

Research Article

Characterization and Transcriptional Profile of Toll-Like Receptors of *Bubalus bubalis* with EndometritisNOHA MOHAMED OSMAN^{1,2*}, AHLAM AHMED ABOU MOSSALLAM², FAWZY REYAD EL SEEDY³, EMAN ROSHDY MAHFOUZ², RUI LI⁴, MICHAL MIKULA⁵

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Abstract | Toll-like receptors (TLRs) are a vital component of innate immune system and perform an important role in disease resistance through recognition of pathogen associated molecular patterns (PAMPs) and initiation of a convenient immune response. Endometritis is the most important disease causing infertility in cattle and buffalo and TLRs play a critical role in elimination of that disease. The present study aims to characterize the transcripts of TLRs: TLR1, TLR2, TLR4, TLR6 and TLR10 in *Bubalus bubalis* with endometritis. Quantitative real-time (q-PCR) assays were performed to detect transcript expression profiles of these TLRs in liver, mammary gland, ovary and uterus of *Bubalus bubalis* with and without endometritis. The result showed that the transcript profiles of TLRs especially TLR2 and TLR4 were significantly differences in mammary glands, ovary and uterus but on the othe hands, there is no significant difference in the transcript profile of TLRs in liver with or without endometritis. TLRs nucleotide sequences have more than 97% nucleotide homology between *Bubalus bubalis* with and without endometritis. Simple modular architecture tool (SMART) analysis revealed the presence of TIR domains and different numbers of leucine rich repeat motifs in tested TLRs. In conclusion, for animals infected with endometritis, there was a significant difference of TLR2 and TLR4 in uterine tissue compared to uninfected animals. Therefore, it is suggested to consider the TLRs results as detection markers for endometritis.

Keywords | Toll-Like receptors (TLRs), *Bubalus bubalis*, Endometritis, Gene expression

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INTRODUCTION

The innate immune system is a global system of host defense against pathogens. Recognition of pathogens relied on a set of germ line-encoded re-

ceptors recognized pattern-recognition receptors (PRRs) as a part of innate immune recognition. These receptors have the ability to recognize conserved pathogen-associated molecular patterns (PAMPs) produced by large groups of pathogens (Akira, 2001;

Ten members of the TLR receptor family are broadly expressed in the mammalian genome (Akira et al., 2006). TLR1, TLR2 and TLR6 recognize bacterial lipids such as lipoteichoic acid (LTA), whereas TLR3, TLR7, and TLR8 as well as TLR9 recognize nucleic acids, often from viruses. TLR9 also identifies bacterial DNA. TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria such as *E. coli*, while TLR5 binds to flagellin. The ligand for TLR10 is still not known (Hirata et al., 2007; Regan et al., 2013).

TLRs are type 1 transmembrane proteins with N terminal extracellular leucine-rich repeat (LRR) and C terminal intracellular TIR (Toll/IL 1 receptor) domain. A TIR domain is required for initiating intracellular signaling and inducing downstream effector molecules, while LRRs are responsible for the recognition of PAMPs. (Akira et al., 2006; Kumar et al., 2009; Dubey et al., 2013).

TLRs are expressed broadly in Antigen-presenting cells, and regulate the activation of both innate and adaptive immunity through recognition of specific patterns of microbial components (Medzhitov, 2001; Matsushima et al., 2007). Their expression and distribution are associated with altered immune responsiveness (Helmy and Grecis, 2003).

TLRs play an important role in regulation of ovulation, fertilization, gestation and parturition, as well as in pathological conditions such as endometritis and mastitis (Kannaki et al., 2011). The endometrium is the first line of defense against microbial pathogens. The endometrial cells have ability to develop innate immune defense in cattle, humans and rodents (Herath et al., 2006; Davies et al., 2008). Endometritis is the most common uterine disease observed in buffaloes slaughtered at abattoirs, and is one of the main causes of infertility in the cattle and buffalo (Azawi et al., 2008; Ajevar et al., 2014).

Microbial disease of the female genital tract is most common, and has greatest economic importance in both humans and animals. Microbial infections of the genital tract cause infertility by disrupting uterine and ovarian function (O'Neill, 2008). Establishment of infection in the endometrium occurs by the interaction between the virulence factors of microbial invaders and the endometrial immune defenses (Sheldon et al., 2010).

Bubalus bubalis population (195 million heads) includes both river and swamp buffaloes. Egyptian buffaloes are of the river type with a population of 4 million heads; it ranks the third after Indian (115 millions) and Pakistani (31.7 millions) buffalo, which plays an important role in the economy of developing countries (FAOstat, 2013). The present study was aimed to characterize the TLRs genes and their transcriptional profile in *Bubalus bubalis* with and without endometritis.

MATERIALS AND METHODS

TISSUE SAMPLES

Tissue samples from four different organs; liver, ovary, mammary gland and uterus, were obtained from thirty animals with and without endometritis from a local abattoir immediately after slaughter and kept on ice until processing. The uteri of animals with endometritis had abnormal secretions as profuse purulent exudate, with signs of inflammation as swelling, redness and hardness (Williams et al., 2005; Sheldon et al., 2006). These animals had no evidence of other diseases based on visual inspection and culture of bacteria using standard microbiological techniques.

