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LOW-COST LIQUID ANIONIC SURFACTANTS IN MASTITIS DETECTION FOR SMALL-HOLD DAIRY BUFFALO FARMERS

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Abstract

The study was conducted to evaluate the effect, consistency, as well as the economic viability of using liquid anionic surfactants (LAS) in mastitis detection in order to provide cleaner milk. A total of forty two (42) milk samples from lactating dairy buffaloes at two (2) different farm locations were used in the study. The experiment was laid out following the Completely Randomized Design with three replications.

Statistical findings showed that CMT and Liquid anionic surfactant with 30 percent to 50 percent dilution level have more or less similar Mastitis detection capability. Based on the economic viability, preparation of liquid anionic surfactants with 30% dilution level is cheaper in terms of cost per sample and cost of producing 1 gallon of test solution when compared to CMT reagent. The result of the laboratory analysis for somatic cell counting also confirmed that Liquid anionic surfactants from 30% to 50% dilution level registered very close results to CMT in detecting types and grades of mastitis infection from dairy buffaloes. Conclusively, 30 % dilution rate can substitute to CMT with regards of mastitis detection for easier and accessible use of the reagent in a shortest possible time at a low-cost.

Keywords: *CMT, liquid anionic surfactant, low-cost, lactating buffalo, mastitis*

Introduction

Mastitis is the most prevalent disease afflicting dairy animals and destroys the milk quality. The teats' exposure to infectious bacterial agents such as *Staphylococcus aureus, Streptococcus agalactiae*, *Streptococcus uberis*; Coliform bacteria: *Escherichia coli, Klebsiella, Aerobacter aerogens*, *Pseudomonas aeruginosa, Coryne bacterium pyogene;* and Mycoplasma organisms frequently leads to infection.

The CMT or California Mastitis Test, the WMT Wisconsin Mastitis Test, Modified Whiteside Test, Somatic Cell Count or SCC and other laboratory methods of mastitis detection are very effective tests but are not readily available in the market. It also requires technical expertise and not affordable to ordinary farmers. Hence, the study focus on developing a low-cost mastitis test which does not require expensive solutions and very practical in detecting mastitis infection at the shortest possible time

The study was conducted to evaluate the effect, consistency, and the economic viability of different levels of liquid anionic surfactants in mastitis detection. A total of 42 milk samples from 11 lactating water buffaloes regardless of age and weight were used. Collection of milk sample for laboratory analysis was done before milk testing using the Calfornia Mastitis Test (CMT) and liquid anionic surfactants (LAS).

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Methodology

Milk testing in different farm locations and milking parlor infrastructure was done early in the morning and late in the afternoon in accordance with the protocol of the dairy laboratory. About 30 ml of milk was drawn from four quarters of the udder into the centrifuge tube. The tubes were labeled with the animal number and teat location and immediately store in the ice chest. The animal number, calving/kidding date, milk yield per day, and the result of CMT and LAS test for each quarter using Mastitis chart were recorded in the sheet provided by the Philippine Carabao Center Dairy laboratory.

Milk samples collected from each experimental animal was submitted to the Philippine Carabao Center Dairy Laboratory- Animal Breeding and Genomics Section for Somatic Cell Count Analysis using Cytometry. The experiment was laid out following the Completely Randomized Design with three replications.

Results and Discussion

Table 1 showed that the use of liquid anionic surfactant at different inclusion levels (30%-50%) is very helpful in detecting sub clinical and clinical mastitis in dairy buffaloes and can be substitute to CMT.

The result of the laboratory analysis for somatic cell count conducted by the Philippine Carabao Center Dairy Laboratory confirmed the tabulated results gathered during the macroscopic evaluation. As per results of the Somatic Cell Counting, different levels of liquid anionic surfactant register very close results to CMT in terms of detecting types and grades of mastitis infection. By justification, the anionic surface-acting agent akyl anyl sulfonate, is an active ingredient that can be found in surfactants and in CMT reagent have the same action in the deoxyribonucleic acid (DNA) of the cell. Both Liquid Anionic Surfactant and California Mastitis Test (CMT) reagent dissolves or disrupts the outer cell wall and the nuclear cell wall of any leukocyte, which are primarily fat (detergent dissolves fat). DNA is released from the nuclei. The DNA will get together to form a stringy mass. As the number of leukocytes increase, the amount of gel formation will increase in a linear fashion (CMT, 2011).

Based on the economic viability, 30% liquid anionic surfactant solution is cheaper in terms of cost of preparation and it is more readily available in the market nationwide compared to California Mastitis Test reagent.

Conclusion

The results concluded that mastitis detection using liquid anionic surfactant is an easy, cheap and a helpful tool in controlling the spread of mastitis by early detection and treatment of the disease. Preparation of the solution is very simple, the component of the mixtures are readily available and affordable. The use of the solution (liquid anionic surfactant) will prevent waste of milk through early detection of the infection that will lead to increased milk production especially among small hold and commercial scale of dairy industries.

Table 1. Test results of the macroscopic evaluation and economic viability using CMT and different dilution levels of liquid anionic surfactant.

Dilution levels	Negative	Trace	Mastitic Grade 1	Mastitic Grade 2	Mastitic Grade 3	Total	Cost per sample (PhP)
T1 – California Mastitis Test Reagent (Control)	34	2	4	1	1	42	1.96
T2 – 30% liquid anionic surfactant + 70% distilled water	34	3	3	1	1	42	0.70
T3 - 40% liquid anionic surfactant + 60% distilled water	31	5	4	1	1	42	0.91
T4 - 50% liquid anionic surfactant + 50% distilled water	32	4	4	1	1	42	1.05

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An Overview of Smart Farming Production Technology for the Advancement of Home-grown Farmers in the Philippines

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ABSTRACT

This article explores the technologies that can be used to establish smart farming in the Philippines, as well as the various smart systems that have been used to aid home-grown farmers. The emergence of smart agriculture and farming is a method that heavily integrates digital technology in order to increase food production while minimizing input costs. The importance of this technology has a significant effect on farmers and investors as a result of technological advancements. It should also be recognized that numerous promotions requiring government funding for the establishment of smart farming technology in the Philippines has been addressed.

Keywords: Smart Farming; Hydroponics; Aquaponics; Aeroponics

INTRODUCTION

In the Philippines, almost half of the population lives in rural areas and relies on agriculture for a living; among them are indigenous people, landless farmers, and fishermen [1]. In general, farmers on different islands in the Philippines operate independently using conventional methods, and their management of farm produce to end-users is facilitated at low prices by middlemen. Micro-propagation protocols for bananas, coconuts, legumes, and oilseed crops are well known [2].

In the first quarter of 2021, the value of agricultural output fell by -3.3 percent at constant 2018 rates. This was attributed to a decrease in livestock and poultry demand. Crops and fisheries, on the other hand, also increased productivity [3]. Despite this condition, the Philippines is working to modernize and improve its agriculture industry, with both the government and private firms encouraging the use of advanced technologies and smart farming practices to raise harvests and reduce losses [4].

Agriculture's creation was a watershed moment in human history. The willingness of fully modern humans to change the atmosphere to produce enough food to support population growth is the first major improvement in the relationship between fully modern individuals and society. Agriculture ushered in a slew of new developments, ranging from the use of fire and cooked food to self-driving machinery [5].

Hence, smart farming is seen as the agricultural future because it produces higher quality crops by making farms more intelligent in sensing their controlling parameters [6].

SIGNIFICANCE OF SMART FARMING TECHNOLOGY

Agriculture routinely uses sophisticated technologies such as robots, temperature and moisture sensors, aerial images, and GPS technology. These cutting-edge devices, precision agriculture, and robotic systems enable businesses to be more profitable, efficient, safe, and environmentally friendly [7].

Thus, technology is critical to the development of the farming industry and the improvement of agribusiness. Researchers have successfully grown crops in deserts and other harsh environments using genetic engineering, which involves inserting traits into established genes in order to produce pest-resistant, drought-resistant, and plant pathogen-resistant crops.

