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Sociodemographic, microbial and phylogentic studies of *Mycobacterium tuberculosis* cases diagnosed in El-Minia governorate, Egypt

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Egypt is considered one of the high-burden countries in WHO's Eastern Mediterranean region. This study determined the sociodemographic, microbial and phylogentic characteristics for *Mycobacterium tuberculosis* cases diagnosed in El-Minia governorate in upper Egypt. Fifty clinical samples were collected (male-to-female sex ratio 1.5 and 50% within the 21- to 40-years old) with complete history of patients; samples were cultured and identified by biochemical tests. DNA was extracted and tested for plc gene. Seven isolates were subjected to sequencing analysis of the amplified *plc* fragment and a phylogentic tree for these strains was formed. Out of 50 samples, 24 samples were culture positive, all grown isolates were identified as *M. tuberculosis* according to physical characters, rate of growth, biochemical tests. Direct polymerase chain reaction (PCR) method based on the amplification of 350 bp region of *plc gene* was used for the detection of *M. tuberculosis*, to differentiate it from *Mycobacterium bovis* in clinical samples. It was found that all isolates were positive for *plc gene*. Direct sequencing of 350 bp region of plc gene revealed many substitution mutations along the entire sequenced fragment. The main cause for such condition may rely on inadequate treatment regimen. The phylogenetic tree of the seven sequenced isolates were classified into three distinct nodes. We concluded that all cases were positive for *M. tuberculosis* and most of them were from rural area. There was different substitution mutations in the *plc* gene which may be due to the intermittent treatment regimens that made the tested strains under stress resulting in mutation.

Key words: *Mycobacterium tuberculosis*, plc gene, phylogentic tree, mutation.

INTRODUCTION

According to WHO (2015), more than 2 billion people, equal to one-third of the world's population are infected with TB. Out of this, 1 in 10 will go on to develop TB during their lifetime. Out of the overall, 13 million TB cases in 2013, 9 million were new cases but consistently 3 million people were not diagnosed and not treated or officially not registered by national TB programmes (NTPs). Many of these missed cases might either die or

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follow some unknown treatment but most of them were thought to be a source of infection for others (WHO, 2015).

In Egypt, tuberculosis is one of the important public health problems. It considers as one of the major public health problems after HCV and bilharziasis. The incidence and prevalence of tuberculosis in Egypt has been declining due to increased efforts of the Ministry of Health and Population (MOHP). Prevalence dropped from 88/100,000 population in 1990 to 24 in 2008, while incidence dropped from 34 in 1990 to 19 in 2008. Although tuberculosis is one of the important public health problems in Egypt, limited information on the *Mycobacterium tuberculosis* genotypes circulating in Egypt is available (Abd-El Aal et al., 2013).

M. tuberculosis and *Mycobacterium bovis* are considered the most important pathogens from the *M. tuberculosis* complex (MTC). Both are highly related TB that can cause infections in humans and other mammals (Vordermeier et al., 2002). *M. tuberculosis* cause mainly active TB among millions of people every year (Brosch et al., 2002), but *M. bovis* has a broader host range causing infections in domestic and wild animals (WHO, 2011). In addition, it was reported that *M. bovis* can infect humans and causing zoonotic TB (Radostits et al., 1994). *M. bovis* is transmited from infected animals to humans by the ingestion of contaminated raw milk or the inhalation of the aerosol released from infected animals (Boulahbal et al., 1978; Cosivi et al., 1998; Michalak et al., 1998; Alexander et al., 2002; Sternberg et al., 2002; Michel et al., 2003; Pavlik et al., 2003; Ocepek et al., 2005; Pavlik et al., 2005; Une and Mori, 2007; Berg et al., 2009). Many studies showed that *M. tuberculosis* can be isolated from domestic or wild animals. As farmers with active TB who share their house with animals can infect animals through their sputum, urine or feces (Michalak et al., 1998; Alexander et al., 2002; Sternberg et al., 2002; Michel et al., 2003). In addition, there are some factors that facilitate the transmission of the disease between animals and humans such as dietary habit of people, close physical contact between humans and animals, rise in the incidence of immunosuppressive diseases, and inadequate disease control measures in animals and humans (Radostits et al., 1994). Our study was done in upper Egypt area which is an agricultural area with climate condition that is suitable for practicing cattle farming and crop cultivation (Ameni et al., 2003, 2008, 2011).

The key to control the spread of tuberculosis includes proper case finding, rapid diagnosis, immediate initiation of effective therapy and contact tracing to arrest further transmission. Recent developments in DNA technology and molecular biology methods have led to rapid detection of mycobacterial infection (Abd-El Aal et al., 2014).

DNA sequencing of variable genomic regions offers a more rapid and accurate identification of mycobacteria compared with conventional phenotypic methods. In addition, it is also capable of providing phylogenic information about the relatedness of the isolated strains. The most routinely used and reliable method of this kind is the amplification and sequence analysis of hyper variable regions of the gene encoding 16S r RNA (Somoskovia et al., 2002).

