COMPARISON BETWEEN FLUORESCENT MICROSCOPY AND DUPLEX PCR TO DETECT MYCOBACTERIUM BOVIS AND MYCOBACTERIUM TUBERCULOSIS IN TUBERCULOSIS SUSPECTED PATIENTS

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Date Received:
March, 20th 2021
Date Revised:
January, 08th 2022
Date Accepted:
January, 13th 2022

This article may be cited as
Tareen S, Rauf A, Tariq N, Khan MA, Shafeeq M, Tareen P, Fahim A. Comparison between fluorescent microscopy and duplex PCR to detect Mycobacterium Bovis and Mycobacterium Tuberculosis in tuberculosis suspected patients. J Postgrad Med Inst 2021;35(4):230-5. https://doi.org/10.54079/jpmi.35.4.2872.

ABSTRACT

Objective: The aim of this study was to compare the gold-standard fluorescent microscopy as a diagnostic technique with the PCR test, an advance molecular technique.

Methodology: A total of 200 suspected pulmonary and extra-pulmonary samples were taken and stored for analysis in Quetta city. Samples were tested for Mycobacterium tuberculosis and Mycobacterium bovis by both the gold standard fluorescent microscopy (auramine-O FM) and molecular technique duplex PCR. By duplex PCR, Mycobacterium tuberculosis's 245bp sequence and Mycobacterium bovis's 500bp sequence was detected by using specific primers.

Results: Among 200 pulmonary and extra-pulmonary samples, fluorescent microscopy detected 31 positive cases, while PCR detected 60 and 2 positive cases for Mycobacterium tuberculosis and Mycobacterium bovis respectively. The PCR analysis showed 28% of male patients and 32% female patients as positive for M. tuberculosis. Moreover, about52/164 pulmonary samples and 8/36 Extra-pulmonary samples were detected to be positive by PCR analysis.

Conclusion: The PCR results were more accurate, rapid, sensitive and specie specific for detection of tuberculosis showing 60 positive cases for Mycobacterium tuberculosis and 2 positives for Mycobacterium bovis with a significant p-value. On the other hand, FM detected Mycobacterium tuberculosis with comparatively lower sensitivity with only 31 positive cases and had failed to distinguish between species.

Keywords: Fluorescent microscopy; Duplex PCR; Mycobacterium bovis; Mycobacterium tuberculosis.

INTRODUCTION

Since prehistoric times Tuberculosis existed and was observed in Egyptian mummies and Hippocrates described it as "Phthisis" 2000 years ago. In history TB has proved itself as a deadly disease and hence termed as "white plaque" because of the fact that one in seven deaths was due to tuberculosis at the first half of 20th Century.[1] It is a highly contagious and is caused by Mycobacterium tuberculosis Complex (MTB-Complex) and most of the cases are due to the specie Mycobacterium tuberculosis.[2] Mycobacterium tuberculosis is aerobic, non- motile and rod- shaped bacilli. Its cell wall possesses waxy cuticle which makes it highly resistant to many antibiotics, disinfectants and Gram staining. It is termed acid fast due to the fact that conventional staining procedures are unable to stain, hence, heat is required to perforate the cell wall and allow the 5% acid alcohol to stain making it unable to decolorize.[3] TB spreads via infected people coughing, talking or sneezing adjacent to anon-infected person.[4] Extra-pulmonary as well as pulmonary TB can be caused by Mycobacterium tuberculosis specie, whereas Mycobacterium bovis which is known for its zoonotic approach typically causes BTB bovine tuberculosis (BTB). M.bovisis also capable of causing TB of Extra-pulmonary and pulmonary origin. The signs and symptoms of Extra–Pulmonary TB varies and depend upon the organ involved, whereas, that of the Pulmonary TB includes the classical symptoms of TB including blood-tinged or dry sputum, fever, weight- loss, chest congestion and night sweats.[5] Mycobacterium tuberculosis mostly attacks host's lungs but is capable of attacking body parts apart from the respiratory system.[6]

