Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 221-230, 2023
DOI: 10.9775/kvfd.2023.28970

RESEARCH ARTICLE

Whole-Genome Sequencing-Based Characterization of *Listeria monocytogenes* from Food and Animal Clinical Cases

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Article ID: KVFD-2023-28970 Received: 05.01.2023 Accepted: 17.04.2023 Published Online: 19.04.2023

Abstract: Listeriosis is a rare but severe foodborne infection caused by *Listeria monocytogenes*. In this study, we performed comparative whole-genome sequencing (WGS) on 28 *Listeria monocytogenes* from seven invasive listeriosis cases in animals and 21 food samples in Türkiye for the first time. Food isolates were delineated into eleven clonal complexes (CCs), namely CC1, CC2, CC3, CC8, CC9, CC20, CC69, CC124, CC155, CC204, ST3002. The isolates from meningoencephalitis cases were associated with CC1, whereas CC9 and CC7 were associated with the isolates from sheep abortus cases. All the isolates carried the *fosX*, *lin*, *norB*, and *sul* genes. In addition, *emrC* (n=15), *bcrC* (n=4), *emrE* (n=2), *qacA* (n=1), *cadA* (n=5) and *cadC* (n=1) genes, conferring resistance to stress and disinfectants were detected. Listeria pathogenicity island (LIPI)-1 and LIPI-2 were distributed in all isolates, but LIPI-3 was closely related to CC1, CC3, and ST3002 isolates. LIPI-4 was not found in any of the *L. monocytogenes* isolates. The Inc18(rep25) and Inc18(rep26) plasmids were found in 16 (57.1%) isolates. A total of 15 different intact prophage genomes ranging from one to three were detected in the genomes of 24 isolates. The hypervirulent CC1 and CC2 clones that pose a significant threat to food safety and public health were detected among food isolates. These findings highlight the importance of continuous surveillance of hypervirulent *L. monocytogenes* strains in different settings.

Keywords: *Food, Genetic diversity, Invasive infection, Listeria monocytogenes, Whole Genome Sequencing*

Gıda ve Hayvan Klinik *Listeria monocytogenes* İzolatlarının Tam Genom Dizilimine Dayalı Karakterizasyonu

Öz: Listeriosis, *Listeria monocytogenes*'in neden olduğu nadir görülen fakat ciddi klinik seyre sahip gıda kaynaklı bir enfeksiyondur. Bu çalışmada, hayvanlardaki invaziv listeriozis vakalarından (n=7) ve farklı gıda örneklerinden (n=21) izole edilen 28 *Listeria monocytogenes* suşunun karşılaştırmalı tam genom dizileme (WGS) ile analizi yapıldı. Gıda izolatları CC1, CC2, CC3, CC8, CC9, CC20, CC69, CC124, CC155, CC204, ST3002 olmak üzere onbir klonal komplekse (CC) ayrıldı. Meningoensefalit vakalarına ait izolatlar CC1'e ait iken, koyun abortus vakalarına ait izolatlar CC9 ve CC7'e ait bulundu. Tüm izolatlarda *fosX*, *lin*, *norB* ve *sul* genleri belirlendi. Ayrıca, değişen oranlarda stres ve dezenfektan direncine aracılık eden *emrC* (n=15), *bcrC* (n=4), *emrE* (n=2), *qacA* (n=1), *cadA* (n=5) ve *cadC* (n=1) genleri tespit edildi. Listeria patojenite adası (LIPI)-1 ve LIPI-2 tüm izolatlarda tespit edilirken; LIPI-3 CC1, CC3 ve ST3002'e ait izolatlar ile yakın ilişkili bulundu. LIPI-4 *L. monocytogenes* izolatlarının hiçbirinde bulunmadı. Inc18(rep25) ve Inc18(rep26) plazmidleri 16 (%57.1) izolatta bulundu. Yirmidört izolatta bir - üç arasında değişen toplam 15 farklı intakt profaj genomu tespit edildi. Bu çalışmada gıda izolatları arasında gıda güvenliği ve halk sağlığı için önemli bir tehdit oluşturan hipervirülent CC1 ve CC2 klonları tespit edilmiştir. Bu bulgular, farklı ortamlarda hipervirülent *L. monocytogenes* suşlarının sürekli izlenmesinin önemini vurgulamaktadır.

Anahtar sözcükler: *Gıda, Genetik çeşitlilik, İnvaziv infeksiyon, Listeria monocytogenes, Tüm Genom Sekanslama*

How to cite this article?