Although the worldwide use of live attenuated vaccine and several antibiotics to prevent and treat tuberculosis, *M. tuberculosis* is considered one of the infectious diseases of high mortality rate. So, it is essential to gain a better understanding of the pathogenicity and the virulence factors of *M. tuberculosis* (Goudarzi et al., 2010).

M. tuberculosis carries different virulence factors, which allow proliferation of the pathogen in the host cell, cell-to cell spread, and evasion of immune response. Among the most known virulence factors, phospholipases C (PLCs) stand out in several intracellular bacteria, including *Clostridium perfringens, Corynebacterium pseudotuberculosis Pseudomonas aeruginosa, Staphylococcus aureus and Listeria monocytogen*. Due to their role in the virulence mechanisms of bacterial pathogenicity, the relevance of PLCs during mycobacterial infection has been the subject of investigation (Assis et al., 2014). Phospholipase C genes include A, B, C and D segments. Three of these genes, *plcA*, *plcB* and *plcC*, are located at 2351 position of the genomic map of *M. tuberculosis* H37Rv, and are organized in tandem (locus *plcABC*). The fourth gene *plcD*, is located in a different region (Goudarzi et al., 2012).

The present study demonstrates demographic, genetic and phylogenetic study of mycobacterium strains that isolated from in-and out-patients treated in the chest hospital at El- Minia governorate.

MATERIALS AND METHODS

Sampling

This study was conducted in El- Minia governorate. A total of 50 early morning sputum samples were collected between March 2011 until December 2011 from patients attending El-Minia chest hospital and suspected to have pulmonary TB through X ray radiography and Mantoux test. The complete case history of these patients were taken. No patient suffered from immunodeficiency illness or treated with immunosuppressant drugs.

Specimen processing and culture

Sputum samples (3 to 5 ml) were homogenized for 15 min in a shaker using an equal volume of 4% NaOH containing 0.5% NALC decontamination solution at room temperature. After centrifugation at 3,000 rpm for 15 min, the deposit was neutralized with 20 ml of sterile distilled water. The samples were again centrifuged at 3000 rpm for 15 min (Petroff et al., 1915; Tripathi et al., 2014). Then samples (N=50) were inoculated on LJ media and liquid medium Middlebrook 7H9. The observations like rate of growth at different

temperature (28, 30, 37 and 42°C), colony character and pigment production (if any) in light and dark (on LJ slants) for colonies were noted.

Identification of *M. tuberculosis*

Smears were prepared using the dissolved sediment from all specimens, stained by Ziehl-Neelson (ZN) method, and examined for presence of Acid-Fast Bacilli (AFB) with a light microscope. Both the samples sediment before culture on LJ, then the sub-cultured samples on LJ media was subjected to microscopical examination. Colonies of *M. tuberculosis* isolates were identified on the basis of niacin production, nitrate reduction, catalase production (semi quantitative and heat resistant), Tween-80 hydrolysis, Iron uptake, Growth in presence of thiophen-2 carboxylic acid hydrazide (TCH), growth on presence of sodium chloride (5%), Arysulfatse test , Urease test.

DNA extraction

One hundred mg (wet weight) of the cell pellet of each isolate was resuspended in 0.5 ml TE buffer, allowed for 2 cycles freezing and thawing. The cells were then homogenized in glass homogenizer, and then incubated 4 h at 37°C with 5 µl lysozymes (final concentration 100 µg/ml). Proteinase-K was added 25 µl/0.5 ml (final concentration 100 µg/ml) and incubated for further 3 h at 56°C with shaking. DNA was then extracted using Trizol reagent.

One milliliter Trizol was added and after 5 min of incubation at room temperature (RT), 0.4 ml of chloroform was added, vortex for 15 s, kept at RT for 3 min. Then centrifuged at 14,000 rpm for 10 min at 4°C. The upper aqueous phase containing RNA was completely discarded and the DNA in the interphase was precipitated with 0.6 ml absolute ethanol and kept at room temperature for 3 min before centrifugation at 4000 rpm for 5 min at 4°C.

The supernatant was completely discarded and the DNA pellet was washed twice with 1 ml of 0.1 sodium citrate in 10% ethanol. At each time, the DNA pellet was kept in the washing solution for 30 min at room temperature with periodical mixing and centrifuged at 4000 rpm for 5 min at 4°C. Following the 2 washes, the DNA was resuspended in 2 ml of 75% ethanol, kept at RT for 20 minutes with periodical mixing and then centrifuged. The DNA pellet was finally dried briefly for 5 min under vacuum and redissolved in 50 µl of 8 mM NaOH. The pH was then adjusted at 8 by adding 115 µl/ml of 0.1 M HEPES. To DNA was added 10 µl of RNAase and incubated at 37°C for 1 h. The mixture was stored at -20°C till used (Sambrook et al., 1989; Soliman et al., 2004). DNA was purified by using Wizard DNA clean up system (Promega, Catalog no. A7280).

