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Development of a Domestic Real-Time Polymerase Chain Reaction (PCR) Test System for the Identification of Strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*

[1]Gulzhagan Chuzhebaeva, [2]Beishova Indira, [1]VadimUlyanov, [1]Kokanov Sabit, [3]Zhumagul Kirkimbaeva, [3]Svetlana Yermagambetova and [3]Batyrzan Mustafin
[1]*Department of Veterinary Sanitation of Kostanai State University, Kostanay, Kazakhstan*
[2]*Department of Biology and Chemistry of Kostanai State University, Kostanay, Kazakhstan*
[3]*Kazakh National Agrarian University, Almaty, Kazakhstan*

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Corresponding Author:
Beishova Indira
Department of Biology and Chemistry of Kostanai State University, Kostanay, Kazakhstan

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Abstract: Pseudotuberculosis and intestinal yersiniosis are among the relatively new infectious diseases that have been particularly intensively studied in the past 25-30 years. Intestinal yersiniosis refers to food zoonoses, i.e., to diseases, the source of infection in which are sick wild, agricultural, domestic animals and the transmission factors-food: milk, meat, vegetables and fruits. The wide distribution of iersiniosis, the variety of the clinic and the laboriousness involved in making the diagnosis made the problem of intestinal iersiniasis on a global scale urgent. The Polymerase Chain Reaction (PCR) method is more sensitive and specific in comparison with microbiological methods and its modification in the Real-time format using a hybridization probe with fluorescent The label allows you to quickly analyze the results without conducting electrophoresis.

INTRODUCTION

Yersinia enterocolitica and *Yersinia pseudotuberculosis*, two species of Yersinia which are enteropathogenic for both animals and humans are spreading around the world and often cause bowel disease in people with moderate and cold climates. *Y. enterocolitica* is the main cause of foodborne illness and causes significant economic losses. Nevertheless, the study of enteropathogenic Yersinia species is quite rare in clinical laboratories because of their specific growth characteristics which make it difficult to isolate them from microorganisms. In addition, the current diagnostic procedures are time consuming and costly which leads to an underestimation of the frequency of intestinal yersiniosis and improper treatment. Diagnosis of acute intestinal infections including those caused by Yersinia, is characterized by low effectiveness of means and methods of their verification. This situation determines the steady attention of scientists to the improvement of bacteriological and the development of immunochemical, molecular-biological methods of laboratory diagnosis of pseudotuberculosis and intestinal yersiniosis. Of particular importance for the selection of the optimal treatment regimen is the early diagnosis of these diseases with the help of highly specific and sensitive test systems[1].

In the European countries and the Russian Federation for the diagnosis of yersiniosis, PCR diagnostic methods are used. Varieties of the PCR method are used for both diagnosis and identification of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* at the serotype level, for the quantitative determination of these microorganisms in food. Methods of PCR analysis based on various

virulence genes, methods of analysis of the Yersinia genome are developed[2-5]. In 2014, Belgian scientists using the PCR methods developed for various genes of virulence and invasiveness of *Yersinia enterocolitica* and *Yersinia enterocolitica*, conducted studies to determine the infection of pigs with yersiniosis at slaughter stations and iersiniyami dangerous for humans. It was found that the incidence of *Yersinia enterocolitica* varies from 5.1-64.4%, *Yersinia pseudotuberculosis* from 2-10%[6]. To determine the prevalence of bioserotypes in various food products, pork and beef from different regions of Malaysia, researchers of three scientific institutes, biochemical studies of isolated Yersinia isolates were carried out with further confirmation by the PCR method. The results of the PCR study showed a high prevalence of *Yersinia enterocolitica* 60.3, 41.7 and 27.9% in products from pork, other foods and pork, respectively[7].

The development of a domestic test system for the identification of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* will allow for the control of this zooanthropo-oonotic infection and timely address food security issues. The use of local, Kazakhstani strains in the development of PCR for the identification of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* will increase the specificity of the reaction, since, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* have many serotypes circulating in different regions and countries.

