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FEATURES OF DIAGNOSIS OF NECROBACTERIOSIS OF COWS BY PCR-RFLP

Abstract

Molecular genetic markers can detect polymorphism at the DNA level. This feature determines the possibility of their widespread use in genetics and breeding. Alleles of the BoLA-DRB3 gene (exon 2) can act as such markers if a statically significant association between the disease and the allele is established. The presence of such DNA markers in the genotype of animals makes it possible to judge the likelihood of disease in postnatal ontogenesis immediately after the birth of a heifer, based on which we can conclude about the conditions of further use of the animal in the main herd.

*According to the results of studying the polymorphism of the BoLA-DRB3 gene in cows of the Ukrainian black and white dairy breed resistant and susceptible to necrobacteriosis, four "informative" alleles were revealed. Two of them *03 and *22 are associated with resistance, and the other two - *16 and *23 with susceptibility to necrobacteriosis.*

The presence of these alleles in the genotype of the animal is determined by testing performed by PCR-RFLP. The method is time consuming, labor intensive and costly. To simplify it, the following technique is proposed.

*Restriction fragments of alleles *03, *16, *22 and *23 for endocluases RsaI, XhoII and HaeIII have the*

*following DNA patterns: **bbb**, **jbd, mba** and **nba**. Due to the peculiarity of the restriction fragments, which is that endonuclease XhoII reveals in these alleles only one pattern **b** with length of 284 bp, the process of determining informative alleles can be simplified.*

*Isolation of DNA from blood samples and amplification of a fragment of the BoLA-DRB3.2 gene with a size of 284 bp is carried out according to the established technique. Next, the restriction of the fragment by endonuclease XhoII and sampling having a pattern **b**. Selected samples are treated with RsaI endonuclease and only those with patterns **b**, **j**, **m** and **n** remain. The next step is to restrict the selected samples with HaeIII endonuclease and select heifers with **bbb** (*03) and **nba** (*23) genotypes. After the first restriction, blood samples without pattern **b** are eliminated from the experimental sample; after the second – two alleles with patterns RsaI + XhoII **jb** (*16) and **mb** (*22) are unambiguously determined, after the third – genotypes **bbb** and **nba**, which correspond to alleles *03 and *23. In total, only 75% of blood samples are typed, which reduces the material consumption, time and cost of work to identify heifers genetically susceptible (resistant) to necrobacteriosis.*

Keywords: *BOLA-DRB3 gene, alleles, polymorphism, necrobacteriosis, cattle, electrophoregram, pattern*

Introduction. Necrobacteriosis of cattle is an infectious disease caused by the polymorphic gram-negative microorganism *Fusobacterium necrophorum*. The crucial role in the emergence and development of necrobacteriosis is played the reduction of the body's natural resistance under the influence of various adverse factors, as well as tissue injury, which provides the necessary conditions for penetration and reproduction of the pathogen. In primary center observed necrotic lesions and ulceration, which leads to the emergence of the sick animals of local functional disorders (lameness).

This is the second most common disease of cattle after mastitis. A large-scale study of nearly 22,5 thousand cows from 191 farms in 17 US states showed that a total of 9,6% of cows (95% SI: 7,3 to 11,9) have lameness. [1]. The cost of economic losses from non-specific lameness (death of animals, premature culling, reduced milk productivity, loss of reproductive function, treatment and prevention costs) is from 76 to 533 dollars [2]. Necrobacteriosis has become especially widespread in Ukraine due to intensive «holsteinization» of cattle [3].

To reduce the risk of necrobacteriosis in the dairy herd it is necessary to include cows genetically resistant to the disease. But in breeding, the assessment of cows for resistance to disease is stretched for several years and is carried out only at the stage of productive use of animals. A number of studies claim that there is a genetic predisposition of cows to necrobacteriosis, which encourages the search for genetic markers associated with this disease [4, 5].

Molecular genetic markers can detect polymorphism at the DNA level. This feature determines the possibility of their widespread use in genetics and breeding. The genes of the Main Histocompatibility Complex (MHC) of cattle provide an immune response to foreign antigens, which causes the presence of associations with resistance (susceptibility) to various diseases. Class II MHC genes in cattle are expressed with varying intensity. The BoLA exists as a system, but in the form of separate clusters; some locus are closely related, while others are relatively remote. There are at least three DRB locus in cattle, but only one DRB3 gene is functional. The study of polymorphism of the locus is important, since this region represents the antigen and the variability in this region can be related to the immune response to various pathogens. In addition, the analysis of polymorphism DRB3 is useful for studying the evolutionary history of MHC in the ruminant species.

