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Analysis of production levels of InlA and InlB invasion factors in *Listeria monocytogenes* isolates collected in the Russian Federation

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Abstract

Background. *Listeria monocytogenes* is characterized by the presence of epidemic hypervirulent clones. A key feature of *L. monocytogenes* is its capacity to invade non-professional phagocytic cells. Hypervirulent clones are strongly associated with the increased production and/or the presence of certain isoforms of invasion factors InlA and InlB.

The **purpose** of the study is to create a test system for InlA and InlB detection and to measure the InlA and InlB production levels in *L. monocytogenes* isolates belonging to clonal groups with different virulence potential.

Materials and methods. The study was performed using 32 *L. monocytogenes* strains belonging to epidemic clones ECII, ECIV, ECVII (clonal complexes CC1, CC2, CC7) and hypovirulent clonal complex CC9. Sequencing of *inlA* and *inlB* genes was performed. The indirect enzyme-linked immunosorbent assay was used to analyze the production levels of InlA and InlB proteins.

Results. The variability of InlA was revealed among strains belonging to the same clonal complex: 3 InlA isoforms were identified among strains belonging to CC7; out of 8 strains belonging to CC9, one strain had a stop codon in the *inlA* gene, leading to the loss of function of the InlA protein. The differences between *inlB* alleles correlated with the specificity of strains belonging to a certain clonal complex. Differences in production levels of invasion factors were measured. In strains belonging to CC9, the InlA production level was 2.5 times as low compared to strains belonging to CC1, CC2, and CC7. In strains belonging to phylogenetically related CC1 and CC2, the InlB production level was on average 4 times as high compared to strains belonging to CC7 and CC9.

Conclusion. The obtained results confirm the variability of major invasion factors both among clonal complexes and strains of the same complex. The increased production of invasion factors InlA and InlB correlates with the potential virulence of strains.

Keywords: *Listeria monocytogenes, InlA, InlB, enzyme-linked immunosorbent assay*

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with «Consensus Author Guidelines for Animal Use» (IAVES, 23 July 2010). The research protocol was approved by the Ethics Committee of the Gamaleya Research Centre of Epidmiology and Microbiology (protocol No. 54, July 24, 2023).

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Научная статья
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Анализ уровня продукции факторов инвазии InlA и InlB у изолятов *Listeria monocytogenes*, выделенных на территории Российской Федерации

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Аннотация

Актуальность. *Listeria monocytogenes* характеризуется наличием эпидемически высоковирулентных клонов. Инвазия в непрофессиональные фагоциты — ключевой момент листериозной инфекции. Формирование высоковирулентных клонов обусловлено повышенной продукцией и/или наличием определённых изоформ факторов инвазии белков InlA и InlB.

Цель исследования — создать тест-систему для обнаружения InlA и InlB и на её основе оценить уровни продукции InlA и InlB у изолятов *L. monocytogenes*, относящихся к клональным группам с различным вирулентным потенциалом.

Материалы и методы. В работе использованы 32 штамма *L. monocytogenes*, относящихся к эпидемическим клонам ECII, ECIV, ECVII (клональные комплексы CC1, CC2, CC7) и гиповирулентному клональному комплексу CC9. Проведено секвенирование генов *inlA* и *inlB*. Для анализа уровня продукции белков InlA и InlB использован непрямой иммуноферментный анализ.

Результаты. Выявлена вариабельность InlA среди штаммов, относящихся к одному клональному комплексу: в том числе среди штаммов, принадлежащих к CC7, выявлены 3 изоформы InlA; из 8 штаммов, принадлежащих к CC9, у одного выявили стоп-кодон в гене *inlA*, приводящий к утрате функциональности белка InlA. Различия между аллелями *inlB* коррелировали с принадлежностью штаммов к конкретному клональному комплексу. Установлены различия в уровне продукции факторов инвазии. У штаммов, относящихся к CC9, уровень продукции InlA был в 2,5 раза ниже по сравнению со штаммами, относящимися к CC1, CC2 и CC7. Уровень продукции InlB был в среднем в 4 раза выше у штаммов, принадлежащих к филогенетически родственным CC1 и CC2, по сравнению со штаммами, относящимися к CC7 и CC9.

Заключение. Полученные результаты свидетельствуют о вариабельности основных факторов инвазии как между клональными комплексами, так и между штаммами одного комплекса. Повышенная продукция факторов инвазии InlA и InlB коррелирует с потенциальной вирулентностью штаммов.

Ключевые слова: *Listeria monocytogenes, InlA, InlB, иммуноферментный анализ*

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Introduction

Listeria monocytogenes causes a dangerous foodborne infection with a high (around 30%) mortality rate in high-risk individuals as well as in domestic and wild animals [1, 2]. The genetic structure of the *L. monocytogenes* species is divided into 4 phylogenetic lineages [3].

Phylogenetic lineage I includes clonal complexes (CCs) CC1, CC2, CC4, and CC6 most frequently associated with listeriosis in humans [4]. Using different methods, CC1 and CC2 strains were assigned to epidemic clones ECII and ECIV, respectively, which were involved in large listeriosis outbreaks in different countries, including Russia. In Europe and the United States, the strains isolated from clinical cases are mostly represented by CC1 strains accounting for 11.4% [5].

