GENETIC POLYMORPHISM OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II GENES IN GOATS

REMYA JOHN V.

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Department of Animal Breeding, Genetics & Biostatistics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

DECLARATION

I hereby declare that this thesis, entitled **"Genetic polymorphism of Major Histocompatibility Complex class II genes in goats"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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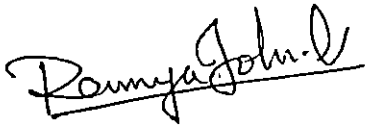

Remya John V.

CERTIFICATE

Certified that this thesis, entitled **"Genetic polymorphism of Major Histocompatibility Complex class II genes in goats"** is a record of research work done independently by **Dr. Remya John V.** under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to him.

Place: Mannuthy

Date: 22-04-2010

Dr. K.C. Raghavan
(Chairman, Advisory Committee)
Professor,
Centre for Advanced Studies in
Animal Genetics and Breeding,
College of Veterinary and Animal
Sciences, Mannuthy, Thrissur

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Dr. Remya John V.** a candidate for the degree of Master of Veterinary Science in Animal Breeding, Genetics & Biostatistics, agree that this thesis entitled **"Genetic polymorphism of Major Histocompatibility Complex class II genes in goats"** may be submitted by **Dr. Remya John V.** in partial fulfilment of the requirement for the degree.

Dr. K. C. Raghavan
(Chairman, Advisory Committee)
Professor
Centre for Advanced Studies in Animal Genetics and Breeding
College of Veterinary and Animal Sciences,
Mannuthy, Thrissur

Dr. K. V. Raghunandanan,
Director,
Centre for Advanced Studies in
Animal Genetics and Breeding,
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. G. Krishnan Nair,
Professor & Head,
Department of Veterinary
Microbiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. Thirupathi Venkatachalapathy,
Associate Professor,
AICRP on Pigs,
Centre for Pig Production and Research,
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

External Examiner
(Dr. M.R. JAHASHANKAR)
Professor & Head
Dept. of Animal Genetics &
Veterinary College, KVA
Hebbal - Bangalore -560

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Remya John V.

Dedicated To

My Beloved family

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Introduction

1. INTRODUCTION

Goat rearing contributes significantly to the rural economy of India. Over the last 15 years, goat population has increased by almost 50 per cent globally whereas, that of cattle increased by only 9 per cent and sheep decreased by 4 per cent. In India, during the last 40 years, the goat population rose by 140 per cent underlining the fact that the goat has emerged as a major livestock species that is enormously increasing in number. Goats play a very vital role in the livelihood security of small and marginal farmers and landless laborers.

India's vast genetic resources in goat is reflected by the availability of about 20 different breeds of goats that have evolved naturally through adaptation to different agro-ecological conditions. Indian goat breeds exhibit enormous variations in growth, fecundity, production of meat, milk and fiber, disease resistance and heat tolerance. Goat production in Kerala is mainly centered to its native breeds, Malabari (or Tellichery) and Attappady Black.

Diseases have always been a major problem in goats, limiting returns from goat rearing. The losses due to disease in goats scaled at national level were estimated to be Rs. 11,720 million per annum (Kumar *et al.*, 2003). Therefore, preventive measures to control disease incidence in goats are essential.

Prevention is the ideal solution for any disease. But prevention is not practical for all diseases. So, the chief defenses employed against pathogens are: vaccination, management measures and use of pharmaceuticals. The first two of these measures are extremely beneficial with many advantages, although they are not effective against all pathogens. In contrast, the use of pharmaceuticals which is currently the dominant method of preventing and treating disease carries several major disadvantages. The first and foremost is the increasing cost of essential drugs which is often beyond the financial capacity of most farmers. Another major problem is the issue of food safety and quality due to non-

compliance of withdrawal periods for drugs and environmental damage caused by drug residues. Development of drug resistant strains is yet another problem especially as the evolution of new drugs with more potential requires extensive research, which impose great economic burden. Use of pharmaceuticals reduces the selective advantage of natural resistance of animals, creating generations of animals with decreased immunity and increased susceptibility. Hence, we need to focus attention on selection for disease resistance *i.e*; selection of animals immune to diseases.

Indiscriminate crossbreeding programmes have caused rapid erosion in the genetic resources of the country. Many breeds of goats which may be treasures in terms of disease resistance qualities are not adequately characterized and evaluated. So there is an urgent need to study the variability in the genes associated with immunity in goats, which may pave the way for selection for disease resistance.

The most reliable and accurate method of selection for disease resistance is the selection based on molecular markers. The most important requirement for marker assisted selection is the identification of genes with the ability to influence disease resistance. The Caprine Major Histocompatibility Complex, called the Caprine Leucocytic Antigens (CLA) are specialized cell surface glycoprotein receptors which control antigen processing and presentation. It provides the major genetic component of disease resistance or susceptibility. These molecules are classified as class I, class II or class III. The characteristic feature of some class I and class II genes is the high degree of genetic polymorphism, which determines the efficiency of antigen binding. Class II molecules are primarily restricted to the surface of immune cells and are responsible for immune regulation. There are different kinds of MHC class II molecules of which DQ and DR subtypes are the most polymorphic and probably play a major role in the development of MHC restricted immune response.

In order to approach the goal of controlled breeding of animals with increased disease resistance, it is advisable to explore the relevant immune mechanisms and their genetic basis. Keeping this in view, this study was undertaken in Malabari and Attappady Black goats with the following objectives:

1. To identify and characterize different allelic variants of Major Histocompatibility Complex (MHC) class II DQA2 and DRB genes.
2. To determine the sequence of replicons of DQA2 and DRB genes.
3. To find out the association of different allelic variants of genes with immune status of goat.

Review of Literature

2. REVIEW OF LITERATURE

Goat rearing is of great importance in rural Indian households and plays a major role in the sustenance of small and marginal farmers as well as landless agricultural labourers. India's vast genetic resources in goat are reflected by the existence of 20 breeds of goats (Acharya, 1982). Goat production in Kerala is mainly centered on its native breeds: Malabari or Tellichery which is known for its prolificacy and Attappady Black reared in the hill tracts of Palakkad district. These breeds have evolved by centuries of selection in the agro climatic conditions of Kerala. The present investigation is aimed at studying the variability in the genes associated with eliciting of immune response in these breeds. The available literature on goats and most relevant details on other species are reviewed under the following headings:

2.1. MOLECULAR MARKERS

Markers revealing variations at DNA level are referred to as molecular markers (Mitra *et al.*, 1999). Molecular markers are capable of detecting variation at the DNA sequence level and possess unique genetic properties that make them more useful than other markers. These variations may be related to genetic differences in economically important traits and disease resistance. Molecular markers are numerous and are distributed ubiquitously throughout the

genome. They follow Mendelian inheritance and are multi allelic. They are unaffected by environmental factors, age and/or sex. The use of molecular markers to define the genetic makeup (genotype) and predict the performance of an animal is a powerful aid in animal breeding (Beuzen *et al.*, 2000). The selection based on these markers is known as Marker Assisted Selection (MAS). Several candidate genes for disease resistance have been studied by various authors. Major Histocompatibility Complex (MHC) genes are one of such candidate gene cluster found to be associated with disease resistance.

2.2 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

In order to trigger an immune response, antigen processing requires not only the fragmentation of antigen molecules inside the cells, but also the binding of these fragments to an appropriate antigen presenting molecule. These antigen presenting molecules are called histocompatibility molecules. They are specialized receptor glycoproteins coded by genes located in the gene complex called the Major Histocompatibility Complex (MHC). Thus MHC can be considered as an organized cluster of genes that control antigen processing and presentation. The MHC therefore provides the major genetic component of infectious or autoimmune disease resistance or susceptibility (Tizard, 1977).

The MHC of vertebrates includes class I and class II genes which encode cell surface proteins with a central function in the immune system (Zinkernagel and Doherty, 1979).The characteristic feature of some class I and class II genes, is the high degree of genetic polymorphism (Nagy *et al.*, 1981) which determines the efficiency of antigen binding.

2.2.1 MHC CLASS II MOLECULES

MHC class II molecules, which are heterodimers composed of two non covalently linked protein chains called alpha (∞) and beta (β) chains, are expressed on the surface of antigen presenting cells. The alpha chains are 31 to

34 kDa, and the beta chains are 25 to 29 kDa. Each chain has two extra cellular domains, a connecting peptide, a transmembrane domain and a cytoplasmic domain. MHC class II molecules have an antigen binding groove that is formed jointly by their α_1 and β_1 domains. The walls of the groove are formed by two parallel alpha helices, and its floor consists of a beta pleated structure (Tizard, 1977).

MHC class II molecules are involved in antigen presentation to $CD4^+$ T cells, which help B cells to produce appropriate immunoglobulins (Andersson, 1990).

There are different kinds of MHC class II molecules, of which DQ and DR subtypes are the most polymorphic and probably play a major role in the development of MHC restricted immune response (Amills *et al.*, 1996).

The caprine lymphocyte antigen (CLA) system i.e; MHC of goat, has been shown to be similar to that of cattle, which has two expressed class II antigens, DQ and DR (Takada *et al.*, 1998).

The ovine MHC class II region is better characterized with evidence of one DRA, four DRB (one coding and three non coding), one DQA1, two DQA2 and one each of the DQB1, DQB2, DNA, DYA, DYB, DMA and DMB genes in the region (Dukkipati *et al.*, 2006).

The major difference is that class I molecules present 'endogenous' antigen to cytotoxic T-lymphocytes, while class II molecules play a critical role in the initiation of the immune response by presenting exogenous antigens to specially stimulate helper T- lymphocytes (Li *et al.*, 2006).

2.2.1.1 MHC CLASS II DRB GENES

A striking feature of MHC class II genes is the extensive polymorphism and this polymorphism is characterized by a large number of alleles at each locus and a large number of amino acid substitutions between alleles.

MHC molecules of DR subtype have been identified as one of principal class II proteins found on the surface of goat cells (Schwaiger *et al.*, 1993a).

In the goat, one DRB gene with 22 different alleles has been described (Schwaiger *et al.*, 1993b), but there is some experimental evidence for the existence of a second caprine DRB locus (Schwaiger *et al.*,1993a; Amills *et al.*, 1995).

Associations of alleles of the bovine major histocompatibility complex DRB3 exon 2 (BoLA DRB*02) with occurrence of disease and production traits have previously been documented (Sharif *et al.*, 1998a, b).However, little is known about the associations between CLA-BRB*02 alleles and the resistance to disease and production traits.

Jugo and Vicario (2000) provided the evidence for the presence of two copies of expressed DRB1 genes in a study on single strand conformational polymorphism and sequence polymorphism of MHC DRB exon II in Latxa and Karrantzar sheep.

Sena *et al.* (2003) studied the polymorphism in MHC DRB (*Bubu-DRB*) and DRA (*Bubu-DRA*) loci in *Bubalus bubalis* belonging to four different breeds, three river buffalo and one swamp buffalo breeds. They found eight alleles of *Bubu-DRB* genes.

The CLA-DRB exon encodes the β1 domain of the DR molecule, which is in close contact with the foreign antigen and displays a very high degree of polymorphism with more than 25 different sequences (Li *et al.*, 2006).

2.2.1.2 MHC CLASS II DQA GENES

In sheep, there are two DQA genes, called DQA1 and DQA2 (Scott *et al.*, 1991). Seven alleles plus a null allele at DQA1 locus and 16 alleles at the DQA2 locus have been identified by RFLP analyses (Wright and Ballingal, 1994).

Escayg *et al.* (1996) reported that the genes encoding MHC class II DQA genes exhibit high levels of polymorphism in sheep in contrast to humans.

Ballingall *et al.* (1998) described the nucleotide and predicted amino acid sequences of the entire coding region of the three transcribed BoLA (Bovine Leucocytic Antigen) DQA3 genes. This provided additional evidence that the BoLA DQA3 locus is distinct from BoLA DQA1 and BoLA DQA2 loci.

Ten sequences of ovine DQA2 have been characterized by Snibson *et al.* (1998) and sequence analysis by Zhou and Hickford (2004) has revealed 14 ovine DQA1 sequences.

Variation in the ovine and caprine DQA2 gene second exon was studied by Hickford *et al.* (2004) and Zhou *et al.* (2005) respectively.

Zidi *et al.* (2008) reported the complete sequence of the coding region of the MHC class I genes in goats. The length of the corresponding open reading frame was 1077 bp encoding a mature protein of 337 amino acids.

2.3 ISOLATION OF GENOMIC DNA

The first step in carrying out genomic analysis studies is the isolation of pure high molecular weight genomic DNA from the sample population.

Sambrook *et al.* (1989) described that the standard way to remove proteins from nucleic acid solution was to extract first with phenol-chloroform and then with chloroform. This procedure takes advantage of the fact that

deproteinisation is more efficient when two different organic solvents are used instead of one.

Aravindakshan *et al.* (1998) compared three methods of DNA extraction, *viz.*, the guanidine hydrochloride method, the high salt method and phenol chloroform method and demonstrated that both high salt and phenol chloroform methods produced good yields of high molecular weight DNA from cattle white blood cells, whereas the guanidine hydrochloride method failed to yield clean DNA.

2.3.1 YIELD AND QUALITY OF DNA

Beckmann *et al.* (1986) reported 300-500 µg genomic DNA per 10 ml of cattle blood. Appa Rao *et al.* (1994) suggested a rapid and simple modified phenol chloroform extraction procedure for isolation of genomic DNA from blood for RFLP studies in livestock animals and obtained a yield of 250- 300 µg DNA from 15 ml of whole blood.

Ahmad *et al.* (1995) reported a modification of the widely used standard proteinase K-phenol extraction method for improving the yield and purity of DNA from frozen blood samples by an initial trypsinisation of whole blood before cell lysis to obtain lymphocytic nuclei and subsequent DNA purification. They reported an increased total yield of DNA as well as improved purity with pre-trypsinised blood samples.

Senthil *et al.* (1996) reported that the mean yields of genomic DNA from 15 ml of cattle blood samples extracted by the phenol and high salt methods were 444.58±21.54 µg and 615.55±20.72 µg, respectively. The ratio of optical density at 260 and 280 nm were more than 1.7 indicating good deproteinisation.

Aravindakshan *et al.* (1998) reported that the mean yields of DNA extracted from 10 ml of whole blood of cattle and buffalo by phenol and high salt methods were 394.50/446.16 and 344.26/432.83 micrograms, respectively.

The ratio of optical densities at 260 and 280 nm was consistently between 1.75 and 1.91, indicating good deproteinisation.

Chithra (2002) reported an average yield of 231.097±11.65 µg DNA from five ml of goat blood. The ratio of optical density at 260 and 280 nm was more than 1.7.

Mathew (2004) and Seena (2006) reported yields of 350.05±10.05 µg and 357±23.057 µg of DNA respectively, from five ml of blood of Malabari goats.

2.4 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction (PCR) was developed by Mullis *et al.* (1986). The technique involves enzymatic amplification of a specific sequence of DNA using two oligonucleotide primers that flank the DNA segment to be amplified resulting in many fold increase in amount of target sequence.

According to Saiki *et al.* (1988) a thermostable DNA polymerase isolated from *Thermus aquaticus* enables *in vitro* DNA amplification to be performed at higher temperature and significantly improves the specificity, yield, sensitivity and the length of the product amplified.

The presence of high concentrations of oligonucleotides can cause priming at ectopic sites, with consequent amplification of undesirable nontarget sequences. Conversely the PCR is extremely inefficient when the concentration of primers is limiting (Sambrook *et al.*, 1989).

Don *et al.* (1991) reported that the appearance of spurious bands can be reduced by adjusting the [mg^{2+}] concentration or increasing the annealing temperature of the PCR.

Bradely and Hills (1997) successfully employed PCR to examine polymorphisms within populations and the technique was not error free. *In vitro*

recombination can also occur when amplifying alleles of single gene loci in heterozygous individuals and the recombination frequency can vary as a function of different polymerases in the extension step of PCR.

2.5 ANALYSIS OF POLYMORPHISM IN DRB AND DQA2 GENES

2.5.1 DRB GENE

2.5.1.1 AMPLIFICATION OF MHC CLASS II DRB GENE SECOND EXON

Amills *et al.* (1995) achieved amplification of the second exon of the caprine DRB gene by performing PCR in a 50 µl reaction mixture containing PCR buffer (50 mM KCl, 10 mM Tris Cl, 1% triton X-100), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 0.5 µM of each primer, 200 ng of genomic DNA and 1.25 U of Taq polymerase. The thermal cycling profile was: hot start for 5 min at 94^0 C followed by 30 cycles of 60 s at 94^0 C, 90 s at 60^0C and 90 s at 72^0C.

Amplification of caprine DRB second exon was carried out in two rounds by Amills *et al.* (1996). The thermal profile of the first round was 94^0 C/5 min and 10 cycles of 94^0 C/1 min, 60^0C/2 min and 72^0C/2 min. For the second round, it consisted of 25 cycles of 94^0C/1 min, 65^0C/0.5 min, 72^0C/0.5 min. The composition of the PCR reaction with a final volume of 100 µl, was 2.5 mM $MgCl_2$, 100 µM of each dNTP, 0.1 µM of each primer, 0.2 ng/ µl of genomic DNA (first round) or 10 µl of the first round amplified product (second round), and 0.025 U/ µl of Taq DNA polymerase.

Ahmed and Othman (2006) carried out the amplification of the second exon of the caprine DRB gene in a PCR cocktail consisting of 1.0 µM upper and lower primers and 0.2 mM dNTPs, 10 mM Tris (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 % gelatin (w/v), 0.1% Triton X-100 and 1.25 units of Taq polymerase. The cocktail was transferred into tubes with 100 ng DNA of goat.

The reaction was cycled for 1 min at 94°C, 1.30 min at 60°C and 2 min at 72°C for 30 cycles.

Li *et al.* (2006) carried out amplification of CLA-DRB*02 in a final volume of 50 µl containing PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1% triton X-100), 1.5 mM $MgCl_2$, 100 µM of each dNTP, 2 µM of each primer, 2U of Taq polymerase and approximately 300 ng of genomic DNA. The amplification conditions comprised an initial denaturation of 95^0C for 4 minutes followed by 30 cycles of denaturation at 95^0 C for 1 min, annealing at 63^0C for 1 min and extension at 72^0C for 1 min, with a final extension at 72^0C for 7 min.

