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Trend of association of *BoLA-DQA1* alleles with FMDV vaccine elicited immune response in crossbred cattle

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ABSTRACT

Major histocompatibility complex molecules play a major role in immunological defense against pathogens. Polymorphism of bovine leukocyte antigen (*BoLA*) *DQA1* gene is being intensively investigated for potential association with economically important diseases of cattle. Accordingly, we investigated the association of *DQA1 Exon 2* polymorphism as evidenced by the variation in the binding pockets with variability in immune response to inactivated trivalent (O, A and Asia1) foot and mouth disease virus (FMDV) vaccine in a closed population of crossbred cattle. Antibody titer of ≥1.8 was set as the cutoff value to distinguish the protected (≥ 1.8) and unprotected (<1.8) animals. Eleven different alleles of over 1% frequency were detected in the population. Allele *0102 occupied highest rank followed by *10011 and *1402 for protective immune response while the allele *1401 ranked lowest for unprotected immune response for all the 3 serotypes. The correlation coefficient (ρ) of overall rank with individual ranks of serotype O, A, and Asia1 was also high in magnitude and positive. The rank correlations were statistically significant for all the serotypes except between O and Asia. Logistic regression analysis revealed that the effect of *DQA1* alleles was nonsignificant on vaccine elicited immune response based on Wald statistics. However, it is evident that odds of protection is high [Exp (β)>1] for a good proportion of DQA1 alleles in a given serotype. The knowledge has potential implications in future selection programmes if integrated with the complete *BoLA* haplotype details and production traits of the herd.

Key words: Bovine leukocyte antigen, Crossbred cattle, DQA1, FMD, Foot and mouth disease

The bovine leukocyte antigen (*BoLA*) Class II genes play a significant role in the genetic control of immune responses. Cattle have approximately 51 characterized alleles of *DQA* gene; out of these, 25 alleles belong to *DQA1* subgroup (Miyasaka *et al*. 2011). Most of the diversity in this gene has been studied using PCR-RFLP; whereas recent advances in sequencing technology have yielded a new approach called sequence based typing (SBT) (Takeshima *et al.* 2007).

Foot and mouth disease virus (FMDV) is the causative agent of foot and mouth disease (FMD) among cloven-hoofed livestock. In a population of susceptible animals, FMDV can lead to high morbidity (100%) and even mortality in young calves. Annual direct loss due to FMD in India has been estimated to the tune of ₹20,000 crores (Pattnaik *et al.* 2012). FMD virus, a single stranded RNA virus, is antigenically diverse having 7 distinct serotypes and many variants within them. In FMDV endemic regions, Africa, Asia and South America, control measures involve regular vaccination with inactivated virus vaccines; unfortunately, these vaccines have low efficacy and require regular boosts (Baxter *et al.* 2010).

A few reports also revealed animal to animal variation in the context of FMDV challenge and immunisation with a variety of vaccine constructs including peptides (Baxter *et al.* 2010, Gowane *et al*. 2013a, Leach *et al.* 2010). Although it is known that the influence of host genetic variation on vaccine response is mediated by polymorphisms in many genes, there has been a renewed focus upon the major histocompatibility complex (*MHC*), due to the important role the *MHC* genes play in host immune response (Sette *et al*. 2003).

Response to a simple FMDV peptide vaccine in cattle was studied vis-a-vis DRB3 polymorphism (Baxter *et al.* 2010, Gowane *et al.* 2013b) in cattle. In this paper, the importance of *BoLA-DQA* polymorphisms were explored with respect to the level of response to the inactivated trivalent (O, A, Asia1) FMDV vaccine in crossbred cattle. The cattle were genotyped at the highly polymorphic exon 2 locus.

