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LIPOPOLYSACHARIDE GENE BASED DEVELOPMENT AND OPTIMIZATION OF DIAGNOSTICS FOR *FRANCISELLA TULARENSIS*

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ABSTRACT

Francisella tularensis (FT) is a Tier 1 select agent due to its low infectious dose and potential threat of being a bio-weapon while causing zoonotic disease in more than 150 mammalian species. Since there are concerns in its culturing at bio-containment deficient labs in developing countries and its subsequent diagnostics and surveillance accordingly, here we proposed a conventional PCR based test of choice that have been developed and optimized for detection of FT using *tul*4 gene specific primers. Annealing temperature and genomic DNA concentration was optimized using a live vaccine strain. Specificity of primer showed FT detection in soil DNA in the presence of other cross-reactive organism. Sensitivity was determined in two fold dilutions with detection limit of up to 320 pg/µL. Utilizing pET28a vector, a construct was prepared containing transformed *tul*4 gene (450bp) showing 100% sequence homology to query gene sequence. These results suggest *tul*4 gene based constructs in vector could be shared to developing countries while developing diagnostic assays as positive control and could potentially be used for lipoprotein purification for ELISA kit development.

Keywords: *tul*4 gene, primer, conventional PCR, specificity, sensitivity, transformation.

INTRODUCTION

Francisella tularensis (FT) is an intracellular gram negative high virulent bacterial which causes tularemia disease in human (Faron *et al.* 2015). Majorly subspecies of FT has been categorized in subspecies *tularensis* (also known as type A), *holarctica* (also known as type B) while subspecies type A further categorized into subtype AI and subtype AII (Sjöstedt 2007; Kugeler *et al.* 2009). Among subspecies of FT, type A is considered to be the highly pathogenic organism which is normally found in North America as compared to type B that is mainly present in Northern Hemisphere (Pechous *et al.* 2009). Infection through inhalation of FT can cause 30-60% mortality if patient remain untreated (STUART and PULLEN 1945). Tularemia disease is majorly present in Scandinavia, North America, Japan, Russia, Turkey, Yugoslavia, Spain, Costa Rica, Sweden, Germany and China and it gradually distributed more than expectation in neighboring countries (Berdal *et al.* 1996; Tärnvik *et al.* 1996; Wicki *et al.* 2000; YanHua *et al.* 2011).

The clinical diagnosis of tularemia is very problematic due to uncommon infection and non specific symptoms. Culturing of FT require specific media, bio-safety level 3 facility and due to laboratory acquired disease, it is not possible in to grow this bacteria and perform biochemical tests for diagnostic purposes in lacking of required facilities and trained persons (Pohanka *et al.* 2008). Therefore, in most of laboratories for traditional and routine diagnosis of tularemia especially in epidemiological studies, is based on quantification of antibodies or detection of pathogen in biopsy. Lipopolysaccharide (LPS) of FT live vaccine strain elite both humoral immunity producing specific antibodies (Sebastian *et al.* 2007) and cellular immunity primarily CD4 T cells as well as interleukin-2 and gamma interferon (Sjöstedt *et al.* 1990; Sjöstedt *et al.* 1990; Ericsson *et al.* 1994). *tul*4 expressed by *Salmonella typhimurium* elicit both humoral and cellular immunity in mice (Sjöstedt *et al.* 1991). In this regards, lipoprotein 17-kDa protein has a significant importance when coated in ELISA plate with concentration of 0.1 to 1 µg per mL for detection of antibodies against FT (Carlsson *et al.* 1979). *tul*4 gene has also same importance in PCR and is the most targeted gene for identification of both *F. tularensis* and *F. philomiragia* isolates due to its conserved nature in all subspecies (Long *et al.* 1993; Sjöstedt *et al.* 1997). According to Russian Federation, export of FT is prohibited in any form either in killed, live or its genome to any country and American Type Culture Collection (ATCC) is also lacking the procedure for transportation of FT to researchers working in other countries (Organization 2007). In present study a

conventional PCR was optimized for detection of *tul*4 gene using primers with NdeI and Xhol enzyme restriction sites and a pET28+tul4 construct was developed which can be used as a positive control in PCR based diagnosis for in house development of diagnostic assays in developing countries. The same construct can also be used for expression of *tul*4 gene and purified lipoprotein can then be used for ELISA kit development.

