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Comparison of Epidemiological Marker Methods for Identification of *Salmonella typhimurium* Isolates from an Outbreak Caused by Contaminated Chocolate

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Plasmid profile analysis, restriction endonuclease analysis, and multilocus enzyme electrophoresis were used in conjunction with serotyping, bacteriophage typing, and biochemical fingerprinting to trace epidemiologically related isolates of *Salmonella typhimurium* from an outbreak caused by contaminated chocolate products in Norway and Finland. To evaluate the efficiency of the epidemiological marker methods, isolates from the outbreak were compared with five groups of control isolates not known to be associated with the outbreak. Both plasmid profile analysis and phage typing provided further discrimination over that produced by serotyping and biochemical fingerprinting. Plasmid profile analysis and phage typing were equally reliable in differentiating the outbreak isolates from the epidemiologically unrelated controls and were significantly more effective than multilocus enzyme electrophoresis and restriction enzyme analysis of total DNA. The greatest differentiation was achieved when plasmid profile analysis and phage typing were combined to complement serotyping and biochemical fingerprinting. However, none of the methods employed, including restriction enzyme analysis of plasmid DNA, were able to distinguish the outbreak isolates from five isolates recovered in Norway and Finland over a period of years from dead passerine birds and a calf.

It has been suggested that many sporadic cases of salmonellosis may actually be part of unrecognized outbreaks which escape detection because of the lack of efficient epidemiological markers (13, 14). Although well-established methods, such as serotyping, bacteriophage typing, and biochemical fingerprinting, have been crucial in identifying outbreaks and tracing sources of infection, these methods are useful only if the organisms have unusual characteristics. In recent years, epidemiological investigations have been substantially improved by the application of molecular techniques, including plasmid profile analysis (PPA), restriction endonuclease analysis (REA) (DNA restriction fragment polymorphism), and multilocus enzyme electrophoresis (MEE). Although isolates may easily lose or gain plasmids, PPA has proved most useful in discriminating between related and unrelated *Salmonella* isolates from outbreaks (8, 13, 15, 18). REA elucidates restriction site heterogeneity of genomic DNA and has been used successfully in epidemiological studies of many bacterial infections (4, 5, 9, 10, 19). MEE, which compares the relative electrophoretic mobilities of a large number of cellular enzymes, has been used to estimate the genetic diversity of natural populations of a variety of bacterial species and has provided extensive data for taxonomic and epidemiological purposes (16, 17).

Salmonellosis is a growing concern to the chocolate industry (6). In 1987, an outbreak of *Salmonella typhimurium* infection, caused by contaminated chocolate products, occurred in Norway and Finland. In the present study, we used PPA, REA, and MEE in conjunction with serotyping, phage typing, and biochemical fingerprinting to trace epidemiologically related isolates from that outbreak. To evaluate the efficiency of the different epidemiological marker systems, five groups of control isolates with no known association with the outbreak were included in the study.

MATERIALS AND METHODS

Outbreak. During the spring of 1987, the Norwegian *Salmonella* Reference Center received a series of *S. typhimurium* isolates, a majority of which were from young children who had not travelled abroad. Since isolates with identical characteristics were obtained simultaneously from most of the medical microbiological laboratories in the country, it was suggested that a common-source outbreak of nationwide distribution was taking place. Epidemiological investigations conducted at the Food Inspection Service Laboratory in Trondheim incriminated a particular chocolate product manufactured by one Norwegian company as a possible vehicle of transmission, and the epidemic strain was subsequently isolated from samples of this product at the stage of retail sale. Ultimately, the epidemic strain was recovered by different laboratories throughout the country from several chocolate products manufactured by the same firm. The company concerned was also supplying chocolate to Finland, and the epidemic strain was eventually recovered from chcolate products and human patients in that country. The outbreak came to an end after the incriminated chocolate products were withdrawn from the market. In Norway, a total of 349 bacteriologically verified cases were recorded. A detailed description of the outbreak will be presented elsewhere.

Bacterial strains. A total of 53 *S. typhimurium* isolates were selected for detailed examination. These strains included one group of outbreak isolates (group 1) and five groups of isolates not known to be associated with the outbreak or with each other (groups 2 through 6). The isolates are listed in Table 1.

