First report of molecular detection of fluoroquinolone resistance-associated \(\text{gyrA}\) mutations in multidrug-resistant clinical \textit{Mycobacterium tuberculosis} isolates in Kuwait

Noura M Al-Mutairi, Suhail Ahmad* and Eiman Mokaddas

**Abstract**

**Background:** Nearly 5% of all \textit{Mycobacterium tuberculosis} strains worldwide are resistant at least to rifampicin and isoniazid (multidrug-resistant tuberculosis, MDR-TB). Inclusion of a fluoroquinolone and an injectable agent (kanamycin, amikacin or capreomycin) in multidrug therapy is crucial for proper treatment of MDR-TB. The incidence of MDR-TB in Kuwait is ~1%. MDR-TB strains additionally resistant to fluoroquinolones and injectable agents are defined as extensively drug-resistant (XDR-TB) strains and have been detected in >55 countries. Infections with XDR-TB strains have very poor prognosis. This study detected the occurrence of \textit{gyrA} mutations associated with fluoroquinolone resistance among MDR-TB strains in Kuwait.

**Findings:** Direct DNA sequencing of quinolone resistance-determining region of \textit{gyrA} gene was performed to detect fluoroquinolone resistance-associated mutations in 85 MDR-TB strains isolated from 55 TB patients and 25 pansusceptible \textit{M. tuberculosis} strains. For isolates exhibiting \textit{gyrA} mutations, 3'-end of \textit{rrs} (16S rRNA) was sequenced for the detection of XDR-TB. Fingerprinting of fluoroquinolone resistant MDR-TB strains was performed by detecting mutations in three (81 bp hot-spot, N-terminal and cluster II) regions of \textit{rpoB}, \textit{katG} codon 315 and \textit{inhA}-regulatory region, polymorphisms at \textit{gyrA} codon 95 and \textit{katG} codon 463 by DNA sequencing and by double-repetitive-element PCR for determining strain relatedness. None of the pansusceptible but six of 85 MDR-TB strains contained \textit{gyrA} mutations. Only \textit{gyrA} codon 94 was mutated in all six (D94A in one and D94G in five) strains. Three of six mutant strains were recovered from the same patient while three other strains represented individual patient isolates. Fingerprinting studies identified all individual patient isolates as epidemiologically distinct strains. All six strains with a \textit{gyrA} mutation contained wild-type \textit{rrs} sequence.

**Conclusions:** Although fluoroquinolones are generally not used for chemotherapy of TB and drug susceptibility testing for second-line drugs is not carried out in Kuwait, four of 55 (7%) individual patient MDR-TB strains contained mutations in \textit{gyrA} gene. The data advocate routine drug susceptibility testing for this important second-line drug for proper management of MDR-TB in Kuwait. Lack of mutations in 3'-end of \textit{rrs} gene that confer resistance to injectable agents reduce the likelihood of occurrence of XDR-TB, at present, in Kuwait.

**Keywords:** \textit{M. tuberculosis} Fluoroquinolone resistance, \textit{gyrA} mutations, Kuwait

* Correspondence: suhail_ah@hsc.edu.kw
Department of Microbiology, Faculty of Medicine, Kuwait University, Kuwait

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Background
Tuberculosis (TB), causing nearly 9 million active disease cases and two million deaths worldwide every year, is a major public health issue [1]. Increasing resistance of Mycobacterium tuberculosis strains to most-effective (first-line) anti-TB drugs and strong association of human immunodeficiency virus (HIV) pandemic with active TB disease are the two major contributors to the current global burden of TB [1-3]. Incomplete/improper treatment of TB patients leads to evolution of drug-resistant M. tuberculosis strains due to chromosomal mutations in genes encoding drug targets [4]. Sequential accumulation of mutations in target genes generate multidrug-resistant (resistant at least to rifampicin and isoniazid) M. tuberculosis (MDR-TB) and extensively drug-resistant (additionally resistant to fluoroquinolones and an injectable anti-TB agent such as kanamycin, amikacin or capreomycin) M. tuberculosis (XDR-TB) strains [4,5]. While proper treatment of drug-susceptible TB has ≥95% cure rate, effective treatment of MDR-TB is difficult in developing countries as it is heavily dependent on rapid diagnosis, supervised aggressive therapy with several (5-6) expensive, toxic and less efficacious (second-line) drugs for 18-24 months [4-7]. Inclusion of a fluoroquinolone and an injectable agent in multidrug treatment regimens have a more favorable outcome in the treatment of MDR-TB [4-8]. Treatment of XDR-TB is far more difficult even in developed countries while in developing countries, XDR-TB is virtually an untreatable disease [8-10]. The prognosis of XDR-TB in HIV-infected TB patients is extremely poor, with fatality rates varying from ~30% in developed countries to nearly 100% in developing countries [8-11]. Hence, all efforts should be made to successfully cure the existing MDR-TB cases to avoid the emergence of XDR-TB [4,6,8,12].

