Characterization of MHC DRB3.2 Alleles of Crossbred Cattle by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

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ABSTRACT : The present investigation was undertaken to study the genetic polymorphism of the DRB3 exon 2 in 75 crossbred cattle by the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique. Five genotypes i.e. *Hae*III-a, *Hae*III-b, *Hae*III-e, *Hae*III-ab and *Hae*III-ae were observed when the 284 bp PCR products were digested with *Hae*III restriction enzyme. The corresponding frequencies of these patterns were 0.53, 0.04, 0.01, 0.38 and 0.04, respectively. Digestion with *Rsa*I restriction enzyme resolved 24 different restriction patterns. The frequencies of these patterns ranged from 0.013 (*Rsa*I-f, *Rsa*I-k and *Rsa*I-c/n) to 0.120 (*Rsa*I-n). The results revealed that the crossbred cows belonged to the *Rsa*I patterns namely b, k, l, a/l, d/s, l/n, l/o and m/n, whose corresponding frequencies were 0.027, 0.013, 0.040, 0.027, 0.040, 0.067, 0.027 and 0.067, respectively. Digestion of the 284 bp PCR product of DRB3.2 gene with *Pst*I in the crossbred cattle did not reveal any restriction site. These results suggested the absence of the recognition site in some of the animals. These results also revealed that the crossbred cows studied were in homozygous as well as heterozygous condition. On the basis of the above results it can be concluded that the DRB3.2 gene was found to be highly polymorphic in the crossbred cattle population. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 9 : 1226-1230)***

Key Words : DRB3.2 Gene, PCR-RFLP, Crossbred Cattle

INTRODUCTION

Indigenous cattle breeds have evolved as a result of natural selection spread over thousands of years. India has a cattle population of 219.6 million heads, which is represented by 30 well-defined breeds (FAO Year Book, 2001). The importance of cattle as a milk producing species is well recognized and its contribution to the economy of the rural sector is remarkable and vital. The crossbreeding programme was launched in the country to improve the productivity of low producing Indian breeds. Although there was a quantum jump in the production potentials of crossbred cattle, these animals were observed to be more susceptible to tropical diseases due to poor immune response.

Immune response is regulated by the major histocompatibility complex (MHC) molecules (antigen presenting molecules) and T-cell receptors (TCRs) molecules (specially recognizing foreign antigen presented by MHC). MHC of vertebrates comprises a group of closely linked genes, which plays a central role in immune surveillance and response (Klein, 1986). The bovine lymphocyte antigen (BoLA) system is known as the major histocompatibility complex of cattle (Amorena and Stone 1978; Spooner et al., 1978; Bernoco et al., 1992). MHC genes encode highly polymorphic cell surface glycoprotein molecules, which represent antigenic peptide to T-cells (Allen et al., 1987), thereby playing an essential role in the immune response to foreign agents. BoLA has been mapped to chromosome 23 and consists of class I, IIa, IIb and III regions (Andersson and Davies 1994). Previously, several methods have been developed for BoLA typing. These include serology (Emery et al., 1987), isoelectric focusing (IEF), direct sequencing (Groenen et al., 1990), (Glass et al., 1992), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Van Eijk et al., 1992), heteroduplex analysis (Sitte et al., 1995), denaturing gradient gel electrophoresis (Aldridge et al., 1998), and PCR sequence based typing (Takeshima et al., 2000). Among these methods, PCR-RFLP developed by Van Eijk and co-workers (1992) has many advantages over the other methods for the genetic analysis of populations, as it requires small amount of genomic DNA and being adaptable to crude DNA preparations. Recently, PCR-RFLP technique has been used to find out the relationship of polymorphism of insulin-like growth factor I gene with growth traits in chicken and to study the association between polymorphism of the fifth intron in lipoprotein lipase gene and productive traits in pigs (Wang et al. 2004 and Zhang et al. 2005). The genetic regulation of host resistance to disease and immune responsiveness is

