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Characterization of *Listeria monocytogenes* Isolated from Chicken Meat: Evidence of Conjugal Transfer of Plasmid-mediated Resistance to Antibiotic

[1]Endang Purwati, [2]Son Radu, [3]Ahmad Ismail, [4]Cheah Yoke Kqueen and [2]Lesley Maurice
[1]Fakultas Peternakan ,Universitas Andalas, Kampus Limau Manis, Padang, Indonesia
[2]Department of Biotechnology, Faculty of Food Science and Biotechnology, University Putra Malaysia, Serdang, Selangor, Malaysia, [3]Department of Biology, Faculty of Science and Environmental, Universiti Putra Malaysia, Serdang, Selangor, [4]Department of Biomedical Science, Faculty of Medicine and Health Science, University Putra Malaysia, Serdang, Selangor, Malaysia

Abstract: *Listeria monocytogenes* isolated from chicken meat were characterized for their antimicrobial susceptibility, plasmid profile, chromosomal polymorphism by RAPD fingerprinting using three primers and to determine whether genetic information coding for antimicrobial resistance in *Listeria monocytogenes* strain may be carried on conjugative R plasmid. A total of 28 strains of *Listeria monocytogenes* isolated from 12 (60%) of the sample analysed, were resistant to three or more antimicrobial agents tested. However, none of the isolates were resistant to norfloxacin. Twelve (42.87%) of the *Listeria monocytogenes* strains contained plasmid DNA bands ranging in size from 2.7 to 54 Kb. Combination of the plasmid profiling and antibiogram could separate the strains into different types. In conjugation studies, tetracycline resistance was transferred from the donor strains *L. monocytogenes* LMC2KLI.6, LMC6KI4.2 and LMC20S3.4 to the recipient strains *L. monocytogenes* LMC7KL4.5, LMC21S3.5 and LMC25K2.1 at frequencies of 2.15×10^{-8}, 3.58×10^{-9} and 3×10^{-9}, respectively. Vancomycin-resistance was transferred to *Listeria monocytogenes* LMC2KL1.6 strain at frequency of 2.15×10^{-8} and novobiocin-resistance was transferred to *Listeria monocytogenes* LMC20S3.4 strain at frequency of 3×10^{-9}. Our results demonstrate that RAPD-PCR fingerprinting method is more sensitive than plasmid profiling and antibiotic resistance patterns with respect to individualization of the isolates used in this study.

Key words: *Listeria monocytogenes*, antimicrobial resistance, plasmids, conjugal transfer, RAPD-PCR, chicken meat

Introduction

In general the bacteria that cause food poisoning do not affect the appearance, aroma, or the flavor of food. Among the most common bacterial causes of the food poisoning is *Listeria monocytogenes.* Listeriosis, caused by the bacterium *Listeria monocytogenes* is a gram-positive and is spread in soft cheeses, undercooked meats including chickens meat, and prepared foods from delicatessen counters (Iida *et al.,* 1998; Inoue *et al.,* 2000). The illness is especially serious for the very young or for pregnant women, who may miscarry or transmit blood infections or meningitis to the baby. In adult, the diseases can progress to central nervous system complications, endocarditis, or pneumonia, and is an especially serious threat to the elderly. Treatment for most bacterial food poisoning include rest, sedation, and replacement of fluid loss if necessary. Antibiotics usually are used in several cases. Some expert have attributed the prescription of antibiotics and the routine use of antibiotics as growth enhances and to treat diseases in livestock, were among practices that encourage the development of drug-resistant bacterial variants (Irenev, 1998; Anonymus, 2000; Franco *et al.,* 2001). Increases in the number and severity of food poisoning cases have led to concern about food inspection and preparation methods, and to consideration to eliminate bacterial contamination (Farber and Peterkin, 1991; De Simon *et al.,* 1992; Brett *et al.*, 1998). In addition, there is a need to asses the transferability of antimicrobial resistance of *Listeria monocytogenes* to establish the possible hazard to public health due to digestion of food-borne resistance strain (Carmina *et al.*, 1996; Endang *et al.*, 1998; CDC, 1999). An RAPD-PCR assay was development for phylogenetic analysis of *Listeria monocytogenes* using short arbitrary oligonucleotide primers was first described in 1990, when DNA polymorphisms amplified by means of 9 or 10-nucleotide primers

were used as genetic markers. Since then the usefulness of RAPD-PCR analysis for epidemiological study of *Listeria monocytogenes* isolates has been confirmed by other groups (Lawrence *et al.* 1995; Wagner *et al.*,1999).