Until 1980, in Russia, lineage II strains isolated from human and animal listeriosis cases as well as from natural sources prevailed; most of them belonged to CC7 (also known as epidemic clone ECVII) [6]. CC7 strains still prevail in natural foci in the European part of Russia; they are also frequently isolated from sporadic cases of listeriosis in humans and animals [7]. Lineage II CC9 strains are often isolated from food and environmental sources; they are also well represented in Russia [7]. In the infectious disease pathology classification in Europe and North America, CC7 strains are assigned to medium virulent strains, while clonal complex CC9 is considered low virulent [8]. The difference in the occurrence frequency among clinical isolates and, consequently, the difference in the potential virulence of strains belonging to different CCs and/or phylogenetic lineages are associated by multiple researchers with the presence of additional virulence factors [9], allelic variation of virulence factors [10], and their expression levels [11].

The initial stage of infection starts with the interaction between Listeria and non-professional phagocytes, involving proteins belonging to the internalin family — InlA and InlB, which promote the *L. monocytogenes* invasion of intestinal enterocytes and epithelial cells through an interaction with target receptors. E-cadherin is a specific receptor for InlA. InlB specifically interacts with two receptors: c-Met and gC1q-R [12]. The presence of functional InlA is sufficient for invasion into enterocytes [13]; the entry into hepatocytes requires InlB [14], and the coordinated action of InlA and InlB mediates the crossing of the placental barrier [15].

Sequencing is performed to identify allelic variation of *inlA* and *inlB* genes. The study results show that clinical strains and, most importantly, strains causing fetal and neonatal infections express full-length InlA more often than strains of food origin. Truncated InlA was detected in most of the hypovirulent CC9 and CC121 isolates [16] and in other clonal complexes of phylogenetic lineage II (for example, CC331, CC199, and CC321) [4].

Earlier it was found that clinical and food isolates differed in *inlA* and *inlB* gene expression levels [11]. The expression of key pathogenicity factors is activated during cell infection and depends on transcriptional regulator PrfA. *L. monocytogenes* has an extensive crosstalk system between σ^B and PrfA regulatory networks, providing optimal expression of genes required in extrahost environments, including repression of genes associated with virulence. Conversely, in intrahost environments, this regulatory network provides elevated expression of genes associated with virulence, enabling successful invasion [17]. The PrfA activity decreases when *L. monocytogenes* is grown in rich media. Hydrophobic adsorbents included in the culture medium activate PrfA and induce enhanced activity of virulence factor genes [18]. However, the mechanisms regulating production levels of the respective proteins-pathogenicity factors remain unknown.

Various techniques are used to detect and measure levels of protein expression, including enzyme activity assays and immunoblotting. Enzyme activity measurement cannot be used if proteins are not biocatalysts. Immunoblotting is a labor-intensive process unsuitable for testing large sample sizes. A test system based on the enzyme-linked immunosorbent assay (ELISA) was developed as an alternative method for the qualitative and quantitative assessment of InlA and InlB. Using this ELISA test system, we assessed *L. monocytogenes* isolates from the collection of the Gamaleya Research Center of Epidemiology and Microbiology (Gamaleya RCEM) for InlA and InlB expression levels and their correlation with the phylogenetic status of the strains.

Materials and methods

Bacterial strains and bacteria culturing conditions

The study was performed using *Listeria* spp. strains from the collection of Gamaleya RCEM (**Table 1**). Microorganisms were grown in a BHI liquid medium (Becton Dickinson) at 37°C with constant shaking at 180 rpm. PrfA was activated by supplementing the BHI broth with 1% (w/v) Amberlite XAD 4 hydrophobic adsorbent (Sigma-Aldrich).

PCR analysis

The collection-derived *L. monocytogenes* isolates were lysed with lysozyme at a concentration of 20 µg/ml for 1 hour at 37ºC; then, the lysates were treated with proteinase K at a concentration of 25 µg/ml at 56ºC for 1 hour. The obtained samples were boiled in a water bath for 10 min. The amplification of *inlA* and *inlB* genes was performed in a Tertsik thermal cycler (DNA-Technology) using TransStart Taq DNA Polymerase (TransGen): stage 1 (1 cycle): 94ºC — 4 min; stage 2 (30 cycles): 94ºC — 30 sec, 52ºC — 30 sec, 72ºC — 2 min; stage 3 (1 cycle): 72ºC — 10 min. Primers were selected using the Ugene v. 40.1

