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gyrA Gene Mutation Conferring Phenotypic Cross-Resistance among Fluoroquinolones (Ofloxacin, Levofloxacin and Gatifloxacin) in Multidrug Resistant *Mycobacterium tuberculosis* Strains Isolated from Pulmonary MDR-TB Patients in Bangladesh

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Abstract

Background and objectives: Fluoroquinolones (FLQs) are an essential component of multidrug resistant-tuberculosis (MDR-TB) treatment regimen but unfortunately the emergence of FLQ resistant MDR-TB cases is challenging the current MDR-TB treatment regimen. FLQ resistance is mainly caused by *gyrA* gene mutation and phenotypic cross-resistance may occur among the different FLQs. A limited number of data exists regarding the cross-resistance phenomenon among FLQs and it appears that resistance to the present class representative FLQ, ofloxacin (OFX), may or may not correlate with complete cross-resistance to other FLQs. So the study was designed to observe if *gyrA* gene mutations confer to the phenotypic cross-resistance among FLQs [OFX, Levofloxacin (LFX) and Gatifloxacin (GFX)] tested. **Methodology:** Sputum samples from 68 diagnosed pulmonary MDR-TB cases were collected. All samples were subjected to Multiplex Real-time PCR for the detection of *gyrA* gene mutations and conventional culture on Lowenstein-Jensen (L-J) media followed by drug sensitivity testing (DST) of culture isolates (MDR-TB strains) by indirect proportion method for the detection of phenotypic resistance pattern to OFX, LFX and GFX. **Results:** Out of the 68 MDR-TB sputum samples 13 (19.11%) had MDR-TB bacilli with mutations in the *gyrA* gene and 11(16.18%) of the MDR-TB culture isolates were found to have resistance

to FLQs by DST. The study observed that 11 MDR-TB samples with *gyrA* gene mutations showed complete phenotypic cross-resistance among OFX, LFX and GFX. **Conclusion:** This study found that mutation in the *gyrA* gene of the MDR-TB bacilli results in the complete cross-resistance among the FLQs (OFX, LFX and GFX) tested. It is therefore of utmost importance to carry out the base line resistance and cross-resistance tests for the individual FLQs prior to initiating the treatment of MDR-TB cases in Bangladesh for successful clinical outcomes.

Keywords

Multidrug Resistant-Tuberculosis (MDR-TB), *gyrA* Gene, Mutations, Fluoroquinolones (FLQs), Cross-Resistance

1. Introduction

Fluoroquinolones (FLQs) are broad spectrum antibiotics which have been successfully used in the treatment of Tuberculosis (TB) since 1984 and have become an essential component of MDR-TB (multidrug resistant-tuberculosis) treatment regimen [1]. When a *Mycobacterium tuberculosis* strain isolated from a patient exhibits resistance to rifampicin (RIF) and isoniazid (INH), it is termed MDR-TB and the patients infected with MDR-TB strains are called MDR-TB patients. Such MDR-TB patients are initiated on a FLQ containing treatment regimen that is very effective [2].

Unfortunately, there is rampant use of the FLQ drugs for undiagnosed respiratory infections in Bangladesh and this has contributed to the emergence of FLQ resistance in *M. tuberculosis* which may in turn influence the clinical outcome of MDR-TB patients [3]. The emergence of FLQ resistant MDR-TB is thus a cause for significant concern. According to the Global Tuberculosis Report, 2014 the FLQ resistance rate was 17% among the MDR-TB strains tested [4].

The FLQs belong to the quinolone class of antibiotics which inhibit bacterial DNA gyrase and topoisomerase IV enzymes. DNA gyrase is an ATP-dependent enzyme which cleaves and reseals double stranded DNA thereby introducing negative supercoils into DNA. This activity is essential for DNA replication, transcription and recombination in bacteria [5] [6]. The DNA gyrase enzyme consists of two GyrA and two GyrB subunits encoded by *gyrA* and *gyrB* genes respectively. Topoisomerase IV is also a heterodimer and consists of ParC and ParE subunits encoded by *parC* and *parE* genes respectively. Many bacterial species possess both DNA gyrase and topoisomerase IV. However, *M. tuberculosis* lacks *parC* and *parE* homologs and the DNA gyrase enzyme appears to be the sole target for the FLQ antibiotics [7]. *M. tuberculosis* acquires resistance to FLQs mainly through mutations in conserved regions of the *gyrA* and *gyrB* genes, referred to as quinolone resistance-determining regions (QRDRs) [8].

