Detection of Drug Resistant Genes of *Mycobacterium tuberculosis* in Sudanese Tuberculosis Patients in Khartoum State Using Multiplex PCR

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Abstract

Tuberculosis is a common contagious disease caused by *M. tuberculosis*. With an estimated 9 million new cases and 2 million deaths every year, tuberculosis represents one of the most serious infectious diseases worldwide. The increased death rate is a result of emergence of new strains of *M. tuberculosis* resistant to some or all current anti-tubercular drugs. The resistance is attributed primarily to improper prescriptions or patient noncompliance. In this study we aimed to detect the frequency of drug resistance genes (**rpoB**, **katG** and **pncA**) in Sudanese tuberculosis patients. Seventy sputum samples were collected from Omdurman Teaching Hospital (Abu Anga) in Khartoum state, Sudan. Sputum samples were disinfected and then DNA was extracted. Multiplex PCR was used to detect drug resistance genes (**rpoB**, **katG** and **pncA**). IS6110 gene was used to confirm the presence of *M. tuberculosis* in the tested samples. Fifty six sputum samples out of 70 were positive for the presence of drug resistance genes, drug resistance genes were detected in 22 (41.3%) for rifampicin, 29 (41.4%) for isoniazid and 33 (47.1%) for Pyrazinamide, also 16 (28.6%) samples had mono drug resistance, 29 (51.8%) had multi drug resistance and 11 (19.6%) had poly drug resistance.

The ability of *M. tuberculosis* for the development of drug resistant gene were found to be associated with many factors, however in this study hypertension and diabetes mellitus were of no significant in the contribution of developing drug resistance P. Value > 0.574. This study provides the first data about Pyrazinamide, isoniazid and rifampicin resistance in Sudan using multiplex PCR. Also representing that multiplex PCR assay use is a rapid, reliable tool and easy to perform for the routine detection of resistant *M. tuberculosis* strains in TB positive patients in Sudan.

Keywords: Mycobacterium tuberculosis; Rifampicin; Isoniazid; Pyrazinamide; Multidrug Resistance

Introduction

Tuberculosis (TB) is a common highly contagious diseases caused by *Mycobacterium tuberculosis* [1]. TB have signs and symptoms including cough, chest pain, hemoptysis and weight loss [2], it is also considered one of most the most challenging disease facing the world now a days; due to continues drug resistance and the lack of available new drug to control the disease [3]. Tuberculosis that infects lungs...
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is called pulmonary TB and that infect bones, lymphoid and genitourinary tract is called extra pulmonary TB [1]. The transmission of TB is mainly through droplets containing bacilli. The infectious dose of *M. tuberculosis* is less than 10 bacteria [4]. World health organization (WHO) has estimated that about one third of world population is infected by *M. tuberculosis* [5]. With an estimated 9 million new cases and 2 million deaths every year, TB represents one of the most serious infectious diseases worldwide [6]. The estimated 8.8 million new cases every year correspond to 52,000 deaths per week or more than 7,000 each day, which translates into more than 1,000 new cases every hour, every day [7,8]. Further contributing to the increased death rate is the emergence of new strains of *M. tuberculosis* resistant to some or all current anti-tubercular drugs [9]. The resistance is attributed primarily to improper prescriptions or patient noncompliance and is often a corollary to HIV infection [10-12].

In most developing countries such as Sudan, although the disease has always been endemic, its severity has increased because of the global HIV pandemic and extensive social restructuring due to rapid industrialization and conflicts [13]. In Sudan there is a high burden of tuberculosis (TB) with a prevalence of 209 cases per 100,000 of the population and 50,000 incident cases during 2009 [6]. Tuberculosis care and treatment is provided by the National Tuberculosis Control Program under the auspices of the Ministry of Health and by a number of non-governmental organizations (NGOs) who provide care to displaced persons, including those living in refugee camps [14]. Treatment is also provided by the private sector [15]. As previously reported MDR-TB was also associated with the geographic region of origin of the patient, being most frequently observed in patients from the Northern regions and least in the Eastern regions of Sudan [16].