Aslantaş Ö, Büyükaltay K, Keskin O, Güllü-Yücetepe A, Adıgüzel A: Whole-genome sequencing-based characterization of *Listeria monocytogenes* from food and animal clinical cases. *Kafkas Univ Vet Fak Derg,* 29 (3): 221-230, 2023.
DOI: 10.9775/ kvfd.2023.28970

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Introduction

Listeria monocytogenes is an opportunistic food-borne pathogen that may cause life-threatening infections in many mammalian species following ingestion [1]. In ruminants, listeriosis is associated with gastroenteritis, abortions, septicemia, central nervous system (CNS) infections (neurolisteriosis), as well as rarely anatomically localized infections such as mastitis, eye infections and keratitis [2]. Infected ruminants are frequent asymptomatic carriers of *L. monocytogenes* in their gastrointestinal tract that allows the pathogen to multiply and spread the environments [3]. Throughout the world, listeriosis most commonly occurs in a sporadic form affecting a single or a few animals or may occur as outbreaks within a farm [4]. In humans, the infection, is frequently linked to consumption of ready-to-eat (RTE) food, occurs mostly in immunocompromised individuals, the elderly people and pregnant women, which manifests itself symptoms similar to those seen in ruminants [5]. Although incidence of listeriosis is low in comparison with other food-borne infections, high fatality rate associated with this infection makes it a major public health concern [6]. *L. monocytogenes* is a ubiquitous microorganism that colonizes a variety of ecological niches, including soil, water, food processing facilities, mammalian intestinal tracts and faeces, making surveillance and control of environmental lifestyle of the pathogen very challenging [7]. In particular, persistence of *L. monocytogenes* in food processing environment for months even years despite sanitation measures applied often results in cross-contamination of the final product, which increases the risk of outbreaks [8]. Presence of same *L. monocytogenes* genotypes were also reported in farm environments and animals [9,10]. Multiple virulence factors (VFs) are key for the adaptation of *L. monocytogenes* to its host and different environmental niches [2].

Molecular epidemiological studies have revealed the variable distribution of genetic and serological subtypes of *L. monocytogenes* with respect to food products and processing environments as well as among human and animal clinical listeriosis cases. Lineage II or serotypes 1/2b, 1/2a, and 1/2c are more frequent in food isolates, and lineage I or serotype 4b is the predominant serotype among clinical isolates. Moreover, clones CC1, CC2, CC4, and CC6 are strongly associated with clinical strains, CC9 and CC121 are hypovirulent food-associated clones [11-14].

Whole genome sequencing (WGS) greatly improved the analysis of bacterial genome sequences and facilitated the evaluation of *L. monocytogenes* isolates from clinical, environmental, and food sources [13,15]. WGS also allows the detection of virulence genes responsible for the pathogenicity of different isolates. So far, four virulence gene clusters, known as Listeria pathogenicity islands (LIPIs) have been identified. LIPI-1 and LIPI-2 contain the key genes responsible for promoting adhesion or binding, invasion, polymerization, and cell-to-cell spread within the host organism [2]. LIPI-3 encodes listeriolysin S (LLS), shows hemolytic and cytotoxic activity associated with the destruction of gut microbiota and thus dysregulates host-microbiota homeostasis during infection [16]. More recently, LIPI-4 gene cluster considered as hypervirulent, strongly linked with neural and placental infections has been described [14].

The objectives of this study were to (i) assess the genomic diversity of *L. monocytogenes* isolates from food and animal invasive infections, (ii) determine the absence and presence of antimicrobial and disinfectant resistance and virulence genes.

Material and Methods

Bacterial Strains

The strains of *L. monocytogenes* tested were isolated from food (n = 21) and animal clinical cases of listeriosis (n = 7) [five from sheep abortus cases and two from CNS cases (one from sheep and one from cattle)] in four provinces of Türkiye over a period of three years (from January 2017 to July 2020). The characteristics and sources of the *L. monocytogenes* strains used in this study are reported in *Table 1*. Species identification was carried out both using MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States) and VIDAS system (Biomérieux, France).

DNA Isolation, Library Preparation and Sequencing

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration was evaluated using fluorometric (Qubit 3.0, ThermoFisher Scientific, Waltham, MA, USA) method. The sequencing libraries of genomic DNA were prepared according to the Illumina protocol and paired-end (2x150 bp) sequencing was performed on a NovaSeq 6000 platform (Illumina, San Diego, USA). After trimming low-quality reads and removing adapter sequences using Trimmomatic v0.36 [17], the quality of both raw reads and trimmed reads was assessed using FastQC (v 0.11.9). The *de novo* genome assembly was conducted using the Shovill pipeline (v 0.9.0) by applying the default parameters [18]. The quality of assemblies was evaluated using QUAST v4.5 [19]. The detailed *L. monocytogenes* sequence parameters used in the present study are listed in *Table 1*. The draft assemblies of all *L. monocytogenes* isolates were deposited in the BIGSdb-Lm database *(https://bigsdb.pasteur.fr/listeria)* under the accession number 86311-86338.

