

Full Length Research Paper

Role of phospholipase C in pathogenesis of Beijing and Non-Beijing strains of *Mycobacterium tuberculosis*

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The Beijing strain of *Mycobacterium tuberculosis* bear high transmission potential and there is a significant correlation between Beijing strain and multidrug resistance. Phospholipase of *M. tuberculosis* plays an important role in its pathogenesis through breaking up phospholipids and production of diacylglycerol (DAG) as an important signaling molecule in infected macrophages as well as a precursor for synthesis of triacylglycerol that acts as an energy store for utilization during long-term dormancy of *M. tuberculosis*. DNA extraction was performed by using CTAB (cetyltrimethylammonium bromide) method from positive culture specimens of tuberculosis patients (Van Soolingen et al., 1991). Spoligotyping was done for differentiation of Beijing strains from non-Beijing strains. Finally Phospholipase C region was detected by polymerase chain reaction (PCR). The current study showed that, 19 (9.5%) of 200 strains were Beijing strain and 181 (90.5%) were non-Beijing strains, corresponding to spoligotyping. Based on PCR assays for *plcA*, *plcB*, *plcC* genes, of Beijing strains, 16(84.2%) strains were positive for *plcA*, and 17 (89.4%) strains were positive for *plcB* and 17(89.4%) strains were positive for *plcC* genes (Goudarzi et al., 2010). In non-Beijing strains 17 strains (9.4 %), 18 (9.9%), 18 (9.9%) were positive for *plcA*, *plcB*, *plcC*, respectively. Considering the majority of Beijing strains possess *phospholipase C* genes, it is possible that *plc* serves a role in pathogenesis and thereby severity of tuberculosis diseases. However, confirmative studies will be needed to verify the exact role of phospholipase C in the pathogenicity of *M. tuberculosis*.

Key words: Tuberculosis, phospholipase c, spoligotyping, polymerase chain reaction.

INTRODUCTION

Mycobacterium tuberculosis the causative agent of pulmonary tuberculosis (PTB) infects one-third of the world population. Tuberculosis remains a leading infectious killer all over the world (Kam et al., 2006). TB has many manifestations that affecting bone, the central nervous system, and many other organ systems, but it is

primarily a pulmonary disease that is initiated by the deposition of *M. tuberculosis*, contained in aerosol droplets, onto lung alveolar surfaces. Despite the knowledge obtained in the last 100 or more years, *M. tuberculosis* does not have classical virulence factors like those which are the major causes of diseases due to other bacterial pathogens, e.g., toxins produced by *Corynebacterium diphtheriae*, *Escherichia coli* O157:H7, *Shigella dysenteriae*, and *Vibrio cholerae*. But in order to develop a better vaccine and more efficient therapeutic agents for tuberculosis prevention and control, it is

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essential to gain a better understanding of the pathogenicity and the virulence of *M. tuberculosis* (Issar, 2003). One of these virulence factors is phospholipase C. Phospholipase C plays a role in the pathogenesis significantly. (Kent et al., 1985; Srinivas et al., 2008). The expression of the different *plc* genes, is important for virulence at any time of the infection. The precise role of phospholipase C in pathogenesis of *M. tuberculosis* is unknown but overall activity of this virulence factor is Hydrolysis of phospholipids, then generates diacylglycerol (DAG), a well-known signalling molecule in infected macrophages as well as a precursor for synthesis of triacylglycerol, that acts as an energy store for utilization during long-term dormancy of *M. tuberculosis* (Srinivas et al., 2008). Phospholipase C genes include A, B, C and D segments. Three of these genes, *plcA*, *plcB* and *plcC*, are located at position 2351 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 (Vera-Cabrera et al., 1997; Ramazanzadeh et al., 2006; Parker et al., 2009).

Functional study of *M. tuberculosis* was demonstrated that all four genes encode functional PLC in *M. tuberculosis*, and each gene contributes to overall PLC activity (Raynaud et al., 2002).

