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Studying Bola Gene in Healthy and Mastitis Animals

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Abstract: Mastitis and pathogen related differences also depend upon the ability of the host's immune defense system. MHC play important role in providing defence against pathogens. Defensin plays a very important role in defending against the various microorganism. Hence, the genes encoding those regions seems to be possible genetic markers for resistant or susceptibility to various microorganisms. In the present study BoLA-DRB3 gene were studied. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method was used for identification of polymorphism in both the genes. Using Hae III restriction enzyme 6 different types of pattern has been observed in cattle, whereas in buffalo 7 types of pattern was observed in healthy and mastitis samples. Using RsaI restriction enzyme 10 different types of pattern has been observed in case of cattle, whereas in buffalo 8 types of pattern was observed in healthy and mastitis samples. Significant differences have been found between cattle and buffalo breed studied. RsaI –I and RsaI-f was found in healthy samples, but was absent in mastitis samples. Collectively, the finding in the present study states the use of alleles HaeIII-c, RsaI-l and RsaI-f as a reference for more detailed mechanistic studies.
Keywords: Mastitis, BoLA gene, BuLA gene, MHC and PCR.

INTRODUCTION

In-spite of various control and prevention strategies used for the treatment of mastitis, it remained the expensive disease of the dairy industry [1]. Existing methods and therapeutics to control the loss due to mastitis are not having long term fruitful results [2].

Hence, alternate approach should be used to improve animals natural genetic resistance to the infectious microorganism [3]. Till date the production goals for dairy animals has focused mostly on increase in milk yield and productivity, but have unnoticed the disease resistant trait in the dairy animals at the time breeding. This led to an rise in the disease susceptible animals [4].

The inclusion of the disease resistant trait in the breeding programme will be very much helpful, as it will be will be directly related to the productivity. Lesser production losses will be there due to decrease in the disease transmission on account of rise in disease resistant population [3]. Another possible benefits from breeding the disease resistant trait is the herd immunity [5]. The breeding for mastitis resistant genes will be helpful to overcome the economic losses due to mastitis.

Mastitis and pathogen related differences also depend upon the ability of the host's immune defense system. Defensin is an assemblage of antimicrobial peptides having antibiotic and cytotoxic activity against microorganisms. Defensin plays a very important role in defending against the various microorganism. Hence, the genes encoding those regions seems to be possible genetic markers for resistant or susceptibility to various microorganisms.

The Major Histocompatibility Complex (MHC) is a compactly interrelated clusters of genes that may be related to host defense by providing resistance or susceptibility. MHC genes are divided into 3 separate classes in mammals i.e. class I, class II and class III. Each of these classes is sub-divided into regions and sub regions having a number of genes and pseudogenes. MHC plays a essential part in the development of antibody and cell-mediated immune responses.

Therefore, they have been associated with resistance and susceptibility to disease causing pathogens and also in the development of autoimmunity [6].

MHC in cattle is well-known as bovine leukocyte antigen (BoLA). MHC play important role in providing defence against pathogens. Polymorphism in bovine leukocyte antigens (BoLA) has been widely explored for marker identification in immunological traits and bovine diseases. MHC genes pose a particular interest to animal breeders and veterinarians. As they provide genetic susceptibility and resistance to various infectious diseases in diverse animal species.

BoLA denotes to both *Bos taurus* and *Bos indicus* and was first reported in 1978 by Amorena and Stone [7] and spooner [6]. BoLA region which is located on chromosome 23 has been subdivided into 2 discrete sub regions i.e. class IIa and class IIb. Functionally expressed DR and DQ genes are present in Class IIa region [8] whereas class IIb genes encodes DM, DN and DO genes. Highest polymorphism is observed in these two genes. In cattle 1 DRA gene and 3 DRB genes are present, but only DRB3 gene is found to be functionally important [9]. Polymorphism in DRB3 gene is important due to its association in various diseases in bovine animals. BoLA class II genes, specifically BoLA-DRB3 gene which is the most polymorphic gene have been linked with variation in disease occurrence [10]. The nomenclature of these genes are created on the basis of amino acid sequences and the format is gene.exon*allele [7]. The BoLA-DRB3.2 gene is reported as highly polymorphic where more than 115 alleles have been identified [11, 12]. The first report of association of disease and MHC in cattle was published in 1984 [13].

MHC in buffalo is similar to cattle it is known as buffalo lymphocyte antigen (BuLA), polymorphism in MHC-DRA and MHC-DRB is observed [14]. In buffalo also three BuLA DBR gene is present i.e. DBR1, DBR2 and DBR3. Among these DBR3 gene is found to be highly polymorphic and associated with the disease condition. Very few reports are there related to the polymorphism in DRB3 gene in buffaloes [14-17].

