Evaluate "Rifampicin Resistance" as Surrogate Marker for Rapid Detection of MDR-TB Using Real-Time PCR Directly on FNAC Samples of Tuberculous Lymphadenitis

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: India has the dubious distinction of having second largest burden of MDR-TB cases in the world. According to WHO, MDR-TB is defined as resistance to isoniazid and rifampicin, the two most important drugs for treatment of TB. "Rifampicin resistance" is recommended as surrogate marker for MDR-TB by WHO, as at least, 90% of all rifampicin-resistant clinical isolates are also found resistant to isoniazid. Localization of genetic alterations in the 81-bp "Rifampicin Resistance-Determining Region" of rpoB gene in 96% of rifampicin resistant strains make it particularly amenable for early detection of MDR-TB by molecular techniques like Real-Time PCR.
Aim: Evaluation of "rifamipicin resistance" as surrogate marker for rapid detection of MDR-TB using Real-Time PCR directly on FNAC samples of tuberculous lymphadenitis (TBLN).
Materials and Methods: Eighty cases of TBLN undergoing anti-tubercular treatment (ATT) and 10 lymphadenitis cases of non-tuberculous origin (controls) were included in the study. To evaluate "rifamipicin resistance" as surrogate marker for rapid detection of MDR-TB, Real-Time PCR and conventional Drug Susceptible Testing (DST) were carried out.

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Results: Eighteen samples were identified as MDR-TB cases by DST. Real-Time PCR picked up mutated ropB gene in 17 cases out of these 18 MDR-TB cases.

Conclusion: "Rifampicin resistance" is an efficient surrogate marker for timely detection of MDR-TB using rapid, accurate and sensitive molecular technique like Real-Time PCR.

1. INTRODUCTION

The spread of isoniazid and rifampicin resistant *M. tuberculosis* known as Multi Drug Resistant [MDR] is a major setback to tuberculosis [TB] control programs worldwide [1]. India is home to the second largest number of MDR-TB cases in the world [2]. Rifampicin resistance is recommended by WHO as "surrogate marker" for detecting MDR-TB because mono-resistance to rifampicin in *M. tuberculosis* is rare [1]. At least 90% of all rifampicin resistant clinical isolates are also resistant to isoniazid [1]. Genetic mutations are mainly responsible for resistance to rifampicin in *M. tuberculosis* [3]. Ninety six percent of rifampicin resistant *M. tuberculosis* strains acquire genetic alterations within 81-bp "Rifampicin Resistance-Determining Region" [RRDR] corresponding to codons 507 to 533 in the *rpoB* gene [4]. This region acts as an ideal target for molecular tests for rifampicin resistance. Real-Time Polymerase Chain Reaction (PCR) is one such accurate, sensitive and rapid molecular method that can yield virtually unlimited copies of specific DNA fragments from minute quantities of the source DNA material in very short time.

The key to prevent the rapid rise and spread of MDR-TB is early detection and appropriate case management. Detection of "rifampicin resistance", the surrogate marker of MDR-TB, using rapid molecular technique like Real-Time PCR could save precious time and lives. This may also help in averting the catastrophic effect of drug resistance on TB control programs extensively relying on rifampicin as the first line drug of Anti-Tubercular Treatment.

In the present study, we evaluated "rifampicin resistance" as surrogate marker for rapid detection of MDR-TB using Real-Time PCR directly on FNAC samples from patients treated with Directly Observed Short Course Treatment [DOTS] regimen. Conventional culture based drug susceptibility testing [DST] by proportion method on LJ medium, according to standard procedures [5] was also performed on all the samples.

1.1 Aim

Evaluation of "rifampicin resistance" (rpoB gene) as surrogate marker for rapid detection of MDR-TB using Real-Time PCR directly on FNAC samples from patients showing persistence of LN or appearance of new LN after 5 months or more of ATT ["failures" as per Drug Resistance in World, WHO Global Report No.3 2004] [1].

