

Characterization of *COX-2* gene using RNA based technique in endometrial epithelial cells of buffalo (*Bubalus bubalis*)

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Abstract: The present study has been reported towards molecular characterization of *COX-2* gene (*PTGS2* gene) in buffalo endometrium. Non-pregnant buffalo uteri were collected from the local abattoir immediately after slaughter and total RNA was extracted. First strand cDNA was synthesized from approximately 2 µg total RNA using iScript cDNA synthesis kit. Following cDNA synthesis, PCR amplification of cDNA was carried out using *COX-2* gene specific primers. To determine the optimum conditions, different concentrations of MgCl₂, template DNA, Taq DNA polymerase, primers, dNTPs as well as different cycling programmes were analysed. Reproducible amplification pattern was obtained using 2 µl cDNA template (50 ng/µl), 2.5 µl 10X Taq Buffer, 0.5 µl dNTP (50 mM), 2.5 µl MgCl₂ (25 mM), 0.5 µl each primer (10 pM/µl), 0.1 µl Phusion DNA polymerase (5 U/µl) and nuclease-free water. The purified PCR amplified product (449 bp) was sequenced using *COX-2* gene specific primers. Nucleotide sequence analysis of PCR amplified product exhibited 100 percent identity with the reference sequence of *Bubalus bubalis COX-2* gene using Clustal Omega Multiple Sequence Alignment Tool. Based on above findings, it is concluded that the optimized PCR conditions and the buffalo *COX-2* gene specific primers can be useful in pursuing further gene expression studies to explore the function of *COX-2* gene in buffalo.

Keywords: Buffalo; *COX-2* gene; DNA; Molecular characterization; PCR

Introduction

Early embryonic mortality is one of the major causes of reproductive failure resulting in reduced pregnancy rate, slower genetic improvement and substantial economic loss to the farmers (Mondal and Prakash, 2002; Tripathi et al. 2018; Tripathi et al. 2021; Mondal et al. 2021). During early pregnancy, the embryo depends on uterine environmental support for survival and growth as it undergoes continual modifications to cope with the needs of embryo. Majority of the losses occur due to failure of signalling exchange at embryo-uterine interface. Prostaglandin F_{2α} (PGF_{2α}) released from the uterus induces luteolysis and reduce the progesterone secretions from the corpus luteum (CL). CL produces progesterone which is needed to stimulate and maintain the endometrial function that is permissive for early embryonic development, implantation, placentation and maintenance of pregnancy (Mondal et al. 2013). Prostaglandins (PGs) are produced by endometrium that plays an important role in ovulation, luteolysis, implantation, maternal recognition of pregnancy and parturition (Dubois et al. 1998; Mondal et al. 2009; Nandi et al. 2012; Lacroix-Pépin et al. 2011). Prostaglandins consist of a diverse family of autocooids derived from cyclooxygenase metabolism of arachidonic acid to PGG₂ and then into PGH₂ which leads to generation of five principal bioactive prostaglandin metabolites; PGE₂, PGF_{2α}, PGD₂, PGI₂ and TXA₂ (Thromboxane A₂) (Breyer and Breyer 2000). The production of endometrial PGs is mainly governed by the rate limiting enzymes i.e., cyclooxygenases (COX-1 and COX-2). The COX-1 and COX-2 are also known as prostaglandin endoperoxide H synthase-1 and 2 (PGHS-1 and PGHS-2), respectively. These enzymes are responsible for the conversion of arachidonic acid into PGH₂, the common precursor of the various forms of PGs including PGE₂ and PGF_{2α}. The downstream enzymes, PGE synthase (PGES) and PGF synthase (PGFS) catalyze the conversion of PGH₂ to PGE₂ and PGF_{2α}, respectively. PGE₂ is luteotrophic in nature, whereas PGF_{2α} acts as the luteolytic agent in ruminant and oxytocin is responsible for its episodic release. In ruminants, oxytocin (OT), progesterone (P4), and estradiol (E2) regulate the uterine secretion of prostaglandin F_{2α} (PGF_{2α}) that causes luteolysis (Mondal et al. 2015a; Mondal et al. 2015b; Mondal et al. 2017).

