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# GENOSEROTYPING OF *LISTERIA MONOCYTOGENES* STRAINS ORIGINATING FROM MEAT PRODUCTS AND MEAT PROCESSING ENVIRONMENTS

S u m m a r y

**Background.** *Listeria monocytogenes* is a foodborne human pathogen and a causative factor of listeriosis, which is an illness with a high mortality rate. Serotyping is a method for differentiating *L. monocytogenes* isolates based on unique combinations of somatic (O) and flagellar (H) antigens on the surface of their cells. Standard serotyping involves agglutination methods, which require using antisera. However, there are also genoserotyping methods which allow to categorise *L. monocytogenes* isolates into particular groups of serotypes (referred to as serogroups) based on genetic analyses. Differentiating *L. monocytogenes* isolates is an important issue in terms of food safety, surveillance and traceability of contamination sources. In this work, we present results of the genoserotyping of 153 *L. monocytogenes* isolates originating from meat products and meat processing environments at Polish processing plants. Two protocols were used for genoserotyping analyses: the first one allows to differentiate between four most common serotypes (1/2a, 1/2b, 1/2c and 4b) and the second one allows to distinguish hipervirulent serovar 4h from other serotypes.

**Results and conclusion.** Results achieved using both methods were consistent and all isolates were categorised into corresponding serogroups within the two methodologies. Most of the isolates (73.9 %) were characterised as members of the IIa serogroup (representing the 1/2a, 3a serovars). The IVb (4b, 4d, 4e) serogroup was the second most common (and comprised 18.3 % of isolates), followed by IIb (1/2b, 3b, 7) and IIc (1/2c, 3c), however, the last two groups were equally numerous (and each of them comprised 3.9 % of all isolates). None of the collected isolates belonged to the serogroup representing the 4a, 4c, 4ab and 4h serotypes.

**Key words**: serotyping, genoserotyping, serotypes, food safety, food surveillance

## Introduction

*Listeria monocytogenes* is a bacterium that can be found in a variety of food products, including meat, fish, milk and vegetables [12, 18]. Heat treatment inactivates

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bacteria of that species, however, products can sometimes be contaminated after processing and due to that fact *L. monocytogenes* can be found in both raw and processed foods [2]. The bacterium can also settle in food processing environments, which may be a cause of food contamination [2, 18]. *L. monocytogenes* is tolerant to high salt concentration (up to 10 %) and low pH (in the range of 3 ÷ 9), as well as is able to proliferate at refrigeration temperatures (minimum 0 ÷ 1 °C), which makes the bacterium challenging to control in food products [2, 12].

*L. monocytogenes* consumed with contaminated food may cause an infection, called listeriosis, which is manifested as acute febrile gastroenteritis (with symptoms such as fever, diarrhoea, muscle pain and headache). However *L. monocytogenes* can cross intestinal barrier, blood-brain barrier and maternal-foetal barrier, leading to invasive listeriosis. Those types of infections can cause septicaemia, maternal-foetal infections (often resulting in abortions or stillbirths) or neurolisteriosis (e.g. in a form of meningitis) [18]. A mortality rate of listeriosis reaches 20 ÷ 30 % [2, 24].

Bacteria that belong to *L. monocytogenes* genus can be differentiated on the basis of their unique combinations of somatic (O) and flagellar (H) antigens on cells' surface [3, 11, 19]. Currently, there are 14 known serotypes (marked with the following symbols: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, 7, 4h) [9, 23], but only four of them (namely 4b, 1/2a, 1/2b, 1/2c) cause more than 95 % listerial cases and comprise more than 95 % isolates from food products [9]. Interestingly, the 1/2a isolate is the most frequently isolated from food, whereas the 4b serotype is responsible for the majority of infections [3, 20].

Standard agglutination methods with antisera can be used for stereotyping *L. monocytogenes* strains, however, the technique is limited by costs, availability and the need for technical expertise. Furthermore, the reproducibility of the results is not always satisfactory [9]. To increase the ease and accessibility of this classification system [3], some authors proposed PCR-based genoserotyping protocols. Genoserotyping is based on detecting particular genes in bacterial genome, which allows to categorise isolates into particular groups of serotypes (referred to as serogroups).