The objective of the present study was to characterize the strains of *Listeria monocytogenes* isolated from chicken meat for their antimicrobial susceptibility, to determine whether a given bacterium harbors plasmid, to determine whether genetic information coding for antimicrobial resistance in *Listeria monocytogenes* strain may be carried on conjugative R plasmid, and to typed the *Listeria* species by random amplified polymorphic DNA (RAPD) analysis

Materials and Methods

Isolation and Identification: Sample of chickens meat purchased in retail markets around Kuala Lumpur (n=5), Subang (n=5), Serdang (n=5), and Kajang (n=5). The samples were collected in sterile plastic bags and transported to the laboratory in boxes containing ice. The samples were kept at 4°C overnight before analysis. Aseptically 25 g of samples were added to the 225 ml Listeria enrichment broth (LEB, Oxoid) in a stomacher bag and homogenized for two minutes in a stomacher (Coldworth Stomacher) and incubated for 24 h at 30°C. After incubation, three loopfulls from LEB were streaked onto the surface of Palcam Agar (Oxoid). The plates were incubated at 30°C for 24-48 h. Presumptive *Listeria* species colonies on Palcam agar were streaked on Tryptone Soya Agar containing 0.6% Yeast extract (TSAYE) for purification and confirmation of *Listeria.* Listeria-like, bluish grey colonies and producing black zone of aesculin hydrolysis, whether β-haemolytic or no on blood agar, were identified by the following examination: gram-staining-positive, catalase-positive, oxidase-negative, motility at 20-25°C as umbrella-like growth on motility semi-solid agar, urea-negative, TSI-produced acid but not gas, voges-proskaur positive, reduction NO_3 to NO_2 positive or negative, hippurate hydrolise positive or negative and Listeria latex slide agglutination test positive (Serobact, Medvet-Australia). Further identification were carried using Microbact 12L *Listeria* identification system (Medvet-Australia) and conventional method of fermentation test of manitol negative, xylose negative, rhamnose positive, salicin positive and the CAMP test was performed with *Staphylococcus aureus* and *Rhodococcus equi* as positive and negative controls, respectively.

Antimicrobial Susceptibility Testing: Disk diffusion tests were performed with antibiotic containing disks obtained from BBL (Becton Dickinson, USA) Microbiology System, Cockeysville, MD, by the method recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (1993). The antimicrobial agents tested included sulphamethoxazole (100 µg), penicillin (10 units), ampicillin (10 µg), carbenicillin (100 µg), cefuroxime (30 µg), chloramphenicol (30 µg), erythromycin (30 µg), gentamicin (10 µg), kanamycin (30 µg), streptomycin (15 µg), tetracycline (30 µg), bacitracin (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), novobiocin (30 µg), ceftazidime (30 µg), spectinomycin (100 µg) and vancomycin (30 µg). Bacteria were suspended in saline to the same visually as a McFarland 0.5 turbidity standard and streaked on Mueller Hinton agar. Plates were incubated for 24 h at 37°C. Characterization of strains as sensitive, intermediate or resistant was based on the size of zones of inhibition surrounding the discs. The multiple antibiotic resistance (MAR) index was calculated according to Krumperman (1983) using the following formula: MAR index = x/y, where x is the number of resistance determinants and y, is the number of antibiotics tested

Plasmid Analysis: Organisms were screened for plasmid DNA by the modified alkaline lysis method of LeBlanc and Lee (1979). Extracted plasmids were electrophoresed for 15 minutes at 300 V and 2 h at 150 V on a 0.7% agarose gel in TBE 1x (89 mM Tris base, 89 mM boric acid, and 2.5 mM disodium EDTA) as described by Sambrook *et al.* (1989). After the gels were stained with ethidium bromide (0.5 µg/ml for 30 min), they were photographed under u.v illumination. The approximate molecular mass of each plasmid was determined by comparison with plasmids of known molecular mass from *E. coli* V517 (Macrina *et al.*, 1979).

