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DETECTING THE PRESENCE OF SALMONELLA IN VARIOUS BIOLOGICAL OBJECTS WITH SIMULTANEOUS SEROLOGICAL TYPING BASIC OPTIONS BASED ON THE POLYMERASE CHAIN REACTION

Today in Ukraine's market there is an increase in the volume of trade of 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. At its disposal today they have only traditional bacteriological methods of isolation and identification of pathogens of toxicoinfection, which is not enough in terms of increasing turnover of products. One of the most dangerous toxicoinfection is salmonellosis.

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. Typing serovar of Salmonella gives an opportunity to get an answer about the source of infection.

The 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 enteritidis, Salmonella enterica typhimurium, Salmonella myphi, Salmonella dublin, Salmonella gallinarum-pullorum by multiplex PCR. The protocol of multiplex PCR was optimization with simples positive DNA matrix.

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).

According to the current classification, *S. Enterica* is divided into six sub-species: *Salmonella enterica*, *Salmonella salamae*, *Salmonella arizonae*, *Salmonella diarizonae*, *Salmonella houtenae* and *Salmonella indica*, which differentiate in the biochemical activity and represent the numbers I, II, IIIa, IIIb, IV and VI, respectively. In the majority of cases of *Salmonella* infection from animal and human serological variants isolated subspecies of *Salmonella enterica* (M.Y. Popoff et al., 2001; 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 may be 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 rooms 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, of which about 216 thousand deaths (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).

MATERIALS AND METHODS

The objects of our study were *Salmonella spp.*, *Salmonella enterica enteritidis*, *Salmonella enterica typhimurium*, *Salmonella typhi*, *Salmonella dublin*, *Salmonella gallinarum*. For the construction of genus-and species-specific primers were set up electronic databases of sequences of essential genes in *Salmonella* contained in the international database GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

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).

Next, the protocol is being followed polymerase chain reaction 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, Maniatis, 1982).

A positive DNA – the matrix used daily broth cultures of *Salmonella* from the museum sector study mycoplasmoses and salmonellosis are grown for meat – peptone broth, and provided for an experiment by Dr. Ekaterina V. Glebova.

Extraction of total nucleic acid was carried out using micro columns. To 450mkl of Extraction buffer was added 100 l of broth culture of *Salmonella* daily. After lysis of the tubes were transferred to microcolumns, centrifuged. This was followed by washing with ethanol followed by extraction of total nucleic acid of TE buffer.

DNA concentration was obtained for the experiment were measured spectrophotometer at 260 nm.

RESULTS AND DISCUSSIONS

In the analysis of these nucleotide sequences of the major genes of 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, including taking into account the requirements of primers was chosen as one, the limited size of 387 bpin length, and called Salm3_4.

For *Salmonella enterica enteritidis* specific motifs were found in the gene SefA. Sequence analysis of this gene has allowed to establish the potential primer pairs 6 of which were further chosen primers flanking portion length 299 bp.

For *Salmonella typhimurium enteris* a specificity possessed the gene fliC. If his analysis revealed eight areas specific to the representative of *Salmonella*, including the chosen one, flanking region 420 bp.

Table 1 – **Nucleotide sequence and PCR product**

Salmonella	Primer	5*–3*	PCR product, bp.
Salmonella spp.	Salm 3	GCTGCGCGCGAACGGCGAAG	387
	Salm4	TCCCGCCAGAGTTCCCATT	
Salmonella enteritidis	Sent F	AAATGTGTTTTATCTGATGCAAGAGG'	299
	Sent R	GTTCGTTCTTCTGGTACTTACGATGAC	
Salmonella typhimurium	Styp F	CCCCGCTTACAGGTCGACTAC	433
	Styp R	AGCGGGTTTTCGGTGGTTGT	
Salmonella typhi	Styphi_F	CACGCACCATCATTTCACCG	738
	Styphi_R	AACAGGCTGTAGCGATTTAGG	
Salmonella dublin	Sdub_F	ACGCGAAATCTGATGGTCTT	203
	Sdub_R	GCCCACCAGTTGTGAAAGGC	
Salmonella gallinarum	Sgal_F	CCGCACAACACATCAGAAAG	97
	Sgal_R	AGCTGCCAGAGGTTACGCTG	

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

For the genome of *Salmonella dublin* serospecific motifs found SeD_A1104. When bioinformatics studies were identified primers flanking the site 203 bp.