FLQ resistance is more commonly caused by *gyrA* gene mutations but mutations in the *gyrB* gene are also found to be associated with FLQ resistance [9] [10].

Ofloxacin (OFX) is a second generation FLQ that has been used as both a first- and second-line anti-TB agent. It was the first FLQ recommended by WHO for inclusion in MDR-TB treatment regimens. Levofloxacin (LFX), another second generation FLQ, is more efficacious than OFX for second-line treatment and is also recommended by WHO for inclusion in MDR-TB treatment regimens [11]. Gatifloxacin (GFX) is a third-generation FLQ that has been used in standard first- and second-line regimens. Inclusion of GFX has successfully shortened the duration of anti-TB treatment regimens [12]. As the newer FLQs like LFX and GFX have become more popular treatment options, an evaluation of cross-resistance between these drugs and the current class representative OFX is crucial. A limited number of studies exist regarding the cross-resistance among the FLQs and it appears that resistance to OFX may or may not results in complete cross-resistance to newer FLQs [13] [14].

In Bangladesh there is scarcity of data regarding this resistance and cross-resistance phenomenon of FLQs, however, it is imperative to have knowledge regarding this matter for the successful treatment of MDR-TB patients. So this study was undertaken to observe the relationship between the *gyrA* gene mutations and phenotypic resistance pattern among OFX, LFX and GFX in MDR-TB strains isolated from pulmonary MDR-TB patients in Bangladesh.

2. Methodology

This cross sectional study was carried out from September 2016 to August 2017 at the Department of Microbiology and Immunology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh and at the National Tuberculosis Reference Laboratory (NTRL), National Institute of Diseases of the Chest and Hospital (NIDCH), Dhaka, Bangladesh.

2.1. Collection of Specimen

Sputum samples from 68 MDR-TB patients (both new cases and patients under MDR-TB treatment regimen) attending the MDR-TB ward of NIDCH were collected after approval by the Institutional Review Board of BSMMU (process number: BSMMU/2017/88).

Inclusion criteria: Pulmonary MDR-TB patients diagnosed by Xpert MTB/RIF assay (Cepheid Inc, Sunnyvale, CA, USA), which detected rifampicin resistance and by the conventional DST (drug susceptibility testing), which detected rifampicin and isoniazid resistance were selected.

Exclusion criteria: Extra pulmonary MDR-TB patients.

2.2. Decontamination of Specimen

All sputum samples were digested and decontaminated with 4% N-acetyl-L-

cysteine-sodium hydroxide [15].

The steps for processing of the specimen, DNA extraction and the Multiplex Real-time PCR assay were carried out in the department of Microbiology and Immunology of Bangabandhu Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh.

2.3. Processing of Specimen for Multiplex Real-Time PCR

Briefly, 1.5 mL of the decontaminated specimen was taken into a new sterile tube and centrifuged at 15,000 × *g* at 25˚C temperature for 15 minutes. The supernatant was discarded and 1 mL of 1 × PBS solution was mixed and centrifuged at 15,000 × *g* for 5 minutes and the supernatant discarded by pipetting. This step was repeated once more.

2.3.1. DNA Extraction

DNA extraction was done on all the processed sputum samples using the Seegene DNA extraction kit (Seegene, Seoul, Korea), according to the manufacturer's instructions. One hundred µL of DNA extraction solution was added to 1.5 mL of pretreated sample using micropipette, in a microcentrifuge tube and vortexed for 30 seconds. Then the lid of the microcentrifuge tube was closed tightly and boiled in a heat block at 100˚C for 20 minutes and then centrifuged at 15,000 × *g* for 5 minutes. 5 µL of the supernatant was taken into the PCR tube and used as the PCR template.

