Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS200-, ribo-, pulsed-field gel electrophoresis and RFLP typing

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Summary. Sixty-two selected strains of *Salmonella* serotype Enteritidis of 33 phage types (PTs), and one strain classified as RDNC, were characterised by four different chromosomally based typing methods to elucidate genetic relationships among strains of different phage types. Based on IS200-hybridisation patterns, two major groups, containing strains of the most commonly encountered phage types, and six minor groups (seven with the RDNC strain included) were observed. IS200 pattern was a stable epidemiological marker in strains of all phage types except PT 6a and 14b. Ribotyping separated strains of the phage types into one major and five minor groups; the pattern of the RDNC strain was not seen with other strains. More than one ribotype was observed among strains of Enteritidis PTs 6, 7, 14b and 21. By pulsed-field gel electrophoresis, strains of 21 of the 33 phage types formed one large cluster when bands > 125 kb were used as the criterion for separation. Among strains belonging to PTs 1, 6, 7 and 14b, more than one pattern was observed by this method. By probing with five random cloned fragments of the Enteritidis chromosome, strains from 27 of 31 phage types examined showed the same hybridisation pattern. With the combined use of four genotypic methods, two groups of strains, representing eight and seven of 33 Enteritidis phage types, were formed; these two groups may be considered as the main evolutionary lines of Enteritidis. Strains of the remaining phage types, and the RDNC strain, belonged to separate groups.

Introduction

Salmonella enterica serotype Enteritidis (S. Enteritidis) is a common cause of foodborne gastroenteritis and the incidence of human salmonellosis caused by strains of this serotype has increased dramatically in many European countries.¹ For epidemiological investigations, Enteritidis can be subdivided by phage typing² and currently 44 different phage types (PTs) have been identified.³ Some of these PTs can be further subdivided by plasmid profiling.⁴

Strains of most serotypes of *Salmonella* contain copies of a 708-bp long, *Salmonella*-specific, insertion element IS200;^{5–7} the function is essentially unknown,⁸ but it seems to insert evenly into different regions of the chromosome.⁹ Based on the copy number of IS200 and the fragments into which it has inserted, Stanley *et al.*¹⁰ grouped Enteritidis strains of different PTs into three evolutionary lines. The patterns obtained for strains within a phage type are stable,^{11,12} and all epidemic strains from the UK have been shown to

carry the same IS200 pattern.¹¹ Therefore, it was concluded that the present epidemic of Enteritidis PT 4 is caused by a single clonal line of bacteria, a suggestion supported by results from other typing methods.¹³

Ribotyping,¹⁴ pulsed-field gel electrophoresis (PFGE),¹⁵ and restriction fragment-length polymorphisms (RFLPs)¹⁶ have also been used to study the phylogeny of bacterial species. Results from IS200-probing and from these other three methods used in parallel may enable a more thorough subdivision of Enteritidis and, at the same time, serve to evaluate results obtained previously from IS200 typing alone.

In the present investigation, type strains of Enteritidis of different PTs² together with a collection of human clinical and poultry isolates from Denmark have been characterised by IS200-typing, ribotyping, PFGE and RFLP. The results have been analysed both in terms of their phylogenetic significance and their applicability to the epidemiology of *Salmonella*.

Table I. Bacterial strains used

Phage type (PT)	Type strain*	Clonal line†	Number of additional strains‡
1	E2331	I	4
2	E2457	II	1
3	P66040	I	0
4	E2187	I	5
4a	P99764	I	0
5	P70001	I	0
6	P99327	I	4
6a	E2408	II	1
7	E1492	I	4
8	E2468	II	5
9	E2402	I	0
9b	NI	—	1
10	E3945	II	0
11	E2109	III	0
12	P95661	I	0
13	E464	II	2
13a	NI	—	1
14	E2387	III	0
14b	NI	—	2
15	E2407	III	0
16	E866	—	0
17	P95940	I	0
18	P89448	III	0
19	E1949	I	0
20	P68147	III	0
20a	NI	—	1
21	P72580	I	1
22	P84357	II	0
23	P88255	II	0
24	P99768	II	0
25	P100613	I	0
31	NI	—	1
32	NI	—	2
RDNC	—	—	1

NI, the type strain was not included.

RDNC, the type strain reacted with the typing phages but lytic patterns did not correspond to any recognised phage type.

* The characteristics of the type strains, kindly provided by Dr B. Rowe, LEP, Central Public Health Laboratory, London, have been described elsewhere.^{2,4}

† Allocation of strains to "clonal lines" of Enteritidis was based on IS200 patterns;¹⁰ strain E866 could not be so assigned.¹⁰

‡ Strains from man and poultry in Denmark.¹⁷

Materials and methods

Bacterial strains, media and culture conditions

Enteritidis strains, listed in table I, were stored in sterile glycerol 15% v/v at -80°C and grown overnight in LB broth or on LB agar plates¹⁸ at 37°C .

