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Full Length Research Paper

Early diagnosis of MDR-TB cases directly on sputum specimens by rapid molecular method

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The control of tuberculosis infection has now become more complicated due to the emergence of multidrug resistant TB (MDR-TB). It takes a longer time of at least 6-8 weeks for diagnosis of MDR-TB by culture and conventional DST. The aim of this study was to test early diagnosis of MDR-TB by rapid molecular method directly from sputum specimens. A total of 56 smear positive sputum specimens were used for the detection of RIF and INH resistant mutation by genotypic assay and compared with phenotypic DST from the same specimens. Out of the 56 specimens, an interpretable result of MDR-TB (RIF^r+INH^r), were obtained from 13/53 (24.5%), by genotypic method and 14/53 (26.4%) from phenotypic method. The majority of common mutation regions were seen in MUT3 64.3% (Ser531lue) in *rpoB* gene, MUT1 24.5% (Ser315lle) in *katG* gene and MUT1 5.6% (Cys-15Thr) in *inhA* gene. Being an equally sensitive and specific method as compared to conventional DST, and short turnaround time, this method is suitable for early diagnosis of MDR-TB, directly on smear positive sputum specimens.

Key words: MDR-TB, mutation, i*nhA* gene, *katG* gene, *rpoB* gene.

INTRODUCTION

Although, Tuberculosis (TB) infection is a curable disease, it still remains a major public health problem worldwide. Worldwide about 16 million people are living with active TB with appearance of 8 million new cases every year and 2 million deaths just due to TB (Miotto, et al., 2008). TB, therefore, has great impact on youths and adults (aged 15-59 years) and constitutes the most common cause of death among adults (Telenti, et al. 1993; Sharma, et al., 2003; Sajduda et al., 2004; Harries and Dye 2006). Efforts to control TB are hampered by expanding of human immunodeficiency virus (HIV) infection and its association with active disease and increasing resistance of Mycobacterium tuberculosis strains to most effective anti-TB drugs (Harries and Dye 2006). Multidrug resistant tuberculosis (MDR-TB) has emerged due to *M. tuberculosis* resistance to at least rifampicin (RIF) and isoniazid (INH), the two most effective anti-TB drugs (Simon and Listiawan 2003). MDR strains develop by sequential acquisition of mutation at different region, usually because of inappropriate treatment of patients (Somoskovi, Parsons et al., 2001). Drug resistance develops due to random genetic mutations in particular genes responsible for resistance in Mycobacterium tuberculosis strains (Hillemann, Rusch-Gerdes et al., 2006). Rifampicin (RIF) acts on RNA polymerase inhibiting the transcription that results into bacterial cell death (WHO 1994; Huang, et al., 2009; Huyen, et al., 2010; Lingala, et al., 2010). Mutation in *rpoB* (within 81-bp core region of the *rpoB* gene corresponding to codons 507-533 incoding 27 amino acids coding for the β-subunit of bacterial RNA polymerase) (Bass, et al., 1994; Ormerod 1999) results into drug resistance diminishing the rifampicin binding to RNA polymerase (Jin and Gross 1989). Mutation of the rifampicin resistant *Mycobacterium tuberculosis* isolates are frequently located in an 81-bp core region (the rifampicin resistance determining region; RRDR) of the *rpoB* gene in up to 95-98% of rifampicin (RIF) resistant strains (WHO ; Hillemann, et al., 2006). Isoniazid (INH), a pro-drug (inactive when it enters inside the bacterial cells), in presence of catalase-peroxidase enzyme is converted into oxidizing organic toxic radicals that inhibits synthesis of mycolic acid on the bacterial cell wall.

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Hence, the molecular mechanism of isoniazid (INH) resistance is more complex than that rifampicin (RIF) (mutation is more common in *katG* gene, *inhA* gene and less common in *kasA*, *ahpC*, and *oxyR* genes) (Bártfai, et al., 2001). However, mutation in the *katG* gene, encoding catalase-peroxidase occurs more frequently and 50 to 95% of INH - resistant strains worldwide contain mutations at the *katG* gene (Ramaswamy and Musser 1998). Since, the fatality rate is much higher in MDR-TB (Zignol, et al., 2006), early identification of MDR-TB strains is crucial for starting of effective chemotherapy and for initiation of infection control measures. The earliest results can be achieved by direct testing of sputum specimens with rapid molecular method.

METHODS

Sputum samples from 56 patients were collected and studied in GENETUP (*German Nepal Tuberculosis Program*) TB research laboratory in Kalimati, Kathmandu Nepal. All 56 sputum samples were processed using NALC-NaOH decontamination method (NaOH final concentration 1%) (Kent and Kubica 1985). After centrifugation and removal of supernatant, the sediment was resuspended in 1.0-1.5 ml of phosphate buffer and used for culture and smear preparation (Auramine stain). The smear grading was done according to WHO guide line for sputum smear microscopy (WHO, 1998). The deposit from the tubes were inoculated on two Lowenstein-Jensen media for culture of *Mycobacterium tuberculosis* and incubated for four weeks.