WGS Characteristics of *L. monocytogenes*

All *L. monocytogenes* sequences were analyzed using the publicly available web-based WGS tools on the BIGSdb-

Table 1. L. monocytogenes sequence parameters

No	BIGSdb id	Contigs	Total Length	Max. Contig Length	Min. Contig Length	Mean Contig Length	StdDev Contig Length	L50 Contig Number	N50 Contig Length (L50)	L90 Contig Number	N90 Contig Lenght	L95 Contig Number	N95 Contig Lenght	GC%
1	86311	17	3.022.337	745.156	238	177.785	232.446	3	513.002	7	224.989	8	97.911	37.79
2	86312	12	2.939.770	1.507.484	238	244.981	442.900	1	1.507.484	4	229.475	5	101.052	37.86
3	86313	16	3.022.564	745.156	238	188.911	236.106	3	518.154	7	97.911	8	97.911	37.79
4	86314	16	2.950.838	848.413	238	184.428	270.484	3	530.836	5	230.756	7	100.203	38.08
5	86315	16	2.950.938	848.173	238	184.434	270.430	3	530.836	5	230.756	7	100.203	38.08
6	86316	15	2.950.898	848.413	238	196.727	268.487	3	530.836	5	230.552	7	100.203	38.08
7	86317	16	2.950.975	848.173	238	184.436	261.841	3	530.836	5	230.756	7	100.203	38.08
8	86318	15	2.964.264	909.192	238	197.618	278.461	2	604.948	4	229.071	6	100.992	37.82
9	86319	15	3.010.367	816.421	221	200.692	289.618	2	774.368	5	228.558	6	101.088	37.83
10	86320	15	2.964.210	909.192	238	197.614	279.456	2	604.948	5	229.071	6	100.992	37.82
11	86321	16	2.964.383	909.192	238	185.274	274.770	2	604.948	5	228.923	6	100.992	37.82
12	86322	23	3.012.987	575.528	217	131.000	175.906	4	397.443	8	167.829	9	100.992	37.73
13	86323	18	2.996.628	1.486.655	238	166.480	335.230	2	430.186	6	99.054	8	75.397	37.86
14	86324	19	2.986.983	1.486.782	238	157.210	336.919	2	430.186	6	99.054	8	75.397	37.85
15	86325	16	2.964.377	909.192	238	185.274	274.769	2	604.942	5	228.923	6	100.992	37.82
16	86326	17	2.957.225	1.243.617	296	173.955	307.671	2	521.587	6	225.190	6	225.190	37.85
17	86327	28	2.877.061	632.635	201	102.753	178.162	3	476.856	7	225.046	8	99.748	37.93
18	86328	20	3.011.077	588.120	238	150.554	190.746	3	439.574	8	99.051	10	71.994	37.83
19	86329	16	2.964.363	909.186	238	185.273	274.773	2	604.942	5	229.071	6	100.992	37.82
20	86330	20	2.956.218	759.675	296	147.811	215.408	3	483.632	7	227.892	8	99.751	37.87
21	86331	16	2.964.368	909.192	238	185.273	274.774	2	604.942	5	229.071	6	100.992	37.82
22	86332	14	2.964.682	909.192	238	211.763	284.144	2	604.942	5	229.071	6	100.992	37.82
23	86333	16	2.964.376	909.192	238	185.274	274.772	2	604.942	5	228.923	6	100.992	37.82
24	86334	17	2.964.487	909.192	238	174.382	270.099	2	604.492	5	228.923	6	100.992	37.82
25	86335	14	2.882.660	1.502.479	238	205.479	386.440	1	1.502.479	5	167.055	6	99.228	37.89
26	86336	24	2.922.545	505.602	259	121.773	160.986	4	331.745	8	207.136	10	96.460	37.88
27	86337	20	3.026.339	745.155	238	151.317	212.219	3	478.320	7	225.128	8	151.808	37.91
28	86338	28	2.877.058	632.635	201	102.753	178.163	3	476.856	7	225.046	8	99.748	37.93

Lm platform *(https://bigsdb.pasteur.fr/listeria*, accessed on 12 July 2022). MLST profiles with the same alleles for seven loci were classified into sequence types (ST) and grouped into clonal complexes (CCs) if at least five out of seven loci were the same as previously described. cgMLST (1748 loci) profiles were grouped into cgMLST types (CTs) and sublineages (SLs), using the cut-offs of seven and 150 allelic mismatches, respectively, as previously described. Allele numbers, CTs, and SLs were determined according to the *Listeria* sequence typing database (BIGSdb-*Lm* platform) [13].

Identification of Virulence and Other Genetic Markers

Assemblies were also screened *in silico* for virulence, antimicrobial, metal, and biocide resistance genes, Listeria Stress Islands as well as the *sigB* and rhamnose operons using the BIGSdb-Lm platform [13]. The presence of premature stop codons (PMSCs) in the *inlA* gene was also investigated. When the BIGSdb-*Lm* database reported that a PMSC mutation was present, the mutation position and the length of the resulting truncated *inlA* protein were specified [13].

Detection of Prophage and Plasmid Sequences

The putative prophage determinants within the genomes of the *L. monocytogenes* were tested, the WGS sequences were analyzed with the PHASTER (PHAge Search Tool Enhanced Release) web server [20]. The criteria for scoring prophage regions (as intact, questionable or incomplete) have been described in PHASTER. If the region's total score was less than 70, it was marked as incomplete, between 70-90 as questionable, and when greater than 90 defined as intact. The presence of plasmid sequences was identified using the PlasmidFinder v2.1 for the specified Gram-positive scheme [21].

