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Research paper

AMPLIFICATION OF A GENOMIC LOCUS SIGNIFICANT IN THE VIRULENCE OF *MYCOBACTERIUM TUBERCULOSIS*

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Abstract:

Ever since the availability of the whole genome sequence, a lot of research have been going on in different countries to understand the pathogenicity and virulence of the strains of *Mycobacterium tuberculosis*. Since India is home to many tuberculosis patients, there is a possibility of the presence of a lot of genetically heterogeneous isolates . One of the important differences which have been pointed out earlier by different studies is the regions of deletions (RDs) in different species and strains of *M. tuberculosis*. Here we have attempted to standardize the amplification conditions for checking the presence of a part of RD1, a region which is deleted in *Mycobacterium bovis* BCG, but present in the genome of *M. tuberculosis* H37 Rv. The patient isolates from South India was used for this study.
Keywords: *Mycobacterium tuberculosis*, RD1, virulence, PCR.

INTRODUCTION

The credit for the world's top killers among infectious disease causing bacteria goes to *M. tuberculosis*, the causative agent of tuberculosis. In the recent years the burden of tuberculosis deaths has increased owing to the increasing number of multidrug resistant strains of the pathogen and also due to its association with HIV. *M. tuberculosis* was first isolated by Robert Koch in 1882 (Koch, 1932). It is an obligate parasite of humans and has mainly an airborne mode of transmission. It is a fastidious, slow growing, strictly aerobic, lipid-rich, hydrophobic, acid-fast rod shaped bacteria. It is an extremely slow growing bacteria. Under optimal conditions, it requires 16-18 hours to undergo one cycle of replication. A lot of variations can be expected in properties such as pathogenicity, host specificity, virulence, adaptation to particular habitats and drug resistance among individual strains within a single bacterial species. Whole genome sequences of the type strain *M. tuberculosis* H37Rv (Cole *et al*, 1998), the clinical strain *M. tuberculosis* CDC1551 (Fleishmann *et al*, 2001) and *M. bovis* (Garnier *et al*, 2003) are already available. Many researchers have used a number of comparative analysis techniques like subtractive hybridization and microarray to identify differences in the genomes of laboratory strains and vaccine strains. Three genomic regions which are absent in *M. bovis* BCG, but present in *M. tuberculosis* were first described using subtractive hybridization (Mahairas *et al*, 1996). DNA microarray based studies between H37Rv and BCG have shown six deletion regions in *M. tuberculosis* H37Rv – RvD1 to RvD5 and TbD1. The present study was an attempt to understand the isolates from Kerala, a state in south India, with respect to the RD1 locus by standardizing the amplification conditions for the genes in RD1 locus.

MATERIALS AND METHODS

Bacterial strains

M. tuberculosis strains H37Rv, H37Ra and *M. bovis* were the type strains used for the present study. The clinical isolates collected from tuberculosis patients in Kerala comprised the field strains in the present study.

DNA isolation

DNA was isolated from cultures grown on LJ slants according to the standard protocol (Jacobs *et al.*, 1991). Culture was scraped off from the slants and suspended in 500µl saline (0.9 % sodium chloride), in a 2ml screw capped tube. The tube was centrifuged at 10,000 rpm for 5 minutes to wash off the medium. The pellet was resuspended in 500µl homogenisation buffer (Tris HCl: 3.63g/100ml, NaCl: 0.58g/100ml, EDTA: 0.22g/100ml, pH 8) and mixed with 0.2mm glass beads. Cells were disrupted using a Mini-Beadbeater (Biospec Products) at 2500 rpm for one minute. It was then extracted twice with 500µl chloroform. The aqueous phase obtained after centrifugation at 12000 rpm for 10 minutes at room temperature was precipitated with 2.5 volume ethanol and 50µl of 3M sodium acetate by incubating at -70˚C for an hour. The DNA was pelleted by centrifuging at 12,000 rpm for 10 minutes at 4˚C. The pellet was washed with 500µl 70% ethanol, dried at room temperature and suspended in TE (Tris HCl, pH 8: 10mM, EDTA pH 8: 1mM). It was then treated with Ribonuclease A (10µg/tube) at 42˚C for 30 minutes. RNase A was removed by extracting once with phenol-chloroform-isoamyl alcohol (25:24:1) and then with chloroform-isoamyl alcohol (24:1). The purified DNA was precipitated with 2.5 volumes of ethanol and $1/10^{th}$ volume 3M sodium acetate [pH 4.8] and dissolved in TE buffer (pH.8.0).

PCR

In order to screen the clinical isolates of *M. tuberculosis* for the presence of the RD1 region primers – RD1 FP and RD1 RP were used as forward and reverse primers respectively. The expected PCR product was 1428 bp. The different thermocycling conditions tried included 35 cycles each of 94°C for 40 seconds, 56 °C to 64°C for one minute and 72°C for half to one and a half minute followed by a final extension of 72°C for 7 minutes.

