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Molecular cloning of IFN-alpha in goat (*Capra hircus*) and black buck (*Antelope cervicapra*) and evaluation of its expression in goat PBM cells

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ABSTRACT

Type I interferons (IFN-α/ß), play a pivotal role linking innate and adaptive immune responses, of which interferon (IFN- α) alpha (leukocytic interferon) that was initially known for antiviral effect, but in recent days recognized as major key elements in priming innate immune response. Bacterial DNA, rich in CpG motifs, triggers the toll-like receptor-9 mediated signaling through IFN- α. The present study reports cloning and characterization of full length ORF of IFN- α in goat and black buck and its expression profile in goat peripheral blood mononuclear cell culture in presence of *Pasteurella multocida* DNA. The open reading frame of IFN-α in these species was 570 bp as in other ruminant species. The expression of IFN- α in presence of *Pasteurella multocida* DNA (rich in CpG motifs) was higher as compared to unstimulated cells. It is plausible that pasteurella DNA induced TLR9 expression, which contributed to rise in the IFN- α transcript in cultured PBM cells of goat.

Key words: *Antelope cervicapra*, *Capra hircus,* Interferon alpha, PBM cells, Real time PCR, TLR9

TLR 9 plays an important role in innate immune responses against invading pathogens, through recognition of infections by a subtle structural difference between eukaryotic and prokaryotic/viral DNA rich in CpG motifs. Upon recognition, TLR9 signaling induces the production of several kinds of downstream proinflammatory cytokines, of which Type I interferons prime the host immune system (Akira *et al.* 2006). Among the Type I interferons, interferon alpha (IFN-α) is secreted 12–24 h upon pathogenic stimulation. IFN- α gene is 570 bp length and sequences is known for only a few species. The present study reports the analysis of this gene in *Capra hircus* and blackbuck (*Antelope cervicapra*) by amplification, cloning into a T/A cloning vector and characterization. Studies showed the induction of Type I interferon by adenovirus encoded small RNA (Yamaguchi *et al.* 2010) and *Streptococcus pneumoniae* DNA (Parker 2011). PBM cells of goat recognized synthetic TLR 9 agonist like CpG ODN type C *in vitro* (Ramesh *et al.* 2010). Here we report the effect of *Pasteurella multocida* DNA (natural source rich in CpG motifs) on IFN-α mRNA expression in goat peripheral blood mononuclear (PBM) cells *in vitro*.

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MATERIALS AND METHODS

Total RNA isolation and cDNA synthesis: Blood was collected aseptically over 2.7% EDTA from healthy, 2–3 year-old, goats of either sex kept in animal shed of Medicine Division, IVRI and male black buck, 2–2 ½ year-old, reared at Deer Park IVRI. Hellabrunn's mixture (ketamine: xylazine, 1: 1.25) at 0.6–1.0 ml was used for tranquilization and capture of black buck as per standard techniques. PBM cells were isolated from blood by density gradient centrifugation over Histopaque1077 according to Baker and Knoblock (1982). Total RNA was isolated from the respective cells, using TRizol reagent. RNA absorbance was determined at 260 and 280 nm to determine quantity and ensure integrity of extracted RNA. cDNA was synthesized using MuMLV reverse transcriptase in total volume 20 µl consisting of 5 µl respective total RNA (1–5µg/ µl), 1 µl of 12–15 mer oligo dT primer (0.5 mg/ml),1 µl of RNase inhibitor (40U/ml), 2 µl of 10 mM dNTPmix, 2 µl of DTT, 2 µl of 10X reverse transcriptase buffer, 1 µl of reverse transcriptase enzyme (5U/ µl) and 6 µl of nuclease free water and incubated for 1 hr at 37°C. cDNA synthesis in both the species was confirmed by

the amplification of 200 bp housekeeping gene glyceradehyde 3-phosphate dehydrogenase (GAPDH) using reported primer pair (Menzies and Ingham 2006).

PCR amplification of IFN-α: Gene specific primer pair including forward 5′-CAATGGCSCYRSCCTGKTCCTT-3′ and reverse 5′- CCAGGTGTGTGTCAKTCCTTYCTCCT-3′. (*K = T or G, S = C or G) was designed based on of cattle, horse, sheep, giant panda, pig, and water buffalo sequences available in NCBI Genbank. For amplifying full length IFN-α gene, PCR reaction consisted of both forward and reverse primers (50pmol) 1.0 µl each, template cDNA of goat /black buck 1.0 µl; dNTP mix (10 mM) 1.0 µl; 10X Taq buffer (without $MgCl_2$) 5.0 µl, 25 mM $MgCl_2$ 4.0 µl and Taq DNA Polymerase (5U/ µl) 0.5 µl and final volume made up to 50 µl using nuclease free water. PCR reaction conditions were as follows: initial denaturation 95°C for 5 min, followed by 35 repeated cycles of 94°C for 45 sec, 60°C for 45 sec, 72°C for 90 sec and final extention at 72°C for 10 min. The PCR was also performed with nested set primer pair to amplify 140 bp internal sequence in order to characterize the gene.

