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A METHOD FOR DETECTING AND TYPING OF SALMONELLA BY MULTIPLEX PCR

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Abstract

Today in Ukraine's market is increasing the volume of trade with livestock products. Also the number of catering services and grocery shops selling ready-made food is growing throughout the country. The veterinary service should have time to check the quality of all of these products. Only traditional bacteriological methods of isolation and identification of pathogens of toxicoinfection, which is not enough in terms of increasing turnover of products, are used today. The one of the most dangerous toxicoinfections is salmonellosis. Typing different *Salmonella* species gives an answer about the source of infection. The aim of our work was to develop a system of identification of *Salmonella* and typing among them five serovars based on the polymerase chain reaction (multiplex PCR). We performed analysis of the nucleotide sequences of the five members of the genus *Salmonella*, on the basis of which a primer designed for the identification of any member of the genus Salmonella with simultaneous typing - *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum by multiplex PCR. The protocol of multiplex PCR was optimization with simples positive DNA matrix.

Key words: salmonella, multiplex PCR, typing DNA.

MULTIPLEX PCR METOD ZA DETEKCIJU I TIPIZACIJU SALMONELA

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Kratak sadržaj

Danas na ukrajinskom tržištu postoji povećanje obima trgovine animalnim proizvodima. Takođe, broj ketering agencija i trgovina mešovite robe koje prodaju gotovu hranu raste u celoj zemlji. Veterinarskoj službi treba dosta vremena za proveru kvaliteta svih tih proizvoda, jer danas na raspolaganju imamo samo tradicionalne bakteriološke metode izolacije i identifikacije uzročnika toksoinfekcija, što nije dovoljno s obzirom na sve veći promet proizvoda. Jedna od najopasnijih toksoinfekcija je salmoneloza. Tipizacija različitih *Salmonella* vrsta daje odgovor o izvoru zaraze. Cilj našeg rada bio je razvijenanje sistema zasnovanog na lančanoj reakciji polimeraze za identifikaciju bakterija iz roda *Salmonella* i tipizaciju pet serotipova (multiplex PCR). Izvršili smo analizu nukleotidnih sekvenci za pet članova roda *Salmonella* i dizajniranje prajmera za identifikaciju bilo kog člana roda *Salmonella* sa istovremenom tipizacijom - *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum metodom multiplex PCR. Optimizacija protokola metode multiplex PCR je izvršena sa jednostavnim pozitivnim matricama DNK.

Ključne reči: *Salmonella*, multiplex PCR , DNK tipizacija

INTRODUCTION

Salmonellosis - one of the most dangerous diseases that is caused by serotypes of bacteria of the genus *Salmonella*, which have mechanisms for habitat and parasitism in the gastrointestinal tract (Althouse et al., 2003; Chiu et al., 2010).

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According to the current classification, *S. enterica* is divided into six subspecies: *Salmonella enterica* subspecies enterica, *Salmonella enterica* subspecies salamae, *Salmonella enterica* subspecies arizonae, *Salmonella enterica* subspecies diarizonae, *Salmonella enterica* subspecies houtenae and *Salmonella enterica* subspecies indica, which differentiate by the biochemical activity and represent the number of subtypes I, II, IIIa, IIIb, IV , and VI , respectively. *Salmonella enterica* subspecies enterica is mostly isolated in the majority of cases of *Salmonella* infection from animal and human (Althouse et al., 2003; Battistuzzi et al., 2004).

Salmonella contamination occurs through the consumption of contaminated food: eggs and egg products, milk and dairy products, meat birds and other animals. Another way of infection is the transfer of infections through tap water, in addition, the sources of infection can be the open water (Bailey, 1998). According to the FAO, 20% of poultry products in the world are contaminated with *Salmonella*, and they can persist for a long time in the animal facilities because they can form a surface film (Vestby et al., 2009; http://www.fao.org/docrep/012/i1133e/i1133e00.htm). Annually on the planet are registered 21 million cases of typhoid fever, and about 216 thousand cases (Zhou and Pollard, 2010).

Worldwide, the monitoring of the incidence of salmonellosis in which tracked various options for its manifestation. As well as a comparison of *Salmonella* strains isolated from humans and animals (Chiu et al., 2009; Chiu et al, 2010; Laupland et al, 2010).

The system of quality control of food, raw materials, based on the use of bacteriological methods of investigation (D'Aoustlnt, 1991).

As an alternative to traditional bacteriological methods for the identification and typing of *Salmonella* proposes the use of polymerase chain reaction (dos Santos, 2001; Zahraei Salehi et al., 2005; Eyigör et al, 2007; Cao et al., 2008; Mirmomeni et al., 2008; Zhou et al., 2010).

Analysis of antigen alleles H1 (i, g, m, r or z10) allowed fast typed serological variants enteritidis, hadar, heidelberg and typhimurium (Hong et al., 2008).

To date, Ukraine has not yet widespread methods of rapid diagnosis of salmonellosis. Typing of the pathogen is an essential component of diagnosis, because it can give an answer about the alleged source of infection. For this reason, the aim of our work was the development of the national test system based on the polymerase chain reaction, which would like to identify and typed some key members of the genus *Salmonella* (Gerylovich, 2011).