TB is second most deadly disease affecting one-third of the global population.[7] As per WHO estimate in the year 2015, the death rate was 1.4 million and

new infection rate was 10.4 million. In the same year the death toll of HIV co-infected with TB was 0.4 million. Although death rate decreased in the year 2000 to 2015 up to 22% but still TB continues to be amongst the top 10 deadly diseases in the world.[8] About 80% of the global TB cases are reported in 22 countries which labels them as high burden countries (HBCs). If we see globally, Pakistan stands 5th amongst these 22 HBCs.[9] The Pashtun belt consisting of specific areas of Afghanistan, Pakistan and neighboring region of Iran has different TB incidence rate from the rest of the world's statistics with females acquiring infection twice commonly than males.[10]

Diagnosis of TB in initial stages is important in term of controlling disease. In order to detect TB infection, different approaches are used; for instance, the initial diagnostic of symptoms, culturing, sputum smear microscopy, tuberculin skin test (TST) and histopathology.[11] Culture method is considered as gold standard for detection. However, this procedure is time consuming and slow as mycobacterium takes six to eight weeks to multiply. In comparison, microscopic method is rapid and low cost but provides low accuracy and specificity for the detection of dead and alive bacteria.[12] The aim of this study was to compare the gold-standard microscopy technique with that of the PCR for the incidence as well as prevalence of Bovine tuberculosis.

METHODOLOGY

In the present prospective study, 200 sputum samples of susceptible patients visiting the Out Patient Department of Fatima Jinnah General and Chest Hospital (FJGCH) with chronic signs of cough, night sweat, fever, weight loss as well as appetite were collected for the study. The sputum samples were stored in wide-open sterile bottles and packed in clean plastic bags & labeled with differentiated IDs. The patients who were on medication were eliminated from research. Before sampling patients were aware of research and approval from ethical review was taken from Sardar Bahadur Khan Women's University board. The initial steps like sample collection, staining (auramine-O), microscopy (fluorescent) heat killing and DNA extraction steps were performed in FJGC and other steps (PCR and Gel Electrophoresis) were performed in Center for Advanced Studies in Vaccinology and Biotechnology (CASVAB), University of Baluchistan, Quetta.

The gold Standard microscopy technique was used to process sample along with PCR diagnosis method. Proper lab safety protocols were followed since suspected sputum sample may contain TB aerosols and can result in infection of tuberculosis by inhaling. Sample were processed using N-acetyl-L-cysteine (NALC), Sodium hydroxide (NaOH) solutions to eliminate contamination & collect TB cells from sample by centrifugation using CDC standard recommended procedure.

Two sets of primers were used in duplex PCR. First sets of primers (INS1: 5'-CGTGAGGGCATCGAGGTGGC-3' and INS2:5'-GCGTAGGCGTCGGTGACAAA-3') were used to detect Mycobacterium tuberculosis and 2nd sets of primers (JB21: TCGTCCGCTGATGCAAGTGC andJB22: CGTCCGCTGACCTCAAGAAAG) were used for Mycobacterium bovis[13,14]. All 200 samples for MTB with 245 bp sequence were amplified using automated applied biosystem thermo-cycler with primer INS1 and INS2. For PCR, total 25µlmixture was prepared in safety cabinet to avoid any type of contamination that hinder PCR process. Likewise, 200 samples were run with primers JB21 and JB 22 for extension of Mycobacterium bovis500bp sequence. Reaction mixtures of volume25µl was prepared with positive and negative controls used in the reaction. The fluorescent microscopy was performed using the Kent & Kubica (1985) recommended protocol for preparing auramine-O stain slides and its analysis.

RESULTS

In the present study, among 200 total patients, 99 (49.5%) were females and 101(50.2%) were male patients. The patients with age ranging between 1 to 80 years old were included. Among them Mycobacterium tuberculosis susceptibility was noted higher in age range of 41-60 years that is 38%and lowest among aged 1-20 years (14%). Other frequencies recorded were24.5% in 61-80 years old and 23.5% in 21-40 aged patients.