PCR amplification of plc gene

Sequences for plcABC from *M. tuberculosis* H37Rv were obtained from GenBank *(http://www.ncbi.nlm.nih.gov/). plc* gene specific primers were 5'-GGATTCCTGGACTGGCGTTG as forward primer and 5'-CCCACCCAAGAAACCGCAC as reverse primer. The two primers include a 350-bp region. The reaction volume was 25 µl containing, 5 µl of the extracted DNA (100 ng/µl), 1 µl of Taq polymerase (5u/µl), 2.5 µl of 10x enzyme buffer, 2 µl of dNTPs, 10 µl of Q-Solution, 0.1 µl of each of the forward and reverse primers, then the volume was completed with bidistilled water to 25 µl.

The mixture was placed in the thermal cycler (T3000 professional BiometraInc, Germany), which was programmed for 40 cycles with initial denaturation at 95°C for three minutes. Each cycle was performed with denaturation at 95°C for one minute, annealing at 56°C for 45 s with an extension at 72°C for one minute. At the end of the last cycle, Final extension at 72°C for 10 min. The PCR product (6 µl aliquot) was separated by electrophoresis in 1% agarose gel at 100 V for 40 min in Tris-acetate buffer visualized by ethidium bromide staining illuminated by UV transilluminator (Sambrook et al., 1989 and Soliman et al., 2004).

Automated DNA sequencing

The PCR product (16 µl aliquot) of 7 isolates were separated by electrophoresis in 1% low melting agarose gel at 30 v for 60 min at 4°C in Tris-acetate buffer then visualized by ethidium bromide staining, illuminated by UV transilluminator to visualize amplified plc gene from each isolate (Sambrook et al., 1989) and then was sliced of and placed separately in 1.5 ml nuclease free tubes. PCR products were purified from gel slices by using Biospin kit catalog no.BSC02S1 .

Sequencing of PCR products were performed using a MJ Research PTC-225 Peltier Thermal Cycler and the Big Dye Terminator sequencing kit v 3.0 (Applied Biosystems) in accordance with the manufacturer's protocol. Subsequent analysis was performed on an ABI PRISM 3730XL Analyzer (Applied Biosystems). Single-pass sequencing was performed on each template using the primer (forward primer) used for PCR amplification. The resulted sequences were aligned using CLC bio software version 6.7.1.

Phylogenetic analysis

The relationships among the sequenced samples were studied by using CLC bio software version 6.7.1. with using maximum likelihood with the following parameter: Starting Tree: Neighbour Joining, Substitution rate model: Jukes Cantor, Include rate variation: Yes, Number of substitution rate categories: 4, Initial Gamma distribution parameter: 1.0, Estimate Gamma distribution parameter: Yes, Estimate substitution rate parameter(s): Yes, Estimate topology: Yes created.

RESULTS AND DISCUSSION

In this study, we evaluated the prevalence of Mycobacterium species in the upper Egypt area which is mainly an agricultural area.

The relation between different parameters (age, sex, Place of Residence, family history, co-morbid disease) and smear microscopy positivity and culture positivity were shown in Table 1. Our results showed that all sputum samples (50) were acid fast bacilli (AFB) positive. Out of the 50 smear positive samples, 24 samples (48%) were culture positive by using modified Petroff's method. The smear positivity was higher than culture positivity in our study which may be due to that Acid-fast bacterium seen in the smear may represent non-tuberculous mycobacteria. Also, smear positive culture-negative specimens can be resulted from Laboratory errors, prolonged specimen decontamination, shortened incubation times of culture, cross-contamination of smears, using water or stains contaminated with acid-fast organisms or obtaining samples from patients under antimycobacterial therapy (Dunlap et al., 2000).

Sharma et al. (2012) reported that culture positivity obtained by modified Petroff's method (46.7%) were less

Table 1. The relation between different parameters and smear microscopy positivity and culture positivity.

Parameter	Smear microscopy positive (N=50)	Culture positive (N=24)
Gender		
Male	20 (40%)	9 (37.5)
Female	30 (60%)	15 (62.5)
Age		
>20	13 (26%)	5 (21%)
21 – 46	25 (50%)	11 (46%)
41-60	9 (18%)	6 (25)
>60	3 (6%)	2 (8%)
Co-Morbid disease		
Yes	5 (10%)	4 (17%)
No	45 (90%)	20 (83%)
Family history		
Yes	15 (30%)	7 (29%)
No	35 (70%)	17 (71%)
Place of residence		
Rural	38 (76%)	20 (83%)
Urban	12 (24%)	4 (17%)

Table 2. The distribution of tuberculosis patients according to age and sex.

Sex	Age in years								Total (n = 50)	
	< 20		21 – 40		41 - 60		> 60			
	No	%*	No	%*	No	%*	No	%*	No	%*
Male	3	6	9	18	7	14	1	2	20	40
Female	10	20	16	32	2	4	2	4	30	60
Total	13	26	25	50	9	18	3	6	50	100

*Percentage was correlated to the total number of tuberculosis patients.

than NALC-NaOH treatment (63.7%). This mean that modified Petroff's treatment method may result in killing mycobacteria in specimens, so the cultures give negative results which may explain the low positivity of culture in our study. On the other hand, Wulandari et al. (2011) reported that although modified Petroff's method using 4% NaOH was more toxic to mycobacteria than NALC-NaOH method, but if the procedure was done properly and carefully, a good rate of decontamination (2-5%) will be obtained. On contrary, maximum number of cultures positivity were obtained by Modified Petroff's method in studies done by Chaudhary and Mishra (2013) and Tripathi et al. (2014).