2.5.1.2 PCR-RFLP ANALYSIS IN DRB GENE

The restriction polymorphism of the caprine DRB second exon has been analyzed by Amills *et al.* (1995) and two different restriction patterns have been found depending on the presence or absence of a *Taq* I and a *Pst* I site at positions 122 bp and 241 bp of the PCR product respectively. Close associations were detected between the presence of *Taq* I and a *Pst* I restriction sites and amino acid substitutions at positions 40 and 78 respectively, suggesting that PCR restriction fragment length polymorphism (RFLP) could be a useful tool in relating amino acid substitutions at critical positions and disease resistance.

A PCR-RFLP typing method for the second exon of the caprine MHC class II DRB gene by using a maximum of three restriction enzymes, corresponding to different combinations of *Rsa*I, *Bsa*I, *Acc*I, *Nde*II, *Hae*III and *Hpa*II was developed by Amills *et al.* (1996). This procedure allows to distinguish unequivocally 18 of the 22 caprine DRB alleles and close associations have been found between RFLPs and amino acid substitutions at positions which are expected to be involved in the formation of antigen recognition site (ARS) of the DR molecule.

Ahmed and Othman (2006) analyzed the genetic polymorphism of MHC class II DRB gene in Egyptian goats by PCR-RFLP method using the enzymes *Taq*I and *Pst*I. Restriction digestion of PCR product by *Taq*I enzyme represented two digested fragments at 122- and 163-bp ('T' restriction pattern) or undigested fragment at 285bp ('t' restriction pattern). After the *Pst*I digestion, the results showed that the frequency of pp pattern (270- and 15- bp restricted fragments) was 29.5% and the frequency of heterozygous Pp pattern (270-, 226-, 44- and 15- bp restricted fragments) was 70.5%, while the PP pattern (226-, 44- and 15- bp restricted fragments) was not displayed in tested goat animals.

Allelic variations in the second exon of the caprine leucocytic antigen-DRB3 gene (CLA-DRB*02) were investigated by Li *et al.* (2006) in a total of 459 animals from 12 Chinese indigenous goat populations. Six alleles and 18 restriction digestion profiles were distinguished by digestion of PCR amplification product of CLA-DRB*02 with *Hae* III.

2.5.2 DQA2 GENE

2.5.2.1 AMPLIFICATION OF MHC CLASS II DQA2 GENE SECOND EXON

Hickford *et al.* (2004) amplified the entire second exon of ovine DQA2 gene by performing PCR in a 20 µl reaction volume containing 50 ng of genomic DNA from whole blood, 0.25 µM each primer, 150 µM dNTP, 1 U of DNA polymerase and 1 X reaction buffer supplied (containing 1.5 mM $MgCl_2$). The thermal profile consisted of denaturation at 94^0C for 2 min, followed by 32 cycles of 94^0C for 30 sec, 59^0C for 30 sec and 72^0C for 50 sec. This was followed by a final extension step at 72^0C for 5min.

Zhou *et al.* (2005) performed the amplification of entire second exon of caprine DQA2 in a 20 µl reaction containing 50 ng of genomic DNA on one 1.2 – mm punch of FTA paper, 0.25 µM of each primer, 150 µM of nucleotides, 2.5 mM of Mg^{2+}, 0.5 U of DNA polymerase, and 1 X reaction buffer. The thermal

profile consisted of 2 min at 94^{0}C, followed by 32 cycles of 94^{0}C for 30 sec, 58^0 C for 30 sec and 72^{0}C for 30 sec with a final extension of 5 min at 72^{0}C.

2.5.2.2 ANALYSIS OF POLYMORPHISM IN MHC CLASS II DQA GENES

Andersson *et al.* (1986) analyzed the polymorphism of DQα gene by Southern blot analysis using human cDNA probes. Highly polymorphic restriction fragment patterns were obtained when genomic DNA, digested with any one of the *Bam*HI, *Eco*RI, or *Pvu*II restriction enzymes, was hybridized with DQα probe and the analysis resolved 9 allelic variants of DQα.

Hickford *et al.* (2000) carried out EcoRV-RFLP and Southern blot hybridization to distinguish copy number and confirmed duplication of the Ovine DQA2 region.

Zhou and Hickford (2004) investigated the variation in the ovine DQA1 gene by amplification of exon 2 using PCR, followed by single strand cofirmational polymorphism (SSCP) analysis, cloning and DNA sequencing. Fourteen novel SSCP patterns, representing 14 different sequences, were identified.

Hickford *et al.* (2004) investigated variation in the ovine DQA2 gene in approximately 2000 sheep from six breeds by single strand conformational polymorphism (SSCP) analysis and 22 DQA2 amino acid sequences were identified.

Zhou *et al.* (2005) studied variation in the caprine DQA2 gene using single strand conformational polymorphism and DNA sequencing and 11 DQA2 alleles were defined by SSCP patterns from 23 goats. All the caprine alleles shared high sequence homology to ovine DQA2 sequences, and exhibited a pattern of polymorphism similar to DQA2 alleles from sheep and cattle but different from caprine DQA1 sequences.

2.6 DNA SEQUENCING

Maxam and Gilbert (1977) reported that DNA could be sequenced by a chemical procedure that broke a terminally labelled DNA molecule partially at each repetition of a base. The lengths of the labelled fragments then identify the positions of that base. They described reactions that cleaved DNA preferentially at guanines, adenines, cytosines and thymines equally and at cytosines alone. When the products of these four reactions were resolved by size, by electrophoresis on a polyacrylamide gel, the DNA sequence could be read from the pattern of radioactive bands.

Sanger *et al.* (1977) explained a method of determining nucleotide sequences in DNA in which 2′, 3′-dideoxy and arabinonucleoside analogues of the normal deoxynucleoside triphosphates, acted as specific chain terminating inhibitors of DNA polymerase. One of the nucleotides was radiolabelled so that the mixture fractionated by electrophoresis could be subjected to autoradiography from which the required sequence could be read.

Chan (2005) documented that faster sequencing methods would undoubtedly lead to faster single nucleotide polymorphism discovery. With the completion of the human genome sequence, there is now a focus on developing new sequencing methodologies that would enable "personal genomics" or the routine study of our individual genomes.

Eid *et al.* (2009) presented single-molecule, real-time sequencing data obtained from a DNA polymerase performing uninterrupted template-directed synthesis using four distinguishable fluorescently labelled deoxyribonucleoside triphosphates (dNTPs). They detected the temporal order of their enzymatic incorporation into a growing DNA strand with zero-mode wave guide nanostructure arrays, which provided optical observation volume confinement and enabled parallel, simultaneous detection of thousands of single-molecule sequencing reactions.

2.7 SEQUENCE ANALYSIS

2.7.1 BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST)

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The programme compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationship between sequences as well as helps to identify members of gene families (Altschul *et al.*, 1990).

Tatusova and Madden (1999) described 'BLAST 2 sequences' as a new BLAST based tool for aligning two protein or nucleotide sequences. BLAST 2 sequences utilized the BLAST algorithm for pairwise DNA-DNA or protein-protein comparison. A World Wide Web version of the programme can be used interactively at the NCBI site (http://www.ncbi.nlm.nih.gov/gorf/bl2.+++html).

2.7.2 CLUSTAL W

Clustal W is a multiple sequence alignment programme for nucleotide and protein sequences. Clustal uses a method called pair wise progressive sequence alignment. This heuristic method first does a pairwise sequence alignment for all the sequence pairs that can be clustered from the sequence set. A dendrograph of the sequence is then done according to the pair wise similarity of the sequences. Finally multiple sequence alignment is constructed by aligning sequences in the order, defined by the guide tree. It is available at http://www2.ebi.ac.uk/clustalw/

Higgins and Sharp (1988) described an approach for performing multiple alignments of large numbers of amino acids or nucleotide sequences. The method was based on first deriving a phylogenetic tree from a matrix of all pair wise sequence similarity scores, obtained using a fast pair wise alignment algorithm. Then the multiple alignments were achieved from a series of pair

wise alignments of clusters of sequences, following the order of branching in the tree.

The current Clustal programs all derive from Clustal W (Thompson *et al.*, 1994), which incorporated a novel position-specific scoring scheme and a weighting scheme for down weighting over-represented sequence groups. Here, the sensitivity of sequence alignment method had been greatly improved for the alignment of divergent protein sequences through sequence weighting, position specific gap penalties and weight matrix choice.

Chenna *et al.* (2003) mentioned that the Clustal series of programmes were widely used in molecular biology for the multiple alignments of both nucleic acid and protein sequences and for preparing phylogenetic trees.

2.7.3 SEQUENCE ANALYSIS OF DRB GENE

Brown *et al.* (1993) reported that amino acid positions 30, 37, 47, 60 and 78 of the β_1 domain of the HLA- DR molecule are involved in the formation of the antigen binding site.

Lewin (1994) reported that *Tyr* substitution at position 78 of the bovine DR molecule has been correlated with a tendency towards susceptibility to persistent lymphocytosis in cattle infected with bovine leukaemia virus.

Stern *et al.* (1994) carried out the X-ray crystallographic analysis of the HLA-DR1 molecule complexed with a haemagglutinin (HA) influenza virus peptide and revealed that position 26 is solvent inaccessible, indicating that it may interact with the bound peptide, and that positions 47 and 78 take part in formation of pockets 7 and 4 respectively.

Amills *et al.* (1995) cloned and sequenced 285 bp fragment of caprine MHC DRB gene second exon and compared the sequence with other caprine, ovine and bovine second exon allelic variants. Nucleotide identities between this sequence (Caae- DRB23) and other caprine DRB alleles ranged from 85.6 %

(Caae- DRB22) to 96.5% (Caae-DRB5). Caae-DRB23 allele had greater similarity to certain bovine and ovine DRB alleles (96.9% of bp identity to Bota-DRB13 and Ovar-DRB5) than to other caprine DRB alleles.

Analysis of the translated caprine DRB sequences by Amills *et al.* (1996) allowed correlation of the presence of the 79, 91, 112, 142,181 and 235 RsaI sites with the occurrence of Tyr residues at positions 26, 30,37,47,60 and 78 of the β_1 domain, respectively.

Sequence analysis of caprine DRB3 gene in Chinese indigenous goats by Li *et al.* (2006) showed that among the 267 nucleotides and 89 amino acid positions, 40 (15%) nucleotide positions and 31 (34.8%) amino acid residues were variable. Four long polymorphic regions and three high variable regions (HVR) were found in the nucleotide and deduced amino acid sequences, respectively.

2.7.4 SEQUENCE ANALYSIS OF DQA GENE

Ballingall *et al.* (1998) identified three sequences of BolA-DQA3 gene. The three sequences were identical within the first exon encoding the signal or leader sequence, but differ by 13 and 7 nucleotides from the corresponding region of the DQA1 and DQA2 genes, respectively.

The importance of specific residues within the antigen binding groove of DQA2 and how antigen-peptide binding ability can be altered with only one or two amino acid changes, has been illustrated in humans by Toussirot *et al.* 1999.

Hickford *et al.* (2004) identified 23 ovine DQA2 sequences from six breeds of sheep. The isolation of three or four DQA2 sequences from a single sheep suggested the existence of up to two DQA2 loci.

Zhou *et al.* (2005) analyzed the DQA2 sequences from 23 Boer goats and found that of the 82 amino acid sites within the α_1 domain of the DQA2, 38

(46%) were polymorphic. In the putative antigen binding region, 14 out of the 19 amino acid sites (74%) were polymorphic. The most polymorphic sites were observed at α14 (D, E, V, T), α68 (I, K, T, A) and α79 (L, R, H, W) which are all included in the putative antigen-binding region. The caprine DQA2 sequences were most similar to the ovine DQA2 sequences, but different from caprine DQA1 sequences.

2.8 MHC POLYMORPHISM AND IMMUNOGLOBULIN LEVELS

The level of immunoglobulin in the blood gives an idea about the disease resistance capacity of animals. The initial passive immunity is dependent upon the colostral transfer of immunoglobulins via intestinal absorption to systematic circulation. The animal starts synthesizing its own immunoglobulins after few weeks only (Brambel, 1970).

Bhargava (1977) reported that in buffaloes, the level of immunoglobulin showed a clear trend of decreasing after the initial peak on the second day. Similar findings were reported by Raja (1977) in cattle.

Nandakumar (1981) reported that the mean immunoglobulin level in goats had an initial peak on the third day which declined gradually by 6-7 weeks, there after it began to rise again.

Raghavan (1983) reported that the peak post colostral level in buffaloes was found to be maximum between 20 -24 hours which started a declining trend till two weeks and slowly started increasing to reach a final concentration at 12 weeks of age. The second increase is attributed to the active synthesis of immunoglobulin by the system.

High serum immunoglobulin may predict high antibody response (Williams and Halliday, 1980; Burton *et al.*, 1989) and serum immunoglobulin concentrations correlate with protection against clinical mastitis and pneumonia in calves (Mallard *et al.*, 1983; Corbeil *et al.*, 1984).

Mallard *et al.* (1989) studied the variation in serum IgG and IgM concentration in three homozygous SLA-defined strains of miniature swine (SLA^a, SLA^c, SLA^d) and recombinant strain SLA^g (AB^cD^d) to analyze the effect of SLA haplotypes on immune response. Least square mean comparison indicated that pigs of the dd, dg and gg haplotypes had significantly higher serum IgG than pigs of the other haplotypes.

Iepema *et al.* (2008) studied serum gammaglobulin concentration in goat kids upto three months of age and found that a highest Ig level was obtained at three months of age.

The study of the variations in the genes responsible for disease resistance could greatly enhance the efficiency of breeding animals that possess innate disease resistance. Furthermore, it will provide new tools to facilitate research into the mechanisms of infection, possibly leading to additional pharmacologic and management approaches for the control of disease transmission. Finally, genetic control of animal diseases can reduce the costs associated with diseases, improve animal welfare, and provide healthy animal products to consumers, and it should be given more attention (Ibeagha-Awemu, 2008).

Materials and Methods

3. MATERIALS AND METHODS

3.1 EXPERIMENTAL ANIMALS

A random sample of 85 Malabari goats and 30 Attappady Black goats in the age group of two to six months formed the materials for this study. The experimental animals were selected from Kerala Agricultural University Goat and Sheep Farm, as well as field units (Thalassery and Badagara) of All India Co-ordinated Research Project on Malabari goat improvement.

3.2 COLLECTION OF SAMPLES

3.2.1 BLOOD

Blood samples, five ml each were collected from jugular vein of goats using sterile disposable syringes, into EDTA coated vacutainers under strict aseptic precaution. The samples were preserved in ice pack and brought to the laboratory where it was stored at -20^{0}C till processing. The samples were used for isolation of DNA.

3.2.2 SERUM

Similarly, five ml of blood was collected into plain vacutainers for serum samples. The tubes were kept at 45 degree slanting position and blood clots were removed. The serum samples thus obtained were centrifuged at 2000 rpm for 2 min to remove all the remaining red blood cells. The supernatent was then separated and stored at -20 ^{0}C till processing. This was used for estimation of immunoglobulins.

3.3 EXTRACTION OF GENOMIC DNA

DNA was extracted from whole blood using the standard phenol chloroform extraction procedure (Sambrook and Russell, 2001), with necessary modifications. The procedure followed was as follows.

1. To five ml of the blood sample, double the volume of ice cold RBC lysis buffer* was added and kept in ice cold condition with occasional mixing for 10 min for complete lysis of red blood cells.
2. The leukocytes were pelleted by centrifuging at 4000 rpm for 10 min, and the supernatent containing lysed RBCs was discarded.
3. The pellet was re-suspended in ice-cold RBC lysis buffer and the above steps were repeated till a clear pellet was obtained.
4. The pellet was then washed twice with Tris buffered saline* by vigorous vortexing followed by centrifugation at 3000 rpm for 10 min.
5. The white blood cell pellet was resuspended in three ml saline EDTA buffer*. The cell suspension was incubated at 50°C in water bath with 0.25 ml of 20 per cent sodium dodecyl sulphate (SDS) and 25 µl of proteinase-K (20 mg/ml) for a minimum of 3 hrs, swirling the viscous solution occasionally.
6. The digested samples were cooled to room temperature, 300 µl of 5 M sodium chloride* was added and mixed. An equal volume of phenol* (pH 7.8) saturated with Tris-hydrochloride was added, mixed by gentle inversion for 10 min and centrifuged at 4500 rpm for 10 min.
7. The aqueous phase containing DNA was collected in fresh tubes, to which an equal volume of saturated phenol: chloroform: isoamyl alchohol (25:24:1) was added. The contents were mixed and centrifuged at 4500 rpm for 10 min.
8. The aqueous phase was transferred into fresh tubes, equal volume of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 4500 rpm for 10 min.

9. The supernatent was transferred to a sterile 50 ml beaker and one tenth volume of 3 *M* sodium acetate* (pH 5.5) was added and mixed.
10. To this mixture, equal volume of chilled isopropyl alcohol was added and the precipitated DNA was spooled out using a fresh micropipette tip, washed in 70 per cent ethanol and air-dried.
11. Dried DNA was re-suspended in 0.5 ml of Tris EDTA buffer * and stored at -20°C.

** Composition and methods of preparation of reagents and buffers are provided in Annexure-I.*

3.4 DETERMINATION OF YIELD, PURITY AND QUALITY OF DNA

Twenty microlitres of the DNA stock solution was diluted to 2 ml with sterile distilled water, giving a dilution of 100 times. Optical densities (OD) were measured at 260 nm and 280 nm using a 2 ml cuvette in a UV spectrophotometer (Jenway, UK). Sterile distilled water was used as blank. Yield and purity of DNA samples were estimated as follows.

3.4.1 YIELD OF DNA SAMPLES

An OD of one at 260 nm wave length corresponds to approximately 50 µg/ml of double stranded DNA. Concentration of DNA stock solution was calculated as follows.

Concentration of DNA stock solution (µg/ml) = OD_{260} x Dilution factor x 50

3.4.2 PURITY OF DNA SAMPLES

The purity of DNA samples was assessed by estimating the ratio between the readings at 260 and 280 nm wave lengths. Pure DNA samples have OD_{260}/OD_{280} ratios of 1.7 to 1.9.

3.4.3 CHECKING QUALITY OF DNA

To determine the quality and molecular weight of DNA samples, 1 µl each of stock solution of DNA was checked electrophoretically using 0.8 percent agarose in 1 x TAE buffer in a horizontal submarine gel electrophoresis unit.

The agarose in 1 x TAE buffer containing 0.5 µg/ml of ethidium bromide was heated until it was a clear solution and was cooled to 50^0C. The comb was kept in proper position in the gel tray on a level surface and the molten agarose was poured carefully into the gel tray avoiding air bubbles. After gelling the comb was removed gently and the gel tray was immersed in the buffer tank with 1 x TAE buffer. From the DNA stock solution 0.5 to 1 µg DNA was mixed with one-sixth volume of 6x gel loading buffer and the samples were loaded into the wells carefully. Electrophoresis was carried out at 80V at room temperature until the bromophenol blue dye migrated more than two-third of the length of the gel. The gel was visualized under a UV transilluminator (Hoefer,USA).

