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Molecular Epidemiology of Antibiotic Resistance of *Salmonella enteritidis* during a 7-Year Period in Greece

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A significant increase in the frequency of isolation of *Salmonella enteritidis* has been observed during recent years in Greece, parallelled by an increasing rate of resistance of this organism to antibiotics. A substantial proportion of ampicillin- and doxycycline-resistant isolates exhibited cross-resistance to drugs of other classes, such as sulfonamides and streptomycin. Isolates of human origin were overall less resistant than those of animal or food-feed origin. Indeed, strains associated with animal infections were characterized by the highest rates of resistance to several antibiotics. These phenotypic data were correlated with genotypic information concerning two distinct populations: isolates from all sources that were resistant only to ampicillin, the drug toward which resistance rates were highest, and a control group of sensitive isolates. Ampicillin resistance was due to a 34-MDa conjugative plasmid. DNA fingerprinting by macrorestriction of genomic DNA revealed two types, A and B, common to both ampicillin-resistant and -sensitive strains, with 80 to 90% of strains being of type A. However, a third type, C, was specific for the sensitive population, representing 17% of those strains. Therefore, although the majority of resistant isolates were genetically related to sensitive ones, there existed a susceptible clone which had not acquired any resistance traits.

Nontyphoidal salmonellae are recognized as one of the principal causes of food poisoning worldwide (5, 16, 25), with an estimated annual incidence of 1.3 billion cases and 3 million deaths (34). Furthermore, they can cause severe infections, such as septicemia and endocarditis (10), empyema (3), and meningitis (7), especially in immunocompromised hosts. They have also been shown, in a population-based surveillance system, to be prevalent pathogens among AIDS patients (4).

In the last decade, there has been an increased incidence of gastrointestinal infections caused by *Salmonella enteritidis*, which has now become the predominant serotype in many countries. According to World Health Organization surveillance data, already in 1987 this serotype was the prevalent serotype in humans with *Salmonella* infection in eight European countries (28).

Several molecular typing methods have been used for epidemiological studies of *S. enteritidis*, including plasmid profiling, ribotyping, hybridization of genomic DNA with the insertion sequence IS*200*, and pulsed-field gel electrophoresis (PFGE) of macrorestricted genomic DNA (8, 17, 22, 26, 27, 32, 34, 36). The latter has been shown to have the highest discriminatory power compared to those of the other methods (22). It can discriminate between strains of the same phage type (27), as well as provide a point of reference for phylogenetic studies, since a PFGE profile can remain stable even after phage type conversion (26).

In Greece, a progressive increase in the prevalence of *S. enteritidis* has been evident (23, 37). However, precise data describing the prevalence and the patterns of resistance of this serotype have not been published until now.

We therefore conducted a study of the distribution of *S. enteritidis* among *Salmonella* strains referred to the National Reference Center for *Salmonella* and *Shigella* from various parts of Greece during a 7-year period. We then tested the susceptibilities of a representative sample of *S. enteritidis* isolates to a variety of antimicrobial agents. Finally, the genetic diversity of strains resistant to ampicillin only, due to carriage of a 34-MDa conjugative resistance plasmid, was assessed by macrorestriction of genomic DNA and was compared to that of sensitive strains.

MATERIALS AND METHODS

Bacterial strains. From a total number of 2,941 strains of *S. enteritidis* referred to the National Reference Center for *Salmonella* and *Shigella* during a 7-year period (1987 to 1993), we randomly selected 350 strains distributed over odd years. Two hundred three strains represented isolates of human origin (196 strains were from patients with gastrointestinal infections and 7 strains were from patients with septicemia). Of the 147 strains from nonhuman sources, 96 were isolated from infected animals (mainly poultry) and 56 were isolated from food and feeds (mainly fresh and frozen chickens).

All strains were identified biochemically by using the API 20E system (bioMerieux sa, Marcy-l'Etoile, France) and were serotyped as described by Kauffman (15) for cell wall (O) and flagellar antigen (H) identification.

