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OPTIMIZATION OF CONDITIONS FOR THE MULTIPLEX PCR FOR DIAGNOSTICS OF HORSE STRANGLES WITH SUBSPECIES DIFFERENTIATION OF *STREPTOCOCCUS EQUI SUBSP EQUI*

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ABSTRACT

As a result of the work performed, the conditions for setting up multiplex PCR with electrophoretic detection for the diagnosis of horse strangles were determined, allowing the identification and differentiation of *S. equi* subsp. *equi* in one reaction. It was found that the developed PCR protocol for the detection and species differentiation of *S. equi* subsp. *equi* with electrophoretic detection in a "multiplex" format has a high specificity and does not lead to amplification of PCR products with DNA of closely related microorganisms, saprophytic microflora, and bacterial pathogens. The sensitivity of the protocols for the detection and species differentiation of *S. equi* subsp. *equi* with electrophoretic detection was assessed. Diluted DNA samples of two S. *equi* subspecies were used as objects of research: *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. DNA samples were diluted by two-fold dilutions, starting from a concentration of 5 ng (which corresponds to 2 million 170 thousand copies in the genomic equivalent) to 1.19 * 10^{-6} ng (which corresponds to 0.71 copies in the genomic equivalent). DNA detection limit for *S. equi* subsp. *equi* was 66 copies in genomic equivalent or 152 fg, DNA of *S. equi* subsp. *zooepidemicus* – 132 copies in genomic equivalent or 305 fg.

Key words: horse strangles, multiplex PCR, sensitivity, specificity, optimization.

INTRODUCTION

Horse strangles (*Adenitis equorum, Coryza contagiosa equorum*) is a bacterial infection of the upper respiratory tract caused by *Streptococcus equi* subspecies *equi*. The classical course of strangles is characterized by fever with pharyngitis and subsequent formation of abscesses in the submandibular and retropharyngeal lymph nodes. The disease affects horses of all ages, but the severity of the disease varies greatly depending on the animal's immune status. Young animals show more pronounced clinical signs with the formation and rupture of abscesses in the lymph nodes, while the course in adult horses is less aggressive and recovery occurs in the early stages of infection. Also, up to 20% of horses can remain asymptomatic carriers, becoming a constant source of infection and maintaining infection in the herd [1,2,3].

The etiological agent of horse strangles is *S. equi* subsp. *equi*. It is believed that *S. equi* subsp. *equi* evolved from *Streptococcus equi* subsp. *zooepidemicus* [4,5,6] which is associated with a wide range of diseases in animals, including humans [7,8,9]. However, despite this, in horses *S. equi* subsp. *zooepidemicus* is usually seen as a commensal of the upper respiratory tract. Commensalism of *S. equi* subsp.

zooepidemicus and genetic identity between subspecies at the level of 97% makes it difficult to diagnose strangles and identify asymptomatic carriers [10, 11].

There is little information in open sources about the level of identification of horse strangles in Kazakhstan. The detailed information given in the dissertation work of AR Sansyzbayev indicates a difficult epizootic situation in the horse strangles in the period 1980-1990. The share of strangles in infectious pathologies of horses exceeded 39%, while the mortality from strangles accounted for 30.6% of mortality in horse breeding [12].

The high proportion of strangles in the infectious pathologies of horses and asymptomatic carriage bring diagnostics to the fore in measures aimed at eliminating and preventing the introduction of infection into farms. The classic diagnosis of strangles is the isolation of a culture of *S. equi* subsp. *equi* from samples of biological material (nasopharyngeal swabs, contents of abscesses). The bacteriological method is highly dependent on the used biological material. So the isolation of pure cultures from abscesses does not cause difficulties, while the isolation of *S. equi* subsp. *equi* from nasopharyngeal swabs is hampered by the presence of saprophytic microflora and leads to success in the case of the dominance of the pathogen over saprophytes [13, 14]. Also, bacteriological diagnostics may be ineffective during the incubation period, in the early stages of the disease, and during the recovery period [15]. The development of molecular genetic diagnostic methods leads to the displacement of classical bacteriology in the diagnosis of several pathogens. Over the past decade, PCR has been widely implemented in clinical practice and is recognized as the gold standard for diagnostics of horse strangles, surpassing the classical method in sensitivity [16, 17]. Thus, the frequency of detection of *S. equi* in nasopharyngeal strangles by PCR is 84% compared to 37% in the bacteriological examination. In this regard, the development of highly sensitive tests is an urgent task.

