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Molecular serotype and evolutionary lineage of *Listeria monocytogenes* isolated from different Nigerian food items

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The molecular serotypes and the evolutionary lineage of *Listeria monocytogenes* isolated from various foods in Nigeria are yet to be documented. Consequently, popular uncooked food items known locally as *Okazi Utazi, Onugbu, Ogbono, Garri* and *Egusi* obtained from plants botanically known as *Gnetum africanum, Gongronema latifolium, Vernonia amygdalina, Irvingia gabonensis, Manihot esculanta Crantz* and *Colocynthis Citrullus,* respectively were analyzed. Molecular serotype of three chosen isolates was determined using multiplex polymerase chain reaction (PCR) serotyping before analysis based on *prfA* virulence gene cluster of *L. monocytogenes* was carried out to establish the evolutionary lineage. There was no *L. monocytogenes* detected in foods from *I. gabonensis, M. esculanta Crantz* and *C. citrullus.* However, the vegetables from *G. africanum, G. latifolium* and *V. amygdalina* showed the presence of the organism and chromogenic tests carried out on the three strains chosen from oxford formulation media indicated that they were not other non-pathogenic strains of *Listeria*. The V3 region of 16S rRNA gene of one strain showed that a close relative of the isolate is a strain implicated in an outbreak of listeriosis. Leafy vegetables could be a major vehicle for transmission of *L. monocytogenes* in Nigeria since this pathogenic bacterium occured in different vegetables analyzed.

Key words: *Listeria monocytogenes,* serotype, lineage, pathogenic potential, Nigerian food.

INTRODUCTION

Listeria monocytogenes is a bacterial foodborne pathogen that can cause listeriosis especially among the young, old aged, pregnant and immunocompromised persons (Cartwright et al., 2013). The presence of *L. monocytogenes* in food remains a major challenge because it is psychrotrophic, relatively tolerant of high

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solute concentrations, resists desiccation and therefore can overcome mild food preservation techniques (McLauchlin et al., 2014). Despite the world-wide reports of outbreaks of food-borne listeriosis, the occurrence of *L. monocytogenes* is still not widely reported in Nigeria due to lack of a comprehensive surveillance system for food-borne pathogens (Nwaiwu, 2015).

Common popular Nigerian foods found in South eastern region namely *Okazi* (also called *Afang* in another region)*, Utazi, Onugbu, Ogbono, Garri* and *Egusi* can be sourced from plants with botanical names *Gnetum africanum, Gongronema latifolium, Vernonia amygdalina, Irvingia gabonensis, Manihot esculanta Crantz* and *Colocynthis Citrullus,* respectively*.* Leaves of *G. africanum* are eaten as a vegetable and also used widely as an ingredient in soups and stews and are important for their nutritional and therapeutic properties (Ali et al., 2011)*.* Also popular is *G. latifolium,* a tropical rain forest plant used as a spice and vegetable and generally believed to possess many medicinal properties (Ugochukwu and Babady, 2002). Equally regarded to have medicinal properties are leaves of *V. amygdalina* which has several health benefits and also used for preparing local bitter leaf soup (Farombi and Owoeye, 2011). In Nigeria *I. gabonensis* is known as the wild mango seeds and it is usually used as a conventional soup thickner (Ekundayo et al*.,* 2013). Another popular soup condiment highly rich in protein is *Egusi* made from melon seeds of *C. citrullus* (Bankole et al*.,* 2006). Most soups are normally eaten with *Garri,* a popular food in Nigeria rich in carbohydrate and derived from the fermentation of mash obtained from the enlarged root of the cassava plant *M. esculanta Crantz* (Okafor et al., 1999).

Reports regarding foodborne listeriosis have increased due to the ability of *L. monocytogenes* to survive in harsh conditions (Hernandez-Milian and Payeras-Cifre, 2014). In order to reduce occurrence, Gillespie et al. (2006) suggested that dietary advice on avoiding high-risk foods should be provided routinely for the elderly and immune-compromised, not just to the pregnant women, these groups are associated with 15% out of 3088 cases of listeriosis reported in England and Wales between 1990-2010 (Awofisayo et al., 2015). In a serological study in Nigeria among different animal species (Oni et al., 1989), the prevalence of agglutinins to 5 serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a and 4b) has been reported but molecular serotypes and evolutionary phylogeny of *L. monocytogenes* from Nigerian foods, animal or environment are still unknown. Although serological (agglutinins) typing correlates well with molecular lineage assignments (Nadon et al., 2001), molecular serotyping methods are now acceptable replacements for sero-grouping procedures (Nightingale et al., 2007).

Many different approaches are now used to evaluate occurrence and evolution of *L. monocytogenes* serotypes. Yin et al*.* (2015) performed multilocus sequence typing (MLST) and multi virulence locus sequence typing (MVLST) for 86 *L. monocytogenes* strains derived from 8 countries from 1926 to 2012 in order to understand the molecular evolution and genetic characteristics of the organism. The scientists identified a total of 13 clonal complexes and noted that polymorphism of housekeeping genes of isolates belonging to one of the clones increased rapidly over time. Furthermore, the phylogenetic analysis carried out showed that serotype 1/2b and 4b strains had an interval-type evolution pattern, while serotype 1/2a and 1/2c strains had a progressive-type evolution pattern. In another study, Pászti et al*.* (2014) used the molecular serotyping method of Doumith et al*.* (2004) and pulsed-field gel electrophoresis (PFGE) to serotype and characterize *L. monocytogenes* from human infections and found 2 serogroups and many pulsotypes. Other workers have used ribotyping and automated ribotyping (Matloob and Griffiths, 2014), Fourier transform infrared spectroscopy and chemometrics (Nyarko et al., 2014) and fluorescent amplified fragment length polymorphism (Amar, 2014) to differentiate *L. Monocytogenes* strains.

Ferreira et al. (2014) has highlighted that the current available data clearly indicate that *L. monocytogenes* persistence at various stages of the food chain contributes to contamination of finished products and continued efforts to integrate data on *L. monocytogenes* persistence is still needed to advance our understanding on the persistence of this important pathogenic bacterium. To this direction, the aim of this study was to establish the molecular serotypes and lineages of *L. monocytogenes* that may be found in common food items in south eastern Nigeria.