MATERIALS AND METHODS

Animals: The study population was a herd of crossbred

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cattle of *Bos indicus* and *Bos taurus,* of which *B. indicus* constituted Indian zebu breed Hariana, whereas *B. taurus* constituted Holstein Friesian (HF) alone or HF in combination with Jersey. Exotic inheritance level was more than 50%. The herd is located at Indian Veterinary Research Institute (IVRI), Mukteswar (Nainital, Uttarakhand) in the temperate himalayan region of India at 29°28'N, 79°38'E, and had an average elevation of 2,171 m (7,123 feet) above the mean sea level (msl). Animals were kept separately according to their age group. The animals were maintained on a semi-intensive system with 5 h on pasture (0800–1300 h) per day, standard ration and water *ad lib*. For newborn calves, milk was supplemented up to 3 months of age and from second week onwards, green grass as well as calf starter feed was provided *ad lib.* The calf starter feed was continued up to 5 months. Calves were sent for grazing after they attained 3 months age. These animals were screened for antibodies against brucellosis, tuberculosis and Johne's disease, and found negative.

Vaccination and sampling: As part of the regular protective scheduled vaccination programme, the animals were vaccinated (2 ml intramuscular) with trivalent (O, A and Asia1) inactivated FMD vaccine. Whole blood (5 ml) was collected aseptically by jugular veinipuncture from the crossbred calves on 0 day (prevaccination) and 28 days post vaccination (DPV) for serum separation. Serum was collected and stored at –20°C until testing. An aliquot of blood (3 ml) was also taken in vacutainer at 0 day for genomic DNA extraction.

Differentiation of infected and vaccinated animals (DIVA): The DIVA test developed in the PDFMD laboratory (http://www.fao.org/ag/againfo/commissions/docs/training/manual/SAARC_Training_Manual.pdf) was employed for the detection of infected animals from the vaccinated animals using the principle that antibodies against non structural proteins (NSP) 3AB3 are produced only in FMDV infected cattle (8–10 days after FMDV infection) but not in FMDV free animals which have been vaccinated with inactivated purified vaccine. In this test, anti-NSP (3AB3) antibody present in the serum sample is measured against purified recombinant 3AB3 antigen. In the present study, DIVA test results on pre-and post-vaccination serum samples were negative, indicating antibodies measured were vaccine response.

LPBE (Liquid phase blocking enzyme linked immunoassay) for detection of antibody against FMDV vaccine: Sera were tested by LPBE (PDFMD Mukteswar, India; http://www.fao.org/ag/againfo/commissions/docs/training/manual/SAARC_Training_Manual.pdf) specific for serotypes O, A and Asia1. Samples were tested in duplicate and plates were read in ELISA reader to obtain optical density (OD) values at 492 nm wavelength (reference 620 nm). Calculated Log_{10} values were recorded. Values more than or equal to 1.8 indicated protection against FMD while those less than 1.8 indicated un-protected response.

Amplification and typing of DQA1 exon 2 alleles: Amplification of BoLA *DQA1* gene was performed by nested PCR reaction with 2 sets of primers specific for *DQA1* exon 2 (Table 1). Optimization of the annealing temperature and PCR protocol was done for the amplification of 355 bp product of gene. First set of primers was used for amplification of 426 bp fragment containing exon II and flanking intron sequences, for the first round 15 cycles were used (Table 2). Reaction mixture contained optimized concentration of Taq buffer 10x, 1.5 mM of $MgCl_2$, 10 mM of dNTPs, 20 picomole of primers, 15 nmol of genomic DNA, and Taq DNA polymerase. Following initial denaturation at temperature 94°C for 2 min, a typical cycle consisted of denaturation at 94°C for 20 sec, annealing at 54°C for 20 sec and extension at 72°C for 40 sec. After 15 cycles, a final extension at 72°C for 4 min was done.

The product of first PCR reaction was used as template for second round of PCR amplifying the DQA1 using second set of primers. The 249 bp of exon II and 94 bp of the 5'-end of intron 1 and 12 bp of 3'-end of intron 2 were amplified using fully nested PCR with DQAintL2 and DQA1–677R primers for first- round PCR and DQAintL3 and DQAexREV ver2.1 primers for the second- round PCR (Table 1). The product of first PCR reaction served as template for the second, where the final volume was 50 µl. Reaction mixture contained optimized concentration of Taq buffer 10x, 1.5 mM of $MgCl_2$, 10 mM of dNTPs, 20 picomole of primers, 1 µl of genomic DNA, and Taq DNA polymerase. Following initial denaturation at temperature 94°C for 2 min, a typical cycle consisted of denaturation at 94°C for 20 sec, annealing at 54°C for 20 sec and extension at 72°C for 40 sec. After 35 cycles, a final extension at 72°C for 4 min was done.

