Original Paper

Major Histocompatibility Complex *Dma* α / β Genes and Their Expressional Diversity in Healthy and Diseased Water Buffalo *Bubalus bubalis*

Leena Rawal^a Deepak Panwar^{a,b} Sher Ali^c

^aMolecular Genetics Laboratory, National Institute of Immunology, Aruna Asaf Ali Marg, New Delhi,

^bDepartment of Zoology, University of Delhi, Delhi, ^cCentre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi, India

Key Words

Bula-*DM* α • *BuLA*-*DM* β • Interacting partners • Quantitative expression • Disease resistance

Abstract

Background/Aims: The major histocompatibility complex (MHC) categorized into three (I, II and III) classes elicits the immunogenic response by presenting exogenous peptides to T cells. The MHC-II DM is composed of *DM* α and *DM* β , two polypeptide chains, both are encoded by separate MHC genes involved in antigen processing and presentation. Despite the acknowledged role of MHC complex in humans, the literature is silent on the organization and expression of these genes in water buffalo *Bubalus bubalis*, an agriculturally important animal species. **Methods:** We deduced the full-length mRNA sequences of *DM* α and *DM* β genes, localized them onto the chromosome 2, assessed their copy number per haploid genome and studied tissue and disease specific expression. **Results:** The Real Time PCR showed higher expression of both the genes and their seven interacting partners in spleen, gonads and spermatozoa. Significantly, upregulation of *DM* α and *DM* β genes and their interacting partners were detected in diseased group of buffaloes as compared to that in healthy ones. **Conclusion:** The upregulation of *Bubalus bubalis* (*BuLA*)-*DM* α and *DM* β genes and their interacting partners reflect their role in regulating immune responses towards the amelioration of diseases. Work on this line would enhance our understanding on the overall roles of MHC locus, allowing development of possible therapeutic treatment strategies.

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L. Rawal and D. Panwar contributed equally to this work.

Sher Ali

Centre for Interdisciplinary Research in Basic Sciences,
Jamia Millia Islamia, New Delhi – 110025 (India)
E-Mail alis@jmi.ac.in, sheralib5@hotmail.com

Introduction

The MHC complex is a cluster of tightly linked genes involved in immunological (host defense) and non-immunological (intercellular communication) functions [1]. The MHC in human referred to as the human leukocyte antigen (HLA) complex is nearly 4 Mb, whereas, the same in cattle is approximately 2.5 Mb, named as BoLA complex [2] and 2 Mb in the mouse known as H-2 complex [3]. The MHC genes have multiple alleles showing co-dominant expression [4].

The MHC genes have been localized on human chromosome 6p21.31 [5], in sheep on 20, in goat and cattle, both on 23 [6] and in mouse on chromosome 17 [7]. The human and mouse MHC have more than 200 genes, including ~128 expressed ones, 96 pseudogenes, and repetitive elements (*SINES*, *LINES*, *LTRs*, and *STRs*). These elements account for about 50% of the sequence in almost all the mammalian species [8, 9]. The MHC complex categorized into three main classes (class I, II and III) is mostly conserved among mammalian species and each one plays well-defined roles. These classes are further divided into regions and sub-regions encompassing number of genes and pseudogenes. The MHC class II molecules are expressed in antigen presenting B cells and macrophages and present antigenic peptide to T-cells, resulting in T_H cell activation. The MHC class II region is composed of non-covalently bound polypeptide chains α and β , encoded by separate A and B genes within the MHC [10]. In general, polymorphic MHC class II molecules present peptide antigens to T cells. Whereas, non-classical MHC class II molecules are usually non-polymorphic and serve as chaperones with their accessory proteins. These proteins assist in folding, transport, loading and editing of antigenic peptides [11]. One of the notable differences in the genomic organization of the ruminants MHCs compared with that of human and mice is the splitting of the class II region into two sub-regions, IIa and IIb, separated by a distance of at least 15 cM (centimorgans) [7]. Significantly, such a large recombination distance between bovine class IIa and IIb genes has not been observed in any other mammalian species. Similar organization of these genes is expected in closely related artiodactyls, sheep and goat [12, 13].

The class IIa region comprises of two clusters of genes, *DR* and *DQ*. Physical and genetic mapping have shown that the bovine and ovine *DR* and *DQ* genes lie in close proximity [14, 15]. The class IIb region is separated from the rest of the MHC by a recombination frequency of ~17%. This region includes the *DM*, *DN* and low molecular weight polypeptide proteasome subunits (*LMP2* and *LMP7*) and transport associated protein encoded by *TAP1* and *TAP2* genes [16, 17]. There are, other class II-like genes, such as *DOB*, *DIB*, *DYA* and *DYB*, whose functions remain unknown [2].

DM is an unconventional Major histocompatibility complex (MHC) class II heterodimer that acts as a chaperone for the B-cell mediated antigen processing and presentation of the MHC class II-restricted T cells. The Human leukocyte antigen (*HLA-DM*) is encoded by two genes *DM α* (major histocompatibility complex, class II, DM alpha) and *DM β* (major histocompatibility complex, class II, DM beta), which map to the MHC class II region [18]. In humans, both *DM α* and *DM β* are found in the MHC region between *DQ* and *DP*. These genes play an essential role in class II-restricted antigen presentation by promoting the exchange of class II-associated invariant-chain peptides (CLIP). These are bound to newly synthesized class II molecules and peptides derived from antigen processing [19]. Thus, the editing function favors display of high-affinity peptides on the surface of antigen presenting cells (APC) and substantially changes the peptide repertoire presented to T cells [20 - 22].