METHODS AND MATERIALS

The objects of our study were *Salmonella spp*., *Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. *Gallinarum*-Pullorum. For the construction of genus-and species-specific primers electronic databases of sequences of essential genes in *Salmonella* contained in the international database GenBank (http://www.ncbi.nlm.nih.gov/genbank/) were analyzed.

Multiple alignment of selected sequences, and their subsequent analysis to select PCR primers was performed using the computer program Bio Edit (v.7.2.4).

The protocol of polymerase chain reaction has been developed on the basis of the primer systems with a certain temperature, the selection of components for the formulation of the multiplex PCR and the identification of the genus *Salmonella spp*. and typing of the five listed above serotypes (Elnifro, 2000; Kaderali, 2007).

One-day-old cultures of *Salmonella* from the museum sector study mycoplasmoses and salmonellosis are grown for meat - peptone medium were used as the source of positive DNA-matrix.

Extraction of total nucleic acid was carried out using micro columns. To 450 μl of Extraction buffer was added 100 μl of *Salmonella* culture. After lysis of the containments from the tubes were transferred to microcolumns and centrifuged. This was followed by washing with ethanol followed by extraction of total nucleic acid of TE-buffer.

DNA concentration was calculated by spectrophotometery at 260 nm.

RESULTS AND DISCUSSIONS

The nucleotide sequences of the major genes were analyzed. The greatest breadth of sample homogeneity and sequenced portions of the gene was detected in *invA* for all members of the genus *Salmonella*. In the computer analysis of the gene sequences *invA* was selected 22 pairs of oligonucleotides - potential pairs of primers for PCR. The PCR product limited by size of 387 bp in length, and olygonucleotides were called Salm3_4.

For *Salmonella enterica* ser. Enteritidis specific motifs were found in the gene *SefA*. Sequence analysis of this gene allowed to establish the potential 6 primer pairs. The primers flanking portion length 299 bp were selected.

The gene *fliC* demonstrated specificity for *Salmonella enterica* ser. Typhimurium. The primers flanking region 420 bp were choosed.

Gene *viaB* contained specific motives for *Salmonella enterica* ser. Typhi. Accordingly, on this basis was chosen area, which limited the targeted gene fragment length 738 bp.

For the genome of *Salmonella enterica* ser. Dublin serospecific motifs were found in SeD_A1104 gene. When bioinformatics studies were identified primers flanking the product of 203 bp.

Finally, gene SG0266 was elected by containing specific motifs for *Salmonella enterica* ser. Gallinarum-Pullorum. Specific primers flanking length of 97 bp region were selected in analyzed area.

Table 1. Nucleotide sequence and PCR product.

Salmonella	Primer	5*-3*	PCR product, bp.
Salmonella spp.	Salm 3	GCTGCGCGCGAACGGCGAAG	387
	Salm4	TCCCGCCAGAGTTCCCATT	
Salmonella enterica ser. Enteritidis	Sent F	AAATGTGTTTTATCTGATGCAAGAGG'	299
	Sent R	GTTCGTTCTTCTGGTACTTACGATGAC	
Salmonella enterica ser. Typhimurium	Styp F	CCCCGCTTACAGGTCGACTAC	433
	Styp R	AGCGGGTTTTCGGTGGTTGT	
Salmonella enterica ser. Typhi	Styphi_F	CACGCACCATCATTTCACCG	738
	Styphi_R	AACAGGCTGTAGCGATTTAGG	
Salmonella enterica ser. Dublin	Sdub_F	ACGCGAAATCTGATGGTCTT	203
	Sdub_R	GCCCACCAGTTGTGAAAGGC	
Salmonella enterica ser. Gallinarum-Pullorum	Sgal_F	CCGCACAACACATCAGAAAG	97
	Sgal_R	AGCTGCCAGAGGTTACGCTG	

After synthesis of primers, we performed optimization of the PCR protocol. As the positive control for PCR we used DNA extracted from the one-day-old culture of *Salmonella* which have been stored in the museum NSC " IECVM ".

The obtained DNA matrix concentration after measuring with a spectrophotometer, we have led to the same concentration and then put PCR.

The first stage was carried out testing each primer pair using the standard composition of the reaction mixture at different temperatures.

To determine optimal temperature parameters PCR was performed with various primer annealing temperature: 58° C, 60° C, 63° C and 65° C. As a result, established the following optimal amplification:

- Initial denaturation - 94° C – 2 min;
- Denaturation- 94° C – 30s;
- Annealing - 63° C – 30s;
- Extension - 72° C – 40s;

