *M. chelonae abscessus* were susceptible to tobramycin and gatifloxacin.



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High degree of resistance was seen to cephalosporins, minocycline, amoxicillin clavulanic acid and trimethoprim-sulfamethoxazole. Hence these drugs should be used only after performing DST.

Keeping in mind the differences in susceptibility patterns of RGM, tentative identification of the commonly encountered species is possible and empiric therapeutic regimens can be decided by the help of DST by the simple and economical disk diffusion method in resource restricted settings. Isolates clearly resistant or sensitive could be reported. However, isolates which show intermediate results should be retested by using broth dilution method.

The primary advantages of the disk diffusion method is the ease of set up and the ability of recognition of mixed cultures that may not be obvious in broth cultures.[12] Limitations include difficulty in interpretation of zones of inhibition, especially when the amount of the drug is near the breakpoint of the drug as seen with 30 µg cefoxitin disk for *M. abscessus* in a study by Broda A *et al*.[7]Also too heavy inoculums can cause falsely resistant interpretation of the disk zone but can be avoided by paying careful attention while matching the turbidity to the Mac Farland standard. Disk diffusion method has not been standardized by CLSI and should be used only for preliminary identification and as an adjunct to broth microdilution. In broth microdilution too variables such as incubation condition and time, inoculums and media do require attention. In addition, the growth of RGM in broth microdilution sometimes gives a hazy appearance instead of a button at the bottom of the well and training is required for interpreting the results. Also imipenem is unstable in broth and makes results difficult to interpret.

Administration of single drug could lead to resistance. Hence multidrug regimen is recommended for treatment of infections caused by RGM. Accurate species identification could reveal biological behaviours of RGM and guide empiric antibiotic therapy.

Communication between the clinician and laboratorian is essential for determining the importance and extent of the identification analysis for a clinical rapidly growing mycobacterial isolate.

**Conclusion**

In the present study all RGM were sensitive to amikacin, clarithromycin and linezolid. High degree of resistance was seen to cephalosporins, minocycline, amoxicillin-clavulanic acid and trimethoprim sulphamethoxazole. Disk diffusion can help in tentative identification of the commonly encountered RGM and help to decide empiric antibiotic therapy.Variability in sensitivity to different antimicrobials exists in all RGM isolates. Resistance data varies geographically and also with time.Therefore, each isolate must be individually evaluated, and it is advisable to perform DST before using the antimicrobial for treatment of infections by RGM.



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