Molecular Cloning and Sequencing of Bovine TNF-α Cytokine Gene from Peripheral Blood Mononuclear Cells

S. D. Audarya[1], A. Sanyal[2], L.K. Pandey[3], J. K. Mohapatra[4] and B. Pattnaik[5]

1, 2,3,4,5 Project Directorate on Foot-and-Mouth Disease (PD-FMD), Indian Veterinary Research Institute Campus, Mukteshwar -Kumaon – 263 138, Uttarakhand, India

Corresponding author e-mail asd_vet@yahoo.com

ABSTRACT

TNF-α is one of the critical cytokine involved in important processes of immune defense system. In the present investigation total RNA from peripheral blood mononuclear cells (PBMCs) from healthy Holstein-Friesian cattle (Bos taurus x Bos indicus) was extracted. Using extracted total RNA complementary DNA (cDNA) was prepared through reverse transcription technique. This cDNA was used in polymerase chain reaction (PCR) for partial amplification of TNF-á cytokine gene with the help of commercially available readymade primers. In the gel electrophoresis, desired TNF-á DNA product of 360 bp was documented. After purifying the resulting PCR product it was inserted into the cloning vector, pGEM®-T Easy Vector System I by T-A cloning technique. Successful transformation of desired amplified DNA product of TNF-á was accomplished in Escherichia coli (JM 109) bacterial cells. Further confirmation of actual success in transformation was achieved on amplifying specific product of TNF-á in PCR when extracted plasmids from transformed Escherichia coli (JM109) cells were used as a template. After treating the plasmids obtained from transformed Escherichia coli (JM109) cells with restriction enzyme, release of the specific inserts in the plasmid was also documented. Finally to reconfirm the results extracted plasmids were sequenced successfully and found to be of TNF-á. The transformed Escherichia coli (JM 109) cells containing TNF-á insert were further preserved. This clone of TNF-á cytokine gene can be used as a positive control to compare and for quantitative analysis of the cytokine TNF-á for healthy, diseased and vaccinated bovines in real time polymerase chain reaction.

Keyword: *Cattle, Cloning, mRNA, RT-PCR, Sequencing, TNF-á*

Besides having tumor cytotoxicity, tumor Necrosis Factor (TNF) is a potent mediator of inflammation as well as many normal physiological functions in homeostasis and health, and antimicrobial immunity as reported by *Sedger et al. (2014).* TNF-α is one of the most potent anti-viral cytokines which acts alone or in synergy with other interferons. *Wong et al. (1986)* and *Sedger et al. (2014)* has reported that TNF-á also induces apoptotic cell death in virus infected cells. Presence of cytokines in blood is one of the first indications of infection according to *Flint et al. (2004).* Secretion of cytokines in response to infection is mediated by cells involved in immune action. Role of cytokines and other related genes in pathogenesis of diseases is not completely known. Cytokines can be targeted to devise a test helpful in diagnosis of the disease. Hence, the present study is planned to identify, amplify, clone and sequence TNF-α cytokine gene so that the generated clone can be used as a positive control in quantitative real time polymerase chain reaction test and related techniques.

METHODOLOGY

Collection Of Blood Sample: Whole blood sample was collected from jugular vein of Holstein Friesian Crossbred cattle reared at Dairy farm, IVRI, Mukteshwar (FMDV uninfected apparently healthy and unvaccinated with no history of FMD) in glass test tube containing 0.1% anticoagulant ethylenediamin- etetraacetic acid, EDTA (Sigma, USA).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs): Isolation of PBMCs was carried out as per the method of *Boyum (1968)*. The collected blood sample was diluted with equal amount of phosphate buffer saline solution (PBS) (5 ml). Diluted blood samples (7 ml) was

carefully and slowly added on the inner-side of the wall of the 15 ml sterile pyrogen free conical polypropylene centrifuge tubes with already added 3 ml of Histopaque®-1077 solution (Sigma-AldrichTM, USA). These tubes were centrifuged at 400xg (1300 revolutions per minute, rpm) for 30 min at room temperature (rt) (SORVALL® RC 3B plus centrifuge with rotor H 4000, USA). Whitish circular band of PBMCs was observed in between upper plasma and lower red cellular layer. The band of PBMCs was transferred to another 15 ml centrifuge tube with 5 ml of Rosewell Parker Memorial Institute-1640 (RPMI-1640, Sigma®,USA) media with the help of glass Pasteur pipette. Then it was centrifuged at rt for 10 min at 250xg (900 rpm). The resulting cell pellet was washed again as per earlier and finally the cell pellet was dissolved in 2 ml of PBS.