Molecular markers, that reveal polymorphism at the level of DNA, are very important markers in the sphere of animal genetics. Currently, for cattle, various genetic markers are suggested. During the selection of a marker for genetic studies, one should take into account its informative and individual level of complexity of typing, for such experiments are conducted on large groups of animals [24, 25].

The BoLA-DRB3 gene is characterized by the presence of highly polymorphic regions

and has one of the highest levels of expression, which indicates its significant role in the development of the immune response. The peculiarity of the gene is due to the formation of the body's immune response to viral and bacterial infections, which is relevant for considering the problems of resistance to disease [6].

Analysis of recent research and publications. Researchers are most interested in studying the relationship between gene BoLA-DRB3 exon 2 alleles (BoLA-DRB3.2) and disease resistance (susceptibility) of cows. One of the main ways to study the polymorphism of this gene is the PCR-RFLP method (restriction fragment length polymorphism).

The alleles of the BoLA-DRB3.2 gene as DNA markers are used in many studies of cattle. They became the most widespread in connection with the search for associations «allele-disease». This method was used to study the distribution of BoLA-DRB3 gene alleles in more than 35 cattle populations and the possibility of their use as DNA markers for leukemia [7,8], mastitis [6, 9], foot and mouth disease [10], and tick-borne diseases [11], necrobacteriosis [12], theileriosis [13], tuberculosis [14], etc.

The BoLA-DRB3 gene polymorphism data are widely used in population genetics. On the basis of the revealed allele frequencies, there were determined the parameters of population-genetic structure of South African [15], Mongolian, Yakut and Kalmyk [16], Argentinian Creole [17], Chilean [18], Philippine native [19], Indian [20], Japanese [21], etc. Many researches is devoted to identifying the associations of the BoLA-DRB3 gene with economic-useful features [22, 23].

The PCR-RFLP method as a tool for studying cattle polymorphism is constantly being improved. Features of the primers used in the formulation of reactions, make it possible to improve the method. Primer HLO-30 flanks exon 2 of the DRB3 gene from the 5'-end and its sequence is complementary to the 5'-terminal region of exon 2 (seven nucleotides) and the adjacent intron region. Primers HLO-31 and HLO-32 flank exon 2 from the 3'-end, while HLO-31 includes part of the introns of the sequence and the 3'-end of exon 2 (eight nucleotides). The HLO-32 primer is completely localized in the 3'-terminal region of exon 2 and partially overlaps with the HLO-31 primer (eight nucleotides). Due to this, it is possible to replace two-stage PCR with one-stage. The result of both methods will be the reaction products represented by one fragment of size 284 bp (281 bp in the case of deletion for some alleles of the gene). The use of single-stage PCR allowed simplifying the method, reducing the analysis time, reducing the likelihood of contamination, as well as reducing the cost of the experiment [18].

Purpose. Development of a simplified method of diagnosis by PCR-RFLP (polymerase chain reaction of restriction fragment length polymorphism) by the BoLA-DRB3 gene of cows resistant or susceptible to necrobacteriosis.

Methodology. The PCR-RFLP method is characterized by high reproducibility of results, which led to its widespread use for the study of the allelic spectrum of the BoLA-DRB3.2 gene.

BoLA-DRB3 typing. The allele frequencies we detected based on the polymorphism analysis of the length of the restriction fragments (PCR-RFLP) of the products of amplification of the exon 2 of the BoLA-DRB3 gene [25, 26].

Isolation of DNA. DNA isolation from the blood was performed using «DIAtomTMDNA Prep200» kits (Isogen Laboratory Ltd.) in accordance with the manufacturer's instructions. DNA isolated from fresh biological material (yield was 5 - 10 mg from 200 ml of whole blood) has high molecular weight (40 - 50 bp) and pure substance (OD260/280 nm = 1,6 - 2,0). The concentration and purity of extracted DNA were assessed by spectrophotometery and electrophoresis in 1% agarose gel. With this purpose, 25, 50, and 100 ng of λ phage DNA and aliquots of solution with an unknown concentration were applied to agarose gel.