The MHC class II genes function in association with other genes inside (local or intra-MHC gene interaction) or outside the MHC region (global or extra-MHC gene interactions). The association of MHC linked *DM α* and *DM β* genes with diseases in human and mouse is well established [23 - 26]. Besides, their associations with disease susceptibility or resistance, the MHC class II genes influence vertebrate mating choice thus affecting reproduction [27, 28]. Earlier studies have reported up regulation of most of the MHC class II genes and complement molecules in intermediate atherosclerotic lesions. This indicates that different immune and inflammatory responses occur at varying stages of the disease progression

[29]. Data from different mammalian species suggests that much remains to be learned about the expression and function of MHC class II genes, especially *DM α* and *DM β* in animals suffering from diseases. Amongst the domesticated species especially buffalo, *DM α* and *DM β* are poorly characterized, despite agricultural and economic importance of this species in the Indian sub-continent and South Asian countries.

In the present study, we report the full-length mRNA sequence of *DM α* and *DM β* genes, their localization on chromosome 2, their tissue and disease specific expression and copy number status. Following this, we conducted *in silico* analysis to deduce interacting partners of *BuLA-DM α* and *DM β* and ascertained their expression across the tissues, spermatozoa and in the blood samples of normal and different diseased groups of buffaloes. Detailed understanding on the *DM α* and *DM β* genes both in healthy (control) and diseased buffalo would help to decipher whether the expression is due to alteration of transcriptional machinery or because of the ailments of the animals. Study on this line would help identifying the immunological basis of diseases in the animals.

Materials and Methods

Sample collection

Blood and different tissues (brain, heart, kidney, liver, lung, spleen, testis and ovary) samples of water buffalo (n=10, age ranging from 5 to 7 years) were collected from local slaughter house at Gazipur, Delhi, India, with the help of an on-site veterinary officer. Buffalo semen samples were procured from an *in-vitro* fertilization (IVF) center (Frozen Semen Production Center, Chak Gajaria), in Lucknow (U.P), India. Blood samples from different diseased groups of buffalo (n=5/group) suffering from mastitis (inflammation of the mammary gland, which is usually correlated with intra-mammary infection by bacteria or fungi), anoestrus (a state of sexual inactivity between two periods of estrus, resulting in inactive ovaries), diaphragmatic hernia (incomplete formation/muscularization of the diaphragm), bloat (indigestion marked by excessive accumulation of gas in the rumen), pneumonia (viral infection of the respiratory tract), hemoglobinuria (presence of free hemoglobin in urine) and pyrexia (high fever) were collected from the local dairy and veterinary hospital. Based on the physical examination and other microbiological tests carried by the veterinary officer the disease was characterized. Additionally, blood samples from healthy buffaloes were used as controls. The sample collection was performed with due permissions from the competent authorities following strictly the guidelines of the Institute's Ethical and Bio-safety committees.

Isolation of RNA and cDNA synthesis

Total RNA was isolated from the blood and tissues using TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH) [30] and that from the semen following the established protocol [31]. The presence of DNA was ruled out by PCR using *β -actin* (*ACTB*; GenBank accession no. DQ661647) primers (Frd: 5'CAGATCATGTTTCGAGACCTTCAA3' and Rev: 5'GATGATCTTGATCTTCATTGTGCTG3'). Following this, ~2 μ g of mRNA was used for cDNA synthesis using high capacity cDNA RT kit (Applied Biosystems, USA). The success of cDNA synthesis was confirmed by PCR using *β -actin* primers. Genomic DNA from the blood was extracted according to standard phenol-chloroform procedure [32].

Molecular cloning

We isolated mRNA sequences of *BuLA-DM α* and *DM β* genes using spleen cDNA as template and set of gene specific primers based on *Bos taurus* major histocompatibility complex, class II, DM alpha (BoLA-

Table 1. Details of primers used for isolating of *Bubalus bubalis* *DM α* and *DM β* mRNA. *Frd – Forward Primer; Rev – Reverse Primer; AT – Annealing Temperature

S.No.	Genes	Primer Sequence (5'-3')	Primers length (mers)	AT (°C)	Amplicon (bp)
1	<i>DMα</i>	Frd- GAAGATATGGATCATGAGCT	20	50	848
		Rev- AACACCTCCTGCTTGGATG	19		
2	<i>DMβ</i>	Frd- TCATTTAGAGAGCATCAAG	19	52	1532
		Rev- CAGGATTCTACCACTTATC	20		

DMA), mRNA (GenBank accession no. NM_001012674.2) and *Bos taurus* major histocompatibility complex, class II, DM beta (BoLA-DMB), mRNA (GenBank accession no. NM_001040481.3), respectively (Table 1). The PCR generated amplicons were cloned into pGEM[®]-T Easy Vector (Promega, USA). Screening for the positive clones were done by colony-PCR. The recombinant clones were then sequenced on 3130xl genetic analyzer using BigDye[®] Terminator v3.1 cycle sequencing kits (Applied Biosystems, USA) [33]. Then the deduced mRNA sequences of *BuLA-DMA* and *DM β* genes were submitted to the NCBI database and their corresponding accession numbers were obtained.

In silico sequence analysis

Homology of deduced mRNA sequences for *DMA* and *DM β* genes was ascertained using the BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) across different species. The open reading frame (ORF) for the candidate genes was obtained using the ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Nucleotide sequences representing complete ORF were translated into protein sequence using ExPASy translation tool (<http://web.expasy.org/translate/>). To identify regions of similarity/variation between *DMA* and *DM β* with that of humans, pairwise sequence alignment was performed through EMBOSS Needle [34].

