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Antimicrobial Drug Resistance and Resistance Factor Transfer among Listeria Species

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Abstract

Ninety four Listeria strains from salted fish samples were examined for resistance to 10 antibiotics and for the occurrence of plasmid DNA. Ten strains of *L. ivanovii*, three of *L. denitrificans*, two *L. innocua* and one *L. seeligeri* contained plasmid DNA ranging in size from 2.7 to 54 kilobases. Antibiotic susceptibility testing of the Listeria strains indicated that all were resistant to two or more antibiotics and that no isolates were resistant to gentamycin, norfloxacin and vancomycin. The incidence of antibiotic resistance was particularly high for *L. innocua*, *L. denitrificans* and *L. welshimeri*. On the other hand, *L. ivanovii* and *L. monocytogenes* exhibited the least resistance. In addition, a selected kanamycin resistant *L. innocua* strain was found to transfer kanamycin resistance with the concomitant transfer of a 54 kilobase plasmid to the recipients, tetracycline-resistant and kanamycin-sensitive *L. innocua* and *L. monocytogenes* strains (intra- and inter-species transfer), which suggested that dissemination of resistance to other strains of Listeria is likely.

Introduction

Listeria species are ubiquitous and associated with disease in animals and food-borne illness in man. Listeria contamination occurs in a wide range of foods such as dairy products, vegetables, raw fish, fermented sausage, meat and poultry (Gellin and Broome 1989; Beuchat *et al.* 1990; Carosella 1990; Marth and Ryser 1990). Although the incidence of the bacterium in food can be minimized by paying particular attention to hygiene during food production, it is difficult to ensure the absence of Listeria from foods that do not receive a final listericidal treatment. Members of the genus *Listeria* are known to have the ability to tolerate environmental stresses. Of particular concern is that *Listeria* spp. is osmotically tolerant and can grow at refrigerated temperatures. For example, it has been reported to grow at more than 10% NaCl (McClure *et al.* 1989).

Salted fish, a very popular food in Malaysia, is made from various fish species marinated in salt (5-10%) as the preservation factor. After marination, the raw fish is dried and most often cooked before consumption. Consumption of contaminated food can cause severe and often fatal infections in susceptible people (Nieman 1980). Thus, there is a need to assess the antimicrobial susceptibility of *Listeria* spp. to establish the possible hazards to public health of certain food-borne multiresistant strains of this bacterium. The incidence of drug resistance in *Listeria* species isolated from salted fish and of the conjugative transferability of kanamycin resistance among *Listeria* spp is presented in this study. The plasmid profiles of these *Listeria* spp. were determined, thereby achieving the characterization of the transferred R plasmid.

Materials and Methods

Bacterial strains and media

Isolates obtained from three species of salted fish from local food outlets in Johor, Malaysia, were used in the study (Table 1). From 25 samples (5 Haruan, *Channa striatus*; eight Lampan, *Puntius javanicus*; and 12 Sepat, *Trichogaster pectoralis*), 94 *Listeria* strains were isolated. The method used was an enrichment procedure followed by plating onto Palcam agar (Merck 11755). Briefly, a 25-g fish sample was homogenized with 225 ml of *Listeria* enrichment broth (LEB) for two minutes in a stomacher (Colworth Stomacher 400). After incubation of the enrichment broth at 35°C for 5–6 h, 0.1 ml was streaked onto Palcam agar, and the samples were further incubated at 35°C for 48 h. For each sample, 10 or more presumptive Listeria colonies were isolated. Suspected Listeria colonies were characterized by using the following criteria: gram reaction (gram-positive bacilli or coccobacilli), motility (umbrella type), presence of catalase (+) and oxidase (-) (Lovett 1988), hemolytic activity (+ or -) and a Microbact 12L *Listeria* Identification System (Medvet, Australia). *Staphylococcus aureus* was used as negative controls in each batch of tests.

Antimicrobial susceptibility testing

Isolates were screened for resistance to ampicillin (10 mg), chloramphenicol (30 mg), erythromycin (15 mg), gentamycin (10 mg), kanamycin (30 mg), nalidixic acid (30 mg), norfloxacin (10 mg), streptomycin (10 mg), tetracycline (30 mg) and vancomycin (30 mg), using commercial discs (BBL Sensi-Disc, Becton Dickinson, U.S.A.). Bacteria were suspended in saline to give a density of 2 McFarland standards, diluted 1:20, and streaked, by the method of Bauer *et al.* (1966), on Tryptic soy agar. Plates were incubated for 24 h at 35°C. Characterization of strains as sensitive, intermediate or resistant was based on the size of the inhibition zones around each disc and the manufacturer's instructions. To determine the level of antibiotic resistance of *Listeria* species isolated, an antibiotic resistance index (ARI) was calculated according to Hinton and Linton (1983) using the following formula: ARI = x/ny, where x is the number of resistance determinants in a population y, and n is the number of antibiotics tested.

