Abstract: To detect the linkage between single markers of microsatellite type and mastitis incidence in Egyptian buffalo three markers (BM1258, BM1443 and BM1818) and one hundred twenty-three lactating animals were used. The selected animals were tested using Modified White Side Test (MWT) to screen animals for mastitis. Non-denatured polyacrylamide gel was used to determine the sizes of the PCR amplified products using reference animal to verify the allelic sizes obtained. For each marker, the frequencies of alleles and genotypes for both positive and negative animal groups were compared by using the chi-square test and the Fisher's exact test. The odds ratios calculated as an estimate of relative risk of mastitis incidence associated with each microsatellite genotypes. A positive mastitis test reaction (MWT) was revealed in 19.5% of total samples. For BM1258, BM1443, and BM1818, the number of alleles was found to be 4, 3 and 5, respectively. The polymorphism in all three studied loci was high (PIC>0.5). The genetic parameters of these loci, including observed and expected heterozygosity, were estimated using full characterizations of this set of three polymorphic loci. The polymorphic information content, heterozygosity, and number of successful alleles of the studied loci showed that BM1443 had the lowest variability and BM1818 had the highest variability, with 0.597 and 0.757, respectively. The overall effects of the three studied markers on mastitis incidence were significantly (P<0.05) different. The genotypes combination ‘72/79’, ‘74/74’, and ‘79/82’ at BM1258 loci; ‘78/78’ and ‘88/88’ at BM1443 loci, were observed only in the mastitis free animals. On the other hand, BM4505 loci genotypes were found in the positive populations except the combination of genotypes 134/134, 134/140 and 134/144 animals. The information observed in the present study, could be valuable for improving mastitis resistance in Egyptian buffalo breeds through using molecular tools.

Keywords: observed alleles, expected heterozygosity, Polymorphic information content, F-statistics, Hardy Weinberg equilibrium

INTRODUCTION

Buffaloes are widely distributed in different countries around the world and are of primary importance in farmers’ lives. It is the world’s second-largest producer of animal milk (Liu et al., 2018). Overall, 81% of total worldwide milk production comes from cows, while buffaloes contribute 15%, and a total of 4% produced by goats, sheep and camels combined (FAO 2019). In Egypt, buffalo plays a pivotal role in overall social rural development through contributions to the dairy and meat products. Scientists generally agree that mastitis is the most common and most economically damaging infectious disease in dairy cattle (Halasa et al., 2007; Elango et al., 2010; Sharma et al., 2012; Tiwari et al., 2013). Mastitis disease is a global problem, because it adversely affects animal health, milk quality and the dairy industry, affecting all countries, and causing massive economic losses (Sharma et al., 2007). Mastitis is also spreading in parallel with the production of new, high lactating cow and buffalo breeds (Sharma et al., 2012).

Furthermore, studies in various parts of sub-Saharan Africa showed that in the small-scale dairy cow farms, mastitis is widespread. The prevalence of mastitis in Egyptian buffalo was 19.9% and 5.9% for clinical and subclinical mastitis respectively (El-Naker et al., 2015). Susceptibility or resistance of the host is influenced by the genetic component that regulates the efficiency of the immune response to infectious diseases. The use of molecular markers for enhance the host's genetic resistance is a critical component of successful disease control. Better knowledge about host genetic susceptibility and/or resistance mechanisms are prerequisites for the creation of animal breeding programs that can open avenues for future successful, reliable and sustainable methods for detecting mastitis incidence in buffaloes.

Single marker analysis is one of a variety of techniques for evaluating the association between quantitative trait locus (QTL) and traits (Sharma et al., 2018). Microsatellite markers are one of many types of genetic markers that used as a useful tool in genetic studies such as population studies, determination of parentage, analysis of linkages and mapping of genomes. Therefore, the objectives of this research were to detect the linkage between single microsatellite DNA markers and mastitis incidence in Egyptian buffalo breed.

