MOLECULAR DIAGNOSIS OF MYCOBACTERIUM TUBERCULOSIS IN PATIENT FROM SPUTUM BY MULTIPLEX PCR IN KARBALA CITY

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ABSTRACT:
The initial differentiation of mycobacteria into sputum is very important. This study was designed to evaluate the usefulness of the newly developed duplex Polymerase chain reaction (PCR) for the hsp65 gene and MPT64-based method in differentiating sputum mycobacteria with fast positive acid bacilli. (AFB) Smear before culture. 50 patient with positive AFB smears were included for analysis. Newly generated Multiplex PCR for the hsp65 gene and MPT64 gene were determined.

The sputum samples used in this study were performed between January 2020 and till the end of July 2020 from patient who admitted to Tuberculosis Health Centers in the clinical and laboratory examinations in kerbala city, the number of sputum was taken from 90 patients who were diagnosed with tuberculosis. First of all, decontamination process was applied to the samples in order to digest the organic residues and to eliminate the bacteria and fungi that cause contamination, the presence of (50) cases of infection that were isolated from clinical cases (12 ) with a percentage of (41%) of the total auditors during the study period extending from January 2020 to Julay 2020, from both sexes and from different groups.

In conclusion : this technique is much faster than the usual method culture uses solid media due to the fact that an algorithm for mycobacteri species identification based on PCR limitations analysis targeting the hsp65 and MPT64 gene. identification method

Keywords: Theileriosis and Babesiosis, Camelus dromedaries, PCR

I. INTRODUCTION:
Tuberculosis is a chronic granulomatous disease caused by Mycobacterium spp. The tubercle bacilli are M. tuberculosis, the agent of the disease in primates, M. bovis in other mammals, and M. avium in birds. Host specificity is relative (1).

Of the 40-odd other Mycobacterium spp., some ("non-tuberculous," "atypical," "anonymous," or saprophytic) cause tuberculosis-like infections. Mycobacterium microti causes tuberculosis of voles; M. leprae causes human leprosy; and M. avium ssp. paratuberculosis causes Johne's disease of ruminants (2).

Mycobacterial cells abound in lipids, especially in their walls. Lipids account for acid fastness and pathogenic and immunologic properties (3).

The surface mycosides (mostly glycolipids and pepti-doglycolipids) determine colonial characteristics, serologic specificities, and bacteriophage susceptibilities. They are considered instrumental in ensuring bacterial survival within macrophages (4).

Subsurface layers of long-chain branched mycolic acids and their esters make up the bulk of cell wall lipids. Acid fastness somehow depends on these cell wall constituents. Mycolic acids are and minerals and vitamins are unsuitable for isolation but support growth from large inocula (5).

Tubercle bacilli are strict aerobes that grow best on complex organic media such as Lowenstein-Jensen's, which contains, among other ingredients, whole eggs and potato flour (6).Generation times of tubercle bacilli range from 12 hours upward, and it may take weeks before colonies are visible (7).
In this study, it was aimed to determine the presence of Mycobacterium tuberculosis complex in the sputum samples of patients diagnosed with pulmonary tuberculosis by Multiplex PCR by targeting the IS6110 insertion gene region, in Tuberculosis Chest Centers in kerbala city.

II. MATERIALS AND METHODS

Decontamination with staining of sputum

The sputum samples used in this study were performed between January 2020 and till the end of July 2020 from patients who admitted to Tuberculosis Health Centers in the clinical and laboratory examinations in kerbala city.

The number of sputum was taken from 90 patients who were diagnosed with tuberculosis. First of all, decontamination process was applied to the samples in order to digest the organic residues and to eliminate the bacteria and fungi that cause contamination.

For this procedure, N-acetyl-L-cysteine (NALC-Mucolytic) and sodium hydroxide (NaOH) method was preferred (8). Specimens were smeared on the slide and stained with the EhrlichZiehl-Neelsen carbolfuchsin method. The slide was kept at room temperature and air dried. The stained smear preparation was examined under a light microscope, and acid-alcohol-resistant bacilli were observed (9).

DNA Extraction and Multiplex PCR assay

DNA extraction of decontaminated sputum samples was performed with a commercial kit (Wizard Genomic DNA Purification kit, Promega). Briefly, 10 mg/ml lysozyme enzyme and 50 mM EDTA were added to the pellets after centrifugation, and the samples were incubated at 37°C for 2 hours with agitator.

The nucleic lysis solution in the commercial kit was added to the mixture and the mixture was kept at 80°C for 10 minutes. The RNAase enzyme, which is also in the kit, was added to the mixture and the mixture was left at 37°C for 30 minutes. Then, protein precipitation solution was added to the mixture and the mixture was kept in ice water for 10 minutes. Afterwards, the supernatant was transferred to a new microcentrifuge tube and an equal volume of isopropranolol was added.

The tubes were kept at room temperature for 1 night for precipitation. Afterwards, DNA pellets were dried by washing with 70% ethyl alcohol.

Pellet DNAs were diluted with 40 µl sterile distilled water, these samples were stored at -80°C until used in Multiplex PCR.

It has been shown that Multiplex PCR is effective in identifying M. tuberculosis complex species from many different clinical samples. Therefore, Multiplex PCR is performed with the obtained DNA using two gene MPT64 (10) and hsp65 (11) region gene.

