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Real-time application of automated ribotyping and DNA macrorestriction analysis in the setting of a listeriosis outbreak

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SUMMARY

A cluster of three cases of listeriosis cases occurred against a background of endemic listeriosis in Western Australia. Human and environmental isolates of *Listeria monocytogenes* obtained during the outbreak investigation were rapidly subtyped by automated ribotyping using an *Eco*RI protocol and a RiboPrinter®. DNA macrorestriction analysis by pulsed-field gel electrophoresis (PFGE) was used to confirm the relatedness of isolates. Serogroup 1/2 predominated among the food samples and the four clinical isolates from the outbreak cluster were also of this serogroup. All isolates from chicken material were serogroup 1/2 and indistinguishable by ribotype pattern. PFGE subdivided strains of this ribotype into four subtypes. The preliminary analysis had an immediate impact on hypothesis generation, environmental health investigations, environmental specimen collection and initial control measures. Sufficient typing data to guide environmental health and disease control initiatives was generated in less than one week by combining automated ribotyping with PCR-based detection of *L. monocytogenes* in suspect foodstuffs and an *L. monocytogenes* DNA probe. There were no further cases of bacteriologically confirmed listeriosis in Western Australia for six months after completion of the investigation.

INTRODUCTION

Listeriosis is an uncommon infection that is potentially fatal in the foetus, newborn infants and immunocompromised adults. The majority of adult infections are foodborne while foetal and neonatal infections are usually acquired by vertical transmission from the mother. The epidemiology of listeriosis is only partially understood particularly in countries where the disease is not notifiable. Case clusters or outbreaks of the infection also occur and are usually attributed to contaminated foods. In a series of studies by the Centers for Disease Control, USA, 11% of refrigerated foods were found to be contaminated by *Listeria monocytogenes* and molecular typing was used to link clinical and food isolates in 33% cases [1, 2]. Ready-to-eat chicken was noted as a particularly common vehicle for *L. monocytogenes*.

Molecular typing methods are widely used to supplement more established typing methods such as phage and serotyping and to support epidemiological investigations of listeriosis. Molecular typing methods used for *L. monocytogenes* include restriction fragment length polymorphism analysis, ribotyping, pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis. In a comparison of three different molecular typing methods, manual ribotyping was less discriminating than PFGE and arbitrarily primed PCR [3]. All three methods had

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high typability and reproducibility. Since then, an *Eco*RI ribotyping protocol has been automated and applied for molecular typing of *L. monocytogenes* in epidemiological studies [4].

Listeriosis in Australia has a similar epidemiology to the rest of the developed world in which occasional case clusters occur against a background of sporadic disease. Outbreaks of listeriosis occurred in Western Australia in 1978–80 and in 1990–1 [5]. During 1997 an extended cluster of 11 *L. monocytogenes* serotype 4 infections prompted molecular typing of the isolates by PFGE and subsequently by restriction fragment length polymorphism analysis of PCR-amplified sequences from the listeriolysin gene using the enzymes *Alu*I and *Mse*I [6]. These methods linked clinical isolates with isolates from chicken products. Following an improvement in local food production conditions, the number of culture-confirmed cases of listeriosis fell to only two in 1998.

We report here a comparison of ribotyping and DNA macrorestriction analysis of *L. monocytogenes* and the application of these methods during a subsequent listeriosis outbreak investigation.

METHODS

Bacterial isolates

Clinical *L. monocytogenes* isolates were obtained from diagnostic microbiology laboratories in Western Australia as part of a continuous surveillance programme. Food isolates were obtained from the Western Australian Food Hygiene Laboratory (PathCentre) and referring environmental microbiology laboratories. A collection of 17 reference strains was provided through the (Australian) National Public Health Laboratory Network.

