Minor contribution of mutations at iniA codon 501 and embC-embA intergenic region in ethambutol-resistant clinical Mycobacterium tuberculosis isolates in Kuwait
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Abstract

Background: Ethambutol (EMB) is a first-line drug for the treatment of tuberculosis (TB). Resistance to EMB in Mycobacterium tuberculosis isolates is mediated by mutations in several genes involved in arabinan synthesis notably three emb (arabinosyl transferase) and iniA (isoniazid-inducible) genes. Most epidemiologically unrelated EMB-resistant M. tuberculosis strains contain mutations at embB codons 306, 406 and 497, embC-embA intergenic region (IGR) and iniA codon 501 (iniA501).

Objective: To develop a more comprehensive molecular screen for EMB-resistance detection among epidemiologically unrelated EMB-resistant M. tuberculosis strains previously analyzed for embB codon 306, 406 and 497 mutations by including analysis of mutations at iniA501 and in embC-embA IGR.

Methods: Fifty consecutive and phenotypically documented EMB-resistant and 25 pansusceptible M. tuberculosis strains isolated from 75 different TB patients over a four-year period in Kuwait were analyzed. Mutations at iniA501 were detected by PCR amplification followed by restriction fragment length polymorphism (RFLP) patterns generated with Hpy 99 I. Direct DNA sequencing was used to confirm RFLP results and for detecting mutations in embC-embA IGR.

Results: Nearly same number of EMB-resistant M. tuberculosis strains were resistant to EMB alone and EMB together with additional resistance to rifampicin and isoniazid (9 of 50, 18% and 11 of 50, 22%, respectively). All the 25 pansusceptible strains contained wild-type sequences at iniA501 and in embC-embA IGR. The analysis of 50 EMB-resistant M. tuberculosis isolates showed that only one strain contained a mutated iniA501 while no mutation was detected in embC-embA IGR in any of the isolate.

Conclusion: Analysis of iniA501 and embC-embA IGR in epidemiologically unrelated EMB-resistant M. tuberculosis isolates in Kuwait indicate that mutations at these locations occur very infrequently and their inclusion for the development of a comprehensive molecular screen will make only minor contribution towards rapid EMB resistance detection.
Background

The tuberculosis (TB) epidemic continues unabated. Despite intense efforts made over the past two decades, the morbidity and mortality associated with TB remain high, with 8 million active disease cases and 2 million deaths occurring worldwide every year [1,2]. Two factors, co-infection with human immunodeficiency virus (HIV) and increasing incidence of infections with drug-resistant strains of Mycobacterium tuberculosis are steadily worsening the problem of TB [3,4]. The latest World Health Organization (WHO)-sponsored study showed that drug-resistant TB among new cases is prevalent in 74 of 77 (96%) settings or countries with resistance to at least one anti-TB drug varying from 0% in some rich, developed countries to >30% in several developing countries [4]. Infections with drug-resistant, particularly multidrug-resistant (MDR) (resistant to at least rifampicin and isoniazid) strains of M. tuberculosis (MDR-TB) are associated with higher mortality [4,5]. Molecular methods for rapidly identifying drug-resistant strains of M. tuberculosis are urgently needed to avoid inadequate treatment since phenotypic drug susceptibility testing by radiometric method requires 4–14 days after the primary culture has been isolated [6]. A simple and rapid line probe assay has been developed recently for detection of resistance of M. tuberculosis to rifampicin and isoniazid (MDR-TB) in cultured isolates and clinical samples [7].

Ethambutol (EMB), an arabinose analogue, is a bactericidal, first-line drug for the treatment of TB. The EMB is often used, along with isoniazid, rifampicin and pyrazinamide, as an alternative to streptomycin, in the four-drug regimens advocated by World Health Organization under the directly observed chemotherapy short-course. Global data on drug resistance patterns have shown that resistance of M. tuberculosis to EMB in newly diagnosed as well as previously treated cases is much less compared to streptomycin [4,8]. Similar pattern is also noted in the rate of resistance of M. tuberculosis isolates to EMB (1.98%) and streptomycin (4.31%) in Kuwait [9]. Since resistance of M. tuberculosis to EMB is also generally associated with resistance to other anti-TB drugs [8,10], early detection of resistance will not only abolish drug-associated adverse reactions, particularly optic neuritis, it will also suggest modifications in therapy regimens.