Table 1. *Listeria* spp. strains used in the study

Strain	Characteristics	Source	Reference
		L. monocytogenes	
EGDe	CC9	Type strain	BIGSdb ID = 1
EGDeΔinlB	*inlB* deletion	Kindly provided by Prof. J. Vazquez-Boland	
EGDeΔinlA	*inlA* deletion		
VIMHA004	CC2	Newborn	BIGSdb ID = 3449
VIMHA007	CC2	Newborn	BIGSdb ID = 3450
VIMHA006	CC2	Newborn	BIGSdb ID = 3606
VIMHA009	CC1	Newborn	BIGSdb ID = 3452
VIMHA011	CC1	Newborn	BIGSdb ID = 3454
VIMHA010	CC1	Newborn	BIGSdb ID = 3453
VIMHA012	CC1	Newborn	BIGSdb ID = 3607
VIMHA017	CC1	Newborn	BIGSdb ID = 3609
L.mo25	CC1	Chicken	BIGSdb ID = 79358
L.mo70	CC8	Chicken	BIGSdb ID = 78808
L.mo78	CC37	Chicken	BIGSdb ID = 79363
VIMPH006	CC7	Carrier	BIGSdb ID = 3464
L.mo71	CC59	Chicken	BIGSdb ID = 78809
GIMC2010:LmcUH8	CC7	Carrier	BIGSdb ID = 42978
GIMC2032:LmcINH-1	CC7	Carrier	BIGSdb ID = 45728
GIMC2007:LmcIH1_3	CC7	Carrier	BIGSdb ID = 42975
766	CC7	Swine	BIGSdb ID = 5803
VIMPR134	CC7	Rodent	BIGSdb ID = 3459
VIMPR422	CC7	Rodent	BIGSdb ID = 3460
VIMPA064	CC7	Newborn	BIGSdb ID = 3455
L.mo84	CC37	Meat	BIGSdb ID = 79367
L.mo49	CC9	Chicken	BIGSdb ID = 79359
L.mo129/3	CC9	Dairy products	BIGSdb ID = 78797
L.mo75	CC9		BIGSdb ID = 79362
GIMC2035:Lmc7218	CC9	Fish	BIGSdb ID = 45731
GIMC2017: Lmc921	CC9	Meat	BIGSdb ID = 42987
L.mo98-20	CC9	Dairy products	BIGSdb ID = 78795
LO28	CC9	Type strain	BIGSdb ID = 3364
		L. ivanovii	
ATCC 19119		Type strain	American Type Culture Collection

software and synthesized by the Syntol Company. For the *inlA* gene, the primers were as follows: InlA1 5'-ggttgaaaagtatactagtagc-3'; InlA2 5'-ggaagatatt-agcccaatttc-3', for the InlB gene: InlBF 5'-gcttatg-gatcctataattcaaaagaag, InlBR 5'-gaaaagctgcagaaaatc-cgccttc. Gel electrophoresis was used for detecting amplification products. Target DNA fragments were purified using a Dia-gene kit (Dia-M).

Sequencing

Samples were prepared for sequencing following the recommendations of the Genome Shared Resource Center. The DNA sequencing was performed using an ABI PRISM BigDye Terminator v. 3.1 reagent kit; the reaction products were analyzed using an automated sequencer — Applied Biosystems 3730 DNA Analyzer. To identify gene sequences, the following primers

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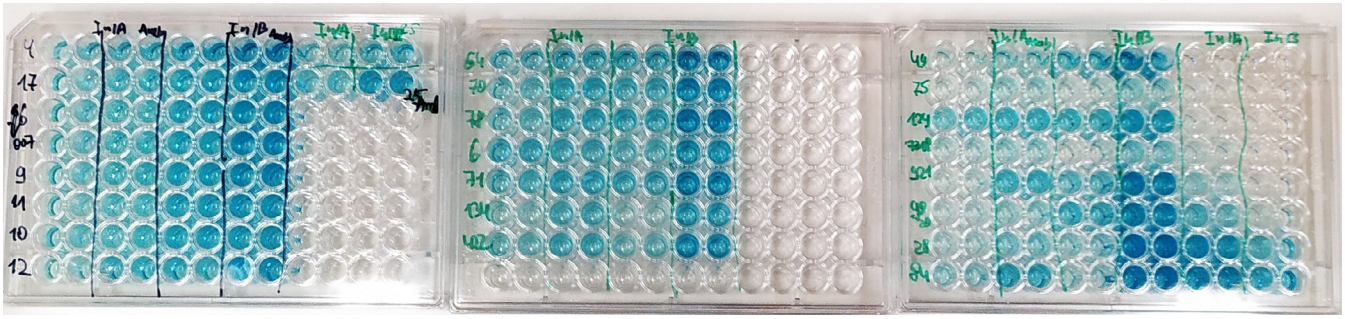

Fig. 1. 96-well plate and test results.

were used: InlA1 5'-ggttgaaaagtatactagtagc-3'; InlA2 5'-ggaagatattagcccaatttc-3'; InlA-R 5'-cttcttttgaattataggatccataagc-3', InlA3 5'-ccaatatcccgggaaaagctat-3', 5'-gcttatggatcctataattcaaaagaag-3', InlBR 5'-gaaaagctgcagaaaatccgccttc-3' InlB1 5'-gaagcaggatcccggataactgcac-3', InlB2 5'-atagcgggttaagttgactgc-3'. The nucleotide sequences were analyzed using the Ugene v. 40.1 software and the BigSdb-Pasteur database[1].

Production of polyclonal monospecific antibodies against InlA and InlB

Rabbits were immunized with purified recombinant InlA and InlB proteins, as previously described [19]. The work with laboratory animals was performed in compliance with ethical principles. The research protocol was approved by the Ethics Committee of the Gamaleya Research Centre of Epidmiology and Microbiology (protocol No. 54, July 24, 2023).

The globulin fraction of hyperimmune sera was precipitated by adding a saturated $(NH_4)_2SO_4$ solution (Ruskhim). The precipitate containing IgG was dissolved in 0.01 M Na-phosphate buffer (pH 6.5) and dialyzed against the same buffer. The obtained solution was passed through a DEAE Sephadex A-50 column and the IgG fraction was collected in the void volume. The affinity column was used to separate antibodies against the respective recombinant InlA and InlB proteins immobilized on an activated BrCN Sepharose (GE Healthcare). The fraction obtained from the DEAE chromatography was washed with 0.3 M NaCl and eluted with 4.5 M $MgCl_2$ (Ruskhim). The obtained samples were dialyzed against phosphate-buffered saline (Sigma-Aldrich), pooled, mixed with glycerol to a concentration of 50% and stored at –20°C.

