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Characterisation of *InlA* truncation in *Listeria monocytogenes* isolates from farm animals and human cases in the province of Quebec

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ABSTRACT

The introduction of *Listeria monocytogenes* into the food production chain is a concern, with numerous grouped cases of listeriosis associated with milk-derived or pork-derived products have been documented. Management of this zoonotic pathogen considers all strains as an equal risk. Recently, a new perspective for characterisation of strain virulence was introduced with the discovery of the unaltered sequence of *InlA* as a determinant of strain virulence; this has also been reported as an infrequent finding among so-called environmental strains, that is, strains isolated from food or from surfaces in food industries. The aim of this study was to differentiate *L monocytogenes* strains isolated from animal cases versus those from human cases and to differentiate clinical strains from environmental ones using a *Caenorhabditis elegans* virulence testing model. In Quebec in 2013/2014, the surveillance of *L monocytogenes* clinical isolates registered a total of 20 strains of animal origin and 16 pulsed-field gel electrophoresis types isolated from human cases. The mixed PCR multiplex agglutination protocol used for geno-serotyping clearly discriminated genogroup IVB strains from bovine and human origins. The presence of a premature stop codon single nucleotide polymorphism in the *inlA* gene sequence in clinical strains and the identical behaviour of particular strains in the *C elegans* model are discussed in this paper from the perspective of industrial management of *L monocytogenes* risk.

INTRODUCTION

Listeria monocytogenes is a great concern both for industrial and public health stakeholders. These bacteria cause listeriosis, a foodborne disease acquired through the consumption of contaminated ready-to-eat products, including milk or meat products. Pork meat has been associated with large-scale outbreaks in the past (de valk and others 2001) and is considered a risk factor, particularly for sensitive populations such as pregnant women, children and the elderly. In the last few years, the increase in human cases (not associated with intensified surveillance), mainly for the elderly, has increased the concern surrounding management of *L monocytogenes* in food in Canada and Europe (Lomonaco and others 2015). One critical question to answer is whether the virulence of *L monocytogenes* has increased. The determinants of virulence have been studied, and there is a large amount of data that allow for discrimination of strains from lineages III or IV from lineages I and II (Ragon and others 2008). Lineages I and II are considered to be the most worrisome because of their potential to cause outbreaks or sporadic listeriosis. Among such strains, discrimination based on pathogenic properties still represents a challenge. A few years ago, the very promising concept of virulence determination through *inlA* gene sequencing was introduced (Van Stelten and others 2010, Van Stelten and others 2011). Published studies revealed the presence of a premature stop codon for internalin A translation. Because this stop codon inactivated this determinant of the first step of pathogenicity in the digestive tract, a key determinant for virulence differentiation of strains appeared to have been discovered. In Quebec, human listeriosis surveillance works hand-in-hand with food surveillance. Few strains isolated from clinical cases in farms (animal primary productions) are available, so we took the opportunity that arose to test virulent strains isolated from animals or humans with clinical signs of listeriosis. After we performed the serovar determination and comparison according to strain origin, we wanted to confirm if the *inlA* sequences were complete in these strains and to test their behaviour in a *Caenorhabditis elegans* infection model, a very attractive but controversial model of pathogenicity testing for *L monocytogenes* (Thomsen and others 2006, Karthikeyan and others 2015)

(see discussion). No relationship between InlA integrity and behaviour of *L monocytogenes* strain in *C elegans* has actually been described. Some strains isolated during a previous study involving pig primary production (Larivière-Gauthier and others 2014) were added to the study's strain collection because only some of them showed stop codon in their *inlA* sequences and because these strains were (based on pulsotype comparison) frequently or not associated with clinical human cases. These additional strains were expected to show opposite virulence potentials. The aim of this study was to analyse *inl*A sequences from strains isolated from clinical forms of human or animal listerioses in Quebec and to compare the behaviour of some selected strains in a *C elegans* virulence model.

MATERIALS AND METHODS

Strain collections

Strains were collected from *L monocytogenes* surveillance in 2013/2014: 20 strains were obtained from animal disease surveillance (laboratory of the Ministry of Agriculture, Fisheries and Food, Quebec (MAPAQ)), among which 13 were included in this study because they satisfied the required 'clinical' criteria (ie, isolated from organs after animal necropsy, excluding isolation from milk and silage) and 16 strains isolated from clinical cases in humans were provided by the Provincial Public Health Laboratory (public health surveillance). The collection was completed with four strains of pig origin isolated in a previous project (Larivière-Gauthier and others 2014). These strains showed a single nucleotide polymorphism (SNP) premature stop codon in the inlA sequence (Pulso1 and Pulso9 strains) or an integrated inlA sequence (Pulso4 and Pulso6 strains). Strains Pulso6 and Pulso9 showed 100% similarity between pulsed-field gel electrophoresis (PFGE) banding patterns (with strains recovered from clinical human cases; strains Pulso1 and Pulso4 have not previously been identified in clinical human surveillance). These added strains were expected to show opposite virulence potentials.

Serotyping

L monocytogenes strains were serotyped based on a combination of multiplex PCR-based geno-serogrouping, completed by detection of *fla*A (Kérouanton and others 2010) and agglutination against discriminatory O serum (OI, OVII, OVIII; Oxoid Thermo Fisher Scientific, Nepean, ON, Canada) (Burall and others 2011).

InlA sequencing

The *inlA* gene sequences were determined after overlapping amplification (Ragon and others 2008) using the Sanger method at the Centre d'Innovation Génome Québec (Applied Biosystems 3730xl DNA analyzer). *InlA* gene sequencing was extensively analysed, and the occurrence and position of SNP before in silico translation of the sequences were compared. Sequences were aligned and screened for mutations causing a premature stop codon or amino acid deletion using ClustalX 2.1 software, with *inlA L monocytogenes* EGD-e (NCBI: NC_003210.1) as reference.

C elegans virulence model

Bacterial strains (Fig 2) were cultured 24 hours at 37°C in BHI (Brain Heart Infusion), acid shocked (0.1 N HCl pH 4.5, one hour) then washed and suspended in phosphate-buffered saline (PBS) (concentration, see Fig 2) before being used in trials. Strain N2 worms, maintained for five days on nematode-growing medium plates and inoculated with *Escherichia coli* OP50 feeding strain at 25°C, were harvested (non-synchronised culture), washed three times in PBS, then inoculated in 96-well plates in PBS and maintained for five more days at 30°C with daily addition of fresh bacteria.

Observation of worm viability (based on worm locomotion and/or pharyngeal pumping and assessed under an inverted phase contrast microscope according to Thomsen and others (2006) allowed determination of a living worm count of 15 mature worms and 30 L1/L2 larvae in each of the tested conditions. The distribution of number of living worms counts (n=8) were compared with control conditions (Mann-Whitney U test; SPSS Statistics V.17, Licenced U de Montreal, α=0.05)

RESULTS

All 16 of the strains of human origin were considered clinical because they were isolated from hospitalised patients. From the 20 strains provided by MAPAQ, only 13 strains strictly associated with clinical listeriosis in animals were included. It should be noted that we excluded strains isolated from milk tanks and silage, all belonging to the 1/2a serovar. Although serotyping revealed that the greater proportion of clinical strains (regardless of origin) belonged to the serovar 1/2a (18/29, 62%), there were still some strains (nine out of 29 with similar proportions from humans and animals, that is, 5/16 and 4/13, respectively) that belonged to the IVB serogroup (a serogroup that contains 4b and 4a/ab serovars). Focusing on this IVB group, a clear distinction appeared where only 4b serovar strains were found in human surveillance, whereas strains shown in ruminant clinical cases all belonged to 4a/4ab serovars (table presented in Fig 1). No SNP occurred on the sequence coding for the InlA LPXTG domain. Moreover, *inl*A sequencing showed that a majority of strains had a complete sequence (table presented in Fig 1). But it should be noted that two out of the 16 strains isolated from clinical listeriosis cases had a premature stop codon; the mutations were expected to induce truncations of InlA at 700 and 762 amino acid (aa) positions, respectively. One of these—which showed the longest InlA size but lost LPXTG domain—was used in the *C elegans* virulence model, as well as another strain presenting a complete sequence (tables presented in

Strain origin	Reference	Age of patient	Isolated from	Geno-serogroup	serotype	*inlA* sequence
Bovine	SHY 13-3735	Calf	Cerebral Trunk	IVB	4ab (4e)	Dele aa
Bovine	STF 13-5405	Adult	Cerebral Trunk	IVB	4ab (4e)	Complete
Bovine	SHY 14-895	nr	nr	IIA	1/2a	Complete
Ovine	STF 15-130	nr	nr	IIA	1/2a	Complete
Bovine	STF 13-5780	nr	nr	IIA	1/2a	Complete
Bovine	STF 13-4520	Aborted foetuse	Invasive multi-organ	IIA	1/2a	Complete
Bovine	STF 14-742	nr	nr	IIA	1/2a	Complete
Caprine	STF 14-2030	Young goat	Invasive multi-organ	IIA	1/2a	Complete
Bovine	STF 14-1682	Calf	Invasive multi-organ	IIA	1/2a	Complete
Ovine	SHY 14-4826	Lamb	Tron cerebral	IVB	4ab (4e)	Complete
Ovine	STF 14-2033	Lamb	Invasive multi-organ	IIA	1/2a	Complete
Bovine	SHY 15-117	nr	Liver	IVB	4ab (4e)	Dele aa
Bovine	SHY 14-4645	Aborted foetuse	Invasive multi-organ	IIA	1/2a	Complete
Swine	**Pulso 1**	**na**	**na**	**IIB**	**1/2b**	**PSC (700) - Ref. (1)**
Swine	**Pulso 4**	**na**	**na**	**IVB**	**4b**	**Complete - Ref. (1)**
Swine	**Pulso 6**	**na**	**na**	**IVB**	**4b**	**Dele aa - Ref. (1)**
Swine	**Pulso 9**	**na**	**na**	**IIB**	**1/2b**	**PSC (700) - Ref. (1)**
Human	ID133477	93	blood	IIA	1/2a	Complete
Human	ID133460	77	nr	IIA	1/2a	PSC (762)
Human	ID133117	90	nr	IVB	4b, (4e)	Complete
Human	ID132843	85	blood	IIA	1/2a	Complete
Human	ID132717	72	blood	IIA	1/2a	Complete
Human	ID132686	73	blood	IIA	1/2a	Complete
Human	ID132415	65	blood	IIA	1/2a	Complete
Human	ID132372	69	blood	IIA	1/2a	Complete
Human	ID131406	78	blood	IIA	1/2a	Complete
Human	ID131393	80	Cerebrospinal fluid	IVB	4b, (4e)	Complete
Human	ID131197	75	blood	IVB	4b, (4e)	Complete
Human	**ID131001**	**73**	blood	**IIb**	**1/2b**	**Complete**
Human	ID130591	83	nr	IIA	1/2a	Complete
Human	**ID130390**	**74**	blood	**IVB**	**4b, (4e)**	**PSC (700)**
Human	ID130364	73	blood	IIA	1/2a	Complete
Human	ID129727	81	blood	IVB	4b, (4e)	Complete

FIG 1: Characterisation of *Listeria monocytogenes* strains of clinical origin. PSC, premature stop codon (aa position). Dele aa: deletion of first amino acids in the protein. na: not applicable; nr: not recorded. Bold: retained for *C elegans* testing.