Electrophoresis was performed in 1x Trisborate (TBE) buffer (89 mM Tris-OH, 89 mM H_3BO_3, 2 mM EDTA) with EtBr (1 μg/ml) added to the gel to stain DNA at constant voltage of 120 V. The concentration of DNA of the test samples were determined by comparing the fluorescence intensity of the aliquots from solutions of unknown concentration and control λ phage DNA.

Amplification. The BoLA-DRB3 exon 2 was amplified by PCR using the single step PCR as modified from Van Eijk [26]. The PCR was carried out using ready-made sets of «GenPakR PCR Core», LLC «Izogen Laboratory» (Russia). The total volume of mixture was 20 μl. The mixture 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. Oligonucleotide primers for amplified of the exon 2 of BoLA-DRB3: HLO-30 (5'-3': TCCTCTCTCTGCAGCACATTTCC) and HLO-31 (5'-3': ATTCGCGCTCAC CTCGCCGCT) for the first round of the reaction were used. DNA (5 μl) was used as a template, regardless of its concentration.

The size of the amplification site was 284 bp (281 bp for alleles with deletion). Primers used for amplified second round: HLO-30 (5'-3': TCCTCTCTCTGCAGCACATTTCC) and HLO-32 (5'-3': TCGCCGCTGCACAGTGAAACTCTC). PCR products of the first round (2 μl) were used for the second round. First stage was started from DNA denaturation at 95°C for 5 min tracked 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 stage was started initial denaturation (95°C aimed at 5 min), was followed by 35 cycles of denaturation (68°C for 30 s), and annealing-extension (72°C for 30 s) and a final extension (72°C for 7 min). Contamination and selfpriming controls were included in each PCR round and 5 μL of the last PCR product were electrophoresed on 1,5% agarose gels in order to check the quality and specificity of DNA fragment amplification.

RFLP Pattern. PCR products were digested separately with three restriction endonucleases: *RsaI, HaeIII, XhoII* (*BstYI*) (Promega, USA, New England BioLabs; SibEnzim, Russia). The restriction fragments we separated by electrophoresis in a 6% or 9% agarose gel (Fig.1). The distribution of restriction sites of endonucleases RsaI, HaeIII and XhoII for exon 2 of the BoLA-DRB3 gene in different allelic variants of the BoLA-DRB3 gene is different, which leads to the formation after processing of gene amplification products of a specific spectrum of DNA fragments that differ in number and length of DNA patterns.

Amplification of exon 2 of the gene by means of a PCR followed by the analysis of restriction fragment length polymorphism and comparison of DNA patterns obtained using the three specified restriction endonucleases allows the identification of 54 alleles of the BoLA-DRB3 gene [26, 27, 28]. The alleles described by the PCR-RFLP method numbered in order from first to fifty-fourth. If alleles that are not included in the nomenclature of PCR-RFLP are detected because of the foregram processing, then the record of the allele without the established nomenclature is carried out in the form of three consecutive letters that correspond to certain restriction patterns *RsaI*, *HaeIII* and *XhoII*.

A total of 293 venous blood samples from three populations of Ukrainian black-and-white dairy cows were studied, and the results established associations between alleles of the BoLA-DRB3.2 gene and cow necrobacteriosis. The research was carried out from 2010 to 2017 in three breeding farms in Khmelnitsky region: LLC «Kozatska Dolyna 2006», agrofirm «Perlyna Podillya» and branch «Ridnyy Kray», engaged in breeding the Ukrainian black and white dairy breed. Blood samples investigated in the Genetics Laboratory of the Institute of Animal Breeding and Genetics of the NAAS of Ukraine and in the Laboratory of Animal Genetics of the Vavilov Institute of General Genetics (Moscow).

