Theoretical and Applied Veterinary Medicine

ISSN 2663-1156 (print)
ISSN 2663-1164 (online)
Theoretical and Applied Veterinary Medicine, 10(1), 3–10
doi: 10.32819/2022.10001

Original researches

BoLA-DRB3 gene as a marker of sensitivity of the white-headed Ukrainian cattle to mastitis

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Received: 10 January 2022
Revised: 28 January 2022
Accepted: 14 February 2022

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Abstract. Mastitis in cows is an important problem in the milk industry. Major histocompatibility complex (MHC), also called bovine lymphocyte antigen (BoLA), is attracting attention due to its association with host immunity. The BoLA system has some equally operating genes that provide antigen presentation by MHC system molecules followed by an immune response to pathogens. Exon 2 of the BoLA-DRB3 gene is the most important and highly polymorphic. Alleles that had a close connection with mastitis have been detected and are considered DNA markers. These play a decisive role in the breeding of cattle to create herds resistant to diseases. Polymorphism of the BoLA-DRB3 gene (exon 2) of the White-Headed Ukrainian cattle breed was studied by PCR-RFLP to search for DNA markers associated with mastitis. In the general sample and group of resistance cows, 28 alleles were found, and in the group of animals prone to mastitis – 21 BoLA-DRB3.2 alleles. The most common variant was BoLA-DRB3.2*24 (12.3%). It also dominated among cows susceptible to mastitis (25.9%). Allele *22 (13%) maximally showed the animal's resistance to the disease. Based on relative risk, it has been reliably established that there are at least two mastitis-sensitive alleles: variant BoLA-DRB3.2*22 ($p<0.05$) associated with resistance, and *24 – with a susceptibility to the disease ($p<0.001$). It was also established that the genotype DRB3.2*11/*24 ($p<0.01$) can be used as a DNA marker of mastitis resistance after additional verification. The obtained results will be useful in the formation of herds of dairy cows resistant to mastitis.

Keywords: DNA; allele; selection; cow; disease resistance

Ген BoLA-DRB3 як маркер чутливості худоби білоголової української породи до маститу

Анотація. Мастит у корів є важливою проблемою молочної промисловості. Головний комплекс гістосумісності (ГКГС, major histocompatibility complex – MHC), у корів це бичачий лімфоцитарний антиген (bovine leucocyte antigens, BoLA), привертає увагу завдяки своєму зв'язку з імунітетом тварини. Система BoLA має кілька генів, які працюють однаково та забезпечують презентацію антигену молекулами системи ГКГС з подальшою імунною відповіддю на патогени. Найбільш важливим і високополіморфним є екзон 2 гена BoLA-DRB3. Виявлено алелі, для яких показаний щільний зв'язок з маститом і які вважаються його ДНК-маркерами. Вони відіграють вирішальну роль у селекції великої рогатої худоби для створення стійких до хвороб стад. Методом ПЛР-ПДРФ (поліморфізм довжин рестрикційних фрагментів) досліджено поліморфізм гена BoLA-DRB3 (екзон 2) великої рогатої худоби білоголової української породи з метою пошуку ДНК-маркерів, асоційованих з маститом. У загальній вибірці та групі резистентних корів виявлено 28 алелей, а в групі тварин, схильних до маститу – 21 алель BoLA-DRB3.2. Найбільш поширеним варіантом був BoLA-DRB3.2*24 (12,3%). Ця алель переважала також, схильних до маститу корів (25,9%). Алель *22 (13%) показана як найбільш асоційована зі стійкістю тварин до хвороби. З огляду на відносний ризик, достеменно встановлено, що існує щонайменше два алелі чутливості до маститу: варіант BoLA-DRB3.2*22 ($p<0,05$), пов'язаний із резистентністю, та *24 – із сприйнятливістю до захворювання ($p<0,001$). Також встановлено, що генотип DRB3.2*11/*24 ($p<0,01$) може бути використаний як ДНК-маркер стійкості до маститу після додаткової перевірки. Отримані результати можуть бути корисними при формуванні стад дійних корів, стійких до маститу.

Ключові слова: ДНК; алель; селекція; корова; стійкість до захворювань

Cite this article: Suprovych, T. M., Suprovych, M. P., Biriukova, O. D., Trach, V. V., Danchuk, O. V., & Grafov, A. V. (2022). BoLA-DRB3 gene as a marker of sensitivity of the white-headed Ukrainian cattle to mastitis. *Theoretical and Applied Veterinary Medicine*, 10(1), 3–10. doi: 10.32819/2022.10001

