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Frequency of Rapid Growing Mycobacteria among Tuberculosis Suspected Patients in Basra- Iraq

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Abstract

Objective: The purpose of this study was to estimate the frequency of rapid growing mycobacteria among tuberculosis suspected patients in Basra governorate and study their resistance to drugs.

Methods: A total of 150 sputum samples were obtained from 150 suspected patients who attended the Advisory Clinic for Chest Diseases and Respiratory (ACCDR) in the Basra Governorate from 01/03/2013 to 1/02/2014. Smears were stained with the Ziehl Neelsen technique and specimens were inoculated on Lowenstein Jensen medium, Identification to species level was achieved on the basis of the growth characteristics, pigment production and conventional biochemical tests. Drug susceptibility was tested to rifampicin, ethambutol, pyrazinamide, isoniazid, and streptomycin using the proportional method.

Results: From 150 sputum samples, 23 isolates were *Mycobacterium tuberculosis* (MTB) (15.33%) and 16 (10.66%) were nontuberculous mycobacteria, seven isolates of them (43.75%), 2 males and 5 females, mean age 40 years, were identified using biochemical tests as rapid growing mycobacteria of which 4 (25%) as *M. chelonae*, 2 (12.5%) *M. abscessus* and 1 (6.2%) *M. smegmatis*. In addition to that, the bacteria were successfully differentiated by Duplex-PCR, as MTB and NTM based on amplification of rpoB gene sequences. Sequencing of 16S rDNA showed matching with 6 biochemically identified ones, and one of the 4 *M. chelonae* was *M. chitae*.

Drug susceptibility testing showed that one *M. abscessus* isolate appeared to be resistant to all antibiotics (TDR), while two isolates of *M. chelonae* showed resistance to ethambutol and rifampicin, while *M. smegmatis* showed weak resistance to pyrazinamide and resistance to rifampicin. Also, all isolates of *M. chelonae* were sensitive to pyrazinamide, isoniazid and streptomycin.

Conclusion: It appears that the rapidly growing mycobacteria represents a high frequency, among non-tuberculosis detected patients, which requires phenotypical and genotypical confirmation on a follow-up, along with the examination of patterns of sensitivity. Implementing Duplex-PCR proved to be decisive for differentiating NTM from MTB.

Keywords: Rapid growing mycobacteria; Non tuberculosis mycobacterium; Suspected tuberculosis patients; TB; Antimicrobial susceptibility testing

Introduction

Non-tuberculousis mycobacteria (NTM) especially rapid growing mycobacteria (RGM) are environmental opportunistic pathogens and their role in human disease is increasingly recognized. In addition, several studies indicate that humans can be infected by NTM from various environmental sources, especially soil and water [1,2].

Runyon classified these organisms as rapidly growing mycobacteria as they can produce mature colonies on agar plates within 7 days, while slowly growing mycobacteria need several more days to grow [3,4].

Identification of rapidly growing mycobacteria is important for clinical and epidemiological studies because of their spread worldwide [2].

Although a system of national disease surveillance, as exists for *Mycobacterium tuberculosis* in developed countries, has not been implemented, infections caused by rapidly growing mycobacteria have been increasingly reported in recent years [5-7].

These rapidly growing organisms have been associated with a wide range of clinical syndromes in both immunocompetent and immunocompromised hosts, ranging from mild, such as infections of skin and soft tissue, to more serious disorders, including osteomyelitis, and lymph node, respiratory tract, bloodstream infections and disseminated infection [6,8].

The differences in susceptibility patterns of species and resistance to first line antituberculosis drugs create challenges in the approach to treatment of these organisms [9].

This study is focused on the isolation and identification of rapidly growing mycobacteria from tuberculosis suspected patients, and testing their drug susceptibility because of their emerging importance in both sporadic infection and outbreak settings.

Materials and Methods

Sample collection

A total of 150 sputum samples were obtained from 150 patients admitted to the ACCDR, during a one year period "March 2013 to February 2014".

All sputa were collected in sterile, screw cap containers. The expectorated sputum was taken by asking the patient to cough deeply into the container, followed by immediate screwing off the cap. Samples were transported to the laboratory within two hours and processed immediately or refrigerated at 4°C as soon as possible [10].

Microbiological examination

The specimens were processed on the same day for microscopy and culture using standard procedures [11]. Smears were stained with the Ziehl Neelsen (ZN) technique. Specimens were inoculated onto Lowenstein Jensen (LJ) medium, after decontamination procedures and concentration, and then incubated at 37°C. Cultures were examined every day for a week and then once a week for eight weeks. Isolates obtained weekly were confirmed as acid-fast bacilli by ZN staining.