Copy number estimation

The copy number of *DMA* and *DM β* genes per haploid genome was calculated by absolute quantitation using SYBR[™] Green assay on Real-Time PCR System-7500 (Applied Biosystems, USA). Real Time assays were performed in a 15 μ l reaction volume containing 7.5 μ l 2x SYBR[™] Green PCR Master Mix (Applied Biosystems, Warrington, UK), buffalo blood genomic DNA (0.5, 1.0 and 2 ng) and forward and reverse primers at final concentration of 1 μ M (Table 2). Assay conditions were followed as recommended by ABI (50°C for 2 min, 95°C for 10 min and 40 cycles for 95°C for 15 s and 60°C for 1 min). Copy number estimation was done using template (blood genomic DNA) and 10-fold serial dilutions of the recombinant plasmid specific to the candidate genes in the range of 30 $\times 10^2$ to 30 $\times 10^7$ copies (assuming haploid genome of bovine animals = 3.3 pg per cell). Reaction specificity was confirmed with the analysis of melting curves. The copies of *DMA* and *DM β* genes in buffalo genome were then extrapolated as per established protocols [35].

Table 2. Details of the primers used for quantitative expression analysis of *DMA* and *DM β* and their interacting genes by Real Time PCR in water buffalo *Bubalus bubalis*

S.No.	Gene ID	Accession number	Primer Sequence (5'-3')	Mers	GC%	T _m	Size of the amplicon (bp)
Relative Expression of <i>DMα</i> and <i>DMβ</i> genes							
1	<i>BuLA-DMα</i>	KJ470674	CAACGTGGCGCTCTCTGA	18	61.1	66.8	61
			CTGGGAAAAGTCGAAGGAGAAAA	23	43.4	66.3	
2	<i>BuLA-DMβ</i>	KJ470675	ACCGTGCAGGTAGCCAAAAC	20	55	54	64
			CATAGCAGGCCAGCATCACA	20	55	54	
Relative Expression of <i>DMα</i> and <i>DMβ</i> interacting partners							
3	<i>DRA</i>	NM_001012677.1	GTTTGACTTTGATGGTGATGAGATTT	26	34.6	65.0	153
			CGCCACACCGTCTCCTTCT	19	63.1	67.8	
4	<i>DRB3</i>	NM_001012680.2	TCTACACCTGCCAAGTGGATCA	22	50	66.7	69
			CAGACCGTGCCCTCCATT	18	61.1	66.3	
5	<i>DQA5</i>	NM_001012675.2	GCCTCGTGGGCATTATGG	18	61.1	66.4	67
			CTGGAAGGGTCACCTGAACGTA	22	54.5	67.2	
6	<i>DQB</i>	NM_001012676.3	CAGCGCCAAGTGGAACTTA	19	57.8	66.3	62
			TGGTGGTTTAGAGCCTCTGTTCT	23	47.8	65.0	
7	<i>DYA</i>	NM_001012678.1	CACTTACGGCACAAATGTCTACCA	24	45.8	66.7	66
			CCATCAAATTCAAACGTGAACGT	23	39.1	65.2	
8	<i>DYB</i>	NM_001012679.1	TGAATCAGCCCAGAGCAAGA	20	50.0	66.1	55
			CAGCCCCAGCACAAAACC	18	61.1	66.8	
9	<i>Ii</i>	D83962.1	TGGATGGCTTGGACTGGAA	19	52.6	66.8	63
			TCATTTCAAACAAGGCCACTGA	23	39.1	65.7	

Chromosomal localization by fluorescence in situ hybridization (FISH)

Approximately, 400 µl of buffalo blood was cultured for chromosome preparation following standard protocols [36]. Fluorescence *in situ* hybridization (FISH) for *BuLA-DMα* and *DMβ* was carried out using bacterial artificial chromosome (BAC) clones corresponding to *Callithrix jacchus* BAC clone (CH259-485P14; GenBank accession no. AC244392.3) and *Pongo abelii* BAC clone (CH276-116A10; GenBank accession no. AC207096) as the probes, respectively, procured from BACPAC Resources Centre, Oakland, California, USA. The DNA from cosmids corresponding to *DMα* and *DMβ* genes were isolated and sequences were confirmed by PCR using gene specific primers. Following this, FISH was conducted onto the metaphase chromosomes and interphase nuclei as per the established protocols [36]. Chromosomal mapping of *BuLA-DMα* and *DMβ* was ascertained following the International System for Chromosome Nomenclature (ISCND 2000) established for Bovids [37].

Expression of *DMα*, *DMβ* and their interacting partners by RT-PCR

Interacting genes of buffalo *DMα* and *DMβ* were uncovered by STRING V10.0 database (<http://string-db.org/>). The mRNA sequences for all the interacting partners were retrieved from NCBI database (<https://www.ncbi.nlm.nih.gov/nucleotide>) and gene specific internal primers designed by Primer Express Software V3.0 (ABI) (Table 2). Following this, RT-PCR was conducted for assessing the expression of *DMα* and *DMβ* and their interacting partners using ~50 ng cDNA from different somatic tissues, gonads and spermatozoa of buffalo. These RT-PCR reactions were validated employing subsequent Real Time PCR.

Quantitative relative expression

To ascertain the quantitative expression of *DMα* and *DMβ* and their interacting partners, we conducted Real Time PCR using cDNA from the somatic tissues, gonads and spermatozoa as template [38]. Thereon, assays were performed using Power SYBR® green (ABI, Warrington, UK) on Real-Time PCR System-7500

Fig. 1. The mRNA sequence of *BuLA-DMα* gene (KJ470674) and its ORF. The deduced mRNA sequence was subjected to ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>), which in turn gave CDS of 786 nucleotides encoding 261 amino acids. Protein sequence corresponding to *BuLA-DMα* is shown under the nucleotide sequence in single letter code. The start and stop codons are highlighted in green and red color, respectively.