The mechanism of action and the molecular genetic basis of resistance to EMB are complex and are not completely defined. The enzymes participating in synthesis and polymerization of cell wall arabinan are implicated as the main target for EMB. These include three homologous and membrane associated arabinosyltransferases encoded by three contiguous genes, embC-embA-embB, isoniazid-inducible genes particularly iniA, acyl carrier proteins and regulatory proteins modulating their expression [11-13]. Mutations in embB particularly involving codon 306 (embB306) and less frequently, codons 406 (embB406) and 497 (embB497) have been identified as most common genetic alterations conferring resistance to EMB in clinical M. tuberculosis isolates [11-14]. The frequency of these mutations in EMB-resistant strains in some studies has been reported to be ~70% [14-17]. However, in epidemiologically unrelated strains, these mutations accounted for EMB resistance in only around 30% EMB-resistant isolates [13,18]. Only one study has so far detected mutations in other emb (embA and embC) or other genes conferring resistance to EMB in epidemiologically unrelated EMB-resistant strains [13]. The data showed that, mutations in embC-embA intergenic region (IGR) (four nucleotide positions), embA (several codons) and iniA (mainly codon 501, iniA501) occurred in ~30% EMB-resistant strains while mutations in eight other genes were either absent or occurred infrequently [13]. However, the role of mutations in these loci in conferring resistance to EMB remain unclear as these mutations, particularly in embC-embA IGR, were found in isolates with other well-characterized EMB resistance conferring mutations [13]. The TCG to TGG mutation at iniA501 most likely confers resistance of M. tuberculosis to EMB as it alters the structural property of the encoded protein [13]. However, the resistance conferring mechanism explaining the role of mutations in embC-embA IGR is not well characterized and the paucity of information on the frequency of mutations in embC-embA IGR in EMB-resistant M. tuberculosis strains from different regions of the world makes it difficult to ascertain their exact role in conferring resistance to EMB. The present study was carried out to detect the frequency of mutations at iniA501 and in embC-embA IGR in 50 consecutive and epidemiologically unrelated EMB-resistant M. tuberculosis strains that were analyzed previously for embB306, embB406 and embB497 mutations [18] in an effort to develop a more comprehensive molecular screen for EMB-resistance detection. The EMB-susceptible isolates were also analyzed simultaneously to ensure that such mutations are not present in EMB-susceptible strains.

Materials and methods

EMB-resistant and -susceptible M. tuberculosis strains

A single TB control unit and the Kuwait National Tuberculosis Reference Laboratory (KNTRL) under the Ministry of Health are responsible for the diagnosis and treatment of all TB patients in Kuwait. Appropriate specimens from suspected TB patients are sent to KNTRL for culture. All active TB cases are diagnosed by culture and susceptibility testing to first line anti-TB drugs is performed on all the isolates identified as M. tuberculosis [19]. All the 50 EMB-resistant M. tuberculosis strains (KE1 to KE50) isolated throughout Kuwait from 50 different TB patients during 2000 to 2003 were included in this study. The clinical...

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background and demographic data of patients yielding EMB-resistant *M. tuberculosis* strains are shown in Table 1. Repeat isolates were recovered from eight TB patients within one to two months of isolation of the first isolate. A total of 25 *M. tuberculosis* strains susceptible to all first-line drugs (pansusceptible strains) were also included. The isolation and identification of *M. tuberculosis* strains from clinical specimens was performed using the mycobacterial growth indicator tube (MGIT) 960 system as described previously [20]. The drug susceptibility testing of *M. tuberculosis* isolates was performed using the BACTEC 460 TB system as reported earlier [6,21]. The isolates were considered EMB resistant when bacterial growth occurred at a concentration of 2.5 μg EMB per ml. Resistance of all the isolates to isoniazid (0.1 μg/ml), rifampicin (2 μg/ml) and streptomycin (2 μg/ml) was also determined.

**Sample preparation for PCR**

The *M. tuberculosis* reference strain H37Rv was used as a control in PCR-RFLP and DNA sequencing. The reference strain and clinical EMB-resistant and pansusceptible *M. tuberculosis* isolates were obtained as heat killed BACTEC liquid cultures. One ml of BACTEC culture was heated with 40 mg Chelex-100 (Sigma) at 95°C for 20 min followed by centrifugation at 12,000 × g for 15 min and the supernatant obtained was used as the source of genomic DNA [22] for the amplification of various gene regions.