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Two isoforms of cyclooxygenase have been identified viz., COX-1 and COX-2, that are encoded by two different genes known as *COX-1* and *COX-2* gene, respectively (Salamonsen and Findlay, 1990; Smith et al. 1996; 2000). The enzyme COX-1 is a constitutively expressed in a range of cell types, conversely COX-2 enzyme is inducible which plays an important function in various pathological and physiological circumstances in animal tissues (Smith et al. 1996; Funk, 2001). The expression and regulation of both the isoforms are tissue and species specific (Arosh et al. 2002). COX-3 has also been identified as a splice variant of COX-1, and it is present mainly in brain and spinal cord (Mondal et al. 2015b). The role of COX-3 is still not known. However, Blitek et al. (2006) reported a possible role in pain sensitivity based on the mechanism of action of acetaminophen (paracetamol), evoked as a selective inhibitor of COX-3. Depending on pathological and physiological circumstances, their expression varies within a specific tissue and species (Kniss, 1999; Funk, 2001; Arosh et al. 2002). Usually, COX-2 is regulated by growth factors, different cytokines and greatly elevated in both acute and chronic inflammation (Simon, 1999). Since, information on characterization and expression profile of *COX-2* gene in buffalo (*Bubalus bubalis*) is lacking. Therefore, in the present study, characterization of *COX-2* gene in the uterine endometrium of buffalo was undertaken.

Materials and Methods

Sample collection

Buffalo uteri were collected from the local abattoir immediately after slaughter and transported to the laboratory on ice. The stages of estrous cycle were determined based on colour, vasculature, size and consistency of CL. Accordingly, uteri were classified into three stages: stage I (days 3 to 5), stage II (days 6 to 15) and stage III (days 16 to 21) of estrous cycle (Ghosh and Mondal, 2006). Uteri were opened longitudinally and carefully cut out from the lamina propria of the intercaruncular endometrium. The endometrium was scrapped out using a sterile surgical blade.

Table 1: Primers used for *COX-2* gene

Primer	Sequence (5'-3')	Melting temp. (°C)	Length
<i>COX-2</i> FP	TCAAGATCACATTTGATTGAGA	62.2	449 bp
<i>COX-2</i> RP	TGTATCCTCCCACAGTCAAAGA	63.4	

Table 2: Primers used for β -actin gene

Primer	Sequence (5'-3' sequence)	Melting temp. (°C)	Length
β -actin FP	GACGACATGGAGAAGATCTGGCA	69.7	341 bp
β -actin RP	GAAGGATCTTATGAGGTAGTCTG	61.5	

Isolation of total RNA from buffalo endometrial tissue

Total RNA was isolated from the buffalo endometrial tissue samples (n= 6) using Trizol (Ambion, Life Technologies, USA) followed by RNeasy Mini Kit (Qiagen, USA). The quality and integrity of the purified RNA was checked through agarose gel electrophoresis, and quantity was measured using nanodrop spectrophotometer (Eppendorf, Germany) at 260 and 280 nm wavelengths. The purified RNA was preserved at -80°C until use.

Synthesis of cDNA using Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The first strand cDNA was synthesized from purified RNA using iScript™ cDNA synthesis kit (Bio-Rad, USA). From each endometrial sample, 2 μ l of total RNA was reverse transcribed using 4 μ l 5 \times iScript reaction mixture and 1 μ l of iScript Reverse Transcriptase to make a final volume of 20 μ l with nuclease free water in a sterile 0.2 ml PCR tube on ice. This reaction mixture was incubated at 25°C for 5 min, later at 46°C for 20 min, after which the reaction was terminated (95°C for 1 min). The cDNA was stored at -20°C.

Designing of primers

Primers for *COX-2* (*PTGS2*) gene (Table 1) and β -actin gene (Table 2) for *Bubalus bubalis* were designed based on the available sequence in NCBI with accession number (NC_037549.1) and (NC_037568.1) respectively using Primer3 software. PCR amplification of cDNA was carried out using β -actin primers to check the quality of the cDNA. Primer sequences used for PCR amplification of coding sequence of *COX-2* gene is given in the Table 1.