Western blot analysis of L. monocytogenes lysates

Cell wall proteins were obtained from an overnight culture grown in a BHI broth supplemented with 1% Amberlite XAD-4. The lysates were separated by SDS-PAGE in a 10% gel under Laemmli denaturing conditions and transferred onto a 0.45 μm pore-size nitrocellulose membrane (Bio-Rad). The nitrocellulose membranes were incubated with obtained antibodies against InlA or InlB, respectively, at 1:10000 dilution for 1 hour. Then, the membranes were washed 3 times with TTBS buffer (Bio-Rad) and anti-rabbit IgG secondary antibodies (at 1:20000 dilution) were added. The target InlA and InlB proteins were visualized on the membrane using the TMB substrate (Thermo Fisher Scientific). GAPDH antibodies were used to control protein loading (at 1:1000 dilution (Thermo Fisher Scientific).

Indirect enzyme-linked immunosorbent assay

Bacterial cells were grown in a BHI medium for 24 hours; then, they were centrifuged and washed three times with phosphate-buffered saline. The obtained samples reached an optical density of OD_{600} = 0.2. To verify that the number of cells has not changed, the cell cultures were seeded in an agar medium. The wells of a 96-well plate were coated with aliquots containing 100 μl of the respective tested *L. monocytogenes* strains. The plates were incubated at 37ºC for 1 hour; then, they were washed three times with Tris-buffered saline containing 0.05% Tween 20 (TTBS) (Ruskhim); the unoccupied sites in the wells were blocked by adding 200 μl of 2% bovine serum albumin (Dia-M) diluted in phosphate-buffered saline for 30 min. Upon completion of the incubation, the blocking buffer was removed and 100 μl of anti-InlB or anti-InlA antibodies were added at 1:4000 dilution in TTBS and incubated for 1 hour at room temperature with continuous shaking at 140 rpm. Then, the wells were washed three times with TTBS and 100 μl of secondary antibodies (goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase, Bio-Rad), were added to TTBS. Then, the plates were washed 6 times with 250 μl of TTBS and developed by adding 100 μl of TMB substrate. To stop the reaction, 100 μl of 2 M H_2SO_4 (Ruskhim) was used. The optical density was measured at the wavelength of 450 nm using an iMark spectrophotometer (BioRad). The InlA and InlB concentration was measured using the calibration curve (**Fig. 1**).

Statistical analysis

All tests were repeated at least 3 times. The statistical analysis included one-way ANOVA and Tukey's test. Values of $p < 0.05$ were considered statistically

[1] URL: https://bigsdb.pasteur.fr/listeria

significant.

Results

Analysis of the occurrence frequency of isolates deposited in the BigSdb-Pasteur database depending on the phylogenetic status and the region of isolation

A total of 111 isolates collected in Russia from farm animals and humans infected with *L. monocytogenes* have been deposited in the BigSdb-Pasteur database. Out of them, 83 isolates were collected in 1958-2019 by researchers of the Somov Research Institute of Epidemiology and Microbiology, the Federal Research Center of Virology and Microbiology, Gamaleya RCEM and the State Scientific Center for Applied Microbiology and Biotechnology, using clinical materials collected from stillborn infants with perinatal listeriosis, from pregnant women without clinical signs of infection, and from samples collected from patients with neurolisteriosis and lung tissue lesions. The analysis of the database showed that human listeriosis cases were mostly caused by strains belonging to CC7 (phylogenetic lineage II), CC1, and CC2 (phylogenetic lineage I) accounting for 16, 11, and 8% of the isolates, respectively. Isolates belonging to CC9 (phylogenetic lineage II) accounted for 2% (**Fig. 2**).

Then, we analyzed the distribution with reference to CCs for isolates collected in Europe (excluding countries of the former Soviet Union) from clinical material of patients with listeriosis. A total of 747 isolates were deposited in the database. Note that 723 isolates represent the same clonal complexes that are identified in Russia. However, the percentage of some CCs among clinical isolates collected in Europe differed from those in Russia. Most of the European isolates belonged to CC1, CC6, and CC2 (23, 15, and 14%, respectively).

Assessment of the inlA and inlB diversity among L. monocytogenes isolates originated from Russia, Gamaleya RCEM collection

The database analysis showed that among phylogenetic lineage II strains prevailing in Russia, strains belonging to CC7 are more common among clinical isolates compared to strains belonging to CC9. To gain an insight into mechanisms responsible for these differences, we sequenced *inlA* and *inlB* genes of 32 strains belonging to the above CCs identified in Russia.

