

# Microbiological and molecular investigation of an increase of human listeriosis in Belgium, 2006-2007

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**In Belgium, the majority of cases of listeriosis are sporadic cases. In this study we present evidence for an episode of listeriosis: a time-linked cluster of cases that occurred in 2006 and 2007, and the identification of identical strains. The episode involved 11 patients, infected with *Listeria monocytogenes* of serovar 4b. The source of infection was not detected.**

## Introduction

*Listeria monocytogenes* is a gram-positive intracellular food-borne pathogen. In some groups (immunosuppressed people, neonates, pregnant women and their unborn children) it can be an important cause of life-threatening bacteraemia and meningitis. Because listeriosis has a long incubation time (three to 60 days), it is often difficult to trace the source of infection. This explains why the vast majority of cases are notified as single cases. Nevertheless some well-documented outbreaks of listeriosis have been reported from Finland [1], France [2], Switzerland [3], the United Kingdom (UK) [4] and United States (US) [5]. In Belgium, one outbreak of listeriosis has been described in 2001 [6]; data from one additional study were inconclusive [7]. In the present study, 36 clinical human strains from hospital laboratories and five food isolates were characterised by serotyping, metal resistance typing and pulsed-field gel electrophoresis (PFGE). This combination provided us with the opportunity to link strains from 11 sporadic cases.

## Methods

Patients were not systematically interviewed about their food habits; clinical data were provided by clinical laboratories.

## Identification and molecular typing

Since 1966, the Belgian *Listeria* Reference Centre (BLRC) has received hospital-isolated strains of *L. monocytogenes* on a voluntary basis. Strain identification is carried out with the api *Listeria* kit (bioMérieux, France). All strains are serotyped according to a standard protocol [8] using a commercial agglutination test (Denka Seiken, Tokyo, Japan) for somatic (O) and flagellar (H) antigens. Strain susceptibility to arsenic and cadmium is determined according to McLaughlin *et al.*

[9]. Molecular typing is performed if a cluster of isolates is suspected based on geographical considerations, on the occurrence of multiple cases within a short period of time or a cluster of isolates with identical serotype.

PFGE is done following the US PulseNet protocol [10] after DNA digestion with *Apal* and *Ascl*. Analysis of the banding pattern is performed with the ImageMaster video documentation system (Amersham Pharmacia Biotech) and Fingerprinting II Informatix software (Bio-Rad). DNA extraction for Random Amplified Polymorphic DNA (RAPD) is done with the QI Amp DNA Mini Kit; primer HLWL 74 and the PCR conditions have been described by Wernars *et al.* [11].

## Isolation of *Listeria monocytogenes* from food

25 g of food sample were added to 225 ml of Half Fraser broth for 24 h at 30°C, followed by the inoculation of 0.1 ml into 10 ml of Frazer broth and incubation for 24 h at 37°C. Following enrichment, 500 µl of the culture was used in the VIDAS LMo2 test (bioMérieux). If positive, 10 µl of the Fraser enrichment broth were subcultured on two media: RAPID L. Mono (Bio-rad) and ALOA (bioMérieux). Colonies characteristic of *L. monocytogenes* were further confirmed with the api *Listeria* kit.

## Results

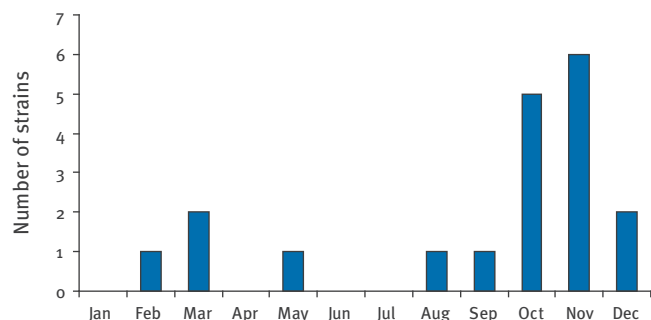
BLRC receives annually between 30 and 68 strains of human clinical listeriosis cases, representing an annual incidence of three to six cases per million inhabitants. This incidence is comparable to the rates reported by other industrialised countries.

In 2006, 56 clinical strains were received: 19 of them were of serovar 4b. Monthly baseline isolations of serovar 4b varied between none and two. However, in October and November 2006, five and six strains were isolated, respectively, suggesting a possible episode of listeriosis (Figure 1). These 11 isolates were first differentiated by metal resistance typing. Three metal resistance types were found: arsenic sensitive – cadmium resistant (SR) (one strain), arsenic and cadmium resistant (RR) (two strains), and arsenic and cadmium

sensitive (SS) (eight strains). It was clear that the episodic strain was of SS phenotype.