2. MATERIALS AND METHODS

The prospective study was conducted on eighty clinico-cytologically confirmed cases of TBLN who had taken 5 months or more of standardized ATT (DOTS regimen) and showed persistence of initial LN or appearance of new LN [failures[1]] at Guru Teg Bahadur Hospital, Delhi, India. Ten cases of non-TBLN consisting of 4 lymphomas, 3 metastatic carcinomas with necrosis [histologically proven] and 3 cases of pyogenic abscess [gram stain positive for cocci] were included as controls. Informed consent was obtained from all patients to utilize their FNA samples for this study. Relevant clinical history and examination findings were recorded. The FNAC material (1.5-2.5 ml) obtained was used for:

i) Cytological diagnosis [Giemsa smears]
ii) Demonstration of *M. tuberculosis* [Zeihl Neelson staining for acid fast bacilli]
iii) Culture [Lowenstein-Jensen slants]
iv) DST for isoniazid & rifampicin by proportion method on LJ medium [5].
v) Real-Time PCR. (A part of the FNA sample was stored at -80° Celsius for Real-Time PCR which was performed only for those samples for which DST results were available).

2.1 Culture

Before culture, the specimens were purified by digestion and decontamination by the N-acetyl-L-cysteine [NALC]-NaOH to prevent overgrowth by non-mycobacterial microorganisms in the culture. *M. tuberculosis* was cultured in the conventional LJ medium at 37°C in 5% CO_2 for 1

week, at 37ºC without CO_2 for another 7 weeks and thereafter were observed once a week for *M. tuberculosis* growth. The fresh growth was then sub-cultured onto LJ medium for DST. DST to the first-line drugs rifampicin [40 μg/ml] and isoniazid [0.2 μg/ml] was performed using proportion method as described in previous study [6]. All inoculations were incubated at 37ºC in 5% CO_2 for 8 weeks. If the number of colonies grown on the drug-containing medium was less than 1% of the number of colonies grown on a drug-free medium, the isolate was defined as susceptible to rifampicin. The isolate was resistant if the number was ≥1% [6,7]. Isolates resistant to both rifampicin and isoniazid were classified as MDR TB [1].

2.2 DNA Extraction

Mycobacterial genomic DNA was extracted from the FNAC samples as described by Van Sooligen et al. [8] with minor modifications. The quality and quantity of DNA was assessed by measuring absorbance ratio at 260/280 nm. An absorbance ratio within 1.8 to 2.0 suggested good quality extraction.

2.3 Real-Time PCR

Real-Time PCR was performed to evaluate rifampicin resistance (rpoB gene) in 30 FNAC samples where DST results were available to facilitate comparison. Using a set of FRET probe [Genosen's Real-time Kit, Genome Diagnostics Pvt. Ltd] targeting codon region 526 to 531 of rpoB gene, the"RRDR" was amplified and the melting temperatures [Tms] of the amplified "RRDR" were obtained on Rotor gene 6000 Real-Time Rotary Analyzer [Corbett Research Australia] using FRET chemistry. This system is an open chemistry platform with 4-6 channel multiplexing capability and multiple excitation sources combined with several detection filters for detecting virtually every known fluorophore. Following amplification, Melting Curve Analysis was performed for characterization of amplimers by their respective melting temperatures. Rate of decrease of fluorescence intensity was plotted to obtain T_m value for each Melt Curve. The change in the T_m was considered an indicator of a mutation and isolates for which the probe had a T_m lower than that for *M. tuberculosis* H37Rv were considered resistant to rifampicin [9]. The T_m of wild rpoB is 73.8-74.8ºC and that of mutant rpoB is 69.8-70.8ºC. A total of 25 μl of PCR mixture was prepared. It included ready to use reaction mixture containing the Genosens' Real-

Time PCR kit, (12.5 μl) PCR buffer (2.5 μl), hot start Taq polymerase, (0.25 μl) deoxynucleotide triphosphate and $MgCl2$ (2.5 μl) and specific primers and probe. Five μl DNA was added. Real-Time PCR was performed in Rotor gene 6000 Real Time Rotary Analyzer [Corbett Research, Australia]. The cycling conditions were denaturing at 95ºC for 10 min [Hold 1], followed by 45 cycles of amplification at 95ºC for 15 seconds, 58ºC for 20 seconds, 72ºC for 20 seconds. The conditions for the melt curve were as follows: Hold 2 at 95ºC for 1 min 0 seconds, Hold 3 at 40ºC for 30 seconds, Hold 4 at 50ºC for 30 seconds. The melt started from 50ºC to 90ºC and the data was acquired on FAM/GREEN and CY5/RED channels. A positive control (MTB h37 Rv wild), negative control (RNAse/DNAse free water) and a non-template control [NTC] were also run. All the 10 negative controls were analyzed similarly on Real-Time PCR.

