Alpha-2-Macroglobulin Gene Polymorphism in Water Buffaloes (*Bubalus bubalis*) with Subclinical Mastitis

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ABSTRACT

The study identified single nucleotide polymorphism (SNP) in the alpha-2-macroglobulin (α2M) gene in water buffaloes with subclinical mastitis and examined its association with its somatic cell score (SCS) via the somatic cell count (SCC) as an indication of presence of mastitis. Buffy coat was separated from the blood samples and was used to extract the genomic DNA. α2M gene was amplified by polymerase chain reaction using a designed primer pair. DNA sequences were aligned with the available sequences in the GenBank and analyzed for presence of SNPs. Result showed that there was a SNP identified along the c.207G>A. However, there was no significant association of the identified SNP with SCS but it can be described as that, the SNP identified was more related to water buffalo than cattle.

INTRODUCTION

Domesticated water buffaloes (*Bubalus bubalis*) are known for their practicality in the livelihood of the community, especially to farmers and agents of research field. In the past, they are primarily known for draft work, secondary for meat production and least of its milk production (NRC, 1981). The worldwide population of the water buffalo is approximately 168 million head and is still increasing (PCAARRD, 2013). However, despite such potential role, small hand tractors are increasingly substituting the buffaloes as an aid in farm power, predominantly in irrigated rice-producing areas and while given this development, the water buffaloes are now being improved as potential source of meat and milk (PCC, 2013). Although water buffalo’s milk is not receiving equal public attention as that of cattle’s milk, it has been proven to possess physiochemical features unlike that of other ruminant species, such as greater content of fatty acids and proteins (D’Ambrosio et al., 2008). Nevertheless, like other mammalian species, they can also suffer from intramammary infections that could cause disturbances in the composition of milk and decrease the sterility of milk, rendering it unsuitable for use in dairy products such as cheese and yogurt.

One of the most commonly encountered diseases is mastitis, which is defined as the inflammation of the mammary glands due to several factors including the presence of bacterial, chemical, thermal, or mechanical injury that affects the composition of the milk (Karthikeyan et al., 2016). The milk produced by animals can be evaluated by several parameters such as expression of genetic markers, milk yield, fat and protein content and somatic cell count (SCC). These are essential in checking for the presence of mastitis and evaluating the milk quality as well (Brasil et al., 2015). Mastitis can be classified into two forms: clinical mastitis (CM) and subclinical mastitis (SCM). SCM is best detected using California Mastitis Testing (CMT) since it cannot be assessed physically and it provides an instant diagnosis that makes it advantageous to farmers even at small-scale farming. Meanwhile, both forms can be quantitatively measured using the well-known method called somatic cell counting (Kahn et al., 2010).

Certain genes play a vital role in host defense mechanism against diseases such as mastitis. Examples of these genes are DRB3 (Kumar et al., 2011), TLR2 (Huang et al., 2011), IL1β and TNF-α (Lahouassa et al., 2007). Another gene with a remarkable impact is the alpha-2-macroglobulin (α2m) gene which encodes for a protein that belongs to the family of major serum proteins synthesized by the body. The α2m has various functions including inhibition of physiological proteinase activity and binding of growth factors, cytokines and disease factors (He et al., 2005). Certain genes, like α2m, play as markers of important roles in host immunity that participate in the improvement of the innate immune response of the mammary gland (Freitas et al., 2015). Hence, detection of such potential genetic markers can improve milk production and quality through effective animal breeding and selection that can serve as long-term benefit to farmers, manufacturers and consumers.

Due to the increasing demand of milk for consumers and increasing potential of water buffaloes in dairy production, there is now a greater attention drawn to them for further improvement. However, just like other mammals, water buffaloes are also susceptible to intramammary infections, such as mastitis. Therefore, an in-depth understanding of mastitis is deemed important. The α2m gene is implicated in conferring resistance to mastitis in several animals. Studies show that polymorphism in this gene may play a role in phenotypic variability in resistance to diseases such as mastitis (Wang et al., 2012). Polymorphisms in this gene are not yet well-elucidated in water buffaloes. The aim of this present research was to detect the presence of α2m gene polymorphism in water buffaloes affected with SCM in order to provide a better understanding on the underlying genetic component of the said gene and a basis of much more effective animal breeding and selection through the α2m gene.