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TABLE 1. Comparison of epidemiological marker methods for differentiating six groups of *S. typhimurium* isolates

Strain[a]	Source	Epidemiological marker[b]					
		SERO[c]	BIO	PHAGE	PPA[d]	REA	MEE
Group 1							
1429/86	Human, Norway	4-12:i:**1,2**	+	U277	1*	+	+
210/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
217/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
221/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
224/87	Human, Norway	4-12:i:**1,2**	+	U277	1*	+	+
225/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
250/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
22/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
270/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
394/87	Human, Norway	4-12:i:**1,2**	+	U277	1	+	+
976/87	Human, Finland	4-12:i:**1,2**	+	U277	1	+	+
977/87	Human, Finland	4-12:i:**1,2**	+	U277	1*	+	+
364/87	Chocolate, Norway	4-12:i:**1,2**	+	U277	1*	+	+
365/87	Chocolate, Norway	4-12:i:**1,2**	+	U277	1	+	+
968/87	Chocolate, Finland	4-12:i:**1,2**	+	U277	1*	+	+
970/87	Chocolate, Finland	4-12:i:**1,2**	+	U277	1	+	+
395/87	Dog, Norway	4-12:i:**1,2**	+	U277	1	+	+
Group 2							
1187/87	Human, 1982	4-12:i:**1,2**	+	U277	2	+	+
1188/87	Human, 1982	4-12:i:**1,2**	+	U277	2	+	+
1189/87	Human, 1982	4-12:i:**1,2**	+	U277	2	+	+
1190/87	Bullfinch,[e] 1982	4-12:i:**1,2**	+	U277	3	+	+
1191/87	Bullfinch, 1982	4-5-12:i:**1,2**	−	U277	3	+	+
1192/87	Bullfinch, 1982	4-12:i:**1,2**	+	U277	2	+	+
1193/87	Bullfinch, 1984	4-12:i:**1,2**	+	U277	1*	+	+
1194/87	Calf, 1984	4-12:i:**1,2**	+	U277	1*	+	+
1195/87	Bullfinch, 1985	4-12:i:**1,2**	+	U277	2	+	+
1196/87	Ground peanuts, 1985	4-12:i:**1,2**	+	U277	2	+	+
1197/87	Bullfinch, 1986	4-12:i:**1,2**	+	U277	2	+	+
1198/87	Bullfinch, 1986	4-12:i:**1,2**	+	U277	1	+	+
Group 3							
106/88	Sparrow hawk,[f] 1982	4-12:i:**1,2**	+	40	1*	+	+
111/88	Tree sparrow,[g] 1982	4-12:i:**1,2**	+	40	1*	+	+
107/88	Bullfinch, 1983	4-12:i:**1,2**	+	40	1*	+	+
108/88	Bullfinch, 1983	4-12:i:**1,2**	+	40	1*	+	+
109/87	Bullfinch, 1983	4-12:i:**1,2**	+	40	1*	+	+
110/87	Greenfinch,[h] 1983	4-12:i:**1,2**	+	40	1*	+	+
71/88	Bullfinch, 1985	4-12:i:**1,2**	+	40	1*	+	+
74/88	Bullfinch, 1987	4-12:i:**1,2**	+	U277	1*	+	+
75/88	Bullfinch, 1987	4-12:i;**1,2**	+	U277	1*	+	+
103/88	Bullfinch, 1988	4-12:i:**1,2**	+	U277	1*	+	+
Group 4							
591/87	Chicken wings	4-5-12:i:1,2	−	10	4	+	+
593/87	Chicken wings	4-5-12:i:1,2	−	10	4	+	+
595/87	Chicken wings	4-5-12:i:1,2	−	66	4	+	+
596/87	Chicken wings	4-5-12:i:**1,2**	−	10	4	+	+
256/87	Human	4-5-12:i:1,2	−	10	4	+	+

Continued

Phenotypic characterization of isolates. Serotyping, biochemical characterization, and antimicrobial susceptibility testing were carried out at the Norwegian *Salmonella* Reference Center. All isolates were serotyped according to standard procedures (7). Thirteen isolates were subjected to a detailed examination which involved 40 biochemical parameters and resistance to 11 antibiotics. The biochemical test reactions were selected among the differentiae recommended by Ewing (7). The results were read after 24 and 48 h. The strains were further tested for susceptibility to antimicrobial agents by using the agar diffusion method with commercial antibiotic tablets (Neo-Sensitabs; Rosco Diagnostica, Taastrup, Denmark). The 11 antibiotics assayed were ampicillin, cloxacillin, cefuroxime, doxycycline, trimethoprim-sulfamethoxazole, tobramycin, amdinocillin, cefotaxime, ceftazidime, azlocillin, and chloramphenicol.

