DEVELOPMENT OF qPCR ASSAY FOR ESTIMATION OF TRANSCRIPTIONAL ABUNDANCE OF CHEMOKINES DURING EMBRYONIC IMPLANTATION IN BUFFALOES

Rajni Kumari[1,*], Rakesh Kumar[2], Shanker Dayal[1], Poolangulam Chinnakkan Chandran[1], Sanjay Kumar[3], Pradeep Kumar Ray[1], Reena Kumari Kamal[1] and Amitava Dey[1]

ABSTRACT

Successful pregnancy is the outcome of a well coordinated embryo- maternal communication events. Few evidences suggest the role of cytokines signaling pathways as mediators of these communications for establishment of pregnancy. In order to investigate the role of cytokines CCL8 and CXCL10 in embryonic implantation during pregnancy, the present study aimed to develop quantitative real time PCR method based on SYBR Green dye chemistry. Primers were designed for the amplification of CCL8, CXCL10 and GAPDH (endogenous control) genes specific to bovines using Primer 3 software. The amplification products for CCL8, CXCL10 and GAPDH (endogenous control) genes yielded fragments of 388, 151 and 81 bp respectively. Purified PCR Products were used for the generation of standard curve for all the three genes. Six scalars tenfold serial dilutions of every PCR product were performed for amplification of genes by optimized protocol. Each sample was run in triplicate along with a no template control for every assay. Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primers dimers. The standards generated linear relationships with regression coefficients: r^2=0.996, 0.993 and 0.992 for CCL8, CXCL10 and GAPDH genes respectively. The method posed to be reliable approach for estimating the relative expression of cytokines CCL8 and CXCL10 in peripheral blood leucocytes in buffaloes.

Keywords: *Bubalus bubalis*, buffaloes, chemokines, embryonic implantation, real time PCR assay

INTRODUCTION

Embryonic implantation is an event which involves complex molecular dialogue between dam and fetus and its success entirely depends on coordinated communications between them. Chemokines are multifunctional molecules and are known to have involvement in these interactions. Any disturbance in this communication network leads to early embryonic losses. These losses contribute to major chunk of repeat breeding cases in dairy animals and thus limit their reproductive potential. Understanding the role of chemokine during embryonic implantation has potential to prevent these losses. For this, it is very important to

[1]Division of Livestock and Fishery Management, Indian Council of Agricultural Research, Research Complex for Eastern Region, Bihar, India, *E-mail: drrajnikumari@rediffmail.com
[2]Indian Council of Agricultural Research, Research Complex for North Eastern Hill Region, Umiam, India
[3]Department of Animal Nutrition, Bihar Veterinary College, Bihar, India

have information on expression profile of important chemokines involved in this process. Recently, role of chemokines, CCL8 (also known as MCP-2) and CXCL10 (also known as IP-10), was elucidated in bovine endometrium on 15th and 18th day of pregnancy (Sakumoto *et al*., 2018). Chemokine (C-C motif) ligand 8(CCL8), also known as monocyte chemoattaractant protein 2(MCP-2), and C-X-C motif chemokine 10(CXCL10) also known as interferon gamma induced protein 10(IP-10) are small cytokines belonging to CC chemokine and CXC chemokine family respectively. These are chemoattaractants and are involved in inflammatory responses and angiogenesis. Data regarding expression profile of these chemokines may also help in determination of pregnancy status in buffaloes. Considering the fact that fluorescence based real time reverse transcription PCR offers an economic, sensitive and reliable approach for quantification of transcripts (Nolan *et al*., 2006), the present study aimed towards the development of assay, for the estimation of transcriptional abundance of chemokines (CCL8 and CXCL10) from whole blood in Murrah buffaloes.

MATERIALS AND METHODS

Experimental animals

The present study was conducted on Murrah breed of buffaloes maintained at livestock farm unit, Division of Livestock and Fishery Management of ICAR-Research Complex for Eastern Region, Patna.

