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Development of a Real-time PCR using Fluorescent Hybridization Probes

AA Abdirassilova[1]*, ZZH Abdel[1], BK Kurmanov[2], AK Kassenova[1], AK Rysbekova[1], DT Yessimseit[1], BZ Abdeliyev[1], TV Meka Mechenko[1], ZHS Dalibayev[1], EZH Begimbayeva[1]

[1]National Scientific Center for Particularly Dangerous Infections Named after M. Aikimbayev (NSC PDI), Almaty, Kazakhstan

[2]University of Florida, Gainesville, USA

ABSTRACT

Objective: This research aims at the domestic development and design of experimental series of assay for plague causative agent determination through the method of real-time multi-locus polymerase chain reaction (PCR) with fluorescent hybridization probes, conducting control tests for refinement of optimal conditions to obtain the maximum sensitivity and specificity of the preparation.

Method: The research group designed fluorescent probes and synthesized the primers with unique nucleotide sequences for detection Yersinia pestis bacteria based on the chromosomal gene YPO2088, plasmid genes pst and caf1, which determine the properties of pesticide activity and capsular antigen synthesis and accordingly are associated with causative agent pathogenicity.

Results: This paper presents the results of the research conducted by the molecular diagnostics and genetics laboratory of research center related to the development of an experimental series of domestically designed polymerase chain reaction assays for plague agent determination through the method of real-time PCR with fluorescent hybridization probes. Based on the selected marker genes, primers YPO2088 F/R, caf1 F/R and pst F/R were developed. To determine their specificity, 49 Y. Pestis strains isolated from various objects, 26 strains of closely related bacteria and 22 non-Yersinia strains were used, as well as 288 suspensions from organs of rodents, fleas and mites obtained from the territories with plague natural foci located in Kazakhstan.

Conclusion: The research results have revealed that primers and probes were specific for Y. pestis, which enables to differentiate them from closely related and heterologous bacterial species, determine the mono- and non-plasmid variants of plague microbe phenotype with assay sensitivity of 100 fg for YPO-2088 F/R primers, and 10 fg for caf1 F/R and pst F/R primers.

Key words: Plague, Yersinia pestis, Real-time polymerase chain reaction, Assay, Primers, Genes, Molecular genetic testing

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Corresponding author: AA Abdirassilova

e-mail✉: esfehani.mohamad3@gmail.com

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INTRODUCTION

At the end of the second decade of the 21st century, plague still remains a topical infection, as evidenced by numerous researches aimed at studying the causative agent itself, its genome, and the patterns of epizootic and epidemic processes of this infection. According to the World Health Organization (WHO), around 2.5 thousand plague cases are reported annually worldwide with no downward trend [1-6]. Over the past few years, the world community has been concerned with the plague epidemiological situation on Madagascar's endemic territory, where since 2015 pulmonic plague epidemics covering 50% of the territory has been registered. The financial cost of anti-epidemic measures amounted to about $ 4 million [6-8].

An important reason for the topical nature of plague is the characteristics of the infectious agent itself as a category A biological agent in the development of bacteriological weapons and bioterrorism means [9-11].

The entire territory of the Republic of Kazakhstan has a very high epidemic potential of the entire group of particularly dangerous infectious diseases of bacterial and viral etiology, including

plague. From 1920 to 2003, in the area of the Central Asian desert focus, 2280 people were infected with plague, 2066 of them died (90.6 %), including people in Kazakhstan–1084 with a mortality rate of 80.0 %. The last cases of plague in the Republic of Kazakhstan were registered in 2003 [12].

In the complex of epidemic preventive measures, one of the priorities is timely, accurate etiological diagnosis of the plague case. The culture method (inoculation of media) is specific, but requires one to two days to obtain a positive result, and six days for a negative result. With a negative result of the bacteriological method, seroconversion (development of antibodies to the plague microbe F1 capsular antigen in the blood) can serve as confirmation of the diagnosis, but in the first five days after exposure to the plague bacteria, seroconversion is unlikely. In the majority of patients, it occurs only during the second week and later (especially with early antibacterial therapy); in 5% of patients, the production of specific antibodies does not occur at all [13].

The most promising methods of plague diagnosis are genetic tests, in particular, polymerase chain reaction (PCR), which are characterized by high sensitivity, specificity, reproducibility and rapidness, the ability to study any kind of material (clinical, samples from environmental objects). PCR is especially important in identifying the first cases of primary pulmonic and septic plague, when the disease has symptoms common to those of many infectious diseases, in the absence of a typical epidemic anamnesis of plague [14,15].

