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Frequency of BoLA-DRB3 alleles in Polish Holstein-Friesian cattle*

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The aim of the study was to describe polymorphism of BoLA-DRB3.2 in Polish Holstein-Friesians. Alleles were identified according to PCR-RFLP procedure using a modification to the van Eijk's proposal [1992]. Restriction patterns were obtained with BstYI, HaeIII and RsaI enzymes. The study presented variability of all the alleles estimated in two herds. 752 animals were genotyped and informative according to previously described RFLP patterns. The most frequent homozygous animals were DRB3.2*24. The frequency was 0.066 and 0.078 in the sampled herds. No DRB3.2*03 nor DRB3.2*28 homozygous animals were found in one herd as opposite to another herd where homozygous animals DRB3.2*03 occurred with frequency 0.26 and animals DRB3.2*28 occurred with frequency 0.59. Homozygous DRB3.2*gba was not observed in that herd. The differences in allele frequencies may be associated with herd production and fitness status.

KEY WORDS: BoLA-DRB3 / Polish Holstein-Friesian / polymorphism / semi-nested PCR

Since the 1950s, the native Polish Black-And-White cows have constantly been upgraded with the Holstein breed of different origins. The consequences of crossbreeding were higher body weight and increase in milk yield. Intensive selection for the complex of production traits has appeared unfavourable for the adaptability of animals. Losses, related to the costs of treatment, and reduction in the productive potential of livestock tend for a breeders to seek new and effective methods of combating and preventing of diseases and disorders. Amongst others the mastitis

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incidence has been substantially elevated [e.g. Hoeschele & Meinert 1990, Sharif *et al.* 1998, Sender *et al*. 2008]. There is a lot of diseases eradication programs related to animal health and livestock production and especially focused on local animal breeds. The study of the genetic diversity of native breeds is necessary to maintain the genetic resources in livestock. It has also been reported that genetic variability exists with regard the resistance to infectious diseases. Some animals are more susceptible than others and the background of this mechanism is strongly associated with the major histocompatibility complex (MHC) genes [Xu *et al.* 1993, Lewin 1994, Juliarena *et al.* 2008, Baxter *et al.* 2010].

The role of MHC in disease resistance in humans is the subject of intensive research. Similar studies in farm animals are much less advanced, but suggest the relationship of MHC with resistance to diseases. Due to the extremely large profound involvement of MHC genes in susceptibility to diseases of animals, a comprehensive study of this issue is important to understand the molecular bases helping to explain the resistance response.

The MHC system has a similar genetic structure among mammalian species, and is organized into three classes. In humans MHC is located on chromosome 6 and takes about 4 Mbp (Mega base pair). In cattle major histocompatibility complex called – BoLA is located on autosom 23 and spans about 2.5 Mbp. [Galal Abdel Hameed K. *et al.* 2006].

The genes located in the MHC class IIa region encode α- and β-chains of MHC class II molecules and are expressed on the surface of antigen-presenting cells [Klein *et al.* 1995]. The main function of class II molecules is to present processed pathogen-derived peptides to T-helper lymphocytes – DC4+ (Th) [Banchereau; Steinman, 1998]. The BoLA class II region consists of 1 DRA, at least 3 DRB loci and multiple DQA and DQB genes [Takeshima *et al.* 2009].

In bovine BoLA-DRB genes have been associated with the regulation of immune response [Andersson *et al.* 1986, Muggli-Cockett *et al.* 1988, Andersson 1990, Glass *et al.* 1991,Van Eijk *et al.* 1992, Dietz *et al.* 1997a, 1997b, Sharif *et al.* 1998, Haeringen 1999, Juliarena *et al.* 2008].

Several methods have been used to detect extensive polymorphism in BoLA-DRB3.2 [Andersson *et al.* 1986, Muggli-Cockett *et al.* 1988, Davies *et al.* 1992]. The PCR-RFLP analysis has been found helpful for typing polymorphism in second exon of DRB3 gene [van Eijk *et al.* 1992, BoLA nomenclature ISAG]. Over time, more than 100 different alleles were found for BoLA-DRB3.2 gene [Da Mota *et al.* 2002].

Such an extensive BoLA-DRB3.2 gene polymorphism requires complex laboratory procedures. The main method based on semi-nested PCR was proposed by van Eijk [1992].

