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Isolation and Characterization of Unidentified *Listeria* Strains Showing Phenotypic and Genetic Similarities to a Novel Group of *Listeria* Species from Malaysian Pekin Ducks

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Abstract: A total of Five Listeria-like strains were isolated from ducks and the environment in Penang, Malaysia between 2009 and 2010. These strains were small Gram-positive rods, utilize dextrose, esculin and maltose and fitted well into the description of *Listeria* spp. 16S ribosomal RNA sequence analysis confirmed a close phylogenetic similarity (99 bootstrap value) to *Listeria* sp. 102, *Listeria fleischmannii* LU2006-1 and *Listeriaceaec bacterium* TTU M1-001 and a more distant relationship to other *Listeria* spp. The five Listeria-like strains also harboured the virulence-associated gene, *hlyA* (haemolysin gene). Plasmid DNA band sizes ranged from 2.1-25 kb and were detected in all isolates. All the isolates were susceptible to ampicillin and nitrofurantoin, but resistant to cefotaxime nalidixic acid and tetracycline. Genotyping by amplified polymorphic deoxyribonucleic acid (RAPD), Enterobacterial Repetitive Intergenic Consensus (ERIC) and repetitive extragenic palindromic (REP) grouped the Listeria-like strains into four similar types at a coefficient of 0.63. This study draws attention to the emerging importance of foodborne pathogens and the need for monitoring changes in antibiotic resistance patterns so that efficient risk and control management strategies can be developed.

Key words: Antibiotic resistance, ducks, haemolysin gene, listeria-like strains, plasmid

INTRODUCTION

In recent times, novel *Listeria* spp. such as *Listeria weihenstephanensis* sp. nov. and *Listeria fleischmannii* sp. nov. have been reported (Halter *et al*., 2012; Bertsch *et al*., 2012). Of all the *Listeria* spp., *Listeria monocytogenes* is the most important one considered in terms of food safety and human health (Adzitey and Huda, 2010). It is responsible for rare foodborne infection howbeit very high mortality (Schlech, 2000). Food safety issues also continue to be an increasingly public health concern worldwide.

Listeria spp. have been isolated from a variety of sources including the intestinal tract of animals, decomposing plant matter, soil, effluents, plants, faeces of animals and humans, processed foods and the processing environments and many more (Hitchins, 2003; Awaisheh, 2009; Eduok *et al*., 2010; Saikia and Joshi, 2010; Adetunji and Arigbede, 2011; Adetunji and Isola, 2011; Alsheikh *et al*., 2012; Nwachukwu and Madubuko, 2013). Studies on the examination of ducks and duck related samples for *Listeria* spp. are limited. However, ducks, their rearing and/or processing environments in Bulgaria and Malaysia have been demonstrated to be contaminated by *Listeria* spp. (Chipilev *et al*., 2010; Adzitey *et al*., 2011, 2013).

Pekin duck production is very important in Malaysia. This is because Malaysia is the third world producer of duck meat (FAO, 2009; Adzitey and Adzitey, 2011). Thus, pekin duck production serves as source of employment and income for some Malaysians and as a source of foreign exchange earner for the Malaysian Government through export of live ducks or processed duck meats (Adzitey *et al*., 2012a, b). The significance of ducks in Malaysia warrants the need to examined ducks and their environments for foodborne pathogens. This study was carried out to isolate and to characterise five Listeria-like strains obtained from pekin ducks and the environment in Penang, Malaysia.

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MATERIALS AND METHODS

Sampling and bacteriological analysis: A total of 531 ducks and their environment samples collected from duck farms and a wet market in Penang, Malaysia were examined between August 2009 and October 2010. Collected samples were analysed for the unidentified *Listeria* strains according to the procedures in FDA-BAM by Hitchins (2003). Briefly, all samples were enriched in Listeria Enrichment Broth and Fraser Broth. Samples in Listeria Enrichment Broth were incubated at 30°C for 24-48 h. Samples in Fraser Broth were first incubated at 30°C for 24 h in half Fraser Broth (containing supplements) and later incubated in Fraser Broth (without supplements) at 30°C for an additional 24 h. After enrichment, 10 μL volumes were streaked onto ALOA and PALCAM agar plates and incubated under aerobic condition at 30°C for 24 to 48 h. Phenotypic characterization of the presumptive *Listeria* spp. was achieved using Microgen™ *Listeria*-ID Identification System (Microgen, UK) and analysed using the software Microgen ID, Version 1.2.5.26 (Microgen, UK).