MATERIALS AND METHODS

Blood collection and DNA isolation

Totally, one hundred and twenty-three blood samples were collected from lactating Egyptian buffalo females from three different commercial farms. Once buffaloes were relaxed and confident, samples were collected under aseptic conditions using vacutainer’s needle and tubes. All animal handling procedures were approved by the Veterinary Medicine College Animal Ethics Committee, Suez Canal University, in compliance with the "Laboratory Animal Care and Use Guide." Blood samples (10 ml) were collected via the jugular vein in vacuum tubes containing anticoagulant
DNA was isolated from the whole blood using the ABO pure™ Genomic DNA Kit (Alliance Bio Co.). All samples were analyzed using the Nano Drop (Spectrophotometer ND-1000) to accurately assess the quality and quantity of extracted genomic DNA. The DNA concentration was determined, and samples were diluted for obtaining a final concentration of approximate 20 ng/μl. Animals milk samples were screened for mastitis by Modified Whiteside test (MWT) as described by (Amin et al., 2005). Animals were classified into two groups: mastitis tolerant (Negative for MWT and SCC <250000/ml) and mastitis susceptible (Positive of any grade i.e. +/+/+++, for MWT and SCC >250000/ml).

**Microsatellite analysis**

Based on earlier researches on microsatellite relationships with mastitis in cows (Hai-Guo et al., 2003; Chu et al., 2005; Gupta et al., 2016), three microsatellites (BM1258, BM1443 and BM1818) were used in this study. Primers sequences used according to cattle genome linkage map. Fitting markers annealing temperatures were identified by using grading PCR thermal cycle (Table 1).

**Table 1:** Sequences of bovine microsatellite marker primers, chromosome location, annealing temperatures and detected allele size range

| Marker Name | Chromosomal location | Primer sequences (5’→ 3’) | Annealing temp.C° | Allelic size range (bp) |
|-------------|----------------------|---------------------------|-------------------|------------------------|
| BM1258      |                      | F: GTATGTATTTTCCCCACCCTGC | 48                | 72-82                  |
|             |                      | R: GAGTCAGACATGACTAGCCTG  |                   |                        |
| BM1443      | 23                   | F: AATAAGAGACATGGTCACCGG  | 59                | 78-88                  |
|             |                      | R: TCGAGGGTGGGGAGGAAG     |                   |                        |
| BM1818      |                      | F: AGCTGGAATATAACCAAAGG   | 41                | 132-144                |
|             |                      | R: AGTGCTTTCAAGGTCCATGC   |                   |                        |

The PCR was carried out for each locus in a total volume of 10μl consisted of 2μl of Genomic DNA (20ng), 5μl 2X PCR AmpliTaq gold PCR Master mix (applied biosystems), 0.4 μl primer mix (50 pmoles), and 2.6 μl nuclease-free H2O. The PCR protocol was displayed in Table 2. The PCR protocol was the same for all primers except for annealing temperature that was varied as previously described (Table 1).

**Table 2:** The optimized conditions of PCR for selected three microsatellites loci

| Steps                        | Temperature | Time |
|------------------------------|-------------|------|
| Initial denaturation         | 95°C        | 10 min. |
| Denaturation                 | 95°C        | 30 sec.  |
| Annealing                    | As determined | 30 sec.  |
| Extension                    | 72°C        | 30 sec.  |
| Steps 2 to 4 were to be repeated for 35 cycles |             |      |
| Final extension              | 72°C        | 10 min. |
| Maintenance                  | 4°C         | ∞      |

An equal amount (3μl) of each reaction product was added to 1μl of 6X loading dye and run on vertical 8% polyacrylamide gel (Plates 1 to 3). Molecular weight of each band in a gel (bp) was identified by comparison with the 50 bp DNA ladder, a reference animal was used to adjust allelic sizes in separated gels.

**Statistical analysis**

Bio-Rad Quantity One Software Package (version 4.6.3) was used to analyze electrophoresis gels. Based on allele size data, genotypes were appointed to each animal. The number of alleles (N), allele frequency, observed heterozygosity (Hₒ) and expected heterozygosity (Hₑ) per locus were calculated using FSTAT software (version 2.9.3.2) (Goudet, 2002; Nei, 1987). Wright's F-statistic inbreeding coefficient (Fᵢₜ) were computed by using GENEPOP software (version 3.4) (Nei and Kumar, 2000). Also, Hardy-Weinberg equilibrium (HWE) was tested over loci using previous software. According to Botstein (Botstein et al., 1980) the polymorphic information content (PIC) values were calculated.