The phenotypic DST was performed from the culture isolates following a proportion method as described by Canetti et al. 1969. The drug concentrations used were as follows: RIF (40 µg/ml), INH (0.2 µg/ml), ethambutol (2.0 µg/ml) and streptomycin (4.0 µg/ml). The quality of all prepared drug media was checked by using H_{37}Rv *M. tuberculosis* control strain. The tubes were incubated at 37^0C for 4 weeks. The "resistant results" were reported on 4^{th} week while "susceptible results" were reported on 6^{th} week. Resistant result was expressed as the percentage of colonies on drug containing media comparing with the growth on drug free medium at the critical concentration of drugs. More than one percent of bacillary population resistant to the both the critical concentration of a drug was considered as resistant (Canetti, et al. 1969).

Molecular assay for drug resistance: The molecular assay for detection of RIF and INH resistant mutations and identification was carried out by using Genotype MTBDR plus kit (*Hain Lifescience GmbH, Nehren, Germany*) according to the manufacturer's instructions (Collins, et al. 1985; Nikolayevsky, et al. 2004).

DNA extraction: A crude DNA was extracted from sputum samples and culture isolates (according to manufacturer's instruction) by heating at 95^0C for 20 minutes in water bath and incubated for 15 minutes in an ultrasonic bath. The content was centrifuged at 12,000 rpm for 5 minutes. A clear supernatant containing DNA genome was transferred to new tube and used for PCR.

Amplification process in multiplex PCR: For amplification, the mixture contained 35µl of the primer nucleotide mix, 5µl of 10x polymerase incubation buffer, 2µl of 25 mM $MgCl_2$, 0.2µl of AmpliTaq Gold polymerase (5 U/l; Applied Biosystems), 3µl of DNA free water and 5µl of the supernatant of the cell lysate, for a final volume of 50µl. The amplification protocol consisted of 15 minutes of denaturation at 95°C, followed by 10 cycles comprising 30 seconds at 95°C and 2 minutes at 53°C, an additional 30 cycles comprising 25 seconds at 95°C, 40 seconds at 53°C, and 40 seconds at 70°C, and then a final extension at 70°C for 8 minutes. Hybridization and detection were performed with a Twincubator (*Hain Lifescience GmbH, Nehren, Germany*). The hybridization procedure included following steps: chemical denaturation of amplification products at room temperature for 5 minutes, hybridization of single-stranded biotin-labeled amplicons to membrane-bound probes at 45°C for 30 minutes, stringent washes, addition of a streptavidin–alkaline phosphatase (AP) conjugate at room temperature for 30 minutes, and an AP staining reaction to detect colorimetric bands. To detect RIF resistance, 8 wild-type (WT) *rpoB* probes encoding amino acids 505 to 533 regions and 4 probes for common mutations were utilized. Probes used for INH resistance detection were designed to recognize one WT S315Thr, with two mutant probes for the highly resistant *katG* gene and two probes specific for WT regions, as well as four mutant probes for the *inhA* gene, which demonstrates low-level resistance. When all WT probes showed positive staining for an isolate and mutant probes demonstrated no staining, the isolate was considered susceptible. In contrast, the isolate was considered resistant when either any one of the WT probes was absent or any one of the mutant probes was present.

RESULTS

Out of the total 56 sputum specimens, 94.6% (53/56) were found smear positive ranged from 2+ to 4+. Among the 56 specimens 94.6% (53/56) were identified as *Mycobacterium* TB complex and 3 specimens were identified as *Mycobacterium* other than TB (MOTT) by Genotype MTBDR plus kit. An interpretable results of total RIF and INH resistant were obtained from 15/53(28.3%) specimens by genotypic method and 16/53(30.1%) from phenotypic method. Of the total 53 specimens 13/53 (24.5%) were found MDR-TB (RIF^r+INH^r); 1/53(1.8%) RIF mono resistant and 1/53 (1.8%) INH mono resistant. The results of phenotypic DST were showed 14/53 (26.4%) MDR-TB (RIF^r+INH^r), 1/53 (1.8%) mono resistant of RIF and 1(1.8%) mono resistant

Table 1. Result of Genotypic test for the detection of RIF and INH resistant in comparison with Phenotypic (conventional DST) testing.