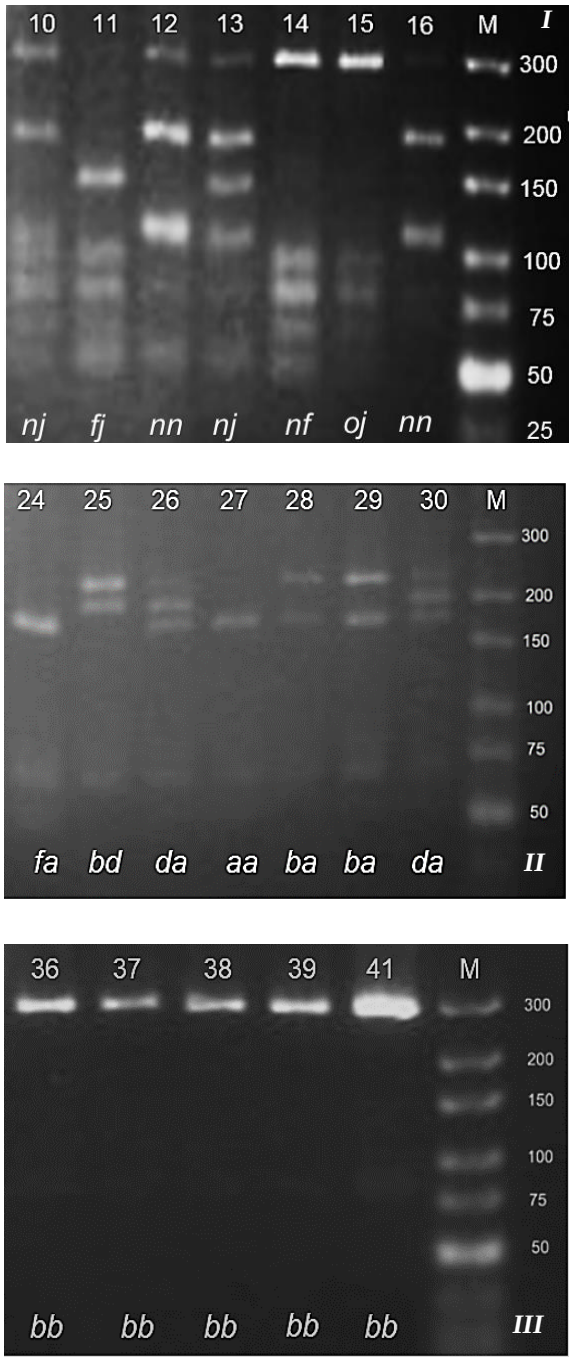

Fig. 1. Fragments of electrophoregrams of the products of the amplification of exon 2 of BoLADRB3 gene from DNA of cows using endonucleases *RsaI* (I), *HaeIII* (II) and *XhoII* (III):
M - marker GeneRuler ™ Ultra Low Range DNA Ladder marker, Fermentas, Canada; numbers of blood samples are indicated at the top; patterns of restriction are indicated at the bottom

Results and discussion. The PCR-RFLP method has a number of disadvantages, namely the complexity and material consumption, which affects the cost of the test for one sample. If you need to test a large herd, the work takes a considerable amount of time and is quite expensive.

Associations in the pair «allele – necrobacteriosis» established based on static analysis. An allele as a unit of genetic information is always a potential DNA marker. It can be transformed into a real (significant) one only when a close connection (association) is established between it and the disease. Alleles for which the value of the Pearson test exceeded the critical level $\chi^2 = 3,84$ (CI = 0,95; dF = 1) were considered significant. The analysis is based on the calculation of a statistically significant relative risk [29].

Previous studies have shown that four alleles can serve as DNA markers of necrobacteriosis: susceptible to the disease are cows that have in the genotype alleles *16 and *23, resistant – *03 and *22 [30].

As mentioned earlier, the method of single-stage PCR-RFLP, which is used to detect alleles of the BoLA-DRB3.2 gene, is performed in the following sequence [9, 25, 26, 29]:

1. Isolation of DNA from blood samples.
2. Amplification of a fragment of the gene BoLA-DRB3.2 size 284 or 281 bp.
3. Restriction of the fragment by endonucleases *RsaI*, *HaeIII*, *XhoII*.
4. Determination of patterns by the number and length of restriction sites.
5. Determination of the allele by a combination of DNA patterns of three restriction enzymes.

Due to some features of combinations of restriction sites of alleles *03, *16, *22 and *23, to identify cows prone to necrobacteriosis, a simplified technique is proposed, which will reduce the cost and speed up testing. It is because some restriction fragments of alleles *03, *16, *22 and *23 have the same sites. For example, these alleles have a common pattern ***b*** with a size of 284 bp, which is opened by endonuclease *XhoII* (table 1). On the electrophoregram, the presence of the site is due to the presence of a track the size of 284 bp (Fig.1c).