Results

WGS-based Typing of *L. monocytogenes*

Overall, all strains were grouped into five PCR-serogroups IIc (comprising serotypes 1/2c, 3c), IIa (1/2a, 3a), IIb (1/2b, 3b), IVb (4b, 4d, 4e) and L. The most frequent PCR-serogroup was IIc (n=9, 32.1%), followed by IIa (n=7, 25%), IVb (n=5, 17.9%), L (n=4, 14.3%), and IIb (n=3, 10.7%) *(Table 2)*. PCR-serogroup IVb was determined in two CNS related isolates and three food isolates. Of the five isolates related with aborted sheep fetuses, four were PCR-serogroup IIc, and one was IIa.

A total of three lineages was identified, Lineage II (17, 60.7%) was the most numerous, followed by Lineage I (7, 25%), and Lineage III (4, 14.3%), respectively. While CNS infection related isolates belonged to Lineage I, all isolates associated with sheep abortus cases belonged to Lineage II. Eleven different STs were identified, ST122 (9, 32.1%) was the most abundant, followed by ST202 (4, 14.3%), ST1 (4, 14.3%), ST20 (2, 7.2%), and ST3002 (2, 7.2%), respectively. Seven STs (25% of all STs) were represented by single isolates. ST3002 was submitted as new ST to the Listeria PasteurMLST database. Ten CCs and one singleton (ST3002) were identified. CC112 (9, 36%) was the most prevalent, followed by CC1 (4, 14.3%), CC69 (4, 14.3%),

Table 2. *Molecular characterization of L. monocytogenes strains tested*

BIGSdb-Lm ID	Source	Province	Phylogenetic Lineage	PCR-Serogroup	ST	CC	SL	cgMLST
86311	Butter	Diyarbakır	I	IIb	3002*	ST3002	SL3	CT11733
86312	Cheddar cheese	Diyarbakır	II	IIa	124	CC124	SL124	CT11738
86313	Cake	Diyarbakır	I	IIb	3002*	ST3002	SL3	CT11733
86314	Ice cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86315	Cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86316	Cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86317	Cream cheese	Diyarbakır	III	L	202	CC69	SL69	CT996
86318	Sausage	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86319	Roasted meat	Diyarbakır	II	IIa	155	CC155	SL155	CT11740**
86320	Meatball	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86321	Lahmacun	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86322	Hamburger	Hatay	II	IIa	204	CC204	SL204	CT11737
86323	Pastry	Hatay	II	IIa	20	CC20	SL20	CT11735
86324	Pizza	Hatay	II	IIa	20	CC20	SL20	CT11736
86325	Cake	Hatay	II	IIc	122	CC9	SL9	CT630
86326	Sausage	Adana	I	IVb	1	CC1	SL1	CT11732
86327	Ice cream	Adana	I	IVb	1	CC1	SL1	CT11730**
86328	Beans	Adana	II	IIa	8	CC8	SL8	CT11739
86329	Raw meat	Adana	II	IIc	122	CC9	SL9	CT630
86330	Cattle Meningoencephalitis	Şanlıurfa	I	IVb	1	CC1	SL1	CT11731
86331	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86332	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86333	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86334	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86335	Sheep aborted fetus	Şanlıurfa	II	IIa	12	CC7	SL7	CT720
86336	Döner	Şanlıurfa	II	IVb	145	CC2	SL2	CT375
86337	Cake	Şanlıurfa	I	IIb	3	CC3	SL3	CT11734
86338	Sheep Meningoencephalitis	Şanlıurfa	I	IVb	1	CC1	SL1	CT11730**

**new ST type, **new cgMLST type*

CC20 (2, 14.3%) and ST3002 (2, 14.3%). The remaining seven (25%) strains, which were mainly recovered from food, were grouped into six CCs, with single isolates in each *(Table 2)*. Minimum spanning tree (MST) analysis based on the cgMLST profiles of 28 *L. monocytogenes* strains was generated using the publicly available web-based WGS tool on the BIGSdb-Lm platform.

The isolates were further divided into 14 different types of cgMLST (CTs), with the most numerous CT630 (10, 35.7%) and CT996 (4, 14.3%). The isolates associated with sheep aborted fetuses belonged to CT630 and CT720, whereas CNS infection related isolates belonged to CT11731 and CT11730. CT11730 and CT11740 were new cgMLST types designated for the first time. cgMLST based analyses results revealed ten different sublineages (SLs). The predominant SLs identified were SL9 (10, 35.7%), SL69 (4, 14.3%), SL1 (4, 14.3%), SL3 (3, 10.7%) and SL20 (2, 3.6%). The remaining SLs (SL24, SL155, SL20, SL8, and SL2) were only determined in one isolate of each. Of the CNS infection related SLs, SL1 was also identified in two food isolates. SLs recovered sheep aborted fetus isolates were SL9 and SL7. Molecular relationship of 28 *L. monocytogenes* strains based on the cgMLST analysis are shown in *Fig. 1*.