3. RESULTS

3.1 ZN Staining

Sixty three percent [51/80] cases were AFB [Acid Fast Bacilli] positive. None of these cases were gram stain positive. None of the 10 negative controls were AFB positive, 3 control samples from pyogenic abscess were gram stain positive for cocci.

3.2 Cytomorphology

The predominant cytomorphological pattern on Giemsa smears was granuloma with necrosis (57/80) followed by necrosis with neutrophilic infiltrates (23/80).

3.3 Culture

M. tuberculosis was isolated in culture in 37**%** [30/80] cases. No other organism was isolated. None of the 10 controls were *M. tuberculosis* culture positive.

3.4 DST Results

The results of DST were divided into following categories:

i)   18 out of 30 cases were resistant to both isoniazid & rifampicin, i.e. MDR strains.
ii) 11 cases were sensitive to both drugs
iii) One case was sensitive to rifampicin but resistant to isoniazid.
iv) 19 cases were resistant to isoniazid.

3.5 Real-Time PCR Results

30 DST positive samples were analyzed for rifampicin resistance (rpoB gene status) by Real-Time PCR. Seventeen out of these 30 samples showed mutated rpoB gene with the probe covering codon 526 and 531of place the gene. The remaining 13 cases had wild rpoB gene. The T_m of the probe for *M. tuberculosis* H37v was 73.8-74.8ºC and that for the mutant rpoB probe was 69.8-70.8ºC. A drop of 4ºc was registered in the Tm for rifampicin resistant isolates. When compared with conventional DST [gold standard], Real-Time PCR picked up 17 of the 18 cases (94.4%) that were found to be rifampicin resistant on drug containing culture media. None of the 11 cases that were sensitive to both the drugs on DST showed mutated rpoB gene.

4. DISSCUSSION

MDR-TB is defined as resistance to isoniazid and rifampicin with or without resistance to other drugs [1]. Rifampicin is the most effective bactericidal drug that helps in shortening the duration of ATT [10]. It inhibits transcription and elongation of bacterial RNA by binding with the bacterial DNA-dependent RNA polymerase [11,12]. This RNA polymerase has 4 subunits but mutations in the rpoB gene coded sub-unit play a significant role in development of resistance. Mutations in this sub-unit prevent rifampicin from binding the bacterial DNA-dependent RNA polymerase [11] making the bacilli resistant to the drug. Random, single step spontaneous genetic mutations are responsible for resistance to rifampicin in M. tuberculosis. High degree of resistance to rifampicin has been reported due to mis-sense mutations at codons 526 to 531of rpoB [3]. The reported frequency of mutation at codon 531 is 29-74%, at 526 is 0-43% and at 516 is 0-38% [13,14].

Importance of rifampicin resistance in MDR-TB is highlighted by the fact that WHO has recommended it as surrogate marker for detecting MDR-TB [1]. Rifampicin resistance has been linked with treatment failure and outbreaks, further adding to its clinical and public health relevance [1]. Also rifampicin resistance is more amenable for genotypic DST because 95- 98% rifampicin resistance occurs via genetic mutations in 81 bp region of rpoB gene [3,4]. As compared to rifampicin resistance, molecular resistance to other anti-TB drugs like isoniazid is more complex and requires assessment of mutations in multiple genes for better correlation with phenotypic DST. Accurate genotypic DST is technically more challenging for the other first-line and second-line anti-TB drugs whereas molecular tests for rifampicin resistance are much more developed [15].