Table 1. Features of the distribution of patterns and restriction sites of alleles BoLA-DRB3.2 associated with necrobacteriosis

Alleles	Restriction patterns (DNA patterns)	Endonucleases and corresponding sizes of restriction sites (bp)		
		RsaI	*XhoII*	*HaeIII*
DRB3.2*03	*b**b**b*	111, 54, 50, 39, 30	284	219, 65
DRB3.2*22	*m**b**a*	111, 104, 69	284	167, 65, 52
DRB3.2*16	*j**b**d*	93, 78, 63, 50	284	190, 65, 29
DRB3.2*23	*n**b**a*	180, 104	284	167, 65, 52

To detect it in blood samples, it is necessary restricted all DNA fragments with XhoII endonuclease. After analysis of electrophoregrams from experimental sample, samples and corresponding animals in which there is no track size of 284 bp are excluded. According to studies, of the 54 alleles, only 32 have a pattern b on XhoII (table 2) [20].

Table 2. Distribution of alleles in which there is a pattern *b* for endonuclease *XhoII*

№	Alleles	DNA patterns *RsaI*+***XhoII***+ *HaeIII*	The proportion of alleles	№	Alleles	DNA patterns *RsaI*+***XhoII***+ *HaeIII*	The proportion of alleles
1	*02	*b**b**a*	0,018	17	*31	*i**b**f*	0,004
2	*03	*b**b**b*	0,053	18	*33	*n**b**f*	0
3	*10	*f**b**a*	0,061	19	*35	*c**b**b*	0
4	*13	*h**b**a*	0,035	20	*36	*l**b**a*	0,031
5	*14	*h**b**b*	0,009	21	*37	*o**b**a*	0,035
6	*15	*i**b**a*	0,018	22	*39	*t**b**a*	0
7	*16	*j**b**d*	0,053	23	*40	*u**b**a*	0
8	*17	*k**b**b*	0	24	*41	*a**b**a*	0,004
9	*18	*l**b**f*	0,018	25	*42	*h**b**f*	0,009
10	*19	*s**b**b*	0,009	26	*43	*k**b**f*	0
11	*20	*l**b**b*	0,009	27	*44	*k**b**i*	0
12	*21	*l**b**e*	0,013	28	*46	*v**b**a*	0
13	*22	*m**b**a*	0,079	29	*48	*w**b**a*	0,018
14	*23	*n**b**a*	0,044	30	*49	*w**b**e*	0
15	*24	*n**b**b*	0,180	31	*50	*x**b**a*	0
16	*28	*o**b**b*	0,075	32	*53	*y**b**a*	0

The next step is to restrict the selected samples with *RsaI* endonuclease to select those with *Rsa* patterns ***b***, ***j***, ***m*** and ***n***. After selection, there are only seven informative options (table 3). Of these, the variant ***jb*** unambiguously indicates the allele *16, and the variant ***mb*** - on *22.

Table 3. Alleles associated with necrobacteriosis having *Rsa*-patterns *b, j, m* and *n* and *Xho*-pattern *b*

Alleles		DNA patterns ***RsaI*+*XhoII***	The proportion of alleles
susceptibility to necrobacteriosis	DRB3.2*16	***jb***-	0,105
	DRB3.2*23	***nb***-	0,096
resistance to necrobacteriosis	DRB3.2*03	***bb***-	0,105
	DRB3.2*22	***mb***-	0,105
neutral	DRB3.2*02	***bb***-	0,035
	DRB3.2*24	***nb***-	0,246
	DRB3.2*33	***nb***-	0

The last step is that blood samples from heifers that have variants of ***nb*** and ***bb*** must be

treated with *HaeIII* restriction enzyme, which will finally detect genotypes with alleles *03 i *23.

Calculations performed according to [20] show that after the first test, about 23% of the sample is eliminated from the sample, after the second - 52% of heifers.

Example. It is necessary to test 100 heifers. Hundred blood samples are taken from which DNA is isolated and is performed amplification of a fragment of the BoLA-DRB3.2 gene. After restriction of the obtained fragments with endonuclease *XhoII* and processing of electrophoregrams in the sample will remain about 77% of samples having a pattern ***b***. The standard normal deviation (CI = 0,95) will be ± 1,6 samples. Next, 77 selected samples are treated with *RsaI* endonuclease. After analysis of electrophoregrams, samples with patterns ***b*** and ***n*** are selected. The approximate number of such samples will be 48 with a normal deviation of ± 1. At the final stage, the selected samples are treated with *HaeIII* endonuclease. After processing the electrophoregrams, heifers with genotypes are selected ***bbb*** (*03) i ***nba*** (*23). Calculations show that such genotypes will be about 20%.