Collection of blood

About 2 ml of peripheral blood was aseptically collected in sterile EDTA coated vacutainers containing 0.5% (10 µl/ml of blood) anticoagulant from jugular vein of buffaloes on day 0 (Day 0 being the day of artificial insemination), and day 24. After collection, the blood samples were immediately processed for extraction of total RNA to prevent degradation of mRNA.

RNA extraction

Extraction of total RNA from the blood samples was carried out using TRIzol reagent. Briefly, 600 µl of blood was transferred to 1.5 ml centrifuge tube to which 900 µl of TRIzol reagent was added with vigorous vortexing to completely dissociate the blood cells. Then 180 µl of chloroform (0.2 volume TRIzol reagent) was added and after vigorous shaking for 15 seconds the mixture was incubated on ice for 15 minutes followed by centrifugation at 13201 x g for 15 minutes at 4°C. interphase. The RNA in aqueous phase was precipitated by adding 500 µl isopropanol and incubation overnight at -20°C. RNA pellet obtained after centrifugation at 13201 x g for 20 minutes was washed with 1 ml of 70% ethanol and again centrifuged at 13201 x g for 10 minutes was carried out. Pellet was then air dried and resuspended in 20 µl of nuclease free water and then kept at 50°C for 15 minutes for proper dissolution of pellet. Isolated RNA was immediately reverse transcribed and was stored at -20°C till used.

Quality and quantity check of RNA

Quality and quantity of RNA was assessed by spectrophotometer. Optical density (O.D.) was determined at wavelengths 260 nm and 280 nm in UV-VIS spectrophotometer against distilled water as blank sample. The ratio between OD_{260} and OD_{280} was observed for each sample. RNA sample with ratio of 2 to 2.2 was considered good and taken for further analysis.

cDNA synthesis

cDNA synthesis was done using commercial cDNA synthesis kit (GCC Biotech, India) as per manufacturer recommendations, and the obtained cDNA was stored at -20°C for further use.

Designing of primers for PCR/RTqPCR

In silico primer designing for target regions of Bovine CCL8 gene (XM_006080459.2PREDICTED: *Bubalus bubalis* C-C motif chemokine 8), CXCL10 gene (XM_006079166.2PREDICTED: *Bubalus bubalis* C-X-C motif chemokine 10), and GAPDH was carried out using Primer 3 software (http://www.primer3.ut.ee). The sequence of primers and their respective nucleotide numbers are given in Table 1. In the present study, GAPDH was selected as internal control to normalize PCRs for the amount of RNA added to the reverse transcription reactions. This internal control gene was properly validated for each experiment to determine that gene expression is unaffected by the experimental treatment.

Amplification of chemokines by conventional PCR

The working solutions of both forward and reverse primers were prepared from stock solutions by adding primer and autoclaved double distilled water to obtain final concentration of 10 pmol for each primer. Template cDNA stock solution was diluted to working concentration of 50 to 100 ng/µl using autoclaved double distilled water. Each PCR reaction cocktail was prepared using PCR Master Mix (2X) (Thermo Fisher). Final reaction cocktail (25 µl) was as follows: 0.5 µl of forward primer and reverse primer, 13.0 µl of PCR Master Mix (2X), 9.0 µl of nuclease free water and 2.0 µl of template cDNA. The contents of PCR reaction cocktail were mixed thoroughly by vortexing followed by brief spin. The PCR amplification was performed using ABI Thermal cycler. Each 0.2 ml tube containing PCR reaction cocktail was kept in Thermal cycler for amplification of targeted region of buffalo CCL8, CXCL10and GAPDH genes. Detailed PCR programme and annealing temperatures are given in Table 2.

Real-Time amplification of chemokines

Amplification was performed inThermal cycler (Applied Biosystem, 7500). The various components of the experiment and cyclic conditions of PCR are described below in Table 2A, 2B and 2C. Samples were run in triplicate along with non template control (NTC). Each run was completed with a melting curve analysis to confirm the specificity of amplification and lack of primer dimmers by the optimized protocol.

Validation of internal control gene

In our study we selected housekeeping GAPDH gene as endogenous control and day 0 samples were used as calibrator sample.

