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DEVELOPMENT OF A REAL-TIME POLYMERASE CHAIN REACTION PROTOCOL FOR DIAGNOSIS OF PASTEURELLOSIS

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ABSTRACT

Pasteurellosis is a zoonotic disease of vertebrate animals, for which the etiological agent is *Pasteurella multocida*. The continuous high prevalence of *P. multocida* and regular periodic outbreaks of pasteurellosis in both wild and domestic animals, especially in the wild saiga populations in Kazakhstan, underscores the importance of developing a local molecular diagnostic system. Accordingly, the goal of the present study was to develop a real-time polymerase chain reaction (PCR) protocol to identify all serovars of *P. multocida*. The envC, ruvB, and QseC genes were chosen as targets, and real-time PCR protocols were developed using three pairs of primers and corresponding fluorescent probes characterized by high efficiency, sensitivity, and specificity. The sensitivity of the developed protocols to the sequences of the envC and QseC genes was 2.38 fg, and that to the ruvB gene was 76 pg. The high specificity of the PCR protocol was confirmed with a collection of samples from 92 species of bacteria and three species of higher eukaryotic organisms. The commercialization of these developed protocols will allow for the production of highly effective PCR test systems, and improve the diagnosis and treatment of pasteurellosis.

Key words: real-time PCR, *Pasteurella multocida*, PCR diagnostics, PCR optimization, PCR specificity, PCR sensitivity

INTRODUCTION

Pasteurellosis is an infectious disease of animals and birds, mainly characterized by poultry cholera, hemorrhagic septicemia in sheep, goats and cattle, atrophic rhinitis in pigs and rabbits, pneumonia and pleuritis in ungulates. The main causative agent of pasteurellosis is *Pasteurella multocida* [1]. *P. multocida* isolates are classified into 5 capsular serogroups (A, B, D, E and F) [2, 3, 4] and 16 LPS serotypes (L1-L16) [5, 6]. It is also worth noting, that complex serovars exist, such as B:L2,5 and B:L3,4 [7].

Hemorrhagic septicemia is caused by isolates from serogroups B and E, and rarely A. Susceptible animals are: cattle, buffaloes, pigs, small cattle, deer, camels, rarely horses. Avian cholera is caused by isolates from serogroups A, F, very rarely D. Susceptible birds are: hens, turkeys, ducks, wild birds. Atrophic rhinitis is caused by isolates from serogroup D, rarely A, and the disease affects pigs and rabbits. Sniffing in rabbits is caused by the pathogen from serogroups A and D. Enzootic pneumonia and convulsive fever are caused by isolates from serogroups A and D, rarely F. Pneumonia occurs in cattle, small cattle and pigs [8].

As is evident, the serogroups do not show monospecificity to one host species, the observation that was confirmed by a new genotyping scheme [9]. Recent studies indicate only presence of genes associated with certain nosologies, for example, atrophic rhinitis is caused by the action of a toxin (product of the *ToxA* gene), and there is a complex of genes common to isolates that cause hemorrhagic septicemia [10].

The greatest danger is pasteurellosis manifests in a form of hemorrhagic septicemia (HS). Hemorrhagic septicemia is a systemic disease which can affect all ungulate animals, and this disease was designated as the most economically important disease in

livestock according to OIE (2009) and FAO (2005). Hemorrhagic septicemia is mainly caused by serotypes B:L2 and E:L2 in Asia and Africa, respectively. As was confirmed [11], isolates circulating in the territory of Kazakhstan belong to serotype B:L2.

Transmission occurs through direct contacts, when carriers or diseased animals secrete large numbers of virulent bacteria directly into the environment through nasal and oral secretions. The pathogen enters a susceptible animal≥s body through the tonsils. Later, a chronic infection occurs in the nasal cavity, paranasal sinuses, middle ears, lacrimal and thoracic ducts of the lymphatic system and lungs. Bacteria multiply in peritonsillar soft tissues, and migrate to local lymph nodes, where the bacteria multiply and spread to surrounding tissues with a blood flow.

Complex infections with other respiratory pathogens, in particular *Bordetella bronchiseptica* or *Mannheimia haemolytica*, significantly enhance colonization rates of *P. multocida*, leading to a more serious disease. Primary infections with respiratory viruses or *Mycoplasma* species also predispose animals to a secondary infection with *P. multocida* or *M. haemolytica*. Environmental conditions, stress and general health conditions of animals also play important roles in determining of disease severity and likelihood of transmission.

After entry into the body, *P. multocida* extremely rapidly proliferates in blood, thus causing a pronounced local edema and necrosis, septicemia with lesions of different organs that without treatment can lead to death within few hours. The septicemia may be asymptomatic until beginning of an acute phase. Otherwise, death may occur within three days [12]. Mortality without urgent antibiotic treatment is close to 100%. Documented mortality during an outbreak of pasteurellosis reaches from 15% [13] to 90% of an animal population. A striking example is the case of 2015, in which about 210 000 animals in the flBetpak Dala saigaffi population died, which amounted to 88% of the total counts of this population, and 62% of the world population of this species [14]. A cause of death of saiga was hemorrhagic septicemia caused by *P. multocida* [15].

A significant damage caused by pasteurellosis to livestock, and a wide geographical distribution and susceptibility of many domestic mammals and birds underscore importance of improving existing methods for diagnosing pasteurellosis. The diagnosis in farm animals is based on clinical signs, determination of pathological changes and bacteriological methods for identification of the pathogen. Efficiency of the classical methods in a diagnostic practice is inferior to modern methods, so that a use of molecular methods is the most effective way to control the disease and reduce economic losses. In recent years, molecular genetics methods for identifying bacteria have proven useful in overcoming limitations of traditional phenotypic procedures. In a guide of the International Animal Health Organization (OIE), a polymerase chain reaction (PCR) method is defined as an alternative diagnostic test and a method for typing of *P. multocida* [16]. One of the main advantages of PCR is its extremely high sensitivity and specificity. The use of the PCR in diagnostics allows a rapid detection and identification of pathogenic microorganisms directly in samples of biological material, as well as in complex or pure cultures. To date, a variety of developed and implemented PCR test systems utilize an electrophoretic detection [17, 18]. The latter method has begun to be replaced by real-time PCR protocols. The real-time PCR allows increasing reliability of diagnosing because of almost complete elimination of a possibility of contamination with previously amplified DNA. The real-time PCR can be used to quantify target DNA in samples, and also reduce duration of an analysis. The real-time PCR is a quick and reproducible alternative to serological methods [19], that increases effectiveness and reliability of a diagnostic study. A use of the real-time PCR method in diagnosing of pasteurellosis in animals will reduce timing of diagnostic tests, and will help to improve anti-epizootic measures.