The multiplex PCR method can also be used in a complex of laboratory methods for routine epizootological examination of the territories endemic to the specific infection, examination for epidemiological indications and for the purpose of typing strains of infectious agents by the presence of target specific genes [16-18]. According to the researchers, the need for rapid genetic tests is also due to the adaptation for use on-the-spot by persons without microbiological training [19].

PCR assay for plague diagnosis should be easy to use, enabling assessment of the pathogenic potential of a *Y. pestis* strain by the presence of relevant DNA fragments. Most of the currently developed PCR-based assays are designed to identify and study isolated strains; the testing procedure includes several stages. The latter complicates the diagnostic process. Many multiplex real-time PCR or quantitative PCR (qPCR) assays, which detect 5-6 DNA fragments at a time, are intended for use only in research laboratories. The Research Institute mainly develops solely experimental primers necessary for current research [20-22].

Commercial diagnostic PCR assays available on Kazakhstan's market are financially inaccessible to most laboratories in sufficient quantity and range. In particular, assays designed for the diagnosis of plague and other highly infectious diseases (HID) are purchased by anti-plague organizations in an amount insufficient to conduct research on a full scale. Centers for sanitary and epidemiological expertise, diagnostic laboratories for infectious hospitals, given the duration and complexity of the supply, have to pre-purchase the required number of foreign commercial PCR assays for HID diagnostics which after the expiration date have to be disposed without use. Moreover, foreign assays are not registered on the territory of the Republic of Kazakhstan due to the complexity of the procedure and financial costs, thus the results of studies that use them have no legal force. The availability of domestically produced preparations would solve this problem. It is worth noting that many foreign assays vary along with sufficiently high sensitivity and low specificity.

In the Laboratory of Molecular Diagnostics and Genetics of the National Research Center for Highly Infectious Diseases, an assay for the detection and identification of plague microbe was previously developed on the basis of standard multiplex PCR with visualization of amplification results by electrophoretic separation of products in agarose gel. The planned research work on the development of an assay based on real-time PCR is supported by the following reasons:

The standard PCR method is a fairly good method in the set of tests for routine epizootological examination of the plague enzootic area, study of *Y. pestis* collection strains. Real-time PCR is the best option for the diagnosis of this infection in humans and domestic animals that are involved in the epizootic process and are potential disease

sources, as well as for the indication of a plague microbe in environmental objects with epidemic complications. Real-time PCR is characterized by greater sensitivity, uncomplicated sample preparation for amplification, shorter analysis time, which is possible due to the ability to read the results during the amplification reaction.

Most research and diagnostic laboratories in Kazakhstan, including anti-plague organizations, are equipped with equipment for real-time PCR.

Commercial assays for the diagnosis of plague using real-time PCR have a high market cost, are not registered in the State Register of Medical Products of the Republic of Kazakhstan, which means that results of the researches where they are applied are lacking legal force.

Scientific novelty of this work consists in the fact that for the first time in Kazakhstan, based on the analysis of the plague agent genome structure, optimal DNA target sequences were determined for the design of original primers for multiplex real-time PCR aimed at identifying the agent by three chromosomal and plasmid genes at once.

MATERIALS AND METHODS

For the study of the qualitative and quantitative characteristics of the developed assay for the detection and identification of plague microbe in real-time PCR were used 49 *Y. pestis* strains isolated from various objects, 26 strains of closely related and 22 strains of heterologous species obtained from the Republican collection of microorganisms of the National Research Center for Highly Infectious Diseases (NRCHID). The effectiveness of the assay was tested on 288 suspensions from organs of rodents, fleas and mites from natural foci of plague in Kazakhstan.

Y. pestis strains are isolates from various field materials extracted during epizootological survey of the territories of natural plague *foci* in Kazakhstan, Russia and Mongolia in different years: North pre-Aral, Ustyurt, Pre-Ustyurt, Ili intermountains, Pre-Balkhash, Taukum, Muyunkum, Trans-Ural, Aryskum-Dariyalyktakyr, Pre-Aral-Karakum, Volga-Ural sand, Volga-Ural prairies, Saryzhaz, Talas, Betpak-Dala, Kyzylkum, Mangyshlak, Altay mountains, Transcaucasian highland. Alongside this, strains of the following closely related and heterologous bacterial species were used: *Yersinia enterocolitica, Y. pseudotuberculosis, Y. kristensenii, Listeria monocytogenes, Shigella flexneri, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, S. typhimurium, Bacillus anthracis, Francisella tularensi, Staphylococcus aureus.*

Phenotypic properties of all used *Yersinia pestis* strains were investigated in the Microbiology and Epidemiology of Plague Laboratory and by the staff of the Republican Collection of Microorganisms and the Depository of Agents of Highly Infectious Diseases of NRCHID by the generally accepted method [23].