Introduction

The biggest problem of modern dairy cattle breeding is associated with mastitis. Mastitis is a disease that affects a large number of dairy cows throughout the world. In most countries, surveys of the incidence of mastitis show comparable figures as about 40% morbidity rate amongst dairy cows & a quarter infection rates as measured by an indirect test of about 25%. On an annual basis, 3 of every 10 dairy cows have clinically apparent inflammation of the mammary gland of the affected cattle, 7% are culled, and 1% die as a sequence of the disease (Nuraddis, 2017; Danchuk et al., 2021). It is the most economically important disease of dairy cattle, accounting for 38% of the total direct costs of the common production diseases. Mastitis is a global problem as it adversely affects animal health, the quality of milk, and the economics of milk production. It is the disease of dairy animals that is responsible for heavy economic losses due to reduced milk yield (up to 70%), milk discarded after treatment (9%), cost of veterinary services (7%), and premature culling of cows (14%) (Sharma et al., 2012). Given the growing pathologies of cattle mammary glands, searching for DNA markers susceptible to mastitis will promote their recovery through genetically balanced selection, culling potentially susceptible animals at the stage of early postnatal ontogenesis and forming a dairy herd of resistant cows.

The main problem with disclosing cattle resistance to mastitis is the total time spent on the cows' evaluation, which can take several years (Danchuk et al., 2020). Searching for reliable signs and marking the resistance or susceptibility of cows to mastitis is quite essential, such that it shortens the assessment time drastically. One of the methods for early detection of mastitis-sensitive animals is the analysis of the BoLA-DRB3 gene polymorphism and the identification of the relationship of its alleles with mastitis.

Resistance to mastitis is associated with alleles of the DRB3 gene of the major histocompatibility complex of cattle (MHC). It occurs because the gene product is directly involved in binding foreign antigens and determines the specifics of the immune response. The gene is highly polymorphic, which makes it possible to use its alleles as DNA markers associated with various diseases (Abdel Hameed et al., 2006). The MHC genes encode classes I and II molecules that present pathogen-derived peptide antigens to T-cells and initiate specific immune responses against parasites. MHC class I receptors capture and present intracellular antigens to cytotoxic T-cells, and MHC class II receptors capture and present intracellular antigens to helper T-cells. High variability of MHC is present at the nucleotide level that translates into a heterodimeric cell surface α and β receptors with broader recognition capabilities of foreign peptides to be presented to T-cells to initiate an immune response (Fernández et al., 2015). BoLA genes consist of one BoLA-DRA gene, three BoLA-DRB genes (with only one, BoLA-DRB3, thought to be functionally important), one or more BoLA-DQA, and BoLA-DQB genes, depending on the haplotype, and some BoLA class I genes. Several early studies have suggested that the progression of mastitis is associated with several BoLA alleles (Takeshima & Aida, 2006; Rupp et al., 2007).

The role of MHC antigens, associated with the immune response to foreign antigens, determines numerous associations with diseases. Many studies are devoted to the establishment of the relation of the BoLA-DRB3.2 gene alleles to such diseases as leukaemia (Nikbakht Brujeni et al., 2016; Farias et al., 2017; Lo et al., 2020), dermatophilosis (Maillard et al., 1996; Ballingall et al., 2004), lameness (Sun et al., 2013), FMD (foot-and-mouth disease) (Lei et al., 2012), tick-borne disease (Martinez et al., 2006; Duangjinda et al., 2013), herpesvirus (Morales et al., 2020), necrobacillosis (Suprovych et al., 2020), etc.

Several studies are devoted to the study of his polymorphism to identify allele variants that can claim the role of DNA markers in connection with mastitis sensitivity (Kelm et al., 1997; Kulberg et al., 2007; Duangjinda et al., 2009; Firouzamandi et al., 2010; Yoshida et al., 2012). Similar studies examined the black-and-white and the red-and-white Ukrainian dairy breeds (Suprovych et al., 2018).

The uniqueness of the BoLA gene is that the single-locus system is superior to multi-locus systems in terms of variability. It makes it possible to perform a thorough genetic analysis with data on the distribution of alleles of only one gene. Gene DRB3 has the highest polymorphism among all studied loci of the major histocompatibility complex of cattle. To date, the IPD-MHC database (https://www.ebi.ac.uk/ipd/mhc/group/BoLA) describes 330 BoLA-DRB3 alleles and this list is constantly updated.

Crossbreeding local Polissia cattle with bulls of the Groningen offspring of the Dutch cattle raised the White-Headed Ukrainian breed (Ernst et al., 1994). The cows of this breed are massive creatures of the milky type and have a strong constitution, with lumpy breasts, straight broad back, thin legs, and large udder with evenly developed quarters. Most cows have a black colour, a white head with "glasses" around the eyes, usually a white belly, and udder.

Long-term selection and breeding hybrids "in itself" led to the formation of a complex of genes that led to high resistance to disease (Efimenko et al., 2008). Therefore, ascertaining allele polymorphism of the BoLA-DRB3 gene of White-Headed cattle is essential for establishing a genetic connection with mastitis. The breed belongs to the small local population and has preserved genetic complex lost in modern commercial cattle. In addition, the study of the population-genetic structure based on the highly informative BoLA-DRB3 gene is of paramount importance for the needs of cattle diversity conservation.

The main task of the study was to study the polymorphism of the BoLA-DRB3 gene and to identify its alleles and genotypes associated with mastitis in White-Headed cows of the Ukrainian breed.