Tuberculosis problem is further complicated by the emergence of multidrug-resistant strains as a consequence of the widespread use and incautious administration of antibiotics (Stavrum et al., 2008; Rohani et al., 2009; Alonso et al., 2010; Niemann et al., 2010). In this condition, the Beijing strain of *M. tuberculosis* has attracted special attention because of its global emergence and resistance to multiple drugs (Ferdinand et al., 2003; Sun et al., 2009; Reece et al., 2010; Cohen-Bacrie et al., 2011). For this reason we selected Beijing strains for our study. Tuberculosis is a major cause of morbidity and mortality in developing countries. In many areas in the developing world, tuberculosis is one of the most common causes of death among adults, so it is certainly the preventable causes of death (Stavrum et al., 2008). Tuberculosis has been a major health problem and a threatening disease in Iran for a long time. These cases concentrate in several areas include Sistan and Baluchestan in East, Golestan in North, Hormozgan in South, Khorasan in North East and Khuzestan in South West (Ramazanzadeh et al., 2006; Rohani et al., 2009). According to the World Health Organization (WHO), the estimated incidence of TB in Iran, is 28 cases per 100000 populations (WHO, 2005). The aim of this study was; 1) determination of drug susceptibility in clinical isolates, 2) determination of spoligotyping pattern of the clinical strain isolated from patients, 3) determination of the extent of Beijing strains prevalence among the clinical isolates, 4) detecting the prevalence of *plcA*, *plcB*, and *plcC* genes

by PCR amplification with specific primers among Beijing and non-Beijing strains.

MATERIALS AND METHODS

Patients and samples

Of all Patients who referred to National Research Institute of Tuberculosis and Lung Disease (NRILT) due to confirmed their pulmonary tuberculosis from August 2008 to August 2010 in Tehran, Iran, 200 patient's possessed *M. tuberculosis* culture positive specimens that were used for this research. All patients had positive acid-fast bacilli (AFB) in their smear microscopy results and their chest X-ray (CXR) had classical picture of TB, that is, upper lobe infiltration with presence of cavities. They had positive PPD test result according to the centers for disease control and prevention (CDC) criteria (CDC 2003) with other laboratory or clinical sign of diseases development. All the patients have signed informed consent.

M. tuberculosis isolation and identification

The pulmonary specimens were collected and processed for culture by digestion, decontamination and concentration following modified Petroff's method and were inoculated into 2 tubes of LJ media. The slants were examined for growth once weekly up to 8 weeks (Kent et al., 1985). All isolates were identified as *M. tuberculosis* by positive reaction in AFB staining, biochemical tests including production of niacin, catalase activity, nitrate reduction, pigment production and growth rate (Rohani et al., 2009).

Spoligotyping

In spoligotyping, all the isolates were studied based on spacer types as described previously by Kamerbeek et al (1997). In spoligotyping the direct repeat (DR) region was amplified by PCR using primers derived from a DR sequence. The amplified DNA hybridized to a set of 43 immobilized oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 by reverse line blotting (Sun et al., 2009; Dong et al., 2010). Spoligotype patterns were designated with hexadecimal codes and/or arbitrary database numbers (Dale et al., 2001). Typing results were analyzed and compared with world spoligotyping database (Sola et al., 2006).

Antibiotic susceptibility

Drug susceptibility testing against isoniazid (INH), rifampicin (RF), streptomycin (SM), ethambutol (ETB); all drugs from Sigma Chemical (St. Louis, MO) were performed by the proportional method on Lowenstein-Jensen media at a concentration of 0.2, 40, 10 and 2.0 µg/ml, respectively (Ramazanzadeh et al., 2006). Briefly 1 McFarland were added to LJ media and result was analyzed according to Clinical and Laboratory Standards Institute (CLSI) guide line after 3 weeks incubation.

DNA extraction and polymerase chain reaction

The isolation of genomic DNA was carried out by the CTAB

Table 1. Oligonucleotide primers used in different PCR assays.