Cloning of IFN-α amplicon: The IFN-α PCR amplicons of goat and blackbuck were resolved in 1% agarose gel incorporated with ethidium bromide along with 100 bp DNA ladder and by visualizing under long range ultraviolet light. The specific band corresponding to exactly PCR amplicons were cut and purified using gel extraction kit as per the manufacturer's instructions. PCR product so obtained was ligated to the pGEMT-easy T/A cloning vector. The ligation mixture consisted of respective purified PCR product (100ng), vector (50ng), 2X ligation buffer and T4 DNA ligase and the reaction volume adjusted to 10 µl using nuclease free water was incubated at 4°C overnight. The ligation mixture was transformed into *E. coli* DH5α competent cells using TSS protocol for blue-white screening. Plasmids were isolated from white recombinant colonies by using commercially available plasmid isolation kit as per manufacturer's guidelines.

The presence of IFN-α gene in recombinant plasmids was ascertained by restriction enzyme (RE) analysis using *Eco*R I to release the insert. Digestion was carried out at 37°C for 4 h in reaction consisting of plasmid DNA 2–5 µl depending on its concentration, 10X RE buffer (2 µl), RE 1 µl (10U/ µl) and volume made up to 20 µl with nuclease free water. The digested products were analysed in 1% agarose gel.

Isolation of Pasteurella multocida DNA: DNA from *Pasteurella multocida* grown in Luria-bertani medium was isolated using column kit as per the manufacturer's guidelines. Purity of DNA was assessed by observing O.D 260/280 ratio >1.8 and was quantified in DNA nanodrop spectrophotometer.

Assessment of IFN-α transcript in cultured goat PBM cells: For assessment of mRNA expression profile of IFN-α, goat PBM cells were isolated as described above and by 2×10^6cells/ml, in RPMI-1640 growth medium were cultured in presence of *Pasteurella multocida* bacterial DNA (10µg/ml) along with unstimulated control cells in quadriplicate at 37°C in a CO_2 incubator. After 18 h of stimulation, the cells were harvested for total RNA isolation and cDNA were synthesized and checked as described above using GAPDH (200bp) primer pair. The real time PCR was carried out using stratagene M×3000P thermolcycler. Internal PCR primers forward 5′-GAAGGCTCAAGCCATCTCTG-3′ and reverse 5′- GGTCAGTGAGCTGCTGATCC-3′ for 140bp of IFN-α along with primers for GAPDH as housekeeping gene were employed in real time PCR. The total reaction mixture of 25 µl consisted forward and reverse primers (10 pmol/µl) 1 µl each, template DNA 1 µl and 12.5 µl QPCR master mix, volume made up to 25 µl using nuclease free water. The PCR condition was as follows, initial denaturation at 95°C for 10 min, denaturation at 94°C for 30 sec, annealing at 56°C for 45 sec, and extension at 72 °C for 40 cycles and last cycle at 95°C for 30 sec, 56°C for 45 sec and gradual increment from 65°C to 95°C @ 2°C per min and lastly for 30 sec at 95°C. The amplification and dissociation curve data was acquired for calculating the initial copy number, a standard curve was made using serial 10-fold dilution of the cloned plasmid containing known amount of initial target, which was used as template in the real-time PCR reactions. Ct values obtained were plotted against initial copy number, and a regression line equation was obtained. The basal expression level was quantified by normalizing each copy of IFN-α against the reference gene GAPDH as number of IFN-α transcript per 1000 copies of GAPDH.

RESULTS AND DISCUSSION

Total RNA isolated for goat and blackbuck were of good quality as evident from O.D 260/280 ratio >1.8. The integrity of the cDNA was ascertained by amplification of housekeeping gene GAPDH (Fig.1, lane.1). The primer pair designed for full length amplification of IFN-α was highly specific as the target gene amplicon of 598bp was obtained (Fig.1, lane.2).The presence of IFN-α gene in the recombinant plasmid was ascertained by the amplification of 140bp product using internal set of primers (Fig.1, lane.3). The digestion of recombinant plasmid with *Eco*RI into fragments of 3 Kbp and 598bp further confirm the presence of IFN-α insert (Fig.1, lane.4). The recombinant plasmids thus characterized were sequenced. Upon sequence analysis, the complete ORF of 570 bp gene sequence of goat and black buck IFN-α was obtained and submitted to NCBI Genbank (Accessions FJ959074 and FJ959075). IFN-α sequences of both the species matched with the published bovine IFN-α sequences suggesting similarity of IFN-α of these species (Chaplin *et al.* 1996, Ramesh *et al.* 2010).