MATERIALS AND METHODS

Animal selection

A total of 60 island-born water buffaloes with complete animal record were used in the study. The majority of the water buffaloes are at least three years old and had at least four full lactations during the period of the study. Although the actual breeding profile of the herd is not known, parent stocks were composed of Bulgarian Murrah water buffaloes imported from the Bulgaria. Lactating water buffaloes diagnosed with CM were excluded from this study since they were no longer included in the milking herd.

Sample herd

The water buffaloes were milked twice daily in a double-five milking parlor equipped with automatic milking machine (DeLaval, Kansas City, Missouri, USA). Teat disinfection is being done before and after milking. The animals were housed under intensive closed feedlot with concrete flooring. Their diet is in variety such as corn silage, specially-formulated concentrates during milking but majority of their diet is composed of fresh roughages.

The somatic cell score (SCS) data used in this study was the average SCC of each sample. It was obtained by averaging the five SCC values gathered from the monthly milk test day. The SCC values were converted to logarithmic values and was used as the basis in identifying which animals will be prioritized for sampling. An SCS of <200,000 cells/ml indicated no mastitis while an SCS of >200,000 cells/ml indicated otherwise. Only the animals with four to five lactations during the research period were considered.

Determination of SCS of milk samples

Data regarding the SCS were obtained by conducting a monthly milk testing of fat and protein content and SCC. The milk samples obtained were analyzed based on the analytical principle of flow cytometry using the equipment Fossomatic™ Minor (FOSS Analytical AS, Hillerod, DK) SCC machine. Samples were classified according to Schalm and Noorlander (1957), with low SCC (<200,000 cells/mL) and high SCC (>200,000 cells/mL) indicative of no mastitis and SCM, respectively.

Blood collection

Each animal was collected with blood samples that were the used in DNA extraction. The animals were properly restrained using a chute in order to minimize the mobility of the animal and facilitate easier collection. All animal care procedures were conducted in accordance with the guidelines approved by the Philippine Carabao Center. Approximately 10 ml of blood was collected from the jugular vein of the animal using a 19 gauge vacuum needle and contained in a vacutainer (BD Vacutainer®, Franklin Lakes, New Jersey, USA) containing sodium heparin. The collected samples were then stored in a cooler and transported in the laboratory for processing.

DNA extraction and quantification

The genomic DNA was extracted using Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, Wisconsin, USA) according to the manufacturer’s instruction. The DNA was eluted using 50 μl of DNA rehydrating solution and stored at -20°C until analysis. The quality and quantity of DNA was assessed using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Concentrated DNA extracts were then diluted to prepare a uniform concentration of 50 ng/μl in each sample into 0.6 μl microcentrifuge tubes.