RNA ISOLATION AND REVERSE TRANSCRIPTION

Total RNA was extracted from liver, ovary, mammary gland and uterus using Easy-RED™ kit (iNtRON Biotechnology) according to the manufacturers' protocols. The RNA quality and purity was determined by NanoDrop 1000 (Thermo Scientific) and cDNA was synthesized as per manufacturer's procedure (RevertAid First Strand Synthesis Kit, Thermo Scientific).

PRIMER DESIGN AND OPTIMIZATION OF RT-PCR

The TLRs coding regions were amplified by designing primers using *Bubalus bubalis* transcripts sequences as following: TLR1 (HQ327989), TLR2 (HM756162), TLR4 (HQ343416), TLR6 (HQ327992), TLR10 (HQ327991) as presented in Table 1 using the Primer3 software (Rozen and Skaletsky, 2000). These primers were used for PCR analysis and Real time quantitative PCR (qRT-PCR). The primers were synthesized by Amersham Pharmacia Biotech Company.

For qPCR analysis, large ribosomal protein (RPL0) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an internal calibrator due to their

Table 1: Primers sequences of TLR1, 2, 4, 6 and 10 used in PCR

Gene	Name	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing	Size(bp)
TLR1	TLR1.1	ATG CCT GAC ATC CTC TCA CT	ACC TCC TTG GAT CGA GGT CC	63.9°C	1165 bp
	TLR1.2	TGA CCC AGG AAA TGA AAT CT	CAG CAG AAA TAC ATT AAC ACG G	63.9°C	1195 bp
	TLR1.3	TCA ACC AGG AAC TGG AAT ACT TGG A	TGG GCA GGG CAT CAA ATG GA	60°C	120 bp
TLR2	TLR2.1	AGA GTG GAG GTC AAA TCA CTG GAC A	TGC CTG AAA CTT GTC GGT GGC C	68.6°C	1329 bp
	TLR2.2	GCC TGT AAG GAT GCC TGG CCC	GGT CAG TCT TGG TCT GGT GGT	68.6°C	1253 bp
	TLR2.3	GGT TTT AAG GCA GAA TCG TTT G	AAG GCA CTG GGT TAA ACT GTG T	60°C	190 bp
TLR4	TLR4.1	CGG CAC AGA CAG AGG GTC ATG C	GCA AAC CTT GAA AAT GGC AGG CAA	62.2°C	1500 bp
	TLR4.2	TTT TGG GAC AAC CAA CCT GAA GCA T	CAA GAA GTG ACA ACC TCC ACC TGA	63.6°C	1342 bp
	TLR4.3	CTT GCG TAC AGG TTG TTC CTA A	CAT AAC TTC TCC AGC TTC CCA G	60°C	153 bp
TLR6	TLR6.1	ATC AGC AGC AAC CCT CCG GG	TGG GTG TGG TAT CTT TAG CAG CCT	67°C	1532 bp
	TLR6.2	ACA CAT GCT TTG TCC TCA GGA ACC A	AAT TTA TCC CCT GCT GCG TTT GGA	65°C	1388 bp
	TLR6.3	AAG TGA ATG ATG ACA ATT GTC	AGT GAC TTC AAT GTC GTT T	60°C	235 bp
TLR10	TLR10.1	TCT GTG GAG CCG CTG CAA GC	TGG ACT TTT TCC AGA GCT GCC AGG	62°C	1654 bp
	TLR10.2	GCC AGC AAC ACA TCC TTG AGG C	GCC TGT AGA GCC CCA CCC ACT	54°C	1270 bp
	TLR10.3	GCT GCA AGC CTG AGG GAT TT	AGG ATT TTA TCA CTG GTT CAC CTC	60°C	93 bp
RPL0	RPL0	CAA CCC TGA AGT GCT TGA CAT	AGG CAG ATG GAT CAG CCA	60°C	220 bp
GAPDH	GAPDH	CCT GGA GAA ACC TGC CAA GT	GCC GTA TTC ATT GTC ATA CCA	60°C	214 bp

threshold cycle (CT) values are close to those of TLRs. Each PCR reaction was performed using 2 µl of 50–60 ng cDNA template, 12.5 µl of RNase DNase free water, 2.5 µl of 2mM dNTPs, 2.5 µl of 10X taq polymerase buffer, and 2.5 µl forward and reverse primers (10 µM), as well as 0.25 µl (1U) of Taq polymerase in 25 µl reaction final volume. The following cycling conditions were used: 3 min. at 94°C; 35 cycles for 1 min at 94°C; 2 min at specific annealing of each primer as in table (1); 1 min at 72°C and a final extension for 10 min at 72°C. Following optimization, the presence of a single product was confirmed on a 1.5% agarose gel by electrophoresis. The gels were inspected by UV light and photographed by Gel documentation system (In Genius, Syngenebioimaging).

The PCR products were purified using (Thermo Scientific, USA) purification kit, according to the manufacturer's instructions. The purified product was sequenced using ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems) in an automatic sequencer (ABI Prism3100, Genetic analyzer (Applied Biosystems)).

SEQUENCE ANALYSIS

cDNAs infected uterus were used to amplify TLR1, TLR2, TLR4, TLR6 and TLR10 genes. Then, the amplified PCR products were sequenced using reverse and forward primers.

Sequence alignment was carried out using NCBI/BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) between coding regions (CDs) and amino acids of tested TLRs in infected uterus of *Bubalus bubalis* with endometritis and *Bubalus bubalis* sequences in GenBank database.

