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Short communication

Prevalence of low-virulence *Listeria monocytogenes* strains from different foods and environments

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ABSTRACT

Various studies have demonstrated variations in the levels of virulence of different *L. monocytogenes* strains. In our laboratory, a plaque-forming assay followed by subcutaneous footpad inoculation of mice enabled us to estimate the prevalence of the low-virulence strains. This value fell from 16.3% to 1.7% with bacteria collected before 1994 and after 1997 respectively. This could be related to the modification in 1997 of the reference method EN ISO 11 290-1 of *Listeria* detection which recommended the use of polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) medium.

The aim of this study was to determine whether the percentage of low-virulence strains detected has changed due to the modification of the detection method recommending the use of the ALOA medium. After analyzing 380 *L. monocytogenes* strains, no increase in the percentage of low-virulence strains could be detected. The prevalence reached only 2.6% (ten of the 380 strains tested). The low virulence of *L. monocytogenes* strains was not related to rare serotypes and was also observed in serotypes usually involved in human disease. Low-virulence strains were found in dairy, meat, ready-to-eat products and also in the environment, highlighting the absence of one specific source. These results are discussed in terms of detection methods and the definition of low virulence.

1. Introduction

The bacterial genus *Listeria* currently comprises six species, but human cases of listeriosis are almost exclusively caused by the species *Listeria monocytogenes*. *Listeriae* are ubiquitous organisms whose main reservoirs are soil, silage and water. Other reservoirs include infected domestic and wild animals. The main route of transmission to both humans and animals is through consumption of infected food or feed (Schlech et al., 1983). Following a sharp decline, the number of listeriosis cases has significantly increased in France: incidence rose from 3.5 human cases per million during the period 2001–2005, to 4.6 in 2006 and 5.6 in 2007 (Goulet et al., 2008). An increase has also been observed in the European Union over the last five years, with a total of 1583 human cases reported in 2006 (EFSA, 2007). Currently, listeriosis mainly occurs among adults and elderly people, with 56% of cases affecting individuals over 60 years of age. *L. monocytogenes* was the most severe causative agent in outbreaks in 2006: Member States reported nine foodborne listeriosis outbreaks during which 17 people died. Soft cheese, mushrooms and dairy products were identified as vehicles in these outbreaks (EFSA, 2007). The *L. monocytogenes* criteria laid down by the Commission Regulation 2073/2005, covering primarily ready-to-eat (RTE) food products, require that: (1) in RTE products intended for infants and for special medical purposes, *L. monocytogenes* must not be present in 25 g; (2) *L. monocytogenes* must not be present at levels above 100 colony forming units (cfu)/g during the shelf life of other RTE products on the market; (3) products which are able to support the growth of the bacterium must not contain *L. monocytogenes* in 25 g when they leave the production plant. At the EU level, the proportion of samples exceeding the legal safety criterion of 100 *L. monocytogenes* cfu/g has most often been observed in ready-to-eat (RTE) fish products (1.7%), followed by cheese (0.1%–0.6%), other RTE products (0.1%–0.4%) and RTE meat products (0.1%) (EFSA, 2007).

L. monocytogenes strains show heterogeneous levels of virulence. Several publications have reported that 10–20% of strains isolated from the food industry are non- or weakly pathogenic in experimental infection of immunocompromised or immunocompetent mice (Conner et al., 1989; Tabouret et al., 1991). Low-virulence strains have also been identified by cellular tests (Pine et al., 1991; Van Langendonck et al., 1998) and more recently by molecular methods such as Polymerase Chain

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Reaction—Restricted Fragment Length Polymorphism (PCR-RFLP) (Rousseaux et al., 2004). Based on a plaque-forming (PF) assay followed by subcutaneous (SC) footpad inoculation of mice, we previously showed that 16.3% of non-clinical isolates identified as *L. monocytogenes* strains are low-virulence (Roche et al., 2001). However, in a second study performed with strains isolated after 2000, the percentage of the low-virulence *L. monocytogenes* strains fell to 1.7% following the same procedure (data not shown). This discrepancy could be related to the modification in 1997 of the reference method EN ISO 11 290-1 related to *Listeria* detection which recommended the use of the polymyxin-acriflavine-LiCl-ceftazidime-aesculin-mannitol (PALCAM) medium (Scotter et al., 2001). We demonstrated that low-virulence strains are poorly detected with PALCAM and Rapid'L.mono media, in contrast to Oxford and Agar *Listeria* according to Ottaviani and Agosti (Gracieux et al., 2003; Vlaemynck et al., 2000).

As the reference EN ISO 11 290-1 was modified in 2005, leading to the replacement of PALCAM medium by ALOA medium, the aim of this study was to determine whether the detection of low-virulence field *L. monocytogenes* strains has increased with the new reference protocol.