MATERIALS AND METHODS

Food items and detection of *L. monocytogenes*

Ground food items of *I. gabonensis, M. esculanta Crantz* and *C. citrullus* and sundried vegetables from *G. africanum, G. latifolium* and *V. amygdalina* (Figure 1) were purchased from a market in Owerri City, south eastern Nigeria after which standard presumptive identification as described by HPA (2007) was carried out. In this study, all media were prepared according to manufacturer's instructions and for primary detection, 25 g of each food item were weighed out into a stomacher bag and made up with 225 ml of half Fraser broth (Oxoid, Basingstoke, United Kingdom; CM895 base plus SR166E supplement) and then homogenized and incubated for 24 h at 30°C.

The incubated half Fraser broth was used for the secondary enrichment by inoculating 0.1 ml into 10 ml of Fraser broth (Oxoid, CM895 base plus SR156 supplement) in a universal bottle. Incubation was carried out at 37°C for 48 h after which 0.1 ml of broth was spread-plated onto *Listeria* oxford formulation (Oxoid; CM856 base and SR140 supplement) agar plates. Colonies that showed typical *L. monocytogenes* morphology were sub-cultured on brain heart infusion (BHI) agar (Oxoid, CM1135) to get pure cultures after which standard Gram stain, motility (Vatanyoopaisarn et al*.*, 2000) and catalase tests were carried out. The isolates were stored at -85°C for future use.

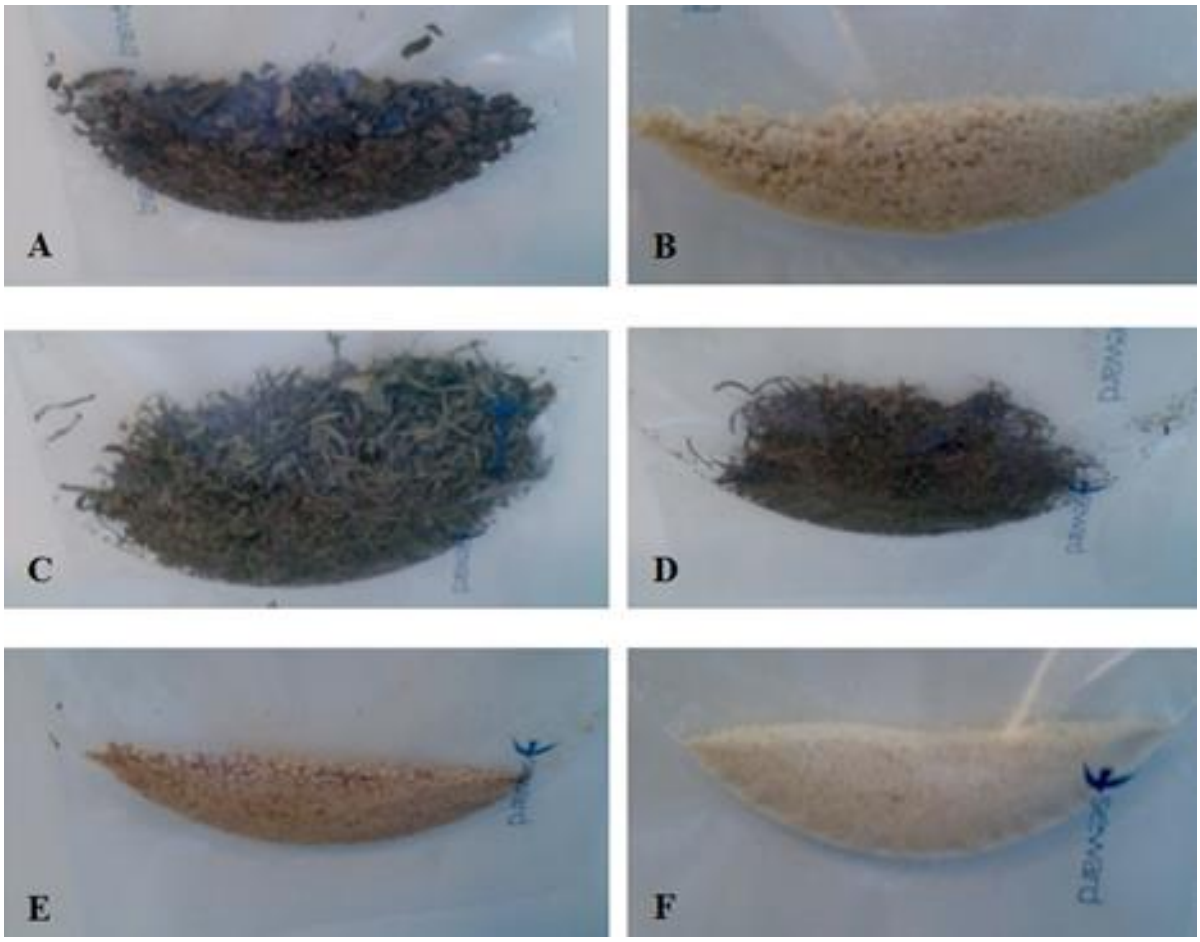

Figure 1. Nigerian food samples in stomacher bags just before homogenization with half Fraser broth. These were *Utazi* from *G. latifolium* (A), *Egusi* from *C. citrullus* (B), *Okazi* from *G. africanum* (C), *Onugbu* from *V. amygdalina* (D), *Ogbono* from *I. gabonensis* (E) and *Garri* from *M. esculanta Crantz* (F).

Separating *L. monocytogenes* from non-pathogenic *Listeria* species

To confirm that selected strains were not non-pathogenic *Listeria*, tests were performed with SwabSURE-ListeriaP kit (TSC, Heywood, United Kingdom). According to the manufacturers, the protocol works by sampling a pre-moistened swab dosed in the kit's TSC neutralising buffer and then followed by incubation for 24-48 h. The appearance of a turquoise blue colour indicates a positive presence for pathogen specific Phospholipase C enzyme. In order to satisfy manufacturer's requirement for tests to be carried out only on surfaces, a test surface to mimic a food contact surface was prepared with a microscope slide as previously described (Mafu et al., 2011) with modification. Instead of cutting glass coupons from the slide, the whole glass microscope slide with plain cut edges measuring 76 × 26 mm (Fisher Scientific) was used. After inoculating a loop of cells from a colony of the test strain to 5 ml of BHI broth in a sterile test tube, the test tube was incubated in a test tube shaker (New Brunswick Scientific) at 30°C for 18 h after which a 100 µl of the broth culture was placed in the middle of the microscope slide and then spread out with an inoculating loop. The slide was air dried for 3 h after which recovery of *L. monocytogenes* from the surface of the slide was carried out according to manufacturer's instructions.