The objects of the research are the primers and fluorescent probes developed for the detection of genes *YPO2088* (chromosomal DNA), *caf1* (*pFra*) and *pst* (*pPst*), assays based on them for multiplex PCR analysis with PCR products detection on the agarose gel, as well as detection of real-time PCR with fluorescent hybridization probes.

Based on the conducted information analysis on *Y. pestis* bacteria identification presented in Pubmed and Gen Bank databases, a number of the most significant species-specificity marker genes were selected. The databases are available on the website of The National Center for Biotechnological Information (NCBI). The properties and characteristics specific to this type of microbe were considered: The distinctive features of virulent strains are encapsulation and pesticide activity. The *caf1* gene, located on the *pFra* plasmid (pMT1) and encoding the capsule antigen F1, and the pst gene, located on the *pPst* plasmid (*pPCP1*) and encoding the bacteriocin of the plague microbe–*pesticin*, were selected [24, 25]. The chromosomal YPO2088 gene (encodes a protein with an unknown function) was selected as the third marker gene due to the existence of plasmid-free "wild-type" strains, which may lack one or both of mentioned plasmids, as well as the possibility of mutations within the target gene sequences. *YPO2088* gene is conserved and specific to *Y. pestis* species. Furthermore, the selection of the chromosomal gene was due to the phenomenon of horizontal genetic material transmission (especially mobile genetic elements) between bacterial cells. In populations of rodents with a colonial way of life, "plague and *pseudotuberculosis* bacteria, and sometimes enteric *yersiniosis* bacteria, can simultaneously circulate in one natural focus,

cause mixed infections and coexist in the body of the same carrier and even a transmitter" [26]. According to Trukhachev et al., plasmid exchange and formation of *Y. pseudotuberculosis* and *Y. enterocolitica* clones with species-specific *Y. pestis* plasmids is possible in conditions of long-term coexistence of closely related species in one macro organism (rodent and ectoparasite-transmitter). The appearance of the *pFra* plasmid in bacteria related to the plague microbe, especially in *Y. pseudotuberculosis*, can lead to errors in the detection and differentiation of *Yersinia* [26].

The primers were designed through online programs "PrimerQuest" and "Primer3Plus". Nucleotide sequences of the selected marker genes were entered into the query window in FASTA format. Additional parameters were set in the program: Melting temperature range (Tm), percentage of GC pairs, primers and amplicon sizes, etc. the program generated several pairs of primers that corresponded to the specified parameters. The selection of primers was carried out according to the main criteria: The length of oligonucleotides is not more than 22 nucleotides, the GC bases permissible percentage is 40-60 %, the melting point (Tm) 56-63 °C and the expected size of amplicons is about 200-500 bp.

Primers *YPO2088 F/R*, *caf1 F/R* and *pst F/R* were developed on the basis of the selected marker genes. The final version of nucleotide sequences is given in Table 1.

The specificity of the developed primers and probes was tested using the BLAST (Basic Local Alignment Search Tool) software package, which allows comparing existing sequences with sequences from the GenBank database.

Synthesis was carried out with subsequent purification of the developed primers. The synthesis was realized by the standard phosphoramidite method with automatic DNA/RNA synthesizer "H6" (K & A Laborgeraete GbR, Germany). For the synthesis, phosphoramidites Glen Research Corporation (USA) and reagents of the company emp BIOTECH (Germany) were used.

Primers attached to the carrier were removed from the columns and placed in screw cap tubes, where 750 µl of concentrated ammonium hydroxide solution was added and incubated at 90°C for 2 hours to release the primers from the carrier.

Purification of aqueous solutions of primers from impurities was carried out on columns Centri Pure N10 (emp BIOTECH GmbH, Germany). Purified aqueous solutions of primers were concentrated to a dry state using the device Centri Vap (Labconco, USA), and then resuspended in deionized water to a concentration of 100 µM. The concentration of primers was measured by the device Nano Photometer® P-300 (Implen).

