Ann. Fac. Medic. Vet. di Parma (Vol. XXVI, 2006) pag. 175 - pag. 182

PRELIMINARY NOTES ON INVASION AND PROLIFERATION OF FOODBORNE *LISTERIA MONOCYTOGENES* STRAINS

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KEY WORDS:
Listeria monocytogenes; virulence; invasion and proliferation, HeLa cells.

Abstract

In this study, virulence properties of *L. monocytogenes* strains isolated from food and food environments were evaluated. In particular, adhesion and invasion efficiencies were tested in a cell culture model (HeLa). Half of the isolates (9/18) exhibited a high invasion index. In particular, the strain isolated from smoked salmon had the highest invasion index. The remaining isolates showed an intermediate invasion index. All environmental isolates belonged to this group. Finally, no isolates revealed a low invasion index. Regarding intracellular growth, all tested isolates had a replication time between 2 and 6 hours. For this reason, they can be considered virulent. In spite of its capability to invade HeLa cells with a medium/high invasion index, a non-haemolytic rabbit isolate did not show any intracellular growth. In conclusion, differences in invasion efficiency and intracellular growth did not seem strictly related to the origin of the strains. Moreover, invasiveness of an organism is not the only requirement for establishing an infection. Virulence of *L. monocytogenes* also depends on ability to grow intracellularly and to spread from cell to cell. For these reasons, PCR detection of known virulence genes has the potential to gain additional insight into their pathogenic potential. A comprehensive comparative virulence characterization of different *L. monocytogenes* strains in studies that include tissue culture models and PCR detection of virulence genes will be necessary to investigate differences in human-pathogenic potentials among the subtypes of this bacterium.

Introduction

Listeria monocytogenes is an ubiquitous bacterium that is responsible for food-borne illnesses in humans. It has been recovered in several countries and from many different foods of animal origin that have been linked to both sporadic cases and outbreaks of listeriosis (Thévenot *et al*., 2006; Kiss *et al*., 2006; Chao *et al*., 2006). Disease incidence in humans is generally low, but given the severity of this illness and the high mortality rate (20-30%), in recent years great attention has been

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addressed to study the virulence properties of this microrganism (Vazquez-Boland *et al*., 2001; Jacquet *et al*., 2002; Doumith *et al*., 2004; Dussurget *et al*., 2004).

Human population responses to exposures to *L. monocytogenes* are highly variable. Disease incidence is dependent on a variety of factors, including the presence of virulence factors, dose, and general health and immune status of the host (Vazquez-Boland *et al*., 2001; Dussurget *et al*., 2004). Numerous methods have previously been applied to determine the virulence of this pathogen, including the use of either immunocompetent or immunocompromised animals, particularly mice (Stelma *et al*., 1987; Roche *et al*., 2001) or chick embryos (Terplan & Steinmeyer, 1989). However, maintaining sufficient numbers of mice or chick embryos for routine analysis is expensive and time-consuming. More recently, different reliable and reproducible cytotoxic tests based on continuous cell lines, such as Caco-2, Vero, Henle407, Hep-2 and HeLa cells, have been developed to distinguish between pathogenic and non-pathogenic *Listeria* spp. (Farber & Speirs, 1987; Pine *et al*., 1991; Braun *et al*., 1998; Van Langendonck *et al*., 1998; Roche *et al*., 2001; Larsen *et al*., 2002).

The purpose of this study was to evaluate the virulence properties of *L. monocytogenes* strains isolated from food and food environments. Virulence was tested in vitro by the invasiveness and growth of the strains in HeLa cell lines.

Materials and methods

Bacterial strains and culture media. This study comprised 21 strains of *L. monocytogenes* (tab. 1). To perform the invasion assay, 3 control strains with different invasion index were used (Jaradat & Bhunia, 2003). Bacteria were grown overnight in brain hearth infusion (BHI) broth (Oxoid, Milan, Italy) at 37°C. For the kinetics of the invasion assay, 1 ml of the overnight culture was used to inoculate 100 ml of fresh BHI broth (4 hours at 37°C under dynamic conditions) in order to obtain microrganisms at the end of the log-phase. At this point, the bacterial concentration was checked by using a spectrophotometer and the number of microorganisms was adjusted to obtain an optical density (OD) at 600 nm equal to 1.5, approximately corresponding to 10^{10} bacteria per ml.