**Primer design and PCR amplification**

Two primer pairs were synthesized. One primer pair (INIAF and INIAR) was used for the amplification of the *iniA* gene (Genbank accession no. Z95324) region around codon 501. Another primer pair (IGRF and IGRR) was synthesized for the amplification of *embC-embA* IGR (Genbank accession no. AL123456) including nucleotide positions that are mutated in EMB-resistant strains [13]. The DNA sequences of the primers, their positions on the target genes and the expected size of the PCR products are shown in Table 2.

The PCR amplification of the target DNA was carried out in a final volume of 50 μl and contained 5 μl of 10× Perkin-Elmer PCR buffer, 10 pmol of the appropriate forward (F) and reverse (R) primers (INIAF and INIAR or IGRF and IGRR, Table 2), 2 μl of template DNA, 0.1 mM dNTPs, 2 units of AmpliTaq DNA polymerase (Perkin-Elmer) and sterile distilled water. The cycling parameters were same as described previously [22]. Following amplification, a portion of the product (10 μl) was run on 2% agarose gels [22] to confirm the amplification of a DNA fragment of expected size. The remaining PCR product was purified by using PCR purification columns (Qiagen) that were used as instructed by the manufacturer. The purified amplicons were used for further studies.

**Detection of mutations at iniA501 by PCR-RFLP**

The mutations at *iniA501* were detected by restriction digestion of amplicons obtained with primers INIAF and INIAR with *Hpy* 99I as described previously [22]. Briefly, the reaction mixture in a final volume of 25 μl contained 5 μl of the purified amplicon, 10.25 μl of sterile water, 6.25 μl of NE Buffer 4, 2.5 μl of 10× BSA and 1 μl (2 units) of *Hpy*99 I (New England Biolabs). After incubation at 37°C for 1 h, the digested products were separated on 2.5% agarose gels. The amplicon from *M. tuberculosis* H37Rv as well as *M. tuberculosis* strains containing TCG at *iniA501* (*iniA501TCG*) yield two fragments of 104 bp and 137 bp while the amplicon from isolates containing TGG at *iniA501* (*iniA501TGG*) yield the original undigested fragment of 241 bp [22]. The results of RFLP for some of the isolates were confirmed by DNA sequencing. The DNA sequencing was performed by using the cycle DNA sequencing kit (DTCS CEQ2000, Beckman Coulter) as described in detail previously [22]. Briefly, the reaction mixtures in a final volume of 20 μl contained, 8.0 μl of purified amplicon, 1.0 μl (3.2 pmol) of primer INIAF or INIAR (Table 2) or an internal primer (INIAS, 5′-CGCT-GGGCCGGATGGAATCGAA-3′), 8.0 μl of the pre-mix reaction components supplied in the kit and 3.0 μl sterile water. The cycling parameters were same as described previously [22]. Reactions products were precipitated and loaded on the DNA sequencer as directed by the manufacturer (Beckman Coulter Model CEQ8800). The DNA sequences of the amplicons, obtained by using IGRF and IGRR primers. The DNA sequencing was carried out, as described above except that primer IGRF or IGRR (Table 2) or an internal primer (IGRS, 5′-CCGCTGATCTGAACCTAGGAAC-3′) was used as sequencing primer.

**Detection of mutations in embC-embA IGR by DNA sequencing**

The mutations at -11, -12, -16 and -43 positions (relative to the translational start point of *embA*) in *embC-embA* IGR [13] were detected by direct DNA sequencing of purified amplicons, obtained by using IGRF and IGRR primers. The DNA sequencing was carried out, as described above except that primer IGRF or IGRR (Table 2) or an internal primer (IGRS, 5′-CGCTGATCTGAACCTAGGAAC-3′) was used as sequencing primer.