In total, only 225 samples need to be typed, which is three quarters of the number that would need to be investigated in the case of 100 samples for three endonucleases. Thus, the material consumption, time and cost of detecting heifers susceptible (resistant) to necrobacteriosis is reduced by 25%.

Conclusions and suggestions. The spread of the PCR-RFLP method, as a tool for genetic research, is constrained by significant costs for its implementation. Our proposed method of simplifying the detection of heifers susceptible or resistant to necrobacteriosis can reduce the number of restrictions by a quarter. However, the method has a drawback. It is impossible to use it for other alleles, as the features of their patterns require an individual approach, which can both simplify and complicate the proposed procedure.

It is necessary to continue the search for BoLA-DRB3.2 alleles associated with other diseases of cows and because of the obtained results to develop new ways to simplify the typing of heifers. Such methods will be individual for each disease, but their search and implementation will reduce the time and cost of research.

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ОСОБЛИВОСТІ ДІАГНОСИТКИ НЕКРОБАКТЕРІОЗУ КОРІВ МЕТОДОМ ПЛР-ПДРФ

Анотація

Молекулярно-генетичні маркери дозволяють виявляти поліморфізм на рівні ДНК. Ця особливість визначає можливість їх широкого використання в генетиці та селекції. Алелі гена BoLA-DRB3(екзон 2) можуть виконувати роль таких маркерів, якщо встановлено статично значиму асоціацію між захворюванням і алелем. Наявність подібних ДНК-маркерів в генотипі тварин дає можливість судити про ймовірність прояву захворювання в постнатальному онтогенезі одразу ж після народження телички, на основі чого можна робити висновок про умови подальшого використання тварини в основному стаді.

*За результатами вивчення поліморфізму гена BoLA-DRB3 у корів української чорно-рябої молочної породи стійких і сприйнятливих до некробактеріозу, виявлено чотири «інформативних» алеля. Два з них *03 та *22 асоціюють з резистентністю, а два інших - *16 і *23 зі сприйнятливістю до некробактеріозу.*

Присутність в генотипі тварини означених алелів визначається тестуванням, яке здійснюється на ПЛР-ПДРФ. Метод займає багато часу, трудомісткий і вартісний. Для його спрощення запропоновано наступну методику.

*Рестрикційні фрагменти алелів *03, *16, *22 і *23 за ендонуклеазами RsaI, XhoII і HaeIII мають наступні ДНК-патерни: **bbb**, **jbd**, **mba** і **nba**. Зважаючи на особливість рестрикційних фрагментів, яка полягає в тому, що ендонуклеаза XhoII відкриває у цих алелів лише один патерн **в** довжиною 284 п.н., процес визначення інформативних алелів можна спростити.*

*Виділення ДНК зі зразків крові та ампліфікація фрагменту гена BoLA-DRB3.2 розміром 284 п.н. проводиться за усталено методикою. Далі виконується рестрикція фрагмента ендонуклеазою XhoII та відбір зразків, які мають патерн **b**. Відібрані зразки обробляються ендонуклеазою RsaI і залишаються лише ті, які мають патерни **b**, **j**, **m** та **n**. Наступний етап полягає в рестрикції відібраних зразків ендонуклеазою HaeIII та відбір теличок з генотипами **bbb** (*03) і **nba** (*23).*

*Після першої рестрикції з дослідної вибірки відсіюються зразки крові, в яких відсутній патерн **b;***

***n**ісля другої – однозначно визначаються два алелі з патернами RsaI+XhoII **jb** (*16) і **mb** (*22); після третьої – генотипи **bbb** і **nba**, які відповідають алелям *03 і *23.*

Сумарно типується лише 75% зразків крові, що зменшує матеріаломісткість, витрати часу і вартість робіт для виявлення теличок генетично сприйнятливих (резистентних) до некробактеріозу.

***Ключові слова:** ген BOLA-DRB3, алелі, поліморфізм, некробактеріоз, ВРХ, електрофореграма, патерн.*

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