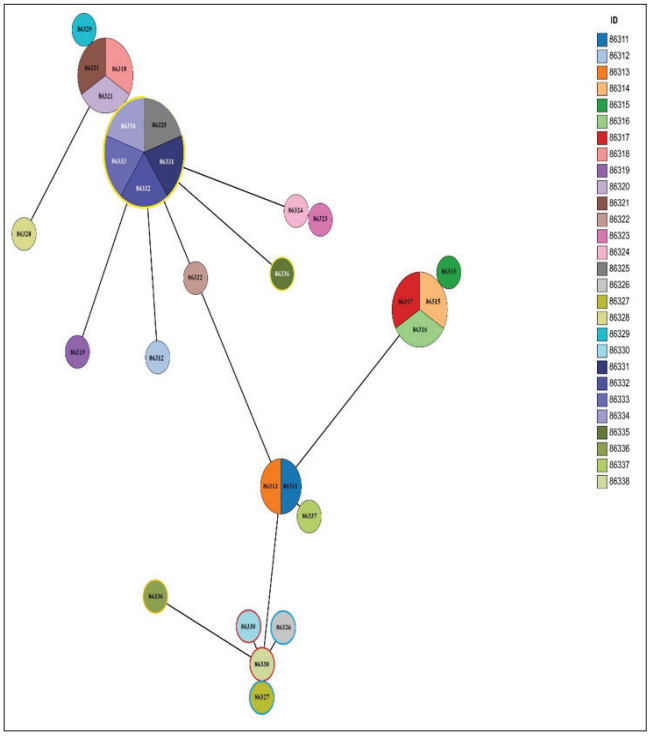

Fig 1. MST based on the cgMLST profiles of 28 *L. monocytogenes* strains studied. Circles with red rim represent *L. monocytogenes* strains from CNS infections. Circles with blue rim represent *L. monocytogenes* strains from foods belonged to CC1. Circles with yellow rim represent *L. monocytogenes* strains from sheep abortus cases

Antimicrobial Resistance and Virulence Gene Profiles

Half of the strains had the same resistance profile with the presence of the *fosX* (fosfomycin), *lin* (antibiotic ABC transporter ATP-binding protein), *norB* (multidrug efflux pump), and *sul* (dihydropteroate synthases) genes, according to the screening screening of *L. monocytogenes* WGS data for 17 antimicrobial resistance genes. Other half of the isolates also carried *mprF* (phosphatidyl glycerol lysyl transferase) gene together with the genes above-mentioned. Acquired antibiotic resistance traits were not detected.

Presence of LIPI-1 was observed in all strains. The LIPI-2 was present in all isolates. However, four strains belonged to ST202 (CC69) did not carry *inlC*, *inlE*, *inlF*, *inlG*, *inlH* and *inlJ* genes. In addition, *inlG* in six isolates and *inlJ* in one isolate were not present. The LIPI-3 virulence genes were identified in seven isolates. LIPI-4 was not found in any of the *L. monocytogenes* studied. The premature stop codon (PMSC) in the *inlA* gene was not present in all the isolates.

The *gltA* and *gltB* genes involved in teichoic acid biosynthesis were found in the only strains belonging to serogroup IVb (n=5) isolates. Similarly, *aut_IVb* gene (autolysin) was only present in the isolates belonging to serogroup IVb.

Benzalkonium Chloride (BC) and Other Tolerance Genes

Of the *bcrABC* gene cassette, encoding benzalkonium chloride resistance protein, *bcrC* was identified in four isolates. The *emrE* gene (encoding the putative small multidrug-resistant (SMR) efflux pump), the *qacA* gene (encoding the quaternary ammonium compound efflux major facilitator superfamily (MFS)), and the *Tn6188 qac* (*ermC*) sequence responsible for benzalkonium chloride tolerance were found in two, one and 15 isolates, respectively. The *cadA* and *cadC* genes, responsible for cadmium resistance, were present in five and one isolate, respectively. All five genes of the stress survival islet 1 (SSI-1) were observed in 21 isolates. The remaining isolates had only one of the SSI-1 islets.

Identification of Prophage Sequences

DNA sequence analysis of 28 *L. monocytogenes* isolates revealed the presence of 41 different intact, questionable or incomplete prophage sequences among the *L. monocytogenes* isolates. Intact prophage sequences were determined in 24 *L. monocytogenes* isolates *(Table 3)*. Phage_Lister_LP_HM00113468 (NC_049900) (n=10, 41.7%) were the most prevalent.

Detection of Plasmids

Plasmids were found in 57.1% (16/28) of the strains *(Table 4)*. pLM330006(pLM33) (rep25) and pLGUG1 (rep26) were detected in 13 (81.3%) and three (18.7%) isolates, respectively.