Polymerase Chain Reaction (PCR) Analysis

The DNA fragment of interest was amplified through PCR technique. The target gene fragment was amplified using polymerase chain reaction (PCR). The primers used to amplify a 304 bp fragment of the α2M gene were: α2mF (forward) 5’-TGTTGCGTGTTGGTCTGGTTTTTT-3’ and α2mR (reverse) 5’-AGAGGTTAGGTTAAAGGACG-3’ (Freitas et al., 2015). The target amplicon covered a part of intron 28, exon 29 and intron 29. The reaction mixture was prepared in a total volume of 20 μl containing: 0.2 M each of forward and reverse primer; 0.8 μl of 2.5 mM iNtRON Biotechnology dNTP mixture; 2 μl of 5X GoTaq® Green Reaction Buffer; and 0.15 μl of GoTaq® DNA Polymerase. The master mix was aliquoted into PCR 8-tube strip with 50 ng of prepared buffalo DNA. The reaction was cycled PCR reaction was performed using the TC-5000 Thermal Cycler (Techne, Stone, Staffordshire, UK) with the following
thermal conditions: initial denaturation for 5 min at 95°C; followed by 38 cycles of denaturation at 95°C, annealing at 60°C and extension at 72°C, each step for 45 sec; and the final extension for 5 min at 72°C. The amplification was verified by gel electrophoresis on 1.7% agarose gel (w/v) in stained with GelRed™ (Biotium, Hayward, California, USA) in 1 X TBE buffer using Invitrogen™ 100-bp ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as a molecular weight marker for confirmation of the PCR length at 100 V for 30 min. After the gel electrophoresis, the amplified product it was viewed and photographed using Fluor Chem E Imaging System (Protein Simple, San Jose, California, USA) with the purpose of confirming that the desired fragment was amplified.

DNA sequence analysis

The PCR products were sent to 1stBASE Laboratories (Seri Kembangan, Selangor, Malaysia) for sequencing using both the forward and reverse primers. Sequence analysis, alignment and polymorphism identification were carried out using Codon Code Aligner program (http://www.codoncode.com/aligner/download.htm).

RESULTS AND DISCUSSION

A total of 60 blood samples were obtained from 60 Bulgarian Murrah water buffaloes in PCC. All these samples showed bands with ampalicon size of 304 bp which matched with the band size of the partial coding sequence of the exon 29 of α2m gene. Out of these samples, 16 PCR products were sequenced and analyzed for SNP. The 16 samples were selected based on the SCC record of the animals. These samples included animals with relatively high SCC (>200, 000 cells/ml) and those with low SCC (<200, 000 cells/ml) compared with others in the sampled population. SCC is a suitable predictor of intramammary infection. Somatic cells can indicate both susceptibility and resistance of the animals to mastitis, thus it can be used to keep track of the level or occurrence of SCM in a milking herd (Sharma et al., 2011).

Computation of SCS via SCC

The mean of SCC data of each lactation period in each animal sample was computed. The SCC average values distribution is often highly skewed and it is usually transformed on a logarithmic scale, using a formula mentioned from previous studies: SCS = log2 (SCC/100,000) + 3 (Dabdoub and Shook, 1994; Rupp and Boichard, 2003; Venturini et al., 2014). In this case, if the value was greater or less than 200, 000 cells/ml, it was translated to greater or less than 2.322 SCS.

Identification of SNPs

The presence of SNP was determined in the 16 samples through DNA sequencing and analysis. Figure 1 shows the gel electrophoresis result, with the samples having bands located near the 304 bp. The PCR products of these samples were subjected to DNA sequencing and yielded 97-99% homology with the reference sequence of the partial coding sequence of the exon 29 of α2m gene obtained from GenBank with accession number KF928932.1.

The SNPs were determined through multiple sequence alignment using Codon Code Aligner v.7.1.2 software. The SNP found in the aligned sequences is shown in Figure 2.

When aligned to the reference sequence, nearly 82% of the animals (13/16) showed polymorphism at c.207G>A, wherein eight (8) animals have SCM while five have no mastitis. The remaining 18% (3/16) showed no polymorphism on that region. Possible association of α2m gene polymorphism with SCS, occurrence of SCM and other production parameters has been reported in a few studies.

Freitas et al. (2015) identified three SNPs in water buffaloes, including one in the partial coding sequence of exon 29, g.241A>G. Although they did not find significant association of this SNP with SCC, their analysis showed that it can be used as a molecular marker for fat and protein production. In another study by Wang et al. (2012), they have found a SNP, also in exon 29 but in dairy cattle at c.3535A>T. Their findings and analysis suggested that the SNP found was located within a recognized exonic splice enhancer and may be the associated with why the α2m gene functions against mastitis by its alternative splicing mechanism. Since many studies in the past have used the data of cattle DNA sequences as reference for studies regarding water buffaloes, it can be considered that the alternative splicing mechanism of exon 29 in the α2m gene found in dairy cattle may also be expressed in the dairy water buffaloes.