The purified PCR products were sequenced by sequencing reaction that exploits di-deoxy chain termination principle. The sequences were obtained using forward and reverse primers (Table 1), ACAATGTTTGATAG TCAATTTC and GGGRACACATACTGTTGGTAG,

Table 1. Primers for the PCR amplification and sequencing of DQA1 exon II

	Orientation	Primer	Sequence
First PCR	Sense	DQAintL2	CACAAATGAAGCCCACAATG
	Antisense	DQA1–677R	CCCTAGGGAAAAGGGAGTGA
Second PCR	Sense	DQAintL3	GCCCACAATGTTTGATAGTC
	Antisense	DQA1ex2REV ver2.1	GGGRACACATACTGTTGGTAGA
Direct sequencing	Sense	DQAintL3.1	ACAATGTTTGATAGTCAATTTC
	Antisense	DQA1ex2REV ver1.1	GGGRACACATACTGTTGGTAGA

respectively. Genotypes were assigned to all animals using BlastN online domain and manually comparing the sequences to previously reported alleles at *MHC* database (http://www.ebi.ac.uk/ipd/*MHC*/*BoLA*/).

Ranking DQA1 exon2 alleles for FMDV vaccine response: All the animals were negative for DIVA indicating that antibody was raised due to vaccination alone. All the pre-vaccination samples were unprotected as their titer was below 1.8. Therefore, 28DPV antibody titer data were normalized for pre-vaccination antibody level. LPBE data which was ordinal in nature was averaged for each allele. Allelic frequency was obtained and only those alleles that had more than 3% frequency were considered for further analyses. Highest score represented the first rank followed by succeeding scores. The ranks were obtained separately for all 3 serotypes as well as for 3 serotypes together. Spearman's Rank correlation coefficient (ρ) was obtained for the ranks of different alleles specific for each serotype and also for overall ranks. The ρ estimate was obtained and significance was tested at 5% and 1% level using SPSS.

Effect of DQA1 alleles on FMDV vaccine elicited immune response: Antibody response, which served as a responding variable was transformed to a binary variable based on whether response is protective (1) or un-protective (0). Logistic regression model was used to associate the various alleles of *DQA1* exon 2 obtained by SBT with post FMDV vaccine antibody titre. The statistical model was as follows:

$$\mathrm{Ln}(P_i/(1-P_i)) = \beta_0 + \Sigma\beta_j BoLA_j + \varepsilon_{ij} \quad [1]$$

where, P_i, probability that animal i was protected with respect to FMDV vaccine titer (0, no protection (titer <1.8); 1, protection (titer >1.8)); β_0, the intercept; β_j the regression coefficients for allele 1, 2, 3,…j; $BoLA_j$, the dummy variables X_1, X_2,…X_j for presentation of effects of allele 1, 2, 3,…j; and ε_{ij}, random error term.

RESULTS AND DISCUSSION

Alleles within the herd: All the animals (48) were sequenced for *DQA1* exon2 using sequence based typing method. In the population 11 alleles were found, however only 7 alleles had more than 3% frequency. Alleles that had less frequency were not taken into consideration to avoid biased results. No new allele was reported from this study. Among the 11 alleles, *10011 showed the highest frequency (40.6%), followed by alleles *10012, *1402 and *12021 with 15.6, 10.4 and 9.4% frequency, respectively (Table 2). Out of the 48 animals screened, 28 were heterozygous and 20 were homozygous indicating variability of DQA1 gene in the present crossbred population. It not only relates to the synthetic structure of the population but also to the importance of this gene in the evolutionary cascade.