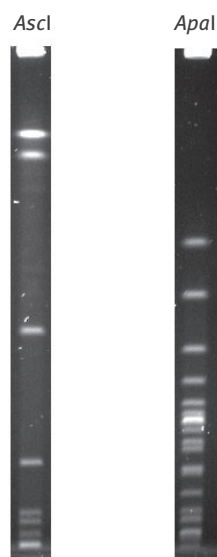
**FIGURE 1**

Clinical isolates of *Listeria monocytogenes* serovar 4b received at the Belgian Listeria Reference Centre, by month, 2006 (n=19)



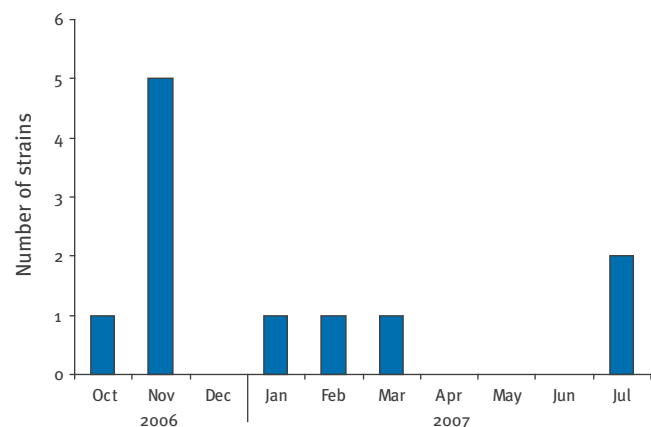
**FIGURE 2**

Pulsed-field gel electrophoresis *AscI* and *ApaI* profiles of *Listeria monocytogenes* pulsovar A



**FIGURE 3**

Monthly distribution of the episodic *Listeria monocytogenes* strain (4b,SS, pulsovar A), Belgium, October 2006-July 2007 (n=11)



The eight strains of phenotype 4b SS were further sub-typed by PFGE. They belonged to three different pulsosvars, with pulsovar A represented by six strains (Figure 2). The remaining two strains belonged to a different pulsovar. One of these pulsosvars (B) was phylogenetic related to pulsovar A with a similarity index of 92,31% (Fingerprinting II Informatix software application).

To evaluate the duration of the episode, the remaining strains of phenotype 4b – SS from 2006 and those isolated in 2007 were subtyped with PFGE. Another five strains (all from 2007) were found with a profile indistinguishable from the episodic strain. The rate of isolation in 2007 was irregular: January (one strain), February (one strain), March (one strain), July (two strains) (Figure 3). The last two cases were geographically linked, indicating a local extension of the episode. Before the onset of the episode, strains with pulsovar A were very uncommon: only one strain was identified in 2005.

A total of 11 cases appear to have been involved in this episode (six in 2006 and five in 2007). The patients' characteristics are outlined in the Table. Of the positive cultures of these patients, nine were from blood and two from cerebrospinal fluid; four cases were pregnant women or newborns. One pregnant woman had a twin stillbirth. The episode was not geographically clustered, as the isolates were received from three different regions in Belgium: Flanders, Wallonia and Brussels.

The episode was first recognised by the BLRC in November 2006. Only on four occasions were patients asked about their food habits. No standardised questionnaire was used. Suspected food samples were taken from the patients' refrigerators or from the same batch of the suspected food at the retail level. Smoked salmon was sampled because in a case of preterm birth, the mother remembered having eaten smoked salmon. Raw beef brains were the only suspected food item in a case of septicaemia. The woman with the twin stillbirth reported having eaten pre-packed lasagne; this food item was suspected after some of her housemates presented with gastroenteritis. However, *L. monocytogenes* could not be detected in any of these samples.

As the contamination source was not found, another line of investigation was followed. We searched the BLRC food isolations collection for a strain with the same characteristics as the episodic strain. Among the food isolates received at BLRC in 2006 and 2007, only six strains were of phenotype 4b-SS. They were subtyped with RAPD. Two strains isolated from poultry preparations matched the episodic strain RADP profile. However, these strains were of a different pulsovar, which excludes them from being involved in the episode.