Extraction of Ribonucleic Acid (RNA): Extraction of RNA from PBMCs was carried out as per the method of Chomczynski and Sacchi (1987) following manufacturer's instructions (TRIZOL® LS Reagent, Invitrogen, USA). Extracted RNA samples were kept at – 80 ïC before its use.

Reverse Transcription (RT): The Complementary DNA (cDNA) was prepared as per the following method for a total volume of 25 µl reaction. In 0.5 ml properly labeled PCR eppendorf tubes, after adding extracted RNA – 8 µl, Oligo dT(15) – 2 µl (1 µg), Random primers – 2 µl (1 µg), tubes were incubated at 70 ïC for 10 min in the water bath. Immediately, tubes were snap chilled for 5 mins on ice. RT Master Mix was prepared as per given below i) 5x RT buffer – 5 µl, ii) dNTP (10 mM/µl) – 2 µl, iii) MMLV-RT (200 U/µl) – 1 µl, iv) Nuclease free water (NFW) – 5 µl. Controls were kept wherever required. After short spin, 13 µl of this RT Master Mix was added into each tube. Tubes were again placed back in the water bath at 42 ïC for 1 hr. Tubes were placed in dry heat at 95 ïC for 10 min to inactivate RT enzyme. Finally, after short spin tubes were stored at – 20 ïC.

TNF-α Primers: Readymade primers for bovine TNF-á cytokine gene (500 pmole of forward and reverse primers in a single tube were commercially obtained (Pierce-Endogen, USA) and used in the investigation.

Polymerase Chain Reaction (PCR): PCR was performed by using commercially procured primers to amplify TNF-α (PCR thermal cycler, Takara, Japan). Positive controls were also kept in the experiment. 3 µl of cDNA was used as a template in PCR reaction volume of 25 µl. In properly labeled 0.2 ml PCR eppendorf tube PCR Master Mix was added (22 µl for each reaction). The PCR Master Mix was prepared as follows i) 10 x buffer – 2.5 µl, dNTP (10 mM/ µl) – 1.0 µl, ii) MgCl2 (25 mM/µl) – 1.5 µl, iii) Primer set (approx. 8.33 pmole/µl) – 1.0 µl iv) NFW – 15.75 µl v) HotStar Taq DNA polymerase (5 U/µl) – 0.25 µl. Template was added and the tubes were kept in the thermocycler at following cycling conditions: i) initial denaturation at 95 ïC for 15 min ii) denaturation at 95 ïC for 30 s, annealing 50 ïC for 30 s and extension 72 ïC for 1 min for 35 subsequent cycles and iii) final extension at 72 ïC for 10 min thereafter iv) hold at 4ïC.

Gel Electrophoresis: 1% agarose gel was prepared in Tris Borate EDTA buffer (TBE, Promega, USA). Before stacking the gel 0.5 µg/ml Ethidium bromide (Electran®, BDH, UK) was added in the gel. 5 µl of amplified cytokine DNA products were loaded in the well using 6x loading dye. PCR marker was also kept to identify the size of the amplified product. Gel documentation system was used to document the picture (Figure 1).

After gel purification of the amplified DNA products with a commercially available kit, molecular cloning and sequencing of TNF-α cytokine gene was performed according to Sambrook et al. (1989) with modifications wherever necessary as per the instructions given by the manufacturer.

Ligation of Purified DNA to the Vector: T-A cloning was employed to clone the desired purified DNA using commercially available vector system (pGEM® - T Easy Vector System I, Promega, USA) according to manufacturer's instructions with slight modifications. Ligation mix was stored at 4 ïC till its use.