Bacterial conjugation: Selected antibiotic-resistant of *Listeria monocytogenes* isolates (LMC2KL1.6, LMC6KL4.2 and LMC20S3.4) harbouring a 54 kilobase plasmid and three small plasmids of 2.7 or 3.0 kilobase in sizes isolated from the chicken meat in this study were used as the donor strains and plasmidless (LMC7KL4.5, LMC21S3.5 and LMC25K2.1) were used as the recipient strains (Table 2).

Donor and recipient cells were grown to mid-log phase (10^7 cfu/ml) in tryptic soy broth (TSB) at

Table 1: Listeria monocytogenes strains isolated from chicken meat

Serotype	No. of isolates	Antibiogram	MAR index	Plasmid size (Kb)	Strains
L. monocytogenes	1	B, CAZ, SXT	0.17	5.1, 54	LMC25K2.1
	1	B, CAX, K, NA, SXT, TE, Va	0.38	5.1, 54	LMC5KL4.1
	1	Am, B, CAZ, NA, P, S, SXT, TE, Va	0.50	54	LMC2KL1.6
	2	Am, B, CAZ, NA, P, S, SXT, TE, Va	0.50	3, 4, 5.6, 54	LMC27K3.1 LMC28K3.4
	1	B, CAZ, CXM, S, NA, P, TE, Va	0.50	4, 5.1, 5.6 54	LMC6KL4.2
	1	Am, B, C, CAZ, B, C, CAZ, CXM, P	0.50	-[c] (ut)	LMC7KL4.1
	1	Am, B, C, CAZ, B, C, CAZ, CXM, P	0.50	-[c] (ut)	LMC21S3.5
	1	B, CAZ, CXM, E, K, NA, P, SXT, TE, Va	0.55	3, 4, 5.1 54	LMC21S3.5
	2	B, CAZ, CB, E, NA, P, S, SXT, TE, Va	0.55	3, 4, 54	LMC3KL3.4 LMC3KL3.6
	2	Am, B, CAZ, E, K, NA, P, S, SXT, TE	0.55	5.1, 5.6, 54	LMC9SB1.5 LMC17S2.3
	5	Am, B, CAZ, CB, GM, NA, BN, P, S, SXT	0.55	-[c] (ut)	LMC15SB5.5 LMC16SB5.6 LMC18SB2.5 LMC19S2.6 LMC26K2.5
	8	B, CAZ, CXM, E, K, NA NB, SPT, SXT, Va	0.55	-[c] (ut)	LMC1KL1.3 LMC8SB1.3 LMC10SB2.4 LMC11SB2.5 LMC12SB4.1 LMC13SB4.2 LMC14SB5.5 LMC23S5.4
	2	B, CAZ, E, K, NA, NB, P, S, SXT, TE, Va	0.61	4, 5.1, 54	LMC20S3.4 LMC22S5.1

[c]Symbols for antimicrobial resistance: Am, ampicillin: B, bacitrain C, chloramphenicol, CAZ, ceptazidime; CB, carbenicillin; CXM, cefuroxime; E, erythromycin, GM, gentamicin; K, kanamycin; NA, nalidixic acid; NB, novobiocin; NOR, norfloxacin, P, penicillin; S, streptomycin; SPT, Spectinomycin; SXT, sulfamethoxazole, TE, tetracycline; Va vacomycin ****Listeria monocytogenes*** (LMC) strains isolated from chicken meat: KL, Kuala Lumpur; SB, Subang; S, Serdang, K, Kajang. ***None detected. Ut, untypable

Table 2: Characterization of transonijugants among *L. monocytogenes* isolated from chicken meat

Donor*	Receipt**	Antibiotic selection ***	Plasmid size (Kb) for donor	Antibiotic resistance Trannferred	Frequency of transfer	Plasmid size detected in transconjugants (Kb)
LMC2 KL1.6	LMC7 KL4.5	TE + S	54.0	TE, Va	2.15×10^{-8}	54
LMC6 KL4.2	LMC25K 2.1	TE + NB	5.1, 5.6 54.0	TE	3.58×10^{-9}	5.1, 5.6, 54.0
LMC2 0S3.4	LMC21S3.5	TE + CXM	4.0 5.1, 54.0	TE, NB	3×10^{-9}	5.1, 54.0

*, **, *** See Table 1 for antimicrobial resistance phenotypes of donor and receipient of the *L. monocytogenes* strains used

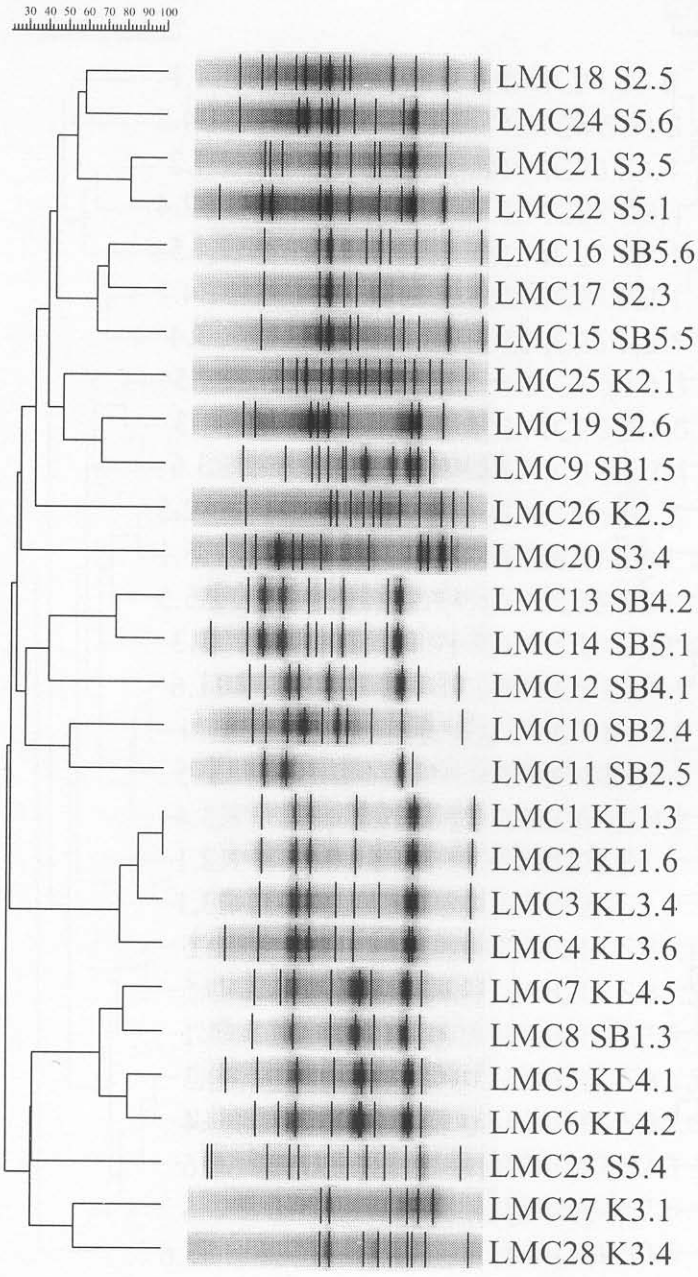

Fig. 1: Dendrogram generated from the RAPD-PCR analysis using primer GEN1-50-01 (5'GTGCAATGAG-3') of the *Listeria monocytogenes* strains. See Table 1 for legends

