Global Veterinaria 9 (1): 08-22, 2012
ISSN 1992-6197

Sequence-Based Typing-Study on the Relationship Between Subclinical Mastitis and BoLA-DRB3.2* Allelic Polymorphism in Egyptian Cows

[1]Ebtihal A. Ibrahim, [2]Nesreen A.T. Allam, [3]Ebtisam E.Z. Kotb,
[1]Galila A. El-Rafey, [1]Mona M. Alaa El-Deen and [1]Manal G. Fadlallah

[1]Department of Biology of Reproduction,
Animal Reproduction Research Institute, El-Haram, P.O. Box 122556, Giza, Egypt
[2]Department of Parasitology and Animal Diseases, Veterinary Research Division,
National Research Center, Dokki, P.O. Box 12622, Giza, Egypt
[3]Department of Mastitis and Neonatal Diseases Research,
Animal Reproduction Research Institute, El-Haram, P.O. Box 122556,Giza, Egypt

Abstract: Mastitis is a multifactorial disease with a complex etiology. Identifying genes associated with reduced mastitis has focused up to date on the Bovine Lymphocyte Antigen gene *DRB* exon 2 (*BoLA-DRB3.2**). The present study tended to monitor this polymorphism in 100 Egyptian Holstein dairy cows from private farm in association with subclinical mastitis. Furthermore, relationships were explored between *BoLA-DRB3.2** variants and immune components including antibodies (IgG and IgA) and interleukin-6. Genetic Polymorphism was investigated by PCR followed by sequencing of amplified products from all cows. Alignments of obtained sequences with corresponding ones released in genbank revealed 3 different alleles designated as *BoLA-DRB3.2*11*, **16* and **24*. However, the three alleles were identified with variant subtypes due to heterologous nucleotides composition from those most homologous records released in genbank. These results indicated a difference existed between Egyptian Holstein breed and foreign populations with regard to genetic composition. As a second goal, allelic frequencies of detected genotypes were 22%, 47% and 31%, respectively, by direct counting in a total sample. Subclinical mastitic cows coding for *DRB3.2*11* genotype were found highly susceptible to *Staphylococcus aureus* (73.8%) then *Streptococcus agalactiae* (71.11%), however, highly resistant to *Escherichia coli* (11.66%) infections. Whereas those coding for genotype *DRB3.2*16* were highly susceptible to coagulase-negative *staphylococci* environmental bacteria (45%) but most resistant to *S. aureus* (2.38%) infections. Additionally, despite individuals possessing the *DRB3.2*24* allele were associated with considerable susceptibility to *E. coli* (61.66%) infection but did not provoke significant resistance to other pathogens included in the study. Therefore, cows having *DRB3.2*11* allele were found with higher tendency to mastitis risk. Statistical analysis of quantitative sandwich ELISA indicated significant increase ($P < 0.01$) in IgA, IgG and IL-6 hence compared with control individuals but could not indicate difference in immune reactions between detected alleles. In continual, *BoLA-DRB3.2** genotype is playing a central role in the development of resistance/susceptibility to infections and development of clinical form of mastitis, subsequently, both antibody- and cell-mediated immunity to clinical form of mastitis in dairy herds. In conclusion, cows carrying alleles *DRB3.2*16* were more resistant to intramammary infection, meanwhile, cows expressing allele **11* showed significant correlation with susceptibility to mastitis despite significant immune reactivity. On the other hand, cows with allele **24* indicated epidemiological role in maintaining source of infection in dairy farm. Further studies are necessary to confirm obtained results before *BoLA-DRB3.2** genotypes can be recommended as marker to be used in selecting the animals at young age before actual expression of the traits of interest.

Key words: Subclinical Mastitis · *BoLa-DRB3.2** Genotypes · Sequencing · Phylogeny · Bovine · Egypt

Corresponding Author: Nesreen A.T. Allam, Department of Parasitology and Animal Diseases, Veterinary Research Division, National Research Center, Postal code: 12622, Dokki, Giza, Egypt.

INTRODUCTION

Mastitis is an inflammatory reaction of the mammary gland resulting mainly; but not exclusively, from the invasion of contagious or environmental pathogens into the teat canal, which is characterized by increased somatic cell count (SCC) in milk [1, 2]. Based on the severity of the inflammatory response, mastitis manifests itself in clinical and/or subclinical forms. *Staphylococcus aureus*, *Streptococcus agalactiae* and *Escherichia coli* are the most common causative agents of mastitis of subclinical character with periodic flare up to clinical symptoms [3-5]. Despite intense research and control strategies, the incidence of mastitis has not been reduced to an acceptable level, which has increased the interest for new control strategies, among these the possible effect of genetics on mastitis [6].

Genetic variation in susceptibility to mastitis has been demonstrated and estimates of its importance have varied considerably, hence little is known about the genes involved in it. Identifying genes that are associated with reduced mastitis has focused up to date on the major histocompatibility complex (MHC) or bovine lymphocyte antigen (*BoLA*) genes [7]. The most promising region of MHC is locus *DRB* where associations with certain haplotypes and reduced SCC and/or clinical mastitis have been described by a number of authors [8-14].

Bovine lymphocyte antigen (*BoLA*) is relatively a large cluster of genes (multigene family) spanning nearly 2.5 Mega base (Mb) of cattle genome. *BoLA* genes is located on the shorter arm of chromosome 23 in cattle organized into three classes (I, II and III) [15]. *BoLA-DRB3* product which is a β chain of MHC class II; that is a glycoprotein expressed on the surface of the antigen presenting cells restricted primarily to B-cell and macrophages, is shown to regulate the immune response where it plays a central role in the development of both antibody- and cell-mediated immunity leading to cellular activation, production of antibodies and lymphokines release [7, 16].

Genotyping of *BoLA* is relatively complex because the genes within this family are extremely polymorphic. The genetic polymorphism of class II α and β genes occurs predominantly in exon 2 encoding the antigen binding site between and within species in both the numbers of loci and alleles [17]. Presently, more than 100 different alleles from exon 2 of the *BoLA-DRB3* gene have been identified [18, 19]. The extensive structural polymorphism of class II molecules is responsible for the differences among individuals in immune response to infectious agents [7, 9, 20, 21]. In cattle, they have been found to be associated with resistance or susceptibility to mastitis [10, 22-26].

Several methods for polymorphism identification in *DRB3* locus of *BoLA* including serological, biochemical and molecular methods have been developed [27-31]. Later on, more efficient methods have been discovered including heteroduplex analysis [32], restriction fragment length polymorphism (RFLP) on PCR products [33], sequence-specific oligonucleotide typing [34], denaturing gradient gel electrophoresis [35], sequencing and single strand confirmation polymorphism (SSCP) [36-38] and sequencing PCR products [12, 39-44].

In Egypt, nationwide studies on mastitis have been conducted showing substantial geographic variation, in addition to, the association of pathogen with bulk milk somatic cell count and barn type [45-47]. The economic importance of the disease is so large that breeding for lower incidence of mastitis seems to be economically profitable [17]. However, in most Egyptian dairy herds despite the framework of performance recording; for assessing the udder health status of herds and better control of mastitis, mastitis resistance has not been a predominant trait in sire selection and/or replacement heifer. Selection of cattle less susceptible to mastitis would be one mean of reducing the impact of this disease [48, 49]. Progress towards a more mastitis resistant cattle population has been hampered due to low heritability and/or low genetic correlations with clinical mastitis of selected traits [16, 50].

Considering the above, the objective of this investigation was to study *DRB3* gene polymorphism pattern within exon 2 and allelic frequency in Egyptian Holstein dairy cows and its association with subclinical mastitis. Furthermore, relationships were explored between *BoLA-DRB3.2* variants and immune components including antibodies (IgG and IgA) and lymphokines (interleukin-6; IL-6) as *in vivo* evaluation for immune response to subclinical mastitis.

MATERIALS AND METHODS

Animals: The present study was carried on one hundred Holstein dairy cows from private farm in Giza governorate, Egypt. Their age ranged 2.5-7 years in different lactation seasons. Animals were managed similarly and fed for high production. All cows were examined periodically to detect clinical and subclinical mastitis.