PCR test systems that detect all serovariants of *P. multocida* have the greatest demand in Kazakhstan≥s diagnostic laboratories. With this regard, the goal of this study was to develop a real-time PCR test-system for diagnosing pasteurellosis in animals and birds.

Materials and methods

Collecting bacterial DNA. DNA from the strain *P. multocida B-0575* was used in the work. The strain *B-0575* is from a collection of the Republican State Enterprise National Reference Center in Veterinary of the Committee of Veterinary at the Ministry of Agriculture. DNA was isolated using the QIAamp DNA Mini Kit manufactured by QIAGEN (USA) according to the manufacturer≥s instructions. DNA concentration was determined by

fluorimetric method using a Qubit 2.0 Fluorometer and the Qubit dsDNA HS Assay Kits.

Selection of target loci for selection of primers. Several bacterial genes that function under a high positive selection pressure [20] were selected as targets for targeting of primers. These genes are related to the group of flhousekeepingffi genes. The selected genes are: *envC* - encodes a metal-chelating subunit found in all enzymes containing molybdenum; *ruvB* - involved in DNA metabolism and recombinations associated with an immune evasion; *QseC* gene - encodes a sensory kinase in the two-component QseBC quorum-sencing system (this transcriptional control system allows pathogens to adapt to different host niches).

Primers design. Sequences of the target genes were downloaded from the NCBI web resource; sequence alignments were produced using the BioEdit Sequence Alignment Editor software [21]. Designing of primers and assessing of likelihood of a formation of undesired secondary structures (such as hairpins and dimers) were done using the PrimerSelect program (DNASTAR). Specificity of the primers, as well as melting temperatures were predicted using the Primer-BLAST web service [22].

The gene sequences were aligned using ClustalW software. Then primers and fluorescent probes (with FAM fluorophores for TaqMan) were devised to target selected genomic regions that are conserved in *P. multocida* but are hypervariable in other species of the *Pasteurella* genus and whole *Pasteurellaceae* family. Probabilities of primers≥ annealing to other genomes (bacterial, viral, animal, plants, etc.) was controlled by using the Primer-BLAST application at the international NCBI database. The primers having stable secondary structures, as well as those complementary to non-target regions, were excluded.

The selected primers were analyzed for a secondary structure formation using PrimerSelect (DNASTAR) software. When selecting fluorescently-labeled probes, the main requirements for the probes were the following: an annealing temperature must be higher than annealing temperatures of primers, probes must lack the G nucleotide at the 5≥-end, stable dimers with primers must be absent, etc.

The nucleotide sequences of primers and fluorescent probes are not presented in this article because they are listed in a patent application, and will be available upon request.

Optimization. Conditions for the real-time PCR for detection of *P. multocida* were optimized using the following variable parameters: concentration of magnesium ions, annealing temperatures, presence of additives that might increase efficiency, specificity and sensitivity of the reaction. The tested additives were betaine, tetramethylammonium chloride, formamide and sucrose.

The invariable components in reaction mixtures for all primer pairs were: primers at 10 pMol, 5 pMol of a corresponding probe, 10 mM Tris-HCl (pH 8.8 at 25°**C**), 50 mM KCl, 0.08% nonidet NP40, dNTP in the concentration 200 mM for each dNTP, 1.5 units of *Taq* polymerase (Alpha ferment, Russia). A PCR amplification program: long denaturation step 95°C - 3 minutes; Cycles (45 sec): 95°C - 20 sec, gradient of primer annealing temperature: 57-67°C - 15 sec, detection step 72°C - 30 sec. The detection was done at a wavelength corresponding to fluorophore FAM.

As a negative control, *E. coli* DNA was used at in the amount 50 ng per the reaction mixture. Also, cattle DNA were used in amount of 500 ng per reaction. In each PCR experiment, one component was optimized, followed by electrophoretic assessment of PCR products.

The optimal composition of a reaction mixture was that allowed detecting of *P. multocida* DNA during the minimal number of cycles, i.e. with the lowest threshold cycle (Ct). Also, values of the coefficient of determination (R^2) and efficiency (E) must be within an acceptable range, as well as non-specific amplification products from negative control (*E. coli* or cattle) DNA must be absent.

Real-time PCR was performed on a CFX-96 thermal cycler (BioRad, USA). The results were analyzed using BioRad CFX Manager 3.0 software (BioRad , USA).

Sensitivity assessment. To assess sensitivity, *P. multocida* DNA was diluted to the concentration 2 ng/µl. DNA concentration was measured using the Qubit dsDNA HS Assay Kit, with triple repetition of results. After that, the DNA was diluted twice in 1xTE buffer (10 mm Tris-HCl, 1 mM EDTA, pH = 8.0) with addition of carrier DNA (fragmented horse DNA at the concentration 10 ng/µl), which was used to stabilize target DNA at low concentrations. A final PCR mixtures contained from 10 ng to 2.38 fg of the target DNA, that is equivalent to 4 million of genomic copies per reaction. Copy numbers were calculat-

ed using an Internet-based service cited in [23].

Assessment of specificity. Specificity of the developed protocols was assessed using a collection of 95 DNA samples, including 92 bacterial species and 3 DNA samples of higher eukaryotic organisms. Species for bacterial strains in the collection were determined by sequence analysis of the *16S rRNA* gene.

RESULTS AND DISCUSSION

Selection of primers. Selection of target DNA and structures of primers is of great importance during developing of PCR protocols. As a rule, a target for selecting the primers is determined based on a goal of amplification. In case that identification of all serovariants of *P. multocida* is required, it is necessary to use as the target the ribosomal region, or genes of the flhousekeepingffi profile [24]. If it is necessary to identify serotypes that can cause certain nosologies, such as pig atrophic rhinitis, it is advisable to identify target genes that cause a specific pathology, for example, genes encoding toxins (*ToxA*), adhesins (*ptfA* and *pfhA*), siderophores (*tonB* and *hgbA*) [25, 26] which are associated with a manifestation of the pathology.