**Molecular fingerprinting of EMB-resistant *M. tuberculosis* isolates**

The molecular fingerprinting of EMB-resistant *M. tuberculosis* isolates was performed by genetic group analysis [23] based on polymorphisms at katG codon 463 (*katG463*) and gyrA codon 95 (*gyrA95*) and by touchdown double-repetitive-element (DRE)-PCR. The presence of Arg463/Leu463 at katG463 and Ser95/Thr95 at gyrA95 were determined by PCR-RFLP as described previously [24,25]. Briefly, the amplification of *katG463* and *gyrA95* DNA regions was carried out as described above except that primers KatG1F (5′-CCCGAGGAATTGGCCGAAGTCGG-3′) + KatG1R (5′-GGTGCGAATGACCTTGCGGTCGGG-3′) [24] and GYRA95F (5′-CCGCTGATCTGAACCTAGGAAC-3′) + GYRA95R (5′-GGGTGGTGTCGGAGTCGTTT-3′) 

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**Table 1:** Background and demographic data of patients yielding EMB-resistant *M. tuberculosis* strains

| Patient No. | Age (years) | Sex | Occupation | Location | Isolation Date | Drug Susceptibility |
|-------------|-------------|-----|------------|----------|---------------|-------------------|
| 1           | 25          | M   | Farmer     | Village 1| 2009-03-15    | EMBsusceptible    |
| 2           | 32          | F   | Student    | Village 2| 2009-04-01    | EMBsusceptible    |
| 3           | 40          | M   | Worker     | Village 3| 2009-04-15    | EMBsusceptible    |
| 4           | 38          | F   | Teacher    | Village 4| 2009-05-01    | EMBsusceptible    |

**Table 2:** Primers used for PCR amplification

| Primer | Sequence (5′→3′) |
|--------|------------------|
| INIAF  | GATGACATGGAATCGAA |
| INIAR  | GGGTTGTACCGTGGATC |
| IGRF   | GATGGAATCGAA      |
| IGRR   | GGGTTGTACCGTGGATC |

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Table 1: Clinical background, resistance patterns and demographic information of patients yielding EMB-resistant *M. tuberculosis* isolates in Kuwait

| Isolate no. | Year of isolation | Clinical specimen | Resistance pattern | Patient demographics |
|-------------|-------------------|-------------------|-------------------|---------------------|
| KE1         | 2000              | Sputum            | E, H, R           | Indian              | SA       | 43       | Male     |
| KE2         | 2000              | Sputum            | E, H, R           | Indian              | SA       | 45       | Male     |
| KE3         | 2000              | Sputum            | E, H              | Egyptian            | ME       | 47       | Female   |
| KE4         | 2000              | Sputum            | E, H, S           | Indian              | SA       | 27       | Male     |
| KE5         | 2000              | Sputum            | E, H              | Indian              | SA       | 42       | Male     |
| KE6         | 2000              | Pus               | E, H              | Sri Lankan          | SA       | 27       | Male     |
| KE7         | 2000              | Abscess           | E, H              | Pakistani           | ME       | 55       | Male     |
| KE8         | 2000              | Sputum            | E, H, S           | Kuwaiti             | ME       | 45       | Male     |
| KE9         | 2000              | Pus               | E, H, S           | Indian              | SA       | 45       | Female   |
| KE10        | 2000              | Sputum            | E, H              | Bangladeshi         | SA       | 40       | Female   |
| KE11        | 2001              | Sputum            | E, H              | Filipino            | SEA      | 35       | Female   |
| KE12        | 2001              | Sputum            | E, H              | Indian              | SA       | 22       | Male     |
| KE13        | 2001              | Sputum            | E, H, R           | Indian              | SA       | 47       | Male     |
| KE14        | 2001              | Sputum            | E, H, R, S        | Indian              | SA       | 27       | Female   |
| KE15        | 2001              | Sputum            | E, H, R, S        | Indian              | SA       | 32       | Male     |
| KE16        | 2001              | FNA               | E, H              | Indian              | SA       | 27       | Female   |
| KE17        | 2001              | Sputum            | E, H              | Indian              | SA       | 30       | Male     |
| KE18        | 2001              | LN                | E, H              | Filipino            | SEA      | 31       | Male     |
| KE19        | 2001              | Sputum            | E, H              | Indian              | SA       | 45       | Male     |
| KE20        | 2001              | Sputum            | E, H              | Egyptian            | ME       | 49       | Male     |
| KE21        | 2001              | Sputum            | E, H, S           | Indian              | SA       | 25       | Male     |
| KE22        | 2001              | Sputum            | E, H, R, S        | Egyptian            | ME       | 27       | Female   |
| KE23        | 2002              | Sputum            | E                  | Kuwaiti             | ME       | 65       | Male     |
| KE24        | 2002              | Sputum            | E, H              | Egyptian            | ME       | 45       | Male     |
| KE25        | 2002              | Pus               | E, H              | Kuwaiti             | ME       | 34       | Male     |
| KE26        | 2002              | FNA               | E, S              | Indian              | SA       | 21       | Male     |
| KE27        | 2002              | Pus               | E                  | Indian              | SA       | 30       | Female   |
CCTATCGCC-3') [25], respectively, were used. The purified katG463 region amplicons were digested with restriction enzyme Nci I to obtain RFLP patterns. The reaction mixture in a final volume of 25 μl contained 2.5 μl of BRL Buffer 8 (Gibco-BRL), 5 μl of amplicon, 2 μl (10 units) of Nci I (Gibco-BRL), and 15.5 μl of sterile water. After incubation at 37°C for 4 h, the digested products were separated on 2.5% agarose gels. The amplicon from M. tuberculosis H37Rv as well as M. tuberculosis strains containing Arg at katG463 (katG463CGG) yield two fragments of 187 bp and 173 bp while the amplified fragment from isolates containing Leu at katG463 (katG463CTG) yield

