Assessment of the pathogenic potential of two Listeria monocytogenes human faecal carriage isolates

Maïwenn Olier, Fabrice H.F. Pierre, J.P. Lemaître, C. Diviès, A. Rousset, Jean Guzzo

ASSESSMENT OF THE PATHOGENIC POTENTIAL OF TWO *LISTERIA MONOCYTOGENES* HUMAN FAECAL CARRIAGE ISOLATES

Keywords : *Listeria monocytogenes*, faecal carriage, virulence, internalin

Maïwenn Olier [1], Fabrice Pierre [1], Jean-Paul Lemaître [1,2], Charles Divies [1], André Rousset [1,2] and Jean Guzzo[1].

[1]Laboratoire de Microbiologie, ENSBANA, 1 Esplanade Erasme, 21000 Dijon, France.

[2] Laboratoire de Microbiologie, Faculté de Pharmacie, 7 boulevard Jeanne d'Arc, 21033 Dijon Cedex, France.

Author for correspondence : Jean-Paul Lemaître. Fax : + 33 3 80 39 66 40

e-mail : jplemait@u-bourgogne.fr

SUMMARY

Two human faeces carriage isolates of *Listeria monocytogenes* isolates (H1 and H2) were compared to reference strains (ScottA and LO28) with regard to their lethality in 14-day old chick embryos, their haemolytic and phospholipases (phosphatidylcholine-phospholipase C and phosphatidylinositol-phospholipase C) activities and their invasiveness toward Caco-2 cells. Experimental infection of chick embryo allowed discrimination of the strains into those exhibiting high virulence (ScottA and H2), those exhibiting slightly attenuated virulence (LO28) and those exhibiting low virulence (H1). A similar percentage mortality and time to death for embryos was observed when they were infected with H2 as was seen with infection by the reference strain ScottA. Therefore, human carriage strain H2 was considered potentially pathogenic. In contrast to H2 and ScottA, H1 exhibited low virulence. Using the tissue-culture cell-line model, it was found that carriage strain H1 was unable to enter Caco-2 cells efficiently, even though it was similar to the virulent strains in terms of the enzymic activities involved in pathogenicity. Detection of the internalins InlA and InlB involved in the internalization of *L. monocytogenes* in the host cells by immunoblot indicated that a truncated form of InlA was produced by H1. Taken together, these data provide a starting point for the study of the behaviour of two types of human faeces carriage strains and their characterization.

Abbreviation: MTD, Mean Time to Death

INTRODUCTION

Listeria monocytogenes is a ubiquitous Gram-positive rod that causes serious infections in humans and animals. The most common route of *L. monocytogenes* infection is through the gastrointestinal tract (Schlech, 1984), as evidenced by several outbreaks of listeriosis caused by ingestion of contamined food materials (Schlech *et al*., 1983). Numerous reports have been published dealing with the prevalence of *L. monocytogenes* in food products (Ryser & Marth, 1999). Despite frequent exposure to this bacteria (Notermans *et al*., 1998), the probability of contracting the disease is low since the incidence range from 1,6 to 6 cases per million population (Rocourt *et al*., 2000).

One explanation of this relatively weak incidence may be the limitation of listeriosis cases to high-risk groups which include pregnant women, neonates and immunocompromised adults. However, few listeriosis cases have been identified in immunocompetent patients (Schlech *et al*., 1983; Goulet *et al*., 1993; Kelly *et al*., 1999) indicating that (1) *L. monocytogenes* has the potential to infect immunocompetent individuals (2) the immunocompetent status alone can not explain the low risk of contamination.

Differences in human-pathogenic potentials of *L. monocytogenes* isolates may also be involved. Most of our knowledge of pathogenicity comes from the many studies carried out in mouse models. Molecular and cellular mechanisms involved in the intracellular life of this bacteria have been identified using complementary *in vitro* tests like tissue culture assays (Gaillard *et al*., 1987) and mutagenesis strategies. Virulence is due to the expression of several genes (Braun & Cossart, 2000) responsible for the pathogen's ability to penetrate, proliferate and spread in cells. The entry process involves the expression of two proteins InlA (Internalin) and InlB encoded by two genes *inlA* and *inlB* respectively, organised as an operon. These surface proteins are both necessary and sufficient for entry into various culture

cell lines (Gaillard *et al*., 1991; Dramsi *et al*., 1995). A second genetic locus is involved in functions essential to the intracellular survivor. This locus encodes listeriolysin O (LLO), phospholipases (Pi-PLC and Pc-PLC) and ActA. All these cell surface and secreted proteins are coordinately regulated by the pleiotropic transcriptionnal activator PrfA.