In most countries, multidrug resistant *M. tuberculosis* (MDR-TB) has increased in incidence and interferes with TB control programs, particularly in developing countries, where prevalence rates are as high as 48% [17-19]. In the past few years, genetic and molecular insights have unraveled the mechanisms involved in the acquisition of drug resistance genes by *M. tuberculosis*, concomitant with the development of various molecular strategies to rapidly detect MDR-TB [20]. Nucleic acid diagnostic techniques, in particular, PCR and multiplex PCR, offered rapid, reliable, and highly sensitive alternative tools for the detection of *M. tuberculosis* [21,22]. The identification of *M. tuberculosis* species by 16S rRNA gene sequence analysis or oligo-hybridization and strain typing as well as detection of drug susceptibility has advanced [23]. The impact of molecular techniques in TB diagnosis provided benefits to the patient in challenging the disease [24].

Mutations in the genome of *M. tuberculosis* that can confer resistance to anti-tuberculous drugs occur spontaneously, with an estimated frequency of $3.5 \times 10^{-4}$ for isoniazid and $3.1 \times 10^{-8}$ for rifampicin [25]. Rapid determination of the antimicrobial susceptibility pattern in clinical isolates of *M. tuberculosis* is important for the early administration of appropriate therapeutic agents for the prevention of additional resistance development [26]. In this context, molecular characterization of drug resistance by identifying mutations in associated genes will be applicable for developing a potential rapid molecular drug susceptibility test as an alternative to conventional methods [27,28].

The main purpose of the study present was to determine the frequency of drugs resistance in TB patients in Sudan and to establish the Multiplex PCR for the detection of *M. tuberculosis* drug resistance genes.

**Materials and Methods**

**Ethical considerations**

The study was approved by the Ethical Review Committee (ERC) of El-Zaaim El-Azahari University; Informed consents were obtained from adult patients, or from parents or legal guardians of children.

**Study area and study periods**

This study was conducted on tuberculosis patients admitted to Omdurman Teaching Hospital (Abu Anga) in Khartoum, Sudan, during the period of February to July 2015, Abu Anga reference hospital for treatment of TB and drugs resistance TB patients.

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**Target population and sample size**

This study was carried out to detect incidence of drug resistance genes among TB patients between 10 to 80 years old. Seventy TB patients who were diagnosed as drug resistant using traditional susceptibility test and were still specific smear positive after two month of treatment were enrolled in this study. The main symptoms in their patients included fever, cough, chest pain, loss of weight, night sweating and hemoptysis.

Participants data included age, gender, geographical origin, underline disease conditions, type of infection and reason of resistance, were collected using a structured questionnaire.

**Sample collection**

Sputum samples were collected from the 70 smear-positive patients in wide, clean, dry sputum container and stored at 4°C until used.

**Digestion and decontamination of sputum samples**

This is was carried out to clean the samples before TB bacillus DNA extraction, sputum samples were digested in 10 ml falcon tube containing 1 gram of N-acetyl cysteine (NALC), 2.9% sodium citrate and 4% Sodium hydroxide (1:1) ratio for 15 minutes. The reaction stopped by addition of a phosphate buffer to a final volume of 10 ml, and then samples were centrifuged at 3500 rpm for 15 minutes. The supernatant was discarded and the pellet was re-suspended in 2 ml phosphate buffer. Samples were heated at 95°C for 30 minute in water bath to kill bacteria and then used for DNA extraction [29].

**DNA Extraction**

The DNA was extracted from the pellet suspension using guanidine hydrochloride method [30]. 2 ml cell lysis buffer, 1ml guanidine hydrochloride (6M Wt 57.32 in 100 ml, Sigma Aldrich-USA), 20 µl proteinase K (Sigma Aldrich-USA), 300µl ammonium acetate (Sigma Aldrich-USA) was added to the sputum samples, then incubated at 37°C overnight, after that 2ml chloroform were added and centrifuged at 3000 rpm for 15 minutes. Then the supernatant was transferred into a new tube and then 10 ml cold absolute ethanol were added and incubated at -20°C overnight. The next day, samples were centrifuged at 3000 rpm for 15 minutes and the supernatant was discharged. Four ml of 70% ethanol were added and centrifuged at 3000 rpm for 15 minutes. The supernatant was discharged and the pellet allowed to dry for up to 2 hours then re-suspended in 200 µl of H2O and stored at -20°C until use.