Determination of PCR efficiencies

PCR amplicons of CCL8, CXCL10 and GAPDH genes were purified by commercial PCR purification kit (Qiagen, Germany). Six scalar tenfold serial dilutions (CO: 100–0.01%) of purified PCR amplicons were made. Amplification efficiencies of CCL8, CXCL10 and GAPDH gene specific PCR amplification reactions was estimated by plotting the standard curves i.e. plotting the curve between log dilution factors and C_t values for each of dilutions in triplicates for all the purified amplicons. C_t values can be used to assess the amount of amplifiable cDNA in the samples.

RESULTS

Amplification of chemokines by conventional and real time PCR

The synthesis of cDNA template in all the samples, was verified by amplification of endogenous control GAPDH gene using conventional PCR. (Figure 1). After all the samples were found positive for cDNA template, both the target genes CCL8 and CXCL10 gene were amplified to yield the amplicons of 388 bp and 151 bp respectively (Figure 2). Later on, real time amplification was performed with same cDNA template for all the three target genes (Figure 3).

Melting curves analysis and primer specificity analysis

Analysis of melting curve revealed three different peaks for GAPDH, CXCL10 and CCL8 genes at 76°C, 78°C and 82°C, respectively. No overlapping was observed between peaks. (Figure 4). The real time amplicons were analysis by agarose gel electrophoresis for confirmation of the amplicon size. The agarose gel electrophoresis revealed amplicons of 388 bp, 151 bp and 81 bp for CCL8, CXCL10 and GAPDH genes respectively similar to the results of conventional PCR. None of the NTC yielded any peak.

The amount of CCL8 on day 24, normalized to GAPDH and relative to day 0 is reported in Table 3.

PCR efficiencies

After the confirmation of gene specific amplification and absence of any nonspecific amplification, efficiencies for all the three PCR were estimated. Analysis of standard curve plot in the assay showed a linear relationship ($r>0.98$) between the logarithm of the dilution factors and the C_t values for the serial template dilutions (Figure 5, 6 and 7). The mean slopes of the log-linear regression plots, which represent the amplification efficiency, resulted to be similar (3.01 ± 0.5; 3.91 ± 0.05 and 3.71 ± 0.05) for CCL8, CXCL10 and GAPDH genes, respectively.

DISCUSSION

Present study was aimed for the development of protocol/assay to be utilized for generation of expression profile of chemokines during embryonic implantation in buffaloes. The assay is based on the relative quantification of chemokines CCL8 and CXCL10 versus endogenous control gene based on SYBR Green chemistry. Use of SYBR Green chemistry has several advantages over Taqman chemistry when, singleplex PCR are performed. The dye is the simplest, sensitive and least expensive compared to the presently known dyes (Simon *et al*.,1998). This dye tends to bind all double-stranded nucleic acid molecules (Whittwer and Kusakawa, 2004), hence the accumulation of primer dimers and the amplification of non-specific PCR products can be detected in SYBR Green (Lekanne *et al*., 2002). The electrophoresis from the amplification fragments of the qPCR amplicons revealed absence of non-specific amplification products unlike the findings of Hatt and Loffler (2012) as a step for further validation and confirmation. In the present study, specificity of amplicons is explained by unique melting temperatures. The melting temperature of a DNA molecule (T_m) is defined as the temperature where half of the DNA helical structure is lost. The T_m depends on size and nucleotide composition of DNA molecule. The melting peaks for GAPDH, CXCL10 and CCL8 genes at 76°C, 78°C and 82°C

Table 1. Sequence of primers and their respective number of bases of CCL8, CXCL10 and GAPDH genes in buffaloes.

Genes	Product Size	Sequence (5′-3′)	No. of base
CCL8	388 bp	F- CGAACACCGAAGCCTTGAAC	20
		R- ACACATCCACTCACAGGAGC	20
CXCL10	151 bp	F- ATTGTCCGTGGACTTCGGTT	20
		R- GCCTCGGTGTAGCTTACAGT	20
GAPDH	81 bp	F-GCGATACTCACTCTTCTACTTTCGA	25
		R-CGTACCAGGAAATGAGCTTGAC	22

Table 2. PCR programme for buffalo CCL8, CXCL10 and GAPDH gene.