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Table 1. PCR-RFLP patterns of the BoLA-DRB3.2 gene with *Hae*III restriction enzyme and their frequencies in crossbred cattle

Observed patterns	Reported patterns	Fragment size (bp)	Genotype frequencies
*Hae*III-a	a	167, 52, 65	0.53
*Hae*III-b	b	219, 65	0.04
*Hae*III-e	e	167, 117	0.01
*Hae*III-a/b	-	219, 167, 52, 65	0.38
*Hae*III-a/e	-	167, 117, 52, 65	0.04

Table 2. PCR-RFLP patterns of the BoLA-DRB3.2 gene with *Rsa*I restriction enzyme and their frequencies in crossbred cattle

Observed patterns	Reported patterns	Fragment size (bp)	Genotype frequencies
*Rsa*I-b	b	111, 54, 50, 39, 30	0.027
*Rsa*I-f	f	141,54, 50, 39	0.013
*Rsa*I-k	k	156,78, 50	0.013
*Rsa*I-l	l	234, 50	0.040
*Rsa*I-n	n	180, 104	0.120
*Rsa*I-o	o	284	0.040
*Rsa*I-s	s	141, 93, 50	0.053
*Rsa*I-a/l	-	234, 50, 78, 33, 30, 39, 54	0.027
*Rsa*I-b/l	-	234, 50, 111, 30, 39, 54	0.093
*Rsa*I-b/o	-	284, 111, 30, 39, 54, 50	0.027
*Rsa*I-c/n	-	180, 104, 111, 30, 50, 93	0.013
*Rsa*I-d/s	-	143, 111, 141, 93, 50, 30	0.040
*Rsa*I-f/n	-	180, 104, 141, 39, 54, 50	0.027
*Rsa*I-f/o	-	284, 141, 39, 54, 50	0.040
*Rsa*I-g/l	-	234, 50, 141, 39, 104	0.053
*Rsa*I-g/n	-	180, 104, 141, 39	0.053
*Rsa*I-g/o	-	284, 141, 39, 104	0.027
*Rsa*I-k/l	-	234, 50, 78, 156	0.027
*Rsa*I-l/n	-	234, 50, 180, 104	0.067
*Rsa*I-l/o	-	284, 234, 50	0.027
*Rsa*I-l/s	-	234, 50, 141, 93	0.053
*Rsa*I-m/n	-	180, 104, 111, 69	0.067
*Rsa*I-n/o	-	284, 180, 104	0.027
*Rsa*I-n/s	-	180, 104, 141, 93, 50	0.027

polygenic. Among the many loci involved in the induction and regulation of immune response, the MHC genes explain a substantial portion of the genetic variation in the response (Biozzi et al., 1974; Mallard et al., 1989). Three DRB genes (DRB1, DRB2 and DRB3) have been identified so far and out of these DRB1 is evidently a pseudo gene and functional expression of DRB2 is still unknown, whereas DRB3 is functionally expressed and highly polymorphic (Davies et al., 1997). Thus far at least 104 alleles have been identified by sequencing exon 2 of the DRB3 gene (Poli et al., 2003). Considering the importance of the DRB gene in controlling the immune response, the present investigation has been undertaken to study the polymorphism of the DRB3.2 gene in a crossbred cattle population.

MATERIALS AND METHODS

The investigation was undertaken on 75 crossbred cows maintained at the Cattle and Buffalo farm, Indian Veterinary Research Institute, Izatnagar. Genomic DNA was isolated from 5 ml of venous blood collected from the jugular vein in a 15 ml sterile polypropylene tube containing 0.5 ml of EDTA (0.5 M) as an anticoagulant by the phenol-chloroform extraction method described by Andersson et al. (1986) with some modifications. The DNA samples were checked for their quality, purity and concentration. The quality of the genomic DNA was checked by using submarine agarose gel electrophoresis. DNA samples of good quality, purity and concentration were used for further analysis.