Preparation of PCR product

The working solutions (100 μ M) of both forward and reverse primers were prepared from stock solutions by adding Milli-Q water (autoclaved). PCR master mix was prepared by adding the reagents in the following order (Table 3) into a sterile 0.2 ml PCR tube. PCR reactions were performed in the thermal cycler

(Eppendorf, Germany) under following general cycle conditions: Initial denaturation at 98°C for 3 min, followed by 30 cycles of denaturation at 98°C for 45 sec, annealing at 59°C for 30 sec, and extension at 72°C for 90 sec and final extension at 72°C for 10 min. The PCR amplified products were stored at -20°C.

Checking of PCR product by agarose gel electrophoresis

After amplification, the PCR product was checked on 1% agarose gel to verify the amplification of the target region. A volume of 5 µl PCR product was mixed with 2 µl of 6X gel-loading dye and loaded slowly in separate wells along with 100 bp DNA ladder in the first well of the gel.

Gel extraction of amplified product

The PCR amplified product was resolved from 1% agarose gel electrophoresis and visualized by ethidium bromide staining. The electrophoresis was performed in 1X TBE buffer at 75 volts for 60 minutes till complete separation. The PCR amplified product (449 bp) was visualized on UV transilluminator and photographed with a gel documentation system (Bio-Rad, USA). PCR product was purified using gel extraction kit (Qiagen, USA) as per manufacturer's instruction.

Sequencing and analysis of PCR product

The purified PCR product was sent for sequencing (Eurofins, Bangaluru) with *COX-2* gene specific primers. The sequence data were analysed using Clustal Omega Multiple Sequence Alignment Tool and NCBI database. The analysis was done against the reference sequence of *Bubalus bubalis COX-2* gene. The obtained nucleotide sequence was also compared with different species by Nucleotide Basic Local Alignment Search Tool (nBLAST) of NCBI database. Clustal Omega Multiple Sequence Alignment Tool and nBLAST was used to calculate the appropriate match for the selected sequences to identify the similarities and differences between sequences.

Results and Discussion

Total RNA was extracted from the buffalo uterine endometrial tissue by Trizol and RNeasy Mini Kit method. The quantitative and qualitative estimation of isolated RNA was checked by Nanodrop spectrophotometer at 260 and 280 nm wavelengths and agarose gel electrophoresis, respectively. RNA concentrations ranged from 510 to 575 ng/µl with an average of 547 ng/µl, and optical density (OD at 260/280 nm) values ranged from 1.91 to 1.99 with an average of 1.93, which indicated that the isolated RNA was of good quality. The quality of isolated RNA was further checked by running on 1% agarose gel electrophoresis. Two distinct 28S and 18S ribosome of RNA bands were observed on agarose gel (Figure 1), which revealed that the RNA was of good quality. The cDNA was synthesized from the reverse transcription of 1000 ng of isolated RNA by using iScript™

Table 3: Composition of reaction mixture for PCR (Thermo Scientific, USA)

Sl. No.	Components	Volumes
1	cDNA Template (50 ng/µl)	2.0 µl
2	10X Buffer	2.5 µl
3	50 mM dNTP mix	0.5 µl
4	25 mM MgCl ₂	2.5 µl
5	Forward Primer (10 pmol/µl)	0.5 µl
6	Reverse Primer (10 pmol/µl)	0.5 µl
7	Phusion DNA Polymerase (5 U/µl)	0.1 µl
8	Milli-Q water (autoclaved)	16.4 µl
	Total Volume	25 µl

cDNA synthesis kit (Bio-Rad, USA) and stored immediately at -20°C until its use.

The quality of the cDNA was analysed by polymerase chain reaction (PCR) using *β actin* gene, NCBI Accession Number (NC_037568.1). A 341 bp amplification was observed for the *β actin* gene (Figure 2) indicated that the cDNA was in good quality. The *COX-2* gene sequence information of *Bubalus bubalis* was retrieved from NCBI database. Gene specific forward and reverse primers for *COX-2* gene coding sequence were constructed using the Primer3 software.