Transformation: Stock of single cell colony of prokaryotic host Escherichia coli (JM 109) grown in Luria Bertani (LB, Sigma-Aldrich, USA) broth was kept at 4 ïC in the laboratory. 50 µl of this stock was taken into 5 ml of freshly prepared LB broth placed in 50 ml glass conical flask (1:100). Flask was kept in shaker cum incubator for overnight incubation at 37 ïC at 180 rpm. Next day, 500 µl of these refreshed Escherichia coli (JM109) cells were put into 50 ml of LB broth placed in 500 ml glass conical flask. Flask was kept in shaker cum incubator for incubation at 37 ïC at 180 rpm for 2 hrs till visible slight turbidity/OD reaches to 0.4 to 0.6. Grown culture was taken in 50 ml centrifuge tube and left on ice in the plastic thermos with lid closed for 20-30 min. Meanwhile new microtips of all capacity and 1.5 ml labeled eppendorf tubes wrapped in alluminium foil were kept at

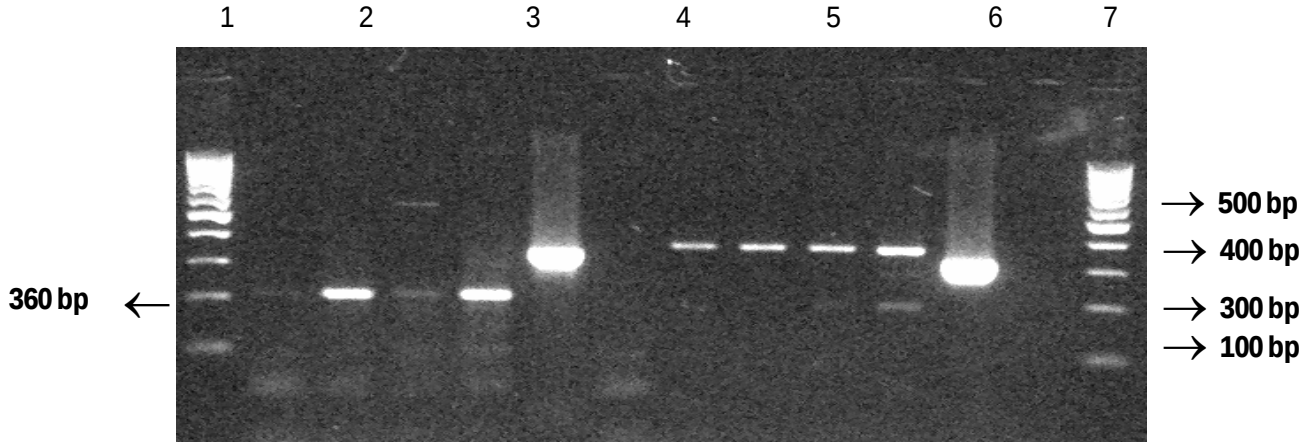

Figure 1. Agarose gel electrophoresis of amplified products

– 40 ïC. Bacterial culture kept on ice was centrifuged for 20 min at 4 ïC for 2000 rpm. Supernatant was discarded; 2 ml of transformation storage solution (TSS) was added in cell pellet while the tube was placed on ice. Gently twice or thrice it was pipette up and down. The tube left on ice with closed lid for 2 hr. 200 µl of competent cells were pipetted in chilled eppendorf tubes placed on ice and 4 µl of ligation mixture was added in the tubes. After gentle tapping with fingers for few seconds tubes were kept on ice for 1 hr. Heat shock was given to the cells for increase in pore size which facilitate entry of DNA into the cells. The eppendorf tubes containing mixture of cells in TSS and ligation mixture was placed in hot water bath at 42 ïC for 55 s. After removing from hot water bath the tubes were immediately placed on the ice for 5 min. 800 µl of solution of competence (SOC) was added in each tube and tubes were placed in shaker cum incubator for 1 and half hr. Autoclaved LB agar was allowed to cool and just before solidification of the agar, 0.5 mM Isopropyl-D-thiogalactopyrinoside (IPTG, Promega USA), 80 µg/ml of X-Gal (Promega, USA) and 100 µg/ml Ampicillin (Ampicillin, Sodium salt, Calbiochem®, Germany) were added. Agar plates were prepared and the culture grown in SOC was poured over it by the pipette and allowed to absorb for 15 min in the laminar flow cabinet in the fashion that the lids of the plates were open. These lid closed agar plates were kept in the room incubator at 37 ïC for overnight. Next day the plates were observed for presence or absence of white and blue colonies.