Outbreak investigation

A cluster of three listeriosis cases occurred in Western Australia one of which resulted in a foetal death *in utero*. A prior food hygiene survey had implicated a specific manufacturer of cooked chicken products and so *L. monocytogenes* isolates from chicken sandwiches and unused finished chicken pieces were characterized by serotyping and automated ribotyping. To culture *L. monocytogenes*, a 25 g sample was processed by stomaching and enriched in two stages; (i) incubation at 30 °C for 24 h in 225 ml half-strength Fraser's broth, and (ii) incubation for 48 h at 37 °C in 10 ml full-strength Fraser's broth before plating on modified Oxford agar (Oxoid) and PALCAM agar (Oxoid). A three tube most probable number method (MPN) was used. The lower limit of detection was 3 and the upper limit 1100 MPN/g. Environmental swabs were enriched by incubation at 30 °C in University of Vermont Medium for 24 h before undergoing a second enrichment stage as above. *L. monocytogenes* detected in foodstuffs by a PCR-based method (BAX-Listeria protocol, Dupont-Qualicon, USA) were confirmed by phenotypic tests and a specific DNA probe (*L. monocytogenes* Accuprobe, Gen-Probe, USA). Presumptive *Listeria* species that proved to be DNA probe negative were identified by API LIST (Biomerieux, France). Serotyping was performed using agglutinating antisera (Difco, USA) for serotypes 1/2 and 4.

Clinical and additional isolates from finished chicken product and samples from the production line were also ribotyped and subjected to PFGE. All isolates were submitted to the Molecular Epidemiology Laboratory with a unique numerical code and no accompanying source information or epidemiological data.

Prospective, laboratory-based surveillance of listeriosis was conducted for a further 12 months to assess the impact of control measures introduced by the Department of Health.

Molecular typing

Ribotyping was performed on live *L. monocytogenes* isolates grown for 18 h in air at 37 °C on 5% horse blood agar using *Eco*RI in an automated ribotyping system (RiboPrinter®, Qualicon) according to the manufacturer's instructions and with proprietary software.

DNA macrorestriction analysis was performed by PFGE as follows. Each isolate was grown in air at 37 °C on blood agar and 10 colonies were used to inoculate TE: 10 mM Tris (pH 7·4) (Gibco, Australia) 0·1 mM EDTA (BDH, Australia) buffer to a turbidity equivalent of MacFarland 5·0 in 150 μl volume. After centrifugation, cells were resuspended in 100 μl of the same buffer and 5 μl of 20 mg/ml lysosyme was added at 37 °C for 15 min followed by 10 μl of 1 mg/ml proteinase K (Promega, USA). Agarose plugs were made from a 1:1 mixture of cell suspension and 1·8% low melting point preparative grade agarose (BioRad, USA). Each plug was suspended in lysis buffer (50 mM Tris pH 7·4, 50 mM EDTA, 1% lauroyl sarcosine (Sigma, USA), containing 400 μg/ml proteinase K for 18 h at 50 °C. After 4×1 h washes with TE buffer,

a 1 h wash with 0·1 TE and a 30 min wash in RE buffer, the plugs were incubated overnight separately with *Sma*I and *Apa*I (Promega) according to the manufacturer's protocol. DNA fragments were separated by electrophoresis on a 1% pulsed field certified agarose gel in a contour clamped homogenous electric field using a CHEF DR II system (BioRad) with 0·5 × TBE buffer at 200 v at 14 °C. The switch times were linearly ramped from 5 to 18 sec over 22 h for *Sma*I and from 4 to 40 sec over 22 h for *Apa*I digests.

Analysis of typing results

During the outbreak investigation the similarity index function in the RiboPrinter® software was used to place a numeric value on the closeness of ribotype match. Digital patterns were also checked by direct visual comparison of the unprocessed ribotype images. Preliminary comparison of DNA macrorestriction patterns was made by direct visual inspection and confirmed by scanning the gels and analysing the image using GelCompar software (version 1.6, Applied Maths, Kortrijk, Belgium). Discrimination indices were calculated according to a previously described method [7]. The entire data set was imported into a molecular typing analysis programme (Bionumerics version 2.01, Applied Maths). Dendrograms of UPGMA type were produced by the software using the DICE coefficient. A composite dendrogram was generated based on the similarity index taken from each typing system.

RESULTS

Comparison of automated ribotyping with DNA macrorestriction

All *L. monocytogenes* isolates were typable using the *Eco*RI automated ribotyping protocol. The discrimination index was high (0·78) and comparable with the DNA macrorestriction protocols used (both 0·80). Three and four isolates respectively were not typable by *Sma*I and *Apa*I macrorestriction due to autodegradation. Both PFGE methods took significantly longer than automated ribotyping. The estimated cost of ribotyping a single isolate of *L. monocytogenes* (AUD 138) was around twice the cost of DNA macrorestriction (AUD 66).

PFGE subdivided strains in the culture collection belonging to ribotype 22-S-5 into 4 *Sma*I and 4 *Apa*I types (Table 1). However, among the clinical case cluster and chicken product isolates all but one isolate belonged to a single *Sma*I and *Apa*I profile. The composite dendrogram produced from the combination of serotype, ribotype and PFGE analysis placed the four clinical isolates from the temporal case-cluster in close proximity (Fig. 1). These isolates were interspersed with environmental isolates collected during the outbreak investigation, and separated into minor branches of the dendrogram due to small differences in the results of separate gels. Three dimensional cluster analysis (data not shown) confirmed that this group of isolates remained as a cluster, and lay at the centre of a much larger cluster that was distinct from other clinical, environmental and type culture isolates.

Outbreak investigation

The four clinical *L. monocytogenes* isolates belonged to serotype 1/2 (Table 1). Prior to the outbreak, *L. monocytogenes* was isolated from four food samples (Table 2). Two non-chicken items contained *L. monocytogenes* serotype 4 in counts that were undetectable in directly plated preparations. The two chicken products contained serotype 1/2 at counts of > 1100 MPN/g. Of the 30 chicken product specimens collected during the outbreak investigation only 2 had counts too low to be detected on direct culture; 12 of the remaining 28 gave counts of > 1100 MPN/g. Culture-based methods took 24–48 h to produce evidence of listeria growth and a further 24 h to confirm the identity of presumptive isolates. The PCR assay detected *L. monocytogenes* within the same working day as food specimen preparation, bringing forward presumptive positive results by 24 h or more. The use of a DNA probe to confirm the identity of presumptive *L. monocytogenes* food isolates also accelerated laboratory reporting and subsequent public health decision-making.

Isolates of *L. monocytogenes* from chicken material were of the same serotype (1/2) and ribogroup (22-S-5) (Table 1, Fig. 1). Further analysis placed the clinical isolates in the same ribogroup as food isolates, with a high similarity index for all isolates from the investigation and this was confirmed by *Sma*I macrorestriction. Each ribotype analysis took 8 h and the time between the first and completion of the last analysis was just over one week. The principal rate-limiting factors were the speed with which samples could be collected, *L. monocytogenes* isolated and its identity confirmed.

Table 1. *Subtyping of 93* L. monocytogenes *isolates by ribotype, PFGE profile and serogroup*