On the other hand regular PCR, as a basic method, may prove sufficiently accurate from the BoLA-DRB3.2 amplification view point. It requires fewer reagents and decreases the risk of making mistakes. It also decreases the time of the analysis because only single PCR is needed.

The final step in the van Eijk's method includes time consuming electrophoresis on the polyacrylamide gel. Nowadays, electrophoresis on the agarose gel, faster and less laborious, can be monitored in order not to loose fast migrating fragments, which can also be supported by visualizing software.

The aim of the study was to describe polymorphism of BoLA-DRB3.2 gene in the Polish Holstein-Friesian cattle with PCR-RFLP method using a modification to the van Eijk's proposal, in order to identify alleles of the gene that might be indicative of animals' performance in marker assisted selection.

Material and methods

Animals

Seven hundred fifty two cows from two commercial herds were sampled to investigate the polymorphism of BoLA-DRB3.2 gene. Animals were routinely recorded for, amongst others, milk, fat and protein yields [kg], fat and protein contents [%], and somatic cell count (SCC). Additionally, bovine viral diarrhoea virus (BVDV) antibodies presence was investigated with the ELISA test. The cows were daughters of 68 and 96 sires in the two herds, respectively with only two sires in common. Performance of the first parity cows was used to characterize the herds' production levels (Tab. 1).

Table 1. Means and their standard deviations (SD) of chosen traits as recorded in first parity cows across herds – pedigree information regards cows of all the parities

Trait	Herd A		Herd B	
	mean	SD	mean	SD
305-days milk yield (kg)	8191	1774	8633	1947
Fat (%)	3.89	0.54	3.86	0.36
Protein (%)	3.38	0.21	3.39	0.20
SCC	2282	2646	586	949
BVDV incidence (%)	82.3		94.2	
Number of sires	68		96	
Number of cows-daughters	223		529	

Genotyping

DNA isolation from blood was performed according to Kanai et al. [1994]. The concentration and purity of extracted DNA were assessed by spectrophotometery and electrophoresis in 1% agarose gel.

The BoLA-DRB3 exon II was amplified by PCR using the single step PCR as modified from van Eijk *et al.* [1992]. The PCR was performed in final volume of 32 µL containing 40 ng of cDNA, 10 pmol of each primer (HLO30; 5'-ATCCTCTCTCTGCAGCACATTTCC-3' and HLO32; 5'-TCGCCGCTGCACAGTGAAACTCTC-3'), PCR-Buffer (20 mM

Tris-HCl pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.25 mM of dNTPs and 1 U of Taq DNA polymerase and water for final volume. The initial denaturation (94°C for 10 min), then 25 cycles as follows denaturation (94°C for 30 s), annealing (65°C for 30 s) and elongation (72°C for 1min). The final elongation was carried out at 72°C for 5 min. PCR products were represented by 284 bp fragments as was expected on the basis of the nucleotide sequence of the gene.

RFLP Patterns

PCR products were digested separately with 3 restriction endonucleases, *Rsa*I, *Hae*III, and *Bst*YI as described by Van Eijk *et al*. (1992). Five units of *RsaI* and *HaeIII* were digested at 37°C for 3 h, and 5 units of *BstYI* at 60°C for 3 h. The restriction fragments were resolved on 3.5% agarose gel. DNA fragments were visualized in UV light and photographed with BioImagine System-Gene Snap (SynGen) using Gene Tools Software. The restriction patterns were compared with previously described in literature [van Eijk *et al.* 1992; BoLA nomenclature, ISAG].

DNA Sequencing

BoLA-DRB3.2 DNA sequence was performed base on the restriction enzyme sites. The sequence was compared with the gene Bank sequences.

Statistical analysis

Allele frequencies were obtained by direct counting. The observed frequencies of heterozygotes ($H^{observed}$), given in Table 2, were obtained directly by dividing the number of heterozygous individuals by the total number of individuals. The expected frequencies of heterozygote ($H^{expected}$) were those expected from the Hardy-Weinberg equilibrium.