Development of probes for multiplex PCR

To develop the probes, the search for unique targets in the genome of the plague microbe was carried out using the software "CLC Genomics Workbench" (CLCbio, Qiagen, Germany) and other support programs. Selection and development of fluorescent probes were realized with the help of online programs "PrimerQuest" (https://eu.idtdna.com/PrimerQuest/Home/Index) and "MPrimer 1.4" (http://biocompute.bmi.ac.cn/MPprimer/). A preliminary assessment of the analytical specificity of the used primers and probes using the online BLAST algorithm indicated the absence of homology of the selected sequences with other microorganisms. The probes contain a fluorophore at the 5′ -end and quencher at the 3′ -end. The characteristics of the developed primers and probes are presented in Tables 1 and 2.

The probes annealing temperature is 67-68°C. Synthesis of the developed fluorescent probes was carried out in the company "SYNTOL" (Russia, Moscow). Probes delivered in lyophilized form were diluted to the concentration of 100 µM.

On the basis of the developed primers, an assay was designed for the detection of plague microbe in multiplex real-time PCR. Components of the assay are: Mixture for PCR, primer pairs *caf1 F/ caf1 R, pst F/ pst R* and *YPO-2088 F/ YPO-2088 R*, probes *caf1, pst* and *YPO-2088*, positive control, negative control. The concentration of primers is 20 pmol/µl, concentration of probes is 10 pmol/µl. The composition of the reaction mixture was optimized. Table 3 shows the volume of components of the reaction mixture per 1 reaction and depending on the number of samples. The total volume of the reaction mixture per analysis is 25 µl. Amplification parameters using the developed primers are shown in Table 4.

Table 1: Nucleotide sequences of primers and probes for the detection of plague microbe species-specific genes.

Primers		Nucleotide sequences	Channel
pst	*F-Primer*	GCGTCAGAGGTGCTGTTCTC	Orange
	R-Primer	CGGTGTCCCTCAGTATATTGCA	
	Probe	ROX-TCATAAGCCTCCTTCCCTCGAAGCA-BHQ-2	
caf1	*F-Primer*	GGCAGCCAGGATTTCTTTGTTC	Yellow
	R-Primer	GGTTACGGTTACAGCATCAGTG	
	Probe	R6G-TCAATTGGTTCCAAAGGCGGTAAAC-BHQ-1	
YPO-2088	*F-Primer*	GGCGGTAATATCGGGATGAGA	Green

Table 2: Characteristics of fluorescent dyes and quenchers.

Fluorescent dye and quencher	λmax, nm (absorption)	λmax of fluorescence and quenching range, nm
FAM	490	520
R6G	520	550
ROX	580	610
BHQ-1	535	480-580
BHQ-2	575	550-650

Table 3: Preparation of reaction mix for triplex real-time PCR.

Component	Volume per 1 reaction (tube), µl	Volume per N reactions (tubes), µl
Mixture for PCR	12.5	12.5 X n
	Primers	
caf1 F	1	1.0 X n
caf1 R	1	1.0 X n
pst F	1	1.0 X n
pst R	1	1.0 X n
YPO-2088 F	1	1.0 X n
YPO-2088 R	1	1.0 X n
	Probes	
caf1	0.5	0.5 X n
pst	0.5	0.5 X n
YPO-2088	0.5	0.5 X n
Volume of the sampling DNA	5	
Total reaction volume	25	

Table 4: Amplification program for primers *YPO2088 F/R, caf1 F/R* and *pst F/R* (multiplex PCR).

Stage	Temperature, °C	Time, min:sec	Cycles number
Initial denaturation	95	02:00	1
Denaturation	95	00:15	45
Annealing	60	00:40	

Thus, the assay was designed to detect the plague microbe in multiplex real-time PCR on three marker genes: *Caf1, pst* and *YPO-2088*.

RESULTS

Preliminary control studies of all strains used in the work were carried out using bacteriological methods: colony morphology on nutrient media, sensitivity to specific bacteriophages, some biochemical tests. Plague microbe strains were studied additionally in the passive hem agglutination reaction on the presence of capsular antigen FI using erythrocyte diagnosticum, as well as standard PCR with a specific assay for the detection of the caf1 gene. The purpose was to confirm the relationship of strains to a particular type of microorganisms. Samples of field material were examined only for the presence of the plague agent.