ME no.	Source	Ribogroup	PFGE *Sma*I	PFGE *Apa*I	Serogroup
454	PHLN	19-S-1	NT	NT	4
470	PHLN	19-S-1	NT	NT	4
455	PHLN	19-S-2	15	21	4
459	PHLN	19-S-2	15	14	4
464	PHLN	19-S-2	15	22	4
467	PHLN	19-S-2	15	16	4
469	PHLN	19-S-2	15	21	4
1057	Clinical	19-S-2	15	19	4
1058	Clinical	19-S-2	16	8	4
1152	Food	19-S-2	15	8	4
1173	Clinical	19-S-2	18	19	4
456	PHLN	19-S-3	8	7	4
458	PHLN	19-S-3	8	5	4
460	PHLN	19-S-3	10	6	1/2
462	PHLN	19-S-3	8a	7	4
466	PHLN	19-S-3	5	1	1/2
468	PHLN	19-S-3	8	7	4
495	Food	19-S-3	9	3	1/2
500	Food	19-S-3	8a	12	4
501	Food	19-S-3	8a	12	4
509	Clinical	19-S-3	19	7	4
513	Food	19-S-3	11	13	1/2
527	Food	19-S-3	8	12	4
671	Food	19-S-3	7	1	4
457	PHLN	19-S-4	4a	9	1/2
514	Food	19-S-4	4	9	1/2
516	Food	19-S-4	4	9	1/2
519	Food	19-S-4	4	9	1/2
520	Food	19-S-4	4	9	1/2
662	Food	19-S-4	14	18	1/2
664	Food	19-S-4	14	18	1/2
461	PHLN	19-S-8	12	10	1/2
465	PHLN	20-S-4	7	11	1/2
463	PHLN	22-S-1	NT	NT	1/2
494	Food	22-S-1	1	15	1/2
499	Food	22-S-1	2	20	1/2
505	Food	22-S-1	2	20	1/2
506	Food	22-S-1	13	4	1/2
517	Food	22-S-1	3	17	1/2
518	Food	22-S-1	1	15	1/2
521	Food	22-S-1	1	15	1/2
522	Food	22-S-1	1	15	1/2
523	Food	22-S-1	1	15	1/2
661	Food	22-S-1	2a	20	1/2
668	Food	22-S-1	1	15	1/2
669	Food	22-S-1	1	15	1/2
670	Food	22-S-1	1	15	1/2
673	Food	22-S-1	1	15	1/2
496	Food	22-S-5	5	1	1/2
497	Food	22-S-5	5	1	1/2
498	Food	22-S-5	5	1	1/2
504	Food	22-S-5	5	1	1/2
507	Food	22-S-5	5	1	1/2
508	Food	22-S-5	6	2	1/2
524	Clinical	22-S-5	5a	1	1/2

Table 1 (*Cont.*)

ME no.	Source	Ribogroup	PFGE *Sma*I	PFGE *Apa*I	Serogroup
525	Clinical	22-S-5	5	1	1/2
526	Clinical	22-S-5	5	1	1/2
658	Clinical	22-S-5	5	1	1/2
659	Clinical	22-S-5	5	1	1/2
665	Food	22-S-5	5	11	1/2
666	Clinical	22-S-5	5	1	1/2
667	Food	22-S-5	7	1	1/2
739	Clinical	22-S-5	5	1	1/2
740	Clinical	22-S-5	5	1	1/2
741	Clinical	22-S-5	5	1	1/2
1054	Food (chops)	22-S-5	8	7	4
1056	Food (chicken)	22-S-5	5	1	1/2
1059	Clinical	22-S-5	5	1	1/2
1060	Clinical	22-S-5	5	1	1/2
1145	Food (chicken)	22-S-5	5	1	1/2
1146	Food (chicken)	22-S-5	5	1	1/2
1147	Food (chicken)	22-S-5	5	1	1/2
1148	Food (chicken)	22-S-5	5	1	1/2
1150	Food (chicken)	22-S-5	5	1	1/2
1151	Food (chicken)	22-S-5	5	1	1/2
1158	Food (chicken)	22-S-5	5	1	1/2
1159	Food (chicken)	22-S-5	5	1	1/2
1153	Food (chicken)	22-S-5	5	1	1/2
1154	Food (chicken)	22-S-5	5	1	1/2
1155	Food (chicken)	22-S-5	5	1	1/2
1156	Food (chicken)	22-S-5	5	1	1/2
1157	Food (chicken)	22-S-5	5	1	1/2
1160	Food	22-S-5	5	1	1/2
1161	Clinical	22-S-5	5	1	1/2
1162	Clinical	22-S-5	5	1	1/2
1163	Clinical	22-S-5	5	1	1/2
515	Food	23-S-3	4	9	1/2
503	Food	25-S-1	4	9	1/2
510	Food	25-S-1	4	9	1/2
511	Food	25-S-1	4	9	1/2
512	Food	25-S-1	4	9	1/2
1053	Food (alfalfa)	50-S-1	17	NT	
1055	Food (chicken)	50-S-3	1	15	1/2