CHARACTERIZATION OF THE TLRs DOMAINS

The different functional domains of TLRs (The transmembrane regions (TM); Leucine-rich repeats (LRR); LRR C-terminal (LRR-CT) and Toll/IL-1 receptor (TIR) was predicted by using simple modular architecture analysis tool (SMART), available at <http://smart.embl-heidelberg.de/> (Schultz et al., 1998) and LRRfinder (www.lrrfinder.com) (Offord et al., 2010). The amino acid sequence of TIR domains of tested TLRs of infected animals with endometritis was subjected to align with TIR amino acid sequence of *Bubalus bubalis* sequences in GenBank database for detecting the percentage of the identity.

QUANTITATIVE REAL TIME PCR (qRT-PCR)

qRT-PCR was used to estimate TLR expression levels in the examined samples. For each sample, cDNA was diluted 1:25 with 10Mm Tris pH 7.5. For each sample, 1.9 µl of diluted cDNA template was combined with 2 µl of 2X Master Mix (Bioline) and 0.1 µl of 10 µM primer set for a final reaction volume 4 µl in MicroAmp optical 384 well reaction plate in triplicates along with no template control (NTC) to all wells. Cycling was performed on 7900HT Real-Time PCR system (Applied Biosystems, Life Technologies Corporation) using the following cycling conditions: 95°C for 10 min (hold), 95°C for 15 sec (melt) and 60°C for 1 min (anneal/extend) for 40 cycles. Dissociation curves were generated to ensure that a single amplicon had been produced.

STATISTICAL ANALYSIS

The expression of candidate genes was evaluated using a $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The Geometric mean of reference genes (RPLP0 and GAPDH) acted as an internal calibrator within each sample to calculate ΔC_t . The $\Delta\Delta C_t$ was calculated by ΔC_t (treatment) - ΔC_t (control). The fold change in expression was then used as a relative measure of gene expression. Data analysis was carried out by non-parametric Mann-Whitney test. Significance was set at $P < 0.05$ and the results were reported as mean \pm SEM.

RESULTS

SEQUENCING OF TLRs GENES

The full-length of TLR1, TLR2, TLR4, TLR6, and TLR10 cDNAs coding regions of *Bubalus bubalis* with endometritis have been submitted to GenBank and their accession numbers are presented in Table 2. The percentage of nucleotide and amino acid identity of *Bubalus bubalis* with and without endometritis is shown in Table 2. There was a highly degree of identity between *Bubalus bubalis* with and without endometritis.

ANALYSIS OF FUNCTIONAL DOMAINS OF TESTED TLRs

The results showed that all TLRs of *Bubalus bubalis* with and without endometritis have LRR and TIR domains and one transmembrane domains (TM) in all tested TLRs except TLR6, which has two transmembrane domains (TM). LRRCT and LRRNT (cysteine flanking regions of LRR at C and N terminals, respectively) were examined in tested TLRs, we

Table 2: Coding DNA, translated amino acids and TIR domain sequence similarity of TLRs between *Bubalus bubalis* with and without endometritis

TLR	CDs identity (%)	Amino acid identity (%)	TIR domain identity(%)	Accession number
TLR1	99.5	99.3	100	KM058706
TLR2	99.6	99.1	99.3	KM058707
TLR4	99.5	98.9	99.3	KM058708
TLR6	99.8	99.2	98.6	KM058709
TLR10	99.5	98.9	97.9	KM058710

Table 3: Leucine-rich repeats in amino acid sequences of tested TLRs

Species	TLR1	TLR2	TLR4	TLR6	TLR10
<i>Bubalus bubalis</i> with endometritis	18	20	21 and 2 predicted overlapped	20	20
<i>Bubalus bubalis</i>	19	20	22 and 2 predicted overlapped	20	20