Table 2 showed the prevalence of tuberculosis in patients according to their age and sex. Females represented 60% while males were 40% which may be due to some factors such as poverty, pregnancy, ignorance, inadequate anta-natal care in pregnancy. Also, Table 2 showed that TB infection was more common in patients aged 21 to 40 (50%) years, and least in patients aged > 60 years (6%) meaning that pulmonary tuberculosis affected productive age group more. Many studies, done in El-Minia, Port Said, Dakahlia showed also that the highest prevalence of TB was among individuals aged 15 to 30 years (El Zeheiry, 2012; George, 2013; Abu Shabana et al., 2015). Also, Ahmad et al. (2015) found that females were more susceptible to infection 56.21% than males 43.79 and 23.54% of pulmonary TB cases were reported in the age between 15 to 24 years. On the contrary, there are many studies reported that males were more affected than females (Acharya et al., 2007; Jethani et al., 2014; Ifeanyi et al., 2015).

By studying the relation between TB infection and the

presence of underlying diseases (Diabetes mellitus, hypertension asthma, chronic kidney disease (ckd)), it was found that about 10% of smear positive cases and 17% of culture positive cases had Co-Morbid diseases. Gupta et al. (2011) found that Diabetes mellitus (DM) was the most underlying risk factor followed by smoking, alcoholism in patients with pulmonary tuberculosis. In a study carried by Golsha et al. (2009), Diabetes mellitus was found to be the most prevalent condition (23.05%) followed by chronic renal failure, corticosteroid consumption and malignancy ranking second, third and forth in the list (5.8, 2.5 and 2% respectively) in patients with pulmonary tuberculosis.

Also, Shetty et al. (2006) and Reis-Santos et al. (2013) found strong relation between TB infection and the presence of diabetes in their studies. Smoking is another factor that increases the chance of infection with TB. It was found that 80% of male cases were smokers for cigarette and Shisha (Goza). Which indicates that smoking may be an important predisposing factor for the infection with *M. tuberculosis.* In agreement with our results, Gupta et al. (2011) reported that 16.9% of pulmonary TB patients were smokers. Also, the same results were showed by Shetty et al. (2006). The present study found that 30% of smear positive cases and 29% of culture positive cases had family history of TB and one or more of the family members were infected. In a study by Abdelwahab et al. (2009) showed that family history is the most important parameter that increase the rate of the infection by *M. tuberculosis.*

Results showed that 76% of smear positive patients and 83% of culture positive patient were from rural area. Most of these cases sharing house with another family members infected with TB and cattles. This high percent in rural area may be attributed to the low socioeconomic level, high family size, and poor socio-economic status. Poor education is associated poor knowledge of tuberculosis, inadequate and delayed availability of health care also associated with the prevalence of tuberculosis. Similar findings have been reported by George (2013) in the El-Minia governorate who explain increasing tuberculosis cases in rural areas due to poverty and bad social conditions and milk sanitation also agricultural workers may acquire occupational infection by bovine bacilli from the infected animals. Hindi (2009) found that rural cases (76.2%) were significantly higher in comparison with urban cases (23.8%) in retrospective study at the Benha chest hospital over the period from 2002 to 2006. Also, Abdelghany (2010) conducted a similar study in the Menoufia governorate (1992 to 2008), he concluded that rural cases (80.05%) were also significantly higher than urban cases (19.95%) during all years of the study. on other hand, Arya et al. (2013) who reported that sputum positivity for *M. tuberculosis* using Zeihl Neelsen staining for samples obtained from rural and urban were nearly the same (15.8 and15.2%).

Although, most of cases were from rural area, physical and biochemical identification tests to the 24 mycobacterial isolates showed that the prevalent strain was *M. tuberculosis* while *M. bovis* was not isolated (Table 3). Ameni et al. (2013) studied the prevalence of Mycobacterium among Ethiopian farms who shared animals in their housed. They found that All mycobacteria (141) isolated from farmers were *M. tuberculosis*, while only five of the 16 isolates from cattle were members of the *M. tuberculosis* complex (MTC) while the remaining 11 were members of non-tuberculosis mycobacteria (NTM). Further speciation of the five MTC isolates showed that three of the isolates were *M. bovis* (strain SB1176), while the remaining two were *M. tuberculosis* strains (SIT149 and SIT53). Also, they found that transmission of TB from farmers to cattle by the airborne route sensitizes the cows but rarely leads to TB. Similarly, low transmission of *M. bovis* between farmers and their cattle was found, suggesting requirement of ingestion of contaminated milk from cows with tuberculous mastitis.