Among the strains belonging to CC7, we identified 3 *inlA* allelic variants; 15 strains had allele 2 of the *inlA* gene, which was presented in the BigSdb-Pasteur database. However, in 2 strains, *inlA* differed from the alleles deposited in the above database. The detected mutations were nonsynonymous, thus suggesting that substitutions may be of functional significance. The VIMPA064 isolate contained 1 nonsynonymous substitution leading to the substitution of glutamine at position 364 for proline in the internalin domain involved in the protein-protein interaction with the E-cadherin eukaryotic receptor. VIMPH006 contained 3 nonsynonymous substitutions at positions 618, 620, and 621 (proline, lysine, and glutamine instead of alanine, glutamine, and serine) outside the internalin domain. None of the

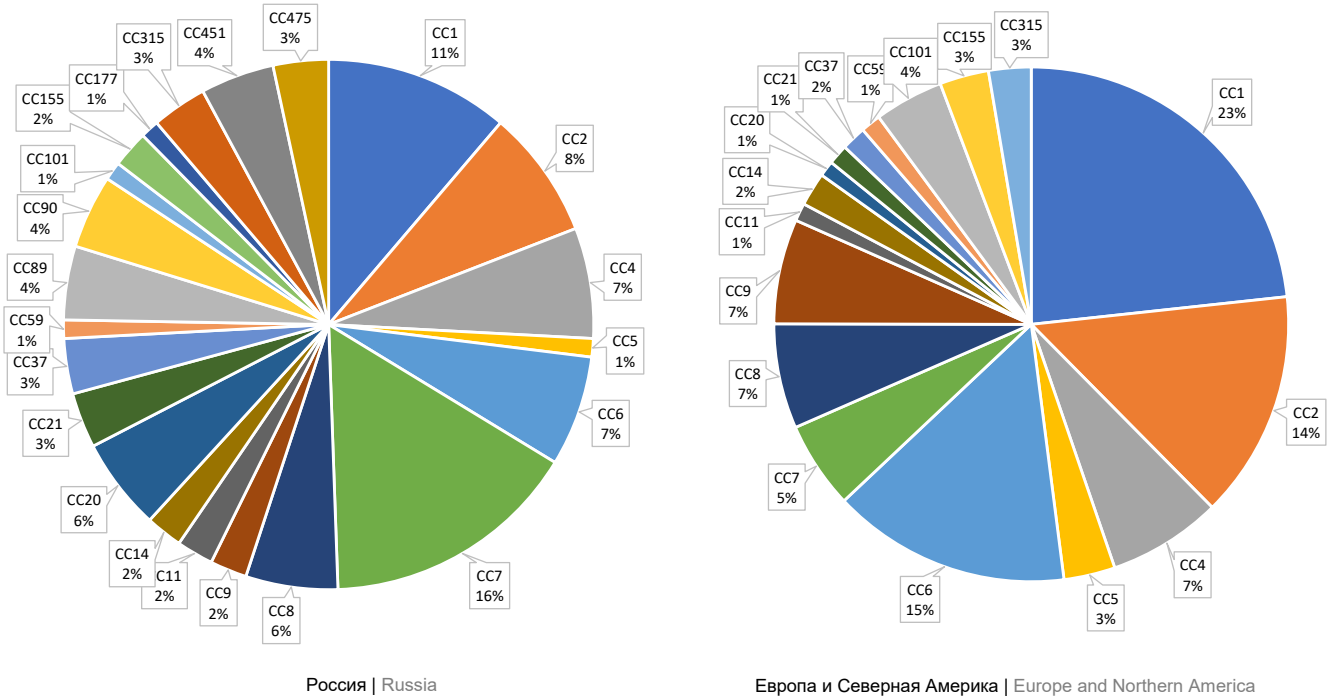

Fig. 2. CC occurrence frequency among *L. monocytogenes* strains from the ListiList database (Institut Pasteur, France), for Russia, countries of Europe and North America.

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alleles of CC7 strains contained premature stop codons.

Among 9 strains belonging to CC9, 1 strain had a stop codon in the *inlA* gene. The stop codon was located outside the internalin domain; however, it prevented the synthesis of the full-length protein and, specifically, the binding of the protein to the bacterial surface, which is essential for the functional activity of InlA as an invasion factor. The other 8 CC9 strains encoded the full-length *inlA* allele 1 variant. The data on sequences of *inlA* genes are available in the GenBank database under accession numbers OQ865090–OQ865119.

The *inlB* gene sequencing in 32 strains did not reveal any specific features within the clonal complexes. Allele 2 was identified for strains belonging to CC7, and allele 1 was identified for CC9 strains.

Thus, the analysis of *inlA* and *inlB* sequences showed that strains belonging to CC7 and CC9 were characterized by intraclonal variability of *inlA*, while *inlB* was conserved for all strains belonging to a specific clone. The alleles identified among highly virulent CC7 strains encoded different InlA isoforms, thus suggesting that there may be differences in their interaction with the target receptor on the surface of human cells. The variant encoded by the alternative *inlA* allele, which was identified among CC9 strains rarely found among clinical isolates, encoded a nonfunctional truncated protein.

Expression levels of the L. monocytogenes InlB pathogenicity factor in strains CC1, CC2, CC7, and CC9

Two ELISA-based test systems were designed to assess the correlation between the production levels of invasion factors InlA and InlB and the occurrence frequency of strains belonging to the above CCs among clinical isolates. The protein concentration was measured using calibration curves from 5 to 1000 ng/ml. Recombinant InlA and InlB were used as reference standards (**Fig. 3**).