To prepare inoculum for infection of cell cultures, 1 ml of the bacterial suspension was centrifuged for 10min at 4000 rpm (Megafuge 1.0R, Heraeus Sepatech, Germany) at 4°C, washed twice in sterile phosphate buffered saline (PBS) (pH 7.3) (Sigma-Aldrich, Milan, Italy), resuspended in its original volume with the cryoprotecting agent (PBS with 20% glycerol) (Larsen *et al*, 2002), and frozen at -80°C. For each experiment, the number of CFU was checked by plating the appropriate dilution on BHI agar.

Cell culture. The continuous cell line HeLa (carcinoma human cervix) was used. Cells were maintained by culture in Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex Biosciences Italia) supplemented with 10% Fetal Bovine Serum (FBS) (Cambrex Biosciences Italia) 1% glutamine (Cambrex Biosciences Italia), and 1% non-essential amino acids (NEAA) (Cambrex Biosciences Italia). Confluent cell monolayers were trypsinized and adjusted to a concentration of 2.5 x 10^5 cells ml^{-1}

in culture medium. One ml cell suspension was dispensed into each well of a 24 well tissue culture plate (Corning Costar) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air, for 24 hours, to obtain a semiconfluent monolayer.

Invasion assay and intracellular growth studies. The semiconfluent monolayers of HeLa cells were routinely inoculated with bacterial suspension adjusted to obtain a multiplicity of infection (MOI) of 100 bacteria per cell (100:1). After a brief centrifugation (3 min at 900 rpm) of the cell culture plate, binding and penetration was allowed to occur for 30 min. at 37°C in 5% CO_2, then cell monolayers were washed twice with PBS and covered with 1 ml of supplemented DMEM containing gentamicin at bactericidal concentration (1%) in order to kill extracellular bacteria (Gaillard *et al*., 1987). After 1 hour, infected cells were washed twice with PBS and lysed with 100 µl of Triton X-100 and CFU of viable bacteria were counted by plating suitable dilution of the lysate on BHI agar incubated for 24 hours at 37°C. The invasion efficiency (Invasion Index, II) expressed as percentage of initial inoculum (T0) was calculated by dividing the number of CFU that invaded the cells (with gentamicin) by the total number of CFU inoculated (without gentamicin) (Jaradat & Bhunia, 2003). For intracellular growth studies, cells were lysed as described above 2, 4, and 6 hours (T2, T4, and T6) after gentamicin had been applied to the infected cells. The proliferation index (Index Growth Culture, IGC) was calculated dividing the number of CFU that invaded the cells at different incubation times (T2-T4-T6) without the initial inoculum (T0), by the initial inoculum (T0). Each isolate was measured in duplicate and results were expressed as mean value.

Results and conclusion

Figure 1 summarizes the results of the invasion assay. Half of the isolates (9/18) exhibited an invasion index higher than the reference strain ATCC 19115 (serotype 4b) described by Jaradat & Bhunia (2003) with a high invasiveness degree. In particular, the strain number 5, isolated from smoked salmon, had the highest invasion index, 17 times greater than the reference strain ATCC 19115. The remaining isolates showed an invasion index intermediate between reference strains ATCC 19115 (high invasiveness) and NCTC 11994 (medium invasiveness). All environmental isolates belonged to this group. Finally, no isolates revealed an invasion index lower than reference strains NCTC 11994 (medium invasiveness) or ATCC 19114 (serotype 4a) (low invasiveness).

Figure 2 displays the results from intracellular growth studies. All tested isolates had a replication time intermediate between 2 and 6 hours. For this reason, as described by Pine *et al*. (1991), they can be considered virulent. After internalisation, strains can be differentiated on the basis of their growth capacity, starting from the 4^{th} hour after invasion (T4). In spite of its capability to invade HeLa cells with a medium/high invasion index, the non-haemolytic rabbit isolate did not show any intracellular growth, having a very low rate of recovery. In fact, it was previously shown that non-haemolytic *L. monocytogenes* mutants are internalized by cell cultures at the same rate as haemolytic strains, but afterwards they are almost completely unable to grow intracellularly (Gaillard *et al*., 1987; Gattuso *et al*. 2000).

Differences in invasion efficiency and intracellular growth did not seem related to the origin of the strains, even if strains isolated from bread-coat chicken meat showed a similar behaviour. However, when interpreting the data, it must be taken into account that the study was conducted on a low number of strains.