Table 3. *Prophages in L. monocytogenes strains tested*

No	BIGSdb id	cgMLST Type	Total Lenght (bp)	Total Number of Prophages Regions	No of Prophages Regions According to the Status			Names of Phages with Intact Status	Characteristics of Phages with Intact Status		
					Intact	Questionable	Incomplete		Size (Kb)	No of Proteins	Position
1	86311	CT11733	3.022.337	5	1	1	3	PHAGE_Lister_B054_NC_009813(44)	65.9	82	445354-511181
2	86312	CT11738	2.939.770	3	1	1	1	PHAGE_Lister_A118_NC_003216(12)	35.6	53	1042532-1078228
3	86313	CT11733	3.022.564	5	1	1	3	PHAGE_Lister_B054_NC_009813(44)	65.9	82	445354-511181
4	86314	CT996	2.950.838	5	2	1	2	PHAGE_Lister_LP_030_2_NC_021539(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	6465-34603
5	86315	CT996	2.950.938	5	2	1	2	PHAGE_Lister_2389_NC_003291(16)	41.3	62	348970-390388
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	6465-34603
6	86316	CT996	2.950.898	5	2	1	2	PHAGE_Lister_LP_030_2_NC_021539(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	44-28182
7	86317	CT996	2.950.975	5	2	1	2	PHAGE_Lister_2389_NC_003291(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	44-28182
8	86318	CT630	2.964.264	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
9	86319	CT11740	3.010.367	5	3	2	0	PHAGE_Strept_315.2_NC_004585(7)	35.3	48	359352-394680
								PHAGE_Lister_B054_NC_009813(65)	48.2	79	455703-503959
								PHAGE_Lister_A006_NC_009815(26)	53	60	34644-87714
10	86320	CT630	2.964.210	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
11	86321	CT630	2.964.383	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
12	86322	CT11737	3.012.987	4	1	2	1	PHAGE_Lister_vB_LmoS_188_NC_028871(23)	24.8	32	550716-575528
13	86323	CT11735	2.996.628	1	1	0	0	PHAGE_Lister_A006_NC_009815(39)	38.2	59	296189-334422
14	86324	CT11736	2.986.983	1	1	0	0	PHAGE_Lister_A006_NC_009815(39)	38.2	59	296189-334422
15	86325	CT630	2.964.377	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
16	86326	CT11732	2.957.225	3	1	1	1	PHAGE_Lister_vB_LmoS_188_NC_028871(31)	48.7	68	252224-300962
17	86327	CT11730	2.877.061	2	0	1	1	-	-	-	-
18	86328	CT11739	3.011.077	3	1	2	0	PHAGE_Lister_LP_HM00113468_NC_049900(38)	47.4	57	392173-439574
19	86329	CT630	2.964.363	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
20	86330	CT11731	2.956.218	4	2	1	1	PHAGE_Lister_2389_NC_003291(48)	37.9	56	153-38085
								PHAGE_Lister_vB_LmoS_293_NC_028929(34)	34.4	47	1223-35650
21	86331	CT630	2.964.368	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
22	86332	CT630	2.964.682	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
23	86333	CT630	2.964.376	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
24	86334	CT630	2.964.487	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
25	86335	CT720	2.882.660	3	1	1	1	PHAGE_Lister_LP_101_NC_024387(47)	43.5	65	983003-1026580
26	86336	CT375	2.922.545	2	0	1	1	-	-	-	-
27	86337	CT11734	3.026.339	6	0	1	5	-	-	-	-
28	86338	CT11730	2.877.058	2	0	1	1	-	-	-	-

Discussion

This study describes the WGS-based characterization of *L. monocytogenes* strains isolated from food and animal clinical cases in Türkiye for the first time. MLST typing revealed that *L. monocytogenes* strains isolated from food were assigned to eleven clonal complexes. The detected CCs in the present study have been also shown to be globally distributed in foods and food-processing environments. As previously reported, CC1 and CC2 are highly associated with clinical human and animal listeriosis cases, but they have also been detected in food products and considered hypervirulent [22,23]. The higher gut colonization ability of hypervirulent *L. monocytogenes* clones, particularly CC1, may lead to prolonged fecal shedding, resulting in persistence dairy cattle farm environment and high frequency contamination of milk and dairy products and thus pose a potential health risk

Tablo 4. *Plasmids in L. monocytogenes strains tested*

No	BIGSdb id	cgMLST Type	Total lenght (bp)	No. of Plasmids	Locus Targeted	Note	Contig	Position	Lenght (bp)
1	86311	CT11733	3.022.337	1	rep25	pLM330006(pLM33)	NODE_9_length_57672_cov_44.684682	34694..36454	1761
2	86312	CT11738	2.939.770	1	rep25	pLM330006(pLM33)	NODE_6_length_48704_cov_35.285329	3761..5521	1761
3	86313	CT11733	3.022.564	1	rep25	pLM330006(pLM33)	NODE_9_length_57728_cov_40.328931	34750..36510	1761
4	86314	CT996	2.950.838	-	-	-	-	-	-
5	86315	CT996	2.950.938	-	-	-	-	-	-
6	86316	CT996	2.950.898	-	-	-	-	-	-
7	86317	CT996	2.950.975	-	-	-	-	-	-
8	86318	CT630	2.964.264	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_40.326559	43465..45225	1761
9	86319	CT11740	3.010.367	-	-	-	-	-	-
10	86320	CT630	2.964.210	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_35.097293	-	-
11	86321	CT630	2.964.383	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_36.925559	-	-
12	86322	CT11737	3.012.987	-	-	-	-	-	-
13	86323	CT11735	2.996.628	1	rep26	repA(pLGUG1)	NODE_8_length_75397_cov_15.714901	-	-
14	86324	CT11736	2.986.983	1	rep26	repA(pLGUG1)	NODE_8_length_75397_cov_16.905055	-	-
15	86325	CT630	2.964.377	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_42.370043	-	-
16	86326	CT11732	2.957.225	-	-	-	-	-	-
17	86327	CT11730	2.877.061	-	-	-	-	-	-
18	86328	CT11739	3.011.077	1	rep26	repA(pLGUG1)	NODE_9_length_79235_cov_25.598162	36670..38478	1809
19	86329	CT630	2.964.363	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_34.140432	43207..44967	1761
20	86330	CT11731	2.956.218	-	-	-	-	-	-
21	86331	CT630	2.964.368	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_34.332148	3503..5263	1761
22	86332	CT630	2.964.682	1	rep25	pLM330006(pLM33)	NODE_8_length_57720_cov_38.563263	34750..36510	1761
23	86333	CT630	2.964.376	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_38.600831	3503..5263	1761
24	86334	CT630	2.964.487	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_38.818954	43207..44967	1761
25	86335	CT720	2.882.660	-	-	-	-	-	-
26	86336	CT375	2.922.545	-	-	-	-	-	-
27	86337	CT11734	3.026.339	1	rep25	pLM330006(pLM33)	NODE_9_length_48469_cov_58.455540	3503..5263	1761
28	86338	CT11730	2.877.058	-	-	-	-	-	-