Moreover, this technology will enhance insect or pest resistance, herbicide or drought tolerance, and disease resistance, providing farmers with a new tool for increasing crop yield. Farmers have used plant breeding and selection techniques to increase crop yield with the assistance of researchers. Technology is also used to protect crops by tracking growth and detecting plant diseases. Without the physical involvement of farmers, automation allows for the consistent distribution of fertilizers, pesticides, and water throughout fields [8].

Lastly, innovative agriculture ensures that new farming and agricultural development models emerge, introducing innovative techniques on how food is produced and distributed. These methods allow more economies and regions to keep up with changing trends and meet the demands of modern living while ensuring sustainably grown food. [9].

SMART FARMING TECHNOLOGY

Hydroponics Farming

Hydroponic farming is a method of growing plants in water without soil using mineral nutrient solutions. The hydroponic gardener controls the nutrient content of the liquid solution used to water the plants [10].

Common Types of Hydroponics System

1. Nutrient Film Technique (NFT)

A method of cultivating plants in which plant roots grow in shallow and circulating hydroponic nutrient layers, allowing plants to receive adequate water, nutrients, and oxygen. Plants grow in layers of polyethylene, with plant roots immersed in nutrient-rich water that is constantly pumped by a pump [11].

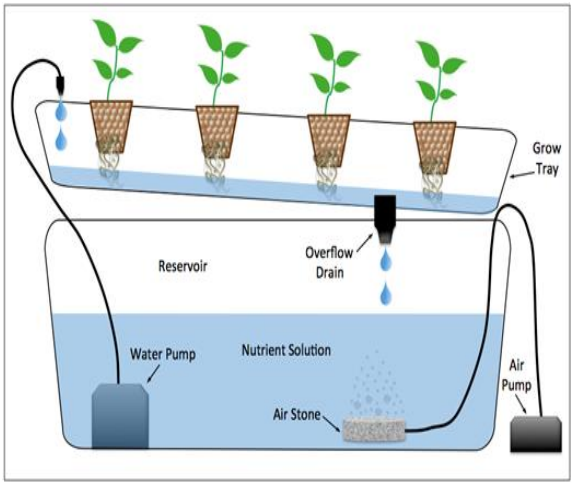

Figure 1. Diagram of the Nutrient Film Technique (NFT) hydroponic system [12]

2. Wick Systems

It is considered the most basic hydroponic device. The Wick system is classified as a passive system, which means it has no moving parts. Your unique Growth Technology nutrient solution is drawn up into the expanding medium through a number of wicks from the bottom reservoir. This device will work with a number of mediums, including perlite, soil, and coco [13].

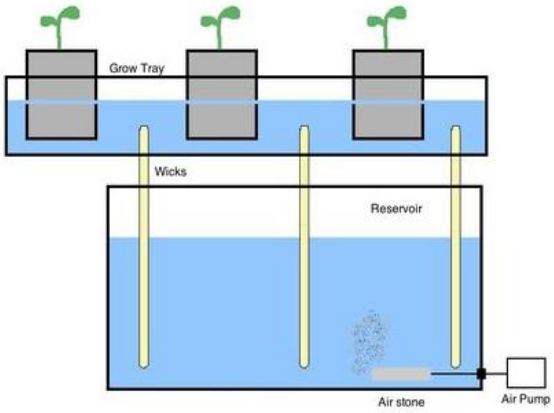

Figure 2. Diagram of the Wick System [14]

3. Deep Water Culture (DWC)

It is a hydroponic method of plant production by suspending the roots of the plant in a solution of oxygenated, rich in nutrients. This system uses rectangular tanks of less than one foot deep filled with a nutrient-rich solution and plants floating on

Styrofoam panels, also known as Deep Flow Technique (DFT), Floating Raft Technology (FRT), or Raceway [15].

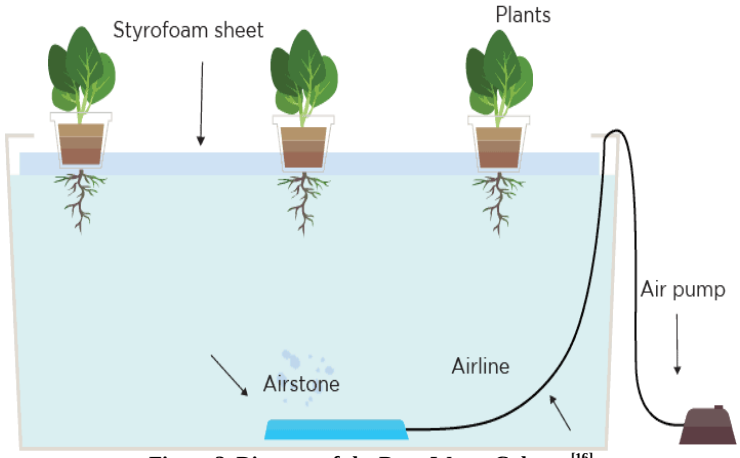

Figure 3. Diagram of the Deep Water Culture [16]

4. Ebb and Flow (Flood and Drain)

It is a hydroponics technique that involves flooding the growth media with nutrient solution for a set period of time, after which the unabsorbed nutrient is returned to the tank. Normally, this hydroponics device uses a timer to fill the water, resulting in inefficient usage of nutrient solution [17].

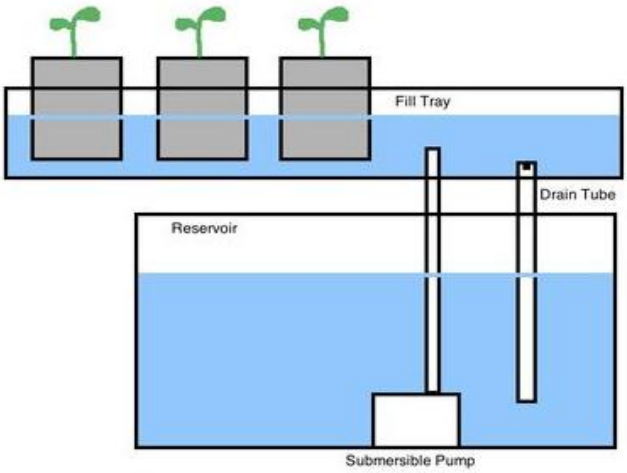

Figure 4. Diagram of the Ebb and Flow [18]

Aquaponics Farming

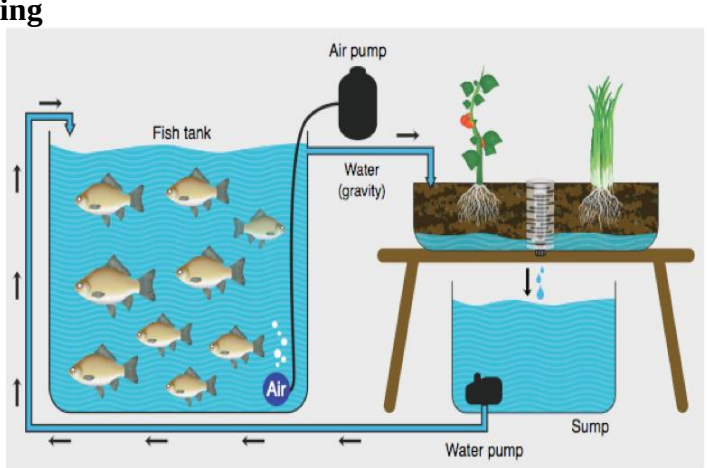

Figure. 5. Diagram of the Aquaponics [19]

In an aquaponics system, water from an aquaculture system is fed into a hydroponic system where by-products' are broken down by nitrifying bacteria first into nitrites and then into nitrates, which are used as nutrients by the plants [19]. A symbiotic relationship between two food production disciplines: (1) aquaculture, the farming of aquatic species, and (2) hydroponics, the cultivation of plants in water without soil. Aquaponics is a closed recirculating device that incorporates the two. A typical recirculating aquaculture system filters and eliminates organic matter ("waste") that accumulates in the water, ensuring that the water is safe for the fish [20].

Aeroponics Farming

In Aeroponics, the nutrient solution is sprayed onto the roots by moving it through misters inside the root region, either continuously or several times per hour [21].