In order to select primers, nucleotide sequences of the *QseC*, *envC* and *ruvB* genes were downloaded from the NCBI database. The genes were from all *P. multocida* strains, as well as from four species belonging to the *Pasteurella* genus, for which whole genomes are currently identified: *Pasteurella dagmatis* ATCC 43325,*Pasteurella bettyae* CCUG 2042, *Pasteurella skyensis* DSM 24204 and*Pasteurella canis* NCTC 11621.

In total, 3 pairs of the primers and corresponding fluorescent probes were selected, the main characteristics of the primers and probes are shown in table 1.

Table 1. List of selected oligonucleotides, probes and their main characteristics

Designation	Annealing temperature calculated by PrimerBlast , °C	Amplifiable product size, bp
envC-F	59	111
envC-R	61	
ruvB-F	60	79
ruvB-R	59	
QseC-F	59	117
QseC-R	58	
probe-envC	66	Not applicable
probe-ruvB	65	Not applicable
Probe-QseC	64	Not applicable

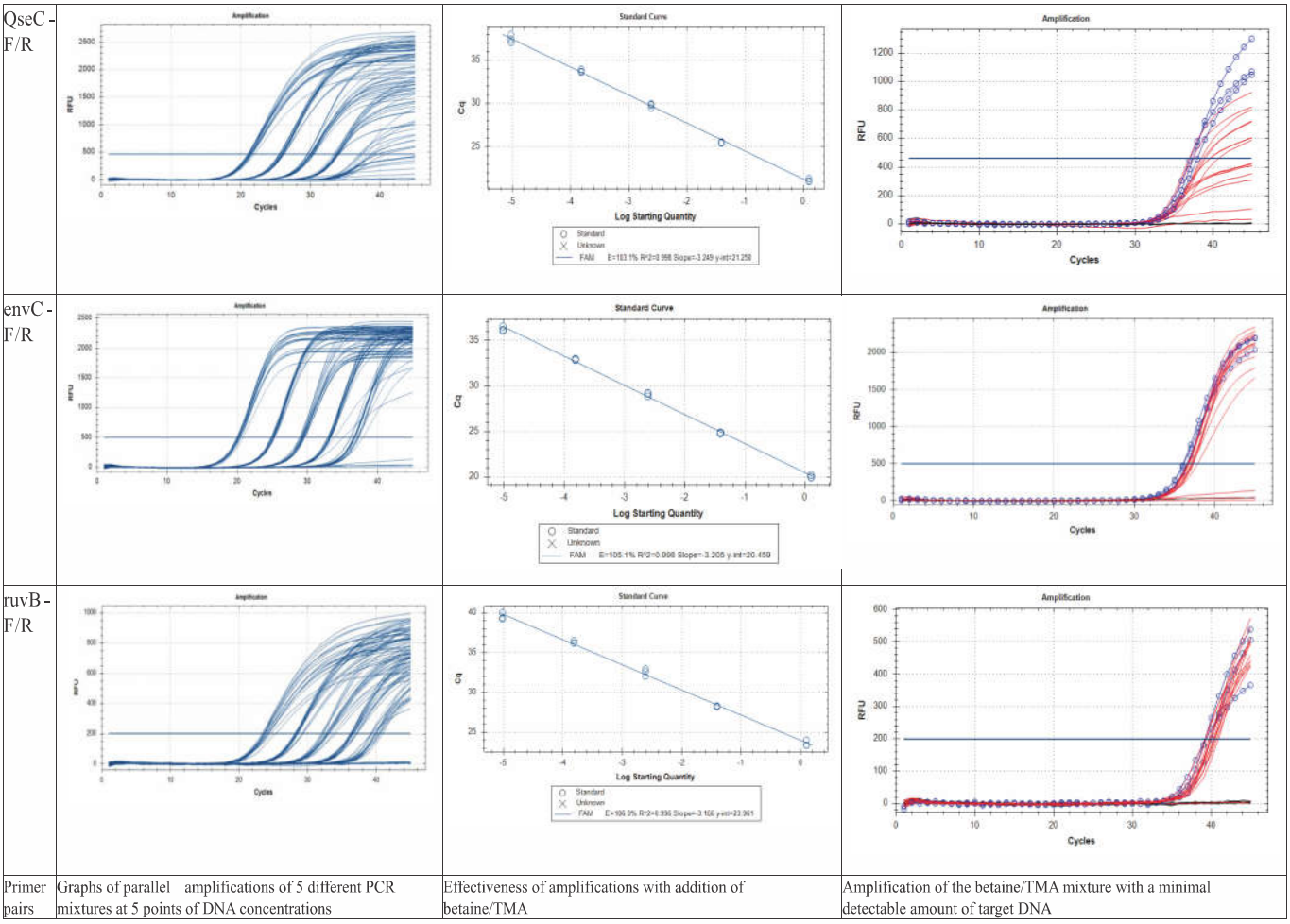
Optimization of the real-time PCR. Much attention during developing of PCR test-systems is given to additives that can affect efficiency, specificity and sensitivity. The majority of commercial PCR kits include specific additives that increase their diagnostic value. Substances such as acetamide, dithiothreitol, and many others like them [27] are expensive; therefore, utilization of inexpensive additives is desired to reduce prices of the PCR kits and ensure their wide distribution.

At the first stage, annealing temperatures of the primers and concentrations of the magnesium ion were optimized. For this purpose, a temperature gradient was used in the range from 57°C to 67°C, and magnesium ion concentrations in a reaction mixture were varied from 2 mM to 4.5 mM with an increment step 0.5 mM.

At the second step of the optimization, dynamics of the reaction was investigated in presence of additives in the reaction mixture. As a result, in one 96-well plate, 5 compositions of the reaction mixtures were tested and compared: without additives (No. 1), with betaine (final concentration 0.2 M) (No. 2), with tetramethylammonium chloride (final concentration 5 nM) (No. 3), with betaine and tetramethylammonium chloride (concentrations similar to the listed above) (No. 4), with formamide (final concentration 2.5%) (No. 5) and with sucrose (final concentration 0.2 M) (No. 6). For each reaction mixture, 5 different amounts of *P. multocida* DNA (1.25 ng, 39 pg, 2.4 pg, 150 fg and 9.53 fg, respectively) or negative control DNA were added to monitor amplification profiles at various concentrations of the input target DNA. Each experiemnt was done in triplicate to avoid possible artifacts or handling errors. This

experiment was repeated three times for each pair of primers.

