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Characterization of Hypervariable Region of Gyra And Gyrb Genes of Mycobacterium Tuberculosis from Jeddah, KSA

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Abstract
Mycobacterium tuberculosis (MTB) is an old enemy of the human race, with evidence of infection observed as early as 5000 years ago.MTB infection is relatively high in Jeddah as compared to other cities of Saudi Arabia due to high influx of people from across the globe for pilgrimage. This study aimed to investigate the phenotypic drug resistance for the first line antituberculous drugs and to explore mutations in fluoroquinolone resistance gyrA and gyrB genes in *Mycobacterium tuberculosis* (MTB) isolates from tuberculosis patients from Jeddah, Saudi Arabia during 2015. Firstly, phenotypic drug susceptibility tests (DST) were performed for first line antituberculous drugs for all the MTB isolates. DST for rifampicin, isoniazid,streptomycin, ethambutol, and pyrazinamide were performed. Then the hypervariable regions of gyrA and gyrB genes were sequenced to identify mutations for Fluoroquinolones (FQs) resistance . Overall, all the MTB isolates were susceptible to Sterpt, RIF and Eth. Where as resistance to INH and Pyr were 2% and 14% respectively. Genotypic resistance revealed mutations in gyrA and gyrB genes among 96% (48/50) of FQ resistant isolates. 96% (48/50) of FQ resistant isolates showed mutations at codons 95 (S95T) and three pattern of double mutations in gyrA gene ; six with E21Q & S95T, and the two with I29S & S95T, and one with A90V & S95T. The mutation in gyrB gene was identified in two of 50 clinical isolates in this study . K421R and T420. For clinical isolates, gyrB mutations appear to be of much rarer occurrence.We conclude that occurrence of mutations at only four codons in gyrA and two codon in gyrB genes among FQ resistant isolates may assist in development of rapid molecular method for FQ resistance detection. Presence of mutations among more than fifty percent of intermediate susceptible FQ MTB isolates could also serve as a predictor for pre-resistant isolates.

1. Introduction

Tuberculosis (TB) remains one of the most serious infectious diseases worldwide, causing 1.8million deaths each year(Bernard et al., 2015). Several millions of people visit the Kingdom of Saudi Arabia Muslims to perform their religious rituals. Big mass gatherings during Hajj and Omra increase the risk for TB transmission. High proportions of these people come from highly endemic TB areas of Asia and Africa region. All these factors lead to increased risk of TB in KSA, which require more public health efforts to cope with these challenges(Al-Otaibi and El Hazmi, 2010). The emergence and transmission of drug-resistant Mycobacterium tuberculosis (MTB) strains further threaten TB control efforts. The treatment of infection due to multidrug-resistant (MDR) TB (i.e., that which is at least resistant to isoniazid and rifampin) requires the use of fluoroquinolones (FQ), since it is correlated with good prognosis .FQ are antibiotics with broad-spectrum antimicrobial activities and are therefore widely used for the treatment of bacterial infections of the respiratory, gastrointestinal, and urinary tracts, as well as of sexually transmitted diseases and chronic osteomyelitis .Unfortunately, the extensive use of FQ has led to the emergence of not only theextensively drug-resistant (XDR) MTB isolates, defined as MDR-TB with resistance to any FQ and at least one of the three injectable second-linedrugs (amikacin, kanamycin, capreomycin) but also the susceptible MTB isolates with FQ resistance(Zhang and Yew, 2009). Disease caused by XDR MTB is associated with very poor treatment outcomes similar to ancient time, when no chemotherapy for TB was available(Wang et al., 2007).The FQ resistance rates in TB patients have been estimated to lie between 0.15 and 30%, depending on the country.

The FQ resistance rates are higher among patients exposed to FQ prior to the diagnosis of TB, and termed as “acquired resistance”. However, in some countries, especially where FQ consumption is very high, FQ resistance is often due to the transmission of strains that are already FQ resistant . FQ resistance is defined by the WHO as resistance of MTB isolates to 2 mg/liter ofloxacin (OFX)(Gandhi et al., 2010) . Theconventional methods for drug susceptibility testing (DST) involve the primary culture of specimens and isolation of MTB in the presence of drug. This process has a long turnaround time of several weeks. Over the past several years, molecular techniques have been developed for the rapid detection of resistance to antituberculosis agents including FQ(Roberts et al., 1983) .The only target of FQ in

MTB is a gyrA gyrB tetrameric enzyme containing two A and two B subunits encoded by the gyrA and gyrB genes, respectively.

2. Literature Review

2.1 Global Epidemiology of Tuberculosis:

According to global TB report of 2015, 22 high-burden countries collectively account for 80% of the global

tuberculosis burden. The countries with the highest TB prevalence reportedly are India (with 2.0 million cases), China (1.3 million), Indonesia (530,000), Nigeria (460,000), and South Africa (460,000); of the estimated 1.37 million cases in HIV-positive persons, 79% were in Africa and 11% in Southeast Asia. Disturbingly, there are an estimated 500,000 cases of multidrug-resistant (MDR) tuberculosis (including 289,000 new cases); of these, 131,000 were in India112,000 in, China, 43,000 in Russia, 16,000 in South Africa, and 15,000 in Bangladesh. Moreover, 55 countries have reported cases of extensively drug-resistant (XDR) tuberculosis which is of a grave concern and highlight a potential threat to our ability to treat tuberculosis, both in individual patients and in the context of a treatment program(Organization, 2010).

2.2 Epidemiology of TB in Saudi Arabia:

Saudi Arabia is the third-largest Arab country with a moderate annual burden of tuberculosis. However, TB is among several infectious diseases that have not been brought under control, despite the government's considerable efforts. This is clearly evidenced by the ongoing transmission of several imported and indigenous clades of *Mycobacterium tuberculosis*. In addition, the country faces the threat from rising proportions of extrapulmonary TB, and drug resistance. One of the most important reasons for persistent of TB is the annual visit of several millions of people to the Kingdom of Saudi Arabia (KSA). The two Holy Mosques in KSA attract huge numbers of Muslims to perform their religious rituals. Big mass gatherings during Hajj and Omra increase the risk for TB transmission(Al-Hajoj, 2012). Moreover, the strong economic position of KSA attracts large numbers of expatriates for work. High proportions of these people come from highly endemic TB areas. All these factors led to increased risk of TB in KSA, which requires more public health efforts to cope with these challenges. Jeddah City is the second largest city in KSA. It is located on the western coast of Red Sea. It is the main gateway to Makkah; thus, it is the port-of-entry for millions of Muslims from all-over the world to perform their religious rituals of Hajj and Omra every year. According to the distribution of primary health care centers, the city is divided to 7 districts, i.e., northern, eastern north, western north, southern, eastern south, western south and middle districts. They constitute a variable mix of nationalities, religions (Muslims and non-Muslims), cultures and socioeconomic levels.

The climate of Jeddah is affected by its location. Warmth and humidity are predominant features during the entire year which increase more in the summer. Its environment has some issues with air and water pollutions particularly in the industrial zones. Some districts have crowded buildings, especially slums in the southern area(Al-Hajoj, 2010).