The plant you want to develop is suspended in an air space with an atmosphere that is either completely closed or semi-closed. As a result, it is best achieved in a closed, regulated environment where you can monitor the amount of light, air, and nutrient-rich water spray that is fed into the plant [22].

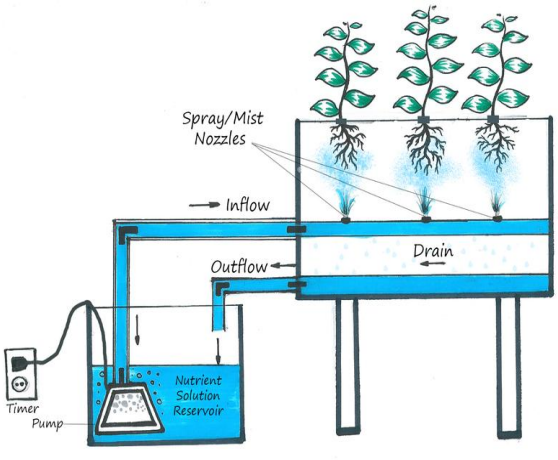

Figure 6. Diagram of Aeroponics [22]

GOVERNMENT SUPPORT TO SMART FARMING

In the Philippines, the local government, led by the Department of Agriculture, is aiming for a 2.5 percent growth this year through further incorporation of technology in agriculture to increase productivity, connectivity, and service delivery to beneficiaries. By focusing on and closely implementing 'Agriculture 4.0,' or the fourth agricultural revolution that encourages the use of smart farming technology, the country would have a better chance of having a better 2021 in terms of agriculture [23].

Agriculture Secretary William Dar released a memorandum to all DA executives, attached agencies and companies, services, and regional offices directing them to "pursue an inclusive approach on these main strategies to accelerate the transition into a new and industrialized Philippine agriculture." [24].

Another agency distinguished in its Labor Market Intelligence report "Soils to Satellites," the Technical Education and Skills Development Authority (TESDA) has been published covering practical topics such as automation in smart greenhouses, agricultural drones, IoT solutions to agricultural problems, and case studies in selected ASEAN countries in smart agriculture applications [25].

CONCLUSION

Some technologies will need to be developed specifically for agriculture, while other technologies already developed for other areas could be adapted to the modern agricultural domain such as autonomous vehicles, artificial intelligence and machine vision and smart farming.

Moreover, as farming in the Philippines faces several problems, proactive solutions like ICT must be implemented together with the full support of the government. Similarly, other major players, such as multinational companies, agricultural and fisheries industry leaders and organizations, and agricultural state

universities and colleges (SUCs), should work together to elevate home-grown farmers in the country.

Lastly, if modern agriculture is applied widely in the near future, millions of farmers will be able to benefit from the acquisition and development of smart farming production technology.

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Mastitis Detection in Holstein Sahiwal Crossbred Cattle (*Bos taurus*) Using Different Brands and Dilution Levels of Liquid Anionic Surfactants

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Abstract. The study was conducted to evaluate the effect, consistency, and economic viability of using different brands and dilution levels of liquid anionic surfactants to detect Mastitis in dairy cattle. Fifty-five milk samples were collected and subjected to macroscopic evaluation at a cattle farm located in Sta. Maria, Bulacan, Philippines. Milk samples were brought to the Philippine Carabao Center National Headquarters and Genepool Science City of Muñoz, Nueva Ecija for Somatic Cell Count. The experiment used 10 treatments with 3 replications arranged in a Completely Randomized Design. The macroscopic evaluation results showed that liquid anionic surfactants at 30%-50% dilution levels could detect Mastitis in dairy cattle. This was validated by the somatic cell count results from PCC Dairy Laboratory. On the other hand, statistical analysis results revealed that the treatments and the control (commercial mastitis test) were comparable except for brand Z at a 40% dilution rate. Brand Z at 30% and 50% dilution rate were found to be consistent among other treatments in detecting Mastitis in dairy cattle. In terms of economic viability, brand X and brand Y at 30% were the cheapest and had the lowest production cost and highest percentage savings.

Keywords: liquid anionic surfactant, mastitis, milk sample, dairy cattle, somatic cell count

Introduction

Milk is considered a complete food being secreted by female mammals to nourish their young. Several problems can occur in this gland, and the most common is mastitis. Mastitis is a common disease in mammals including dairy livestock industry, which is very detrimental since it decreases the quantity and quality of milk production (Syamsi et al., 2019).

Mastitis is a prevalent disease that affect the mammary gland causing inflammation that is painful to dairy animals and reduces milk quality. Poor management practices or environmental micro-organisms cause the disease. The dairy industry paid more attention to subclinical and clinical Mastitis because of its negative effect on the animal and dairy farms' economic status worldwide. Through early detection of Mastitis, treatments can be employed immediately to reduce the infection and the transmission to other cows.

Although commercial mastitis tests are available, their use in the Philippines are limited. These test kits are expensive and not accessible to some dairy farmers. Moreover, local farmers are not trained of conducting mastitis test for milk. It is necessary to find a material that has the same effect but is able to accommodate the ease of application in farmers.

Given that liquid anionic surfactant does not require expensive solutions, instruments, and technical laboratory skills, it is also accessible; hence, it is efficient to detect mastitis infection in the shortest possible time. Despite improvements made in animal production and health, particularly in prevention by early detection of contagious disease and therapeutic medicine, this study was conducted to determine the efficacy of liquid anionic surfactant as a reagent for mastitis test.

Materials and Methods

Scope and Delimitation of the Study

This study was conducted from January to May 2020. Milk samples were collected at a dairy cattle farm located in Sta. Maria, Bulacan. Milk samples were brought to the Philippine Carabao Center National Genepool Headquarters, Science City of Muñoz, Nueva Ecija for Somatic Cell Count. The study was limited to mastitis detection in dairy cattle using different brands and dilution levels of liquid anionic surfactants.

Experimental Design

A total of 55 milk samples from dairy cattle were used in this study. The experiment was laid out following the Completely Randomized Design (CRD). Treatment with a corresponding dilution level was replicated three (3) times. The 3-day sample collection represented the three (3) replications.

There were ten treatments used in the study as shown in Table 1.

Table 1. Brands and dilution levels of liquid anionic surfactants used in the study

Treatments	Brands and dilution levels of liquid anionic surfactants
1	commercial mastitis test (Control)
2	30% Brand X + 70% Distilled water
3	40% Brand X + 60% Distilled water
4	50% Brand X + 50% Distilled water
5	30% Brand Y + 70% Distilled water
6	40% Brand Y + 60% Distilled water
7	50% Brand Y + 50% Distilled water
8	30% Brand Z + 70% Distilled water
9	40% Brand Z + 60% Distilled water
10	50% Brand Z + 50% Distilled water

Preparation of the Test Solution

All the necessary equipment used were thoroughly cleaned, disinfected, and air-dried to prevent contamination of milk samples.

Commercial Mastitis Test and Liquid Anionic Surfactant Test Solution Preparation

On the preparation of the Commercial Mastitis Test Solution, the diluting distilled water was first tested by using 1-2 drops of reagent concentrate. The second step was mixing 10% of reagent and 90% water and was stirred gently in a beaker to avoid bubbles. Lastly, the mixture was transferred in the empty bottles and appropriately labeled.

Liquid Anionic Surfactant Test Solution preparation was similar to that of the commercial solution. To make a 500 ml test solution, different percentage of brands of liquid anionic surfactants and distilled water was computed to the desired volume of the test solution. The distilled water's computed amount volume was measured for the different dilution levels using a beaker, then measured the computed amount volume of liquid anionic surfactant and added into the prepared water solution. The mixture was stirred gently to avoid the excessive formation of bubbles. Lastly, the solution was carefully transferred into clean empty container bottles and labeled. The same procedure was employed following the treatments specified in the experiment.

