Molecular Detection of Mycobacterium Other Than Tuberculosis among Patients with Pulmonary Infection

Mogahid M Elhassan, PhD* Al Wadah Y Eltikeina, BSc, MSc** Ramia M Saleh, BSc, MSc** Miskelyemen A Elmekki, PhD* Mohamed E Hamid, PhD***

Objective: Isolation of Mycobacterium other than tuberculosis (MOTTs) among patients with pulmonary infection and evaluation of the usefulness of polymerase chain reaction (PCR) as a confirmatory test.

Design: Descriptive laboratory based study.

Setting: National Health Laboratory (Stack), Elasha'ab Teaching Hospital, Abo Anga Hospital, Ebrahem Malek Hospital and Academy Charity Hospital.

Method: One hundred and seventy-one sputum samples were collected from suspected pulmonary tuberculosis patients, males and females with different ages. Informed consent was taken.

Sputum samples were inoculated on LJ medium and organisms were identified according to their Ziehl-Neelsen stain, cultural characteristics and biochemical proprieties. The rapidly growing isolates were subjected to PCR.

Result: In this study, males were found to be more affected than females 125 (73.1%). The patients between 21-50 years were the most affected with TB and NTM. On LJ medium, 40 (23.4%) of the isolates gave characteristic growth of *Mycobacterium tuberculosis* and were identified according to their Ziehl-Neelsen stain and cultural characteristic. Ten (5.8%) isolates were identified as rapid growers, 6 out of which were identified as MOTTs according to their indirect Ziehl-Neelsen stain, cultural characteristics and biochemical proprieties. On PCR, six of the ten rapid growers showed a band typical in size (136 bp) to target *rpoB* gene as indicated by the standard DNA marker.

Conclusion: The results revealed clearly the importance of conventional methods including Z.N stain and culture techniques in the diagnosis of TB and NTM. As well as it proved the effectiveness of PCR as a sensitive, specific and rapid diagnostic and confirmatory test.

Bahrain Med Bull 2011; 33(2):

---

* Associate Professor and Head Department of Microbiology
Sudan University of Science and Technology
** Assistant Professor of Immunology and Head Department of Parasitology
Federal Ministry of Health, Khartoum, Sudan
*** Associate Professor and Consultant of Microbiology
Tuberculosis Research Group, Department of Microbiology
College of Medicine, King Khalid University, KSA
Email: mogahidelhassan@yahoo.com; greensdan70@yahoo.com

Non-tuberculous mycobacteria (NTM), also known as environmental mycobacteria, and mycobacteria other than tuberculosis (MOTTs); are defined as mycobacteria which do not cause tuberculosis or leprosy. This group of organisms includes true pathogens, opportunistic pathogens, colonizers, and contaminates. Based on their rate of growth, these organisms can be categorized into two major groups: the slow-growing mycobacteria which require 1 to 12 weeks before exhibiting growth on solid media and the rapid-growing mycobacteria, which require 72 hour to 1 to 2 weeks to grow on solid media. The rapidly growing species of mycobacteria are so named due to their shorter generation time compared to the slowly growing mycobacterial species[1].

Thers is a perception among many clinicians and public health tuberculosis workers that new cases of NTM lung disease may significantly exceed TB cases in their communities or regions. It was observed that non-tuberculous mycobacteria were far more frequently isolated from pulmonary specimens than was *Mycobacterium tuberculosis*[2].

The diagnosis of mycobacterial infection is accomplished by culture-based identification as growth properties and colonial morphology can be used for the preliminary identification. The definitive identification of mycobacteria can be achieved using variety of techniques. Biochemical tests represent the standard method for identifying mycobacteria, but because results are not available for at least 3 weeks or more, most laboratories do not rely on these tests. Mycobacterium species can also be identified through chromatographic analysis of their characteristic cell wall lipids[3].

However, during the past several years, many molecular methods have been developed for direct detection, species identification, and drug susceptibility testing of mycobacteria. These methods can potentially reduce the diagnostic time from weeks to days[4].

Because the recent global resurgence of mycobacterial infections, there is an increasing demand for rapid, sensitive, and specific diagnostic methods for the detection and identification of *Mycobacterium tuberculosis* and non-tuberculous mycobacteria (NTM) in a clinical setting[5]. In addition, mixed infections of *Mycobacterium tuberculosis* and NTM have been reported; therefore, it has become important to be able to differentiate between the two during the early stage of the diagnostic procedure[6].

The aim of this study is to isolate *Mycobacterium* other than tuberculosis (MOTTs) among patients with pulmonary infection and evaluation of the usefulness of polymerase chain reaction (PCR) as a confirmatory test.

METHOD

This is a descriptive cross sectional laboratory based study, which was conducted from December 2009 to May 2010. The study subjects were 171 patients with pulmonary infection who were suspected to have tuberculosis. Clinical data were collected by medical technicians using structured questionnaire after informed consent was taken.