Saifi et al. (2013) reported that 80% *M. tuberculosis*, 6% *M. bovis* and 14% NTM strains were obtained from positive cultures isolates. Species identification by conventional cultural methods and biochemical tests are of limited value because of the comparatively low yield of the culture and long time to grow. Even the so-called rapid growers may require 1 to 3 weeks to grow. The differentiation between *M. tuberculosis* and *M. bovis* is

Table 3. Results of physical and biochemical identification of the isolated acid fast bacilli.

Test or property	Result
Number of isolates	24
Zeal Nielsen staining	Acid fast bacilli
Growth at 28°C	-ve
Growth at 35°C	+ve
Growth at 37°C	+ ve
Growth at 42°C	±Ve
Colony morphology	Rough
Dark pigmentation	non-chromogen
Photo pigmentation	non-chromogen
Niacin production	+ve
Nitrate reduction	+ve
Tween 80 hydrolysis 10 days	-ve
Catalase semiquantitative < 45	-ve
Catalase PH7.0 -68°C	-ve
Arylsulfatase (3days)	-ve
Arylsulfatase (2 weeks)	-ve
Urease	+ ve
Iron uptake	-ve
Growth on TCH (2µg/ml)	+ve
Growth on NaCl 5%	-ve

*Percentage was correlated to the total number of tuberculosis patients.

Table 4. The distribution of Mycobacterium tuberculosis infection according the type of patients and sex.

Type of infection / Sex	New case	%*	Reactivation	%*	Relapsed	%*
Male	18	36	1	2	1	2
Female	26	52	4	8	-	-
Total	44	88	5	10	1	2

*Percentage was correlated to the total number of tuberculosis patients.

not always conclusive by Niacin production and PZA sensitivity tests. To differentiate between *M. tuberculosis* complex and non-tuberculous mycobacteria, PCR was a very good tool. If it was used in conjunction with culture and biochemical tests; it would give best results as already mentioned by Gupta et al. (2010).

Results shown in Table 4 showed that 88% of patients were new cases, while 10% were reactivated cases. Most of the reactivated cases were for female which may be due to pregnancy, the presence of active cases in their houses. One of these females was sharing house with 4 family members active cases.

Also, some of the reactivated cases were suffering from depression due to losing someone they love in their families or due to the presence of psychological stress of any other reason. The relapsed case for a patient who was treated from TB infection with his sister in the same time. He did not complete his course of treatment because he was a prisoner. In addition, We found that all new cases were treated by first line regimen that include pyrazinamide, Ethambutol, Rifampicin, Isoniazide. The relapsed case was treated by first line regimen plus streptomycin.

The standard regimen for treatment of new cases of pulmonary tuberculosis consists of 6 months treatment, with four drugs in the initial phase including isoniazid, rifampicin, pyrazinamide, and either ethambutol or streptomycin, followed by two drugs in the continuation phase including isoniazid and rifampicin. For retreatment cases (reactivated, relapsed), the recommendation is a 9-month standard regimen starting with five drugs including isonazid, rifampicin, pyrazinamide, ethambutol and streptomycin for the initial 3 to 4 months, followed by isoniazid and rifampicin in the continuation phase (Tam, 2006). WHO recommended drug susceptibility testing (DST) at the start of therapy for all previously treated patients. As, previously treated patients are defined by their likelihood of MDR-TB. The retreatment regimen with first-line drugs is ineffective in MDR-TB; it is therefore critical to detect MDR-TB promptly so that an effective regimen can be started. So, samples for culture and drug susceptibility testing (DST) should be obtained from all previously treated TB patients at or before the start of treatment. DST should be performed for at least isoniazid and rifampicin (WHO, 2010).

In our study, we found that 60% of reactivated cases were treated by first line regimen and other 40% were treated with first line regimen plus streptomycin. It mean that not all reactivated cases were treated by one regimen and no drug susceptibility testing was done that may suggest the presence of multi drug resistant-TB (MDR) strains in the hospital. As all patients in our study were in the same hospital and treated in the same period.

Plc gene is considered as one of the virulence factor for *M. tuberculosis*, the 350 bp region of this gene was amplified and sequenced. All the 24 grown isolates gave nearly the same amplicon size (Figures 1, 2 and 3 represents a selected sample of the amplified plc gene from some isolates). Direct PCR method based on the amplification of 350 bp region of plc used for detection of *M. tuberculosis*, to be differentiated it from *M. bovis* in clinical samples. The result of our study is in accordance with the study made by Behr et al. (1999). Also, Vera-Cabrera et al. (2001) in their study found out that the main genetic difference known between *M. tuberculosis* and *M. bovis* was the presence of the mtp40 sequence. mtp40 was part of the mpcA gene, which encodes a phospholipase C.

Goudarzi et al. (2010) reported that Phospholipase C genes could be important in pathogenesis of Beijing strains of *M. tuberculosis*. The Beijing MTB strain has attracted special attention because of its global emergence and resistance to multiple drugs.