With the aim to evaluate "rifampicin resistance" as surrogate marker, the versatile rapid molecular technique of Real-Time PCR was used directly on 30 FNAC samples. Conventional DST using the proportion method [5] was also performed as it is considered "gold standard". By DST, 18 samples were found resistant to both isoniazid and rifampicin i.e. MDR-TB, 11 cases were susceptible to both the drugs and 1 case was sensitive to rifampicin but resistant to isoniazid. Thus 18 cases were found to be rifampicin resistant by the conventional DST. On analysis by Real-Time PCR using rpoB probe that covered RRDR [codons 531 and 526] of rpoB gene we could detect 17 of 18 [94%] rifampicin resistant isolates. Thus by both the methods, none of the rifampicin resistant cases were found to be sensitive to isoniazid. The study corroborates the fact that" rifampicin resistance" is an excellent surrogate marker for MDR-TB.

In the study, none of the cases found to be "rifampicin sensitive" by DST were revealed as "rifampicin resistant" by Real-Time PCR. Thus no false positives were observed with Real-Time PCR. Although the study cohort was limited, yet it brings out the high sensitivity of Real-Time PCR for detection of drug resistance. Sensitivity of Real-Time PCR for detecting rifampicin resistance has been reported at 82.8% by Mokrousov et al. [16] and 97.5% by Ruíz et al. [17].

In this study the sensitivity of Real-Time PCR was found to be 94.4% and the positive predictive value was one.

Although in recent years, several molecular techniques have been applied to detect mutations associated with anti-tubercular drug resistance, but most of these techniques have been evaluated in cultures. Very few studies in the literature have been published for direct detection of resistance in clinical samples [16,17,18,19,20,21]. The study also supports the fact that Real-Time PCR can be successfully applied on clinical samples directly (without the need for culture), as we were able to pick up 94% rifampicin resistant cases from FNA samples of TBLN.
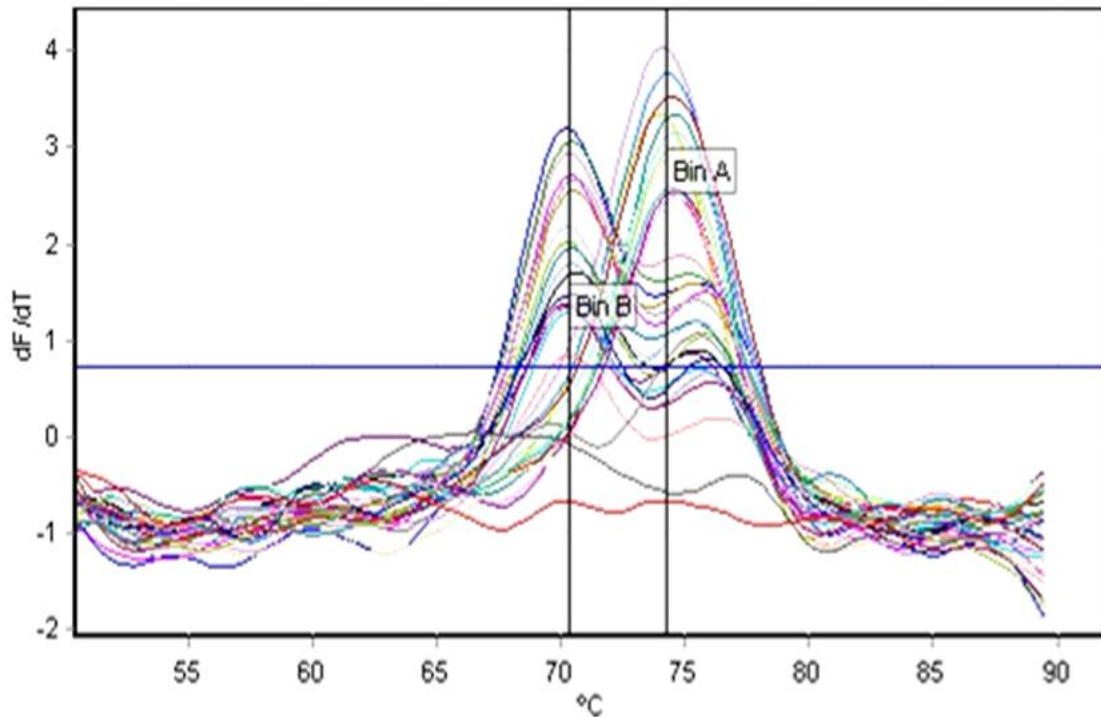
**Fig. 1. Real time PCR results showing two distinct peaks (Tm) for wild & mutant rpoB Gene.
(Melt data for Melt A. Green)**