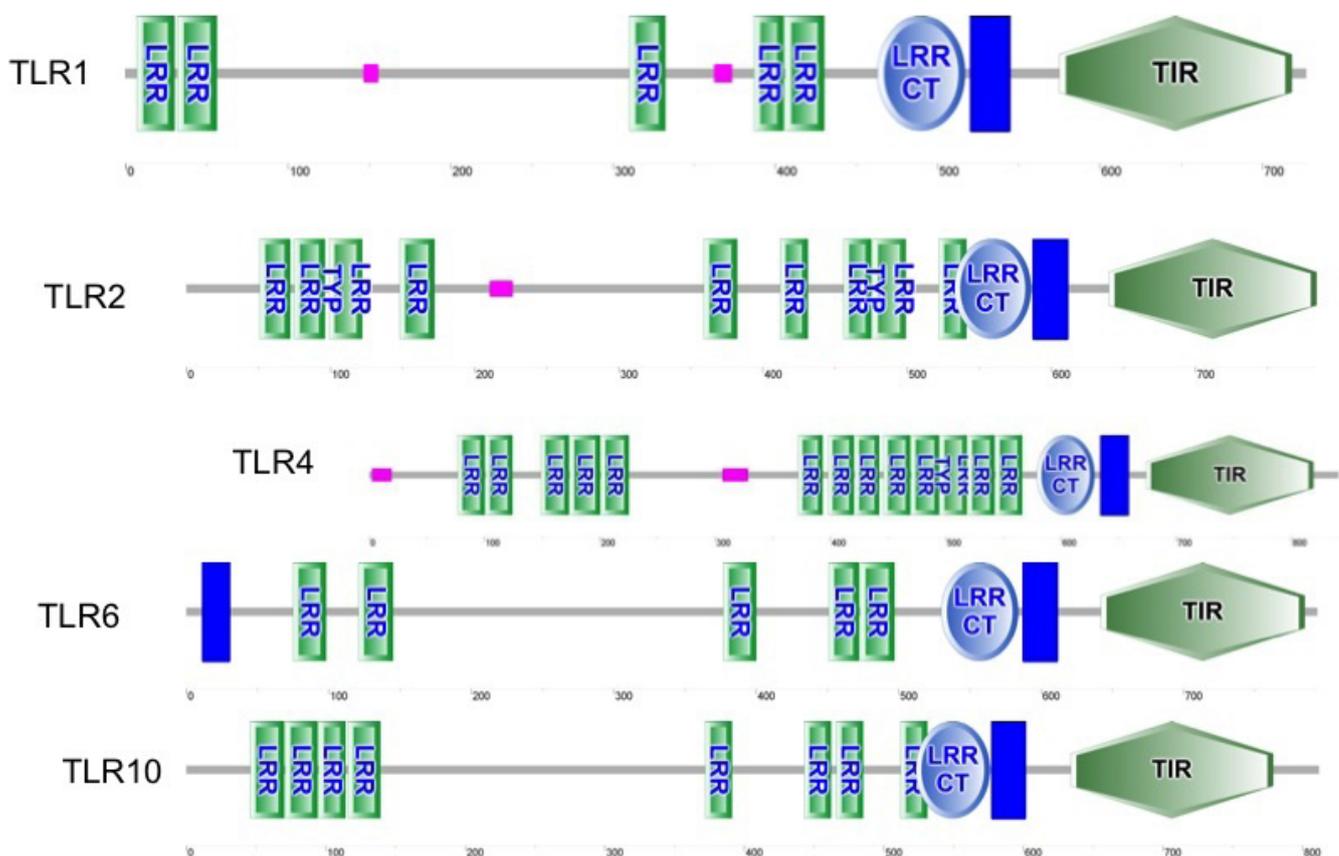


Figure 1: predicted functional domains in tested TLRs of *Bubalus bubalis*, with and without endometritis (vertical bar is transmembrane domain (TM))

found all TLRs contain LRRCT, while LRRNT was absent in all TLRs as shown in Figure 1.

Next, we used LRR finder to predict the repeat number of LRR motifs present in TLRs. As shown in Table 3, we found an equal number of LRRs (20) in TLR2, TLR6 and TLR10 *Bubalus bubalis* with and without endometritis but on the other hand TLR1 of *Bubalus*

bubalis with and without endometritis has (18) and (19) LRR respectively, while TLR4 of *Bubalus bubalis* with and without endometritis has (21) and (22) LRRs with 2 LRRs predicted overlapped respectively.

RESULTS OF EXPRESSION OF TLRs GENES

As shown in Figure 2D, our results revealed that TLR1 expression levels increased six-fold in the uterine tis