Seven isolates were subjected for sequencing analysis of the PCR amplified plc fragment. Sequence alignment revealed that many substitution mutations (SNPs) were found along the entire sequenced fragment denoting the heterogeneity of the isolated strains (Figure 4). Although these strains were isolated from the same governorate and within a short period of time therefore homogeneity of the isolated mycobacterial strains were assumed to be, and that in contrary of the data presented here. The main cause for such condition may rely on inadequate treatment regimen such as intermittent dosing during the initial intensive treatment phase (Li et al., 2005; Burman et al., 2006) or discontinued treatment (Nolan et al., 1995; Bradford et al., 1996). In a study by Ramaswamy and Musser (1998) found that inadequate treatment and non-compliance lead to the development of selected mutated resistant and MDR-TB. Drug stress induced

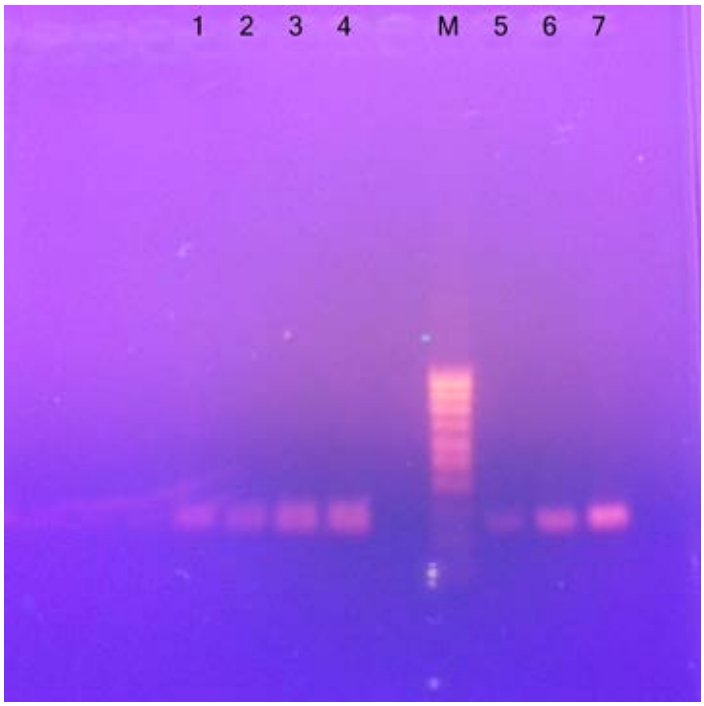

Figure 1. Amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 4 represents isolate number 1, 2, 7, 9 and Lane 5 to 7 represents isolate number 10 , 11 and 12.

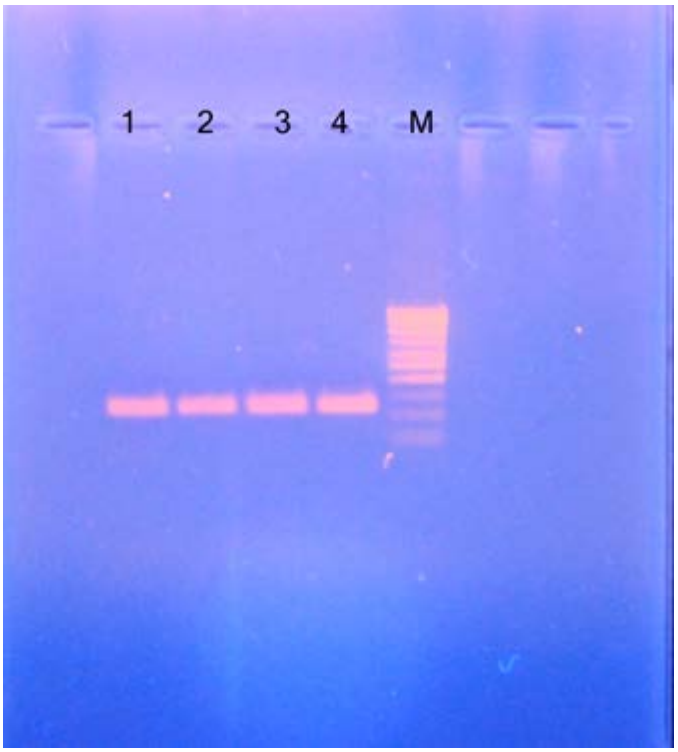

Figure 2. amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 4 represents isolate number 13, 21, 22 and 31.