Samples

Milk Samples

Field Evaluation by California Mastitis Test: California mastitis test (CMT) is a cow-slide test to obtain a rough data on farm impression of udder health. Scores represent four categories: 0 = negative, +1 = trace, +2 = weak positive, +3 = distinct positive and 4 = strong positive. Quarters milk samples collected from 100 cows were strictly standardized: after stripping about 10 ml of milk, visual inspecting and evaluating the milk with California Mastitis Test (CMT) was carried out [51]. The first milk sample was taken for SCC evaluation, afterwards, aseptic samples were collected for bacteriological analysis using standard procedures [51]. The samples were transported to the laboratory at 4°C. Samples destined for bacteriological testing were stored at -20°C until further use [52], those for SCC evaluation were kept at 4°C [53]. Before sample collection, the udder and teats of each cow were examined by visual inspection and palpation and abnormal findings were recorded.

Milk Somatic Cell Counts Determination: Milk samples of all quarters kept at 4°C were analyzed within 24 h after collection. For evaluation, they were pre-warmed at 37°C for 10 min then automatically measured using SOMA-COUNT 150 (Bentley, USA) [54]. The log10 (SCC) values had classified milk samples into two categories, normal cows (SCC below 4×10^5 cells/ml) and apparently normal subclinical mastitic cows (SCC above 4×10^5 to 8×10^5 cells /ml) [55]. For SCC controls, ten bacteriologically negative milk samples were randomly selected.

Isolation and Characterization of Pathogenic Bacteria: Milk specimens representing investigated population were laboratory studied. After thawing at 37°C for 5 min, equal volumes of 10 µl of each sample were simultaneously plated on Nutrient agar, Edward agar, Mannitol salt agar, Salmonella/Shigella agar (SS agar), 5% Sheep-blood agar and MacConkey agar (Biolife Laboratories, Milano, Italy) and incubated aerobically at 37°C [56]. Single colonies were obtained for isolation. The isolates were identified based on colony morphology, color, hemolytic activity, Gram stain and standard biochemical tests [53].

Antibiotic Susceptibility Patterns: Antibiotic susceptibilities of isolates were determined by disk diffusion method on Muller Hinton agar plate (Oxoid) [57]**.** The following commercial antibiotic disks (Oxoid) were used in illustrated concentration: AMC (Amoxicillin 20 µg & Clavulanic acid 10 µg), AK (Amikacin 30 µg), AMP (Ampicillin 10 µg), AS (Ampicillin 10 µg & Sulbactam 10 µg), E (Erythromycin15 µg), G (Gentamycin 10 µg), OB (Cloxacillin 5 µg), OT (Oxytetracycline 10 µg), OFX (Ofloxacin 5 µg), P (Pinicillin 10 Units/disc) and S (Streptomycin 10 µg). Inhibition zone diameter of each antibiotic disk was measured and compared with standard zone chart according to manual of the supplier [58].

Blood Samples: One hundred cows were bled for both DNA dependent assays and immune parameters serological estimation. Approximately 10 ml of venous blood sample/cow was collected in equal volumes in sterile tubes; one containing 0.5 ml of 0.5 M EDTA (Sigma Aldrich) as anticoagulant for DNA isolation and the other one was anticoagulant-free for serum separation. Samples were kept at -20°C till used.

Oligonucleotide Primers Design: PCR oligonucleotide primers were designed with reference to Sigurdardottir and co-workers [59] and modifications of those published by van Eijk and co-workers [34] for PCR amplification of the *DRB3* exon 2. Primers HL30 (5`-ATCCTCTCTCTGCAGCACATTTCC-3`) and HL32 (5`-TTTAATTCGCGCTCACCTCGCCGCT-3`) (Metabion International AG, Martinsried/Deutschland) could amplify specific *BoLA-DRB3.2** gene fragment sized 284 bp by PCR.

Genomic DNA Isolation: DNA was extracted from anticoagulated blood by modification of the standard procedure involving digestion with proteinase K and extraction with phenol: chloroform: isoamyl alcohol followed by precipitation with absolute ethanol [60]. The working DNA concentration was adjusted to 100 ng/ µl.

Amplification of *BoLA-DRB3.2 Fragments by Single Step PCR:** All of the PCR assays were performed in a total volume of 25 µl. Each PCR mix prepared with 1 µl of genomic DNA (100 ng/ µl), 50 pM/ µl of each primer, 20 µl of Ready TaqMix Complete Mater Mix (AllianceBio, USA) and nuclease free water (Qiagen) to complete the total volume of the reactions. PCR steps were performed in a PTC-100™ Thermal Cycler (MJ Research Inc., USA). The thermal cycling profile was as follows: initial denaturation at 94°C for 2 min then 30 cycles of 92°C for 1 min, 66°C for 1 min and 1 min of elongation at 72°C. Final extension was carried out at 72°C for 10 min. followed by cooling down to 4°C [59, 13]. A reagent blank (containing all the components of the reaction mixture

with Mill Q water instead of genomic DNA) run as controls in every PCR procedure. Amplified products from the PCR were electrophoresed on 2% agarose gels in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.3, Sigma Aldrich) and stained with ethidium bromide (Sigma Aldrich). A 100 bp ladder (Axygen Bioscince) was used as DNA marker with each gel. The stained gels were viewed using a standard UV transilluminator (312 nm, Spectronic).

Sequencing of *BoLA-DRB3* Exon 2 PCR Products: Each amplicon was purified for sequencing using the QIAquick Spin PCR Purification kit (Qiagen) according to the manufacturer's instructions. Sequencing reactions were performed with the Dye Terminator DNA sequencing kit on an ABI 3100 DNA sequencer (Applied Biosystems, USA), as described by the manufacturer. Each sequencing reaction was repeated at least three times in both the forward and reverse directions before being accepted for analysis.

Construction of Phylogenetic Tree Based on Genotypes of *BoLA-DRB3.2:** Sequences of *BoLA-DRB3.2** amplified fragments were aligned using Nucleotide BLAST program of NCBI [61] for sequence homology searches against genbank databases. The obtained sequences were assembled using ChromasPro 1.49 beta (Technelysium Pty Ltd, Tewantin, Australia). Multiple sequences were aligned using the ClustalW 1.8® program [62]. Aligned sequences were edited by BioEdit sequence alignment editor (V. 7.0.9.0). Rooted phylogenetic tree was constructed with the neighbor joining method (NJ) [63] to infer the phylogenetic relationships between Egyptian present amplicons and others published in genbank. The evolutionary distance was calculated by the Maximum Composite method [64]. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 198 positions in the final dataset. The phylogenetic tree was linearized assuming equal evolutionary rates in all lineages [65]. The tree was drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree and were in the units of the number of base substitutions per site. Phylogenetic tree was constructed by MEGA4 [66].

Immune Parameter Analysis by Sandwich ELISA: Serum concentrations of two immunoglobulin' isotypes IgA (Uscn Life Science Inc., Wuhan) and IgG (Immunotek, ZeptoMetrix corporation, NY, USA) and interleukin-6 (IL-6) immune mediator (Antibodies-online, Germany) as indirect indication of humeral and cellular immune responses to subclinical mastitis were determined. Quantitative sandwich ELISAs were carried out utilizing coated plates according to manufacturer's instructions.

Statistical Analysis: Data of immune parameters were analyzed using T-test according to Sendecor and Cochran [67].

RESULTS

Udder Health Impression and Somatic Cell Count Analysis: Samples of apparently healthy cows with low SCC bulk milk tank (4×10^5 - 8×10^5 cells / ml), were CMT positivity (+1 & +2) and were found in udder quarters of 100 cows, including controls (less than 4×10^5 cells / ml).

Prevalence of Infections with Different Pathogenic Bacteria in Subclinical Mastitic Cows: Both contagious and environmental bacterial pathogens were isolated from subclinical mastitic cows in two clinical forms: single (detected within 27% of examined animals) and mixed (detected within 63% of examined animals) bacterial infections. From the 187 bacterial isolates characterized during the present study; environmental microorganisms (100/187) had higher prevalence recording 53.48% (32.09% (60/187) for *E. coli* and 21.39% (40/187) for CNS) than contagious bacteria (87/187) which recorded 46.52% prevalence within bacterial infections (22.46% (42/187) for *S. aureus* and 24% (45/187) for *Str. Agalactiae*).

The epidemiology of different types of infections in the studied population was determined. In single infection type (27/100); 18, 4, 3 and 2 were the prevalence of *S. aureus, E. coli, Str. agalactiae* and CNS among cases, respectively. In mixed form of infection (63/100), the lowest percentage (3%) was recorded in simultaneous infection with *S. aureus, Str. agalactiae, E. coli* and CNS, while absence of *S. aureus* from the mixture triggered the highest percentage (25%). Cows mixed infected with the environmental microorganisms (*E. coli* and CNS) recorded 10%, meanwhile, 7% was reported for contagious bacteria (*S. aureus* and *Str. agalactiae*). *S. aureus* infections recorded 8% when detected with *E. coli* but the percentage decreased to 6% when *Str. agalactiae* was added to the mixture of bacterial isolates. However, lower percentage was reported when *S. aureus* was removed from the mix and only *Str. agalactiae* and *E. coli* were the dominant types during infection.