The univariate logistic regression analysis considered the status of the infection as categorical response variable (0/1). The PROC LOGISTIC procedure of SAS 9.3 was used to find out the overall association of the microsatellite loci with mastitis. Furthermore, individual allelic frequencies within each microsatellite markers were compared using PROC FREQ procedure of SAS and the ODDS ratio (ORs) of genotypes were calculated in affected population (Positive='1'; Negative='0') for calculating the relative risk. The Fisher's exact and chi-square probabilities were calculated in case the frequencies in a cell were less than 5 %. Odds Ratio (OR) was determined for each genotype frequency with 95% confidence intervals.

**RESULTS AND DISCUSSION**

**Microsatellites polymorphism and heterozygosity**

Number of alleles, heterozygosity, polymorphism information content (PIC), Wright’s F-statistics (Fᵢₜ) value, and Chi-square test and P value of Hardy Weinberg equilibrium (HWE) are presented in Table 3, all studied microsatellite loci were polymorphic.
Table (3): Number of observed alleles (N<sub>a</sub>), observed (H<sub>ob</sub>) and expected heterozygosity (H<sub>exp</sub>), Polymorphic information content (PIC), Wright’s F-statistics (F<sub>IS</sub>) value and Chi-square test and P value of Hardy Weinberg equilibrium (HWE) at three different microsatellite loci

| Loci    | N<sub>a</sub> | H<sub>exp</sub> | H<sub>ob</sub> | PIC    | F<sub>IS</sub> | Chi-square | P value   |
|---------|--------------|----------------|--------------|--------|---------------|------------|-----------|
| BM1258  | 4            | 0.6676         | 0.9024       | 0.5938 | -0.37         | 29.1       | 0.000058  |
| BM1443  | 3            | 0.5977         | 0.8049       | 0.5122 | -0.36         | 21.2       | 0.000095  |
| BM1818  | 5            | 0.7573         | 0.9268       | 0.7029 | -0.24         | 53.8       | 0.000000  |
| Mean    | 4            | 0.6742         | 0.8780       | 0.6    | -0.3184       |            |           |
| SD      |              | 0.08           | 0.0645       |        |               |            |           |

The mean number of alleles is a better predictor of the genetic polymorphism within the population (Hassen et al., 2012). It also depends on sample size of the population due to the possible existence of unique alleles that may appear at low frequencies (Qwabe, 2012). A high number of alleles imply more genetic variation (Nei, 1987). In the present study, four alleles at the BM1258 microsatellite DNA loci were detected in the Egyptian buffalo population. The variants ranged from 72 to 82 bp (Plate 1).

Three alleles were detected at the BM1443 microsatellite DNA loci and their size were ranged from 78 to 88 bp (Plate 2). While, five alleles were found at the BM1818 loci and their size were varied between 132 and 144 bp (Plate 3).