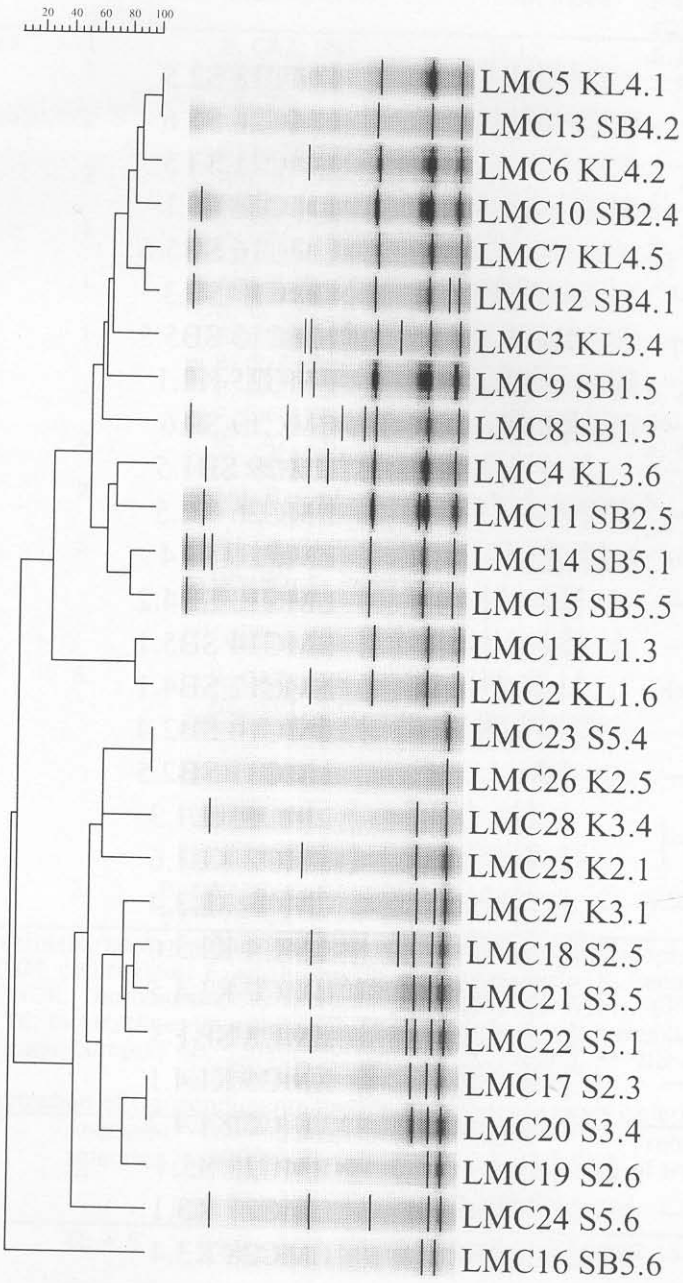

Fig. 2: Dendrogram generated from the RAPD-PCR analysis using primer GEN 1=50-02. (5'CAATGCGTCT-3') of the *Listeria monocytogenes* stains. See Table 1 for legends