Results and discussion

BoLA-DRB3.2 genotyping

Of 752 genotyped animals all genotypes appeared informative according to previously described RFLP patterns. The amplification resulted in DNA fragment with size of 284 base pair. After digestion with the restriction nucleases the amplified fragments smaller than 60 bp were difficult to separate on the gel during electrophoresis. Especially bands 50/51, 50/54 which is b/p patterns combination of RFLP-*Rsa*I were undistinguishable. We also found that different *Rsa*I genotypes be/fp, bj/cy, fj/sy, hn/im or gi/fn, had the same restriction pattern. However, those alternative genotypes in conjunction with RFLP-*Hae*III and -*Bst*YI patterns allow correct allele and genotype identification (e.g. faa is the DRB3.2*08 allele). Typical patterns of restriction of the amplified PCR product with the endonucleases *Rsa*I, *Hae*III and *Bst*YI are show in Figure 1.

Table 2. BoLA-DRB3.2* allele frequencies of Polish Holstein-Friesian cattle (herds A and B)

Herd A (n=223)			Herd B (n=529)		
allele	n	frequency	allele	n	frequency
*02	13	0.0288	*03	27	0.0255
*08	59	0.1305	*08	145	0.1370
*10	10	0.0221	*10	22	0.0208
*11	14	0.0310	*12	22	0.0208
*16	34	0.0752	*16	51	0.0482
*22	56	0.1239	*22	93	0.0879
*23	37	0.0819	*23	36	0.0340
*24	65	0.1438	*24	219	0.2070
*28	14	0.0310	*28	62	0.0586
*36	17	0.0376	*gba	34	0.0321
*gba	17	0.0376	*jbb	32	0.0302
*jba	12	0.0265	*nbd	33	0.0312
*jbb	13	0.0288			
*nbd	17	0.0376			
Others[1]	68	0.1637	Others[1]	282	0.2666
Total	446		Total	1058	

*Nomenclature homepage (http://www.projects.roslin.ac.uk/bola/drb3pcr.html).
[1]Alleles with frequency lower than 0.02.

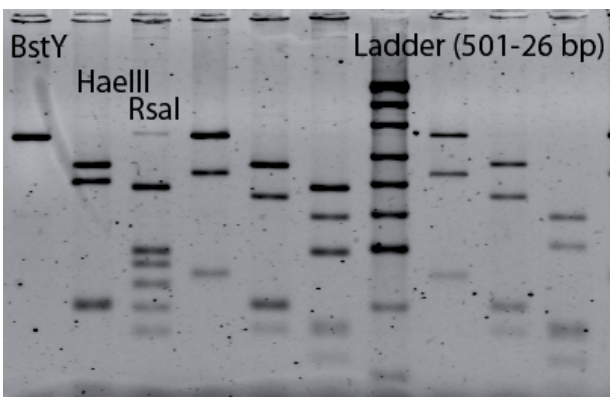

Fig. 1. Exon 2 amplification products of gene BoLA-DRB3.2 digested by endonucleases *Rsa*I *Hae*III, and *Bst*YI.

Combining the three restriction patterns (RFLP-*Rsa*I, -*Hae*III and –*Bst*YI) allowed recognition of 28 known DRB alleles, 12 without established nomenclature, and more than 40 which either occurred in small numbers (average of 3 copies in population) or were unclear to attribute them to known patterns. There were differences in distribution of alleles in investigated herds, however, in both herds allele DRB3.2*24 was the

most frequent 14.4% and 20.7% in herds A and B, respectively. The second most frequent allele was DRB3.2*08 with frequency 0.131 in herd A and 0.137 in herd B. Allele distribution is strongly associated with herd. For instance, animals with alleles DRB3.2*14, DRB3.2*18, DRB3.2*41, DRB3.2*baa, DRB3.2*ibb, DRB3.2*mea, and DRB3.2*pb occurred only in herd B. In contrary, alleles DRB3.2*06 and DRB3.2*15 were found in herd A only. Allele frequencies for A and B herds are given in Table 2.

In the studied population the observed heterozygosity (Ho) was in accordance with Hardy-Weinberg equilibrium (Tab. 3).