MATERIALS AND METHODS

Primer designing: For optimization of conventional PCR, a genomic DNA of reference *Francisella tularensis* live vaccine strain (LVS strain) was used. Before primer designing *tul*4 gene sequence from *Francisella tularensis* subsp. *holarctica*LVS (FTL_0421), *Francisella tularensis subsp. holarctica* OSU18 (FTH_0414), *Francisella tularensis subsp. tularensis* SCHU S4 (FTT_0901) and *Francisella tularensis subsp. novicida* U112 (FTN_0427) was aligned using NCBI online tool and was found 99-100% similarity in all subspecies. Primers *tul*4F –TCG-ATC-ATA-TGA-AAA-AAA-TAA-TTA-AGC-TTA-GTC-T 3' and *tul*4R TAC-GCT-CGA-GTT-AAA-TAT-TTA-TTG-AAT-CAA-GAA-GC with product size of 450 base pairs were designed using online software Oligoanalyzer 3.1 (https://www.idtdna.com/calc/analyzer) against *tul*4 gene encoding lipoprotein in *Francisella tularensis.*

PCR optimization: A series of touchdown and gradient PCR was carried out. Thermo-cycler machine was programmed with touchdown PCR to find exact annealing temperature of primer. Program was design in such a way that thermo cycler decreased annealing temperature 1°C per cycle from 60 to 50°C followed by 25 cycles at 50 °C as directed by (Roux 2009). PCR product was confirmed through 1.5% agarose gel with using 50 bp DNA ladder as marker. Later, another gradient PCR was programmed with a temperature gradient of 50 to 60°C during annealing step to find out the exact annealing temperature of primers and each was run in duplicate. Samples were run on gel with clear and sharp band observed at 55°C. Conditions of PCR was finalized as initial denaturation at 95°C for 3 minutes followed by 35 cycles at 95°C for 30 sec, 55°C for 30 sec, 72°C for 45 sec and after that final extension at 72°C for 2 minutes. Reaction mixture of 50 µL was used including 0.62 µL of 100 mM $MgSO_4$ (BioLab, UK), 1 µL of dNTPs mixture containing 10 mM each dNTP (BioLab, UK), 5 µL 10X thermopol reaction buffer (BioLab, UK), 0.37 µL vent DNA polymerase enzyme containing 2 U/mL (BioLab, UK), 0.37 µL of each primer, 1.5 µL genomic DNA, and 40.7 µL nuclease free water.

Primers sensitivity: The DNA quantification was carried out through Nanodrop 2000 (Thermo scientific, USA) and Biophotometer plus spectrophotometer (Eppendrop, Germany). Genomic DNA of LVS was twofold diluted in nuclease free water with initial DNA concentration of 164 ng/µL and PCR was performed as per optimized conditions (George 2005; Woubit *et al.* 2012).

Primer specificity: Genomic DNA from different soil type and LVS was diluted two fold keeping 1 ng/µL as initial quantity in extracted soil DNA. During primer BLAST on NCBI website forward primer showed 100% similarity with *Staphylococcus aureus* so specificity of primer was further checked with genomic DNA extracted from soil and was mixed with DNA of *Staphylococcus aureus* followed by PCR based amplification.