The aim of this work was to develop a PCR protocol for setting up multiplex PCR for the diagnosis of horse strangles with subspecies differentiation of *Streptococcus equi* subsp *equi.*

Materials and methods

We used inactivated cultures of streptococci *S.equi subsp. equi* and *S. equi subsp. zooepidemicus.*

DNA concentration was measured using a Qubit® dsDNA HS Assay Kit (Invitrogen) on a Qubit 2.0 fluorometer (Invitrogen). The method is based on measuring the fluorescence of an intercalating dye that binds to a double-stranded DNA molecule. The method is characterized by a higher degree of accuracy in comparison with spectrophotometric measurement methods. Measurement using reagent kits was performed according to the manufacturer's instructions.

The selection and verification of specific primers were carried out using the PrimerSelect (DNASTAR), BioEdit, FastPCR programs, and the PrimerBlast web resource (NCBI) (https://www.ncbi.nlm.nih.gov/tools/primer-blast/), the sequences were aligned using BioEditSequenceAlignmentEditor software 1997-2013. The choice of target DNA and the structure of primers are given great attention in the development of a PCR protocol. When selecting primers, the main parameters were taken into account: close annealing temperatures of forward and reverse primers, primer length from 18-25 bp, low probability of secondary structures formation [18]. Also, when selecting primers, the high genetic identity of two *S. equi* subspecies was taken into account: *S. equi* subsp. *equi* and *Streptococcus equi* subsp. *zooepidemicus* [19].

The evaluation of the specificity of the developed protocol was carried out on collection of samples of bacterial DNA, including, *Streptococcus equi subsp. equi*

№36328, Streptococcus equi subsp. equi №38060, Streptococcus equi subsp. equi 37989, , Streptococcus equi subsp. zooepidemicus №36298, Streptococcus equi subsp. zooepidemicus №37899, Streptococcus oralis, Brucella spp (6 isolates), *Bacillus spp* (15 isolates), *Campylobacter spp* (13 isolates), *Lactobacillus spp* (8 isolates), *Pseudomonas spp* (12 isolates), *Acinetobacter johnsonii, Aeromonas bivalvium, Agrobacterium tumefacie, Alcaligenes aquatilis, Clostridium haemolyticum, Delfitia tsuruhatensis, Dermacoccus nishinomiyaensis, Devosia neptunia, Escherichia coli, Ensifer adhaerens, Enterococcus durans, Erwinia amylovora, Exiguobacterium aurantiacum, Halomonas nitritophilus, Klebsiella michiganensis, Klebsiella pneumonia, Listeria innocua, Lysinibacillus xylanilyticus, Mannheimia haemolytica, Microbacterium hydrocarbonoxydans, Ochrobactrum tritici, Pediococcus acidilactici, Pediococcus pentosaceus, Rahnella aquaticus, Rhizobium agrobacterium, Rhizobium pusense, Rhodococcus opacus, Rummeliibacillus pycnus, Salmonella enteritidis, Salmonella enterica, Serratia plymuthica, Serratia proteamaculans, Sinorhizobium meliloti, Sphingomonas aerolata*. The species of bacteria included in the collection was determined by sequence analysis of the *16S rRNA* gene.

