Original Research Article

Species and virulence determination of *Listeria monocytogenes* isolated from goat meat in Port Harcourt, Nigeria

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A B S T R A C T

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The speciation and virulence determination of four *Listeria monocytogenes* strains isolated from goat meat alongside *L. monocytogenes* PCM 2191 serovar 01/2 was done using internalin genes *inlA, inlB, inlC* and *inlJ* primers individually. The *inlA* and *inlB* gene primers formed the expected bands of 800bp and 884bp respectively with the genomic DNA of *L. monocytogenes* PCM 2191 serovar 01/2 but not with genomic DNA of the 4 *L. monocytogenes* isolated from goat meat. The *inlJ* gene primer generated a 238bp fragment with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2 and the DNA of 3 of the 4 isolated *L. monocytogenes* while *inlC* gene primer generated a 517bp fragment with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2 and 2 of the 4 isolated *L. monocytogenes*. Only the DNA of 1 of the 4 isolated *L. monocytogenes* alongside *L. monocytogenes* PCM 2191 serovar 01/2 formed the expected fragment with the *inlJ* and *inlC* gene primers. The inability of the isolated strains to produce the expected fragments with the *inlA* and/or *inlB* gene primers remained to be unravelled.

Introduction

Listeria monocytogenes, a Gram-positive bacterium has emerged as a significant foodborne pathogen, causing serious illness in infants, pregnant women, elderly and immnuno-suppressed individuals, with symptoms ranging from septicemia, meningitis, encephalitis, abortions, to occasional death (Lee et al.,2012; Liu, 2006; Mead et al., 1999). *L. monocytogenes* mortality rate is highest of all foodborne pathogens, and is usually 20-30%, but has been reported to be as high as 50% (Lindbäck et al., 2011; Bhunia, 2008). It has been estimated to cause approximately 1600 human invasive listeriosis cases in the United States each year; of these, as many as 1455 cases annually are predicted to result in hospitalization and 255 cases are expected to result in death, making *L. monocytogenes* one of the leading causes of death due to foodborne illness in the United States (Scallen et al., 2011). The ability to survive and/or proliferate under

stress contributes to the persistence of *L. monocytogenes* both in foods and food-processing environments, elevating the risk of transmission of this pathogen through foods to humans (Bergholz et al., 2012).

Although *L. monocytogenes* is an important foodborne pathogen, the species encompasses a spectrum of strains with varied virulence and pathogenicity (Liu et al., 2007). There are 13 distinct O-antigenic patterns, which comprise the serovars; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab, and 7. Of which, 1/2a, 1/2b and 4b are responsible for 98% of the outbreaks and serotype 4b is considered the most virulent; a subpopulation of serotype 4b also exists and one or more of those subtypes are considered as epidemic clones (Bhunia 2008; Jadhav et al., 2012). *L. monocytogenes* are grouped into three lineages based on their ribopatterns and their association with outbreaks, namely: lineage I (serotypes 1/2b, 3b, 3c, and 4b) from human clinical listeriosis, lineage II (serotypes 1/2a, 1/2c, and 3a) from food isolates and animal clinical cases, and less common lineage III (serotypes 4a and 4c, as well as some 4b) from animal and environmental specimens (Jadhav et al.,2012; Bhunia,2008; Nadon et al., 2001).

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood, with most of the available information derived from interpretation of epidemiological, clinical, and histopathological findings and observations made in experimental infections in animals, particularly in the murine model (Vazquez-Boland et al., 2001). Despite being pathogenic at the species level, *L. monocytogenes* is in fact made up of a spectrum of strains or

genotype with varying pathogenic potential; some of which are highly pathogenic and sometimes deadly while others are relatively avirulent and cause little harm in the host (Liu, 2006).

