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Nonsense-mutated *inlA* and *prfA* not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan

Satoko Handa-Miya, Bon Kimura *, Hajime Takahashi, Miki Sato, Tatsuya Ishikawa, Kazunori Igarashi, Tateo Fujii

Department of Food Science and Technology, Faculty of Marine Science, Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

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Abstract

InlA is a surface protein participating in the entry of *Listeria monocytogenes* into mammalian non-phagocytic cells. PrfA is a positive regulatory factor that regulates the expression of a set of virulence genes. Recent studies revealed that some *L. monocytogenes* strains have a truncated form of these proteins because of nonsense mutations in their sequences, and these truncations contribute to the significant reduction in virulence of this pathogen. In this study, sequence analyses of *inlA* and *prfA* among *L. monocytogenes* isolated from ready-to-eat seafood revealed that only one out of 59 isolates had a nonsense-mutated *inlA* and all had non-mutated *prfA*. This indicated that these strains could be fully virulent based on the sizes of these proteins.

Keywords: *Listeria monocytogenes*; Internalin A; Ready-to-eat; Seafood

1. Introduction

Listeria monocytogenes is an ubiquitous bacterium that can cause serious listeriosis infections in humans and animals. Both sporadic and epidemic cases of human listeriosis are mainly of food-borne in origin and have an associated mortality rate as high as 20–30% (Mead et al., 1999). Healthy adults are generally asymptomatic or develop only mild symptoms with simple gastroenteritis (Grif et al., 2001; Rocourt et al., 2000). However, infection in high-risk individuals, such as pregnant women, newborn infants, and immunocompromised people, can result in serious outcomes such as spontaneous abortion, septicemia, and meningoencephalitis. *L. monocytogenes* is therefore a public concern in terms of food safety and regulations to control this organism have been established in many countries. However, acceptable levels of this organism in ready-to-eat foods are defined differently from country to country. The United States adopted a zero-tolerance policy for all ready-to-eat foods whereas the EU allows 100 CFU/g of this pathogen at the best-before date for some classes of foods (European Commission, 2005). Establishing a definitive universal policy on acceptable levels of this organism is definitely required, and to this end, risk analysis is necessary to understand the actual dose response. However, it should be noted that these policies have been established based on the hypothesis that all *L. monocytogenes* strains are equally pathogenic, despite the heterogeneity of pathogenicity that has been reported to exist among isolates. This is indicated by most of the human listeriosis cases having been caused by strains of certain serotypes, such as 1/2a, 1/2b and 4b (Schuchat et al., 1991). Specifically, the strains of serotype 4b have been responsible for most food-borne epidemic listeriosis cases and the majority of sporadic cases (Farber and Peterkin, 1991; Schuchat et al., 1991). The varying levels of virulence were also demonstrated by virulence tests using chick embryo, various human cell lines, and mouse injection test (Bhunia et al., 1994; Nørrung and Andersen, 2000; Pine et al., 1991; Roberts et al., 2005; Roche et al., 2003; Roche et al., 2001; Stelma et al., 1987; Tabouret et al., 1991; Van Langendonck et al., 1998).

* Corresponding author. Tel./fax: +81 3 5463 0603.
E-mail address: kimubo@kaiyodai.ac.jp (B. Kimura).

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The factors for this heterogeneity in virulence have been elucidated by various molecular methods. The observation that some *L. monocytogenes* isolates express a truncated non-functional form of internalin A (InlA) is of particular importance (Jonquières et al., 1998). InlA allows the pathogen to invade non-phagocytic cells, such as human intestinal epithelium cells (Gaillard et al., 1991), but strains expressing truncated InlA show a significant reduction in invasive ability into Caco-2 cells compared to ones lacking the nonsense mutation (Olier et al., 2005; Olier et al., 2002; Rousseaux et al., 2004). A truncated form of InlA was shown to be widely distributed in food isolates and less so in clinical isolates (Jacquet et al., 2004), indicating the critical role of InlA in the pathogenesis of human listeriosis. Reasons for strains of serotype 4b posing high risks for humans is still unknown, but having the intact form of *inlA* gene, rather than a nonsense-mutated form found in other strains, may play a partial role (Jacquet et al., 2004). In another recent study, truncation of this protein in a number of clinical and food isolates was confirmed using isolates from the United States (Nightingale et al., 2005a). Moreover, nonsense mutations were also found in the *prfA* gene (Roche et al., 2005), which regulates expression of a set of virulence factors. Although only three isolates were found to have nonsense-mutated *prfA*, all of them failed to enter human adenocarcinoma cells and were either avirulent or hypovirulent to mice because of their truncated PrfA proteins (Roche et al., 2005).