Furthermore, the country falls behind the global targets set by WHO for the success rate of TB treatment. The country needs more population-based research studies, centralized and easily accessible clinical data registries, and centralized research and diagnostic facilities. The global burden of TB particularly with MDR is increasing and has become a major health challenge(Abubakar et al., 2013).

2.3 Resistant TB; A Serious Threat to Global Health:

The prevalence of MDR-TB ranged from 0% to 22% among newly diagnosed cases and from 0% to 60% among previously treated cases reportedly(Organization, 2014). In addition, since 2002, 45 countries have reported cases of XDR-TB, i.e., TB that is resistant not only to isoniazid and rifampicin but also to at least one fluoroquinolone and to any of the following injectable second-line drugs: kanamycin, amikacin, or capreomycin. Of the MDR isolates tested for second line drugs, 0%–30 % were found to be XDRs. Drug-resistant TB poses a major threat to control of TB worldwide. By the end of 2014, data on anti-TB drug resistance were available for 153 countries, accounting for more than 95% of the world's population and estimated TB cases. Eighty of these countries have continuous surveillance systems, while the others rely on epidemiological surveys(Gandhi et al., 2006). According to 2014 and 2015, drug resistance global surveys an estimated 3.3% (95% CI: 2.2–4.4%) of new cases and 20% (95%CI: 14–27%) of previously treated cases have MDR-TB; these levels have remained virtually unchanged in recent years. In 2014, there were an estimated 480 000 (range: 360 000–600 000) new cases of MDR-TB worldwide, and approximately 190 000 (range:120 000–260 000) deaths from MDR-TB. Among patients with pulmonary TB who were notified in 2014, an estimated 300 000 (range: 220 000– 370 000) had MDR-TB. More than half of these patients were in India, China and the Russian Federation. Extensively drug-resistant TB (XDR-TB) has been reported by 105 countries. On average, an estimated 9.7% (95% CI: 7.4–12%) of people with MDR-TB have XDR-TB. There was major progress in coverage of drug susceptibility testing (DST) between 2013 and 2014. Worldwide, 12% of new bacteriologically- confirmed TB cases and 58% of previously treated TB patients were tested for drug resistance in 2014, up from 8.5% and 17% respectively in 2013 (representing proportional increases of 43% and 223%, respectively). Coverage was highest in the European Region (97% of new cases). In the South-East Asia and Western Pacific regions combined, two-thirds of previously treated cases underwent testing(Organization, 2013). Globally in 2014,123 000 patients with MDR -TB or rifampicin resistant tuberculosis (RR-TB) were notified, of whom about 75% lived in the European Region, India, South Africa or China. Globally, only 50% of patients on MDR-TB treatment were successfully

treated, largely due to high rates of mortality and loss to follow-up. Despite progress in responding to the challenge of drug resistant TB, serious detection and treatment gaps remain. Intensified efforts to close these gaps are urgently required(Jenkins et al., 2014).

2.4 Drug resistance TB in Saudi Arabia:

Empirical anti-tuberculosis therapy used in Saudi Arabia usually includes three to four first line drugs including isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin as recommended by WHO. The prevalence of MDR-TB (defined by WHO as resistant with at least two first-line anti tuberculosis drugs i.e. rifampicin (RIF) and isoniazid (INH) or more) within different regions of Saudi Arabia (Khan et al., 2001). Studies have shown drug resistance TB for single and multi anti-TB agents including rifampicin (RIF)-, isoniazid (INH), pyrazinamide (PZA), ethambutol (ETB), streptomycin (STR) and second line drugs.

Table (1): Drug Resistance TB in Saudi Arabia:

	Drug resistance					MDR-TB	Reference
City	RIF	INH	PZA	ETB	STR	%	
Jeddah	20.8	28.7	7.9	6.9	22.8	25	(al-Mazrou,Khoja et al.1997)
Riyadh	2.8	9.1	5	2.8	1.6	11.8	(Arya,2002)
Jazan	43	80	S	NA	53	44	(Ellis, al-Hajjar et al.1996)
Dammam	0.2	6	S	S	0.7	7	(Al-Rubaish, Madania et al.2001)

2.5 FQs resistance among MTB

The fluoroquinolones (FQs) were introduced into clinical practice in Taiwan in 1986(Wang et al., 2007). FQs have broad-spectrum antimicrobial activity and so are widely used for the treatment of bacterial infections. In contrast to many other antibiotics used to treat bacterial infections, the FQs have excellent in vitro and in vivo activity against MTB. FQs are recommended for use as prophylactic treatment of patients exposed to multidrug-resistant tuberculosis for treatment of proven MDR-TB, for empirical treatment of TB disease in settings with high rates of MDR-TB and for patients with severe adverse reaction to first-line agents. Recent studies showed that previous FQ use and MDR-TB were associated with FQ resistance in MTB isolates, therefore it is crucial to maintain information about the FQ susceptibility in different patient populations to guide selection of the most appropriate treatment(Chan et al., 2007). This is especially important for those patients with recurrent TB after treatment, those with MDR-TB as well as those who have previously received FQ therapy for infections other than TB. Fluoroquinolones kill M. tuberculosis via double-stranded breaks in DNA, by binding to DNA gyrase. DNA gyrase consists of two A and two B subunits encoded by the gyrase A (*gyrA*) and gyrase B (*gyrB*) genes, respectively. The interaction between fluoroquinolones and DNA gyrase occurs in a conserved region of *gyrA* (codons 74 to 113) and *gyrB* (codons 500 to 538) known as the quinolone resistance-determining region (QRDR)(Mdluli and Ma, 2007). Fluoroquinolone resistance in M. tuberculosis is frequently conferred by mutations in the QRDR of *gyrA* or *gyrB* .However, up to 58% of fluoroquinolone-resistant M. tuberculosis isolates lack known resistance mutations .Most studies of fluoroquinolone-resistant M. tuberculosis have limited their assessment of mutations to the QRDR of *gyrA* and/or *gyrB*. A systematic review of all studies of genotypic(Devasia et al., 2011).

2.6 Conclusion:

On the basis of the current findings, we can conclude that the Pyrazinamide first line TB drug resistance is prevalent in Jeddah. This finding indicates that prudent use of the first line drugs is warranted to prevent further development of resistance and to preserve the usefulness of these valuable drugs. Genotypic data of this study showed mutations in the gyrA and the gyrB genes some of which have been associated with resistance to FQs in M.tuberculosis. This may assist in rapid detection of anti-tuberculosis drug resistance and thus use of appropriate regimens for treatment. However, further investigation with larger sample size from across the country is required to ascertain the findings of this study.

3: Materials and Methods

3.1 Materials:

3.1.1: Samples

Mycobacterium tuberculosis (MTB) isolates were collected from clinical microbiology laboratory of King Abdulaziz university hospital.

3.1.2: Demographics of Patients

Demographic information including age and gender from whom the samples were collected, was gathered from the hospital records.