The first PCR-based genoserotyping methodology, established by Borucki and Call [3], included four pairs of primers and allowed to categorise bacteria into five serotype groups. However, PCR reactions need to be held separately, not in multiplex PCR, which increases analysis time. First multiplex-PCR serotyping protocol was proposed by Doumith et al. [9]. It allows to differentiate 4 major *L. monocytogenes* serovars (namely 4b, 1/2a, 1/2b, 1/2c) and therefore the technique has discriminatory power similar to traditional serotyping (as 95 % of isolates belongs to one of those four serotypes). Specifically, the methodology allows to differentiate serogroups: IIa (including the 1/2a, 3a serovars), IIb (1/2b, 3b, 7), IIc (1/2c, 3c), IVb (4b, 4d, 4e) and serogroup L (including all other *Listeria sensu stricto* species and remaining serotypes

of *L. monocytogenes*: 4a, 4c, 4ab and 4h) [5,13]. Although the protocol of Doumith et al. [9] is commonly accepted, the 4h serovar, which has been discovered recently, cannot be distinguished from other serovars by this protocol. The isolates of the 4h serovar are considered hipervirulent, posing a threat to food safety [23], and due to that fact Feng et al. [10] proposed an assay in which the 4h serovar is specifically distinguished from other serotypes [10]. There are also other protocols regarding genoserotyping of *L. monocytogenes* strains in multiplex-PCR reactions [6,8,15], a serotyping scheme using a combination of an antibody-based serogrouping and a multiplex PCR assay [4] and protocols for real-time PCR [1,21].

In general, the identification of genoserotypes is one of the methods of differentiating *L. monocytogenes* strains, which is an important issue in terms of food safety, food surveillance and traceability of contamination sources. Alía et al. [1] stated that carrying out the correct differentiation of *L. monocytogenes* serotypes is of utmost importance, as the epidemiology and persistence of this bacterium in meat processing plants may be related to its serotype [1].

Our hypothesis is that *L. monocytogenes* isolates originating from Polish meat products and meat processing environments present diverse genoserotypes. The aim of this research was to determine the genoserotypes of *L. monocytogenes* strains of that origin and additionally to compare the results obtained with two genoserotyping protocols – the one that is commonly accepted, proposed by Doumith et al. [9], and the modern one, put forward by Feng et al. [10].

## Materials and methods

### *Microorganisms*

Strains of putative *Listeria* spp. originating from Polish meat products (raw and processed ones) and meat processing environments in Poland were obtained during routine microbiological quality analyses. Environmental samples were obtained from surfaces both being in contact with food (e.g. knife surface, workers' gloves or transport boxes) and not coming into contact with food (e.g. shoe soles, floors or walls of a facility). The samples were collected between October 2020 and November 2021. Microorganisms were isolated on plates with selective agar media (OXFORD or ALOA). Bacteria from one colony of typical appearance were restreaked on Brain Heart Infusion (BHI) (POCH, Poland) agar plate and incubated at 37 °C for 24 hours prior to DNA isolation. Overall, 153 *L. monocytogenes* isolates were obtained from the samples delivered, 45 of which originated from the environment and 108 originated from food products. Details regarding the origin of the samples are presented in Fig. 1.

*DNA isolation*

Microorganisms were removed from 24-hour BHI agar plate culture with a sterile inoculation loop and subjected to DNA isolation procedure. Isolation was performed with a commercially available kit Genomic Mini (A&A Biotechnology, Poland), in line with the manufacturer's instructions. DNA concentration was measured with a Spectrophotometer ND-1000 (NanoDrop, USA).