Gene	Designation	Sequence (5'-3')	Product size	Ref.
<i>plcA</i>	<i>plcA</i> -F	5'TCG AAC GCC GGG AGA TTA CC 3'	450bp	11
	<i>plcA</i> -R	5'GCA GGA AGG CAG GGC AAG TG3		
<i>plcB</i>	<i>plcB</i> - F	5'-TCC GGC GAA TGC ACC TTG GCT CAC-3'	1300bp	11
	<i>plcB</i> -R	5'-CGG CAG GCA GGC GGA ATC AGA ACA-3'		
<i>plcC</i>	<i>plcC</i> -F	5'-GGG CGG CAA AGG CGG ACC AAG AG-3'	500bp	11
	<i>plcC</i> -R	5'-AAG CCG AAA TAC ACG AGG GAG AGC-3'		

*PLC: phospholipase C genes, *plcA*:phospholipase C_A, *plcB*: phospholipase C_B, *plcC*: phospholipase C_C.

(cetyltrimethylammonium bromide) method (Van Soolingen et al., 1991). Briefly bacteria were harvested from the Lowenstein–Jensen slopes, heat-killed at 80°C for 20 min and then incubated with lysozyme followed by digestion with proteinase K in 10% SDS. A further incubation with CTAB/NaCl and sedimentation by using chloroform/isoamyl alcohol were the next steps. The total DNA was precipitated using isopropanol.

PCR of *plcABC*

The PCR amplification of *plcA*, *plcB*, and *plcC* genes was performed using previously published primers by Sarah Talarico et al (Talarico et al., 2004). The sequences of gene-specific primers were as indicated in Table 1. The amplification reaction was performed in a 25 volume reaction mix containing. All PCR mixtures were prepared in a volume of 2.5 µl PCR buffer, 1 mM MgCl₂, 0.5 µm each of four dNTP, 2 µm each of primers, 1.5 U of Taq polymerase, 300 ng of extracted DNA from sample and run at 94°C for 1min. PCR program was as follows : 26 cycles: 94°C for 30s, annealing at 62, 65 and 67°C for 30 s in *plcA*, *plcB* and *plcC* genes, respectively and extension at 72°C for 150 s and final extension at 72°C for 10 min. The amplification products, together with 100 bp ladder fragment, were electrophoresed on 1% agarose gel and were stained and visualized by using ethidium bromide.

Data analysis

Statistical analysis was done with SPSS (version 11.5). Chi-square and Fisher exact test were applied: P value < 0.05 was considered significant. In addition, all figures including percentages in tables and text were rounded down if they were <0.5, and were presented as whole numbers if they were ≥0.5.

RESULTS

Of all referred patients with pulmonary TB to MRC (Mycobacteriology research center), 200 patients 108 (54%), male and 92 (46%), female with mean age 49 ± 18 (varies 10 to 88) years that were positive for MTB culture then were analyzed by spoligotyping. According to spoligotyping, 181 (90.5%) were defined non-Beijing and 19 (9.5%) were Beijing strains (Table 3). There were no

relationship between gender and type of TB (Beijing and non Beijing). Due to resources limitation, the antibiotic susceptibility test was performed only for Beijing strains. According to drug susceptibility testing, 63% of isolates were resistant to INH followed by 58% for RIF, while 95% and 84% of isolates were sensitive to ETB and ST respectively. Fifty three percent of studied Beijing MTB was resistant to both INH and RF while this rate was 5% for INH and ST (detail are summarized in Table 2). In MTB any isolate resistant to Isoniazid and Rifampin is considered as multi drug resistant (MDR) (WHO, 2010). We found 53% (10 of 19) Beijing isolates and 10% (18 of 181) non-Beijing strains were categorized as MDR Of studied isolates. Overall 14% of studied isolated regardless to spoligotyping were considered as MDR. 26% of Beijing strains were sensitive to all applied antibiotics whereas 21% were resistant to one drug (Table 2). Using PCR assays for *plcA*, *plcB*, *plcC* genes, among Beijing strains, 16 strains (84.2%) were positive for *plcA*, 17 (89.4%) for *plcB* and 17 (89.4%) for *plcC* genes and in non-Beijing strains 17strains (9.4%), 18 (9.9%), and 18 (9.9%) were positive for *plcA*, *plcB*, and *plcC* respectively. Interestingly rate of studied phospholipases were extremely statically significant among Beijing and non-Beijing isolates (p< 0.0001). The standard strain H37RV(ATCC 27294) was used as a positive control and sterile water as a negative control in each reaction.