DISCUSSION

While there have been many instances where, with the aid of molecular typing methods, a food item has been implicated as a source of *L. monocytogenes* infection, there has as yet been no report of real-time application of molecular typing in a listeriosis outbreak investigation. It has therefore been difficult to assess the practical contribution of molecular epidemiology to outbreak control. A lack of real-time experience and the relatively high cost of molecular typing methods have prevented public health agencies from recognizing their value in the management of food-borne infection.

Ribotyping has been used previously to analyse *L. monocytogenes* isolates from apparent listeriosis case clusters and to link human infection with suspected food sources [4, 8–10]. The time taken to produce a result and the high level of technical skill required make the method unsuitable for routine public health applications. DNA macrorestriction has been much more widely used for public health investigations and is now the primary method for collaborative epidemiological surveillance [11–13]. The automation of ribotyping has permitted centres equipped with a RiboPrinter® to perform molecular typing more quickly and with considerable reproducibility. Recent experience with *Eco*RI ribotyping of

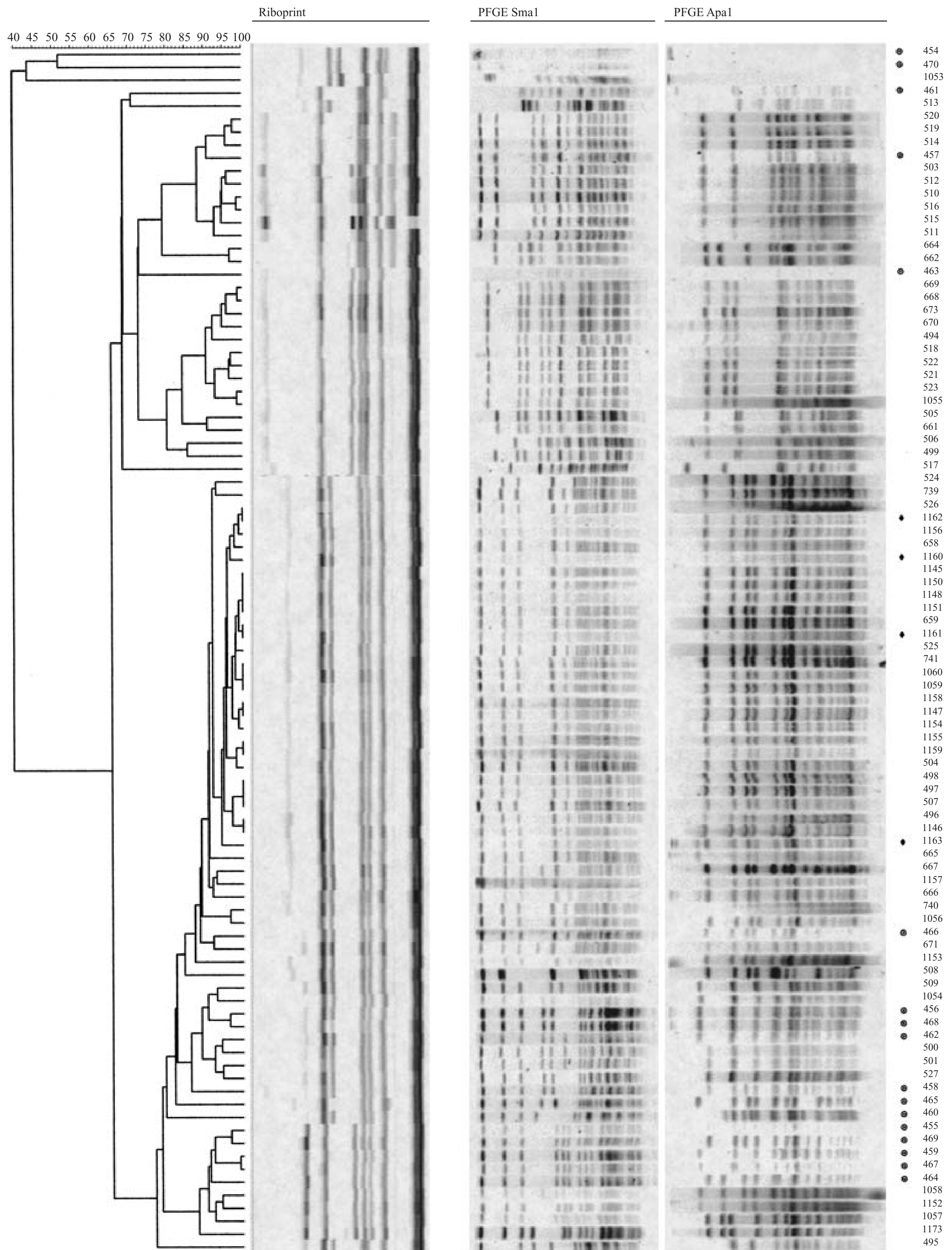

Fig. 1. Composite dendrogram of *Eco*RI ribotype patterns and PFGE with *Sma*I and *Apa*I. Gel images have been included to allow direct visual comparison. Food isolates (unmarked), clinical isolates (◆) and strains from nationally representative collection (⊙) are shown.

Table 2. *Foodstuffs and source of environmental specimens positive for* L. monocytogenes

Date	Isolate	Foodstuff	Source	Count*	Serogroup
3/7/00	097/00	Alfalfa & chives	Supermarket 1	<3	4
3/10/00	098/00	Raw chops	Manufacturer 1 via Laboratory 1	<3	4
28/3/00	118/00	Chicken sandwich	City EHO 1	>1100	1/2