The virulence testing protocols developed to enhance the laboratory assessment of *L. monocytogenes* virulence include: in vivo bioassays, in vitro cell assays, and assays for key virulence-associated proteins and their corresponding genes (Liu et al., 2007). While the in vivo mouse virulence assay offers a comprehensive assessment of all known and uncharacterized virulence determinants of *L. monocytogenes*, its utility as a routine technique is questionable due to its expense and its use of animals (Liu et al., 2007). In addition to the genes located in the virulence gene cluster, an independent operon harboring two genes, *inlA* and *inlB*, has been detected in *L. monocytogenes* by transposon-induced mutagenesis and isolation of noninvasive mutants (Gaillard et al., 1991).

With *L. monocytogenes* consisting of a diversity of strains, the availability of subtyping procedures to track individual strains involved in listeriosis outbreaks, and to examine the epidemiology and population genetics of *L. monocytogenes* bacteria, is integral to control and prevention programmes aimed at listeriosis (Liu, 2006). The present study is aimed at speciation and determining the presence of virulent genes in *L. monocytogenes* isolated from goat meat in Port Harcourt, Nigeria.

Materials and Methods

Listeria moncytogenes strains

The *L. monocytogenes* PCM 2191 serovar 01/2 was obtained from the Polish

Collection of Microorganisms, Poland, while the four *L. monocytogenes* were isolated from goat meat using Fraser broth and PALCAM agar (Oxoid, England) and confirmed on the basis of their conventional and polymerase chain reaction (PCR) (Eruteya et al., 2014).

Extraction of *Listeria* DNA (deoxyribonucleic acid)

DNA was extracted by the boiling method without Triton X-100 (Hitchins et al., 2004). Cells were harvested by centrifugation (Eppendorf centrifuge 5418, Germany) of overnight brain heart infusion broth culture of *Listeria* in 2ml Eppendorf tube at 10,000rpm for 2min and the supernatants discarded. The pellets were re-suspended in 1ml sterile distilled water and re-centrifuged after vortexing (Vortexer 59A, Denville scientific INC, Taiwan)at 10,000rpm for 5min. the supernatants were again discarded and the pellets re-suspended in200µl sterile water and vortexed. The suspensions were heated for 10min in a boiling bath (100°C) (Grant GLS400, Grant Instrument, England). After cooling and vortexing, the mixture were centrifuged at 10,000rpm for 5min. the supernatants were then transferred to a pre-labeled 1.5ml Eppendorf tube while the sediments were discarded. The DNA extracted was stored in deep freezer (-20°C) until further analysis.

Species and virulence-specific determination of *Listeria monocytogenes*

Oligonucleotide primers from L. monocytogenes internalin genes *inlA, inlB, inlC* and *inlJ* described by Liu *et al.* (2007) for species-specific recognition (*inlA* and *inlB* primers) and virulence determination (*inlC* and *inlJ* primers) all synthesized by Biomers.net GmbH, Germany were employed (Table 1).

PCR was conducted in thermocycler (Mastercycler-Eppendorf, Vapo-product,Germany) in a volume of 25µl, containing 2.5 µl 10×PCR buffer, 1.5 µl MgCl$_2$, 0.5µl dNTP (deoxynucleoside triphosphate), 0.25 µl each of appropriate primer, 0.1µl AmphiTaq DNA polymerase (All products of Solis BioDyne), 1.5µl of appropriate DNA preparation and 18.65µl sterile distilled water. Amplification following an initial denaturation at 95°C for 3min was performed in 30 cycles, at 95°C for 30s, 55°C for 60s and 72°C for 60s. A final extension was performed for 10min at 72°C. A 8µl aliquot of PCR product mixed with a loading dye (10mM, EDTA, 10% glycerol, 0.015% bromo phenol blue and 0.017% sodium dodecyl sulphate(SDS), made up to 100ml) were checked in an ethidium bromide stained 1.5% agarose (Fermentas, Life Science, Germany) and the gel read in a UV transilluminator (GenoSens 1500, Clinx Science Instruments Co.Ltd, China). Reaction mixture with the DNA of *L. monocytogenes* PCM 2191 serovar 01/2(Polish Collection of Microorganisms, Poland) template serve as positive control while a reaction mixture with no DNA template was incorporated as a negative control in each reaction.