DISCUSSION

TB is considered as a major worldwide health problem, particularly in developing countries where high incidence of diseases is quite common. Also there is a concept that understanding of TB pathogenesis is important in disease control and prevention. Phospholipase is well known virulence factor in some microorganisms such as *Listeria* spp., *Bacillus* spp., *Pseudomonas* spp., *Clostridium* spp. and *Mycobacterium* spp. (Marquis et al., 1997; Agaisse et al., 1999). Recently, there are limited studies which

Table 2. Result of antibiotic susceptibility tests for Beijing strains.

Antibiotics	INH	RF	ST	EB	INH+RF	INH+SM	RIF+ST	RIF+EB	INH+EB
Sensitive	7(37%)	8(42%)	16(84%)	3(16%)	9(47%)	18(95%)	19(100%)	18(95%)	18(95%)
Resistant	12(63%)	11(58%)	3(16%)	1(5%)	10(53%)	1(5%)	0 (0%)	1(5%)	1(5%)
Total	19	19	19	19	19	19	19	19	19

* INH: Isoniazid, RF: Rifampin, SM: streptomycin, ETB: ethambutol.

Table 3. The frequency of various phospholipase C genes in Beijing and non-Beijing strains of *M. tuberculosis*

PLC	Type of plcC			
	Number	plcA	plcB	plcC
Beijing	19(9.5%)	16(84.2%)	17(89.4%)	17(89.4%)
non-Beijing	181(90.5%)	17(9.4%)	18(9.9%)	18(9.9%)
P value		0.001	0.001	0.001

*PLC: phospholipase C genes, plcA:phospholipase C_A, plcB: phospholipase C_B, plcC: phospholipase C_C.

propose PhospholipaseC (plcC) as a putative virulence factor for TB. In the present study, our aims are detecting prevalence of phospholipase C and related segments including A, B and C genes in Beijing and non-Beijing strains of *M. tuberculosis*. TB isolates were classified to Beijing and non-Beijing strains based on spoligotyping. Beijing strains were believed to be more virulent (Sun et al., 2009). According to our results, 9.5% of isolates were defined as Beijing strains which were different with Saudi Arabia 4.5% and central Asia 50% (Cox et al., 2005; Al Hajoj and Rastogi, 2010). According to drug susceptibility testing for Beijing strains we found that 63% of isolates were resistant to INH followed by 58% for RF. We found 53% Beijing isolates and 10% non-beijing strains were resistant to both INH and RF (MDR) ($p < 0.05$). This findings were accordance with those studies which showed Beijing strains were more resistant, than non-Beijing (Alonso et al., 2010; Cohen-Bacrie et al., 2011). Our results were far different from the study in the North East Iran during 2001 to 2002, from 80 isolates of patients with pulmonary tuberculosis, only 3% (compare to 14% of current study) were MDR whereas 71% were sensitive to all drugs including INH, ETB, RF, and SM (Namaei et al., 2006). This contrast may be is due to change of TB feature during time or place even in close region. Our results were similar to central Asia where 58% of Beijing strains were MDR, 52% resistance to INH, 27% resistance to RF (Cox et al., 2008) and far different from Sweden and South East Asia which low number of MDR, 24 and 20% respectively, was detected among Beijing strains followed by 73 and 19% resistance to INH