} 40 cycles

- Final extension - 72° C – 5min

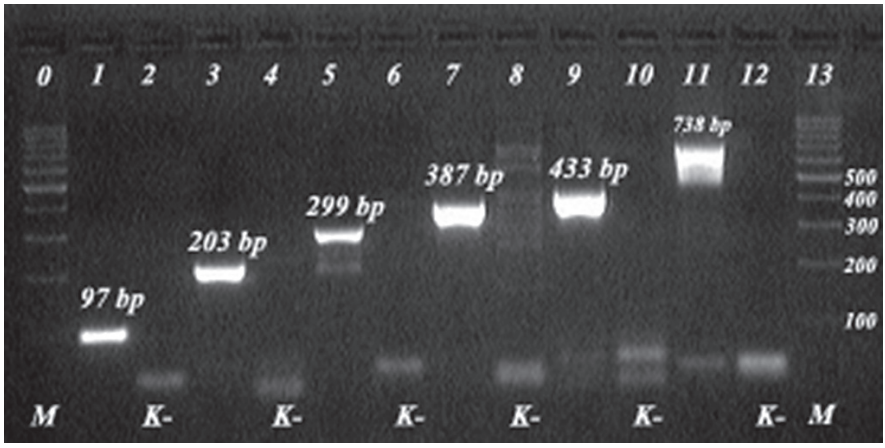

Figure 1. Electropherogram of results of initial testing of primers with positive DNA template: Track number 1 - corresponds the *Salmonella enterica* ser. Gallinarum-Pullorum aplicon (97 bp) lane number 3 - corresponds to the amplicon for *Salmonella enterica* ser. Dublin – (203 bp), lane number 5 - corresponds to the amplification *Salmonella enterica ser.* Enteritidis – (299 bp), lane number 7 - corresponds to the amplicon for *Salmonella* spp. – (387 bp), track number 9 - corresponds to the amplicon for *Salmonella enterica ser.* Typhimurium – (433 bp), track number 11 - corresponds to the amplicon for *Salmonella enterica* ser. Typhi – (738 bp.)

To set up the multiplex PCR, the optimization of reaction was performed using the basic sets for the amplification produced by Thermo Scientific (Lithuania).

Table 2. The composition of the reaction mixture for multiplex PCR

№	Components	
1	10×DreamTaq Buffer	2,5 µl
2	dNTP Mix, 2 mM each	2,5 µl
3	25 mM MgCl2	0,5 µl
4	Primer Styphi_Forward, (conc. 40 pM)	40,0 pM
5	Primer Styphi_Reverse, (conc. 40 pM)	40,0 pM
6	Primer Styp _ Forward, (conc. 40 pM)	20,0 pM
7	Primer Styp _ Reverse, (conc. 40 pM)	20,0 pM
8	Primer Salm_3 Forward, (conc. 40 pM)	20,0 pM
9	Primer Salm_4 Reverse, (conc. 40 pM)	20,0 pM
10	Primer Sent_ Forward, (conc. 40 pM)	10,0 pM
11	Primer Sent_ Reverse, (conc. 20 pM)	10,0 pM
12	Primer Sdub_ Forward, (conc. 20 pM)	10,0 pM
13	Primer Sdub_ Reverse, (conc. 20 pM)	10,0 pM
14	Primer Sgal_ Forward, (conc. 20 pM)	10,0 pM
15	Primer Sgal_ Reverse, (conc. 20 pM)	10,0 pM
16	Template DNA	10 pg – 1 µg
17	DremTaq DNA Polymerase	10,0 U
18	Water, nuclease-free	to 25,0 µl
Total volume		25, 0 µl

We have increased the time of denaturation of DNA to 45s for multiplex PCR-protocol establishment. The annealing of primers was also prolonged to the 45s, elongation time was increased to 1 minute. Final elongation was 10 min :

- Initial denaturation - 94° C – 2 min;
- Denaturation- 94° C – 45s;
- Annealing - 63° C – 45s;
- Extension - 72° C – 1min;

} 40 cycles

- Final extension - 72° C – 10 min

This mode is enabled to carry out the amplification of the expected fragments (Fig. 2) in a single reaction.

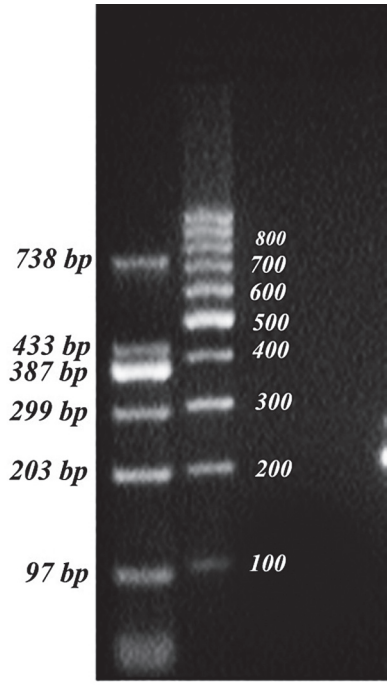

Figure 2. Electropherogram results Multiplex - PCR

CONCLUSIONS

The primer system, which allows simultaneous identification of a multiplex - PCR its five core members (*Salmonella enterica* ser. Enteritidis, *Salmonella enterica* ser. Typhimurium, *Salmonella enterica* ser. Typhi, *Salmonella enterica* ser. Dublin, *Salmonella enterica* ser. Gallinarum-Pullorum) has been developed.

Multiplex PCR protocol could be applied in the laboratories for identification and typing of *Salmonella* in the shortest possible time. Also, the system can be convenient for monitoring *Salmonella* contamination of various objects, while typing their main representatives.

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