Agarose gel electrophoresis

A 0.7% agarose gel was prepared by weighing the required amount of electrophoresis grade agarose into a known volume of electrophoresis buffer - 1X TBE (Tris: 5.4g/100ml, boric acid: 2.75g/100ml, 0.5M EDTA: 2ml/100ml) and was melted in a microwave oven. After cooling to about 50˚C, Ethidium bromide was added to a concentration of 0.5 g/ml, mixed well and poured into a gel tray without any air bubbles. The electrophoresis tank was filled with 1X TBE buffer and the gel was placed on it. The buffer level should be a little above the gel. 2µl of DNA, mixed with 1µl loading dye (bromophenol blue: 2.5mg/ml, xylene cyanol: 2.5mg/ml, glycerol: 0.4%) was loaded into the wells. The electrophoresis tank was connected to a power pack and resolved at 8V/cm till the Bromophenol Blue in the tracking dye reached 2/3 of the gel. The DNA in the gel was observed by placing the gel on a transilluminator using UV light.

Results and Discussion

The passaging of *M. tuberculosis* H37Rv and *M. bovis* BCG for several decades outside the human host have induced changes in the genome of the pathogen and have also altered their

virulence characteristics. Earlier studies using subtractive hybridization (Mahairas *et al*, 1996) and microarrays (Behr *et al*, 1999) have revealed the presence of sixteen RDs (regions of deletion ranging in size from 2 kb to 12.7 kb) in *M. tuberculosis* H37Rv which are absent in *M. bovis* BCG. Deletions were also reported in H37Rv – RvD1 to RvD5 and TbD1 (Brosch 1998) All these results points to the fact that generation of deletions may be a major mechanism for creating genetic diversity among the members of the complex. This prompted us to screen the clinical isolates of *M. tuberculosis* from Kerala for differences in the RD regions. Since RD1 is the most important region of difference and is deleted in all the sub strains of *M. bovis* BCG, this specific region was chosen for our study. The loss of RD1 is one major genetic event that contributes to the attenuation of BCG (Pym *et al*, 2002)http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1262720/ - B13.

Our preliminary screening of the clinical isolates of *M. tuberculosis* from Kerala was carried out using primers specific to RD1. RD1 is a genomic region deleted in *M. bovis* BCG, but is present in *M. tuberculosis*- both in the lab strain H37Rv and also in clinical isolates. Regions of deletion RD1-16 were genomic deletions found in *M. bovis* strains (Behr *et al* 1999, Brosch *et al*, 1999). Thermocycling was done to partially amplify RD1. The RD1 region is about 9.5 kb long and spans nine open reading frames. The details of the genes encoded by this locus is given in table 1. As a preliminary study, we tried to amplify part of this region which was 1428 bp long. This amplicon of 1428 bp spanned three ORFs in the RD1 region- part of Rv3871, full length Rv3872 and part of Rv3873.The primers and their corresponding genomic loci in *M. tuberculosis* H37Rv are depicted in Table 2.

In order to standardize the amplification conditions, different annealing temperatures and extension times were tried out. The annealing temperatures tried ranged from 56°C to 64°C. Extension times tried ranged from 30 seconds to 150 seconds. Maximum amplification without any nonspecific products was obtained at an annealing temperature of 64°C and by using an extension time of one minute. We have checked for the presence of RD1 in a representative sample of 10 clinical isolates from Kerala. All of them showed positive result (Figure 1). This confirms the significance of the RD1 region in the virulence of the pathogen.

RD1 was implicated in the virulence of *M. tuberculosis* for several years. Previous studies have shown that RD1 contributes to bacterial virulence, the generation of protective immunity (Pym *et al.*, 2003; Brodin *et al.*, 2006) and granuloma formation (Volkman *et al.*, 2004). RD1 has been shown to participate in caspase-1-dependent cytokine production via induction of the potassium ion efflux in infected macrophages (Kurenuma *et al*, 2009). More recent research have shown that RD1 contributes to the increase in cytosolic Ca^{2+} levels in infected macrophages, and the RD1-dependent Ca^{2+} influx leads to calpain activation and IL-1α secretion from infected macrophages (Yang *et al* 2014). All these studies underline the significance of RD1 in the virulence and pathogenicity of *M. tuberculosis.* Our present study of the clinical isolates from South India confirms the presence of RD1 locus in these isolates. In order to get more conclusive data, more number of clinical isolates have to be screened.

genome contributes to the maturation and secretion of IL-1α from infected macrophages through the elevation of cytoplasmic calcium levels and calpain activation. FEMS Pathogens and Disease. 70 (1), 51-60.