**Multiplex Polymerase chain reaction (Multiplex- PCR)**

The Multiplex PCR performed by processing the extracted DNA from sputum using primers specific for the different resistance genes of *M. tuberculosis* [31]. PCR primers designed for each target region (Table 1). These primers were designed for the IS6110, rpoB, katG and pncA genes. The reaction was performed in 25 µl total, that included: 5 µl master mix (iNtron iTaq, Korea), 2 µl of mixed primers, 5 µl extracted DNA and 13 µl distilled water. The DNA amplified in thermo-cycling conditions using PCR machine (Techno-Japan) follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 5s and extension at 72°C for 60 seconds, with a final extension at 72°C for 5 minutes.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Drug name</th>
<th>Primer sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rpoB</td>
<td>rifampicin</td>
<td>F:5’-CGAGGTGCGGCTGGAAAAC-3’</td>
<td>721</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:5’-GTCGTCGTGCCTCCAGGAAGG-3’</td>
<td></td>
</tr>
<tr>
<td>PncA</td>
<td>Pyrazinamide</td>
<td>F:5’-GACGTATGGGCGCGTGTTGA-3’</td>
<td>498</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:5’-CCATCAGAGGCTGCAAACC-3’</td>
<td></td>
</tr>
<tr>
<td>KatG</td>
<td>isoniazid</td>
<td>F:5’-GAGCCCGATGAGCTTCATTTG-3’</td>
<td>569</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:5’-GTCCTGGCGGCTGGTTAGC-3’</td>
<td></td>
</tr>
<tr>
<td>IS6110</td>
<td>Mtb</td>
<td>F:5’-GGATCTCTGCGGACGGTAGGCGTGG-3’</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:5’-CCTGTCCGGGACCCACCCCGGCAA-3’</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1:** List of primers used in multiplex PCR.

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Five micro liters of the amplified product was subjected then to direct analysis by gel electrophoresis in 1.5% agarose. The product visualized by staining with 0.15% Ethidium bromide using UV gel documentation system (Biometra, GmbH-Germany), the PCR amplicons sizes estimated by comparison to 100bp molecular size marker.

**Statistical analysis**

Generated data was analyzed using the statistical packages for social science (SPSS) version 21.

**Results and Discussion**

This study aimed to assess and determine the presence of drug resistance genes in *M. tuberculosis* patients in Sudan. The multiplex PCR was able to detect the drug resistance genes to rifampicin, isoniazid and Pyrazinamide. The frequency of *M. tuberculosis* drug resistance genes detected were as follow; rifampicin resistance 22 (41.3%), isoniazid resistance 29 (41.4%) and Pyrazinamide 33 (47.1%) (Table 2), these results were lower than previously reported in Sudan [32] where rifampicin resistance were 57.1% and isoniazid 64.4%, and also in Iran [33] were 83.3% were rifampicin resistant and 60.0% were isoniazid resistant. Another study conducted by Akos., *et al.* (2006) [34] showed 96.2% resistance rate for rifampicin and 84.2% for INH resistance, while in china [35] detected resistance rate 92% for rifampicin and 100% for isoniazid, and 97.4% for rifampicin, 60.6% for isoniazid and 75.0% for Pyrazinamide in USA [31]. These results are indicating the decrease in TB resistance against anti-tuberculous drugs, whereas when compared to previous studies in other regions in Sudan our results were higher than those done by Hassan., *et al.* (2012) in northeastern Sudan [36] were rifampicin 8% and isoniazid 13% and in Mexico [37], which 20% were rifampicin-resistant and 40% were isoniazid-resistant.