to humans [18]. Moura et al. [22] reported that CC1 was the most prevalent clonal group associated with human listeriosis cases in the world and was strongly associated with cattle and dairy products. In addition, the teichoic acid biosynthesis genes *gltA* and *gltB* and the invasion gene *aut_IVb* were only detected in isolates belonging to CC1 and CC2. Other CCs, despite, were defined as hypovirulent or intermediate clones with low clinical frequency [14], invasive infections caused by these clones were also reported in humans [24,25] and animals [26,27].

L. monocytogenes strains have many virulence factors that determine their pathogenic potential and all of which have an important role at various stages of the infection cycle. The current study looked for 69 potential virulence markers that could be used to predict the level of virulence in *L. monocytogenes* isolates. Based on virulence markers, it was suggested to classify *L. monocytogenes* isolates as putatively hypo-virulent, with unknown virulence potential, and putatively hypervirulent [8]. Markers were identified across the isolates suggesting that most virulence markers are ubiquitous across *L. monocytogenes* strains. Intact LIPI-1, which harbors Prf-A dependent virulence cluster genes that are critical in the infectious cycle of *L. monocytogenes*, was observed in all isolates. In the current study, the LIPI-3, which is associated with promoting the virulence capabilities of *L. monocytogenes*, was found in four isolates from ST1 from serogroup IVb belonging to lineage I, but was also present in two isolates from lineage I belonging to ST3002 which was identified as the new ST, and one isolate from lineage I belonging to ST3. The LIPI-3 carries a gene encoding the hemolytic and cytotoxic factor known as listeriolysin S, which contributes to the intracellular survival of *L. monocytogenes* in human polymorphonuclear neutrophils [8]. Painset et al. [28] and Chen et al. [29] reported similar findings and revealed that

LIPI-3 is ubiquitous to lineage I, which was also observed in the present study. The recently described pathogenicity island LIPI-4 that confers hypervirulence by enhancing the invasion of the CNS and placenta was not detected among the *L. monocytogenes* isolates. LIPI-4 was reported to be the most prevalent in CC4 strains in Western countries and in CC87 strains in Asia [2].

Except for *L. monocytogenes* strains belong to CC69 (ST202), the rest of the isolates generally carried the virulence genes examined. However, the known adhesion and invasion related genes, such as *inlC, inlE, inlF, inlG*, *inlH, inlJ*, *ami*, *vip*, and *aut* were not found in genomes of CC69 strains, which suggests a possible limitation of the invasiveness and virulence of these *L. monocytogenes* strains [30,31]. The *inlA* gene was found in all of the isolates in the current study. A recent study showed that the truncation of the gene *inlA* due to premature stop codon resulted in reduced invasiveness in *L. monocytogenes* strains [32]. Therefore, it has been noted that this mutation may serve as a marker of hypovirulence. None of the isolates showed mutations in the *inlA* gene leading to premature stop codon (PMSC).

Various resistance genes, *lin* (lincosamides), *mprF* (cationic antimicrobial peptides), *fosX* (fosfomycins), *norB* (quinolones) and *sul* (sulfonamides) were found in the WGS of *L. monocytogenes* isolates using the BIGSdb-Lm database (https://bigsdb.pasteur.fr/listeria/, accessed on 12 July 2022). Hanes and Huang [33] reported that the genes *fosX* and *lin* were present in nearly every *L. monocytogenes* isolate, presence of other resistance genes other than *fosX* and *lin* changed according to country or isolation sources (clinical, environmental or other).

In the current study, two isolates from central nervous system (CNS) infection belonged to CC1. It was shown that CC1 was the most prevalent CCs isolated from rhombencephalitis-associated cases in ruminants [1,23]. Dreyer et al.[1] reported that neurotropism of *L. monocytogenes* strains belonged to this clone was associated with their hyperinvasiveness and increased intracellular replication. Furthermore, cgMLST analyses of *L. monocytogenes* ST1 strains revealed two different cgMLST types (CT11730 and CT11731), were not previously reported among ruminant rhomboencephalitis isolates. This could be explained by the fact that such strains have not been previously isolated or such sequences have not been submitted to the PasteurMLST BIGSdb-*Lm* database.