The kit's pre-moistened swab with foam tip was removed from its holding tube making sure that it did not touch any surface before swabbing the surface of the microscope glass slide in 3 planes (horizontal, vertical and diagonal) whilst rotating swab between forefinger and thumb. After swabbing the surface of the slide, the swab was inserted into the media tube placed on a test tube rack and then incubated horizontally at 37°C for 48 h after which the tube was observed for change of colour.

Resuscitation of control strains

Four strains of *L. monocytogenes* with known serotype and lineage were used as control strains for this study, among them two reference strains were included (Table 1). The strains were maintained under cryo preservation at -85°C and these were obtained from the culture collection of Division of Food Science, University of Nottingham, UK. Cryo preservation beads containing the strains were removed from the -85°C freezer and streaked on to BHI media. Colonies that emerged after 48-72 h incubation at 37°C were used. To ensure that there was no contamination during recovery of the strains, the organisms were Gram stained and also grown on *Listeria* selective Oxford medium to confirm typical colonies of *L. monocytogenes*.

Table 1. Control strains representing the major serotypes of *L. monocytogenes* used in this study.

Strain	Serotype	PCR lineage	Source	Reference
Lm 4	1/2b	I	Bovine	Lawrence et al. (1995)
Lm 27	1/2c	II	Food environment	Lawrence et al. (1995)
Lm 10403S*	1/2a	II	Human	Bishop and Hinrichs (1987)
Lm ATCC23074*	4b	I	Human	ATCC catalogue

*****Reference strain.

Extraction of genomic DNA

This was carried out as described by Pitcher et al. (1989). To the sterile test tubes, 5 ml of BHI broth was added and inoculated with a colony of of *L. monocytogenes*. The test tubes were placed in a test tube shaker (New Brunswick Scientific) and incubated at 37°C for 24 h. A slight modification to the protocol was introduced by pelleting 1.5 ml of the incubated culture in an Eppendorf at 13000 xg for 1 min with a microfuge (Biofuge 13; Heraeus Sepatech) instead of centrifuging at 1000 xg for 15 min to obtain a suitable cell pellet. The process was repeated to obtain a bigger pellet following which 1 ml of ice cold lysis buffer (25 mM Tris-HCl 8.0, 10 mM EDTA, 50 mM sucrose, Fisher Scientific, UK) was added. The mixture was then treated with 50 $mgml^{-1}$ lysozyme (Sigma) and incubated for 1 h at 37°C after which 0.5 ml lysis solution (5 M guanidium thiocynate, 0.1 M EDTA, Fisher Scientific; 0.5% Sakosyl, Sigma) was added, mixed well and left at room temperature for 5 min. The lysate was cooled on ice for 30 min and 0.25 ml of ammonium acetate was added, vortexed and incubated again on ice for another 10 min. Chloroform : isoamyl alcohol (24:1 ratio, 0.5ml, Fisher Scientific, UK) was added, vortexed and spun for 10 min at 13000 xg.

The upper phase 850 µl was removed and introduced into a clean Eppendorf tube following which exactly 0.54 volumes of cold isopropanol were added. The mixture was centrifuged at 13000 xg for 20 s and the visible pellet was washed 3 times in 70% ethanol. The pellet was resuspended in 50 µl TE (10mM Tris, 1mM EDTA pH7.0). To remove RNA, 1 µl (50 $mgml^{-1}$) of lyophilized RNAse was added to the suspension and incubated at 37°C for 30 min. The integrity of the DNA was checked by horizontal electrophoresis in 0.7% agarose in TAE buffer (40 mM Tris base, 20 mM glacial acetic acid 1 mM EDTA, pH 8, Fisher Scientific) containing ethidium bromide (0.5 $mgml^{-1}$) and viewed under ultraviolet light (ImageMaster® VDS system (Amersham Biosciences,No.80-6254-80). The concentration (ng/µl) was measured by placing 1 µl of DNA sample onto NanoDrop spectrophotometer (Thermo Fisher Scientific Inc.,USA) after which the DNA was stored at -20°C till its subsequent use in PCR reactions.

Molecular serotyping

Serotyping was carried out using the multiplex PCR protocol described by Doumith et al. (2004) using the primer pairs No. 5-9 (Table 2). A final volume of 50 µl containing 1 U of Taq polymerase (ABgene), 10 ng DNA template, 0.2 mM deoxyribonucleoside triphosphate (Promega), 2 mM $MgCl_2$ (ABgene) and 5 µl of 1X PCR buffer (ABgene) was used. Primers were added at the following concentration: 1 µM for *lmo*0737, *ORF*2819 and *ORF*2110; 1.5 µM for *lmo*1118 and 0.2 µM for *prs.* PCR was performed with an initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 0.40 min, 53°C for 1.15 min and 72°C for 1.15 min and one final cycle of 72°C for 7 min in a thermocycler (Techne 312). Following PCR reaction, the product (5 µl) was mixed with 2 µl of loading buffer and separated on a 2% agarose gel in TAE buffer. Staining with ethidium bromide enabled visualization of PCR fragments under UV light. After scoring the PCR products against a 100bp ladder, serotypes were assigned to the test strains based on genes amplified by the corresponding reference strain.

Evolutionary lineage analysis

Lineage group identification based on *prfA* virulence gene cluster of *L. monocytogenes* developed by Ward et al*.* (2004) was used by amplifying allelic specific oligonucleotides (ASO) using primer pair No. 2-4 (Table 2). Amplifications were performed in 50 µl volumes with 0.5 µM concentrations of each primer, 2 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 0.5 U of Taq polymerase (Abgene) and 100 ng of DNA. Amplifications consisted of 25 cycles of 15 s at 94°C, 10s at 56°C and 10 s at 72°C following which amplification products were resolved in 2% (wt/vol) agarose gel and scored relative to 100 bp DNA size ladder before evolutionary lineages were assigned to the test strains based on genes amplified by the corresponding reference strain.