Identification to species level was achieved on the basis of the growth characteristics, including growth in less than 7 days, growth at 37°C, growth in the presence of NaCl 5%, pigment production, Niacin production, pyrazinamidase, urease, nitrate reduction test, catalase test, heat-stable catalase (pH 7, 68°C), Tween 80 hydrolysis, growth on MacConkey agar, arylsulfatase test, and colony morphology.

Genetic identification

DNA was extracted by DNA PrepMate-M (Bioneer) according to the manufacture's instructions and DNA was detected on agarose gel electrophoresis [12]. 16S rDNA sequencing was done in Nicem company (USA) and Macrogen company (Korea).

Duplex PCR: RNA polymerase gene (rpoB) primers

The isolates were subjected to identification by using Duplex PCR (two pairs of primers) in order to amplify rpoB gene which is listed in Table 1.

Gene	Primer type	Primer sequence (5'-3')	Size of product
rpoB gene	Tbc 1F	5'-CGTACGGTCGGCGAGCTGATCCAA-3'	235 bp
	TbcR5 R	5'-CCACCAGTCGGCGCTTGTGGGTCAA-3'	
	M5 F	5'-GGAGCGGATGACCACCCAGGACGTC-3'	136 bp
	RM3 R	5'-CAGCGGGTTGTTCTGGTCCATGAAC-3'	

Table 1: rpoB gene primers.

Thermal cycling condition

The PCR method for amplifying the rpoB gene was conducted as reported by Singh et al. [13] (Table 2).

Steps	Temperature	Time	No. of cycles
Initial denaturation	95°C	5 min	1
Denaturation	94°C	20 Sec	
Annealing	55°C	20 Sec	40
Extension	72°C	40 Sec	
Final extension	72°C	5 min	1
TM = Melting temperature, TA = Annealing temperature			

Table 2: Program used in PCR amplification for rpoB gene.

Drug susceptibility tests

The *in vitro* antimicrobial susceptibility of these RGM was performed according to the proportional method [14]. Five antibiotics were used as rifampicin 1 µg/ml, ethambutol 2 µg/ml, pyrazinamide 0.25 µg/ml, isoniazid 0.2 µg/ml, streptomycin 2 µg/ml.

The results were read after three weeks of incubation at 35°C. The isolate was recorded as resistant when growth in the critical concentration of the drug is more than 1% the growth of the Middlebrook 7H10 medium without antibiotic. When the case is opposite, the isolate is considered as susceptible. The resistance percent was calculated by the following Equation 1 [15]:

$$\frac{Number\ of\ colonies\ on\ the\ drug}{Number\ of\ colonies\ on\ the\ control} * 100 = \%\ \ resistance \quad (1)$$

Results

Of one hundred fifty tuberculosis suspected patients, attended the ACCDR who were suffering from upper respiratory tract infections, twenty three isolates were MTB (15.33%) and sixteen (10.66%) were NTM, seven of them (43.75%) were rapid growing mycobacteria, which included 4 (25%) as *M. chelonae*, 2 (12.5%) *M. abscessus* and 1 (6.2%) *M. smegmatis* (Figures 1 and 2) (Table 3).

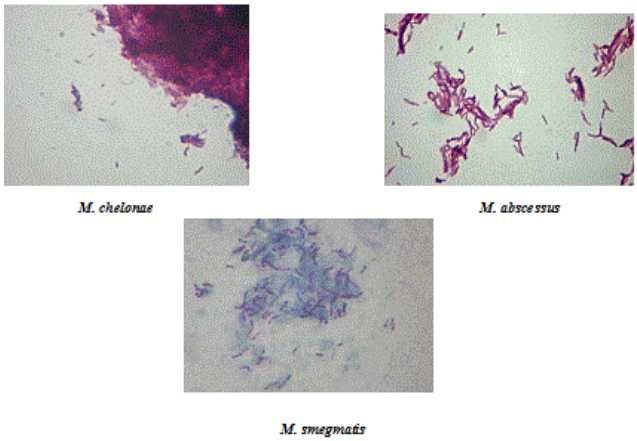

Figure 1: Direct examination of rapid growing mycobacteria on ZN stain under immersion oil (X100).