Materials and methods

The object of study. Blood was sampled from the Ukrainian White-Headed breed (n = 77) raised at Podolsky Master Farm (Antoniny, Krasyliv district, Khmelnytskyi region). Molecular genetic studies were carried out at the Genetics Laboratory of the Institute of Breeding and Genetics of Animals nd. a. M.V. Zubets of NAAN.

Determination of mastitis. Both mastitis-prone and resistant cows were selected based on a study of somatic cell counts (SCC) in milk. Animals of at least three consecutive SCC studies from the first to the fifth lactation were considered. Cows with SCC< 200 thousand cells/ml were classified as healthy (n = 50). Animals with SCC > 300 thousand cells/ml in two consecutive tests or cows with SCC > 500 thousand cells/ml in any single test were classified as mastitis (n = 27).

BoLA-DRB3 typing. Isolation of DNA. Allele frequencies were determined by analysis of restriction products obtained after exon 2 of the BoLA-DRB3 gene amplification. We performed DNA isolation from blood samples using the DiatomTM DNA Prep 200 kit (Isogen Laboratory Ltd.) in agreement with the manufacturer's instruction manual (Van Eijk et al., 1992, Sulimova, 2004). DNA secluded from fresh biological material (yield was 5–10 mg from 200 ml of whole blood) has a high molecular weight (40-50 base pairs) and pure substance (OD260/280 nm = 1.6–2.0). We used spectrophotometric analysis and electrophoresis to control the concentration and purity of extracted DNA. For this purpose, 25 ng (50 or 100) of λ phage DNA and aliquots of solution with an unknown concentration were applied to agarose gel. The electrophoresis was made in 1X Trisborate (TBE) buffer (89 mM Tris-OH, 89 mM H3BO3, 2 mM EDTA) and EtBr (1 μg/ml) added to the gel at a constant voltage of 120 V. DNA concentration was determined by comparing the fluorescence intensity of the aliquots of unknown concentration

and controlled lambda phage DNA. The gels were analyzed under ultraviolet rays on the transilluminators UVT and Biocom and were photographed using a gel document system MINITRON.

Amplification. The BoLA-DRB3 exon 2 was amplified by using single-step PCR. The PCR we carried out using ready-made sets of GenPak™ PCR Core (Isogen Laboratory Ltd.). The total volume of mixture (20 µl) contained 60 mM Tris-HCL (pH 8.8), 2.5 mM $MgCl_2$, 20 mM KCl, 15 mM $(NH_4)_2SO_4$, 10 mM mercaptoethanol, 0.1% Triton X-100, 0.2 mM dNTP, 10 units of Klentaq DNA polymerase, 10 pM of each primer, template DNA. Amplification of the BoLA-DRB3 exon 2 was carried out with oligonucleotide primers for the first round of the reaction HLO-30 (5'-3': TCCTCTCTCTGCAGCACATTTCC) and HLO-31 (5'-3': ATTCGCGCTCACCTCGCCGCT). DNA (5 µl) was used as a template, regardless of its concentration. Primers used for the amplified second round: HLO-30 (5'-3': TCCTCTCTCTGCAGCACATTTCC) and HLO-32 (5'-3': TCGCCGCT GCACAGTGAAACTCTC). PCR products of the first round (2 µl) were used for the second one. First period: DNA denaturation at 95 °C for 5 min followed by 10 cycles with denaturation (94 °C for 1 min), annealing (62.5 °C for 2 min) and elongation (72 °C for 1 min) and a final extension at 72 °C for 7 min. Second period: initial denaturation (95 °C for 5 min.), was followed by 35 cycles of denaturation (68 °C for 30 sec), and annealing-extension (72 °C for 30 sec) and a final extension (72 °C for 7 min). Each PCR round was accompanied by contamination and self-priming control. Five µL of the previous PCR product were electrophoresed on 1.5% agarose gels to check the quality and specificity of DNA fragment amplification. After the second round of nesting PCR, electrophoresis was carried out in 1.8% agarose to assess the quality and concentration of the resulting fragment.

RFLP pattern. Products of PCR were processed separately in three restriction endonucleases: RsaI, HaeIII, and XhoII (Promega, New England BioLabs and SibEnzim). The restriction fragments were separated by electrophoresis in 2% agarose and 9%–12% polyacrylamide gels. To estimate the fragment length, a marker of molecular scales GeneRuler™ Ultra Low Range DNA Ladder made by Fermentas, Latvia, was used.

Amplification of exon 2 followed by the analysis of restriction fragment length and comparison of DNA patterns obtained using the three specified restriction endonucleases allows the identification of 54 nomenclature alleles (Van Eijk et al., 1992; Gelhaus et al., 1995; Maillard et al., 1999). Besides, identified other alleles not described by the PCR-RFLP method, were designated as "without established nomenclature" (Oprzadek et al., 2012). They were identified through a set of patterns detected with the help of each of the three restriction endonucleases.