CC9 (ST122) and CC7 (ST12) were detected an abortion-associated CCs in the current study. CC7 was reported in Latvia [27] and in Slovenia [23] from abortus cases, despite not being amongst the three most common clones. In contrast, CC7 (7/46) were reported as the most prevalent CCs among *L. monocytogenes* isolates from different animal the clinical cases (abortus, bacteremia, CNS infection) from the USA [26]. CC9 is considered as food-associated hypovirulent MLST clones which were rarely implicated in clinical listeriosis cases. The genes of LIPI-1 and internalin family are the main virulence factors involved in the pathogenesis of *L. monocytogenes*. These genes are required for the intestinal infection stage, the entry into the host cells, and the adaptation to an intracellular lifestyle [17]. These genes were observed in all the isolates from abortus cases. Indeed, even if prevalence rate is low, invasive infections caused by CC9 have been reported both in humans [24,25] and animals [27]. It could be argued that the emergence of infections caused by CC9 could be related with the predisposing factors as well as virulence factors.

Like many bacterial species, *L. monocytogenes* strains are known to harbor plasmids with frequencies reaching as high as 92%. To date, plasmids obtained from *L. monocytogenes* strains have been shown to contain genes that confer resistance to disinfectants, heavy metals, antimicrobials, biotics. In addition, plasmids have been reported to carry the genes related with oxidative, osmotic, and heat stress [34]. The most common plasmid was Inc18(rep25), and only three strains carried Inc18(rep26). These plasmids contain genetic determinant for cadmium resistance [35]. Environmental and foodborne isolates have been reported to harbor plasmid in higher rates than clinical isolates [36,37]. Similarly, plasmids were detected with food-related isolates, none of the isolates belonged to CC1, CC2 and CC3 strains did not harbor plasmids.

WGS analysis revealed 15 different intact prophages across the *L. monocytogenes* isolates. Multiple prophages were observed in some isolates, supporting the previous studies that prophages were highly prevalent in the genomes of *L. monocytogenes* [38,39]. The prophages have been regarded as an important contributor to the evolution and virulence of *L. monocytogenes* [40,41], conferring an ecological advantage for persistence and survival over time [38]. This demonstrates that prophage diversification is a driving force for the adaptation to specific environmental niches and genetic evolution of *L. monocytogenes* strains [34].

Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC) are the factors that could contribute to the persistence and survival of *L. monocytogenes* in food processing environments, through the activity of various efflux pumps encoded by *brcABC* cassette, *qacA*, *qacC*, *qacH*, *emrE* and *emrC* [42]. QACs, like the factors that could contribute to the persistence and survival of *L. monocytogenes* in food processing environments, through the activity of various efflux pumps encoded by *brcABC* cassette, *qacH*, *qacA*, *qacC*, *emrE* and *emrC* [7]. In the present study, *emrC* gene (53.6%), was first described on plasmid pLMST6 [43], was most frequently detected in *L. monocytogenes* isolates, followed by *bcrC* (14.3%), *emrE* (7.1%), and *qacA* (3.6%). Similarly, *emrC*

(40%) was the most frequently detected gene responsible for BC resistance in *L. monocytogenes* strains originating from food chain in South Africa [39]. In contrast, a higher prevalence for *qacH* were reported in France (18.8%) [44], Norway (22%) [45], and Czechia [42]. In the present study, two chromosomal major efflux pump genes, *mdrL* and *lde*, were detected in all *L. monocytogenes* isolates. The high prevalence of QAC efflux genes may give *L. monocytogenes* an advantage for survival and persistence in their specific environments [46].

The WGS analysis performed in the current study revealed the genetic diversity of *L. monocytogenes* strains from different food and clinical cases of animals in Türkiye. Novel ST and cgMLST types were also identified, which were previously not present in the *Listeria* database. Several hypervirulent strains were detected among the food-associated isolates belonging to CC1 and CC2, which could present a major public health threat. *L. monocytogenes* strains from CNS infections belonged to CC1, whereas strains from maternal-neonatal infections belonged to CC9 and CC7. According to our knowledge, this is the first study based on WGS analysis of *L. monocytogenes* from different food and animal invasive infections in Türkiye. The findings of this study also highlight the importance of WGS to provide more detailed genetic information on *L. monocytogenes* obtained from different sources. The present study also shows the necessity of implementation of effective hygienic procedures to prevent contamination of food with *L. monocytogenes*.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (Ö. Aslantaş) on reasonable request.

Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee Funding Support

Funding support

This study was supported by the Scientific Research Fund of Hatay Mustafa Kemal University with a grant number of 21.GAP.051.

Conflict of Interest

The authors declared that there is no conflict of interest related to this study.

Author Contributions

ÖA and OK planned, designed, and supervised the research procedure, AGY and AA performed all microbiological experiments, ÖA and KB performed bioinformatic analyses, and ÖA wrote the manuscript. All authors have read and approved the manuscript.

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