Oligonucleotides sequences were obtained from company (Macrogen company, Korea) as table (1):

<table>
<thead>
<tr>
<th>oligonucleotided primers</th>
<th>Sequence</th>
<th>PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPT64.</td>
<td>F 5-ACCGAACACTCATTTCCGC-3</td>
<td>771 bp</td>
</tr>
<tr>
<td></td>
<td>R 5-CTACTCCCCGGAGGAATTTCG-3</td>
<td></td>
</tr>
<tr>
<td>Hsp65</td>
<td>F 5-TCGTCGAGAAGGTACATCGGA-3</td>
<td>195 bp</td>
</tr>
<tr>
<td></td>
<td>R 5-TCACTGCAGCACCACGGTGAGA-3</td>
<td></td>
</tr>
</tbody>
</table>

PCR master mix preparation

Mixture containing Taq DNA polymerase (Promega/USA) enzyme was prepared, 40 µl of PCR mix and 7 µl of sample DNA were placed in microcentrifuge tubes one primer (5 picomole) was added to mixture then complete the final size by ddH2O. The samples were then run as 1 cycle of preheating at 94°C for 5 min, and 45 cycles.
consist of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min, and elongation at 72 °C for 1 min. PCR with the final extension at 72 °C for 10 min.

The resulting PCR products were electrophoresed on a 1.5 % agarose gel containing 0.5 g/ml ethidium bromide. After electrophoresis, the gels were examined with ultraviolet rays in the imaging system. The statistical relationship between the results of Multiplex-PCR experiments performed in direct sputum was determined using the chi-square ($\chi^2$) test.

### III. RESULTS AND DISCUSSION:

#### Incidence rate with ages using clinical examination

The current study showed, as shown in Table (2), the presence of (50) cases of infection that were isolated from clinical cases (12) with a percentage of (41%) of the total auditors during the study period extending from January 2020 to July 2020, from both sexes and from different groups, all of these cases are confirmed infections by specialized doctors through clinical symptoms and stages of disease development that occurred to the injured during the periodic follow-up during the treatment period, and (70 samples) were collected for healthy people at a rate of (52%), there was no significant differences in the ages of subjects.

<table>
<thead>
<tr>
<th>Patient</th>
<th>N</th>
<th>Mean</th>
<th>S. D</th>
<th>S. E</th>
<th>P. V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Health</td>
<td>70</td>
<td>42.04</td>
<td>17.57</td>
<td>2.10</td>
<td></td>
</tr>
</tbody>
</table>

#### Gender Relationship with the Sample Environment (Urban, Rural)

The results of our study indicated in Table (3) the effect of the type of environment (urban, rural) on gender, and the results showed that there were no significant differences ($P>0.05$) between the injured males of urban and rural residents, nor between females of urban and rural residents.

<table>
<thead>
<tr>
<th>البيئة</th>
<th>العدد والنسبة</th>
<th>الجنس</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Rural</td>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Vertical %</td>
<td>57.1%</td>
<td>42.9%</td>
</tr>
<tr>
<td></td>
<td>Horizontal %</td>
<td>23.5%</td>
<td>37.5%</td>
</tr>
<tr>
<td>Urban</td>
<td>26</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Vertical %</td>
<td>72.2%</td>
<td>27.8%</td>
</tr>
<tr>
<td></td>
<td>Horizontal %</td>
<td>76.5%</td>
<td>62.5%</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Vertical %</td>
<td>68.0%</td>
<td>32.0%</td>
</tr>
<tr>
<td></td>
<td>Horizontal %</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
</tbody>
</table>

#### Acid fast staining smear microscopy

The results of Table (4) showed the relationship of gender with categories +1 (from 10-99 sticks / 100 microscopic fields), +2 (from 1-10 sticks / microscopic field) and +3 (more than 10 sticks / microscopic field). There were no significant differences between category +1 for males and females, while category +2 recorded a significant increase in males with 12 samples and a rate of 66.7% compared with females with 6 samples at a rate of 33.3%, while category +3 recorded a significant increase in males with 12 samples and a percentage of 100 % compared with the females who recorded zero samples at 0%. This result was agreement with (13).

<table>
<thead>
<tr>
<th>شكل</th>
<th>العدد والنسبة</th>
<th>AFS smear microscopy</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 +</td>
<td>2 +</td>
<td>3 +</td>
</tr>
</tbody>
</table>

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Tuberculosis (TB) is increasing worldwide and is one of the leading causes of death among adults in developing countries. In 1993, the World Health Organization declared tuberculosis a "global health emergency" (14). In Indonesia, tuberculosis is a major public health problem with a prevalence of 0.29 and 5.6 of the 7.5 million new cases of tuberculosis worldwide in 1990. Pulmonary tuberculosis, a chronic infectious disease caused by Mycobacterium tuberculosis, is characterized by prolonged cough, haemoptysis, chest pain and shortness of breath. Systemic manifestations of the disease include fever, weakness, loss of appetite, weight loss, weakness, and night sweats (15).

The two genes were detected using Multiplex PCR assays, As shown in the figure (1).

It is recently reported a method based on Multiplex PCR to identification of mycobacteria tuberculosis amplification of the hsp65 gene and MPT64. sequence (12). Through this study, it was tried to distinguish mycobacteria from the collected future sputum amplification by Multiplex PCR. Our data show that duplex PCR for the hsp65 gene was followed by a direct overlapping sequence this method is promising for the identification of mycobacteria species sputum shows a positive AFB smear. despite this how to identify tuberculosis in lower numbers In diagnosis, it was comparable or better in comparison Non mycobacterium tuberculosis regardless of collection time or presence tuberculosis during or after anti-tuberculosis treatment (16).

In addition, this method is much faster than the usual method culture uses solid media due to the fact that previously introduced an algorithm for mycobacteri species identification based on PCR limitations analysis targeting the hsp65 and MPT64 gene (17).
REFERENCE: