Whole genome sequencing for typing and characterisation of *Listeria monocytogenes* isolated in a rabbit meat processing plant

Federica Palma, Frédérique Pasquali, Alex Lucchi, Alessandra De Cesare, Gerardo Manfreda

Department of Food and Agricultural Sciences, Alma Mater Studiorum-University of Bologna, Bologna, Italy

Abstract

Listeria monocytogenes is a food-borne pathogen able to survive and grow in different environments including food processing plants where it can persist for month or years. In the present study the discriminatory power of Whole Genome Sequencing (WGS)-based analysis (cgMLST) was compared to that of molecular typing methods on 34 *L. monocytogenes* isolates collected over one year in the same rabbit meat processing plant and belonging to three genotypes (ST14, ST121, ST224). Each genotype included isolates indistinguishable by standard molecular typing methods. The virulence potential of all isolates was assessed by Multi Virulence-Locus Sequence Typing (MVLST) and the investigation of a representative database of virulence determinant genes. The whole genome of each isolate was sequenced on a MiSeq platform. The cgMLST, MVLST, and *in silico* identification of virulence genes were performed using publicly available tools. Draft genomes included a number of contigs ranging from 13 to 28 and N50 ranging from 456298 to 580604. The coverage ranged from 41 to 187X. The cgMLST showed a significantly superior discriminatory power only in comparison to ribotyping, nevertheless it allows the detection of two singletons belonging to ST14 that were not observed by other molecular methods. All ST14 isolates belonged to VT107, which 7-loci concatenated sequence differs for only 4 nucleotides to VT1 (Epidemic clone III). Analysis of virulence genes showed the presence of a full-length *inlA* version in all ST14 isolates and of a mutated version including a premature stop codon (PMSC) associated to attenuated virulence in all ST121 isolates.

Introduction

Listeria monocytogenes is a food-borne pathogen adapted to survive, grow and ultimately persist in different environments (Gray *et al*., 2006). In food processing plants, *L. monocytogenes* has been repeatedly isolated from food samples as well as food processing environment for months or years (Stasiewicz et al., 2015; Véghová et al., 2017; Leong et al., 2014). Through the contamination of food, *L. monocytogenes* can be transmitted to humans where it can switch from saprophyte to intracellular pathogen associated to approximately 2000 confirmed cases of Listeriosis each year in Europe, with a fatality rate of 17 % (EFSA and ECDC, 2016).

Within *L. monocytogenes* biodiversity, isolates of lineage II and serotype 1/2a have been isolated from food and food processing plants more frequently than isolates belonging to lineage I (Orsi et al., 2011). Clonal complex (CC) 121 belonging to serotype 1/2a has been described as the most prevalent clone with a strong association to food origin. In particular, CC121 showed an overall prevalence of 17.6% over 6633 tested isolates of *L. monocytogenes* collected over nine years from food and clinical sources, with a statistical significant over-representation in food sources in comparison to clinical ones (92.9% vs 7.0%) (Maury et al., 2016). Comparable values of prevalence of human CC121 (11/116, 9.5%) were observed also within the Institute Pasteur *L. monocytogenes* database (http://bigsdb.pasteur.fr/listeria/). The low frequency of *L. monocytogenes* in humans might be linked to the attenuated virulence of this CC. Premature Stop Codons (PMSC) in key virulence genes, such as *inlA* and *prfA*, as well as production of truncated InlA and PrfA proteins, were associated to attenuated virulence (Nightingale et al., 2005; Van Stelten et al., 2011; Cruz et al., 2014; Kanki et al., 2015). Compared to CC121, CC14 has been isolated rarely in food. Maury and colleagues found 92 isolates belonging to CC14 over 6633 tested isolates (1.4%) (Maury et al., 2016). Lower detection values were described also in 19 meat processing plants located in Northern Italy (5.7% over 69 tested isolates) in comparison to CC121 (23%) (Morganti et al., 2015). Besides a low prevalence, CC14 was described to be associated to higher infection rates. The clinical frequency of CC14 reached a not negligible 29.5% among isolates of clinical sources (Maury et al., 2016). Within CC14, a ST14 strain isolated from a case of invasive listeriosis was molecular characterised as belonging to epidemic clone (EC) III (corresponding to Multi-Virulence Locus Type 1 (VT1)) previously associated to a sporadic case occurred in United States in 1988, as well as a multi-state outbreak occurred in United States in 2000 (Mammina et al., 2013; Kathariou et al., 2002) (https://sites.google.com/site/mvlst-database/protocol-for-mvlst).