3.5 TEMPLATE DNA PREPARATION FOR PCR

Template DNA for PCR was prepared by diluting the DNA stock solution in sterile triple distilled water to a concentration of 50 ng/ µl and was stored at -20^0C.

3.6 SELECTION, RECONSTITUTION AND DILUTION OF PRIMERS

The primers for the Major Histocompatibility Complex (MHC) class II DRB and DQA2 genes were selected from published reports of Ahmed and Othman (2006) and Zhou *et al.* (2005) respectively and were custom synthesised (Integrated DNA technologies Pvt. Ltd., U.S.A).

DRB Forward primer: 5' TAT CCC GTC TCT GCA GCA CAT TTC 3'
DRB Reverse primer: 5' TCG CCG CTG CAC ACT GAA ACT CTC 3'

DQA2 Forward primer: 5'CTT CCT GCT CCT CAC CCT CAC 3'
DQA2 Reverse primer: 5'AAAGAG AAG TAG AAT GGT GGA CAC TT 3'

The primers obtained in lyophilised form were centrifuged at 10,000 rpm for 10 min and were reconstituted in sterile distilled water to a concentration of 200 pM/ µl. The tubes were kept at room temperature with occasional shaking for one hour. The tubes were spun briefly to pellet down the insoluble particles, if any and the stock solution was distributed into 10 µl aliquots and stored at -20^{0}C. At the time of use, the aliquots were thawed and further diluted 10 fold before using for PCR.

3.7 PCR-RFLP ANALYSIS

3.7.1 DRB LOCUS

The PCR-RFLP analysis was carried out at DRB locus by digesting the amplified product with *Taq*I and *Pst*I restriction enzymes to analyse the polymorphic pattern.

3.7.1.1 SETTING UP OF PCR

A master mix was prepared just before setting up the PCR assay combining 10x PCR buffer, 50 mM $MgCl_2$, dNTP mix, primer pairs, Taq DNA polymerase and sterile distilled water in such a way to get a final concentration of 1 x PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 10 pM of each primer and one unit Taq DNA polymerase in a total volume of 20 µl in 200 µl reaction tubes.

To each reaction tube 19 µl of master mix and 1 µl (50 ng) of template DNA were added. The tubes were spun briefly and placed in the thermal cycler. The standardized thermal cycling profile consisted of an initial denaturation of 3 min at 94 ^{0}C followed by 30 cycles of 1 min each at 94 ^{0}C, 56.7 ^{0}C and 72 ^{0}C. This was followed by a final extension of 7 min at 72 ^{0}C. The PCR amplified products were stored at -20 ^{0}C till analysed.

3.7.1.2 CHECKING OF TARGET DNA FOR AMPLIFICATION

The PCR products were checked by agarose gel electrophoresis to confirm the amplification before analysing for polymorphism.

Two microlitre aliquots of the PCR products were checked electrophoretically using one per cent agarose gel in 1 x TAE buffer. As a DNA size marker 100 bp DNA ladder was electrophoresed with the samples in a separate well.

3.7.1.3 DIGESTION OF AMPLIFIED PRODUCTS WITH *Taq* I

A master mix containing all the ingredients except the PCR product was prepared. Eight microlitres of the amplified products were taken in PCR tubes and seven microlitres of the master mix were added into each tube. The composition of master mix was as follows:

10X assay buffer	-	1.5 µl
BSA (0.1 µg/ µl)	-	0.1 µl
*Taq*I (20U/µl)	-	0.5 µl
Distilled water	-	4.9 µl
Total	-	7 µl

Digestion was carried out at 65°C for 3 hrs in a final volume of 15 µl in a dry bath. Following the digestion, the enzyme was inactivated by incubating the tubes at 80^0C for 20 min and the digested products were stored at 4^0C till analysed.

3.7.1.4 DIGESTION OF AMPLIFIED PRODUCTS WITH *Pst* I

A master mix containing all the ingredients except the PCR product was prepared. Eight microlitres of the amplified products were taken in PCR tubes and seven microlitres of the master mix was added into each tube. The composition of master mix was as follows:

10X assay buffer	-	1.5 µl
BSA (0.1 µg/ µl)	-	0.1 µl
Pst I (20U/µl)	-	0.8 µl
Distilled water	-	4.6 µl
Total	-	7 µl

Digestion was carried out at 37°C for $3^1/_2$ hrs in a final volume of 15 µl in a dry bath. Following the digestion, the enzyme was inactivated by incubating the tubes at 80^0C for 20 min and the digested products were stored at 4^0C till analysed.

3.7.2 DQA2 LOCUS

PCR-RFLP analysis was carried out at DQA2 locus by digesting the amplified product with *EcoR*V restriction enzyme to analyse the polymorphic pattern.

3.7.2.1 SETTING UP OF PCR

A master mix was prepared just before setting up the PCR assay combining 10x PCR buffer, 50 mM $MgCl_2$, dNTP mix, primer pairs, Taq DNA polymerase and sterile water in such a way to get a final concentration of 1 x PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 10 pM of each primer and one unit Taq DNA polymerase in a total volume of 10 µl in 200 µl reaction tubes.

To each reaction tube 9 µl of master mix and 1 µl (50 ng) of template DNA were added. The tubes were spun briefly and placed in the thermal cycler. The standardized thermal cycling profile consisted of an initial denaturation of 3 min at 94 ^{0}C followed by 30 cycles of 1 min each at 94 ^{0}C, 59.5 ^{0}C and 72 ^{0}C. This was followed by a final extension of 7 min at 72 ^{0}C. The PCR amplified products were stored at -20 ^{0}C till analysed.

3.7.2.2 CHECKING OF TARGET DNA FOR AMPLIFICATION

The PCR products were checked by agarose gel electrophoresis to confirm the amplification before analysing for polymorphism.

Two microlitres aliquots of the PCR products were checked electrophoretically using one per cent agarose gel in 1 x TAE buffer. As a DNA size marker 100 bp DNA ladder was electrophoresed with the samples in a separate well.

3.7.2.3 DIGESTION OF AMPLIFIED PRODUCTS WITH *EcoR*V

A master mix containing all the ingredients except the PCR product was prepared. Eight microlitres of the amplified products were taken in PCR tubes and seven microlitres of the master mix was added into each tube. The composition of master mix was as follows

PCR product	-	8.0 µl
10X assay buffer	-	1.5 µl
BSA (0.1 µg/ µl)	-	0.1 µl
EcoRV (20U/µl)	-	0.5 µl
Distilled water	-	4.9 µl
Total	-	15 µl

Digestion was carried out at 37°C for $3\frac{1}{2}$ hrs in a final volume of 15 µl in a dry bath. Following the digestion, the enzyme was inactivated by incubating the tubes at 80^0C for 20 min and the digested products were stored at 4^0C till analysed.

3.8 SEPARATION OF RESTRICTION FRAGMENTS

The digested DNA fragments of DRB and DQA2 second exons were separated by electrophoresis in 2.5 per cent agarose gels in 1 x TAE buffer containing ethidium bromide at 55 V for 3 hrs. As a DNA size marker pUC19/

*Msp*I digest was electrophoresed with the samples in a separate well. The gels were visualized and the images were documented in a gel documentation system (Bio rad Laboratories, USA).

3.9 ALLELIC AND GENOTYPIC FREQUENCIES OF DRB AND DQA2 LOCI

The allelic and genotypic frequencies at DRB and DQA2 loci were calculated by direct counting method for Malabari and Attappady Black goats separately, as well as for the pooled population. The variation of the allelic frequencies among the two populations was analyzed by the *Chi*-square test of significance as described by Snedecor and Cochran (1994), considering the allelic frequencies in a 2×2 table using the formula,

$$\chi^2 = \frac{(ad-bc)^2 N}{(a+c)(b+d)(a+b)(c+d)}$$

where a, b, c and d are allelic frequencies in different populations and N is the total number of alleles observed.

The distribution of *TaqI*/ *Pst*I and *EcoR*V genotypes in Malabari and Attappady Black goats was checked for Hardy-Weinberg equilibrium by *Chi*-square test comparing the observed and expected frequencies.

3.10 DNA SEQUENCING

Gel purification, cloning and sequencing of the amplified products of the second exon of DRB (285 bp) and DQA2 (305 bp) genes were carried out commercially (Bioserve Hyderabad, Pvt. Ltd.) by the dideoxynucleotide sequencing method using an automated DNA sequencer (Applied Biosystems, USA).

3.11 SEQUENCE ANALYSIS

3.11.1 DNA SEQUENCE ANALYSIS

The DRB (285 bp) and DQA2 (305 bp) second exon sequences obtained through sequencing were analysed by BLAST search at NCBI site for homology using BLASTn programme (http://www.ncbi.nlm.nih.gov/BLAST). The BLASTn programme compares a nucleotide query sequence against nucleotide sequences in the database. The DRB (285 bp) and DQA2 (305 bp) second exon sequences of Malabari and Attappady Black goats were submitted as query sequences and selected the MegaBLAST programme for searching highly similar sequences from the DNA database. The sequences of second exons DRB or DQA2 gene obtained from each breed were compared using BLAST2 programme.

3.11.2 SUBMISSION OF THE SEQUENCES TO GENBANK

The sequences of DRB and DQA2 gene second exon of Malabari and Attappady Black goats were submitted to the GenBank using BankIt option (http://www.ncbi.nlm.nih.gov/BankIt/) at the NCBI site and the unique accession numbers for the new sequences were obtained.

3.11.3 PROTEIN SEQUENCE ANALYSIS

Amino acid residues of the polypeptide chains of DRB and DQA2 second exon were predicted using the programme ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). The protein sequence was analysed by using the BLASTp programme at the NCBI site. This programme compares an amino acid query sequence against protein sequences in the data base. The predicted amino acid sequence was entered as query sequence and selected the protein database for the search.

A multiple sequence alignment of the predicted protein sequences of the Malabari and Attappady Black goats was carried out by the widely used computer programme EBI tool ClustalW (http://align.genome.jp/). This programme is available from European Bioinformatics Institute (EBI) ftp server. The input sequences were loaded in FASTA format.

3.12 IMMUNOGLOBULIN CONCENTRATION

3.12.1 ZINC SULPHATE TURBIDITY TEST

Immunoglobulin concentration in serum samples was calculated using Zinc sulphate turbidity test and standard curve (McEwan *et al.*, 1970).

3.12.1.1 PREPARATION OF WORKING SOLUTION OF $ZnSo_4$:

Working solution of zinc sulphate was prepared by diluting 4.1 ml of 5 per cent $ZnSo_4.7H_20$ solution to one litre of freshly boiled and cooled double distilled water.

3.12.1.2 PROCEDURE

1. Test tubes were arranged in three rows.
2. First two rows of tubes were named 'Test tubes' and the third row was named 'control tubes'.
3. Six ml of working solution of $ZnSo_4$ was poured into each test tube and a similar volume of distilled water in control tubes.
4. 0.1 ml of each serum sample diluted to 1 in 4 with distilled water was poured into each of the tubes.
5. Tubes were shaken gently and allowed to stand at room temperature for an hour.
6. Turbidity developed in tube was read in spectrophotometer at a wavelength of 595 nm. Adjustment was made against $ZnSo_4$ solution.
7. The reading of the control was subtracted from the average of the test solutions to arrive at the OD of each individual serum sample.

8. OD values were converted to gamma globulin concentration (mg/ml) of serum with prediction equation developed from standard curve.

3.12.1.2 STANDARD CURVE FOR SERA:

Bovine immuno gamma globulin (Sigma-Aldrich chemicals, Bangalore) was dissolved in pooled pre colostral kid sera, to give concentration ranging from 4 to 120 mg/ml. Pre colostral serum after dissolving the gamma globulin was diluted to 1 in 4 with distilled water. The standard solutions were then subjected to zinc sulphate turbidity test. The value obtained for the control was subtracted from average of observed values of the test solutions to arrive at the net OD values. Net value for OD was the average of three replications. A standard curve was plotted using the OD values. Linear prediction equation for finding out the immunoglobulin concentration from OD values was prepared from the standard curve using SPSS statistical package. The prediction equation obtained was

$Y = -5.05 + 113.493X$

Where,

Y= Immunoglobulin concentration

X= OD value

3.12.2 LEAST SQUARE ANALYSIS

The effect of age, breed and *Taq*I, *Pst*I, *Eco*RV genotypes on immunoglobulin concentration was worked out using least square analysis of variance as described by Harvey (1960) for non-orthogonal data. Model used was

$$Y_{ijklmn} = \mu + A_i + B_j + T_k + P_l + E_m + e_{ijklmn}$$

Where

Y_{ijklmn} = n^{th} observation of m^{th} *Eco*RV genotype of l^{th} *Pst*I genotype of k^{th} *Taq*I genotype of j^{th} breed of i^{th} age group

μ = General mean

A_i = Effect of i^{th} age group

B_j = Effect of j^{th} breed

T_k = Effect of k^{th} *Taq*I genotype

P_l = Effect of l^{th} *Pst*I genotype

E_m = Effect of m^{th} *EcoR*V genotype

e_{ijklmn} = Random error

Results

4. RESULTS

The results obtained in the present study aimed at PCR-RFLP analysis and sequence analysis of DRB and DQA2 genes and the association of different allelic variants with immune status in Malabari and Attappady Black goats are presented under the following heads:

4.1 Isolation of genomic DNA

4.2 Major Histocompatibility Complex (MHC) class II genes

4.2.1 PCR-RFLP analysis

4.2.1.1 PCR-RFLPs in DRB gene

4.2.1.2 PCR-RFLP in DQA2 gene

4.3 DNA sequencing

4.3.1 Sequence analysis of DRB gene

4.3.2 Sequence analysis of DQA2 gene

4.4 Effect of allelic variants on Immunoglobulin levels

4.1 ISOLATION OF GENOMIC DNA

The DNA used in this study was isolated from venous blood samples of 85 Malabari goats and 30 Attappady Black goats using phenol chloroform extraction method.

4.1.1 YIELD, QUANTITY AND QUALITY OF DNA

The average (mean ± SE) yield of DNA obtained from 5 ml of blood was 245.762±26.593 µg. The ratios of optical density at 260 and 280 nm were more than 1.7. On agarose gel electrophoresis the DNA samples were found to be of high molecular weight and appeared as single bands without sheared fragments.

4.2 MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II GENES

In the present study, PCR-RFLPs were carried out in the second exons of Caprine Major Histocompatibility Complex class II DRB and DQA2 genes.

4.2.1 PCR RFLP ANALYSIS

4.2.1.1 PCR RFLPs IN DRB GENE

The PCR reactions were set up for the amplification of the second exon of Major Histocompatibility Complex class II DRB gene using primers given by Ahmed and Othman (2006).

4.2.1.1.1 AMPLIFICATION OF DRB GENE BY PCR

For efficient amplification of the target sequence individual reaction components, time and temperature parameters were optimized. The DNA isolated from blood samples of goat was amplified by PCR at the DRB locus without any major non-specific amplified products (Fig. 4.1). In all the animals the size of the amplified product was approximately 285 bp.

4.2.1.1.2 PCR-RFLP ANALYSIS IN DRB GENE USING TaqI

The amplified DNA was digested with restriction enzyme *Taq*I and the restriction fragments were resolved by agarose gel electrophoresis. Upon electrophoresis three restriction digestion patterns could be observed (Fig. 4.2). These allowed the identification of two alleles *viz.*, t (163 and 122 bp fragments) and T (undigested fragment of 285 bp). The gene and genotypic frequencies of DRB/ *Taq*I polymorphism in Malabari and Attappady Black goats are given in Table 4.1.

The gene frequencies for T and t alleles in the Malabari goat were 0.57 and 0.43 and in Attappady Black the frequencies were 0.60 and 0.40, respectively.

In Malabari goat the genotypic frequencies were observed as 0.30 (TT), 0.55 (Tt) and 0.15 (tt), whereas in Attappady Black the frequencies were 0.33 (TT), 0.53 (Tt) and 0.13 (tt).

The populations of Malabari and Attappady Black goats were found to be in Hardy-Weinberg equilibrium by comparing the observed frequencies with expected frequencies using chi square test (Table 4.1). A comparison of the DRB/*Taq*I allele frequencies between Malabari and Attappady Black goats showed that the frequencies of T and t alleles observed in the both populations did not differ significantly (Table 4.1).

4.2.1.1.3 PCR-RFLP ANALYSIS IN DRB GENE USING Pst I

The amplified DNA was digested with restriction enzyme *Pst*I and the restriction fragments were resolved by agarose gel electrophoresis. Upon electrophoresis two restriction digestion patterns could be observed (Fig. 4.3). These allowed the identification of two alleles *viz*., P (270 and 15 bp fragments) and p (226, 44 and15 bp fragments). The gene and genotypic frequencies of DRB/*Pst*I polymorphism in the Malabari and Attappady Black goats are presented in Table 4.2.

The gene frequencies for P and p alleles in the Malabari goat were 0.73 and 0.27 and in Attappady Black the frequencies were 0.60 and 0.40, respectively.

In Malabari goat the genotypic frequencies of DRB/ *Pst*I polymorphism were observed as 0.46 (PP), 0.54 (Pp) and 0.00 (pp), whereas in Attappady Black the frequencies were 0.20 (PP), 0.80 (Pp) and 0.00 (pp).

The populations of Malabari and Attappady Black goats were found not to be in Hardy-Weinberg equilibrium by comparing the observed genotypic frequencies with expected frequencies (Table 4.3). A comparison of the DRB/*Pst*I allele frequencies between Malabari and Attappady Black goats showed that the frequencies of P and p alleles observed in the both populations did not differ significantly (Table 4.2).

In the present study, DRB/*Taq*I polymorphism was observed as three patterns with two alleles having a frequency of 0.57 (T) and 0.43 (t) in Malabari and 0.60 (T) and 0.40 (t) in Attappady Black goats. DRB/*Pst*I polymorphism showed two genotypes with two alleles, the gene frequencies of which were 0.73 (P) and 0.27(p) in Malabari and 0.60 (P) and 0.40 (p) in Attappady Black goats. There was no significant difference in allele frequencies between breeds with regard to both loci.

4.2.1.2 PCR RFLP IN DQA2 GENE

PCR reactions were set up for the amplification of the second exon and parts of flanked intronic sequences of Major Histocompatibility Complex class II DQA2 gene using primers given by Zhou *et al.* (2005).

