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BIOCHEMICAL, MICROBIOLOGICAL AND MOLECULAR DETECTION OF MASTITIS IN ASSOCIATION WITH LACTOFERRIN GENE IN WATER BUFFALO (*BUBALUS BUBALIS*)

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Abstract:

The study aimed to identify the incidence of clinical and subclinical mastitis in fresh buffaloes' milk using two different methods, the California Mastitis Test and Somatic Cell Count and its possible relation in detecting lactoferrin gene in water buffaloes milk using polymerase chain reaction method. A total of nine milk samples were collected from different breeds of water buffalo from selected farms in Central Luzon, Philippines. All the samples was underwent California Mastitis Test (CMT) and Somatic Cell Count (SSC) and lastly to Polymerase Chain Reaction (PCR) method to detect the lactoferrin gene present in milk. The study showed that there was a strong relation between California Mastitis Test and Somatic Cell Count in identifying incidence of mastitis in the milk of water buffalo. Although the detection of lactoferrin gene is mostly likely seen in milk positive with mastitis, the study also revealed that lactoferrin gene were present in non mastitic animals.

Keywords: Mastitis, California mastitis test, somatic cell count, lactoferrin gene

INTRODUCTION

Buffaloes *(Bubalus bubalis)* are an integral part of Philippine agriculture. They play an important role in the economy as suppliers of milk and meat and for draft purposes. Farmers utilize buffaloes for land preparation and in transporting farm inputs and products. Aside from meat and milk they provide, buffaloes are tethered in the backyard during the night and in open field during the day which favors direct infection of the animal from the soil, grasses and any contaminated materials in the pastures.

One of the many infections encountered and the most common is mastitis which can be described to be acute or chronic according to duration and severity. In such cases, buffaloes with mastitis exhibit redness of the udder among other clinical signs observed. Other indications, mastitis can be observed in milk which appears to be watery and the presence of flakes and clots.

Genetic make-up of buffaloes is necessary factor in determining its resistance or susceptibility to mastitis one method is to find out the genetic characteristics of water buffaloes specifically the lactoferrin gene. Lactoferrin is an iron binding glycoprotein which plays an essential role in antimicrobial defense and a potential candidate gene including water buffaloes. Lactoferrin (Lf) gene is found in milk and external secretions of the body such as saliva, bile, tears, semen and also in blood.

Due to the importance of the lactoferrin in inhibiting the growth of microbes, studying lactoferrin in dairy animals should be attempted. Advances in doing this attempt through the process of biological science (biotechnology) will give information about the traits of the buffaloes with regards to its lactoferrin gene. Investigation of lactoferrin gene is a tool that can be utilized in selection process. Molecular biotechnology enables selection up to the DNA level, by finding the composition of the nucleotide(s) of the lactoferrin gene suspected of affecting a high level of lactoferrin. Hence, the study aimed to detect subclinical mastitis in water buffaloes' milk using California Mastitis Test (CMT); identify Somatic Cell Count (SSC) of milk using Fosscombi methods of analysis; and detect the lactoferrin gene of different breeds of buffalo.

MATERIALS AND METHODS

Time and place of the study

The study was conducted from July 2017 to December 2017. The collection of blood samples of two different breeds of buffalo was done at Philippine Carabao Center, Central Luzon State University, Science City of Munoz,

Nueva Ecija. Milk samples were submitted for PCR amplification at the Animal Health Laboratory of Phillippine Carabao Center National Headquarters.

Sample collection

A total of nine water buffaloes from three different breeds were sourced out from Philipine Carabao Center wherein 50 ml of milk samples were collected from each of them. The udder and teats were cleaned by brushing dirt, debris or bedding particles. The udder and teats were then dried thoroughly with dry clean cloth. Sterile gloves were worn in collecting milk. Three to four stream of milk were initially removed to minimize chances of sample contamination from bacteria in the teat end. Milk samples were put in conical tube immediately before the samples were taken. Caution was observed by not letting the debris or dirt enter to the container. The cap was kept from touching the floor to prevent contamination. The cap was kept upside down to prevent the inside of the cap from touching so that debris may not contaminate the inside of the cap. The collection vial was held at a 45° angle to keep the debris (hair, manure, dirt) from accidentally falling into the collection vial (conical tube). The teat was never touched the collection vial or cap. Rapid collection of the samples is recommended. Samples were labeled and the collected vial (conical tube) were immediately placed on ice and kept refrigerated until delivered to the laboratory.

California Mastitis Test (CMT)

Foremilk was drawn from each quarter into separate cups of a four-cup plastic paddle. The paddle was tilt to equalize milk quantities in the cups. One to two drops of diluted CMT was added to each cup paddle. After the paddle was rotated to mix the diluted CMT and the milk samples were observed within 20 seconds after mixing.