Whole Genome sequencing (WGS) based analyses have recently revealed a valuable potential in pathogen fingerprinting and identification of novel gene/features related to specific phenotypes. Based on the nucleotide sequence of the whole genome, an unprecedented discrimination power has been achieved by core genome Multi Locus Sequence Typing (cgMLST). This is particularly relevant in studies of repeatedly re-isolated strains showing high genetic similarity (same Pulsed Field Gel Electrophoresis (PFGE) profile or 7-locus Multi Locus Sequence Typing (7-locus MLST)) in which a superior discrimination

Correspondence: Frédérique Pasquali, Department of Food and Agricultural Sciences, Alma Mater Studiorum-University of Bologna, via del Florio 2, 40064 Ozzano dell'Emilia (BO), Italy.
Tel: +39.051.2097862 - Fax: +39.051.2097852.
E-mail: frederique.pasquali@unibo.it

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power is required, especially to differentiate true persistent strains to sporadic strains as well as strains repeatedly reintroduced into the food processing plant through contaminated raw materials (Ferreira et al., 2014). Moreover, starting from the same sequence data, WGS offers the great potential for multiple investigations of novel genetic determinants as well as genetic determinants already known as linked to specific phenotypes *(i.e.* virulence).

In a previous study on prevalence of *L. monocytogenes* in four Italian rabbit meat processing plants (A, B, C, D), isolates sharing the same 7-locus MLST (ST), Multi Locus Tandem Repeat Analysis (MLVA) and *Apa*I-PFGE profiles were repeatedly collected over time from carcasses, meat cuts, meat products and the food processing environment (De Cesare *et al*., 2017). This study provided an interesting dataset to investigate further specific ST-types over time. For this purpose, a specific focus was put on ST14 (belonging to CC14) and ST121 (belonging to CC121) isolates, repeatedly collected over one year and six month, respectively, from different rabbit meat and environmental sources of the same processing plant (A). The aims of the present study were: 1) to evaluate the discriminatory power of cgMLST in comparison to molecular typing methods; 2) to characterize the virulence potential of ST14 and ST121 sequenced isolates by comparative genomic analyses.

Materials and Methods

L. monocytogenes isolates included in the present study are a subset of isolates collected over one year within a previous study on prevalence of *L. monocytogenes* in rabbit meat processing plants (De Cesare et al., 2017). In particular, isolates sharing the same genotype and collected more than six times over a period of more than six months from different sources (rabbit carcasses, rabbit meat cuts, rabbit meat products and food processing environment) in the same plant were considered as potentially persistent. With this definition, 33 isolates of *L. monocytogenes* isolates of lineage II, serotype 1/2a and belonging to two 7-locus Multi Locus Sequence Types (MLST) ST14 and ST121 were selected as potentially persistent. An additional sporadic isolate, belonging to lineage I, serotype 1/2b, ST224 was also included for backward comparability. Isolates sharing the same 7–locus MLST were indistinguishable also by *Apa*I-Pulsed Field Eletrophoresis (PFGE), automated ribotyping and Multi Locus Variable number tandem repeat Analysis (MLVA) (De Cesare et al., 2017).

Whole-genomic DNA was extracted using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). The purified DNA concentration and the quality parameter ratio 260/280 were measured by BioSpectrometer fluorescence (Eppendorf). The whole genome of selected 34 *L. mono-*

Table 1. Sequence type and assembly statistics of the 34 sequenced *Listeria monocytogenes* isolates.