Among 200 pulmonary (sputum and BAL) and extra-pulmonary (ascitic fluid, CSF, gastric lavage, pleural fluid, puss and tissue) samples were collected as given in Figure No.1. Fluorescent microscopy detected 31 positives among 200 but could not be differentiated on specie level and no case of Mycobacterium bovis were detected on FM (Figure No. 2). On the other hand, PCR detected 60 positives for Mycobacterium tuberculosis and 2 positives for Mycobacterium bovis, one each of male and female patients with a significant p-value of 0.001(Figure No. 3& Table No. 1).

Out of 101 male patients, 28(27.7%) patients were positive for M. tuberculosis, whereas among 99 female patients, 32(32.3%) patients were positive for M. tuberculosis on PCR. Analysis clearly showed that frequency of female patients (32.3%) to male patients (27.7%) was visibly high and P-value of 0.471 was noted that is non-significant. Pulmonary and extra-pulmonary samples were collected in which 52/164 pulmonary samples were PCR positive and other 112 were negative. Whereas about 8/36 extra-pulmonary samples were detected PCR positive and other 28 were found negative. The P-value is 0.261 which is non-significant as given in Table No. 2.

Table 1: Comparison of Fluorescent Microscopy Versus PCR Showing Prevalence of Both Species; Mycobacterium Tuberculosis and Mycobacterium Bovis

Technique	Mycobacterium tuberculosis		Mycobacterium bovis		X^2	P-value
	Positive	Negative	Positive	Negative		
FM	31	169	0	0	82.353	0.001
PCR	60	140	2	198		

Table 2: Distribution of Pulmonary and Extra Pulmonary Samples on PCR

Sample Type	PCR		X^2	P-value
	Positive	Negative		
Pulmonary	52(32%)	112(68%)	1.265	0.261
Extra Pulmonary	8(22.2%)	28(77.8%)		

Table 3: Age-Wise Prevalence of Mycobacterium Tuberculosis on PCR.

Age Groups	PCR		X^2	P. Value
	Negative	Positive		
1 – 20	19 (67.9%)	9(32.1%)	0.990	0.112
21 – 40	33(70.2%)	14(29.8%)		
41 – 60	53(69.7%)	23(30.3%)		
61 – 80	35(71.4%)	14(28.6%)		

The prevalence of Mycobacterium tuberculosis was found different in various age groups, but results indicated that age group 1-20 years was the most affected with 32% cases positive on PCR (Table No. 3) with a P-value 0.990, which is non-significant.

DISCUSSION

The effective and timely management of tuberculosis as well as its prevention can only be achieved through the use of new approaches to detect the presence of Mycobacterium in sputum samples. Thus, the scientists have targeted the mycobacterial DNA identification through real-time PCR in the specimens being studied[15]. Beside this, the routine conventional methods for detection of mycobacterial species depends on microscopic examination of the smears along with culture but have drawback of lack of sensitivity and required prolonged duration[16].

