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Genetic Polymorphism of *BoLA-DRB3* Exon 2 in Egyptian Buffalo

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ABSTRACT

The role of the *BoLA-DRB3* gene in the development of an immune response system and its highly polymorphic nature make it one of the most important candidate genes in disease resistance studies. PCR-RFLP was used in this study to detect the genotypic polymorphism of *BoLA-DRB3* exon 2 in 50 Egyptian buffalo. The PCR amplified fragments at 284-bp were digested by two restriction enzymes, *Rsa*I and *Bst*YI. Digestion of the amplified fragments using *Rsa*I endonuclease yielded six alleles (B, F, I, K, L and O) with frequencies that ranged from 6% (I allele) to 40% (L allele). The presence of six alleles resulted in 14 different genotypes from the tested buffalo. These genotypes included three homozygous genotypes (B/B, L/L and O/O) with 6, 18 and 8% frequency, respectively, while the remaining 11 genotypes were heterozygous: B/F (2%), B/K (2%), B/L (6%), F/I (2%), F/L (10%), F/O (2%), I/K (2%), I/L (8%), K/L (8%), K/O (14%) and L/O (12%). Digestion of the PCR products with *Bst*YI yielded three alleles and five genotypes in Egyptian buffalo. The three alleles A, B and D showed 24, 62 and 14% frequency, respectively while the five genotypes included three homozygous genotypes AA (6%), BB (34%) and DD (4%) and two heterozygous genotypes AB (36%) and BD (20%). In conclusion, genetic polymorphism of *BoLA-DRB3* exon 2 in Egyptian buffalo displayed a total of 9 alleles and 19 genotypes distinguished by digestion of PCR products with two endonucleases. The results suggest that absence of the mastitis susceptibility alleles C and S in tested Egyptian buffalo and the presence of mastitis resistance alleles L in 40% and B in 11% of animals.

Keywords: *Bst*YI, *BolA-DRB3.2*, PCR, RFLP, *Rsa*I

INTRODUCTION

Major histocompatibility complex (*MHC*) of farm animals comprises of a group of closely linked genes, most of which are polymorphic and play a central and important role in the immune responsiveness and resistance/susceptibility to diseases that involve immune intervention (Kumar *et al.* 2008).

The Bovine leukocyte antigen (*BoLA*) is a gene of *MHC* in cattle (Parnian *et al.* 2006). There are two major classes of *MHC* molecules, both of which consist of A and B chains, but from different sources. *MHC* class *I* molecules (*MHC I*) consist of one membrane-spanning A chain (heavy chain) produced by *MHC* genes, and one B chain (light chain or b2-microglobulin) produced by the b2-microglobulin gene. *MHC* class *II* molecules (*MHC II*) consist of two membrane-spanning chains, A and B, of similar size and both produced by *MHC* genes. *MHC* molecules are cell surface proteins, which take active part in host immune reactions and involvement of *MHC* class *I* and *II* in response to almost all antigens (Gomase *et al.* 2009). *MHC II* molecules are of central important for adaptive immunity. Defective *MHCII* expression causes a severe immunodeficiency disease called bare lymphocyte syndrome (Krawczyk and Reith 2006).

The *MHC* class *II* genes are highly polymorphic and located either closely linked (as in human) or in two distinct chromosomal locations (as in cattle). *MHC* subclass *IIa* containing *DR* and *DQ*, while subclass *IIb* containing *DI, DY* and *DO* genes (Muggli-Cockett and Stone 1989; Ellis and Ballingall 1999).

There are at least three bovine *BoLA-DRB* genes which are *DRB1*, *DRB2* and *DRB3*, but only *BoL-ADRB3* was reported to be functional. The *BoLA-DRB1* gene is a pseudogene and the *BoLA-DRB2* gene is transcribed at a very low level (Lewin *et al.* 1999). The role of the *BoLA-DRB3* gene is the development of an immune response and it is highly polymorphic, therefore it is a candidate gene in disease resistance studies in different farm animals (Miretti *et al.* 2001). DRB3 exon 2 alleles were found to be associated with resistance or susceptibility to various diseases in cattle (Xu *et al.* 1993; Zanotti *et al.* 1996; Dietz *et al.* 1997; Sharif *et al.* 1998; Lewin *et al.* 1999; Baxter *et al.* 2008).