3.1.3: DNA extraction

-loopful of M. tuberculosis culture

-Lowenstein–Jensen medium
-1.5 mL tube that contained
-400 mL of TB lysis buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA (pH 8.0))

3.1.4: Polymerase Chain Reaction (PCR) amplification of gyrA and gyrB genes :

The primers for PCR amplification were designed with reference to gyrA and gyrB gene sequences The gyrA gene was amplified with the
-forward primer (CCGGATCGAACCGGTTGAC)
-reverse primer (GTTAGGGATGAAATCGACTG)
The gyrB gene was amplified using the
- forward primer (GTCGTTGTGAACAAGGCTGTG)
-reverse primer(GTGGAAATATGTTGGCCGTC)
- Qiagen HotStrat PCR kit was used to amplify gyrA and gyrB genes for 50 MTB isolates.
-Agaros gel
-Ethedium promide
-PCR product
-Tris Acetate EDTA (TAE) buffer
-Electrophoresis machine

3.1.5: Purification of *gyrA* and *gyrB* genes PCR products

-H_2O
-Centerifuge
-Plate
-pipettes

3.1.6: *gyrA* and *gyrB* gene sequencing and analysis

- Taq Polymerase
- Forward or Reverse Primer
- dNTPs
- ddNTPs
- Buffer
- PCR product

3.2 Methods:

3.2.1 Sampling:

The MTB confirmed 50 samples were collected from clinical lab of King AbdulAziz University hospital, Jeddah from between January December 2015. Selected MTB isolates were grown on Lowenstein-jensen (LJ) medium. Phenotypic drug susceptibility testing (DST) for the first line drugs i.e. Isoniazid (INH),Rifampicin (RIF), Streptomycin, Pyrazinamide and ethambutol was performed by conventional indirect drug MTB drug susceptibility testing. This method involves inoculation of drug containing media with a pure culture grown from the TB patients samples. Indirect phenotypic tests is currently regarded as the "gold standard" of drug susceptibility testing for MTB. For all antituberculosis drugs except PZA, Middlebrook 7H10 medium (Becton Dickinson and Company, Sparks, MD) supplemented with oleic acid-dextrose-catalase (OADC; Becton Dickinson and Company) of pH 6.6 is used. Toprepare 500 ml medium solution, the amount of Middlebrook 7H10 powder recommended by the manufacturer is added to 450 ml distilled water. Subsequently, 2.5 ml glycerol (about 87%; BDH Laboratory Supplies, Poole, England) is added and the solution is placed in a water bath at 100°C until the agar is completely dissolved. This solution is sterilized for 10 min at 121°C. After cooling to 50°C in a water bath, 50 ml OADC, preheated to the same temperature, is added. One milliliter of agar is solidified to check the final pH of the medium, which should amount to 6.6 ± 0.2.For the preparation of about 50 DST plates, 2.5 liters of Middlebrook 7H10 medium, supplemented with OADC, is prepared. In 23 bottles, dilutions of antituberculosis drugs in this medium are prepared with the following concentrations: 0.1, 0.2, 0.5, 1, and 2 μg/ml INH (isonicotinic acid hydrazide; Sigma Chemical Co., St. Louis, MO) 1, 2, 5, 10, and 20 μg/ml SM (streptomycin sulfate; Sigma); 1, 2, 5, 10, and 20 μg/ml EMB (ethambutol dihydrocloride; Sigma). The medium with antituberculosis drugs from these 23 bottles and from two bottles containing medium without drugs (for the control wells) is transferred in 2.5-ml amounts into 25-well plates by using a dispenser developed at the RIVM .With this device, the medium is transferred from the bottles into the plates through 50 small plastic hoses by an electronic pump. To solidify the medium, the plates are left at room temperature for approximately 1 h. The plates are stored at 4°C until use.Because PZA testing requires growth medium of another pH (see below), separate DST plates are prepared containing 10, 20, 50, and 100 μg/ml of this drug. Before adding OADC to the dissolved 7H10 medium, the pH is lowered by adding 0.95 ml of 3 M HCl to achieve a final pH of 5.7 ± 0.15.

3.2.2 DNA extraction:

A loop full of MTB culture from LJ medium was transferred to a1.5-mL tube that contained 1.0 mL sterilized

water. The tube was centrifuged at 10,000 for 5 min. The supernatant was discarded and the sediment was resuspended in 400 ìL of TB lysating buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA [pH 8.0]) heat inactivated at 85°C for 30 min, and centrifuged at 10,000 g at 4°C for 10 min The supernatant was transferred to another 1.5-mL tube and stored at –70°C until use.

3.2.3 PCR amplification of *gyrA* and *gyrB* genes:

The final volume of 25µl, contain 12.5µl of green master mix , 1µl of reverse primer, 1µl of forward primer and 1µl of BSA , 7.5µl of sterilized water and 2 µl DNA extraction. The tube was placed in PCR for the amplification.

3.2.3.1: Stages of amplification program

i) Initial denaturation at 94ºC for 5min

ii) 35 cycles each consisted of 3 stages: denaturation at 94ºC for 1min, annealing at 57°C for 45s and extension at 72°C for 1min.

i) The last extension step at 72°C for 10 min.

3.2.3.2: Visualization of PCR product:

2% agarose gel for electrophoresis was prepared using following stpes:

- 2 g of agarose was dissolved in 100 mL of 1 x TAE buffer.
- 0.5 ìl of Ethidium bromide was added.
- The mixture was heated in microwave for 1 min to completely dissolved agarose powder.
- Cooled the solution to 60ºC.
- Pour cooled solution into gel casting tray with comb, then wait about 30min till the gel solidifies.
- Remove the comb and used the gel well for loading samples for electrophoresis.

Mixture was loaded on to the 2% agarose gel. A total of 5 ìl of diluted marker was also loaded on the same gel. Agarose gel was placed in the electrophoresis apparatus and attached to the power supply set up as a voltage of 100 volt and electrophores ed for about 60 min. The resolved bands were visualized on the gel documentation system using UV light.

3.2.4: Sequencing of the FQ resistance genes:

DNA sequencing is atechnique used to determine the order of nucleotide in a gene.After getting the acquired band size of amplicons for (*gyrA* and *gyrB)* genes, were purified and were sequenced .

First of all sample preparation according to the sequencing technology was done for DNA sequencing as follws: .

PCR template purification

1- Labelling of DNA fragments using the chain termination method with energy transfer

2-Dye-labelled di-deoxynucleotides and DNA polymerase

3-Capillary electrophoresis

4-Fluorescence detection that provides four colour plots to reveal the DNA sequence.

3.2.5: Sequence analysis:

Sequences segment for gyrA and gyrB genes were analyzed using BLAST tool from NCBI website: www.ncbi.nlm.nih.gov/blast/

4:Result:

4.1: Demographic data of patients

4.1.1:patients' information

Following demographic information for all the patients from whom the TB samples were collected was obtained from the patients' records.