Statistical analysis. We calculated allele frequencies (P_A) considering the number of homozygotes and heterozygotes. We used the χ^2 test to check both actual and expected distributions of alleles and genotypes. The strength of communication in allele-disease

Table 1 – Polymorphism of alleles of the BoLA-DRB3.2 gene cows of White-Headed Ukrainian breed.

Alleles	Resistance (*n = 50*)			Susceptibility (*n = 27*)			Total (*n = 77*)		
	PA	*SE*	χ^2	*PA*	*SE*	χ^2	*PA*	*SE*	χ^2
*02	0.01	0.01	1.85	0.019	0.018	0.83	0.013	0.009	1.45
*03	0.09	0.029	8.25**	0.056	0.031	1.10	0.078	0.022	4.99*
*06	0.01	0.01	1.85	0.037	0.026	0	0.019	0.011	0.74
*08	0.01	0.01	1.85	0.074	0.036	4.12*	0.032	0.014	0.03
*10	0.01	0.01	1.85	0.019	0.018	0.83	0.013	0.009	1.45
*11	0.04	0.02	0.05	0.148	0.048	35.4***	0.078	0.022	4.99*
*12	0.03	0.017	0.09	0.019	0.018	0.83	0.026	0.013	0.27
*13	0.1	0.03	11.6***	0.019	0.018	0.83	0.071	0.021	3.57
*14	0.01	0.01	1.85	0.019	0.018	0.83	0.013	0.009	1.45
*15	0.08	0.027	5.49*	0.093	0.039	9.06**	0.084	0.022	6.64**
*16	0.04	0.02	0.05	0.019	0.018	0.83	0.032	0.014	0.03
*19	0.02	0.014	0.69	0.019	0.018	0.83	0.019	0.011	0.74
*22	0.13	0.034	24.9***	0.037	0.026	0	0.097	0.024	10.7**
*23	0.09	0.029	8.25*	0.019	0.018	0.83	0.065	0.020	2.39
*24	0.05	0.022	0.57	0.259	0.060	140***	0.123	0.027	21.5***
*26	0.02	0.014	0.69	0.019	0.018	0.83	0.019	0.011	0.74
*32	0.02	0.014	0.69	0.019	0.018	0.83	0.019	0.011	0.74
*33	0.02	0.014	0.69	0.074	0.036	4.12*	0.039	0.016	0.03
*35	0.02	0.014	0.69	0	0	3.57	0.013	0.009	1.45
*36	0.02	0.014	0.69	0.019	0.018	0.83	0.019	0.011	0.74
*39	0.02	0.014	0.69	0	0	-	0.013	0.009	1.45
*45	0.04	0.02	0.05	0	0	-	0.026	0.013	0.27
*54	0.03	0.017	0.09	0	0	-	0.019	0.011	0.74
*mdb	0.02	0.014	0.69	0	0	-	0.013	0.009	1.45
*iab	0.02	0.014	0.69	0	0	-	0.013	0.009	1.45
*gbb	0.02	0.014	0.69	0.019	0.018	0.83	0.019	0.011	0.74
*fbd	0.02	0.014	0.69	0	0	-	0.013	0.009	1.45
*naa	0.01	0.01	1.85	0	0	-	0.006	0.006	2.39

P_A – frequency of alleles; *SE* – standard error; *p<0.05; **p<0.01; ***p<0.001.

and genotype-disease pairs is determined based on the relative risk (*RR* – relative risk, f_b – frequency of gene carriers among susceptible animals, f_k – frequency of gene carriers in resistant animals). It determines the likelihood of the disease in animals having a suitable allele (genotype) compared to animals that do not have them.

$$RR=f_b\ (1-f_k)\ /\ f_k\ /\ (1-f_b) \qquad (1)$$

The reliability of the obtained result was monitored by Pearson's test and checked by calculating the expected frequencies to limit the experimental sample size (Suprovych et al., 2018). Statistical data processing was performed in the standard package Microsoft Excel 2013 using the GenAlEx 6.503 package (http://biology-assets.anu.edu.au/GenAlEx/Download.html). Verification of the normal distribution of allelic frequencies was performed based on Shapiro-Wilk and Kolmogorov-Smirnov tests by use of the standard package IBM SPSS Statistics V24.0 (https://www.ibm.com/support/knowledgecenter/ru/SSLVMB_24.0.0/spss/product_landing.html).

Results

Table 1 demonstrates the results of allele frequency detection for the general sample, both mastitis-resistant and susceptible. We identified 28 alleles in the total sample. In addition, 28 similar variants were found in the genotypes of resistant cows, but only 21 were among susceptible animals. There are nine "consolidating" alleles, of which *03, *15, and *24 have a frequency of more than 5% in all three samplings. The most common variant of the White-Headed breed was BoLA-DRB3.2*24 (12.3%). It also dominated among mastitis-susceptible cows (25.9%). The allele *22 (13.0%) was the most definite in the disease-resistant animals. The degree of cumulation in this group is the lowest because the five "consolidating" alleles in total occupied only 44% of all identified options in healthy animals. For six variants in the sample of susceptible cows, this figure is 70.4%, and in general, for the breed, seven alleles with a frequency of more than 5% had 59.6%. We also found five alleles "without established nomenclature", the total share of which was 6.5%.