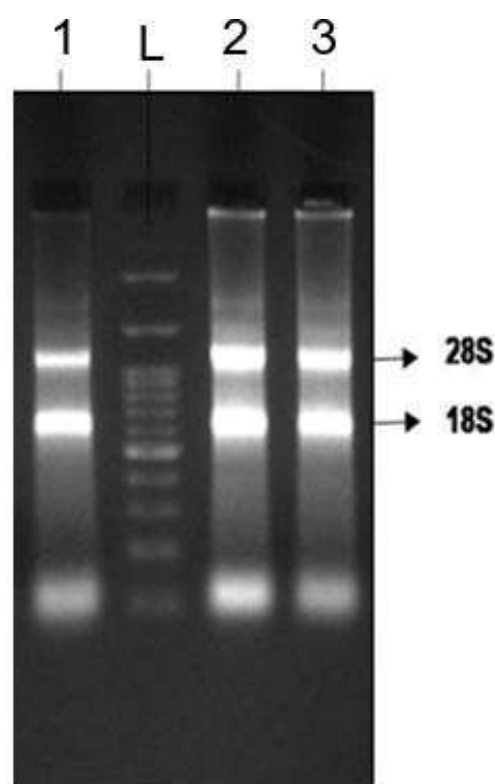


Fig. 1 Agarose gel electrophoresis exhibited amplification at 28S and 18S of RNA

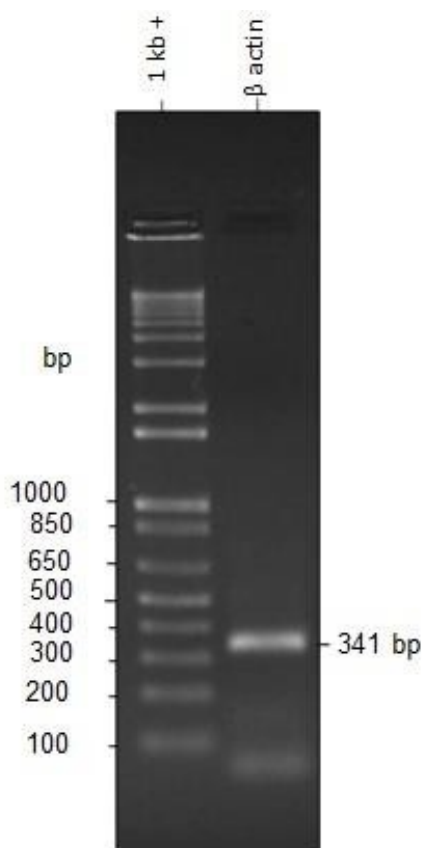


Fig. 2 Agarose gel electrophoresis of PCR product for β -actin gene (Lane 1: 1 kb DNA ladder; Lane 2: β -actin gene)

The PCR was performed to amplify the *COX-2* gene. The PCR product showed amplification of 449 bp (Figure 3) on agarose gel after electrophoresis. The amplified PCR product of 449 bp was cut, weighed and purified using QIAquick Gel Extraction kit (Qiagen, USA) and subsequently examined by electrophoresis in agarose gel stained with ethidium bromide.

Sequence analysis of PCR amplified product

The purified PCR product of 449 bp was sent for DNA sequencing (Eurofins, Bangalore). Custom DNA sequencing was done by Sanger sequencing. After sequencing of 449 bp PCR product, a length of 360 bp was matching with the *Bubalus bubalis COX-2* sequence along with primer binding. Clustal Omega Multiple Sequence Alignment Tool was used to study the appropriate match for the selected sequences to ascertain the similarities and difference between target and reference sequences. The sequence analysis using Nucleotide Basic Local Alignment Tool (nBLAST) revealed that there was 100 percent homology of each with nucleotide sequence (Figure 4 and Figure 5) with the *Bubalus bubalis COX-2 (PTGS2)* gene (Accession Number: NC_037549.1). Multiple sequence alignment of nucleotide sequence obtained