Several studies showed heterogeneity in the virulence of *L. monocytogenes* isolates (Del Corral *et al*., 1990; Brosch *et al*., 1993; Wiedmann *et al*., 1997; Van Langendonck *et al*., 1998; Norrung & Andersen, 2000; Barbour *et al*., 2001) without systematic correlation between the level of virulence and type characteristics (serovars, phagovars, ribovars, DNA macrorestriction patterns) or origin (human, animal, category of food, environment) of the strain. These studies dealt generally with comparison between strains originated from clinical and food environments. However, little attention has been paid to *Listeria monocytogenes* human carriage strains.

Detection of *Listeria monocytogenes* carriage strains has been demonstrated particularly in human faeces. The majority of epidemiological investigations focused only on the rate of faecal carriage *L. monocytogenes* strains; it was estimated between 1 and 6% of the general population (Durst & Zimanyi, 1976; Kampelmacher & Van Noorle Jansen, 1972; Schuchat *et al*., 1993). In order to better understand their occurrence, the characterization of such strains is needed. The well characterized LO28 strain was recovered from faeces of healthy pregnant women. Analysis of the LO28 strain has shown that a mutation in *inlA* resulted in a truncated protein released in the culture medium (Jonquières *et al*., 1998). Surprisingly this human carriage strain has shown a high level of virulence in the mouse model.

In this work, in order to explain the lack of listeriosis symptoms, the virulence of two human faecal carriage strains H1 and H2 was compared to that of the well known reference strains, ScottA and LO28.

Three different approaches were considered in order to find out the reasons that could explain the differences observed in the degrees of virulence. First of all, the pathogenicity of the isolates was characterized by inoculating 14-day old chick embryos via the chorioallantoic route, as this test appears to be a reliable alternative to the mouse bioassay for the assessment of the pathogenicity of *Listeria spp*. (Terplan & Steinmayer, 1989; Notermans *et al*., 1998). Secondly, the comparison of their virulence associated determinants including haemolytic and phospholipases (Pc-PLC and Pi-PLC) activities were analysed. Thirdly the evaluation of their efficiency for adhesion, invasion and growth within human Caco-2 intestinal cells was investigated. At the same time, we analysed the production of InlA and InlB proteins in order to characterize further the internalization process of these human faecal carriage strains.

METHODS

Listerial strains.

All *Listeria monocytogenes* strain were isolated from humans. *Listeria monocytogenes* strains Scott A (CIP 103575, serotype 4b), an epidemic strain, isolated from a 1992 French human outbreak and LO28 (serotype 1/2c), a carriage strain recovered from the faeces of a healthy pregnant woman were picked as reference strains. In our laboratory, two faecal carriage *L. monocytogenes* strains, H1 (serogroup 1) and H2 (serogroup 4), were originally isolated from two patients human without listeriosis, a pregnant woman carrier of H1 for which the twin new-borns were healthy, and a 3-year old infant carrier of H2. (Rousset *et al*., 1994).

Listeria welshimeri strain was also used as a negative control in the virulence assays. It was also isolated in our laboratory from faeces of a healthy human (Rousset *et al*., 1994).

Culture medium and growth conditions

Overnight cultures of *Listeria* cells grown at 37°C in brain-heart infusion (BHI) broth (Biomérieux) were used for the chicken embryo bioassays and cell culture assays. For western blot analysis, protein samples were derived from cultures that reached the stationary phase at 37°C in MCDB 202 (Cryo Bio Systems) supplemented with glucose at 50 mg l^{-1}, 10% Yeast Nitrogen Base w/o amino acids (YNB, Difco) and 1% trace elements (Cryo Bio Systems).

