Evaluation of DNA vaccines encoding *M. Tb* gene *Bfrb* and *Mpt32* in mice model

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Abstract: Tuberculosis (TB) is a life threatening infectious disease which is prevalent throughout the world. *Mycobacterium bovis* based Bacille Calmette–Gue´rin (BCG) is the only vaccine available against TB however, this (single) vaccine is not enough to eradicate it. Furthermore, numbers of researches from different parts of the World have shown its efficacy as variable. Hence other (better) vaccines like DNA vaccines are needed in addition to BCG in order to achieve desired goal of TB eradication. The current study was aimed to develop subunit based DNA vaccines against TB and to check their efficacy. Two constructs *Bfrb-pND14 and Mpt32-pND14* were made and used as DNA vaccines. Endotoxin free DNA preparations were made and used in immunization studies. Twenty Balb/c female mice of age eight weeks were used in trial. Two experimental groups each comprising eight animals were used to inoculate *Mpt32-pND14* and *Bfrb-pND14* vaccines respectively. A group of four animals was used as negative control. Animals were bled through tail periodically and finally through cardiac puncture before euthanization. Antibodies were confirmed through dot blot and Agar Gel Immuno Diffusion test (AGID). All the animals immunized with both vaccines were found positive as tested through dot blot and AGID. The results of this study have indicated that both the *M. tb* genes have produced strong immune response in mice model through pND14 vector and proved themselves as good subunit based DNA vaccines.

Keywords: *Bfrb*, *Mpt32***,** DNA vaccines, subunit vaccines, tuberculosis**,** pND vector.

INTRODUCTION

Tuberculosis (TB) is one of the major life threatening global infectious disease that causes death. It is caused by various strains of *Mycobacterium tuberculosis* (Ducati *et al.,* 2006). According to an estimate, one-third of the world's population is carrier of TB bacteria, but only 10% of them develop active TB. Although, this disease was well managed and near to eradication in Europe and North America but emergence of AIDS led to its re-born. Since 1980s, this disease is on continuous rise and its spread is most concentrated in Southeast Asia and sub-Saharan Africa. At present, the11 high-burden countries include: Ethiopia, Cambodia, China, India, Pakistan, Brazil, Myanmar, Philippines, Uganda, Viet Nam and Zimbabwe (WHO Report 2015). The bacterium is inhaled as airborne droplets generated by sneezing and coughing of patients, and this disease usually affects respiratory tract (Perkins *et al.,* 2006).

Bacille Calmette–Gue´rin (BCG) is the only licensed vaccine which provides highly variable protection against pulmonary TB (McShane, 2011). It was introduced a century ago and still in use for immunizing people against TB (Kallenius *et al*., 2007). However, a number of studies have shown the inefficiency of BCG and least protection against pulmonary TB. Over the last few years several works have been started to introduce new yet efficient vaccines like DNA vaccines that may address the problems especially in human and livestock industry (Simth *et al*., 1999; Kristensen *et al*., 2000 and Johansen *et al*., 2000). DNA vaccines have been successfully tried against several other disease including influenza, respiratory syncytial virus (RSV), corona virus, HIV, Hepatitis, TB and many other diseases (Davis, *et al*., 1996; Giri *et al*., 2004). DNA vaccines induce long term immune response as compared to live-attenuated vaccines and no risk of re-activation of microbe is associated (Xu *et al*., 2014). DNA vaccines are based on state of the art molecular biology techniques and antigen used are well characterized. This novel approach holds the promise to treat diseases like tuberculosis HIV, cancers and may more. Furthermore, it is the cheapest and safe method of vaccination (Chu *et al*., 2016).

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The present was study aimed to develop economically feasible DNA vaccine against *M.tb*.

MATERIALS AND METHODS

Animal Ethic Statement

All experimental procedures employed during the conduct of this study were approved by the University Ethical Committee for Animals Welfare, University of Peshawar, Khyber Pakhtunkhwa, Pakistan. At every stage all possible measures were taken to protect animals to suffer from side effects, by monitoring them daily.

Bfrb-pND14 and Mpt32-pND14 constructs

Bfrb-pND14 and Mpt32-pND14 clones were obtained from the depository of Biochemistry and Molecular Biology Lab, University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpuar, Pakistan. The clones were streaked on ampicillin positive LB agar plates and incubated for overnight at 37˚C and later transfer single colony from each plate to LB broth, and kept the tubes at constant shaking of 250 rpm and 37˚C for overnight.