A sensitivity assessment was performed on DNA samples of two types of streptococci, *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus*. The DNA concentration was adjusted to 5 ng. For each DNA sample, a series of double dilutions were performed from 5 to $1.19*10^{-6}$ ng in µl (Table 1). 5 µl of DNA was used in the reaction mixture, which in terms of the number of copies corresponded from 2 million 170 thousand to 0.71 copies in the reaction. The copy rate was calculated using an Internet resource (http://cels.uri.edu/gsc/cndna.html) [20].

Table 1. Range of DNA Concentrations for Sensitivity Testing

Hole	The amount of DNA in the reaction, ng	Number of genomic copies in reaction, copy in genomic equivalent	Hole	The amount of DNA in the reaction, ng	Number of genomic copies in reaction, copy in genomic equivalent
A1	5	2170000	E2	0,001220703	529,7851563
B1	2,5	1085000	F2	0,000610352	264,8925781
C1	1,25	542500	G2	0,000305176	132,4462891
D1	0,625	271250	H2	0,000152588	66,22314453
E1	0,3125	135625	A3	7,62939E-05	33,11157227
F1	0,15625	67812,5	B3	3,8147E-05	16,55578613
G1	0,078125	33906,25	C3	1,90735E-05	8,277893066
H1	0,0390625	16953,125	D3	9,53674E-06	4,138946533
A2	0,01953125	8476,5625	E3	4,76837E-06	2,069473267
B2	0,009765625	4238,28125	F3	2,38419E-06	1,034736633
C2	0,004882813	2119,140625	G3	1,19209E-06	0,710487366
D2	0,002441406	1059,570313	H3	Negative control	Negative control

The analysis of amplified target DNA fragments was performed by separation in agarose gel (agarose concentration from 1.5 to 2.5 %, depending on the length of the analyzed fragment), in the presence of an intercalating agent – ethidium bromide, which was used for further DNA visualization. Electrophoresis was performed in a PowerPac horizontal electrophoresis chamber and a BioRadElectrophoreticbath current source. 1x TAE buffer was used as the electrode buffer. The results were documented using the GelDoc documentation system (Bio-Rad) with the QuantityOne software (Bio-Rad).

The molecular sizes of the analyzed DNA samples were determined by comparing their electrophoretic mobility in the gel with the mobility of markers - a DNA fragment of known molecular weight. “DNALadder 1kb” (Fermentas) was used as a molecular weight marker.

RESULTS AND DISCUSSION

Closely related streptococcal species *S. equi* subsp. *equi* and *S. equi* subsp. *zooepidemicus* were identified by polymerase chain reaction using oligonucleotide primers designed according to the species-specific genes encoding superoxide dismutase A (sodA), integrated region ICESe2, *CRISPR*-associated genes.

As a species marker of *S. equi*, the nucleotide sequence of a species-specific segment of the inner part of the gene, encoding superoxide dismutase, sodA, is used. Superoxide dismutase A (sodA) is a widespread gene region in *Streptococcus* spp, therefore this region was included in analyzes to obtain a positive/negative result at the genus level. Species-specific sequence variation of this gene has already been used to identify various gram-positive bacteria, including bacteria of the genus *Streptococcus*. The sodA gene sequences have been previously published. However, due to the close relationship of the sodA gene sequences of both *S. equi* subspecies, the designed oligonucleotide primers did not allow differentiation of *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi*, since both belong to the pyogenic group of the genus *Streptococcus*. The nucleotide sequence of this gene allows the selection of primers to the conserved regions between *S. equi* subsp. equi and *S. equi* subsp. *zooepidemicus*, which at the same time are variable in other streptococcal species. As a target for subspecies differentiation, *S.equi* subsp. *equi*, in most cases, SeM, SeeI, SeeH, SeeL are used, which encode super antigenic toxins L and M [21]. An alternative marker for the selection of primers for subspecies differentiation of *S. equi* subsp. *equi* is the integrated region ICESe2. For subspecies differentiation, *S. equi* subsp. *zooepidemicus*, it is advisable to use CRISPR-associated genes (SZO14370-SZO14430) deleted from all known strains of *S. equi* subsp. *equi* [22]. In this regard, the following genetic markers were used for the selection of primers: sodA (gene encoding superoxide dismutase) - to differentiate *S. equi* from other streptococci species [23]; ICESe2 region - for differentiation of *S. equi* subsp. *equi* [24]; CRISPR-associated genes - for differentiation of *S. equi* subsp. *zooepidemicus* [25].
The main characteristics of the primers are shown in Table 2.