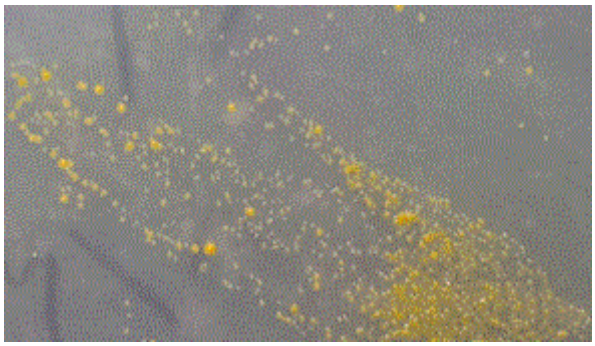

Figure 2: Rapid growing mycobacteria growth on the Middlebrook 7H10 medium.

Tests	*M. chelonae* 4 isolates	*M. abscessus* 2 isolates	*M. smegmatis* 1 isolate
Growth in less than 7 days	+	+	+
Growth at 37°C	+	+	+
Photo reactive pigment	-	+	-
Pigment in dark	-	-	+
Growth in presence of NaCl 5%	+	+	+
Growth on MacConkey agar	+	-	-
Niacin production	-	-	-
Nitrate reduction	+	-	+
Arylsulfatase (3 days)	+	+	-
Tween 80 hydrolysis	+	-	+
Heat stable catalase	-	-	-
Pyrazinamidase	-	+	+
Urease	-	+	+

Table 3: Biochemical tests of rapid growing mycobacteria.

Genetic identification

Amplifying universal 16S rDNA: The extracted DNAs for the isolates were subjected to PCR for amplifying universal 16S rDNA gene. PCR products for the universal 16S rDNA primers gave bands on an agarose gel at the position 1500bp when compared with standard molecular DNA ladder (Figure 3).

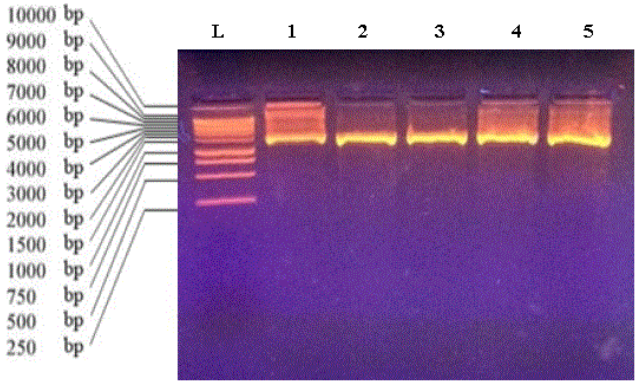

Figure 3: Agarose (2 %) gel electrophoresis of universal 16S rDNA PCR products for mycobacterial isolates under UV transilluminator. Lane 1: (L): (250 bp-10000bp) DNA ladder. Lane1 to 5: of 16S rDNA bands (1500bp) for bacterial isolates.

DNA amplification and duplex PCR: The isolates were identified by D-PCR method for differentiating between *Mycobacterium tuberculosis* (MTB) and NTM based on amplification of rpoB gene sequences. The results showed two bands, 235 bp band for MTB and 136 bp band for NTM (Figure 4).

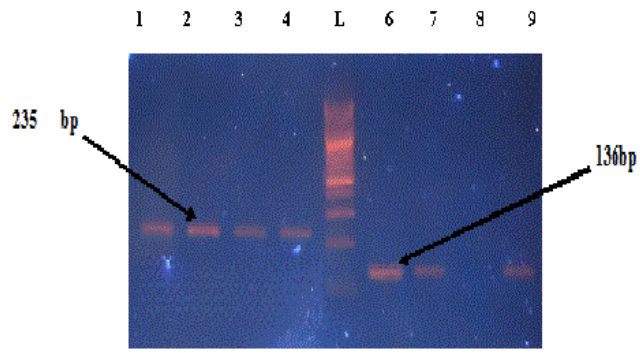

Figure 4: D-PCR assay results performed with culture isolates of MTB and NTM. Two amplicons of different sizes (235 and 136 bp) were amplified from MTB (lanes 1 to 4) and NTM (lanes 6 to 9) strains by a single D-PCR. Lane: L, ladder DNA (100-1500 bp ladder).

Sequencing for Universal 16S rDNA Gene: The isolates have been identified using 16S rDNA sequencing, the results showed that 6 RGM

matched with biochemical results, as follows: *M. chelonae* (n=3), *M. abscessus* (n=2) and *M. smegmatis* (n=1), while one *M. chelonae* did not match, and gave *M. chitae*.

