ISOLATION- DRUG RESISTANCE PROFILE AND MOLECULAR CHARACTERIZATION OF INDIGENOUS TYPICAL AND ATYPICAL MYCOBACTERIA

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ABSTRACT

One hundred and fifty mycobacterial isolates from different pathological Labs. of Karachi were collected and screened as acid fast. On the bases of phenotypic and biochemical results, it was found that, 58.66% isolates were typical mycobacteria while 41.33% belonged to atypical mycobacteria. The individual percentages of different mycobacterial species include: *M. xenopi* 35%, *M. thermoresistible* 19 %, *M. terrae complex* 6 %, *M. marinum* 6 %, *M. fortuitum* 6 %, *M. kansasii* 25 % and *M. tuberculosis* 58.66 %. The sensitivity of mycobacterial isolates was determined against 5 first line, 3 second line and 1 third line anti-tuberculosis drugs. The highest number of the isolates (typical and atypical mycobacteria) offered resistance against isoniazid and streptomycin. Clarithromycin was found to be the drug of choice as regards the drug sensitivity in case of atypical mycobacterial isolates. A total of 40 isolates were subjected to PCR based identification and differentiation of 16S rRNA gene(s). Accordingly, 37.5% isolates were identified as typical mycobacteria while 25% were identified as atypical mycobacteria. These findings carry significance because a detailed research based identification (PCR and Multiplex PCR based) regarding indigenous mycobacteria has been reported for the first time in Pakistan. However, both the approaches (conventional and molecular methods) have experimental importance while identifying these organisms.

Keywords: Atypical mycobacteria, Typical mycobacteria, Drug sensitivity profile, conventional methods, Multiplex PCR,

INTRODUCTION

The 22 high tuberculosis burden countries (HBCs) account for approximately 80% of the estimated new tuberculosis (TB) cases being reported each year (Floyd *et al*., 2002). According to the world health organization (WHO), nearly 2 billion people (one third of the world's population) are being exposed to the TB pathogen annually, 8 million people fall ill, while 2 million people die from the disease worldwide. Almost 50 % of MDR-TB (multi drug resistant tuberculosis, defined as resistant to the two most effective first line anti TB drugs i.e. rifampicin and isoniazid.) cases worldwide are estimated to occur in China and India. In 2008, MDR-TB caused an estimated 150,000 deaths (Falzon *et al*., 2010). The problem has further been aggravated by the AIDS factor (Chum *et al*., 1996; Mac-Arthur *et al*., 2001). According to Gandhi *et al* (2010), mortality from MDR and XDR-TB (extensively drug resistant TB defined as MDR-TB plus resistance to a fluoroquinolone and at least one second-line injectable agent: amikacin, kanamycin and/or capreomycin) in their high HIV-prevalence region (Tugela Ferry, South Africa) is extraordinarily high, especially during the first month. As in many other countries, mycobacterium multidrug-resistant strains have also become a major issue. The incidence of MDR-TB was found increased in Pakistan from 14% to 47% within seven years i.e. 1999 to 2006 (Tanveer *et al*., 2008). Drug-resistant tuberculosis was first observed in 1948 after the first trials of streptomycin for the treatment of TB (Robert, 2000). Drug-resistant TB is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs. Worldwide emergence of drug-resistant TB has changed views about the way we treat infections caused by *Mycobacterium tuberculosis (M. tuberculosis)*. This change reflects our understanding of the failures of standard regimens in patients suffering from drug-resistant strains (Becerra *et al.,* 2000; Espinal *et al*., 2000*).* Atypical mycobacteria cause neither TB nor leprosy, but they do cause pulmonary diseases resembling TB (Van Crevel *et al*., 2001). The distribution and the incidence of disease caused by them are not fully understood in most parts of the world. These organisms are widely distributed in nature (Kazda, 1983) and have been isolated from natural water, tap water, soil water used in showers and surgical solutions. In United States, most of the isolates reported include *M. avium, M. kansasii and M. fortuitum* (O' Brien *et al*., 1987). There have been some reports of atypical mycobacteria from Japan (Tsukamura *et al*., 1988). In most of the studies from India *M. tuberculosis* has been found as major cause of mycobacterial infections and the proportion of atypical mycobacteria has been considered low. Thus, species like *M. fortuitum, M. avium and M . scrofulaceum* have reportedly been isolated by Sachdev *et al*. (2002). Stratmann *et al*. (2002) had used polymerase chain reaction (PCR) techniques for the detection and rapid identification of the clinically relevant mycobacteria

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using methods for concentration and detection of *M. avium, M. intracellulare* from the clinical specimens and *M. paratuberculosis* from clinical specimens and milk. Roth *et al* (2000) reported PCR assays using genus/ group specific amplification followed by restriction analysis for the detection of gene regions like 65 KDa and rRNA gene region which have been found useful for identification of different mycobacteria. Ruiz *et al*. (2002) declared the merits of PCR amplification followed by hybridization for the detection of *M. avium, M. chelonae, M. scrofulaceum, M. ulcerans* and other mycobacteria.