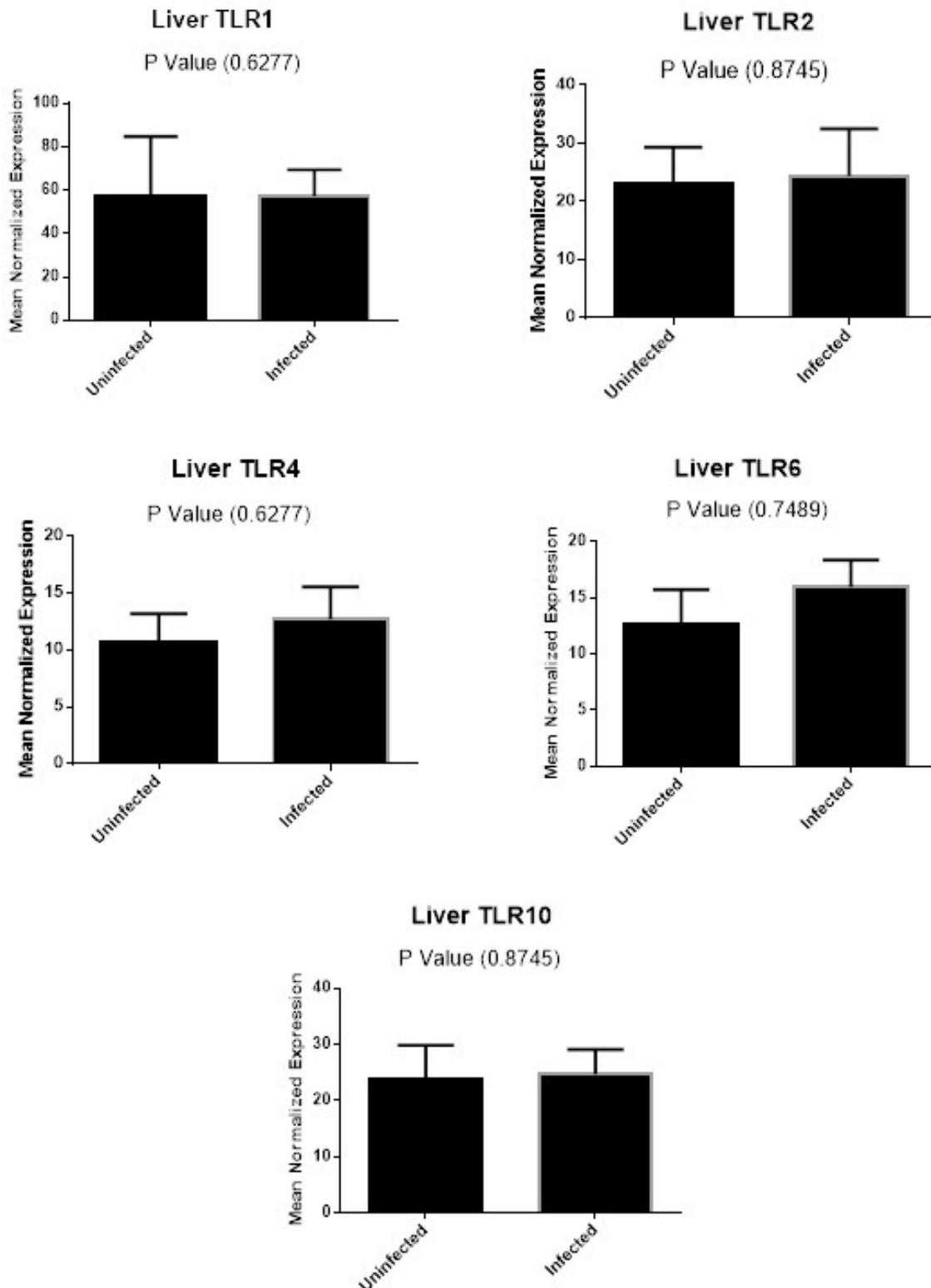


Figure (2A): Expression levels of TLR1,TLR2, TLR4,TLR6 and TLR10 genes in liver tissues collected from animals with and without endometritis. The expression levels of these genes were normalized to RPL0 and GAPDH and calibrated with the average of control. Expression levels were obtained from three pooled biological replicate samples \pm S.E.M.