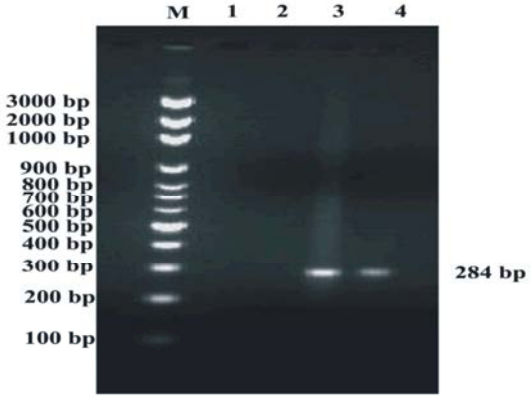

Fig. 1: PCR products amplified from coding sequences of MHC class II *DRB3* gene exon 2 (*BoLA-DRB3.2** sequences) representing Egyptian Holstein cattle during the present study electrophoresed in 2% agarose gel stained with ethidium bromide.
Lane M: Molecular weight marker (100-3000 bp).
Lanes 1 & 2: Control negative preparations.
Lanes 3 & 4: Specific molecular sized 284 bp amplicons representing *BoLA-DRB3.2** alleles obtained from Egyptian cows genomes.

Antibiogram Pattern: It was observed that variant bacterial isolates are highly resistant to cloxacillin, erythromycin, ampicillin, ofloxacin, penicillin and streptomycin recording 100, 90, 85, 80, 75 and 70%, respectively. While different susceptibility were recorded with oxytetracyclin (50%) and gentamycin (50%). In contrast, they were highly sensitive to amikacin (70%), ampicillin/sulbactam (80%) and amoxicillin/clavulanic acid (90%).

Statistical Analysis of Immune Globulins and Interleukin Reactivity by Sandwich ELISA: Optical density read during quantitative sandwich ELISA on sera indicated significant ($P < 0.01$) reactivity of IgA and IgG and IL-6 immune mediators to subclinical mastitis compared with control ones. In control group 227.62 ± 2.23, 762.84 ± 2.2 and 123.25 ± 1.1 were reported for IL-6, IgG and IgA, respectively. On the other hand, 252.64 ± 3.16, 895.11 ± 3.16 and 159.17 ± 1.31 were calculated as significant means compared to controls in subclinical mastitic group for IL-6, IgG and IgA at $P < 0.01$, respectively.

Phylogenetic Tree Construction Based on Genotypes of *BoLA-DRB3.2 and Analysis of their Frequency in Relation to Bacterial Pathogens and Host Immune Response:** The applied single step PCR method successes in amplifying 284 bp specific fragments from studied population as expected on the basis of nucleotide sequence of *BoLA-DRB3.2** gene exon 2 (Figure 1).

The present design was successful in identifying the allelic polymorphism of *BoLA-DRB3.2** in studied Egyptian Holstein cows when followed by direct sequencing then alignments on NCBI genbank. Alignments of obtained sequences with corresponding ones released in genbank identified 3 different alleles designated; BoLA-*DRB3.2*11*, *BoLA-DRB3.2*16* and *BoLA-DRB3.2*24* (Figures 2 & 3). Allelic frequencies were determined by direct counting in the total sample (n=100). The most frequent one was allele **16* (47%), followed by alleles **24* (31%) and **11* (22%) (Table 1). It worth to be mentioned that cows designated as control group of the study were found carrying the *BoLA-DRB3.2*16*

Table 1: Allelic frequencies of detected *BoLA-DRB3.2** genotypes in relation to prevalence of bacterial pathogens among subclinical mastitis correlated to SCC and CMT in studied Egyptian Holstein cows.

		*BoLA-DRB3.2** Alleles Frequency in Studied Cows (n=100 cows)			Udder Health Impression (n=100 cows)	
Isolates from Subclinical		*DRB3.2*11*	*DRB3.2*16*	*DRB3.2*24*		
Mastitic Cows (n= 187 isolates)		22% (22/100)	47% (47/100)	31% (31/100)	SCC	CMT
S. aureus	22.46%	73.8% Φ	2.38%	23.8%	4×10^5 to 8×10^5 cell/ml	+1 to +2
	(42/187)	(31/42)	(1/42)	(10/42)		
E. coli	32.09%	11.66%	26.66%	61.66% Φ		
	(60/187)	(7/60)	(16/60)	(37/60)		
Str. agalactiae	24.06%	71.11% Φ	4.44%	24.44%		
	(45/187)	(32/45)	(2/45)	(11/45)		
CNS	21.39%	40%	45% Φ	15%		
	(40/187)	(16/40)	(18/40)	(6/40)		
Bacteria-Free	0%					
(control group)	(0/187)	-/-	10 ∑	-/-	< 4×10^5 cells/ml.	+1

Φ Highest prevalence of specific bacteria within specific *BoLA-DRB3.2** allele.

∑ Control group cows (n=10) used during present study were all coding for *DRB3.2*16* allele.

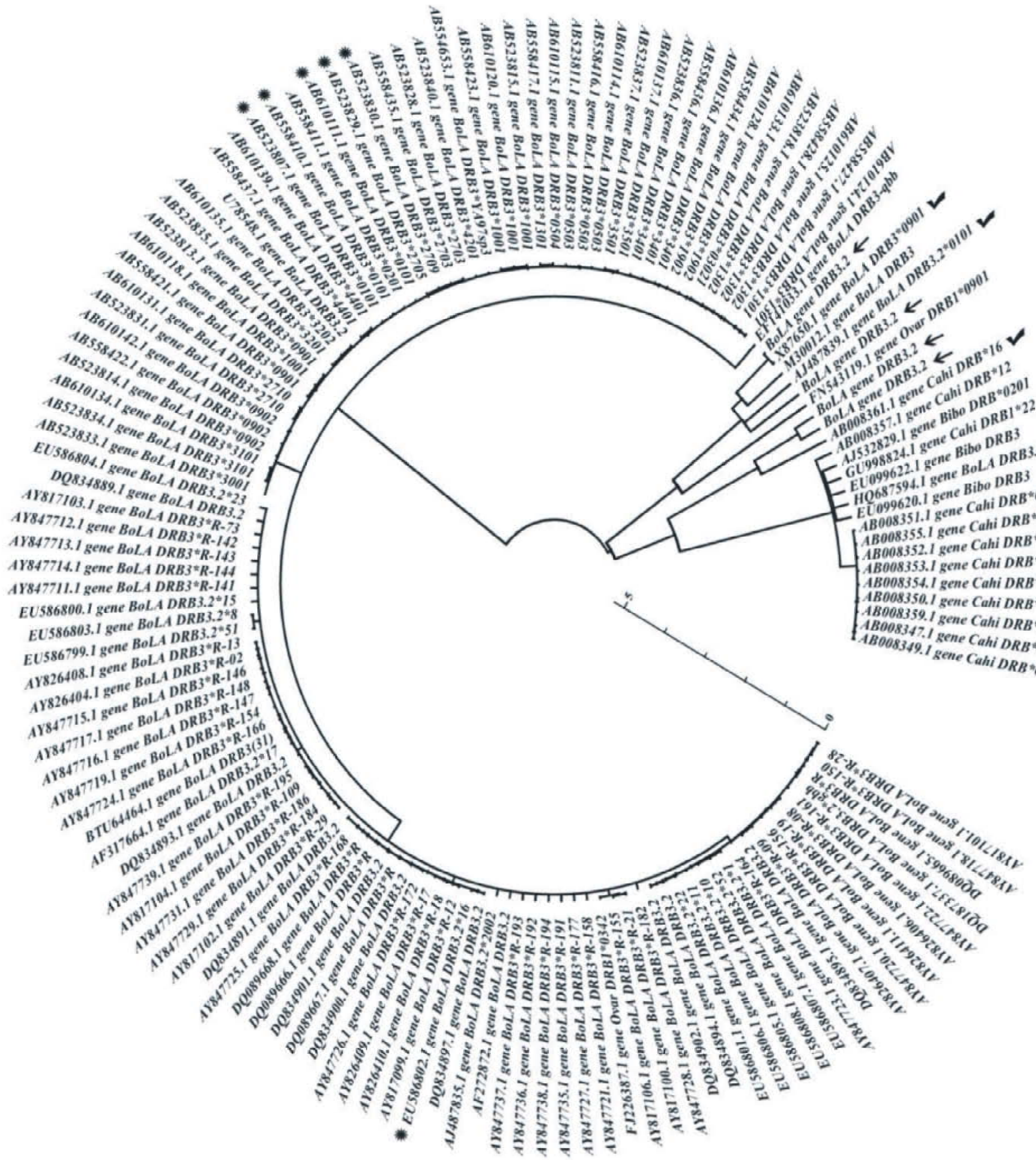

Fig. 2: A neighbor-joining phylogenetic tree showing genotypes of MHC class II *DRB3* gene exon 2 of different taxa with the sum of branch length = 34.13081705. The genbank accession numbers of used published sequences are next to each record. The related genotypes of *BoLA-DRB3.2** sequences are labeled, where the novel genotypes amplified from Egyptian Holstein cattle are marked with (?). While the highly identical or related genotypes are marked with (v). Finally, the related but less identical records to Egyptian genotypes are marked with (*).

allele (10/47) as shown in table 1. The prevalence of each bacterial infections within each allele recorded in the study in relation to SCC and CMT results were shown in table 1.