GENE NAME	GENE LENGTH	COORDINATES		DESCRIPTION
		START	END	
Rv3871	1776	4348827	4350602	Conserved hypothetical protein
PE35	300	4350745	4351044	PE- family related protein

PPE68	1107	4651075	4352181	PPE family related protein
esxB	303	4352274	4352576	10 KDA culture filtrate antigen EXSP(LHP) (CFP 10)
esxA	288	4352609	4352896	6 KDA early secretory antigenic target esxa(ESTA-6)
Rv3876	2001	4353010	4355010	Conserved hypothetical proline and alanine rich protein
Rv3877	1536	4355007	4356542	Probable conserved transmembrane protein
Rv3878	843	4356693	4357535	Conserved hypothetical alanine rich protein
Rv3879c	2190	4357593	4359782	Hypothetical alanine and proline rich protein

Table 1: Details of the RD1 locus in *Mycobacterium tuberculosis* with the gene name, genome position and the encoded proteins of the nine ORFs (Source: Tuberculist)

Table 2: Details of the PCR primers depicting the forward and the reverse primer sequences, the genome coordinates in the type strain *M. tuberculosis* H37Rv and the amplification product size.

Name of the primer	**Sequence of the primer**	**Genomic coordinates in *M. tuberculosis* H37Rv**		**PCR product size**
		Start	**End**	
RD1FP	5' TCGATGGAAAAGCACCATCTGA 3’	4349655	4349676	1428 bp
RD1RP	5' CACAGCATGGTGATCACTCCCT 3’	4351082	4351061	

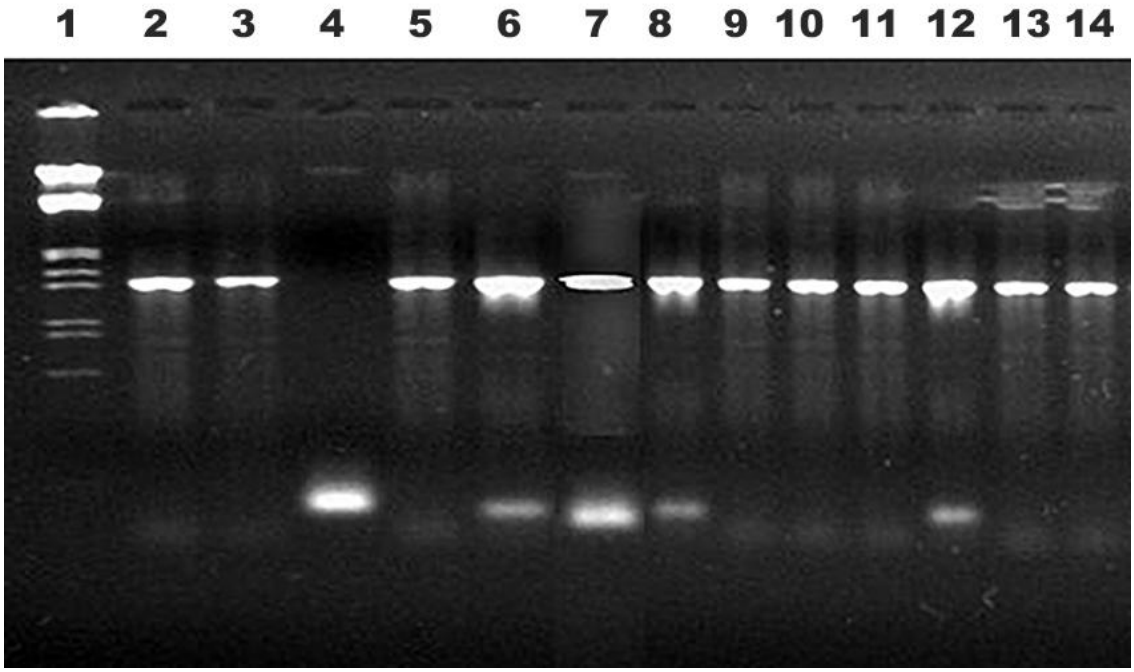

Figure 1. PCR of 10 representative clinical isolates of *M. tuberculosis* from Kerala using primers RD1FP and RD1RP. Lane 1: Marker DNA (λ DNA double digest *Hind* III/*Eco*RI, lane 2: H37Rv, lane 3: H37Ra, lane 4: *M. bovis* BCG, lanes 5-14: Clinical isolates RGTB 29, 37, 40, 43, 55, 60, 70, 86, 87, 93 respectively depicting the 1428 bp amplification product.

CONCLUSION

The virulence of *Mycobacterium tuberculosis* depends on various factors. One of the most significant determinants is the genes present in the RD1 locus. This study was carried out to standardize the thermocycling conditions for RD1 PCR and screen the clinical isolates of *M. tuberculosis* from Kerala to check for the presence of RD1. All the clinical isolates tested were positive for the locus implying the relevance of this region in the virulence of this pathogen.

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