Mastitis Testing Using Different Test Solutions

Mastitis testing on commercial reagent and Liquid Anionic Surfactant had the same procedure. It was done strictly using the following procedures. First, raw milk was collected and placed in a bottle. Five ml of milk from each quarter was placed into the commercial reagent paddle with four compartments labeled as A, B, C, and D. Second, an equal amount of commercial reagent and LAS solution with different brands and dilution levels was added to each quarter in the paddle. Then, the paddle was rotated in a circular motion to mix the milk and the solution thoroughly. Visible reaction disintegrated after about 20 seconds. The reaction was scored visually according to the gel formation: the more gel formation, the higher the score. Results were recorded accurately per animal and per teat.

Collection of Milk Samples for Somatic Cell Count

Before milk collection, 60 pieces of the centrifuge tube, permanent marker, ball pen, record book, and ice chest were prepared. The procedure of collection was done following the protocol of the dairy laboratory. Strictly 50 ml of milk was drawn from four quarters of the udder into the centrifuge tube. The tubes were labeled with the cow number and teat location and immediately store in the ice chest. The cow number, calving date, milk yield per day, and the result of the commercial reagent test for each quarter were recorded in the sheet provided by the Philippine Carabao Center dairy laboratory.

Laboratory Analysis

Milk samples were collected from the four quarters of the udder from each experimental animal and subjected to macroscopic evaluation.

Table 2. Guide to macroscopic evaluation

No	Result Symbol	Meaning	Description	Remarks
1	-	Negative	Free from the gelatinous formation	Negative of Mastitis
2	-+	Trace	Slight thickening of the mixture. Trace reaction seems to disappear with a continuous rotation of the paddle. If all four quarters read trace, there is no infection. If one or two quarters read trace, infections are possible.	Possible Mastitis Infection
3	+	Weak Positive	A distinct precipitate formed, and milk become slightly viscous, but no tendency of gel formation	Sub-clinical Infection (Grade 1 Mastitis)
4	++	Distinct Positive	The mixture thickens immediately with some gelatinous formation formed.	Serious Mastitis Infection (Grade 2 Mastitis)
5	+++	Strong Positive	A gelatinous formation will be formed and causes the mixture's surface to become convex, and a thick mass is formed in the middle of the paddle while doing swirling and titling. When the mixture is poured out, the whole mass formation will drop without leaving any remains or if there is a presence of little sticky mixture will be left but will drop also	Serious Mastitis Infection (Grade 3 Mastitis)

Note: Formation of gel involves the binding of leukocytes due to the anionic of the liquid antibacterial detergent on the milk. Gelatinous formation usually indicates the quarter of the cow's udder is positive or infected with Mastitis. Source: McCurnin's Clinical Textbook for Veterinary Technicians

Table 3. Somatic cell range and interpretation

Test result Cmt code	Equivalent somatic cell range (cells/ml)	Interpretation
(-) Negative	0-200,000	Healthy Udder
(-+) Trace	> 200,000 – 400 , 000	Sub-clinical Infection. If all four quarters read trace, there is no infection. If one or two quarters read trace, infections are possible.
(+) Grade 1 weak positive	400,000 – 1,200,000	Sub-clinical Infection
(++) Grade 2 distinct positive	1,200,000 -5,000,000	Serious Mastitis Infection
(+++) Grade 3 strong positive	Over 5,000,000	Serious Mastitis Infection

Source: Philippine Carabao Center, National Gene Pool Headquarters – Dairy Laboratory, Science City of Muñoz, Nueva Ecija.

Milk samples were graded the guides in Table 2. Milk samples were also submitted immediately to the Philippine Carabao Center National Gene Pool Headquarters – Dairy Laboratory, Science City of Muñoz, Nueva Ecija, Philippines for Somatic Cell Count Analysis using Cytometry. Based on their recommendations, milk samples were declared infected with mastitis or not using Table 3 as guide.

Data Gathered

The following data were gathered:

1. ***Macroscopic Evaluation Results*** are tabulations in Mastitis Detection using the Commercial reagent and different brands of liquid anionic surfactants.
2. ***Somatic Cell Count Results*** are results of the milk samples collected from the different quarters of the udder of experimental animals that was conducted by PCC.
3. ***Cost of Producing Liquid Anionic Surfactant Agent*** is the computed expenses of producing test solutions.

Data Analysis

All the data gathered were statistically analyzed using the Analysis of Variance (ANOVA) for Completely Randomized Design (CRD). The mean of the results was compared employing the Least Significant Difference test at a 0.01 level of confidence.

Results and Discussion

Mastitis Detection by Macroscopic Evaluation

Results of the macroscopic evaluation for milk samples using commercial mastitis test and different Brands of Liquid Anionic Surfactants (LAS) at different dilution rates are presented in Table 4.

Table 4 shows that Brands X, Y, and Z at 30%-50% dilution rate were able to detect different levels of positive Mastitis like CMT. Brand Z at 30% and 50% dilution level has the highest ability to detect mastitis at its highest grade, i.e., grade 2.

The ability of the LAS to detect mastitis was due to its anionic surface-acting agent akylanylsulfonate – an active ingredient found in surfactants that have the same action in the deoxyribonucleic acid (DNA) reagent which dissolves or disrupts the outer cell wall and the nuclear cell wall of any leukocyte, which are primarily fat. DNA is released from the nuclei. DNA will get together to form a stringy mass. As the number of leukocytes increases, the amount of gel formation also increases linearly.

This is parallel to the result of the study conducted by Gangan et al. (2018) wherein liquid anionic surfactants can be used to detect clinical and subclinical mastitis in dairy animals.

Effectiveness of Liquid Anionic Surfactants at Different Dilution Levels

Presented in Table 5 is the comparison among the different treatments which includes the different brands and dilution levels of liquid anionic surfactants.

Analysis of variance revealed that all treatments have no significant difference to the commercial mastitis test except for treatment 9 (Brand Z at a 40% dilution rate).

This is parallel to the result of the study by Gangan *et al.* (2018) wherein liquid anionic surfactants produced comparable result to commercially available mastitis test reagents.

Consistency of Using Liquid Anionic Surfactants in Mastitis Detection

Results on macroscopic evaluation reveal that the different liquid anionic surfactants and dilution levels were consistently able to detect mastitis dairy cattle's fresh milk. Statistical analysis of variance in Table 4 also reveals the consistency of the liquid anionic surfactants at 30%-50% dilution rate to commercial mastitis test except treatment 9 (brand Z at 40% dilution rate).

Table 4. Results of the macroscopic evaluation using CMT and different brands and dilution levels of liquid anionic surfactants

Treatments	Mastitis grade				
	Negative	Trace	Grade 1	Grade 2	Grade 3
Treatment 1	35	15	5	0	0
Treatment 2	33	18	4	0	0
Treatment 3	31	19	5	0	0
Treatment 4	30	20	5	0	0
Treatment 5	29	21	5	0	0
Treatment 6	31	19	5	0	0
Treatment 7	29	21	5	0	0
Treatment 8	28	23	3	1	0
Treatment 9	18	35	2	0	0
Treatment 10	28	22	4	1	0

Table 5. Mean grade for mastitis detection using the CMT 5-point hedonic scale

Treatments	Treatment id	Means
1	Commercial mastitis test	1.46[a]
2	Brand x at 30 % dilution rate	1.40[a]
3	Brand x at 40 % dilution rate	1.46[a]
4	Brand x at 50 % dilution rate	1.49[a]
5	Brand y at 30 % dilution rate	1.48[a]
6	Brand y at 40 % dilution rate	1.48[a]
7	Brand y at a 50 % dilution rate	1.50[a]
8	Brand z at a 30 % dilution rate	1.50[a]
9	Brand z at a 40 % dilution rate	1.68[b]
10	Brand z at a 50 % dilution rate	1.50[a]

Note: Means followed by the same letter are not significantly different at a 1% level of significance. P(>F) = 0.0005. CV (%) = 3.39

Table 6. Cost efficiency of using different brands of liquid anionic surfactants (LAS) at different dilution levels

Brand and Dilution rate	Cost per gallon (₱)	Cost of CMT/LAS Per 5ml (₱)	Savings from the use of LAS (₱)	Percentage of savings from the use of LAS (%)
Commercial reagent	1,136	1.42		
Brand X at 30%	268	0.335	1.085	323.88
Brand X at 40%	344	0.43	0.99	230.23
Brand X at 50%	420	0.525	0.895	170.48
Brand Y at 30%	268	0.335	1.085	323.88
Brand Y at 40%	344	0.43	0.99	230.23
Brand Y at 50%	420	0.525	0.895	170.48
Brand Z at 30%	304	0.38	1.04	273.68
Brand Z at 40%	392	0.49	0.93	189.80
Brand Z at 50%	480	0.6	0.82	136.67

Therefore, all the treatments were consistent in mastitis detection but treatments 8 and 10 (Brand Z at 30% and 50% dilution rate) were found to be the most consistent among all treatments. This was confirmed by the Somatic Cell Count result (Appendix B) and antibacterial component of the brand can kill bacteria. This result confirms De Guzman *et al*. (2019) that 30% and 50% of brand Z were also consistent in detecting mastitis.