4.2.1.2.1 AMPLIFICATION OF DQA2 GENE BY PCR

For efficient amplification of the target sequences individual reaction components, time and temperature parameters were optimized. The DNA isolated from blood samples of goats was amplified by PCR at the DQA2 locus without any major non-specific amplified products (Fig. 4.4). In all the animals the size of the amplified product was approximately 300 bp.

4.2.1.2.2 PCR-RFLP ANALYSIS IN DQA2 GENE USING EcoRV

The amplified DNA was digested with restriction enzyme *EcoRV* and the restriction fragments were resolved by agarose gel electrophoresis. Upon electrophoresis three restriction digestion patterns could be observed (Fig. 4.5). These allowed the identification of two alleles *viz.*, E (236 and 64 bp fragments) and e (undigested fragment of 300 bp). The gene and genotypic frequencies in the Malabari and Attappady Black goats are shown in Table 4.3.

The gene frequencies for E and e alleles in the Malabari goat were 0.55 and 0.45 and in Attappady Black the frequencies were 0.67 and 0.33, respectively.

In Malabari goat the genotypic frequencies of DQA2/*EcoRV* polymorphism were observed as 0.15 (EE), 0.79 (Ee) and 0.06 (ee) whereas in Attappady Black the frequencies were 0.37 (EE), 0.60 (Ee) and 0.03 (ee).

The population of Attappady Black goats was found to be in Hardy-Weinberg equilibrium while population of Malabari was in not in equilibrium by comparing the observed frequencies with expected frequencies (Table 4.3). A comparison of the allele frequencies between Malabari and Attappady Black goats was carried out employing the *Chi*-square test. It showed that the frequencies of E and e alleles observed in the both populations did not differ significantly (Table 4.3).

DQA2/*Eco*RV polymorphism has shown the presence of three restriction digestion patterns with two alleles with a frequency of 0.55 (E) and 0.45 (e) in Malabari goat and 0.67 (E) and 0.33 (e) in Attappady Black goats. Breed effect was not found to be significant in allele frequencies.

Table 4.1 Genotype and allele frequencies of DRB/*Taq*I polymorphism in Malabari and Attappady Black goats.

Population	Source	Genotype frequency			χ^2 value (df=2)	Allele frequency		χ^2 value (df=1)
		TT	Tt	Tt		T	t	
Malabari (85)	Number Observed	0.30 (25)	0.55 (47)	0.15 (13)	1.19^{NS}	0.57 (97)	0.43 (73)	0.185^{NS}
	Number Expected	0.33	0.49	0.18				
Attappady Black (30)	Number Observed	0.33 (10)	0.53 (16)	0.13 (4)	1.94^{NS}	0.60 (36)	0.40 (24)	
	Number Expected	0.36	0.48	0.16				

Figures in parenthesis are actual numbers
^{NS} – Not significant
df – degrees of freedom

Table 4.2 Genotype and allele frequencies of DRB/*Pst* I polymorphism in Malabari and Attappady Black goats.

Population	Source	Genotype frequency			χ^2 value (df=2)	Allele frequency		χ^2 value (df=1)
		PP	Pp	Pp		P	p	
Malabari	Number Observed	0.46 (39)	0.54 (46)	0.00 (0)	11.04*	0.73 (124)	0.27 (46)	2.098^{NS}
	Number Expected	0.53	0.40	0.07				
Attappady Black	Number Expected	0.20 (6)	0.80 (24)	0.00 (0)	14.41**	0.60 (36)	0.40 (24)	
	Number Observed	0.36	0.48	0.16				

Figures in parenthesis are actual numbers
^{NS} – Not significant
*– $P \leq 0.05$
**–$P \leq 0.01$
df – degrees of freedom

Table 4.3 Genotype and allele frequencies of DQA2/*EcoRV* polymorphism in Malabari and Attappady Black goats.

Population	Source	Genotype frequency			χ^2 value (df=2)	Allele frequency		χ^2 value (df=1)
		EE	Ee	ee		E allele	e allele	
Malabari	Number Observed	0.15 (13)	0.79 (67)	0.06 (5)	40.47^{**}	0.55 (93)	0.45 (77)	2.602^{NS}
	Number Expected	0.30	0.50	0.20				
Attappady Black	Number Expected	0.37 (11)	0.6 (18)	0.03 (1)	3.89^{NS}	0.67 (40)	0.33 (20)	
	Number Observed	0.45	0.44	0.11				

Figures in parenthesis are actual numbers

^{NS} – Not significant

** – $P \leq 0.01$

df – degrees of freedom

4.3 DNA SEQUENCING

Amplified products of DRB and DQA2 genes from Malabari and Attappady Black goats (two samples each from both breeds corresponding to different *Pst*I and *Eco*RV genotypes) were gel eluted, cloned and sequenced commercially.

4.3.1 SEQUENCE ANALYSIS OF DRB GENE

4.3.1.1 DNA SEQUENCE ANALYSIS OF DRB GENE

The sequencing of the amplified products of DRB gene form Malabari and Attappady Black goats revealed four different DRB alleles having 285 bp each. Three of them were novel alleles where as the fourth one was having 100% nucleotide identity with Caae-DRB1 allele from Saanen (Capra aegagrus-Accession# U00192). Nucleotide sequences of all the four alleles of MHC class II DRBI second exon in Malabari and Attappady Black goats are depicted in Fig. 4.6.

BLAST2 analysis showed that the sequences of second exons of DRB gene in Malabari goats had only 89 per cent identity with variations at 30 positions and the sequences from Attappady Black goats had 92 per cent identity with variations at 22 positions. The nucleotide sequence at the polymorphic *Taq*I site at position 122 bp was 'TCGA' (t allele) in the first sample of Malabari goat where as it was 'ACGA' (T allele) in the second sample. In Attappady goats, the sequences at position 122 bp were 'ACGA' and 'GCGA' (both correspond to T allele), respectively as against 'TCGA' (t allele) which is the recognition sequence for *Taq*I. The *Pst*I RFLP resulted from the presence of a polymorphic *Pst*I site at position 241bp of the amplified fragment. The nucleotide sequences at this polymorphic site in the sequenced samples were 'CTGCAG' (p allele) and 'GTGGAG' (P allele) in both Malabari and Attappady Black goats. Another non-polymorphic *Pst*I site is located at position 15 bp, in the forward primer complementary region.

The BLASTn analysis of the nucleotide sequence of the second exon of DRB gene of Malabari goat is given in Table 4.4. The sequence from Malabari goat has revealed 98, 95, 94 and 93 per cent identity with wild goat (*Capra aegagrus;* Accession # U00183.1), sheep (*Ovis aries;* Accession # FM209041.1), Indian cattle (*Bos indicus*; Accession # DQ834889.1) and European cattle (*Bos Taurus;*, Accession # AJ487835.1), respectively.

The BLASTn analysis of the nucleotide sequences of the second exon of DRB gene of Attappady Black goat is presented in Table 4.5. The sequence from Attappady Black goat has revealed 99, 95, 92 and 93 per cent identity with Saanen (*Capra aegagrus;* Accession # U00196.1), sheep (*Ovis aries;* Accession # FM998807.1), Indian cattle (*Bos indicus*; Accession # DQ834889.1) and European cattle (*Bos Taurus;*, Accession #AB523835.1), respectively.

Table 4.4 Results of BLASTn showing the percentage of identity of the MHC class II DRB second exon nucleotide sequence of Malabari goat with other species.

Accession	Description	Maximum Score	Total score	Query coverage (per cent)	Maximum identity (per cent)
AY496935.1	*Capra hircus* (DRB3)	460	460	96%	96%
FM209041.1	*Ovis aries* Ovar-DRB*0702	438	438	96%	95%
U00183.1	*Capra aegagrus* Caae-DRB01	427	427	84%	98%
AY212157.1	*Rupicapra pyrenaica* Rupy-DRB*09	411	411	96%	93%
AJ487835.1	*Bos taurus* BoLA-DRB3*2002	411	411	96%	93%
DQ834889.1	*Bos indicus* BoLa-DRB3	409	409	94%	94%
AY009506.1	*Oryx leucoryx* Orle-DRB*2	394	394	82%	97%
AJ920403.1	*Ovis dalli*, HLA-DRB*8 exon 2	394	394	87%	95%

Table 4.5 Results of BLASTn showing the percentage of identity of the MHC class II DRB second exon nucleotide sequence of Attappady Black goat with other species.

Accession	Description	Maximum Score	Total score	Query coverage (per cent)	Maximum identity (per cent)
AF376813.1	*Capra hircus* DRB3	446	446	88%	98%
FM998807.1	*Ovis aries* Ovar-DRB*0308	438	438	96%	95%
U00196.1	*Capra aegagrus* Saanen Caae-DRB5	453	453	87%	99%
AY212156.1	*Rupicapra pyrenaica* Rupy-DRB*08	416	416	96%	93%
AB523835.1	*Bos taurus* DRB3*3201	398	398	94%	93%
AF324847.1	*Ovis canadensis* DRB*8	383	383	87%	94%
DQ834889.1	*Bos indicus* BoLa-DRB3	392	392	94%	92%
AJ920398.1	*Ovis dalli* HLA-DRB*3	383	383	87%	94%

The sequencing of the DRB gene second exon form Malabari and Attappady Black goats revealed four different DRB alleles having 285 bp each. The sequence analysis has shown that these alleles were more similar to *Capra aegagrus* (98 to 99 per cent) DRB alleles than to *Capra hircus* DRB alleles (96 to 98 per cent). The sequencing has also revealed the base changes responsible for the presence or absence of *Taq*I and *Pst*I restriction sites in DRB alleles of Malabari and Attappady Black goats.

4.3.1.2 GENBANK ACCESSION NUMBERS

The new nucleotide sequence data of Malabari and Attappady Black goats MHC class II DRB gene second exon determined has been submitted to the GenBank DNA database and obtained the unique Accession Numbers: GU998821, GU998822, GU998823, GU998824.

4.3.1.3 PROTEIN SEQUENCE ANALYSIS OF DRB GENE

The Major Histocompatibility Complex class II DRB gene second exon in Malabari and Attappady Black goats has an open reading frame (ORF) of 279 nucleotides encoding a protein of 93 amino acid residues. The single letter code representing the amino acids and their triplet codons are given in the Annexure-IV.

ClustalW multiple nucleotide sequence alignment of the protein sequences from Malabari and Attappady Black goats is depicted in Figure 4.7. The protein sequences from Malabari goats had a homology score of 77 per cent and that from Attappady goats had a score of 86 per cent.

The results of BLASTp analysis of protein sequence of the second exon of DRB gene in Malabari goat with other species are presented in Table 4.6. The protein sequence from Malabari goat has revealed 93, 89, 88 and 87 per cent identity with goat (*Capra hircus*; Accession # AAS46246.1), Indian cattle (*Bos indicus*; Accession # ABH09477.1), goat antelope (*Rupicapra pyrenaica*;

Accession #AAP50510.1) and European cattle (*Bos taurus;*, Accession # BAA08219.1), respectively.

The results of BLASTp analysis of protein sequence of the second exon of DRB gene in Attappady Black goat with other species are presented in Table 4.7. The sequence from Attapady Black goat has revealed 98 per cent identity with wild goat (*Capra aegagrus*; Accession #AAB51098.1), 95 per cent identity with goat (*Capra hircus*; Accession #AAK59169.I) and 89 per cent identity with goat antelope (*Rupicapra pyrenaica*; Accession #AAP50508.1).

Table 4.6 Protein sequence analysis by BLASTp showing percentage of identity with MHC class II DRB second exon of Malabari goat.

Accession	Description	Maximum Score	Total score	Maximum identity (per cent)
AAP50510.1	*Rupicapra pyrenaica*	171	171	88
BAA08219.1	*Bos Taurus*	168	168	87
AAS46246.1	*Capra hircus*	167	167	93
ABU80531.1	*Bison bonasus*	167	167	84
AAA31572.1	*Ovis aries*	166	166	86
ABA29015.1	*Bubalus bubalis*	166	166	84
ABH09477.1	*Bos indicus*	165	165	89
ACB97636.1	*Bos taurus x Bos indicus*	164	164	85

Table 4.7 Protein sequence analysis by BLASTp showing percentage of identity with MHC class II DRB second exon of Attappady Black goat.

Accession	Description	Maximum score	Total score	Maximum identity (per cent)
AAP50508.1	*Rupicapra pyrenaica*	173	173	89
AAK59169.1	*Capra hircus*	173	173	95
AAA16794.1	*Ovis aries*	168	168	88
BAA08217.1	*Bos taurus*	162	162	83
ACB97633.1	*Bos taurus x Bos indicus*	161	161	82
CAD58719.1	*Bison bonasus*	161	161	81
ABA29015.1	*Bubalus bubalis*	157	157	80
AAB51098.1	*Capra aegagrus*	169	169	98

Protein sequence analysis of the DRB second exon in Malabari and Attappady Black goats showed that it codes for a polypeptide of 93 amino acid residues. The alleles from Malabari showed a homology of 77 per cent and alleles from Attappady Black goats showed a homology of 86 per cent. The protein sequences were found to be more similar to caprine (93 to 98 per cent) and rupicaprine (88 to 89 per cent) protein sequences in the BLASTp analysis.

4.3.2 SEQUENCE ANALYSIS OF DQA2 GENE

4.3.2.1 DNA SEQUENCE ANALYSIS OF DQA2 GENE

The sequencing of the amplified products of DQA2 gene from Malabari and Attappady Black goats revealed four different DQA2 alleles. Three alleles had 304 bp and the fourth one had 300 bp. Two of them were novel alleles where as the third allele was having 100% nucleotide identity with DQA2 allele

of Boer goat (*Capra hircus*; Cahi-DQA2*0101 allele, Accession# AY829349.1) and the fourth one was having 100% nucleotide identity with another DQA2 allele from Boer goat (*Capra hircus*; Cahi-DQA2*0104 allele, Accession# AY829354.1). Nucleotide sequences of all the four alleles of MHC class II DQA2 second exon in Malabari and Attappady Black goats are depicted in Fig. 4.8.

BLAST2 analysis showed that the sequences of second exons and the flanking intronic sequences of DQA2 gene in Malabari goats had 91 per cent identity with variations at 25 positions and the sequences from Attappady Black goats had only 90 per cent identity with variations at 29 positions. The *EcoR*V RFLP resulted from the presence of a polymorphic *EcoRV* site at position 234 bp of the amplified fragment. The nucleotide sequences at this polymorphic site in the sequenced samples were 'GATATT' (e allele) and 'GATATC' (E allele) in both Attappady Black and Malabari goats.

The BLASTn analysis of the nucleotide sequence of the second exon of DQA2 gene in Malabari goat with other species is presented in Table 4.8. The sequence from Malabari goat has revealed 94, 93, 91 and 90 per cent identity with sheep (*Ovis aries;* Accession # AY312380.1), goat (*Capra hircus;* Accession # AY829351.1), European cattle (*Bos taurus;*, Accession # U80868.1) and European Bison (*Bison bonasus;* Accession # EU153371.1), respectively.

The BLASTn analysis of the nucleotide sequence of the second exon and the flanking intronic sequences of DQA2 gene in Attappady Black goat with other species is presented in Table 4.9. The sequence from Attappady Black goat has revealed 95, 94 and 90 per cent identity with sheep (*Ovis aries;* Accession #AY312380.1), goat (*Capra hircus;* Accession #AY829351.1) and Indian cattle (*Bos indicus*; Accession #EU395629.1), respectively.

Table 4.8 Results of BLASTn showing the percentage of identity of the MHC class II DQA2 second exon and the flanking intronic nucleotide sequence of Malabari goat with other species.

Accession	Description	Maximum Score	Total score	Query coverage (per cent)	Maximum identity (per cent)
AY312380.1	*Ovis aries* OLA-DQA2*0501	473	473	100%	94
AY829351.1	*Capra hircus* Cahi-DQA2*0201	451	451	100%	93
EU153371.1	*Bison bonasus* (Bibo-DQA)	372	372	91%	90
EU395629.1	*Bos indicus* (BoLA-DQA)	368	368	95%	89
DQ092804.1	*Bos taurus x Bos indicus* (BoLA-DQA)	368	368	95%	89
DQ868981.1	*Bubalus bubalis* BuLA-DQA*07	351	351	95%	88
U80868.1	*Bos taurus* (BoLA-DQA2) allele 6	315	315	76%	91
Z79514.1	*B.primigenius* BoLADQA1	265	265	72%	88

Table 4.9 Results of BLASTn showing the percentage of identity of the MHC class II DQA2 second exon and the flanking intronic nucleotide sequence of Attappady Black goat with other species.

Accession	Description	Maximum Score	Total score	Query coverage (per cent)	Maximum identity (per cent)
AY312380.1	*Ovis aries* OLA-DQA2*0501	484	484	100%	95%
AY829351.1	*Capra hircus* Cahi-DQA2*0201	462	462	100%	94%
EU395629.1	*Bos indicus* (BoLA-DQA)	379	379	95%	90%
DQ092804.1	*Bos taurus x Bos indicus* (BoLA-DQA)	379	379	95%	90%
DQ868981.1	*Bubalus bubalis* BuLA-DQA*07	363	363	95%	89%
EU153371.1	*Bison bonasus* (Bibo-DQA)	372	372	91%	90%
AY442305.1	*Bos taurus* BoLA-DQA2	324	324	81%	90%
Z79522.1	*B.primigenius* BoLADQA1	303	303	72%	91%

The sequencing of the DQA2 gene second exon and the flanking intronic sequences form Malabari and Attappady Black goats revealed four different alleles three of which were having 304 bp each and the fourth one having 300 bp. The sequence analysis has shown that these alleles were more similar to *Capra hircus* (93 to 94 per cent) DQA2 and *Ovis aries* DQA2 alleles (94 to 95 per cent). The sequencing has also revealed the base changes responsible for the presence or absence of *EcoRV* restriction site in DQA2 alleles of Malabari and Attappady Black goats.

4.3.2.2 GENBANK ACCESSION NUMBERS

The new nucleotide sequence data of Malabari and Attappady Black goats MHC class II DQA2 gene second exon and flanking intronic sequences determined has been submitted to the GenBank DNA database and obtained the unique Accession Numbers: HM122040, HM122041, HM122042, HM122043.

4.3.2.3 PROTEIN SEQUENCE ANALYSIS OF DQA2 GENE

The Major Histocompatibility Complex class II DQA2 gene second exon in Attappady Black and Malabari goats had an open reading frame (ORF) of 246 nucleotides encoding a protein of 82 amino acid residues. The single letter code representing the amino acids and their triplet codons are given in the Annexure-IV.