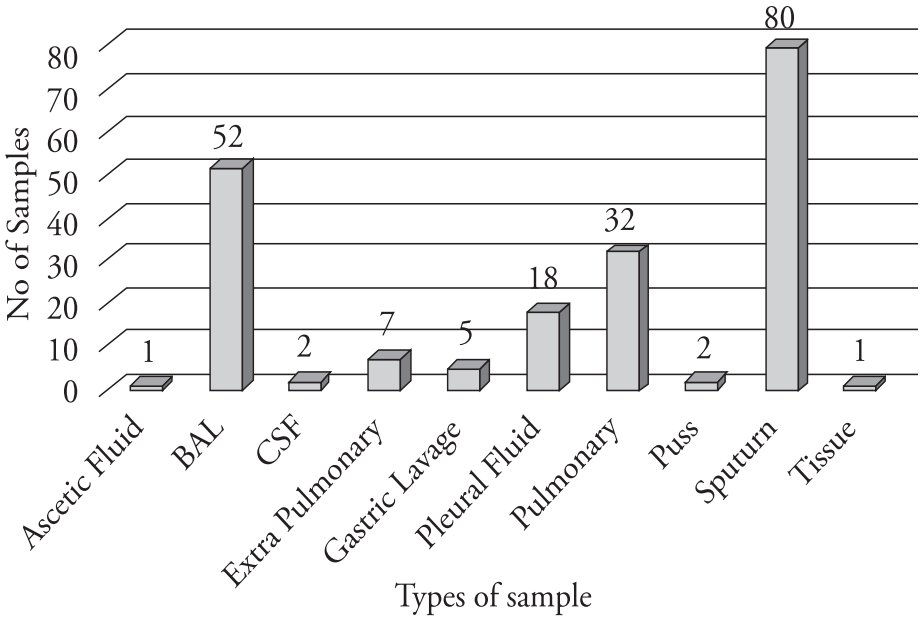

Figure 1: Graph Showing the Percentage of Different Sample Types of the Suspected Tb Patients

Because of low sensitivity of microscopy technique, Pakistan does not practice this technique in proper diagnosis. Because, if patient is not diagnosed on time then infection may spread to surrounding population and can affect other healthy individuals. To lower this risk, rapid, specific and sensitive techniques are mandatory in laboratories. This study aims for detection of rapid, accurate, specie-specific (like BTB) technique. Molecular techniques make specie-specific identification possible. Larger number of smear negative cases creates doubts and can be resolved by steadfast molecular technique like PCR.

First line drugs for treating MTB are not effective on Bovine TB. The present study

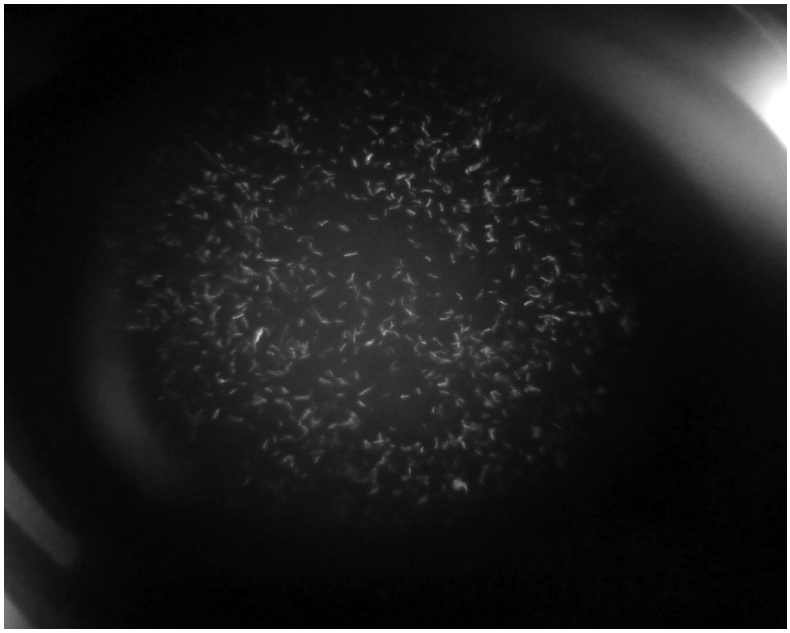

Figure 2: Fluorescence Microscopy of Afb Via Auramine-O Dye.
Fluorescent smear microscopy: The specimen on microscopic slide stained with auramine-O fluorochrome dye and visualized under dark field microscope with 40X objective showing positively for Mycobacterium tuberculosis AFB and graded as 3+ positive results.