2.3.2. Multiplex Real-Time PCR Procedure

Real-time PCR was carried out on all the extracted DNA from samples using Anyplex™ II MTB/XDR detection kit (Seegene, Seoul, Korea) in Bio-Rad's 96 well PCR plate (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's instructions.

The Anyplex II MTB/XDR kit identifies *Mycobacterium tuberculosis* Complex (MTC) and mutations conferring resistance to FLQ (*gyrA*) and ISL (*rrs* and *eis* promoter region). The kit is capable of detecting up to 13 XDR associated mutations (7 FLQ and 6 ISL resistance mutations) utilizing the DPO™ (dual priming oligonucleotide) and TOCE™ (tagging oligonucleotide cleavage and extension) technologies for the simultaneous real-time detection of multiple point mutations with high specificity.

Each PCR solution consisted of 5 µL of the extracted DNA added to 15 µL of master mix (4 × TOCE oligo mix for XDR, 4 × Anyplex PCR master mix, and RNase-free water). For positive control 5 µL of MTB/DR PC, for negative control 5 µL of RNase-free water and for the wild type control 5 µL of MTB/DR WTC were added in separate wells containing master mix, during each PCR run. The plate was sealed with plate cover and then spun in the centrifuge very briefly to mix the contents of the wells properly and to remove air bubbles. The blunt end of a sharpie was used to seal the gaps between the wells in order to prevent evaporation of the contents of the wells during the PCR process. The plate was then loaded on the plate holder in the PCR machine and plate lay out was de-

signed in the software. The PCR run method was then set in the machine. Amplification was performed using the CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) and the melting-curve data analyses and interpretation were performed automatically with a connected computer, using Seegene viewer software, version 2.0 (Seegene Technologies, Concord, CA, USA).

Culture and drug sensitivity testing (DST) were done in the National Tuberculosis Reference Laboratory (NTRL) of the NIDCH, Dhaka, Bangladesh.

2.4. Conventional Culture and Drug Sensitivity Testing (DST) by Indirect Proportion Method

Conventional culture was done on Lowenstein-Jensen media (L-J media). Preparation of L-J media and inoculation of the media were done as per standard procedure [15]. At first two drops of the decontaminated pellets from each sputum sample were inoculated on the slants of 2 sets of L-J media. The inoculated media were then incubated at 37˚C. The bottles were examined after 72 hour to exclude contamination. If no contamination was observed the bottles were then examined weekly for 8 weeks. If no growth appeared after 8 weeks of incubation the culture was reported as culture negative. If one of the 2 media bottles was found to be contaminated, the result was interpreted from the other bottle. On appearance of visible colonies as shown in **Figure 1**, the colony morphology, rate of growth and pigment production were studied. The growth was confirmed as Mycobacterium by Ziehl-Neelsen staining and as MTC by Capilla 64 test (SD Bioline TB Ag MPT64 RAPID® test, SD Bioline Kit, Standard Diagnostics, Inc., Yongin, Korea).

DST by indirect proportion method on L-J media was done for the FLQs viz. OFX, LFX and GFX. To prepare the drug containing media each drug was added at a concentration of 4 μg/ml for OFX, 1 μg/ml for LFX and 1 μg/ml for GFX to

Figure 1. Growth of MDR-TB bacilli on L-J media by conventional culture.

the L-J media. P-nitrobenzoicacid (PNB) was taken as control. Resistance was defined when greater than 1% of growth was obtained on the drug containing medium compared to the number of colonies obtained on the control medium [15] [16].

3. Results

All of the 68 sputum samples collected from MDR-TB patients were tested by both Multiplex Real-time PCR and conventional culture and DST by indirect proportion method. Mutations of the *gyrA* gene were detected in 13 (19.11%) of the MDR-TB samples whereas FLQ resistance was detected in 11 (16.18%) of the MDR-TB culture isolates (**Table 1**). Out of the 13 MDR-TB samples with *gyrA*-gene mutations; 11 (84.62%) showed phenotypic cross-resistance among OFX, LFX and GFX, whereas, the remaining 2 (15.38%) did not show phenotypic resistance to any of the FLQs tested (**Table 2**).