Viable counts were performed on Acriflavine (10 mg l^{-1}, Sigma) - Ceftazidime (50 mg l^{-1}, Glaxo Wellcome) Agar (ACA) plates incubated 48h at 37°C.

Chicken embryo methodology

Chicken embryos.

Fertile eggs were purchased from IFFA-CREDO. Eggs were incubated for 14 days as described by Buncic & Avery (1996) at 37.5°C in a rotary egg incubator (Grumbach S84 model).

Chorioallantoic inoculation of embryos by the technique of Buncic *et al*. (1996).

Strains of *L. monocytogenes* were grown in BHI at 37°C to an OD_{560} of 1.0 and harvested by centrifugation at 6000 × g for 10 min at room temperature. Cell pellets were gently suspended in phosphate buffer saline (PBS, Invitrogen) at pH 7.2 to obtain an initial density of 3×10^7 to 3×10^8 c.f.u. ml^{-1}. Serial dilutions were prepared in PBS, and embryos were inoculated with 0.1 ml of a 10^{-5} dilution *via* the chorioallantoic membrane after boring an artificial hole through the shell. The inoculum dose was confirmed by enumeration using ACA plate count. At least five embryos were used per strain tested. Lots were inoculated with sterile PBS in each experiment to control chick embryos' viability.

Inoculated eggs were incubated in a horizontal position at 37.5°C and the vitality of the embryos was monitored daily for 6 days by transillumination (CL TH model); death was recognized by a loss of all visible blood vessel structure, a gelling of the suspending liquids, and an absence of any reflexive movement of the embryo. The mean time to death (MTD) and the mortality rate was calculated to appreciate the level of virulence for each strain tested.

Strain characterization

Test strains were spot inoculated (10^9 bacteria/spot) on appropriate agar plates for the observation of three distinct enzymatic activities :

Haemolytic activity was tested on Bacto Colombia blood agar base EH medium (mBAP, Difco) supplemented with 5% (v/v) saline-washed horse red blood cells. Plates were incubated for 48h at 37°C after inoculation and observed for blood cells lysis zone around the colonies (Fujisawa & Mori, 1994).

Phosphatidylcholine-specific phospholipase C (Pc-PLC) activity was tested by inoculating bacteria on BHI agar supplemented with 5% (v/v) fresh egg yolk (Coffey *et al*., 1996). Plates were incubated for 48h at 37°C and observed for opacity zones of degraded egg yolk lecithin around the colonies.

Phosphatidylinositol-specific phospholipase C (Pi-PLC) activity was determined as described by Notermans *et al*. (1991). After growth at 37°C on TY agar (1% tryptone, 0.5% yeast extract, 1% NaCl, 1% agar), plates were covered with 1% L-α-phosphatidylinositol substrate (Sigma) in 0.7% agarose. Plates were reincubated at 37°C and observed for turbid haloes (the insoluble diacylglycerol) around the colonies for 6 days.

Cells

The human colon carcinoma cell line Caco-2 (ECACC No. 86010202) was used between passages 36 and 42. The cells were routinely grown in 25 cm^2 plastic tissue culture flasks (Greiner) at 37°C in a humidified atmosphere of 5% (v/v) CO_2 air. The culture medium was Dulbecco's modified Eagle minimum essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, and antibiotics (100 U penicillin ml^{-1}, 100 µg streptomycin ml^{-1}). All reagents were purchased from Invitrogen (Life Technologies).

Infection of Caco-2 cells

Confluent cell monolayers were trypsinized and adjusted on 24-well tissue culture plates (Greiner) to obtain almost semiconfluent monolayers after 3 days of incubation at 37°C (10^6 cells per well). Cells were incubated in medium without antibiotics for 12h and were washed with fresh medium just before used. After overnight growth in BHI, *Listeriae* were centrifuged (6000 x g, 10 min at room temperature) and resuspended in DMEM before infection. Caco-2 monolayers were infected with 300 µl bacterial suspension (multiplicity of infection (m.o.i.) of 100 bacteria per cell) according to Van Langendonck *et al*. (1998).