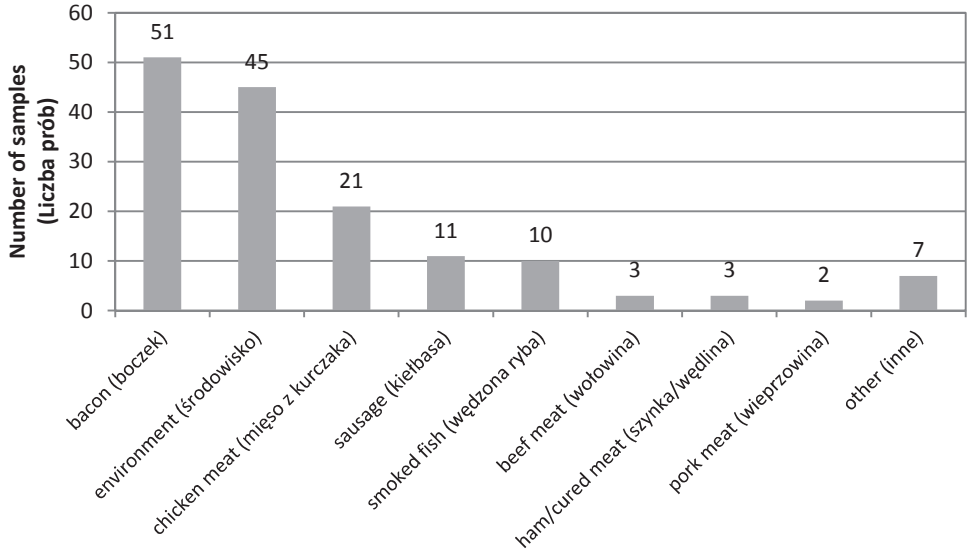


Fig. 1. Bar chart showing the sources of the samples included in the study
Rys. 1. Wykres słupkowy obrazujący źródła pochodzenia prób uwzględnionych w badaniu

*Confirmation of species affiliation*

Species were confirmed as *L. monocytogenes,* using two genetic methods, namely RFLP-PCR by Paillard et al. [16] and multiplex PCR by Li et al. [14], according to the protocols described in the references, with minor modifications. PCRs were performed in 10 µL final volume using RUN polymerase (A&A Biotechnology, Poland). Restriction digestions were performed using HhaI and XmnI (ThermoFisher Scientific, USA) enzymes with a corresponding Tango buffer. Only microorganisms confirmed as *L. monocytogenes* with both techniques were included in the study.

*Genoserotyping*

Genoserotyping was performed with multiplex PCR reactions, according to two protocols proposed by Doumith et al. [9] and Feng et al. [10], with minor modifica-

tions. Primers, whose sequences are presented in Table 1, were synthesised to order by Genomed company.

In genoserotyping protocol according to Doumith et al. [9], the multiplex mixture contained 1U of *Taq* RUN polymerase (A&A Biotechnology) and compatible reaction buffer at recommended concentration, 0.2 mM dNTPs (A&A Biotechnology) and 1.5 µM of *lmo1118*, 1.0 µM of: *lmo0737*, ORF2819 and ORF2110, and 0.2 µM of *prs* final concentration for each primer. DNA was added in the amount of 10 ng per reaction. The mixture was filled with water to reach the final volume of 10 µL. Negative control sample with PCR-grade water used instead of DNA was employed in each reaction set. PCR was performed in T-Gradient thermocycler (Biometra) with the following programme: initial denaturation at 94 °C for 3 min; 35 cycles of: 94 °C for 24 s, 53 °C for 69 s, 72 °C for 69 s; and a final step of 72 °C for 7 min.

In the genoserotyping protocol proposed by Feng et al. [10], the multiplex mixture was prepared in a similar way as described above, however, different primers were used, namely: *LMxysn_1095*, *lmo1083* and *smcL*, all at final concentration of 0.25 µM. DNA isolated from *L. ivanovii* ATCC 19119 was used as a positive control for *smcL* amplicon, as well as PCR-grade water was used as a negative control sample in each reaction set. PCR was performed with the following programme: initial denaturation at 95 °C for 5 min; 30 cycles: at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final step at 72 °C for 10 min.

Table 1. Primers used in the study and their sequences
Tabela 1. Startery użyte w badaniu i ich sekwencje

| Primers' name<br>Nazwa starterów | Primers' sequence (5' → 3')<br>Sekwencja starterów (5' → 3') | Product size (bp)<br>Rozmiar produktu (pz) | Reference<br>Źródło |
|---|---|---|---|
| *lmo0737* | F: AGGGCTTCAAGGACTTACCC<br>R: ACGATTTCTGCTTGCCATTC | 691 | Doumith et al. protocol [9] |
| *lmo1118* | F: AGGGGTCTTAAATCCTGGAA<br>R: CGGCTTGTTCGGCATACTTA | 906 | |
| ORF2819 | F: AGCAAAATGCCAAAACTCGT<br>R: CATCACTAAAGCCTCCCATTG | 471 | |
| ORF2110 | F: AGTGGACAATTGATTGGTGAA<br>R: CATCCATCCCTTACTTTGGAC | 597 | |
| *prs* | F: GCTGAAGAGATTGCGAAAGAAG<br>R: CAAAGAAACCTTGGATTTGCGG | 370 | |
| *LMxysn_1095* | F: AATACTTGGACAGACGGAACGC<br>R: TCATCTGGCTCTTTTAGAACCG | 602 | Feng et al. protocol [10] |
| *lmo1083* | F: CACAAATGGTCTTGACGGGG<br>R: TTTGCGCGTGATTTTAGTGG | 390 | |
| *smcL* | F: CACAGACCATTGTGGTGACTTG<br>R: CGGTGCTTTCATTTTTTTACTC | 889 | |

Reaction products were separated on 2 % agarose gel (dyed with ethidium bromide) in 1 × TAE buffer and visualised using Gel Doc Imaging System (Bio-Rad).