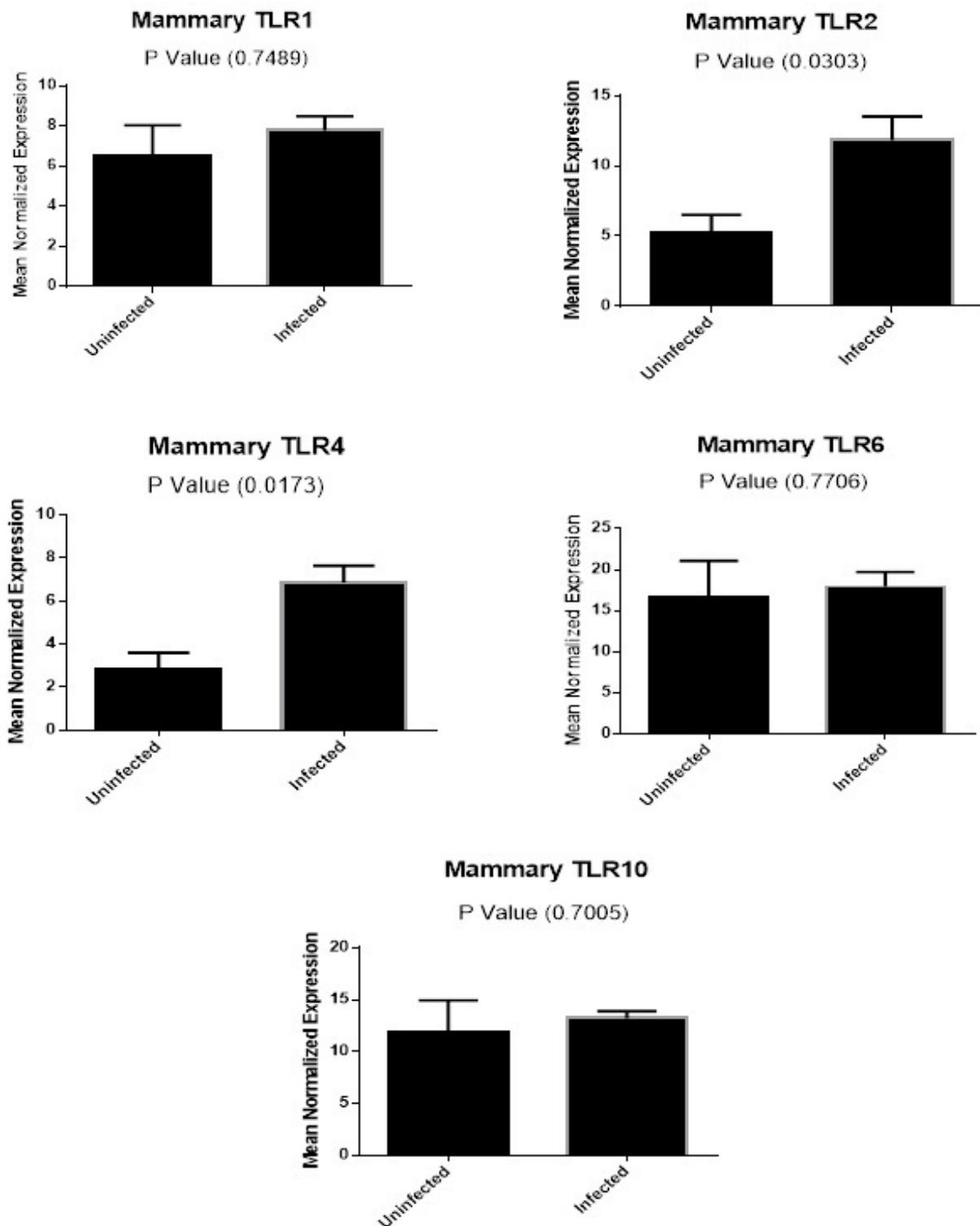


Figure (2B): Expression levels of TLR1,TLR2, TLR4,TLR6 and TLR10 genes in mammary gland tissues collected from animals with and without endometritis. The expression levels of these genes were normalized to RPL0 and GAPDH and calibrated with the average of control. Expression levels were obtained from three pooled biological replicate samples \pm S.E.M.

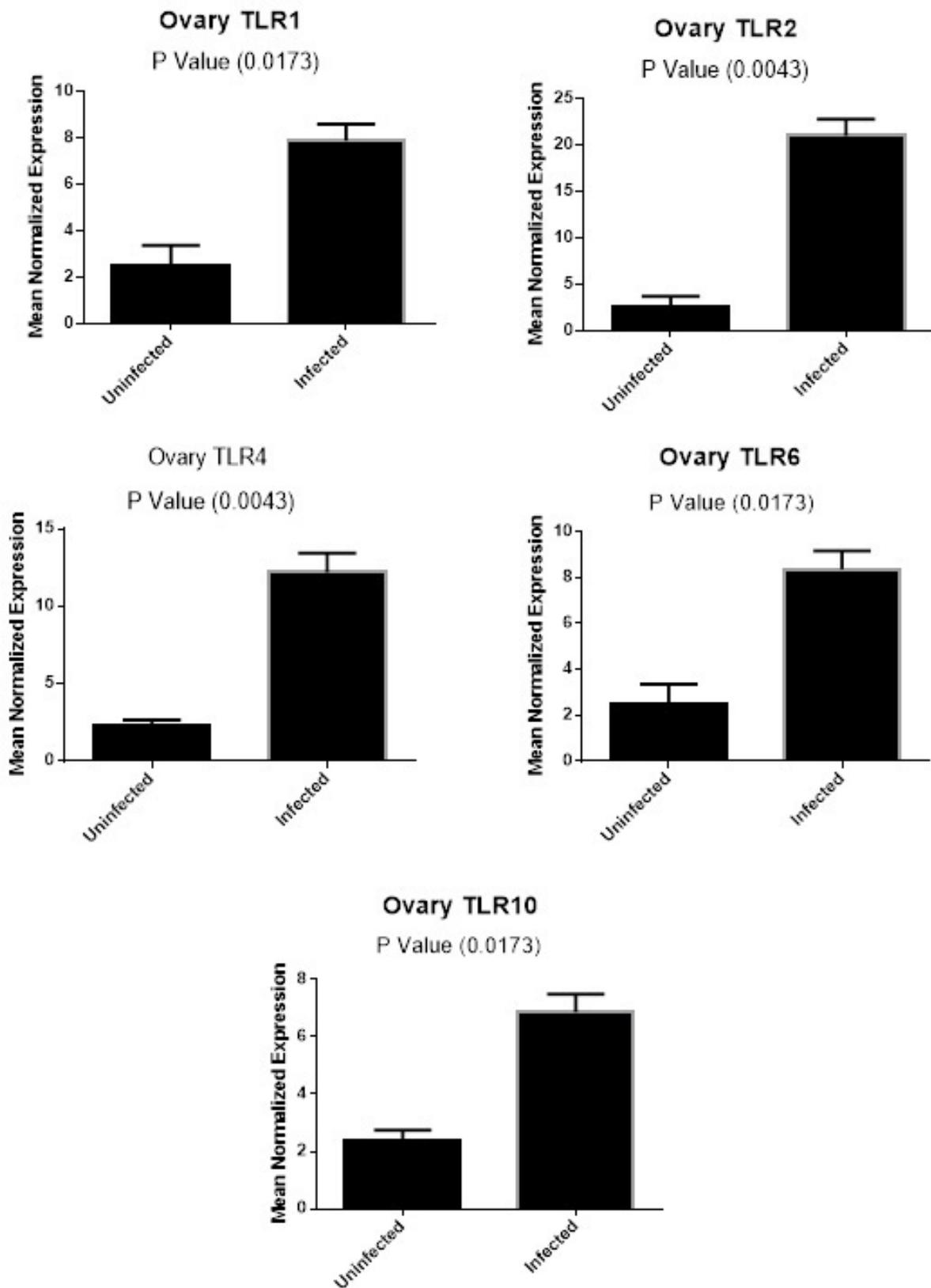


Figure (2C): Expression levels of TLR1,TLR2, TLR4,TLR6 and TLR10 genes in ovarian tissues collected from animals with and without endometritis. The expression levels of these genes were normalized to RPL0 and GAPDH and calibrated with the average of control. Expression levels were obtained from three pooled biological replicate samples ± S.E.M.