Table 5 presents the number of strains and field material samples used, and the results of control studies (bacteriology, passive hemagglutination reaction and classical PCR). For more information, the table presents the results of specific studies of the material using the developed assay.

The species identity of all strains stated in the passports was confirmed. Immunological analysis of plague microbe DNA samples on the presence of capsular antigen FI revealed 11 so-

Table 5: Number of strains and field materials used, results of control and specific studies on PCR assay effectiveness for detection and identification of plague microbe in real-time.

Sampling material	Material amount	Control studies results (positive, absolute count)			Real-time specific studies results (positive, absolute count)		
		Bacteriology	passive hemagglutination reaction	Classical PCR	caf1 F/R (Yellow)	pst F/R (Orange)	YPO2088 F/R (Green)
Yersinia pestis strains	49	49	38	49	41	39	49
Closely related strains	26	26	-	-	–	–	–
Heterologous species strains	22	22	-	-	–	–	–
Rodent suspensions	16	–	–	–	–	–	–
Fleas suspensions	220	–	–	–	1	1	1
Mites suspensions	52	–	–	–	2	2	2

called afraction strains. According to the results of studies of plague microbe strains in classical PCR, the target fragment of the caf1 gene was not detected in 10 out of 11 afraction strains.

The study of field material samples in PCR with the plague detection of results with gel electrophoresis gave negative results. The main characteristics of any assay for the detection of infectious agents are the specificity (response only to specific targets) and sensitivity (the smallest detectable number of targets) of the assay with their use.

Sensitivity assessment studies on the developed assay for real-time PCR were conducted on the *Y. pestis* strain with typical cultural, morphological, antigenic properties. Fragments of all target genes were found in the strain genome: *YPO2088, caf1* and *pst*. A cell suspension of 1 X 109 in 2 ml of 0.9 % sterile sodium chloride solution was prepared according to the standard turbidity sample of 10 units of the Tarasevich State Research Institute for Standardization and Control. By ten-fold dilutions in 0.9% sodium chloride solution, a suspension of 1 X 106 was obtained. DNA with a concentration equal to 1 ng as measured on the device Nano Photometer® P-300 (Implen, Germany) was isolated from the suspension. A series of *Y. pestis* DNA ten-fold dilutions were performed to obtain different concentrations: From 1 ng to 10 ag (Table 6). The real-time PCR was conducted with the obtained DNA dilution by using the designed assay. The results are shown in Figure 1 and Table 7.

According to the PCR results, the sensitivity of the assay detecting plague microbe specific genes is 100 fg per the pair of primers *YPO-2088 F/R*, in terms of microbial cells, this corresponds to 100 microbial cells per 1 ml. For primers caf1 F/R and *pst* F/R, the lowest DNA concentration that they detected was equal to 10 fg (from 10 and above microbial cells per 1 ml).

Studies on specific identification of fragments of marker genes of the plague microbe by primers of the designed assay for real-time PCR were conducted on plague microbe strains, including a fraction strains.

Studies have revealed the absence of a target fragment of the *pst* gene in 10 DNA samples. Only eight (8) out of 11 a fraction strains did not comprise the caf1 gene. In other three strains, the negative results of passive hem agglutination reaction can be explained by the low level of capsule antigen synthesis, insufficient sensitivity of the method. Two of these three strains were isolated on the territory of the Transcaucasia highland plague center, where strains with a very low epidemic potential circulate in the populations of the Common Vole (*Microtus arvalis*) [27]. The third strain was isolated on the territory of the Volga-Ural prairies plague center that was active in the early 20th century, where the main infection carrier is a little ground squirrel (*Spermophilus pygmaeus*). Highly virulent *Y. pestis* subsp strains circulate in this area. The causes of the decrease in the level of F1 secretion can be different, ranging from unfavorable conditions of strain cultivation to point mutations in DNA, and require study. The specificity of the PCR assay for detection of the plague agent was also assessed on DNA samples of collection strains of three closely related *Yersinia* species, as well as of nine heterologous species of eight genera. Some of the results of the research are presented in Table 8 and Figure 2.

According to the figures (in all three figures only exponential curves of positive controls– red lines) and the tables, developed primers for the detection of a plague agent showed the

Table 6: *Y. pestis* DNA dilutions.