Extraction of plasmid DNA

Plasmid DNA was extracted from each overnight grown culture. The plasmid DNA was extracted by using EZ-10 Spin column Plasmid DNA MiniPreps Kit (cat# BS414) and DNA was stored at -20˚C. DNA was confirmed by 1% TAE agarose gel with 1kb DNA ladder *(*Cat# 11700, NORGEN, Canada). Both the constructs were further confirmed by restriction digestion. The enzymes Kpn I and Pst I were used for confirmation of *Mpt32-pND14* clone and enzymes EcoR1 for *Bfrb-pND14* clones.

Endotoxin free plasmid DNA extraction

The confirmed DNA samples were subjected to endotoxin free plasmid DNA extraction, by GeneJet Endo-free Plasmid Maxiprep kit (K0861) provided by Thermo Scientific, USA. This protocol enabled to isolate constructs on large scale and reduced endotoxin level up to <0.1 EU/µg plasmid DNA. The extracted DNA was quantified by spectrophotometer and stored at -20˚C for further use.

Animal trial

Specific pathogen free female Balb/c mice of age eight weeks were obtained from animal house facility of faculty of Pharmacy, The Islamia University of Bahawalpur. Total twenty animals were used in study and further divided into three groups. Two experimental groups comprising eight animals and a group of four animals was used as negative control (normal saline). The DNA was injected through intramuscular (IM) and intradermal (ID) routes. A total 125 µg plasmid DNA was injected in each animal. Each animal was injected with 50 µg/leg DNA through IM route and 25 µg at the base of tail through ID route.

Post vaccination immune response

All animals were bled through tail before inoculations and periodically at three weeks interval till nine weeks post inoculations. Antibodies were confirmed through dot blot and Agar Gel Immunodiffusion (AGID) Test. Finally, the animals were bled through cardiac puncture before euthenization.

Agar gel immunodiffusion test (AGID)

Agar plates were made by dissolving 1gram noble agar in normal saline buffer (pH 7.2) and wells of 4mm diameter were made by well cutter (Ouchterlony and Örjan, 1949). The *Mycobacterium tuberculosis* lysate was added in central well and antisera from vaccinated and control animals were added in surrounding wells. Plates were incubated at 37˚C for overnight. Lines of precipitation were seen and results were noted (fig. 2).

Dot blot analysis

Nitrocellulose (NC) paper of (0.45µm) pore size was cut into 2cm square pieces and labeled with ball point. A sample of 2µL of bacterial lysate was taken and place in center of each strip of NC paper. The dot was allowed to dry and then soaked in blocking buffer for 1 h. Later the membrane was washed thrice with wash buffer under constant shaking condition. Again this membrane was incubated for one hour with alkaline phosphatase conjugated secondary Antibody. Now it was again washed as indicated earlier. Then the membrane was placed in substrate solution till the spots became visible. After obtaining the required color of spots stop solution was added in order to stop the reaction and incubated for 15 minutes. Now the membrane was washed with double distilled water and allowed to dry and results were noted (Cardona-Castro *et al*., 2011).

RESULTS

Confirmation of Mpt32-pND14&Bfrb-pND14 construct

Bfrb-pND14 and Mpt32-pND14 constructs were confirmed by PCR and later by restriction digestion. Fig. 1 represents the sizes of each construct. Restriction enzyme Pst I was used to confirm *Mpt32-pND14* clone and EcoR I and Kpn I were used to confirm *Bfrb-pND14* clone in separate reactions.

Confirmation of anti Mpt32 and Bfrb antibodies through AGID

Serum antibodies from vaccinated animals were first analyzed by Agar Gel Immuno Diffusion test (AGID) and later by dot blot. Blood samples were collected from all animals pre and post vaccination and sera were separated. *Mycobacterium tuberculosis* (*M. tb*) whole cell lysate was

used as an antigen and placed in middle well of agar plates during AGID test. All the serum samples including positive and negative controls were added in designated surrounding wells (fig. 2). The plates were incubated at 37˚C for overnight and results were obtained. According to AGID results, all the vaccinated animals with *Bfrb-pND14* and *Mpt32-pND14* were positive. Fig 2A & 2B represents the results. The intensity and distance of line of precipitation from serum wells represents the quantity of antibody from each vaccinated animal. Although this is a rough estimation but good enough to see the clear difference from non-vaccinated animals (figs. 2A & 2B).