Antibiotic test: Antibiotic test was done using the disk diffusion method (Bauer *et al*., 1996) and the results were interpreted according to the CLSI guidelines (CLSI, 2006). The isolates were examined against 15 antibiotics. Pure cultures were grown overnight in Trypticase-Soy Broth supplemented with 0.6% yeast extract (TSB, Merck, Germany) at 37°C and the concentration was adjusted to 0.5 McFarland turbidity. One hundred μL of the suspension was spread plated onto Mueller Hinton agar (MHA, Oxoid, UK) supplemented with Defibrinated Horse Blood (Oxoid, UK) using a cotton swab. Three or two antimicrobial disks were placed on the surface of MHA and the plates were incubated at 37°C for 48 h.

Plasmid DNA extraction: A pure colony of the unidentified *Listeria* strain was inoculated into 5 mL TSB and incubated with vigorous shaking at 37°C for 16 to 18 h. After wards, 3 mL of the culture with cell density adjusted between 1.6 and 1.9 at 600 nm was centrifuged for 5 min at 1000 x g. The pellets obtained were dried and subjected to plasmid DNA extraction/purification using Promega Wizard® plus Minipreps DNA Purification System (Madison, USA) by following the manufacturer's instructions available at http://www.promega.com /tbs/tb225/tb225. Extracted plasmids (10 μL) were stained with EZ-Vision® One DNA Dye (2 μL), loaded on a 0.7% agarose gel and electrophoresed at 90 V for 1h 30 min. Plasmid DNA bands were visualized using UV transilluminator (Bio-Rad Gel Imaging System, Bio-Rad, USA). Lambda DNA/HindIII marker was used as the molecular weight marker and plasmid DNA size was determined using the detect button of the NTSYSpc Version 2.2 computer software.

DNA extraction kit: A pure colony of the unidentified *Listeria* strain was inoculated into 10 mL TSB and incubated at a temperature of 37°C overnight. One mL of the overnight culture was centrifuged for 2 min at 14,000×g. Pelleted bacterial cells were resuspended thoroughly in 480 μL of 50 mM EDTA, added with 120 μL 10 mg mL^{-1} lysozyme and incubated at 37°C for 30-60 min before being subjected to DNA extraction using Wizard® Genomic DNA Purification Kit (Promega, Madison, USA) by following the manufacturer's instructions available at http://www.promega.com/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol/. Extracted DNA was adjusted to 100 ng μL^{-1} before being used.

Detection of iap gene, hylA gene and preparation of Listeria strains for sequencing: The method of (Bubert *et al*., 1992; Chen and Knabel, 2007) was used to confirm the genus, *Listeria* spp. while the method of (Jallewar *et al*., 2007; Paziak-Domanska *et al*., 1999) was used to identify the presence of haemolysin gene. *Listeria monocytogenes* 62313 was used as positive control. The method of (Inglis and Cohen, 2004) was adapted to amplify the 16S rRNA sequences of the unidentified *Listeria* strains. The primers, PCR preparations and conditions used are presented in Table 1. Sequencing of the unidentified *Listeria* strains was done using Eurofins MWG Operon sequencing service, Germany (<www.eurofinsdna.com>). Sequenced data were queried against NCBI genetic database available at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>. Amplicons (10 μL) were stained with EZ-Vision® One DNA Dye (2 μL), loaded on a 2% agarose gel and electrophoresed at 90 V for 1h 30 min. HyperLadder Bioline I or 100 bp DNA ladder was used as the molecular weight marker and the amplicons were visualized under UV transilluminator gel imaging system (Bio-Rad Gel Imaging System, Bio-Rad, USA).