Sequencing of the V3 region of 16S rDNA

Sequencing for an isolate of interest was carried out by PCR amplification of the V3 region of the 16s rDNA (Muyzer et al., 1993; Coppola et al*.,* 2001) using primer pairs No. 1 (Table 2). A 1 µl of DNA template (100 ng/µl) was used and the reaction mixture (50 µl) for amplification contained 1.25 U of Taq DNA polymerase (Fisher Scientific) 5 µl of 10X PCR buffer (Fisher Scientific,), 0.2 mM of the deoxynucleotide triphosphates, 0.2 pmol/µl of forward and reverse primers and 2.5 mM of magnesium chloride.

DNA was denatured for 5 min at 94°C followed by initial annealing temperature of 66°C, which was decreased 1°C every cycle for 10 cycles after which another 20 cycles were performed at 56°C. Elongation was performed at 72°C for 3 min before a final extension at 72°C for 10 min was carried out. The PCR fragments generated were cut out from the gel and cleaned with a purification kit (Qiagen) and then sequenced (MGW Eurofins, Germany). Sequence generated was used for a search on the databases of National Center for Biotechnology Information (http://ncbi.nlm.nih.gov) using the BLAST program after which the sequence was submitted to EMBL archives and given accession number LN832629.

RESULTS AND DISCUSSION

Detection and identification of *L. monocytogenes*

It is known that the ability of *L monocytogenes* to hydrolyze esculin to esculetin (Qadri et al., 1980) and the

Table 2. Primers pairs used in this study.

Gene	Forward (top line) and reverse primers (5'- 3')	≈PCR product (bp)	Reference
V3 16S rDNA	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTCG	200	Coppola et al. (2001)
*actA*1	AATAACAACAGTGAACAAAGC TATCACGTACCCATTTACC	373	Ward et al. (2004)
*plcB*2	TTGTGATGAATACTTACAAAC TTTGCTACCATGTCTTCC	564	"
*actA*3-f *plcB*3-r	CGGCGAACCATACAACAT TGTGGTAATTTGCTGTCG	277	"
lmo0737	AGGGCTTCAAGGACTTACCC ACGATTTCTGCTTGCCATTC	691	Doumith et al. (2004)
lmo1118	AGGGGTCTTAAATCCTGGAA CGGCTTGTTCGGCATACTTA	906	"
ORF2110	AGTGGACAATTGATTGGTGAA CATCCATCCCTTACTTTGGA	597	"
ORF2819	AGCAAAATGCCAAAACTCGT CATCACTAAAGCCTCCCATTG	471	"
prs	GCTGAAGAGATTGCGAAAGAAG CAAAGAAACCTTGGATTTGCGG	370	"

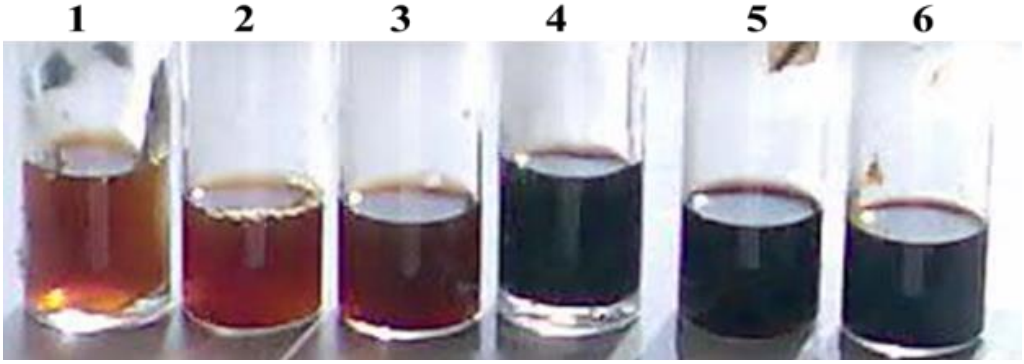

Figure 2. Secondary enrichment with Fraser broth. 1, Ogbono (from *I. gabonensis*); 2, egusi (*C. citrullus*); 3, garri(*M. esculanta Crantz*); 4, utazi (*G. latifolium*); 5, Okazi (*G. africanum*); 6, Onugbu (*V. Amygdalina*).

subsequent reaction of esculetin with ferric ions which results in blackening of media is used for the identification of the organism. After secondary enrichment, there was complete blackening of the Fraser broth for food samples from *G. latifolium, G. africanum, V. Amygdalina* whereas broth samples of food from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* did not blacken (Figure 2).

All broths were subcultured on Oxford agar before discarding, irrespective of colour change. After incubation at 30°C for 48 h colony types that showed black colouration of media were selected and Gram

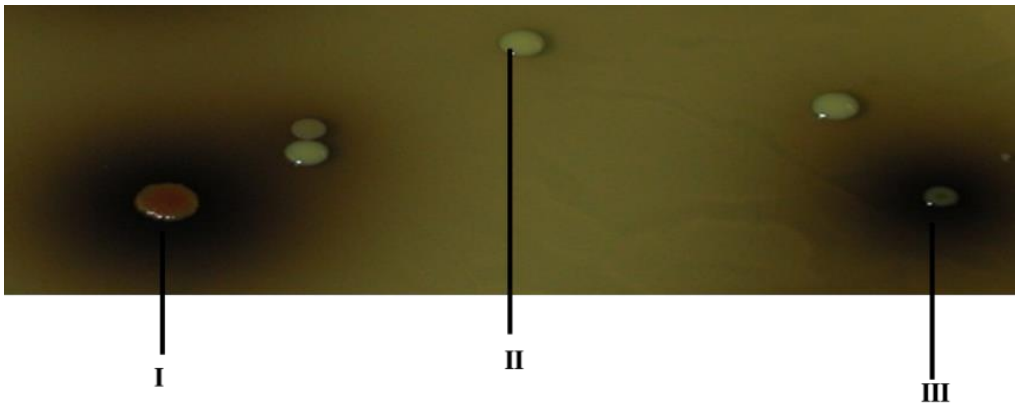

Figure 3. Three colony types isolated from leaves of *G. latifolium* growing on Oxford formulation agar showing aesculin (I and III) and non- aesculin (II) hydrolyzing colonies after 48 h incubation at 37°C.

stained. Colonies (Colony III, Figure 3) consistent with *L. monocytogenes* morphology were found to be Gram positive rods, had a doughnut like appearance on Oxford media and were about 0.5 µm in width and 1-1.5 mm in length whereas staining of aesculin producing colonies with non-conforming *L. monocytogenes* morphology (Colony I, Figure 3) showed Gram positive cocci shaped organisms similar to *Enterococcus* species that are known to be also capable of aesculin hydrolysis (HPA, 2010).