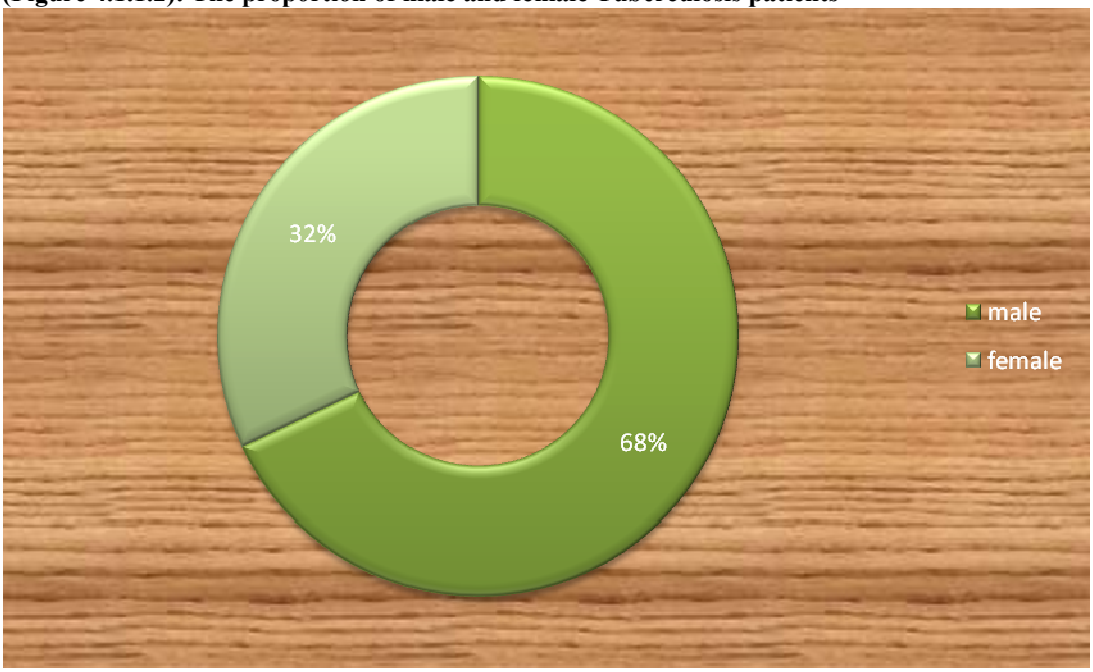
4.1.1.1: Gender of the patients

Out of 50 patients, 34 (68%) were male and 16 (32%) were female (Figure 1.1.2.1).

4.1.1.2: Age of the patients

The age range of the patients in this study group was between 2 days to 91 years. Overall, mean age of the patients was **(42)** years. Whereas, the mean age of male patients was **(43)** years and female was **(41)** years, which was comparable.

(Figure 4.1.1.2): The proportion of male and female Tuberculosis patients



4.2.: Bacterial Sample:

4.2.1.: Drug Susceptibility Testing (DST)

M. tuberculosis were tested for DST for the following drugs figure (4.2.1.1):

- Isoniazid
- Rifampin
- Ethambutol
- Streptomycin
- Pyrazinamide

Information of DST result of each the sample has been shown in Table **(4.2.1.1) . Overall, all the tested** isolates 50 (100%) were susceptible to Streptomycin, RIF and Eth. Whereas, 49 (98%) and 43 (86%) isolates were susceptible to INH and Pyr respectively.

(Figure 4.2.1.1): Drug Susceptibility Testing :

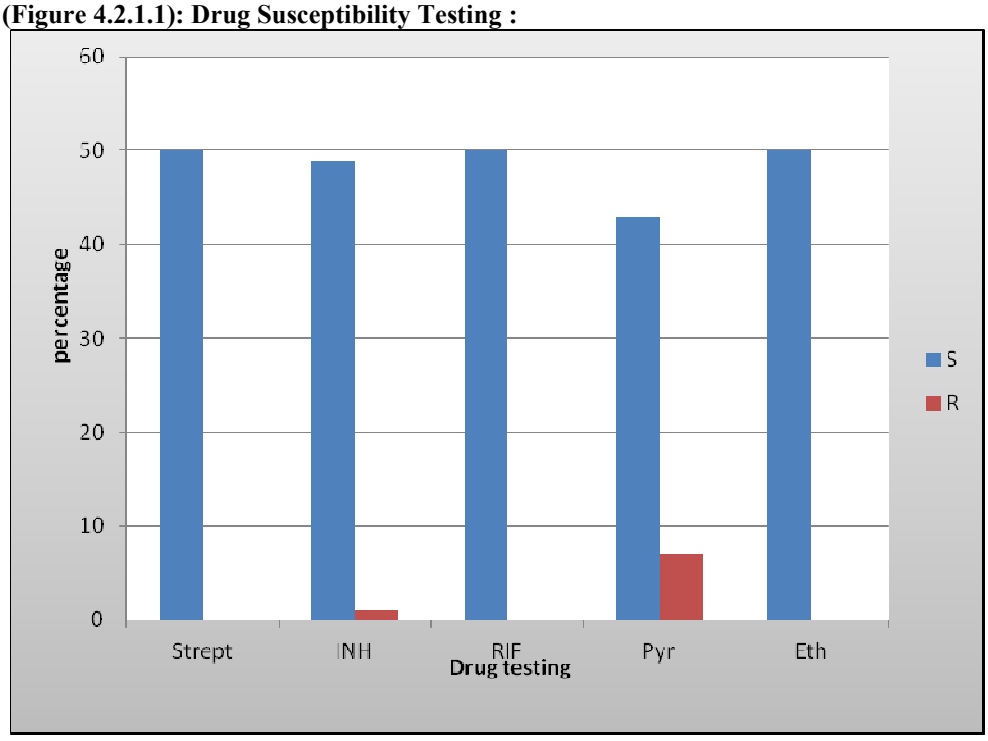


Table 4.2.1.2: Drug Susceptibility Testing of individual Bacteria:

Phenotypic DST result					
ID of MTB	Sterpt.	INH	Eth	Pyr	RIF
1	S	S	S	S	S
2	S	S	S	S	S
3	S	S	S	R	S
4	S	S	S	S	S
5	S	S	S	S	S
6	S	S	S	S	S
7	S	S	S	S	S
8	S	S	S	S	S
9	S	S	S	S	S
10	S	S	S	S	S
11	S	S	S	S	S
12	S	S	S	S	S
13	S	S	S	S	S
14	S	S	S	S	S
15	S	S	S	S	S
16	S	R	S	R	S
17	S	S	S	S	S
18	S	S	S	S	S
19	S	S	S	S	S
20	S	S	S	S	S
21	S	S	S	S	S
22	S	S	S	S	S
23	S	S	S	S	S
24	S	S	S	S	S
25	S	S	S	R	S
26	S	S	S	S	S
27	S	S	S	S	S
28	S	S	S	R	S
29	S	S	S	S	S
30	S	S	S	S	S
31	S	S	S	S	S
32	S	S	S	S	S
33	S	S	S	S	S
34	S	S	S	S	S
35	S	S	S	S	S
36	S	S	S	S	S
37	S	S	S	R	S
38	S	S	S	R	S
39	S	S	S	S	S
40	S	S	S	S	S
41	S	S	S	S	S
42	S	S	S	R	S
43	S	S	S	S	S
44	S	S	S	S	S
45	S	S	S	S	S
46	S	S	S	S	S
47	S	S	S	S	S
48	S	S	S	S	S
49	S	S	S	S	S
50	S	S	S	S	S
Abbreviations: S′=Susceptible, R″=Resistance					