Plasmid Extraction from Transformed Cells: Plasmid was extracted from white colonies from plates by commercial plasmid extraction kit (AuPrepTM plasmid extraction kit, Life technologies, USA). The tubes containing eluted plasmids were properly labeled. Plasmid DNA concentration was measured using spectrophotometer. (NanoDrop 1000, Thermo Scientific Spectrophotometer, USA) and these tubes were kept at – 80 ïC till its use. Plasmids were checked in 1 % agarose gel using TBE buffer as per mentioned earlier and documented.

Checking The Plasmid For Insert: Plasmids with inserts were checked either by conducting polymerase chain reaction using bacterial colony/plasmid PCR) or treating plasmids with restriction enzyme.

Colony Or Plasmid PCR: 200 µl transformed bacterial cultures grown in LB broth with 100 µg/ml antibiotic were taken in the 0.5 ml centrifuge tubes and centrifuged at 2000 g for 2 min at rt. Resulting bacterial pellets were resuspended in 100 µl of NFW and placed in the hot water bath at 90 ïC for 10 min. Later the tubes were centrifuged at 4000 g for 2 min. Resulting supernatants (2 µl) or extracted plasmids (1 µl) were used in the PCR (12.5 µl total volume of the PCR reaction) as per earlier. Gel electrophoresis was done in 1% agarose gel as per mentioned earlier and results were documented.

Restriction Enzyme Treatment: After adding the following items in the 0.5 ml tubes for each plasmid the tubes were placed in the hot water bath for 2 hr at 37 ïC, i) EcoRI or appropriate restriction enzyme – 0.75 µl ii) 10x buffer – 1.00 µl and iii) Plasmid – 8.25 µl. 1 % agarose gel with ethidum bromide was prepared in the TBE. Gel was stacked and 5 µl of restriction enzyme treated plasmids were loaded in the wells cut into the gel. Suitable PCR marker was loaded in a well. The gels were visualized for specific release of the inserts and documented using gel documentation system (Figure 2).

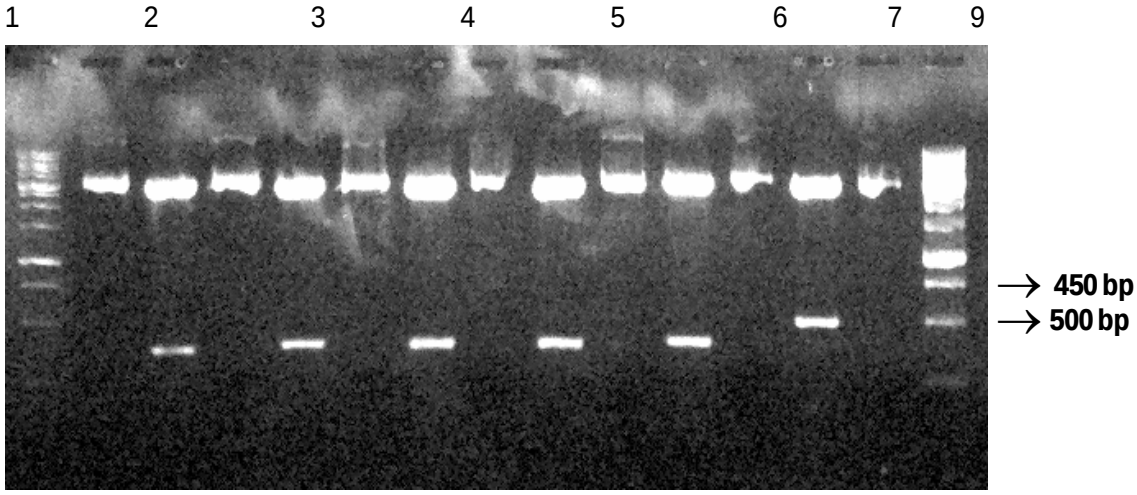

Figure 2. Agarose gel electrophoresis of plasmids with cloned insert before and after restriction enzyme digestion
(Lanes: 1, 3, 5, 7 - Uncut plasmid with TNF-α insert; 2, 4, 6, 8 - Cut plasmid with release of TNF-α insert and 9 - GenRuler™ 1 kb DNA Ladder, Fermentas)