This virulence attenuation mechanism sheds light on questions about the rate of listeriosis cases. Although *L. monocytogenes* is widely present in ready-to-eat foods (Gombas et al., 2003), the number of cases of human infection is relatively low. This is also true in Japan where almost no food-borne listeriosis cases have been reported to date, although *L. monocytogenes* is known to be prevalent in many kinds of foods (Okutani et al., 2004). Despite significant consumption of these foods in Japan, it should be particularly noted that raw fish and ready-to-eat raw fish products have never been implicated in listeriosis in humans. This may be due to the low cell number of *L. monocytogenes* in these foods. Or, the presence of *L. innocua*, which is commonly found in foods (Karunasagar and Karunasagar, 2000), enhances host protective immunity against this pathogen (Vázquez-Boland et al., 2001). Alternatively, previous outbreaks simply have escaped recognition since *Listeria* detection from patients with diarrhea has not been routinely performed (Makino et al., 2005). However, the possibility that non-virulent or virulence-attenuated strains are prevalent in these foods cannot be ignored. Therefore, it is of extreme importance to determine whether truncation of virulence or virulence-associated genes could be a new tool for assessing risk of consuming food products contaminated with *L. monocytogenes*. Thus, we investigated *L. monocytogenes* isolates in ready-to-eat seafoods in this study to determine whether virulence-related genes *inlA* and *prfA* have nonsense mutations that leads to the truncated form of their respective proteins, InlA and PrfA. As sample foods, we specifically selected fish roe and minced tuna, since these have high levels of *L. monocytogenes* contamination (Handa et al., 2005) and risk assessment of these foods is urgently required.

2. Materials and methods

2.1. Bacterial isolates

The 59 seafood isolates used in this study are summarized in Table 1. A total of 10 isolates were from a previous study (Handa et al., 2005) and an additional 49 were selected from 64 isolates obtained from 531 ready-to-eat raw seafood retail products obtained in 61 different grocery stores in and around Tokyo between October 2004 and July 2005. The remaining15 isolates were excluded from further analyses because isolation sources (sampling date and store number), EcoRI ribotyping (Bruce, 1996) or MLST (Maiden et al., 1998; Zhang et al., 2004) data suggested that they were clonal isolates of other isolates already included in our list. Strains of the same serotype, ribotype and MLST profile were included in this study when the food samples were obtained on different dates or different stores.

2.2. Serotyping

Serotyping was carried out with commercial *Listeria* antiserum (Denka Seiken, Tokyo, Japan). O-antigen determination strains were grown on brain heart infusion agar (Becton Dickinson, Sparks, MD, USA) for 24 h at 35 °C. Cells were suspended in 0.2% sodium chloride and heated at 121 °C for 30 min followed by centrifugation at 3000 rpm for 20 min and resuspended in 0.5 ml of 0.2% sodium chloride. Slide agglutination tests using polyvalent type O-antiserum were performed first, followed by typing with individual O-antiserum. H-antigen strains were determined using the tube agglutination test. Briefly, sample cultures were incubated in semiliquid BHI medium (0.2% wt/vol agar) at room temperature (20–25 °C) for 24 h, repeated four times. The samples were incubated in a semiliquid BHI medium in Craigie tubes for 24 h followed by removal to BHI medium for an additional 24-h incubation. H-antigen type was determined after mixing two drops of antiserum with 0.5 ml of cell suspension with 1% formalin and incubating at 50 °C for 1 h.

2.3. Lineage designation

L. monocytogenes has been grouped into 3 distinct phylogenetic lineages based on genotypings such as sequencing analysis, ribotyping, and PCR-restriction fragment length polymorphisms (Rasmussen et al., 1995; Wiedmann et al., 1997). Each of the 59 strains used in this study was categorized into one of these 3 lineages using a method described previously (Ward et al., 2004). This method used multiplex PCR to produce a lineage-specific sized band on electrophoresis gels.

2.4. MLST (multilocus sequence typing)

Partial regions of 6 different virulence and virulence-associated genes were selected for MLST analysis according to Zhang et al. (2004) since they have reported a high discriminatory power of this method. DNA sequencing for each locus was performed