2. Materials and methods

2.1. Strains and culture conditions

This study analyzed 380 *L. monocytogenes* strains isolated on ALOA medium in accordance with the reference EN ISO 11 290-1 modified in 2005. All isolates were from independent sources and had different isolation dates. They originated from 3 laboratories: 1) the Faculté de Pharmacie, Rennes, for the oyster farms ($n=166$), 2) AFSSA from Maisons-Alfort for surveillance plans of the Direction Générale de l'Alimentation DGAL ($n=92$) and different food chains (seafood, dairy products and others) ($n=73$), and 3) the Centre National de Référence des *Listeria*, Paris, for food or its environment included in French and/or European notification in 2006 ($n=58$). The strains were maintained in storage medium (Sanofi Pasteur, Bio-Rad, Ivry sur Seine, France) at 4 °C. For analysis, they were cultured for 8 h in brain heart infusion (BHI) broth (Difco, Becton Dickinson, Meylan, France) at 37 °C.

In Fig. 1, the strains were grouped into dairy products (16%), meat products (31%), seafood products (23%), RTE products (2%), environmental strains (sea-water and sediments) (27%), and food-manufacturing plants (1%). In the dairy products, only 8 strains of the 59 isolates originated from milk samples, the others were from processed food (cheese). All meat products were processed. For the sea-food products, 60 of the 86 isolates were from oysters. Forty-two percent of the *L. monocytogenes* strains were of serotypes 1/2a and 3a (PCR profile IIa), 18% of serotypes 1/2b, 3b and 7 (PCR profile IIb), 13% of serotypes 1/2c and 3c (PCR profile IIc) and 27% of serotypes 4b, 4d, 4e (PCR profile IVb) (Doumith et al., 2004).

2.2. Cell line

The human adenocarcinoma cell line HT-29 (ECACC No. 85061109, Salisbury, UK) (Fogh and Trempe, 1975) was grown in 75 cm^2 plastic tissue culture flasks (Nunc, Life Technologies, Cergy Pontoise, France) in culture medium (Dulbecco's Modified Eagle Medium 4.5 g/L glucose, DMEM; Life Technologies) supplemented with 10% (v/v) fetal calf serum (Life Technologies) and 2 mM L-glutamine (Life Technologies). Antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin; Sigma, Saint-Quentin Fallavier, France) were routinely added to the culture medium except for the virulence assays. Cells were maintained in a humidified incubator (at least 90% RH) (Heraeus, Les Ulis, France) at 37 °C under 5% (v/v) CO_2.

2.3. Quantification of the virulence of L. monocytogenes strains

The levels of virulence of *L. monocytogenes* strains were assessed as previously described (Roche et al., 2001).

In brief, the virulence level was first estimated by a PF assay. Trypsinized HT-29 cells (3×10^4) deposited in 96-well tissue culture plates (Falcon, AES, Combourg, France) were incubated for 4 days to obtain almost confluent monolayers, and then in the medium without antibiotics for 24 h (Velge et al., 1994). Bacterial concentrations standardized turbidimetrically ($OD_{620\ nm}$) were diluted appropriately in DMEM after overnight growth on BHI agar slopes. Cells were infected with 2 to 7 log *Listeria* per well for 2 h at 37 °C in a humidified

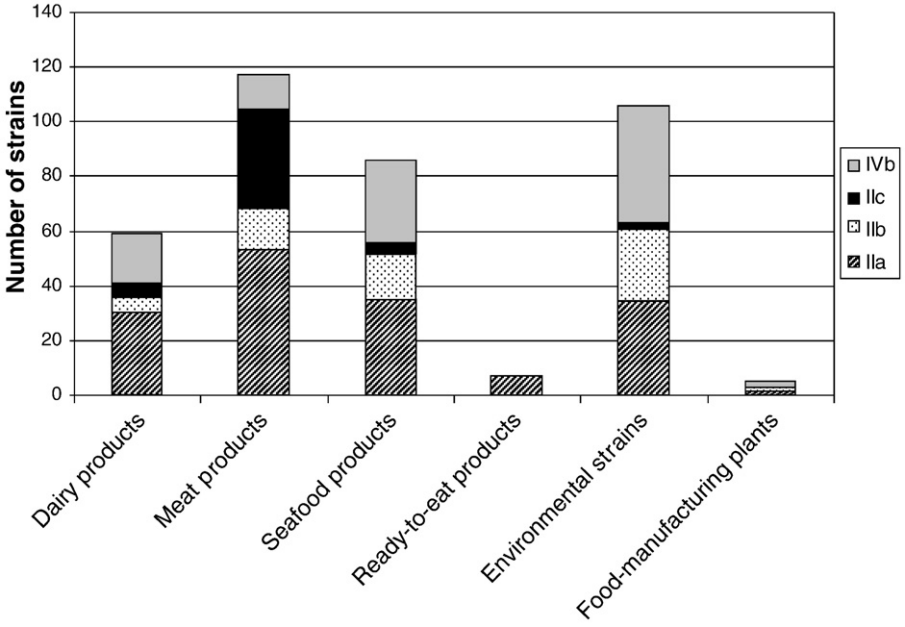

Fig. 1. Distribution of each serotype PCR profile in the different sources: serotype PCR profiles IIa supplied for serogrouping (1/2a and 3a) ▨; serotype PCR profiles IIb supplied for serogrouping (1/2b, 3b and 7) ⁙; serotype PCR profiles IIc supplied for serogrouping (1/2c and 3c) ■; serotype PCR profiles IVb supplied for serogrouping (4b, 4d, 4e and 4ab) □.