Finally, SG0266 was elected genome with specific motifs for *Salmonella gallinarum-pullorum*. On the basis of selected area, bounded by specific primers length of 97 bp.

After synthesis of primers we performed optimization of the PCR protocol. As a positive control during PCR using DNA extracted from the daily broth 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:

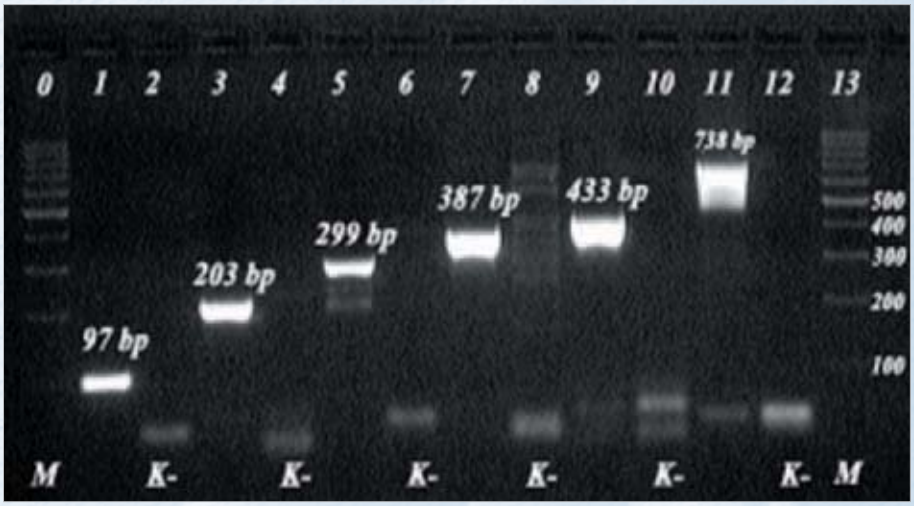

Fig. 1. Electrophoregram results of initial testing of primers with positive DNA template: Track number 1 – corresponds to *Salmonella gallinarum* pliconlength of 97 bp, lane number 3 – corresponds to the amplicon for *Salmonella dublin* – a length of 203 bp, lane number 5 – corresponds to the amplification *Enterica* for *Salmonella enteritidis* – a length of 299 bp, lane number 7 – corresponds to the amplicon for *Salmonella spp*. – 387 bp in length, track number 9 – corresponds to the amplicon for *Enterica Salmonella typhimurium* – 433 bp in length, track number 11 – corresponds to the amplicon for *Salmonella typhi* – a length of 738 bp.

- Initial denaturation – 94 °C – 2 min;
- Denaturation – 94 °C – 30 s;
- Annealing – 63 °C – 30 s; 40 cycles
- Extension – 72 °C – 40 s;
- Final extension – 72 °C – 5 min

To place a multiplex-the optimization of PCR reaction was performed using the basic sets for the amplification produced by Thermo Scientific (Lithuania).

When conducting a multiplex PCR – positive samples we initially failed to obtain products for all primer pairs designed, prompting the need for further optimization of the multiplex PCR protocol. To do this, we have increased the time to denature the DNA 45 s. The annealing of primers to the 45 s, elongation time was increased to 1 minute. Final elongation was 10 min:

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 $MgCl_2$	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**