This confirms that relying on black coloration of Oxford media for selection and enumeration purposes as shown in some reports (Nwaiwu, 2015), may result in false positives (Fraser and Sperber, 1988). No isolate fitting *Listeria* morphology was found in food items obtained from *I. gabonensis, M. esculanta Crantz* and *C. Citrullus.* One potential *L. monocytogenes* isolate which was catalase positive and showed tumbling motility at 25°C (Vatanyoopaisarn et al., 2000) was selected for each food sample from the vegetables *G. latifolium, G. africanum, V. amygdalina* and designated LmNG1, LmNG2 and LmNG3, respectively and stored at -85°C for future use. Overall, only these 3 isolates were presumptively identified as *L. monocytogenes* and selected for molecular processing.

Assessing pathogenicity potential of isolated strains

To indicate if isolates are pathogenic *L monocytogenes*, a test was carried out using SwabSure ListeriaP kit, a chromogenic kit described by the manufacturers as being capable of differentiating pathogenic *L monocytogenes* and *L. ivanovii* from other commensal microorganisms including *L. innocua.* Test with the kit after swabbing the surface of a microscope glass slide containing dried cultures produced the turquoise blue colour for positive identification indicating the presence of pathogen specific phospholipase C enzyme (Notermans et al., 1991).

Molecular analysis of selected strains

According to Gasanov et al. (2005) early typing methods differentiated isolates based on phenotypic markers (motility, hemolysis, sugar fermentation) but these phenotypic typing methods are being replaced by molecular tests, which reflect genetic relationships between isolates and are more accurate. Further confirmation and determination of the serotypes and lineage of the isolated strains were ascertained by PCR multiplex serotyping and lineage analysis. These methods are still widely used (Warren et al., 2015) by other investigators. Strains representing the four major serotypes 1/2a, 1/2b, 1/2c and 4b of *L. monocytogenes* (Liu and Busse, 2009) and strains representing Lineage I and II were used as control (Table 1).

Genomic DNA template which had approximately 2.0 (260/280 nm) absorption ratio after Nano-Drop nucleic acid measurements was used for all PCR reactions. The result of the multiplex PCR serotyping carried out is shown in Figure 4. The control and test strains amplified *prs* gene (approximately 370 bp). In addition to *prs*, the control strain that is 4b amplified *ORF*2110 and *ORF*2819 while strains that were serotype 1/2a amplified *lmo* 0733. The strain that is 1/2c also amplified *lmo* 0733 in addition to *lmo*1118 and strains of 1/2b serotype amplified *ORF*2819. A summary table of the result gained is in Table 3. When all the amplification patterns were considered using the grouping scheme shown by Doumith et al. (2004), it was found that *L. monocytogenes* isolated from food item of *G. latifolium* and that of *G. africanum* were Group 3 (1/2b, 3b,7) whereas the strain from *V. amygdalina* food sample was grouped into Group 1 (1/2a, 3a). There was no prevalence found in groups 2 (1/2c, 3c) or 4 (4b, 4d and 4e). The amplification patterns corresponded to the control strains Lm4 (1/2b) and Lm10403S (1/2a) which indicated that the new strains LmNG1 and LmNG2 are1/2b serotype whereas LmNG3 is serotype 1/2a.

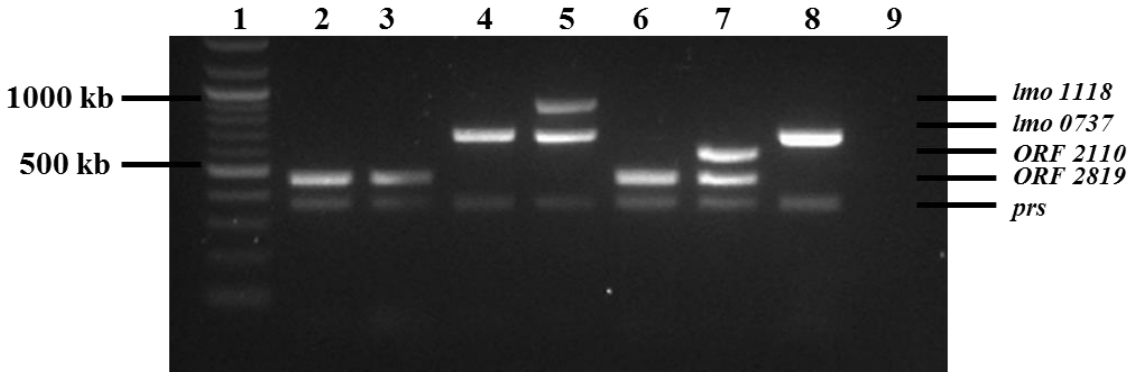

Figure 4. Multiplex PCR serotyping by amplification of different *L. monocytogenes* genes. The PCR products were separated on 2.0% agarose gel and scored against a 100 bp ladder, Lane 1 = 100 kb ladder; lane 2 = LmNG1; lane 3 = LmNG2; lane 4 = LmNG3; lane 5 = LM27; lane 6 = Lm4; lane 7 = LmATTC23074; lane 8 = Lm10403S; lane 9 = negative control.

Table 3. Summary of serotype assignment based on multiplex PCR serotyping of *L. monocytogenes* strains isolated from Nigerian vegetables. Serotypes were assigned to the strains based on the pattern of genes amplified. Strains of known serotypes were used as control.