According to several studies, SCS is an indicator of mastitis and associated with SNPs found in other genes. Ju et al. (2015) analyzed the role of SNP in the alternative splicing of neutrophil cytosolic factor 4 (NCF4) gene in dairy cattle and mastitis susceptibility characterized by SCS and inflammation. They have found a novel splice variant designated as NCF4-TV and a novel SNP g.18174 A>G. As their confirmatory analysis, they have constructed a two mini-gene splicing assay to demonstrate whether the SNP caused the splice variant and the results suggested that the NCF4-TV could be due to the presence of the SNP which was associated with SCS and increased risk of mastitis in dairy cattle.

Selvan et al. (2016) carried out a study about cluster of differentiation 14 (CD14) gene SNPs with association with CM in dairy cattle. There were nine combined genotypes found but only the A1AC typed animals were recognized to be least susceptible to mastitis when compared to other combined genotypes.

Since there is a limitation with references concerning the α2m gene in B. bubalis, the exact position of the exon 29 of α2m gene was located using B. bubalis alpha-2 macroglobulin (α2m), exon 29 and partial coding sequence and Bos taurus alpha-2-macroglobulin (α2m), mRNA reference assemblies, both found in the GenBank database with the accession numbers NM_001109795.1 (4938bp) and KF928932.1 (304bp), respectively. Using Standard Nucleotide BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the two sequences mentioned was aligned. The result of the alignment showed that the exon 29 of α2m gene was positioned at the region 3534...3714, while the bait region was located at 692...703, thioester region at 1009...1012 and inhibitory regions at 706...711, 721...725 and 732...737.
In a previous research conducted by Wang et al. (2012), the exon 29 of α2m gene carries splice variants that chiefly influences the protein through the bait region, inhibitory region and thioester region. In another related study, Lin et al. (2012) and Meyer et al. (2012) have said that when the bait region is cleaved, the configurational change in the framework of the alpha macroglobulins is activated which will be followed by the entrapment of the unwanted proteases in the body and that mechanism can be attributed to the susceptibility of the remarkable sequence of amino acids contained in the said region.

The presence of SNP at c.207G>A along the partial exon 29 of α2m gene was demonstrated in this study, However, the observed mean SCS from the SNP variant is apparently not associated with the occurrence of polymorphism. According to the results collected, it showed that the SNP found was more commonly found in animals with greater SCS but it does not guarantee the exclusivity of this SNP to lesser resistance to mastitis since the SNP is also present in animals with lesser SCS. Thus, possibly, this SNP is not a functional mutation against mastitis.

Conclusions

All the 60 samples showed amplification of the desired α2m gene fragment. Among the 16 DNA sequences analyzed, there are 13 animals with SNP at c.207G>A. However, the occurrence of SNP is not associated with that of their corresponding SCS. It is therefore recommended to conduct a study using samples coming from different herds with varying severity of intramammary infections, including both CM and SCM, when determining SNPs of the α2m gene. Another factor to consider is to study the whole exon 29 in the α2m gene as well as other candidate genes in relation to mastitis and other milk quality parameters in water buffaloes.

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REFERENCES


Table 1: Primers for exon 29 of α2m gene (Freitas et al., 2015)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>α2mF (forward)</td>
<td>5’-TGTGCCTGGTTCCGTTTTTA-3’</td>
</tr>
<tr>
<td>α2mR (reverse)</td>
<td>5’-AGAGGGTAGGTTATAAGAGC-3’</td>
</tr>
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Fig. 1: α2m gene primer for water buffalo amplified the desired portion of the DNA with an ampicicon size of 304 bp. Lane M-100 bp ladder; Lane 1-8 samples.

Fig. 2: SNP identified at the region c.207G>A.