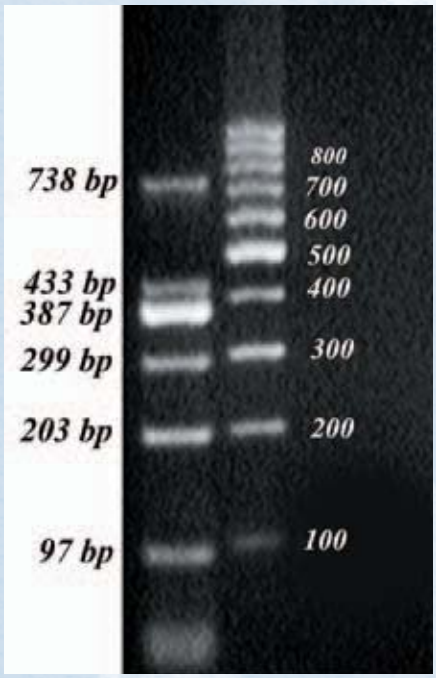

Fig. 2. Electrophoregram results Multiplex – PCR

Initial denaturation – 94 °C – 2 min;
Denaturation – 94 °C – 45 s;
Annealing – 63 °C – 45 s; 40 cycles
Extension – 72 °C – 1 min;
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.

CONCLUSIONS

The studies we were able to get a primer system, which allows the use of both in the test to detect the genetic material of biological material of the genus Salmonella with simultaneous identification of a multiplex – PCR its five core members: *Salmonella enteritidis*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella dublin*, *Salmonella gallinarum-pullorum*.

This development can be applied in the laboratory, if necessary 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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Виявлення наявності сальмонел у різних біологічних об'єктах з одночасним типуванням основних серологічних варіантів на основі полімеразної ланцюгової реакції. *Б.Т. Стегній, А.П. Герілович, В.Л. Ареф'єв, Е.В. Глєбова*

Сьогодні на ринку України спостерігається зростання обсягів торгівлі сировиною тваринного походження. Також збільшується кількість місць громадського харчування й продуктових магазинів, які реалізують готові харчові продукти. Ветеринарна служба повинна встигати контролювати якість цієї продукції. У її розпорядженні на сьогодні є лише традиційні бактеріологічні методи виявлення й ідентифікації збудників токсикоінфекцій, яких в умовах збільшення товарообігу продукції недостатньо. Однією з найбільш небезпечних токсикоінфекцій є сальмонельоз.

Результатом нашої роботи стало розроблення системи ідентифікації сальмонел і типування серед них п'яти серологічних варіантів на основі полімеразної ланцюгової реакції. Типування серологічного варіанта сальмонели дає можливість одержати відповідь про джерело інфекції.

На основі аналізу нуклеотидних послідовностей п'яти представників роду *Salmonella* розроблені праймери для їх ідентифікації з можливістю одночасного типування серед них – *Salmonella enterica enteritidis, Salmonella enterica typhimurium, Salmonella typhi, Salmonella dublin, Salmonella gallinarum-pullorum* за допомогою мультиплекс ПЛР.

Выявление наличия сальмонелл в различных биологических объектах с одновременным типированием основных серологических вариантов на основе полимеразной цепной реакции. *Б.Т. Стегний, А.П. Герилович, В.Л. Арефьев, Е.В. Глебова*

Сегодня на рынке Украины наблюдается рост объемов торговли сырьем животного происхождения. Также повсеместно увеличивается количество точек общественного питания и продуктовых магазинов, реализующих готовые продукты. Ветеринарная служба должна успеть проконтролировать качество этой продукции. В её распоряжении на сегодняшний день есть только традиционные бактериологические методы выделения и идентификации возбудителей токсикоинфекций, которых в условиях увеличения товарооборота продукции становится недостаточно. Одной из наиболее опасных токсикоинфекций является сальмонеллёз.

Результатом нашей работы была разработка системы идентификации сальмонелл и типирование среди них пяти серологических вариантов на основе полимеразной цепной реакции. Типирование серологического варианта сальмонеллы позволяет получить ответ об источнике инфекции.

На основе анализа нуклеотидных последовательностей пяти представителей рода *Salmonella* разработаны праймеры для их идентификации с возможностью одновременного типирования среди них – *Salmonella enterica enteritidis, Salmonella enterica typhimurium, Salmonella typhi, Salmonella dublin, Salmonella gallinarum-pullorum* в мультиплекс ПЦР.