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Expression and molecular cloning of interferon stimulated genes in buffalo (*Bubalus bubalis*)

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ABSTRACT

Buffalo, the most important livestock species in tropical India, remains to be a poor breeder mainly due to embryonic mortality (65%) occurring mostly between 16 and 18 days of pregnancy. Early and accurate diagnosis of pregnancy can thus become a boon for successful herd management in buffalo. However, most of the currently available methods allow diagnosis only after 30 days post AI. Interferon tau (IFNT), the first pregnancy recognition signal in ruminants is one such molecule, which stimulates expression of various Interferon stimulated genes (ISGs) in the peripheral blood mononuclear cells (PBMC's) concomitant with IFNT signaling which occurs around maternal recognition of pregnancy (MRP). Hence, the study was planned to demonstrate the expression dynamics of ISGs (OAS1, MX1, MX2 and ISG15) in PBMCs during peri-implantation period in buffalo and also molecular cloning and expression of suitable ISG coded protein (s) in suitable host. Blood was collected from two groups of multiparous buffaloes: Group1: (n = 10) inseminated/pregnant (Experimental) and Group2: (n = 10) anestrous/non pregnant (Control). The expression profile of ISGs was then analyzed using real time qPCR. Expression profile of most ISGs was observed to increase through day 14 to day 20 post AI and declined thereafter. On the basis of differential gene expression at day 18 post AI, OAS1 and MX2 were identified as suitable ISG candidate biomarkers for accurate pregnancy diagnosis within 18 days post AI. Molecular cloning and expression of selected ISGs in a suitable prokaryotic expression vector was done thereafter. Bulk expression of the recombinant proteins was done and purified by affinity chromatography and confirmed by Western blot using Mouse Monoclonal His-probe antibodies. To conclude, as OAS1 and MX2, showed distinct differential expression at day 18 post AI, they may serve as ideal biomarkers for detection of early pregnancy in buffalo.

1. Introduction

India is highly privileged in terms of possessing the largest buffalo population which is an indispensible livestock species, found mostly in the tropical and subtropical regions of the world. Buffaloes are the most important dairy animal of the Indian subcontinent in terms of milk yield with high fat content, good feed conversion efficiency and relatively low maintenance requirements, yet they experience problems related to reproduction especially high calving interval, late puberty, and high incidence of anestrous. The reproductive efficiency could be improved with early detection of pregnancy following artificial insemination (AI). Many methods of pregnancy diagnosis, both direct and indirect, are being practiced in bovine species. To date, none actually qualify as the ideal pregnancy diagnosis method due to the limitations they inherit. Per-rectal palpation of the amniotic vesicle and slipping of the chorio-allantoic membranes between the thumb and forefinger is the most widely practiced method for early pregnancy diagnosis in large dairy animals even today. Another important tool for early pregnancy diagnosis is *trans*-rectal real-time B-mode ultrasonography for visualizing placentomes or identifying fetus through imaging fluid in the uterine lumen [9,19], but it has practical difficulties under field conditions.

The indicators of pregnancy are some proteins also called pregnancy markers, which are embryo specific and depict presence

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and viability of an embryo. They are secreted as a signal from the conceptus during the peri-attachment period and facilitate in maternal recognition of pregnancy. One such important signal to the maternal system to sustain pregnancy is pregnancy associated glycoproteins (PAGs). Available methods utilizing PAGs compromise accurate pregnancy diagnosis with the false positives because some PAGs have a long half-life time and hence, can be detected in serum for many weeks post-partum [17]. Studies on levels of progesterone, PAGs and early pregnancy factor are some of the common clinically practiced pregnancy detection methods in bovines, and each has its own benefits and limitations. Another important signaling molecule in ruminant is interferon-tau (IFNT), which is secreted from the trophectoderm of the conceptus beginning at the blastocyst stage [37] and increases with elongation of the conceptus [12]. Early studies clearly demonstrate that a portion of the IFNT secreted into the uterine cavity enters the uterine vein and directly up-regulates the expression of interferon-stimulated genes in both peripheral blood cells and the CL [14]. The expression level of interferon-tau stimulated genes (ISGs) in peripheral blood mononuclear cells (PBMCs) has recently opened up opportunities for research communities to look for novel pregnancy biomarker molecules. Transcripts of ISG15, MX2 and OAS1 gene such as interferon-stimulated protein 15 kDa (ISG15) and myxovirus resistance protein (MX) 1 and 2 [48], 2′-5'-oligoadenylate synthetase (OAS1) [21] in PBMCs is stimulated by IFNT, which is used as a biomarker of pregnancy in ruminants. The expression of ISG15, MX2 and OAS1 mRNA has been reported to be higher in PBMCs within 24–48 h of initial IFNT signaling in pregnant sheep [53]. The gene expression levels of ISG15, MX1 and MX2 during maternal recognition of pregnancy (MRP) are also found to be greater in pregnant compared with non-pregnant cows [33]. In heifers, a greater fold increase for peripheral MX2 -gene was observed from d 0 to 18 of pregnancy when compared to non-pregnant animals [49].