Strains were from the following microbiological laboratories in Greece and were kindly provided by their directors, as indicated in parentheses: Laboratory of Bacteriology, Infectious Diseases Hospital, Thessaloniki (B. D. Danielides); General State Hospital, Athens (K. Polychronopoulou); P. and A. Kyriakou Children's Hospital, Athens (V. Deliyianni); Veterinary Institute of Food Hygiene, Thessaloniki (I. Kaniou-Gregoriadou); Institute of Infectious and Parasitic Diseases, Thessaloniki (S. Tsitsamis); Department of Avian Clinical Pathology, Veterinary School, Thessaloniki (E. Artopios); Department of Microbiology, Veterinary School, Thessaloniki (O. Papadopoulos); Tzanneio Hospital, Athens (C. Nikolopoulou); University Hospital, Crete (I. Tzelentis); Sismanogleio Hospital, Athens (E. Papafrangas); Hygeia Hospital, Athens (V. Alikouli); Laiko Hospital, Athens (A. Avlami); Veterinary Institute, Crete (E. Anastasiadou); Department of Avian Pathology, National Institute of Agricultural Re-

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TABLE 1. Distribution of main nontyphoidal *Salmonella* serotypes from all sources referred to the National Reference Center for *Salmonella* and *Shigella* during the study years over a 7-year period (1987 to 1993)

Source	*Salmonella* serotype	No. (%) of isolates per year			
		1987	1989	1991	1993
Human	*S. enteritidis*	201 (25)	636 (67)	949 (81)	907 (78)
	S. infantis	24 (3)	79 (8)	15 (1)	13 (2)
	S. newport	375 (47)	15 (2)	3 (1)	5 (1)
	S. typhimurium	13 (2)	116 (12)	110 (10)	160 (14)
	All other	185 (23)	108 (11)	95 (8)	113 (9)
	Total[a]	798 (100)	954 (100)	1,172 (100)	1,198 (100)
Animal	*S. enteritidis*	12 (4)	24 (15)	83 (59)	35 (63)
	S. gallinarum	208 (69)	80 (51)	13 (9)	5 (9)
	S. typhimurium	38 (13)	18 (12)	13 (9)	11 (20)
	All other	41 (14)	34 (22)	33 (23)	5 (9)
	Total	299 (100)	156 (100)	142 (100)	56 (100)
Food-feed	*S. enteritidis*	8 (13)	51 (20)	16 (12)	19 (41)
	S. sofia	4 (7)	44 (18)	23 (17)	1 (2)
	S. typhimurium	9 (14.5)	27 (11)	17 (13)	3 (6)
	S. virchow	12 (19)	11 (5)	14 (11)	2 (4)
	All other	29 (47)	119 (47)	64 (48)	22 (47)
	Total	62 (100)	252 (100)	134 (100)	47 (100)

[a] Number of *Salmonella* sp. strains.

search, Thessaloniki (I. Papanikolaou); and Aghia Olga Hospital, Athens (S. Kitsou).

Susceptibilities to antibiotics. Susceptibilities to 15 antibiotics (see Table 2) were determined by the disk diffusion method on Mueller-Hinton agar (Oxoid Ltd., Hampshire, United Kingdom) according to the standards outlined by the National Committee for Clinical Laboratory Standards (18). Disks were provided by a commercial source (Oxoid Ltd.).

Escherichia coli ATCC 25922 was used as a reference strain. Resistance was determined according to reference zone diameter interpretative standards (18). Isolates with an intermediate zone of inhibition were considered susceptible.

Transfer of resistance. Conjugation experiments were carried out in broth as described previously (37). Transconjugants were selected on MacConkey agar containing ampicillin (10 μg/ml) and/or streptomycin (500 μg/ml) and rifampin (90 μg/ml). Previously described *S. enteritidis* strains were used as positive control markers for the conjugative transfer of antibiotic resistance (37).

Plasmid DNA isolation and restriction endonuclease analysis. Plasmid DNA was extracted from the transconjugants by the method described by Olsen (21) and was digested with the *Eco*RI restriction endonuclease (Boehringer Mannheim Biochemicals, Mannheim, Germany) according to the instructions of the manufacturer. The digests were subjected to electrophoresis through 0.8% agarose, stained with ethidium bromide, and documented under UV illumination by the BIO-PROFIL (Vilber Lourmat, Marne La Valle, France) imaging analysis system. *Hin*dIII digests of bacteriophage lambda DNA provided linear DNA size markers.