Results and Discussion

The application of molecular techniques has facilitated the identification and characterization of major virulence-associated genes and proteins in *L. monocytogenes* (Liu, 2006).

The results of the PCR shows that the internalin genes *inlA* (Fig. 1) and *inlB* (Fig. 2) primers performed individually

only formed the expected band of 800bp and 884bp respectively with genomic DNA of the *L. monocytogenes* PCM 2191 serovar 01/2 but not with the genomic DNA of the 4 isolated *L. monocytogenes.* The *inlC* gene primers(Figure 3) produced the expected band of 517bp with the genomic DNA of the *L. monocytogenes* PCM 2191 serovar 01/2 and 3 of the 4 isolated *L. monocytogenes* while the *inlJ* gene primers (Figure 4) also produced the expected band of 238bp with the genomic DNA of the *L. monocytogenes* PCM 2191 serovar 01/2 and 2 of the 4 isolated *L. monocytogenes.*

With *L. monocytogenes* comprising a diversity of strains of varying pathogenicity, the ability to precisely track the strains involved in listeriosis outbreaks and speedily determine their pathogenic potential is critical for the control and prevention of further occurrences of this deadly disease (Liu,2006). The phenotypic subtyping methods are generally less sensitive, have low differentiation ability and are not easy to reproduce, whereas the genotypic approaches are more sensitive and reliable (Shuckken et al., 2003).

Internalin A (inlA) interacting with epithelial cadherin (E-cadherin) and internalin B (inlB) interacting with Met, gClq-R, and proteoglycan receptors aids *L. monocytogenes* invasion into hepatocytes and endothelial cells (Bhunia, 2008).
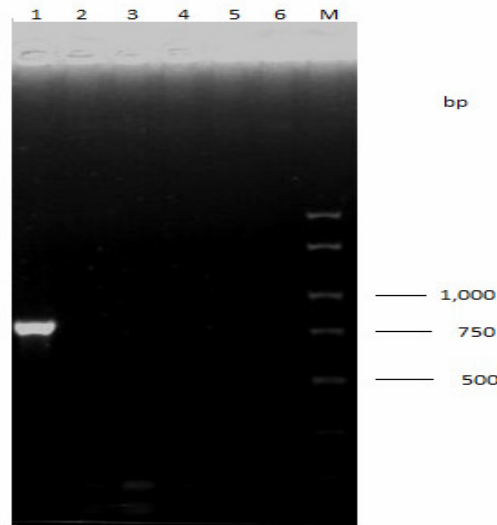
The result of this investigation shows the inability of the genomic DNA of the isolated *L. monocytogenes* to form the expected band of 800bp and 884bp with the *inlA* and *inlB* respectively. The genomic DNA of the *L. monocytogenes* PCM 2191 serovar 01/2 however, produced the 800bp and 884bp bands with the *inlA* and *inlB* primers. Liu et al (2007) reported the failure of serotypes 4a-4e

strains to produced expected band of 884bp with *inlB* primers whereas, the 36 strains tested produced the 800bp band with *inlA* primers both individually and in multiplex. This findings of Liu *et al* (2007) was in agreement with earlier report by Poyart *et al.* (1996), that *inlA* were consistently present in 68 *L. monocytogenes* they examined, regardless of the origin, serovar and virulence of the isolates. They also reported that the size of the amplified DNA fragments of repeats A and B of inlA were constant in all 68 isolates of *L. monocytogenes.* However, during routine confirmation of serotype designations of serotype 4b *L. monocytogenes* by multiplex PCR, Lee et al (2011) observed certain isolates with atypical profiles.