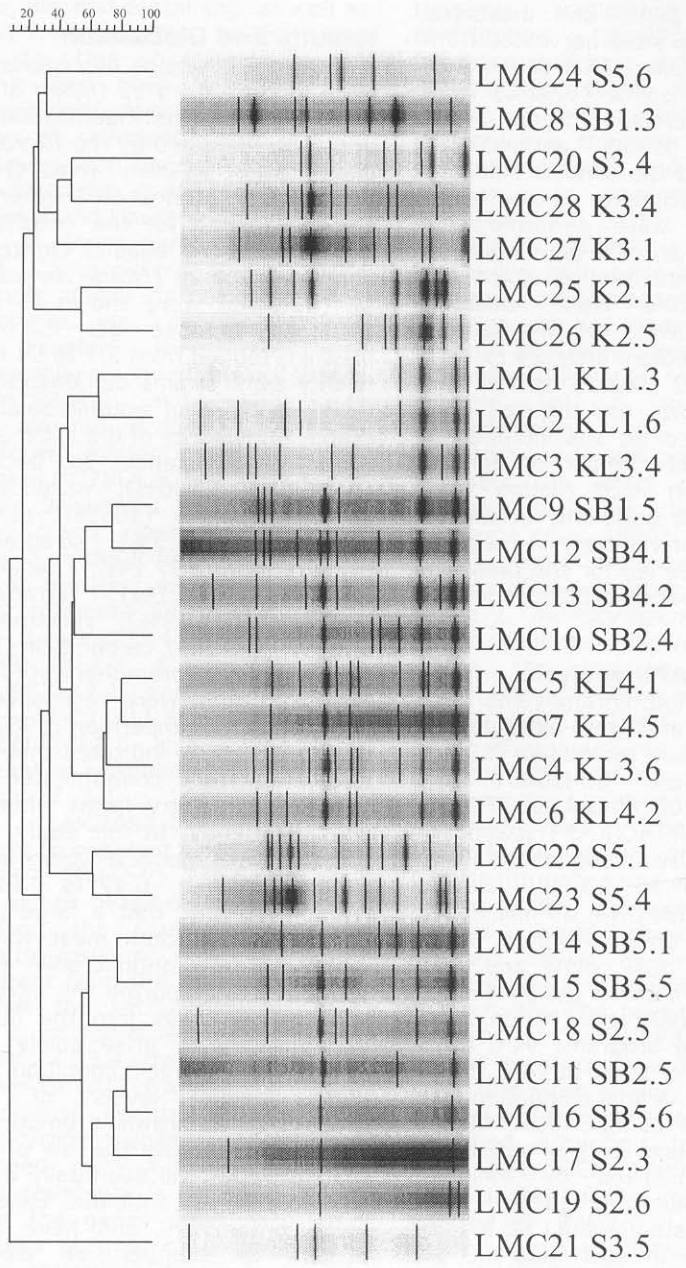

Fig 3: Dendrogram generated from the RAPD-PCR analysis using primer GEN 1-50-10 (5'CCATTTACGC-3') of the Listeria monocytogenes strains. See Table 1 for legends

35°C. A 0.5 ml sample of the donor strains was added to 1.0 ml of the recipient sample on a tryptic soy agar (TSA) plate, and incubated overnight at 35°C. Bacteria were harvested from the TSA plate and a ten-fold serial dilutions of each mating mixtures in saline (0.80%) were spread on plates supplemented with appropriate concentration of the donors antimicrobial resistance phenotypes and the counter-selective antibiotic to which the recipients was resistant (Table 2). Plate counts were performed for estimates of donor and recipient population on TSA plates containing antibiotic to which the donor or recipient strains were resistant, respectively. Colonies growing on this double-inhibitor-supplemented medium after 24 to 48 h of incubation at 35°C were scored as presumptive transconjugants, and the frequency of transfer was calculated as the number of transconjugants per initial number of donors. Ten transconjugants from each mating were picked and tested for their antibiotic resistance. Tetracycline, novobiocin, or vancomycin-resistant transconjugants were screened for the presence of plasmid by the method of LeBlanc and Lee (1979).

DNA preparation and RAPD analysis: Prior to amplification by PCR method, chromosomal DNA of the *Listeria* species isolates were extracted by the mini-preparation method by William (1990). The primers used were GEN15001 (5'-GTGCAATGAG-3'), GEN15002 (5'-CAATGCGTCT-3') and GEN15010 (5'-CCATTACGC-3') (Genosys Biotechnology, USA). PCR reactions for the RAPD assays were performed in 25 µl volumes containing 20 ng of genomic DNA, 25 mM $MgCl_2$, 2.5 µl 10x buffer, 1 µl (20 pmol) of primer, 0.5 µl of 10 mM each of dATP, dCTP, dGTP and dTTP and 1.0 unit of *Taq* polymerase. Amplifications were carried out in a thermal cycler (Perkin Elmer 2400) using the following program: 94°C for 1 min followed by 45 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. A final elongation step was performed at 72°C for 10 min. After PCR, 10 µl aliquots of product were electrophoresed in 1.2% agarose gels, followed by ethidium bromide staining and photographed under UV light. DNA molecular size standards (1 Kb ladder, Promega) were included in each agarose gel electrophoresis run.