PFGE of macrorestricted genomic DNA. Plug preparation was done by a modification of a published protocol (11). Briefly, cell lysis with lysozyme (Sigma) at 37°C was followed by a proteinase K (Sigma) treatment at 55°C and DNA digestion with *Xba*I (New England Biolabs) at 37°C. Electrophoresis through a 1% agarose gel in 0.5× TBE (Tris-borate-EDTA) was performed with a CHEF DRIII apparatus (Bio-Rad). Conditions were 14°C, 6 V/cm, 120° switch angle, and a run time of 4 h followed by 14 h, with two linear switch time ramps of 25 to 65 s and 5 to 24 s, respectively. The gel was stained in 0.5 μg of ethidium bromide per ml and was photographed (Polaroid) under UV illumination. Lambda phage DNA concatamers (New England Biolabs) were used as DNA size markers. The isolates' chromosomal fingerprints were compared by eye and assigned to PFGE types and subtypes according to published guidelines (33).

RESULTS

Table 1 presents the distribution of the main serotypes of nontyphoid salmonellae referred to the National Reference Center for *Salmonella* and *Shigella* during a 7-year period. *S. enteritidis* was, overall, the dominant serotype among isolates from human, animal, and food-feed sources, especially in the later years of the study. An increase in its incidence in strains of human origin was clearly observed, ranging from 25% in 1987 to 78% in 1993, parallelled by a corresponding trend in strains of animal origin.

An increasing rate of resistance of isolates of this serotype to several antibiotics was also obvious and was most noticeable during the later years (Table 2). Resistance to ampicillin replaced resistance to doxycycline as the main resistance trait after 1991, with resistance to streptomycin, sulfonamides, and nitrofurantoin being less pronounced but showing significantly increasing trends. A substantial proportion of ampicillin- and doxycycline-resistant isolates exhibited cross-resistance to drugs

TABLE 2. Antibiotic resistance rates of *S. enteritidis* strains tested during a 7-year period (1987 to 1993)

Antibiotic	% Resistance among the indicated isolates in the following years[a]:											
	1987			1989			1991			1993		
	h (n = 34)	a/f (n = 16)	Total (n = 50)	h (n = 64)	a/f (n = 36)	Total (n = 100)	h (n = 47)	a/f (n = 53)	Total (n = 100)	h (n = 58)	a/f (n = 42)	Total (n = 100)
Ampicillin	9	12	10	6	36	17	57	62	60	34	76	52
Amoxicillin + clavulanic acid	0	6	2	2	6	3	13	6	9	0	0	0
Cefalothin	0	0	0	2	6	3	13	7	10	0	0	0
Cefamandole	0	0	0	2	6	3	13	6	9	0	0	0
Ceftriaxone	0	0	0	0	0	0	2	0	1	0	0	0
Streptomycin	6	0	4	17	19	18	23	21	22	24	19	22
Gentamicin	0	0	0	0	0	0	0	4	2	0	0	0
Kanamycin	6	0	4	2	11	5	6	4	5	7	7	7
Chloramphenicol	3	0	2	3	6	4	11	4	7	3	5	4
Doxycycline	18	19	18	39	39	39	51	51	51	29	45	36
Ciprofloxacin	0	0	0	0	0	0	0	2	1	0	0	0
Sulfonamides[b]	3	12	10	19	19	19	34	47	40	21	21	21
Sulfamethoxazole + trimethoprim	3	0	2	6	14	9	11	7	9	7	7	7
Colistine	4	12	7	6	14	9	13	13	13	19	7	14
Nitrofurantoin	12	0	8	11	19	14	21	49	36	17	26	21

[a] h, isolates of human origin; a/f, isolates of animal and food origin. Numbers in parentheses represent the number of strains tested from each year's collection.
[b] The disk consisted of sulfadiazole (37%), sulfadiazine (37%), and sulfamerazine (26%).

TABLE 3. Distribution of *S. enteritidis* resistance phenotypes during a 7-year period (1987 to 1993)

Resistance phenotype[a]	No. (%) of isolates from the following sources:		
	Human	Animal-food	Combined
AMP	20 (15)	19 (19)	39 (17)
DOX	22 (16)	9 (9)	31 (13)
STM, SUM	7 (5)	5 (5)	12 (5)
FUR	0 (0)	6 (6)	6 (3)
AMP/DOX	6 (4)	11 (11)	17 (7)
AMP/FUR	3 (2)	5 (5)	8 (3)
AMP/STM, SUM	9 (7)	2 (2)	11 (5)
DOX/STM, SUM	11 (8)	4 (4)	15 (6)
DOX/FUR	5 (4)	3 (3)	8 (3)
AMP/DOX/STM, SUM	2 (1)	5 (5)	7 (3)
AMP/DOX/STM, SUM+	12 (9)	19 (19)	31 (13)
Other	37 (28)	14 (14)	51 (22)
Total resistant isolates	134	102	236

[a] AMP, ampicillin; DOX, doxycycline; STM, streptomycin; SUM, sulfonamides; STM, SUM, streptomycin and sulfonamides in combination; FUR, nitrofurantoin; +, other antibiotics.