ClustalW multiple sequence alignment of the protein sequences from Malabari and Attappady Black goats is depicted in Figure 4.9. The analysis showed that the protein sequences from both Malabari and Attappady Black goats had a homology score of 82 per cent.

The results of BLASTp analysis of protein sequence of the second exon of DQA2 gene in Malabari goat with other species are given in Table 4.10. The sequence from Malabari goat has revealed 91 per cent identity with sheep (*Ovis aries;* AAP79484.1), 85 per cent identity with European cattle (*Bos taurus;*

Accession # AAB62013.1) and 84 per cent identity with goat (*Capra hircus*; AAV88371.1).

The results of BLASTp analysis of protein sequence of the second exon of DQA2 gene of Attappady Black goat with other species are given in Table 4.11. The sequence from Attappady Black goat (sample 2) has showed 100 per cent identity with goat (*Capra hircus*; Accession # AAV88369.1), 97 per cent identity with *Ovis aries* (Accession #AAP79479.1), and 85 per cent identity with European cattle (*Bos taurus;*, Accession #AAB62013.1).

Table 4.10 Protein sequence analysis by BLASTp showing percentage of identity with MHC class II DQA2 second exon of Malabari goat.

Accession	Description	Maximum Score	Total score	Maximum identity (per cent)
AAP79484.1	*Ovis aries*	160	160	91
AAB62013.1	*Bos taurus*	152	152	85
ABW71894.1	*Bison bonasus*	149	149	81
ACA13571.1	*Bos indicus*	147	147	81
AAZ04540.1	*Bos taurus x Bos indicus*	147	147	81
AAV88371.1	*Capra hircus*	148	148	84
ABI29906.1	*Bubalus bubalis*	145	145	80
AAV66431.1	*Bison bison*	143	143	84

Table 4.11 Protein sequence analysis by BLASTp showing percentage of identity with MHC class II DQA2 second exon of Attappady Black goat.

Accession	Description	Maximum score	Total score	Maximum identity (per cent)
AAV88369.1	*Capra hircus*	173	173	100
AAP79479.1	*Ovis aries*	169	169	97
AAB62013.1	*Bos Taurus*	155	155	85
ACA13571.1	*Bos indicus*	151	151	81
AAZ04540.1	*Bos taurus x Bos indicus*	150	150	81
ABD98454.1	*Bubalus bubalis*	149	149	80
ABW71893.1	*Bison bonasus*	149	149	80
AAV66431.1	*Bison bison*	148	148	85

The analysis of deduced protein sequences of the DQA2 second exon in Malabari and Attappady Black goats showed that it codes for a polypeptide of 82 amino acid residues. The alleles from both Malabari and Attappady Black goats showed a homology of 82 per cent. The protein sequences were found to be more similar to caprine (84 to 100 per cent) and ovine (91 to 97 per cent) protein sequences in the BLASTp analysis.

4.4 EFFECT OF ALLELIC VARIANTS ON IMMUNOGLOBULIN (Ig) LEVELS

The immunoglobulin concentration measured in 115 goats by zinc sulphate turbidity test and standard curve method ranged from 29.849 to 179.206 mg/ml. The mean immunoglobulin concentration obtained was 66.78±05.65 mg/ml. Least square means of immunoglobulin concentration according to age, breed and genotypes of DRB and DQA2 genes are shown in table 4.12.

Table 4.12 shows the means of immunoglobulin concentration along with standard error according to the age and breed of goats. Age had significant effect on immunoglobulin (Ig) level. The mean Ig level at 60-90 days, 90-120 days, 120-150 days and 150-180 days were 61.20±08.655, 77.38±07.255, 54.44±09.587 and 74.10±10.082 mg/ml respectively. The highest Ig level was found at 90-120 days and the lowest at 120-150 days. The least square mean of Ig level was higher in Malabari goat (72.31±06.548 mg/ml) than in Attappady Black (61.26 ±08.650 mg/ml). However breed had no significant effect on Ig level.

4.4.1 EFFECT OF GENOTYPES

4.4.1.1 EFFECT OF DRB GENOTYPES

Least square means of immunoglobulin concentration along with standard error according to different DRB genotypes are shown in table 4.12. The least square means for *Taq*I genotypes were 69.08±06.768 mg/ml (TT), 70.250±06.059 mg/ml (Tt) and 61.01±09.389 mg/ml (tt). The highest Ig levels were found in goats having heterozygous (Tt) genotype. However analysis had shown that the effect of different *Taq*I genotypes on Ig level was not significant. The effect of *Pst*I genotypes on immunoglobulin level was significant. The least square means for *Pst*I genotypes were 58.71±7.057 mg/ml (PP) and 74.85±05.780 mg/ml (Pp). The Ig level was significantly higher in animals having heterozygous genotype (Pp) than those having homozygous (PP) genotype.

4.4.1.2 EFFECT OF DQA2 GENOTYPES

Least square means of immunoglobulin concentration along with standard error in accordance with different DQA2 genotypes are presented in table 4.12. The least square means for *EcoRV* genotypes were 77.63±06.966 mg/ml (EE), 68.12 ± 04.835 mg/ml (Ee) and 54.60±13.182 mg/ml (ee). In

contrast to the results obtained for DRB genotypes, the highest Ig levels were found in goats having homozygous (EE) genotype. However the effect of different *EcoRV* genotypes on Ig level was not significant.

Table 4.12 Least square means of immunoglobulin concentration according to age, breed and genotypes of DRB and DQA2.

Effect	**Ig level mg/ml (mean±SE)**
Age*	
60-90 days	61.21±08.655[ab]
90-120 days	77.38±07.255[b]
120-150 days	54.44±09.587[a]
150-180 days	74.10 ±10.082[ab]
Breed	
Malabari	72.31±06.548
Attappady	61.26±08.650
Taq I genotypes	
TT	69.08±06.768
Tt	70.25±06.059
Tt	61.01±09.389
Pst I genotypes*	
PP	58.71± 07.057[a]
Pp	74.85±05.799[b]
EcoRV genotypes	
EE	77.63±06.966
Ee	68.12±04.835
Ee	54.60±13.182

*P≤ 0.05

Values with same superscripts do not differ significantly.

Least square analysis to find out the effects of DRB and DQA2 genotypes on Ig level has shown that *Pst*I genotypes of DRB gene had a significant effect on the Ig level in Malabari and Attappady Black goats.

The study on PCR-RFLP analysis and sequencing of DRB and DQA2 genes were conducted using 85 Malabari and 30 Attapady Black goats. The analysis of polymorphism of the second exon of DRB was carried out using *TaqI* and *PstI* restriction enzymes and that of DQA2 gene second exon was carried out using *EcoRV* restriction enzyme. It was found that the frequencies of different alleles were not significantly different between Malabari and Attappady Black goat populations. The DNA and protein sequence analysis of the DRB and DQA2 gene has shown the high degree of polymorphism in these regions. Only DRB/*Pst*1 genotypes significantly affected immunoglobulin levels in goats.

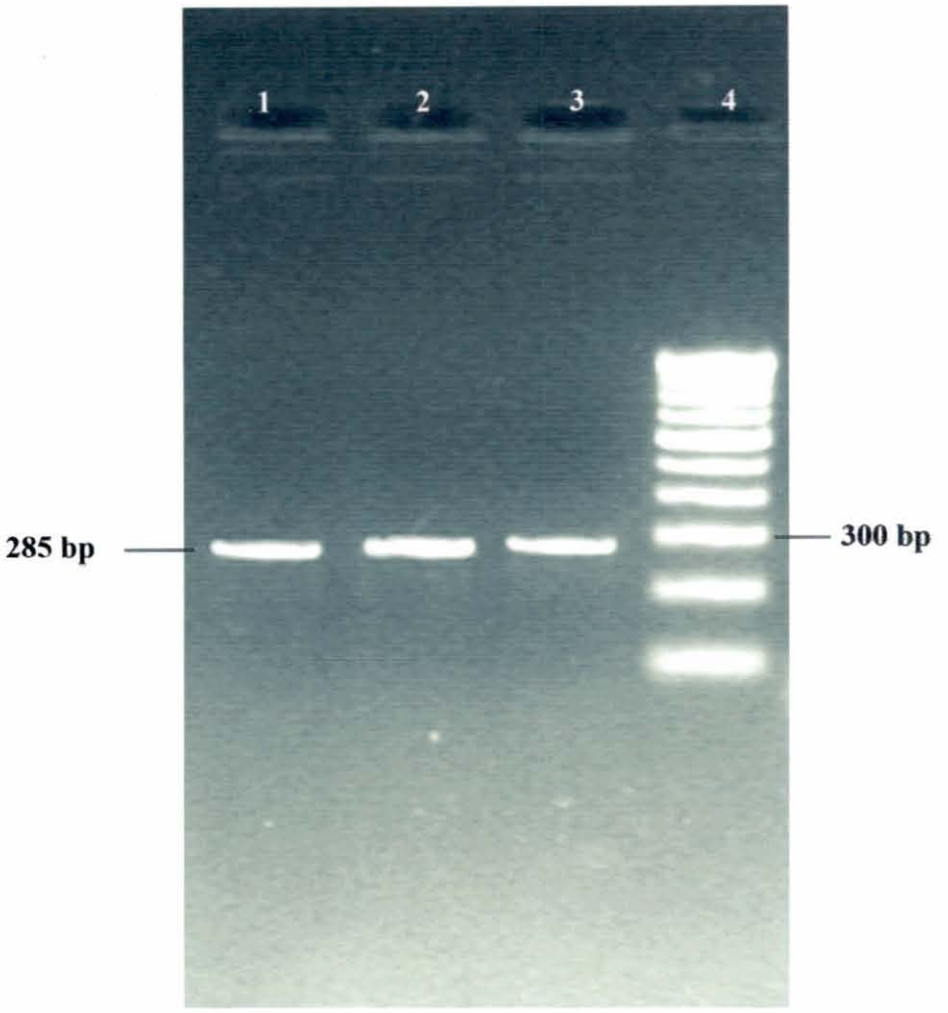

Fig. 4.1 **The 285 bp fragment of second exon of Major Histocompatibility Complex class II DRB gene amplified from genomic DNA by PCR in Malabari and Attappady Black goats on 1 per cent agarose gel. Lane 4: 100 bp ladder as DNA size marker.**

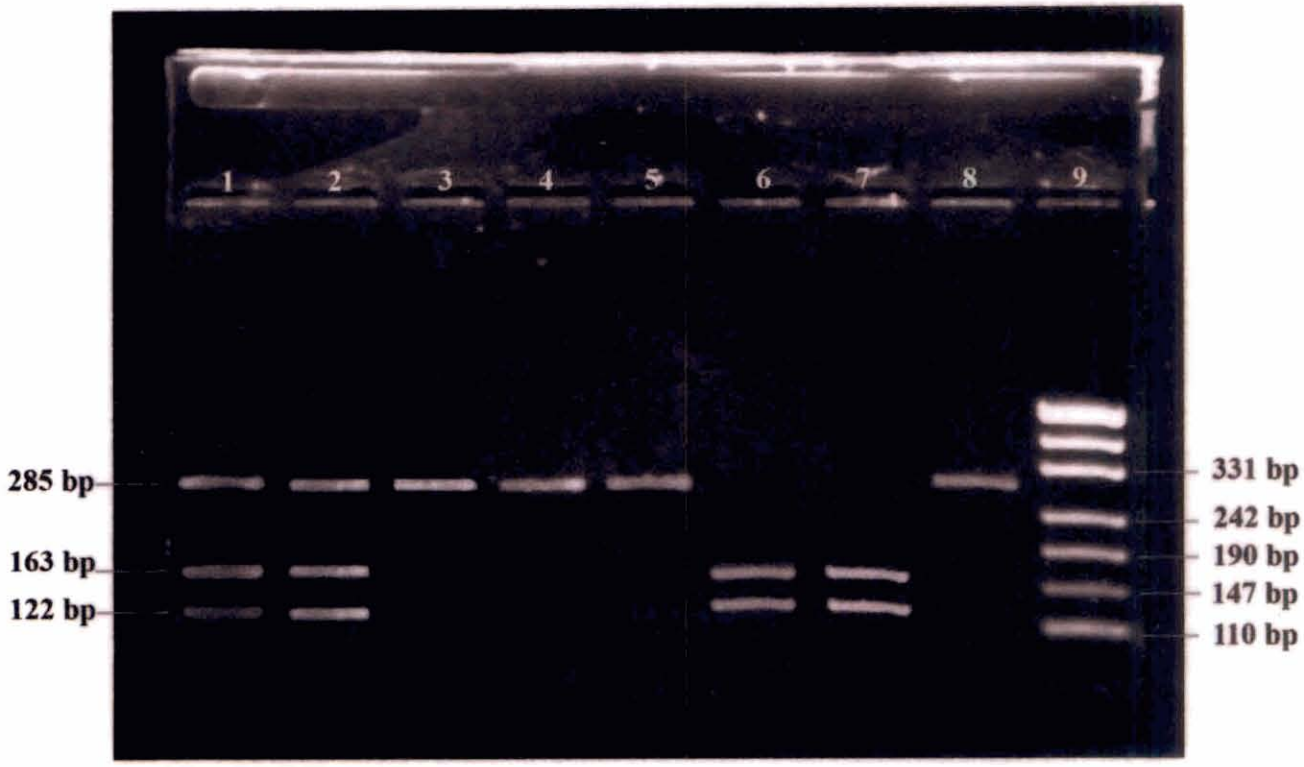

Fig. 4.2 **The DRB/*Taq*I genotypes resolved on 2.5 per cent agarose gel. Lane 1-2: 'Tt' genotype- 285, 163 and 122 bp fragments. Lane 3-5 and 8 : 'TT' genotype - undigested fragment of 285 bp. Lane 6-7 : 'tt' genotype - 163 and 122 bp fragments. Lane 9: pUC 19 DNA/ Msp I Digest as DNA size marker.**

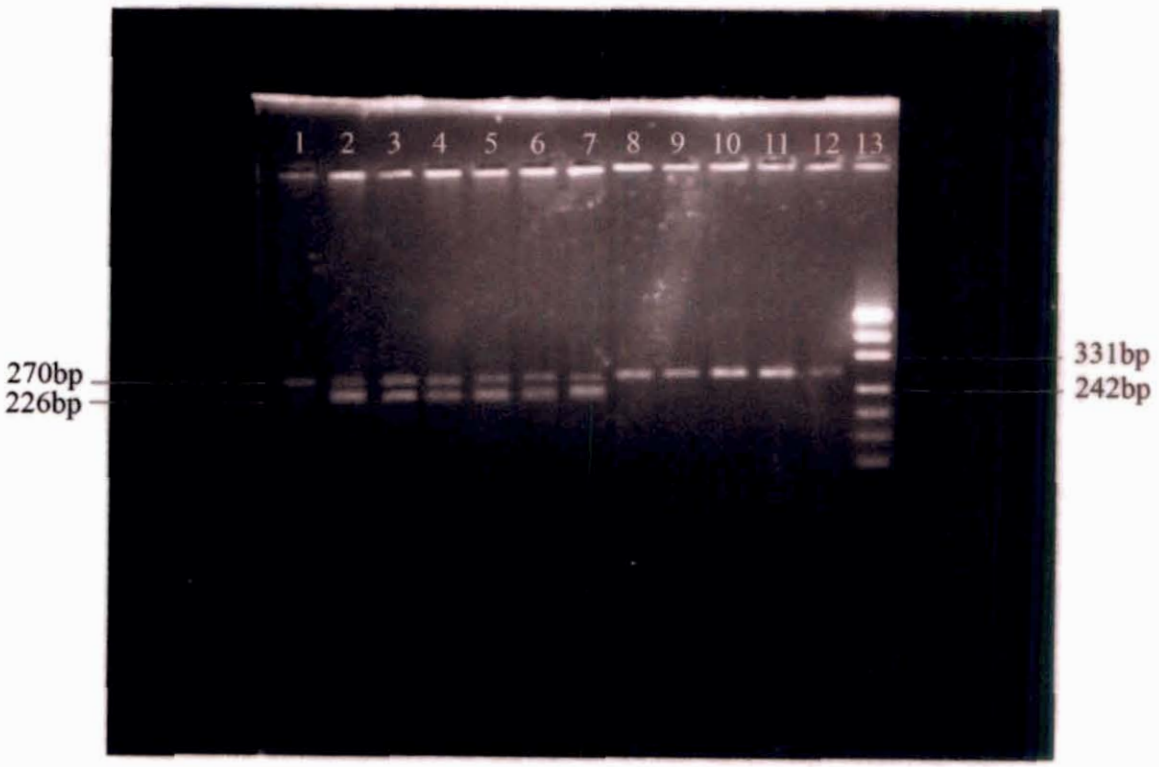

Fig. 4.3 **The DRB/*Pst*I genotypes resolved on 2.5 per cent agarose gel. Lane 1-7: 'Pp' genotype - 270, 226, 44 (not demonstrated) and 15 (not demonstrated) bp fragments. Lane 8 - 12 : 'PP' genotype - 270 and 15 (not demonstrated) bp fragments.Lane 13: pUC 19 DNA/ Msp I Digest as DNA size marker.**