Transformation of tul4 gene into pET28a vector: In order to check transformation of tul4 gene PCR product was gel eluted and restricted with *Xhol* and NadI restriction enzyme in 100 µL reaction mixture containing 20 µL PCR product, 10 µL reaction buffer, 1 µL of each enzyme and 68 µL nuclease free water. Reaction mixture was incubated at 37°C for 1 hour and then heat inactivated at 65 µC for 20 minutes followed by cleaned up with gel elution kit. pET-28a plasmid (Novagen) was restricted under similar conditions and gel eluted. After gel elution both restricted plasmid and product were confirmed through 1% gel separately. Plasmid was dephosphorylated in PCR tube containing 40 µL plasmid vector, 4.5 µL Antarctic phosphatase enzyme buffer (Biolab, USA) and 1 µL Antarctic phophatase buffer (Biolab, USA) and incubated at 37 °C for 1 hour followed by heat inactivation at 72 °C for 20 minutes. For ligation both vector and PCR products were mixed with 1:3 and 1:7 followed by incubation at 16°C overnight and then heat inactivated at 65 °C for 20 minutes. Resulting vector was transformed into *E.coli BL21* competent cell in Luria Bertani broth (Becton, Dickinson and Company, USA) having 20µg/ml kanamycin using rubidium chloride (Green and Rogers 2013).

Screening of transformed colonies: After overnight incubation, Plasmid extraction was carried out from overnight culture using E.Z.N.A.® Plasmid Mini Kit (OMEGA, USA). Extracted plasmid was run with control plasmid on 1% agarose gel. After size confirmation, transformed plasmid was restricted with *Xhol* and NadI enzymes. After restriction analysis, insert was gel eluted and was confirmed through Sanger based sequencing.

RESULTS

Reliability of PCR product detection: During optimization of annealing temperature, band of PCR product was obtained through touchdown PCR in a range of 60°C to 50 °C. To find exact annealing temperature, another gradient PCR showed same band size at 55°C using same composition of reaction mixture as given above. Finally a conventional PCR using same annealing temperature (55°C) was run and results of post PCR agarose gel electrophoresis showed exact PCR product size (450 bp) as shown in Figure 1.

Sensitivity: Sensitivity of primer was found excellent for detection of FT and PCR products run on 2% agarose gel showed detection level up to 320 pg/µL.

Specificity of primers: During specificity test, soil DNA was extracted was counted as 65 ng/µL. In start, tenfold serial dilution of soil DNA keeping 1 ng/µL of LVS DNA showed no band may be because of some inhibitors. Heat treatment of soil DNA at 65 °C for 20 minutes was carried out to inactivate nuclease enzymes. Before doing PCR, genomic DNA of *Staphylococcus aureus* was added to soil extracted DNA to check miss pairing as because of 100% similarity of forward primer. Results of PCR on optimized condition showed exact band size (450 bp) with no nonspecific fragment on 2% agarose gel as given below in figure 2.

Screening of Tul4 gene transformed colonies: Restricted plasmid showed successful elution of tul4 gene insert with exact size (450bs) as given in figure 03 along with tul4 gene insert control.