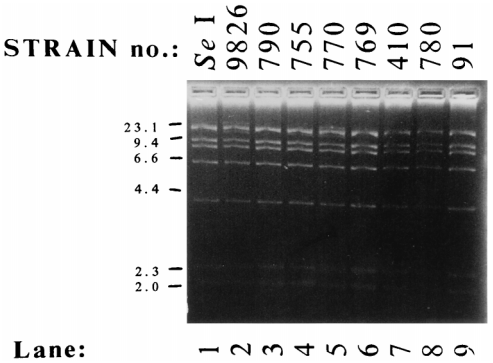

FIG. 1. *Eco*RI digestion patterns of the 34-MDa ampicillin resistance plasmid from selected strains. *Se* I, an *S. enteritidis* strain described previously (37). The positions of electrophoretic migration of bacteriophage λ *Hin*dIII fragments (in kilobases) are indicated to the left of the gel.

of other classes, such as sulfonamides and streptomycin (Table 3). Strains of human origin were in general less resistant to ampicillin, doxycycline, and sulfonamides than strains of other origins (Table 2). In addition, with the exception of doxycycline, strains originating from animal infections were more resistant than those of food-feed origin: resistance rates to ampicillin were 63.6% for animal strains and 37.5% for food-feed strains; those to streptomycin were 26.4 and 14.3%, respectively; those to sulfonamides were 41.7 and 10.7%, respectively; and those to nitrofurantoin were 36.3 and 8.9%, respectively.

In order to obtain some information about the molecular mechanism of ampicillin resistance, 38 strains of human, animal, and food-feed origin which were resistant only to this antibiotic and which were selected to represent isolates from all contributing laboratories were tested for their ability to transfer resistance in a conjugation assay. All except five strains transferred this trait to recipient cells; a 34-MDa plasmid could subsequently be isolated from all transconjugants. When the plasmid DNA was digested with the restriction endonuclease *Eco*RI, all patterns obtained were identical and were indistinguishable from that of a 34-MDa conjugative plasmid which had been previously isolated and characterized from the majority of Greek clinical ampicillin-resistant strains (Fig. 1) (37).

Ampicillin-resistant strains were then subjected to DNA fingerprinting by macrorestriction of genomic DNA with *Xba*I, followed by PFGE (Table 4). Two distinct DNA fragment profiles, PFGE types A and B, were observed, representing 90 and 10% of the isolates, respectively (Fig. 2; compare, for example, lane 5 to lane 6). Type A could be subdivided further into four subtypes. Subtype A1 represented the majority (85%) of type A strains and differed from subtype A2 in that the latter lacked a band in the 48.5-kb region (Fig. 2; compare lane 24 to lane 23). Subtype A3 differed from subtype A1 in the 250- to 330-kb region (Fig. 2; compare lane 25 to lane 23), while subtype A4 differed from subtype A1 by having a doublet instead of a single band in the 194-kb region (Fig. 2; compare lane 28 to lane 27).

The same analysis was performed with a matched set of sensitive strains (Table 4). In this group, in addition to PFGE types A (80% of strains) and B (3% of strains), a third type, type C (17% of strains), was present. Type C had not been observed in the ampicillin-resistant population (Fig. 2; compare, for example, lane 18 to lanes 14 and 15). Furthermore, within type A, although subtype A1 was still dominant (90%), subtypes A2 to A4 were not observed. Instead, two new subtypes, subtypes A5 and A6, specific for the sensitive population, were seen (Fig. 2). Subtype A5 differed from subtype A1 in the regions of 260 and 75 kb (Fig. 2; compare lane 12 to lane 11), while subtype A6 displayed an additional band in the 48.5-kb region (Fig. 2; compare lane 22 to lane 23). All three PFGE types, types A, B, and C, were obtained from isolates of animal or animal product origin as well as from isolates of human origin (Table 4).