*Data analysis*

Microsoft Excel 2007 software was used to analyse the data and prepare the charts.

## Results and discussion

In this study, we present the results of the genoserotyping of 153 *L. monocytogenes* isolates originating from meat products and processing environments. Experiments were performed in line with two genoserotyping protocols. The results achieved by employing both methodologies, which are presented in Fig. 2, were consistent and coherent in the case of all isolates, meaning that all of the samples were categorised into corresponding serogroups within the two methodologies. Hypothesis that isolates originating from Polish meat products and meat processing environments present diverse genoserotypes was confirmed by both methodologies.

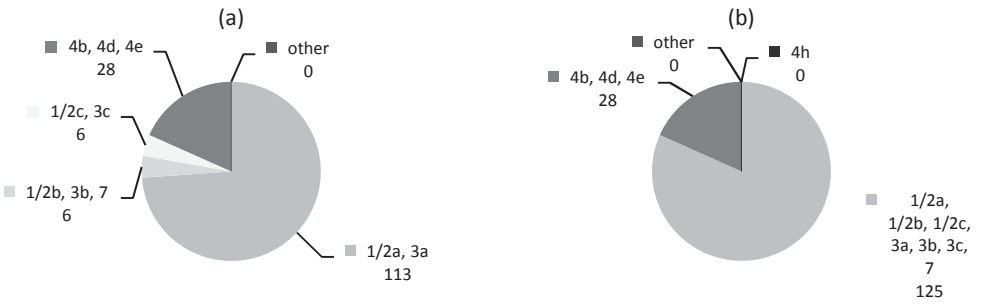


Fig. 2. Pie chart representing the results of genoserotyping acc. to: (a) Doumith et al. [9]; (b) Feng et al. [10] protocol

Rys. 2. Wykres kołowy przedstawiający wyniki genoserotypowania otrzymane według protokołu: (a) Doumith i wsp. [9]; (b) Feng i wsp. [10]

In the protocol according to Feng et al. [10], none of the isolates was identified as the 4h serovar, as well as none of the isolates was classified as a member of the serogroup consisting of the other serovars (4a, 4ab and 4c). Owing to that, in the case of our samples, the methodology of Doumith et al. [9] allowed to achieve more detailed results, as one serogroup (comprising the 1/2a, 1/2b, 1/2c, 3a, 3b, 3c and 7 serovars) from the protocol of Feng et al. [10] was further separated into three distinct groups in the protocol of Doumith et al. [9] (as can be seen in Fig. 2). Detailed results of serogrouping achieved in the protocol of Doumith et al. [9], with separation into samples from food products and processing environments, are presented in Table 2.

Most of the isolates included in the study (73.9 %) belonged to the IIa serogroup, whereas the IVb serogroup was the second most common (and comprised 18.3 % of all of the samples). Six isolates (3.9 %) were identified as belonging to the IIb serogroup, of which 4 were detected in food (these were samples from brawn (Polish *salceson*), pork meat, Vienna sausage and chicken meat) and 2 were detected in a processing environment (isolates originated from knife surface and shoe sole). All isolates (6 - 3.9 %) characterised as the IIc serogroup were identified in food products. Their origin was as follows: Vienna sausages (3 samples), chicken meat (2 samples) and white steamed sausage. None of the collected isolates belonged to the L serogroup, representing the remaining 4a, 4c, 4ab and 4h serovars.