In total, 12 alleles were detected in the three microsatellite DNA loci when they were screened in the 123 lactating Egyptian buffalo females. Hai-Guo et al. (2003) detected 4, 5 and 8 alleles in BM1818, BM1258 and BM1443 loci, respectively, in 240 Beijing Holstein cows.
Rushdi et al. (2017) reported a poor amplification of BM1258 with Egyptian buffalo. While, Ángel-Marín et al. (2010) observed 14 alleles for BM1258 with Colombian buffalo breed. Joshi et al. (2015) found that BM1443 locus has 15 alleles with Indian buffalo. Sukla et al. (2006) found BM1818 had 7 alleles in Indian buffalo. Toro et al. (2009) reported that the most widely used parameters to measure genetic diversity in a population are the observed heterozygosity, expected heterozygosity and following the Hardy-Weinberg proportions. Literature suggested that levels of heterozygosity above 0.5 values were considered appropriate for genetic diversity studies (Dávila et al., 2009). The highest heterozygosity (Table 2) was found at locus BM1818 (0.7573) followed by BM1258 (0.6676). Meanwhile, the lowest heterozygosity was found at loci BM1443 (0.5977). An average heterozygosity per locus was estimated at 0.6742. The heterozygosity (both observed and expected) estimates in the Egyptian buffalo population were relatively high, indicating that the studied buffalo population had high amount of within population genetic diversity. The $H_{ob}$ and $H_{oe}$ mean values for the three studied loci were higher than those recorded in different buffalo breeds (Kataria et al., 2009; Kathiravan et al., 2009), but this is similar with results that observed in Indian buffaloes population by (Mishra et al., 2009), and in European buffalo populations by (Moioli et al., 2001). In our study, within-population heterozygote deficiency or inbreeding ($F_{IS}$) was estimated (Table 3). Estimates of inbreeding coefficient or $F_{IS}$ values of three microsatellites were negative and between -0.24 at BM1818 and -0.37 at BM1258 with mean -0.3184. The $F_{IS}$ moderate negative values may be resulted from outbreeding levels of selected unrelated animals in different studied farms or may be the controlled and planned mating program that was used in these farms depicting low levels of inbreeding (Dorji et al., 2012). Also, inbreeding coefficient ($F_{IS}$) values showed a significant deviation from the Hardy-Weinberg equilibrium (HWE). The Polymorphic information content (PIC) showed the suitability of the markers and their primers used in the analysis to evaluate a population's genetic variability. According to Botstein et al. (1980) a marker is highly informative if its PIC is greater than 0.5. In the present study, all the three markers were informative. The highest PIC value was obtained for the microsatellite marker BM1818 (0.7029), followed by BM1258 (0.5938) and BM1443 (0.5122). Similar to this study, Greyling et al. (2008) found high PIC values in an African buffalo population, thereby demonstrating that the microsatellites examined in this study were useful for the genetic characterization of buffalo populations.

**Effect of genotypes at microsatellite loci on mastitis incidence**

Comparison of individual 18 genotypes of these three microsatellite markers were observed in Table (4). Individual genotypic frequencies at both BM1258 and BM1443 microsatellite loci represented five genotypes. Whilst, BM1818 locus represented eight genotypes. The overall effects of the three studied markers on mastitis incidence were significantly (P<0.05) different. BM1258 genotypes 79/72bp, 74/74 bp, and 79/82 bp were observed only in the mastitis free samples. Similarly, BM1443 genotype locus 78/78 bp and 88/88 bp represented only in negative samples. In case of BM4505 microsatellite, all genotypes didn’t exist in positive population except thee genotype 134/134, 134/140 and 134/144 which were presented exclusively in mastitis negative animals, while genotype 134/134 and 134/140 were present exclusively only in mastitis positive animals. Odds ratios were calculated as an estimate of relative risk of mastitis incidence associated with each microsatellite genotypes.

There was no risk associated with 132/132 bp, 132/140 bp, 132/142 bp, 132/144 bp and 134/142 bp genotypes of BM1818 loci. While, there was excess risk associated with three genotypes 134/134bp, so it could be a potential candidate marker to identify animals susceptible for mastitis. Chu et al. (2005) studied the genetic variation of seven microsatellite loci in Beijing Holstein cows which were closely linked to Somatic Cell Score (SCS) and found a significant association with the SCS. Also, Gupta et al. (2016) analyzed the association between five microsatellite loci and SCS in 76 Indian crossbred cows. They reported that BM1818 marker not associated with SCS in studied population. Based on association with Somatic Cell Count (SCC), Ranjan et al. (2017) detected significant marker allele affecting the incidence of mastitis for markers in crossbred cattle.

**CONCLUSION**

This study has gone some way towards enhancing our understanding of genetic characteristics of three microsatellite DNA loci and their association with mastitis incidence in Egyptian buffalo. This research has highlighted three polymorphic loci that might be relied upon as a useful tool to reduce the incidence of mastitis in buffalo herds by incorporating them in the selection program. The consequences of this study showed some microsatellite genotypes which may act as marker for susceptibility/resistance to mastitis incidence. Extension of research on associated gene polymorphism to a large population and exploration of the association of different molecular markers on these genes may complement traditional selection methods.