Analysis of allelic polymorphism is to identify "informative" alleles, among which there are "consolidating" and "significance". Alleles that found with a frequency of at least 5%, we consider "consolidating". The threshold of 5% is conditional but usual for a criterion of informativeness (Maguire et al., 2002; Mohammadi et al., 2009). The allele frequency limit set at 0.95 is arbitrary. On the one hand, it contributes to defining those genes in which allele variability is quite common (Cavalli-Sforza & Bodmer, 1981). On the other hand, in multiple loci, the threshold of 5% is a significant sign of the pedigree of the allele.

With the directed selection, there is a consolidation (accumulation) of several gene variants and the elimination of rare alleles. The cumulation of 6-7 variants with a total frequency of more than 75% indicates that most animals have them in their genotypes, which makes it possible to consider a set of these alleles as a genetic feature of the breed. Thus, large-scale studies of the polymorphism of Holstein cows in the USA and Canada found six "consolidating" alleles BoLA-DRB3.2*08, *11, *16, *22, *23, and *24. Their total frequency was more than 75%. A similar set of "consolidating" alleles was found in "holsteinization" cattle. That options are predominant in Iranian (Nassiry et al., 2005), Argentine (Juliarena et al., 2008), Polish (Oprzadek et al., 2018), Colombian (Morales et al., 2020), and other "holsteinization" cattle breeds.

The information on the number and proportion of animals with "significance" alleles in the genotype is always important. It is essential in genetic studies because these alleles are widespread or, on the contrary, rare and have a reliable deviation from a normal distribution. Therefore, the associated properties (economically beneficial traits, resistance or susceptibility to the disease, etc.), as a rule, are associative. Such alleles must meet the condition $\chi^2 \geq 3.84$ for *CI*=95%. In this case, the null hypothesis that the obtained data conform to a normal distribution is rejected. A "significance" allele is a potential DNA marker because it indicates a statistically significant level of a genetically determined relationship with a particular phenotypic trait. Depending on the share among the total number, "significance" alleles are defined as "major" (widespread) or "minor" (rare). Other options are defined as "neutral".

In all experimental samples, we found nine "significance" variants of different levels of significance in the χ^2 test. All of them turned out to be "major" alleles. In each sampling, there are five options (Table 1). Only one BoLa-DRB3.2*15 allele had a statistically significant deviation from a normal distribution in three samples. It is not suitable for the role of the DNA marker, because there is an antagonism between opposite signs: the marker cannot be associated with alternative states of a phenomenon or system.

Forty-one genotypes (1.88 average frequency) were found in 77 cows (Table 2). In resistant and susceptible animals, 28 and

Table 2 – Map of genotypes of the BoLA-DRB3 gene of the White-Headed Ukrainian breed.

Genotype	P_G			Genotype	P_G			Genotype	P_G		
	Σ	r	c		Σ	r	C		Σ	r	c
*02/*22	0.013		0.037	*11/*19	0.013		0.037	*15/*gbb	0.039	0.04	0.037
*02/*23	0.013	0.02		*11/*24	0.078	0.02	0.185	*16/*22	0.026	0.04	
*03/*22	0.026	0.04		*11/*26	0.013		0.037	*16/*mdb	0.026	0.04	
*03/*24	0.013		0.037	*11/*32	0.026	0.04		*19/*iab	0.026	0.04	
*03/*26	0.026	0.04		*11/*54	0.013	0.02		*22/*22	0.026	0.04	
*03/*33	0.052	0.04	0.074	*12/*15	0.026	0.04		*22/*24	0.026	0.04	
*03/*39	0.026	0.04		*12/*22	0.026	0.02	0.037	*22/*54	0.026	0.04	
*03/*fbd	0.013	0.02		*13/*13	0.026	0.04		*23/*24	0.013		0.037
*06/*24	0.026		0.074	*13/*23	0.078	0.1		*23/*45	0.026	0.02	
*06/*45	0.013	0.02		*14/*24	0.026	0.02	0.037	*24/*32	0.013		0.037
*08/*10	0.026	0.02	0.037	*15/*16	0.013		0.037	*24/*33	0.026		0.074
*08/*15	0.026		0.074	*15/*24	0.013		0.037	*24/*fbd	0.013	0.02	
*08/*36	0.013		0.037	*15/*35	0.026	0.04		*45/*naa	0.013	0.02	
*11/*13	0.013		0.037	*15/*36	0.026	0.04					

P_G – frequency of genotypes; Σ – all herd; r – resistance; c – susceptibility.

Table 3 – Checking the normality of the frequency distribution of alleles and genotypes.