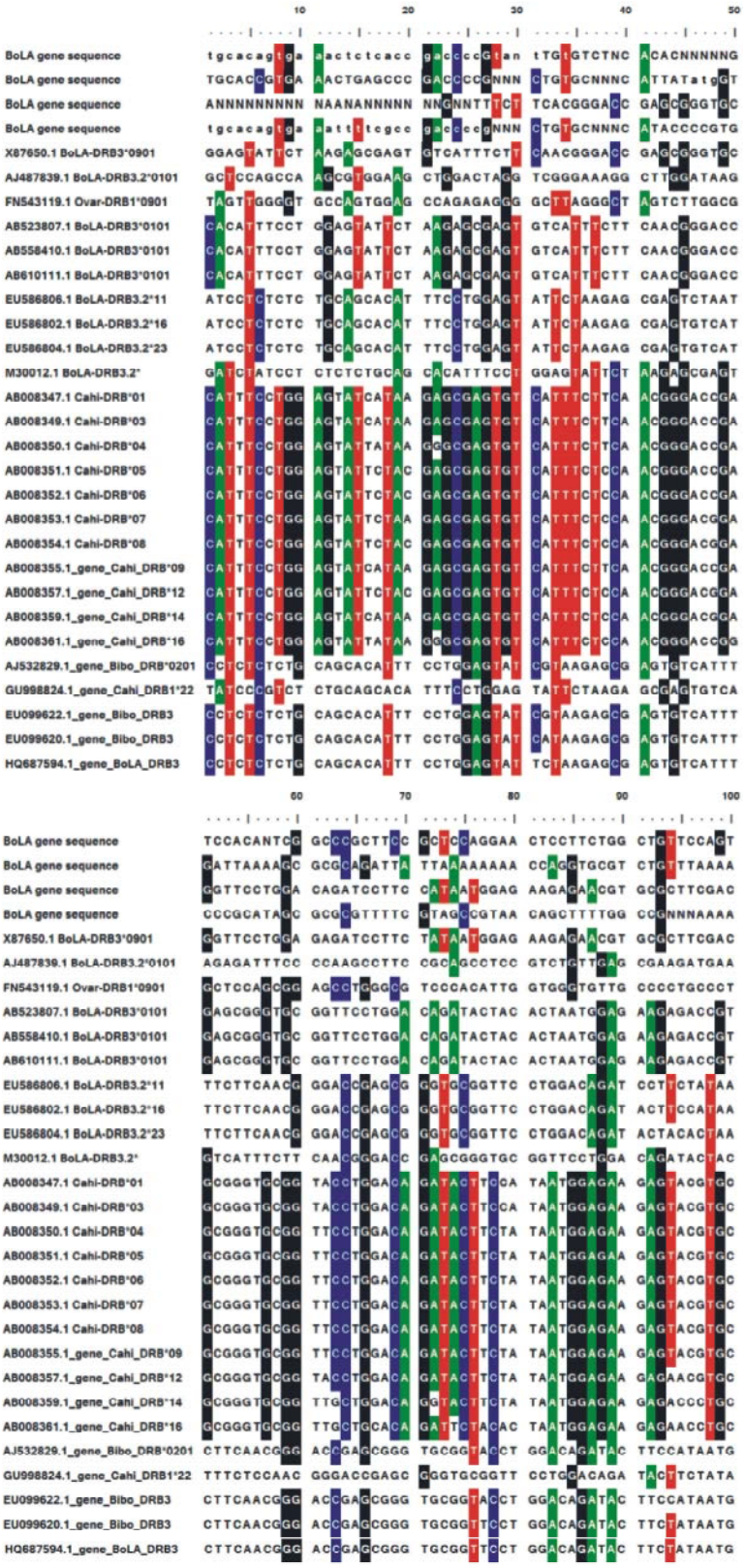

Fig. 3: Continued

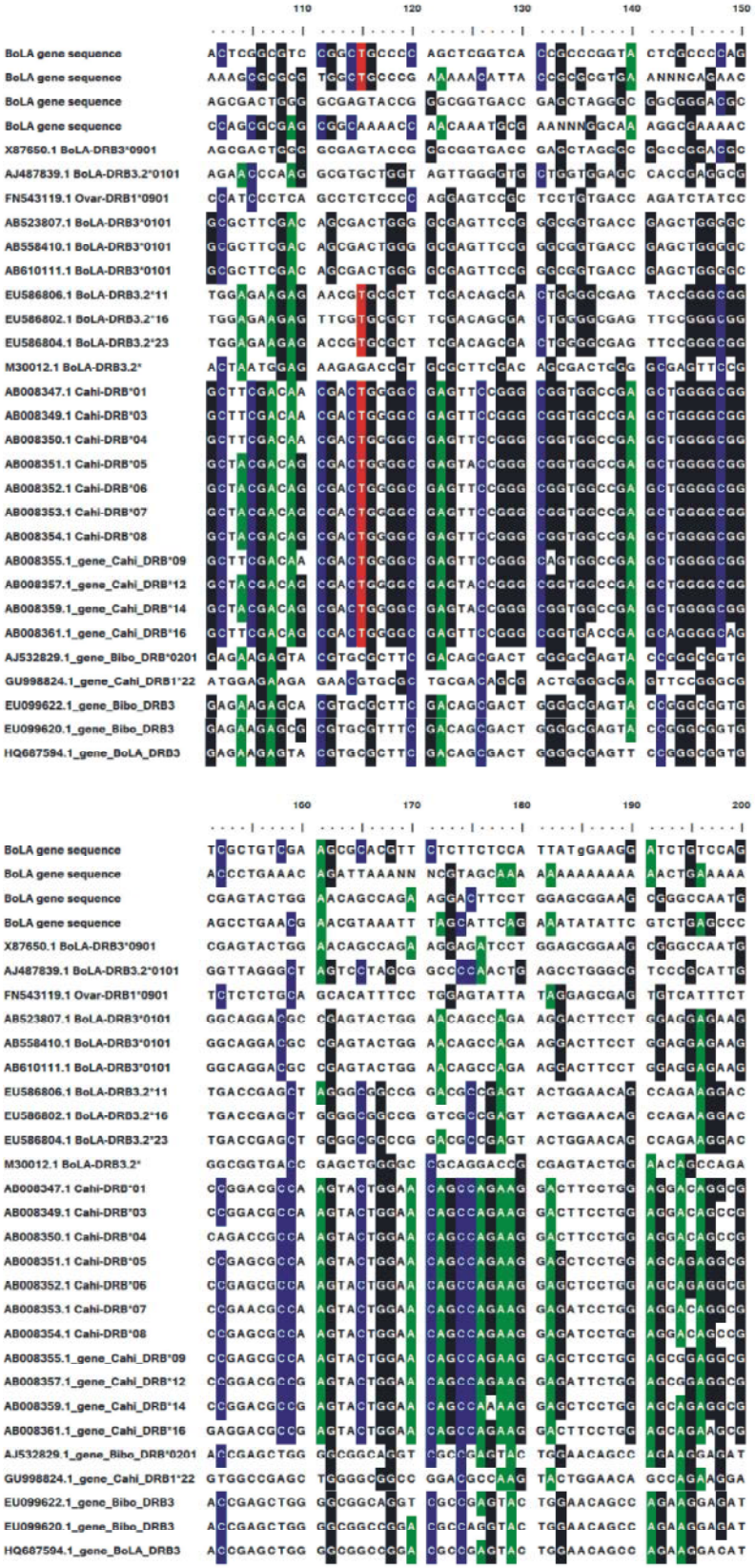

Fig. 3: Continued

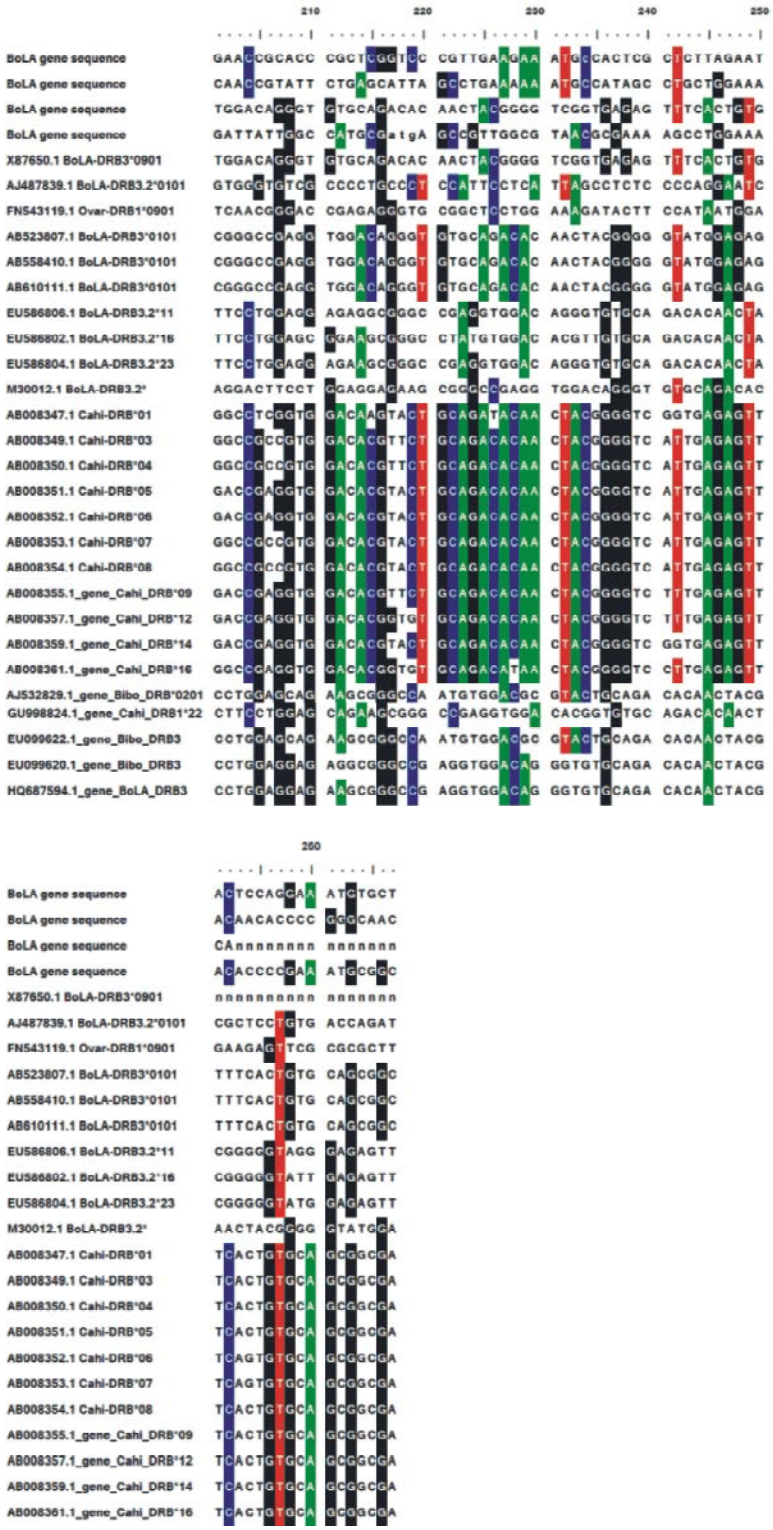

Fig. 3: Sequence alignment of *BoLA-DRB3.2** genotypes amplified from Egyptian Holstein cattle during the present study. Colored blocks revealing evolutionary relationship of MHC class II *DRB3* gene exon 2 of novel Egyptian genotypes to identical and/or related *BoLA-DRB3.2** genotypes from different taxa. The genbank accession numbers of used published sequences are shown next to each record.

DISCUSSION

In the present study, analysis of *BoLA-DRB3.2** alleles polymorphism in Egyptian subclinical mastitic Holstein cattle by construction of phylogenic tree; to identify the evolutionary relationship of the sequences of local breed with those previously released in genbank resulted in identification of three *DRB3.2** alleles which were *BoLA-DRB3.2*11, *16* and *24*. These are similar to those recorded in earlier studies by Dietz and coworkers [9, 20] who carried out polymorphism studies on the *BoLA-DRB3.2** locus in population of Holstein cows. The research team reported that *BoLA-DRB3.2*8, *11, *16, *22, *23* and *24* were the six most frequently detected alleles. Also Sharif and his team [10] reported the same alleles in addition to *BoLA-DRB3.2*3* genotype. In another study carried out on Iranian Holstein cows, the most frequent alleles were *BoLA-DRB3.2*8, *11, *16* and *24* [68]. Therefore, the three recorded alleles in the current work belong to the most common alleles identified in Holstein cattle. However, the revealed topology from the constructed phylogenic tree, illustrated that the obtained alleles homologous to *DRB3.2*16* and *24* are new subtypes different from those recorded in genbank (Figures 2 & 3). Since sequences of the two genotypes samples were more identical to allele *DRB3.2*16* from goat genome AB008361.1 (*Cahi-DRB *16*) than to that of bovine origin (*BoLA-DRB3.2*16*: EU586802.1) as shown in figures (2 & 3). While sequence of *BoLA–DRB3.2* of the other genotype was found closure to that previously recorded for allele *24*, subtype *3.2*0101* (Accession on: AJ487839.1) than the other three records of *3.2*0101* (Accession number: AB610111.1, AB558410.1 and AB523807.1, respectively) as shown in figures (2 & 3). Additionally, the analyzed sequence of *BoLA* gene locus 3 of the remaining Egyptian cows genotype sequence sample was identified as allele *BoLA-DRB3.2*11* with higher nucleotides identity to subtype *3.2*0901* of allele *BoLA-DRB3.2*11* recorded in genbank with accession number X87650.1 (Figures 2 & 3). The recorded differences in the results of the present investigation from previous ones done on Holstein cows may largely be due to the long term adaptation of examined population to different geographical and climatic conditions [72]. Therefore, *BoLA-DRB3.2** genotypes polymorphism information obtained from the research herd seems reprehensive of regional Holstein population.

BoLA-DRB3.2 alleles potentially affect many traits to immunity, milk somatic cell score and mastitis [9]. Many obtained results confirmed the proposition that *BoLA-DRB3.2** genotypes can be used as a marker for somatic cell concentration in milk in consequence as a marker of susceptibility/resistance to mastitis in dairy cows [70]. The obtained results are in disagreement with Dietz and coworkers [9, 20] and Kelm and his research team [23] who found that *BoLA-DRB3.2*16* allele had a significant effect on higher SCC. Whereas Starkenbureg and coworkers [24] found association between allele *16* and lower SCC. In continuity, alleles *DRB3.2*11*(homologous to *3.2*0901*) and *DRB3.2*24* (homologous to *3.2*0101*) obtained in the present study correlated with low SCC. *BoLA-DRB3.2** is characterized by a large number of gene variants which corresponds to the actual variability within *DRB3.2* genes, this great variability is likely to be the reason for contradictory results [70]. The influence of *BoLA* alleles among pathogenic stimuli showed that *S. aureus* and *Str. agalactiae* tend to cause more sustained increase in SCC but *E. coli* usually cause immediate increases in SCC that may quickly subsides. Nonetheless, the non-significant effect of SCC as marker in the present study might be due to the fact that animals were all apparently healthy with subclinical form of mastitis and therefore, the count of somatic cells was independent of genotypes of *BoLA-DRB3.2** in studied cattle.

