International Journal of Agricultural Technology 2024 Vol. 20(5):1967-1978
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Association of TLR4 polymorphism with subclinical mastitis in early lactating riverine buffalo

Medina, N. P.[1], de Guia, A. C. M.[2*], Rieza, M. L. K.[1] and Mingala, C. N. M.[2,3]

[1]College of Veterinary Science and Medicine, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines; [2]Livestock Biotechnology Center – Philippine Carabao Center, Science City of Muñoz, Nueva Ecija, Philippines; [3]Institute of Graduate Studies, Department of Animal Science, College of Agriculture, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines.

Medina, N. P., de Guia, A. C. M., Rieza, M. L. K. and Mingala, C. N. M. (2024). Association of TLR4 polymorphism with subclinical mastitis in early lactating riverine buffalo. International Journal of Agricultural Technology 20(5):1967-1978.

Abstract Association of polymporphism of the TLR4 gene with the occurrence of subclinical mastitis in early lactating riverine buffaloes is determined. The buffalo samples TLR4 nucleotide sequence had 98% similarity with that of Bubalus bubalis from 96% to 97% homology with *Bos taurus* and *Bos indicus*. The buffalo samples showed a high degree of similarity in their amino acid sequences, averaging 96.2%, with those of the small ruminant species *Ovis aries* and *Capra hircus*. When compared to *Bubalus bubalis*, the translated amino acid sequences from the buffalo samples had an 80% similarity. However, the amino acid sequences of *Bos indicus*, *Bos taurus*, *Ovis aries*, and *Capra hircus* exhibited a lower similarity, averaging around 72%. Maximum likelihood algorithm phylogenetic analysis showed that the buffalo sample and *Bubalus bubalis* clade together, that separated from the clade of *Bos indicus* and *Bos taurus* which further separated with the clade of small ruminants. RFLP analysis showed three possible genotypes using *Rsa*I restriction enzyme. Genotype A and B had a high odds ratio of 6.86 with significant association, there was 5.4 times greater probability that occurrence of subclinical mastitis can happen than genotype C. Therefore, genotypes A and B were found to be associated with the incidence of subclinical mastitis in buffaloes.

Keywords: PCR, Phylogenetic analysis, Clade, Restriction enzyme

Introduction

The water buffalo can be developed to be a contributor for agricultural economy thru milk production, however, the potential of these animals has seldom been recognized. One of the reasons behind is that the majority of those who have a stake in rearing buffalo are generally underprivileged, hence not able to project its impact on their livelihood and well-being. Smallholder-farmers

[*] **Corresponding Author**: de Guia, A. C. M.; **Email**: arrenchristian@gmail.com

happens to be the main economic beneficiaries owning 99% of this animal resource.

In this regard, the great impact of buffalo in the farmer's livelihood carries a great problem as several diseases happen to infect these milk producing animals. Mastitis is one of the most prevalent and expensive diseases in the dairy industry because it adversely impacts milk production. Economically, it results in higher costs and reduced income from milk production compared to that of healthy dairy animals.

Sharma *et al.* (2011) aforementioned that mastitis is a persistent inflammatory reaction of the udder tissue resulting to an increased somatic cell count and is associated with bacterial infections such as *Staphylococcus aureus*, *Streptococcus agalactiae* and other bacteria.

Subclinical mastitis impacts dairy producers' final products by lowering milk yield, degrading milk quality, and impairing reproductive performance. Buffaloes exhibiting a high Somatic Cell Count (SCC) during the initial milk test, a sign of subclinical mastitis, are estimated to lose over 1,500 pounds of milk per animal. Research presented at the 2015 National Mastitis Council Annual Meeting by Kirkpatrick and Olson (2015) concluded that the cost of subclinical mastitis frequently surpasses that of clinical mastitis.

Toll-like receptors (TLRs) could serve as genetic markers for subclinical mastitis. These innate immune receptors are highly conserved and found in a wide range of organisms, from insects (Alfano *et al*., 2014) to mammals (Medzhitov *et al*., 1997). As key elements of the pattern recognition receptor (PRR) system, TLRs identify molecular structures known as pathogen-associated molecular patterns (PAMPs) from invading pathogens.