Figure 1 shows the values of effic iency and determination coefficients. The lowest threshold cycle values were observed with the PCR reactions containing TMA and betaine, with the DNA amount 9.53 fg. Efficiencies of the reactions were 103.1%, 105.1% and 106.9%, and the relative fluorescence units (RFU/OEF) were 1200, 2000 and 600 for the *QseC*, *envC* and *ruvB* genes, respectively.



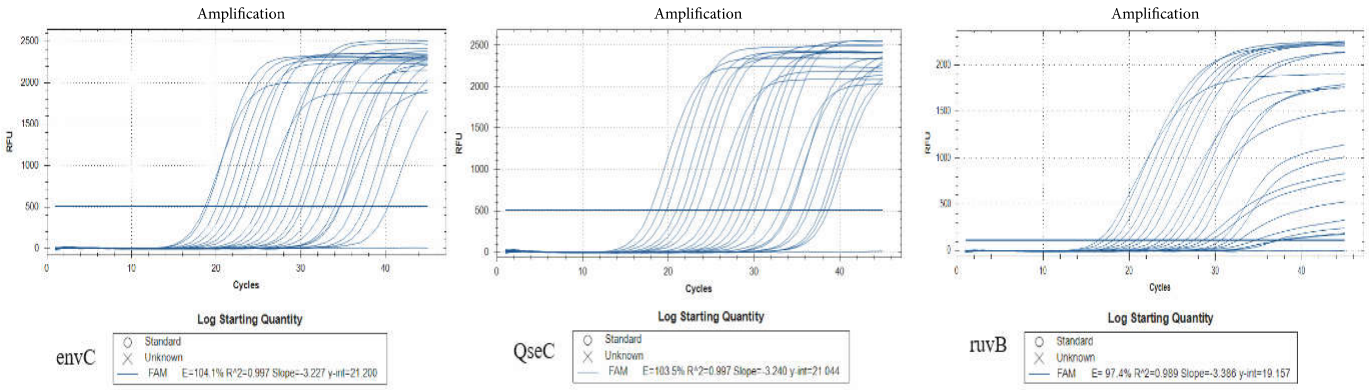
Primer pairs	Graphs of parallel amplifications of 5 different PCR mixtures at 5 points of DNA concentrations	Effectiveness of amplifications with addition of betaine/TMA	Amplification of the betaine/TMA mixture with a minimal detectable amount of target DNA

Fig. 1. Optimization of a composition of PCR mixtures using 5 different additives

Thus, the optimal compositions of the reaction mixtures for the primer pairs QseC-F/R, envC-F/R and ruvB-F/R with the corresponding fluorescent probes include: magnesium chloride 3.5 mM, 4.5 mM and 4 mM, respectively; primers at 10 pMol each, a corresponding probe at 5 pMol, 10 mM Tris-HCl (pH 8.8 at 25°C), 50 mM KCl, 0.08% Nonidet NP40, dNTP at the concentrations 200 mM each, 5 nM tetramethylammonium chloride, betaine at the concentration of 0.2 M, 1.5 units of *Taq* polymerase (Alpha ferment, Russia). The optimized PCR amplification program is: long denaturation at 95°C - 3 min; 45 cycles: 95°C - 20 sec, 60°C, 61°C, 58°C (respectively) - 15 sec, and the detection step (FAM) 72 °C - 30 sec.

Sensitivity assessment. Based on a literature review, highly sensitive diagnostic PCR protocols are those protocols which allow detecting target DNA in amounts of from 50 pg to 50 fg [28]. However, for real-time PCR, the sensitivity depends on a level of fluorescence, especially when detecting low DNA concentrations.

As shown in figure 2, for the primer pairs envF/R, QseC the PCR detected of low target DNA amounts equal 2.38 fg. This corresponds to one genomic copy of *P. multocida* DNA in the reaction mixture. For the primer pair ruvB F/R, the protocol sensitivity was also 2.38 fg; however, with DNA amounts below 76 pg, RFU values were below 250, and the amplification curve did not have a logarithmic form. Therefore, a detection limit for a given pair of primers is defined as 76 pg, which is equivalent to 30 genomic of *P. multocida* DNA.



Fluorescence signal detection curves with a *P. multocida* DNA concentration from 10 ng to 2.38 fg in the reaction, with two-fold dilutions (from left to right). envC - PCR reaction with primers to the envC gene; QseC - PCR reaction with primers to the QseC gene; ruvB - PCR reaction with primers for the ruvB gene.

Fig. 2. Graphs of the real-time PCR reactions during assessing sensitivity of PCR protocols

As a result, the PCR protocols designed to detect *P. multocida* in real time for the pairs of primers envC-F/R and QseC-F/R have the sensitivity of 1 copy genomic, and for the pair of primers ruvB - 30 genomic copies. Three pairs of primers (envC-F/R, QseC-F/R and ruvB-F/R) showed efficiencies of 104.1%, 103.5% and 97.4%, respectively, when target DNA is present in amounts from 10 ng to 2.38 fg.