Phage typing was done at the National Public Health Institute in Helsinki, Finland, using the extended typing scheme of Anderson et al. (1). The phage lysis patterns of 19 of the isolates were confirmed by the World Health Organization Collaborating Centre for Phage Typing and Resistance of Enterobacteria, Colindale, England (courtesy of B. Rowe).

Plasmid analysis. Bacteria were cultured overnight at 37°C

TABLE 1—*Continued*

Strain[a]	Source	Epidemiological marker[b]					
		SERO[c]	BIO	PHAGE	PPA[d]	REA	MEE
Group 5							
958/86	Human	4-5-12:i:1,2	−	120	5	−	+
1043/86	Human	4-12:i:**1,2**	−	104	6	−	+
1077/86	Human	4-5-12:i:1,2	−	15a	7	−	+
1069/86	Human	4-5-12:i:**1,2**	−	15a	8	−	+
Group 6							
7/87	Human	4-5-12:i:1,2	−	93	9	−	+
18/87	Human	4-5-12:i:1,2	−	93	10	−	+
39/87	Human	4-5-12:i:**1,2**	−	93	10	−	+
809/87	Human	4-5-12:i:**1,2**	−	8	11	−	+
902/87	Human	4-5-12:i:1,2	−	108	12	−	+

[a] Groups: 1, 17 outbreak isolates from Norway and Finland, 1987; 2, 12 isolates from Finland, 1982 to 1986; 3, 10 avian isolates from Norway; 4, 5 isolates from imported chicken and from a related human case; 5, 4 isolates from travellers returning from abroad; 6, 5 isolates from workers at oil-drilling rigs in the North Sea, 1987.

[b] The isolates were compared by serotyping (SERO), biochemical fingerprinting (BIO), phage typing (PHAGE), plasmid profile analysis (PPA), restriction endonuclease analysis of total DNA (REA), and multilocus enzyme electrophoresis (MEE). +, Same characteristics as the outbreak isolates (group 1); −, differs from the outbreak isolates.

[c] The dominant H-antigen phase is in boldface type.

[d] The numbers 1 to 12 indicate 12 different plasmid profiles (see Fig. 1). *, Strains carrying plasmids with identical *Taq*I and *Hae*III restriction sites.

[e] *Pyrrhula pyrrhula*.

[f] *Accipiter nisus*.

[g] *Passer montanus*.

[h] *Carduelis chloris*.

in Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy broth with 0.6% yeast extract. The plasmid profile was investigated by using a small-scale modification of the alkaline lysis technique of Birnboim and Doly (3) as described by Maniatis et al. (12), followed by electrophoresis for 3 h at 120 V on a 0.7% horizontal agarose gel in Tris-borate buffer (12). Plasmid DNAs from *Escherichia coli* VA517 (11) and J5 (RI) were included as molecular weight standards.

Seventeen plasmid preparations were analyzed by restriction endonuclease digestion, using the enzymes *Taq*I and *Hae*III under conditions recommended by the supplier (Toyobo Co. Ltd., Osaka, Japan). The resulting digests were subjected to electrophoresis for 1 h at 200 V on a 7.5% vertical polyacrylamide gel, using a Mini-Protean II slab gel cell (Bio-Rad Laboratories, Richmond, Calif.).

REA of total DNA. Bacteria were cultured at 37°C overnight in Luria broth. Total DNA was extracted, purified, and subsequently cleaved with the restriction enzyme *Hin*dIII, as described previously (10). The resultant DNA fragments were separated by electrophoresis for 20 h at 40 mA (constant current) on a 4% vertical polyacrylamide gel (10).

MEE. MEE was performed by the methods described by Selander et al. (16). Each isolate was grown overnight in 100 ml of nutrient broth (Difco Laboratories, Detroit, Mich.) at 37°C with agitation. Cells were harvested by centrifugation, suspended in 1 ml of buffer (0.01 M Tris, 0.001 M EDTA, pH 6.8), and sonicated for 1 min with ice-water cooling. After centrifugation at 20,000 × *g* for 20 min at 4°C, the supernatant (lysate) was stored at −70°C. Lysates were electrophoresed on starch gels and selectively stained for 18 metabolic enzymes as previously described (16). Buffer system A was used for electrophoresis of malate dehydrogenase, malic enzyme, 6-phosphogluconate dehydrogenase, isocitrate dehydrogenase, glucose 6-phosphate dehydrogenase, adenylate kinase, carbamate kinase, and two peptidases. Buffer system C was used for electrophoresis of alcohol dehydrogenase, indophenol oxidase, glutamic-oxaloacetic transaminase, and mannose phosphate isomerase. Glutamate dehydrogenase, phosphoglucomutase, two alkaline phosphatases, and fumarase were electrophoresed in buffer system D.