Isolate ID	ST	No. of contigs	N50	Genome size (bp)	Fold coverage
LM1	14	13	579785	3022050	84,03
LM2	14	14	456298	3022428	41,78
LM3	14	13	580466	3022420	114,86
LM5	14	15	579913	3022168	57,83
LM8	14	14	580478	3023685	176,68
LM9	14	13	580478	3023308	121,54
LM10	14	14	580478	3023687	167,18
LM11	224	13	511384	2925197	159
LM15	14	13	580472	3022932	146,98
LM16	14	14	580454	2986892	131,2
LM17	14	14	580454	2986900	149,23
LM18	14	14	580454	2986900	187,12
LM19	14	15	580471	3023022	172,15
LM22	14	13	580454	3022659	79,03
LM27	121	26	529438	3060499	120,24
LM29	121	28	530140	3061007	131,41
LM31	121	25	530140	3060579	127,78
LM35	14	13	580472	3022414	106,18
LM39	14	15	580604	3022475	120,54
LM41	14	13	580472	3022475	126,39
LM44	14	13	580478	3022308	144,31
LM46	14	13	580478	3022405	85,42
LM47	14	13	580478	3022625	106,27
LM50	121	24	530364	3068560	111,58
LM51	121	25	530139	3060573	137,29
LM53	121	21	530354	3062194	139,38
LM54	14	13	580472	3022424	150,5
LM55	14	13	580478	3022427	142,63
LM56	14	13	580478	3022415	121,34
LM57	14	13	580478	3022426	134,73
LM58	14	13	580478	3022498	123,61
LM59	14	13	580478	3022821	135,9
LM60	14	13	580472	3022629	132,49
LM61	14	13	580478	3022408	108,5

ST, sequence type.

Table 2. Simpson's ID of Whole Genome Sequencing-based analysis in comparison with molecular typing methods on the 34 sequenced *Listeria monocytogenes* isolates.

Typing method	N profiles	Simpson's ID	CI (95%)
cgMLST	5	0.439	(0.253-0.624)
Ribotyping	2	0.214	(0.045-0.383)
ApaI-PFGE	3	0.348	(0.170-0.525)
MLST	3	0.348	(0.170-0.525)
MLVA	3	0.348	(0.170-0.525)

CI, confidence interval; MLST, multilocus sequence typing; MLVA, multiple-locus variable number tandem repeat analysis.

cytogenes isolates was sequenced using Illumina MiSeq platform (TrueSeq library, paired-end reads). Reads were quality checked and *de novo* assembled using the INNUca pipeline (https://github.com /INNUENDOCON/INNUca).

Briefly, INNUca calculates if the sample raw data fulfill the expected coverage (minimum default 15X). Then, after a read quality analysis using FASTQC and trimming using TRIMMOMATIC, *de novo* draft genome assembly is performed with SPAdes, which is subsequently improved using PILON to correct bases and fix misassemblies.

Core genome Multi Locus Sequence Typing (cgMLST) was inferred based on *in silico* sequence alignment of 1748 loci of corresponding core genes. Briefly, after alignment, similarly to 7-locus MLST, an allele number is assigned to each locus. A cluster type, representative of all allele numbers, is then assigned (Moura et al., 2016).

The Multi Virulence Locus Sequence Type (MVLST) was inferred based on *in silico* sequence alignment of seven virulence determinant gene loci: *clpP, dal, inlB, inlC, lisR, prfA* (https://sites.google.com /site/mvlstdatabase) (Zhang et al., 2004).

Analyses of virulence was performed using VirulenceFinder 1.5 (https://cge.cbs.dtu.dk/services/VirulenceFinder) (Joensen *et al*., 2014). With this tool a BLAST search of a database of 82 *L. monocytogenes* virulence determinant genes was applied to all ST14 and ST121 draft genomes included in the present study, as well as to 23 publicly available genomes of *L. monocytogenes* belonging to ST14 and ST121 and isolated from humans or food processing plants. Publicly available genome of EGD-e (Genbank accession number NC_003210.1) was included as reference. The default parameters used were 90% ID threshold and 60% of minimum length. Upon detection of no gene the analysis was repeated with 85% ID threshold and 20% minimum length. Alignment of *inlA* and *prfA* genes was performed by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo).

The discriminatory power of each typing method was assessed by the Simpson's Index of Diversity (ID). ID values with P<0.05 were considered statistical significant different (Hunter and Gaston, 1988).

Results

Thirty four isolates of *L. monocytogenes* of ST14, ST121 and ST224 collected over one year of sampling in a rabbit meat processing plant from environment, meat cuts and meat products were sequenced and *de novo* assembled. Extracted DNA had a yield ranging from 14.2 to 81.3 µg/mL and a 260/280 ratio ranging from 1.81 to 1.88. The generated draft whole genomes sizes ranged from 2,925,197 to 3,068,560 bp. Draft genomes included a number of contigs ranging from 13 to 28 and N50 ranging from 456298 to 580604. Finally, coverage ranged from 42 to 187X (Table 1).