The 284 bp fragment consisting of the 267 bp exon 2 region of the DRB3 gene and the flanking intron of 17 bp present in the genomic DNA of crossbred cattle was amplified by employing the corresponding primer pairs (forward and reverse) as described by Van Eijk et al. (1992). The details of the primer sequences are as follows:

P_1 5'-ATC CTC TCT CTG CAG CAC ATT TCC-3' (24 bp)
$P_2$5'-TTT AAA TTC GCG CTC ACC TCG CGC CT-3' (26 bp)
P_3 is same as P_1 (24 bp)
P_4 5'-TCG CCG CTG CAC AGT GAA ACT CTC-3' (24 bp)

PCR-RFLP analysis

For each sample, 10 µl of the PCR product was digested with five units of restriction enzymes (*Hae*III, *Rsa*I and *Pst*I) in a final volume of 20 µl at 37°C for overnight. The restriction fragments were resolved on 4% agarose gel electrophoresis at 30 volt for 4 h in 1×TBE buffer and documented through photography using a gel documentation system. 50 bp DNA ladder was used to estimate the size of the fragments.

RESULTS AND DISCUSSION

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique for BoLA DRB3 typing is based upon the extensive polymorphism that is present in exon 2 of the BoLA DRB3 gene, part of which can be detected with restriction endonucleases. PCR-RFLP is a rapid and sensitive method for the detection of polymorphisms in a functionally relevant domain of the BoLA DRB3 gene and is useful for studying the evolutionary changes and DRB polymorphisms in cattle and other Bovidae. The analyses of the BoLA DRB3.2 allelic patterns are presented in Tables 1 and 2.

The digestion with *Hae*III revealed four restriction sites, which resulted in five distinct restriction patterns namely *Hae*III-a, *Hae*III-b, *Hae*III-e, *Hae*III-a/b and *Hae*III-a/e (Figure 1). The major fragment sizes of these patterns were 167, 65, 52 for *Hae*III-a; 219, 65 for *Hae*III-b; 167, 117 for

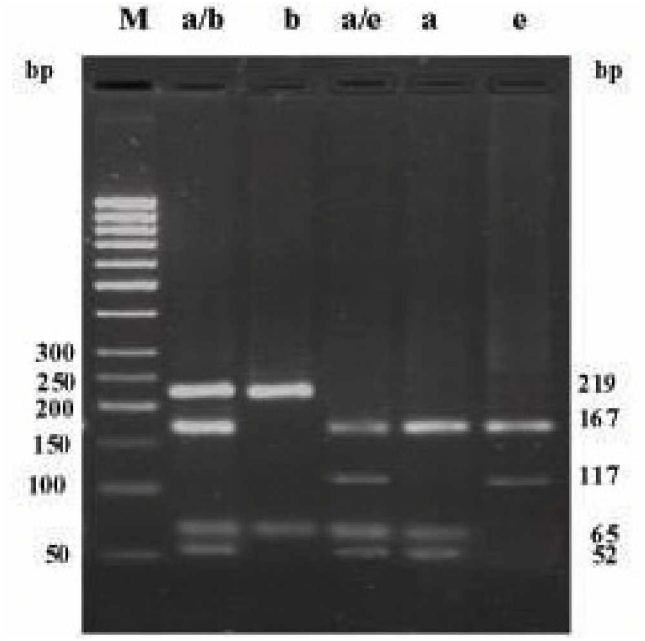

Figure 1. Electrophoretic mobility of RE fragments obtained by digestion of the BoLA-DRB 3 exon 2, 284 bp amplified PCR products with *Hae*III in crossbred cattle. Lane M: 50 bp DNA ladder.