The PrfA regulator controls the transcription of both genes encoding InlA and InlB proteins [12]. When *L. monocytogenes* are grown in nutrient media, the PrfA activity increases in the presence of hydrophobic adsorbents (activated carbon or Amberlite XAD4), reaching levels typically demonstrated by bacteria during infection [18]. To analyze changes in the invasin levels depending on the PrfA status, we measured InlA and InlB levels without activation and with activation of the PrfA regulon, depending on the presence of the Amberlite XAD4 adsorbent in the medium. Without PrfA activation, the InlA level in *L. monocytogenes* strains ranged from 5 to 65 ng/ml (**Fig. 4**). On average, the strains belonging to CC1, CC2, and CC7 demonstrated higher InlA levels compared to the strains belonging to CC9 (53.5 ± 5.3 ng/ml compared to 21.1 ± 3.2 ng/ml; $p < 0.05$). The L.mo129-3 strain belonging to CC9 was an exception, demonstrating the level comparable with CC1, CC2, CC7 strains (59.1 ± 1.5 ng/ml).

The medium supplemented with the Amberlite XAD4 adsorbent and the resulting activation of PrfA led to increased InlA concentration in all strains by 31% on average. In some strains the concentration reached the highest levels: VIMHA017 (CC1) — 99 ng/ml, VIMHA007 (CC2) — 93 ng/ml, L.mo71 — 107 ng/ml (CC7), L.mo129-3 (CC9) — 90 ng/ml.

Among the strains, InlB levels ranged from 15 to 958 ng/ml without activation of the PrfA regulon (**Fig. 5**). On average, the concentration in CC1 and CC2 strains was 740.5 ng/ml, being significantly higher than in CC7 and CC9 strains (209.5 and 177.9 ng/ml, respectively). However, compared to InlA, the InlB level in L.mo129-3 strain did not reach values typical of CC1 and CC2 strains and was twice as low ($p < 0.05$).

When PrfA was activated, the InlB concentration reached the highest levels of 1,353 ng/ml for CC1 and

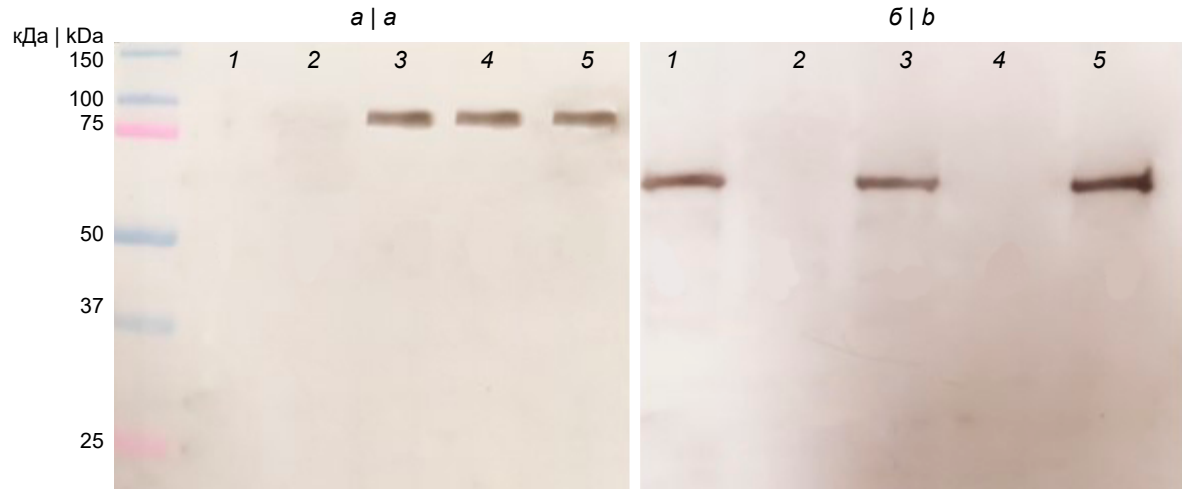

Fig. 3. Immunoblot of *Listeria* spp. cell lysates.

a — monospecific antibodies against InlA; *b* — monospecific antibodies against InlB. *1 – L. monocytogenes* EGDe Δ*inlA*; *2 — L. ivanovii* ATCC 19119; *3 — L. monocytogenes* EGDe (CC9); *4 — L. monocytogenes* EGDe Δ*inlB*; *5 — L. monocytogenes* L.mo49 (CC9).

CC2 strains, 917 ng/ml — for CC7 strains, 835 ng/ml — for CC9 strains. The highest InlB levels were demonstrated by CC7 strains (a 3.29-fold increase compared to the levels without activation). In the strains belonging to CC9 and phylogenetic lineage I (CC1, CC2), the InlB levels increased 2.93 and 1.54 times, respectively, in the presence of the adsorbent.

Discussion

In *L. monocytogenes*, like in most of the other pathogenic bacteria, clonal groups and lineages differ in their virulence potential [20, 21]. For example, phylogenetic lineage II strains are frequently associated with food products, while phylogenetic lineage I strains are commonly found in clinical isolates. However, the distribution among sources is different even within phylogenetic lineages. For example, phylogenetic lineage II strains belonging to CC7 are more frequently isolated from clinical samples than strains belonging to CC9 [20]. The underlying causes of this heterogeneity must be identified to understand the mechanisms of evolution and development of highly virulent strains.

In some pathogenic bacteria such as enteropathogenic Yersinia, the differences in virulence are associated with the presence of additional pathogenicity factors, which can be located on plasmids and mobile genetic elements [22]. The search of additional pathogenicity factors in *L. monocytogenes* strains belonging to clonal complex CC1 resulted in the discovery of LLS bacteriocin, which, presumably allows Listeria to survive longer in the presence of normal microbiota, though it is not a true pathogenicity factor and is absent in other highly virulent strains belonging to other CCs [23].