The *inlJ* gene primers (Figure 3) produced the expected band of 238bp with *L. monocytogenes* PCM 2191 serovar 01/2 and 3 of the 4 isolated *L. monocytogenes.* The putative internalin gene *lmo*2821, designated *inlJ* has been confirmed to be a novel internalin gene directly involved in *L. monocytogenes* virulence (Sabet et al., 2005). Being present in *L. monocytogenes* strains/serotypes that are capable of causing human listerial outbreaks and mouse mortality but absent in avirulent, non-pathogenic strains/serotypes (Doumith et al., 2004; Liu, 2004).
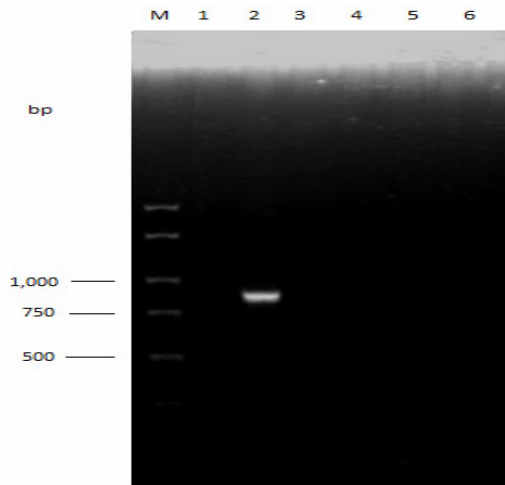
According to Liu (2006), *inlJ* represents the target of choice for laboratory differentiation of virulent from avirulent *L. monocytogenes* strains. Liu *et al* (2007), has observed some uncommon *L. monocytogenes* strains/serotypes (lineage IIIB strains F2-086 and R2-142) with virulence potential but lacking the *inlJ* gene.

Fig.1 The PCR products obtained when genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 total and isolated *L. monocytogenes* were subjected to PCR using *in1A*-F and *in1A*-R primer combination
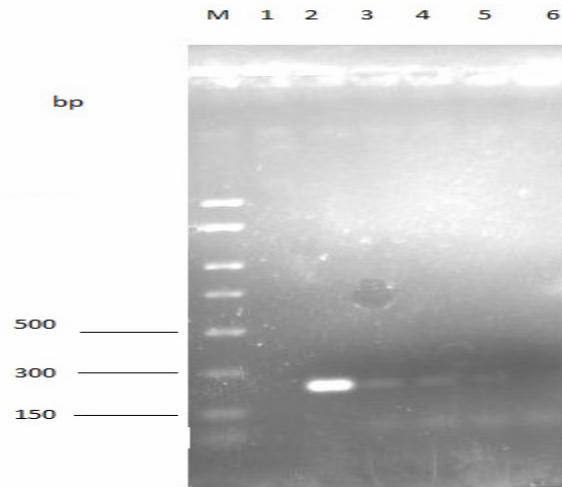

for *L.monocytogenes* species-specific recognition. PCR conditions were as follows: 30 cycles, each at 95^0C for 30s, 55^0C for 60s, and 72^0C for 60s.Lanes: 1, *L. monocytogenes* PCM 2191 serovar 01/2; 2-5, isolated *L.monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standard. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

Fig 2 The PCR products obtained when total genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 and isolated *Listeria monocytogenes* were subjected to PCR using *in1B*-F and *in1B*-R primer combination for *L.monocytogenes* species-specific recognition.
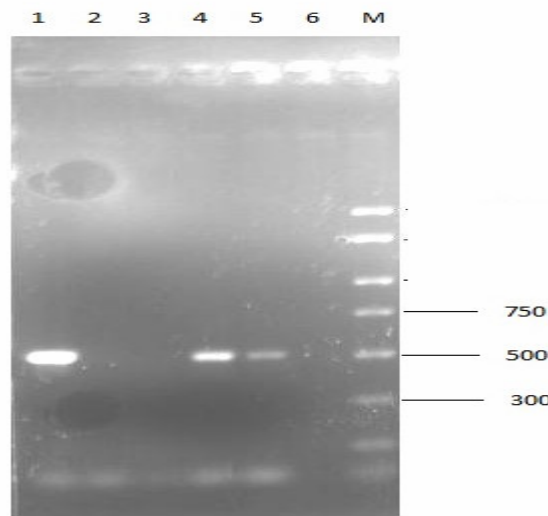

PCR conditions were as follows: 30 cycles, each at 95^0C for 30s, 55^0C for 60s, and 72^0C for 60s.Lanes: M, molecular weight standard; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocytogenes* PCM 2191 serovar 01/2; 3-6,isolated *L.monocytogenes* . PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

Fig.3 shows the PCR products obtained when total genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 and isolated *Listeria monocytogenes* were subjected to PCR using *in1J*-F and *in1J*-R primer combination for virulence determination of *L.monocytogenes*.