Fluoroquinolones (FQs), particularly new generation compounds (such as moxifloxacin and gatifloxacin) and injectable aminoglycosides (kanamycin and amikacin) and cyclic peptide (capreomycin) have excellent bactericidal activity against M. tuberculosis and are crucial for proper management of MDR-TB patients [4,8,12,13]. Widespread emergence of MDR-TB has accelerated development of rapid drug susceptibility testing (DST) procedures for these important second-line drugs to help design effective treatment strategies. The cellular target of FQs in M. tuberculosis is DNA gyrase, a type II topoisomerase consisting of two A and two B subunits encoded by gyrA and gyrB genes, respectively [14]. Mutations in a small region of gyrA, called quinolone resistance-determining region (QRDR) and, less frequently, in gyrB are the primary mechanism of FQ resistance in M. tuberculosis [14-16]. Analysis of QRDR alone by genotypic tests has been suggested as sufficient for rapid identification of vast majority of FQ-resistant M. tuberculosis strains as additional targeting of gyrB did not enhance the sensitivity significantly [16,17]. The molecular basis of resistance of M. tuberculosis strains to injectable agents such as aminoglycosides (kanamycin and amikacin) and cyclic peptide (capreomycin) has also been determined [4,18,19]. Nearly 90% to 95% of M. tuberculosis strains resistant to one or more of the injectable agents contain mutations near the 3’-end of rrs (16S rRNA) gene involving nucleotide positions A1401, C1402, and G1484 [4,17-19].

The rate of MDR-TB is quite low (~1%) and FQs and injectable agents are rarely used for chemotherapy of TB in Kuwait [20]. This study was carried out to detect the occurrence of gyrA mutations associated with fluoroquinolone resistance among MDR-TB strains. The 3’-end of rrs gene was also sequenced among isolates containing gyrA mutations to detect the occurrence of XDR-TB in Kuwait.

Methods
Bacterial isolates and susceptibility testing
A total of 4926 M. tuberculosis strains were isolated from TB patients during the study period (January 2001 to June 2008) at National Tuberculosis Reference Laboratory in Kuwait. A total of 110 M. tuberculosis isolates were analyzed in this study. These included 85 MDR-TB strains isolated from 55 TB patients (representing all available MDR M. tuberculosis strains). Twenty-five drug-susceptible M. tuberculosis strains (isolated from 25 patients) were also included to ensure that fluoroquinolone resistance-conferring mutations in the gyrA gene are not found in pansusceptible M. tuberculosis strains. M. tuberculosis H37Rv was used as a control.

Isolation and identification of M. tuberculosis isolates was performed by using MGIT 960 system (Becton Dickinson) and multiplex PCR as described previously [20,21]. The phenotypic DST against first-line drugs (isoniazid, rifampicin, ethambutol, and streptomycin) was carried out by BACTEC 460 TB system as described in detail previously [20].

DNA extraction for molecular assays
One ml of MGIT 960 culture of reference or clinical M. tuberculosis isolate was heated with 40 mg Chelex-100 (Sigma-Aldrich) at 95°C for 20 min and then centrifuged at 12,000 × g for 15 min [22]. For a PCR, 2 μl of supernatant was used as a source of DNA.

Detection of mutations in QRDR of the gyrA gene by DNA sequencing
The QRDR of the gyrA gene was amplified by touchdown PCR by using GYRAF (5’- CGCGACTACATCGACTATGC-3’) and GYRAR (5’-GGGATGAA
ATCGATGTCTTCCTCG-3’) as amplification primers and the reaction and thermal cycling conditions as described in detail previously [23]. The 400 bp amplicons were purified by using PCR product purification kit (Qiagen) and sequenced by using cycle DNA sequencing kit (DTCS CEQ2000, Beckman-Coulter) and GYR-AFS (5’-CGGGTGTCTATGCAATGTTC-3’) or GYRARS (5’-GGCTTCGGTGACCTCATC GGCC-3’) as internal sequencing primer. Although PCR products were purified, internal primers were used for sequencing of PCR amplicons to avoid interference from trace amounts of primer dimers, if still present. The detailed methodology of DNA sequencing was same as described in detail previously [23]. Nucleotide and amino acid sequences of the amplified products were compared with corresponding sequences from susceptible strain M. tuberculosis H37-Rv using BLAST. DNA sequencing of QRDR also revealed the polymorphism (S95 or T95) that occurs at gyrA codon 95 among M. tuberculosis strains [24].