### Table 1: Clinical background, resistance patterns and demographic information of patients yielding EMB-resistant M. tuberculosis isolates in Kuwait (Continued)

| Patient Code | Year | Specimen | Drugs | Ethnicity | Region | Age | Sex |
|--------------|------|----------|-------|-----------|--------|-----|-----|
| KE28         | 2002 | BAL      | E, H  | Bangladeshi | SA     | 32  | Male |
| KE29         | 2002 | GS       | E, R  | Syrian     | ME     | 57  | Female |
| KE30         | 2002 | Sputum   | E, H, R, S | Nepalese | SA | 23  | Female |
| KE31         | 2002 | FNA      | E     | Indian     | SA     | 31  | Male |
| KE32         | 2002 | Sputum   | E     | Kuwaiti    | ME     | 25  | Female |
| KE33         | 2002 | Sputum   | E, H  | Pakistani  | ME     | 38  | Male |
| KE34         | 2002 | Sputum   | E, H  | Filipino   | SEA    | 27  | Female |
| KE35         | 2002 | Sputum   | E     | Indian     | SA     | 29  | Male |
| KE36         | 2003 | LN       | E     | Yemani     | ME     | 31  | Male |
| KE37         | 2003 | Pus      | E     | Kuwaiti    | ME     | 23  | Male |
| KE38         | 2003 | FNA      | E, H, S | Pakistani | ME | 23  | Male |
| KE39         | 2003 | Sputum   | E     | Indian     | SA     | 26  | Male |
| KE40         | 2003 | FNA      | E     | Indian     | SA     | 30  | Male |
| KE41         | 2003 | Sputum   | E, H  | Kuwaiti    | ME     | 18  | Female |
| KE42         | 2003 | LN       | E, H  | Indian     | SA     | 25  | Female |
| KE43         | 2003 | FNA      | E, H  | Kuwaiti    | ME     | 37  | Male |
| KE44         | 2003 | Sputum   | E, H  | Pakistani  | ME     | 54  | Male |
| KE45         | 2003 | PF       | E, H  | Indian     | SA     | 45  | Male |
| KE46         | 2003 | Sputum   | E, H, R, S | Indian | SA | 47  | Male |
| KE47         | 2003 | Pus      | E, H, R, S | Iranian | ME | 35  | Male |
| KE48         | 2003 | Sputum   | E, H, R, S | Egyptian | ME | 24  | Male |
| KE49         | 2003 | FNA      | E, H, R, S | Egyptian | ME | 65  | Female |
| KE50         | 2003 | PF       | E, H  | Indian     | SA     | 46  | Male |

*FNA, fine needle aspirate; LN, lymph node; BAL, bronchoalveolar lavage; GS, gastric secretion; PF, pleural fluid

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

*SA, South Asia; ME, Middle East; SEA, Southeast Asia
the original undigested fragment of 360 bp [24]. The purified gyrA95 region amplicons were digested with restriction enzyme \( \text{Ale} \) I to obtain RFLP patterns. The reaction mixture in a final volume of 25 \( \mu \)l contained, 2.5 \( \mu \)l of NE Buffer 4, 1.25 \( \mu \)l of 10× BSA (New England Biolabs), 6.0 \( \mu \)l of the amplified DNA, 1 \( \mu \)l (5 units) of \( \text{Ale} \) I and 14.25 \( \mu \)l of sterile water. After incubation at 37°C for 3 h, the digested products were separated on 2.5% agarose gels. The amplicon from \( M. \) tuberculosis H37Rv as well as \( M. \) tuberculosis strains containing Ser at gyrA95 (\( \text{gyrA95AGC} \)) yield the original undigested fragment of 322 bp while the amplified fragment from isolates containing Thr at gyrA95 (\( \text{gyrA95ACC} \)) yield two fragments of 212 bp and 110 bp [25].