Table 2. Results of serotyping acc. to protocol of Doumith et al. (2004)
Tabela 2. Wyniki serotypowania wg protokołu Doumith i wps. (2004)

| Serogroup / Serogrupa | | All isolates Wszystkie izolaty | | Isolates from food products Izolaty pochodzące z żywności | | Isolates from food processing environment Izolaty ze środowiska produkcyjnego żywności | |
|---|---|---|---|---|---|---|---|
| Symbol | Serovars Serowary | Number Liczba | % | Number Liczba | % | Number Liczba | % |
| IIa | 1/2a, 3a | 113 | 73.9 | 82 | 75.9 | 31 | 68.9 |
| IIb | 1/2b, 3b, 7 | 6 | 3.9 | 4 | 3.7 | 2 | 4.4 |
| IIc | 1/2c, 3c | 6 | 3.9 | 6 | 5.6 | 0 | 0.0 |
| IVb | 4b, 4d, 4e | 28 | 18.3 | 16 | 14.8 | 12 | 26.7 |
| L | 4a, 4c | 0 | 0.0 | 0 | 0.0 | 0 | 0.0 |
| | Sum / Suma: | 153 | | 108 | | 45 | |

Out of 51 isolates originating from bacon, 47 belonged to the IIa serogroup and 4 to the IVb serogroup. Chicken meat samples (21 in total) represented three distinct serotypes, of which 18 samples were characterised as the IIa serogroup, 2 isolates belonged to the IIc serogroup and 1 isolate belonged to the IIb serogroup. Sausages (11 samples) also contained diverse *L. monocytogenes* isolates, as 6 samples (3 from dried fermented sausages (Polish *kabanos*), 2 from Vienna sausages and one from Silesian sausage) were characterised as the IIa serogroup, 4 samples (3 from Vienna sausages and one from white steamed sausage) belonged to the IIc serogroup and one sample (from Vienna sausage) was classified under the IIb serogroup. In contrast to the abovementioned group of products, all 10 samples from smoked fish contained isolates characterised as the IVb serogroup.

In general, our findings are consistent with most of literature reports. The results of genostereotyping according to protocol of Feng et al. [10] have not been published to date, however, many authors have used the protocol of Doumith et al. [9]. For ex-

ample, Psareva et al. [17] examined *L. monocytogenes* isolates collected between 2001 and 2020, originating from meat, poultry, dairy, and fish products from the Central European part of Russia. Out of 40 samples, 22 (55.0 %) belonged to the IIa serogroup; 11 (27.5 %) belonged to the IVb group; 4 (10.0 %) isolates belonged to the IIc group and 3 (7.5 %) to the IIb group [17], which makes the prevalence of particular serogroups similar to those achieved in our research.

On the other hand, Wang et al. [22] performed traditional serotyping of *L. monocytogenes* isolates from pork samples available at supermarkets in Wuhan, China. The 1/2a serovar comprised 45 out of 63 of all samples (71.4 %), the 1/2c serovar comprised 12 samples (19.0 %) and the 1/2b serovar comprised 6 samples (9.5 %). Other serovars were not detected [22]. That study demonstrated that the 1/2a serotype was the most commonly prevalent in food products, however, the authors did not detect any isolate which belonged to the 4b, 4d or 4e serovars, whereas in our case, the IVb serogroup was the second most common. Similarly to findings of Wang et al. [22], according to Coban et al. [7], who used the protocol of Doumith et al. [9], the IVb serogroup was the least numerous (1 sample – 1.0 %) in their study. The authors serogrouped 103 isolates originating from poultry samples from the retail markets and slaughterhouses in Turkey. However, the majority of *L. monocytogenes* strains (78 samples – 75.7 %) were characterised as the IIa serogroup (which is in agreement with our findings), followed by the IIc serogroup (15 samples – 14.5 %) and the IIb group (6 samples – 5.8 %) [7].

Chen et al. [5] performed genoserotyping of 102 isolates from food samples purchased at retail in U.S. FoodNet sites using the protocol of Doumith et al. [9]. The IIa serogroup was the most prevalent, with 46 isolates (45.1 %), while the second most common serogroup was IIb, with 28 samples (27.5 %), followed by the IVb group, with 20 (19.6 %) samples, and the IIc group, with 5 (4.9 %) samples. Interestingly, the authors also identified 3 isolates (2.9 %) which were characterised as the L serogroup (comprising the remaining serovars: 4a, 4ab, 4c and 4h) [5]. Microorganisms of that serogroup were not identified in this study.

In contrast to the aforementioned reports in which the IIa serogroup was the most common among isolates, Simonavičienė et al. [20], who used the protocol of Doumith et al. [9], have found that the IVb segrogroup was the most prevalent in cold smoked fish (salmon) products from Lithuania. Out of 37 *L. monocytogenes* isolates, 35 (90.5 %) belonged to the IVb serogroup, whereas only 2 samples (5.4 %) were identified as members of the IIa serogroup [20]. However, these results are also in agreement with our findings, as only one serogroup, namely IVb, was identified in all 10 samples originating from smoked fish included in our study.