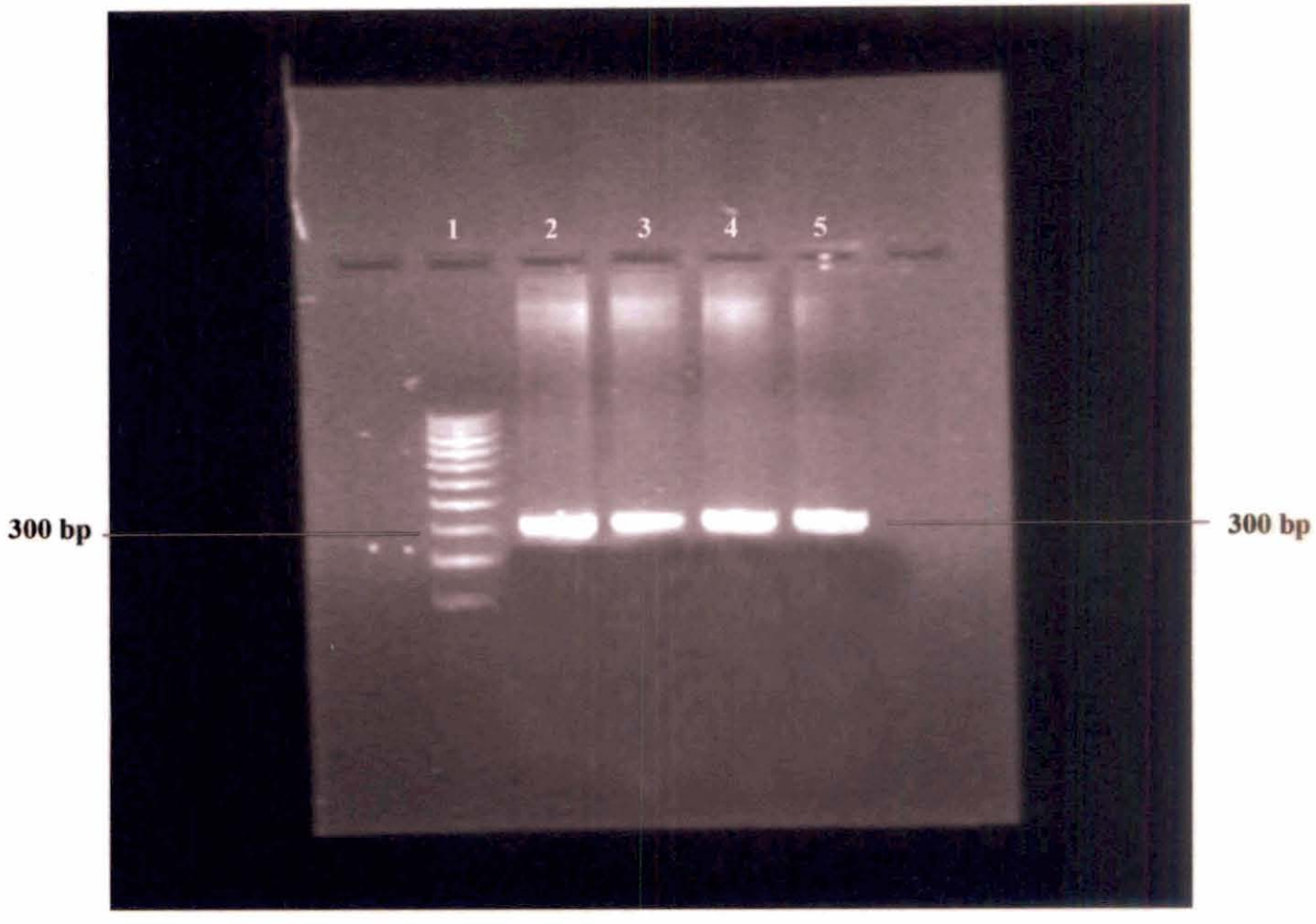

Fig. 4.4 **The 300 bp fragment of second exon and flanking introns of Major Histocompatibility Complex class II DQA2 gene amplified from genomic DNA by PCR in Malabari and Attappady Black goats on 1 per cent agarose gel. Lane 1: 100 bp ladder as DNA size marker.**

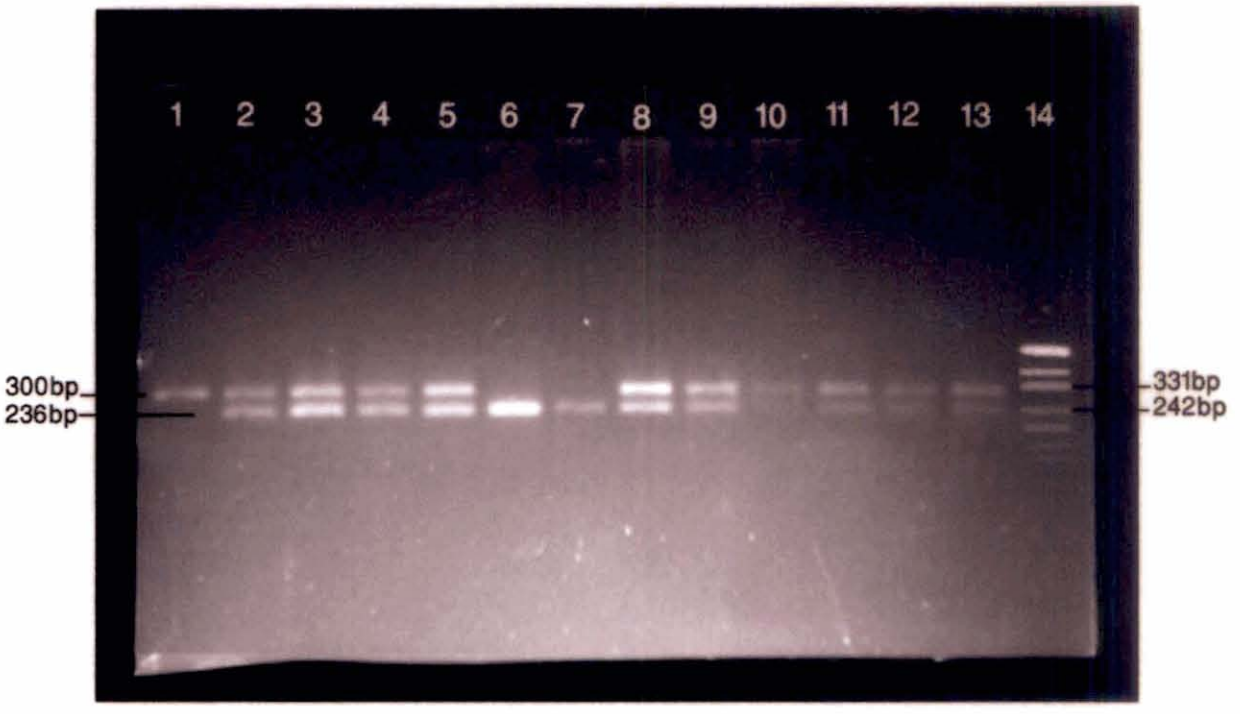

Fig. 4.5 **The DQA2/EcoRV genotypes resolved on 2.5 per cent agarose gel. Lane 1: 'ee' genotype - undigested fragment of 300 bp. Lane 2-5, 8-13: 'Ee' genotype - 300, 236 and 64 (not demonstrated) bp fragments. Lane 6-7: 'EE' genotype- 236 and 64 (not demonstrated) bp fragments. Lane 14: pUC 19 DNA/ Msp I Digest as DNA size marker.**

>MALABARI 1
TATCCCGTCT**CTGCAG**CACATTTCCTGGAGTATGCTAAGAGCGAGTGTCATTTCTTCAGTGGGA
CCGAGCGGGTGCGGTTCCTGGACAGATACTTCCATAATGGAGAAGAGTTCGTGCGCT**TCGA**CAG
CGACTGGGGCGAGTTCCGGGCAGTGACCGAGCTGGGGCGGCCGGACGCCGAGTACTGGAACAGC
CAGAAGGACTTCCTGGAGAGCAGGAGGACCGCGGTGGACACGTA**CTGCAG**ATACAACTACGGGG
TCGGTGAGAGTTTCAGTGTGACTTGAGAG
>MALABARI 2
TATCCCGTCT**CTGCAG**CACATTTCCTGGAGTATTCTAAGAAAGAGTGTCATTTCTCCAACGGGA
CGGAGCGGGTGCGGTACCTGGACAGATACTTCTATAATGGAGAAGAGTACGTGCGCT**ACGA**CAG
CGACCGGGGCGAGTTCCGGGCGGTGGCCGAGCTGGGGCGGCCGGAGGCCGAGTACTGGAACAGC
CAGAAGGAGATCCTGGAGCAGAAGCGGGCCGAGGCGGACACGGT**GTGCAG**ACACAACTACGGGG
TCATTGAGAGTTTCAGTGTGACTTGAGAG
>ATTAPPADY BLACK 1
TATCCCGTCT**CTGCAG**CACATTTCCTGGAGTATACTAAGAAAGAGTGTCGTTTCTCCAACGGGA
CGGAGCGGGTGCGGTTCCTGGACAGATACTTCTATAATGGAGAAGAGTACGTGCGCT**ACGA**CAG
CGACTGGGGCGAGTTCCGGGCGGTGGCCGAGCTGGGGCGGCCGGATGCCGAGTACTGGAACAGC
CAGAAGGAGATCCTGGAGGACAGCCGGGCCGCCGTGGACACGTA**CTGCAG**ACACAACTACGGGG
TCGGTGAGAGTTTCAGTGTGACTTGAGAG
>ATTAPPADY BLACK 2
TATCCCGTCT**CTGCAG**CACATTTCCTGGAGTATTCTAAGAGCGAGTGTCATTTCTCCAACGGGA
CCGAGCGGGTGCGGTTCCTGGACAGATACTTCTATAATGGAGAAGAGAACGTGCGCT**GCGA**CAG
CGACTGGGGCGAGTTCCGGGCGGTGGCCGAGCTGGGGCGGCCGGACGCCAAGTACTGGAACAGC
CAGAAGGACTTCCTGGAGCAGAAGCGGGCCGAGGTGGACACGGT**GTGCAG**ACACAACTACGGGG
TCTTTGAGAGTTTCAGTGTGACTTGAGAG

Fig 4.6 Nucleotide sequence of various alleles of MHC class II DRB second exon obtained in Malabari and Attappady Black goats. Allelic variations in the *Taq* I (green) and *Pst* I (blue) recognition sites are marked in bold letters.

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CLUSTAL W (1.81) multiple sequence alignment


MLDRB 2 YPVSAAHFLEYSKKECHFSNGTERVRYLDRYFYNGEEYVRYDSDRGEFRAVAELGRPEAE
ABDRB 2 YPVSAAHFLEYSKSECHFSNGTERVRFLDRYFYNGEENVRCDSDWGEFRAVAELGRPDAK
ABDRB 1 YPVSAAHFLEYTKKECRFSNGTERVRFLDRYFYNGEEYVRYDSDWGEFRAVAELGRPDAE
MLDRB 1 YPVSAAHFLEYAKSECHFFSGTERVRFLDRYFHNGEEFVRFDSDWGEFRAVTELGRPDAE
        ***********:*.**:* .******:*****:**** ** *** ******:*****:*:


MLDRB 2 YWNSQKEILEQKRAEADTVCRHNYGVIESFSVT
ABDRB 2 YWNSQKDFLEQKRAEVDTVCRHNYGVFESFSVT
ABDRB 1 YWNSQKEILEDSRAAVDTYCRHNYGVGESFSVT
MLDRB 1 YWNSQKDFLESRRTAVDTYCRYNYGVGESFSVT
        ******::**. *: .** **:**** ******
```

Fig 4.7 Comparison of the deduced amino acid sequences of the MHC class II DRB gene second exon of Malabari (MLDRB 1 & MLDRB 2) and Attappady Black goats (ABDRB 1 & ABDRB 2). The variant amino acids are shown in bold red letters.

>MALABARI 1
CTTCCTGCTCCTCACCCTCACTTACAGCTGACCACGTTGGCTCCTATGGCACAACTATCTACCA
ATCTCATGGTCCCTCTGGCCAGTTCACCCAGGAATTTGATGGAGACGAGTTGCTTTATGTGGAC
CTAGGGAAGAAGGAGACTGTCTGGCGGCTGCCTATGTTTGGTGAATTAACAAGTTTTGACCCTC
AAGGTGCACTGAGTAACATAGCTACAGCAAAACACAACTTG**GATATT**CTGTCTAAATGCTCCAA
CTGTACTCCAGTTATCAATGGTATGTGTCCACCATTCTACTTCTCTTT
>MALABARI 2
CTTCCTGCTCCTCACCCTCACTTACAGCTGACCACGTTGGCTCCTATGGCACAGTTATCTACCA
ATCTCATGGTCCCTCTGGCCAGTTCACCCAGGAATTTGATAGAGACGAGCTGTTTTATGTGGAC
CTGGAGAAGAAGGAGACTGTCTGGCGGCTGCCTATGTTTAGCCAGTTTGCAGGTTTTGACCCTC
AAGGTGCACTGAGTAACATAGCTACAGCGAAACACAACTTG**GATATC**ATGACTAAATTGCACAA
CTTTACCCCAGTTATCAACGGTAAGTGTCCACCATTCTACTTCTCTTT
>ATTAPPADY BLACK 1
CTTCCTGCTCCTCACCCTCACTTACAGCTGACCACGTTGGCTCCTATGGCACAACTATCTACCA
ATCTCATGGTCCCTCTGGCCAGTTCACCCAGGAATTTGATGGAGACGAGTTGCTTTATGTGGAC
CTAGGGAAGAAGGAGACTGTCTGGCGGCTGCCTATGTTTGGTGAATTAACAAGTTTTGACCCTC
AAGGTGCACTGAGTAACATAGCTACAGCAAAACACAACTTG**GATATT**CTGTCTAAATGCTCCAA
CTGTACCCCAGTTATCAATGGTAAGTGTCCACCATTCTACTTCTCTTT
>ATTAPPADY BLACK 2
CTTCCTGCTCCTCACCCTCACAGCTGACCACGTTGGCATCTATGGCGCAGACCTCTACCAATCT
CATGGTCCCTCTGGCCAGTACACCCACGAATTTGATGGGGACGAGCTGTTTTATGTGGACCTGG
GGAAGAAGGAGACTGTCTGGCGGCTGCCTATGTTTGGTGAATTCACAAGTTTTGACCCGCAAGG
TGCACTGAGTGAAATAGCTAAAGCAAAACACAACTTG**GATATC**ATGATTAAACGTTCCAACTTT
ACCCCTGTTATCAATGGTAAGTGTCCACCATTCTACTTCTCTTT

Fig 4.8 Nucleotide sequence of alleles of MHC class II DQA2 second exon and parts of the flanked intronic sequences obtained in Malabari and Attappady Black goats. Allelic variations in the *EcoR*V (green) recognition site are marked in bold letters.

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CLUSTAL W (1.81) multiple sequence alignment


MLDQA2 1 DHVGSYGTTIYQSHGPSGQFTQEFDGDELLYVDLGKKETVWRLPMFGELTSFDPQGALSN
ABDQA2 1 DHVGSYGTTIYQSHGPSGQFTQEFDGDELLYVDLGKKETVWRLPMFGELTSFDPQGALSN
ABDQA2 2 DHVGIYGADLYQSHGPSGQYTHEFDGDELFYVDLGKKETVWRLPMFGEFTSFDPQGALSE
MLDQA2 2 DHVGSYGTVIYQSHGPSGQFTQEFDRDELFYVDLEKKETVWRLPMFSQFAGFDPQGALSN
         **** **: :********:*:*** ***:**** **********.::::********:

MLDQA2 1 IATAKHNLDILSKCSNCTPVIN
ABDQA2 1 IATAKHNLDILSKCSNCTPVIN
ABDQA2 2 IAKAKHNLDIMIKRSNFTPVIN
MLDQA2 2 IATAKHNLDIMTKLHNFTPVIN
         **.*******: *  * *****
```

Fig 4.9 Comparison of the deduced amino acid sequences of the MHC class II DQA2 gene second exon of Malabari (MLDQA2 1 & MLDQA2 2) and Attappady Black goats (ABDQA2 1 & ABDQA2 2). The variant amino acids are shown in bold red letters.

Discussion

5. DISCUSSION

The most reliable and accurate method of selection for disease resistance is the selection based on molecular markers. In goats, Caprine Leucocytic Antigen (CLA) or Caprine Major Histocompatibility Complex (MHC) is one of the most important molecular markers that can influence disease resistance. This work focuses on genetic polymorphism and sequence analysis of Major Histocompatibility Complex class II DRB and DQA2 genes in goats and the association of different allelic variants with immune status of the goats. The topic is discussed under the following heads:

5.1 Isolation of genomic DNA

5.2 Major Histocompatibility Complex (MHC) class II genes

5.2.1 PCR-RFLP analysis

5.2.1.1 PCR-RFLPs in DRB gene

5.2.1.1 PCR-RFLP in DQA2 gene

5.3 DNA sequencing

5.3.1 Sequence analysis of DRB gene

5.3.2 Sequence analysis of DQA2 gene

5.4 Effect of allelic variants on Immunoglobulin levels

5.1 ISOLATION OF GENOMIC DNA

Phenol chloroform extraction procedure (Sambrook *et al.*, 1989) was used for the isolation of high molecular weight DNA from blood samples. This procedure is a common and efficient technique for DNA isolation from blood (Tantia *et al.*, 2004; Araujo *et al.*, 2006).

5.1.1 YIELD AND QUALITY OF DNA

The mean yield of DNA from 5 ml samples of goat blood extracted by phenol-chloroform method was 245.762±26.593 µg/ml. Chithra (2002) reported a similar yield of 231.097±11.65 µg of DNA from 5 ml of goat blood. Mathew

(2004) and Seena (2006) reported higher yields of 350.05±10.05 µg and 357±23.057 µg of DNA, respectively from five ml of blood of Malabari goats.

Beckman *et al.* (1986) and Senthil *et al.* (1996) reported 300-500 µg and 444.58±21.54 µg, respectively per 10 ml of whole blood of cattle using the phenol chloroform method. The yield of DNA per unit volume of blood was high in goat as compared to that of cattle. This might be due to the high leucocytic count in goat (Swenson, 1996) and better suitability of this procedure for goat blood. The ratios of optical density at 260 and 280 nm were more than 1.7 indicating good deproteinisation. Chithra (2002) reported similar ratio in goats.

5.2 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) CLASS II GENES

Major Histocompatibility Complex class II molecules are dimeric glycoproteins composed of two noncovalently linked α and β chains. Each molecule contains two extracellular domains, a transmembrane segment and a cytoplasmic tail (Amills *et al.*, 1995). MHC class II molecules are found on the surface of B cells and antigen presenting cells such as macrophages, dendritic cells and Langerhans cells. They are involved in antigen presentation to $CD4^+$ T cells, which help B cells to produce appropriate immunoglobulins (Andersson, 1990). There are different kinds of MHC class II molecules, but the DQ and DR subtypes are the most polymorphic both in man and domestic species, and probably play a major role in development of MHC restricted immune response.

5.2.1 PCR RFLP ANALYSIS

5.2.1.1 PCR RFLPs IN DRB GENE

By analyzing the available sequences of DRB gene (Amills *et al.* 1996; Li *et al.* 2006), it was found that the enzymes *Taq* I and *Pst* I cut the sequence of DRB second exon at different polymorphic sites (at 122 bp and 270 bp,

respectively). So these two enzymes were selected for the present PCR-RFLP analysis in DRB second exon.

5.2.1.1.1 AMPLIFICATION OF DRB GENE BY PCR

PCR parameters used for the amplification of caprine DRB gene second exon are given in Table 4.1. Annealing temperature and time optimized in this study was 56.7^{0}C for 60 sec for 30 cycles. Ahmed and Othman (2006) used 30 cycles of 60^{0}C for 90 sec and Amills *et al.* (1996) used 10 cycles of 60^{0}C for 120 sec followed by 25 cycles of 65^{0}C for 30 sec for optimum annealing. In all the animals the size of the amplified product was 285 bp indicating conservation of DNA sequence at this locus. The finding of this study is in close agreement with the reports made earlier by Ahmed and Othman (2006) in Egyptian goats and Amills *et al.* (1995) in Spanish goats.