The typing of EMB-resistant \( M. \) tuberculosis isolates was performed by touchdown DRE-PCR as described previously [26]. Briefly, the reaction mixtures in a final volume of 50 \( \mu \)l contained \( 1 \times \) Perkin-Elmer PCR buffer II, 2.5 mM MgCl\(_2\), 10 pmol each of the four primers (\( \text{IS6110-5'R} \), \( \text{IS6110-3'F} \), \( \text{PGRS-5'R} \), \( \text{PGRS-3'F} \)) [26], 2 \( \mu \)l of template DNA, 0.2 mM dNTPs and 2 units of AmpliTaq DNA polymerase. The cycling parameters were same as described previously [26]. The amplified products (20 to 25 \( \mu \)l) were resolved on 2% agarose gels. The \( M. \) tuberculosis isolates belonging to different genetic groups and/or yielding unique patterns of DNA amplified fragments in DRE-PCR were considered as genotypically distinct strains.

### Table 2: Primer sequences and their locations in target genes used for PCR amplification of \( \text{iniA501} \) and \( \text{embC-embA IGR} \)

| Primer pair | Primer name | Primer sequence | Target gene | Direction/position | Target region | Amplicon size (bp) | Mutations detected by |
|-------------|-------------|-----------------|-------------|--------------------|---------------|-------------------|----------------------|
| 1           | INIAF       | 5'-CGCTGGGCGGGA TGGAATCGAA-3' | iniA | F, 1403–1424 | Codon 501 | 241 | Hpy 99 I RFLP |
|             | INIAR       | 5'-ACGAAGCGGCGGC ACATTGGGCTT-3' |             | R, 1643–1621 |             |             |                      |
| 2           | IGRF        | 5'-GTCAACGGGGT GACCCCTACTA-3' | IGRc | F, 29061–29084 | -11 to -43d | 261 | Sequencing |
|             | IGRR        | 5'-GTTACAGGGTT GACCCCTACTA-3' |             | R, 29321–29298 |             |             |                      |

The DNA sequencing data for the mutant strain reported in this study have been deposited in EMBL under the accession number AJ973188.

### Results and Discussion

A total of 50 EMB-resistant strains of \( M. \) tuberculosis were isolated from 50 different TB patients throughout Kuwait during the year 2000 to 2003 and all the isolates were included for analysis. Twenty of these isolates were from patients of Middle-Eastern origin (including seven Kuwaiti nationals), 27 from patients of South-Asian origin and three from patients of Southeast Asian countries (Table 1). All the isolates were recovered from HIV-negative adult TB patients (18 to 65 years) and included 15 female and 35 male patients. The clinical specimens yielding these strains included sputum (n = 29), pus (n = 6), fine needle aspirate (n = 7), lymph node (n = 3), pleural fluid (n = 2), abscess (n = 1), bronchoalveolar lavage (BAL) (n = 1), and gastric secretion (n = 1) (Table 1). In addition, 25 pansusceptible clinical \( M. \) tuberculosis isolates were also included in the study. Nine, 12 and four of these isolates were from patients of Middle-Eastern (including three Kuwaiti nationals), South Asian, and Southeast Asian origin, respectively. The clinical specimens yielding pansusceptible strains included sputum samples (n = 29), pus (n = 6), fine needle aspirate (n = 7), lymph node (n = 3), pleural fluid (n = 2), abscess (n = 1), bronchoalveolar lavage (BAL) (n = 1), and gastric secretion (n = 1) (Table 1). In addition, 25 pansusceptible clinical \( M. \) tuberculosis isolates were also included in the study. Nine, 12 and four of these isolates were from patients of Middle-Eastern (including three Kuwaiti nationals), South Asian, and Southeast Asian origin, respectively. The clinical specimens yielding pansusceptible strains included sputum samples (n = 15), pus (n = 6), fine needle aspirate (n = 3), and tissue biopsy (n = 1). The data are consistent with previously reported observations that majority (~80%) of \( M. \) tuberculosis infections in Kuwait occur in foreign-born expatriate workers mostly within the first few years of their migration even though all expatriates entering Kuwait are screened for TB (chest radiograph) as well as for HIV infection [9,27].
Only nine of 50 (18%) EMB-resistant strains were monoresistant to EMB while the remaining (41 of 50, 82%) isolates were additionally resistant to at least one more first-line drug. Most (39 of 41) of the latter isolates were resistant to isoniazid or without additional resistance to other drugs. Twenty-four (48%) M. tuberculosis strains were resistant to two drugs while eight (16%) isolates were resistant to three drugs (including three MDR-TB strains). Only eight (16%) EMB-resistant strains were also resistant to the other three first-line drugs. All the isolates were resistant to EMB on first isolation indicating that the patients were already infected with EMB-resistant strains. Furthermore, nearly same number (9 of 50, 18% and 11 of 50, 22%) of EMB-resistant M. tuberculosis strains were resistant to EM only or EM together with additional resistance to rifampicin and isoniazid (MDR-TB strains). Worldwide, monoresistance to EM is rare and majority of EM-resistant strains particularly those originating from TB endemic countries are MDR-TB strains [8,17,28].