Various methods are reported to study the genetic polymorphism in MHC genes like Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP), PCR-RFLP [18], Single Stand Conformation Polymorphism (SSCP-PCR), Sequence based typing [19], Sequencing using high throughput sequencer using genomic DNA, custom amplicons, cDNAs and cloned PCR products.

Looking at the importance of MHC it becomes important to study the polymorphism so as to study the immune response genes on resistant or susceptibility to an disease [20]. Hence in the present study the influence of bovine lymphocyte antigen (BoLA) complex polymorphism on mastitis was investigated.

MATERIALS AND METHODS

Sample collection and screening for mastitis

Quarter milk samples were aseptically collected from farm located in Anand district of Gujarat, India. The entire udder of each cattle was thoroughly washed with 0.01% potassium permanganate solution (1:1000) and clean with 70% alcohol to allow drying [21]. Samples were collected after discarding the first 2 ml of milk. Thirty ml of milk was kept in the refrigerator at 4ºC for DNA extraction. The remaining samples were used for mastitis diagnosis. Clinical mastitis was diagnosed by visible signs and symptoms. In subclinical mastitis, where no visible symptoms occur, hence California Mastitis Test (CMT) and Somatic Cell Count (SCC) were performed. CMT was carried out by mixing equal volume of milk and CMT reagents and mixed together by swirling the paddle and results were noted within 30 seconds. Somatic cell count of quarter milk samples was done using Fossmatic Minor (Foss Analytical, Denmark). The results of SCC and microbiological culture examination of infected quarters were interpreted following the International Dairy Federation (IDF) guidelines. Healthy samples were selected with minimum 3 lactation and no previous history of mastitis. After the screening, about 8-10 ml of blood were collected from the infected and healthy animals. Samples were stored in EDTA-coated vacutainer tubes

DNA isolation

Genomic DNA from blood was isolated using QIAamp DNA blood mini kit (Qiagen, Germany) as per the manufacturer's protocol. The DNA was then precipitated, washed and re-suspended in 150 µl TE buffer. Concentrations of resulted total DNA were measured by Qubit flurometer (Life Technologies, USA) and purity was determined by A_{260}/A_{280} ratio using spectrophotometer (Nanodrop-1000, Thermo Fisher, USA). DNA integrity was verified by running the samples on 0.8% agarose gel electrophoresis after staining with ethidium bromide under ultraviolet light. Samples were stored at -20°C till further processing.

BoLA gene amplification

The exon 2 of BoLA- DRB3 gene (284 bp) was amplified by polymerase chain reaction (PCR) using primers with two sequential reactions, the first round using primers HLO30 (5´-ATC CTC TCT CTG CAG CAC ATT TCC-3´) and HL031 (5´TTT AAA TTC GCG CTC ACC TCG CCG CT 3´), followed by a second reaction using primers HLO30 (5´-ATC CTC TCT CTG CAG CAC ATT TCC-3´) and HLO32 (5´-TCG CCG CTG CAC AGT GAA ACT CTC-3´), as described by [22]. The reaction mixture for polymerase chain reaction is described in Table 1 and the amplification condition is describes in Table 2. PCR product was visualized on 2.0% agarose gel.

PCR-RFLP

The final product obtained was of 284 bp in length and it was digested with 2 restriction endonucleases i.e. *RsaI* and *HaeIII* separately. From each sample 10 µl of the PCR product was digested with 5 units of restriction enzymes *Hae*III and RsaI making final volume up to 20 µl and kept overnight at 37°C. To confirm the digestion of the targeted PCR amplification, initially 5 µl of digested product was mixed with 1 µl gel loading dye and electrophoresis was carried out on 2.0 % agarose gel containing ethidium bromide at 100V for 30 to 45 minutes. Along with the samples 50 bp DNA molecular weight marker was also run (GeneRuler, MBI Fermentas). The product was then visualized U.V light under the gel documentation system.

Table-1: Reaction mix for polymerase chain reaction

	Component	Volume	Final Concentration
Reaction Mix	Buffer	5 µl	5X
	dNTP Mix	1	10 mM
	$Mgcl_2$	0.5	1.5 mM
	F Primer	1 µl	0.2 mM
	R Primer	1 µl	0.2 mM
	Enzyme	1 µl	1.5 U
	Water (PCR grade)	14.5	
	Template (DNA)	1	50 ng
	Total Volume	25 µl	

Table-2: PCR conditions

Steps	Temperature ^oC	Time	Number of cycles
Initial denaturation	94	5 minute	
Denaturation	94	1 minute	20 cycle for HL031 and 30 cycle for HLO32
Annealing	60 for HL031 and 65 for HL032	30 seconds	
Extension	72	30 seconds	
Final extension	72	5 minute	

RESULTS AND DISCUSSION

A total of 96 animals were taken for the current study from cattle and buffalo. A total of 12 healthy samples and 80 mastitis samples were collected. Healthy group was categorized by animals having 3 or more lactation, with no prior cases of mastitis and somatic cell count less than 200 cells/µl. Mastitis samples were identified as per the IDF guidelines.