Alfano *et al*. (2014) noted that toll-like receptors are crucial for innate immunity, as they identify pathogens and trigger appropriate immune responses. Pathogens express several signal molecules, known as pathogen-associated molecular patterns (PAMPs), which are vital for their survival and pathogenicity. When TLRs recognize PAMPs, they initiate a range of antimicrobial immune responses by inducing various inflammatory cytokines.

The occurrence of subclinical mastitis (SCM) is a pervasive and economically significant infectious disease affecting bovines worldwide. This condition results in decreased milk production, adverse alterations in milk composition, and higher expenses related to management strategies. The resulting negative impact on farmers' income underscores the urgent need for sustainable means to manage the disease. While the use of teat sealants and farm hygiene practices involving various disinfectants has proven to be costly, antimicrobial therapy has demonstrated success. However, the emergence of

antimicrobial resistance limits its efficacy, prompting the exploration of alternative options.

In addressing disease resistance in animals, there is a growing trend to investigate specific genes associated with immunity. The TLR4 gene, in particular, has become a focus of attention. Information obtained from studying this gene could serve as a valuable genetic marker in selection programs for mastitis resistance, aiding in the targeted selection of animals with desirable traits. Furthermore, insights gained from such studies contribute to establishing trends in understanding the nature of genetic variation in immune responses.

Focusing on early lactating riverine buffalos, the study was specifically aimed to determine the association between subclinical mastitis and the TLR4 gene. To achieve this, the study characterized TLR4 gene exon 3, determined the genotype variation using restriction fragment length polymorphism (RFLP), and investigated the association of TLR4 gene polymorphism with the occurrence of subclinical mastitis.

Materials and methods

Sampling procedure

A total of 37 riverine buffaloes, in their 2nd to 4th month of lactation, were chosen from the Philippine Carabao Center's gene pool. The animals' housing, feeding, and overall management adhered to the dairy center's standards.

Collection of samples

DNA was obtained from milk samples collected after manually milking test animals from a single teat to gather 30 ml of milk. The milk was then transferred into a 50 ml vial and stored in a cooler. If immediate processing was not feasible, the milk was refrigerated and processed the following day.

Subclinical mastitis determination

The raw milk samples from the animals underwent somatic cell count determination using the California Mastitis test. Equal amounts of test reagent and milk were combined and gently mixed. The reaction was then evaluated on a scale ranging from N (mixture remains unchanged) to 3 (almost-solid gel forms) (Table 1). Milk containing less than 200,000 cells/mL was classified as non-mastitic and of high quality. Milk with 200,000 cells/mL or more was

considered sub-clinically mastitic, considering the absence of visible mammary inflammation and no apparent changes in milk consistency or color.

Table 1. California mastitis test scoring values (Jasper, 1967)

CMT Score	Somatic Cell Range	Interpretation
N (Negative)	0-200,000	Healthy Quarter
T (Trace)	200,000- 400,000	Subclinical Mastitis
1	400,000- 1,200,000	Subclinical Mastitis
2	1,200,000 – 5,000,000	Serious Mastitis Infection
3	Over 5,000,000	Serious Mastitis Infection

DNA extraction

DNA extraction from milk samples followed the Promega protocol with modifications: Initially, 20 mL of raw milk was centrifuged at 4500 rpm for 20 minutes. Following centrifugation, the fat layer and supernatant were discarded, and the pellet was resuspended in 10 mL of 1X PBS solution (pH 7.4). This suspension underwent centrifugation again at 4500 rpm for 25 minutes, with repeated centrifugation steps until the milk clarified (typically three times).

From the falcon tube containing the pellet, 1 mL of milk was collected and transferred to a 2 mL microtube. To this microtube, 1 mL of PBS was added and centrifuged for 1 minute. The supernatant was removed, and this process was repeated. Subsequently, 1 mL of cell lysis solution was added, followed by centrifugation at 14,000 rpm for 1 minute. Then, 200 µL of protein precipitation solution (Promega) was added and centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred to a 1.5 mL microtube, mixed with 500 µL of isopropanol, and centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded, and 700 µL of ethanol (EtOH) was added, followed by centrifugation at 14,000 rpm for 1 minute. This step was repeated, and the microtube was dried. After drying, rehydrating solution was added and incubated at 65°C for 10 minutes. Finally, the sample was stored at 4°C in a refrigerator.