In 3-5% of rifampicin resistant isolates, additional mechanisms like rifampicin permeability and novel mutations in alternate sub units of RNA polymerase may confer resistant phenotype [3,22]. In our study also Real-Time PCR could not detect mutation in the single sample that was resistant to rifampicin by DST. The probable reason could be that novel mutations in alternate sub units of RNA polymerase may have been missed by the probe which detected mutation in the "hot spot" region of the rpoB gene. Using probes with higher resolving power may further improve the specificity. However, our study corroborates findings from earlier studies from South India which have also found that codon 531 and codon 526 are most frequently involved in mutations [23,24].

Culture is the most specific method for diagnosis but is not very sensitive [<50%] and is also time consuming [results available only after 4-8 weeks] [25]. In the study too, culture based results were available after 6-8 weeks, but Real-Time PCR results were available within hours of DNA extraction. Culture requires viable organisms [10 to 100 viable organisms per sample]. This is often a problem in partially treated patients and pauci-bacillary cases [26]. Detection of *M. tuberculosis* DNA in clinical samples of such cases by Real-Time PCR is a promising approach for rapid diagnosis of the disease as this technique is independent of the viability of organism. Extra-pulmonary TB is even more difficult to diagnose as samples are frequently obtained using invasive or semi-invasive methods and may not have adequate number of bacilli [25].

Most of the studies [27,28] have reported culture positivity for FNAC samples of TBLN varying from 17-55.5%. Thus culture may not be available in many cases where DST needs to be performed before initiating appropriate treatment. In our study culture was positive in only 37% (30/80) cases, which limited the DST to only 30 out of the total 80 cases. Fifty cases could not be evaluated further. Reasons for low culture positivity in the study could be presence of scant bacilli and bactericidal substances as majority of the patients were receiving ATT and use of harsh decontamination procedure [29]. PCR positivity in culture negative samples highlights the importance of this technique in detection of drug resistance cases where culture based methods may not be available. Thus the

main advantages of Real-Time PCR are rapid detection [1.5-2.0 hours after DNA extraction], quantitative analysis, ability to pick up very minute quantity of DNA independent of the viability of organism and a lower risk of contamination.

In a study by Payanandan et al. [30] the incidence of MDR-TB in Thailand was found to be same as that of rifampicin resistance, establishing that rifampicin resistance can be effectively used as surrogate marker for MDR-TB. In this study rifampicin resistance in 17 out of 18 MDR-TB cases was detected by rpoB probe targeting codons 531 and 526 indicating that rifampicin resistance could be effectively used as a proxy for MDR-TB, averting cost of analyzing the status of isoniazid resistance in suspected cases.

The alarming rise in MDR-TB is threatening to weaken TB control programs worldwide. There is an emergent need for rapid diagnosis of MDR-TB for early initiation of treatment, improved prognosis and interrupting transmission of MDR strains which is not possible with the conventional DST due to longer turn-around-time. Rifampicin resistance, surrogate marker for MDR TB, can be easily detected by targeting the 81-bp RRDR of rpoB gene using molecular methods like Real-Time PCR [31,32,33] that can yield rapid results for timely initiation of treatment of MDR-TB particularly in countries like India with high prevalence.

5. LIMITATIONS OF THE STUDY

In developing country like ours with high case burden of MDR TB, relatively faster, reliable and readily usable technique like Real-Time PCR could be of more value for timely & accurately detecting MDR-TB. We tried to assess the possibility of utilizing Rifampicin resistance (rpoB gene) as surrogate marker for MDR-TB using Real-Time PCR directly on FNA samples. It also needs to be mentioned that sequencing of rpoB gene was not a part of our study. The only limitation we feel regarding use of Real-Time PCR based methods are requirement of appropriate equipments and reagents, standardization of techniques, and availability of trained human resources.

6. CONCLUSION

The study underlines importance and usefulness of "rifampicin resistance" as surrogate marker for MDR TB using Real-Time PCR directly on FNAC samples to screen both naïve as well as treated TB cases to save valuable time.

ETHICAL APPROVAL

Protocol of the study was presented to the institutional ethical committee before undertaking the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/9929