Specificity assessment. The developed real-time PCR protocol has been tested on a collection of DNA from various bacterial species. The collection includes strains of the following bacterial species: *Acinetobacter johnsonii, Aeromonas bivalvium, Agrobacterium tumefacie, Alcaligenes aquatilis, Arthrobacter polychromogenes, Bacillus amyloliquefaciens, Bacillus aryabhattai, Bacillus atrophaeus, Bacillus axarquiensis, Bacillus cereus, Bacillus licheniformis, Bacillus mojavensis, Bacillus mucilaginosus, Bacillus mycoides, Bacillus pseudomycoides, Bacillus pumilus, Bacillus safensis, Bacillus simplex, Bacillus sonorensis, Bacillus stratosphericus, Bacillus subtilis, Bacillus tequilensis, Bacillus thuringensis, Brucella abortus, Brucella canis, Brucella melitensis, Brucella ovis, Brucella suis, Campylobacter coli, Campylobacter concisus, Campylobacter gracilis, Campylobacter hyointestinalis, Campylobacter jejuni, Campylobacter mucosalis, Campylobacter rectus, Campylobacter showae, Campylobacter upsaliensis, Clostridium haemolyticum, Clostridium novyi, Delfitia tsuruhatensis, Dermacoccus nishinomiyaensis, Devosia neptunia, Escherichia coli, Ensifer adhaerens, Enterococcus durans, Erwinia amylovora, Exiguobacterium aurantiacum, Halomonas nitritophilus, Klebsiella michiganensis, Klebsiella pneumonia, Lactobacillus brevis, Lactobacillus casei, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus pontis, Lactococcus garvieae, Listeria innocua, Lysinibacillus xylanilyticus, Mannheimia haemolytica, Mannheimia glucosidal, Mannheimia varigena, Microbacterium hydrocarbonoxydans, Ochrobactrum tritici, Pediococcus acidilactici, Pediococcus pentosaceus, Pseudomonas chengduensis, Pseudomonas fluorescens, Pseudomonas mandelii, Pseudomonas marginalis, Pseudomonas mucedolens, Pseudomonas plecoglossus, Pseudomonas putida, Pseudomonas reactants, Pseudomonas syringae, Rahnella aquaticus, Rhizobium agrobacterium, Rhizobium pusense, Rhodococcus jostii, Rhodococcus kroppenstedtii, Rhodococcus opacus, Rummeliibacillus pycnus, Salmonella enteritidis, Salmonella enteric, Serratia liquefaciens, Serratia plymuthica, Serratia proteamaculans, Sinorhizobium meliloti, Sphingomonas aerolata, Stenotrophomonas maltophilia, Stenotrophomonas pneumonia, Streptococcus salivarius,Pasteurellamultocida.*

Also DNA from horse (*Equus ferus caballus*), hen (*Gallus gallus domesticus*) and cattle (*Bos taurus taurus*) was included in the tests as negative controls.

Bacterial DNA, except DNA of *P. multocida* was used in amounts 5-50 ng in a reaction, which corresponded to genomic equivalents of 8 042 000 genomic copies per reaction. DNA from higher eukaryotes was used in amounts 200 ng per reaction. As a result of the assessment of specificity for all primers pairs with the corresponding DNA probes, only specific amplification of *P. multocida* DNA was observed.

DISCUSSION

To prevent economic losses and the spread of pasteurellosis, it is necessary to improve diagnostics using sensitive and specific methods. Direct determination of the pathogen DNA, high specificity and sensitivity, the versatility of the procedure, the rel

atively small time spent on the analysis, made the PCR method indispensable.

The development of classical PCR involves optimization: the concentration of magnesium ions, the type and concentration of the used polymerase, the composition of the buffer used, the temperature of annealing of the primers, the ratio of primers, the ratio of primers to probes, the addition and concentration of additives, the amplification program, etc. [29]. The strategy used by us [30], is similar to the generally accepted PCR optimization methods.

The use of additives significantly increases the yield and specificity of PCR, and is a recommended element [31]. Due to the low price and the qualitative effect on amplification, betaine, dimethyl sulfoxide (DMSO), formamide, tetramethylammonium chloride (TMA), sucrose, polyethylene glycol, glycerol, and bovine serum albumin are most widely used. Betaine and DMSO have long been used by researchers for PCR optimization, especially for amplification of GC-rich sequences, however, in our practice, DMSO did not shows a significant effect on the dynamics of real-time PCR. Also in our practice, the use of betaine with TMA contributed to a stable yield and high specificity of PCR. Therefore, the additives we selected were precisely: betaine, TMA, formamide, and sucrose.

Efficiency is an important criterion for characterizing a PCR protocol. Physically, the amplicons are doubled during each cycle, therefore, the maximum efficiency of the PCR protocols is 100%, and however, the inappropriateness in signal detection leads to ± 5% error. Another important characteristic is also the coefficient of determination (R^2). An R^2 value >0.980 provides good confidence in correlating Cq [32]. The most accurate assessment of the effectiveness of PCR is the serial dilution of DNA and the construction of a standard curve. The use of five selected concentrations of serially diluted DNA and the construction of a standard curve at five points allowed us to accurately assess the sensitivity and effectiveness, both at high and low DNA concentrations (Figure 1, column: graphs of parallel amplifications of 5 different PCR mixtures at 5 points of DNA concentrations).

Optimization of diagnostic PCR for the detection of pathogens [33] introduces new requirements for the validation of the developed protocols. These include: diagnostic (analytical) specificity and sensitivity, precision, reproducibility [34, 35].

In developing diagnostic PCR for the detection of a specific microorganism, bioinformatics methods, such as the Primer-BLAST tool and alignment of the target gene of the target organism with phylogenetically close ones [36], in practice do not guarantee absolute specificity [37], and it is always a hypothesis. The standard methodology for assessing the specificity of the developed PCR protocols is performing a reaction to DNA collections, however, the number and content of sample collections varies depending on the capabilities of the laboratory itself. Therefore, the size of the DNA collection is not standardized and in various works DNA collections from a dozen to several hundred specimens are observed. As for the content, the best way would be to use phylogenetically closely related microorganisms. The difficulty of using a DNA collection is the accuracy of the identified collection species. The DNA collection we used was identified by the *16S rRNA* gene sequence with at least 99% identity, which is considered a highly species-specific collection.

A standard assessment of the PCR protocol sensitivity is serial DNA dilution. The fragmented DNA of horse has been used to stabilize the dilution. Several studies maintained that real-time PCR is a more sensitive method compared to classical PCR [38]. Highly sensitive PCR protocols allow detect of DNA in an amount from 1-4 equivalents (target molecules) to 100 copies [39]. Our results indicate a high sensitivity of the developed protocols that allows to detect minimal amounts of *P. multocida* DNA in clinical samples.

For PCR diagnostics, both of all serovariants and isolates leading to various manifestations of pasteurellosis, many options have been proposed. In PCR detection of *P. multocida*, depending on the purpose of the study, capsule-specific, disease-specific, and species-specific PCR protocols are used.