PCR conditions were as follows: 30 cycles, each at 95^0C for 30s, 55^0C for 60s, and 72^0C for 60s.Lanes: M, molecular weight standard; 1, control reaction (all reagent ingredients except chromosomal DNA); 2, *L. monocutogenes* PCM 2191 serovar 01/2; 3-6, isolated *L.monocytogenes*. PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

Figure.4 The PCR products obtained when total genomic DNA from *L. monocytogenes* PCM 2191 serovar 01/2 and isolated *Listeria monocytogenes* were subjected to PCR using *in1C*-F and *in1C*-R primer combination for virulence determination of *L.monocytogenes*.

PCR conditions were as follows: 30 cycles, each at 95^0C for 30s, 55^0C for 60s, and 72^0C for 60s.Lanes: 1, L. *monocytogenes* PCM 2191 serovar 01/2; 2-5, isolated *L.monocytogenes*; 6, control reaction (all reagent ingredients except chromosomal DNA); M, molecular weight standard . PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide.

The *inlC* gene primers (Figure 4) produced the expected band of 517bp with *L. monocytogenes* PCM 2191 serovar 01/2 and 2 of the 4 isolated *L. monocytogenes.* According to Engelbrecht *et al* (1996) internalin gene *inlC* is a known virulence protein in L. monocytogenes with a role in the post-intestinal stages of listerial infection. Only 1 of the 4 isolated *L. monocytogenes* and the *L. monocytogenes* PCM 2191 serovar 01/2 produced the expected 517bp and 238bp bands with the internalin *inlC* and *inlJ* primers respectively.

The presence of the *inlC* and/or *inlJ* genes in a given *L. monocytogenes* strain implies its potential virulence and its ability to cause mouse mortality via the intraperitioneal route, it does not necessarily indicates the certainty of the strains harbouring these genes to produce disease in humans via the conventional oral ingestion (Liu et al., 2007). This notwithstanding, any strain of *L. monocytogenes* should be considered potentially pathogenic for humans (Jacquet et al., 2002).

The inability of the genomic DNA of *L. monocytogenes* isolated from goat meat to produce the expected 800bp and 884bp with the internalin *inlA* and/or *inlB* gene primers respectively remains to be unraveled since they produced the 517bp and/or 238bp with the internalin *inlC* and *inlJ* gene primers and one with both primers. This may just open a new frontier that may be employed in the control of virulent *L. monocytogenes* through genetic engineering with strains having these genes.

Acknowledgement

References

Bergholz, T.M., Bowen,B., Wiedmann,M. and Boor,K.J. 2012. Listeria monocytogenes shows temperature-dependent and –independent responses to salt stress, including responses that induce cross-protection against other sresses. Appl. Environ. Microbiol. 78,2602-2612.

Bhunia A.K. 2008. Foodborne microbial pathogens: mechanisms and pathogenesis. NY: Springer, New York. Pp. 165-182.

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C. and Martin, P. 2004. Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–3822.

Engelbrecht, F., Chun, S.K., Ochs, C., Hess, J., Lottspeich, F., Goebel, W. and Sokolovic, Z. 1996. A new PrfA-regulated gene of Listeria monocytogenes encoding a small, secreted protein which belongs to the family of internalins. Mol. Microbiol. 21, 823–837.

Eruteya, O.C., odunfa, S.A. and Lahor, J. 2014. Listeria spp. in raw cow and goat meat in Port Harcourt, Nigeria. British Biotechnol. J. 4, 204-214.