Strain	Multiplex PCR fragment amplification					Sero group classification	Source
	***lmo*1118 (906 bp)**	***lmo*0737 (691 bp)**	***ORF*2110 (597 bp)**	***ORF*2819 (471 bp)**	***Prs* (370 bp)**		
ATCC23074	-	-	+	+	+	4b	UoN
Lm4	-	-	-	+	+	1/2b	UoN
Lm27	+	+	-	-	+	1/2c	UoN
Lm10403S	-	+	-	-	+	1/2a	UoN
LmNG1	-	-	-	+	+	1/2b	*G. latifolium*
LmNG2	-	-	-	+	+	1/2b	*G. africanum*
LmNG3	-	+	-	-	+	1/2a	*V. amygdalina*

+ = Amplified; -, not amplified; UoN = University of Nottingham.

The lineage classification performed showed that strains that are serotype 1/2b and 4b amplified fragments of *actA1* **(**373bp) expected for lineage I, while strains that are serotypes 1/2a and 1/2c amplified fragments of *plcB2* (564bp) expected for lineage II (Figure 5). The lineage classification of all the strains under study is shown in Table 4. There was no amplification of lineage III specific sequence showing that no lineage III strain was found in this study. Isolates LmNG1 and LmNG2 were found to be lineage 1 whereas LmNG3 was found to be lineage II. Isolates LmNG1 and LmNG2 had the same amplification with control strain Lm4 (1/2b) whereas strain LmNG3 had the same lineage with reference strain Lm10403S (1/2a).

Over 90% of the human listeriosis cases are caused by *L. monocytogenes* serotypes 1/2a,

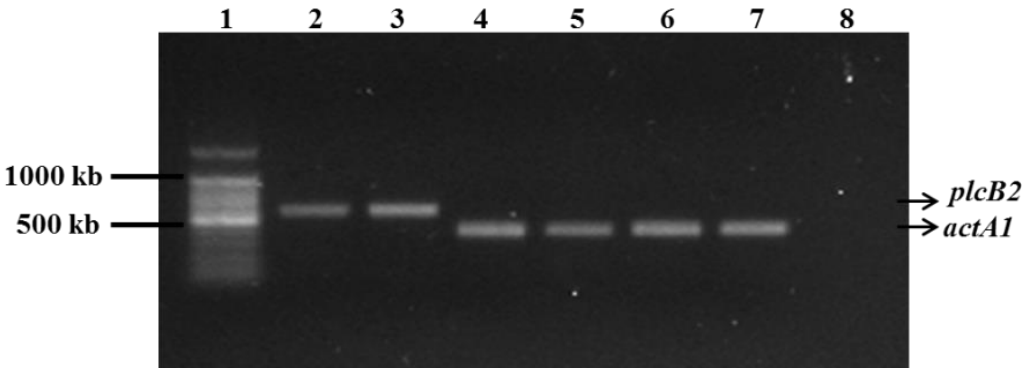

Figure 5. Allelic specific oligonucleotide amplification to determine evolutionary lineage of new isolates from Nigerian food items. PCR products were separated on 2.0% agarose gel. Lane 1= 100 kb ladder; lane 2 = LmNG3, lane 3 = Lm10403S; lane 4 = LmNG2; lane 5 = LmNG1; lane 6 = Lm4; Lane 7 = Lm23074; lane 8 = negative control.

Table 4. Lineage classification of *Listeria monocytogenes* strains.

Strain	Serotype	ASO-PCR amplification			Lineage classification	Source
		actA1	*plcB2*	*actA3*		
ATCC23074	4b	+	-	-	I	UoN
Lm27	1/2c	-	+	-	II	UoN
Lm4	1/2b	+	-	-	I	UoN
Lm10403S	1/2a	-	+	-	II	UoN
LmNG1	Unknown	+	-	-	I	*G. latifolium*
LmNG2	Unknown	+	-	-	I	*G. africanum*
LmNG3	Unknown	-	+	-	II	*V. amygdalina*

1/2b and 4b strains (Laksanalamai et al., 2014) and all the serotypes are spread over at least 4 (I-IV) evolutionary lineages (Combrouse et al., 2013). Serotype 1/2a is common in the environment while serotype 1/2b and 4b are isolated from many clinical samples. Previous reports show that isolates from human listeriosis cases are common in lineage I whereas lineage II strains are prevalent in food isolates and lineage III and IV rarely occurs (Orsi et al., 2011). The occurrence of Lineage II isolate (serotype 1/2a) in sample from *V. amygdalina* is in line with predominance of serotype 1/2a in food and environmental samples but the strains recovered in samples from *G. latifolium* and *G. africanum* were least expected because they have the serotype and lineage associated more with clinical samples. However, recovery of Lineage 1 serotypes (mostly associated with clinical samples) from food is no longer unusual because the serotype 1/2b belonging to lineage 1 has now been isolated from other vegetables (Cordano and Jacquet, 2009; Sant'Ana et al., 2012).

The isolation of *L. monocytogenes* from food product of *G. latifolium* used to prepare local salads was least expected given the anecdotal antimicrobial status of *G. latifolium* in south eastern Nigeria. Hence, the serotype 1/2b isolate from *G. latifolium* was analyzed to determine its phylogeny. After using the BLAST program and comparing sequences producing significant alignment, *L. monocytogenes* strain N2306 (Accession CP011004) involved in an outbreak of listeriosis in Switzerland was the top hit among 100 strains with 99% match (denotes percent identity of sequence query alignment) and the same Expect value (E-value). The full genome sequence of strain N2306 is on the NCBI data base hence a future study to carry out genome sequencing of the new isolate (Accession LN832629) for comparison would be beneficial.

Yin et al. (2015) found that *L. monocytogenes* strains from temporally and geographically unrelated outbreaks in different countries were clustered in the same subgroup of phylogenetic tree, and suggested that they have similar virulence genes and genetic characteristics due to adaptation. Therefore, the food isolate from *G. latifolium* might be a potential causative agent for an outbreak because the closest relative of the isolate in a geographically unrelated region has been implicated in an outbreak of listeriosis. Also, the isolate is serotype 1/2b and the ability of this serotype to cause outbreaks has been reported, while this was listed among serotypes

that caused 24 listeriosis outbreaks in the United States for the period 1998-2008 (Cartwright et al., 2013).