Buffalo *MHC* genes have been mapped to chromosome 2p (Iannuzzi *et al.* 1993). Compared to other ruminant species, buffalo *MHC* loci are poorly characterized. Few studies for detection of the genetic polymorphisms of *BoLA-DRB3* have been investigated in buffalo (Aravindakshan *et al.* 2000; Acharya *et al.* 2002; De *et al*. 2002; Ahmed and Othman 2006; Kumar *et al.* 2008).

In studies of variation in disease susceptibility, the ability to characterize *MHC* polymorphism and determine its relationship to immune responsiveness is an impact factor and required reliable and sensitive genotyping techniques (Sawhney *et al.* 2001; Behi *et al.* 2007). From various molecular techniques used to investigate the genetic polymorphism of *MHC* loci, PCR-RFLP has been reported a successful technique for determination of the genetic polymorhism of *BoLA-DRB3* gene in cattle (van Eijk *et al.* 1992; Sawhney *et al.* 2001; Parnian *et al.* 2006).

Based on the association between *BoLA-DRB3* exon 2 gene locus with various immunological traits, the study of the polymorphisms at this locus is potentially important. Our study aimed to use the PCR-RFLP method with two different restriction enzymes for detection of the genetic variability of *BoLA-DRB3* exon 2 in Egyptian buffalo.

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MATERIALS AND METHODS

Animals

A total of 50 blood samples of healthy and unrelated Egyptian buffalo were collected from different farms in Menoufia and Kafr el sheikh.

Genomic DNA extraction

Genomic DNA was extracted from the whole blood by phenol-chloroform method described by John *et al.* (1991) with minor modifications. Ten ml of blood taken on EDTA was mixed with 25 ml cold sucrose-triton (Merck, Germany) and the volume was completed to 50 ml by autoclaved double distilled water. The solution was mixed well and the nuclear pellet was obtained by spinning and discarding the supernatant. The nuclear pellet was suspended in lysis buffer (10 mM Tris base (Sigma Aldrich, Germany), 400 mM NaCl (Ran Baxy, Newdelhi, India) and 2 mM sodium EDTA (Sigma Aldrich) pH 8.2, with 20% sodium dodecyl sulfate (SDS) (Merck) and proteinase K (10 mg/ml, Bioron, Germany), and incubated overnight in a shaking water-bath at 37°C.

Nucleic acids were extracted once with phenol (Merck), saturated with Tris-EDTA (TE) buffer (10 mM Tris, 10 mM NaCl and 1 mM EDTA), followed by extraction with phenol: chloroform: isoamyl alcohol (25: 24: 1, Loba Chemie-com, India) until there was no protein at the interface. This was followed by extraction with chloroform: isoamyl alcohol (24: 1).

To each extraction, equal volume of the solvent was added, followed by thorough mixing and centrifugation for 10 min at 2000 rpm. The top layer was carefully transferred to another Falcon tube for the next extraction. To the final aqueous phase, 0.1 vol of 2.5 M Na acetate (Sigma Aldrich) and 2.5 vol of cold 95% ethanol (Loba Chemie-com) were added. The tubes were agitated gently to mix the liquids and a fluffy white ball of DNA was formed. The DNA was picked up with a heat-sealed Pasteur pipette and washed briefly in 70% ethanol.

The DNA was finally dissolved in an appropriate volume of 1X TE buffer. DNA concentrations were determined and diluted to the working concentration of 50 ng/µl, which is suitable for polymerase chain reaction.