**Fingerprinting of MDR-TB strains carrying gyrA mutation**

Fingerprinting of MDR-TB strains carrying gyrA mutation was carried out by direct DNA sequencing of hotspot, N-terminal and cluster II regions of rpoB gene, inhA-regulatory region and katG codon 315 to detect mutations conferring resistance to rifampicin and isoniazid, as described in detail elsewhere [25]. The genetic group [24] of the isolates was determined by detecting the polymorphism at gyrA codon 95 (described above) and at katG codon 463 (L463 + T95, Group I; R463 + T95, Group II and R463 + S95, Group III). The presence of R463/L463 at katG codon 463 was detected by PCR amplification with KATG463F and KATG463R primers followed by restriction digestion with Nci I, to generate RFLP patterns, as described previously [26]. Further fingerprinting of the isolates was carried out by double-repetitive-element (DRE)-PCR and isolates yielding unique patterns were classified as genotypically distinct strains [27].

**Detection of mutations at 3’-end of rrs gene by DNA sequencing**

The 3’-end of rrs (16S rRNA) gene that is mutated in nearly 90% to 95% of M. tuberculosis strains exhibiting resistance to injectable agents (kanamycin, amikacin and capreomycin) [17-19] was also amplified by touchdown PCR by using 16S3F (5’-GCAGATGCGCCGAGGT TAAACGAA-3’) and 16S3R (5’-CAACAGTGTGTT GGTGCCCA-3’) as amplification primers and the reaction and thermal cycling conditions described previously [23]. The 403 bp amplicons were purified and sequenced by using 16S3FS (5’-ATCCCTAAAAAGC CGGTCTCAGT-3’) or 16S3RS (5’-CTCCTTAGAAA GGAGGTGATCCA-3’) as internal sequencing primer. Again, internal primers were used for sequencing to avoid interference from trace amounts of primer dimers, if still present along with of PCR amplicons of rrs gene. The detailed DNA sequencing protocol has been described in detail previously [23]. Nucleotide and amino acid sequences of the amplified products were again compared with corresponding sequences from susceptible strain M. tuberculosis H37-Rv using BLAST.

**Nucleotide sequence accession numbers**

The DNA sequencing data reported in this study have been deposited in EMBL under accession numbers FR734170-FR734173.

**Results**

Phenotypic DST data for 85 MDR-TB strains showed that 21 (25%) isolates were resistant to isoniazid and rifampicin only while seven (8%), 23 (27%) and 34 (40%) isolates were additionally resistant to streptomycin, ethambutol and to streptomycin and ethambutol, respectively (Table 1). Of the 55 patients yielding MDR-TB strains, only two were Kuwaiti nationals while the remaining 53 patients were expatriate workers or their family members. The countries of origin for the 53 expatriate patients included India (n = 24), Philippines (n = 10), Egypt (n = 7), Bangladesh (n = 2), Indonesia (n = 2), Syria (n = 2), Iraq (n = 2), Ethiopia (n = 1), Nepal (n = 1), Nigeria (n = 1) and Pakistan (n = 1). The date of arrival in Kuwait was not available for the expatriate patients. All M. tuberculosis isolates were recovered from HIV-seronegative adult TB patients between the ages of 21 to 65 years and were resistant to the indicated drugs on first isolation; however, information on prior treatment of expatriate patients with anti-TB drugs was not available. The 25 M. tuberculosis isolates were susceptible to all first-line drugs (pan susceptible strains).

**Table 1 Resistance patterns and presence of fluoroquinolone resistance-associated gyrA mutations in 110 M. tuberculosis strains tested.**

| Resistance pattern of M. tuberculosis isolate | No. of isolates tested | No. of isolates with gyrA mutation |
|---------------------------------------------|-----------------------|-----------------------------------|
| None                                       | 25                    | 0                                 |
| H, R                                       | 21                    | 0                                 |
| H, R, S                                   | 7                     | 0                                 |
| H, R, E                                   | 23                    | 4*                                |
| H, R, S, E                                | 34                    | 2                                 |
| Total                                      | 110                   | 6                                 |