Ranking DQA1 alleles and association with FMDV vaccine elicited immune response: Present study revealed that in the crossbred cattle, for serotype O, allele *DQA1**0102 occupied highest rank followed by *1402 and *10011 while allele *1401 ranked lowest. Similarly for serotype A, *DQA1**0102 occupied highest rank followed by *1402 and *10011 while the lowest rank was occupied by allele *1401. For serotype Asia1, again the highest rank was occupied by allele *DQA1**0102 followed by *10011 and *12012 while the allele*01401 ranked lowest. Based on overall ranks for all the 3 serotypes, allele *0102 occupied the highest rank followed by *10011 and *1402 while the allele *1401 ranked lowest (Table 2). Spearman's Rank correlation coefficient (ρ) of *DQA1* alleles based on serotype specific immune response between the ranks of serotype O with A (0.99) and Asia1 (0.75) and between A and Asia1 (0.78) was positive. The correlation coefficient (ρ) of overall rank with individual ranks of serotype O, A, and Asia1 was also high in magnitude and positive. The rank correlations were statistically significant (P<0.05) for all the serotypes except between O and Asia1 (Table 3). Results indicated concordance between the ranks of alleles for all the serotypes. Therefore, we conclude that selection using any 1 of the 3 serotypes can be reliably extrapolated to other 2 serotypes.

Logistic regression analysis revealed that the effect of *DQA1* alleles was nonsignificant on vaccine elicited immune response based on Wald statistics (P>0.05). However, it is evident that odds of protection is high [Exp (β)>1] for a good proportion of DQA1 alleles in a given serotype. Though the overall odds of protection is statistically nonsignificant, alleles having higher odds of

Table 2. Ranks of DQA1 alleles based on FMDV vaccine elicited immune response in crossbred cattle

Allele	[a]Frequency (%)	[b]O	[b]A	[b]Asia1	[c]Over all
*0101	2.1	–	–	–	–
*0102	7.3	1	1	1	1
*0203(1)	2.1	–	–	–	–
*0401	1.0	–	–	–	–
*10011	40.6	3	3	2	2
*10012	15.6	6	6	6	6
*12011	1.0	–	–	–	–
*12012	5.2	5	4.5	3	5
*12021	9.4	4	4.5	4	4
*1401	5.2	7	7	7	7
*1402	10.4	2	2	5	3

[a]Frequency of each allele; [b]each column with serotype name signifies ranks of each allele for vaccine response (serotype specific); [c]overall rank of allele for 28DPV LPBE titer.

Table 3. Rank correlation for the ranks of DQA1 alleles for FMDV vaccine response

Serotypes	O	A	Asia1	overall
O	1.000	0.991**	0.750	0.964**
A	–	1.000	0.775*	0.955**
X1	–	–	1.000	0.857*

** Spearmans rank correlation is significant at the 0.01 level (2–tailed). * Correlation is significant at the 0.05 level (2–tailed).

protection, for instance *0102 and *10011, are likely to show practical and clinical importance because of the consistency between rank correlation and high odds of protection for all the three serotypes. As it is also said that 'absence of evidence is not evidence of absence' (Altman *et al.* 1995), in this study the estimates of odds of protection for different alleles clearly indicates deterministic distribution of alleles for either protection or non-protection. For serotype O, A and Asia1, the odds of protection estimates for all the alleles were above the cut off value (1.00) (Table 4). However, for the higher ranking alleles, the odds of protection were significantly higher. For serotype O, highest odds were for *0102, *1402 and *10011 when allele *1401 was considered for comparison being the lowest ranking allele. For serotype A, also these 3 alleles gave highest odds of protection. For serotype Asia1, alleles *0102 and *10011 gave highest odds of protection (Table 4). Taken together, the concurrence between odds of protection estimate and Spearman's rank correlation test strongly suggested *DQA1* allele specific effect on protective response to FMDV vaccine.