Culture

Two tubes of Lowenstein Jensen medium were inoculated with 20 µl of the sediment that was obtained from the digestion and decontamination of the sputum sample by modified Petroff's method[12]. One of the two tubes contained glycerol while the other tube contained pyruvic acid to isolate the *Mycobacterium bovis* species, if present. All cultures were incubated at 37°C for 8 weeks before being discarded. Growth was monitored daily during

the first week to observe the presence of rapid growers which if present will show growth within 7 days, and then the growth was observed weekly up to the 8th week.

Isolates were primarily identified depending on their growth rate (slow or rapid), pigment production, biochemical tests (catalase, nitrate reduction, sensitivity to para-nitrobenzoic acid (PNB) 500 mg/L and sensitivity to thiophene-2-carboxylic acid hydrazide (TCH) 5 mg/L).

Polymerase Chain Reaction (PCR)

Polymerase chain reaction was performed according to Eisenach et al to confirm the isolation results[8].

DNA was extracted from Cultures by boiling. Two to three loops full of cultured isolates were added to one ml of sterile water into Eppendorf tubes and the suspension was mixed gently and boiled in water bath for 5 minutes, then centrifuged at 12000 rpm for 5 minutes, the supernatant was transferred to new Eppendorf tubes and kept in -20ºC till use.

RESULT

Among the 171 patient, 125 (73.1%) were males, while 46 (26.9%) were females. All the ages were found to be affected with tuberculosis, the highest frequency was among age group 21-50 years 117 (68.4%), followed by age group >50 years 31 (18.1%) while the lowest frequency was age group <20 years 23 (13.5 %). All patients affected with NTM were found in middle ages.

Thirty-seven (21.6%) showed positive result. The growth rate of the isolates ranged between 3 days and 5 weeks. Most of the isolates showed visible growth after 2 weeks. Ten out of 171 isolates were identified as rapid growers of mycobacteria, while the growth rate of 40 isolates ranged from 2 to 5 weeks and they were identified as slow growers and considered belonging to MTC species.

Forty (23.4%) showed MTC colonies, 10 (5.8%) were considered rapidly growing mycobacteria and 121 (70.8%) samples revealed no growth. Colonies were visible within 7 days at primary culture. Some old colonies appear greenish by absorbing malachite green egg medium. The rapidly growing was confirmed by some of their phenotypic and genotypic characteristics.

Smears from rapid growers (10 isolates) were all found positive for acid fast bacilli (AFB).

Six of ten samples that showed rapid growth confirmed as MOTTs by further identification. Selected biochemical test were performed, these test include nitrate reduction, catalase production and growth in thiophene carboxylic acid hydrazide and para nitro benzoic acid, see table 1.

Table 1: Result of Biochemical Tests

NO	Code No	Catalase test at R.T	Catalase test at 68°C	Nitrate	BNP	TCH
1	43E	+ve	-ve	+ve	+ve	+ve
2	43 AK	-ve	-ve	+ve	-ve	+ve
3	44 E	+ve	-ve	-ve	-ve	+ve
4	59	-ve	+ve	-ve	-ve	+ve
5	27	+ve	-ve	-ve	+ve	-ve
6	42	+ve	-ve	-ve	-ve	-ve

The 6 isolates which showed rapid growth confirmed by culture characteristic and biochemical test as MOTTs were subjected to PCR. These isolates showed a band typical in size (136 bp) to the target gene (*rpoB*) as indicated by the standard DNA marker. As shown in Figure 1.

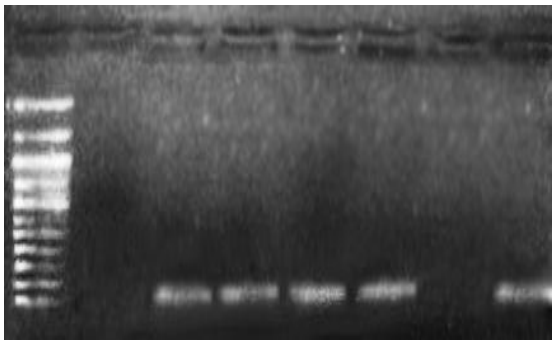

Figure 1: The Amplicon of MOTTs after First Run of PCR on 1% Agarose Gel as Follow: Lane 1=DNA Marker; 2=Control Negative; 3=Control Positive; 1, 2, 3, and 5=136bp Band of NTM; 7= Negative (No Band)

DISCUSSION

In the last few decades, isolation of MOTTs has been increasing, ranging between 30-50% among mycobacterial isolates. Although the clinical significance remains to be continuously evaluated, emerging MOTTs infections are frequently reported. Traditional clinical assessments, acid fast bacilli staining (AFB) and culture methods accompanied with biochemical identifications are not satisfactory for accurate and precise diagnosis.