Adhesion was allowed to occur for 30 min at 37°C (Santiago *et al*., 1999), then cell monolayers were washed five times to remove nonadherent bacteria. Adherent bacteria were harvested after lysis of cell monolayers with 0.5 ml Triton X-100 (0.1% in cold PBS) for 10 min. Colony forming units (c.f.u.) of viable bacteria were counted by plating suitable dilutions of the lysates on ACA plates, and incubating for 48h at 37°C.

For entry and intracellular growth assays, monolayers were infected with bacteria for 2h at 37°C. After incubation, nonadherent bacteria were removed by three washes. Then, cell monolayers were covered with fresh DMEM containing gentamicin at bactericidal

concentration (100 µg ml^{-1}) to kill extracellular bacteria. After a contact time of 1.5h at 37°C, the rate of entry was determined by plate counts following washed Caco-2 cell lysis (0.5 ml Triton X-100 at 0.1% in cold PBS). It was considered that 3.5h after onset of infection, the bacterial counts were the number of bacteria internalized. To determine the rate of intracellular growth, DMEM supplemented with gentamicin at 5 µg ml^{-1} was used and the incubation time was increased to 19.5h (Van Langendonck *et al*., 1998).

Preparation of culture supernatants and extraction of bacterial surface proteins

The technique of Kocks *et al*. (1992) was used. Following growth of culture in MCDB 202 supplemented with 50 mg l^{-1} glucose, 10% YNB and 1% traces element, bacteria were removed by two centrifugations (6000 x g, 10 min at room temperature) to obtain the culture supernatant proteins. Supernatant proteins were precipitated overnight by addition of 16% (v/v) trichloroacetic acid (TCA) at 4°C.

For sodium dodecyl sulfate (SDS)-extracts, cell pellets were washed in Tris/HCl, 10mM, pH 8, immediately resuspended in 1% SDS (v/v) in Tris/HCl, 10 mM, pH 8, and incubated for 15 min with gentle shaking (1000 rpm). The suspension was then centifugated, bacterial surface proteins which were found in this supernatant were precipitated with 16% (v/v) TCA and allowed to stand at 4°C overnight.

All precipitated protein fractions were washed twice in 90% acetone, suspended in Laemmli sample buffer (Laemmli, 1970) and heated at 95°C for 7 min prior to SDS-PAGE and Western blotting analysis.

SDS-PAGE and immunoblotting

SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli with 12.5% polyacrylamide gels (Laemmli, 1970). Equal amounts (10 µg) were loaded in each lane of the gel. After electrophoresis (90 min, 20 mA), proteins were transfered to nitrocellulose sheet (PROTEAN®, Schleicher and Schuell) using the semi-dry electroblotting method. The membrane was stained with 0.2% Ponceau red to confirm the transfer efficiency. Membrane hybridizations with antibodies were performed with diluted (1:1000) mouse monoclonal antibodies directed against InlA (Mab L7-7) or InlB (Mab B4-6) (Braun *et al*., 1997 & 1999; Mengaud *et al*., 1996a). For immunodetection, bound primary antibodies were revealed with anti-mouse immunoglobulin-horseradish peroxidase conjugate (Sigma) diluted at 1:1000 and peroxydase activity was detected using the ECL kit (Amersham Pharmacia).

RESULTS

Virulence of *L. monocytogenes* strains for chick embryos

The chick embryo model was chosen to study the virulence of *Listeria* strains from various origins. The inoculum dose, determined by plate count, was ranging from 1.5-2.5 $\log_{10}$ c.f.u. per egg. Inoculation of 14-day chick embryos *via* the chorioallantoic fluids distinguished highly virulent strains having 100% mortality rate with a mean time to death (MTD) ranging from 2 to 3 days, from strains having a lower mortality rate with a higher MTD (> 3.5 days) (figure 1).

As expected, the epidemic strain, ScottA, was fully pathogenic for 14-day old chick embryos (mortality 100%), whereas the asymptomatic carriage reference strain, LO28, was slightly deficient in pathogenicity (mortality 80%). *L. welshimeri*, a non virulent species for human, was non virulent for chick embryos (mortality 0%) and was used as a control strain.

In comparison with these reference strains, H2, a carriage strain, was found to be highly virulent at the same extent as the epidemic strain ScottA. In contrast, the virulence of strain H1 was strongly attenuated for virulence, since 4 out of 5 chick embryos inoculated with this strain were still alive after 6 days.