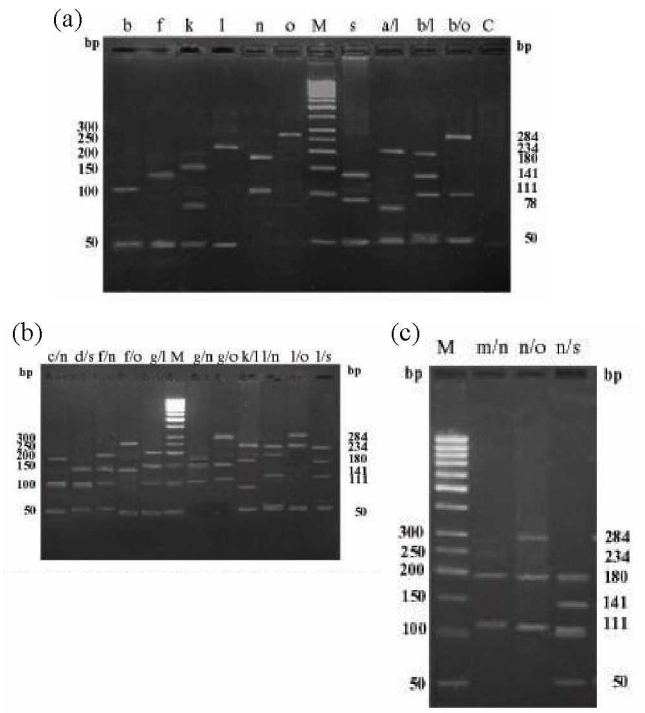

Figure 2. (a), (b), (c) Electrophoretic mobility of RE fragments obtained by digestion of the BoLA-DRB 3 exon 2, 284 bp amplified PCR products with *Rsa*I in crossbred cattle. Lane M: 50 bp DNA ladder.

*Hae*III-e; 219, 167, 65, 52 for *Hae*III-a/b and 167, 117, 52, 65 base pair for *Hae*III-a/e, respectively. The frequencies of these PCR-RFLP patterns of the BoLA DRB3.2 genotypes were 0.53, 0.04, 0.01, 0.38, and 0.04, respectively (Table 1). The high frequency (53%) of *Hae*III-aa genotype might be due to natural selection favoring the fixation of allele 'a' in homozygous condition or mild inbreeding might have occurred in the herd of crossbred cattle due to the small number of breedable cows and the use of a limited number of sires for breeding. The frequency of *Hae*III-a/b (38%) genotype was next to the frequency of *Hae*III-aa, whereas the frequencies of *Hae*III-bb and *Hae*III-a/e genotypes were low and the same (4%). The frequency of genotype *Hae*III-ee was only 1%, which was very low. The low frequencies of these genotypes namely *Hae*III-bb, *Hae*III-ee and *Hae*III-a/e may be due to natural selection against these genes in the herd.

Van Eijk et al. (1992) described 30 BoLA alleles identified by digestion of PCR products with *Rsa*I, *Bst*YI and *Hae*III restriction enzymes. Thereafter, the PCR-RFLP technique for typing of BoLA DRB3 was restricted to mostly these three restriction enzymes. The restriction sites observed in the present study are similar to the patterns reported by Van Eijk et al. (1992), Gelhaus et al. (1995) and Aravindakshan et al. (1999). These workers have reported 6, 8 and 10 restriction patterns with *Hae*III, respectively. Most of the patterns observed in the present study were in agreement with those reported by Singh (2001) and De (2000) in buffaloes. However, Sharma et al. (2004) could observe only three *Hae*III restriction patterns in Rathi cattle. In the present investigation, three out of the five patterns were found in the homozygous condition and the remaining two were in the heterozygous condition. De (2000) identified all the previously reported alleles by digestion with *Hae*III and *Bst*YI restriction enzymes.