Economic Viability

Presented in Table 6 is the economic viability of using different brands and dilution level of liquid anionic surfactants in detecting subclinical and clinical mastitis in dairy cattle.

Table 6 showed that brand X and brand Y at 30% dilution level obtained the lowest cost per gallon (₱ 268) and cost per sample (₱ 0.335) among all treatments including commercial reagent. In terms of the amount saved using LAS and percentage savings from using commercial reagent, brand X and brand Y at 30% dilution level acquired the highest savings and percentage savings among all treatments with ₱ 1.085 and 323.88%.

Similarly, Dela Cruz *et al.* (2019) stated that using liquid anionic surfactants in detecting Mastitis ruminants was viable because of its availability in the nationwide market and has cheaper cost compared to commercial reagent

Conclusions

Based on the results of the study, the researchers concluded that Brand Z at 30% and 50% dilution levels is the most consistent surfactant solution in detecting mastitis on fresh dairy cattle milk, while Brands X and Y at 30% dilution level are the cheapest solutions to use.

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Original Article

Molecular characterization of lactoferrin gene as genetic marker to subclinical mastitis in water buffaloes (*Bubalus bubalis*)

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Abstract

The study characterized the lactoferrin (Lf) gene in different water buffalo breeds and determined its association with subclinical mastitis (SCM). A total of seventy five (75) and five (5) milk and blood samples, respectively were collected for the conduct of this study. Amplified Lf gene demonstrated a 2224 base pairs (bp) molecular weight. Nucleotide and amino acid sequence of Lf gene of riverine- and swamp-type water buffaloes revealed 98.83% and 98.29% identity, respectively with that of *Bubalus bubalis* Lf gene in the GenBank. Phylogenetic studies showed that Lf genes of both types of water buffaloes grouped with Lf gene of water buffalo sequences registered in the GenBank. Three water buffalo genotypes were documented using the restriction enzymes *AluI* and *HaeIII*. The *AluI* can produce three genotypes (AA, AB, BB) resulting in three cuts of 561, 217 and 123 bp. Using *HaeIII*, three restriction patterns were observed producing three genotypes as well with four fragment sizes of 561, 318, 112, and 70 bp. Based on the statistical analysis, no direct association has been established between the Lf genes of water buffalo with SCM using *AluI* and *HaeIII* restriction enzymes although a higher number of animals with genotype BB belongs to those with SCM group. The results of the study merit for further studies regarding the BB genotype of Lf gene as a possible resistance gene marker for SCM in water buffaloes.

Keywords: *Bubalus bubalis*, Lactoferrin, Subclinical mastitis

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Introduction

Domesticated water buffaloes (*Bubalus bubalis*) are major milk-producing animals in several countries and contribute significantly to global milk production (FAO, 2016). Unlike dairy cows, water buffaloes are resistant to most dairy diseases even with stressful environments and settings of low feeding (Sahin et al., 2016a; Sahin et al., 2016b; Sahin et al., 2017). Distinct anatomical features, such as long narrow teat canal,

teat skin less prone to chapping and sores, thicker epithelium and keratin layer and tighter sphincter of streak canal, and absent milk cisterns may be the reason for this relative resistance (Thomas et al., 2004). However, buffaloes that are managed intensively, mastitis begins to occur even with these distinct anatomical features (Preethirani et al., 2015). The impact of mastitis is comparable to that of dairy cows involving production losses, culling and treatment costs, and decreasing animal health and welfare (Hogeveen and Van der Voort, 2017; Fagiolo and Lai, 2006; Halasa et al., 2007).

Mastitis is an economically significant infectious disease of dairy animals. Losses are due to decrease quantity and quality of milk, heightened by medicine and labor cost. A retrospective study on the prevalence of subclinical mastitis (SCM) conducted in the Philippines from September 2006 to June 2009 showed 42.76% with recurrence of 75% in water buffaloes (Salvador et al., 2012). In reducing economic losses, lowering the cases of SCM in farms is a vital strategy. To understand the mechanism of the susceptibility of animals to mastitis, genetic traits for milk production should be considered. Thus, genetic markers in the selection of animals for breeding has also been considered (Gholizadeh et al., 2008).

Lactoferrin (Lf), also known as "red protein from milk", is an iron-binding protein that can be found in most bodily fluids. Lf is released by neutrophils and inflamed tissues and has relationship with innate immunity due to its direct antimicrobial property as it limits bacterial proliferation and adhesion in the process of killing the microbes (Walker, 2010; Van Der Strate et al., 2001; Valenti and Antonini, 2005; Legrand et al., 2005; Legrand et al., 2008; Siqueiros-Cendon et al., 2014).

Information about Lf gene in water buffaloes (*Bubalus bubalis*) is limited. Genetic characterization of the gene would augment the ability to understand its role in the occurrence and onset of mastitis (Pawlik et al., 2009). The potential of Lf gene as a genetic marker for mastitis resistance in dairy animals needs to be elucidated to validate its role (Shimazaki and Kawai, 2017).

Identification of nucleotide polymorphism as a marker-associated resistance or tolerance to mastitis may be used as a tool in improving the performance of water buffaloes. Molecular characterization and genetic diversity of Lf gene of cattle have been associated with different production trait and diseases, thus, have been incorporated for selection criteria and production (Sharma et al., 2015). With this, it is rational to study the relationship of Lf gene with resistance to mastitis for water buffaloes.

The objective of the study is to characterize and identify Lf gene as a genetic marker for SCM resistance in water buffaloes by molecular analysis and association of nucleotide polymorphisms with the presence of SCM.

Material and Methods

Sample collection

A total of seventy-five (75) milk samples from riverine type water buffaloes on the 2nd month of lactation and five (5) blood samples from swamp-type water buffaloes were collected for the study. Fifty (50) and twenty-five (25) samples were non-mastitic and mastitic, respectively. There were no alterations made on the overall management of the water buffaloes included in the study.

The test animals were manually milked from the udder and pooled to collect 30 mL of milk sample for RNA extraction. The milk was collected in a 50 mL conical tube and was placed in a cooler with ice transported to the laboratory and processed on the same day of collection. For samples that were not processed on the same day were stored in a freezer (-20 °C) until used.

For the five swamp-type water buffaloes, RNA was extracted from blood as these animals were not lactating. Five mL of blood was collected from the jugular veins of the animals using EDTA tube. Samples were processed on the same day of collection.

RNA extraction

RNA extraction from milk and blood samples from the water buffaloes was done following the Promega protocol with modifications. For milk, 30 mL of raw milk was centrifuged at 4,000 rpm for 20 mins. After centrifugation, the fat layer and the supernatant were discarded, and afterward, the pellet was suspended in 1 mL PBS (phosphate-buffer-saline, a pH of 7.4). One mL of PBS was mixed again with the pellet and was centrifuged for 14,000 rpm for 1 min. This process was repeated twice and the supernatant was removed to collect the pellet.