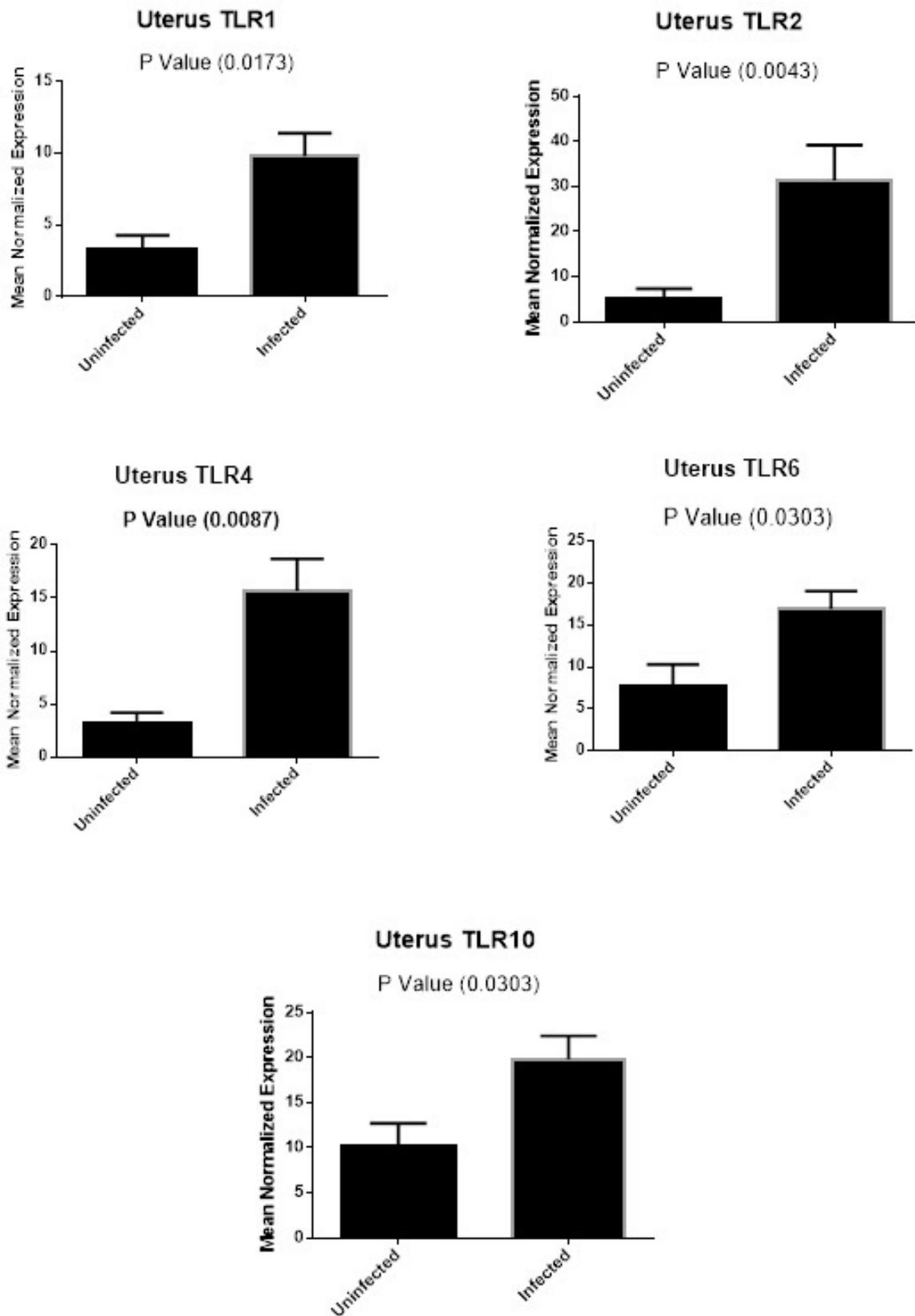


Figure (2D): Expression levels of TLR1,TLR2, TLR4,TLR6 and TLR10 genes in uterine tissues collected from animals with and without endometritis. The expression levels of these genes were normalized to RPL0 and GAPDH and calibrated with the average of control. Expression levels were obtained from three pooled biological replicate samples ± S.E.M.

sues of *Bubalus bubalis* with endometritis compared to those in the uterine tissues of animals without endometritis ($P=0.0173$). Similarly, the expression levels of both TLR6 and TLR10 were higher with nine-fold increase for TLR6 ($P=0.0303$), and an almost ten-fold increase for TLR10 ($P=0.0303$) in the uterine tissues of animals with endometritis compared to those in the uterine tissues of animals without endometritis. In addition, TLR2 showed twenty-six-fold increase in the uterine tissues of animals with endometritis compared to those in the uterine tissues of animals without endometritis ($P=0.0043$). Moreover, TLR4 showed a significant increase in the uterine tissues of animals with endometritis compared to those in the uterine tissues of animals without endometritis, with a twelve-fold increase ($P=0.0087$).

In the ovarian tissues, the expression profile of candidate genes showed that the expression of TLR1, TLR6 and TLR10 had the same level of expression between animals with and without endometritis ($P=0.0173$, Figure 2C). In addition, there was a significant increase in the expression levels of TLR2 and TLR4 genes in the ovary tissues of animals with endometritis compared with those without endometritis ($P=0.0043$; Figure 2C).