It was found that the number of TB infected males was significantly higher than females. Four out of 6 patients who were diagnosed as MOTTs were males and 2 were females. Similar results were reported by Elhassan et al, who found that 73.3% were males and 27.7% were females among TB infected patients[9]. Bellamy et al, who suggested that an X chromosome susceptibility gene contributes to high susceptibility of males to TB than females as observed in many different populations[10]. Moreover, according to job type, males are more exposed to the source of infection than females.

It was found that young patient between 21-50 years were the most affected with TB and MOTTs, Kennedy and Weber found that the majority of the affected patients were middle aged[11].

Four out of 6 MOTTs were ZN positive and 2 samples were ZN negative, this finding is similar to the results obtained by Fowler et al who found that in a total of 98 patients were investigated, 10 grew MOTTs on first sputum culture; none were smear-positive[12]. The clinical relevance of this is not clear, but it demonstrates that the mycobacterial load in the current authors' patients was low.

Ten (5.8%) isolates were considered as rapid grower, 6 (3.5%) were identified as MOTTs by conventional method, the finding was similar to the results obtained by Grubek-Jaworska who found that among the 445 smear-positive or/and culture-positive patients, 142 subjects (31.9%) were found to be infected with *Mycobacterium tuberculosis, and 27* (8.9%) were NTM[13].

CONCLUSION

The infection caused by *Mycobacterium* other than tuberculosis is clinically similar to tuberculosis. Therefore, physicians should pay attention to the occurrence of NTM among patient with lung infection in order to describe the suitable therapy.

Author Contribution: All authors share equal effort contribution towards (1) substantial contributions to conception and design, acquisition, analysis and interpretation of data; (2) drafting the article and revising it critically for important intellectual content; and (3) final approval of the manuscript version to be published. Yes

Potential Conflicts of Interest: No

Competing Interest: None, **Sponsorship:** None

Submission date: 05.12.2010 **Acceptance date:** 15 April 2011

Ethical approval: Approved by "The ethical committee of the Federal Ministry of Health" and "The ethical Committee of the research council of College of Medical Laboratory Science"

REFERENCES

1. Kenneth JR, George RC. Sherris Medical Microbiology: An Introduction to Infectious Diseases. 4th ed. New York: McGraw-Hill, 2004; 439-54.
2. Marras TK, Chedore P, Ying AM, et al. Isolation Prevalence of Pulmonary Non-Tuberculous Mycobacteria in Ontario. Thorax 2007; 62(8): 661-6.
3. Murray PR, Rosenthal KS, Pfaller MA. Medical Microbiology. 6th ed. London: Mosby, 2008; 366-77.
4. Soini H, Musser J. Molecular Diagnosis of Mycobacteria. Clin Chem 2001; 47 (5): 5809-814.
5. Bloom BR, Murray CJ. Tuberculosis: Commentary on a Re-Emergent Killer. Science 1992; 257(5073): 1055-64.
6. Libanore M, Bicocchi R, Ghinelli F. Mixed Bronchial Infection Due to Mycobacterium Tuberculosis and *Mycobacterium Avium*-Intracellulare in an AIDS Patient. Infection 1992; 20(5): 298-9.
7. Polansky F, Viznerova A, Hejdova E, et al. Comparison of Laryngeal Smear and a Modified Petroff's Method. Cesk Epidemiol Mikrobiol Immunol 1971; 1:43-7.

8. Eisenach KD, Cave MD, Bates JH, et al. Polymerase Chain Reaction Amplification of a Repetitive DNA Sequence Specific for Mycobacterium Tuberculosis. J Infect Dis 1990; 161(5): 977-81.
9. El Hassan MM, Magzoob E, El Rahman MF, et al. The Occurrence of *Nocardia* Species among Patients with Pulmonary Infection. Bahrain Med Bull 2010; 32(1): 30-3.
10. Bellamy R, Beyers N, McAdam KP, et al. Genetic Susceptibility to Tuberculosis in Africans: A Genome-Wide Scan. Proc Natl Acad Sci USA 2000; 97(14): 8005-9.
11. Kennedy TP, Weber DJ. Nontuberculous Mycobacteria: An Underappreciated Cause of Geriatric Lung Disease. Am J Respir Crit Care Med 1994; 149(6): 1654-8.
12. Fowler SJ, French J, Screaton NJ, et al Nontuberculous Mycobacteria in Bronchiectasis: Prevalence and Patient Characteristics. Eur Respir J 2006; 28(6): 1204-10.
13. Grubek-Jaworska H, Walkiewicz R, Safianowska A, et al. Nontuberculous Mycobacterial Infections among Patients Suspected of Pulmonary Tuberculosis Eur J Clin Microbiol Infect Dis 2009; 28(7): 739-44.