MATERIALS AND METHODS

Strains of *Yersinia enterocolitica* and 2 strains of *Yersinia pseudotuberculosis*; Isolation of DNA. Samples of the material received were plated on a specialized broth for the growth of yersinia, after the appearance of growth, 1 ml was taken from the broth to isolate the DNA. Isolation was performed using the commercial kit "PureLink® Genomic DNA Kits" produced in the USA. The procedure for DNA isolation was performed according to the instructions attached to the DNA extraction kit "PureLink". The DNA concentration was determined using a Halo DNA master Dynamica spectrophotometer ("Dynamica GmbH", UK). Before introducing into the reaction mixture, the DNA isolated from the cultures was diluted to 10 ng mL^{-1}. Selection of specific primers and fluorescently-labeled probes. The search for nucleotide sequences for the selection of specific primers was performed using the GenBank NCBI online program (http://www.ncbi.nlm.nih.gov/GenBank). The alignment of the nucleotide sequences was performed using the Vector NTI advance program complex. The primers and probes were operative using Oligo 6.71.

PCR and analysis of results: Amplification was carried out using the QuantStudio 5 Real-Time PCR System ("Applied Bio systems™", USA). The composition of the reaction mixture consisted of: primer F (10 pmol) 1.5 μL, primer R (10 pmol) 1.5 μL, dNTP 2 mM 3 μL, BuferKCl 10× 3 μL, MgCl2 25 μM 3 μL, deionized water 12.8 μL, Taq Polymeraza 0.2 μL, DNA 5 μL. As the reaction progressed, the dependence of the fluorescence level on the cycle number was determined for each sample and a corresponding graph was plotted using the software attached to the amplifier. A threshold analysis method was used to determine Cq values. Primers and fluorescently-labeled probes were synthesized in ZAO "Sintol" (Moscow).

PCR was performed in accordance with the following amplification program: pre-denaturation 95°C-4 min; 95°C-30 s, 52°C-30 s; 72°C-20 s (30 cycles), post-replication 72°C-5 min. Cloning positive controls with the primers chosen earlier, amplification was carried out with obviously positive samples. Then, the amplified region was inserted into the plasmid T-vector (T-vector-has a T segment at the ends (thymine), the length of the vector was 3 kb). The amplification was incubated with the polymerase, dNTA was added and placed for 45 min at 72°C, for the synthesis at the A-adenine ends. The resulting inserts were incubated with the T vector at 30°C for 30 min. with addition of ligase and ligase buffer. The concentration of inserts was 2-4 times greater than the vector.

Preparation of cells: *E. coli* cell cultures with the ΔM13 mutation were used. From this culture, chemically competent substances were obtained under the influence of temperature (hit-shock, rapid heating). The frozen cell suspension was placed in 1.5 mL of the tube in a volume of 50 μL+0.5-5 μL of the vector with the insert and left on ice for an h, during which time the vectors were adsorbed on the cell surface. Then it was sharply heated in a prepared water bath (42°C) for 30 sec-1.5 min while in the cytoplasmic membranes pores were formed, through which the vectors penetrated. The resulting suspension was transferred to an enriched nutrient medium and incubated for one hour with periodic shaking (to ensure aeration). They made a medium (1.5-2%) containing antibiotic (ampicillin) on LB. Incubated for 12-24 h, after incubation, only white colonies were selected (because only the vector with insertion was integrated in them). Plasmid DNA was isolated by commercial kits and tested for its quality in a 0.7-1% agarose gel. At the same time, two bands (twisted plasmid and torn fragment) were seen in the upper part of the staircase as well as in the lower part was seen bend-short sections of RNA. Evaluation of the molecular weight of the fragments was carried out using DNA markers with a molecular mass of 50 base pairs GeneRuler. The results of electrophoresis were visualized on the gel-documenting system QUANTUM.

To establish the length of the plasmid and its purification, it was placed on restriction. The restriction

site was in the inserted site. After this, the ring plasmid becomes linear and one band (3 kB+amplified section) is visible on the fore face. After that, the concentration of DNA was measured. As a result, a positive control sample was obtained. As a PC, a plasmid construct with an insert size of 235 bp was used for *Yersinia enterocolitica*, 333 bp for *Yersinia pseudotuberculosis* flipped with inverted sequences homologous to the primer used to amplify the internal control sample. One specific fragment for each primer pair was used to clone positive controls.

RESULTS AND DISCUSSION

Search for specific markers in the genome of the microorganisms: The genome of microorganisms of the genus Yersinia consists of one round chromosome, 4.552.107 bp in length and the virulence plasmid PYV, 69.704 bp in length, the entire genome contains a total of 4.021 coding sequences (CDS). 76.6% of which can be annotated with known or predictable functions. The chromosome contains 3935 genes of which 3012 had definite functions and 923 were closest to the prospective proteins of the public database. In addition, 85 pseudogenes were found as well as 71 genes encoding tRNA and 7 rRNA operons. The pYV plasmid was found to carry 86 genes encoding proteins and 6 pseudo genes. Proposed unique genomic islands.