(no=53)

Types of assay used	Result for RIF mono resistance (no%)	Result for INH mono resistance (no%)	Result for MDR (no%)	Total resistant RIF and INH (no%)
Genotypic assay	1(1.8)	1(1.8)	13(24.5)	15(28.3)
Phenotypic assay (conventional DST)	1(1.8)	1(1.8)	14(26.4)	16(30.1)

Table 2. Mutation pattern of *rpoB* gene associated RIF resistant by Genotyping method.

no=14

Genes	Falling wild type probe	Type of mutation	Location of codons	Nucleotide changes	Amino acid changes	No.% of mutation
rpoB	*rpoB*WT3/WT4	MUT1	D516V D516Y	GAC→GTC	Asp→Val	4(28.6)
	*rpoB*WT7	MUT2A	H526Y	CAC→TAC	His→Tyr	1(7.1)
		MUT2B	H526D	CAC→GAC	His→Asp	0(0)
	rpoB WT8	MUT3	S531L	TCG→TTG	Ser→Lue	9(64.3)
					Total	14(100.0%)

Table 3. Mutation pattern of katG gene and inhA gene associated INH resistant Genotyping method.

no=15

Genes	Falling wild type probe	Type of mutation	Location of codons	Nucleotide changes	Amino acid changes	No.% of mutation
katG	*katG* WT, T1	MUT1	S315T1	AGC-ACC	Ser→Thr	13(92.8)
	katG WT,T1	MUT2	S315T2	AGC-AAC	Ser→Ile	0(0)
inhA	i*nhA* WT	MUT1	C15T	UGC- ACC	Cys→Thr	1(7.1)
					Total	14(100.0%)

of INH. (Table 1).

Among the total 15 RIF resistant cases by phenotypic method, the RIF resistant associated mutation in *rpoB* genes were found 14/15 (93.3%) by genotypic method. Of the total 14 cases, the distribution of mutation genes in various regions were showed 9/14 (64.2%) in *rpoB* MUT3 and amino acid changed were Ser531Lue; 4/14 (28.5%) in *rpoB* MUT1, Asp516Val and 1/14 (16.6%) in MUT2A, His526Tyr (Table 2).

Similarly, among the 15 INH resistant cases by phenotypic method, the INH resistant associated mutation in *katG* genes were found 14/15 (93.3%) by genotypic method. Of the total 14 cases, the distribution of mutation in *katG* gene showed 13/14 (92.8%) in MUT1 and amino acid changed was Ser315Thr, and in *inhA* gene was showed 1/14 (7.1%) in MUT1 amino acid changed was Cys-15Thr (Table 3). In comparison to the results of phenotypic (conventional) DST (Table 1), the genotypic DST results showed 93.3% of sensitivity, 100% of specificity, 100% of positive predictive value and 97.4% negative predictive

Table 4. Sensitivity and specificity of Genotype MTBDRplus DST comparison with Phenotypic(conventional)DST as a gold standard.

	RIF	INH	MDR
Sensitivity	93.3%	93.8%	93.3%
Specificity	100.0%	100.0%	100.0%
Positive predictive value	100.0%	100.0%	100.0%
Negative predictive value	97.4%	97.4%	97.5%