Samples		*dF*	$\Sigma\chi^2$	Criteria			
				Kolmogorov-Smirnov		Shapiro-Wilk	
				indicator	*p**	indicator	*p*
Alleles	all herd	28	74.6	0.272	<0.001	0.766	<0.001
	susceptibility	21	207	0.306	<0.001	0.628	<0.001
	resistance	28	78.1	0.295	<0.001	0.743	<0.001
Geno-types	all herd	41	109	0.36	<0.001	0.636	<0.001
	susceptibility	19	77.8	0.394	<0.001	0.454	<0.001
	resistance	28	64.0	0.376	<0.001	0.549	<0.001

dF – number of degrees of freedom; *Lilliefors significance correction.

21 variants are presented respectively. Common to the general sample, groups of resistant and susceptible cows were 6 genotypes BoLA-DRB3.2*11/*24, *03/*33, *12/*22, *14/*24, *08/*10 and *15/*gbb. Only two homozygotes were found in alleles *13 and *22 among resistant animals.

The most common in the breed were three variants *03/*33 (P_G =0.052), *11/*24 and *13/*23 (P_G =0.078). Genotype *13/*23 was most common among healthy cows (P_G =0.1). In the sample of animals susceptible to mastitis, there were four genotypes with a frequency of more than 5%: *11/*24 (P_G = 0.185) and three variants *03/*13, *06/*24, *08/*15 with the same frequency P_G = 0.074.

The uneven distribution of genotypes allows for detecting "significance" variants and searching for DNA markers based on them. Finding markers based on genotypes is carried out by a

Table 4 – Detection of alleles of the gene BoLA-DRB3.2 associated with resistance and susceptibility to mastitis in cows of the White-Headed Ukrainian breed.

Alleles	P_A	*RR*	χ^2	Checking for a limited sample ****			
				(a+b)(a+c) *N*	*(a+b)(b+d)* *N*	*(c+d)(a+c)* *N*	*(c+d)(b+d)* *N*
*02	0.013	1.88	0.2	0.7	1.3	26.3	48.7
*03	0.078	0.57	0.63	4.21	6.86	22.8	42.2
*06	0.019	3.92+	1.37	1.05	1.99	25.9	48.1
*08	0.032	8.52+	4.74*	1.75	3.44	25.3	46.8
*10	0.013	1.88	0.2	0.7	1.3	26.3	48.7
*11	0.078	4.84+	6.23*	4.21	8.42	22.8	42.2
*12	0.026	0.6	0.19	1.4	2.49	25.6	47.4
*13	0.071	-6.5-	3.8	3.86	5.86	23.1	42.9
*14	0.013	1.88	0.2	0.7	1.3	26.3	48.7
*15	0.084	1.19	0.08	4.56	7.94	22.4	41.6
*16	0.032	-2.26-	0.53	1.75	3.05	25.3	46.8
*19	0.019	0.92	0	1.05	1.91	25.9	48.1
*22	0.097	-4.39-	3.86*	5.26	7.6	21.7	40.3
*23	0.065	-5.71-	3.17	3.51	5.45	23.5	43.5
*24	0.123	9.69+	16.5***	6.66	14.6	20.3	37.7
*26	0.019	0.92	0	1.05	1.91	25.9	48.1
*32	0.019	0.92	0	1.05	1.91	25.9	48.1
*33	0.039	4.17+	2.85	2.1	4.05	24.9	46.1
*35	0.013	-2.84-	1.11	0.7	1.25	26.3	48.7
*36	0.019	0.92	0	1.05	1.91	25.9	48.1
*39	0.013	-2.84-	1.11	0.7	1.25	26.3	48.7
*45	0.026	-5.32-	2.28	1.4	2.39	25.6	47.4
*54	0.019	-4.05-	1.69	1.05	1.83	25.9	48.1
*mdb	0.013	-2.84-	1.11	0.7	1.25	26.3	48.7
*iab	0.013	-2.84-	1.11	0.7	1.25	26.3	48.7
*gbb	0.019	0.92	0	1.05	1.91	25.9	48.1
*fbd	0.013	-2.84-	1.11	0.7	1.25	26.3	48.7
*naa	0.006	0.6	0.55	0.35	0.64	26.7	49.4

P_A – frequency of alleles; *RR* – relative risk; + susceptibility to mastitis, - resistance to mastitis; *a* – susceptible animals with an allele; *b* – resistant animals with an allele; *c* – susceptible animals in which there is no allele; *d* – resistant animals in which there is no allele. *p<0.05; **p<0.01; ***p<0.001; ****expected frequencies must be greater than 5.

Table 5 – Checking BoLA-DRB3 alleles for the significance of the connection with mastitis sensitivity.

Alleles	P_A	*RR*	χ^2	Fisher's exact test	Pearson coefficient
*08	0.032	8.52	4.74	0.044	0.241
*11	0.078	4.84	6.23	0.014	0.271
*13	0.071	-6.5	3.8	0.042	0.217

P_A – frequency of alleles; *RR* – relative risk.

method similar to DNA markers based on BoLA-DRB3 alleles. As "significant" for the entire herd manifested four genotypes: *15/*gbb ($p<0.05$), *03/*33 ($p<0.01$), *11/*24 i *13/*23 ($p<0.001$). With a high level of reliability ($p<0.001$) among resistant animals, variant *11/*24 was found, and *13/*23 was found among susceptible animals.