For blood, 500 µL of the buffy coat was aspired after centrifugation at 4,000 rpm for 5 min and transferred into the 2 mL microtube, and then added with 2-3 volumes of 1 ml of 0.14 NH_4Cl. The mixture was mixed by vortex and centrifuged at 14,000 rpm for 1 min. The supernatant was removed and the process

was repeated twice to collect the pellet.
The pellet from the milk and the blood was added with 1 mL cell lysis solution and was subjected to vortex to mix the pellet and cell lysis. After this, it was centrifuged to 14,000 rpm in 1 min. The supernatant was discarded and 500 µL of nuclei lysis and 300 µL protein precipitation solution was added.
After discarding the supernatant, isolated cells were dissolved with 1 mL Trizol reagent and incubated for 10 min at room temperature. The solution was decanted and pellets were washed by 1 ml 75% ethanol and centrifuged for 5 min at 8,000 rpm at 4°C. Pellets were dried and reconstituted with 30 µL diethyl pyrocarbonate (DEPC) treated water.

Reverse transcription PCR (RT- PCR)
Complementary DNA (cDNA) was done using the cDNA Synthesis Kit (Takara Bio, Inc, Japan). One µL of oligo dT random primers were mixed with 1 µL dNTP, 5 µL of RNAse free water and 3 µL of RNA template. The solution was incubated for 5 min at 65°C before running for PCR. The prepared RNA primer mix was mixed with 4 µL 5X buffer, 0.5 µL RNAse inhibitor, 1 µL reverse transcriptase, and 4.3 µL RNAse free water. This was subjected to PCR run for segment 1, 10 min at 30°C; segment 2, 45 min at 50°C; and segment 3, 5 min at 95°C. The synthesized cDNA was tested for *β-actin* gene amplification to ensure cDNA synthesis protocol.

PCR primers
Three primer sets were used to amplify the Lf gene (Table 1). These primers were designed using the Primer3 server (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the bubaline sequence (Accession number: JF825526) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).
The designed primers were analyzed for self-annealing and loop properties using Oligo analyzer software (https://sg.idtdna.com/calc/analyzer). Primers forming hairpin loops, self-annealing, dimers, cross dimer, and repeats were avoided as much as possible. Suitable primers were checked using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure that it amplifies the Lf gene.

Table-1: Nucleotide and amino acid sequence percentage similarity of Lf gene in ruminant species regarding swamp-type and riverine-type water buffaloes.

Primers	Nucleotide Sequence	Expected Product Size
Primer 1 - F	TAGCCATGAAGCTCTTC	835 bp
Primer 1- R	GAGTACGGACAACACCGGGC	
Primer 2 - F	GCTTCTCTGCCTAAACAACAC	1008 bp
Primer 2 - R	GTCTCAGCACACAATCTAGG	
Primer 3 - F	AACATCCCCCATGGGCCTG	694 bp
Primer 3 - R	TTTCTTCGATCGGACGGA	

Gene amplification
All PCR assays were performed in 20 µL reaction volume containing 2 µL of genomic DNA template, 10 pmol of each primer and PCR master mix. The amplification of the Lf gene was carried out in a thermocycler (SimpliAmp, Thermofisher) under optimized conditions. Briefly, initial denaturation at 94˚C for 4 min, followed by 40 cycles consisting of denaturation at 94˚C for 30 sec, annealing at 57˚C for 1 min and extension at 72˚C for 45 sec, with a final extension at 72˚C for 4 min.
After amplification, 1 µL of the PCR product was electrophoresed on 1.5 % agarose gel containing 1X TAE buffer at 70 volts for 30 min and visualized under ultraviolet light using UV transillumination advanced imaging system. A 1kb plus DNA ladder (Invitrogen, CA, USA) was used as a ladder to determine the sizes of the PCR products.

Nucleotide sequencing and phylogenetic analysis
Lf products were submitted for sequencing at the Philippine Genome Center. DNA sequences were assembled using Mega 7 software. The forward and reverse sequences for each primer were assembled to form contigs of the respective regions. The gene sequences were compared with the Lf mRNA sequences to annotate different exonic regions putatively to identify SNPs in respective regions. The contiguous Lf gene nucleotide sequence was subjected to Basic Local Alignment Search Tool (BLAST) at the NCBI database to determine the sequence similarity with the corresponding regions of other species.
Nucleotide sequences were aligned and translated to the amino acid sequence using the MEGA 7 software.

The phylogenetic tree was constructed using the neighbor-joining method. Confidence in the groups was estimated by a bootstrap of data using 1000 replications. Phylogenetic trees were constructed using MEGA 7 software (Tamura et al., 2011).

RFLP analysis

The Lf PCR new set of primers sequence in water buffaloes (F-5' ATCCTTCGCCCGTTCTTGAG 3' and R-3' CGCCGAATCTACTTTTGAGG 5') was designed to amplify the ligand-binding region in exon 2, 3 and 4 of Lf gene in water buffaloes in which the putative area is located with an amplicon size of 561 bp.

Restriction enzymes *AluI and HaeIII* were used based on the suggested enzymes by Sequence Manipulation Suite: Restriction map (http://www.bioinformatics.org/sms2/rest_map.html) (Stothard, 2000).

Restriction fragment length polymorphism (RFLP) was conducted by preparing the reaction mixture composed of 7 µl of PCR product, 5.15 µl of sddH_2O, 0.35 RFLP buffer and 0.075 µL for each enzyme. Samples were incubated at 37°C for 4 hr. Restriction fragments were resolved on 2% agarose gel in horizontal electrophoresis. The restriction-digested gene fragments were visualized on a UV transilluminator (FlourChem E by ProteinSimple ™) and photographed.

Association of Lf gene to SCM

The amplified fragment of the Lf gene from the milk of water buffaloes showed different genotypes based on the exhibited banding patterns from *AluI* and *HaeIII* restriction enzymes. The different genotypes were associated based on the result of the CMT test of each of the milk samples. A total of 62 samples from water buffaloes were used in studies that associated expression of Lf gene and the occurrence of SCM.

CMT analysis

CMT was used as an indirect method of measuring Somatic Cell Count (SCC) as the indicator of intramammary infection as it has the advantage of being quick, cheap, and simple "animal side" test.

All milk samples collected were subjected to CMT. A small amount of milk (approximately 5 mL) was put into the paddle. The paddle was tilted almost vertically so that only 2 to 1 teaspoon of milk remains in each cup. An equal amount of CMT reagent was added to the milk and swirled for about 15 seconds. Observation of the reaction was done immediately to see if there is any thickening of the milk. The thicker the mixture, the higher the SCC. In goats and water buffaloes, the reaction scores are: N (negative) with no reaction and with estimated SCC of below 200,000 cells/mL; T (trace) with slight slime, tends to disappear with continued swirling with 150,000 to 500,000 cells/mL; 1 when there is distinct slime but without gel with 400,000 to 1,500,000 cells/mL; 2 when there are immediate gel formation and moves as a mass during swirling with 800,000 to 5,000,000 cells/mL; and 3 when gel develops a convex surface and adhere to the bottom of the cup with cell count estimated above 5,000,000 cells/mL (Rahman et al., 2010).

CMT scores for all animal subjects were classified as non-mastitic if the CMT score result is 1 or lower and mastitic if the CMT score result is 2 or higher.

Statistical analysis

Univariate analysis on the possible association between the genotypic frequency and the occurrence of SCM was examined using Chi-square (X^2) test analysis for the goodness of fit (Petrie and Watson, 2006).

$X^2 = E\,(O-E)^{2E}$

Where,

O= Observed Frequencies

E= Expected Frequencies

Results and Discussion

A study on the detection and molecular characterization of Lf gene in blood and milk samples of water buffaloes (riverine- and swamp-types) was undertaken. Out of all samples collected, one sample of each type of buffalo was sent for sequencing.

Figure 1 shows that the target primers 1, 2, and 3 were amplified and generated an amplicon size of 835 bp, 1008 bp, and 694 bp, respectively.

Sequence analysis of Lf Genes

In water buffaloes, swamp-type Lf mRNA sequence resulted in an average of 2224 bp (LC425118.1) while 2226 bp (LC425117.1) in riverine-type water buffalo.

Table-2: Nucleotide and amino acid sequence percentage similarity of Lf gene in ruminant species regarding swamp-type and riverine-type water buffaloes.