Polymerase chain reaction (PCR)

The amplification of buffalo *BoLA-DRB3* exon 2 was achieved using primers (Sigma Aldrich) designed from cattle sequence (van Eijk *et al.* 1992):

HL030: (5' -ATC CTC TCT CTG CAG CAC ATT TCC- 3')

HL031: (5' -TTT AAA TTC GCG CTC ACC TCG CCG CT- 3').

A PCR cocktail consists of 1.0 µM upper and lower primers and 0.2 mM dNTPs (Biotechnology, Cairo, Egypt), 10 mM Tris (pH 9), 50 mM KCl (Ran Baxy), 1.5 mM $MgCl_2$ (Sigma), 0.01% gelatin (Merck), 0.1% Triton X-100 (Merck) and 1.25 units of *Taq* polymerase (Bioron). The cocktail was aliquoted into tubes with 100 ng DNA of buffalo. The reaction ran in a Programmable Thermal Controller (PTC-100), MJ Research Inc. The reaction was cycled for 1 min at 94°C, 2 min at 60°C and 2 min at 72°C for 30 cycles.

RFLP and agarose gel electrophoresis

20 µl of PCR product were digested with 10 units of *Rsa*I or *Bst*YI (Fermentas, Germany) restriction enzyme in a final reaction volume 25 µl. The reaction mixture was incubated at 37°C in water bath overnight. After restriction digestion, the restricted fragments were analyzed by electrophoresis on 8% polyacrylamide (Sigma Aldrich)/1X TBE gel stained with ethidium bromide (Sisco Research, India). The 100-bp ladder (Biotechnology) was used as molecular size marker. The bands were visualized under UV light and photographed with yellow filter on black and white film. The *BoLA-DRB3.2* alleles defined according to allelic nomenclature by van Eijk *et al.* (1992).

RESULTS AND DISCUSSION

Most of the polymorphisms of the *BoLA-DRB3* gene are located in exon 2, which encodes the peptide-binding cleft, and its sequence differences play a role in variability of immune responsiveness and disease resistance (Baxter *et al.* 2008).

The *BoLA-DRB3* exon 2 alleles in cattle have been found to be associated with resistance or susceptibility to various diseases (Dietz *et al.* 1997) like mastitis (Sharif *et al.* 1998), persistent lymphocytosis by bovine leukemia virus (Lewin *et al.* 1999; Kabeya *et al.* 2001), cystic ovarian disease, retained placenta and milk fever (Sharif *et al.* 1998). Associations have been observed for resistance to dermatophilosis in Brahman cattle of Matinique (Maillard *et al.* 1996) and immune response to foot and mouth disease (Glass *et al.* 1991; Lewin *et al.* 1999). The *DRB3* exon 2 polymorphism has also been observed to be associated with milk protein traits (Starkenburg *et al.* 1997). More than 100 different alleles from exon 2 of the *BoLA-DRB3* gene have been identified (da Mota *et al.* 2004).

PCR-RFLP was used in this study to screen the genetic polymorphism of *BoLA-DRB3* exon 2 in 50 Egyptian buffaloes. PCR was done using primers designed from a cattle sequence. The PCR-amplified fragments for all tested animals were at 284 bp. The restriction fragment length polymorphisms resulted from the digestion of the PCR-amplified fragments by two restriction enzymes, *Rsa*I and *Bst*YI.

Genetic polymorphism detected by *Rsa*I

Digestion of the amplified fragments using *Rsa*I yielded six alleles, each of which had a characteristic band as showed in **Table 1**. These alleles were B, F, I, K, L and O. The frequency of alleles ranged from 6% for the I allele to 40% for the L allele. The presence of six alleles resulted in 14 different genotypes from tested buffalo. These genotypes included three homozygous genotypes of B/B, L/L and O/O with 6, 18 and 8% frequency, respectively. The remaining 11 genotypes showed heterozygous variation: B/F (2%), B/K (2%), B/L (6%), F/I (2%), F/L (10%), F/O (2%), I/K (2%), I/L (8%), K/L (8%), K/O (14%) and L/O (12%) (**Table 2**).