Table 1. *Listeria* strains used in the study.

Serotype	No. of isolates	Antibiogram[a]	Plasmid size (Kb)	Strain No.[b]
L. ivanovii	30	ApErTeKm	3	S22, S221
		ApErTe	3	H, L10, L101, L211, S12, S13, S411, S121
		ApEr	-	S14, S41, S131, L13, L14, L15, L16, L17, L21, L22, L23, L34, L39, L101, L121, L131, H11
		ApErTe	-	S2, S24, S222
L. innocua	20	ApTeCm	-	L35
		ApErKmNaSm	3, 54	L11, L38
		ApCmErTeNaS	-	S3, S21, S31, S32, S36, S51, L8, L31, L33, L37, L221, L331, H1, H15, H21, H111, H221
L. welshimeri	20	ErNaSmTe		S4, S41, S43, S52, S53, S54, S241, S441, S551, L55, L56, L57
		ApErKmNaSmTe	-	S1, S5, S6, L7, L71, H51, H52, H57 L12, S23, S231
L. denitrificans	16	ApErNaSmTe	2.7, 54	L19, L22, L40, L44, L45, L111, L191, L233
		ApErNaSmTe	-	H25, H29, S29, S47, S48
L. seeligeri	5	ApErKmNaSmTe	3, 54	L2111
		ApErNaSmTe	-	L331, H14, H15, H16
L. monocytogenes	3	ApTe	-	S15
		ApCmErSm	-	S16, S141

[a]Symbols for drug resistance: Ap, ampicillin, Cm; chloramphenicol, Er, erythromycin; Gm, gentamycin; Km, kanamycin; Na, nalidixic acid; Nor, norfloxacin; Sm, streptomycin; Te, tetracycline; and Va, vancomycin

[b]Source of isolation of strains: H, Haruan (*Channa striatus*); L, Lampan (*Puntius javanicus*); and S, Sepat (*Trichogaster pectoralis*)

Plasmid analysis

Organisms were screened for plasmid DNA by the method of LeBlanc and Lee (1979). Extracted plasmids were electrophoresed for 2 h at 35 mA on a 0.7% agarose gel in TBE (89 Mm Tris base – 89 mM boric acid – 2.5 mM disodium EDTA) as described by Meyers *et al.* (1976). After the gels were stained with ethidium bromide (1.5 mg/l for 30 min), they were photograph under u.v. illumination. The approximate molecular mass of each plasmid was determined by comparison with plasmid of known molecular mass from *E. coli* V517 (Macrina *et al.* 1978).

Bacterial conjugation

Each potential donor was incubated overnight in Tryptic soy broth (TSB) at 35°C, and recipient strains were incubated in the same medium at 35°C to an equal density. Overnight donor and recipient cultures were mixed at a 1:2 ratio on a Tryptic soy agar (TSA) plate. Matings were performed at 35°C. Bacteria were harvested from the TSA plate and ten-fold serial dilutions of each mating mixture in saline (0.85%) were spread on plates supplemented with 30 mg of tetracycline or chloramphenicol per ml and 30 mg of selected drugs per ml to which the potential donors were resistant. Colonies growing on this double-inhibitor-supplemented medium after 24 h 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 or more transconjugants from each mating were picked and tested for their biochemical characteristics, antibiotic resistance and screened for plasmid.

Results

Ninety four bacterial strains isolated from 25 samples of salted fishes were identified as members of the genus *Listeria* using the Microbact 12L *Listeria* Identification System (Medvet, Australia) (Table 1). Salted fish in Johor, Malaysia, harboured the six *Listeria* species: *L. ivanovii*, *L. innocua*, *L. denitrificans*, L. *monocytogenes*, *L. seeligeri* and *L. welshimeri* with a clear domination of *L. ivanovii*. All isolates were tested for their resistance to each of the ten antibiotics tested. Frequencies of antibiotic-resistance among these isolates are shown in Table 2. Most of the *Listeria* spp. isolates displayed resistance towards erythromycin (99%), ampicillin (87%), tetracycline (78%), nalidixic acid (65%) and streptomycin (63%). Percentages of resistance of chloramphenicol and kanamycin-resistant isolates were 21 and 15 respectively. However, none was resistant to gentamycin, norfloxacin and vancomycin. The level of antibiotic resistance among the six *Listeria* species (Table 2) were compared and according to the antibiotic resistance index (ARI) calculated for each species, *L. ivanovii* and *L. monocytogenes* (ARI = 0.25 and 0.27 respectively) seemed to be the lowest antibiotic resistant species. This is related to their higher susceptibility to chloramphenicol and nalidixic acid. A total of 16

Table 2. Antibiotic resistance among *Listeria* species isolates from fermented fish.