Polymerase chain reaction

Primers utilized for PCR targeting TLR4 were adapted from Medina's (2019) work. The forward primer sequence is AATGCCCCTACTCAACCT, and the reverse primer sequence is CTTCGCAGAGTCAATCCA, designed to amplify the exon 3 region of the gene, yielding a 105 base pair amplicon. The primer designs underwent analysis using Oligo Analyzer software (https://sg.idtdna.com/calc/analyzer) to assess properties such as self-annealing, hairpin loops, dimers, cross-dimers, and repeats, with efforts made to avoid these

issues. Additionally, the primers were validated using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm specific targeting of the intended gene.

Gene amplification

PCR was conducted using a SimpliAmp thermocycler. Each PCR assay utilized a 20 µL reaction volume consisting of 2 µL of genomic DNA template, 10 pmol of each primer, and PCR master mix. The amplification cycles were performed in a thermocycler optimized for this study (Table 2).

Following amplification, 1 µL of the PCR product was electrophoresed in a 1.5% agarose gel containing 1X TAE buffer at 70 volts for 30 minutes and visualized under UV light using an advanced UV transillumination imaging system. To confirm the size of the amplified products, a 1kbplus DNA ladder was run concurrently as a marker.

Table 2. PCR profile for TLR4 (Medina *et al*., 2019)

PCR Cycles	TLR4	
	Temp (ºC)	Time (m/s)
Initial denaturation	94	2 m
35 Cycles of		
Denaturation	94	30 s
Annealing	54	30 s
Extension	72	45 s
Final extension	72	8 m

Gene sequencing and analysis

The TLR4 DNA products underwent sequencing, and sequences were assembled using MEGA (Version 7.1). Forward and reverse sequences from each primer were aligned to construct contigs of their respective regions. These gene sequences were then compared with the Bubalis bubalis TLR4 sequence to annotate potential SNPs in specific regions.

The DNA sequence was translated into amino acids using MEGA 7 software and compared with the Bubalis bubalis sequence to identify any changes in buffalo regions. The contiguous TLR4 nucleotide sequence was analyzed using Basic Local Alignment Search Tool (BLAST) at the NCBI database to determine homology with corresponding regions in other species. A phylogenetic tree was constructed using the neighbor-joining method, with group confidence estimated through bootstrap analysis with 1000 replications. Genetic distances and phylogenetic trees will be further analyzed using MEGA 7 software.

Polymorphism analysis

The amplified TLR4 PCR products (15 µL) from study 1 underwent endonuclease digestion. Restriction enzymes capable of fragmenting the DNA were selected using Sequence Manipulation Suite: Restriction map. Each enzyme reaction mixture (20 µL) was then incubated at 37°C for 4 hours. The resulting restriction fragments were separated using horizontal electrophoresis on 2-3% agarose gel.

The gene fragments digested by restriction enzymes were visualized using a UV transilluminator (Flour Chem E by ProteinSimple™, USA) and photographed. Variations in fragment lengths produced by different enzymes, indicative of polymorphisms in specific genes, were analyzed and compared in riverine buffalo.

Statistical analysis

A univariate analysis was conducted to investigate the potential association between genotypic frequency and the incidence of subclinical mastitis, utilizing Chi-square test analysis. Odds ratios were calculated to assess the magnitude of this association.

Results

Sequence analysis of TLR4

Five DNA samples extracted from milk were sequenced to amplify the ligand binding region of exon 3 of the TLR4 gene in buffaloes, resulting in a 105 bp TLR4 nucleotide sequence. Comparative analysis using sequences from other ruminants in the NCBI GenBank database revealed an average of 100 identical nucleotide pairs. The TLR4 nucleotide sequence from buffalo samples showed 98% similarity with *Bu. bubalis* (XM_006057215.2), 96% to 97% homology with *Bo. taurus* (XM_005210586.4), and 96% to 97% similarity with *Bo. indicus* (XM_019966825.1) (Table 3). BLASTx analysis indicated an average of 80% similarity in translated amino acid sequences (Table 4).