## Discussion

In Belgium, the majority of listeriosis cases are reported as single cases. Clinical laboratories mandatorily report cases to the community health authorities. However, these authorities rarely receive information on strain characteristics, which does not facilitate linking sporadic cases. Clinical laboratories may submit their isolates to the BLRC. It is estimated that the BLRC receives approximately 70% of the total number of clinical isolates in Belgium. As the BLRC performs strain typing, clusters of identical strains are easily recognised.

The combination of serotyping, metal resistance typing and PFGE led to the identification of 11 identical isolates. The episodic strain was of serovar 4b, sensitive to arsenic and cadmium and belonged to pulsovar A. Six of them were isolated within a period of a few weeks which is exceptional for a small country like Belgium. Besides the cluster isolations in 2006, the episodic strain was isolated from a further five patients in 2007, indicating a long extension of the episode which went on until July 2007.

The source of contamination was not detected. Two factors may have contributed to this failure: no systematic interviewing of the patients and unsuccessful food sampling. During this episode only four patients were contacted by community health inspectors and only three different food samples were taken which proved to be negative for *L. monocytogenes* in 25 g. On the other hand, the episodic strain was not present in the BLRC collection of food strains, which therefore could not provide a clue for potential suspected food items to be investigated.

Not finding the implicated food is not exceptional. In 1987, 23 cases of listeriosis in the UK were attributed to a strain of unusual serotype designated 4b(X); the implicated food was not identified [12]. In the Netherlands, cluster analysis based on serotyping and

PFGE showed one cluster of 15 listeriosis cases without the identification of a clear source of infection [13].

Serovar 4b is not unusual. In Europe and North-America, most published outbreaks of listeriosis in the past 20 years have involved 4b [14]. In addition, strains of serovar 4b tend to be overrepresented in perinatal listeriosis, suggesting that they may have special virulence attributes for pregnancy and breach of the blood-placenta barrier. In the cluster described here, four of the 11 cases were pregnancy-related.

It is presumed that the episodic strain was particularly virulent because it involved a relatively high number of pregnancy-related cases and meningitis cases, four of 11 and two of 11 respectively. According to annual data from the BLRC, strains from cases with maternal-neonatal listeriosis represent 10% of the total number of clinical strains; a similar proportion is observed for cases with meningitis.

It is generally accepted that persons with an underlying disease are more susceptible to contracting listeriosis. In this episode, information from only three patients was available. As it not mandatory for clinical laboratories to report to the BLRC, a lot of epidemiological data are ignored.

During this episode of listeriosis, we received a human *Listeria* strain closely related to the episodic strain. This strain was of serovar 4b, sensitive to arsenic and cadmium but represented another pulsovar (B). Pulsovar B differs from pulsovar A by only three bands in the *Apal* profile. According to Tenover *et al.* [15], two profiles that differ by only three bands are considered as closely related. However, the similarity index between the two profiles was 92.31% and thus lower than 95%, the minimum level for highly related strains. Therefore this strain was excluded from the episode.

## TABLE

Patients' characteristics of the listeriosis episode, Belgium, October 2006-July 2007 (n=11)

Isolation date	Region	Age (years)	Sex	Isolation site	Symptoms	Underlying disease
October 2006	Flanders	48	Female	Blood	Septicaemia	NA
October 2006	Flanders	52	Female	CSF	Meningitis/coma	Lupus
November 2006	Brussels	65	Male	CSF	Meningitis	NA
November 2006	Wallonia	81	Male	Blood	NA	Heart disease
November 2006	Wallonia	Neonate	Female	Blood	Preterm birth	NA
November 2006	Flanders	Neonate	Male	Blood and ear	Septicaemia/preterm birth	NA
January 2007	Flanders	58	Male	Blood	Septicaemia	NA
February 2007	Flanders	36	Female	Blood	Septicaemia/twin stillbirth	NA
March 2007	Flanders	Neonate	Female	Blood	Septicaemia	NA
July 2007	Wallonia	59	Male	Blood	NA	Cancer
July 2007	Wallonia	54	Female	Blood	Septicaemia	NA

CSF: cerebrospinal fluid; NA: not available.

This episode would have passed unnoticed had not the BLRC performed strain typing. Efficient monitoring of listeriosis requires systematic interviewing of the patients using a standardised questionnaire. Close cooperation between community health inspectors, the Belgian Federal Agency for the Safety of the Food Chain (FASFC) and the BLRC would result in a rapid linking of sporadic cases and enhance the chance of finding the infection source in outbreaks.

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