4.2.2: Polymerase Chain Reaction (PCR) for fluoroquinolones resistance genes :

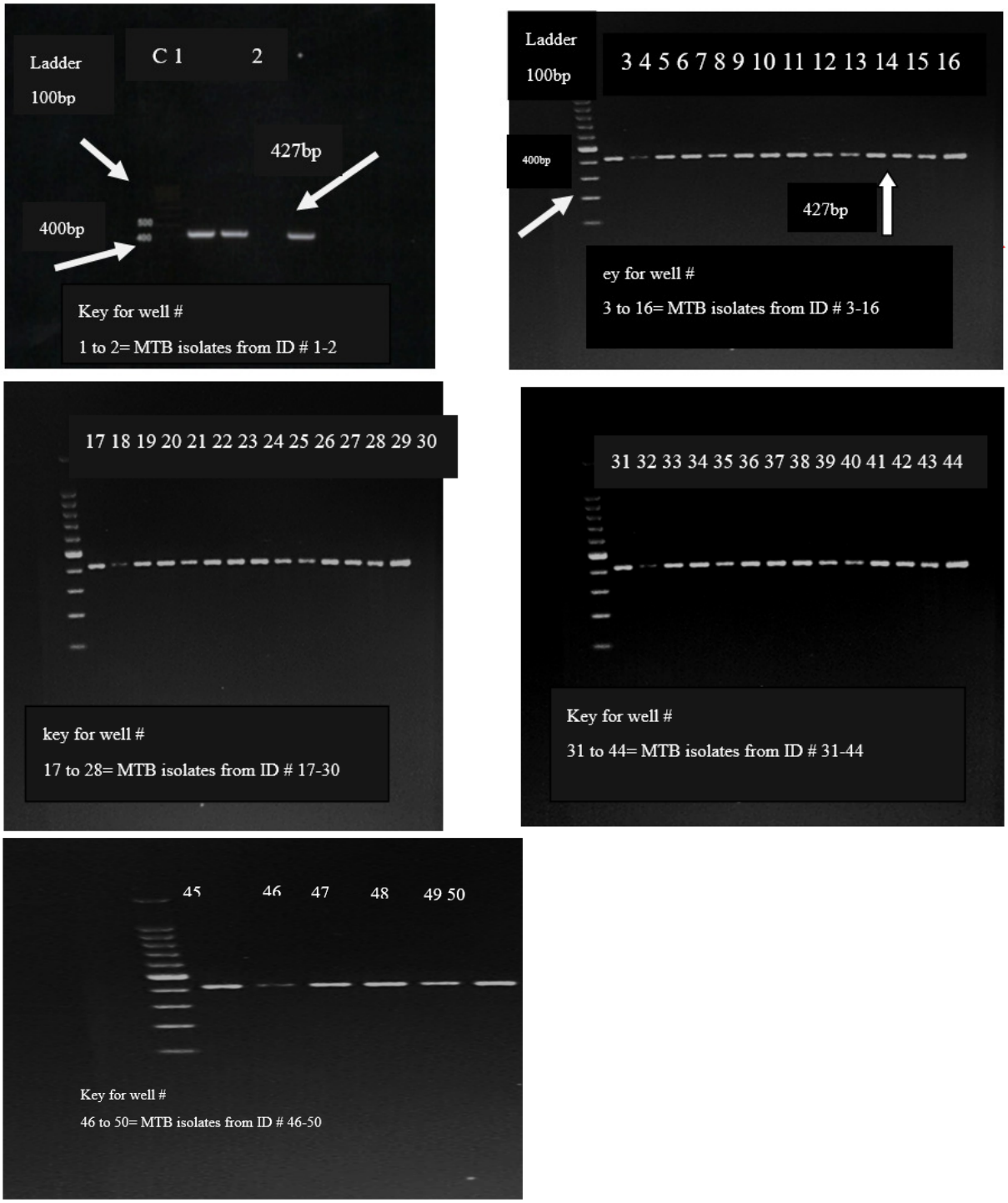
The resolved bands were visualized on the gel documentation system using UV light shown in figure (4.2.2.1) and (4.2.2.2) .

4.2.3: Sequencing of the FQ resistance genes:

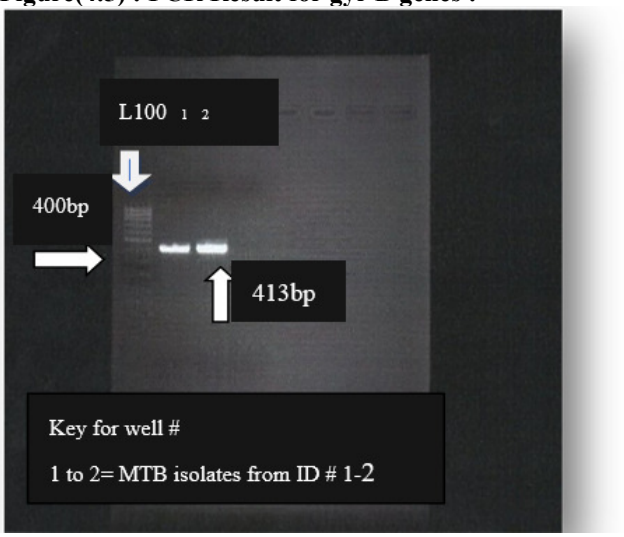
After getting the acquired band size of amplicons for (*gyrA* and *gyrB)* genes, PCR were purified and were sequenced.

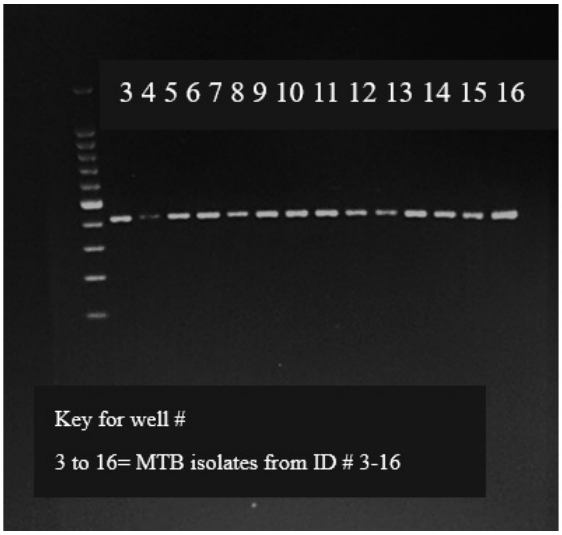
Sequencing of the *gyrA* gene revealed that 6/50 (12%) isolates had a mutation at codon 21, while 2/50 (4%) had variable mutations at codon 29 ,and 1/50 (2%) had variable mutations at codon 90. Forty-eghit strains had an AGC-to-ACC polymorphism at codon 95. No *gyrA* mutation was detected in two isolates. As the codon 95 mutation is reportedly not associated with drug resistance . Sequencing of the *gyrB* genes had mutations only in two strains at codon 421 & 420 (Table 4.2.3.1).