Concentration in microbial cells/ml	Amount of DNA in 1 µl
106	1 ng
105	100 pg
104	10 pg
103	1 pg
102	100 fg
101	10 fg
1	1 fg
-	100 ag
-	10 ag

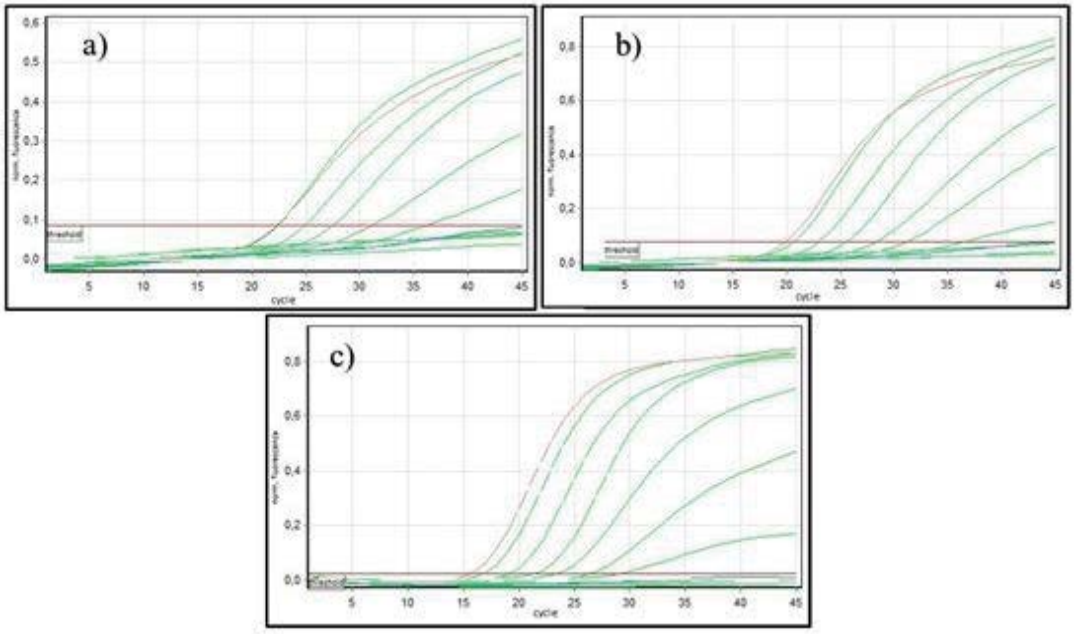

Figure 1: Assessment of real-time PCR sensitivity, a series of *Y. pestis* DNA ten-fold dilutions to obtain various concentrations: From 1 ng to 10 ag. Channels fluorescence curve: A: FAM (green) for the pair of primers *YPO-2088* F/R; B: R6G (Yellow) – *caf1* F/R; C: ROX (Orange)–*pst* F/R.

Table 7: Results of sensitivity of real-time PCR with designed multiplex assay for plague microbe detection and identification.

Concentration in microbial cells/ml	Amount of DNA in 1 µl	Results of PCR with primers		
		YPO-2088 F/R	caf1 F/R	pst F/R
106	1 ng	+	+	+
105	100 pg	+	+	+
104	10 pg	+	+	+
103	1 pg	+	+	+
102	100 fg	+	+	+
101	10 fg	-	+	+
1	1 fg	-	-	-
-	100 ag	-	-	-
-	10 ag	-	-	-

absence of nonspecific reactions with DNA of non-target bacteria species: *Y. enterocolitica, Y. pseudotuberculosis, Bacillus anthracis*. Similar results were obtained with DNA strains of other heterologous species of bacteria used in the study.

The effectiveness of the developed assay for the detection and identification of plague microbe in real-time PCR was tested on samples of biological material collected on the enzootic plague area in Kazakhstan. Fragments of all target genes *YPO2088, caf1* and *pst* were found in three samples (Table 7). In one fleas suspension collected on the territory of Zhambyl region, and in two mites suspensions of *Hyalomma asiaticum* species collected in Aktobe region. Preliminary studies of the field material used in the work by generally accepted methods of epizootological

Table 8: Results of assessment of specificity of real-time PCR with designed multiplex assay for plague microbe detection and identification.