S. No.	Steps	Temperature	Time	No of Cycles
1	Initial denaturation	95°C	3.0 minutes	One
2	Denaturation	94°C	30 seconds	35
3	Annealing	58°C	45 seconds	35
4	Extension	72°C	60 seconds	35
5	Final extension	72°C	5.0 minutes	-
6	Hold	4°C	Forever	-

Table 2A. Cycling conditions for qPCR (Thermocycling condition).

Stage	Repetition	Temperature	Time
1	1 Cycle	50°C	2 minutes
2	1 Cycle	95°C	10 minutes
3	40 Cycle	95°C	15 Seconds
4	40 Cycle	58°C	1 minute

Table 2B. Cycling conditions for qPCR (Denaturing condition).

Stage	Repetition	Temperature	Time
1	1 Cycle	95°C	15 Seconds
		60°C	20 Seconds
		95°C	15 Seconds
		60°C	15 Seconds

Table 2C. Reaction mixture for SYBR Green based Real time PCR for amplification of Buffalo CCL8, CXCL10 and GAPDH gene.

Reaction mix	Volume (µl)
Master mix (2X)	10.0
Forward primer (1 pm/µl)	0.5
Reverse primer (1 pm/µl)	0.5
Nuclease free water	7.0
Template	2.0
Total	20

Table 3. Data analysis using 2-ddCT method.

Sample	CCL8 C_T	GAPDH C_T	dC_T	ddC_T	Normalized CCL8 amount relative to caliberator 2^{-ddCT}
Day 0 sample (Caliberator)	28.2	21.8	-	0±0.07	1.0
	28.6	21.2	-	-	-
	27.9	21.2	-	-	-
	28.1	21.4	-	-	-
	28.2	21.3	-	-	-
Average	28.2±0.02	21.3±0.01	6.9±0.17	-	-
Technical Replicates for Day 24 sample	25.6	22.1	-	-3.18±0.04	9.063
	25.9	21.7	-	-	-
	25.2	21.8	-	-	-
	25.1	22.0	-	-	-
	25.8	21.8	-	-	-
Average	25.52±0.02	21.8±0.05	3.72±0.07	-	-

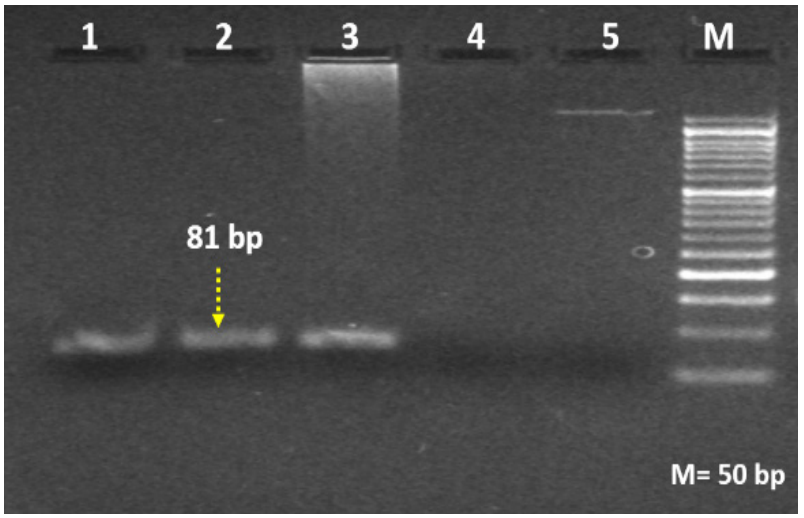

Figure 1. Amplification of endogenous control of GAPDH gene.
Lane 1-3: GAPDH; Lane-4: NTC.