(Applied Biosystems, USA). All the qPCR reactions were performed in triplicates employing universal cycling conditions, similar to those used for copy number estimation. Similarly, expression of *DMα* and *DMβ* genes and their seven interacting partners in disease specific samples was carried out using cDNA isolated from blood lymphocytes of different disease groups of buffalo and same set of real time primers (Table 2). *GAPDH* was taken as an internal control for each reaction. Primers giving single peak in dissociation curve and a standard curve having a slope value of -3.3 to -3.5 with a regression coefficient (R^2) value >0.99 were considered to be accurate.

Interpretation of data: Statistical analysis

The qPCR data of all the genes quantified was subjected to student t-test to determine the significance employing GraphPad Prism v5 (GraphPad Software, Inc.). All the results were presented as the mean $\pm$ SEM of three independent experiments and statistical significant values ($P < 0.05$) were considered.

Results

Isolation of mRNA sequences

Upon analysis of sequencing data, *Bubalus bubalis DMα* mRNA of 848 bp and *DMβ* mRNA of 1532 bp, were deduced from the different overlapping fragments. Following

Fig. 2. The mRNA sequence of *BuLA-DMβ* gene (KJ470675) and its ORF. The deduced mRNA sequence was subjected to ORF finder (<https://www.ncbi.nlm.nih.gov/orffinder/>), which in turn gave CDS of 789 nucleotides encoding 262 amino acids. Protein sequence corresponding to *BuLA-DMβ* is shown under the nucleotide sequence in single letter code. The start and stop codons are highlighted in green and red color, respectively.

1	TCATTTAGAGAGCATCAAGGGGCCCCGGGCAATACCACATTCCCAGTTAGTGTGTGGAGATCCTCTACCCAG
73	GCTGGGACTTCTGGGGTAGACGAAGACCCAGGGCCCCGGGAGAGCTGGTCCAGGGGACCGAACTCCCGGTG
145	TCTCCCCAGAGCAGAGCACTGACTGCTCCTCTGCTGCTGCTGGGCTCAGCCTGGGCTGCACCGCAGCA
	M T V L L S L L L G L S L G C T A A
216	GGTGGTTTTGTGGCACACGTGGAAGCACCTGTGTGTGGACGATGATGGGAATCCGAAGACTTCTCGTAT
	G G F V A H V E S T C V L D D D G N P K D F S Y
288	TGCATCTCCTTCAACAAGGATTGCTGACCTGCTGGGATCCCTGCAGACAGTATGGTTCTCGTGAATTT
	C I S F N K D L L T C W D P L Q T S M V P R E F
360	GGGGTGCTGAACGGCTTGGCCCGGTACCTCTCACAGTTCCTCAACAATAACAGTGACCTGATCCTTCGCTTG
	G V L N G L A R Y L S Q F L N N S D L I L R L
432	TCCAGTGGTCTGCAGAACTGTGCCGCGCACACCCAGCCCTTCTGGAGCTCTCTGACCCACAGGACACGCCCA
	S S G L Q N C A A H T Q P F W S S L T H R T R P
504	CCGACCGTGCAGGTAGCCAAACCACTCCTTTTAACACGAGGAGTCTGTGATGCTGGCCTGCTATGTGTGG
	P T V Q V A K T T P F N T R E S V M L A C Y V W
576	GGCTTCTACCCAGCGGACGTGGCCATCACGTGGAGGAGGAACGGGAGGAGTCTCCACACGGCAGGGCC
	G F Y P A D V A I T W R R N G Q E V L P H G R A
648	CAGAGGATCGTCCAGCCCAACGGAGACTGGACATACCAGACCGTCTCCCATCTGGCCACAACCCCTCTTTT
	Q R I V Q P N G D W T Y Q T V S H L A T T P S F
720	GGGGATACCTACACCTGTGTGGTTCGAGCATTGGGGCTCCTGAACATCCTCCAGGACTGGACTCCTGGG
	G D T Y T C V V E H I G A P E L I L Q D W T P G
792	CTGTTGCCTGCGCAGACGGTGAAGGTTTCTGTGTCTCTGGTAACGCTGGTCTGGGTCTCATCGTCTTCGTT
	L L P A Q T V K V S V S L V T L V L G L I V F V
864	TTCGGTCTGCGCAGTGGCGGAGGCCACCTCCTCCGGCTACATTTCTCCCGGGTCCACTTATCCAGAA
	F G L R S W R R A T S S G Y I F L P G S T Y P E
936	GGTCAGCACAATTGATAGAGGCAGAACCTACAGCTTTCCCTCCAGTGAGAAGGAGTTCTGAACCTTTGGG
	G Q H N
1008	GATGCTGCTGGGCCCCCTCCAGCTCCTCCAGCCCTCCACATCTTCTGGACCCATATGGTTTCTCTATTTAATT
1080	CTTTGAATTTCCAGTCTTCCCTATGTGAGGGCTTTCTGGTGGCTCAGTGGTAAAGAAATCCGCTGCAAT
1152	ACGGGAGACCTGTGTGTGATCCCTGGGTTGGGAAGATCCCTGGAGAAGGGAACGGCTACCCACTTCAATAT
1224	TCTGGCCTGGAGAATTCATGGACTGTAAAGTCCATGGGGTCACGAAGAGTTGGACATGACTGACGCACTTT
1296	CACCTTCACTTCACTTCACTTCACTTTCCTTATGTGAACCTTAGTAACCTGGGGAACTCATCTCGGTAATAA
1368	ATATTCTGTAACCAAGGTGCTGTCATATGCAGAGCCAGGGCTAAATATCTGGGGTGAATATCATCTGGGG
1440	AGGAAGGTCAAAGACCTCTTGGGGCCCCCTGTGTACAGAGGGCGTGAGTCACTGTTGATTGTTCTCTG
1512	AGATAAATGGTAGAATCCTG--1532

Fig. 3. Pairwise sequence alignment of *DMα* CDS between buffalo (*DMα_B*) and human (*DMα_H*). The two sequences showed 83.1% identity at CDS. A single dot denotes semi-conservative amino acid residues.