respectively. In South East Asia 99% isolates were sensitive to RIF (Agdamag et al., 2003; Ghebremichael et al., 2010). Various studies have been suggested that phospholipases are found in a wide variety of Gram-positive and Gram-negative bacteria and are generally categorized by substrate specificity. Of different classes of phospholipase, phospholipase C plays significant role in bacterial pathogenesis such as interference with signal transduction and modulating the host immune responses (Marquis et al., 1997; Agaisse et al., 1999; Marquis and Hager, 2000). Although bacterial PLCs are thought to be key virulence factors in several infectious diseases, the pathogenic mechanisms are quite varied. For example, clostridial α -toxin is a PLC with haemolytic and lethal dermonecrotic and platelet-aggregating properties whilst that of *Listeria monocytogenes* functions to allow the organism to escape from intracellular phagolysosomes. Purified PlcH preparations from *Pseudomonas aeruginosa* are cytotoxic since injection into mice causes hepatonecrosis and renal tubular necrosis. This PlcH also inhibits the bacterium-induced neutrophil respiratory burst by interfering with a protein kinase C-dependent, non-p38 kinase-dependent pathway (Marquis and Hager, 2000; Gomez et al., 2001; Korbsrisate et al., 2007). Very limited studies demonstrated that phospholipaseC may have a role in pathogenesis of *M. tuberculosis*. An investigation indicated that the expression of the *plc* genes of *M. tuberculosis* is also up regulated in macrophages during host infection (Bakala N'gom et al., 2010). These findings may support the idea that the Phospholipases C plays a role in mycobacterium infection severity. Marquis et al.

(1997) revealed the disruption of the *plcABCD* or *plcABC* genes, impaired the ability of *M. tuberculosis* to multiply in the lungs and spleen of infected mice. This study suggests that phospholipases C are required for the full virulence of *M. tuberculosis* (Marquis and Hager, 2000). The other study showed a limited role for *plcD* in the virulence of the tubercle bacillus compared to other types of *plc*, as they demonstrated that three out of seven studied clinical isolates were deficient in the expression of *plcD* (Vera-Cabrera et al., 1997). Then we thought if *plc* were important for pathogenesis, it should be more prevalent in Beijing strains which were known as a more virulent TB strains. Surprisingly we found out that *plc a*, *b* and *c* were more prevalent in Beijing strains, 84% *plcA*, 89% *plcB*, and 89% *plcC* in comparison with Non-Beijing ones, (9.4%) *plcA*, 9.9% *plcB* (%), and 9.9% *plcC*. The correlation of Beijing strains with presence of *plc A*, *B* and *C* were considered extremely statically significant ($p=0.0001$). Thereby our data suggests that *plc* probably plays important role in pathogenesis of *MT* and presences of *plc* may leads to emerge of high virulent strains. Future genomic and proteomic studies will need to address how the variations of these genes among clinical isolates relate to the pathogenicity and virulence of *M. tuberculosis* by correlating genetic polymorphisms of the isolates with clinical and epidemiological data of the patients from whom the isolates were obtained. Identification of genes that will show genetic polymorphisms having a strong association with the important clinical and epidemiological characteristics of the patients can provide information useful for a rational selection of genes for future *in vitro* and *in vivo* functional analysis and allow for a more focused search for virulence factors of *M. tuberculosis*, which will ultimately contribute to the development of better vaccines and therapeutic agents for tuberculosis prevention and control.

Conclusion

High number of Beijing strains in our population has been made TB status in alarming situation and needs urgent consideration. Respecting to presence of remarkable number of MDR among Beijing strains, current study supports that Beijing strains are more virulent strains. *Plc* is extremely frequent in being strains which support the possible role of this gene in TB pathogenesis and disease severity. However, confirmative experiments are necessary to verify the precise role of phospholipase C in pathogenesis of *M. tuberculosis*.

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