The obtained data of subclinical mastitic cows accompanied with the detection of four major pathogens isolated on specific media, where *Str. agalactiae* and *S. aureus* are considered among the contagious pathogens, while *E. coli* and CNS are ranked among the environmental ones as previously had been classified [9, 20, 71]. Infections caused by *S. aureus* are subclinical in nature with periodic flare-up of clinical symptoms. While *Str. agalactiae*, *E. coli* and CNS cause milder form of mastitis with slight increase in milk cells count [69]. The obtained results indicated that cows carrying alleles *DRB3.2*16* were more resistant to intramammary infection caused by major pathogens, meanwhile, cows expressing allele *11* showed significant correlation with susceptibility to developing mastitis despite significant immune reactivity. On the other hand, cows whose genome coding for allele *24* did not exhibit clear cut between resistance or susceptibility which indicated epidemiological role in maintaining source of infection for these major bacterial pathogens in dairy farm that mostly characterize subclinical and chronic form of infections. The difference here may have genetic base related to microorganisms, host status, their interaction, environmental effect on both of them, as well as farm management [69].

Yoshida *et al*. [72] reported that susceptibility/ resistance to mastitis causing pathogens is due to various amino acids substitution. They also reported that *BoLA-DRB3.2*0101* (**24*) and **16* are considered to have specific susceptibility to all 4 mastitis pathogens isolated in the present study [73]. Which contradict with the obtained results in the present study where alleles *DRB3.2*11*(homologous to *3.2*0901*) and *DRB3.2*24* (homologous to *3.2*0101*) were more susceptible to mastitis infectious causes than *DRB3.2*16* genotype. Present observations could be explained by previous theory illustrated that different pathogens are present in different environments and the same allele may respond differently to alternative pathogens during various time of evolution [27]. Additionally, pressure from rapidly evolving pathogens is largely responsible for generating and maintaining this diversity which may relate to the molecular structure and antigen presenting capacity of specific allele [21]. Suitable antibiotics were used as prophylactic doses in studied cases and kept under observation to detect the flaring up of subclinical to clinical mastitis [74-77]. Amikacin, ampicillin/sulbactam and amoxicillin/clavulanic acid were the drugs of choice on recommendations for prophylaxes as well as treatment of clinical individuals.

BoLA-DRB3 gene analysis in cattle is of special interest due to its high functional importance as one of the key genes controlling the immune response of the individuals to viral, bacterial and parasitic infections accompanied by high level of polymorphism [78]. *BoLA-DRB3.2** alleles are significantly associated with humeral and cellular [21], as well as, innate and adaptive immunity [79] which reflects variations in class II surface protein represented by genotypes possessed by cows; these surface proteins may perform their role with differing efficiencies or respond differently to the environmental challenges [78]. The present investigation revealed a significant increase in IL-6, IgA and IgG levels. Interlukine-6 is one of the most important mediator of fever and acute phase response, which is derived from activated T lymphocytes and macrophages in response to specific microbial molecules referred to as pathogen associated molecular pattern (PAMPs) [78]. The increase in IL-6 in inflamed mammary glands correlates with the level of inflammation and tissues damage [80, 81]. IgA is the predominant class of immunoglobulins present on mucosal surface constitutes an important defense mechanism against microbial infections within inflamed udder. The increase in its level indicated passive transfer of IgA from the serum to the mammary secretion which was the first line of defense against pathogens [82]. Meanwhile, the increase in IgG level may be due to the presence of indirect association between *DRB3.2* alleles and immune function including IgG [26]. Although there are no results directly correlating *BoLA* complex with the number of mast cells, however, there is some indication of an association between *BoLA-DRB3.2* alleles **11, *24, *12, *3* and **28* and increased IgG2 concentration and between allele **26* and decreased IgG2 [20]. More information was added by Rupp and coworkers [21], they elucidated that *DRB3.2*16* and **24* are associated with high antibody immune response. Whereas Dietz and his group [9] stated that allele *DRB3.2*11* is associated with increased level of IgG2 concentration. Association between single immune response variable and many alleles may reflect the homeostatic nature of the immune system response to variation in *DRB3* genotype or the surrounding loci.

To our knowledge, there are no previous studies on *BoLA–DRB3.2** genotypes in Egyptian Holstein breed. This study tended to utilize direct sequencing of PCR products amplified from locus 3 to identify allele frequency of *BoLA-DRB3.2** genes. Since mastitis is a multifactorial disease with a complex etiology, these discrepancies might be explained by the difference in causative organism, genetic background, micro-environmental factors and interaction between environment and genetic background. The results obtained in this study highlighted the hypothesis of *DRB3* being a marker for susceptibility/resistance to mastitis in dairy cows. Where justifying the superior quality of cows carrying alleles *DRB3.2*16* found more resistant to infection, meanwhile, cows expressing allele **11* showed significant susceptibility for mastitis. In addition, the dangerous role played by other genotype (**24*) in maintaining infection in dairy farm hence characterized by subclinical form of infections. However, in spite of the small number of the animals that have been genotyped in the present study, the three alleles identified had variant subtypes due to different nucleotides composition from those released in genbank. These results indicated the difference existed between breeds and populations of cattle with regard to genetic composition as well as frequencies of specific *BoLA-DRB3.2** alleles. In continual, further studies are necessary to confirm these results before *BoLA-DRB3.2** genotypes can be recommended as marker used to select the animals at young age before actual expression of the traits of interest.

REFERENCES

1. El Seedy, F.R., M. El-Shabrawy, A.S. Hakim, S.F. Syame and N.M. Osman, 2012. Advanced techniques used for isolation and characterization of *Staphylococcus aureus* isolated from mastitic buffaloes. Global Veterinaria, 8(2): 144-152.
2. Dufour, S., A. Fréchette, H.W. Barkema, A. Mussell and D.T. Scholl, 2011. Invited review: effect of udder health management practices on herd somatic cell count. Journal of Dairy Science, 94: 563-579.
3. Bruno, D.R., P.V. Rossitto, R.G. Bruno, M.T. Blanchard, T. Sitt, B.V. Yeargan, W.L. Smith, J.S. Cullor and J.L. Stott, 2010. Differential levels of mRNA transcripts encoding immunologic mediators in mammary gland secretions from dairy cows with subclinical environmental Streptococci infections. Veterinary Immunology and Immunopathology, 138(1-2): 15-24.
4. Laport, M.S., P.R. Marinho, O.C. Santos, P. de Almeida, M.T. Romanos, G. Muricy, M.A. Brito and M. Giambiagi-Demarval, 2012. Antimicrobial activity of marine sponges against coagulase-negative *Staphylococci* isolated from bovine mastitis. Veterinary Microbiology, 155(2-4): 362-368.
5. Zaki, M.M., H.Y. El Zorba and H.A. Kaoud, 2010. Environmental Organisms as Risk Factors in the Occurrence of Mastitis in Dairy Buffaloes with Suggested Methods of Control. A Field Study. Global Veterinaria, 5(2): 97-105.
6. McDougall, S., K.I. Parker, C. Heuer and C.W. Compton, 2009. A review of prevention and control of heifer mastitis via non-antibiotic strategies. Veterinary Microbiology, 134(1-2): 177-185.
7. Axford, R.F.E., S.C. Bishop, F.W. Nicholas and J.B. Owen, 2000. The major histocompatibility complex and its role in disease resistance and immune responsiveness. Eds., CAB International, Wallingford, Oxfordshire, UK.
8. Schmutz, S.M., T.G. Berryere, J.W. Robbins and T.D. Carruthers, 1992. Resistance to *Staphylococcus aureus* mastitis detected by DNA marker. In the Proceeding of 31st Annual Meeting of the National Mastitis Council, Arlington, VA, National Mastitis Council, Inc, Madison, Wisconsin, pp: 124-133.
9. Dietz, A.B., N.D. Cohen, L. Timms and M.E.Jr. Kehrli, 1997a. Bovine lymphocyte antigen class II alleles as risk factors for high somatic cell counts in milk of lactating dairy cows. Journal of Dairy Science, 80: 406-412.
10. Sharif, S., B.A. Mallard, B.N. Wilkie, J.M. Sargeant, H.M. Scott, J.C.M. Dekkers and K.E. Leslie, 1998. Association of the bovine major histocompatibility complex *DRB3* (*BoLA-DRB3*) alleles with occurrence of disease and milk somatic cell score in Canadian dairy cattle. Animal Genetics, 29: 185-193.
11. Gilliespie, B.E., B.M. Jayarao, H.H. Dowlen and S.P. Oliver, 1999. Analysis and frequency of bovine lymphocyte antigen *DRB3.2* alleles in Jersey cows. Journal of Dairy Science, 82: 2049-2053.
12. Takeshima, S., Y. Nakai, M. Ohta and Y. Aida, 2002. Characterization of *DRB3* alleles in the MHC of Japanese Shorthorn cattle by polymerase chain reaction-sequence-based typing. Journal of Dairy Science, 85: 1630-1632.
13. Behl, J.D., N.K. Verma, R. Behl, M. Mukesh and S.P.S. Ahlawat, 2007. Characterization of genetic polymorphism of bovine lymphocyte antigen *DRB3.2* locus in kankrej cattle (*Bos indicus*). Journal of Dairy Science, 90: 2997-3001.
14. Fernandez, I.G., J.G. Rios Ramirez, A.G. Vazquez, R.U. Arvizu and R.A. Alonso Morales, 2008. Polymorphism of locus *DRB3.2* in populations of Creole cattle from northern Mexico. Genetics and Molecular Biology, 31(4): 880-886.
15. da Mota, A.F., M.L. Martinez and L.L. Coutinho, 2004. Genotyping *BoLA-DRB3* alleles in Brazilian cattle (*Bos indicus*) by temperature gradient gel electrophoresis (TGGE) and direct sequencing. European Journal of Immunogenetics, 31: 31-35.
16. Rupp, R. and D. Biochard, 2003. Genetics of resistance to mastitis in dairy cattle. Veterinary Research, 34: 671-688.
17. Abdel Hameed, K.G., G. Sender and M. Mayntz, 2006. Major histocompatibility complex polymorphism and mastitis resistance- a review. Animal Science Papers and Reports, 24(1): 11-25.
18. http://www.projects.roslin.ac.uk/bola/
19. Maillard, J.C., C. Renard, P. Chardon, I. Chantal and A. Bensaid, 1999. Characterization of 18 new *BoLA-DRB3* alleles. Animal Genetics, 30: 200-203.
20. Dietz, A.B., J.C. Detilleux, A.E. Freeman, D.H. Kelly, J.R. Stable and M.E. Jr. Kehrli, 1997b. Genetic association of bovine lymphocyte antigen *DRB3* alleles with immunological traits of Holstein cattle. Journal of Dairy Science, 80: 400-405.
21. Rupp, R., A. Hernandez and B.A. Mallard, 2007. Association of bovine leukocyte antigen (*BoLA*) *DRB3.2* with immune response, mastitis and production and type traits in Canadian Holsteins. Journal of Dairy Science, 90: 1029-1038.