DNA isolation and PCR amplification

Blood samples were collected from all the selected animals. DNA was isolated and visualized by agarose gel electrophoresis (Figure 1). All the PCR products of the amplified DRB3.2 generated 284bp fragment (Figure 2). DRB3 gene has been extensively assessed as a potential marker gene for linked with various diseases in bovine animals. It may have significant association for antigen binding sites and useful in determining susceptibility and resistant to disease. PCR- RFLP method was used for identification the occurrence of different alleles in BoLA-DBR3 gene in healthy and affected cattle and buffalo.

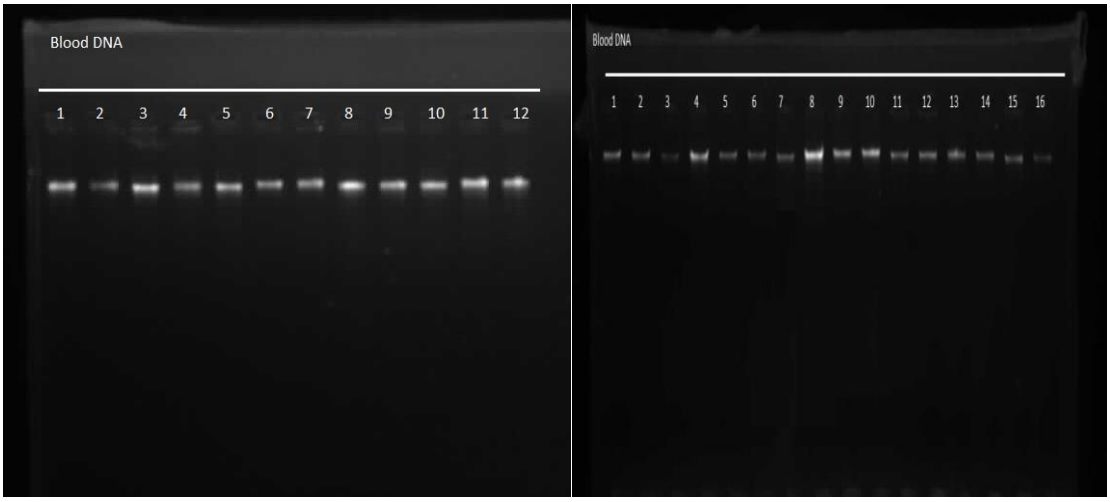

Fig-1: Genomic DNA from blood a. Cattle and b. Buffalo

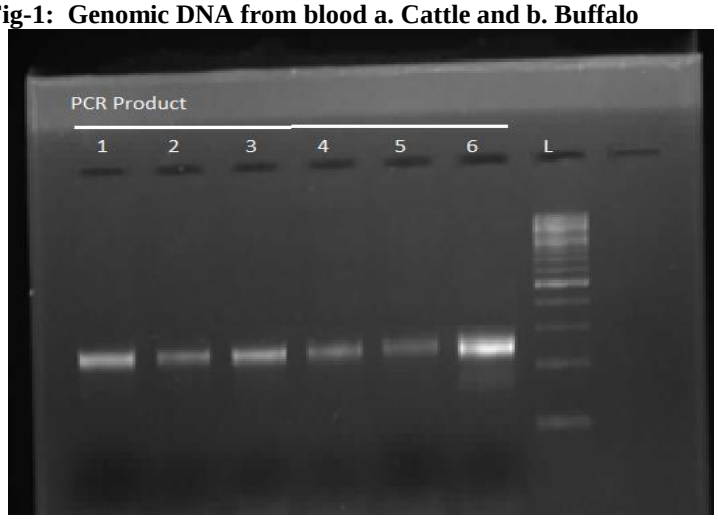

Fig-2: Gel showing amplified product of BoLA DRB3 (284bp)
(Lane 1-6 PCR product of DRB3 gene L= 100bp ladder)