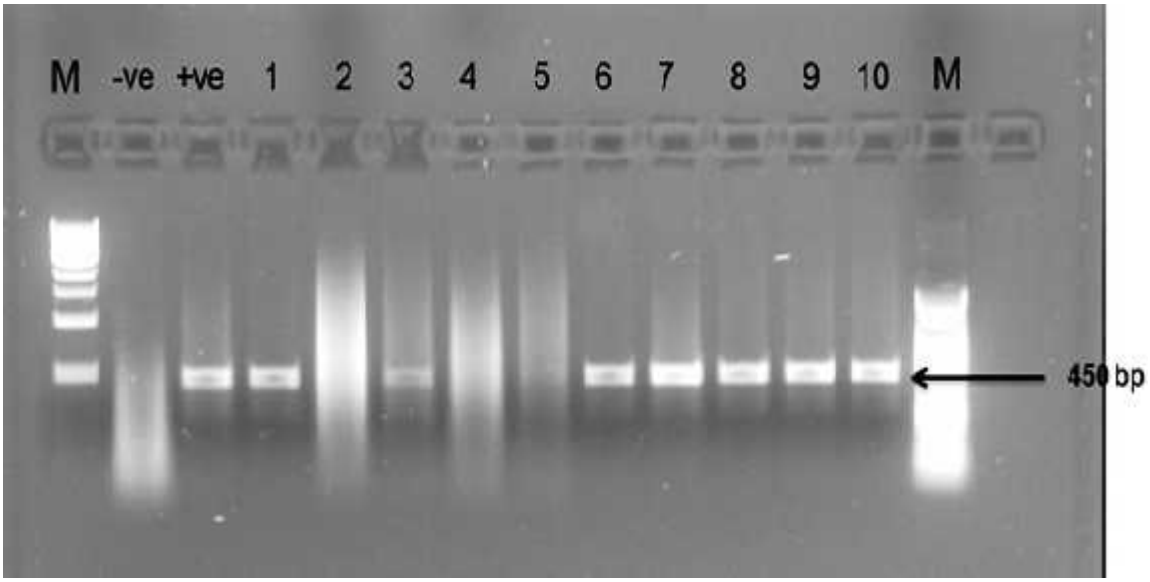

Figure 1.Agarsose gel electrophoresis of Tul4 gene (450 bp) using 1 KB (right side) 50 bp (left side) DNA ladder

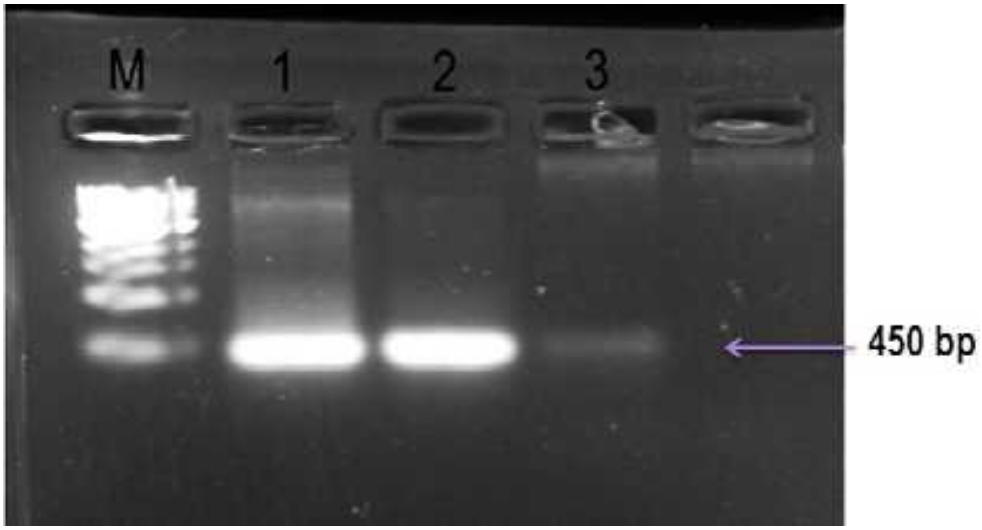

Figure 2.Primer specificity for detection of tul4 gene (450 bp).