22. Dietz, A.B., J.C. Detilleux, A.E. Freeman, D.H. Kelly, J.R. Stable and M.E. Jr. Kehrli, 1997c. Genetic association between parameters of innate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science, 80: 1767-1775.
23. Kelm, S.C., J.C. Detilleux, A.E. Freeman, M.E.Jr. Kehrli, A.B. Dietz, L.K. Fox, J.E. Butler, I. Kasckovics and D.H. Kelley, 1997. Genetic association between parameters of inmate immunity and measures of mastitis in periparturient Holstein cattle. Journal of Dairy Science, 80(8): 1767-1775.
24. Starkenburg, R.J., L.B. Hansen, M.E.Jr. Kehrli and H. Chester-Jones, 1997. Frequencies and effects of alternative *DRB3.2* alleles of bovine lymphocyte antigen for Holsteins in milk selection and control lines. Journal of Dairy Science, 80: 3411-3419.
25. Kumar, S., M.L. Sangwan and Rupender, 2008. Polymorphism in DRB3 exon 2 by PCR-RFLP and its association with mastitis in *Nili-Ravi* breed. Indian Journal of Biotechnology, 7: 398-400.
26. Firouzamandi, M., J. Shoja, A. Barzegari and E. Roshani, 2010. Study on the association of *BoLA-DRB3.2* alleles with clinical mastitis in Iranian Holstein and *Sarabi* (Iranian native) cattle. African Journal of Biotechnology, 9(15): 2224-2228.
27. Nascimento, C.S., A.L.S. Azevedo, M.A. Machado, M.L. Martinez, M.V.G.B. Silva, M.F.M. Guimarães, A.L. Campos, R.L. Teodoro, R.S. Verneque, S.E.F. Guimarães and D.A.A. Oliveira, 2006. Association of *BoLA-DRB3* alleles with milk constituents and somatic cell score in *Gyr* cattle. In the 8th World Congress on Genetics applied to Livestock production, 13-18 Aug, Belo Horizonte, MG, Brazil.
28. Watkins, D.I., J.A. Shadduck, C.E. Rudd, M.E. Stone, H.A. Lewin and N.L. Letvin, 1989. Isoelectric focusing of bovine major histocompatibility complex class II molecules. European Journal of Immunogenetics, 19: 567-570.
29. Davies, C.J. and D.F. Antczak, 1991. Prodution and characterization of alloantisera specific for bovine class II major histocompatibility complex antigens. Animal Genetics, 22: 417-434.
30. Davies, C.J., L. Andersson, I. Joosten, P. Mariani, L.G. Gasbarre and E.J. Hensen, 1992. Characterization of bovine MHC class II polymorphism using three typing methods: serology, RFLP and IEF. European Journal of Immunogenetics, 19: 253-262.
31. Glass, E.J., R.A. Oliver, J.L.W. Williams and P. Millar, 1992. Alloreactive T-cell recognition of bovine major histocompatibility complex class II products defined by one-dimensional isoelectric focusing. Animal Genetics, 23: 97-111.
32. Paswan, C., B. Bhushan, B.N. Patra, P. Kumar, A. Sharma, S. Dandapat, A.K.S. Tomar and T. Dutt, 2005. Characterization of MHC *DRB3.2* alleles of crossbred cattle by polymerase chain reaction-restriction fragment length polymorphism. Asian-Australian Journal of Animal Science, 18: 1226-1230.
33. Sitte, K., I.J. East, M.F. Lavin and E.C. Jazwinska, 1995. Identification and characterization of new *BoLA-DRB3* alleles by heteroduplex analysis and direct sequencing. Animal Genetics, 26: 413-417.
34. Van Eijk, M.J.T., J.A. Stewart-Haynes and H.A. Lewin, 1992. Extensive polymorphism of the *BoLA-DRB3* gene distinguished by PCR-RFLP. Animal Genetics, 23: 483-489.
35. Sitte, K., I.J. East and E.C. Jazwinska, 1996. Detection of a common *BoLA-DRB3* deletion by sequence-specific oligonucleotide typing. Animal Genetics, 27: 271-273.
36. Aldridge, B.M., S.M. McGuirk, R.J. Clark, L.A. Knapp, D.I. Watkins and D.P. Lunn, 1998. Denaturing gradient gel electrophoresis: a rapid method for differentiating *BoLA-DRB3* alleles. Animal Genetics, 29: 389-394.
37. Jugo, B.M. and A. Vicario, 2000. Single-stand conformational polymorphism and sequence polymorphism of MHC-DRB in Laxta and Karrantzar sheep: Implication for Caprine phylogeny. Immunogenetics, 51: 887-897.
38. Jugo, B.M. and A. Vicario, 2001. Lymphocyte antigens in sheep: linkage to the MHC class II *DRB* I gene. European Journal of Immunogenetics, 28: 451-458.
39. Pipalia, D.L., C.G. Joshi, D.N. Rank, B.P. Brahmkshtri and J.V. Solanki, 2004. PCR-SSCP typing of MHC in cattle and buffaloes. Indian Journal of Animal Science, 74(6): 637-639.
40. Aida, Y., M. Niimi, M. Asahina, K. Okada, Y. Nakai and K. Ogimoto, 1995. Identification of a new bovine MHC class II *DRB* allele by nucleotide sequencing and an analysis of phylogenetic relationships. Biochemical and Biophysical Research Communications, 209: 981-988.