<i>DMα_B</i>	1	ATGGATCATGAGCTGAGCCAGGAAGCTGCGCTGCTACGGCTGCTCTCCTTTCTGTGGCTG	60
<i>DMα_H</i>	1	ATGGGTATGAACAGAACCAAGGAGCTGCGCTGCTACAGATGTTACCACTTCTGTGGCTG	60
<i>DMα_B</i>	61	CTGCCCCTGTCTGGACCGCCCTGAAGCTCCTGCTCCAGGGTGGAGGGATGAGCTACAA	120
<i>DMα_H</i>	61	CTACCCCACTCTGGGCGCTCCCTGAAGCTCCTACTCCAATGTGGCCAGATGACCTGCAA	120
<i>DMα_B</i>	121	AACCACACGTTCTGCACACAATGTACTGCCAGGACTGGAGCCCCAACGTGGCGCTCTCT	180
<i>DMα_H</i>	121	AACCACACATTTCTGCACACAGTGTACTGCCAGGATGGGAGTCCCACTGTGGGACTCTCT	180
<i>DMα_B</i>	181	GAGTCTACGACGAGGACCACTTTTCTCTTCGACTTTTCCAGAGCATCCGAGTGCCT	240
<i>DMα_H</i>	181	GAGGCTACGACGAGGACCACTTTTCTCTTCGACTTTTCCAGAACACTCGGGTGCCT	240
<i>DMα_B</i>	241	CGCTTGCTGAATTTGCTGACTGGGCTCACCAGCATGGAGATCTTCTGACATATATGTTT	300
<i>DMα_H</i>	241	CGCTTGCTGAATTTGCTGACTGGGCTCAGGAACAGGAGATGCTCTGCCATTTTATTTT	300
<i>DMα_B</i>	301	GACAAAGGTTTTTGCCGGCCATGATCGAGGAAATGGCCCACTTGAAGGGCAATC	360
<i>DMα_H</i>	301	GACAAAGAGTTCTGCGAGTGGATGATCCAGCAAAATAGGGCCAAACTTGATGGGAAATC	360
<i>DMα_B</i>	361	CCAGTGTCTAGAGGGTTCCCATCGTTTCAGGTGTTTACACTGAAGCCCTGGAGTTTGGC	420
<i>DMα_H</i>	361	CCGCTGTCCAGAGGGTTTCTTATCGTGAAGTGTTCACGCTGAAGCCCTGGAGTTTGGC	420
<i>DMα_B</i>	421	AAGCCCAACACTTGTCTGTTTCATCAGTAATCTCTTCCACCCACTGACCGTGAAC	480
<i>DMα_H</i>	421	AAGCCCAACACTTGTCTGTTTGTCTAGTAATCTCTTCCACCCACTGACAGTGAAC	480
<i>DMα_B</i>	481	TGGCAGCATCAGTCGGCATCTGTGAAGGAGCGGGCCCACTTTTGTCTCTCGGTTGAT	540
<i>DMα_H</i>	481	TGGCAGCATCATTCCTCCCTGGAAGGATTTGGGCTACTTTTGTCTCAGCTGTGAT	540
<i>DMα_B</i>	541	GGACTTACCTTCCAGGCTTTTCTACTTAAACGTACACCAGCACCCTGATCTTTTC	600
<i>DMα_H</i>	541	GGACTCAGCTTCCAGGCTTTTCTACTTAAACTTACACCCAGAACCTTCTGACATTTTC	600
<i>DMα_B</i>	601	TCTTGATCGTGACTCATGAGATCGATGGCTACACAGCAATCGCTTTTGGGTGCCCCAG	660
<i>DMα_H</i>	601	TCCTGCATTGTGACTCACGAAATGACCGCTACACAGCAATTCCTATTGGGTACCCCGG	660
<i>DMα_B</i>	661	AACGCGCTGCCCTCTGATCTTCTGGAGAATGTGCTGTGCGGCATGGCCTTTGGCTGGGT	720
<i>DMα_H</i>	661	AACGCACTGCCCTCAGATCTGCTGGAGAATGTGCTGTGCGGCATGGCCTTTGGCTGGGT	720
<i>DMα_B</i>	721	GTGCTGGGCATCATTTGTTGGCTTGGTCTCATCATCTACTGCCGAAAGCCGTGCTTAGGG	780
<i>DMα_H</i>	721	GTGCTGGGCATCATCTGTTGGCTTGGTCTCATCATCTACTGCCGAAAGCCGTGCTTAGGG	780
<i>DMα_B</i>	781	GGCTGA	786
<i>DMα_H</i>	781	GAATGA	786

this, retrieved sequence were deposited to NCBI database and subsequently, the accession numbers were obtained for *BuLA-DMα* (GenBank accession no. KJ470674) and *BuLA-DMβ* (GenBank accession no. KJ470675). Also, analysis of the sequences revealed an open reading frame (ORF) encoding CDS of 261 and 262 amino acids corresponding to *DMα* and *DMβ* proteins, respectively (Fig. 1 and 2). Also, *in silico* analysis and pairwise alignment of buffalo *DMα* and *DMβ* genes with that of human showed 83.1% and 79.5% similarity at their CDS, respectively, (Fig. 3 and 4).