Knowledge of the biosynthetic loci encoding capsule proteins allowed the development of serogroup-specific PCR analyzes [4], which are still used to characterize isolates. The protocols for the detection of toxigenic isolates of both classical PCR [40, 41] and real-time [42] have long been developed and abound with many analogues. For the diagnosis of avian cholera, classic PCRs targeting the *hyaC-hyaD* [43] and *pls* [44] genes have been well established. For the diagnosis of pneumonic pasteurellosis of pigs and ungulates, serotype A protocols are used in combination with toxA-specific protocols протоколами [45, 46]. For the diagnosis of hemorrhagic septicemia, the developed PCR protocol [47] is used, which remains

specific for HS-causing cultures of serogroup B with the predominant somatic antigen of serotype 2 or 5. As an alternative,there are specify real-time PCR developed by [48], which based on esterase target gene and can specifically detected ST122, ST63, ST147 and ST162 associated with HS.

The most urgent task is the detection of all serovariants using species-specific PCR protocols. Initially, PCR targets were genes of the ribosomal region, such as: tRNA-intergenic spacer [49], *23S rRNA* [50]. Currently, to detect and verify isolates belonging to the genus Pasteurella from clinical samples, the PCR protocol of the amplifying gene fragment KMT1 is used. Despite widespread use, the PCR protocol gives a false positive result for isolates belonging to the species Mannheimia haemolytica as a result of close phylogenetic kinship [51]. Therefore, this protocol is verified by subsequent sequencing of both this fragment and the *16S rRNA* gene fragment.

CONCLUSION

Outbreaks of pasteurellosis are recorded annually in Kazakhstan. The outbreaks are common in populations of wild animals and sometimes lead to local epizootics. In this regard, developing of domestic test-systems is an urgent task. We developed a real-time PCR protocol for diagnosing of pasteurellosis in animals and birds. Their efficiency with the targeted *QseC*, *envC*, and *ruvB* genes averaged 105%. The protocols were tested on a DNA collection including a variety of opportunistic and pathogenic microorganisms, resulting in 100% specificity. Sensitivity of the developed protocols was also high, and amounted to 2.38 fg for the *QseC* and *envC* genes and 76 pg for the *ruvB* gene in the PCR reaction.

Thus, the real-time PCR protocols were developed for the diagnosis of pasteurellosis in animals and birds caused by all *P. multocida* serovars, with high specificity and sensitivity. Implementing of the developed protocols in a form of commercial test-systems will improve pasteurellosis diagnostics, and will be favorable to improve the epizootic situation.

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REFERENCES

1. Mutters R., Ihm P., Pohl S., Frederiksen W., Mannheim W. Reclassification of the Genus Pasteurella Trevisan 1887 on the Basis of Deoxyribonucleic Acid Homology, with Proposals for the New Species *Pasteurella dagmatis, Pasteurella canis, Pasteurella stomatis, Pasteurella anatis,* and *Pasteurella langaa. International Journal of Systematic and Evolutionary Microbiology*, 1985, vol. 35, pp. 309–322.

2. Carter G. R. Pasteurellosis: *Pasteurella multocida* and *Pasteurella hemolytica. Advances in Veterinary Sciences*, 1967, vol. 11, pp. 321–379.

3. Rimler R. B., Rhoades K. R. Serogroup F, a new capsule serogroup of *Pasteurella multocida. Journal of Clinical Microbiology* 1987, vol. 25, pp. 615–618.

4. Townsend K. M., Boyce J. D., Chung J. Y., Frost A. J., Adler B. Genetic organization of *Pasteurella multocida* cap Loci and development of a multiplex capsular PCR typing system *Journal of Clinical Microbiology*, 2001, vol. 39, pp. 924-929.

5. Heddleston K. L., Gallagher J. E., Rebers P. A. Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Diseases,* 1972, vol. 16, pp. 925–936.

6. Harper M., John M., Turni C., Edmunds M., St Michael F., Adler B., et al. Development of a rapid multiplex PCR assay to genotype *Pasteurella multocida* strains by use of the lipopolysaccharide outer core biosynthesis locus. *Journal of Clinical Microbiology,* 2015, vol. 53, pp. 477–485. http://dx.doi.org/10.1128/JCM.02824-14.

7. Veken J. W., et al. Binding of bovine transferrin by *Pasteurella multocida* serotype B:2,5, a strain which causes haemorrhagic septicaemia in buffalo and cattle. *FEMS microbiology letters*, 1994, vol. 115, no. 2-3, pp. 253-257.

8. Wilkie I. W., Harper M., Boyce J. D., Adler B. Pasteurella multocida: Diseases and Pathogenesis. *Current Topics in Microbiology and Immunology,* 2012, vol. 361, pp.

9. Peng Z., Liang W., Wang F., Xu Z., Xie Z., Lian Z., Hua L., Zhou R., Chen H. and Wu B. Genetic and Phylogenetic Characteristics of *Pasteurella multocida* Isolates From Different Host Species. *Frontiers in Microbiology*, no. 9, pp. 1408. http://dx.doi.org/10.3389/fmicb.2018.01408.

10. Moustafa A. M. et al. Comparative genomic analysis of Asian haemorrhagic septicaemia–associated strains of *Pasteurella multocida* identifies

more than 90 haemorrhagic septicaemia-specific genes. *PLoS One*, 2015, vol. 10, no. 7.

11. Orynbayev M., Sultankulova K., Sansyzbay, A., Rystayeva R., Shorayeva K., Namet A., Ferei-douni S., Ilgekbayeva G., Barakbayev K., Kopeyev S. and Kock, R. Biological characterization of Pasteurella multocida present in the Saiga population. *BMC microbiology,* 2019, vol. 19, no. 1, pp. 37.

12. Kharb S. and Charan S. Mouse model of haemorrhagic septicaemia:dissemination and multiplication of *Pasteurella multocida* B:2 in vital organs after intranasal and subcutaneous challenge in mice. *Veterinary Research Communications*, 2013, vol. 37, no. 1, pp. 59ć63.

13. Hedau M., Kurkure N. V., Jangir B. L., Chopde N. A., Kalorey D. R. and Bhandarkar A. G. Pathological diagnosis of an outbreak of pasteurellosis in buffaloes of Vidharbha region in India.*Indian Journal of Veterinary Pathology*, 2017, vol. 41, no. 1, pp. 38-40.