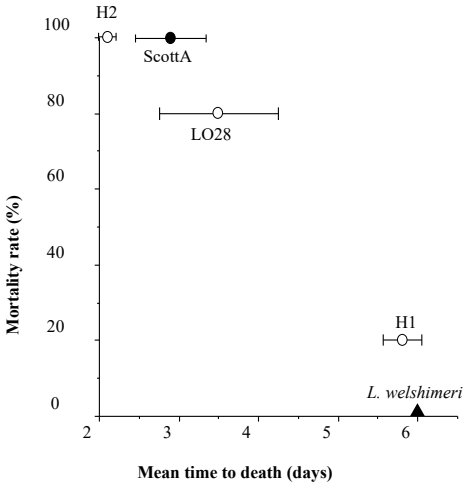

Figure 1. Mortality rate and mean time to death of chick embryos inoculated with the human *Listeria monocytogenes* reference epidemic strain ScottA (●), the *L. monocytogenes* carriage strains (○) and the *L. welshimeri* strain (control) (▲). For each strain, approximately 1.5-2.5 log c.f.u. were inoculated in 5 14-day-old chick embryos, for which the vitality was monitored daily for 6 days. Error bars depict standard deviations resulting from the mean time after which each of the five embryos died. The mortality rate was expressed as a percentage of the number of embryos that died, out of a total of five. Mean time to death was calculated for the five embryos.

Enzymatic potential for virulence of carriage strains

Table 1 summarizes the characteristics of the 6 cultures studied, regarding their origin, the serogroup, the haemolitic activity on horse blood, and the Pc-PLC and Pi-PLC activities.

As expected, *L. welshimeri* tested negative in the three enzymatic assays, since this non pathogenic *Listeria* species does not possess the corresponding genes. All of the *L. monocytogenes* strains tested showed clear haemolysis on colombia blood agar base supplemented with horse blood. Carriage strains supported similar high levels of haemolytic activity, whereas ScottA demonstrated the lowest haemolytic activity.

In the same way, *L. monocytogenes* carriage strains exhibited lecithin degradation (Pc-PLC activity), and were positive for Pi-PLC activity. Surprisingly, the epidemic strain ScottA, again gave the lowest activity for these two phospholipase activities compared with all other *L. monocytogenes* strains.

Table 1. Strains of *Listeria* used in this study, and their characteristics

Species /category of isolate	**serogroup**	**Haemolytic activity**	**Pc-PLC activity**	**Pi-PLC activity**
***Listeria monocytogenes* reference epidemic strain**				
ScottA	4 b	+	+	+
***Listeria monocytogenes* carriage strains**				
LO28	1/2 c	+++	+++	+++
H1	1	+++	+++	+++
H2	4	+++	++	++
Listeria welshimeri	4	-	-	-

Symbols : +++, strong activity ; ++, moderate activity ; +, weak activity ; -, negative activity

Efficiency of human *Listeria monocytogenes* carriage strains infection within Caco-2 cells

The ability of *L. monocytogenes* strains to adhere, enter and multiply into the intestinal epithelial cells was evaluated using an *in vitro* model with human enterocyte-like Caco-2 cells. Results of kinetics for ScottA, LO28, H1 and H2 are summerised in figure 2. Results are expressed as percentage of adhesion, entry and intracellular growth, in order to take into account the differences in initial cell numbers between strains (between 3 x 10^6 and 5 x 10^7 bacteria per well). The rate of replication was calculated from the percentages of entry and intracellular growth.