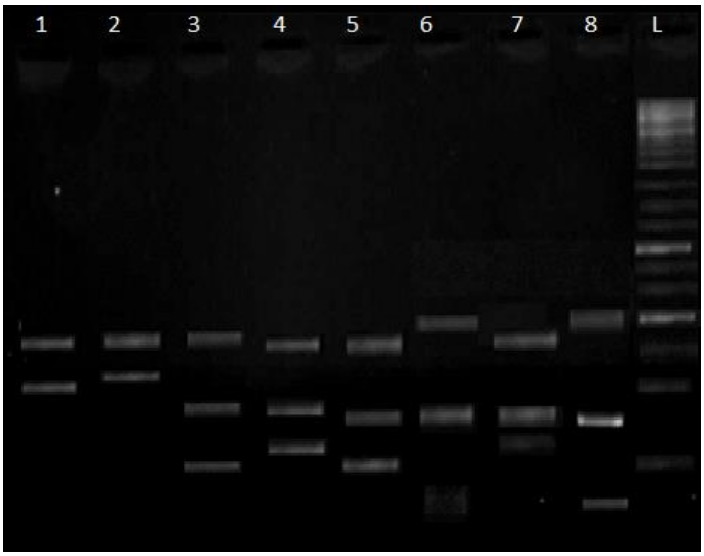

Fig-3: Hae III restriction patteren generated from amplified product of DRB3 exon 2 fragment in cattle
(Lane 1-8 PCR RFLP pattern L= 50bp ladder)

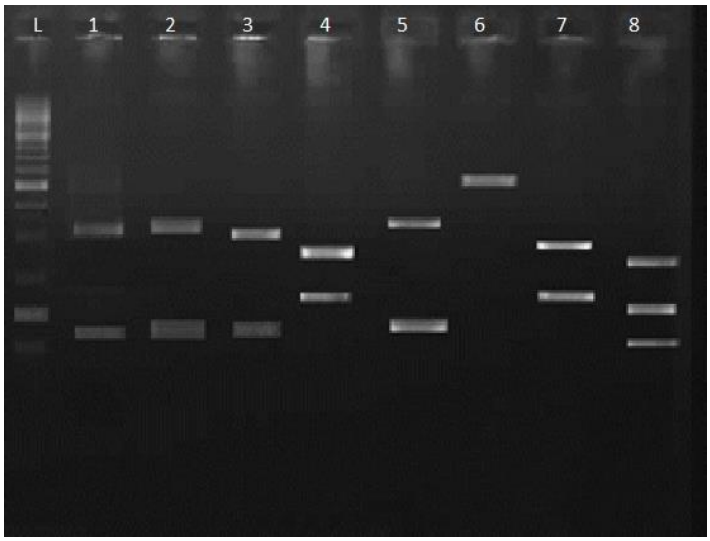

Fig-4: Hae III restriction patteren generated from amplified product of DRB3 exon 2 fragment in buffalo
(Lane 1-8 PCR RFLP pattern L= 50bp ladder)

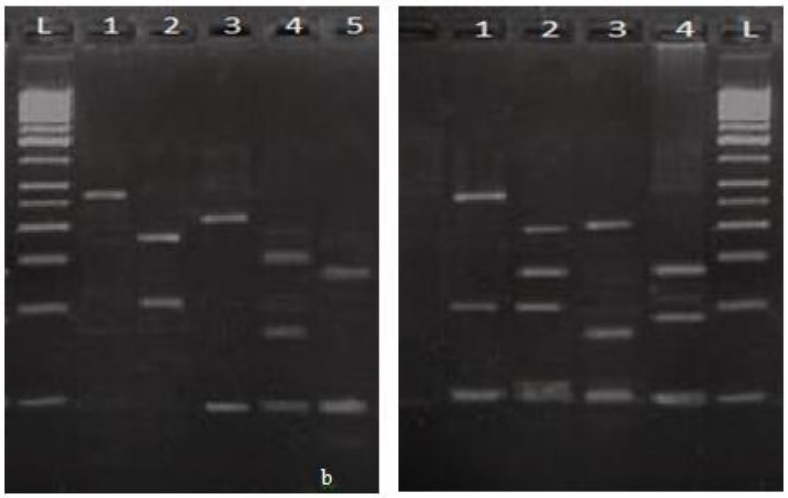

Fig-5: RsaI restriction patteren generated from amplified product of DRB3 exon 2 fragment in cattle and buffalo
a. Cattle samples **b.** Buffalo samples