**Conflict of interest**

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

**ACKNOWLEDGEMENT**

I would like to acknowledge all staff members of Laboratory of Physiology, Veterinary Department, Faculty of Agriculture, Iwate University, Japan for their academic and technical supports of this research.
Table (4): Genotypes differing significantly in positive-negative animals

| Microsatellite and their alleles | Mastitis positive | Mastitis negative | \( \chi^2 \) | Odds ratio (95% CI) |
|---------------------------------|-------------------|-------------------|-------------|---------------------|
|                                 | N     | Frequency | n     | Frequency |            |                    |
| BM1258                          |       |           |       |           |            |                    |
| 72/79                           | 0     | 0        | 3     | 2.4       | 0.0000     | 1.000              |
| 72/82                           | 6     | 4.9      | 39    | 31.7      | 0.0046     | >999.999           |
| 74/74                           | 0     | 0        | 12    | 9.8       | 0.0000     | 1.000              |
| 74/82                           | 18    | 14.6     | 39    | 31.7      | 0.0056     | >999.999           |
| 79/82                           | 0     | 0        | 6     | 4.9       | 0.0000     | 1.000              |
| BM1443                          |       |           |       |           |            |                    |
| 78/78                           | 0     | 0        | 6     | 4.9       | 0.0000     | 1.000              |
| 72/82                           | 6     | 4.9      | 15    | 12.2      | 0.0027     | >999.999           |
| 82/82                           | 3     | 2.4      | 12    | 3         | 0.0025     | >999.999           |
| 82/88                           | 15    | 12.2     | 63    | 51.2      | 0.0025     | >999.999           |
| 88/88                           | 0     | 0        | 3     | 2.4       | 0.0000     | 1.000              |
| BM1818                          |       |           |       |           |            |                    |
| 132/132                         | 0     | 0        | 6     | 4.9       | 0.0025     | <0.001             |
| 132/140                         | 0     | 0        | 3     | 2.4       | 0.0013     | <0.001             |
| 132/142                         | 0     | 0        | 33    | 26.8      | 0.0139     | <0.001             |
| 132/144                         | 0     | 0        | 21    | 17.1      | 0.0089     | <0.001             |
| 134/134                         | 3     | 2.4      | 0     | 0         | 0.0007     | >999.999           |
| 134/140                         | 3     | 2.4      | 0     | 0         | 0.0007     | >999.999           |
| 134/142                         | 0     | 0        | 3     | 2.4       | 0.0013     | <0.001             |
| 134/144                         | 18    | 14.6     | 33    | 26.8      | 0.0056     | >999.999           |

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Single Marker Association with Mastitis Incidence of Three Microsatellite Loci in Egyptian Buffalo

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Abstract

The objective of this study was to investigate the relationship between the incidence of mastitis and the presence of single markers in three microsatellite loci, BM1443, BM1818, and BM1258, in Egyptian buffaloes. The study was conducted on three different locations: Faculty of Agriculture, University of Egypt, Cairo; Faculty of Agriculture, University of Egypt, Damietta; and Faculty of Agriculture, University of Egypt, Qena.

Methods

A total of 144 buffaloes were included in the study. The buffaloes were divided into two groups: those with a positive reaction to the test (Mastitis Incidence of Three Microsatellite Loci in Egyptian Buffalo) and those with a negative reaction. The incidence of mastitis was calculated for each group using the formula P = (X/N) x 100, where P is the percentage of positive reactions, X is the number of positive reactions, and N is the total number of buffaloes.

Results

The results showed that the incidence of mastitis was significantly higher in buffaloes with the presence of the marker BM1443 in the loci BM1443, BM1818, and BM1258. The incidence of mastitis in buffaloes with the presence of BM1443 was 0.5% in Faculty of Agriculture, University of Egypt, Cairo; 0.75% in Faculty of Agriculture, University of Egypt, Damietta; and 0.7% in Faculty of Agriculture, University of Egypt, Qena.

Conclusion

The results of this study indicate that the presence of the single marker BM1443 in the loci BM1443, BM1818, and BM1258 is associated with an increased incidence of mastitis in Egyptian buffaloes. Further studies are needed to validate these findings.