Listeria species	Percentage isolate resistant to:[a]										ARI[b]
	Ap	Cm	Er	Gm	Km	Na	Nor	Sm	Te	Va	
L. ivanovii	100	0	100	0	7	0	0	0	43	0	0.25
L. innocua	100	90	100	0	10	100	0	100	60	0	0.59
L. welshimeri	40	0	100	0	40	60	0	100	100	0	0.44
L. denitrificans	100	0	100	0	0	100	0	100	100	0	0.50
L. seeligeri	100	0	100	0	20	100	0	100	100	0	0.52
L. monocytogenes	100	0	67	0	0	0	0	67	33	0	0.27

[a] See Table 1 for drug resistance symbols.
[b] ARI, antibiotic resistance index.

strains; 10 *L. ivanovii*, 3 *L. denitrificans*, 2 *L. innocua* and one *L. seeligeri* were found to contain plasmid DNA ranging in sizes from 2.7 to 54 kilobases. The resistances of a single *L. innocua* strain L38 ($Ap^rEr^rKm^rNa^rSm^rTe^s$) was shown to be the result of the presence of resistance plasmid as single-step conjugation resulted in transfer of the plasmid DNA (Fig. 1) and the associated resistance (kanamycin) to the recipients, *L. innocua* L35 ($Ap^rTe^rKm^s$) and *L. monocytogenes* S15 ($Ap^rTe^rKm^s$). Frequencies of conjugal transfer in the plate mating were 3.3×10^{-3} and 4×10^{-3} transconjugants per donor cell in the mating mixture, respectively (Table 3).

Discussion

The results presented here provide information on the incidence and distribution of antimicrobial resistance, plasmid profiles and conjugal transfer of kanamycin resistance and plasmid DNA among *Listeria* species isolated from salted fish samples in Johor, Malaysia. Antimicrobial susceptibility analysis revealed that all the strains were resistant to at least two or more of the 10 antibiotics tested, although none showed resistance towards gentamycin, norfloxacin and vancomycin. Thus, our results concur with data among *Listeria* spp. reported elsewhere (Quentin *et al.* 1990; *Facinelli et al.* 1991; Poyart-Salmeron *et al.* 1992; Charpentier *et al.* 1993; Facinelli *et al.* 1993; Hardon *et al.* 1993; Charpentier *et al.* 1995), who isolated *Listeria* spp. with multiple antibiotic resistances from food and clinical sources; two different environments where antimicrobial prophylaxis has provided the selective pressure necessary for selection of microorganisms resistant to these valuable agents (Levy 1987). Differences were found in antibiotic susceptibility depending on the species. *L. ivanovii* and *L. monocytogenes* seemed to be the species most susceptible to antibiotics tested. The most striking difference among the six species was seen in resistance to nalidixic acid followed by kanamycin. To the best of our knowledge, the high percentage (60 to 100%) resistance to nalidixic acid has not yet been described in *Listeria* species. The reasons for this observation are unclear at the moment. In addition, in conjugation studies (Table 3), resistance to nalidixic acid was not transferable indicating that resistance is due to a yet-undefined determinant. Thus, with regards to resistance determination, it can be concluded that resistance among *Listeria* species was a serious problem as all (100%) of the strains exhibited

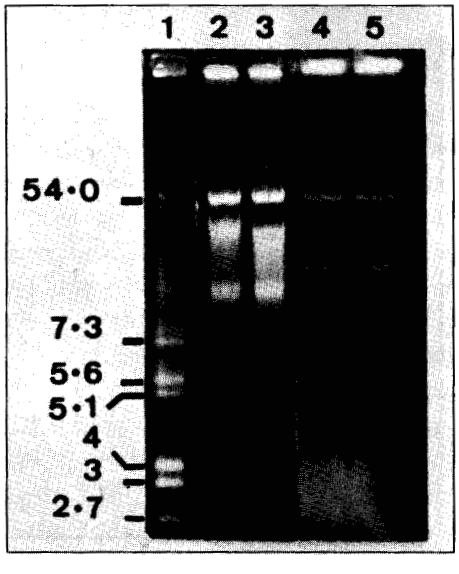

Fig. 1. Agarose (0.7%) gel electrophoresis of plasmid DNA from Listeria strains and their respective transconjugants. Lanes: 1, molecular mass standard plasmids of *E. coli* V517 in kilobase pairs (Kb); 2, donor *L. innocua* L38; 3, transconjugant of donor L38 (*L. innocua* L35); 4, donor *L. innocua* L38; 5, transconjugant of donor L38 (*L. monocytogenes* S15).

Table 3. Genetic transfer of kanamycin resistance among *Listeria* species.