The *Rsa*I restriction enzyme digestion of the amplified 284 bp fragment of the DRB3.2 gene resulted in 24 different restriction patterns (Figures 2a, 2b and 2c). The frequencies of these patterns ranged from 0.013 to 0.120 (Table 2). The *Rsa*I restriction digestion generated seven homozygous and seventeen heterozygous patterns. It was observed that approximately 31% of the total crossbred cows were homozygous for the restriction sites of this enzyme, whereas, about 69% of the crossbred cows were found to be heterozygous. Among the homozygotes, the frequency of genotype *Rsa*I-nn (12%) was highest, which may be due to the selective advantage of this allele followed by the *Rsa*I-ss genotype (5.3%). The other *Rsa*I genotypes namely *Rsa*I-ll, *Rsa*I-bb and *Rsa*I-oo had the genotype frequencies as 4%, 2.7% and 4%, respectively but the genotypes *Rsa*I-ff and *Rsa*I-kk had the lowest frequencies (1.3% and 1.3%). Crossbred cows having heterozygous *Rsa*I patterns namely a/l, d/s, l/n, l/o and m/n were present in very low frequencies of 0.027, 0.040, 0.067, 0.027 and 0.067, respectively. Among the heterozygous genotypes, *Rsa*I-b/l showed the highest frequency (9.3%) and *Rsa*I-c/n

the lowest frequency (1.3%). It was found that the alleles *Rsa*I-n (*Rsa*I-c, *Rsa*I-f, *Rsa*I-g, *Rsa*I-l, *Rsa*I-m, *Rsa*I-o and *Rsa*I-s) and *Rsa*I-l (*Rsa*I-a, *Rsa*I-b, *Rsa*I-g, *Rsa*I-k, *Rsa*I-n, *Rsa*I-o and *Rsa*I-s) shared the maximum number of heterozygotes with seven alleles. The results of the present investigation of PCR–RFLP homozygous patterns of DRB3 exon 2 with *Rsa*I are comparable to those reported by Van Eijk et al. (1992) and Gelhaus et al. (1995). These workers have reported 19 and 21 distinct *Rsa*I restriction patterns, respectively. De (2000) reported that the *Rsa*I restriction enzyme showed 15 different patterns in Indian cattle breeds.

Poli et al. (2003) reported that among the three DRB genes, DRB3 appeared to have the highest expression and displayed a high degree of polymorphism with at least 104 alleles identified by sequencing exon 2. A high degree of polymorphism at the BoLA-DRB3.2 locus had been reported in high yielding exotic cattle breeds. Gilliespie et al. (1999) also studied the polymorphism of the BoLA-DRB3.2 gene by digestion with *Rsa*I, *Hae*III and *Bst1*I restriction enzymes and reported 24 alleles in Jersey cows. Similarly, Maillard et al. (1999) typed 37 of the 47 official PCR-RFLP alleles including 18 new ones, which included seven PCR-RFLP patterns of which four were new *Rsa*I patterns in their study of the zebu (*Bos indicus*) population of Martinique and the allelic frequencies of these patterns ranged from 0.004 to 0.358.

The restriction digestion of the 284 bp PCR product by *Pst*I did not reveal any restriction site for this enzyme. This showed the absence of any cutting site for *Pst*I in the DRB3.2 gene in crossbred cattle. The results of the present investigation are in agreement with the findings of Dechamma et al. (1998) and Sharma et al. (2004) as they also concluded that the DRB 3.2 gene had no internal restriction site for the *Pst*I restriction enzyme in zebu cattle, which resulted in the monomorphic pattern for this gene in crossbred cattle also.

It may be concluded that PCR-RFLP may be used to study the polymorphism of the BoLA DRB3.2 gene because of its sensitivity for the determination of polymorphism in a functionally relevant domain. Crossbred cattle showed a moderate level of polymorphism with respect to the patterns reported by various previous workers. It was also found that the crossbred cows studied were homozygous as well as heterozygous for the sites of digestions by *Hae*III and *Rsa*I restriction enzymes. The results of the present investigation suggested that the DRB 3.2 gene is highly polymorphic in nature in our crossbred cattle population.

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