The PCR amplification of iniA501 DNA region from 25 pansusceptible and 50 EM-resistant M. tuberculosis strains yielded an amplicon of 241 bp, as expected. The Hpy 99 I digestion patterns of purified amplicons showed that all the 25 pansusceptible and 49 EM-resistant strains contained TCG at iniA501 (iniA501TCG) while only one EM-resistant strain contained a mutated iniA501. The results of PCR-RFLP for three pansusceptible strains containing iniA501TCG, three EM-resistant strains also containing iniA501TCG and one EM-resistant strain containing a mutated iniA501 were confirmed by DNA sequencing. The solitary isolate with a mutation at iniA501 contained iniA501TCG (Table 3). Likewise, the PCR amplification of DNA region encompassing embC-embA IGR from 25 pansusceptible and 50 EM-resistant M. tuberculosis strains also yielded an amplicon of 261 bp, as expected. Direct DNA sequencing of the purified amplicons showed that all the 25 pansusceptible and 50 EM-resistant strains contained wild-type sequences including nucleotide positions -11, -12, -16 and -43 [12] relative to the translational start point for embB gene (Table 3). The repeat isolates recovered from eight TB patients yielded the same resistance pattern and the same results for iniA501 and embC-embA IGR analyses as the parent isolate (data not shown).

Consistent with previously reported data showing that majority of rifampicin-resistant and isoniazid-resistant M. tuberculosis infections in Kuwait occur in foreign-born TB patients as a result of reactivation of previously acquired infection [21,29,30], genetic group analysis and fingerprinting patterns obtained in DRE-PCR showed that majority of EMB-resistant M. tuberculosis strains were also unique strains (data not shown).

Majority (~70%) of EMB-resistant M. tuberculosis strains from several geographical locations around the world have been shown to contain mutations within embB gene particularly at embB306, embB406 and embB497 [14-17]. However, in most of these studies, all or consecutive EM-susceptible and EMB-resistant M. tuberculosis strains from a single location and/or isolated over a specified period of time were not included and the proportion of MDR-TB strains or strains resistant to several anti-TB drugs was unusually high [15-17]. When the 50 phenotypically documented EMB-resistant M. tuberculosis strains used in this study were tested for genotypic resistance detection targeting mutations at embB306, embB406 and embB497, only 38% (19 of 50) isolates contained a resistance conferring mutation [17]. These findings suggested that mutations at other codon positions within embB gene or in other loci are responsible for conferring resistance to EM in majority of EM-resistant M. tuberculosis strains in Kuwait and their identification will be crucial for the development of a molecular screen for rapid detection of majority of EMB-resistant strains.