We have proposed an alternative hypothesis on the role of variability of major pathogenicity factors as the main mechanism responsible for development of highly virulent *L. monocytogenes* strains and other sapronotic pathogens [24]. Natural InlB isoforms differ from each other both in the kinetics of activation of intracellular signaling pathways and in the binding constant to their target receptors c-Met and gC1qR [25, 26]. Strains that differed only in the InlB isoform differed in their ability to support gastrointestinal infection in mouse-like rodents, while InlB isoforms were responsible for different invasion of these strains into animal epithelial cells (sheep kidney cells, shKEC) [10]. In our study, we demonstrated the variability of another invasion factor — the InlA protein among strains belonging to phylogenetic lineage II CCs: CC7 and CC9. Among the analyzed strains, we identified one strain with a nonfunctional InlA protein, thus demonstrating a relatively insignificant role of CC9 in the clinical spread of listeriosis and being consistent with the findings of other researchers [27].

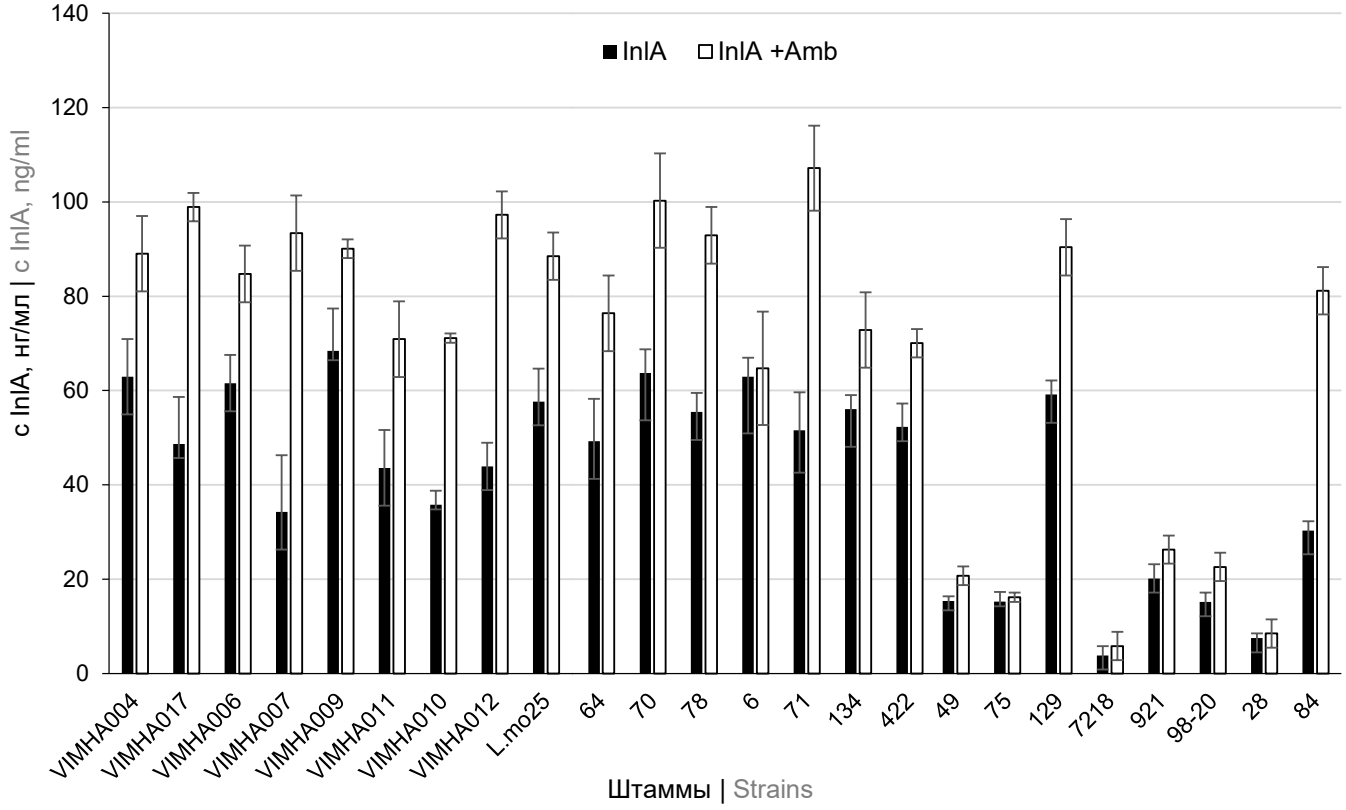

Fig. 4. Levels of InlA isolates belonging to different CCs.

The InlA concentration was measured using the new ELISA test system. Bacteria were grown in a BHI medium without and with the addition of 1% Amberlite XAD4.