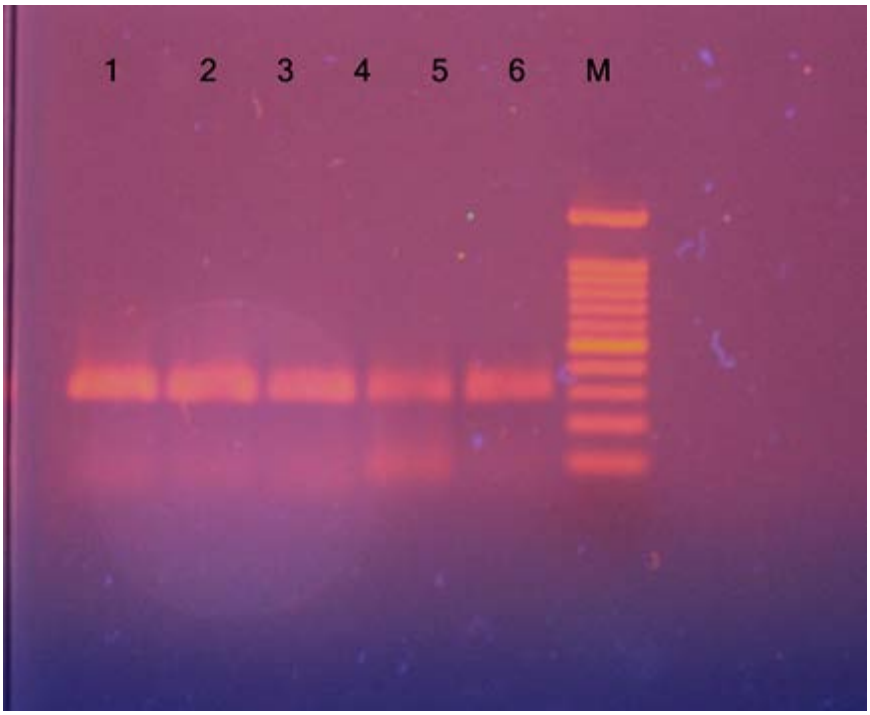

Figure 3. Amplified plc gene migrating about 350 bp from isolated *M. tuberculosis* strains, M= 100 bp DNA ladder, Lane 1 to 6 represents isolate number 32, 38, 39, 40, 41, and 42.

mutation is one of the main methods by which the mycobacteria can escape the devitalizing state caused by the drug or the immune system to survive in the tissues and because plc gene is one of the important virulence factor as described earlier so it would be reasonable to be target for mutations .

Unsurprisingly, the phylogenetic tree (Figure 5) sub classed the 7 sequenced isolates into 3 distinct nodes: node 1 (isolates 31 and 1), node 2 (with isolates 43 and 22), and node 3 (isolates 49, 40 and 2). Each node contain samples for patients that may share some socio-demographic characters and differ in some. As patients of node 2 differ in their marital state (43 is married while 22 is single) such condition may arise from different source of infection, different responses to the drug regimen, non-adherent to the drug regimen. Such cause would raise a question about the usefulness of the same drug regimen to all patients.

Table 5 showed that most of patients were from rural area, they all shared in the treatment regimen which was first line consisting of isoniazid, rifampicin, pyrazinamide and ethambutol.

LIMITATIONS OF THE STUDY

This study has certain limitations; the sample size was limited as it covered only a proportional number of the total reported cases during the study period. Also 7 selected isolates were sequenced only. Other studies on large sample size may be needed to give precise data about the prevalence of mycobacterial strains in El-Minia governate and on other governates in Egypt. In future, we can study the prevalence of MDR strains in the environment of the hospital.

Conclusion

(1) *M. tuberculosis* was the prevalent strain in El Minia governate.
(2) Tuberculosis was most prevalent in female especially at age group 21-40 due to some factors such as poverty, pregnancy, ignorance, inadequate anta-natal care in pregnancy, the lack of female education, carelessness.
(3) Most of cases were from rural area which may be due to their lifestyle, ignorance, large number of family members sharing the same house and they can share their houses with animals. In addition to the presence of psychological stress.
(4) Smoking, Family history of TB infection, Co morbid disease (especially Diabetes mellitus) are an important predisposing factors for the infection with *M. tuberculosis*.
(5) To choose the effective treatment regimen and detect the presence of Multi-drug resistance (MDR) strains, drug susceptibility tests should be done to the reactivated,