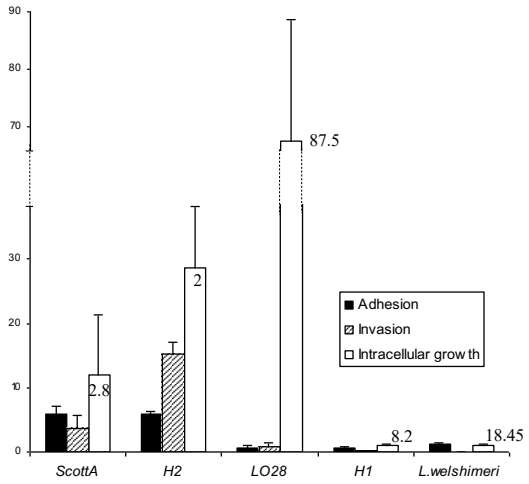

Figure 2. Listerial adhesion to, entry into and intracellular growth in Caco-2 cells, and rate of replication of listerial cells in Caco-2 cells. For adhesion (solid bar), entry (grey bars) and intracellular growth (open bars) rates, the number of viable bacteria was determined after different times of infection, by cell lysis and inoculation onto ACA plates using appropriate dilutions. Results are expressed as the mean percentage (of initial inoculum) of viable recovered bacteria per well from two experiments analysed in duplicate. Values above the intracellular growth columns (open bars) represent the rate of replication. The rate of replication was calculated by dividing the intracellular growth rate by the entry rate. Vertical bars depict SD.

Under these conditions, the kinetics of Caco-2 cell infection were similar for *L. monocytogenes* strains that were fully pathogenic for chick embryo, i. e. the reference epidemic strain ScottA and the H2 carriage strain. These strains were the most efficient to enter in Caco-2 cells but had the lowest rate of replication: after 3.5h of contact with Caco-2 cells, the amount of bacterial cells internalized was 3.7 and 15.2% for ScottA and H2 respectively; after 21.5h of infection, the rate of replication were 2.8 and 2.0 for ScottA and H2 respectively.

In contrast, *L. welshimeri* strain, showed low adhesion values (1.3% of deposited bacteria) and extremely low entry values (0.06% of deposited bacteria), even though its intracellular growth ability inside the Caco-2 cells was higher than that of ScottA and H2 (rate of replication of 18.45 after 21.5h of infection).

Among the carriage strains with attenuated virulence towards chick embryo, the weakly attenuated strain LO28 and the strongly attenuated strain H1 showed results that were closer to *L. welshimeri's* behaviour than *L. monocytogenes* ScottA's behaviour. They was characterized by extremely low adhesion percentages (< 1% of deposited bacteria) and consecutively, low entry levels, which were about 0.8% for LO28 and 0.11% for H1 in 3.5h of infection. However, bacteria that reached the Caco-2 cytoplasm had a high rate of replication (87.5 for LO28 and 8.2 for H1 respectively after 21.5h of infection). The relatively high intracellular multiplication level observed for LO28, H1 and *L. welshimeri* within Caco-2 cells might be related to the relatively low entry level. However, cell infection performed with a higher multiplicity of infection showed that these strains exhibited the same intracellular rate of replication (data not shown). Therefore, this high rate of replication for LO28 (rate of multiplication > 80) might be the reason why mortality rate for chick embryos reach 80% compared to the H1 one (mortality 20%), for which this rate of replication is ten times lower.

The lower level of invasion and dissemination of strains LO28 and H1 compared to ScottA and H2 could be due, at least partially, by an impaired ability to adhere. This lead us to examine in LO28 and H1 the synthesis of two surface proteins (InlA and InlB) implicated in adhesion mechanisms.

Analyses of *L. monocytogenes* human carriage strains surface proteins (Internalin (InlA) and InlB) required for entry into epithelial cells

The presence of InlA and InlB in H1, H2, ScottA and LO28 was analysed in culture supernatants and SDS-extracts by using two distinct specific monoclonal antibodies (Mabs), L7.7 for the production of InlA (Mengaud *et al*., 1996a) and B4-6 for the production of InlB (Braun *et al*., 1997) (Figure 3).