The cgMLST showed a superior discriminatory power in comparison to ribotyping, 7-loci MLST and MLVA (0.439 vs 0.214, 0.348 and 0.348 respectively) although statistically significant only in comparison to ribotyping (P=0.01) (Table 2, Figure 1). In particular, by WGS based method, ST14 isolates were differentiated in three clusters, whereas the same isolates were indistinguishable by *Apa*I-PFGE, automated ribotyping and MLVA (De Cesare et al., 2017). According to cgMLST results, one cluster gathered the majority of putative persistent ST14 sequenced *L. monocytogenes* isolates (CT1701). The other two clusters (CT1702 and CT1703) included only one isolate each (LM15,

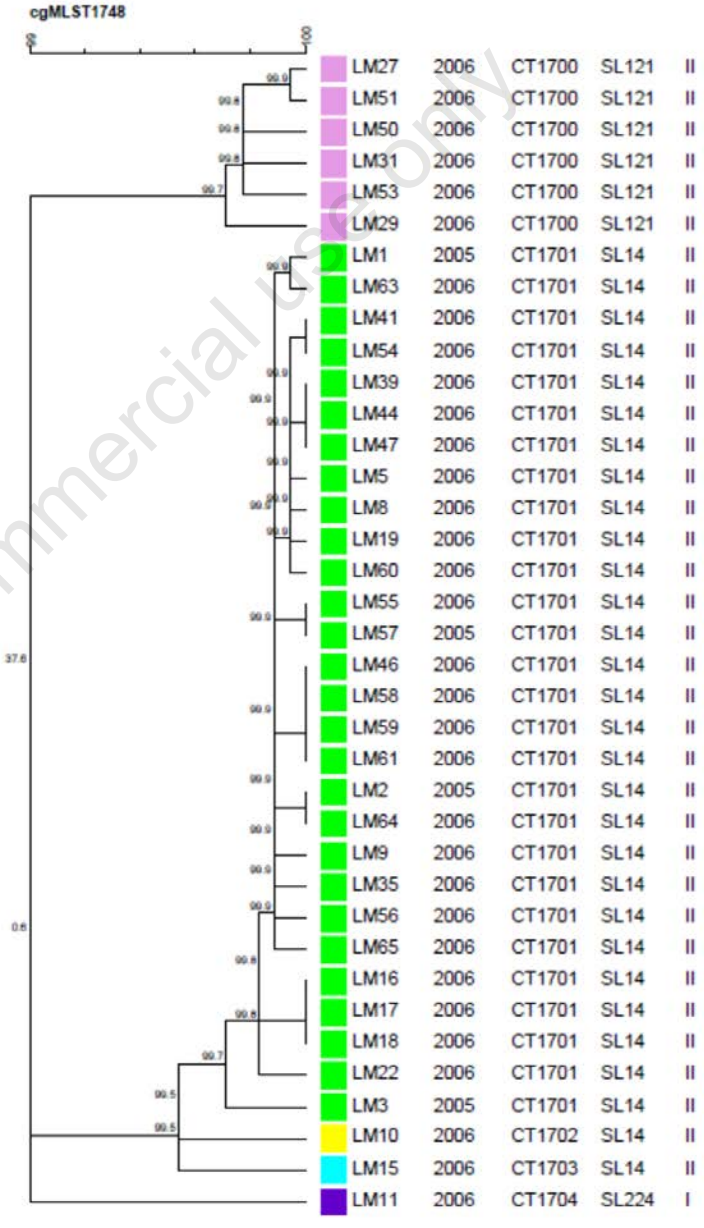

Figure 1. Whole Genome Sequencing-based analysis of the 34 sequenced *Listeria monocytogenes* isolates.

LM10). Out of the 1748 loci of the cgMLST scheme, these two singletons carried 12 and 11 different loci respectively in comparison to CT1701. This finding suggests that three ST14 strains circulated in the rabbit-meat processing plant during the time of sampling: one persistent strain and two sporadic ones. All ST121 isolates were indistinguishable by cgMLST, confirming the high similarity of these isolates already suggested by molecular typing methods.