RAPD, ERIC and REP analyses: The C-06 (10-mer) primer 5'-GAACGGACTC-3' was selected for RAPD after a panel of 20 random primers (designed and manufactured by 1st BASE) had been screened. ERIC and REP were done

Table 1: Primer sequences, PCR preparations and conditions used in this study

Target	Primer sequence	PCR preparation (25 μL)	PCR conditions	Reference (s)
Listeria spp.	F-ATGAATATGAAAA AAGCAACR-TTATACG CGACCGAAGCCAAC	12.5 μL GoTaq mastermix, 1μL 25 mM $MgCl_2$, 7.5 μL nuclease free water, 2 μL template DNA and 1 μL of each primer mix	5 min at 95°C prior to 15 cycles of 1 min at 94°C, 1 min with a touchdown from 55°C to 51°C (three cycles per temperature) and 1 min at 72°C, followed by 15 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C and one final cycle of 8 min at 72°C	Bubert *et al.* (1992) Chen and Knabel (2007)
Haemolysin gene	F-GCAGTTGCAAGC GCTTGGAGTGAA R-GCAACGTATC CTCCAGA GTGATCG	12.5 μL GoTaq mastermix, 2 μL 25 mM $MgCl_2$, 3.5 μL nuclease free water, 2 μL template DNA and 2.5 μL of each primer	2 min at 95°C, followed by 35 cycles of 15 s at 95°C, 30 s at 60°C and 1.30 min for 72°C and a final cycle of 10 min at 72°C	Jallewar *et al.* (2007) Paziak-Domanska*etal.* (1999)
Listeria spp. 16S rRNA	F-AGAGTTTGATCCT GGCTCAG R-TACGG (C/T)TACCTTGTTA CGACT	12.5 μL GoTaq mastermix, 2.5 μL 25 mM $MgCl_2$, 3 μL nuclease free water, 5 μL template DNA and 2 μL of each primer	95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 58°C for 1 min and 72°C for 2 min; terminating at 72°C for 10 min	Inglis and Cohen (2004)
RAPD	GAACGGACTC	12.5 μL GoTaq mastermix, 6.25 μL nuclease free water, 2.5 μL 25mM $MgCl_2$, 2.5 μL template DNA and 1.25 μL primer	95°C for 2 min, followed by 35 cycles at 95°C for 30 s, at 45°C for 30 s and at 72°C for 1 min and a final temperature of 72°C for 7 min	Adzitey *et al.* (2013)
ERIC	F-ATGTAAGCTCCTG GGGATTCAC R-AAG TAAGTGACTGGGGT GAGCG	12.5 μL GoTaq mastermix, 6.25 μL nuclease free water, 2.5 μL 25 mM $MgCl_2$, 2.5 μL template DNA and 0.5 μL of each primer	95 for 2min, followed by 30 cycles at 90°C for 30 s, 50°C for 30 s, 52°C for 1 min and 72°C for 1 min and a final temperature of 72°C for 8 min	Jersek *et al.* (1999)
REP	GCGCCGICATGCG GCATT	12.5 μL GoTaq mastermix, 5.5 μL nuclease free water, 2 μL 25mM MgCl2, 2.5 μL template DNA and 2.5 μL primer	2 cycles at 94°C for 5 min, 33°C for 5 min and 68°C for 5 min, followed by 30 cycles at 94°C for 1 min, 45°C for 1 min and 68°C for 2 min and a final cycle at 68°C for 16 min	Tiong *et al.* (2010)

according to Jersek *et al.* (1999) and Tiong *et al.* (2010), respectively with slight modification. The PCR preparations, conditions and primers used are presented in Table 1. Amplicons (10 μL) were stained with EZ-Vision® One DNA Dye (2 μL), loaded on a 1.5% agarose gel and electrophoresed at 90 V for 1h 30 min. VC 1 kb and 100 bp DNA ladders were used as the molecular weight marker and the amplicons were visualized under UV transilluminator gel imaging system (Bio-Rad Gel Imaging System, Bio-Rad, USA).