Genomic islands are included in the T3SS cluster, the ATP transport system, the insecticidal toxin complex (TC), the cholera vibrio RTX gene, the colicin E2 protein gene, the flagellar (Flag-2) gene cluster, the respiratory respiratory gene complex. Pathogenicity islands (HPI), Yersinia secretion genes (yts1 and YSA). As target genes, ypo2088, a gene encoding a methyl transferase and a PLA gene encoding a plasminogen activator, an invasive locus (ail) protein attachment gene and a ribosomal RNA (16sRNA) gene, a Hypothetical Protein (HP) gene, a gene coding sequence enzyme, rhenyl-phosphate, alpha-N, acetyl-glucosamenyl transferase (rfe).

Selection of specific primers and fluorescently-labeled probes: The next stage, after the search for target genes was the selection of specific primers. For this purpose, the selected genes were aligned, alignment was performed with target organisms and the closest related microorganisms. In the study of the alignment results, in some cases, no specific sites were found for the targeted organisms, in other situations when the appropriate sites were found, inadequate results of the "BLAST analysis" were obtained, or the melting temperature was not suitable for the requirements of PCR, also we have eliminated oligonucleotides containing defective regions (so-called dimers, tails and loops). As a result, sections of the ail and HP genes proved to be satisfactory in all requirements (Fig. 1).

As a result, after the above actions were performed, the primers and probes necessary for the identification of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* were selected and meet all the requirements for oligonucleotides. The list of matched sequences is given in Table 1. In the future, for each stage of amplification, the temperature and time were selected experimentally. The temperature of the denaturation stage was chosen between 92-95°C, annealing between 58-66°C and the elongation between 70-75°C. Based on the optimal time and temperature parameters chosen during the experiments for all the stages of amplification, the following mode was developed for PCR:

- Pre-denaturation: 95°C-4 min
- Denaturation: 95°C-30 s
- Annealing: 58°C-30 s 30 cycles
- Synthesis: 72°C-20 s
- Post-replication 72°C-5 min

After the time and temperature conditions of the amplification were determined, the content of the components in the reaction mixture was optimized and the PCR was perfected. The concentration of MgCl2 largely determines the specificity and sensitivity of PCR, affects the primer annealing, the denaturation of the sample, in addition, MgCl2 is necessary to maintain the activity of Taq polymerase. However, an excess of magnesium chloride can cause the production of a non-specific amplificate. In the case of a change in concentration, a qualitative or quantitative change in the resulting amplification occurs. To select the optimal concentration of magnesium chloride, reaction mixtures with the

Table 1: List of selected oligonucleotides

Types	Target gene	Oligonucleotide	Sequence	Length
Yersinia enterocolitica	ail	Upper primer	5´-ACTCGATGATAACTGGGGAG-3´	20
		Lower primer	5´-CAATGTGTCTGAACTTTCTTCAC-3´(gtgaagaaagttcagacacattg)	23
		Probe	5´-(BHQ1)-TAACATATTCGT(FAMdT)GATGCGGAAAGAT GGCC-3´ (ggccatctttccgcatcaacgaatatgtta)	30
Yersinia pseudotuberculosis	HP	Upper primer	5´-GACTACACCGGTGAAATAGACA-3´	22
		Lower primer	5´-AGCAATGTGTCTGAACTTTCTTCA-3´ (tgaagaaagttcagacacattgct)	24
		Probe	5´-(BHQ1)- GAGTTGGTTCGCAG(FAMdT)AAATCCGCCAGC -3´	27