Sequencing: Absolute confirmation of cloned DNA inserts in the plasmids was done by sequencing in capillary method as per the instructions given by the manufacturer (3130 Genetic Analyzer, USA). After adding following items per well into MicroAmp™ optical 96-well plate (AB Applied Biosystems, Singapore), it was sealed with MicroAmp™ optical adhesive film (AB Applied Biosystems, USA). Plasmid DNA concentration was measured by NanoDrop. 200 ng of plasmid DNA was used in the sequencing. Big Dye® enzyme for sequencing was from AB Applied Biosystems, UK. Sequencing reaction was as per below Big Dye® enzyme Terminator v1.1, v3.1 5x sequencing buffer – 0.5 µl, Big Dye® Terminator v1.1, v3.1 5x sequencing buffer – 1.75 µl, Primer M13R/T7 promoter – 1.5 µl, NFW –4.25 µl and Template (plasmid DNA-about 200 ng) – 2 µl. The plate was kept in the thermocycler and cycling conditions were used as under a) Initial activation at 95 ïC for 1 min b) Denaturation at 96 ïC for 10 s, Annealing at 45-50 ïC for 05 s followed by extension at 60 ïC for 4 min for 25 subsequent cycles and finally hold at 4 ïC (Annealing temp 45 ïC for M13R and 50 ïC for T7 promoter). After PCR reaction was completed, purification of sequencing extension products was carried out as follows. 12 µl of Mix I (10µl of Milli-Q water and 2 µl of 125 mM EDTA (pH 8.0) was added into each well with subsequent addition of 52 µl of Mix II (2µl of Sodium acetate, pH 4.6 and 50 µl of absolute ethanol). After sealing the plate, contents in the plate were mixed by inverting and the plate was left to incubate for 15 min at rt. The plate was centrifuged for 30 min at 3000 g. Supernatant was discarded by inverting the plate on paper towels. Plate was centrifuged in inverted position at 180 g for short duration to remove residual supernatant. After adding 100 µl of 70 % ethanol the plate was centrifuged at 3000 g for 5 min and the step was repeated. Again the plate was centrifuged in inverted position at 180 g for short duration to remove residual supernatant. 10 µl of Hi-Di formamide was added in each well and the plate was placed in the centrifuge and short spin was given. Sequencing products were denatured at 95 ºC for 5 min, immediately placed on ice and short spin was given. Electrophoresis was carried out by placing the plate between plate base and plate retainer and loaded in ABI 3130 genetic analyzer. Data collection software (v3.0- Applied Biosystems) was used to control and monitoring of the run. KB base caller v1.4 software was used to generate DNA sequence data. Data collection software (v3.0- Applied Biosystems) was used to control and monitor the run. KB base caller v1.4 software was used to generate DNA sequence data.

RESULTS AND DISCUSSION

TNF is one of the most potent anti-viral cytokines which acts alone or in synergy with interferons. It also induces apoptotic cell death in virus infected cells according to *Wong et al. (1986)* and Sedger et al. (2014). In the present experiment TNF-α cytokine gene was amplified, identified, cloned and sequenced successfully. After sequencing, generated data was analyzed in the blast and the sequence match was found with AF348421