*H, isoniazid; R, rifampicin, S, streptomycin; E, ethambutol

*3 of 4 M. tuberculosis isolates were recovered from the same TB patient
All 110 isolates were identified as *M. tuberculosis* by a multiplex PCR that yielded 2 DNA fragments of ~473 bp and ~235 bp derived from *oxyR* and *rpoB* genes, respectively, as expected (data from seven selected MDR-TB strains are shown in Figure 1). The PCR amplification of QRDR of *gyrA* gene from reference strain *M. tuberculosis* H37Rv as well as from all 25 pan-susceptible and 85 MDR *M. tuberculosis* strains yielded an expected amplicon of ~400 bp. The DNA sequencing data of all 25 pan-susceptible *M. tuberculosis* strains showed complete concordance with wild-type *gyrA* sequence from reference strain except for polymorphism (S95 or T95) at *gyrA* codon 95, which is not associated with fluoroquinolone resistance [24]. The DNA sequencing data from 85 MDR-TB strains showed nucleotide (and amino acid) changes in QRDR of the *gyrA* gene in six isolates while the remaining 79 strains contained wild-type sequences except for *gyrA* codon 95 (Table 1). Among the six MDR-TB strains, nucleotide changes were detected only at *gyrA* codon 94, with five strains containing GAC94G (D94A) mutation and one isolate containing GAC94GG (D94G) mutation. Interestingly, FQ resistance in MDR-TB strains was associated with additional resistance to ethambutol. Thus, all MDR-TB strains with *gyrA* mutations were resistant to three or all four first-line drugs (Table 1). Three of six FQ-resistant strains were isolated from the same patient within a period of two months while the remaining three isolates were recovered from three separate TB patients. Thus, four of 55 (7%) individual patient MDR-TB strains contained mutations in QRDR of the *gyrA* gene.

The year of isolation and results of fingerprinting studies for the four individual patient MDR-TB strains containing FQ resistance-associated *gyrA* mutations are presented in Table 2. The isolates were recovered over a four-year period (2004 to 2008) from male expatriate patients originating from India (n = 3) and Bangladesh (n = 1). Three patients developed pulmonary TB while the fourth patient had extrapulmonary form of the disease. Fingerprinting of the isolates based on specific mutations in hot-spot, N-terminal and cluster II regions of *rpoB*, *katG* codon 315 and *inhA*-regulatory region and genetic group analyses showed that all four isolates were distinct strains (Table 2). All four isolates also yielded unique DRE-PCR patterns. The two repeat isolates recovered from one patient (no. 3) exhibited the same fingerprinting patterns as the first isolate from this patient (data not shown).

**Discussion**

Kuwait, an Arabian Gulf country with nearly 25 cases per 100,000 population is a low TB incidence country [28]. However, it has a large expatriate population originating from TB endemic countries of South-Southeast Asia. Nearly 600 patients are diagnosed with mycobacterial infections every year. Nearly 95% of mycobacterial infections are caused by *M. tuberculosis* (TB) while the remaining 5% are caused by non-tuberculous mycobacteria [29]. The non-tuberculous mycobacterial infections are more common among Kuwaiti patients, however, ~80% of TB patients are expatriate workers or their family members [20,29]. This is despite the fact that all expatriates are screened for TB and HIV on entry and are allowed to stay in Kuwait only if they exhibit no obvious signs (suspected lesions on chest radiograph) of active TB disease or previous exposure to active disease. Furthermore, all non-infectious TB cases are sent back (after anti-TB therapy of 4-8 weeks, if required) to their respective countries for further management [20]. While global proportion of MDR-TB has been estimated to be ~5.3% among all cases [2,3], only ~1% of *M. tuberculosis* isolates in Kuwait are MDR-TB strains and nearly all of these strains are isolated from expatriate patients [20]. The incidence of extrapulmonary TB in Kuwait is relatively high, accounting for 44% of all TB cases and majority of pulmonary TB patients have cavitary lesions in the upper lobes [20,28]. These findings together with the low incidence of TB in Kuwait are consistent with observations that majority of active TB disease cases in foreign-born persons occur as a result of reactivation of prior (latent) infection, usually within the first few years of their migration [30]. Previous fingerprinting studies

**Figure 1** Species-specific identification of *M. tuberculosis* isolates by multiplex PCR Representative agarose gel of amplicons of multiplex PCR from 7 selected multidrug-resistant *M. tuberculosis* isolates (lanes 1-7) showing *M. tuberculosis*-specific amplification of two fragments of 473 bp and 235 bp (marked by arrows) from *oxyR* and *rpoB* genes, respectively. Lane M is 100 bp DNA ladder and the position of migration of 100 and 600 bp fragments are marked.
have also shown that vast majority of MDR-TB cases among expatriate patients in Kuwait have unique patterns [31,32]. Since fingerprinting patterns are highly variable in countries that have a low incidence of active TB disease and immigrants originating from high incidence countries [33], these observations also support reactivation of previously acquired infection as the major mechanism for active TB disease in most patients in Kuwait.