41. Davies, C.J., L. Andersson, S. Mikko, S.A. Ellis, E.J. Hensen, H.A. Lewin, N.E. Muggli-Cockett, J.J. van der Poel and G.C. Russell, 1997. Nomenclature for factors of the *BoLa* system, 1996: report of the ISAG *BoLA* Nomenclature Committee. Animal Genetics, 28: 159-246.
42. Russell, G.C., C.J. Davies, L. Andersson, S.A. Ellis, E.J. Hensen, H.A. Lewin, S. Mikko, N.E. Muggli-Cockett and J.J. van der Poel, 1997. *BoLA* class II nucleotide sequences. Animal Genetics, 28: 169-180.
43. Takeshima, S., M. Ikegami, M. Morita, Y. Nakai and Y. Aida, 2001. Identification of new cattle *BoLA-DRB3* alleles by sequence-based typing. Immunogenetics, 53: 74-81.
44. Miltiadou, D., A.S. Law and G.C. Russell, 2003. Establishment of a sequence-based typing system for *BoLA-DRB3* exon 2. Tissue Antigens, 62: 55-65.
45. Jeong, H.J., M.S.A. Bhuyan, J.S. Lee, S.L. Yu, B.C. Sang, D. Yoon, J.T. Jeon and J.H. Lee, 2007. Characterization of *BoLA-DRB3.2* alleles in *Hanwoo* (Korean cattle) by sequence based typing (SBT). Asian-Austuralian Journal Animal Science, 18, (The Free Library, 1st Dec.) http://www.thefreelibrary.com/
46. El-Rashidy, A.A., L. Berier, A.S. Amin Mostafa and M.S. Tawfik, 1986. Etiology and diagnosis of subclinical mastitis among dairy herd in Egypt. Journal of Egyptian Medical Association, 48: 289-296.
47. Abdel-Fattah, A.A., A.S. Amin and R.H. Hamouda, 2008. Detection of *E.coli* O_{157}:H_7 in bovine milk and fecal samples by the polymerase chain reaction assay. Journal of Egyptian Medical Association, 68(3): 83-92.
48. El-Khodery, S.A. and S.A. Osman, 2008. Acute *coliform* mastitis in buffaloes (*Bubalus bubalis*): clinical findings and treatment outcomes. Tropical Animal Health and Production, 40(2): 93-39.
49. Nash, D.L., G.W. Rogers, J.B. Cooper, G.L. Hargrove, J.F. Keown and L.B. Hansen, 2000. Heritability of clinical mastitis incidence and relationships with sire transmitting abilities for somatic cell score, udder type traits, productive life and protein yield. Journal of Dairy Science, 83(10): 2350-60.
50. Stear, M.J., S.C. Bishop, B.A. Mallard and H. Raadsma, 2001. The sustainability, feasibility and desirability of breeding livestock for disease resistance. Research in Veterinary Science, 71: 1-7.
51. Shook, G., 2001. Breeding, selection and somatic cell counts: where are we today? National Mastitis Council Annual Meeting Proceeding, pp: 113.
52. National Mastitis Council (NMC), 1999. Laboratory Handbook on Bovine Mastitis. Revised Ed, National Mastitis Council Inc, Madison.
53. Hogan, S.J., R.N. Gonzalez, J.R. Harmon, S.C. Nickerson, S.P. Oliver, J.W. Pankey and L.K. Smith, 1999. Laboratory Handbook on Bovine Mastitis. National Mastitis Council Inc., WD Hoard, Fort Atkinson, USA.
54. Quinn, P.J., M.E. Carter, B.K. Markey and G.R. Carter, 2000. Clinical Veterinary Microbiology. London: Mosby-Year Book Europe Limited, pp: 120-121.
55. International Dairy Federation (IDF), 1999. Suggested interpretation of mastitis terminology. Bulletin of the International Dairy Federation, pp: 338.
56. Hamann, J., 2003. Definition of the physiological cell count threshold based on changes in milk composition. IDF Mastitis Newsletter, 25: 9-12.
57. Quinn, P.J., B.K. Markey, M.E. Carter, W.J.C. Donnelly, F.C. Leonard and D. Maguise, 2002. Bacterial causes of bovine mastitis: Environmental microorganism infrequent associated with bovine mastitis. In the Veterinary Microbiology and Microbial Diseases (1st ed.), Blackwell Science Ltd., Blackwell Publishing Company, Section IV, 81: 465-475.
58. Oxoid Manual, 2000. The oxoid manual of culture media ingredients and other laboratory services, (10th ed.), Wade Road Basingstoke, Oxoid Limited, Hampshire, England.
59. Sigurdardottir, S., C. Borsch, K. Gustafasson and L. Andersson, 1991. Cloning and sequence analysis of 14 *DRB* alleles bovine MHC by PCR. Animal Genetics, 22: 199-209.
60. Sambrook, J., E.F. Fritscgh and A. Meniates, 1989. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York.
61. http://www.ncbi.nlm.nih.gov/BLAST/
62. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22: 4673-4680.
63. Saitou, N. and M. Nei, 1987. The neighbor-joining method: a new method for sequences. Journal of Molecular Biology, 16: 111-120.
64. Tamura, K., M. Nei and S. Kumar, 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA), 101: 11030-11035.

65. Takezaki, N., A. Rzhetsky and M. Nei, 2004. Phylogenetic test of the molecular clock and linearized trees. Molecular Biology and Evolution, 12: 823-833.
66. Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution, 24: 1596-1599.
67. Snedecor, G.W. and W.C. Cochran, 1982. Statistical Methods (8th ed.). Iowa State University Press, Ames, USA.
68. Mohammadi, A., M.R. Nassiry and J. Mosafer, 2009. Distribution of *BoLA-DRB3* allelic frequencies and identification of a new allele in the Iranian cattle breed *sistani* (*Bos indicus*). Russian Journal of Genetics, 45(2): 224-229.
69. Abdel Hameed, K.G., G. Sender and A. Korwin-Kossakowska, 2008. An association of *BoLA* alleles *DRB3.2*16* and *DRB3.2*23* with occurrence of mastitis caused by different bacterial species in two herds of dairy cows. Animal Science Papers and Reports, 26(1): 37-48.
70. Katarzyna, W.M., M. Kmiec, I. Kowalewska-Luczak and M. Warlinski, 2010. *DRB3* gene polymorphism and somatic cell count in milk of Jersey cows. Journal of Animal and Veterinary Advances, 9(9): 1295-1300.
71. Radostits, O.M., K.E. Leslie and J. Fetrow, 1994. Herd Health. In: Food Animal Production Medicine. W.B. Saunders Company Ltd, London, United Kingdom, pp: 229-273.
72. Yoshida, T., H. Mukoyama, H. Furuta, Y. Kondo, S.N. Takeshima, Y. Aida, M. Kosugiyama and H. Tomogane, 2009a. Association of *BoLA-DRB3* alleles identified by a sequence-based typing method with mastitis pathogens in Japanese Holstein cows. Animal Science Journal, 80(5): 498-509.
73. Yoshida, T., H. Mukoyama, H. Furuta, Y. Kondo, S.N. Takeshima, Y. Aida, M. Kosugiyama and H. Tomogane, 2009b. Association of the amino acid motifs of *BoLA-DRB3* alleles with mastitis pathogens in Japanese Holstein cows. Animal Science Journal, 80(5): 510-519.
74. Bhatt, V.D., M.S. Patel, C.G. Joshi and A. Kunjadia, 2011. Identification and antibiogram of microbes associated with bovine mastitis. Animal Biotechnology, 22(3): 163-169.
75. Ahmed, W.M., S.I. AbdEl-Moez and G.M. Nabil, 2008. Observations on sub-clinical mastitis in buffalo-cows with emphasis on measuring of milk electrical resistance for its early detection. Global Veterinaria, 2(1): 41-45.
76. Beheshti, R., J. Shaieghi, B. Eshratkhah, J.G. Ghalehkandi and N. Maheri-Sis, 2010. Prevalence and Etiology of Subclinical Mastitis in Ewes of the Tabriz Region, Iran. Global Veterinaria, 4(3): 299-302.
77. Bedacha, B.D. and H.T. Menghistu, 2011. Study on prevalence of mastitis and its associated risk factors in lactating dairy cows in batu and its environs, Ethiopia. Global Veterinaria, 7(6): 632-637.
78. Sharifzadeh, A. and A. Doosti, 2011. Polymorphism of *BoLA-DRB 3.2* gene in Iranian native cattle by using PCR-RFLP method. Journal of Cell and Animal Biology, 5(3): 47-52.
79. Nassiry, M.R., F.E. Shahroodi, J. Mosafer, A. Mohammadi, E. Manshad, S. Ghazanfari, A.M.R. Mohammad and G.E. Sulimova, 2005. Analysis and frequency of bovine lymphocyte antigen (*BoLA-DRB3*) alleles in Iranian Holstein cattle. Genetika Journal, 41(6): 817-822.
80. Bannreman, D.D., 2009. Pathogen dependent induction of cytokines and other soluble inflammatory mediators during intrammary infection of dairy cows. Journal of Animal Science, 87(13): 16-25.
81. Sakemi, Y., Y. Tamura and K. Hagiwara, 2011. Interlukin-6 in quarter milk as a further prediction marker for subclinical mastitis. Journal of Dairy Research, 78(1): 118-121.
82. Song, X.M., J. Perez-Casel, M.C. Fontain and A. Potter, 2002. Bovine immunologlobulin A (IgA)-binding activities of the surface-expressed Mig protein of *Streptococcus dysagalactia*. Microbiology, 148(7): 2055-2064.