5.2.1.1.2 PCR-RFLP ANALYSIS IN DRB GENE USING Taq I

The amplified DNA was digested with *Taq* I restriction enzyme and the fragments were separated by agarose gel electrophoresis. The amplified 285 bp fragment was restricted into a 163 bp fragment and a 122 bp fragment when a polymorphic *Taq* I site was present. This allowed the identification of two alleles *viz.*, t (163 and 122 bp fragments) and T (undigested fragment of 285 bp).

In Malabari goats among the 85 tested animals, 25 TT, 47 Tt and 13 tt genotypes were found giving rise to genotype frequencies of 0.30, 0.55 and 0.15, respectively where as in Attappady Black goats among the 30 tested animals, the corresponding frequencies were 0.33 (10 TT), 0.53 (16 Tt) and 0.13 (4 tt), respectively. The gene frequencies were 0.57 for 'T' and 0.43 for 't' alleles in Malabari while 0.60 (T) and 0.50 (t), respectively in Attappady Black. Similar restriction patterns had been observed by Ahmed and Othman (2006) who found 't' (0.60) allele to be more frequent over 'T' (0.40) allele in Egyptian goats.

Amills *et al.* (1995) observed only Tt and tt restriction patterns in Spanish breeds of goat with a frequency of 0.65 for 't' allele and 0.35 for 'T' allele.

The gene frequencies of the different alleles of DRB/*Taq*I polymorphism in Malabari and Attappady Black breeds were not significantly different. This indicates the genetic homogeneity between these two goat breeds of Kerela in disease resistance with respect to the DRB locus.

The populations of Malabari and Attappady Black goats investigated in the present study were under Hardy-Weinberg equilibrium with respect to the genotypic frequencies of DRB/T*aq*I genotypes. So it can be concluded that no mutation or natural selection is acting on the DRB/T*aq*I polymorphic site and all the three genotypes are having equal genetic disease resistance capacity and chance of survival. To investigate further on the amino acid differences between these alleles and their relationship to disease resistance, DNA sequence analysis was carried out at DRB locus second exon in both the breeds.

The analysis of DRB/*Taq*I polymorphism in Malabari and Attappady Black breeds of goats has shown a similar pattern of polymorphism in both breeds with regard to disease resistance at the DRB/ *Taq*I site. It is also evident that no genotype of these loci has any selective advantage over others as evidenced by the Hardy-Weinberg equilibrium status.

5.2.1.1.3 PCR-RFLP ANALYSIS IN DRB GENE USING Pst I

The amplified DNA was digested with *Pst* I restriction enzyme and the fragments were separated by agarose gel electrophoresis. The amplified 285 bp fragment has a non polymorphic *Pst* I site located at position 15 bp, in the forward primer complementary region suitable for control of *Pst* I digestion giving rise to two fragments of 270 bp and 15 bp. The 270 bp fragment was restricted into a 226 bp fragment and a 44 bp fragment when a polymorphic *Pst* I site was present at position 241 bp of the amplified fragment. This allowed the

identification two alleles *viz.*, P (270 and 15 bp fragments) and p (226, 44 and15 bp fragment).

In Malabari goats among the 85 tested animals, 39 PP and 46 Pp genotypes were found giving rise to genotype frequencies of 0.46 and 0.54, respectively where as in Attappady Black goats among the 30 tested animals corresponding frequencies were 0.20 (6 PP) and 0.80 (24 Pp), respectively. Out of the three possible genotypes *viz.*, PP, Pp and pp, the pp genotype was not observed in the present study. This finding is in close agreement with the findings made earlier by Ahmed and Othman (2006) in Egyptian goats. Amills *et al.* (1995) reported all the three genotypes viz., PP, Pp and pp in Spanish breeds of goats. It is suspected that absence of 'pp' genotype might be due to the lethal effect of the P*st* I homozygote (pp) or due to the linkage of a lethal gene to P*st*I 'p' allele in Malabari and Attappady Black goats. Chitra (2002) and Mathew (2004) reported a similar finding in polymorphism of the growth hormone gene in Malabari goats.

The gene frequencies were 0.73 for 'P' and 0.27 for 'p' alleles in Malabari while it was 0.60 (P) and 0.40 (p), respectively in Attappady Black goats. The predominance of 'P' allele over 'p' allele in the present study was found to be in agreement with the findings of Ahmed and Othman (2006) who got a frequency of 0.65 (P allele) and 0.35 (p allele) and with that of Amills *et al.* (1995) who got a frequency of 0.59 (P allele) and 0.41 (p allele). The gene frequencies of the different alleles of DRB/ P*st*I polymorphism in Malabari and Attappady Black breeds did not differ significantly. This indicates the genetic homogeneity between these different goat breeds of Kerala with respect to the DRB locus.

The populations of Malabari and Attappady Black goats investigated in the present study were not under Hardy-Weinberg equilibrium for DRB/*Pst*I genotypes. It is suspected that the natural selection process acting against the 'pp' genotype through reduced viability or early embryonic death may be a

possible reason for the disagreement of the Hardy-Weinberg equilibrium (Chitra, 2002) for the DRB/*PstI* locus. However, further detailed investigations are required to confirm this finding.

The analysis of DRB/ P*stI* polymorphism has revealed existence of two digestion patterns (PP, Pp) while the 'pp' pattern was absent in both Malabari and Attappady Black goats which is believed to be due to the lethal effect of 'pp' genotype which has prevented the survival of that genotype in the studied population. This natural selective force acting against 'pp' genotype has resulted in significant changes in the expected genotypic frequencies and the populations were found to be not in Hardy-Weinberg equilibrium.

5.2.1.2 PCR RFLP IN DQA2 GENE

Variations in the ovine and caprine DQA2 gene second exon was investigated by Single Strand Conformational Polymorphism (SSCP) by Hickford *et al.* (2004) and Zhou *et al.* (2005), respectively. By analyzing the available caprine DQA2 sequences it was found that the enzyme *Eco*RV cut the DQA2 second exon at a polymorphic site (position 236 bp). So, *Eco*RV restriction enzyme was selected for the present PCR-RFLP analysis in DQA2 second exon.

5.2.1.2.1 AMPLIFICATION OF DQA2 GENE BY PCR

PCR parameters used for the amplification of caprine DQA2 gene second exon are given in Table 4.4. Annealing temperature and time optimized in this study was 59.5 ^{0} C for 60 seconds for 30 cycles where as Zhou *et al.* (2005) used 32 cycles of 58^{0} C for 30 seconds. In all the animals the size of the amplified product was around 300 bp indicating conservation of DNA sequence at this locus. The finding of this study is in close agreement with the reports made earlier by Zhou *et al.* (2005).

5.2.1.2.2 PCR-RFLP ANALYSIS IN DQA2 GENE USING EcoRV

The amplified DNA was digested with EcoRV restriction enzyme and the fragments were separated by agarose gel electrophoresis. The amplified 305 bp fragment was restricted into a 236 bp fragment and 69 bp fragments when a polymorphic EcoRV site was present. This allowed the identification of two alleles viz., 'E' (236 and 69 bp fragments) and 'e' (undigested fragment of 305 bp).

In Malabari goats among the 85 tested animals, 13 EE, 67 Ee and 5 ee genotypes were found, giving rise to genotype frequencies of 0.15, 0.79 and 0.06, respectively where as in Attappady Black goats among the 30 tested animals, the corresponding frequencies were 0.37 (11 EE), 0.60 (18 Ee) and 0.03 (1 ee), respectively. The gene frequencies were 0.55 for 'E' and 0.45 for 'e' alleles in Malabari while 0.67 (E) and 0.33 (e), respectively in Attappady Black goats.

The gene frequencies of the different alleles of DQA2/EcoRV polymorphism in Malabari and Attappady Black breeds were not significantly different. This indicates the genetic homogeneity between these different goat breeds of Kerala in disease resistance with respect to the DQA2 locus.

The population of Malabari goat investigated in the present study was not under Hardy-Weinberg equilibrium where as population of Attappady Black goat was in equilibrium, with respect to the genotypic frequencies of *Eco*RV genotypes. The *chi*-square value was highly significant in Malabari goats since there was a discrepancy between the observed and expected frequencies. An excess of heterozygotes can result from selective elimination of homozygotes (Falconer and Mackey, 1996). Heterozygotic advantage obtained in this study was consistent with earlier reports of Chitra (2002) and Mathew (2004) in growth hormone gene of goats. The presence of duplicate copies of DQA2 gene in goats (Zhou *et al.*, 2005) may be a possible cause for this.

To know further about the base changes creating *Eco*RV polymorphism and variations in the amino acid sequences related to changes in the antigen binding site, DNA sequencing and sequence analysis was carried out at the DQA2 second exon.

Thus, the analysis of DQA2/*Eco*RV polymorphism in Malabari and Attappady Black goat populations revealed existence of all the three possible restriction patterns with a clear heterozygotic advantage (genotype frequency of 0.79 for 'Ee' in Malabari goats) which is expected to be due to existence of duplicate copies of DQA2 gene.

5.3 DNA SEQUENCING

Amplified products of DRB and DQA2 gene second exon from Malabari and Attappady Black goats were gel eluted, cloned and sequenced at the DNA Sequencing Facility, Bioserve, Biotechnologies (India), Pvt. Ltd. Hyderabad. The nucleotide sequence was determined by the dideoxy-chain termination method using M13 Reverse primer.

5.3.1 SEQUENCE ANALYSIS OF DRB GENE

Gel purification and cloning of PCR amplimers representative of the unique RFLP patterns followed by DNA sequencing revealed three novel sequences and a fourth sequence which showed 100 percent nucleotide similarity with Saanen (*Capra aegagrus*; Caae-DRB1 Accession # U00196.1).

In the present study, it was found that the T*aq* I site is generated by substitution of adenine or guanine with thymine at position 122 bp. The presence of the T*aq* I site was found to be associated with a TTC codon (Phe) at position 40 (122 bp) while its absence was associated with TAC codon (Tyr) at the same position in Malabari goats and with a TAC codon (Tyr) or TGC codon (Cys) in Attappady Black goats. The result obtained in Malabari goats was found to be in agreement with finding of Amills *et al.* (1995) who reported that absence of

*Taq*I site was always associated with a TAC codon where as the presence of TGC codon (as in Attappady Black goats) was not reported early.

Tyr is considered as one of the amino acids to play an important role in HLA-DR molecule, which is involved in the formation of the antigen-binding site (Brown *et al.*, 1993). In cattle, Tyr substitution at position 78 of the bovine DR molecule has been correlated with a tendency towards susceptibility to persistent lymphocytosis affected with bovine leukemia virus (Lewin, 1994).

According to Amills *et al.* (1995) the *Pst*I site could be generated by substitution of guanine or thymine by cytosine at position 237 bp and the presence of the *Pst*I site could be associated with a TTC (Phe) or TAC (Tyr) codon at position 78 (237 bp) while its absence was associated with GTG (Val) or TGT (Cys) at the same position. In the present study the presence of *Pst* I site was found to be associated with a TAC (Tyr) codon at position 78 (237 bp) and the absence of *Pst* I site was found to be associated with a GTG (Val) codon in the sequenced samples of both Malabari and Attappady balck breeds of goat. *Pst* I site was observed to be polymorphic in sheep, cattle and pigs displaying all the three restriction patterns and when present was associated with a TAC codon at position 78 and when absent was associated with a GTG codon at the same position (Amills *et al.*, 1995).

The 285 bp nucleotide sequences obtained from Malabari goats for MHC class II DRB gene second exon were found to be having 89 per cent identity with variations at 30 positions and those from Attappady Black goats were having 92 percent identity with variations at 22 positions. This shows the high variability of this region within the breeds (Amills *et al.*, 1995; Li *et al.* 2006).

The 93 amino acid composition of the MHC class II DRB gene second exon deduced from the sequences obtained from Malabari and Attappady Black goats were found to be having an identity score of 77 and 86 percent, respectively with twenty three amino acid replacements at positions 12, 14, 17, 19, 20, 27, 33, 38, 41, 45, 52, 58, 60, 67, 68, 71, 72, 74, 75, 76, 79, 82, 87 in

aligning the four sequences. Two hyper variable regions (HVR) were found at around position 12 to 20 and 67 to 76. Presence of HVR at position 56 to 74 was reported earlier by Davies *et al.* (1997) in cattle, Takada *et al.* (1998) in sheep, Marsh *et al.* (2001) in human and Li *et al.* (2006) in Chinese indigenous goats. Of the four sequences obtained, three amino acid substitutions were found at positions 12 (Ser, Thr, Ala), 38 (Tyr, Asn, Phe), 41 (Tyr, Cys, Phe), 71 (Gln, Asp, Ser), 72 (Lys, Ser, Arg) and 87 (Ile, Phe, Gly). Schwaiger *et al.* (1993) reported four amino acid substitutions at position 70 (Gln, Ser, Arg and Asp), three at position 71 (Arg, Ser, Lys), two at position 73(Ala, Thr), three at position 74 (Ala, Glu, Asn), and four at position 78 (Tyr,Val,Cys and Phe). This extensive polymorphism is explained by the fact that the side chains of the amino acid residues 70, 71, 74 and 78 are expected to be included in the antigen binding groove of the DR molecule which interact with the processed foreign antigen (Brown *et al.*, 1993).

The codon positions 52-56 (corresponding to positions 51-56 of CLA-DRB*02) were 'AELGR' in three of the sequences and 'TELGR' in one of the sequences. This position located at the transition between β sheet and the α helix is encoded by a nucleotide sequence, which is believed to promote recombination both between different alleles and between different loci (Gyllensten *et al.*, 1991). Li *et al.* (2006) found that amino acid sequence at this position was 'TELGR' in caprine (CLA-DRB*02 alleles), ovine (OLA-DRB*02) and bovine (BoLA-DRB3*02) sequences, which was found to be highly conserved in primate DRB genes. The result obtained in this study shows a clear variation from this observation.

The amino acid sequences deduced from the nucleic acid sequences from Malabari and Attappady Black goats were more similar to other caprine DRB alleles than bovine or ovine DRB alleles where as Amills *et al.* (1995) reported that *Capra aegagrus* DRB allele (Caae-DRB 23) was more similar to ovine and bovine DRB alleles than caprine alleles. Close similarities between the DRB polymorphisms of different species have been reported when

comparing human DRB polymorphism with cattle (Andersson *et al.*, 1991), dogs (Sarmiento *et al.*, 1990) and pigs (Gustaffson *et al.*, 1990). This phenomenon may be explained by the maintenance of short ancestral motifs that emerged before speciation events (Klein, 1987) or by convergent evolution (Andersson *et al.*, 1991).

In short, the analysis of second exon of DRB gene in Malabari and Attappady Black goats revealed that the *Taq* I site was generated by substitution of adenine or guanine with thymine at position 122 bp and the *Pst*I site was generated by substitution of guanine with cytosine at position 237 bp in the sequenced samples. The 93 amino acid composition of the DRB gene second exon revealed amino acid replacements at 23 positions with two hyper variable regions at positions 12 to 20 and 67 to 76.

5.3.2 SEQUENCE ANALYSIS OF DQA2 GENE

Gel purification and cloning of PCR amplimers representative of the unique RFLP patterns followed by DNA sequencing revealed two novel sequences. The third and fourth sequences showed 100 percent nucleotide similarity with Cahi-DQA2*0101 allele (Accession# AY829349.1) and with Cahi-DQA2*0104 allele (Accession# AY829354.1), respectively, of Boer goat breed.

In the present study, it was found that the *Eco*RVsite is generated by substitution of thymine with cytosine at position 239 bp. The presence of the *Eco*RV site was found to be associated with an ATC codon at position 70 (239 bp) while its absence was associated with ATT codon at the same position in Malabari goats and Attappady Black goats. Both these codons code for amino acid isoleucine. The *Eco*RVsite was created by a silent mutation which involves transition of thymine by cytosine.

The 304 bp nucleotide sequences obtained from Malabari goats for MHC class II DQA2 gene second exon were found to be having 91 per cent identity with variations at 25 positions and those from Attappady Black goats were

having 90 percent identity with variations at 29 positions. This shows the high variability of this region within the breed itself. A similar finding was reported by Zhou *et al.* (2005) while studying the polymorphism of DQA2 gene in Boer goats.

The 82 amino acid composition of the MHC class II DQA2 gene second exon deduced from the sequences obtained from Malabari and Attappady Black goats were found to be having an equal identity score of 82 percent. Of the 82 amino acid sites, 21 (25.6 percent) were polymorphic; however, in the putative antigen-binding region (Reche and Reinherz, 2003), 10 out of the 19 AA sites (52.6%) were polymorphic. The most polymorphic sites were observed at positions 9 (T, D, V), 72 (S, I, T) and 74 (C, R, L) of which positions 9 and 74 are included in the putative antigen binding region. Variation in this region may affect the antigen binding groove and antigenic peptide binding ability, and hence peptide specificity.

The identification of four alleles from four of the sequenced samples indicate a high level of polymorphism in the caprine DQA2 gene, and it might be expected that more alleles may be found as other goats from diverse breeds are studied. The role of this variation in defining susceptibility to disease remains to be identified.

The analysis of the second exon and flanking intronic sequences of DQA2 gene from Malabari and Attappady Black goats revealed that the *Eco*RV site was created by a silent mutation which involves transition of thymine by cytosine at position 239 bp and the 82 amino acid composition of second exon of DQA2 gene revealed amino acid replacements at 21 positions of which 10 replacements were in the putative antigen binding site which will alter the antigen processing capacity.

5.4 IMMUNOGLOBULIN LEVELS

The immune response is regulated by genes within the Major Histocompatibility Complex in concert with non-MHC genes (Rosenstreich *et al.*, 1982; Vaiman, 1987). Antibody diversity, affinity, and quantity are each under separate genetic regulation. The combined influences of these genes are critical in controlling the humoral immune response and serum immunoglobulin concentration may reflect in their combined effects (Mallard *et al.*, 1989).

To study the relationship of MHC genotypes with Ig levels, animals (2 to 6 months) were classified into four age groups viz., 60-90 days, 90-120 days, 120-150 days and 150-180 days and immunoglobulin levels were estimated. The effect of age and *Pst* I genotypes on immunoglobulin concentration were found significant.

The highest Ig level was found at 90-120 days (77.38±07.255). Iepema *et al.* (2008) studied serum gammaglobulin concentration in goat kids upto three months of age and found that the highest Ig level was obtained at three months of age and also that the effect of colostrum on serum gammaglobulin concentration was significant at 28 days and was not significant at 56 and 86 days. This indicates that the high level of Ig at three months obtained in this study might be due to an increase in the active Ig synthesis during that period.