Antibiotic susceptibility: The drug susceptibility results showed that, One *M. chelonae* isolate was resistant to EMB and one was MDR (RIF and EMB). *M. smegmatis* isolate was MDR (RIF and PZA), while one isolate of *M. abscessus* appeared to be resistant to all antibiotics tested. It was from a thirty year male, non smoker, with no previously documented lung disease, and lives in the poor socio-economic district. The rest isolates were sensitive (Table 4).

Isolate No.	Mycobacterial species	Antibiotics	Resistant percent
48	*M. chelonae*	RIF	95.70%
		EMB	4.20%
114	*M. chelonae*	EMB	2%
50	*M. abscessus*	RIF	94%
		STR	24%
		EMB	4%
		INH	10%
		PZA	8%
110	*M. smegmatis*	RIF	100%
		PZA	14.20%

Table 4: Resistance percentage of the RGM isolates against some antibiotics.

Discussion

Non-tuberculous mycobacterial infections are becoming increasingly common. Among them, the rapidly growing organisms such as *Mycobacterium chelonae*, *Mycobacterium fortuitum* and *Mycobacterium abscessus* are widespread in nature and in hospital environments [16].

The results showed that the rates of infection in Iraq are considered lower than the rates in the world based on previous studies [17-19].

To the best of our knowledge, this is the first study conducted in Iraq for the isolation and identification of NTM with human infection, a previous study was on environmental sites [1].

The results of the present study showed that, the prevalence of *M. chelonae* was 42.8%, *M. abscessus* 28.5% and *M. smegmatis* 14.2% of RGM for all isolates from pulmonary samples. *M. chelonae* and *M. abscessus* have similar phenotypes and antimicrobial susceptibility profiles [20,21]. The profile of antibiotics of the RGM isolates appeared that, most isolates have the ability to resist one or more antimicrobial drugs (Table 4), One isolate of *M. abscessus* was resistant to all antimycobaterial drugs and this bacterium is deemed to be one of the most virulent and resistant species of rapidly growing mycobacteria. Many researchers found that, this bacterium is usually resistant to imipenem, ciprofloxacin, cotrimoxazole, linezolid, and doxycycline, but is susceptible only *in vitro* to amikacin and clarithromycin [22-25].

Depending on sputum smear only for diagnosis is not decisive as positive sputum smear may, in fact, due to an NTM that is then erroneously treated with standard anti-TB medications. As many NTM is resistant to first-line anti-TB medications, most of these cases would be considered failures, and subsequently treated with the second line regimen. On failure of the latter regimen, the patients are reported as chronic cases [26].

The mycobacterial cell wall functions as an efficient protective barrier and limits the entry of drug molecules to their cellular targets [27]. A major porin of *M. smegmatis*, MspA, forms a tetrameric complex with a single central pore [28]. Deletion of the porins MspA and MspC raised the resistance to β-lactam antibiotics without changing its β-lactamase activity. Hydrophilic fluoroquinolones such as norfloxacin, and chloramphenicol, diffuse through porins in mycobacteria [29].

The cell wall barrier alone is not enough to explain the intrinsic drug resistance of these bacteria. Drug efflux is a drug resistance mechanism contributed to intrinsic or acquired resistance in a wide range of bacteria [30]. *M. smegmatis* LfrA was the first multidrug efflux pump confirmed in mycobacteria [31]. It produces low-level resistance to fluoroquinolones and other toxic compounds such as ethidium bromide [31,32]. EfpA, Tap, and P55 are the three other major facilitator super family (MFS) pumps found in several mycobacterial species, and of these pumps, Tap and P55 are known to give low-level resistance to aminoglycosides and tetracyclines [33]. Mmr (a small multidrug resistance family (SMR) pump) and DrrAB (an ATP-binding cassette super family (ABC) exporter) were reported in MTB [34]. These exporters produce low-level resistance to certain antimicrobial agents [35]. Physiological adaptations appearing in the host can also lead to antibiotic tolerance [36].

Conclusion

An early identification of MTB and NTM is highly recommended, so that a correct antibiotic treatment can be initiated. The Duplex PCR proved to be the right differential technique. Concurrently, emerging drug resistance is indicated here by the appearance of one *M. abscessus* isolate resistant to all antimycobacterials pressing for more effective drugs.

Also, it is worthwhile to suggest the implementation of the monophasic – biphasic culture setup of tuberculosis broth and LJ agar for rapid recovery of MTB [1].

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