14. Convention on Migratory Species (2015). Available at: https://www.cms.int/sites/default/files/document/Saiga%20MOS3_Overview_Report_of_Conse rvation_Status_Eng.pdf

15. Kock R., Orynbayev M., Robinson S., Zuther S., Singh J., Beauvais W., Morgan R., Kerimbayev A., Khomenko S., Martineau M., Rystaeva R., Omarova Z., Wolfs S.,Hawotte F., Radoux J., Milner-Gulland. J. Saigas on the brink: multi-disciplinary analysis of the factors influencing a mass die-off event. *Science Advances*, 2018. Available at: http://dx.doi.org/ 10.1126/sciadv.aao2314.

16. OIE, World Organisation for Animal Health. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Paris, France, 2012, vol. 7, pp.1440.

17. Al-Maary K. S, Dawoud T. M, Mubarak A. S, Hessain A. M, Galal H. M, Kabli S. A, Mohamed M. I. Molecular characterization of the capsular antigens of *Pasteurella multocida* isolates using multiplex PCR. *Saudi journal of biological sciences*, 2017, vol. 24, no. 2. pp. 367-370.

18. Rajkhowa S. Development of a novel multiplex PCR assay for rapid detection of virulence associated genes of *Pasteurella multocida* from pigs. *Letters in applied microbiology,*2015, vol. 61, no. 3, pp. 293-298.

19. Arumugam N. D., Ajam N., Blackall P. J., Asiah N. M., Ramlan M., Maria J., Yuslan S., Thong K. L. 2011. Capsular serotyping of *Pasteurella multocida* from various animal hosts ć a comparison of phenotypic and genotypic methods*Tropical Biomedicine*, 2011, vol. 28, no. 1 pp. 55-63.

20. Cao P., Guo D., Liu J., Jiang Q., Xu Z. and Qu L. Genome-Wide Analyses Reveal Genes Subject to Positive Selection in*Pasteurella multocida. Frontiers in Microbiology*, 2017, vol. 8, pp. 961.

21. Hall T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*, vol. 41, no. 41, pp. 95-98.

22. US National Library of Medicine National Institutes of Health. Available at:http://www.ncbi.nlm.nih.gov/tools/primer-blast.

23. Calculator for determining the number of copies of a template. Available at:http://cels.uri.edu/gsc/cndna.html.

24. Miflin J. K., Blackall P. J. Development of a 23S rRNA-based PCR assay for the identification of *Pasteurella multocida. Letters in applied microbiology*, 2001, vol. 33, pp. 216-221.

25. Rajkhowa S. Development of a novel multiplex PCR assay for rapid detection of virulence associated genes of *Pasteurella multocida* from pigs. *Letters in applied microbiology*2015, vol. 61, pp. 293-298.

26. Lichtensteiger C. A., Steenbergen S. M., Lee R. M., Polson D. D. and Vimr E. R. Direct PCR analysis for toxigenic Pasteurella multocida.*Journal of clinical microbiology,* 1996, vol. 34, pp. 3035-3039.

27. Simonović A., Trifunović-Momčilov M., Raspor M., Cingel A. D., Bogdanović M., Dragićević M. and Subotić A. Dimethyl sulfoxide improves sensitivity and specificity of RT-PCR and qRT-PCR amplification of low-expressed transgenes.*Archives of Biological Sciences*, 2012, vol. 64, no. 3, pp. 865-876.

28. Klee S. R., Tyczka J., Ellerbrok H., Franz T., Linke S., Baljer G. and Appel B. Highly sensitive real-time PCR for specific detection and quantification of Coxiella burnetii. *BMC Microbiology*, 2006, vol. 6, no. 1, pp. 2.

29. Roux K. H. Optimization and troubleshooting in PCR*Cold Spring Harbor Protocols,* 2009, vol. 2009, no. 4.

30. Amirgazin, A. O., Shvedyuk, V. B., Kuibagarov, M. A., Karibaev, T. B. and Shevtsov, A. B. PCR optimization protocol algorithm for identification of microorganisms using Pasteurella multocida. *Eurasian Journal of Applied Biotechnology,* 2017, no. 3, pp. 33-42.

31. Innis M., Gelfand D. Optimization of PCR: conversations between Michael and David. *PCR ap-*

plications, 1999, pp. 3-22.

32. Bustin S., Huggett J. qPCR primer design revisited. *Biomolecular detection and quantification,* 2017, vol. 14, pp. 19-28.

33. Sachse K., Frey J.*PCR detection of microbial pathogens.* Springer Science & Business Media, 2003, 216 pp.

34. López L. F. et al. Standardization and validation of real time PCR assays for the diagnosis of histoplasmosis using three molecular targets in an animal model. *PloS one*, 2017, vol. 12, no. 12.

35. Marino A. M. F. et al. Development and validation of a real-time PCR assay for the detection of Toxoplasma gondii DNA in animal and meat samples. *Journal of Veterinary Diagnostic Investigation,* 2017, vol. 29, no. 2, pp. 203-207.

36. SantaLucia Jr J., Hicks D. The thermodynamics of DNA structural motifs. *Annual Review of Biophysics and Biomolecular Structure,* 2004, vol. 33, pp. 415-440.

37. SantaLucia J. Physical principles and visual-OMP software for optimal PCR design. *PCR primer design*, 2007, pp. 3-33.

38. Scherrer S., Frei D., Wittenbrink M. M. A novel quantitative real-time polymerase chain reaction method for detecting toxigenic Pasteurella multocida in nasal swabs from swine. *Acta Veterinaria Scandinavica*, 2016, vol. 58, no. 1, 83 pp.

39. Forootan A. et al. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular detection and quantification*, 2017, vol. 12, pp. 1-6.

40. Lichtensteiger C. A., Steenbergen S. M., Lee R. M., Polson D. D., Vimr E. R. Direct PCR analysis for toxigenic *Pasteurella multocida*. *Journal of Clinical Microbiology*, 1996, vol. 34, pp. 3035ć3039.

41. Nagai, S., Someno, S., Yagihashi, T. Differentiation of toxigenic from nontoxigenic isolates of *Pasteurella multocida* by PCR. Journal of Clinical Microbiology, 1994, vol. 32, pp. 1004ć1010.