Phylogenetic analysis of TLR4

The phylogenetic analysis revealed that the TLR4 nucleotide sequence from the buffalo sample (TEB7) clustered closely with *Bu. bubalis* (XM_006057215.2), showing an 87% bootstrap value. It formed a distinct clade

with *O. aries* (DQ922636.1) and *C. hircus* (DQ922635.1) TLR4 sequences (Figure 1). In contrast, it separated from the clade containing *Bo. taurus* (XM_005210586.4) and *Bo. indicus* (XM_019966825.1) TLR4 sequences, which had a 77% bootstrap value. The phylogenetic tree suggested a shared ancestry of the TLR4 gene sequence among the analyzed ruminant species.

Table 3. Nucleotide percentage similarity of TLR4 in ruminant species

Species	Buffalo 4	Nucleotide Buffalo 6	Sequence Buffalo 7	Buffalo 8	Buffalo 9
Bubalus bubalis (XM_006057215.2)	98%	98%	98%	98%	98%
Bos taurus (XM_005210586.4)	97%	96%	96%	97%	97%
Bos indicus (XM_019966825.1)	97%	96%	96%	97%	97%
Ovis aries (DQ922636.1)	98%	95%	95%	98%	95%
Capra hircus (DQ922635.1)	98%	95%	95%	98%	95%

Table 4. Amino acid percentage homology of TLR4 in ruminant species

Species	Buffalo 4	Nucleotide Buffalo 6	Sequence Buffalo 7	Buffalo 8	Buffalo 9
Bubalus bubalis (XM_006057215.2)	81%	81%	81%	76%	81%
Bos taurus (XM_005210586.4)	71%	71%	71%	76%	71%
Bos indicus (XM_019966825.1)	71%	71%	71%	76%	71%
Ovis aries (DQ922636.1)	71%	71%	71%	76%	71%
Capra hircus (DQ922635.1)	71%	71%	71%	76%	71%

Polymorphism analysis of TLR4 gene in buffalos

RFLP analysis conducted on the 105 bp TLR4 gene revealed 8 nucleotide polymorphisms at locations 22, 48, 53, 54, 56, 57, 63, and 73. (Figure 2) Functional polymorphisms were identified at loci 22, 53, 54, 56, and 57. The resulting patterns from *Rsa*I enzyme digestion (patterns A, B, and C) indicated genetic diversity within the buffalo population.

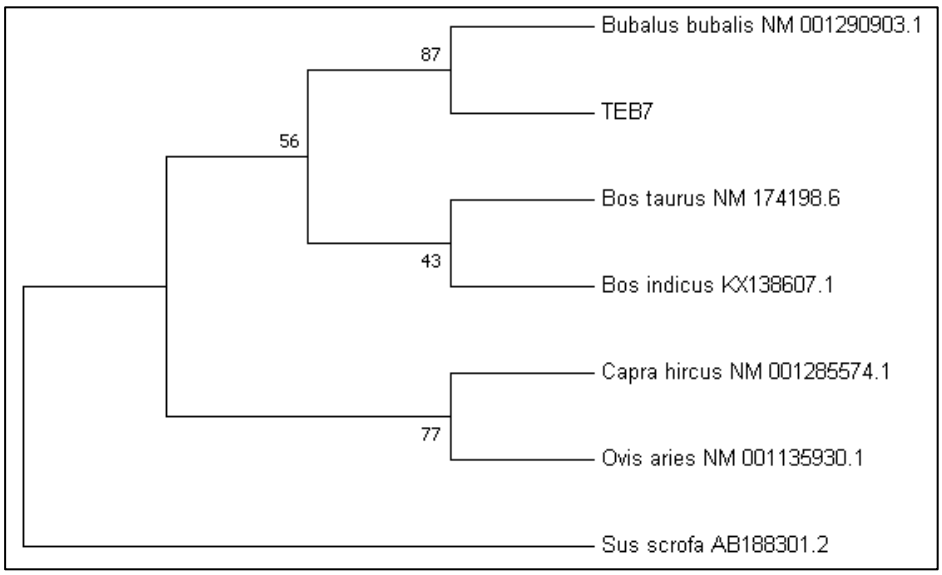

Figure 1. Phylogenetic tree showing relationship between buffalos and other ruminants' TLR4 nucleotide sequence, *Sus scrofa* as an out-clade