MATERIALS AND METHODS

A total of 150 (including typical and atypical) mycobacterial isolates were procured from different pathological labs of Karachi (table 1). Phenotypic identification of typical and atypical mycobacteria was performed by different methods as described in ASM manual (Pfyffer *et al*., 2003) that included rate of growth, pigment production, colonial morphology and growth at different temperatures (37^{o}C, 45^{o}C and room temperature i.e., 20-25^{o}C) while the selection of specific biochemical tests for identification was made on individual basis after the growth rate, pigment production and colonial morphological features were noted as described by Niemann *et al*. (2000). In addition, tests based on Tween-80 hydrolysis, nitrate reduction , urease production, niacin accumulation, heat stable catalase and semi quantitative catalase production, growth on MacConkey's agar(without crystal violet), and pyrazinamidase production were done (Kent and Kubica, 1985). Drug resistance profile was done by resistance ratio method in which different drugs were selected for susceptibility testing (Acharya *et al*., 2008). Serial two fold dilutions of the drugs, first line (isoniazid, rifampicin, streptomycin, ethambutole and pyrazinamide) second line (ciprofloxacin, amikacin and sparfloxacin) third line (clarithromycin) were made in Lowenstein Jensen (LJ) medium. Molecular based identification and differentiation of typical and atypical mycobacteria were done by EZTBPCR kit from MBDr Diagnostics (Bio Diagnostic Research Company, Malaysia). Thermo stabilized PCR mix with specific primers was used for the detection of typical and atypical mycobacteria However, multiplex-polymerase chain reaction was also used by Kapur *et al*. (1995). As described by Gopinath and Singh (2009). Thus, a number of amplified products were obtained. All the protocols were followed according to the manufacturer's recommendations. In short, for PCR, 15µl of DNAse RNAse free water was added to each thermo stabilized PCR mix tube. Extracted DNA sample (5 µl template DNA) was added to the thermostable PCR mix. Positive & negative controls were also run. Tubes were placed in PCR machine (Thermo Electron Corporation, USA). Results were analyzed after gel electrophoresis of the amplified DNA; TB DNA ladder and 100 bp DNA ladder (Promega; 0.13µg µl^{-1}) were used for comparison of the results (Sambrook *et al*., 1989).

RESULTS

On the bases of phenotypic and biochemical results it was concluded that out of one hundred and fifty isolates, 58.66 % were typical mycobacteria while 41.33 % were atypical mycobacteria. The individual percentages of different mycobacterial species include: *M. xenopi* - 35%; *M. thermoresistible* – 19 %; *M. terrae complex* – 6 %; *M. marinum* – 6 %; *M. fortuitum* – 6 %; *M. kansasii* – 25 % and *M. tuberculosis*- 58.66 % (fig. 1). According to resistance ratio method (table 2) rifampicin resistance was offered by 6.2 % and 9.68 % of typical and atypical respectively, while 93.8 % and 90.32 % were found sensitive respectively. Isoniazid resistance (26 % isolates) was shown by typical while 59.9 % resistant isolates belonged to atypical mycobacteria while sensitivity was offered by 74 % and 40.1% isolates respectively. Similarly resistance against streptomycin was shown by 22 % (typical) and 16.9 % (atypical) isolates while sensitivity was offered by 78% and 83.1% of the isolates respectively. Ethambutol resistance was offered by 14.2 % and 15.9 % of the typical and atypical mycobacterial isolates (respectively), 85.8 % and 84.1% of the isolates were found sensitive against ethambutol. Resistance against pyrazinamide was offered by 32.9 % of typical and atypical mycobacterial isolates while equal number (67.1 %) of both the types were found sensitive. Multidrug resistance was offered by 6 % and 2 % of the typical and atypical isolates respectively. Over all highest resistance (32.9 % by both the types) was offered against pyrazinamide, followed by isoniazid resistance by typical 26 % and 59.9 % by atypical, 22 % and 16.9 % for streptomycin, 14.2% and 15.9% for ethambutol and 6.2 % and 9.68% for rifampicin (for both typical and atypical mycobacterial isolates respectively). According to our findings, the most suitable drug to control both typical and atypical mycobacteria is rifampicin (fig. 2). Although, the atypical isolates were found more resistant to isoniazid and rifampicin however, the frequency of MDR was low among the atypical (fig. 2). In case of second line drugs, the highest number of isolates(typical mycobacteria) offered resistance against ciprofloxacin while the atypical offered highest resistance against amikacin. The second line drug of choice to control (as per *in vitro* investigations) TB infection by atypical mycobacteria has been found to be sparfloxacin, while clarithromycin (third line) can be rated as the drug of choice for both the mycobacterial types (fig. 3). On the basis of genotypic results, 37.5 % and 25 % of the isolates were identified as typical and atypical mycobacteria respectively. Figure 4 presents the DNA bands of different isolates (sized using Mycobacterium DNA ladder). Lane wise description of the bands is given in fig. 4.