The results presented in this study showed that mutations at iniA501 and in embC-embA IGR in EMB-resistant M. tuberculosis strains in Kuwait also occur rather infrequently (1 of 50, 2%). Thus, their inclusion in the molecular screen will have only minor contribution towards detection of EMB resistance in Kuwait. Only one previous study has detected the occurrence of mutations at iniA501 and in embC-embA IGR in EMB-resistant M. tuberculosis strains [13]. Although the authors reported that mutations at iniA501 and in embC-embA IGR occurred in nearly 20%

| Susceptibility to ethambutol | No. of isolates | No. of isolates with iniA501 as Wild-type (TCG) | No. of isolates with wild-type nucleotides at -11, -12, -16 and -43 in embC-embA IGR Mutant (TGG) |  |
|-----------------------------|----------------|------------------------------------------|--------------------------------------|---|
| Susceptible                 | 25             | 25                                       | 0                                    | 25 |
| Resistant                   | 50             | 49                                       | 1                                    | 50 |

Table 3: Occurrence of mutations at iniA501 and embC-embA IGR in 25 pansusceptible and 50 EM-resistant clinical M. tuberculosis strains isolated in Kuwait
(15 of 75) of EMB-resistant strains, majority of isolates with mutations at iniA501 and all the isolates with a mutation in embC-embA IGR also contained other well characterized EMB resistance-conferring mutations, particularly at embB306, embB406 and embB497 [13]. Previous studies have shown that drug-resistant M. tuberculosis strains isolated from TB patients with limited previous exposure to first-line anti-TB drugs usually contain single point mutations in resistance conferring genes while strains from previously treated patients usually contain multiple mutations in target genes [15,18,20,31-33]. The susceptibility of EMB-resistant strains to other first-line drugs and the HIV status of the TB patients in the study that analyzed mutations at iniA501 and in embC-embA IGR was not indicated [13]. However, the presence of mutations in two or more genes involved in EMB resistance in 25% (19 of 75) EMB-resistant strains and the geographical origin of the tested isolates indicates that the isolates were either MDR-TB strains or strains additionally resistant to multiple first-line drugs [13]. These observations are also consistent with the role of mutations at iniA501 and in embC-embA IGR in conferring resistance to EMB.

The TCG to TGG mutation at iniA501 most likely confers resistance of M. tuberculosis to EMB by altering the structural property of the encoded protein [13]. However, the resistance conferring mechanism explaining the role of mutations in embC-embA IGR in EMB resistance is not well characterized. The suggestion that C to T mutations at -12 and -16 positions lead to creation of TATA box-like sequences [13] seems less likely since these elements are found in promoter elements while the hot-spot DNA segment is immediately upstream of the translational start point for EmbA and putatively involves ribosomal binding site. Furthermore, other characteristic features of promoter elements (such as -35 element sequences) are also absent in the surrounding DNA region. An alternative (titration) mechanism to explain the role of mutations in embC-embA IGR is summarized below and most likely involves premature termination of some transcripts originating from embCAB promoter before embA and embB genes are transcribed. The embC-embA IGR of 85 nucleotides is rather long for a polycistronic bacterial mRNA. A closer look at embC-embA IGR and N-terminal region of embA indicates that it contains an L-shaped transcriptional terminator typically found in mycobacteria including M. tuberculosis [34]. The RNA transcript involving -19 to +23 nucleotides (relative to translational start site of embA) can form a hairpin structure that is stabilized by nine G:C pairs including the two ‘C’ residues (at -12 and -16 positions) that are mostly mutated in some EMB-resistant strains [13]. Thus, mutations of -12C or -16C residues will decrease the stability of the hairpin structure resulting in decreased termination of transcripts and consequently, increased expression of embA and embB encoded gene products. These assumptions are further supported by occurrence of nearly all the mutations in embC-embA IGR in isolates with a mutated embB gene. The above also explains the absence of these mutations in EMB-resistant M. tuberculosis isolates in Kuwait as such mutations are more likely to occur in TB patients with previous history of prolonged therapy with anti-TB drugs [15,18,20,31-33].

Conclusion

In conclusion, the results presented in this study showed that mutations at iniA501 and in embC-embA IGR in EMB-resistant M. tuberculosis strains isolated from TB patients in Kuwait occur infrequently. Consequently, inclusion of mutation detection at these loci in the molecular screen for detecting majority of EMB-resistant M. tuberculosis strains will contribute minimally towards this goal in Kuwait as well as, perhaps, in other countries. Further studies from other geographical regions are clearly warranted to validate our observations.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

SA and EM participated in the design of the study, analyzed the data and drafted the manuscript. They also arranged financial support for the study. A-AJ carried out the experimental work. All authors have read and approved the final manuscript.

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