In order to investigate the virulence potential of 33 ST14 and ST121 isolates, repeatedly isolated over one year and six month, respectively, in a rabbit meat processing plant, MVLST was assessed along with the *in silico* detection of 82 Listeria virulence genes. In order to investigate potential associations of presence/absence of genes with STs or origin, virulence genes were additionally investigated in the reference genome EGD-e as well as in 23 publicly available genomes of *L. monocytogenes* belonging to ST14 and ST121 and isolated from humans or food processing environment. Regarding MVLST results, all sequenced ST14 isolates belonged to virulence type VT107, whereas all ST121 to VT94. Regarding *in silico* detection of virulence genes, the reference genome EGD-e and the analyzed ST14 and ST121 genomes carried 82, 80 and 80 virulence genes respectively indicating that those genes are core virulence genes of *L. monocytogenes*. The genes *inlF* and *lmo2026* were not detected in any of the 56 ST14 and ST121 genomes analyzed. The gene *inlJ* was not found or found truncated in all 56 genomes analyzed. All ST14 carried a full-length version of the gene a*ctA* (1920 nucleotides), which was detected truncated in all the ST121 genomes. The gene *ami* was detected in a truncated version in some ST14 and ST121 genomes.

No mutations were found in *prfA* genes of all sequenced genomes as well as of *inlA* of ST14 genomes. In contrast, the *inlA* gene of all ST121 genomes showed a mutation leading to a premature stop codon (PMSC) predicting the translation of a truncated InlA protein of 492 aa instead of the full-length InlA of 800 aa (Figure 2). This mutation has been already described as PMSC of type 6 which was specifically associated to attenuated virulence of *L. monocytogenes* (Van Stelten et al., 2010).

No relevant differences in carriage of virulence genes were found in relation to food or human origin.

Discussion

Whole-genome based analyses have recently revealed an unprecedented potential as a powerful tool for multiple analyses, including typing and gene characterization of food-borne pathogens. In the present study, WGS revealed its great potential as a one-serve-all approach. Based on the same genome sequence data, cgMLST typing, in silico MVLST and in silico detection of 82 virulence genes were performed in parallel. However, it must be noted that, in comparison to standard molecular techniques, this approach requires great bioinformatics skills, standardized protocols, agreed cut off and parameter values in order to obtain robust and comparable results.

The cgMLST revealed a superior discriminatory power in comparison to molecular tools, although significant only in comparison to ribotyping. The cgMLST confirmed only in part results of molecular typing, suggesting that most, but not all, of the ST14 isolates, collected over one year in the same rabbit-meat processing plant, belonged to the same persistent event. Two cgMLST clusters included only one isolate each, suggesting the detection of these two isolates as a sporadic event in the rabbit-meat processing plant.

Persistent *L. monocytogenes* isolates collected in food processing plants represent a public health concern, due to their potential transfer to humans via the food chain. In order to investigate the virulence potential of sequenced ST14 and ST121 *L. monocytogenes* isolates, MVLST as well as an *in silico* detection of 82 virulence determinant genes was carried out. All ST14 belonged to VT107. This VT is particularly interesting since the concatenated sequence of the 7 virulence loci differs to VT1 (Epidemic Clone III) for only 4 nucleotides, suggesting the high similarity of sequenced ST14 isolates to this epidemic clone (Murugesan et al., 2015). Interestingly, three years after sampling of the rabbit-meat processing plant, a case of invasive Listeriosis in the North of Italy associated to ST14 strain belonging to VT1 has been described (Mammina et al., 2013).

Eighty out of 82 virulence determinant genes were found in the 57 *L. monocytogenes* genomes analyzed. This finding suggests that the presence/absence of these 82 genes can hardly explain the high diversity between ST14 and ST121 which have been shown to occur with a high and low frequency respectively in clinical samples (Maury et al., 2016, Morganti *et al*., 2015). The genes *inlF* and *lmo2026* were not detected. The gene *inlF* belong to the internalin family, which includes genes exclusive of the genus Listeria and associated to the adehesion and invasion of host cells (Vázquez-Boland et al., 2001). The gene *lmo2026*, was recently identified as an internalin gene, *inlL*, associated to biofilm formation and adhesion to mucin (Popowska et al., 2017). Besides presence/absence of virulence genes the regulation of expression as well as the presence of truncated genes or the presence of mutations leading to truncated versions of their translated proteins might have a crucial role in the virulence potential of ST14 and ST121. Regarding truncated virulence genes, the genes *inlJ*, *actA* and *ami* were found truncated. The gene *inlJ* belong to the internalin family (Vázquez-Boland et al., 2001; Sabet et al., 2008). The *actA* gene found truncated in all ST121 genomes, encodes the surface protein ActA, the factor responsible for actin-based motility and cell-to-cell spread (Smith and Portnoy, 1997). The gene *ami* codes for an autolysin which contributes to the adhesion of *L. monocytogenes* to eukaryotic cells by anchoring its cell wall (Milohanic et al., 2001). Regarding point mutations, a premature stop codon (PMSC), already described as associated to attenuated virulence of ST121, was detected in all the sequenced ST121 genomes (Van Stelten et al., 2010). The attenuated virulence potential of sequenced ST121 isolates is also supported by the detection of a truncated version of the gene *actA*, which was described as indispensable for *L. monocytogenes* pathogenicity (Smith and Portnoy, 1997). An attenuated virulence of ST121 might explain the low frequency of its corresponding clonal complex CC in clinical samples (Maury *et al*., 2016).