Table-3: Frequency of RFLP pattern in cattle

Enzyme	Restriction Patterns	Fragment size (bp)	Allele frequency	
			Healthy	Disease
HaeIII	HaeIII-a	284	0	0.1
	HaeIII-b	219,65	0.17	0.28
	HaeIII-d	167,117	0.33	0.2
	HaeIII-f	141,78,65	0.17	0.08
	HaeIII-g	141,93,50	0	0.05
	HaeIII-h	180,65,39	0.17	0.3
RsaI	RsaI-a	284	0	0.05
	RsaI-b	234,50	0.33	0.15
	RsaI-c	234,50,111	0	0.18
	RsaI-d	141,54,50,39	0.17	0.05
	RsaI-e	141,93,50	0	0.1
	RsaI-i	180,104	0.17	0.08
	RsaI-j	284,234,50	0	0.08
	RsaI-k	284,180,140	0.17	0.23
	RsaI-l	234,50,141,93	0.17	0
	RsaI-m	180,140,141,39	0	0.1

Hae III restriction patterns

PCR-RFLP was performed using enzyme Hae III of 284 bp of DRB3 exon 2, which generated various combination of patterns (Figure 3 and 4). The RFLP pattern were typed as HaeIII a-h in cattle and buffalo. In cattle 6 different types of pattern has been observed, whereas in buffalo 7 types of pattern was observed in healthy and mastitis samples (Table 3 and 4). HaeIII-c was found in healthy buffalo samples, but was absent in mastitis samples. The allelic frequency in pattern c was 0.33. So, it can be related to the resistant to infection. No association has been found in DRB3 exon 2 and mastitis in cattle using Hae III restriction enzyme.

Table-4: Frequency of RFLP pattern in buffalo

Enzyme	Restriction Patterns	Fragment size (bp)	Frequency (%)	
			Healthy	Disease
HaeIII	HaeIII-a	284	0	0.05
	HaeIII-b	219,65	0.17	0.28
	HaeIII-c	197,85	0.33	0
	HaeIII-e	156,78,50	0.33	0.3
	HaeIII-f	141,78,65	0	0.13
	HaeIII-g	141,93,50	0	0.08
	HaeIII-h	180,65,39	0.17	0.2
RsaI	RsaI-a	284	0	0.08
	RsaI-b	234,50	0.33	0.28
	RsaI-c	234,50,111	0	0.23
	RsaI-d	141,54,50,39	0.17	0.1
	RsaI-e	141,93,50	0	0.18
	RsaI-f	156,78,50	0.33	0
	RsaI-i	180,104	0.17	0.03
	RsaI-j	284,234,50	0	0.13

RsaI restriction patterns

PCR-RFLP was performed using enzyme Hae III of 284 bp of DRB3 exon 2 generated various combination of patterns (Figure 5). The RFLP pattern were typed as RsaI a-m in cattle and buffalo. In cattle 10 different types of pattern has been observed, whereas in buffalo 8 types of pattern was observed in healthy and mastitis samples (Table 3 and 4). RsaI-l found in cattle healthy samples, but was absent in mastitis samples. Similarly, in buffalo RsaI-f was found in healthy samples, but was absent in mastitis samples. The allelic frequency in pattern l and f was 0.33 respectively. Hence these patterns may be associated with mastitis resistant. Due to lack of healthy samples having more than 2 lactation and with no prior cases of mastitis, so less number of healthy samples were incorporated in the present study.

Van Eijk *et al.* reported different BoLA alleles for first time by the digestion of PCR products with RsaI, BstYI and HaeIII restriction enzyme in European cattle [18]. Thereafter, the typing of the BoLA DRB3 using the PCR RFLP method was restricted to mostly by these three restriction enzyme. Various studies have reported high polymorphism in the DRB3.2 from cattle [23] and buffalo [24].

Significant associations have been made between BoLA genes with disease prevalent during early lactation i.e. associated with resistant to *S.aureus* resistant [25], and with innate and adaptive immunity in cattle [26]. Several reports are there for different allele of DRB3.2 linked to subclinical mastitis susceptibility [27], increase prevalence of subclinical mastitis caused by *S. dysgalactiae* [28], higher SCC [29], with severe cases of mastitis [10]. The DRB3.2 gene locus has previously been reported to be associated with various disease in animals like bovine leukemia virus [30] tick resistance [31] and clinical mastitis [32]

CONCLUSION

The finding in the present study states the use of alleles HaeIII-c, RsaI-l and RsaI-f as a reference for more detailed mechanistic studies. It does not states that mastitis resistant genes selection should be based on the above mention genes or various other alleles of BoLA. The evidence on a various genes may be beneficial in the identification and selection for disease resistant and good health of the animals. DRB3.2 gene may play anvital role in association with bovine mastitis and various alleles differencing in healthy and disease animals may be associated with providing resistant to various infectious microorganisms. The extensive polymorphism exhibited by this gene may have important implication for antigen binding and useful in determining susceptibility and resistant to disease.

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