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Direct detection of *rpoB* and *katG* gene mutations of *Mycobacterium tuberculosis* in clinical samples

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ABSTRACT

Objectives: To study the *rpoB* and *katG* gene mutation rate and its markers.
Methods: Cross-sectional study methods were used to study Tuberculosis. A total of 45 sputum samples were collected from Annapurna Neurological Institute and Allied sciences. Then, acid fast bacilli staining were performed. Positive and negative samples were carried for conventional polymerase chain reaction identification and electrophoresis.
Results: Out of 45 samples, 3 were acid fast bacilli positive and the rest were negative. Male participants were more as compare to female participants and the mutation in *rpoB* and *katG* gene was found similar *i.e.* 6.66% among the total samples.
Conclusions: We can conclude that genetic mutation in *Mycobacterium tuberculosis* can be identified directly from the clinical samples. However, we have carried this study in less sample size and to validate research on large number of sample is recommended.

1. Introduction

Tuberculosis (TB) is caused by bacteria *Mycobacterium tuberculosis,* which most affects the lung and is preventable and curable. In 2015, there were an estimated 10.4 million new (incident) TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children [1]. In 2015, there were a predictable 480 000 incidences of multidrug-resistant TB (MDR-TB) and a supplementary 100 000 people with rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment [1]. Many social & economic factors including poverty, drug addiction and lack of health care facilities are compounding factors for causation of tuberculosis [2,3]. Isoniazid and rifampicin are the antibiotics advised first-line drugs and are the first medicines used to treat all persons with TB disease. Although there are advances in TB therapy over the past century, there were an estimated nine million new cases and 1.1 million deaths in 2013 [4].

Usually drug resistance occurs when bacteria become resistant to the drugs which are used to treat TB. It means that the drug no longer can kill the pathogens of TB. The most important way to measure the TB is quickly diagnosis with recommendation, treatment guidelines, monitoring the patients report frequently and full completion of the therapy. Isoniazid resistance (INH-R) is more complex and is related with mutations in one or more genes, such as the genes encoding catalase-peroxidase [*katG* gene (codon 315)] and the enoyl-acyl-carrier protein reeducates enzyme, which is involved in mycolic acid biosynthesis [*inhA*

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(−15 nucleotide)] [5,6]. The prevalence of the *katG* (AGC-ACC) mutation among MDR-MTB strains in the world varies, being low in area with low TB incidence and high in area with high TB incidence [7,8]. Most INH-resistant clinical isolates become resistant by losing or altering *katG* activity, nevertheless, only *katG* mutations do not result in all observed INH resistance [7–10].

Due to lack of chronic and infectious disease management policies, diseases such as tuberculosis, cancer, diabetes and cardiovascular disease do not fall under government priorities in Nepal. Direct detection of mutated genes of *Mycobacterium tuberculosis* from clinical specimen is rare in Nepal as there are only few researches carried out to detect *rpoB* and *KatG* gene mutations in MTB from clinical isolates. This led us develop this research so that we can understand the current level of mutation of *rpoB* & *katG* gene from direct sample in Nepal and help in screening and treatment. Tuberculosis is one of the greatest widespread infectious diseases in Nepal and have a serious threat to the health and growth of the people of this country [10,11]. In Nepal, about 60% of adults and 45% of the overall population have been infected with TB. Nearly 2% of people are ill every year [11].

Many developing and sub-Saharan countries are heavily burdened with MDR-TB disease and cases of resistant strains to first line drugs are increasing [12]. However, routine diagnostic tests do not type to strain level and this has complicated management of MDR-TB patients. Previously, no direct detection of *rpoB* and *katG* gene study in Nepal has determined the strains in human MDR-TB isolates. But this type of study gives great value to TB patients in proper diagnosis of the diseases.

2. Materials and methods

2.1. Study setting

The study was carried out in Decode Genomics and Research Center Pvt. Ltd., Sinamangal Kathmandu, Nepal. The hospital was chosen because of large number of visit of tuberculosis patients as according to hospital research laboratory record. Ethical approval from patients, hospital and Nepal Health Research Council was taken. Hospitalized patients of both sexes were included in the study.

2.2. Sample size

We collected sample at least 45 patients visiting the hospital. This is a cross-sectional study and the sample about this size will be sufficient to identify the *rpoB* & *katG* gene mutation from clinical samples.

2.3. Sample collection

In total, 45 samples were collected from Annapurna Neurological Institute of Allied Sciences from Kathmandu, Nepal. Sputum of hospitalized patient was taken as study sample. Spot and early morning samples of sputum were collected in 2 sterile wide mouth containers and were processed and graded on the same day as per National Tuberculosis Control Programme (NTCP) guidelines. Only sputum samples were included in the study and the volume was 5 mL or more. Samples were labeled as saliva mucoid, purulent, mucopurulent or blood stained according to their physical appearance.

2.4. Sample handling and DNA extraction

Collected sample was first decontaminated by Petroff's modified method (Table 1), and Acid Fast Bacilli (AFB) staining was carried out. Both positive and negative AFB results were processed for DNA extraction. Collected patient's sputum samples were vortexed and decontaminated by Modified Petroff's method. Four mL of the decontaminated sputum were homogenized for 15 min in a shaker using an equal volume of 4% NaOH because modified Petroffs method is an excellent simplified decontamination technique. After 15 min, centrifugation at 3 000 rpm, the deposit was neutralized with 20 mL of sterile distilled water. The samples were again centrifuged at 3 000 rpm for 15 min [13]. From the pellet, DNA was extracted which was processed for polymerase chain reaction (PCR) for the detection of TB using *MPB64* and *IS6110* primers. Gel Electrophoresis was performed to detect the PCR amplification product.

2.5. Primers amplification conditions

The compositions of reaction mixtures and PCR conditions for each of three different *rpoB* codons and *katG*315 codon were as described by [14], with few modifications. For *rpoB* MAS-PCR each 25-μL reaction mixture contained purified DNA sample (0.5 μL), $MgCl_2$ (2.5 mM for *rpoB*526- and *rpoB*531-PCR or 1.5 mM for *rpoB*516- PCR), 1 U of recombinant *Taq* DNA polymerase (Solis Bordyne, Estonia). The reactions of *rpoB*526-PCR and *rpoB*531-PCR were performed in different condition as attached in Table 1.

3. Results

Of the 45 samples collected, 60% were from the male patients and 40% were from the female patients. Most cases of

Table 1
Master Mix final concentration, PCR condition used in MTB, *rpoB* and *katG* genes.

Genes	Master mix component				PCR condition				
	Master mix	DNA	Primers	Distilled water	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation
MTB	12.5 μL	0.5 μL	*IS6110*, FP(10 pmol/μL)$^{-1}$ RP(10 pmol/μL)$^{-1}$	9 μL	95 °C for 5 min	94 °C for 50 s	56 °C for 45 s	72 °C for 1 min	72 °C for 2 min
rpoB	12.5 μL	0.5 μL	OR(10 pmol/μL)$^{-1}$ OF(10 pmol/μL)$^{-1}$ IF(10 pmol/μL)$^{-1}$	9 μL	96 °C for 5 min	94 °C for 50 s	65 °C for 50 s	72 °C for 1 min	72 °C for 3 min
katG	10 μL	1 μL	OF(10 pmol/μL)$^{-1}$ OR(10 pmol/μL)^{1} IR(10 pmol/μL)$^{-1}$	11 μL	96 °C for 5 min	95 °C for 1 min	66 °C for 40 s	72 °C for 1 min	72 °C for 3 min

Final volume of 25 μL; Cycle 25.