Table 1: Procurement of the isolates from different pathological labs of Karachi

Labs and hospitals	Essa's lab	SIUT	Ehsan Ullah's Lab	OICD
No of collected isolates	10	100	20	20
No of collected biological sample	50	0	0	0

Key: NCI - No. of collected isolates, NCB - No. of collected biological sample, ESSA'S LAB - Dr. Essa's Laboratories, SIUT - Sindh Institute of Urology and Transplantation, Ehsan Ullah's Lab - Dr. Ehsanullah Laboratories, OICD - Ojha Institute of Chest Diseases.

Table 2: Drug (first line) resistance profile of typical and atypical mycobacteria

S. No.	Drugs	Typical mycobacteria			Atypical mycobacteria		
		R(%)	S (%)	MDR (%)	R (%)	S (%)	MDR (%)
1	Rifampicin	6.2	93.8	6 (5.68)	9.68	90.32	2 (3.2)
2	Isoniazid	26	74		59.9	40.1	
3	Streptomycin	22	78		16.9	83.1	
4	Ethambutol	14.2	85.8		15.9	84.1	
5	Pyrazinamide	32.9	67.1		32.9	67.1	

Key: S - sensitive, R - resistance. The results are based on the resistance ratio method.

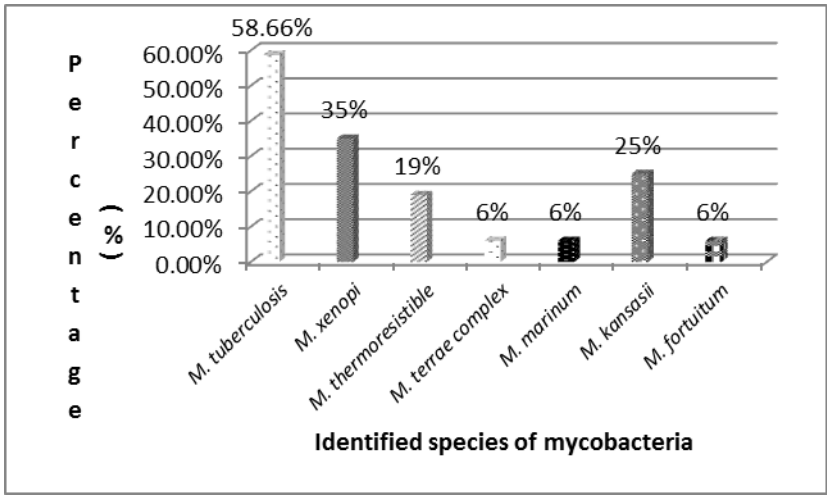

Fig. 1: Identification of mycobacteria up to species level.