*Rsa*I restriction enzyme was used to investigate the genetic polymorphism of *BoLA-DRB3* exon 2 in Jaffarabadi and Mehsani buffaloes (Acharya *et al.* 2002). The authors reported the presence of 10 alleles in Jaffarabadi breed and seven alleles in Mehansi breed. Five alleles were common between the two breeds (D, G, I, K and O). Four out of the 10 alleles in Jaffarabadi breed were displayed in the Egyptian breed, whereas the shared alleles between Egyptian buffalo and Mehansi breeds were four out of seven alleles in the Mehansi breed. This result suggested that the Egyptian buffalo is genetically closer to Mehansi breed than to Jaffarabadi breed.

Kumar *et al.* (2008) studied the genetic polymorphism of *BoLA-DRB3* exon 2 and its association with mastitis in Indian Nili-Ravi buffalo using PCR-*Rsa*I. They reported the presence of six alleles in healthy animals; B, C, F, L, O and S. Four of them are reported in our animals (B, F, L and O). They also reported the absence of alleles B and L in mastitis-animals; these two alleles were found in Egyptian buffalo with 11% frequency for the B allele and 40% frequency for the L allele. The authors found that the mastitis-animals carried alleles C and S with high frequencies. In Egyptian buffalo, these two alleles were absent. These results suggested that Egyptian buffalo could be highly resistant against mastitis due to absence of the mastitis susceptibility alleles C and S and presence of the L allele in 40% of animals and the B allele in 11% of animals.

Genetic polymorphism detected by *Bst*YI

The second restriction enzyme used in our study was *Bst*YI. It was used for the first time to study the polymorphism of

Table 1 Allele frequencies of *BoLA-DRB3* exon 2 with *Rsa*I in Egyptian buffalo.

Allele	Type of Appearance	B	F	I	K	L	O
Characteristic band		111 bp	141 bp	180 bp	156 bp	234 bp	284 bp
No. of animals	Homozygous	3	-----	-----	-----	9	4
	Heterozygous	5	8	6	13	22	14
	Total	8	8	6	13	31	18
Frequency of appearance (%)		11	8	6	13	40	22

Table 2 Genotype frequencies of *BoLA-DRB3* exon 2 with *Rsa*I in Egyptian buffalo.

Genotype	B/B	B/F	B/K	B/L	F/I	F/L	F/O	I/K	I/L	K/L	K/O	L/L	L/O	O/O
No. of animals	3	1	1	3	1	5	1	1	4	4	7	9	6	4
Frequency of appearance (%)	6	2	2	6	2	10	2	2	8	8	14	18	12	8

Table 3 Allele frequencies of *BoLA-DRB3* exon 2 with *Bst*YI in Egyptian buffalo.

Allele	Type of appearance	A	B	D
Characteristic band		199 bp	284 bp	197 bp
No. of animals	Homozygous	3	17	2
	Heterozygous	18	28	10
	Total	21	45	12
Frequency of appearance (%)		24	62	14

Table 4 Genotype frequencies of *BoLA-DRB3 exon 2* with *Bst*YI in Egyptian buffalo.

Genotype	A/A	A/B	B/B	B/D	D/D
No. of animals	3	18	17	10	2
Frequency of appearance (%)	6	36	34	20	4

BoLA-DRB3 exon 2 in buffalo. Digestion of the amplified fragments using *Bst*YI yielded three different alleles (**Table 3**) and five genotypes (**Table 4**) in 50 tested Egyptian buffalo. The three alleles were the A allele in 21 animals, the B allele in 45 animals and the D allele in 12 animals. These alleles were observed either as homozygous or heterozygous genotypes. The homozygous genotypes were AA, BB and DD with 6, 34 and 4% frequency, respectively, while the heterozygous genotypes were AB with 36% frequency and BD with 20% frequency.