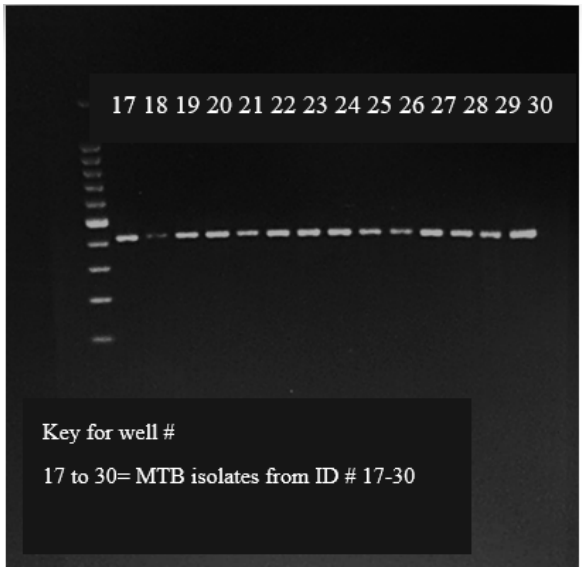
Figure(4.3) :PCR Result for gyr A genes :

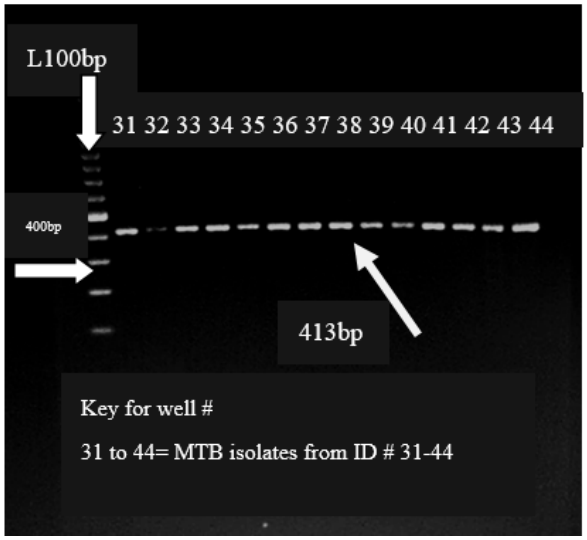


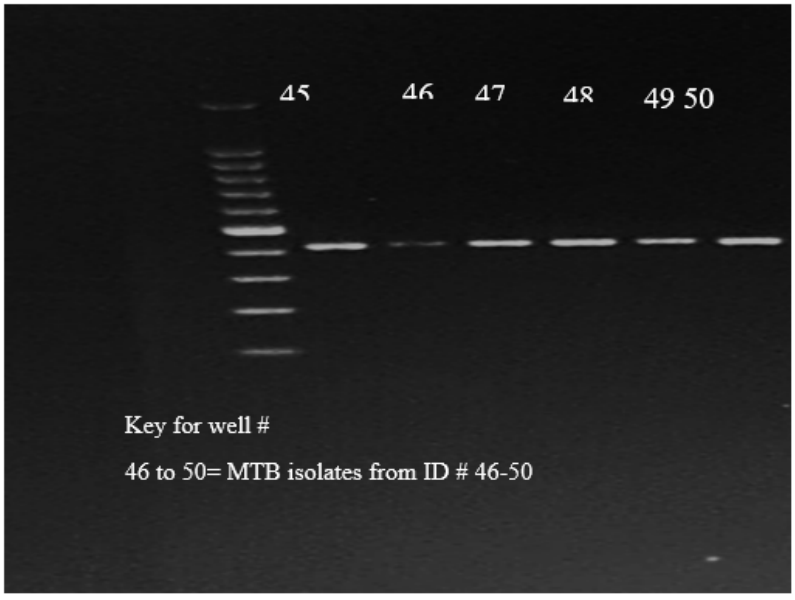
Figure(4.5) : PCR Result for gyr B genes :



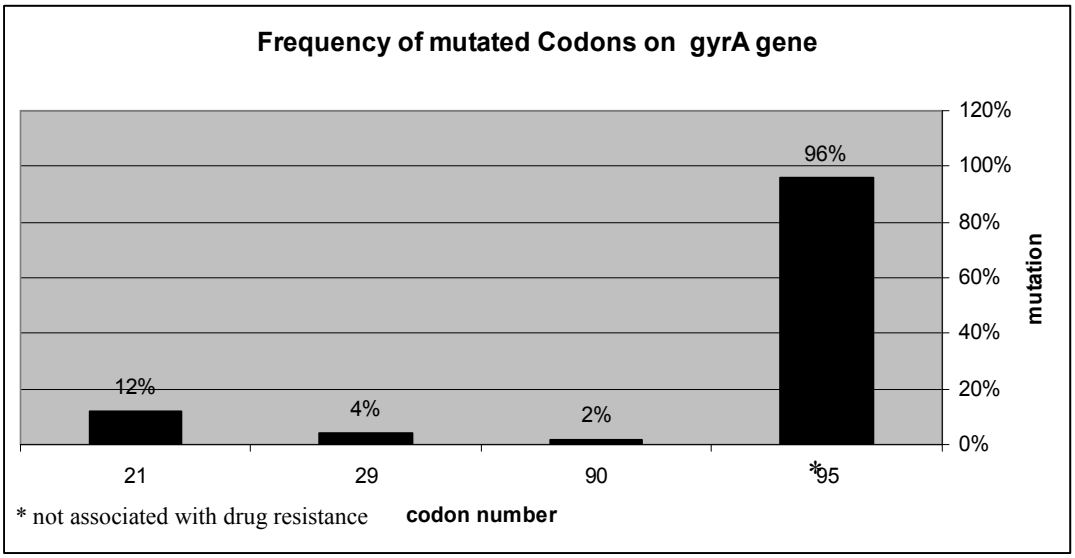








Figure(4.5): Codon mutation in *gyrA* and *gyrB* genes:



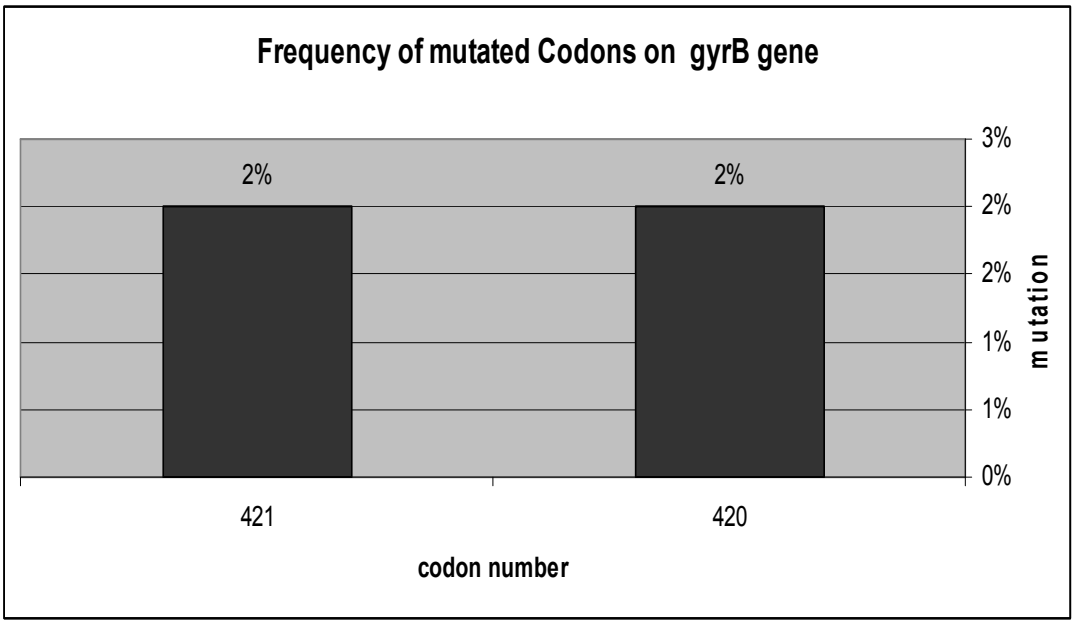


Table 4.2.3.1: Mutations detected by sequencing on *gyrA* & *gyrB* of individual MTB isolates:

Isolate ID	gender	gyrA		gyrB	
		Codon Number	Mutation to	Codon Number	Mutation to
1	F	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
2	M	95	AGC (S) → ACC (T)	-	-
3	F	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
4	M	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
5	F	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
6	M	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
7	M	21	GAG (E) → CAG (Q)	-	-
		95	AGC (S) → ACC (T)		
8	M	95	AGC (S) → ACC (T)	421	AAG (K) → AGG (R)
9	M	95	AGC (S) → ACC (T)	-	-

Isolate ID	gender	gyrA		gyrB	
		Codon Number	Mutation to	Codon Number	Mutation to
10	F	95	AGC (S) → ACC (T)	-	-
11	F	95	AGC (S) → ACC (T)	-	-
12	F	95	AGC (S) → ACC (T)	-	-
13	M	95	AGC (S) → ACC (T)	-	-
14	M	95	AGC (S) → ACC (T)	-	-
15	M	-	-	-	-
16	F	95	AGC (S) → ACC (T)	-	-
17	M	95	AGC (S) → ACC (T)	-	-
18	M	29	ATC (I) → TCC (S)	-	-
		95	AGC (S) → ACC (T)		
19	F	95	AGC (S) → ACC (T)	-	-
20	M	95	AGC (S) → ACC (T)	-	-
21	M	29	ATC (I) → TCC (S)	-	-
22	F	95	AGC (S) → ACC (T)	-	-
23	F	95	AGC (S) → ACC (T)	-	-
24	M	95	AGC (S) → ACC (T)	-	-
25	M	95	AGC → ACC (S) (T)	-	-

Isolate ID	gender	gyrA		gyrB	
		Codon Number	Mutation to	Codon Number	Mutation to
26	F	95	AGC (S) → ACC (T)	-	-
27	M	95	AGC (S) → ACC (T)	420	GCA (A) → ACA (T)
28	M	95	AGC (S) → ACC (T)	-	-
29	M	95	AGC (S) → ACC (T)	-	-
30	M	-	-	-	-
31	M	95	AGC (S) → ACC (T)	-	-
32	F	95	AGC (S) → ACC (T)	-	-
33	F	95	AGC (S) → ACC (T)	-	-
34	F	95	AGC (S) → ACC (T)	-	-
35	M	95	AGC (S) → ACC (T)	-	-
36	M	95	AGC (S) → ACC (T)	-	-
37	M	95	AGC (S) → ACC (T)	-	-
38	F	95	AGC (S) → ACC (T)		
39	M	95	AGC (S) → ACC (T)	-	-
40	F	95	AGC (S) → ACC (T)	-	-
41	M	95	AGC (S) → ACC (T)	-	-
42	M	95	AGC (S) → ACC (T)	-	-
43	F	95	AGC (S) → ACC (T)	-	-
44	M	95	AGC (S) → ACC (T)	-	-
45	M	95	AGC (S) → ACC (T)	-	-
46	M	90 95	GCG (A) → GTG (V) AGC (S) → ACC (T)	-	-
47	M	95	AGC (S) → ACC (T)	-	-
48	M	95	AGC (S) → ACC (T)	-	-
49	M	95	AGC (S) → ACC (T)	-	-
50	M	95	AGC (S) → ACC (T)	-	-

Abbreviations:
A'=Alnine, E'=Glutamic Acid, I'=Isoleucine, K'=Lysine, Q'=Glutamine, R'=Arginine, S'=Serine, T'=Threonine, V'=Valine.

5: Discussion

This was a two pronged study aimed to investigated resistance patterns of the first line anti-tuberculosis drugs and molecular detection of resistance of a second line drug FQ of MTB isolates. These MTB isolates were collected at the central referral MTB center in Jeddah at King Abdul-Aziz University Hospital during 2015. The demographic data of MTB patients from this study revealed that the frequency of male and female patients suffering from MTB infection were 68% male and 32% were females. Also, the demographic finding revealed that the MTB infection is common among the middle age individuals as compared to old and very young people. These observations may be explained by the more social activities practiced by male in our society. Also the outgoing activities are more among middle age people, which increase the possibility of their contact and exposure to sources of infection resulting in more number of male patients suffering from tuberculosis.

Overall, the findings of the first line anti-tuberculosis drug resistance in this study revealed that the Pyr was the drug with the highest percentage of resistance (14%) followed by INH (2%).However this was encouraging to note that rest of the first line drugs i.e. Strep,, RIF and Ethfrom MTB isolates from this study did not reveal any resistance. This drug resistance pattern observed in present study is almost similar to that reported in other studies conducted in Saudi Arabia. Such as studies conducted in Riyadh by Kordy et al, and Al-Orainey et al have reported similar findings. However , findings from a study conducted in Saudi Arabia showed most common a resistance in Riyadh (13.2%) but < that in Gizan (44%).

This variation in the percentages of resistance to each of the anti-tuberculosis drugs may be due to heterogeneous population residing in different regions of Saudi Arabia. On the other hand comparison of our findings with other studies show that the resistance to INH varied from 27to 47%, RIF from 15 to 59% Strept 22 to 47% and Eth from 4 to 10% (Al-Hajoj and Alrabiah, 2004) Further, MDR-TB has been reported a serious problem of tuberculosis which not only hinder the successful treatment of the TB patients but also increases the risk of transfer of MDR-TB to other individuals in a community.