Table 1. Scoring and interpretation of CMT (California Mastitis Test)

CMT score	Test appearance
Negative	Mixture liquid, no precipitate
T (trace)	Slight precipitate, tends to disappear with paddle movement
1(weak positive)	Distinct precipitate but does not gel with paddle movement
2(distinct positive)	Distinct gel formation
3 (strong positive)	Strong gel formation that tends to adhere to paddle. Forms distinct central peak

Source: *McCurnin's Clinical Textbook for Veterinary Technicians*

Somatic cell count analysis

The raw milk samples from the water buffalo with CMT scores of 1 or higher were subjected for somatic cell count determination using Fosscombi methods of analysis of PCC, Dairy Laboratory.

DNA extraction

Milk samples (50ml) were collected in each different breeds of water buffalo and were placed in a conical tube and centrifuged for about 20 minutes under 6000rpm (rate per minute) .Genomic DNA was extracted from milk of water buffaloes according to the procedure used by the PCC. DNA extraction was initially processed in a way that 1000 µl milk sample was placed in a 1.5 ml MCT (Micro Centrifuge Tube), added by 1000 µl ammonium chloride (NH4Cl) then centrifuged at a speed of 14000 rpm for 1 minute. Formed supernatant was discarded, until white pellet was obtained in two repetitions.1000µl cell lysis was added and centrifuged for 14000 rpm for a minute. Supernatant was discarded by pipetting. 300µl Nuclei lysis solution was again added. Then addition of 100 µl protein precipitate until homogenized. New 1.5 MCT with 500 µl isopropanol was again prepared and clear liquid from the MCT was transferred and mix by inversion. Then centrifuged at the speed of 14000 rpm for 1 minute. 500 µl 70% ethyl alcohol (ETOH) was added after supernatant was discarded and centrifuged for 1 minute with same speed of 14000 rpm. Air dried for 30 minute then rehydrated by DRS (DNA Rehydration Solution) of 30 µl. It is incubated for 10 minutes at 65 °C and store at 4 °C.

Polymerase Chain Reaction Amplification

Primers were designed for the amplification of complete CDS of buffalo lactoferrin gene using Primer3 online software based on the reported bovine lactoferrin gene sequence (Accession No.NM_180998). Full-length buffalo lactoferrin gene PCR product was generated using the cycles: initial denaturation at 95°C for 5 minutes in 40 cycles, and final denaturation of 95 °C for 30 seconds, Annealing for PCR product was at 57 °C, initial elongation at 72 °C for 45 sec followed by final elongation at 72°C for 7 minutes. Reagent for the DNA amplification was generally performed using a DNA mixture consisting performed in 110.4 µl total volume and contained 54µl sdH20, 24 µl of buffer, 9.6 µl of dNTPs,and 2.4 µl of primer 1 forward 5′ CTTGGAGCCCTTGGACTGT -3′ and reverse 5′ CCAACCTGTGTCAACTGTG -3′, primer 5 forward 5′-ATTGTGTGCTGGCGATGACC -3' and reverse 5'-GAGCATTTTTTCAGGTTGGCA -3' one unit of Taq DNA polymerase in 12x reaction.

PCR reaction

Promoter regions from a portion of the first repetitive domain of the buffalo Lactoferrin gene were amplified by PCR. PCR was carried out in a final reaction volume of 110.4 µl a master mix for minimum of 9 samples was prepared and was aliquoted to 9.2 µl in each PCR tube. 1.0µl genomic DNA (100 ng) was added in each tube to make a final volume of 10.2.

Table 2. Components used for its reaction volume

PCR components	Volume (µl)	Final concentration
Sodium hydroxide	4.5 µl x 12	54µl
Buffer	2.0 µl x 12	24 µl
Magnesium chloride	0 .80 µl x 12	9.6µl
dNTPs	0 .50 µl x 12	6.0 µl
Forward	0 .20 µl x 12	2.4 µl
Reverse	0 .20 µl x 12	2.4 µl
Taq DNA polymerase enzyme	1.0 µl x12	12 µl
Total	9.2 µl	110.4 µl (master mix)

PCR protocol

The PCR protocol followed for the amplification of desired fragment of DNA is shown in Table 3. All the reactions were carried out in 0.2 ml thin walled PCR tubes. PCR tubes containing mixture were tapped gently and quickly spinned at 10,000 rpm for few seconds. The tubes were placed in a Master cycler gradient (Bio-Rad, USA) and subjected to PCR. One reaction tube without DNA was maintained as negative control.

Table 3. The total running time for PCR amplification is .1hour and 45 minutes for the whole 40 cycles.