Presence of *L. monocytogenes* in only sundried vegetables from *G. latifolium, G. africanum, V. amygdalina* might be due to the fact that sundried leafy vegetables were not ready to use when they were purchased and may still have environmental and other post-harvest debris present which were not readily visible. Also, the vegetables still needed a final wash before it could be cooked for food. Washing reduces the soil present in fresh food and may lower the level of the organisms present. Furthermore, the prevalence of *L. monocytogenes* might come from post-harvest contamination and moreover, most vegetables sold in Nigeria are not pre-packed under controlled atmosphere.

Fresh vegetables are increasingly recognized as a source of food-borne outbreaks in many parts of the world (Tromp et al., 2010) and prevalence of *L. monocytogenes* has been severally reported in vegetables. Pre-harvest prevalence of pathogenic *L. monocytogenes* in selected vegetables from agricultural farm samples has been reported in a previous study (Soni et al., 2014) and many workers have found the post-harvest occurrence of *L. monocytogenes* in vegetables. Little et al. (2007) found that 4.8%, out of 2686 samples of pre-packed mixed vegetable salads in UK were contaminated with *L. monocytogenes* and Aparecida de Oliveira et al. (2010) used immunoassay *Listeria* rapid test and 16s rRNAreal-time PCR assay to detect *L. monocytogenes* in minimally processed leafy vegetables.

Conclusions

The molecular analysis of the three strains isolated from different popular vegetables from Nigeria indicates that the major *Listeria* serotypes and evolutionary lineages associated with listeriosis could occur in popular Nigerian leafy vegetables. To the best of the knowledge of the author, this is the first time that the major *L. monocytogenes* molecular serotypes have been linked with popular Nigerian vegetables. The harsh process of obtaining food products from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* and the intrinsic and extrinsic factors in play from farm to fork appears to be unfavourable to *L. monocytogenes* denoting that occurence of the organism is unlikely if good food processing and preservation practices are applied and maintained after harvest.

Popular Nigerian leaf vegetables from *I. gabonensis, M. esculanta Crantz* and *C. citrullus* could be a major vehicle for transmission of the pathogenic organism in Nigeria based on the study. Therefore, further genomic investigations and increased surveilance would be a major concern due to the finding that the closest relative of the sequenced isolate has been implicated in an outbreak of listeriosis.

Conflict of interest

The author declares that there is no conflict of interest.

REFERENCES

Ali F, Assanta MA, Robert C (2011). *Gnetum africanum*: a wild food plant from the African forest with many nutritional and medicinal properties. J. Med. Food 14(11):1289-1297.

Amar C (2014). Fluorescent amplified fragment length polymorphism (fAFLP) analysis of *Listeria monocytogenes*. Meth. Mol. Biol. 1157(2):95-101.

Aparecida de Oliveira M, Abeid Ribeiro EG, Morato Bergamini AM, Pereira de Martinis EC (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. Food Microbiol. 27(1):19-23.

Awofisayo A, Amar C, Ruggles R, Elson R, Adak GK, Mook P, Grant KA (2015). Pregnancy-associated listeriosis in England and Wales. Epidemiol. Infect. 143(2):249-256.

Bankole SA, Ogunsanwo BM, Osho A, Adewuyi GO (2006). Fungal contamination and aflatoxin B-1 of 'egusi' melon seeds in Nigeria. Food Control 17(10):814-818.

Bishop DK, Hinrichs DJ (1987). Adoptive transfer of immunity to *Listeria monocytogenes*: The influence of in vitro stimulation on lymphocyte subset requirements. J. Immunol. 139(6):2005-2009.

Cartwright EJ, Jackson KA, Johnson SD, Graves LM, Silk BJ, Mahon BE (2013). Listeriosis outbreaks and associated food vehicles, United States, 1998-2008. Emerg. Infect. Dis. 19(1):1-9.

Combrouse T, Sadovskaya I, Faille C, Kol O, Guérardel Y, Midelet-Bourdin G (2013). Quantification of the extracellular matrix of the *Listeria monocytogenes* biofilms of different phylogenic lineages with optimization of culture conditions. J. Appl. Microbiol. 114(4):1120-1131.

Coppola S, Blaiotta G, Ercolini D, Moschetti G (2001). Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese. J. Appl. Microbiol. 90(3):414-420.

Cordano AM, Jacquet C (2009). *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: Prevalence and strain characterization. Int. J. Food Microbiol. 132(2-3):176-179.

Doumith M, Buchrieser C, Glaser, P, Jacquet CP, Martin P (2004). Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. 42(8):3819-3822.

Ekundayo FO, Oladipupo OA, Ekundayo EA (2013). Studies on the effects of microbial fermentation on bush mango (*Irvingia gabonensis*) seed cotyledons. Afr. J. Microbiol. Res. 7(34):4363-4367.

Farombi O, Owoeye O (2011). Antioxidative and chemopreventive properties of *Vernonia amygdalina* and *Garcinia biflavonoid.* Int. J. Environ. Res. Public Health 8(6):2533-2555.

Ferreira V, Wiedmann M, Teixeira P, Stasiewicz MJ (2014). *Listeria monocytogenes* persistence in food-associated environments: Epidemiology, strain characteristics, and implications for public health. J. Food. Prot. 77(1):150-170.

Fraser JA, Sperber WH (1988). Rapid detection of *Listeria* spp. in food and environmental samples by esculin hydrolysis. J. Food Prot. 51(10):762-765.

Gasanov U, Hughes D, Hansbro PM (2005). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. FEMS Microbiol. Rev. 29(5):851-875.

Gillespie IA, McLauchlin J, Grant KA, Little CL, Mithani V, Penman C, Lane C, Regan M (2006). Changing pattern of human listeriosis, England and Wales, 2001-2004. Emerg. Infect. Dis. 12(9):1361-1366.

Hernandez-Milian A, Payeras-Cifre A (2014). What is new in listeriosis? Biomed. Res. Int. Vol. 2014, Article ID 358051, 7 pages.

HPA (2007). Health Protection Agency, UK. Identification of *Listeria* species and other non-sporing gram-positive rods (except *corynebacterium*) Issue no: 2 Standards Unit, Evaluations and Standards Laboratory. Reference no: BSOP ID 3i2. Available at: http://www.hpa-standardmethods.org.uk/pdf_sops.asp.