Figs 1,2). Four more strains (different PFGE type) from porcine origin were added; some had been recurrently detected in production (and food) but had never been associated with human cases (table presented in Fig 2; Pulso1 and Pulso4 strains). The two others were kept because of their 100% homology with strains involved in clinical human cases (sporadically for strain Pulso9 or recurrently for strain Pulso6; table presented in Fig 2). After five days, unfed *C elegans* worms were almost all dead. Using *E coli* OP 50 as a control, viability was maintained at 74% after five days, both in larvae and in mature worm forms. By comparison, *L monocytogenes* lowered the viability of the worms and significantly so for the larvae forms (Mann-Whitney U test, $P<0.05$).

Condition	Concentration (UFC/mL)	serovar	*InlA*	Mean viability n=8 (+/- SD)			
	Daily 100µL			Larvae (living worms; n=30)		Mature (living worms; n=15)	
				D0	D5	D0	D5
E coli OP50	$6.0.10^{6}$			30	22.3 (1.6)	15	11.4(1.3)
L. monocytogenes InlA 3 h s	$6.0.10^{9}$	1/2 b	Comp.	30	16.0 (4.9)	15	10.1(2.2)
L. monocytogenes InlA 7 h s	$1.6.10^{9}$	4b	PSC 700	30	16.0 (3.9)	15	9.2 (1.6)
L. monocytogenes pulso 1 s	$4.0.10^{9}$	1/2 b	PSC 700	30	15.0 (4)	15	8.3 (3.4)
L. monocytogenes pulso 4 s	$4.9. 10^{9}$	4b	Comp.	30	16.0 (5.0)	15	10.1 (2.3)
L. monocytogenes pulso 6 h s	$4.3.10^{9}$	4b	Comp.#	30	17.4 (4.2)	15	11.1(2.4)
L. monocytogenes pulso 9 h s	$5.0.10^{9}$	1/2b	PSC 700	30	15.1 (4.2)	15	10.1(2.9)
S Typhimurium 14 028	$1.2.10^{7}$			30	9.4 (3.3)	15	2.5 (2.1)
No bacteria				30	2.3 (1.8)	15	0 (0.2)

FIG 2: *Listeria monocytogenes* virulence on *Caenorhabditis elegans* model isolated in human (h) or pig (s). h s: strains from animal origin related to human cases (identical pulsed-field gel electrophoresis profile). Comp: complete InlA; PSC 700: truncated InlA due to the presence of a 700 aa position premature stop codon. Comp.#: complete but with substitution of first amino acids. aa, amino acid.

No differences in virulence behaviour were revealed using this model to compare the *L monocytogenes* strains (Fig 3 and Fig 4). The strain that was expected to be the least virulent (Pulso1) showed the largest decrease in value for worm viability for both forms (50% and 55%, respectively, for larvae and mature worms) (table presented in Fig 2).

DISCUSSION

L monocytogenes virulence determination is a current research concern, and many authors have contributed to the advancement of this endeavour. A quite recent proposition (relevant when considering the capital role of Inl*A* in pathogenicity) is that the truncation of Inl*A*, induced by the presence of a premature stop codon in *inlA*, is associated with lower virulence properties (Nightingale and others 2008). Moreover, a great proportion of truncated forms were shown in strains isolated from food or food-related environments but not in *L monocytogenes* strains involved in clinical human cases (Van Stelten and others 2010). Discussion of the relevance of this virulence determinant arose when it was demonstrated that some truncated forms can express virulence in animal models (Van Stelten and others 2011, Holch and others 2013). Our analyses confirm that truncated forms can be found in clinical human cases in Quebec. The truncation positions, induced by SNP stop codon (700 and 762 aa) were previously reported (Kovacevic and others 2013). For the 700 aa truncation position, the entire anchorage region (between 700 and 800 aa), including the determinant proline in the LPXTG motif, was not translated. So, at least for the strain harbouring the 700 aa truncated InlA, pathogenicity was expected to be extremely low. The clinical origin of such strains led us to question the dose of ingested inoculum. In studies that demonstrated, in experimental conditions, a maintenance of virulence for strains harbouring truncated Inl*A*, high doses of inoculum were used (Van Stelten and others 2011, Holch and others 2013). High-dose ingestion of *L monocytogenes* could be observed in outbreaks, giving an opportunity for a strain harbouring Inl*A* truncated form to cause disease. We suggest that *inlA* analysis