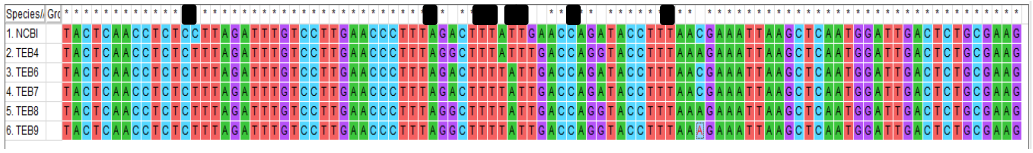

Figure 2. Site of nucleotide polymorphisms. Areas with black shade show the polymorphic loci

Association of TLR4 gene polymorphism in the occurrence of subclinical mastitis in buffalos

The RFLP analysis of TLR4 gene PCR products using *Rsa*I enzyme digestion revealed three distinct patterns (Figure 3). In a sample population of 37 buffaloes, 3 samples displayed a single-band pattern, 4 samples exhibited a two-band pattern, and 30 samples showed a three-band pattern. Genotypic frequencies were analyzed using Chi-square (χ^2) tests, indicating a significant association between the occurrence of subclinical mastitis and TLR4 gene polymorphism in buffaloes. Genotypes A and B were more prevalent among buffaloes testing positive for subclinical mastitis, while genotype C was more common in those testing negative.

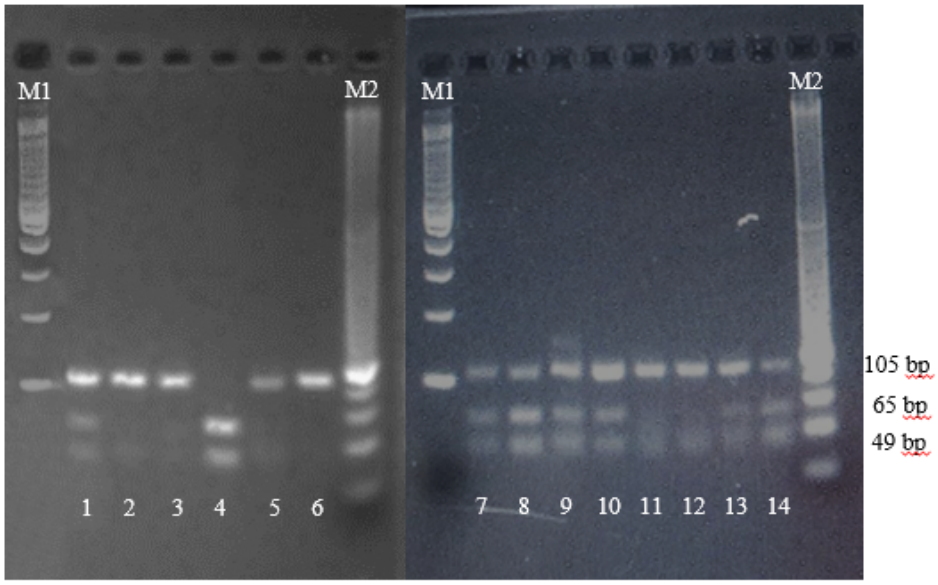

Figure 3. Restriction patterns observed by digestion of TLR4 gene PCR products using *Rsa*I in 2% agarose gel. M1 – 100 bp ladder: M2 – 25 bp ladder; Samples 1, 7, 8, 9, 10, 13, and 14- three bands; Samples 2, 3, 5 and 6 - one band; Sample 4 – two bands

Detection of subclinical mastitis

Using the California Mastitis Test (CMT), 11 buffalo milk samples were identified as positive for subclinical mastitis, whereas 26 samples were classified as negative (Table 5). The lower prevalence of subclinical mastitis was linked to the specific lactation stage.

Table 5. Percentage frequency of CMT scores

CMT Score	n	Percent (%)
N	26	70.27%
T	5	13.51%
1	3	8.10%
2	3	8.10%
3	0	0
Total	37	100%

Association of TLR4 gene polymorphism in occurrence of subclinical mastitis

The Chi-square (χ^2) test assessing the goodness-of-fit for the study and the odds ratio analysis to examine the potential association between genotypic

frequency and the occurrence of subclinical mastitis are presented in Table 6. Genotypes A and B corresponded to the one and two-band patterns, while genotype C corresponded to the three-band pattern in the RFLP using *Rsa*I restriction enzyme. The total frequency of genotypes A and B combined was 6, whereas genotype C occurred 31 times. Numerically, genotypes A and B were more prevalent among buffaloes that tested positive for subclinical mastitis, whereas genotype C was more common among those that tested negative.