The genotypic distinctions observed between isolates were confirmed by a second DNA fingerprinting method: PCR with the ERIC-1R enterobacterial repetitive intergenic consensus sequence (38) as the primer and an annealing temperature of 25°C (data not shown). Strains that belonged to PFGE types A, B, and C could also be clearly differentiated by this method;

TABLE 4. Distribution of PFGE types according to ampicillin resistance, source, and year of isolation

Year	Source	No. of isolates of the following ampicillin resistance phenotype and PFGE type:															
		Resistant								Sensitive							
		A1	A2	A3	A4	A5	A6	B	C	A1	A2	A3	A4	A5	A6	B	C
1987	Animal	1												2			
	Food																
	Human	2								2							
1989	Animal																
	Food	2						2		4							2
	Human							1									1
1991	Animal	3	1	1						3							
	Food									1					1		
	Human	10			1					7							
1993	Animal	4						1		3							1
	Food	3								2							
	Human	4	2							4							3
Total		29	3	1	1			4		26				2	1	1	6

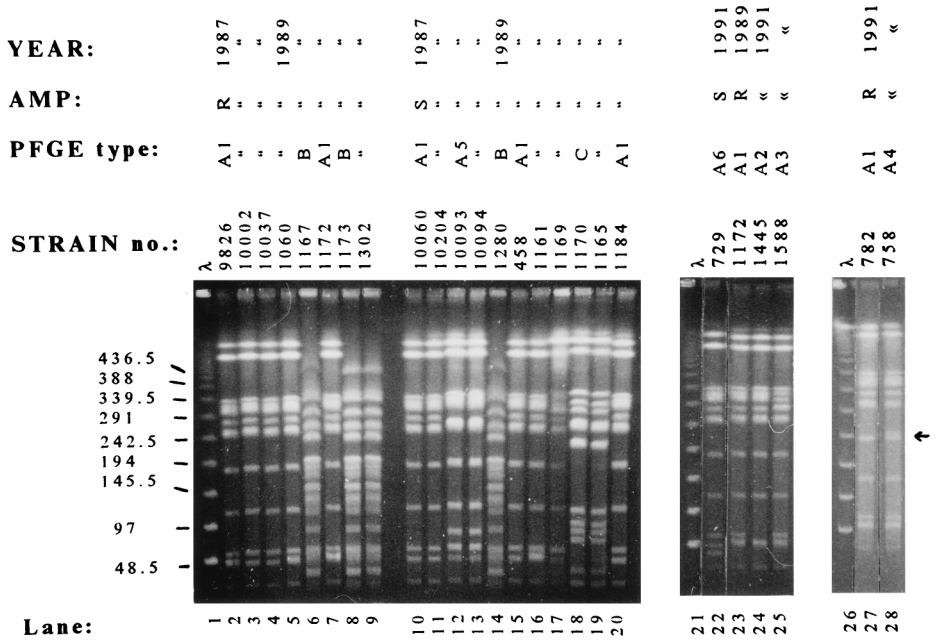

FIG. 2. PFGE patterns of selected ampicillin-resistant and -sensitive strains. AMP, susceptibility to ampicillin; R, resistant; S, sensitive. The sizes (in kilobases) of bacteriophage λ concatamers (λ; lanes 1, 21, 26) are indicated to the left of the gel. The arrow to the right of the gel indicates a doublet in strain 758. Lanes 1 to 20, 21 to 25, and 26 to 28 are from three different gels; λ concatamers and a strain of PFGE subtype A1 are included in each gel for easy cross-reference.

however, under the conditions used, strains belonging to distinct PFGE type A subtypes could not be discriminated.

DISCUSSION

Since 1987, there has been a steady increase in the total number of *Salmonella* spp. from humans, animals, and foodstuffs reported to the National Reference Center for *Salmonella* and *Shigella* in Athens, Greece. A noticeable increasing trend in the rate of isolation of *S. enteritidis* from humans established it as the major serotype from 1989 onward, with a similar increasing trend being seen for isolates from animal and food sources; these were accompanied by an increase in antibiotic resistance rates from 1987 to 1991. However, from 1991 to 1993, resistance rates generally seemed to have reached a plateau or even decreased. This may be due to the introduction of restrictive measures on antibiotic usage in farming. Overall, strains of human origin were the least resistant, while those isolated from animal infections were more resistant than those from food-feed sources; these findings are somewhat different from those reported for most other European countries and for other parts of the world (2, 12, 35).