Table 2. Characteristics of selected primers

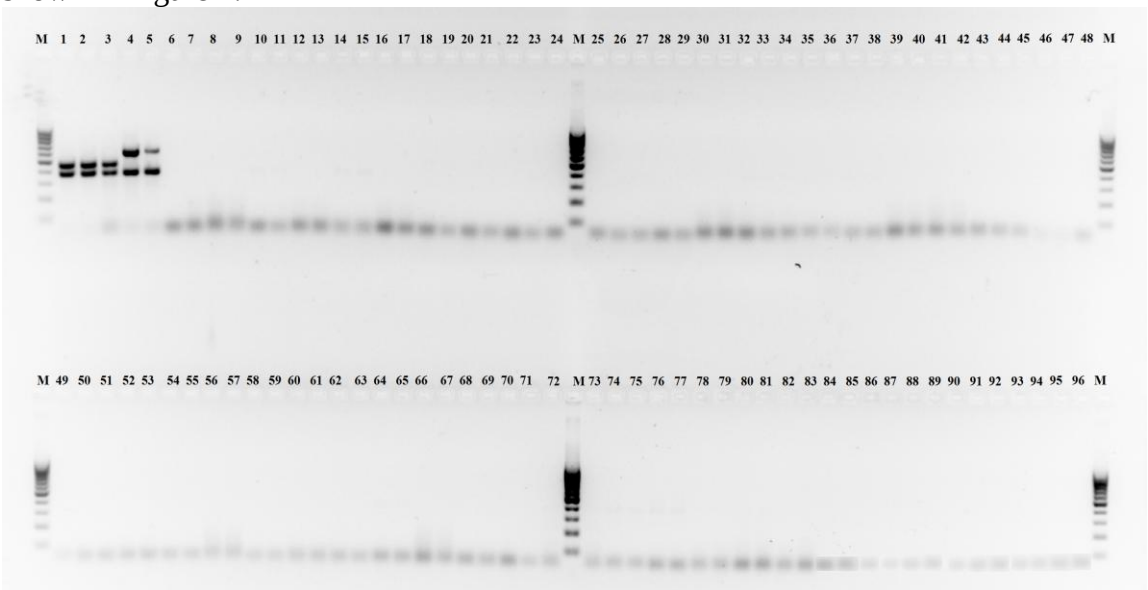
Name of primers	Target gene	Specificity	Sequence	Annealing temperature, °C	PCR product size, bp
S.equi_381_F	*sodA*	*S.equi*	tgacggatattcaggagcaact	57.65	381
S.equi_381_R	*sodA*	*S.equi*	atctgcgattgccttgaggaa	58.93	
S.equi. subsp *equi_472 _F*	ICESe2	*S.equi subsp equi*	taagcaccatgccacctatg	61.73	472
S.equi. subsp *equi _472 _R*	ICESe2	*S.equi subsp equi*	ttgccctctgagattggtgt	59.29	
S.equi. subsp *zooepidemicus_ 619_F*	CRISPR	*S.equi subsp zooepidemicus*	ctatatttttgccgttgcttgat	60.61	619
S.equi. subsp	CRISPR	*S.equi subsp*	gcaaccaaacatcg	58.21	

zooepidemicus _619_R		*zooepidemicus*	gagaaac		

The conditions for setting up PCR with electrophoretic detection for detecting and differentiating *Streptococcus equi* subsp. *equi* were determined. A "multiplex" Protocol has been developed that allows identification and differentiation of *S. equi* subsp. *equi* in a single reaction.