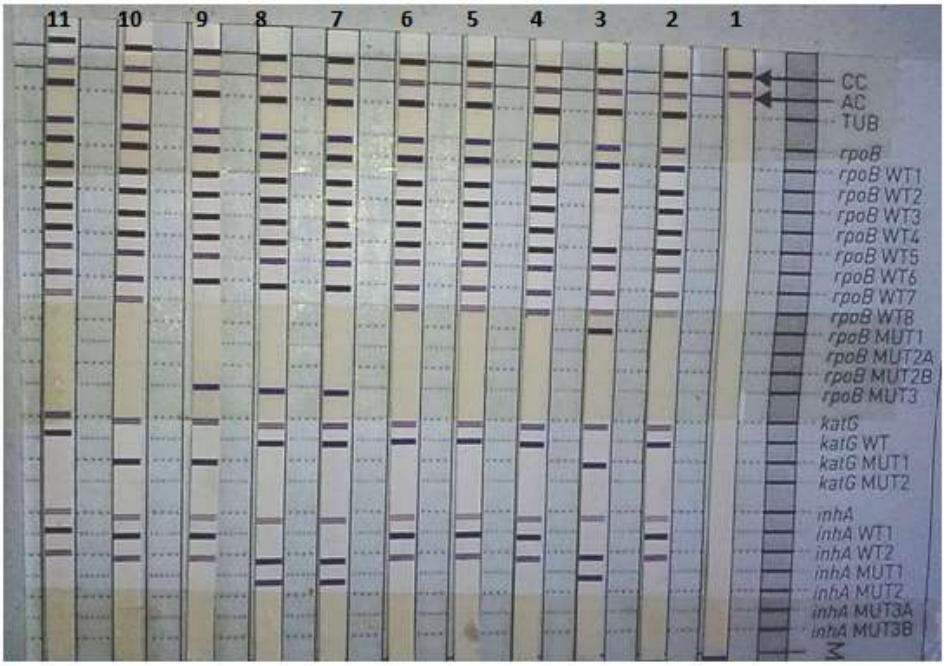

Figure 1. Rapid Genotype® MTBDR plus test results. *Lane-1*: Negative control; *Lane-2,6,11*: *M. tuberculosis* sensitive to RIF and INH; *Lane-3*: mutation *rpoB* MUT1 (Asp516Val), *katG* MUT1 (Ser315Thr), *inhA* MUT1 (Cys15Thr); *Lane-7*:mutation *rpoB* MUT3 (Ser531Lue); Lane8: mutation *inhA* MUT1 (Cys15Thr);*Lane-9*: mutation *rpoB* MUT3 (Ser531Lue), *katG* MUT1 (Ser315Thr), *Lane 10*: mutation *katG* MUT1 (Ser315Thr).

value for RIF mono resistance. For INH mono resistance, the sensitivity, specificity, positive predictive value and negative predictive value showed 93.3%,100%,100% and 97.4 % respectively. Similarly, for the MDR (RIF^r+INH^r), the sensitivity, specificity, positive predictive value and negative predictive value showed 92.8%, 100.0%, 100% 97.5% respectively(Table 4).

DISCUSSION

In this current study, we performed the rapid molecular assay (*Genotype MTBDR plus, Hain Life Science*) for early detection of resistance to the most important anti-TB drugs rifampicin and isoniazid directly on smear positive sputum specimens. In Nepal, uses of molecular assay for diagnosis of anti-TB drugs resistance are not widely available. But the major advantages of rapid molecular assay contribute early diagnosis of MDR-TB and substantially reduction of time as comparison to phenotypic (conventional) DST which takes at least 4 to 8 weeks. In present study, the positivity of sputum smear microscopy was graded 2+ to 4+ positive, but there was no significant correlation between degree of intensity of

the bands on hybridization probes with smear positivity high grading results of AFB sputum microscopy (Hillemann, et al., 2005). However the lower readability rates of molecular assay is associated with lower AFB grading in smear microscopy (Nikolayevskyy, et al., 2009). Of the total 15 RIF resistant and 15 INH resistant cases by phenotypic(conventional)DST, the spectrum of mutations associated with resistance to RIF was found 14/15 (93.3%) and INH was found 14/15 (93.3%) by genotypic method. Such a high rate of mutation was also reported by (Hillemann, et al. 2006; Nikolayevskyy, et al. 2009; Lingala, et al. 2010). In this study there was no significant difference found in the RIF and INH resistance 16/53 (30.1%) by phenotypic DST and 15/53(28.3% by rapid genotypic methods. Similar results were also reported by (Telenti,et al., 1993; Hillemann, et al., 2005). The majority of common mutations in *rpoB* gene associated to RIF resistant were observed in MUT1 (Asp516Val), MUT2 (His526Tyr) and MUT3 (Ser531Lue). Similarly, the common mutation observed in *katG* gene and *inhA* gene associated INH resistant was MUT1(Ser315Thr) and MUT1(Cys-15Thr) respectively which have also been reported by previous studies (Huang, et al., 2009; 2010). The concordant result of genotypic and phenotypic resistant RIF (93.3%) and INH (93.3%) gene observed in this study were very similar with the findings reported by other investigators (Ahmad and Mustafa 2001; Lu, et al., 2009; Huyen, et al. 2010). On the other hand, the discordant results 1/16 (6.2%) of phenotypic and genotypic resistant found in this study was also consistent with previous report (Miller, et al., 1994; Barnard, et al., 2008). The discordant results in RIF (n=1) and in INH (n=1) could be due to either presence of uncommon mutation which may be present outside the RIF and INH resistance regions. The additional molecular mechanisms were not able to be detected by this rapid genotypic method used in this study (Ozkutuk, et al. 2007; Khadka, et al., 2011). Such undetectable mutation regions could be further studied by nucleic acid sequencing technique which can detect all types of mutation regions. The sensitivity, specificity, predictive positive value and predictive negative value were showed 93.3%, 100.0%, 100% and 97.4% for RIF resistance and 93.3%, 100%, 100% and 97.4% for IHN resistance by this method as reported by other investigator (Nikolayevskyy, Balabanova et al., 2009; Khadka, Rai et al., 2011).

CONCLUSION

The rapid molecular method is a sensitive and specific tool for diagnosis of RIF and IHN resistance and MDR-TB. Being a short turnaround time, easy to set up in low cost, even in small level TB laboratory, this method is suitable for rapid diagnosis of drug resistant TB, directly on smear positive sputum specimens.

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