A comparison of the expression levels of TLR1, TLR2, TLR4, TLR6 and TLR10 genes in mammary gland tissues from animals with and without endometritis were performed. As shown in Figure 2B, the expression levels of TLR1 ($P=0.7489$), TLR6 ($P=0.7706$) and TLR10 ($P=0.7005$) genes in the mammary gland tissues had no significant difference between animals with and without endometritis. Conversely, TLR2 showed a highly significant increase in animals with endometritis compared to animals without endometritis, with a 6.6 fold increase ($P=0.0303$). Similarly, TLR4 showed a highly significant increase in the mammary glands of animals with endometritis compared to those from animals without endometritis, with a 4.0 fold increase ($P=0.0173$).

Finally, we carried out a comparison for the expression levels of TLR1, TLR2, TLR4, TLR6 and TLR10 genes in liver tissues from animals with and without endometritis. As shown in Figure 2A, these tissues had similar expression levels for TLR1, TLR2, TLR4, TLR6 and TLR10.

DISCUSSION

In the present study, we reported the first sequencing data of TLRs genes and their expression in *Bubalus bubalis* with endometritis. Toll-like receptors consider the initial defence against microbes in endometrium. Their characterizations are important to evaluate the innate immune system. TLR1, TLR2, TLR4, TLR6 and TLR10 cDNA, translated amino acids and TIR domain sequences of *Bubalus bubalis* with endometritis were compared with *Bubalus bubalis* without endometritis. TLRs cDNA nucleotide sequences of *Bubalus bubalis* with and without endometritis were 98 to 99% identical. Furthermore, TLR6 of *Bubalus bubalis* with and without endometritis had an insertion of nucleotide triple 'TTA' resulting in the addition of tyrosine amino acid (Y) at position 181 compared with *Bos Taurus* (Carl et al., 2003; Dubey et al., 2013).

TIR domain has an important role in activation of inflammatory response, in cooperation with the adaptor protein MyD88 (Kawai and Akira, 2007). We found that the identity of TIR sequences between with and without endometritis ranged from 97 (TLR10) to 99.3 (TLR2 and TLR4) and 100% (TLR1). TLR2 recognizes peptidoglycan cell wall components of Gram-positive bacteria, while TLR4 ligands include Gram-negative bacteria. Both of TLR2 and TLR4 have an important role in regulation of innate immune response (Herath et al., 2006; Davies et al., 2008).

The number and structure of LRRs of TLRs have a significant effect on the function of pathogen recognition receptor system among species (Kumar et al., 2009; Dubey et al., 2013). Peter et al. (2009) reported that deletion of LRR motif leads to loss of functional integrity of the ligand-binding domain of murine TLRs. In addition, (Dubey et al., 2013) demonstrated that the differences in the number of LRRs among buffalo and cattle may lead to possible differences in the specific recognition of TLR ligands between these species. In our study we found that the number of LRR domains of *Bubalus bubalis* with endometritis and their distribution is similar to *Bubalus bubalis* without endometritis except TLR1 and TLR4.

Bacterial infection of the female genital tract is common in animals especially after parturition causing infertility and sometimes mortality (Davies et al., 2008). The endometrium is the first defence against

pathogens. PAMPs were recognized by TLRs which consider as key components of innate immune system which lead to increase expression of TLRs (Akira, 2006). TLRs expression were evaluated in liver, mammary gland, ovary and uterus of *Bubalus bubalis* with and without endometritis. The results showed no significant difference in TLRs expression in liver tissues between buffaloes with and without endometritis but on the other hand the expression of TLRs in mammary gland showed no significant difference in the expression levels of TLR1, TLR6 and TLR10 between animals with and without endometritis. Conversely, TLR2 and TLR4 genes expression significantly increased in mammary glands of animals with endometritis compared to animals without endometritis. In Ovarian tissue, TLR1, TLR6 and TLR10 had the same level of significant difference ($P=0.0173$). Similarly, TLR2 and TLR4 had the same level of significant difference ($P=0.0043$). This indicates that the expression levels of several TLRs vary significantly in association with the degree of infection in animal (Sharma et al., 2006; Deborah et al., 2008; Chapwanya et al., 2009; Priyanka et al., 2012). The presence of uterine infection due to pathogenic infection had a localized effect on ovarian function (Sheldon et al., 2002). This is hypothesis is supported by (Williams et al., 2008) who stated that pathogenic agents such as *E.coli* has the ability to produce endotoxin lipopolysaccharide (LPS), which is released from infected uterine tissue to ovarian tissue and peripheral circulation leading to disruption of the hypothalamus, pituitary and ovary functions in animals.

Previous studies demonstrated that the expression levels of TLRs family members increase upon endometritis. Specifically, the mRNA levels of TLR2 and TLR4 increase in endometritis infection of bacterial origin (Ozinsky et al., 2000; Goldammer et al., 2004; Azawi et al., 2008; Davies et al., 2008). With the agreement of these data, we detected overexpression of all five examined TLR genes, particularly TLR2 (26.4 fold) and TLR4 (12.4 fold), in infected uterine tissues with endometritis compared to the uninfected ones.

CONCLUSION

Our data demonstrated that the variations in nucleotides and amino acids sequence between *Bubalus bubalis* with and without endometritis are not associ-

ated with endometritis. In addition, there is an overexpression of examined TLRs genes especially TLR2 and TLR4 in animals with endometritis compared to animals without endometritis specifically in uterine tissue.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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