Work was carried out to optimize the conditions for setting up multiplex PCR with electrophoretic detection to detect *Streptococcus equi* using the following primers *S.equi_381*_F/*S.equi_381*_R, *S.equi.* subsp *equi_472_F*/*S.equi*.subsp *equi _472 _R*, *S.equi*.subsp *zooepidemicus _619*_F/*S.equi*.subsp *zooepidemicus _619*_R. The composition of the reaction mixture was determined: primers *S.equi*_381_F, *S.equi*_381_R 30 pmol per reaction, primers *S.equi.* subsp *equi*_472_F and *S.equi.* subsp *equi*_472_F 45 pmol; primer .*S.equi*.subsp *zooepidemicus _619*_F - 90 pmol, primer *S.equi*.subsp *zooepidemicus _619*_F - 60 pmol; 75 mMTris-HCl (pH 8.8 при 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% (volume/volume) Twin 20; dNTP at a concentration of 200 μM each; 2 units of Taq polymerase, magnesium ions 2.5 mM. PCR amplification program: long-term denaturation and activation of the enzyme at 95°C for 3 minutes; 42 cycles 95°C - 30 seconds, 60°-30 seconds, 72°C - 1 minute; final elongation for 5 minutes at 72°C.

The specificity assessment was performed on the collection of DNA samples. The DNA of non-target microorganisms was used at a concentration of 1 ng/μl. DNA of *S. equi* subsp *equi* and *S. equi* subsp *zooepidemicus* with concentrations of 0.1 ng/μL was used as a positive control; DNA of *Streptococcus oralis* with a concentration of 1 ng/μL was used as a genetically related species. Electrophoretic accounting of PCR results is shown in Figure 1.



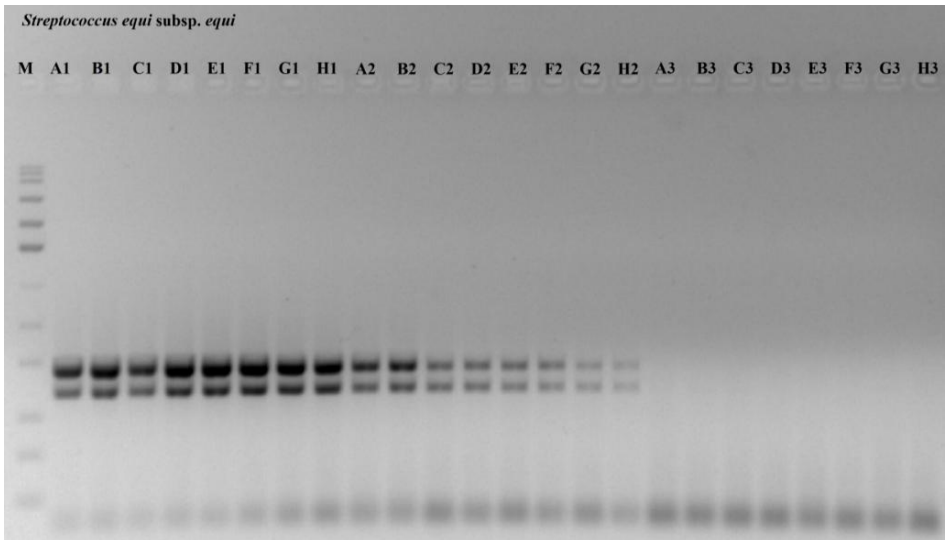
1-96 - DNA samples according to the list; M - molecular weight marker (100-1000 bp, step 100 bp)

Fig. 1. Electropherogram assessment of the specificity of the developed conditions for setting PCR on the collection of DNA samples

As a result, it was found that the developed PCR protocol for the detection and species differentiation of *S.equi* subsp. *equi* with electrophoretic detection in a "multiplex" format has high specificity and does not lead to amplification of PCR products with DNA from closely related microorganisms, saprophytic microflora, and bacterial pathogens.