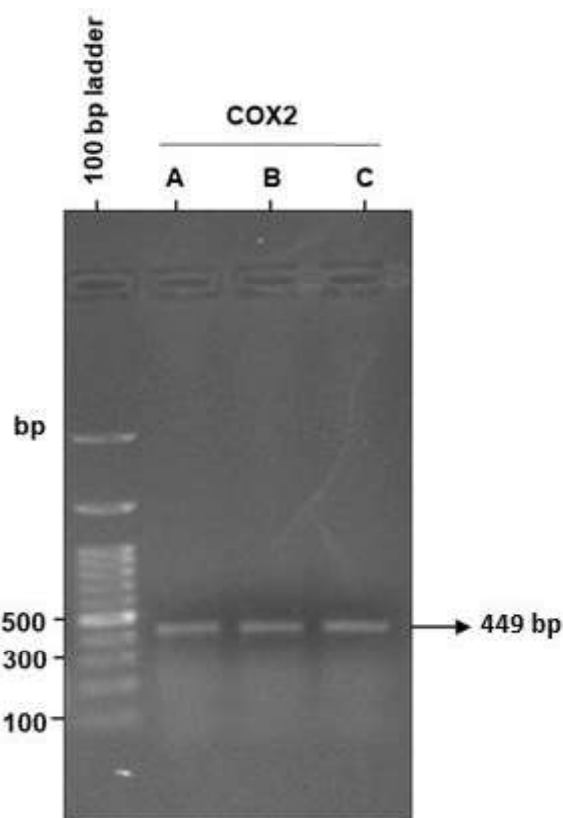


Fig. 3 Agarose gel electrophoresis of PCR product for *COX-2* gene (Lane 1: 100 bp DNA ladder; Lane 2, 3 and 4: *COX-2* gene)

for 360 bp sequence of PCR product showed 100% homology with the *Bubalus bubalis COX-2* gene (Figure 5). All these results clearly indicated that the PCR amplified product was from the *Bubalus bubalis COX-2* gene.

These sequence analysis showed 100, 98.32, 96.64 and 91.60 percent identical with cattle, goat, sheep and pig, respectively. Asselin et al. (1997) demonstrated that cDNAs of *COX-2* gene were cloned and sequenced in endometrial epithelial cells of *Bubalus bubalis*. The *COX-2* gene was found 84, 86, and 87 percent of homology in relation to rat, guinea pig and human, respectively. Liu et al. (2001) reported cloning and characterization of the full-length bovine *COX-2* or *PGHS-2* cDNA of 1.8 kb size. Comparative analysis revealed that the amino acid sequence of the bovine protein was very similar to that of other mammalian homologs, being 89, 87, 87, 89, 90, 86 and 86 percent identical to human, rat, mouse, horse, rabbit, guinea pig and mink *PGHS-2* gene. Tsai et al. (1996) cloned a PCR product of 553 bp into a pCRTM II vector and subsequently sequenced the cloned product. The sequence was compared and found to be 87.5 and 86.2 percent identical with human and mouse *PGHS-2*, respectively. Sakaram et al. (2010) amplified the full length cDNA of *DRA* gene in Murrah buffalo and analysed to identify the

Fig. 4 Sequence of PCR amplified product (449 bp). Blue colour indicates *COX-2* specific primers used for sequencing, Purple colour indicates 360 bp sequence obtained from 449 bp PCR product