Among the *Pst*I genotypes the highest Ig level was found in the heterozygous 'Pp' genotype (74.85±05.799). High serum Immunoglobulin might predict high antibody response (Williams and Halliday, 1980; Burton *et al.*, 1989) where as serum immunoglobulin concentration is correlated with protection against clinical mastitis and pneumonia in calves (Mallard *et al.* 1983; Corbail *et al.*, 1984). Serum immunoglobulin concentration might be a useful index of resistance to infectious disease for which antibody mediates protection (Mallard *et al.* 1989). The high Ig level is an indication of increased disease resistance which in turn indicates that heterozygous (Pp) genotype has increased efficiency to process antigens.

Least square analysis had shown that age and *Pst*I genotypes significantly affected the Ig level in goats. The *Pst*I heterozygous genotype had the highest Ig level which indicated increased efficiency of 'Pp' genotype to process antigens.

The PCR-RFLP analysis of DRB and DQA2 genes were conducted using 85 Malabari and 30 Attappady Black goats and sequencing of different alleles from both breeds were carried out. All the three DRB/*Taq*I genotypes had an equal chance of survival as a natural selective force (may be a lethal effect) acted against and eliminated the DRB/*Pst*I 'pp' genotype in the populations of Malabari and Attappady Black goats studied. A clear heterozygotic advantage was observed in the DQA2/*Eco*RV polymorphism which is believed to be due to gene duplication at the DQA2 locus. The base changes at *Taq*I, *Pst*I and *Eco*RV sites in Malabari and Attappady Black goats was analyzed from the sequencing results. The 93 and 82 amino acid compositions of DRB and DQA2 second exons revealed amino acid replacements at 23 and 21 positions, respectively many of which are present at the antigen binding region and hence affect peptide specificity. *Pst*I genotypes significantly affected the Ig level in goats and the heterozygote genotype was found to have higher efficiency in antigen processing.

Summary

6. SUMMARY

Marker assisted selection (MAS) based on molecular markers is the most reliable method for selection for disease resistance. The foremost requirement for MAS, is the identification and characterization of genes that can influence disease resistance. Major Histocompatibility Complex molecules (MHC) are cell surface receptors involved in antigen presentation and play a key role in genetic basis of disease resistance. Of the different types of MHC genes, DQ and DR subtypes are the most polymorphic and potential candidates in development of immune response. Keeping this in view, a study was undertaken to analyze the sequence and polymorphism of Major Histocompatibility Complex class II DRB and DQA2 genes in Malabari and Attappady Black goats.

Genomic DNA was isolated from venous blood samples of 85 Malabari and 30 Attappady Black goats using phenol chloroform extraction method. The mean yield of DNA was 245.762±26.593 µg. The ratio of optical densities at 260 and 280 nm was above 1.7 indicating good deproteinisation. The DNA samples on agarose gel electrophoresis were found to be of high molecular weight and appeared as single bands without sheared fragments.

PCR-RFLPs were carried out in the second exons of Caprine Major Histocompatibility Complex (MHC) class II DRB and DQA2 genes. Second exon of major histocompatibility complex class II DRB gene was amplified using primers based on available caprine sequences. In all the animals the size of the amplified product was approximately 285 bp. Analysis of polymorphism of DRB gene second exon was carried out using restriction enzymes *Taq*I and *Pst*I. DRB/*Taq*I polymorphism was observed as three patterns which allowed the identification of two alleles *viz.*, t (163 and 122 bp fragments) and T (undigested fragment of 285 bp). The gene frequencies for T and t alleles in the Malabari goat were 0.57 and 0.43 and in Attappady Black the frequencies were 0.60 and

0.40 respectively. The populations of both breeds were found to be in Hardy-Weinberg equilibrium with regard to DRB/*Taq*I polymorphism.

DRB/*Pst*I polymorphism showed two genotypes with two alleles, *viz.*, P (270 and 15 bp fragments) and p (226, 44 and15 bp fragment), the gene frequencies of which were 0.73 (P) and 0.27(p) in Malabari and 0.60 (P) and 0.40 (p) in Attappady Black goats. The populations of Malabari and Attappady Black goats were found not to be in Hardy-Weinberg equilibrium. With regard to both DRB/*Taq*I and DRB/ *Pst*I loci, there was no significant difference in allele frequencies between breeds.

Amplification of DQA2 second exon and the flanking intronic sequences was done and the size of the amplified product was approximately 300 bp. Analysis was done by using the enzyme *Eco*RV and three restriction digestion patterns were observed which allowed the identification of two alleles *viz.*, E (236 and 64 bp fragments) and e (undigested fragment of 300 bp). The gene frequencies for E and e alleles in the Malabari goat were 0.55 and 0.45 and in Attappady Black the frequencies were 0.67 and 0.33 respectively. The population of Attappady Black goats was found to be in Hardy-Weinberg equilibrium while population of Malabari was not in equilibrium. Breed effect was not found to be significant in allele frequencies.

The sequencing of the DRB gene second exon form Malabari and Attappady Black goats revealed four different DRB alleles having 285 bp each. The sequence analysis showed that these alleles were more similar to *Capra aegagrus* (98 to 99 percent) DRB alleles than to *Capra hircus* DRB alleles (96 to 98 percent). The sequencing revealed that the *Taq* I site was generated by substitution of adenine or guanine with thymine at position 122 bp. The presence of *Pst*I site was found to be associated with a TAC (Tyr) codon at position 78 (237 bp) and the absence of *Pst* I site was found to be associated with a GTG (Val) codon in the sequenced samples.

Protein sequence analysis of the DRB second exon in Malabari and Attappady Black goats showed that it codes for a polypeptide of 93 amino acid residues. The alleles from Malabari and Attappady Black goats showed a homology of 77 and 86 percent, respectively with amino acid replacements at 23 positions. The protein sequences were found to be more similar to caprine (93 to 98 percent) and rupicaprine (88 to 89 percent) protein sequences in the BLASTp analysis.

The sequencing of the DQA2 gene second exon and the flanking intronic sequences from Malabari and Attappady Black goats revealed four different alleles, three of which were having 304 bp each and the fourth one having 300 bp. The sequence analysis has shown that these alleles were more similar to *Capra hircus* (93 to 94 percent) DQA2 and *Ovis aries* DQA2 alleles (94 to 95 percent). The sequence analysis revealed that the presence of the *Eco*RV site was associated with an ATC codon at position 70 (239 bp) while its absence was associated with ATT codon at the same position in Malabari and Attappady Black goats.

Protein sequence analysis of the DQA2 second exon in Malabari and Attappady Black goats showed that it codes for a polypeptide of 82 amino acid residues. The alleles from Malabari and Attappady Black goats showed an equal homology of 82 percent with amino acid replacements at 21 positions of which 10 replacements were in the putative antigen binding site which may alter the shape of antigen binding groove and hence peptide specificity. The protein sequences were found to be more similar to caprine (84 to 100 percent) and ovine (91 to 97 percent) protein sequences in the BLASTp analysis.

Least square analysis has shown that age and *Pst*I genotypes significantly affect the Ig level in Malabari and Attappady Black goats. The *Pst*I heterozygous

genotype had the highest Ig level which indicates increased efficiency of 'Pp' genotype to process antigens.

The analysis of DRB/*Taq*I and DQA2/*Eco*RV polymorphism in Malabari and Attappady Black goat populations revealed existence of all the three possible restriction patterns. A clear heterozygotic advantage was observed in DQA2/ *Eco*RV polymorphism. The analysis of DRB/ P*st*I polymorphism revealed existence of only two digestion patterns in both breeds which is believed to be due to the lethal effect of recessive genotype. Breed was not a significant source of variation for allelic frequencies of any of the polymorphisms studied. The sequence analysis of DRB and DQA2 gene second exon in Malabari and Attappady Black goats revealed the base substitutions at the T*aq* I site, *Pst* I site and *Eco*RV site. The analysis of the amino acid compositions of DRB and DQA2 second exons revealed amino acid replacements at 23 and 21 positions respectively, many of which are present at the antigen binding region and hence affect peptide specificity. The study has shown that very high degree of polymorphism exists in the caprine Major Histocompatibility Complex class II DRB and DQA2 genes which may play a major role in the differences in immune status among animals.

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Annexures

ANNEXURE – I

COMPOSITION OF REAGENTS AND BUFFERS USED IN THE STUDY

Agarose (0.8%)

Weighed 0.8 g of agarose powder and mixed with 100 ml of 1X TBE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

Agarose (2.5 %)

Weighed 2.5 g of agarose powder and mixed with 100 ml of 1X TAE buffer in a conical flask. Solution was heated in a microwave oven until boiling and cooled slowly.

EDTA (0.5*M*, pH 8.3)

Dissolved 18.61 g of EDTA (disodium, dihydrate) in 80 ml of distilled water by bringing the pH to 8.3 with NaOH solution and volume made upto 100 ml. Stored at room temperature after filtration and autoclaving.

Ethidium Bromide (10 mg/ml)

Dissolved 100 mg of Ethidium bromide in 10 ml of distilled water. Solution was stored at 4 °C in a dark coloured bottle.

Gel loading buffer

Bromophenol blue	0.25 %	50 mg
Xylene cyanol	0.25 %	50 mg

Sucrose	40 %	8 g

Components were mixed and stirred well in 20 ml distilled water and stored at 4 °C.

Phenol (Saturated, pH 7.8)

Commercially available crystalline phenol was melted at 65 °C in a water bath. Hydroxyquinolone was added to a final concentration of 0.1 percent. To the molten phenol, 0.5 *M* Tris HCl (pH 8.0) was added in equal volume and stirred for 30 min in a magnetic stirrer. The contents were transferred into a separating funnel. Lower phenolic phase was collected and mixed with equal volume of 0.1 *M* Tris HCl (pH 8.0) and stirred again for 30 min. The phenolic phase was collected and extraction repeated with 0.1*M* Tris HCl (pH 8.0) until the pH of phenolic phase was more than 7.8. Finally 0.1 volume of 0.01 *M* Tris HCl (pH 8.0) added and stored in a dark bottle at 4 °C.

RBC lysis buffer

Ammonium chloride	150 mM	8.0235 g
Potassium chloride	10 mM	0.7455 g
EDTA	0.1 mM	0.0372 g

Dissolved the contents in distilled water and volume made up to 1000 ml. Stored at 4 °C after filtration and autoclaving.

Sodium acetate

Dissolved 40.824 g of Sodium acetate in 70 ml of distilled water and pH adjusted to 5.5 with glacial acetic acid. Volume was made up to 100 ml, autoclaved and stored at room temperature.

Sodium chloride (5 M)

Dissolved 29.22 g of sodium chloride in 80 ml distilled water and volume made up to 100 ml. Solution filtered and stored at room temperature.

Sodium chloride- EDTA (SE) buffer (pH, 8.0)

Sodium chloride	75 mM	4.383 g
EDTA	35 mM	9.306 g

Dissolved the contents in 900 ml distilled water and pH adjusted to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4°C.

Tris Acetate EDTA (TAE) buffer (50X)

Tris base	48.4 g
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	20 ml
Distilled water up to	1000 ml

Autoclaved and stored at room temperature.

Tris-Borate (TBE) buffer pH 8.3, 10X

Tris base	108.0 g
Boric acid	55.0 g
EDTA	9.3 g

Dissolved in 700 ml of distilled water and pH adjusted to 8.3. Volume was made up to 1000 ml, autoclaved and stored at room temperature.

Tris Buffered Saline (TBS) pH 7.4

Sodium chloride	140 mM	8.18 g
Potassium chloride	0.5 mM	0.0373 g
Tris base	0.25 mM	0.0303 g

Dissolved in 900 ml of distilled water and pH adjusted to 7.4. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Tris EDTA (TE) buffer (pH 8.0)

Tris base	10 mM	1.2114 g
EDTA	0.1 mM	0.3722 g

Dissolved in 900 ml of distilled water and adjusted the pH to 8.0. Made up the volume to 1000 ml, filtered, autoclaved and stored at 4 °C.

Tris 1*M* (pH 8.0)

Tris base	121.14 g

Added distilled water up to 1000 ml, pH adjusted to 8.0, filtered and stored at room temperature.

ANNEXURE – II

SOURCES OF IMPORTANT CHEMICALS AND ENZYMES USED IN THIS STUDY

(A) CHEMICALS

Agarose (Low EED)	- Bangalore Genei Pvt. Ltd.
Ammonium chloride	- SRL, Bombay
Boric acid	- SRL, Bombay
Chloroform	- Merck
Crystalline phenol	- Merck
Di-sodium hydrogen orthophosphate	- SRL, Bombay
dNTPs	- Finn Enzymes
EDTA	- SRL, Bombay
Ethanol	- Merck
Ethidium bromide	- BDH lab, England
6 X gel loading buffer	- Bangalore Genei Pvt. Ltd.
Glacial acetic acid	- BDH-E, Merck (India) Ltd.
Hydroxy quinolone	- Qualigens Chemicals, Mumbai
Isoamyl alcohol	- Merck
Isopropyl alcohol	- SRL, Bombay
Potassium chloride	- SRL, Bombay
Sodium acetate	- SRL, Bombay
Sodium chloride	- SRL, Bombay

Sodium dodecyl sulphate (SDS)	- SRL, Bombay
Tris base	- SRL, Bombay

(B) PRIMERS

Integrated DNA Technologies

(C) MOLECULAR MARKERS

pUC18/ *Msp*I digest	- Bangalore Genei Pvt. Ltd.
100 bp DNA ladder	- Bangalore Genei Pvt. Ltd.

(D) ENZYMES

Taq DNA polymerase	- Bangalore Genei Pvt. Ltd.
Proteinase-K	- Bangalore Genei Pvt. Ltd.
Restriction endonucleases-	
*Taq*I, *Pst*I, *Eco*RV	- New England Biolabs Pvt. Ltd.

(E) BIOLOGICALS

Bovine immunogamma globulin - Sigma-Aldrich chemicals, Bangalore.

ANNEXURE – III

ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide BLAST
BLASTp	Protein BLAST
bp	base pair
Da	Dalton
DNA	Deoxyribo Nucleic Acid
dNTP.	Deoxyribo Nucleotide Triphosphate
EDTA	Ethylene Diamine Tetraacetic Acid
ExPASy	Expert Protein Analysis System
FAO	Food and Agricultural Organization
g	gram
hr	hour
i.e.	that is
M	molar
MAS	Marker Assisted Selection
mg	milligram
$MgCl_2$	Magnesium chloride
min	minute
m*M.*	millimolar
NCBI	National Center for Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
p*M*	Pico moles
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute

SSCP	Single Strand Conformation Polymorphism
µg	microgram
µl	microlitre
°C	degree Celsius

ANNEXURE -IV

ABBREVIATIONS AND TRIPLET CODONS OF AMINO ACIDS

Amino Acid	Abbreviation		Codons
	3 letter	Single Letter	
Isoleucine	Ile	I	ATT, ATC, ATA
Leucine	Leu	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	Val	V	GTT, GTC, GTA, GTG
Phenylalanine	Phe	F	TTT, TTC
Methionine	Met	M	ATG
Cysteine	Cys	C	TGT, TGC
Alanine	Ala	A	GCT, GCC, GCA, GCG
Glycine	Gly	G	GGT, GGC, GGA, GGG
Proline	Pro	P	CCT, CCC, CCA, CCG
Threonine	Thr	T	ACT, ACC, ACA, ACG
Serine	Ser	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Tyr	Y	TAT, TAC
Tryptophan	Trp	W	TGG
Glutamine	Gln	Q	CAA, CAG
Asparagine	Asn	N	AAT, AAC
Histidine	His	H	CAT, CAC
Glutamic acid	Glu	E	GAA, GAG
Aspartic acid	Asp	D	GAT, GAC
Lysine	Lys	K	AAA, AAG
Arginine	Arg	R	CGT, CGC, CGA, CGG, AGA, AGG
Termination	Ter	X	TAA, TAG, TGA

GENETIC POLYMORPHISM OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II GENES IN GOATS

REMYA JOHN V.

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Faculty of Veterinary and Animal Sciences
Kerala Agricultural University, Thrissur

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Department of Animal Breeding, Genetics & Biostatistics
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR-680651
KERALA, INDIA

ABSTRACT

The study was conducted in 85 Malabari and 30 Attappady Black goats to analyze the PCR-RFLP polymorphism and the sequences of Major Histocompatibility Complex class II DRB and DQA2 genes.

The analysis of DRB/*TaqI* polymorphism in Malabari and Attappady Black breeds of goats showed similar restriction patterns in both breeds with no significant changes in allele frequencies. It was also found that all the three genotypes had an equal survival capacity with respect to DRB/*TaqI* site as both the populations were under were under Hardy-Weinberg equilibrium.

The analysis of DRB/ P*stI* polymorphism revealed existence of two digestion patterns (PP, Pp). The 'pp' pattern was absent in both Malabari and Attappady Black goats probably due to the lethal effect of 'pp' genotype which has prevented the survival of that genotype in the studied population. This natural selective force acting against 'pp' genotype resulted in significant changes in the expected genotypic frequencies and the populations were found to be not in Hardy-Weinberg equilibrium.

The analysis of DQA2/*Eco*RV polymorphism in Malabari and Attappady Black goat populations revealed existence of all the three possible restriction patterns with a clear heterozygotic advantage (genotype frequency of 0.79 for 'Ee' in Malabari goats) which is expected to be due to existence of duplicate copies of DQA2 gene.

The sequencing of the DRB gene second exon form Malabari and Attappady Black goats revealed four different DRB alleles having 285 bp each and were more similar to *Capra aegagrus* (98 to 99 percent) DRB alleles in BLASTn analysis. The sequencing of the DQA2 gene second exon and the flanking intronic sequences form Malabari and Attappady Black goats revealed four different alleles, three of which were having 304 bp each and the fourth one having 300 bp. The

sequencing revealed the base changes responsible for the presence or absence of *TaqI*, *PstI* and *EcoRV* restriction sites in Malabari and Attappady Black goats.

The 93 and 82 amino acid compositions of DRB and DQA2 second exons revealed amino acid replacements at 23 and 21 positions respectively, many of which are present at the antigen binding region and hence affect peptide specificity. *Pst*I genotypes significantly affected the Ig level in goats and the heterozygotic genotype was found to have higher efficiency in antigen processing.

The present study has helped to understand the variations existing in the second exon of DRB and DQA2 loci in Malabari and Attapady Black breeds of goats which has a role in the disease resistance and immunity.