RAPD fingerprint pattern analysis: The banding patterns generated by RAPD-PCR were analyzed with GelCompar software (Version 4.1, Applied Maths, Kortrijk, Belgium). The arrangement of the RAPD-PCR profiles into dendrogram was accomplished by the unweighted pair group method with arithmetic averages (UPGMA).

Results and Discussion

A total of 28 strains of *Listeria monocytogenes* were isolated from 12 (60%) of the 20 samples analysed using the modification of FDA method and were identified by the Microbact 12L *Listeria* identification system (Medvet-Australia). The twenty-eight strains of *Listeria monocytogenes* were examined for the occurrence of plasmid DNA and tested against eighteen antimicrobial agents. Of the 28 *Listeria monocytogenes* strains 12 (42.86%) were shown to harbour plasmid DNA by agarose gel electrophoresis. The plasmids ranged from 2.7 to 54 Kb (Table 1). The twenty-eight strains demonstrated resistance to three or more of antimicrobial agents tested (Table 1). Majority of the *Listeria monocytogenes* displayed resistance to bacitracin (100%), ceptazidime (100%), nalidixic acid (100%), sulfamethoxazole (100%), penicillin (64.3%), vancomycin (64.3%), streptomycin (57.1%), erythromycin (57.1%), kanamycin (57.1%), novobiocin (57.1%), tetracycline (46.4%), cefuroxime (42.9%), spectinomycin (35.7%), ampicillin (32.1%), carbenicillin (25%), gentamicin (17.9%) and chloramphenicol (14.3%) (Table 1). However, none were resistance to norfloxacin. According to Krumperman (1983), a MAR indices of 0.2 and above indicated that bacterial isolates originated from contaminated areas such as commercial poultry farms where antibiotics are used frequently. In this study, the MAR indices value of the *L. monocytogenes* from chicken meat ranged between 0.17 to 0.61 (Table 1). The results indicated that a large proportion of the bacterial on chicken meat was resistant to a variety of the antimicrobial tested. However, Manie (1998) reported the introduction of drug-resistance isolates into the human food chain was unlikely to arise solely from processing under unhygienic condition. The use of subtherapeutic levels of antibiotics for prophylaxis as growth promoters continue to be of concern because the law of evolution dictate that microbes will eventually develop resistance to practically any antibiotic. Listeriae are common food contaminant and have clearly begun to acquire a number of different antibiotic resistance genes, many of which associated with conjugative elements (Hadon *et al.*, 1993).

Antibiotic resistance is often determined by genetic information of plasmid origin and that the correlation between antibiotic-resistance and plasmid profile may indicate that the genetic information is plasmid-borne (Marilyn, 1996;

Manie *et al.*, 1998). Looking through the antibiotic resistance patterns of the 28 strains of *L. monocytogenes*, plasmid containing as well as plasmidless strain were resistant to the same antibiotic (Table 1). This observation is not unexpected as it has been reported that antibiotic resistance in *Listeria* species can be due to acquisition of self-transferable plasmid and conjugative transposon (Poyart-Salmeron *et al.*, 1990; Poyart-Salmeron *et al.*, 1992; Marilyn *et al.*, 1996; Lemonut and Hauspie, 2001).