Step	Temperature	Time
Initial denaturation	95°C	5 minutes
Denaturation	95°C	30 seconds
Annealing	57°C	30 seconds
Extension	72°C	45 seconds
Final extension	72°C	7 minutes

Agarose gel electrophoresis

Electrophoresis were carried out by using 4 µl PCR product on 1.5% agarose gel at 100 volts for 30 minutes. Gel were prepared by heating 1 g agarose, was dissolved in 100 ml 0.5 XTAE (tris acetate EDTA) solution, then added by 2 µl Gel Red during steering process until a clear solution resulted. Liquid solution was poured into a printer and a comb was placed near the gel edge. The gel was hardened. After that, by removing the comb, 4 µl PCR product was formed in the wells of the gel .The gel was into the electrophoresis gel tray already containing a buffer solution, and then electricity was passed through. The DNA molecules with a negative charge, at neutral pH. The gel electrophoresis was completed and the agarose gel was taken to determine the length(s) of the DNA band(s) gel was visualized under the UV trans-illuminator and photographed using gel documentation system.

RESULTS AND DISCUSSION

Nine samples of milk from different breeds of water buffaloes were subjected to California Mastitis Test (CMT). The changes in color that resulted from the test were generally more intense in sample than that of the infected ones. When mixture remains liquid, it showed negative result and a purple mixture. When there is a gel formation, it displayed positive and the mixture appears deep purple.

Table 4. Results for CMT (California Mastitis Test)

SAMPLES	CMT SCORE
BR-1	+ (T) trace
BR-2	+ (1) positive, grade 1 level for California Mastitis Test
BR-3	+ (2) positive, grade 2 level for California Mastitis Test
IT-1	(-) negative
IT-2	(-) negative
IT-3	+ (T) trace
CB-1	(-) negative
CB-2	(-) negative
CB-3	(-) negative

The results in the detection of subclinical mastitis using CMT in different breeds of water buffalo was shown in table 1. IT-3 and BR-1 exhibited trace level of mastitis wherein, slight precipitate tends to disappear with paddle movement. BR-2 showed grade (1) subclinical mastitis means that there was a distinct precipitate but does not gel with paddle movement also BR-3 showed grade (2) subclinical mastitis means that there is a distinct gel formation on the milk samples. The rest of the samples showed negative results in California Mastitis Test.

Subclinical mastitis is an infection of the mammary gland of the cow, which occurs without obvious clinical signs such as abnormal milk, udder swelling or tenderness, or systemic signs such as fever (Leigh, 1999). In addition, subclinical mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils, into the mammary gland. The migration of more immune cells into the mammary gland causes deterioration of the blood-milk barrier and mammary epithelium damage, and subsequently leads to death of epithelial cells

Table 2 showed the result for somatic cell count in nine different breeds of water buffalo. All milk samples from Bulgarian showed positive result for subclinical mastitis because of its high level of somatic cell than normal count. One sample from Italian breeds (No. 2014929) showed positive result for subclinical mastitis and all samples from crossbreed have no significant value for subclinical mastitis. Somatic cells are normal constituent of milk and only when they become excessive do they indicate a problem.

Table 5. Result and interpretation for somatic cell counts in water buffaloes.

Samples	A (Front Left Teat) Cells/ml	B (Front Right Teat) Cells/ml	C (Back Left Teat) Cells/ml	D (Back Right Teat) Cells/ml
BR1	13,000	32,000	135,000	2,752,000
BR2	488,000	15,000	70,000	67,000
BR3	50,000	26,000	23,000	726,000
IT1	49,000	44,000	85,000	26,000
IT2	27,000	35,000	36,000	36,000
IT3	25,000	17,000	29,000	345,000
CB1	28,000	30,000	20,000	41,000
CB2	21,000	21,000	16,000	13,000
CB3	34,000	35,000	39,000	58,000

Somatic cells are composed of leucocytes (75 %) and epithelial cells (25 %). Leucocytes (white blood cells) increase in milk in response to infection or injury while increase in epithelial cells is the result of infection or injury. The number of cells reflects the severity of mastitis. Somatic cells are expressed either as cells/ml of milk SCC.

According to Tripaldi *et al.* (2010) that in buffalo as in other species, total SCC is a valid indicator of udder inflammation and a value of 200,000 cells/mL should be used as the threshold value for early identification of an animal affected by subclinical mastitis. Somatic cell count (SCC) in milk constitutes a good diagnostic tool that allows early detection of either subclinical or acute form of mastitis (Green *et al.* 2004; de Haas *et al.* 2004).

DETECTION OF LACTOFERRIN GENE THROUGH POLYMERASE CHAIN REACTION

The detection of lactoferrin gene in milk of nine different breeds of water buffalo were likewise subjected into polymerase chain reaction. Lactoferrin gene was visualized under the UV trans-illuminator and photographed using gel documentation system.