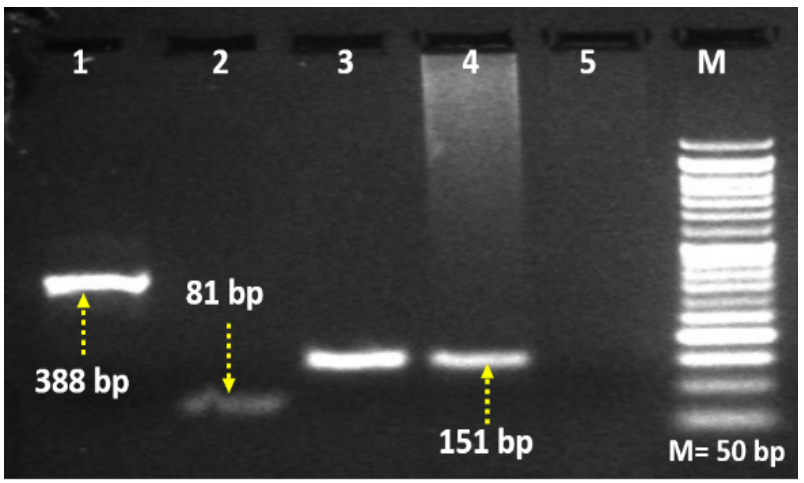

Figure 2. Amplification of CCL8, CXCL10 and GAPDH gene.
Lane 1: CCL8; Lane 2: GAPDH; Lane 3-4: CXCL10

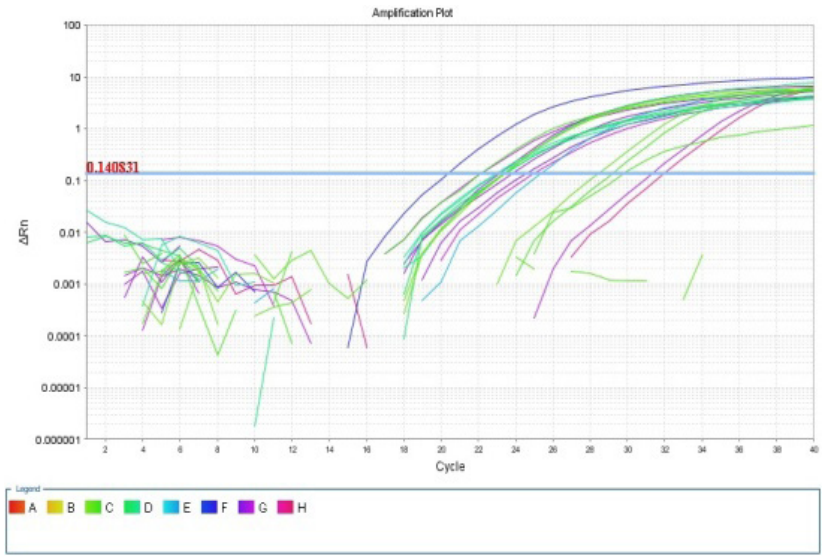

Figure 3. Real time amplification of CCL8, CXCL10 and GAPDH gene.

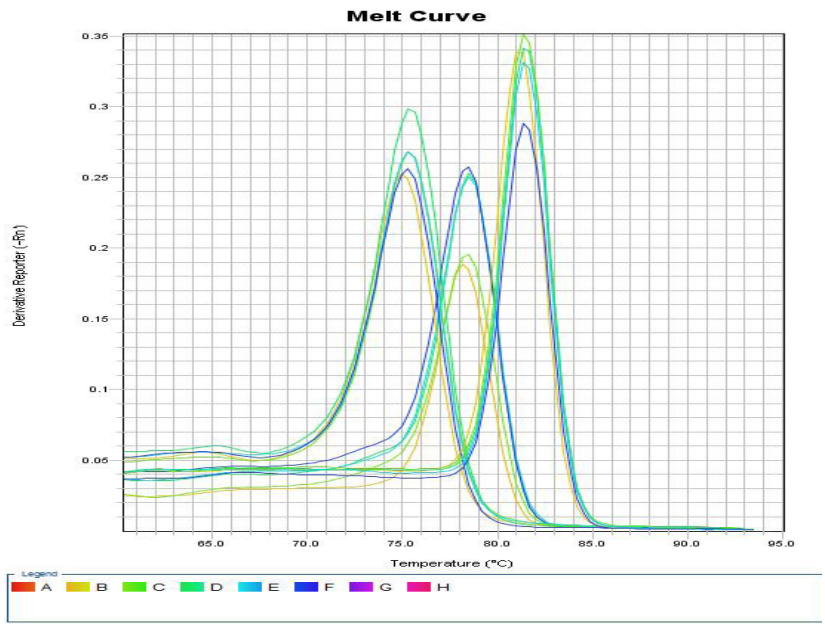

Figure 4. Melt curve analysis for CCL8, CXCL10 and GAPDH gene.