Copy number and chromosomal localization

The *BuLA-DMα* and *BuLA-DMβ* were found to have 1 copy each per haploid genome uncovered by Real Time PCR. Representative amplification plots along with their corresponding dissociation and standard curves for *DMα* and *DMβ* genes are shown in Fig. 5A and B, respectively. Chromosomal mapping using FISH showed the presence of *DMα* and *DMβ* on the short arm of buffalo sub-metacentric chromosome 2 with corresponding signals on the interphase nuclei (Fig. 6A and B).

Interacting genes and their relative expression across tissues

Seven interacting partners of *DMα* and *DMβ* genes were retrieved through STRING V10.0 database. These included, class II, *DY* alpha (*DYA*), *DY* beta (*DYB*), *DR* alpha (*DRA*),

Fig. 4. Pairwise sequence alignment of *DMβ* CDS between buffalo (*DMβ_B*) and human (*DMβ_H*). The two sequences showed 79.5% identity at CDS. A single dot denotes semi-conservative amino acid residues.

<i>DMβ_B</i>	1	ATGACTGTC---CTCCTGTCGCTGCTGCTGGGCTCAGCCTGGGCTGCACCGCAGCAGGT	57
<i>DMβ_H</i>	1	ATGA---TCACATTCTGCGCTGCTGCTGGGCTCAGCCTGGGCTGCACAGGAGCAGGT	57
<i>DMβ_B</i>	58	GGTTTTGTGGCACACGTGGAAAGCACCTGTGTGTGGACGATGATGGGAATCCGAAAGAC	117
<i>DMβ_H</i>	58	GGCTTCGTGGCCCATGTGAAAGCACCTGTCTGTGGATGATGCTGGGACTCCAAAGGAT	117
<i>DMβ_B</i>	118	TTCTCGTATTGCATCTCCTTCAACAAGGATTTGCTGACCTGCTGGGATCCCTGCAGACC	177
<i>DMβ_H</i>	118	TTCACTACTGCATCTCCTTCAACAAGGATCTGCTGACCTGCTGGGATCCAGAGGAGAAT	177
<i>DMβ_B</i>	178	AGTATGGTTCTCTGTAATTTGGGGTGTGAACGGCTTGGCCCGTA---CCTCTCACAG	234
<i>DMβ_H</i>	178	AAGATGGCCCTTGCGAATTTGGGGTGTGAATAGCTTGGC---GAATGCTCTCTCACAG	234
<i>DMβ_B</i>	235	TTCTCAACAATAACAGTGA-CCTGATCCTTCGCTTGTCAGTGGTCTGCAGAACTGTGC	293
<i>DMβ_H</i>	235	CACCTCAACCA-AAAAGACACCTGATGCAGCGCTTGCGAATGGGCTTCAGAATTGTGC	293
<i>DMβ_B</i>	294	CGCGCACACCCAGCCCTTCTGGAGCTCTTGACCCACAGGACACGCCACCGACCGGTGCA	353
<i>DMβ_H</i>	294	CACACACACCCAGCCCTTCTGGGGATCACTGACCAACAGGACACGGCCACCATCTGTGCA	353
<i>DMβ_B</i>	354	GGTAGCCAAAACCACTCCTTTTAACACGAGGAGTCTGTGATGCTGGCCTGCTATGTGTG	413
<i>DMβ_H</i>	354	AGTAGCCAAAACCACTCCTTTTAACACGAGGAGCCTGTGATGCTGGCCTGCTATGTGTG	413
<i>DMβ_B</i>	414	GGGCTTCTACCCAGCGGACGTGGCCATCAGTGGAGGAGGAACGGGACAGGAGTCTCCC	473
<i>DMβ_H</i>	414	GGGCTTCTATCCAGCAGAAGTACTATCAGTGGAGGAAGAACGGGAAGCTTGTCATGCC	473
<i>DMβ_B</i>	474	ACACGGCAGGGCCAGAGGATCGTCCAGCCCAACGGAGACTGGACATACCAGACCGTCTC	533
<i>DMβ_H</i>	474	TCACAGCAGTGCACAAAGACTGCCAGCCCAATGGAGACTGGACATACCAGACCCCTCTC	533
<i>DMβ_B</i>	534	CCATCTGGCCACAACCCCTCTTTTGGGGATACCTACACCTGTGTGGTCGAGCACATTGG	593
<i>DMβ_H</i>	534	CCATTTAGCCTTAACCCCTCTTACGGGACACTTACACCTGTGTGGTAGAGCACACTGG	593
<i>DMβ_B</i>	594	GGCTCCTGAACCTCATCTCCAGGACTGGACTCCTGGGCTGTTGCCTGCGCAGACGGTGAA	653
<i>DMβ_H</i>	594	GGCTCCTGAGCCATCCTTCGGGACTGGACACCTGGGCTGTCCCATGCAGACCCTGAA	653
<i>DMβ_B</i>	654	GGTTTCTGTGTCTCTGGTAACGCTGGTCTGGGTCTCATCTCTTCGTTTTCGGTCTGCG	713
<i>DMβ_H</i>	654	GGTTTCTGTGTCTGCACTGACTCTGGGCTGGGCTCATCTCTCTCTTGGTGTGAT	713
<i>DMβ_B</i>	714	CAGTTGGCGGAGA---GCCACCTCCTCCGGCTACATTTTCCTC---CCGGGGTCCACTTA	767
<i>DMβ_H</i>	714	CAGCTGGCGGAGAGCTGGCCA---CTCTAGTTACA--CTCCTCTTCTGGGTCCAATTA	767
<i>DMβ_B</i>	768	TCCAGAAGGTTCAGCACAATT---GA	789
<i>DMβ_H</i>	768	TTCAGAAGGATGGCACATTTCCTAG-	792