Samples and controls	Results of PCR with primers		
	YPO-2088 F/R	caf1 F/R	pst F/R
Y. pseudotuberculosis	–	–	–
Y. enterocolitica	–	–	–
Bacillus anthracis	–	–	–
Negative control	–	–	–
Positive control	+	+	+

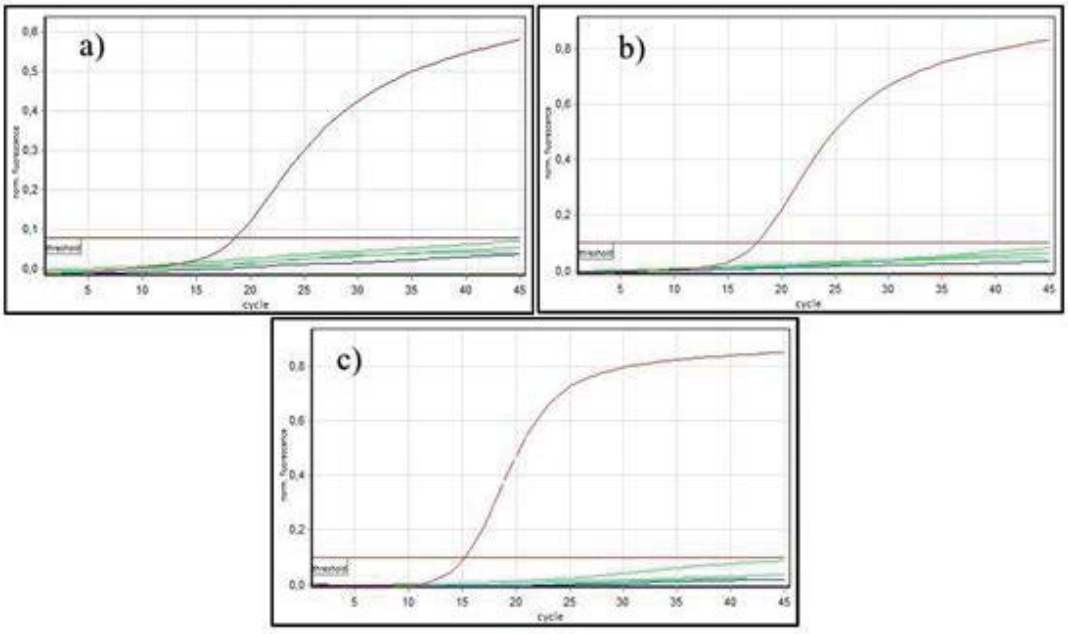

Figure 2: Assessment of real-time PCR specificity on the DNA samples of collective strains of closely related species *Y. pseudotuberculosis* and *Y. enterocolitica, Bacillus anthracis* strain. Channels fluorescence curve: FAM (Green) for the pair of primers *YPO-2088* F/R; b) R6G (Yellow) – caf1 F/R; c) ROX (Orange)–pst F/R.

examination (bacteriological, serological) did not reveal the presence of a plague microbe or its traces (specific antibodies). PCR analysis with detection of results by electrophoresis also gave a negative result. Such low efficiency of laboratory researches corresponds to a sharp decrease in the level of plague epizootic process activity on enzootic territories of the country in 2018-2019. According to experts, climate change towards warming could be one of the causes of depression of parasitic systems, including in natural foci of plague on all continents [28,29]. However, the results of our studies have shown that the developed assay for the detection and identification of plague microbe has sufficient sensitivity and specificity.

DISCUSSION

The designed new assay is aimed at detection and identification of the plague agent on the basis of multiplex real-time PCR on three species-specific plasmid and chromosomal genes. The assay is able to specifically identify not only typical Yersinia pestis strains, but also strains with different variants of the species-specific plasmid content–monoplasmid and non-plasmid ones, occurring not only among laboratory but also among "wild-type" strains. The assay possesses a high sensitivity, revealing DNA at a concentration of 100-10 fg/µl, which in terms of the concentration of microbial cells corresponds to 100 to 10 microbial cells per 1 ml. This plays important role for the detection and identification of the plague agent in biological material and in samples from environmental objects containing a small amount of the microbe, both during surveys for epidemiological indications, and during routine surveys of enzootic plague areas. Good sensitivity and specificity of the assay for multiplex real-time PCR acquire particular importance when the artificial nature of plague epidemic process is suspected, when not only speed, but also the accuracy of the result is vital. The etiological basis of the epidemic in these cases is usually laboratory strains of the pathogen with altered properties. Most likely,

the changes may relate to the DNA marker areas, most often used as targets in genetic diagnosis. In the case of the plague microbe, these are the plasmid genes *caf1* and *pst*.