27/3/00	120/00	Chicken sandwich	City EHO 2	>1100	1/2
7/4/00	127/00	Cooked diced chicken	Manufacturer 1	>1100	1/2
17/4/00	134/00	Chicken sandwich	City EHO 3	1100	1/2
28/4/00	138/00	Chicken sandwich	Coffee shop 1	210	1/2
2/5/00	141/00	Cooked diced chicken	Manufacturer 1	>1100	1/2
2/5/00	142/00	Cooked diced chicken	Manufacturer 1	>1100	1/2
12/5/00	154/00	Chicken roll	Coffee shop 2	>1100	1/2
23/5/00	162/00	Chicken sandwich	Coffee shop 3	>1100	1/2
1/6/00	166/00	Chicken & salad sandwich	City EHO 4	>1100	1/2
1/6/00	168/00	Chicken sandwich	City EHO 4	>1100	1/2
2/6/00	173/00	Cooked chicken meat	Manufacturer 1	240	1/2
2/6/00	174/00	Cooked chicken meat	Manufacturer 1	75	1/2
9/6/00	175/00	Cooked chicken meat	Manufacturer 1	>1100	1/2
9/6/00	176/00	Cooked chicken meat	Manufacturer 1	>1100	1/2
13/6/00	183/00	Cooked chicken	Manufacturer 1 via Laboratory 2		4
13/6/00	184/00	Cooked chicken	Manufacturer 1 via Laboratory 2		1/2
13/6/00	185/00	Cooked chicken	Manufacturer 1 via Laboratory 2		1/2
13/6/00	186/00	Cooked chicken	Manufacturer 1 via Laboratory 2		1/2
13/6/00	187/00	Cooked chicken	Manufacturer 1 via Laboratory 2		1/2
13/6/00	188/00	Cooked chicken	Manufacturer 1 via Laboratory 2		1/2
21/6/00	190/00	Cooked chicken meat	Manufacturer 1	<3	1/2
21/6/00	191/00	Cooked chicken meat	Manufacturer 1	23	1/2
16/6/00	196/00	Cooked chicken	Manufacturer 1	>1100	1/2
16/6/00	197/00	Cooked chicken	Manufacturer 1	>1100	1/2
16/6/00	198/00	Cooked chicken meat	Manufacturer 1	>1100	1/2
16/6/00	199/00	Cooked chicken meat	Manufacturer 1	>1100	1/2
20/8/00	201/00	Cooked diced chicken	Manufacturer 1	23	1/2
20/6/00	202/00	Cooked diced chicken	Manufacturer 1	4	1/2
20/6/00	203/00	Cooked diced chicken	Manufacturer 1	15	1/2
21/6/00	204/00	Cooked diced chicken	Manufacturer 1	<3	1/2
28/6/00	244/00	Cooked diced chicken	Manufacturer 1	43	1/2
30/6/00	246/00	Cooked diced chicken	Manufacturer 1	7	1/2
4/7/00	247/00	Cooked diced chicken	Manufacturer 1	33	1/2