Table 3. The value of observed heterozygosity in the Polish Holstein-Friesian cattle

Herd	PIC	Ho	Allele diversity	χ^2	P
A	0.9231	0.7793	0.9274	3000	0.0001
B	0.9158	0.8479	0.9201	12587	0.0001

The fourteen most frequent alleles (with frequency higher than 0.02) from herd A, and twelve from herd B accounted for more than 83% and more than 73%, respectively. The distribution of DRB3.2 homozygous animals is shown in table 3. The most frequent homozygous animals were DRB3.2*24; the frequency was 0.0663 and 0.0775 for A and B herd, respectively. We did not notice homozygous DRB3.2*03 and DRB3.2*28 animals in herd A, contrary to herd B where homozygous animals DRB3.2*03 occurred with frequency 0.0255 and DRB3.2*28 with frequency 0.586. Homozygous DRB3.2*gba was not observed in herd B (Tab. 4).

Table 4. The frequency of homozygous animals for BoLA-DRB3.2 genotype

Herd A			Herd B		
homozygotes	n	frequency	homozygotes	n	frequency
DRB3.2*08	4	0.0177	DRB3.2*03	7	0.0078
DRB3.2*16	5	0.0221	DRB3.2*08	13	0.0245
DRB3.2*22	10	0.0442	DRB3.2*16	4	0.0076
DRB3.2*23	5	0.0221	DRB3.2*22	10	0.0189
DRB3.2*24	15	0.0663	DRB3.2*24	41	0.0775
DRB3.2*gba	11	0.0486	DRB3.2*28	2	0.0038

In our study the BoLA-DRB3.2 polymorphism was determined in Polish Holstein-Friesian cattle. Three enzymes were applied to determine polymorphism in DRB3.2 locus. Some DNA fragments with small restriction size differences were difficult to separate. For example, the *b* RFLP-*Rsa*I pattern (band size in bp: 30, 39, 50, 54, 111) is hard to distinguish from *p* pattern (band size in bp: 30, 39, 50, 51, 111). Miretti *et al.* [2001] noticed that *r* and *c RsaI* restriction patterns are indistinguishable because they differ only with 3 bp in the 90 bp fragment. In Similar disadvantages occurred for

RFLP-*Bst*YI patterns *a, c, d* and RFLP-*Hae*III patterns *a, c, f*. For this reason PCR-RFLP is an inadequate method for some DRB3 alleles and genotypes, and therefore other more sensitive molecular tools, such like SBT-PCR are required. Nassiry *et al.* [2005] demonstrated that the BoLA-DRB3.2 locus is highly polymorphic in the studied herd. Comparison of the restriction patterns obtained with use of three endonucleases made it possible to identify 26 alleles of gene DRB3.

The allele frequencies found in our study were in good agreement with previous studies performed in various breeds [Dietz *et al.* 1997a,b; Sharif *et al*. 1998; Takeshima *et al*. 2003; Nassiry *et at.* 2005; Rupp *et al.* 2007]. In our population we did not find alleles described previously by van Eijk *et al*. [1992] – BoLA-DRB3.2*1, *3, *4, *5, *6, *7, *9, *15, *17, *18, *19, *20, *27, *29, and *30 (table 4). In research conducted on Iranian population of Holstein bulls the seven most frequent alleles (BoLA-DRB3.2*8, *11, *16, *22, *24, *28, and *51) comprised 80% of all the alleles [Parnian *et al.* 2006]. Similar frequencies were observed in our population, although allele DRB3.2*51 was observed with frequency lower than 2%, and because of it, it was not taken into consideration in further analysis. In both herds the DRB3.2*24 allele was present at the highest frequency. Similar data was reported for various populations e.g. Iranian Holstein population [Parnian *et al*. 2006] and USA Holstein population [Dietz *et al.* 1997a], whereas in general the DRB3.2*08 is prevalent in Holsteins [Kelm *et al*. 1997]. It is interesting to highlight that in investigated population of the Polish Holstein-Friesian cattle occurred also the *ibb allele, previously found in Jerseys [Gillespie *et al*. 1999]. It was shown that most breeds differ in the spectrum of alleles and the allele frequency profile. For example, the six most frequently detected alleles in Jeresy cattle: BoLA-DRB3.2*8, *10, *17,*21,*36 and *ibe, accounted for approximately 74% of the alleles in the population, whereas the six most frequently detected alleles (BoLA-DRB3.2*8,*9, *21, *27, *7, *24) accounted for 70% of the alleles in the Japanese Shorthorns [Takeshima *et al.* 2003]. Alleles *22, *24, *11, *16, *18, *23, *8, and *27 are the most frequent in Russian Black Pied cattle [Sulimova *et. al.* 1995].