DR beta (DRB3), *DQ alpha (DQA5)*, *DQ beta (DQB)* and invariant chain (*Ii*) (Fig. 7). The RT-PCR analysis using gene specific internal primers of *BuLA-DMα* and *DMβ* genes and their interacting partners *DYA*, *DYB*, *DRA*, *DRB3*, *DQA5*, *DQB* and invariant chain (*Ii*) across the different tissues and spermatozoa of buffalo detected amplicons in the range of 55-153 bp (Fig.8). These genes showed highest expression in spleen and relatively lower in other tissues.

Tissue and disease specific quantitative expression

The RT-PCR results were substantiated by quantitative expression for *DMα*, *DMβ* and their interacting genes across the tissues, spermatozoa and in the diseased animals using Real Time PCR. Quantitative expression analysis showed the highest expression of *DMα*, *DMβ* and their interacting genes in spleen followed by testis, ovary and spermatozoa and lowest expression in heart and kidney compared to that of the calibrator tissue (Fig. 9A-I). In disease

Fig. 5. Copy number status of *BuLA-DMα* (A) and *BuLA-DMβ* (B) genes assessed by Real Time PCR. Standard curves with slope values of -3.25 and -3.55 for *DMα* and *DMβ* genes, respectively. Dissociation curves showing single peak, substantiating primer specificity with the target DNA. Delta Rn vs Cycle plots of the standard plasmid and genomic DNA of water buffalo.

Fig. 6. Localization of *DMα* and *DMβ* genes on buffalo sub-metacentric chromosome using Fluorescence in situ hybridization. Note the signals corresponding to *DMα* (A) and *DMβ* (B) genes shown by arrows on the interphase nuclei {A (i-v) and B (i-v)}, respectively and on the short arm of sub-metacentric chromosome 2 {A (vi) and B (vi)}. The images shown are at the scale of 20 μ m.

conditions, the expression of *DMα* and *DMβ* along with their interacting genes was upregulated as compared to that in healthy (control) ones. In mastitis condition, 10-17 folds higher expression of *DMα*, *DMβ*, *DYA* and *DYB* genes was observed. *DRA* and *DRB3* showed highest expression in buffaloes suffering from anoestrus. Moreover, *DQA5* and *DQB* showed maximum expression in buffaloes affected with diaphragmatic hernia (Fig. 10 and Table 3). On the other hand, not much variation in the expression of invariant chain (*Ii*) was

Fig. 7. Diagrammatic interaction network showing interacting partners of *DM α* and *DM β* genes, deduced using STRING software (10.0). Interactions across the all interacting partners (*DYA*, *DYB*, *DRA*, *DRB3*, *DQA5*, *DQB* and Invariant chain (*Ii*)) of *DM α* and *DM β* are demonstrated in molecular action network.

Fig. 8. RT-PCR based expression of *DM α* and *DM β* genes and their interacting partners in different tissues and spermatozoa of buffalo. The gene name, sample IDs and corresponding amplicon size are given on the left, top and right of the panels, respectively. The quality and quantity of the cDNA samples were normalized using *GAPDH* primers shown at the bottom. For size marker, 50 bp ladder was used.

observed between the healthy and diseased animals (Fig. 10 I). The higher expression of *DM α* and *DM β* along with their interacting partners (except *Ii*) suggests the active involvement of these genes in triggering the immune response to ameliorate the possible impact of the diseases.

Discussion

The MHC is present across all the vertebrate species and show high level of polymorphism. Although individual MHC genes were found to have clear orthologues among ruminants and

Fig. 9. Quantitative expression of *DMα* and *DMβ* genes and their interacting genes in different tissues and spermatozoa of buffalo. Representative expression of *DMα* and *DMβ* genes (A-B) and their seven interacting partners, *DYA*, *DYB*, *DRA*, *DRB3*, *DQA5*, *DQB* and *li* across the tissues are given in panels (C-I). The bar represents the expression levels of the genes in folds. The gene IDs are mentioned on top left corner and sample IDs, below the panels. Testis was taken as the calibrator for all the genes. The error bars indicate the reproducibility of the experiments. Note the significantly higher expression in spleen. Results are expressed as mean $\pm$ SEM of three independent experiments. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

Table 3. Relative expression of *DMα* and *DMβ* genes in *Bubalus bubalis* suffering from different diseases. Cb- denotes calibrator tissue (expression value 1). The diseased samples having maximum expression have been italicized