Commercial diagnostic assays for real-time PCR, that are available on the Kazakhstan's market, are designed for detection of plague microbe by one gene, often by *caf1* and pst genes located on *pFra* (*pMT1*) and *pPst* (*pPCP1*) plasmids, respectively. The existence of natural mono- and non-plasmid strains of *Y. pestis*, the possibility to form the *Y. pseudotuberculosis* and *Y. enterocolitica* clones with species-specific *Y. pestis* plasmids as a result of horizontal transmissions in long-existing mixed infections in rodent populations may be the cause of false negative and/or false positive results of PCR studies. The optimal technological solution to this problem is simultaneous detection of at least two or three targets in one multiplex PCR, including chromosomal genes. In this work, primers were used for marker sequences of three genes: *caf1 (pFra)*, responsible for capsular antigen production, *pst (pPst)*, determining the synthesis of bacteriocin of the plague microbe–*pesticin*, chromosomal *YPO2088*, encoding a protein with an unknown function.

Y. pestis, the etiologic agent of plague, is a recently emerged clone of *Yersinia pseudotuberculosis* that may have diverged as recently as ~*3300YBP* [30]. This recent emergence has led to an overall lack of genetic diversity in this pathogen, making many subtyping methods commonly used in other species of little or no use for differen-tiating among strains of *Y. pestis*. Complicating the search for genetic di-versity is the fact that older more genetically diverse populations within *Y. pestis* are also geographically restricted [31-32] and, therefore, are often not well represented in strain collections used for subtyping studies. In contrast, the most geographically wide-spread population, which often dominates strain collections, displays even less genetic diversity than *Y. pestis* as a whole, due to its very recent emergence. This population, ORI (classically referred to as *biovarorientalis*), consists of a highly successful clone within *Y. Pestis* [33] that emerged as recently as ~205 *YBP* [34], and then spread around the globe in the late 1800s and early 1900s [35]. The very recent emergence and consequent low genetic diversity of this population have further restricted the number of useful subtyping methods when discrimination within this population is desired. Despite these challenges, the historical significance, high virulence, and potential threat of *Y. pestis* have led to many subtyping efforts targeted at identifying, classifying, and/or tracking this pathogen. Early subtyping efforts included phenotypic assays, serotyping, phage typing, and plasmid analysis. Various fragment-based subtyping methods have also been utilized, including restriction fragment length polymorphism (RFLP) based approaches, insertion sequence (IS) element based approaches, different region (DFR) analysis, locally collinear block (LCB) linking modes, random amplification of polymorphic DNA (RAPD), repetitive element sequence based-polymerase chain reaction (REP-PCR), and variable-number tandem repeat (VNTR) analysis. Fragment sequencing-based subtyping methods that have been used include 16S analysis, multi-locus sequence typing (MLST), and clustered regularly interspaced palindromic repeats (CRISPR) analysis. Most recently, whole genome sequencing has allowed for large scale single nucleotide polymorphism (SNP) based approaches and the development of highly accurate phylogenies for *Y. pest* is on a global scale, which may eventually render many other subtyping methods obsolete. This article reviews various subtyping efforts, their efficacy for various applications and current usefulness, and the future of *Y. Pestis* subtyping.1.2. Evolutionary context of subtyping Successful subtyping depends upon at least three major compo-nents: The organism being subtyped, the subtyping method being used, and the scientific question being asked. The successful interaction of these three components (i.e., the success of a subtyping method in answering a particular scientific question for a specific organism) is impacted by several factors, including the type and amount of variation being detected, the mutation rate(s) associated with that variation, the evolutionary rate, the propensity for convergent evolution, population age, clonality/recombination rate, and selective versus neutral variation, among others. Continued use/widespread adoption of any par-ticular subtyping method is further dependent on the reliability, dis-criminatory power, cost, and difficulty of the method, as compared to other developed methods.

CONCLUSION

The proposed assay for the detection and identification of the plague agent based on multiplex real-time PCR with fluorescent hybridization probes specifically identifies only bacteria of the Y. pestis species. Studies have shown the absence of nonspecific reactions with DNA of non-target bacteria species. The sensitivity of the PCR method using this assay was equal to 10-100 microbial cells/ml. The preparation planned to be released has great commercial potential. In the case of registration in the State Register of Medical Products, it is possible to produce products competitive in the market of Kazakhstan, which will be used by consumers for the etiological diagnosis of plague in humans and animals; detection and identification of *Y. pestis* strains; indication of plague microbe in environmental objects; genetic typing of isolated *Y. pestis* strains.

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