In conclusion, invasiveness of an organism is not the only requirement for establishing an infection. Virulence of *L. monocytogenes* also depends on ability to grow intracellularly and to spread from cell to cell. For these reasons, PCR detection of known virulence genes has the potential to gain additional insight into their pathogenic potential. A comprehensive comparative virulence characterization of different *L. monocytogenes* strains in studies that include tissue culture models and PCR detection of virulence genes will be necessary to investigate differences in human-pathogenic potentials among the subtypes of this bacterium. Furthermore, a better understanding of the virulence characteristics of *L. monocytogenes* could prevent product recalls due to subtypes that do not present a public health risk.

Table 1: Bacterial strains.

Strain	Source
1	smoked salmon
2	smoked salmon
3	smoked salmon
4	smoked salmon
5	smoked salmon
6	smoked salmon
7	bread coat chicken meat
8	bread coat chicken meat
9	bread coat chicken meat
10	bread coat chicken meat
11	rabbit meat
12	pork fermented sausage
13	pork fermented sausage
14	pork fermented sausage
15	pork fermented sausage
16	smoked salmon environment
17	fermented sausage environment
18	chicken meat environment
ATCC 19114 (4a)	reference strain (ovine brain)
ATCC 19115 (4b)	reference strain (human)
NCTC 11994 (4b)	reference strain

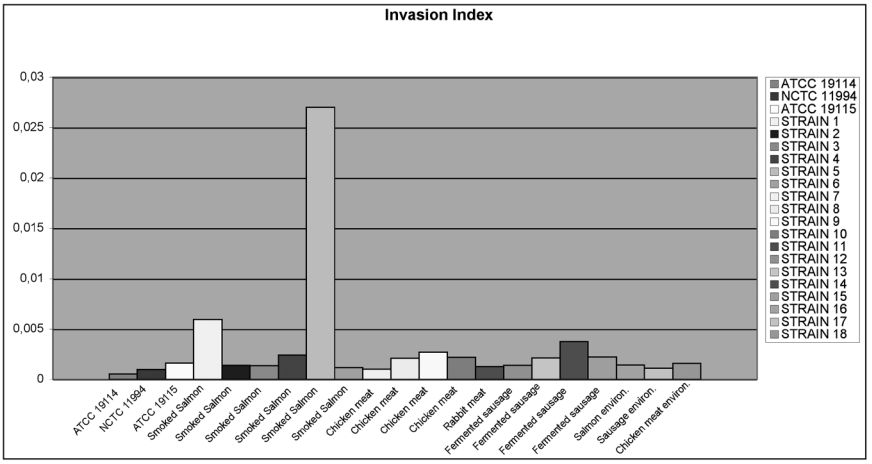

Figure 1: Invasion index of L. monocytogenes strains.

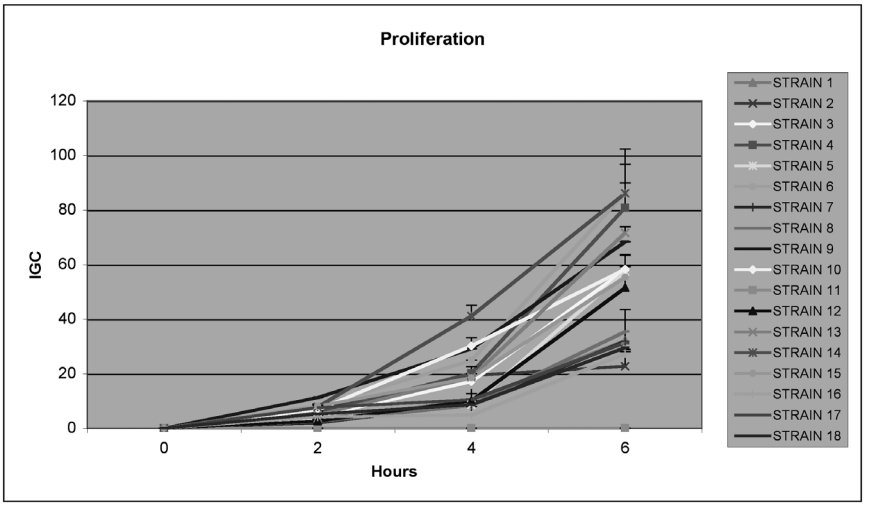

Figure 2: Growth rate of Listeria monocytogenes strains tested.

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