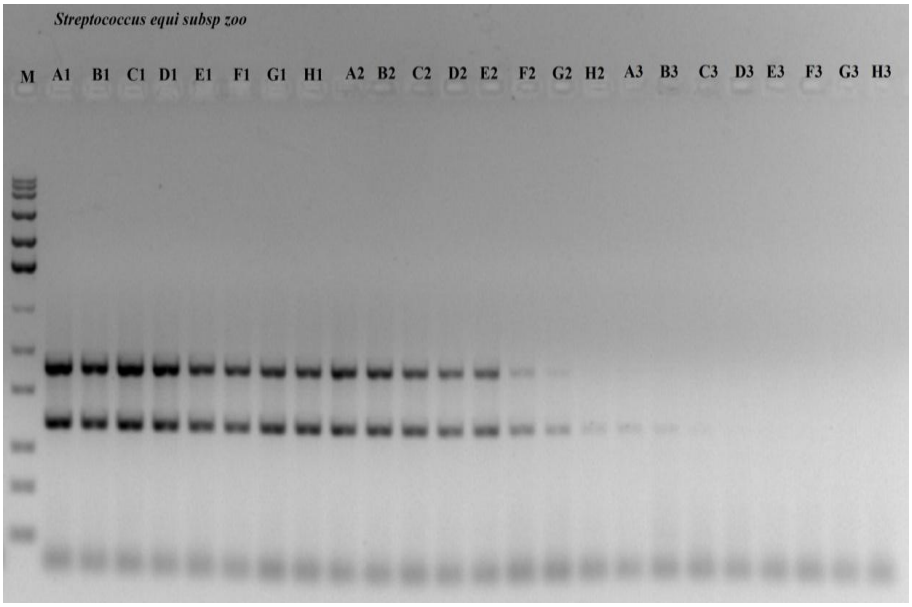
At the next stage, the sensitivity of *S. equi* subsp. *equi* detection and species differentiation protocols with electrophoretic detection was evaluated.

The results of evaluating the sensitivity of the PCR Protocol with electrophoretic detection are shown in figures 2 and 3.



A1-G3 dilution of *S. equi* subsp. *equi* DNA according to table 3; H3 - negative control, #SM1293, marker of molecular weight from 100 to 10000 bp.

Fig. 2. The sensitivity of the PCR protocol with DNA of S.equisubsp.equi



A1-G3 dilution of *S.equi subsp.zooepidemicus* DNA according to table 12; H3 - negative control, #SM1293, marker of molecular weight from 100 to 10000 bp.

Fig. 3. The sensitivity of the PCR protocol with DNA of *S. equi subsp. zooepidemicus*

As a result of the analysis, the limiting sensitivity of the PCR protocol with electrophoretic detection for *S. equi* subsp. *equi* DNA was 66 copies in genomic equivalent, for *S. equi* subsp. *zooepidemicus* DNA was 132 copies in genomic equivalent.

Thus, the Protocol of differentiation of *S. equi* subsp. *equi* by PCR method developed based on optimized PCR conditions is better because the methods are specific to only one type of microorganism (*S. equi* subsp. *equi*) and have a greater sensitivity. The introduction of this method in practice will reduce the time of diagnosis

to several hours, reduce the cost of diagnosis, will identify sick animals, which will have a positive impact on the epizootic situation.

CONCLUSIONS

The developed variants of PCR protocols are promising for the production of a fast, specific and sensitive test system for the detection and differentiation of *S. equi* subsp. *equi*. An optimized protocol allows purification of PCR products and is not inferior to commercial analogs in results.