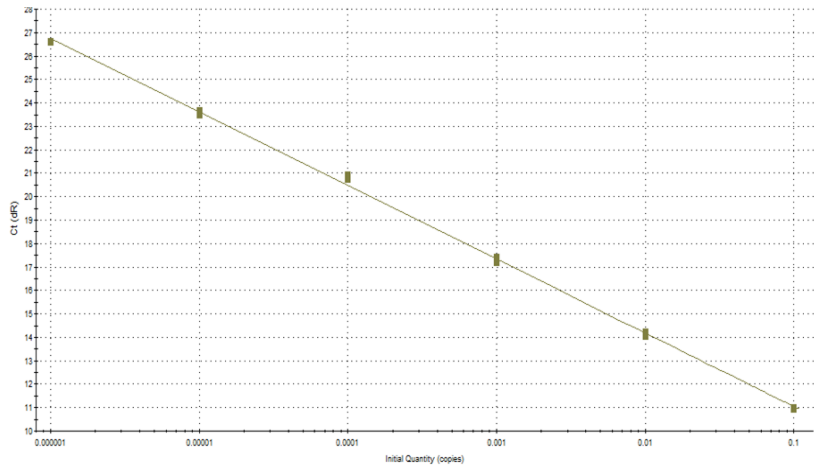

Figure 5. Standard curve for CCL8 gene specific qPCR.

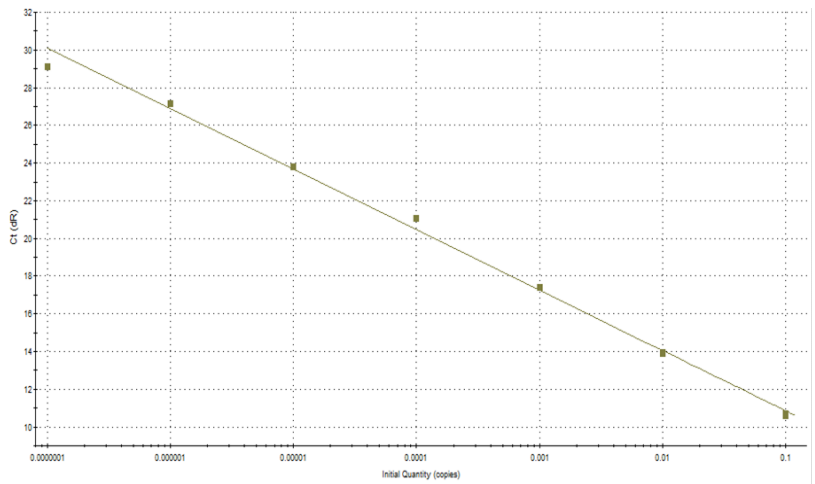

Figure 6. Standard curve for CXCL10 gene specific qPCR.