RESULTS

Phenotypic characterization. All isolates recovered from human patients and chocolate products in Norway and Finland during the outbreak lacked the O-antigen factors 1 and 5. The phase 2 H-antigens 1,2 predominated, whereas the phase 1 determinant i was suppressed and required phase reversal to become detectable. To determine additional phenotypic parameters suitable for identification of the epidemic strain, 3 isolates from the chocolate outbreak were compared with 10 epidemiologically unrelated isolates in a battery of 51 tests comprising 40 biochemical characteristics and resistance to 11 antibiotics. All isolates were susceptible to the antibiotics tested, but the outbreak isolates differed from the controls by a positive reaction for xylose (after 2 days of incubation) and negative reactions for inositol and rhamnose. All subsequent isolates were consequently tested for these characteristics. All strains which showed the characteristic antigenic pattern of the epidemic strain exhibited these biochemical properties.

Ten outbreak isolates from patients ($n = 5$) and chocolate ($n = 5$) in Norway were phage typed and found to exhibit the same uncommon phage lysis pattern as isolates obtained from the outbreak in Finland (phage type U277; B. Rowe, personal communication). A small but consistent number of isolates with this phage type have been isolated in Finland since 1982 from dead passerine birds, especially bullfinches (*Pyrrhula pyrrhula*), and from about 30 patients, many of which had fed wild birds. One isolate was obtained from ground peanuts imported as feed for wild birds, and another isolate was recovered from a calf. Twelve of these strains were selected for detailed examination (group 2). With one exception, none of them could be distinguished from the outbreak isolates by means of serological, biochemical, or phage lysis characteristics (Table 1).

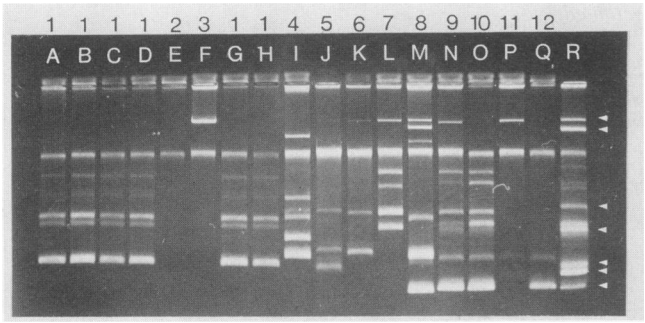

FIG. 1. Agarose gel electrophoresis of partially purified plasmid DNA from *S. typhimurium*. The numbers 1 to 12 represent 12 different plasmid profiles (see Table 1). Lanes: A to D, strains 224/87, 976/87, 364/87, and 968/87 (group 1); E to F, strains 1187/87, 1190/87, and 1193/87 (group 2); H, strain 75/88 (group 3); I, strain 591/87 (group 4); J to M, strains 958/86, 1043/86, 1077/86, and 1069/86 (group 5); N to Q, strains 7/87, 18/87, 809/87, and 902/87 (group 6); R, standard plasmids (top to bottom: 93.6, 54.1, 7.2, 5.1, 3.0, 2.7, and 2.1 kilobases).

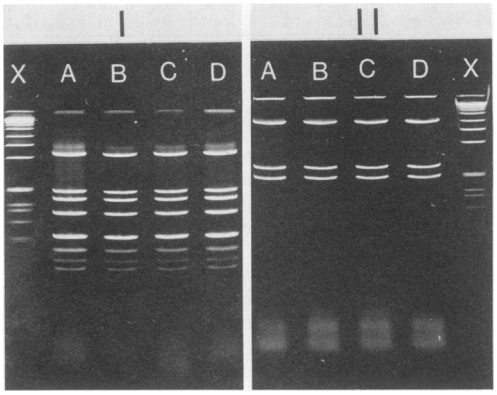

FIG. 2. Polyacrylamide gel electrophoresis of *Taq*I and *Hae*III digests of plasmid DNA from *S. typhimurium*. (I) *Taq*I cleavage patterns. (II) *Hae*III cleavage patterns. Isolates were from a patient from the chocolate outbreak in Norway (group 1) (lane A), a chocolate product from Norway (group 1) (lane B), a bullfinch from Finland (group 2) (lane C), and a bullfinch from Norway (group 3) (lane D). In lane X is a size marker (1-kilobase DNA ladder; Bethesda Research Laboratories, Gaithersburg, Md.).