DNA fingerprints positions were defined as presence of DNA band (a score '1') and absence of DNA band (a score '0'). The scores were entered into NTedit and then inserted into NTSYSpc Version 2.2 computer software for the construction of dendogram based on simple matching coefficient and UPGMA (unweighted pair-group arithmetic average clustering).

RESULTS AND DISCUSSION

Of the 531 duck and their environmental samples examined, five (0.94%) were positive for the unidentified *Listeria* strains. These strains were isolated from duck faeces (3 isolates, L15F, L18F and L22F), duck cloaca (1 isolate, LIC) and pond water (1 isolate, L1P). The strains were Gram positive, catalase positive and rod shaped bacteria. They utilize esculin, trehalose, methyl-d-glucoside, ribose and rhamnose. However, they do not utilize arabitol, mannitol, tagatose, methyl-d-mannoside, glu-1-phos and xylose. The iap gene (Fig. 1a) and the virulence-associated gene h*ylA* (Fig. 1b) were detected in all the five isolates. 16S ribosomal RNA sequence analysis confirmed a close phylogenetic similarity to *Listeria* sp. 102, *Listeria fleischmannii* LU2006-1 and *Listeriaceaec bacterium* TTU M1-001 and a distant relationship to *Listeria grayi* strain ATCC 25400, *Listeria seeligeri* strain ATCC 35967, *Listeria monocytogenes* strain R1653, *Listeria innocua* strain ATCC 33090, *Listeria marthii* strain NR-9579, *Listeria ivanovii* strain CLIP 12510, *Listeria welhenstephanensis* sp. nov isolate WS 4560 and *Listeria rocourtiae* strain CIP109804 (Fig. 2). The isolation of strains of *Listeria* not showing 16S rDNA sequence similarity to known strains suggests the potential for the emergence of new foodborne pathogens resulting from human activity. Novel *Listeria* species like *Listeria weihenstephanensis* sp. nov. has been isolated from water plant (*Lemna trisulca*) of a German fresh water pond (Halter *et al*., 2012) and *Listeria fleischmannii* sp. nov. has been isolated from cheese in Switzerland (Bertsch *et al*., 2012) have been reported.

The antibiotic resistant of the five unidentified *Listeria* strains is showed in Table 2. The five unidentified *Listeria* strains exhibited 100% resistant to cefotaxime, nalidixic acid and tetracycline but 100% susceptible

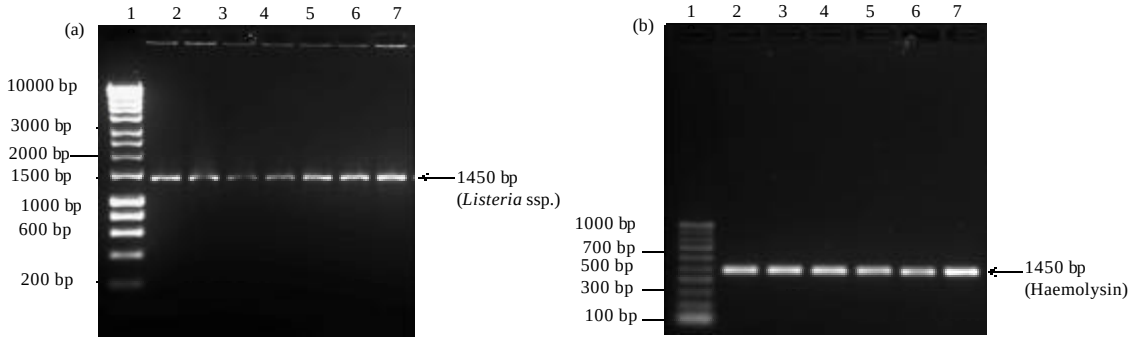

Fig. 1(a-b): Agarose gel eletrophoresis showing results for *Listeria* spp. (iap) gene, Lane 1: HyperLadder Bioline I; lanes 2-6: unknown *Listeria* strains, 1450-bp fragment; lane 7, *Listeria monocytogenes* positive control, haemolynsin (*hyIA*) gene detection by PCR, Lane 1: 100 bp ladder viviantis; lanes 2-6: unknown *Listeria* strains, 456-bp fragment; lane 7; *Listeria monocytogenes* positive control