4. Discussion

Emergence of multidrug resistant-tuberculosis is a major roadblock in the TB control programme in Bangladesh. FLQs are one of the major second line drugs used in the treatment of MDR-TB but the phenomenon of cross-resistance within the FLQs is common [13]. Despite current knowledge, the association between the resistance levels of FLQs and the different mutations remains debatable. Knowledge of the cross-resistance pattern between OFX and other FLQs is a must in order to customize effective MDR-TB treatment regimen.

In this study we found 19.11% of FLQ resistance among the MDR-TB strains by detection of the *gyrA* gene mutation using Multiplex Real-time PCR assay and 16.18% of FLQ resistance by conventional culture and DST by indirect proportion

Table 1. Results of the *gyrA* gene mutations and FLQ sensitivity of the MDR-TB strains present in the sputum samples of the pulmonary MDR-TB patients (n = 68).

Detection Method	Number (n)	Percentage (%)
Real-time PCR assay		
gyrA gene mutations present[a]	13	19.11
gyrA gene mutations absent	55	80.89
Total	**68**	**100**
Conventional culture and DST		
Culture negative[b]	16	23.53
Culture positive[c]	52	76.47
(FLQ resistant + FLQ sensitive)	(11 + 41)	(16.18 + 60.29)
Total	**68**	**100**

Note: PCR: Polymerase chain reaction, DST: Drug sensitivity testing, FLQ: Fluoroquinolone. a. *gyrA* gene mutations present: indicates FLQ resistance in the MDR-TB strains detected by molecular method. b. Culture negative: no growth on L-J media by conventional culture. c. Culture positive: growth on L-J media which were further tested for sensitivity to FLQs (Ofloxacin, Levofloxacin and Gatifloxacin) by indirect proportion method. The "Culture Positive" values in the table are the sum of the FLQ resistant and FLQ sensitive values respectively.

Table 2. Distribution of the FLQs (OFX, LFX and GFX) resistance pattern among the MDR-TB culture isolates with mutation in the *gyrA* gene (n = 13).

Serial number of the MDR-TB culture isolates	*gyrA* gene mutation	DST report		
		OFX resistance	LFX resistance	GFX resistance
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	–	–	–
6	+	+	+	+
7	+	+	+	+
8	+	–	–	–
9	+	+	+	+
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
13	+	+	+	+

Note: "+" indicates presence and "–" indicates absence of characteristic. DST: drug sensitivity testing, OFX: Ofloxacin, LFX: Levofloxacin, GFX: Gatifloxacin.

method. The findings of our study correspond with the global FLQ resistance rate of 17% among MDR-TB strains, reported by The Global Tuberculosis report, 2014 [4]. Similarly, a study conducted in New Delhi, India by Singhal *et al.* (2016) found 17.1% of FLQ resistance among MDR-TB patients and another hospital-based study from Wuhan, China by Chen *et al.* (2017) found 22% of OFX resistance in TB suspects [17] [18]. A study from Pakistan reported an increase in the level of FLQ resistance in *M. tuberculosis* from 17.4% in 2005 to 42.9% in 2009 [11]. This likely occurred due to the over prescription of FLQs which takes place in certain regions of the world, especially in the resource-limited countries, where FLQs are readily available over the counter [19].

Bangladesh being a TB endemic country, this potentially high proportion of FLQ resistance among MDR-TB patients is of significance. It is important to note that MDR-TB patients are treated with second-line anti-TB drugs and FLQs are one of the essential drugs in the treatment of MDR-TB. However, resistance to FLQs is known to be associated with poor treatment outcomes among MDR-TB patients [20].