* MPN/g.

L. monocytogenes serotype 4 suggests that the automated method works well and can also be performed with a variety of different restriction enzymes in addition to *Eco*RI [4, 14].

A previous comparison of *L. monocytogenes* typing by multilocus enzyme electrophoresis (the method used for the 1990/1 WA listeriosis outbreak) and conventional ribotyping suggested that neither method was sufficiently discriminating for subtyping serotypes 1/2 and 4 [9]. Furthermore, a more recent comparison of five different typing methods found that a combination of ribotyping and PFGE was the most discriminating for *L. monocytogenes* typing [10]. It is therefore noteworthy that automated ribotyping and PFGE of our isolate collection gave highly similar discrimination indices and strain relationships.

The additional discrimination achieved by composite analysis with Bionumerics software further enhanced our ability to identify a cluster within our extensive library of previously analysed isolates.

Human listeriosis is one of the less common food-borne infections but it remains a significant potential cause of death *in utero*, in newborns, in the elderly and the immunocompromised. Although *L. monocytogenes* is a common contaminant of chicken carcasses, chicken products are not often reported as a major vehicle for human listeriosis and, then, usually as a cause of sporadic disease [2, 15]. The mismatch between *L. monocytogenes* demonstrable in prepacked sandwiches and other ready-to-eat foods and clinical listeriosis has already been noted [16]. The finding of high counts of *L. monocytogenes* in chicken sandwiches and an unopened bag of cooked chicken pieces was evidence of a failure in food hygiene practice at some point in the process. The first ribotyping results helped link the isolates obtained from these diverse items and these data accelerated the process of epidemiological hypothesis development, securing the co-operation of the product's manufacturer. The similarity of the clinical *L. monocytogenes* isolates to these food isolates gave momentum to the environmental health investigation at the production plant and underpinned recommendations for additional control measures. These included recall of unsold product, steam cleaning of the production plant and an additional heat treatment at the end of the production line.

It was six months before another case of listeriosis was recorded in Western Australia. In view of the fact that salmonellosis and campylobacter infection have also been linked with the consumption of contaminated poultry products in Western Australia, it will be interesting to see whether environmental control measures introduced to deal with this cluster of cases alter the incidence of other food-borne infections.

It was noted that one of the two ribotypes (19-S-2) of *L. monocytogenes* found in the chicken product was also represented in the panel of national typing strains. Examination of archived ribotype data revealed two clinical isolates with this ribotype from infections diagnosed previously in WA. Other studies have shown that a given type of *L. monocytogenes* can be widely distributed, and that different types may be present in the same contaminated environment [8, 17]. We were able to confirm from the manufacturer that raw chicken had been obtained from the Eastern States to maintain continuity of product output.

Integration of a rapid molecular typing system with the test repertoire of a public health laboratory enabled a more rapid response to a case-cluster of listeriosis than had been possible previously. Though we have shown how a combination of complementary molecular microbiology methods might impact on the investigation and control of one cluster of a specific food-borne infection, it remains to be seen how effective this approach is when applied to long term prospective surveillance or to other food-borne pathogens.

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