Conclusions

Although not yet fully standardized, Whole genome Sequencing (WGS) can be applied for multiple investigations, such as typing and prediction of virulence. In the present study, WGS-based analyses revealed higher discriminatory power in comparison to molecular typing methods, although significant only in comparison to ribotyping, on 34 *L. monocytogenes* isolates collected over one year in a rabbit meat processing plant. In particular 25 ST14 and 6 ST121 isolates were confirmed to belong to two persistent strains. Moreover, based on the same genome sequence used for typing purposes, *in silico* analyses were useful to predict the potential virulence of *L. monocytogenes* isolates. Multi Virulence Locus Sequence typing (MVLST) results, as well as analysis of virulence genes, suggest a higher virulence potential of ST14 sequenced isolates in comparison to ST121. Additional *in vitro* investigations should be

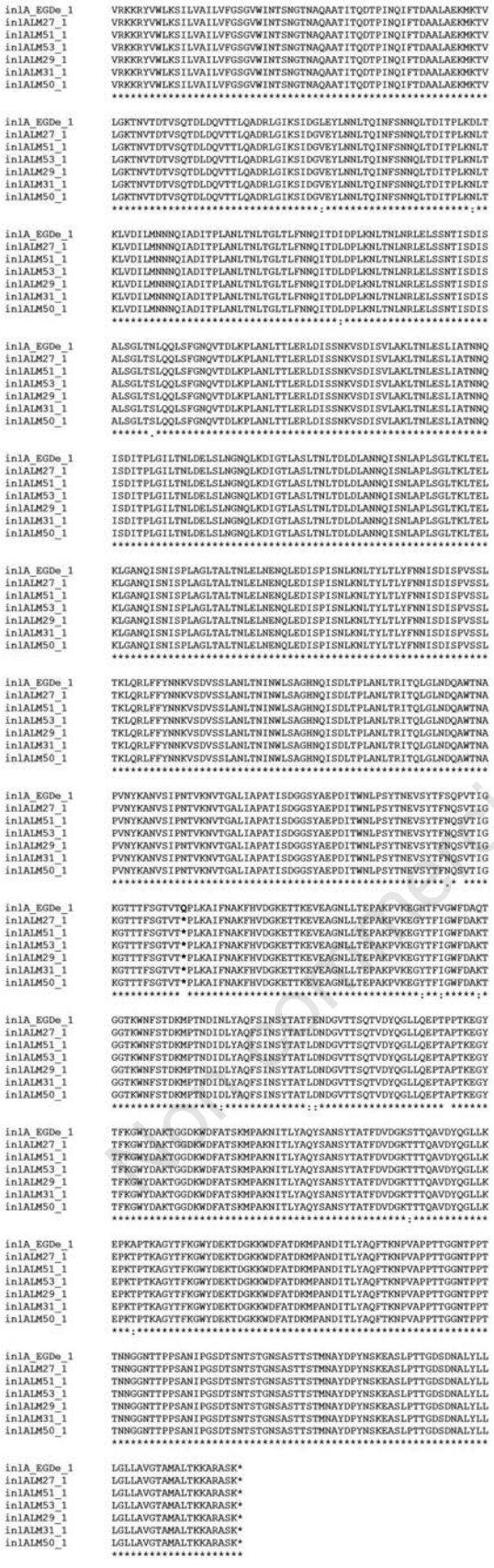

Figure 2. Predicted amino acid sequence alignment of InlA in ST121 *Listeria monocytogenes* isolates and EGD-e (GenBank accession number NC_003210.1). The mutation leading to the truncated InlA protein is highlighted in bold.

performed to confirm the virulence of ST14 and ST121 isolates.

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