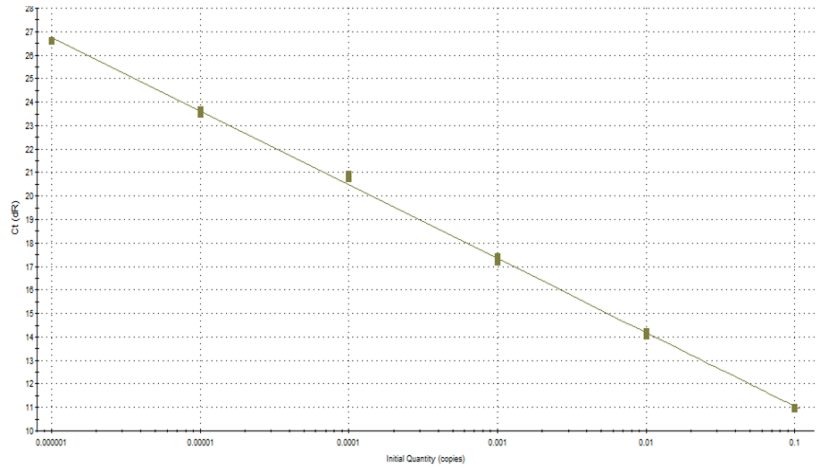

Figure 7. Standard curve for GAPDH gene specific qPCR.

is in accordance with their respective amplicon size i.e., 81 bp, 151 bp and 388 bp . As expected, the amplicons with more size had high melting temperature.

The present study uses ddC_T model for relative quantification. For the calibrator sample ddC_T equals zero as per the norms. Normalizing to an endogenous control (GAPDH gene in the present study) provides a method for correcting results for differing amounts of input RNA. Therefore, this is very useful in the cases where, where precious RNA samples are very limited in amount and cannot be afforded for measurement of input RNA. PCR efficiency is an indication of how well the PCR reaction has proceeded. Slope of -3.32 corresponds to 100% efficiency. Since in our study, PCR efficiencies of chemokine genes and endogenous control were found similar, it can be said that primers are working well and relative quantification can be performed using this assay. The present study fulfills both the conditions of the design and evaluation of the assay i.e., (i) Selection and validation of endogenous control gene (ii) ensuring the similar efficiencies of PCR for dilutions of RNA/cDNA for target and endogenous control genes (Livak and Schmittgen, 2001) and thus qualifies for relative quantification assay of chemokines.

CONCLUSION

The amplification efficiencies of the PCR reaction targeted for CCL8, CXCL10 and GAPDH genes were found to be similar as evident from standard curves and therefore the present study can be used further for relative quantification of chemokines (CCL8 and CXCL10) taking GAPDH as endogenous control.

ACKNOWLEDGEMENTS

I acknowledge the support rendered by network project on buffalo. Improvement for carrying out the research on animals reared under the project.

REFERENCES

Deprez, R.H.L., A.C. Fijnvandraat, J.M. Ruijter and A.F.M. Moorman. 2002. Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal. Biochem*., **307**(1): 63-69. DOI: 10.1016/s0003-2697(02)00021-0

Sakumoto, R., K.G. Hayashi, S. Fujii, H. Kanahara, M. Hosoe and T. Furusawa. 2017. Possible roles of CC- and CXC- chemokines in regulating bovine endometrial function during early pregnancy. *Int. J. Mol. Sci*., **18**(4): 742. DOI: 10.3390/ijms18040742

Simon, C., P. Caballero-Campo, J.A. Garcia-Velasco and A. Pellicer. 1998. Potential implications of chemokines in reproductive function: an attractive idea. *J. Reprod. Immunol*., **38**(2): 169-193. DOI: 10.1016/s0165-0378(98)00031-x

Nolan, T., E.R. Hands and A.S. Bustin. 2006. Quantification of mRNA using real-time RT-PCR. *Nat. Protoc*., **1**(3): 1559-1582. DOI: 10.1038/nprot.2006.236

Hatt, J.K. and F.E. Loffler. 2012. *Quantitative real-time PCR (qPCR) detection chemistries affect enumeration of the Dehalococcoides 16S rRNA gene in groundwater. J. Microbiol. Meth*., **88**(2): 263-270. DOI: 10.1016/j.mimet.2011.12.005

Livak, K.J. and T.D. Schmittgen. 2001. *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods*, **25**(4): 402-408. DOI: 10.1006/meth.2001.1262

Wittwer, C.T. and N. Kusakawa. 2004. Real-time PCR. p. 71-84. *In* Persing, D.H., F.C. Tenover, J. Versalovic, J.W. Tang, E.R. Unger, D.A. Relman and T.J. White. (eds.) *Molecular Microbiology: Diagnostic Principles and Practice*, ASM Press, Washington, USA.