When six strains (3 strains harbouring plasmid DNA 54 Kb and 3 plasmidless strains) were examined for their ability to transfer their resistance phenotypes via conjugation were observed to transfer their resistance phenotypes to recipient. Thus these strains (LMC2KL1.6, LMC6KL4.2 and LMC20S3.4) harbouring large plasmid of the 54 Kb were chosen to perform mating with selected plasmidless strains (LMC7KL4.5, LMC21S3.5 and LMC25K2.1). Table 2 showed the variation in resistance patterns and plasmid content of donor strains as well as the R factor transferred. The frequencies of transfer, expressed as the number of transconjugants per donor colony forming unit (CFU) were: 3.4×10^{-9} to *L. monocytogenes* LMC2KL1.6, 4.2×10^{-9} to *L. monocytogenes* LMC25K2.1 and 3×10^{-9} to *L. monocytogenes* LMC21KL1.6. The *L. monocytogenes* LMC2KL1.6 also transferred vancomycin-resistant to recipient LMC5K2.1 at a transfer frequency of 3.4×10^{-8} and the *L. monocytogenes* LMC20S3.4 transfered novobiocin-resistant to recipient LMC21S3.5 at a frequency of 3×10^{-9} (Table 2). The 54 kilobase plasmid of the donor was detected in the tetracycline-resistant, the novobiocin-resistant and the vancomycin-resistant transconjugants. Thus, it was apparent from the results obtained in this study that the tetracycline-resistance phenotype of the *L. monocytogenes* LMC2KL1.6, LMC6KL4.2 and LMC20 S3.4 strain was mediated by the 54 kilobase plasmid. Tetracycline is broad-spectrum antimicrobial agents. However, all structural of tetracycline resistance genes that have been described, carry a single structural gene. Tetracycline resistance is normally due to the acquisition of the genes often associated with either a mobile plasmid or a transposon. These tetracycline resistance determinants are distinguishable both genetically and biochemically. Tetracycline-resistant bacteria are found in pathogens, opportunistic and normal flora species can be isolated from man, animals, food and environment (Poyart-Salmeron *et al.*, 1992).

Vancomycin is a glycopeptide antibiotic is active against a wide range of gram-positive bacteria. However, resistance to vancomycin is contributing significantly to increasing importance of bacterial pathogens and in more general terms to the growing concern about the impact of antimicrobial resistance. The VanA phenotype is characterized by inducible, mostly plasmid-mediated and transferable high level resistance to vancomycin (Carosella, 1990; Biavasco *et al.*, 1996).

The results obtained in this study demonstrated the ability of natural plasmid conjugal transfer among *L. monocytogenes* isolated from poultry. Isolation of *L. monocytogenes* containing R plasmid and demonstration of the ability of the plasmidless isolates to act as recipient for R plasmid is an important step toward understanding the impact when *L. monocytogenes* are present in sufficiently high numbers can effect significant transfer of R plasmids including chimeric plasmid (Poyart-Salmeron *et al.*, 1990).

Initially, ten primers were screened using a sub-sample of ten isolates to detect polymorphism within *L. monocytogenes*. However, only three oligonucleotides showed DNA polymorphism within the isolates tested. These three discriminating primers are GEN15001, GEN15002 and GEN15010. The G+C content of these are 50% where *Listeria* spp. have an average genome GC content of 50-52%. They were chosen to analyze all of the 28 *L. monocytogenes*. The remaining primers (GEN15003, 04, 05, 06, 07, 98, and 09) gave bands with only some of the isolates or had poor reproducibility and were not examined further. Figures 1, 2 and 3 showed the RAPD-PCR profiles of the *L. monocytogenes* isolates obtained using primers GEN15001, GEN 15002 and GEN15010, respectively. The possible number of RAPD-PCR patterns was estimated on the basis of changes in one or more clear bands or band size with molecular sizes ranging from 250 to 10000 base pairs (bp) (data not shown). All isolates could be classified according to types using primers GEN15001, GEN 15002 and GEN15010. The combination of the result with three primers showed 27 RAPD-PCR types among the 28 *L. monocytogenes* isolates. Thus, the sensitivity of the RAPD-PCR was higher than antibiotic resistance pattern and plasmid profiling for fingerprinting, and that the high discriminating power of RAPD-PCR will undoubtedly be of great help in epidemiological studies.

The presence of R plasmids in *Listeria monocytogenes* isolated from chicken meat is

indicative of potential human health hazard, since these plasmid-containing bacteria can be transferred to human through the consumption of contaminated poultry meat or poultry related products. RAPD in combination with antibiotic resistance patterns and plasmid profiles can provide substantial epidemiological data about *Listeria monocytogenes*.

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