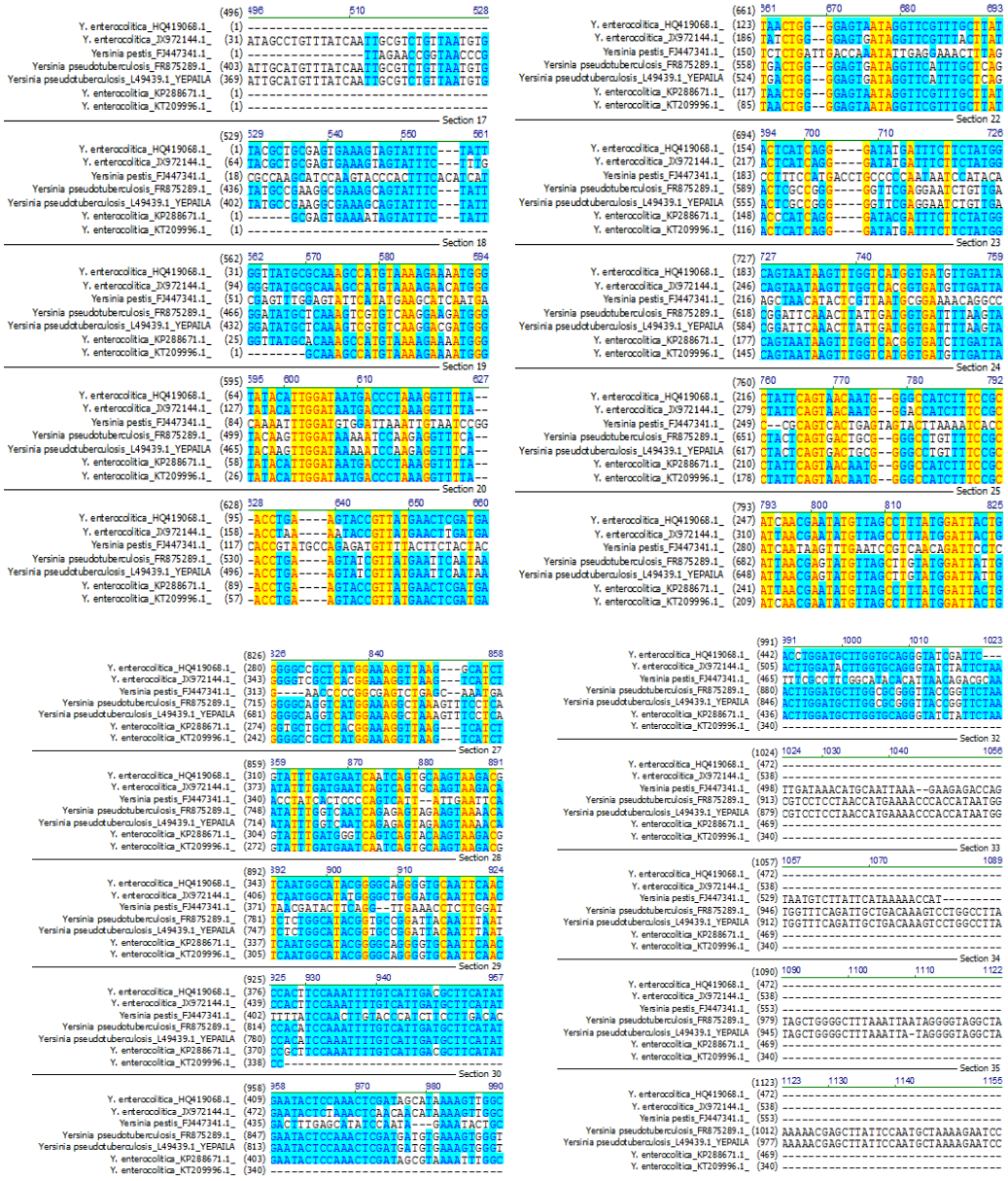

Fig. 1: Alignment of fragments of the geneail of microorganisms of the genus Yersinia (yellow areas indicate complete coincidence of nucleotides, blue-partial matching, green-50% coincidence, colorless areas-unique sequences)

following concentration of MgCl2 were used: 1.5-4.5 mm (Fig. 2). 1-1.5 mM, 2-2 mM, 3-2.5 mM, 4-3 mM, 5-3.5 mM, 6-4 mM, 7-4.5 mM. The results of the experiments (Fig. 2) showed that the concentration of MgCl2 influenced the amplification process in all cases. The best concentration of magnesium chloride was that in which the lowest value of C (T) (the threshold cycle) and the largest value of fluorescence were observed. As can be seen from Fig. 2, the most optimal concentration is 2.5 mM. In all cases, the value of the fluorescence of the IC probe exceeded by 2.5 times the level of its background fluorescence which indicates the absence of inhibition of PCR. In embodiments where the Observed formation of a specific PCR product is less than the VC signal several times compared to variants in which the formation of a specific product does not occur. The DNA results of *Y. enterocolitica* and *Y. pseudotuberculosis* spore cultures using quantitative PCR using the developed