The detected frequencies of alleles and genotypes were uneven for all sampling. The reliable difference in the distribution of the obtained values from the normal allows using non-parametric criteria in the analysis. This difference is achieved by comparing the sample under study with the normal distribution using the tests of Kolmogorov-Smirnov and Shapiro-Wilk (Table 3).

The calculated values of the criteria indicate a statistically reliable deviation from the normal distribution of frequencies of alleles and genotypes of the BoLA-DRB3 gene of White-Headed Ukrainian cows ($p<0.001$ for all variants).

The detection of mastitis-sensitivity alleles of the BoLA-DRB3.2 gene was carried out based on a relative risk indicator. The results of the calculations are given in Table 4.

In terms of *RR*, 16 alleles were worth considering for possible association with mastitis. Many options are due to the lack of "minor" alleles and a weighty number of "neutral" variants. Therefore, when calculating the *RR* using the Woolf-Haldane correction, an error occurs in rare alleles, which sometimes exceed the limits of ± 2.

The reliability of the detected association was controlled in two ways: by χ^2 and by the formulas of verification for the limitedness of the experimental sample. The first test showed that statistically significant associations with mastitis ($\chi^2 > 3.84$ for $CI = 0.95$) had four alleles BoLA-DRB3*08, *11, *22, and *24. Only the last two options withstand a test for limited sampling. It means that there are at least two alleles for the White-Headed Ukrainian breed, which can be used as DNA markers. The genetic relationship with mastitis resistance had allele BoLA-DRB3.2*22 (P_A=0.097; *RR*=-4.39; $p<0.05$), with susceptibility – *24 (P_A=0.123; *RR*=9.69; $p<0.001$). Two other variants of BoLA-DRB3.2*08 and *11 could not withstand the sample size check. Allele *13 had a lower than critical value of the chi-square test ($\chi^2 = 3.8$). With a minimal decrease in the accuracy of biological research (*CI*=0.949), it can be included in the list of associative variants.

Checking the possibility of using alleles BoLA-DRB3.2*08, *11, and *13 as DNA markers were performed according to Fisher's exact test, and the strength of the association – by Pearson coefficient (https://medstatistic.ru/calculators/calchi.html) (Table 5).

Candidates for DNA markers withstand Fisher criterion checks ($p<0.05$) and show the average strength of the association according to the Pearson coefficient, which requires additional research on the possibility of using them as DNA markers associated with mastitis.

Identifying genotypes associated with udder disease has some difficulty with limited statistical material. Most of the 41 genotypes among the studied animals were found once (Table 2). Of the six variants common to the general sample, groups of resistant and susceptible cows, only three genotypes were characterized by a reliable deviation from the normal distribution. Checking "significant" genotypes showed that only one variant of BoLA-DRB3.2*11/*24 (*RR*=-11.1; χ^2=6.66) can claim to be a mastitis resistance marker (Table 6).

Fisher's exact test (0.017) and Pearson's coefficient (0.282) confirm the possibility of using this genotype as a DNA marker associated with mastitis resistance.

Discussion

Numerous previous studies of the association of DRB3.2 loci with udder diseases have mixed results. Thus, the associations of mastitis-susceptibility to alleles 24 and *26 ($p<0.01$) and *07 ($p<0.01$) and *08 ($p<0.05$) in Ukrainian black-and-white and red-and-white cows, respectively, were found. Resistance to the disease had animals with alleles, respectively, *13 ($p<0.05$) and *22 ($p<0.05$) and *22 ($p<0.001$) and *24 ($p<0.05$) (Suprovych et al., 2018).

In the large study (blood and milk samples from 1100 Holstein cows collected from different U.S. farms), the allele DRB3*16 ($p\leq0.05$) was proven to be closely related to the increased risk of intramammary infection determined by high levels of somatic cells in milk. This allele was found in the genotypes of all 258 cows diagnosed with acute mastitis. The DRB3.2*08 allele ($p<0.04$) was associated with increased SCC in first lactating cows, and the DRB3.2*23 allele ($p<0.02$) was associated with high SCC in cows after the second lactation (Dietz et al., 1997).

The work of Japanese researchers for 714 heads of 26 herds of the Holstein breed established that alleles DRB3.2*08 and DRB3.2*16 are related to susceptibility, and DRB3.2*22, DRB3.2*23 and DRB3.2*24 – with mastitis resistance (Yoshida et al., 2012). Genotyping of the DRB3.2 locus of Norwegian red cows ($n = 523$) showed that BoLA-DRB3.2 variants *13, *18, *22, and *27 had a significantly higher frequency in the group of cows with low clinical mastitis, whereas the alleles *03, *09, *11 and *26 had a higher frequency in cows of the group with high protein content in milk. Alleles *22 and *26 are associated with increased susceptibility to

Table 6 – Detection of genotypes BoLA-DRB3.2 associated with resistance and susceptibility to mastitis in cows of the White-Headed Ukrainian breed.