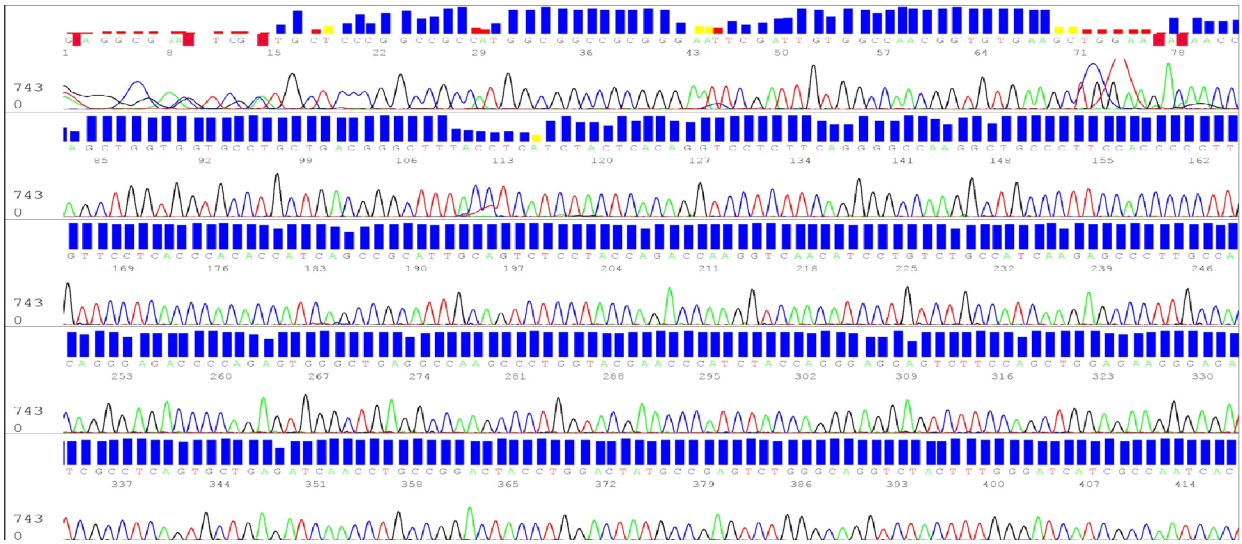

Figure 3. Electropherogram data of cloned TNF-α cytokine gene in the vector (TNF-α clone sequence starts in between position 50-57)

nucleotide data. The TNF-α clone was named as PDFMD-Mahanadi/Bovine TNF-α clone and stored in the laboratory after proper labeling. The generated sequence in the experiment for TNF-α was; GTGGCCAACGGTGTGAAGCTGGAAGACAACCA GCTGGTGGTGCCTGCTGACGGGCTTTACCTCATCTA CTCACAGGTCCTCTTCAGGGGCCAAGGCTGCCCTT CCACCCCCTTGTTCCTCACCCACACCATCAGCCG CATTGCAGTCTCCTACCAGACCAAGGTCAACATCCT GTCTGCCATCAAGAGCCCTTGCCACAGGGAGA CCCCAGAGTGGGCTGAGGCCAAGCCCTGGTA CGAACCCATCTACCAGGGAGGAGTCTTCCAGCT GGAGAAGGGAGATCGCCTCAGTGCTGAGATC AACCTGCCGGACTACCTGGACTATGCCGAGT CTGGGCGGTCTACTTTGGGATCATCGCC (Figure 3). *According to Zhang et al. (2006)* during persistence, TNF-α mRNA expression in carrier cattle was much higher than in non-carrier cattle. This clone of TNF-α cytokine gene can be used as a control to compare quantitative analysis of the cytokine for healthy, diseased and vaccinated bovines in real time polymerase chain reaction in future.

Paper received on : September 20, 2014
Accepted on : October 23, 2014

REFERENCES

A. Boyum (1968). "Separation of leukocytes from blood and bone marrow." Scandinavian *J. of Clinical Lab. Investigation*, **21**:77.

G.H.W. Wong and D.V. Goeddel (1986). "Tumour necrosis factors á and â inhibit virus replication and synergize with interferons." *Nature*, **323**:819-22.

J. Sambrook, E.F. Fritsch and T. Maniatis (1989). "Molecular Cloning, a laboratory manual." 2nd edition. CSHL Press, New York (USA).

L.M. Sedger and M.F. McDermott (2014). "TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants – past, present and future." Cytokine Growth Factor Review, http://dx.doi.org/10.1016/j.cytogfr.2014.07.016.

P. Chomczynski and N. Sacchi (1987). "Single step method of RNA isolation by acid guanidium thiocyanate – phenol – chloroform extraction." Analytical Biochemistry, 162:156-159.

S. Konnai, T. Usui, K. Ohashi and M. Onuma (2003). "The rapid quantitative analysis of bovine cytokine genes by real-time RT-PCR." Veterinary Microbiology, 94:283-294.

S. J. Flint, L.W. Enquist, V.R. Racaniello and A.M. Skalka (2004). Principles of Virology: molecular biology, pathogenesis, and control of animal viruses, 2nd edn, (ASM Press, Washington D C).

Z. Zhang, J.B. Bashiruddin, C. Doel, J. Horsington, S. Durand and S. Alexandersen (2006). "Cytokine and toll-like receptor mRNAs in the nasal-associated lymphoid tissues of cattle during Foot-and-Mouth disease virus infection." Journal of Comparative Pathology, 134:56-62.100 bp

• • • • •