The utility of ISGs for the purpose of pregnancy detection in dairy cattle has been demonstrated recently [14,21,49]. [21] found that blood progesterone and ISG15 were the acceptable results as a method of pregnancy diagnosis if samples were collected over a series of days. Therefore, we hypothesized that interferon stimulated gene products would be suitable biomarkers for pregnancy detection in buffaloes. Hence, the study was planned to demonstrate the transcriptional abundance of ISGs (OAS1, MX1, MX2 and ISG15) in PBMCs during peri-implantation period in buffalo and to identify most suitable ISG candidate(s) having differential gene expression during peri-implantation period. As no validated ELISA for the detection of early maternal recognition of pregnancy in buffalos is available as on date, thus, molecular cloning and expression of OAS1 and MX2 were tried to obtain the recombinant ISG coded protein(s) for elicitation of antibodies for further ELISA development in future.

2. Materials and methods

2.1. Animals and sampling

Apparently healthy buffaloes of animal farm under IVRI, Izatnagar were used for research. The animals were kept in a dry, clean and well-ventilated shed and reared under strict management and proper hygienic conditions throughout the study. First group (Group 1), comprised of 10 pregnant animals, chosen as per heat detection and insemination schedule followed in IVRI, livestock farm. Total 10 non pregnant/anestrous animals were selected in second group (Group 2), serving as control animals. Blood samples were collected using heparin (10 IU/ml) as anticoagulant by jugular vein puncture under sterile conditions on days-10, 12, 14, 16, 18, 20, 22 and 24 post-artificial inseminations (AI). Precautions were taken to minimize the effect of ribonuclease activity while processing.

2.2. Total RNA extraction, quality determination and quantitative RT-PCR

The PBMC from the blood was collected using Histopaque-1077 [5,6,7,11] and the PBMC pellet was re-suspended in 500 μl DEPC treated PBS and transferred to a 2 ml nuclease-free (DEPC treated) microcentrifuge tube. Total RNA was isolated using Trizol reagent (Invitrogen, USA). The RNA samples were treated with RNase free DNase and subsequently DNase was inactivated by heating at 56 °C for 10 min and immediately chilled at 4 °C. The quality of RNA was checked by A260/A280 ratio and quantified using Nanodrop spectrophotometer. Integrity of the total RNA was checked using denaturing agarose gel (1%) electrophoresis and visualization under UV light. Isolated RNA samples were free from the protein contamination as the OD_{260}:OD_{280} values were more than 1.8. The concentrations of the RNA samples were in the range of 200–2000 ng/μl. Two intact bands of 28 S and 18 S with smearing indicated good quality and intactness of RNA. One microgram of total RNA from different samples were reverse transcribed to cDNA using Thermo scientific cDNA synthesis kit according to manufacturers instruction using oligo dT primers.

The primers of OAS1, ISG15, MX2, MX1, GAPDH and Ribosomal protein S15a (RPS15A) were designed using the mRNA sequence taken from NCBI website by New-DNA Star, Genetool, Oigo analyzer software. The sequences, annealing temperature and expected PCR product length are presented in Table 1. Quantitative real-time PCR was performed using Ssofast Eva Green® qPCR kit, Biorad, USA. Each sample was run in triplicate in 20 μl reaction mixture which consisted of 10 μl Eva green mix, 0.5 μl each of forward (0.25 μM) and reverse primer (0.25 μM), 1 μl of cDNA and 8 μl nuclease-free water. The real-time PCR (MxPro3005 P Stratagene, Agilent Technologies, USA) was run with initial denaturation at 95 °C for 30 s followed by 40 cycles of denaturation at 95 °C for 5 s, annealing for 10–12 s and extension at 72 °C for 10 s. The optimum annealing temperatures for different genes are tabulated in Table 1. Real time PCR efficiencies were determined by amplification of a standardized dilution series and slopes were obtained. To assess the specificity of amplified product, dissociation curve for each gene was generated at temperature of 65–95 °C. Results were expressed as changes in threshold cycle values (CT) which reflects the cycle number when the fluorescence of reporter dye is higher than background. The threshold, automatically adjusted by instrument, was used for generating CT values.

2.3. Molecular cloning, prokaryotic expression and purification of recombinant OAS1 & MX2

For amplifying target DNA, both forward and reverse primers were designed by adding the sequence identical to the end of the vector adjacent to the cloning site as per- Expresso Rhamnose SUMO Cloning and Expression System. The primer details for both r. OAS1 and r. MX2 have been presented in Table 2. PCR amplification of desired coding sequence for both OAS1 and MX2 genes were done by using cDNA from Day 18 pregnant buffalo as template by Taq Polymerase (GCC Biotech). The samples were run in 50 μl reaction mixture which consisted of 38 μl PCR-nuclease free water, 5 μl 10× Taq buffer, 1 μl dNTP mix (10 mM), 1.25 μl each forward and reverse primers (10 μM), 1 μlTaq DNA Polymerase and 2.5 μl DNA template. The PCR (Quanta biotech S-96 End PCR) was run with initial denaturation at 94 °C for 3 min, followed by 40 cycles of denaturation at 94 °C for 30s, annealing at 60 °C for OAS1 and 64 °C for MX2 for 30 s and extension at 68 °C for 2 min, followed by final