HPA (2010). Health Protection Agency, UK. Aesculin hydrolysis test. National Standard Method BSOP TP Issue 2 Available at: http://www.hpastandardmethods.org.uk/pdf_sops.asp.

Laksanalamai P, Huang B, Sabo J, Burall LS, Zhao S, Bates J, Datta AR (2014). Genomic characterization of novel *Listeria monocytogenes* serotype 4b variant strains. PLoS ONE 9(2):e89024.

Lawrence LH, Hill HA, Linton M (1995) DNA sequence divergence within the iap gene of *Listeria monocytogenes* as a means of characterizing strains. In: XII International symposium on problems of listeriosis, Perth, Australia.

Little CL, Taylor FC, Sagoo SK, Gillespie IA, Grant K, McLauchlin J (2007). Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. Food Microbiol. 24(7-8):711-717.

Liu D, Busse H-J (2009). *Listeria* In: Liu D, (Ed) Molecular detection of food-borne pathogens. CRC Press, United Kingdom. pp. 207-220.

Mafu AA, Plumety C, Deschênes L, Goulet J (2011). Adhesion of pathogenic bacteria to food contact surfaces: Influence of pH of culture. Int. J. Microbiol. 2011(2011):972494.

Matloob M, Griffiths M (2014). Ribotyping and automated ribotyping of *Listeria monocytogenes.* Meth. Mol. Biol. 1157:85-93.

Mclauchlin JR, Rees CED, Dodd CER (2014). *Listeria monocytogenes* and the genus *Listeria.* In: Rosenberg E, Delong EF, Lory S, Stackebrandt E, Thompson F (Eds.), The Prokaryotes: Firmicutes and Tenericutes, 4th edition, Springer-Verlag, Berlin. pp. 241-259.

Muyzer G, De Waal EC, Uitterlinden AG (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59(3):695-700.

Nadon CA, Woodward DL, Young C, Rodgers FG, Wiedmann M (2001). Correlations between molecular subtyping and serotyping of *Listeria monocytogenes*. J. Clin. Microbiol. 39(7):2704-2707.

Nightingale K, Bovell L, Grajczyk A, Wiedmann M (2007). Combined *sigB* allelic typing provide improved discriminatory power and reliability for *Listeria monocytogenes* molecular serotyping. J. Microbiol. Meth. 68(1):52-59.

Notermans SH, Dufrenne J, Leimeister-Wächter M, Domann E, Chakraborty T (1991). Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and non-pathogenic *Listeria* species. Appl. Environ. Microbiol. 57(9):2666-2670.

Nwaiwu O (2015). An overview of *Listeria* species in Nigeria. Int. Food Res. J. 22(2):455-464.

Nyarko EB, Puzey KA, Donnelly CW (2014). Rapid differentiation of *Listeria monocytogenes* epidemic clones III and IV and their intact compared with heat-killed populations using Fourier transform infrared spectroscopy and chemometrics. J. Food Sci. 79(6):M1189-1196.

Okafor N, Azubike C, Ibenegbu C (1999). Carriers for starter cultures for the production of garri, a fermented food derived from cassava. World J. Microbiol. Biotechnol. 15(2):231-234.

Oni OO, Adesiyun AA, Adekeye JO, Sai'du SN (1989). Sero-prevalence of agglutinins to Listeria monocytogenes in Nigerian domestic animals. Revue Élev. Méd. Vét. Pays Trop. 42(3):383-388.

Orsi RH, Den Bakker HC, Wiedmann M (2011). *Listeria monocytogenes* lineages: Genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301(2):79-96.

Pászti J, Király J, Füzi M (2014). Serogrouping and pulsed-field gel electrophoresis analysis of *Listeria monocytogenes* isolates from cases of human infection in Hungary 2004-2012 molecular typing of *Listeria monocytogenes* in Hungary. Acta Microbiol. Immunol. Hung. 61(1):71-78.

Pitcher DG, Saunders NA, Owen RJ (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. 8(4):151-156.

Qadri SM, DeSilva MI, Zubairi S (1980). Rapid test for determination of esculin hydrolysis J. Clin. Microbiol. 12(3):472-474.

Sant'Ana AS, Igarashi MC, Landgraf M, Destro MT, Franco BD (2012). Prevalence, populations and pheno- and genotypic characteristics of *Listeria monocytogenes* isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. Int. J. Food Microbiol. 155(1-2):1-9.

Soni DK, Singh M, Singh DV, Dubey SK (2014). Virulence and genotypic characterization of *Listeria monocytogenes* isolated from vegetable and soil samples. BMC Microbiol. 14:241.

Tromp SO, Rijgersberg H, Franz E (2010). Quantitative microbial risk assessment for *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in leafy green vegetables consumed at salad bars. J. Food. Prot. 73(10):1830-1840.

Ugochukwu NH, Babady NE (2002). Antioxidant effects of *Gongronema latifolium* in hepatocytes of rat models of non-insulin dependent diabetes mellitus. Fitoterapia 73(7-8):612-618.

Vatanyoopaisarn S, Nazli A, Dodd CE, Rees CE, Waites WM (2000). Effect of flagella on initial attachment of *Listeria monocytogenes* to stainless steel. Appl. Environ. Microbiol. 66(2):860-863.

Ward TJ, Gorski L, Borucki MK, Mandrel RE, Hutchins J, Pupedis K (2004). Intraspecific phylogeny and lineage group identification based on *prfA* virulence gene cluster of *Listeria monocytogenes*. J. Bacteriol. 186(15):4994-5002.

Warren J, Owen AR, Glanvill A, Francis A, Maboni G, Nova RJ, Wapenaar W, Rees C, Tötemeyer S (2015). A new bovine conjunctiva model shows that *Listeria monocytogenes* invasion is associated with lysozyme resistance. Vet. Microbiol. 179(1-2):76-81.

Yin Y, Tan W, Wang G, Kong S, Zhou X, Zhao D, Jia Y, Pan Z, Jiao X (2015). Geographical and longitudinal analysis of *Listeria monocytogenes* genetic diversity reveals its correlation with virulence and unique evolution. Microbiol. Res. 175:84-92.