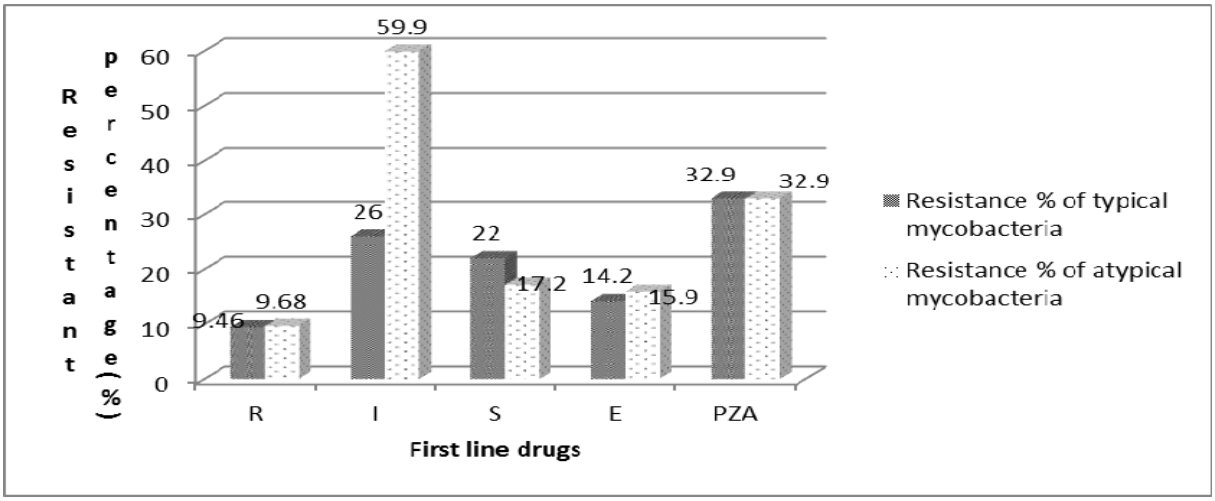

Fig. 2: Comparison of first line drug resistance pattern of typical and atypical mycobacteria.
Key: R=Rifampicin, I=Isoniazid, S=Streptomycin, E=Ethambutol, PZA= Pyrazinamide

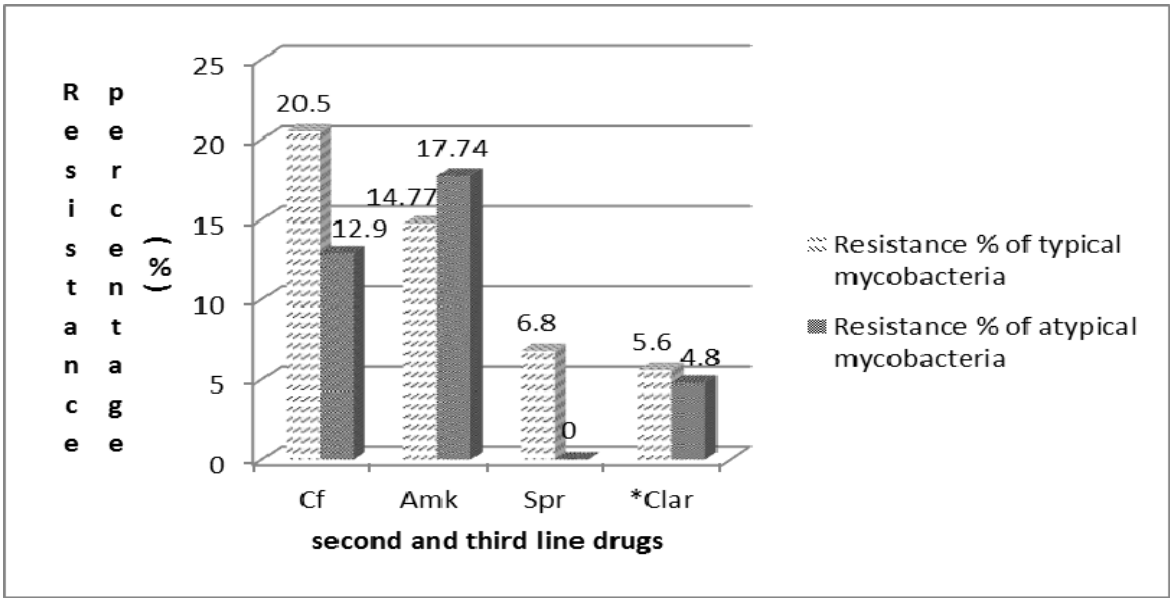

Fig. 3: Comparison of second and third line drug resistance pattern of typical and atypical mycobacteria.
Key: Cf=Ciprofloxacin, Amk=Amikacin, Spr =Sparfloxacin, *Cla=Clarithromycin (A third line drug)