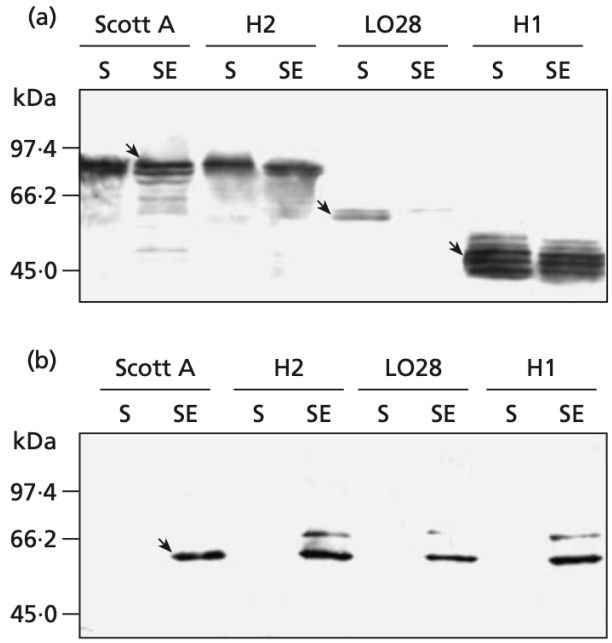

Figure 3. Western blot analyses of supernatant proteins (S) and SDS-extract proteins (SE) from strains ScottA, H2, LO28 and H1. (a) Identification of the InlA polypeptide analysed by immunoblotting with Mab L7.7 (Mengaud *et al*., 1996a). (b) Identification of the InlB polypeptide analysed by immunoblotting with Mab B4-6 (Braun *et al*., 1997).
Molecular mass standards are indicated on the left of each image.

As expected, InlB produced by the carriage strain H1 and H2 and the reference strains ScottA and LO28 had an apparent estimated molecular weight of 60 KDa in the SDS-extracts fractions.

In culture supernatants and SDS-extract fractions from H2 and ScottA, InlA was produced with a molecular weight of 80 KDa. In contrast, InlA from strains H1 and LO28 had apparent molecular weights of approximately 47 KDa and 60 KDa respectively; these two proteins were therefore considered as truncated. The presence of truncated form of InlA in strain LO28 has already been observed by Jonquières *et al*. (1998). Taking into account that Mab L7.7 is specific for the N-terminal part of InlA, these authors suggested the absence of the N-terminal part of InlA, and so, the absence of the cell wall anchoring motif, and could lead to the release of the surface protein in the culture medium. This hypothesis is supported by the fact that there was a higher amount of InlA detected in the LO28 culture supernatant

than in its SDS-extract fraction. Nevertheless, InlA is detected in large quantities in supernatants of all strains in our experiments. We can note furthermore that the amounts of InlA are lower for LO28 than for the other strains.

So, if the human carriage strain H2 seems to be potentially as pathogenic as a known pathogenic strain ScottA, the other carriage strain H1, as LO28 carriage reference strain, produced a truncated form of InlA, for which the function is consequently probably affected. These results could explain their reduced ability for adhesion on Caco-2 cells and consequently, reduce probably their ability to be fully pathogenic for the chick embryo.

DISCUSSION

The present study focused on the characterization of the entry mechanisms of two *L. monocytogenes* carriage strains to determine if the lack of virulence could be linked to one or several components implicated in internalization into host cells, the first step of infection.

Firstly we have assessed the ability of the chick embryo test to classify our isolate according to their level of virulence. Our results confirmed the occurrence of *L. monocytogenes* strains with attenuated virulence. To our knowledge the present investigation demonstrated, for the first time, an attenuated virulence for LO28. This highlights the importance of the choice of the *in vivo* model used for the study of pathogenicity. In fact, the chick embryo assay could be a better model than the mouse or rat model which are not considered appropriate for evaluating the virulence of *L. monocytgenes* strains like LO28. Indeed, as noted by Lecuit *et al.* (2000), cells expressing mouse or rat E-cadherin (mEcad) do not allow InlA-dependent entry, whereas cells expressing human E-cadherin (hEcad), guinea pig or chicken E-cadherin (LCAM) are totally permissive to invasion by InlA expressing bacteria.

Furthermore, althought both H1 and H2 strains seem to be avirulent for humans, H2 was fully virulent for chick embryos with levels similar to those of the epidemic strain ScottA. For H2, the differences of virulence against human cells and chick embryo cells may be due to a low dose of ingested bacteria for the human and/or to the performance of the human immune system. In comparison, H1 seems to have a reduced capacity to invade both human and chick embryo cells.This led us to examine more precisely the virulence associated enzymatic properties of the strains, in order to determine if these differences in pathogenicity could be correlated with differences in enzymatic activities.