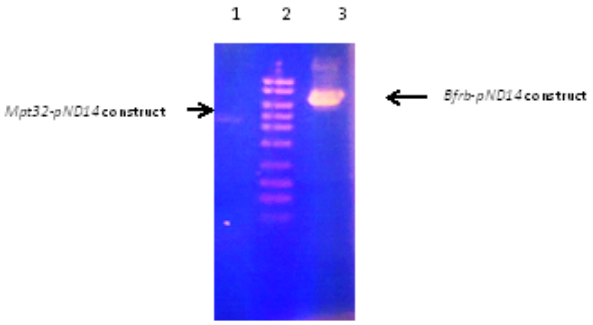

Fig. 1: Extraction of Plasmid DNAs. Lane 1. Confirmation of plasmid DNA (*Mpt-pND14*). Lane 2. 1 kb DNA ladder (Cat# 11700, NORGEN, Canada). Lane 3. Confirmation of plasmid DNA (*Bfrb-pND14*).

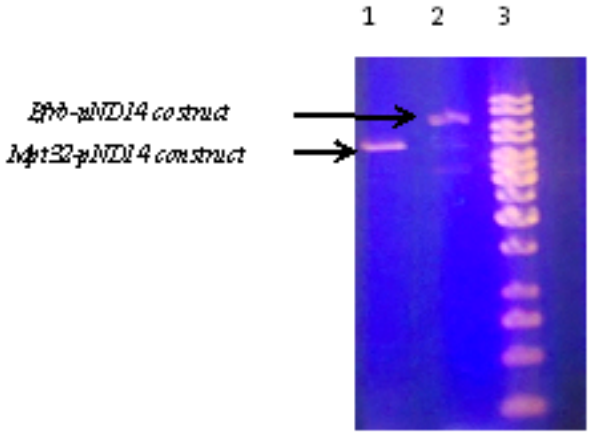

Fig. 2: Endotoxin free preparation of Plasmid DNA (*Mpt32-pND14*&*Bfrb-pND14*).Lane 1. Plasmid DNA *Mpt32-pND14*. Lane 2. Plasmid DNA *Bfrb-pND14*. Lane 3. 1kbDNA ladder (Cat# 11700, NORGEN, Canada).

Confirmation of anti Mpt32 and Bfrb antibodies through dot blot

Anti Mpt32 and Bfrb antibodies were also confirmed through dot blot. Fig 3A represents the results of *Mpt32-pND14* vaccinated group. The clear difference in color intensity of dot 1 with other dots indicates the difference in positive and negative reactions. Similar results were obtained from *Bfrb-pND14* vaccinated animals (fig. 3B).

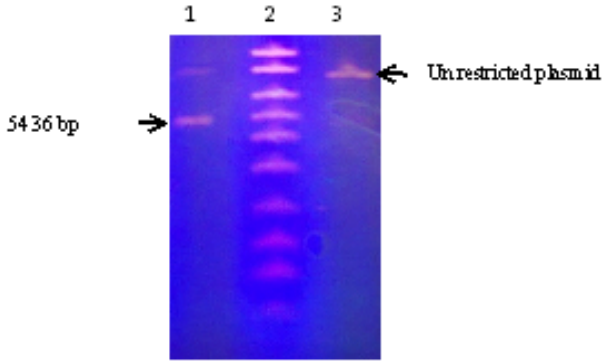

Fig. 3: Restriction digestion of *Bfrb-pND14* construct. Lane 1. *Bfrb-pND14* digestion with EcoR1 enzyme. Lane 2. 1kb DNA marker (Cat# 11700, NORGEN, Canada). Lane 3. *Bfrb-pND14* plasmid DNA.

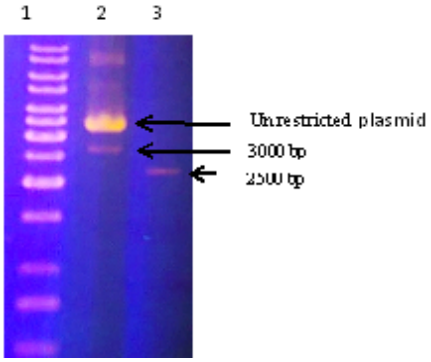

Fig. 4: Restriction digestion of *MPT32-pND14* construct. Lane 1. 1kb DNA marker (Cat# 11700, NORGEN, Canada). Lane 2.*Mpt32-pND14* restriction digestion with Pst1 enzyme. Lane 3. *Mpt32-pND14* digestion with Kpn1 enzyme.