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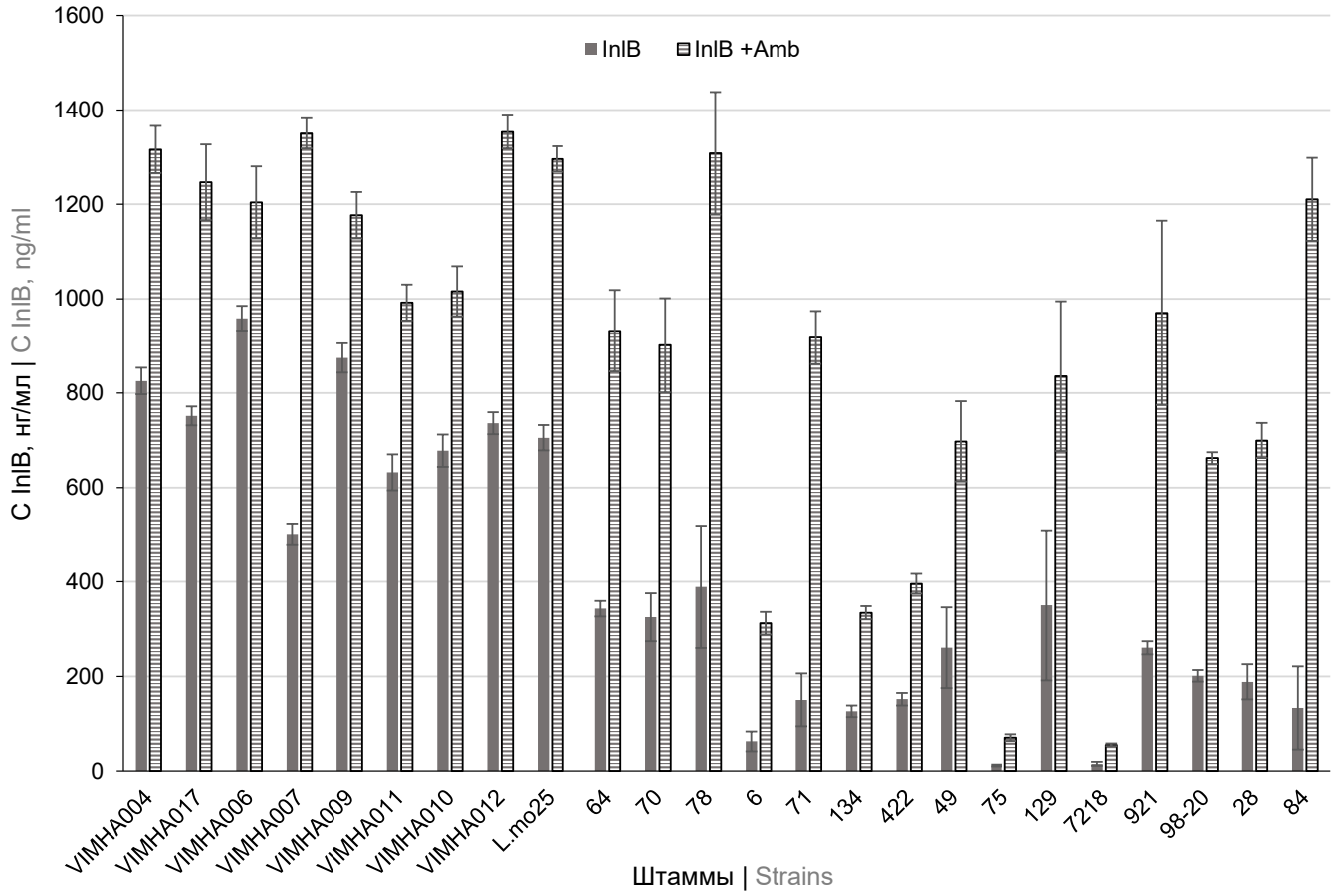

Fig. 5. Levels of InlB isolates belonging to different CCs.
The InlA concentration was measured using the new ELISA test system. Bacteria were grown in a BHI medium with and without 1% Amberlite XAD4.

The production level of major pathogenicity factors can be seen as another mechanism playing a significant role in the virulence potential of a strain [11]. It was previously found that the InlB production level affects the interaction of *L. monocytogenes* with macrophages [28]. In *L. monocytogenes*, the production of pathogenicity factors is controlled by the PrfA protein, which lacks activity in the growth environment outside the host (low temperature, presence of plant sugars and hydrophobic peptides) [22]. This regulatory mechanism is justified, as it eliminates the redundancy of the synthesis of pathogenicity factors for sapronotic pathogens, when they exist in the abiotic environment. At the same time, high production levels of factors required for crossing the intestinal epithelial barrier should have a positive effect on bacterial virulence. Following this hypothesis, this study demonstrated that the production level of the InlB invasion factor without PrfA activation was 4 times as high in phylogenetic lineage I strains (CC1 and CC2) compared to phylogenetic lineage II strains (CC7, CC9). The InlA production levels, without and with PrfA activation, in CC1, CC2, CC7 isolates were higher than the levels in CC9 isolates. This is consistent with the fact that CC1, CC2, CC7 are most often found in clinical samples in Russia. In CC9 strains, which are most common for food products, the InlA level was 2.5 times as low, except for the L.mo129-3 strain, which demonstrated levels similar to those observed in CC1 and CC2. Thus, our study has shown that strains highly virulent for humans are characterized not only by specific isoforms, but also by increased production of InlA and InlB. Our findings confirm the results obtained by other researchers who have found that clinical isolates differ from food isolates in *inlA* expression levels [11].

Conclusion

In total, our findings are consistent with the epidemiological monitoring data and demonstrate possible mechanisms of the formation of *L. monocytogenes* CCs with different virulence potentials. The obtained results are of fundamental importance; they can also serve as a basis for classifying newly collected isolates into virulent and hypovirulent clones.

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