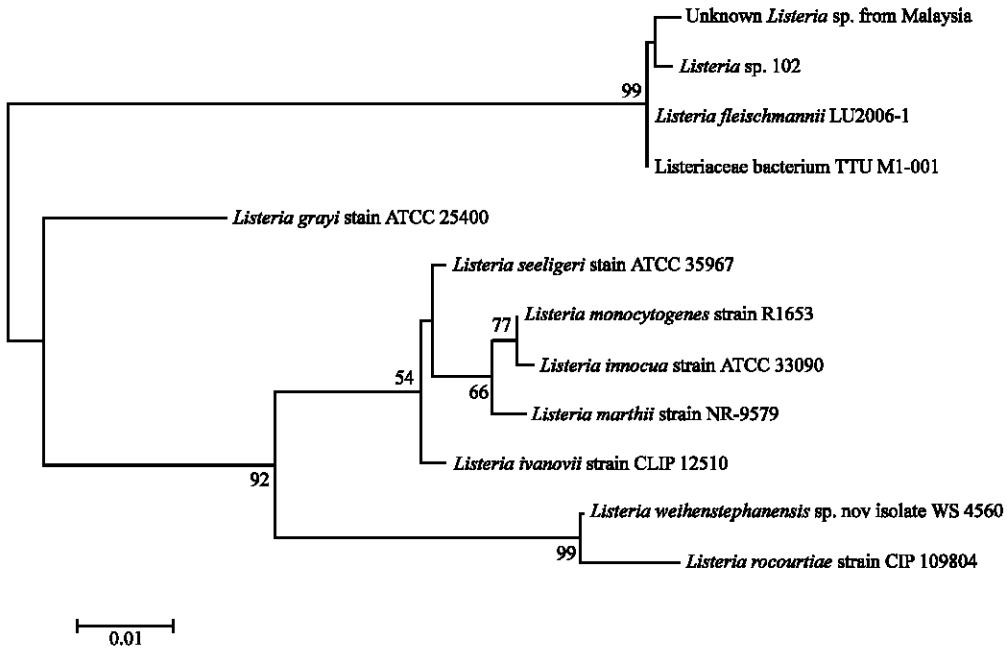

Fig. 2: Phylogram inferred from maximum likelihood analysis of a 1215 bp region of the 16S rDNA coding sequence in selected members of Listeriaceae. The analysis was conducted in MEGA5. Values on the branches indicate bootstrap values based on 1000 bootstrap replicates. Bootstrap values less than 50 are not shown

to ampicillin and nitrofurantoin. Furthermore, resistances≥60% was observed for cephalothin, gentamicin and streptomycin, while susceptibilities≥60% was observed for suphamethoxazole/trimethoprim and chloramphenicol. The unidentified *Listeria* strains exhibited 60% intermediates to erythromycin, ofloxacin and vancomycin. Resistant of foodborne pathogens to antibiotics have been linked to the use of antibiotics in the treatment of humans, animals and for purposes such as improved feed conversion in animals (Krumperman, 1983; Singh *et al*., 2010). The antibiotic resistance profile, multiple antibiotic resistance index and plasmid DNA size of the unknown *Listeria* strains is presented in Table 3. The strains exhibited 5 different antibiogram patterns that is, TeNaCxt, TeNaCxtKfSCn, TeNaSxtCtxKfSCn, TeNaCxtKfNorSCnCip and TeNaSxtCtxCKfENorSCnOfxCip with MAR index ranging from 0.2 to 0.8. One isolate was resistant to as many as 12