Donor[a]	Recipient[b]	Antibiotic selection	Plasmid size (Kb)	Frequency of transfer	Plasmid size detected in transconjugants (Kb)
L. innocua (L38)	*L. monocytogenes* (S15)	Te + Km	3, 54	3.3×10^{-3}	3, 54
		Te + Na		ND	ND
		Te + Sm		ND	ND
		Te + Er		ND	ND
L. ivanovii (L21)	*L. monocytogenes* (S15)	Te + Er	ND	ND	ND
L. innocua (L38)	*L. innocua* (L35)	Te + Km	54	4×10^{-3}	54
		Te + Na		ND	ND
		Te + Sm		ND	ND
		Te + Er		ND	ND
L. seeligeri (L2111)	*L. innocua* (L35)	Cm + Km	3, 54	ND	ND
		Cm + Na		ND	ND
		Cm + Sm		ND	ND
		Cm + Er		ND	ND
L. denitrificans (L19)	*L. innocua* (L35)	Cm + Na	2.7, 54	ND	ND
		Cm + Er		ND	ND
		Cm + Sm		ND	ND

[a], [b]. See Table 1 for antibiotic resistance phenotypes of donor and recipient strains used.
ND - None detected

resistance. Since not only epidemic but also sporadic cases of listeriosis may be transmitted by contaminated food (Poyart-Salmeron *et al.* 1992), both clinical and food isolates should be monitored for the emergence of antibiotic-resistant strains.

Antibiotic resistance is often determined by genetic information of plasmid origin. The correlation between antibiotic resistance and plasmid profile may indicate that the genetic information is plasmid-borne. Our *Listeria* strains isolated from food sources contain plasmids ranging in sizes from 2.7 to 54 kilobases. Elsewhere, other groups of researchers (Perez-Diaz *et al.* 1982; Fistrovici and Collin-Thompson 1990) have reported a very low frequency of plasmid carriage by *Listeria* species. However, their findings differed from the report of Kolstad *et al.* (1990) on the plasmid screening of *Listeria* spp. isolated from foods: where 107 of 135 strains examined contained plasmid DNA.

Antibiotic resistance in *Listeria* species is due to acquisition of two types of movable genetic elements, self-transferable plasmids and conjugative transposon (Poyart-Salmeron *et al.* 1990; Poyart-Salmeron *et al.* 1992). In five independent experiments, the kanamycin resistance of a plasmid-containing isolate, *L. innocua* L38 could be transferred to *L. monocytogenes* S15 or *L. innocua* L35 by conjugation. As could be expected, isolate with no plasmid, *L. ivanovii* L21 did not exhibit drug resistance transfer.

Analysis of the plasmid content of donor and transconjugants strains allowed us to associate a specific plasmid (54kb) with the kanamycin resistance transferred (Table 3 and Figure 1), though mobilization of low-molecular-weight (nonconjugative) plasmid of 3 kb in donor strain *L. innocua* L38 was detected. These data suggest a different genomic environment for kanamycin, nalidixic acid, streptomycin, chloramphenicol and erythromycin resistances in *Listeria* species used in the present study: in *L. innocua* L38, *L. seeligeri* L2111, *L. denitrificans* L19 and *L. ivanovii* L21 the chloramphenicol, erythromycin, nalidixic acid and streptomycin resistances are located in the chromosome. Published reports showed resistance to chloramphenicol, macrolide-lincosamid-streptogramin, tetracyline and streptomycin in *Listeria* species are usually plasmid-mediated (Robert *et al.* 1996). Thus, we report here on our observation of conjugal transfer of a plasmid-mediated kanamycin resistance at frequencies 10^{-3} per donor from a *L. innocua* isolate.

With respect to food safety, even though *Listeria* is prevalent in the ready -for-market salted fish products, as evidence by the finding of this study, these products have not been implicated as a source of listeriosis. No report is available to indicate the incidence of human listeriosis in Malaysia, and the prevalence of *Listeria* in foods is not well understood. Elsewhere, available reports indicated that infections due to ingestion of foodborne, multiresistant strains of *Listeria* has been regarded as unlikely; due to the general susceptibility of food isolates to antibiotics (Charpentier *et al.* 1995), and it has been suggested that multiple resistance might be acquired *in vivo* (Poyart-Salmeron *et al.* 1990; Quentin *et al.* 1990; Fistrovici and Collin-Thompson 1990; Schuchat *et al.* 1991), in the gastrointestinal tract or the vagina/cervix of human and animals. However, our findings show that multiple resistant strains of *Listeria* spp. are already present in food sources and that human infection due to antibiotic-resistant strains in Malaysia may stem from food sources. Thus, there is every

reason to be concerned at reports of *Listeria* species exhibiting resistance to several antibiotics.

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