The occurrence of BoLA-DRB3.2 allele in different cattle breeds is shown in Table 5.

BoLA polymorphism information from the investigated herds is representative for the regional Holstein population. These differences may be largely due to the long-term adaptation to different geographical and climatic conditions.

Several associations between a particular alleles of the BoLA genes and resistance/susceptibility to infectious diseases in cattle were reported, particularly diseases that are prevalent during early lactation. In several studies the relationship was investigated between BoLA class II alleles and different mastitis indicators [Sharif *et al*. 1998, Kullberg *et al*. 2007, Rupp *et al*. 2007, Sender *et al.* 2008]. For example Schmutz *et al*. [1992] indicated that one BoLA-DRB3.2 gene pattern in a study of Holstein cows was associated with resistance to *Staphylococcus aureus*, but Galal Abdel Hameed K., *et al*. [2008] showed no associations between cows carrying allele *16 and *23 and

Table 5. The number of alleles in different breeds of cattle

References	Breed	Number of animals	Allele with high frequency
Sulimowa *et. al.* 1995	Black Pied	200	BoLA-DRB3.2*22, *24, *11, *16, *18, *23, *8
Giovamabattista *et al*. [1996]	Argentine	194	BoLA-DRB3.2* 5, *15,*18*,20
Golijow [1996]	Aberdeen Angus	65	BoLA-DRB3.2* 36. *8, *4, *15,*22
Dietz *et al*.[1997a]	Holstein	1100	BoLA-DRB3.2* 3,*7,*8,*16,*22,*23,*24,*27
Dietz *et al*. [1997b]		835	
Sharif *et al*. [1998]		115	
Takeshima *et al*. [2003]		471	
Nassiry *et al*. [2005]		250	
Present work [2010]		752	
Udina *et al*. [1998]	Ayrshire	129	BoLA-DRB3.2* 8,*28,*0,7
Udina *et al*. [1998]	Black Pied	127	BoLA-DRB3.2* 22.*24,*11,*16
Ripoli *et al*. [2004]	Saavedreno	125	BoLA-DRB3.2* 16, *36, *08, *11
Duangjinda *et al*. [2009]	Zebu × Holstein	409	BoLA-DRB3.2* 16,*51,*23,*11
Mohammdi *et al*. [2009]	Sistani	65	BoLA-DRB3.2* 8,*10,*11,*20

susceptibility/resistance to sub-clinical *S. aureus* or CNS mastitis. However genotype *23/- was found related to increased susceptibility to sub-clinical mastitis causes by *Streptococcus dysgalactiae*.

BoLA-DRB3.2*3 and *11 were associated with lower SCC, whereas alleles *22 and *23 were associated with higher SCC. The results of associations between BoLA-DRB3.2 and production traits were, in some cases, antagonistic. BoLA-DRB3.2 alleles *11 and *23, which are associated with increased production traits, were associated with lower and higher SCC, respectively [Firouzamandi *et al*. 2010]. In present work BoLA-DRB3.2*22 allele (associated with a lower risk of cystic ovarian disease) was found with the frequency 12% and 8.8%. The alleles associated with the resistance to mastitis and to bovine leukemia virus infection BoLA-DRB3.2* 11 and *23 are detected with the frequencies 3.2% and 8.2% respectively.

It was found in association studies that allele DRB3*10 had an effect increasing the milk yield, [Starkenburg *et al.* 1997, Duangjinda *et al.* 2008], while the presence of DRB3*22 allele was shown to decrease milk production. Noticeably, the mastitis-susceptibility alleles tended to be associated with increasing milk yield, whereas mastitis-resistance alleles tended to be associated with decreasing milk yield. However, the study by Duangjinda *et al*. [2008] revealed that DRB3*10 could be used as a candidate gene marker for improving milk production with moderate mastitis resistance. The herds in our study differ with regard to milk yield and SSC

and the differences in BoLA-DRB3.2 allele frequency may also be associated with the performance levels.

The results of this study demonstrated that BoLA-DRB3.2 in the Polish Holstein-Friesian cattle is highly polymorphic. There is also an indication that differences in frequency of particular alleles may be correlated with differences in production and fitness status of herds but further research is needed.

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