Gaillard, J.-L.,Berche,P., Frehel, C., Gouin, E. and Cossart,P. 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell. 65, 1127–1141.

Hitchins, A.D., Jinneman, K.C., Yoshitomi, K.J., Blackstone, G.M.K., Thammasouk, K., Johnson, J.M. and Feist, M.D. 2004.

Multiplex real–time PCR to simultaneously detect *Listeria* spp. and *L. monocytogenes* from a variety of food enrichments, abstr. 49. XV International Symposium on Problems of Listeriosis (ISOPOL), Uppsala, Sweden.

Jacquet, C., Gouin, E., Jeannel, D., Cossart, P. and Rocourt, J. 2002. Expression of ActA, Ami, InlB, and listeriolysin O in Listeria monocytogenes of human and food origin. Appl. Environ. Microbiol. 68,616–622.

Jadhav, S., Bhave, M and Palombo, E.A. 2012. Methods used for the detection and subtyping of *Listeria monocytogenes.* J. Microbiol. Methods. 88, 327-341.

Lee, **S.,** Ward, T.J., Graves, L.M., Wolf, L.A., Sperry, K., Siletzky, R.M. and Kathariou, S. 2011. Atypical Listeria monocytogenes serotype 4b strains harboring a Lineage II- Specific gene cassette. Appl. Environ. Microbiol. 78,660-667.

Lee,S., Ward, T.J., Siletzky, R.M. andKathariou, S. 2012. Two Novel Type II Restriction-Modification Systems Occupying Genomically Equivalent Locations on the Chromosomes of *Listeria monocytogenes* Strains. Appl. Environ. Microbiol. 78, 2623-2630.

Lindbäck, T., Secic, I. and Rørvik, L.M. 2011. A Contingency Locus in *prfA* in a *Listeria monocytogenes* Subgroup Allows Reactivation of the PrfA Virulence Regulator during Infection in Mice. Appl. Environ. Microbiol. 77, 3478–3483.

Liu, D. 2004. Listeria monocytogenes: comparative interpretation of mouse virulence assay. FEMS Microbiol. Lett. 233, 159–164.

Liu, D. 2006. Identification, subtyping and virulence determination of Listeria monocytogenes, as an important pathogen. J. Med. Microbiol. 55, 645-659.

Liu, D., Lawrence, L.M., Austin, F.W. and Ainsworth, A.J. 2007. A multiplex PCR for species- and virulence-specific determination of *Listeria monocytogenes*. J. Microbiol. Methods. 71, 133-140.

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., Griffin, P. M. and Tauxe, R. V. 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. 5, 607-625.

Nadon, C. A., Woodward, D.L., Young, C., Rodgers, F.G. and Wiedmann, M. 2001. Correlations between molecular subtyping and serotyping of Listeria monocytogenes. J. Clin. Microbiol. 39, 2704–2707.

Poyart, C., Trieu-Cuot, P.and Berche, P. 1996. The inlA gene required for cell invasion is conserved and specific to Listeria monocytogenes. The Microbiol. 142, 173–180.

Sabet, C., Lecuit, M., Cabnes, D., Cossart, P. and Bierne, H. 2005. LPXTG protein InlJ, a newly identified internalin involved in Listeria monocytogenes virulence. Infect. Immunol. 73, 6912–6922.

Scallen, E., Hoekstra, R.M., Angulo, F.T., Tauxe, R.V., Widdowson, M.A. and Roy, S.L. 2011. Foodborne illness acquired in the United State- major pathogens. Emerg. Infect. Dis. 17, 7-15.

Shuckken, Y.H., Grohn, Y.T. and Wiedmann, M. 2003. Part VI: *Listeria monocytogenes*, epidemiology of listeriosis. In: Torrence, M.E., Isaacson, R.E. (Ed.), Microbial Food Safety in Animal Agriculture: Current Topics. Wiley-Blackwell, Hoboken, pp. 221-232

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehlan, J. and Kreft, J. 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev.14, 584–640.