These observations suggested that the epidemic strain could have been introduced into the avian wildlife fauna in Finland several years ago through imported peanut feed. To ascertain whether the epidemic strain could be recovered from Norwegian birds, we examined 10 isolates of *S. typhimurium* obtained from wild birds in Norway in the period 1982 to 1988 (group 3). Although all of these isolates were indistinguishable from the outbreak isolates by serological and biochemical parameters, only three showed the characteristic phage lysis pattern (Table 1). The latter three isolates were obtained in 1987 and 1988 from dead bullfinches from the same county where the incriminated chocolate factory is located. The remaining seven group 3 strains belonged to phage type 40, which differed significantly from phage type U277 by 20 of 31 lysis reactions tested. Thus, it is unlikely that the discrimination of phage types 40 and U277 reflects a nonspecific variation within the method.

Analysis of plasmid DNA. Surprisingly, all of the 10 isolates obtained from dead passerine birds in Norway from 1982 through 1988 (group 3) exhibited the same plasmid profile as the 17 outbreak isolates examined (group 1), even though seven of these isolates belonged to a different phage type (Table 1 and Fig. 1). REA (*Taq*I and *Hae*III) failed to uncover any differences between plasmid DNA from these strains and from five outbreak isolates (Table 1 and Fig. 2). On the other hand, only three of the group 2 isolates, all of which belonged to the same rare phage type as incriminated in the outbreak, showed the characteristic plasmid profile. All three isolates were indistinguishable from the outbreak isolates as regards *Taq*I and *Hae*III restriction sites of plasmid DNA. The isolates in question were recovered in 1984 and 1986 from dead bullfinches and a calf in Finland.

The outbreak isolates exhibited a distinct plasmid profile which was markedly different from those detected among the epidemiologically unrelated isolates of groups 4 to 6. (Table 1 and Fig. 1). Each of four isolates from travellers returning from abroad (group 5) were characterized by a distinct plasmid profile. Likewise, four plasmid patterns were recorded among five isolates from employees at oil-drilling rigs in the North Sea (group 6). Four isolates from imported chicken wings and one single epidemiologically related human isolate (group 4) showed identical plasmid profiles distinct from those of the outbreak isolates.

REA of total DNA. The DNA fragment patterns obtained by polyacrylamide gel electrophoresis of *Hin*dIII digests of total DNA preparations, consisted of approximately 50 bands (Fig. 3). Although all of the 17 outbreak isolates included in group 1 were identical by this method, they could not be differentiated from any of the control isolates in groups 2, 3, or 4 (Table 1). In contrast, the strains of groups

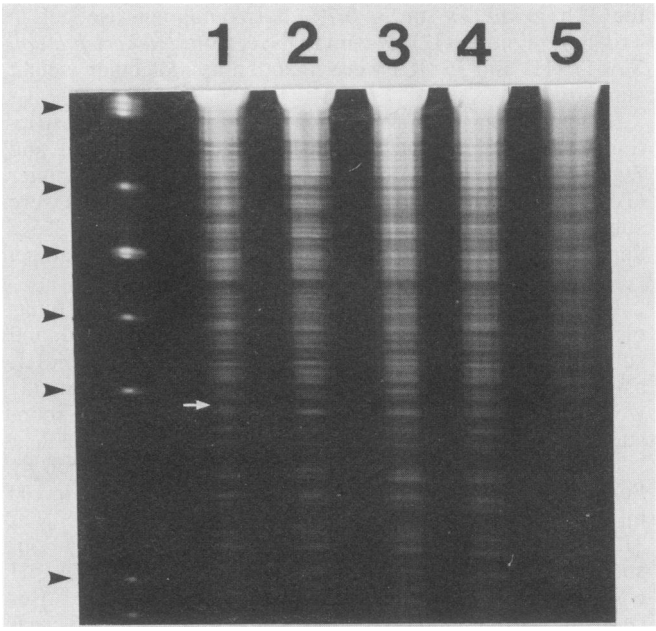

FIG. 3. Polyacrylamide gel electrophoresis of *Hin*dIII digests of whole-cell DNA from *S. typhimurium*. Isolates were from a patient from the chocolate outbreak in Norway (group 1) (lane 1), a patient from an oil-drilling rig in the North Sea (group 6) (lane 2), a traveller returning from abroad (group 5) (lane 3), a patient from the chocolate outbreak in Finland (group 1) (lane 4), and a veterinarian handling infected chickens (group 4) (lane 5). The arrow indicates the location of a band which is present in lanes 1, 4, and 5 but not in lanes 2 or 3. The size marker at left is a *Pst*I digest of lambda phage DNA (the following fragments are indicated [top to bottom]: 4.8, 2.8, 2.5, 2.0, 1.7, and 1.2 kilobases).