In conclusion, scientific data is not entirely consistent as regards the detailed proportions and prevalence rate of particular serogroups of *L. monocytogenes* isolates,

however, in most cases the IIa serogroup (or the 1/2a serovar) is the most prevalent, which was also a result of our findings. In general, the 1/2a serotype is commonly considered a serotype the most frequently isolated from food products [3]. The discrepancies in the proportions of particular serogroups probably result from examining a different type of samples, originating from different sources, obtained at distant geographical locations and collected during different time frames.

## Conclusions

1. *L. monocytogenes* isolates occasionally appearing in meat products and a processing environment were genetically diverse, as they were assigned to different serogroups. The prevalence rate of particular serogroups (and thus serotypes) is not equally distributed among isolates.
2. The IIa serogroup (comprising the 1/2a, 3a serovars) was the most common in our study and comprised 73.9 % of all samples. The IVb serogroup (comprising the 4b, 4d, 4e isolates) was the second most common and comprised 18.3 % of all isolates. The IVb serogroup was also the only one detected in all 10 samples of smoked fish included in our study.
3. Both genoserotyping protocols proposed by Doumith et al. [9] and Feng et al. [10] are useful tools to differentiate *L. monocytogenes* isolates. The methodologies present results that were consistent (in the case of all tested isolates) and complementary, as the protocol by Feng et al. [10] allows to distinguish the 4h serovar from other serovars, whereas the protocol by Doumith et al. [9] separates 7 serovars which fall into one group in the protocol by Feng et al. [10], into three serogroups.

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## GENOSEROTYPOWANIE IZOLATÓW *LISTERIA MONOCYTOGENES* POCHODZĄCYCH Z PRODUKTÓW MIĘSNYCH I ŚRODOWISKA PRODUKCYJNEGO

S t r e s z c z e n i e

**Wprowadzenie.** *Listeria monocytogenes* jest ludzkim patogenem związanym z żywnością oraz czynnikiem wywołujący listeriozę, czyli chorobę o wysokim odsetku śmiertelności. Serotypowanie jest metodą różnicowania izolatów *L. monocytogenes* w oparciu o unikatowe kombinacje antygenów somatycznych (O) i rzęskowych (H) na powierzchni ich komórek. Klasyczne serotypowanie jest wykonywane z wykorzystaniem metod aglutynacyjnych, które wymagają użycia przeciwciał. Istnieją jednak metody genoserotypowania, które pozwalają zakwalifikować izolaty *L. monocytogenes* do poszczególnych grup serotypów (nazywanych serogrupami) na podstawie analiz genetycznych. Różnicowanie izolatów *L. monocytogenes* jest ważnym zagadnieniem w kontekście bezpieczeństwa żywności, kontroli i śledzenia źródeł skażeń. W niniejszej pracy przedstawiamy wyniki genoserotypowania 153 izolatów *L. monocytogenes* pochodzących z produktów mięsnych i środowiska produkcyjnego w polskich zakładach przetwórstwa. Do przeprowadzenia analiz genoseroptyu wykorzystano dwie metodyki: pierwsza pozwala na rozróżnienie czterech najczęściej występujących serotypów (1/2a, 1/2b, 1/2c oraz 4b), natomiast druga pozwala rozróżnić hiperwirulentny serowar 4h od innych serotypów.

**Wyniki i wnioski.** Otrzymane oboma metodami wyniki były zgodne i wszystkie izolaty zostały zakwalifikowane do odpowiadających sobie serogrup w obrębie obu metodyk. Większość izolatów (73.9 %) została scharakteryzowana jako należąca do serogrupy IIa (reprezentującej serowary 1/2a, 3a). Serogrupa IVb (4b, 4d, 4e) była drugą najbardziej liczną (zawierała 18.3 % izolatów), a następnie IIb (1/2b, 3b, 7) oraz IIc (1/2c, 3c), przy czym ostatnie dwie grupy były równoliczne (i każda z nich zawierała 3.9 % wszystkich izolatów). Żaden z zebranych izolatów nie należał do serogrupy reprezentującej serotypy 4a, 4c, 4ab i 4h.

**Słowa kluczowe**: serotypowanie, genoserotypowanie, serotypy, bezpieczeństwo żywności, kontrola żywności