Our results demonstrate that both human carriage strains H1 and H2, have the enzymatic potential to be fully pathogenic since levels of the three enzymatic activities (haemolytic and phospholipases activities) observed for the two strains were higher than those of epidemic reference strain ScottA. Furthermore, there was no quantitative correlation between enzymatic activities required for virulence and the lethality for chick embryo or human. This was previously reported with the mouse model (Kathariou *et al*., 1988; Del Corral *et al.,* 1990; Erdenlig *et al*., 2000). These results suggested that either the production or activity of virulence factors in the host is different from that observed *in vitro* or that other putative virulence factors are responsible for this difference in degrees of virulence.

In this way, we decided to follow *in vitro* the steps of infection of host epithelial cells in order to discover what impaired function was responsible for the differences in pathogenicity. Virulent strains for the chick embryo, i. e. ScottA and H2, entered Caco-2 cells with great penetration efficiency, while strains that were attenuated for virulence with the chick embryo, i. e. LO28 and H1, showed low entry level.

Our results demonstrated on one hand significant differences in the behaviour of these two carriage strains in the kinetics within Caco-2 cells, whereas they had equivalent enzymatic potential, sufficient to induce pathogenicity. Non virulent strains (according to

results obtained with tissue culture cell model) with pathogenic enzymatic potential have already been described (Pine *et al.,* 1991; Van Langendonck *et al.,* 1998; Pandiripally *et al.,* 1999), indicating the absence of relation between *in vitro* assays and levels of enzymatic activities implicated in virulence.

On the other hand, compared to *in vivo* tests, human enterocyte like cell line Caco-2 seems to be a reliable alternative model, allowing elucidation of an altered function in the progress of the bacteria through the host cell. Then as LO28, H1 entered Caco-2 cells poorly, with levels as low as those found for *L. welshimeri.* This low entry of *L. monocytogenes* LO28 was reported previously (Mengaud *et al*., 1996b). It has been already attributed to a truncated form of InlA (Jonquières *et al*., 1998) but this unfunctional InlA did not affect its virulence in a mouse model.

Western blot analysis showed that, as LO28, our strain H1 had indeed a truncated InlA with a molecular weight of 47 KDa instead of 80 KDa, whereas H2's InlA was entire. Otherwise, anti-InlB allowed to confirm that there were no problems concerning the production of InlB from the *locus inlAB*.

Taking into acccount that both our strains with attenuated pathogenicity for human or chick embryo displayed a truncated form of InlA, these results supported the hypothesis of a critical role of InlA in clinical forms of listeriosis.

Furthermore, in LO28, a nonsense mutation was responsible of the truncated form of H1 InlA (data not shown). This confirms clearly the hypothesis of Chakraborty *et al*. (1994), who were working however with environmental strains. Indeed, these authors showed that naturally occuring virulence–attenuated isolates were detected in hosts only for short periods of time as a result of mutations occuring within virulence genes. They suggested that such carriage strains, similar to strain H1 described in our study, could induce protection to subsequent infection by more pathogenic variants like H2. Manohar *et al*. (2001) reinforced

this point of view by showing that gut colonization of mice with actA-negative mutants of *Listeria monocytogenes* could stimulate a humoral mucosal immune response. These findings could explain partly the widespread resistance in humans exposed to *L. monocytogenes*. Another reason for the absence of listeriosis in carrier H2 could either be due to additional physiological factors or compounds not involved for its internalization or its growth within cells but linked to colonization or survival through the mucosa of the intestine (Barbour *et al*., 2001; Cotter *et al*., 2001). Moreover, the microbial interactions between *L. monocytogenes* and enteric commensal bacteria could explain the absence of listeriosis, as suggested by Zachar and Savage (1979) and Cebra (1999).

Finally, this study allowed us to characterize two opposite behaviours for these two human faecal carriage strains. One is attenuated for virulence in chick embryo and can not be translocated through the intestinal wall on account of a truncated InlA. The other carriage strain should be considered potentially pathogenic in regard to its virulence for the chick embryo, its abilities to express virulence associated determinants and its invasion efficiency within Caco-2 cells. Indeed, this strain may have encountered particular circumstances within the host intestinal tractus that prevented the listeriosis. Whether truncation of Internalin within human carriage *L. monocytogenes* is a rare event or not remains to be investigated.

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