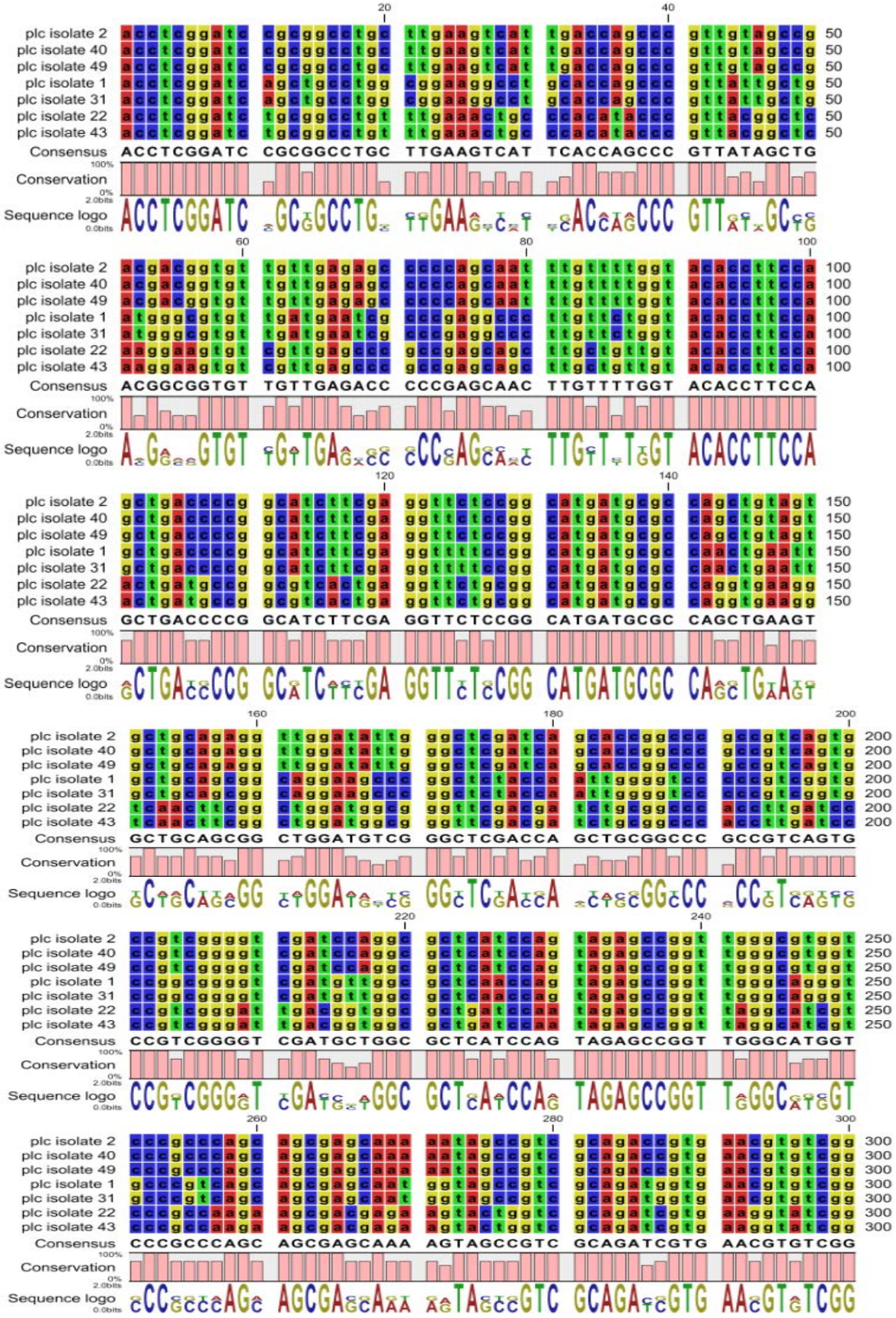

Figure 4. The sequence alignment of the plc gene amplified from 7 selected Mycobacterium *tuberculosis* isolates.

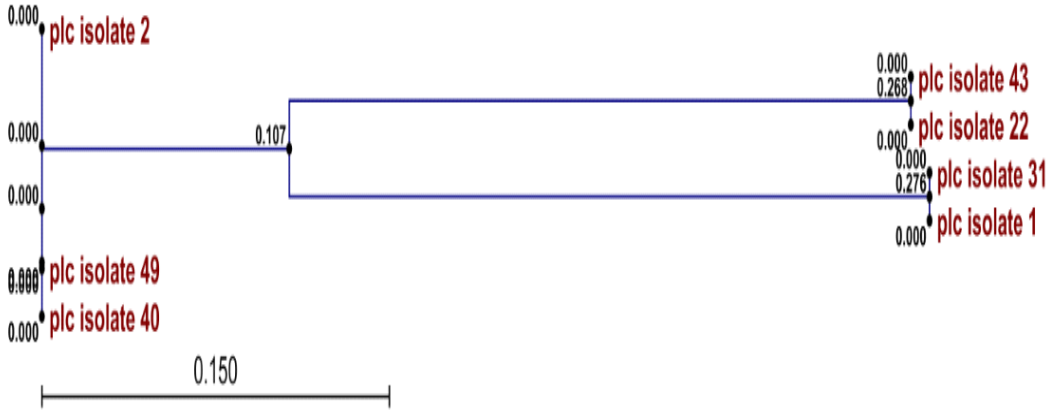

Figure 5. The phylogenetic tree of the sequenced plc gene amplified from the 7 selected *M. tuberculosis* isolates.

Table 5. Socio-demographic data for the tested cases in each node of the phylogenetic tree.

	Sample no.	Sex	Age	Place of residence	Marital state	Presence of house animals	Treatment	Co-morbid disease	Family history	Case
Node 1	1	F	58	Rural	Married	Yes	First line	Yes	No	New
	31	F	21	Rural	Married	yes	First line	No.	yes	Reactive case
Node 2	43	F	20	Rural	Married	Yes	First line	No	No	New
	22	F	24	Urban	Single	No	First line	No	No	New
Node 3	2	F	21	Rural	Married	No	First line	No	No	New
	49	M	59	Urban	Married	No	First line	No	No	Reactive case
	40	M	44	Rural	Married	No	First line	Yes	No	New

F: Female, M: Male.

relapsed cases and patient who had family history of TB infection before the beginning of treatment.

(6) There was different substitution mutations in the plc gene which may be due to the intermittent treatment regimens that made the tested strains under stress resulting in mutation.

Conflict of Interests

The authors have not declared any conflict of interests.

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