Genotypes	*RR*	χ^2	*SE*	checking for the limited number of genotypes **			
				(a+b)(a+c) *N*	*(a+b)(b+d)* *N*	*(c+d)(a+c)* *N*	*(c+d)(b+d)* *N*
*03/*33	0.521	0.413	0.013	2.59	1.4	47.4	25.6
*11/*24	-11.1	6.66*	0.016	3.89	1.79	46.1	24.9
*15/*gbb	1.08	0.004	0.011	1.95	1.09	48.1	25.9

RR – relative risk; *SE* – standard error; *a* – susceptibility animals + genotype; *b* – resistant animals + genotype; *c* – susceptible animals without genotype; *d* – resistant animals without the genotype.
*$p<0.01$; **expected frequencies should be higher than 5.

clinical mastitis, while alleles *07, *11, *18, and *24 affect positively on resistance to udder inflammation (Kulberg et al., 2007).

In the Chinese Holstein breed, alleles DRB3.2*1201 (*08) and *2703 (*23) are associated with mastitis resistance, but alleles *1501 (*16), especially in a homozygous state, are relevant to a possible mastitis disease (Lin Jyun-Hong, 2011).

The review (Behl et al., 2012) analyzed studies in which researchers studied the associations between BoLA-DRB3.2 alleles and mastitis. It is shown that variants *03, *08, *11, *13, *1501 (*16), *18, *22, *23 and *0101 (*24) indicate resistance to the disease, and variants *1201 (*08) and *1101 (*22) – on the predisposition to mastitis. There is uncertainty for the allele *1401 (* 27) because it had the opposite immune status for breast disease in different studies.

The analysis of data sets of different authors and their research results showed that some alleles, declared as markers of susceptibility to mastitis, manifest themselves differently depending on the breed. The reason is that mastitis belongs to the factor diseases of different aetiologies. Dozens of pathogens are known to cause acute intramammary infections. Some researchers specify them and look for a link between alleles and specific pathogens (Sharif et al., 2000; Rupp et al., 2007; Pashmi et al., 2009; Yoshida et al., 2012; Oprzadek et al., 2018). Accurate assessment of the association in a pair of "allele–mastitis" requires considering many components that affect the result: the presence and number of pathogens, their species composition, features of livestock, etc. It is almost impossible to estimate the complexity of such complex research.

Gene polymorphism is maintained at a population level. If one individual can carry only two variants of the gene, the set of alleles in the population may be very diverse, and their combination within the genotypes varies. It promotes the recognition of a wide range of foreign antigens. Therefore, the information about the connection of genotypes with the disease is no less substantial. The association of the genotype with the disease is informative for the individual sensitivity to a specific pathology and is of practical importance for forming the dairy herd.

Studies that present the relation between BoLA-DRB3.2 genotypes and diseases are limited. In the study of Polish Holstein cattle (n = 525) establishing associations between the drb3 locus alleles and the content of somatic cells in milk, the authors indicate the informativeness of two variants BoLA-DRB3.2*16 and *23 in connection with mastitis and the manifestation of their consequences for the breeding value of cows and SCC. If these alleles are considered genetic markers for establishing phenotypic resistance (susceptibility) to mastitis, then the analysis of any herd is simplified. It is enough to study only three genotypes – *16/*A, *23/*A, and *16/*23 (*A – any allele type in the population) (Sender et al., 2008).

There is an assumption that allele variants of the BoLA-DQA1 gene are associated with mastitis resistance. Its alleles *0101 and *10012 are associated with susceptibility to streptococcal mastitis. Homozygotes *0101/*0101 are susceptible to streptococcal mastitis and *10011/*10011 – to mastitis caused by *E. coli* (Takeshima et al., 2007). Some authors suggest that with mastitis resistance, haplotypes rather than genotypes are associated (Kulberg et al., 2007).

Conclusions

In our study, 28 alleles of the BoLA-DRB3 gene confirm the high level of its polymorphism. The distribution of allele frequencies and genotypes in the White-Head Ukrainian breed is uneven. Two alleles have reliable associations by the magnitude of relative risk of mastitis and may be used as DNA markers. There is a genetic relationship between the BoLA-DRB3.2*22 allele and mastitis resistance, as well as the *24 allele and mastitis susceptibility. The genotype DRB3.2*11/*24 can claim to be a marker of resistance to mastitis.

The results of the study of the BoLA-DRB3 gene polymorphism of the White-Headed Ukrainian breed complement the knowledge base on the major histocompatibility complex of the cattle. Detected DNA markers of sensitivity to mastitis are substantial for breeding programmes implementation because they allow forming the herd resistant to udder diseases at the stage of early postnatal ontogenesis.

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