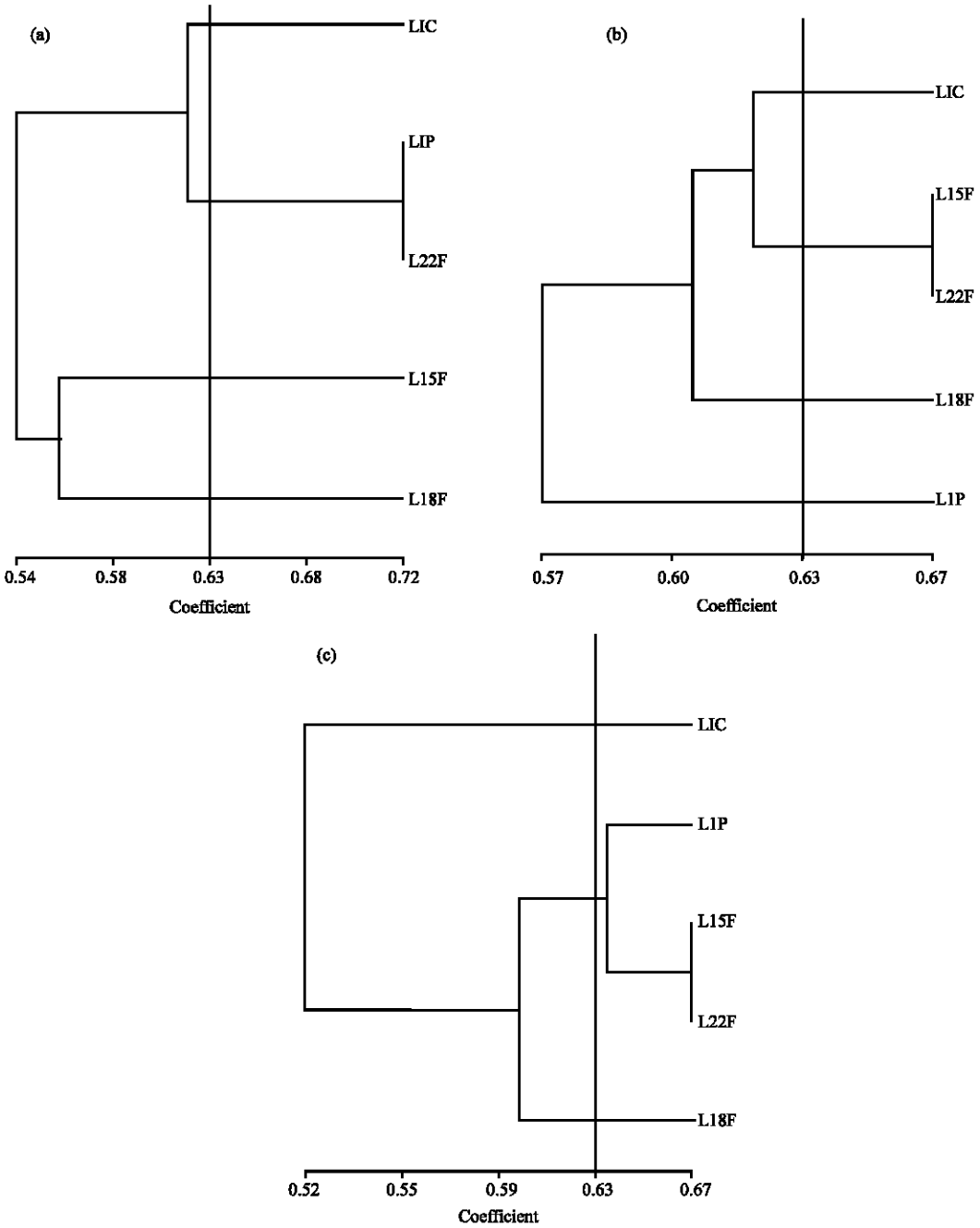

Fig. 3(a-c): Dendogram showing the genetic relatedness of unknown *Listeria* strains according to, (a) RAPD, (b) REP and (c) ERIC

antibiotics out of the 15 examined (MAR index of 0.80). One isolate each was also resistant to 7 (MAR index of 0.53) and 8 (MAR 0.47) antibiotics. Krumperman (1983) reported that isolates with high MAR index originated from environments where antimicrobials are often used. Furthermore, multidrug resistant pathogens have been suggested to be more pathogenic compared to non-multidrug resistant pathogens (Foley and Lynne, 2008). Resistance of foodborne pathogens to multiple antibiotics is a concern worldwide and makes it difficult to treat infected patients (Adzitey *et al*., 2012a). Plasmid sizes ranged from 2.1-25 kb and were detected in all isolates. One isolated each haboured one, three and four plasmid DNA. The rest of the two isolates haboured six plasmid