In cattle, van Eijk *et al.* (1992) described PCR-RFLP genetic polymorphism of *BoLA-DRB3* exon 2 in bovine using three different restriction enzymes: *Hae*III, *Rsa*I and *Bst*Y1. A total of 30 alleles were distinguished by digestion of PCR amplification products with these three endonucleases. They reported 19 alleles with *Rsa*I, 5 alleles with *Bst*YI and 6 alleles for *Hae*III. The same three restriction enzymes were used for genotyping *BoLA-DRB3* exon 2 in Holstein bulls of Iran (Parnian *et al.* 2006) and Kankrej Indian's cattle (Behi *et al*. 2007). Seventeen alleles were identified in Holstein bulls with frequencies ranging from 1 to 21%. Sixteen alleles were similar to those reported previously by van Eijk *et al.* (1992) and one was a new allele. In Kankrei cattle 24 *BoLA-DRB3.2* alleles were identified with frequencies ranging from 1 to 22%. Twenty-one alleles of a total of 24 alleles were reported before and 3 alleles were new.

Six of the *Rsa*I and three of the *Bst*YI alleles reported in bovine by van Eijk *et al.* (1992) were observed in Egyptian buffalo tested in this work.

In addition to the two restriction enzymes – *Rsa*I and *Bst*Y1 – used in this study, *Hae*III endonuclease was used for genotyping the *BoLA-DRB3* exon2 in buffalo. Acharya *et al.* (2002) reported four *Hae*III alleles (A, B, D and E) in seven different genotypes (A/A, A/B, A/E, BB, B/E and EE) in Jaffarabadi breed. They also recorded the same four alleles but in nine different genotypes in Mehsani breed: A/A, A/B, A/D, B/B, B/D, B/E, D/D, D/E and E/E. The same results were reported by Kumar *et al.* (2008) in Nili-Ravi breed. They observed that the digestion of PCR product with *Hae*III resulted 6 genotypes (A/A, E/E, D/D, A/B, B/D and B/E) with the frequency ranging from 0.04 to 0.28 and four alleles (A, B, D and E) with the frequency ranging from 0.08 to 0.6.

The *Hae*III patterns of the 2nd exon of the Egyptian buffalo *BoLA-DRB3* were characterized by three polymorphic sites at 167 bp (A allele), 190 bp (D Allele) and at 219 bp (B allele) whereas 10% of tested animals did not show any restriction site (Ahmed and Othman 2006). Egyptian buffalo showed a high percentage of homozygous restriction genotypes (80%) compared with heterozygous genotypes (10%). The homozygous genotypes D/D, BB and AA displayed 32, 44 and 4% frequency, respectively, while the heterozygous genotypes A/D and B/D displayed 4 and 6% frequency, respectively. These results suggest that Egyptian buffalo carries a new pattern, which was not detected in Jaffarabadi, Mehsani or Nili-Ravi breeds. The B allele of *Hae*III in Egyptian buffalo was the most frequent allele as that found in Nili-Ravi breed (60%, Kumar *et al.* 2008) and Mehansi breeds (64%, Acharya *et al.* 2002).

CONCLUDING REMARKS

The role of the *BoLA-DRB3* gene in the development of an immune response system and its highly polymorphic nature make it one of the most important candidate genes in disease resistance studies. In Egyptian buffalo, the genetic variations of *BoLA-DRB3.2* showed six alleles and 14 genotypes by *Rsa*I digestion and three alleles with five genotypes by *Bst*Y1 digestion, while in our previous study (Ahmed and Othman 2006) *Hae*III digestion showed four alleles and five genotypes. A total of 13 alleles and 24 genotypes were recorded in Egyptian buffalo *BoLA-DRB3.2* using three different restriction enzymes. The results of the present study suggested that absence of the mastitis susceptibility alleles C and S and presence of mastitis resistance allele L in 40% and allele B in 11% of animals.

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