5 and 6 constituted a homogeneous cluster which differed from that of groups 1 to 4 by at least four DNA bands. No significant differences in banding patterns were detected within groups 5 and 6, except for one strain which lacked a single band of approximately 2 kilobases.

MEE. MEE did not reveal any differences among the *S. typhimurium* isolates included in groups 1 to 6. All 18 enzymes assayed were monomorphic. Thus, all isolates had identical multilocus genotypes (Table 1).

DISCUSSION

The detection of the present outbreak of *S. typhimurium* infection was the result of the national *Salmonella* surveillance system in which all isolates from medical and food inspection laboratories are routinely serotyped and examined biochemically by one reference institution. Although serological and biochemical tests were sufficient to distinguish the outbreak isolates (group 1) from three groups of epidemiologically unrelated controls (groups 4 to 6), these methods failed to differentiate the epidemic strain from two clusters of isolates recovered in Finland (group 2) and Norway (group 3) over a period of 7 years (Table 1).

PPA and phage typing are valuable and convenient tools of considerable versatility in epidemiological tracing. In a comparative study of *S. typhimurium* from 20 outbreaks, Holmberg et al. (8) concluded that PPA appeared to be at least as specific as phage typing in identifying epidemiologically related isolates. In accordance with their conclusions, we found that PPA enabled effective discrimination between group 1 isolates and 9 of the 12 isolates constituting group 2, even though all of these strains belonged to the same rare phage type. Likewise, group 1 isolates were easily distinguished from the controls included in groups 4 to 6. On the other hand, PPA failed to distinguish the outbreak isolates from group 3, although seven of these isolates could be differentiated on the basis of phage lysis patterns. Thus, both phage typing and PPA provided further discrimination over that produced by serotyping and biochemical fingerprinting. The greatest differentiation was achieved when PPA and phage typing were combined to complement serotyping and biochemical fingerprinting.

Although REA and MEE have been used successfully to subtype many species of bacteria (4, 16, 17), these methods were less efficient than PPA or phage typing in discriminating among the *S. typhimurium* strains included in the present study. Whereas a total of 12 different plasmid profiles were detected, REA of total DNA recognized only two distinct groups, and MEE grouped all strains together. Recent results, however, show that it is possible to differentiate *S. typhimurium* by MEE (2). The observation that strains containing identical restriction sites and identical multilocus genotypes showed different plasmid profiles might not be unexpected. Plasmids may be acquired, rearranged, or even lost, although the frequency of such events during the course of an outbreak would probably be low (8, 13). REA and MEE, on the other hand, are based mainly on analysis of the chromosome, a genetic unit that is more stable. Minor differences between REA and MEE might be expected because small mutations or base rearrangements may alter a restriction site without resulting in an amino acid substitution affecting the enzyme electrophoretic mobility (18). Although plasmid carriage may be unrelated to the underlying chromosomal structure, PPA provides a convenient epidemiological marker system that complements the other methods. PPA is not invariably useful, however (13). Although this method provided important additional discrimination, it failed to differentiate the epidemic strain from a series of isolates recovered over a period of years from dead passerine birds (group 3). This observation is not unique. Holmberg et al. (8) mentioned that a *S. typhimurium* strain, identical by plasmid profile, phage type, and antimicrobial resistance pattern, was recovered from dead sparrows in the Rocky Mountains in March of 1978 and from two separate outbreaks in New York City and Virginia 7 months later. The interrelationship among these strains was unclear. In the present study, *S. typhimurium* isolates which were indistinguishable by all methods employed were recovered from (i) human patients and chocolate products in Norway and Finland during the outbreak in 1987, (ii) three dead bullfinches in Norway in 1987 and 1988, and (iii) two dead bullfinches and a calf in Finland in 1984 and 1986. Although there is no known epidemiological relationship among these strains, the question remains whether the epidemic strain was originally derived from an avian wildlife reservoir extending over large areas in the Nordic countries.

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