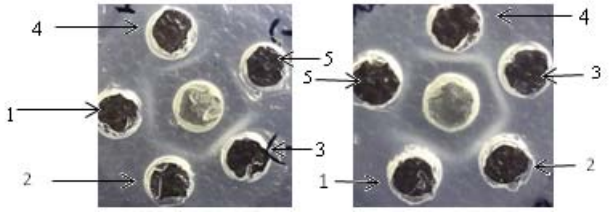

Fig. 5: Testing of anti Mpt32 and Bfrb antibodies through AGID test. A) Confirmation of anti Mpt32antibodies. Central well. Lysed antigen. Well 1, 2 & 3. Sera tested from Mpt32-pND14 vaccinated animals. Well 4. Positive control serum. Well 5. Negative control serum. B)Confirmation of anti Bfrbantibodies. Central well. Lysed antigen. Well 1, 2 & 3. Sera tested from Bfrb-pND14 vaccinated animals. Well 4. Positive control serum. Well 5. Negative control serum.

DISCUSSION

Tuberculosis (TB) is a major global health problem. To control TB is a difficult task and one of the major reason is unavailability of good vaccine(s). BCG is the only

licensed vaccine but its efficacy is greatly variable throughout the world (McShane, 2011; Andersen and Doherty, 2005). Practically no TB vaccine is available for human and livestock since last hundred years. Advances in mycobacterial genetics and genome sequencing have highlighted the potential of some genes including *cfp 10*, *hspx*, *esat6*, *Ag85 A, B and C*, *Mpt32*and *Bfrb* as a candidate for DNA vaccine. Some of these genes are already tested as DNA vaccines (Shahzad *et al.,* 2013; Gao *et al*., 2010) while others are in process to use. According to results of this study, *M. tb* gene *Mpt32* and *Bfrb* were successfully amplified and cloned in mammalian expression vector pND14 through standard biotechnological procedures. The clones were confirmed through restriction digestion and sequence analysis and finally subjected to in vivo trial. All the vaccinated mice found positive through dot blot and agar gel immunodiffusion (AGID) test. The change in intensity of color on nitrocellulose (NC) membrane and position and intensity of line of precipitation in AGID test indirectly tells about titer of antibody from vaccinated animals. Both *Mpt32* and *Bfrb* genes have produced strong humoral immune response and proved themselves as a good candidate for subunit based DNA vaccines. Liu *et al*., (2011) conducted similar work and confirm the clones of Pro685A, pcD685A, Esat6 and Ag85A through restriction digestion with BamH I and EcoR I. Nakayama and Aruga (2015) have suggested the use of plasmid based DNA vaccines HIV, dengue fever, Ebola virus, malaria, TB and viral influenza. According to them DNA vaccines hold better potential to control these infections. Along with DNA vaccines, efforts are made to develop rBCG vaccines e.g. Sugawara *et al.* (2006) and Romano *et al.*, (2006) have reported the use of rBCG in different animal models and these vaccines have produced better immune responses as compared to BCG. Many DNA vaccines are used along with BCG e.g. Gu *et al.* (2016) used combination of DNA and protein booster after BCG and found good increase in immunogenicity of BCG. Similar study was done by Oksanen *et al*. (2016), and they have used combination of Ag85B, Esat6 and RpfE DNA vaccines along with BCG and found better protection from BCG. DNA vaccines are stable, well characterized, consistent in their expression, does not need booster and easy to produce, that's why these vaccines hold the promise to use in near future.

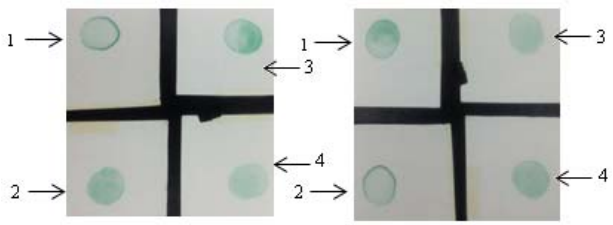

Fig. 6: Testing of anti Mpt32 and Bfrb antibodies through Dot Blot. A) Confirmation of anti Mpt32 antibody. Dot 1.Blot from negative control serum. Dot 2. Blot from positive control serum. Dots 3-4. Blot from *Mpt32-pND14* inoculated group. B) Confirmation of anti Bfrb antibody. 1) Positive control serum. 2) Negative control serum. 3-4) sera tested from animals vaccinated with *Bfrb- pND14*.

CONCLUSION

The results of this study show that both the genes have good antigenic potential and they have induced strong humoral immune response in mice model. By keeping in view the potential of these antigens, DNA vaccines based on these antigens alone or in combination of other DNA vaccines or BCG can act as good therapeutic tool against TB.

ACKNOWLEDGEMENTS

All the authors are thankful to the authorities of Faculty of Pharmacy, The Islamia University of Bahawalpur, Pakistan for providing animal house facility. Furthermore, we are thankful to Dr. Asma, Head of Pathology Section, Bahawal Victoria Hospital, Bahawalpur for providing support in collection of *M. tb* samples.

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