Figure 1. Gel Electrophoresis of PCR Products using Primer 1 and 5

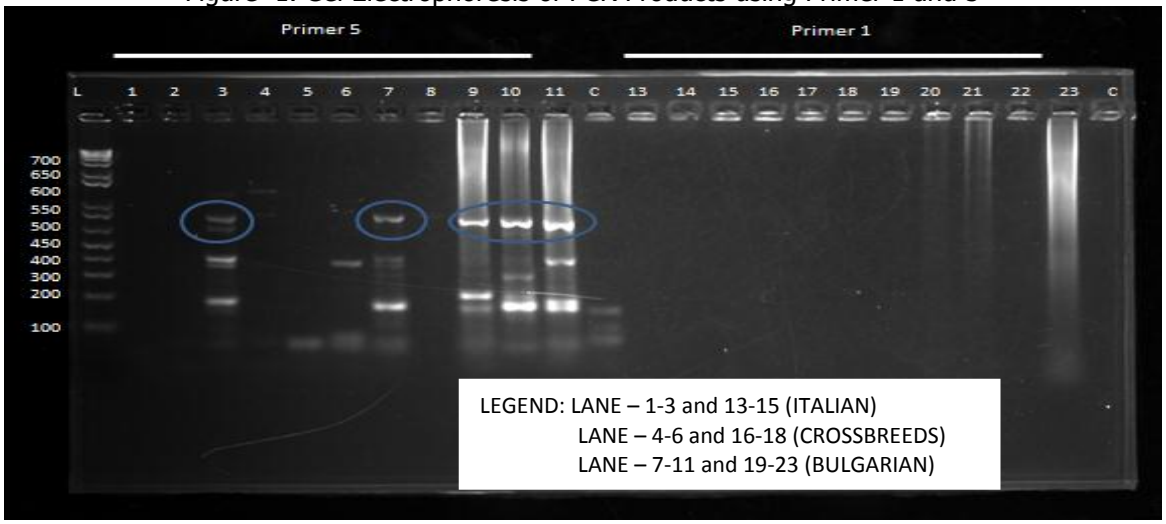

Figure 1 shows the gel electrophoresis of PCR product whereas lane 1-9 are PCR product of milk comes from different breeds of water buffaloes including another 2 (10-11) lane for PCR product of blood and a negative control used primer 5 forward 5'-ATTGTGTGCTGGCGATGACC -3' and reverse 5'-GAGCATTTTTTCAGGTTGGCA -3' while lane 13-24 are PCR product for primer 1.forward 5' CTTGGAGCCCTTGGACTGT -3' and reverse 5' CCAACCTGTGTCAACTGTG -3',. Result showed that primer 5 with lane number (3, 7, 9,) PCR product of milk of buffalo positively amplified lactoferrin gene while lane (10, 11) PCR product of blood fully expressed bonded which indicative of positively detected lactoferrin genes of buffalo with 500-550 base pair, on the other hand figures also showed that there is No expression of lactoferrin gene in primer 1. Hence, the results also showed expression of non- specific gene having 200 up to 400 base pair. Since Gene Blasting is not part of the conducted study the detected gene with 200-400bp are still unidentified.

Results of the three methods conducted in the whole study. In whereas 3 samples (BR-1, BR-3 and IT-3) exhibited positive in all methods conducted. Lactoferrin may have therapeutic potential in mastitis (Diarra *et al.,* 2002a). it may substitute the practice of using antibiotic which root problems due to residues in milk and the danger for occurrence of resistance. Lactoferrin is a constituent of the natural defense system of humans and animals. LTF is present in milk and such external secretion of the body as saliva, bile, tears and sperm. It is released by secondary granules of neutrophils and epithelial cells in high concentration in reaction to inflammatory stimuli (Baggiolini *et al.,* 1970; Harmon and Newland, 1980; Brock, 1995; Lonnerdal and Iyer, 1995; Nuijens *et al.,* 1996; Aguila and Brock, 2001; Plaffi *et al.,* 2003).

SUMMARY AND CONCLUSION

The study aimed to detect incidence of subclinical mastitis among different breeds of water buffalo and it was identified through different methods. California Mastitis Test (CMT), Somatic Cell Count (SCC) and Polymerase Chain Reaction (PCR). A total of nine different breeds of water buffalo were used (Bulgarian, Italian and crossbreed) in the study. Milk samples were collected from selected water buffaloes and were subjected to different test to detect possible subclinical mastitis, such as CMT and SCC. The results for the two methods were correlated by the detection and presence of lactoferrin gene in milk of water buffalo using Polymerase chain reaction (PCR). The results revealed that the number of positive result in subclinical mastitis using CMT were the same number of positive result in somatic cell count and can be associated for the detection of lactoferrin gene. From nine milk samples, four samples showed positive results on both CMT and SCC and PCR detected lactoferrin gene on 4 samples.

The study concluded that there was a correlation between CMT and SCC in identifying incidence of subclinical mastitis and also the study proved that lactoferrin gene were also present in mi of water buffaloes with or without subclinical mastitis.

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