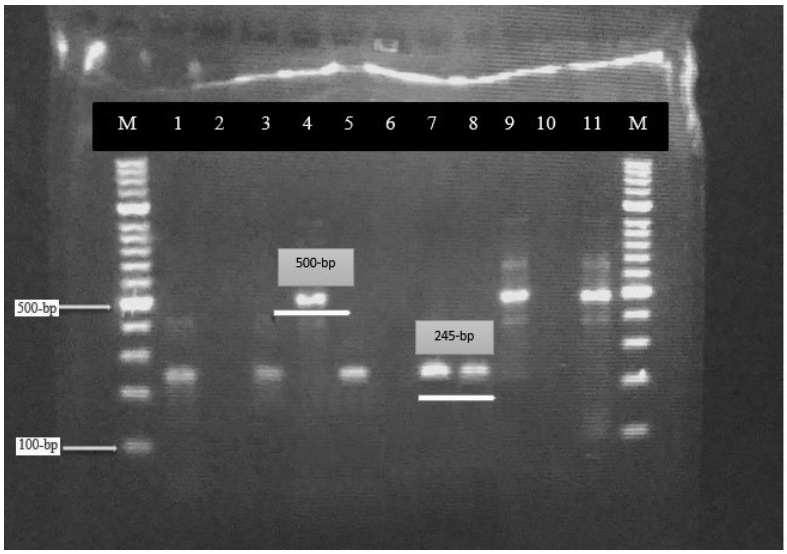

Figure 3: PCR Analysis for Mycobacterium Tuberculosis (245bp) and Mycobacterium Bovis (500bp) Detection.
Lanes M: 1Kb marker, Lane 1, 11: Positive control (245bp left and 500bp right), Lane 2, 10: Negative control(500bp right and 245bp left); Lane 4 and 9 positive for Mycobacterium bovis; Lane3,5,7 and 8 positive for Mycobacterium tuberculosis and Lane6 negative for Mycobacterium tuberculosis amplification.

aims for rapid identification M. Bovisin order for the cure to be possible timely. The results revealed that the infection rate of M. Bovis is 1% in humans in pulmonary samples as only two such cases (one male and a female patients) were seen in the present study.

In this study, the results of microscopy (florescence) and molecular technique (PCR) were analyzed. Its how's clear difference between these techniques, where the analysis via PCR showed30% positive and FM showed15.5% positives. Other studies also show the better compatibility of PCR results over microscopy[17,18]. PCR detection of MTB and M. Bovis is based on specific sequence extension based on primers INS and JB respectively. The highly significant statistical difference between FM and PCR was noticed ($P<0.005$).

According to Mostaza et al. (2007), 17% smear negative cases of TB were transmitted and showed great human heath damage, and 14.5% negative microscopy results were later confirmed positive when run via PCR[19]. In 2007, Oberoi and Aggarwal observed 60% negative microscopy results[20] due to which the control of TB had been a challenge, and much accurate methods had to be adopted.

The present study also indicated the prevalence of TB disease in females (32.3%) in comparison to males (27.7%). Similarly, few of the other studies which were conducted in Pakistan have shown similar findings[21,22]. This may be due to inadequate access of health facilities available to females and their low acquaintance to outdoor. Results are opposite in Russia, where TB prevalence is high in males as compared to females[23,24]. The reasons behind might be due to high smoking rate and frequent male gatherings compared to females.

The prevalence of tuberculosis was also observed in distinguished age groups and

high prevalence was noted in age group 1-20 i.e. 32.1% and secondly 41 to 60 years age group i.e. 30.3%. That might be due to lower preventive measures adopted by younger population or the exposure is frequent to infectious persons. The high risk of TB prevalence in comparatively high age groups (i.e.,20-40 years) is reported in other studies[25,26]. This factor affects economic development where TB is endemic, and it may upsurge due to crowding, malnourishment, family histories and poor socio- economic status.

CONCLUSION

The PCR results were more accurate, rapid, sensitive and specie specific for detection of tuberculosis with 60 positive cases to Mycobacterium tuberculosis and 2 positive to Mycobacterium bovis with a significant P-value. On the other hand, FM detected Mycobacterium tuberculosis with comparatively lower sensitivity with only 31 positive cases and failed to distinguish between species.

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Author's Contribution

ST conception and design of study, statistical analysis & interpretation of data. AR data acquisition and manuscript writing and editing. NT conception and design of study, statistical analysis & interpretation of data. MAK & MS drafting and critical revision of manuscript and final approval of manuscript. Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interest

Authors declared no conflict of interest

Grant Support and Financial Disclosure

None

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.