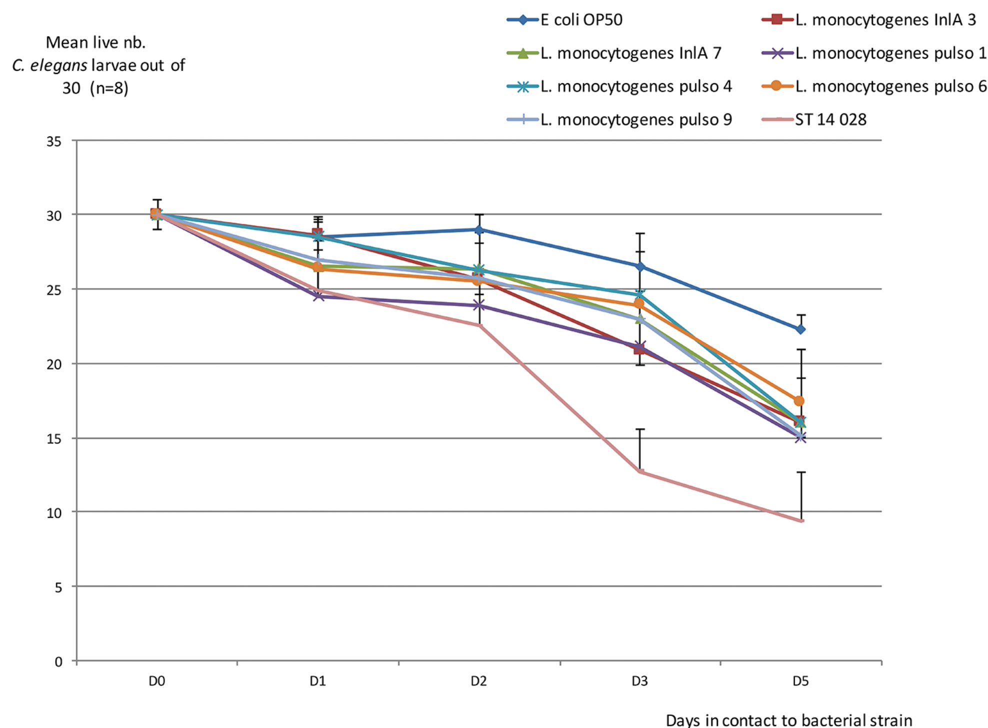

FIG 3: Evolution of mean live number *Caenorhabditis elegans* larvae out of 30 (n=8). Mean (n=8) number of live *C elegans* larvae out of 30 after one to five days in contact with bacterial strain (see Fig 2). Bars: SD.

alone is not sufficient to characterise the virulence potential of *L monocytogenes* strains.

Looking for a complementary test that avoids labour intensive and ethically questioned experimentations on mammal models, we considered the *C elegans* model for comparing the virulence of our characterised strains. The *C elegans* model was first proposed in 2006 and confirmed in 2007 (Thomsen and others 2006, Forrester and others 2007) when studies showed that the worm is as sensitive to pathogenic *L monocytogenes* as humans. But Guha and others questioned the model in 2013 (Guha and