The high incidence of resistance to doxycycline, sulfonamides, and nitrofurantoin among nonhuman isolates is of special concern. These classes of antimicrobial agents are normally administered to poultry in Greece either as therapeutic agents or in subtherapeutic doses in premixed form. As has been shown previously, such regimens increase the susceptibility to infections due to antimicrobial agent-resistant strains of *Salmonella* (24, 29, 30), thus aggravating the cost of potential treatment. Ampicillin resistance presents an additional problem not restricted to veterinary and food industry concerns, since, after 1991, the highest rates of resistance of human as well as animal isolates were to ampicillin. It is known that ampicillin, tetracycline, and streptomycin resistance are plasmid mediated (9, 31, 37). In a recent study from Greece, ampicillin resistance was found to be associated in the majority of human clinical strains with a 34-MDa conjugative plasmid belonging to the N incompatibility group (37). In the present study, a plasmid indistinguishable from the 34-MDa conjugative plasmid was shown to confer ampicillin resistance to all but five animal and food-feed strains, as well as human strains. The exceptions were two strains from poultry and three strains from humans. We presume that the resistance of these latter strains resided either on the chromosome or on nonconjugative plasmids. Our results therefore support the hypothesis that resistance originating in animal strains can be transmitted to infected humans (6, 13, 14, 20). This is a potentially serious public health hazard not only because of the consequent problems regarding therapy but also because of the risk of resistance spreading to other enterobacterial organisms, including normal flora, through the in vivo passage of plasmids (1). The spread of antibiotic resistance has additional implications since, although it is not considered a virulence factor, it may assist in the establishment and persistence of the organism in the host (19). Furthermore, the transmissibility of antimicrobial resistance and virulence factors by conjugation may contribute to the development and dissemination of pathogenic *Salmonella* strains (14).

Even though 87% of the ampicillin-resistant strains harbored the same plasmid, they were not genetically identical. DNA fingerprinting by PFGE of macrorestricted genomic DNA revealed two distinct clones, represented by PFGE types A and B. Type A could be further divided into four subtypes on the basis of the presence or absence of specific DNA bands; we cannot exclude the possibility that some of the low-molecular-weight bands may arise from silent plasmids.

The same two clones were also represented in the sensitive population. However, a third PFGE type, type C, was only seen among sensitive strains, at a frequency of 17%. Although subtype A1 was again the dominant subtype here, subtypes A2 to A4 were absent; instead, two new subtypes, subtypes A5 and A6, specific for the sensitive isolates, were observed. Interest-

ingly, one subtype A6 and two subtype A1 strains were isolated from imported French and Dutch frozen chicken meat, indicating the wide geographical spread of clone A. We have recently observed a fourth distinct PFGE type, type D, among doxycycline-resistant isolates (unpublished data).

Finally, clones of all three types, types A, B, and C, were isolated from animal sources as well as from humans, supporting the animal origin of human strains, given that animal products are the sole source of *S. enteritidis* for humans.

Thus, our extended molecular study of *S. enteritidis* isolates from different sources spanning a 7-year period corroborates reports that there is generally poor genetic variability among *S. enteritidis* strains (17, 22, 32, 34, 36). Strains have been shown previously to be clonally related even when they had originated from distant geographical locations (34) or unrelated outbreaks (36). Since, ultimately, all strains are derived from a common ancestor, poor genetic variability within members of a specific species must be explained by low mutation rates and/or the expansion and dissemination of a small number of clones. However, in agreement with the observations of Olsen et al. (22) and Powell et al. (27), our results indicate that the resolution afforded by PFGE can reveal small differences at the DNA level, even within clones. We therefore believe that strains which are identical with respect to their PFGE types must be considered clonally related. However, this does not resolve the problem of precise epidemiological linkage, since genetically indistinguishable strains could be recovered from a large number of sources. This emphasizes once again that typing methods on their own cannot always provide conclusive answers to epidemiological questions.

However, we have also demonstrated the existence of a sensitivity-specific PFGE type. Since ampicillin resistance was plasmid mediated, this clone, type C, would either have to be resistant to plasmid uptake or never have been exposed to a plasmid reservoir.

In conclusion, the present study has highlighted a potential public health hazard by showing that phenotypically and genotypically indistinguishable antibiotic-resistant *S. enteritidis* strains can be isolated from animals and humans. Ninety percent of ampicillin-resistant isolates belonged to a single clone, which was also represented in the sensitive population. However, a clone specific for the sensitive group, which included 17% of strains, was also observed, suggesting the possibility that antibiotic resistance may not spread in an entirely promiscuous fashion among the entire *S. enteritidis* population.

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