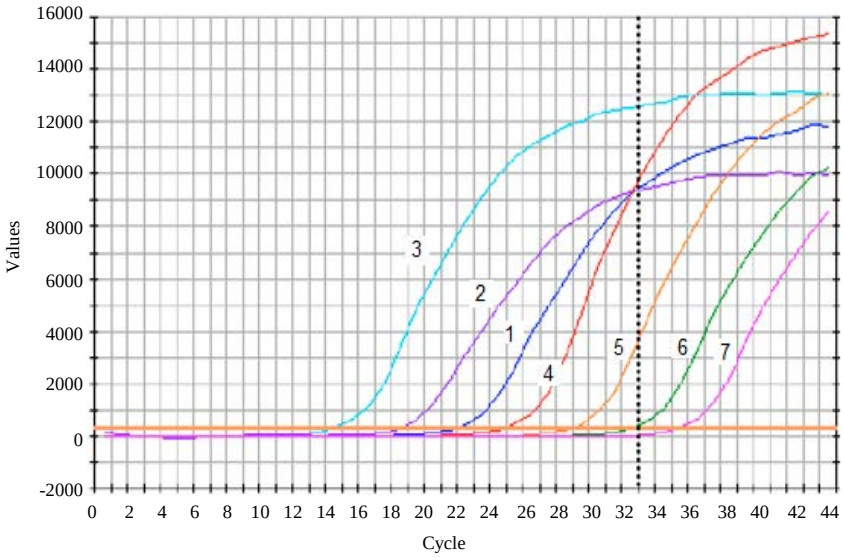

Fig. 2: Optimization of MgCl2 concentration

Table 2: Results of quantitative PCR using the developed primers and probes for *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. HEX-internal control. "-" negative control

Examples	Cq (FAM)	Cq (HEX)
(*Y. enterocolitica*)		+ (33.1)
(*Y. enterocolitica*)		+ (32.8)
(*Y. enterocolitica*)		+ (31.5)
(*Y. enterocolitica*)		+ (30.9)
(*Y. enterocolitica*)		+ (31.7)
(*Y. enterocolitica*)	+ (22.5)	+ (33.3)
(*Y. enterocolitica*)	+ (22.4)	
(*Y. enterocolitica*)	+ (21.3)	
(*Y. enterocolitica*)	+ (21.6)	
(*Y. enterocolitica*)	+ (20.9)	
-		+ (32.5)
(*Y. pseudotuberculosis*)	+ (20.1)	+ (30.2)
(*Y. pseudotuberculosis*)	+ (22.9)	+ (32.1)
(*Y. pseudotuberculosis*)	+ (20.9)	
(*Y. pseudotuberculosis*)	+ (21.6)	+ (30.5)
(*Y. pseudotuberculosis*)		+ (32.3)
(*Y. pseudotuberculosis*)		+ (30.7)
(*Y. pseudotuberculosis*)		+ (33.6)
(*Y. pseudotuberculosis*)		+ (34.1)
(*Y. pseudotuberculosis*)		+ (30.2)
(*Y. pseudotuberculosis*)		+ (31.2)

primers are shown in Table 2. In some samples there is no signal from internal control, apparently, this may be due to competitive inhibition of a specific sample. Thus, none of the pairs of primers showed a cross-reaction with other related species.

Evaluation of the effectiveness of PCR-RV. To evaluate the effectiveness, sensitivity of the reaction and the presence of potential inhibitors, a standard procedure was used in the reaction mixture. For this purpose, PCR-PB was carried out for consecutive tenfold dilutions of specific genomic DNA. Each concentration (in the range of 101-106 pg DNA per reaction) was analyzed in four replicates. The sensitivity of the test systems was about 10 pg of specific DNA per reaction which is about 200 copies, taking into account the size of the genome of microorganisms of the genus Yersinia. The efficiency of PCR was determined experimentally by successive dilutions of the sample of the analyzed DNA. Each dilution was analyzed in several replicates. The efficiency of the reaction was 96% in the experiments which is a very high efficiency value for PCR diagnostic systems.

CONCLUSION

As a result, we developed a test system that allows detecting microorganisms of the genus Yersinia: *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*, these pathogens are widespread in all regions including in Northern Kazakhstan. The test system has the necessary specificity and sensitivity, excludes false-positive results.

Also, selected probes (labeled with a fluorescant label) allow working in Real-Time mode which excludes from the diagnostic process the electrophoresis stage, i.e., does not require the use of carcinogenic substances that are used for electrophoretic separation of PCR products. Those. the results can be read already during the PCR reaction.

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