Specifically, the occurrence of subclinical mastitis in genotypes A and B was 4, compared to 2 instances of non-occurrence. The odds ratio indicated a significant association with a value of 6.86, suggesting that buffaloes with genotype A or B were 6.86 times more likely to develop subclinical mastitis than those with genotype C. Thus, animals with genotype A or B are considered more susceptible to subclinical mastitis than those with genotype C.

Conversely, among subclinically mastitic buffaloes, genotype C was observed 7 times, compared to 24 occurrences in non-subclinically mastitic buffaloes. Genotype C exhibited an odds ratio value of 0.15 without significant association, indicating that buffaloes with genotype C were 0.15 times less likely to develop subclinical mastitis compared to those with genotype A or B. Therefore, animals with genotype C are considered more resistant to subclinical mastitis than those with genotype A or B.

Table 6. Frequency of *Rsa*I-based genotypes in buffalos

Genotype	Category		Total	Odds Ratio
	SCM (-)	SCM (+)		
A/B	2	4	6	6.86*
C	24	7	31	0.15

*Significant association was found, $p<0.05$

Discussion

The intricate relationship between the TLR4 gene and subclinical mastitis in buffaloes are revealed by our comprehensive study, adds significant depth to understandgenetic factors influencing mastitis susceptibility. It observed genetic diversity in the TLR4 gene among buffalo populations, showcased through sequence analysis and RFLP, and underscored the complexity of the host's immune response mechanism. Such diversity may contribute to variations in disease resistance and resilience within the studied buffalo population (Kenchaiwong *et al*., 2023).

The phylogenetic analysis provided insights into the evolutionary relationships of TLR4 gene sequences among different ruminant species. The clustering of buffalo TLR4 nucleotide sequences with those of *Bubalus bubalis*

and their common ancestral origin suggested a conserved genetic foundation (Mossallam *et al*., 2023). This shared ancestry not only highlights the evolutionary significance of the TLR4 gene but also hints at the potential conservation of certain immune response mechanisms across diverse ruminant species (Chen *et al*., 2024).

The nucleotide polymorphisms were identified particularly those with functional consequences at loci 22, 53, 54, 56, and 57, offer valuable information for understanding the potential phenotypic effects on subclinical mastitis resistance. These polymorphisms may influence the immune response, affecting the severity and frequency of mastitis incidents. The association between specific genotypes (A and B) and a higher incidence of subclinical mastitis opposed to genotype C, further emphasized the importance of genetic factors in mastitis susceptibility.

The application of RFLP using the *Rsa*I enzyme enabled the differentiation of genotypes, revealing distinct patterns (A, B, and C) in the buffalo population. This genetic diversity was evidenced by the varied band patterns which may serve as a foundation for future breeding programs that is aimed to enhance mastitis resistance. The identification of genotypes associated with higher susceptibility or resistance to subclinical mastitis is opened avenues for targeted selective breeding, facilitating the development of more robust and disease-resistant buffalo populations.

The detection of subclinical mastitis through CMT corroborated the genetic findingswith 11 buffalo milk samples classified as positive and 26 as negative. The low prevalence of subclinical mastitis is possibly attributed to the lactation stage, aligned with existing knowledge that somatic cell count tended to increase in late lactation. This further emphasized the dynamic nature of mastitis susceptibility influenced by both genetic and environmental factors (Ebrahimie *et al*., 2021; Cobirka *et al*., 2020).

In the broader context, our study contributes to the ongoing discourse on mastitis resistance in livestock. The comparison with studies by Gulhane and Sangwan (2012) and Wang *et al.* (2007) provides a nuanced perspective on TLR4 gene polymorphisms and their varied associations with mastitis resistance in different species. The inconclusive findings from Haris *et al.* (2016) regarding TLR4 genotypes and somatic cell scores underline the need for further research to unravel the complex genetic landscape governing mastitis susceptibility.