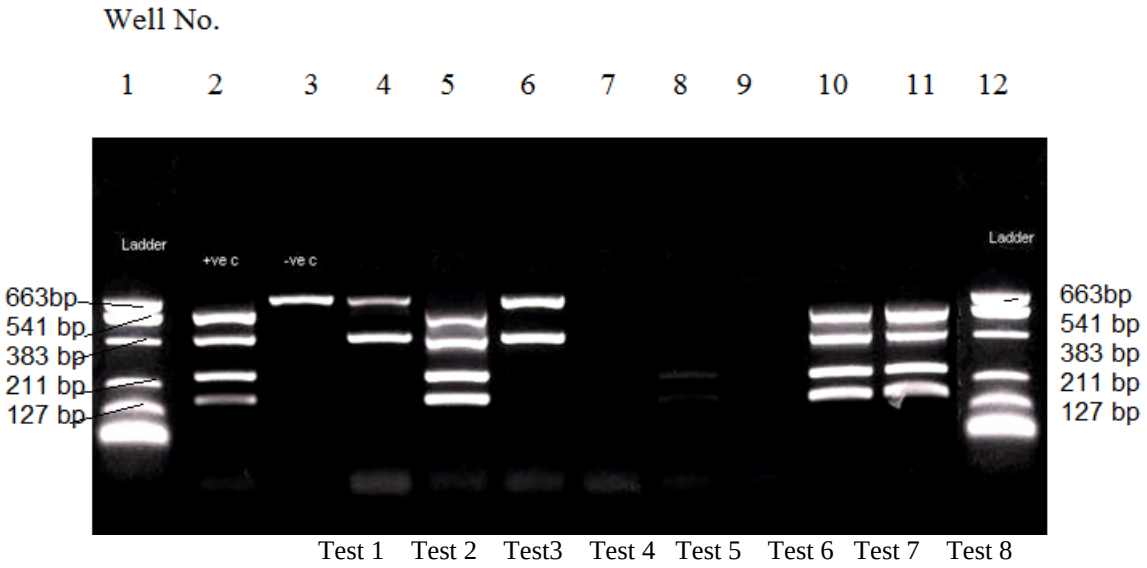

Fig. 4: PCR based differentiation of typical and atypical mycobacteria
Key: Lane 1 and 12: MTB DNA ladders.
Lane No.2: positive control (showed 541, 383, 211 and 127 bp bands).
Lane No.3: negative control (showed only 663 bp band, PCR control.
Lane Nos. 5, 10, 11 are indicative of *M .tuberculosis* as they showed all 541, 383, 211 and 127 bp bands; lane No.4 and 6 showed DNA products that gave bands at 663 and 383 bp positions which are specific for atypical mycobacteria.

DISCUSSION

Nontuberculous or atypical mycobacteria are opportunistic pathogens frequently found in water sources, soil, dust, and air. Infections from animals i.e. cattles (Bollo *et al*., 1998), dogs (Wallace, 1989) and cats (Ngan *et al*., 2005) have been described. These organisms are implicated in infections of the skin, bones and soft tissues. Immunocompromised patients are most susceptible to the disseminated infection caused by nontuberculous mycobacteria (Porat & Austin, 2008; Prendiki *et al*., 2008). Although, diseases caused by these organisms are uncommon (compared with the classical tuberculosis) but a significant increase in pulmonary and non pulmonary infections by mycobacteria has been observed during the last two to three decades (Wolinsky

1979; Wayne and Sramek 1992). Our results show that the frequency of atypical is lower compared to the typical mycobacteria and the results are in agreement with the findings of Agarwal (2001). However, the prevalence of atypical mycobacteria varies from country to country and region to region. Mawak *et al*. (2006) reported 61.54 % *M.tuberculosis*, 15.38 % *M. bovis* and 23.08% as atypical mycobacteria. Among them 20.69 % were classified as *M. avium* while *M. kansasii* and *M. fortuitum* percentage was found to be the same i.e. 3.45%. Reports are available that the drug susceptibility profile of atypical is usually quite different from typical mycobacteria. Firstly, these organisms are frequently sensitive at high concentrations of antimycobacterial drugs (Wallace *et al*., 1990). These results are also in agreement with the ones reported by Katoch and Mohan (2001); according to them first, higher cut off values for deciding sensitivity or resistance are needed. Secondly, rapidly growing mycobacteria are usually resistant to rifampicin and isoniazid, whereas these are found sensitive to drugs like new generation macrolides, cephalosporins and sulphones (Katoch, 2004). These observations are partially in agreement as we have reported amikacin resistant isolates (figure 3). Javaid *et al.* (2008) reported the prevalence of MDR among untreated patients in NWFP as 2.5 %, whereas we found 6 % and 2 % for typical and atypical mycobacterial isolates respectively which is a major cause of concern and should be addressed through effective TB control programme, preferably with DOTS strategy. The multiplex PCR method could not clearly differentiate *M. tuberculosis* complex and NTM strains. In addition, restriction fragment length polymorphism analysis and direct sequencing of the amplicon of NTM could be used to supplement species identification. Moreover, the standard culture method (considered a gold standard) proved to be 100-fold more sensitive than the EZTBPCR Test Kit.

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