On comparison of our findings in terms of MDR-TB reported from across the globe, it shows that our findings are different with other studies reported from Chandigarh, Tamil Nadu state. Mumbai, and Gujarat showing 25 to 30% ofMDR-TB (Songara et al., 2015) Whereas our study reveal no MDR-TB among our MTB isolates. However, this observation may be due to limited selection of MTB sample from one hospital or due to absence of MDR-TB isolates in our community. This needs to be further explored with larger sample size from across the Jeddah first and then the entire country. The second part of investigation for FQ resistance genes in this study revealed that 48 of 50 (96%) MTB clinical isolates showed mutations in the QRDR of the *gyrA* gene,and the other two strains had no mutations in the *gyrA* and *gyrB* genes, a finding consistent with what has been previously, reported on fluoroquinolone resistance. Further investigation of FQ resistance QRDR of *gyrA* gene among *M. tuberculosis* isolates showed mutations in codons21, 29, 90, and 95 predominantly among 96% of the isolates. All but two MTB isolates had no mutation in the *gyrB* gene, which is in agreement with previous studies demonstrating that fluoroquinolone resistance of MTB are mostly attributed to the mutations of the *gyrA* gene, whereas *gyrB* mutations are rare (Chen et al., 2012).This finding largely corroborate the findings

of other Studies (Yew et al., 2003) .This study reports three patterns of double mutations in nineMTB clinical isolates; six with E21Q & S95T, and the two with I29S & S95T, and one with A90V & S95T. Previous reports on double mutations mainly focused on codons 90 and 94.

The comparative analysis of MTB isolates from various region of Saudi Arabia exhibited thatthe strains isolated from Jeddah, showed more double Mutations in the *gyrA* gene, which may indicate that frequency of mutations in the *gyrA* gene vary in different regions of Kingdom.

In this study most of the mutations found in the hypervariable region of *gyrA* gene were shown to be associated with phenotypic FQ resistance in previous studies (Wang et al., 2007). Other resistance mechanisms, such as mutations found in *gyrA* or *gyrB* genes outside of the QRDR decreased cell-wall permeability to the drug and efflux pumps probably accounting for the fluoroquinolone resistance in MTB isolates without mutations in hypervariable regions of *gyrA* and *gyrB* genes which needs further investigations. The total mutation frequency of the *gyrA* gene (18%) in this study compared with other studies is lower than those of Shanghai (89.5%) 12 and Russia (83%).Mutation pattern of *gyrA* gene and prevalence of each pattern in this study exhibit some discrepancy with those reported from the other regions. Furthermore, mutation T80A from Rwanda, mutation G88C from Russia and mutation G88A from Taiwan were not found in our data, but these two mutations in G88C and G88A were described in clinical strains in France. These major patterns are similar to those reported in previous studies from other regions but the frequency of each pattern is quite different. Among the 38 patterns of single codon mutation in our data, one patterns (S95T) has been shown not to be associated with resistance to FQs. However, different substitutions of amino acid at codon 95 has been shown to be associated with different MTB lineages and genotypes and thus has been shown to serve as genetic marker.. The codon 95 (AGC-ACC) polymorphism observed in this study has been shown to be associated with lineages or genotypes having various members of Mycobacterium species in addition to tuberculosis. In addition MTB strains with codon 95 (AGC-ACC) mutation have been found to have important link with outbreaks (Ameni et

al., 2011) Thus, finding MTB isolates with this polymorphism may indicate more TB cases due to enhanced transmission ability of the circulating MTB isolates in the kingdom. This study has observed double codons mutations in *gyrA* gene among the MTB isolates tested. Mutations among nine MTB clinical isolates; six with E21Q & S95T, and the two with I29S & S95T, and one with A90V & S95T. Previous reports on double mutations mainly focused on codons 90 and 94.

The comparative analysis of MTB isolates from various region of Saudi Arabia exhibited that the strains isolated from Jeddah, showed more double mutations in the *gyrA* gene, which may indicate that frequency of mutations in the *gyrA* gene vary in different regions of Kingdom. Such as study from Riyadh shows prevalent single mutation on codon (Farhat et al., 2013). In contrast with the single codon mutation, the double codons mutation is relatively rare and frequently correlated to high-level resistance to FQs. Similarly double mutation has also been reported in the study from Taiwan (G88A with B94Y) as well. However studies from Hong Kong, Rwanda and Russia have not reported any double mutations in their studies. The mutation in *gyrB* gene was identified in two of 50 clinical isolates in this study. Similarly mutation in *gyrB* gene was also found in the studies from Taiwan, France and Russia (Yin and Yu, 2010).

However, the mutation pattern of *gyrB* gene observed in this study , (A420T & K421R) is different from that reported in studies from Taiwan (N538D), France (N510D) or Russia (N510K, A515V, A515T and Q549H).The low prevalence of *gyrB* gene mutation may reveal that genetic alterations of this gene are secondary to that of *gyrA* gene in terms of impact on FQ susceptibility of MTB.

In summary, significant variations in terms of drug rsistance pattern and the type prevalence of mutation at specific codons of *gyrA* and *gyrB* genes are exhibited by MTB isolates in this study as compared to MTB isolates from other parts of the world. Some possible reasons for the diversity may be as follows: firstly, different geographical environment gives rise to different pressure of natural selection on the corresponding strain. Secondly, the differences of the treatment regimes containing FQ bring out the differences of imposed drug selection. Thirdly, several mutation patterns which are correlated to a certain FQ drug resistance may be filtered because the selected FQ drug is different in the antibiotics susceptibility test.

6: Conclusion and Recommendations

Conclusion

On the basis of the current findings, we can conclude that the Pyrazinamide first line TB drug resistance is prevalent in Jeddah. This finding indicates that prudent use of the first line drugs is warranted to prevent further development of resistance and to preserve the usefulness of these valuable drugs. Genotypic data of this study showed mutations in the gyrA and the gyrB genes some of which have been associated with resistance to FQs in M.tuberculosis. This may assist in rapid detection of anti-tuberculosis drug resistance and thus use of appropriate regimens for treatment. However, further investigation with larger sample size from across the country is required to ascertain the findings of this study.

Recommendations

This study reveals first line drug resistance pattern and characterization of FQ resistance genes among MTB isolates from Jeddah. Following recommendations are being made based on the findings of this study:

1. It is encouraging to find that MTB isolates from this study were susceptible to almost all the first line antituberculosis drugs, however, study with larger sample size from across the Jeddah and then Kingdom is required to ascertain this findings and to take future actions.
2. FQs is an important antituberculosis drug for the treatment. Presence of resistance mutations in FQ resistance genes warrant that these drugs should be used with some caution to reduce the selective pressure for development of resistance to FQs among MTB isolates.
3. Also, molecular testing of FQs resistance could be an essential method in Kingdom of Saudi Arabia to prevent spread FQ resistance. This study reveal existence of few FQ drug resistance mutations in *gyrA* and *gyrB* genes which is encouraging and may be used for rapid molecular detection methods for FQ resistance among MTB isolates in the region.
4. Codon 95 polymorphism of *gyrA* gene has been shown to be associated with various MTB lineages and genotypes. MTB isolates from this study show codon 95 (AGC-ACC) polymorphism which have been found to have important link with MTB strains causing outbreaks and have enhanced transmission ability. This is an important finding for the control of tuberculosis in the Kingdom. Thus needs further investigations.

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