42. Scherrer S., Frei D., Wittenbrink M. M. A novel quantitative real-time polymerase chain reaction method for detecting toxigenic *Pasteurella multocida* in nasal swabs from swine. *Acta Veterinaria Scandinavica*, 2016, vol. 58, no. 1, pp. 83.

43. Gautam R. et al. Specific identification of Pasteurella multocida serogroup-A isolates by PCR assay. *Research in veterinary science,*2004, vol. 76, no. 3, pp. 179-185.

44. Kasten R. W., Carpenter T. E., Snipes K. P., Hirsh D. C. Detection of *Pasteurella multocida*-specific DNA in turkey flocks by use of the polymerase chain reaction. *Avian Diseases*, 1997, vol. 41, pp. 676ć682.

45. Vougidou C. et al. Distribution of the ompA-types among ruminant and swine pneumonic strains of *Pasteurella multocida* exhibiting various cap-locus and toxA patterns. *Microbiological research*, 2015, vol. 174, pp. 1-8.

46. Djordjevic S. P. et al. Demonstration that Australian Pasteurella multocida isolates from sporadic outbreaks of porcine pneumonia are non-toxigenic (toxA-) and display heterogeneous DNA restriction endonuclease profiles compared with toxigenic isolates from herds with progressive atrophic rhinitis. *Journal of medical microbiology*, 1998, vol. 47, no. 8, pp. 679-688.

47. Townsend K. M., Frost, A. J., Lee, C. W., Papadimitriou J. M., Dawkins H. J. Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *Journal of Clinical Microbiology*, 1998, vol. 36, pp. 1096-1100.

48. Petersen A., Bisgaard M., Townsend K., Christensen H. MLST typing of *Pasteurella multocida* associated withhaemorrhagic septicaemia and development of a real-time PCR specific for haemorrhagic septicaemia associatedisolates. *Veterinary Microbiology*, 2014, vol. 170, pp. 335-341.

49. Catry B., Baele M., Opsomer G., De Kruif A., Decostere A., Haesebrouck F. tRNA-intergenic spacer PCR for the identification of Pasteurella and Mannheimia spp. *Veterinary Microbiology,* 2004, vol. 98, pp. 251ć260.

50. Miflin J. K., Blackall P. J. Development of a 23S rRNA-based PCR assay for the identification of Pasteurella multocida. *Letters in Applied Microbiology*, 2001, vol. 33, pp. 216ć221.

51. Deressa A, Asfaw Y, Lubke B, Kyule M. W, Tefera G, Zessin K. H. Molecular detection of Pasteurella multocida and Mannheimiahaemolytica in sheep respiratory infections in Ethiopia. *The International Journal of Applied Research in Veterinary Medicine*, 2010, vol. 8, no 2, 101 p.

ПАСТЕРЕЛЛЕЗДІ ДИАГНОСТИКАЛАУ ҮШІН НАҚТЫ УАҚЫТ РЕЖИМІНДЕ ПТР ХАТТАМАСЫН ӘЗІРЛЕУ

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ТҮЙІН

Пастереллез омыртқалы жануарлар мен құстардың зооноздық ауруы, этиологиялық агенті *P. multocida* болып табылады. *P. multocida* жоғары басымдылығы, сондай-ақ, жабайы және үй жануарларында, әсіресе Қазақстандық ақбөкен популяцияларында байқалатын мерзімдік өршулерді тіркеу пастереллезді диагностикалауға арналған отандық ПТР тест-жүйелерін әзірлеуді өзекті етеді. Осы жұмыстың мақсаты *Pasteurella multocida* барлық серонұсқаларын анықтау үшін нақты уақыт режимінде ПТР хаттамасын әзірлеу болып табылады. Зерттеу нысаны ретінде *envC, ruvB* және *QseC* гендері таңдалды. Нәтижесінде жоғары тиімділігі, сезімталдығы және өзгешелігімен сипатталатын, 3 жұп праймерді және тиісті флуоресцентті зондтарды пайдалана отырып, нақты уақыт режимінде ПТР хаттамалары әзірленді. *Envc* және *QseC* гендерінің тізбектеріне әзірленген хаттамалардың сезімталдығы 2,38 фг құрады, ruvB гені үшін тиісінше 76 фг құрады. Жоғары өзгешелігі бактериялардың 92 түрі мен жоғары эукариотикалық ағзалардың 3 түрі сынамаларының коллекциясында расталды. Әзірленген хаттамаларды өндірістік практикаға енгізу жоғары тиімді ПТР тест-жүйесін шығаруға мүмкіндік береді.

Негізгі сөздер: нақты уақыт режимінде ПТР, *Pasteurella multocida,* ПТР диагностикасы, ПТР оңтайландыру, ПТР өзгешелігі, ПТР сезімталдығы.

РАЗРАБОТКА ПРОТОКОЛА ПЦР В РЕЖИМЕ РЕАЛЬНОГО ВРЕМЕНИ ДЛЯ ДИАГНОСТИКИ ПАСТЕРЕЛЛЁЗА

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АБСТАКТ

Пастереллёз - зоонозная болезнь позвоночных животных и птиц, этиологическим агентом которой является *P. multocida*. Высокая преваленция *P. multocida* и регистрация периодических вспышек как диких, так и домашних животных, особенно наблюдаемых в Казахстанских популяциях сайги, актуализирует разработку отечественных ПЦР тест-систем для диагностики пастереллёза. Цель настоящей работы являлась разработка протокола ПЦР в режиме реального времени для выявления всех сероварианто *Pasteurella multocida*. В качестве мишеней были выбраны гены: *envC, ruvB* и *QseC*. В результате были разработаны протоколы ПЦР в режиме реального времени с использованием 3 пар праймеров и соответствующих флуоресцентных зондов, характеризующиеся высокой эффективностью, чувствительностью и специфичностью. Чувствительность разработанных протоколов к последовательностям генов *envC* и *QseC* составила 2,38 фг, для гена *ruvB* составило 76 пг, соответственно. Высокая специфичность была подтверждена на коллекции образцов 92 видов бактерий и 3 видов высших эукариотических организмов. Внедрение разработанных протоколов в производственную практику позволит выпускать высокоэффективные ПЦР тест-системы.

Ключевые слова: ПЦР в режиме реального времени, *Pasteurella multocida*, ПЦР диагностика, ПЦР оптимизация, специфичность ПЦР, чувствительность ПЦР.