Association of DQA1 alleles with FMDV vaccine elicited immune response: There were 7 alleles with more than 3% frequency in the population studied (Table 2). Reason behind more number of alleles owes to the synthetic structure of this population as described in the earlier section. Alleles *0102, *1402 and *10011 always ranked high and gave better odds of protection in our study (Tables 2, 4). With regards to DRB3 gene, a study was conducted in our lab (Gowane *et al*. 2013b) that reported similar allelic effect on the vaccine elicited immune response in crossbred cattle. However, for DQA1 gene there was no study till now in India, with regards to FMDV vaccine elicited immune response. Earlier report showed that most of BoLA class II haplotypes which were examined to be associated with FMDV- specific T cell and antibody response, a few haplotypes were associated with low or non-responsiveness even in the presence of other haplotypes which conferred responsiveness. There was consistent qualitative difference between haplotypes, which suggested that different BoLA DR and DQ molecules bind different fragments of the 40–mer peptide with different affinities (Glass *et al.* 1994). In another study, the Ag-presenting function of DQ molecules in 2 heterozygous animals were analyzed, 1 of which carried a duplicated haplotype and it was compared for the class II isotype specificity of T cell clones recognizing a putative vaccinal peptide from foot and mouth disease virus (FMDV15) (Glass *et al.* 2000). Previous report showed first time that bovine T cells can recognize Ag in the context of DQ molecules, indicating that inter haplotypes pairings of DQA and DQB molecules form functional restriction elements. Both animals showed distinct biases to usage of particular restriction elements (Glass *et al.* 2000). In our study, those animals which are high responder, majority are heterozygous. It may be due to the fact that the extra restriction elements simply increase the chance of high affinity binder for the given peptide. Similar analysis worked on the identification of MHC restriction and anchor residues of FMD disease virus derived bovine T cell epitopes in 2 cattle, report indicated that the epitope located on FMDV protein 1A can be presented by MHC class II DQ molecules encoded by DQA allele *22021 and DQB allele *1301 and present first evidence of the binding motif of this particular DQ molecule (Gerner *et al*. 2009). However, in our study, these alleles were not present.

Genetic variability in response to vaccination is likely to become an even more significant factor in designing ideal "safe, synthetic" vaccines (Glass *et al.* 2004). The genes identified might also be important for disease resistance traits, and could potentially provide the tools to select "good responders". However, the genes underlying variation in vaccine response have not been greatly explored in livestock (Glass *et al.* 2004). *BoLA-DQA1* is one of the most polymorphic genes within the bovine *MHC* locus. Most of the associations found in this work, although are important but are not statistically significant and thus not absolute. Role of environmental determinants as well as other *MHC* and non-*MHC* genes therefore cannot be neglected, especially DRB3 as reported earlier (Gowane *et al.* 2013b). Role of QTLs and non-genetic factors have been studied in detail showing the role of non-*MHC* genes and environmental factors on FMDV vaccine response (Leach *et al.* 2010).

Response to the inactivated trivalent FMDV vaccine in

Table 4. Estimates of Wald statistics and b exponential for effect of DQA1 alleles on FMDV vaccine elicited response against serotype O, A and Asia1

Parameter	O			A			Asia1		
	Wald	Sig.	Exp(β)	Wald	Sig.	Exp(β)	Wald	Sig.	Exp(β)
Allele	4.232	0.645		4.135	0.658		5.980	0.425	
*0102	1.528	0.216	5.333	1.528	0.216	5.333	0.000	0.999	2.154E9
*1402	1.165	0.280	4.000	1.165	0.280	4.000	0.000	1.000	1.000
*10011	1.528	0.216	4.211	0.772	0.379	2.783	0.000	0.999	2.376E8
*10012	0.088	0.766	1.455	0.088	0.766	1.455	0.000	1.000	1.000
*12012	0.462	0.497	2.667	0.462	0.497	2.667	0.000	1.000	1.000
*12021	0.796	0.372	3.200	0.009	0.923	1.143	0.000	1.000	1.000
Constant	1.537	0.215	0.250	1.537	0.215	0.250	0.000	0.999	0.000

Reference allele: *1401.

the studied population was variable that can be explained by environmental and genetic factors. There can be several factors responsible for this variation of which some are environmental and some genetic in nature. Exploring the genetic variability of *DRB3* gene in the host has revealed deterministic trend for distribution of alleles in either protected or un-protected category in this crossbred population. This paper supports the idea that variation in the vaccine elicited response might occur due to polymorphism in the *DQA1* locus of cattle apart from *DRB3*. Although statistically significant differences were not observed in the odds of protection by different alleles, magnitude of β exponential estimates and ranking of alleles for vaccine response generated antibody titre support the findings. Selection of individuals to be parents for next generation on the basis of protective alleles is the next logical step in the direction of creating the resistant stock; however, complete information with regards to different components of *BoLA* are necessary such as haplotype combination of *DQA* with *DRB*, and several *DQB* genes.

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