TLR9 expression was identified in different tissues like in gonads and reproductive tract (Girling and Hedger 2007), endometrium, faetal tissues (Singh 2008), lungs (Sethi *et al.* 2011) and plasmacytoid dendritic cells and B cells (Liu 2005).

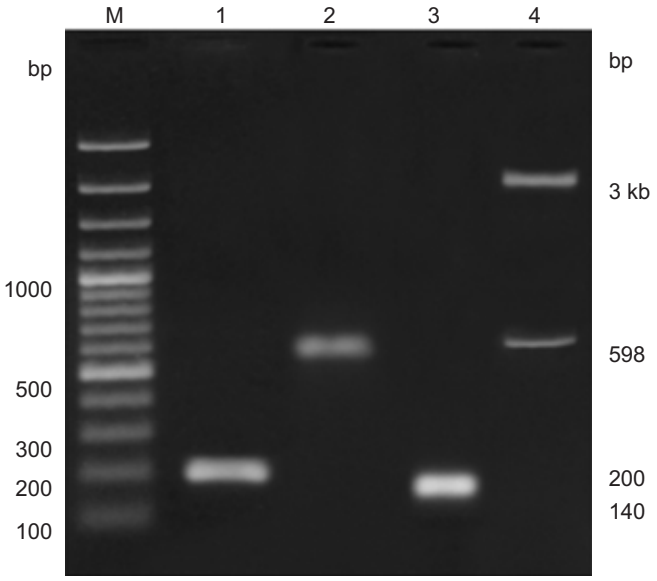

Fig.1. Characterization of IFN-α of goat depicting lane M: 100 bp DNA ladder, lane 1, GAPDH 200bp; lane 2, PCR amplicon IFN-α 598bp, lane 3, Nested PCR product 140bp; lane 4, Insert release of 598 bp and pGEMT-Easy vector of 3 kb (Also similar pattern have been obtained for the blackbuck IFN-α).

Though the TLR9 expression is cell- and tissue- specific (Spies *et al.* 2003), the immunological cascade activity of this novel PRRs is elicited through the Type I (IFN- α and IFN-β) interferons. The expression of IFN-α was identified in goat PBM cells with 1380 copies per 1000 copy of GAPDH. *Pasteurella multocida* DNA produced an increase in the expression of IFN-α in the present study (1778.27 copies of IFN-α per 1000 copy of GAPDH). Increased expression of IFN-α may be attributed to the abundance of the pathogenic CpG motifs in *Pasteurella multocida* DNA as reported in chickens (Herath *et al.* 2010), which increased TLR 9 expression in PBM cells (Singh 2008). Parker *et al.* (2011) reported that *Streptococcus pneumoniae* DNA initiates the Type I Interferon signaling in the respiratory tract of mice suggesting the importance of TLRs in the pulmonary defenses. In addition viral derived RNA also induced the Type I interferons from immune cells (Yamaguchi *et al.* 2010). The response of goat PBM cells towards the synthetic TLR 9 agonist with respect to downstream production of Type I interferons like IFN-α has been observed (Ramesh *et al.* 2010). However, this is the first report suggesting the response of the goat PBM cells towards the natural TLR9 agonist like *Pasteurella multocida* DNA which are rich in CpG motifs.

The IFN-α sequence of goat and black buck obtained from present study and also the observation of IFN-α production in response to *Pasteurella multocida* DNA will pave way for investigation of the innate immune responses in conjunction with adaptive immune cells in these species to safeguard them from the infectious diseases in India.

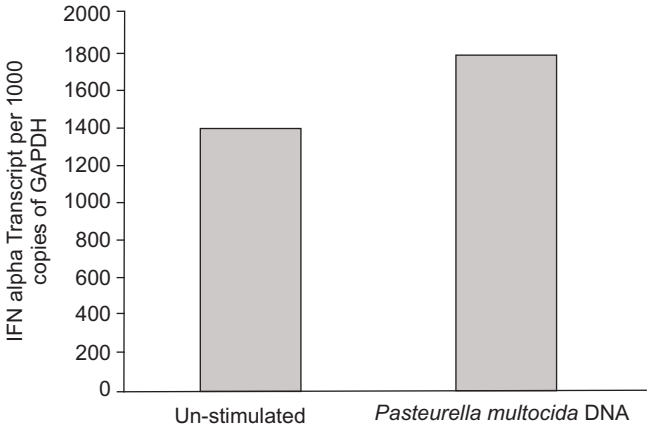

Fig. 2. Relative abundance of IFN-a transcript in PBM cells stimulated using *Pasteurella multocida* DNA along with unstimulated cells, depicted as number of IFN-a transcript per 1000 copies of GAPDH.

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