SPECIES (Accession Number)	NUCLEOTIDE SEQUENCE		AMINO ACID SEQUENCE	
	Swamp (LC425118.1)	Riverine (LC425117.1)	Swamp	Riverine
B. bubalis (JF825526.1)	98.29%	98.83%	97%	98%
B. Taurus (FJ589071.1)	95.42%	95.98%	96%	96%
B. indicus (GU059864.1)	95.28%	95.84%	95%	96%
B. grunniens (DQ387455.1)	95.35%	95.91%	95%	96%
C. hircus (U53857.1)	90.69%	91.19%	91%	92%
O. aries (NM_001024862.1)	92.66%	93.23%	93%	94%
E. caballus (NM_001163974.1)	74.06%	74.37%	75%	75%
S. scrofa (M81327.1)	73.69%	74.01%	74%	73%
H. sapiens (U076343)	70.76 %	71.98 %	69%	71 %

The riverine Lf gene nucleotide sequence was aligned with other ruminants' sequence of Lf gene using the database from GenBank. The 14 aligned sequences showed an average of 2127 identical pairs, 31 transitional pairs, and 18 tranversional pairs. Between riverine-type, swamp-type water buffalo, and *B. bubalis* (JF825526.1) nucleotide sequence, the average identical pairs were 2221 bp with 3 transversional pairs. This showed that there is a high similarity of sequence between the two types of water buffaloes

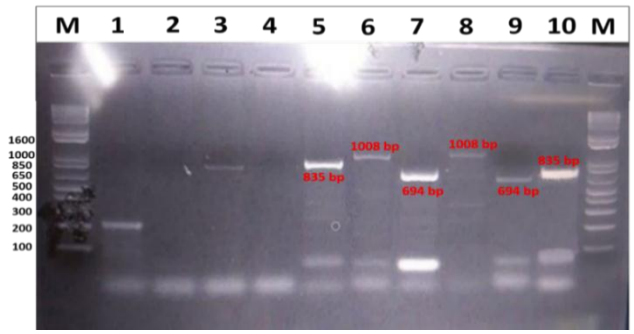

Figure-1: Amplification of Lf gene of swamp-type and riverine-type water buffaloes using primers 1, 2, and 3. Lane M (1kb plus ladder); Lanes 1-4 unamplified PCR products. Lane 5 and 10 (Primer 1); Lane 6 and 8 (Primer 2); Lane 7 and 9 (Primer 3)

The nucleotide BLAST of Lf coding sequence of the riverine and swamp samples revealed a high percentage of similarity between the query sequence and other species of Lf sequence available in the NCBI database (Table 2). Riverine-type Lf gene nucleotide sequence was 98.83% similarity with that of *B. bubalis* (JF825526.1), 95.98% similarity with *B. taurus* (FJ589071.1), 95.91% with *B. grunniens* (DQ387455.1) and 95.84% *B. indicus* (GU059864.1). The lower similarity was observed with small ruminant species such as *O. aries* (NM_001024862.), *C. hircus* (U53857.1) and other species such as *E. caballus* ((NM_001163974.1), *S. scrofa* (M81327.1), and *H. sapiens* (U076343).

Similarly, the protein BLAST result presented a high similarity of translated amino acid sequences of riverine-type Lf to *B. bubalis* (JF825526.1). A lower similarity in water buffaloes Lf amino acid sequence was observed with *B. taurus*, *B. grunniens*, *C. hircus* and *O. aries*, respectively.

Phylogenetic analysis

The maximum likelihood NJ algorithm with 1000 bootstrap resampling revealed the clustering of water buffaloes. Swamp-type water buffalo Lf gene clustered together under a single clade with *B. bubalis* sequence from GenBank being related to swamp-type water buffaloes but more descendants of riverine-type water buffaloes. *B. taurus* and *B. grunniens* can also be descendants of the bubaline Lf while *C. hircus* and *O. aries* are distant relatives.

However, the phylogenetic tree analysis (Figure 2) showed a similar evolutionary origin of riverine-type and swamp-type water buffaloes in consonance with *B. bubalis* Lf generated from the Genbank. This, therefore, could indicate minimal variation on the susceptibility or resistance to diseases.

Polymorphism analysis

The coding sequences of Lf gene in water buffaloes were analyzed for differences in nucleotides and functional amino acid substitution. Furthermore,

restriction enzyme capable of digesting the sequence to identify the nucleotide polymorphism and distinguish the genotype has been identified and results are herein presented.

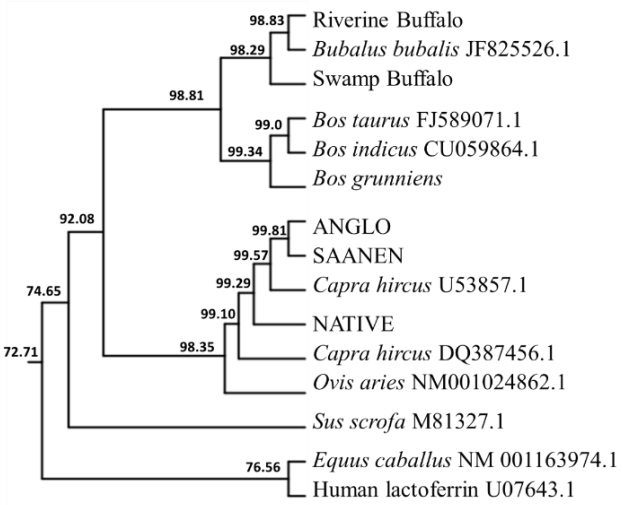

Figure-2: Phylogenetic tree showing the relationship of Lf gene between water buffaloes, goats, other ruminants' and human

To develop rapid recognition of polymorphic site of Lf gene, restriction enzymes *AluI* and *HaeIII* were used to digest 561 bp that was amplified by the primer 2. This 561 bp segment covered exons 2 and 3 of the coding region. SMS Restriction Digest (http://www.bioinformatics.org) application was used for the four samples with a complete Lf gene nucleotide sequence.

The availability of these restriction patterns on extracted Lf gene was examined by using a PCR product that was subjected to *AluI* and *HaeIII* digestion and electrophoresed. Results showed a restriction pattern that was almost the same base on the conceptualized expected pattern. From the 2224 bp nucleotide sequence, it revealed three-nucleotide polymorphism in riverine-type at locus 514, 857, and 1039 (Table 3) with two amino acid substitutions and in swamp-type water buffalo there are four nucleotide polymorphism in the Lf coding sequence at locus 315, 817, 1039 (Table 4), and 1488 with two amino acid substitutions.

The nucleotide polymorphism at locus 857of two types of water buffalo Lf gene sequences resulted in a functional amino acid substitution from threonine to isoleucine. However, there is no amino acid substitution at locus 1039, although there are changes in their nucleotides.

Lee et al. (1997), Martin-Burriel et al. (1997), Li and Chen (1999) and Kaminski et al. (2006) reported that polymorphisms in Lf gene reportedly occur in the coding and regulatory regions and polymorphisms are encoded in exons and introns.

Table-3: Nucleotide polymorphism in Lf gene of riverine-type water buffalo and corresponding sites of amino acid substitution

Base location		Nucleotide polymorphism		Amino acid location	Amino acid substitution	
1	514	C	T	202	L (Leucine)	P (Phenylalanine)
2	857	C	T	286	T (Threonine)	I (Isoleucine)
3	1039	C	T	347	Same	

Table-4: Nucleotide polymorphism in Lf gene of swamp-type water buffaloes and corresponding sites of amino acid substitution

Base location		Nucleotide polymorphism		Amino acid location	Amino acid substitution	
1	315	G	C	105	E (Glutamic acid)	D (Aspartic acid)
2	857	C	T	286	T (Threonine)	I (Isoleucine)
3	1039	C	T	347	Same	
4	1488	C	T	496	Same	

The results of this study corroborated with the result of previous Lf gene studies. In addition, Seyfert et al. (1996) mentioned that the number of exons in the gene and the number of amino acids encoded by 15 of the 17 exons are identical among them. The major differences lie in exons 2, where bovine and other animals' Lf contains one or two amino acids less than human Lf. However, Rupp and Boichard (2003) reported that there is still no strict association found between known Lf gene polymorphisms and mastitis susceptibility.