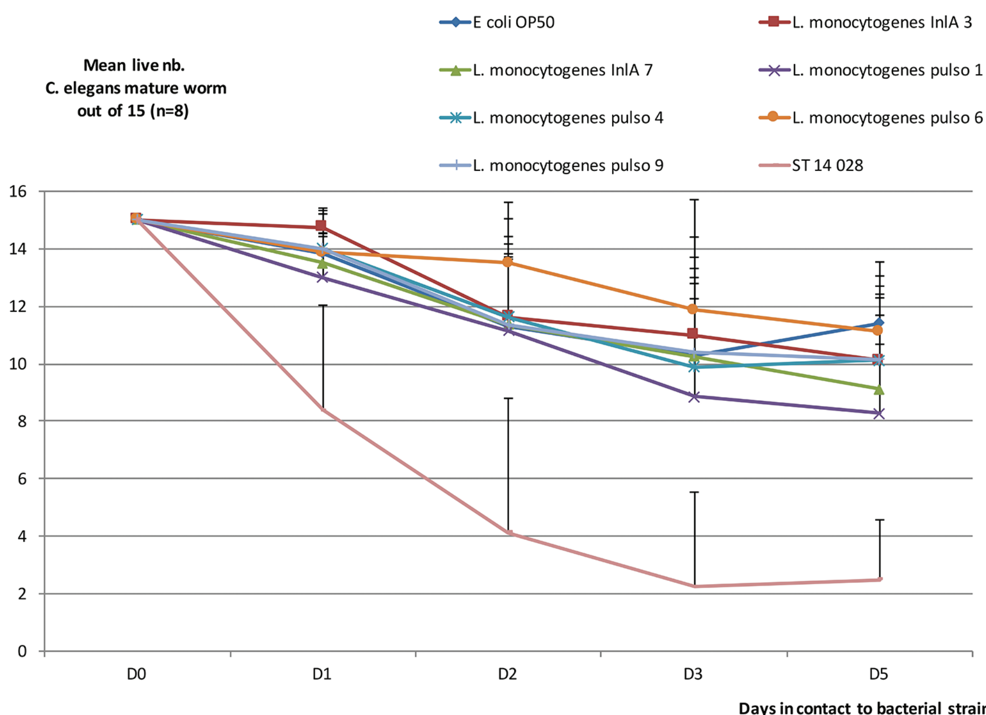

FIG 4: Evolution of mean number of live mature *Caenorhabditis elegans* out of 15 (n=8). Mean (n=8) number of live *C elegans* mature out of 15 after one to five days in contact with bacterial strain (see Fig 2). Bars: SD.

others 2013) after failing to obtain loss of viability of worms inoculated by *L monocytogenes*. That same year, Neuhaus and others (2013) confirmed that primed strains (acid shocked) increase the lethality to the worms and ultimately confirmed the worm model as able to compare virulence for food-derived strains (Karthikeyan and others 2015). Our results, when incubating worms at 30°C with acid-shocked *L monocytogenes* (to promote prfA-driven virulence factors in the pathogen), confirmed a decrease of viability of worms in contact with *L monocytogenes* (in a lesser proportion than *Salmonella* Typhimurium). Contrary to the work of Karthikeyan and others (2015), the worms did not show any morphological changes when they were submitted to our *L monocytogenes* strain inoculations. The loss of viability was solely based on immobility and loss of pharyngeal pumping activity, in accordance with Neuhaus and others (2013). In our conditions, on both the mature form and the young larvae, *L monocytogenes* showed differences in virulence regardless of origin (environmental or clinical) and in their *inlA* integrity.

CONCLUSION

Using complete serotyping, differences in IVB geno-serogroup between clinical strains from bovine and human origins were shown, with serovar 4b found only in human clinical case strains. The virulence analysis tools used in this study (*inl*A sequencing and *C elegans* model) on both confirmed virulent and presumed less virulent strains could not univocally established the public health risk associated with *L monocytogenes* strains. No pig clinical case strains were provided by the MAPAQ laboratory, but serovar 4b strains were previously found in the collection of strains from healthy pigs in primary production (Larivière-Gauthier and others 2014). As calls for farm to fork strain traceability continue to be made, accurate surveillance is needed to enlarge the collection of strains in animal surveillance, particularly from pig production. It should be mentioned that we did receive all the collected *L monocytogenes* strains isolated in the MAPAQ laboratory, and we observed that they mainly originated from bovine clinical surveillance (where *L monocytogenes* present economical relevance).

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Contributors All authors equally contributed to the conception of the study; data acquisition was equally distributed among Philippe Fravalo, Tamazight Cherifi and Kersti Neira laboratory work. The paper was written by Philippe Fravalo after analyses and discussions of the results done by all authors.

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