Acknowledgements

The authors would like to thank the DA-BPO for funding this project and the Biosafety and Environment Section of DA-PCC for allowing this research to be conducted in their facility.

References

Alfano, F., Peletto, S., Lucibelli, M. G., Borriello, G., Urciuolo, G., Maniaci, M. G., Desiato, R., Tarantino, M., Barone, A., Pasquali, P., Acutis, P. L. and Galiero, G. (2014). Identification of single nucleotide polymorphisms in toll-like receptor candidate genes associated with tuberculosis infection in water buffalo (*Bubalus bubalis*). BMC genetics, 15:139.

Chen, Y. H., Wu, K. H. and Wu, H. P. (2024). Unraveling the Complexities of Toll-like Receptors: From Molecular Mechanisms to Clinical Applications. International Journal of Molecular Sciience, 25:5037.

Cobirka, M., Tancin, V. and Slama, P. (2020). Epidemiology and Classification of Mastitis. *Animals* (Basel), 10:2212.

Ebrahimie, E., Mohammadi-Dehcheshmeh, M., Laven, R. and Petrovski, K. R. (2021). Rule Discovery in Milk Content towards Mastitis Diagnosis: Dealing with Farm Heterogeneity over Multiple Years through Classification Based on Associations. Animals, 11:1638.

Gulhane, A. B. and Sangwan, M. L. (2011). Polymorphism in TLR4 gene and its association with mastitis in murrah buffaloes. Indian Journal of Biotechnology, 11:330-332.

Harris, R. A, Bajo, M, Bell, R. L, Blednov, Y. A., Varodayan, F. P., Truitt, J. M., de Guglielmo, G., Lasek, A. W., Logrip, M. L., Vendruscolo, L. F., Roberts, A. J., Roberts, E., George, O., Mayfield, J., Billiar, T. R., Hackam, D. J., Mayfield, R. D., Koob, G. F., Roberto, M. and Homanics, G. E. (2017). Genetic and Pharmacologic Manipulation of TLR4 Has Minimal Impact on Ethanol Consumption in Rodents. Journal of Neuroscience. 37:1139-1155. doi: 10.1523/JNEUROSCI.2002-16.2016. Epub 2016 Dec 16. PMID: 27986929; PMCID: PMC5296793.

Jasper, D. E. (1967). Proceedings of National Mastitis Council (adapted). Dairyman, Calif., 04, p. 32.

Kenchaiwong, W., Pongthaisong, P., Kananit, S., Duangjinda, M. and Boonkum, W. (2023) An Analysis of DNA Sequence Polymorphism in the Swamp Buffalo Toll-like Receptor (TLR2) Gene. Animals (Basel), 13:2012.

Kirkpatrick, M. A. and Olson, J. D. (2015). Somatic cell counts at first test: more than a number. In proceedings. National Mastitis Council Annual Meeting, 53-56. Retrieved from http://www.qscoutlab.com/what-is-subclinical-mastitis/

Medina, N. P., De Guia, A. C. M., Venturina, V. M. and Mingala, C. N. (2019). Molecular characterization of MHC II DRB3 gene of swamp- and riverine-type water buffaloes. Journal of Advanced Veterinary and Animal Research. 6:308-314. doi: 10.5455/javar.2019.f349. PMID: 31583227; PMCID: PMC6760514.

Medzhitov, R., Preston-Hurburt, P. and Janeway, Jr. C. A. (1997). A human homologue of the drosophila toll protein signals activation of adaptive immunity.

Mossallam, A., El Nahas, S. M., Osman, N. M., El-Hassan T., Shahwan, E. H. T. and Sabry, N. M. (2023). Sequence analysis of *TLR4* gene in river buffalo (Egyptian breed) and SNPs association with Mastitis. Bulletin of the National Research Center, 47:110.

Sharma, N., Singh, N. K. and Bhadwal, M. S. (2011). Relationship of somatic cell count and mastitis: an overview.

Wang, X., Xu, S., Xue, G., Ren, H. and Chen, J. (2007). Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle Journal of Genetenics and Genomics, 34: 406-412.

(Received: 25 January 2024, Revised: 10 July 2024, Accepted: 12 July 2024)