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STREPTOCOCCUS EQUISUBSP EQUI ТҮРАСТЫН САРАЛАУМЕН ЖЫЛҚЫ САҚАУЫН ДИАГНОСТИКАЛАУҒА АРНАЛҒАН МУЛЬТИПЛЕКСТІ ПТР ЖҮРГІЗУДІҢ ШАРТТАРЫН ОҢТАЙЛАНДЫРУ

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ТҮЙІН

Жүргізілген жұмыс нәтижесінде *S. equi* subsp *equi-ді* бір реакцияда сәйкестендіруге және саралауға мүмкіндік беретін жылқы сақауын электрофоретикалық детекциямен диагностикалау үшін мультиплекстік ПТР орнату шарттары анықталды. *S. equi* subsp *equi-ді* сәйкестендіруге және саралауға арналған мультиплекстік форматта әзірленген ПТР хаттамасы жоғары ерекшелікке ие және жақын туыстас микроорганизмдердің, сапрофиттік микрофлораның және бактериялық қоздырғыштардың ДНҚ-мен ПТР өнімдерінің амплификациялануына әкелмейді. *S. equi* subsp *equi-ді* сәйкестендіруге және саралауға арналған хаттамалардың электрофоретикалық детекциясымен сезімталдығына баға берілді Зерттеу нысандары ретінде *S. equi-дің* екі түрастының: *S. equi*: *S. equi* subsp. *equi* және *S. equi* subsp. *zooepidemicus* сұйылтылған ДНҚ үлгілері пайдаланылды. ДНҚ сынамалары 5 нг концентрациясынан бастап (геномдық эквивалентте 2 миллион 170 мың данаға сәйкес келеді) 1,19 * 10-6 нг-ға дейін (бұл геномдық эквивалентте 0,71 данаға сәйкес келеді) екі еселік сұйылту әдісі арқылы сұйытылды. S. *equi* subsp. *equi* үшін ДНҚ анықтау шегі геномдық эквивалентте 66 көшірме немесе 152 фг, *S. equi* subsp. *zooepidemicus* үшін - геномдық эквиваленттегі 132 көшірме немесе 305 фг.

Негізгі сөздер: жылқы сақауы, мультиплексті ПТР, сезімталдылық, ерекшелік, оңтайландыру.

ОПТИМИЗАЦИЯ УСЛОВИЙ ПОСТАНОВКИ МУЛЬТИПЛЕКСНОЙ ПЦР ДЛЯ ДИАГНОСТИКИ МЫТА ЛОШАДЕЙ С ПОДВИДОВОЙ ДИФФЕРЕНЦИАЦИЕЙ *STREPTOCOCCUS EQUISUBSP EQUI*

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АБСТРАКТ

В результате проведенных работ определены условия постановки мультиплексной ПЦР с электрофоретической детекцией для диагностики мыта лошадей, позволяющие проводить выявление и дифференциацию *S. equi* subsp. *equi* в одной реакции. Установлено, что разработанный ПЦР протокол выявления и видовой дифференциации *S. equi* subsp. *equi* с электрофоретической детекцией в «мультиплексном» формате обладает

высокой специфичностью и не приводит к амплификации ПЦР продуктов с ДНК близкородственных микроорганизмов, сапрофитной микрофлоры, а также бактериальных патогенов. Была проведена оценка чувствительности протоколов выявления и видовой дифференциации *S. equi* subsp. *equi* с электрофоретической детекцией. В качестве объектов исследования использовали разведенные образцы ДНК двух подвидов *S. equi*: *S. equi* subsp. *equi* и *S. equi* subsp. *zooepidemicus.* Образцы ДНК разводили методом двукратных разведений, начиная с концентрации 5 нг (что соответствует 2 млн. 170 тыс. копий в геномном эквиваленте) до $1{,}19*10^{-6}$ нг (что соответствует 0,71 копиям в геномном эквиваленте). Предел обнаружения ДНК S. *equi* subsp. *equi* составил 66 копий в геномном эквиваленте или 152 фг, ДНК *S. equi* subsp. *zooepidemicus* - 132 копий в геномной эквиваленте или 305 фг.

Ключевые слова: лошадиный мыт, мультиплексный ПЦР, чувствительность, специфичность, оптимизация.