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TCAAGATCACATTTGATTGAGAGTCCGCCAAC TTATAATGTGCACTACAGCTATAAAAGCTGG
GAAGCCTTTTCTAACCTGTCTTATTATACCAGAGCTCTTCTCCGGTGCCTGATGACTGCCCA
ACACCCATGGGTGTGAAAGGGAGGAAAGAGCTTCTTGATTCAAAGAAGTTGTAAGGAAAGTA
CTTCTAAGAAGAAAGTTCATTCTGATCCCCAGGGCACAATCTGATGTTTGCATTCTTTGCC
CAGCACTTCAACCATCAATTTTTCAAGACAGATTTTGAACGAGGACCAGCTTTCACCTAAGGGA
AAGAACCATGGGTGGACTTAAATGCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTG
CGCCTTTTCAAGGATGGAATAATGAAATATCAGATGATTAATGGAGAGATGTATCCTCCACA
GTCAAAGA
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Fig. 5 Multiple alignment of PCR amplicon (449 bp) for *Bubalus bubalis COX-2* gene sequence

Download GenBank Graphics

PREDICTED: Bubalus bubalis prostaglandin-endoperoxide synthase 2 (PTGS2), mRNA

Sequence ID: [XM_025285468.1](#) Length: 3387 Number of Matches: 1

Range 1: 348 to 707 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
665 bits(360)	0.0	360/360(100%)	0/360(0%)	Plus/Plus
Query 1	TAATGTGCACTACAGCTATAAAAGCTGGGAAGCCTTTTCTAACCTGTCTTATTATACCAG	60		
Sbjct 348	TAATGTGCACTACAGCTATAAAAGCTGGGAAGCCTTTTCTAACCTGTCTTATTATACCAG	407		
Query 61	AGCTCTTCTCCGGTGCCTGATGACTGCCCAACCCATGGGTGTGAAAGGGAGGAAAGA	120		
Sbjct 408	AGCTCTTCTCCGGTGCCTGATGACTGCCCAACCCATGGGTGTGAAAGGGAGGAAAGA	467		
Query 121	GCTTCTGATTCAAAGAAGTTGTAAAAAAGTACTTCTAAGAAGAAAGTTCACTCTCGA	180		
Sbjct 468	GCTTCTGATTCAAAGAAGTTGTAAAAAAGTACTTCTAAGAAGAAAGTTCACTCTCGA	527		
Query 181	TCCCCAGGGCACAATCTGATGTTTGCACTTTTGGCCAGCACTTCACCCATCAATTTT	240		
Sbjct 528	TCCCCAGGGCACAATCTGATGTTTGCACTTTTGGCCAGCACTTCACCCATCAATTTT	587		
Query 241	CAAGACAGATTTTGAACGAGGACCAGCTTTCACCTAAGGGAAAGAACCATGGGTGGACT	300		
Sbjct 588	CAAGACAGATTTTGAACGAGGACCAGCTTTCACCTAAGGGAAAGAACCATGGGTGGACT	647		
Query 301	AAGTCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTGCGCCTTTTCAAGGATGG	360		
Sbjct 648	AAGTCACATTTATGGTGAATCTTTAGAGAGACAGCATAAGCTGCGCCTTTTCAAGGATGG	707		

genetic variability. Naskar et al. (2012) cloned and characterized MHC (*Bubu*)-*DRB* cDNA in water buffalo. Gul et al. (2023) performed molecular cloning, expression and structural characterization of growth hormone-receptor (GHR) and its extracellular domain as growth hormone binding protein (GHBP) from the liver of Nili-Ravi buffalo. Medina et al. (2023) conducted molecular characterization of TLR4 genes of swamp and riverine types of water buffaloes to determine unique genotypic characteristics specific to each type of water buffalo and provide baseline information for explaining differences in disease resistance between each type. Pramanik et al. (2022) conducted characterization of α^s1 -casein gene in buffalo at the molecular level to determine complete α^s1 -casein cDNA sequence.

Conclusion

In the present study, *COX-2* gene was characterized in the endometrial epithelial cells of buffalo. The purified PCR product (449 bp) was sequenced using buffalo *COX-2* gene specific primers that was subjected to sequence analysis. Nucleotide sequence analysis of PCR amplified product exhibited 100 percent identity with the reference sequence of *Bubalus bubalis COX-2* gene. The optimized PCR conditions and the buffalo *COX-2* gene specific primers used in the study can be useful in pursuing further gene expression studies to explore various functions of the *COX-2* gene in buffalo.

Acknowledgements

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