Resistance to FLQs is most frequently attributed to mutations within the conserved regions of *gyrA* gene which are the QRDRs. In this study we observed the correlation between *gyrA* mutations and the resistance pattern among FLQs (OFX, LFX and GTX). Out of the 13 MDR-TB sputum samples with *gyrA* gene mutations; 11 (84.62%) attributed phenotypic cross resistance between OFX and the newer FLQs (LFX and GFX). A study by Von Groll *et al.* (2009) found that

40 out of a total of 41 strains shared the same cross-resistance pattern for all three FLQs viz. OFX, GFX and Moxifloxacine (MXF). The remaining one strain was susceptible to OFX but resistant to GFX and MXF and it harboured a mutation in an Asn-533 → Thr region which is a mutation of the *gyrB* gene [21]. Another study conducted by Mamathaa and Shanthia (2018) from India, observed that 88% of the MDR-TB isolates exhibited a similar DST pattern for all three FLQs tested (OFX, MXF and LFX). However, in eight isolates the cross-resistance among FLQs was not complete [22]. Willby *et al.* (2015) stated that the presence of mutations within the *gyrA* gene QRDRs correlated well with increased MICs (minimum inhibitory concentrations) of OFX, LFX and MXF. They observed that the level of LFX and MXF resistance was dependent on the specific *gyrA* gene mutations present and that a high degree of cross-resistance occurred among MXF, LVX and OFX when a MIC > 0.5 μg/ml was used [23].

In this study we found 2 (15.38%) MDR-TB sample with the *gyrA* gene mutation that did not show phenotypic resistance to any of the FLQs tested. A possible explanation for this may be the fact that most laboratories performing DST for *M. tuberculosis* test only at the critical concentration recommended for that specific testing method. Strains harbouring mutations leading to a higher MIC level than the wild-type strains and equal to or slightly less than the critical concentration would test susceptible with conventional testing methods. However, molecular assays can identify these mutations that result in borderline resistance levels and alert clinicians to possible treatment complications and in some cases the genetic information obtained could be useful in tailoring treatment regimens for the specific patients [9].

The newer FLQs like LFX, GFX and MXF are becoming more common components of TB treatment regimens. The degree of cross-resistance between OFX and these newer FLQs has not been thoroughly investigated. The use of newer FLQs for the management of OFX resistant MDR- and XDR-TB is recommended [23]. In this study we observed that mutations in the *gyrA* gene conferred cross-resistance among the three FLQs tested; OFX, LFX and GFX. The cause of this complete cross-resistance among OFX, LFX and GFX may be associated with the specific locus or loci of mutations in the *gyrA* gene. Malik *et al.* (2012) in their study observed that the A74S + D94G double mutation in the *gyrA* gene conferred complete cross-resistance among OFX, LFX, MXF and ciprofloxacin [9].

It is important to note that the most frequent mutations in clinical isolates are found at codons 90 (A90V), 91 (S91P) and 94 (D94G, D94A, D94N and D94Y) of *gyrA* gene [13] [21] [24]. Every generation of FLQs has the same drug target which is the DNA gyrase enzyme [25]. This enzyme contains a drug-binding pocket called the quinolone-binding pocket (QBP), which consists of both amino acid residues and DNA nucleotides [26] [27]. Mutations in the conserved regions of the QRDRs of the *gyrA* and *gyrB* genes which encode DNA gyrase changes the structure of the QBP and this may result in broad cross-resistance to all FLQs [28] [29].

5. Limitations

Determination of FLQ MICs and correlation between FLQ MICs and specific mutations within the *gyrA* gene by sequencing could not be done in this study due to limitation of time, budget and resources. DST of MXF could not be done because the drug was unavailable during the study period.

6. Conclusion

In this study a significant proportion of MDR-TB cases were resistant to FLQs which is a major concern and obstacle in the successful treatment of MDR-TB patients in Bangladesh. We also observed that mutation in the *gyrA* gene of the MDR-TB strains lead to a complete cross-resistance among the FLQs (OFX, LFX and GFX) tested. It is therefore paramount to carry out the base line resistance and cross-resistance tests for the individual FLQs prior to initiating the treatment of MDR-TB patients in Bangladesh for successful clinical outcomes.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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