S.No.	Gene	Relative expression (in folds 2 ^{-ΔΔC_t})											
		Control Samples					Samples from diseased buffalo						
		Control 1	Control 2	Control 3	Control 4	Control 5	Mastitis	Anoestrus	Diaphragmatic Hernia	Blot	Pneumonia	Hemo- globinuria	Pyrexia
1	<i>BuLA-DMα</i>	Cb	1.1609	1.0271	1.2402	1.2094	17.1674	12.8883	16.2256	9.2682	10.9381	2.7188	11.0051
2	<i>BuLA-DMβ</i>	Cb	0.7328	0.6858	0.7534	2.3861	10.6634	6.0927	7.4415	4.3343	3.6560	3.0230	5.5228
3	<i>DYA</i>	Cb	1.14925	1.0048	1.2972	1.2810	11.2503	7.4163	10.0414	3.6334	3.3152	1.3542	6.5346
4	<i>DYB</i>	Cb	1.0866	1.2596	1.0418	1.4247	14.7720	13.6383	7.7387	7.6480	2.8918	2.4332	6.2506
5	<i>DRA</i>	Cb	0.7187	0.1101	0.0745	0.0857	3.4621	10.997	2.6953	0.8176	1.3649	0.9145	1.0588
6	<i>DRB3</i>	Cb	0.0615	0.1100	0.0068	0.0085	2.1797	4.8356	0.9139	0.3146	0.7244	0.1425	0.8423
7	<i>DQA5</i>	Cb	1.1024	1.0660	1.0955	1.0535	9.0179	14.933	15.0365	2.1929	7.8152	7.0284	8.7488
8	<i>DQB</i>	Cb	0.05571	0.48999	0.0901	0.0912	1.8579	2.5994	3.4519	1.3012	1.8198	1.1024	1.4256
9	<i>li</i>	Cb	1.6466	1.4724	1.8063	2.1299	2.3654	2.8333	2.9876	3.1368	3.0084	3.7411	2.7738

other mammalian species, the MHC of buffalo is still not characterized. The class II region genes are closely linked in humans and other mammals examined, and variants of these genes are generally in strong linkage equilibrium [39]. The HLA genomic alterations generated by copy number variations (CNVs) are known to be associated with different autoimmune diseases, and has been reported in some human MHC related genes [40]. However, in the present study, copy number analysis showed no CNVs in *DMα* and *DMβ* genes in the diseased samples of buffalo. Though, the role of these CNVs in infectious and autoimmune diseases

Fig. 10. Quantitative expression of *DM α* and *DM β* genes across the different diseased groups of buffalo. The quantitative expression carried out using the cDNA isolated from the blood lymphocytes of different disease group of animals. Note the enhanced expression of *DM α* and *DM β* (A-B) and their interacting partners (C-I) in diseased animals compared to that in the healthy (control) ones. The control sample was taken as the calibrator. Results are expressed as mean $\pm$ SEM of three independent experiments. ***p<0.001, **p<0.01, *p<0.05. D. Hernia: Diaphragmatic Hernia; Hb. urea: Hemoglobinuria.

has not yet been elucidated, their possible detection in a species could be correlated with the susceptibility or resistance to a disease.

Constitutive expression of class II MHC is restricted to “professional” antigen-presenting cells but can be induced in various tissues by gamma interferon (IFN- γ) [41, 42]. Our study showed highest expression of *DM α* and *DM β* genes and their interacting partners in spleen suggesting their roles in immuno-transaction. Higher expression of these genes was also observed in testis, spermatozoa and ovary in buffalo. This is in accordance with earlier studies reporting the presence of cells expressing class II MHC molecules within the mice corpus luteum indicating their roles in reproduction, mate selection, pregnancy maintenance and fertility [43, 44].

The MHC partly determines the response of an individual to antigens of infectious organisms and has been implicated in susceptibility/resistance to infectious diseases including development of autoimmunity [7]. So far, neither the involvement nor the expression of *DM α* , *DM β* genes and that of their interacting partners in the context of diseases in buffalo has been studied. Similarly, in bovines no report is available on the autoimmune disorder, if any. In the present study, *DM α* , *DM β* , *DYA* and *DYB* showed highest expression in buffaloes suffering from mastitis. As the function of *DYA* and *DYB* genes is not yet established, our expression based studies reflect their possible chaperonic functions similar to *DM α* and *DM β* genes in antigen presentation. Invariant chain (*Ii*) is reported to be a chaperone for MHC class II polypeptides in the endoplasmic reticulum (ER) [27]. In the absence of *DM*, many MHCII proteins

do not exchange peptides and remain bound to CLIP (a remnant of the invariant chain), with the result, DM-deficient cells remain defective in antigen presentation [45 - 47]. For instance, in human, breast cancer cells express breast cancer metastasis suppressor 1 (*BRMS1*) gene and in response to this, high expression of MHC genes (*HLA-DQB1*, *DRB1*, *DRB5*, *DM β* , *DQA1*, *DRA*, *DRB4* and *DM α*) was observed. This immune response was triggered to eliminate or reduce the metastasizing potential of the abnormal cells [48]. In other carcinomas, high *Ii* expression is associated with poor prognosis and disease progression [49, 50], suggesting that *Ii* interferes with CD4+ T-cell recognition of endogenously derived tumor peptides [51]. We observed low level expression of *Ii* accompanied with high expression of *DM α* and *DM β* genes and their interacting partners in the animals suffering from different diseases. Our observations support earlier studies, where reduced CLIP levels in acute myeloid leukemia (AML) patients, enhanced the endogenous leukemia-associated antigens (LAA) presentation leading to improved leukemia-specific T-cell immunity [52]. Therefore, we infer that the enhanced expression of *DM α* and *DM β* genes and their interacting partners in diseased animals inhibit the expression of *Ii* thereby facilitating antigen presentation resulting in heightened immune response to combat the disease. In the absence of available vaccine, study on this line is of importance towards the development of new strategies for protection of the animals against infectious diseases.

Conclusion

The tissue and disease specific expression of *DM α* and *DM β* genes and their interacting partners in buffalo established their role in immuno-transaction in imparting disease resistance to the species. Present study provides a much-needed road map to establish a correlation between the genes from the MHC locus and their expressional diversity in healthy and diseased animals. Work on this line would be a useful towards evolving the strategies for better animal health.

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Disclosure Statement

The authors declare no conflict of interest.

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