Table 2: Percentage antibiotic resistant isolates of the unidentified *Listeria* strains

		Unidentified *Listeria* strains			
Antimicrobial	Dose (µg)	*n/5	R (%)	I (%)	S (%)
Ampicillin (Amp)	10	0	0.0	0.0	100.0
Cefotaxime (Ctx)	30	5	100.0	0.0	0.0
Cephalothin (Kf)	30	4	80.0	0.0	20.0
Chloramphenicol (C)	30	1	20.0	0.0	80.0
Ciprofloxacin (Cf)	10	2	40.0	40.0	20.0
Erythromycin (E)	15	1	20.0	60.0	20.0
Gentamicin (Cn)	10	4	80.0	0.0	20.0
Nalidixic acid (Na)	30	5	100.0	0.0	0.0
Nitrofurantoin (F)	300	0	0.0	0.0	100.0
Norfloxacin (Nor)	10	2	40.0	20.0	40.0
Ofloxacin (Ofx)	5	1	20.0	60.0	20.0
Streptomycin (S)	10	4	80.0	0.0	20.0
*Sxt		2	40.0	0.0	60.0
Tetracycline (Te)	30	5	100.0	0.0	0.0
Vancomycin (Va)	30	0	0.0	60.0	40.0

n: No. of resistant, S: Susceptible, I: Intermediate, R: Resistant, *Suphamethoxazole/trimethoprim (Sxt) 22 µg

Table 3: Antibiotic resistance profile, multiple antibiotic resistance index and plasmid size of the unidentified *Listeria* strain

Listeria spp.	Source	Antibiotic resistant profile	MAR index	Plasmid size (kb)
L1C	Carcass rinse	TeNaCxt	0.20	24.9, 20.0,14.8,4.4, 3.1, 2.1
L1P	Pond water	TeNaCxtKfSCn	0.40	15.1
L15F	Faeces	TeNaSxtCtxKfSCn	0.47	14.6, 4.4, 3.0, 2.1
L18F	Faeces	TeNaCxtKfNorS CnCip	0.53	14.4, 5.6, 2.9
L22F	Faeces	TeNaSxtCtxCKfE NorSCnOfxCip	0.80 3.1, 2.2	23.2, 19.4, 14.4, 4.4,

DNA each. Emergence of resistant bacterial foodborne pathogens to antibiotics reflects evolutionary processes that take place as animals are exposed to antibiotics (Levy, 1994; Witte, 2004). Resistance of bacterial foodborne pathogens to antibiotics can also occur by inheritance, horizontal gene transfer, which is more likely to happen in locations of frequent antibiotic use (Witte, 2004).

Characterization of the five *Listeria* strains by RAPD, REP and ERIC is shown in Figure 3a, 3b and 3c. RAPD, REP and ERIC analyses showed some level of similarities and differences among the five strains. At a co-efficient of 0.63, each of the genotyping method grouped the isolates into four types. The types were Rapd type 1 (L1C), Rapd type 2 (LIP, L22F), Rapd type 3 (L15F) and Rapd type 4 (L18F) for RAPD; Rep type 1 (LIC), Rep type 2 (L15F, L22F), Rep type 3 (L18F) and Rep type 4 (L1P) for REP; and Eric type 1 (L1C), Eric type 2 (L1P), Eric type 3 (L15F, L22F) and Eric type 4 (L18F) for ERIC. The typing methods successfully typed all the unknown *Listeria* strains and revealed relatively greater genetic diversity among the 5 isolates. There was also a good agreement among RAPD, REP and ERIC typing tools. For instance, LIC and L18F were grouped as singletons (single isolate) by all the typing methods.

It is possible that these unknown *Listeria* strains represent a novel species; however, further work is needed to establish that. This study also draws attention to the emerging importance of this foodborne pathogen and the need for monitoring changes in antibiotic resistance patterns so that efficient risk and control management strategies can be developed.

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