<table>
<thead>
<tr>
<th>Gender</th>
<th>katG</th>
<th>pncA</th>
<th>rpoB/katG</th>
<th>rpoB/pncA</th>
<th>katG/pncA</th>
<th>rpoB/katG/pncA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>female</td>
<td>5</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 20</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>21 - 40</td>
<td>6</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 40</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>3</td>
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<tr>
<td>Area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>East Sudan</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>North Sudan</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>West Sudan</td>
<td>5</td>
<td>1</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>South Sudan</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

*Table 2: Prevalence of resistance within demographic subgroups gender, age and area.*

Our results concerning the type of resistance whether for a single type of anti-tuberculous drug i.e. mono resistance 16 (28.6%), and for more than two types of anti-tuberculous drug i.e. multi drug resistance 29 (51.8%) are comparable with previous study done in Northeastern Sudan [32,36]. Other type of drug resistant was those who showed a resistant against two types of anti-tuberculous drug which is known as poly drug resistance 11 (19.6%) (Table 3).
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<table>
<thead>
<tr>
<th>Type of Resistance</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono resistance</td>
<td>16</td>
<td>28.6</td>
</tr>
<tr>
<td>Poly resistance</td>
<td>11</td>
<td>19.6</td>
</tr>
<tr>
<td>Multidrug resistance</td>
<td>29</td>
<td>51.8</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>56</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

*Table 3: Resistance among population.*

In this study we observed no statistical association between drug resistance and other factors that might be as factors for the development of this resistance including hypertension and diabetes mellitus. P. Value > 0.574, also we find hypertension and diabetes mellitus has partial relation with some of resistance to specific drug like Pyrazinamide and some find combine with isoniazid resistance which might be due to genetic association or other factors [38] (Table 4). However, drugs resistance in male was higher than in female, in this context this may be due to nature of working or physiological differences [39]. Moreover, resistance was higher in western Sudan rather than in other States in Sudan based on the different geographical region of patients, this might be due to the varied degree of awareness of people against tuberculosis or may be due to the availability of specialized centers for the diagnosis and treatment of tuberculosis in that certain region [37].

<table>
<thead>
<tr>
<th><em>rpoB</em></th>
<th>Genotypic Results</th>
<th>katG</th>
<th>pncA</th>
<th>rpoB/katG</th>
<th>rpoB/pncA</th>
<th>katG/pncA</th>
<th>rpoB/katG/pncA</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of previous disease</td>
<td>Diabetes</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>No disease</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Type of case</td>
<td>Primary</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Secondary</td>
<td>7</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Response of resistance</td>
<td>Defaulter</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Failure</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
</tbody>
</table>

*Table 4: Resistance among the sub group history of previous infection, type of cause and reason of resistance.*

In addition, we also observed that most of the cases were primary TB patients, this reveals that resistance to drugs by *M. tuberculosis* are being continuously transferred through certain strains rather than non-resistance strains [40] (Table 4).

Based on the obtained results, we found out that if we were to meet the goal of controlling the spread of drug-resistant tuberculosis, the time frame of many weeks required for detection, identification, and drug susceptibility testing of MTBC strains by microbiological testing must be shortened. This is best achieved by the introduction of modern molecular methods that can be applied directly to clinical specimens [31].

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**Figure 1:** Results of agarose gel electrophoresis of PCR amplicons produced from the multiplex PCR. Lane MM: molecular marker size 100 bp, Lane A: Negative control, Lane B: *IS6110* positive control (200bp), Lane C and Lane F: *pncA* (498bp)/*katG* (569bp)/*IS6110* (200bp), Lane D: *katG* (569bp)/*rpoB* (721bp)/*pncA* (498bp)/*IS6110* (200bp), Lane E: *IS6110* positive sample (200bp), and Lane G: *rpoB* (721bp)/*IS6110* (200bp).

**Conclusion**

Incidence and existence of drug resistance in Sudan was documented through the detection of *rpoB*, *katG* and *pncA* genes among TB positive patients in Sudan using the Multiplex PCR. Generally, these findings are useful for future studies since there is little available information about MTB drugs resistance in Sudan. The findings of the present study show that the recently develop multiplex PCR assay is a suitable molecular method for detecting the resistance genes. The assay offers a simple protocol that is compatible with routine work flow and can be completed within 48 hours.

**Acknowledgements**

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**Conflict of Interests**

The authors declared that they have no competing interests.

**Bibliography**


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