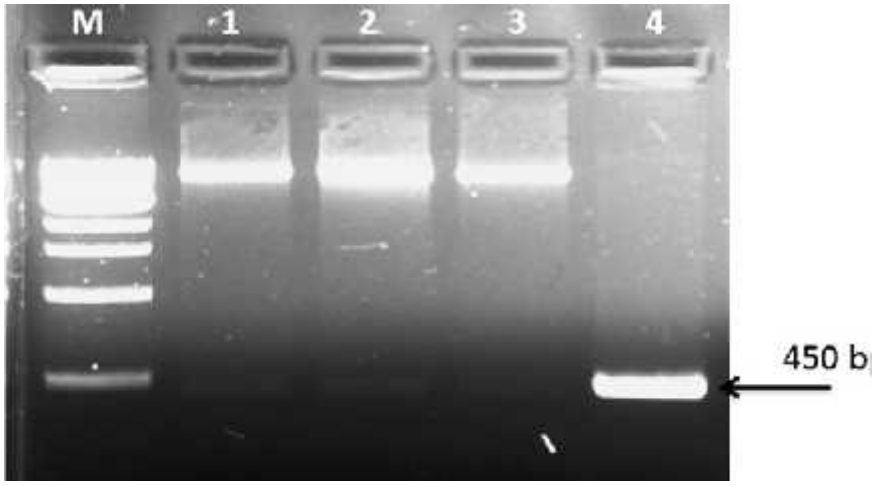

Figure 3.Restriction analysis of pET28a+tul4. Well # 1-3 (Restricted plasmid with tul4 gene insert); well # 4 (positive control of tul4 gene).

DISCUSSION

*Francisella tularensis*is highly virulent and during diagnosis of a clinical case in laboratory via culturing, non-vaccinated staff prone to a high threat (Burke 1977). As the nutritional requirements of FT is known but there is a lack of experience in culturing of FT in routine base diagnosis (Johansson *et al.* 2000). Due to hazardous nature of culturing of FT in lab, PCR is the only way for rapid diagnosis and as *tul*4 gene is highly conserved among different species of FT so PCR on the basis of *tul*4 gene gives highly sensitivity and having no significant similarity with other prokaryotic and eukaryotic sequences in gen bank (Forsman *et al.* 1994; Johansson *et al.* 2000). Interestingly, our data covered all three points of simplicity, sensitivity with detection limit of 2.5 ng/µL and specificity with no non specific band and smear formation. Diagnosis on the basis of PCR test is of good choice as compared to serological tests in tularemia cases (Sjöstedt *et al.* 1997). In a diagnostic lab, for proper diagnosis and detection of an organism, three things 1) reliability and simplicity of a test 2) specificity and 3) sensitivity are majorly important (Hübschen *et al.* 2004). According to Emanuel *et al.*, (2003), *tul*4 gene primer has limit of detection up to 200 fg/µL on BioSeeq thermocycler platforms but it mainly depend on rise in threshold level, primer ratio, annealing temperature, *Taq* enzyme and probe level. During specificity, heat treatment at 80 °C for 20 minutes of soil DNA sample prior PCR improved detection level and nuclease enzymes became inactivated. According to previous study, fetal calf serum contain heat stable nuclease enzymes which degrade DNA, can be inactivated at 70 °C for 30 minutes while nuclease enzymes in serum and plasma from mouse and human inactivated at 55 °C (von Köckritz-Blickwede *et al.* 2009). Soil of different types has different physical and chemical nature and has different organic and inorganic composition which is a great challenge in extraction of good quality DNA (Whitehouse and Hottel 2007). In our study, it was showed that in absence of high quality DNA extraction kit, improvement in detection of FT can be carried out with simple heat inactivation of nuclease enzymes present in environmental samples or patient serum. As FT is potential source of bioterrorism and previously in both east and west, this organism has been used as bio-weapon in different wars. In future, high infectivity and aerosolized weaponization of this organism attracted its usage as bio-weapon (Organization 2004). These problems become a great hurdle in transport of FT live culture, killed or in genomic form from country to country and researcher usually obtains culture in their own country for research work. A vector (*tul*4+pET28a) constructed in this study can solve this problem and can be transported to other country as a DNA control for diagnosis purposes and for protein expression followed by purification of His-tagged tul4 protein which can help in ELISA kit development by coating 96 well plate with His-Tagged *tul*4 protein (Kaur *et al.* 2012). Heat shock protein and lipoprotein of FT are considered best candidates for subunit vaccine production and has a significant role in inducing protective immunity (Fulop *et al.* 1995; Conlan *et al.* 2002; Cole *et al.* 2009).

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