The FQs play an important role in the treatment of MDR-TB since their inclusion in therapy regimens improves treatment outcome. Resistance of MDR-TB strains to FQs is associated with poor treatment outcome and is also one of two key defining conditions of XDR-TB [4,7-10,12]. Thus, there is a pressing need for rapid DST of MDR-TB strains against FQs to improve clinical management. However, like many other countries [14], routine DST for second-line drugs has not been implemented in the National Tuberculosis Control Program in Kuwait, mainly due to low rate of MDR-TB [20]. Before planning for routine phenotypic and/or genotypic DST for FQs and other second-line drugs in Kuwait, this study was carried out to detect the occurrence of gyrA mutations associated with FQ resistance among MDR-TB strains.

Our data showed that four of 55 (7%) individual patient MDR-TB isolates in Kuwait contained mutations in QRDR of the gyrA gene. Interestingly, all MDR-TB strains with a gyrA mutation were additionally resistant to ethambutol with/without additional resistance to streptomycin. Previous studies have also noted an association of FQ resistance in M. tuberculosis strains with additional resistance to several first-line drugs [15,17].

| Patient no. | Nationality | Clinical specimen | Year of isolation | Resistance pattern | Mutation in QRDR of gyrA gene | Fingerprinting data from analysis of rpoB<sup>a</sup>, inhA-RR<sup>b</sup>, Genetic group<sup>c</sup> |
|-------------|-------------|-------------------|-------------------|-------------------|------------------------------|--------------------------------------------------|
| 1           | Indian      | Pleural fluid     | 2004              | H, R, S, E        | GAC94GGC/D94G               | Wild-type<sup>d</sup> WT                          |
| 2           | Bangladeshi | Sputum            | 2006              | H, R, S, E        | GAC94GCC/D94A               | 9S26Q + D16G WT                                   |
| 3*          | Indian      | Sputum            | 2006              | H, R, E           | GAC94GGC/D94G               | 1572F WT                                         |
| 4           | Indian      | Sputum            | 2008              | H, R, E           | GAC94GGC/D94G               | 5S31L -17G→T                                      |

<sup>aH, isoniazid; R, rifampicin, S, streptomycin; E, ethambutol.</sup>
<sup>bAll four were male patients and all individual patient isolates contained AGC315ACC mutation in katG codon 315 and yielded unique patterns in DRE-PCR.</sup>
<sup>cThree (hot-spot, N-terminal and cluster II) regions of rpoB gene were sequenced to detect rifampicin resistance-conferring mutations and codon numbering system of rpoB gene from Escherichia coli is used [4].</sup>
<sup>dInhA-RR, inhA regulatory region sequencing data.</sup>
<sup>eGenetic groups were assigned based on polymorphisms at katG codon 463 and gyrA codon 95L/463/T95, Group I; R463/S95, Group III</sup>
<sup>fThis isolate either contained a mutation in other regions of the rpoB gene or in other genes involved in rifampicin resistance [25].</sup>
<sup>gTwo repeat isolates recovered from this patient exhibited identical patterns.</sup>
strains. A limitation of the present study is that phenotypic DST for FQs was not performed. Previous studies from several geographical locations have shown that nearly 70% to 100% of FQ-resistant *M. tuberculosis* strains contain mutations in QRDR of the *gyrA* gene while FQ resistance in other isolates is associated with mutations outside of QRDR of *gyrA* gene or due to *gyrB* gene mutations [15, 17, 34-38]. It is, therefore, probable that phenotypic DST may have identified few (one or two) additional FQ-resistant MDR-TB strains in Kuwait. Thus, phenotypic DST should be performed as an important preliminary tool to determine the real prevalence of FQ resistance in Kuwait.

The absence of mutations in 3’-end of *rrs* gene, which confer resistance to kanamycin, amikacin or capreomycin [17-19], among the six FQ-resistant MDR-TB strains is reassuring as it decreases the possibility of the presence of XDR-TB in Kuwait. This is most likely due to very infrequent use of these injectable agents for the treatment of TB in Kuwait.

**Conclusions**

The detection of fluoroquinolone resistance-associated mutations in *gyrA* gene in four of 55 (7%) individual patient MDR-TB strains strongly suggest the need for routine drug susceptibility testing for this important second-line drug for proper management of MDR-TB in Kuwait. However, lack of mutations in 3’-end of *rrs* gene that confer resistance to injectable agents is encouraging and rules out, at least for now, the presence of XDR-TB in Kuwait.

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**Authors’ contributions**

SA and EM designed the study and NMA carried out the experiments. All authors contributed in manuscript writing. All of the authors have read and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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