Restriction enzyme *AluI* cuts AG/CT nucleotide sequence was used to examine polymorphisms in the nucleotide sequence of Lf gene via banding patterns and were compared to the Lf gene sequences of water buffaloes in the GenBank. It was revealed that *AluI* can produce three genotypes at the nucleotide sequence of the 561 bp amplicon. Genotype AA produced two bands of 561 and 123 bp size; one cut was also produced in genotype AB of 217 and 123 bp, and genotype BB that produced two cuts resulting in 561, 123, and 217 bp amplicon sizes (Figure 3).

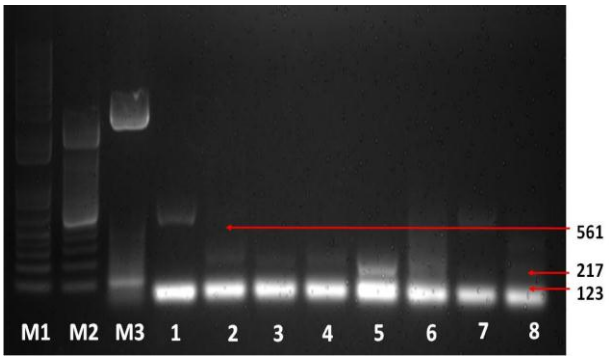

Figure-3: Restriction patterns of Bubaline Lf gene PCR product using *AluI* in 2% agarose gel. M1. 1000 bp ladder, M2. 100 bp ladder. M3. 25 bp ladder, Lanes 1 and 7 genotype AA (fragment size: 561, 123 bp), Lanes 2, 3, 4 and 5-genotype AB (217, 123 bp), Lanes 6 and 8-genotype BB (size: 561, 217, 123, bp).

Figure 4 shows the result of the digestion of water buffalo Lf gene PCR products using *HaeIII* enzyme. From the conceptualization four restriction patterns from nucleotide sequence were observed, genotype AA had one cut with a fragments sizes of 561 and 70 bp; genotype AB with three cuts of 561, 318, 112, and 70 bp, and genotype BB with two cuts of 318, 112, and 70 bp.

No recent studies supported in the three different genotypes found in the riverine water buffalo Lf gene using *HaeIII* and *AluI* restriction enzyme, hence this study will be the baseline for other researchers in identifying mastitic resistance genotypes.

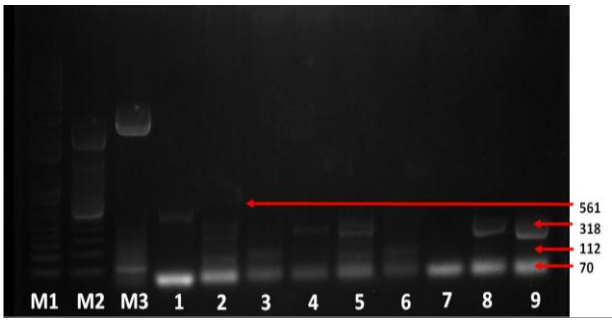

Figure-4: Restriction patterns of Bubaline Lf gene PCR products using *HaeIII* in 2% agarose gel. M1. 1000 bp + ladder, M2 100 bp ladder. M3 25 bp ladder, Lane 1 and 7, genotype AA (fragment size: 561, 70 bp), Lanes 2, 3, 5 and 6, genotype AB (fragment size: 561, 318, 112 and 70 bp), Lanes 4, 8 and 9, genotype BB (fragment size: 318, 112 and 70 bp)

Association of Lf Gene to SCM

From the total of 75 milk samples, only 62 milk samples were used in for RFLP analysis.

The 561 bp putative ligand-binding domain was targeted in this segment of the study. Table 5 shows the percentage frequency of Lf *AluI*-based genotype in non-mastitic and sub-clinically mastitic riverine water buffalo.

Table-5: Percentage frequency of Lf *AluI*-based genotypes in non-mastitic and SMC riverine-type water buffaloes

Genotype	Category of animal				Total	
	Non- mastitic		SMC			
	%	(n)	%	(n)	%	(n)
AA	66.67	14	33.33	7	34.4	21
AB	57.14	12	42.86	9	34.4	21
BB	52.63	10	47.37	9	32.2	19
Total	59.02	(36)	40.98	(25)	100	61

* bp = base pair

From a total of 61 milk samples tested, 40.98% (n=25) were considered sub-clinically mastitic, while 59.02 % (n=36) were non-mastitic. Genotype AA was found to be more frequent in non-mastitic animals, although Chi-square analysis did not show any significant difference with that of genotype. Furthermore, Statistical analysis of the result could not provide concrete evidence that resistance to SCM was due to the specific genotype.

The Lf gene in water buffaloes has a bactericidal and bacteriostatic activity that could lessen the bacterial population in the milk through phagocytic killing (Valenti and Antonini, 2005; Legrand et al., 2008). This may not be enough to sequester the microbial flora in the mammary gland of the animals. Underlying factors such as climate, housing system, type of bedding, and rainfall and wetness in the vicinity of dairy premises interact to influence the degree of exposure of teat and tend to increase mastitis pathogens that cause SCM (McEwen and Cooper, 1947).

Table 6 shows the percentage frequency of Lf *HaeIII*-based genotype in non-mastitic and SCM riverine water buffalo. From a total of 62 milk samples tested, 35.48% (n=22) were considered SCM, while 64.52% (n=40) were not mastitic. Genotype AA and AB were found to be more frequent in non-mastitic animals, however, Chi-square analysis did not show any significant difference with these genotypes.

The inflammation of the mammary gland induces epithelial cells to secrete Lf. The regulatory region of this gene is useful in the expression of the other antimicrobial proteins with the more potent anti-inflammatory action than Lf itself. Furthermore, Lf cannot work alone thus, animal genetics must coincide with good management practices to prevent or minimize the possible occurrence of SCM in a dairy herd.

Both digestion enzyme used may not have shown a significant association of the investigated genotype, but the presence of extensive polymorphic nucleotide in Lf gene can still be investigated using other restriction enzymes that could target the said polymorphic loci.

Table-6: Percentage frequency of Lf *HaeIII*-based genotype in non- mastitic and sub-clinically mastitic riverine-type water buffaloes

Genotype (fragment sizes)	Category of animal				Total	
	Non- mastitic		Sub-clinically mastitic			
	%	(n)	%	(n)	%	(n)
AA	28.57	15	71.43	6	33.87	21
AB	70.00	14	30.00	6	32.26	20
BB	47.62	10	52.38	11	33.87	21
Total	35.48	(22)	64.52	(40)	100	62

* bp = base pair

Conclusion

The whole mRNA coding sequence of water buffaloes had an average molecular weight of 2224 bp. Both nucleotide and translated amino acid sequences of Lf in water buffaloes were highly similar (99%) and phylogenetic analysis found an evolutionary relationship with water buffaloes from NCBI GenBank. The Lf gene of riverine water buffaloes established three different genotypes (AA, AB, and BB) using restriction enzymes *AluI* and *HaeIII*. No direct relationship existed between Lf gene and SCM. These initial findings are applicable in the field of immunity and disease resistance. The incidence of association between restriction sites and clinical parameter converts PCR-RFLP into a powerful tool in relating specific amino acid substitution at a critical position to possible disease resistance. Moreover, the selection of disease resistance genes will provide potential avenues for improving the health status of the animal and increasing productivity.

Lactoferrin is a very polymorphic gene that merits further investigation and its significance on disease resistance and susceptibility. Polymorphism of this gene may be useful as a selection marker for resistance against SCM. The result of this study may not statistically prove that lactoferrin can be a marker for SCM. However, the study also points out that most of the water buffaloes with SCM fall under the BB genotype.

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Contribution of Authors

Waminal YO: Designed and conducted the study and prepared the manuscript of the draft

Tubalinal GASP: Conducted the study and prepared the manuscript draft

Mingala CN: Designed and supervised the study and prepared and approved the final version of the manuscript draft