Southern analysis

Total DNA was isolated as described by Christensen *et al.*¹⁹ Approximately $1\text{ }\mu\text{g}$ was digested with *Pvu*II (IS200), *Pst*I (IS200 and RFLP) or *Sma*I (ribotyping) (all from Boehringer Mannheim). DNA was transferred to Hybond-N hybridisation membranes (Amersham) by vacuum blotting and hybridised with 11-dUTP-digoxigenin (Boehringer Mannheim)-labelled probes as described previously.²⁰ Plasmid DNA was isolated and analysed by agarose-gel electrophoresis (AGE) as before.²⁰

Fig. 1. Patterns observed among Enteritidis strains after hybridisation with insertion element IS200. Lane 1, *Hind*III-digested λ DNA as size marker. Lanes 2–9, patterns after digestion with *Pvu*II; 2, type I (PT 1); 3, type II (PT 2); 4, type III (PT 11); 5, type IV (PT 14); 6, type V (PT 16); 7, type VI (PT 18); 8, type VII (PT 14b); 9, type VIII (RDNC). Lanes 10–15, patterns after digestion with *Pst*I: 10, type I (PT 1); 11, type II (PT 2); 12, type IV (PT 11); 13, type V (PT 16); 14, type III (PT 9b); 15, type VI (RDNC).

IS200

Plasmid pIZ45, containing a 300-bp internal fragment of insertion element IS200,⁶ was kindly provided by Dr J. Casadesús, Department of Genetics, University of Seville, Spain. The fragment was excised after digestion with *Eco*RI–*Hind*III and re-cloned in vector pGEM3 (Scandinavian Diagnostic Systems) by standard cloning techniques.¹⁸

For use as a hybridisation probe, vector-free IS200 was generated by polymerase chain reaction (PCR) with oligonucleotides complementary to 26 and 25 bases of the promoter sequences T7 and SP6, as primers. PCR assay conditions have been described.²¹ Generated PCR product was subjected to AGE in agarose (Litex LSL) 1.2% w/v in $1\times$ TAE buffer¹⁸ and purified with a DNA-isolation kit (Prep-a-Gene, BioRad). IS200 probe was labelled with digoxigenin by random priming.²²

Ribosomal RNA probe

Ribosomal RNA from *Escherichia coli* (Sigma) was purchased and labelled with digoxigenin by use of reverse transcriptase (Boehringer Mannheim), as described by Christensen *et al.*¹⁹

Random cloned DNA probes

Chromosomal DNA from strain P132344/1²³ of Enteritidis PT 4 was digested with *Eco*RI and *Hind*III; resulting fragments were cloned in *E. coli* strain

Table II. Grouping of Enteritidis strains by IS200 type, ribotype, PFGE and RFLP

Phage type (PT)	IS-200type*			Ribotype <i>Sma</i> I	NotI PFGE-type bands		RFLP <i>Pst</i> I
	<i>Pvu</i> II	<i>Pst</i> I	Combined		All	> 150 kb	
1	I	I	I	I	I†	I†	I
2	II	II	II	II	II	I	I
3	I	I	I	III	III	I	II
4	I	I	I	II	IV	I	I
4a	I	I	I	II	IV	I	I
5	I	I	I	I	V	II	I
6	I	I	I	II†	VI†	I†	I
6a‡	I	I	I†	I	VI	I	I
7	I	I	I	II†	VII†	I†	I
8	II	II	II	II	II†	I	I
9	I	I	I	II	VIII	III	I
9b	I	III	III	II	IX	IV	I
10	II	II	II	III	II	I	I
11	III	IV	V	I	X	V	I
12	I	I	I	II	XI	VI	I
13	II	II	II	II	IV	I	I
13a	II	II	II	II	II	I	ND
14	IV	IV	VI	IV	XII	VII	III
14b	II/VII	IV	VII/VIII	V/VI	XIII/XIV	VIII/IX	ND
15	IV	IV	VI	I	I	I	I
16	V	V	IX	VII	XV	X	IV
17	I	I	I	II	XI	VI	I
18	VI	IV	X	II	XI	VI	I
19	I	I	I	III	III	I	II
20	III	IV	V	I	X	V	I
20a	I	I	I	I	I	I	I
21	I	I	I	II†	IV	I	I
22	II	II	II	II	II	I	I
23	II	II	II	II	II	I	I
24	II	II	II	II	II	I	I
25	I	I	I	II	IV	I	I
31	I	I	I	II	IV	I	I
32	I	I	I	I	I	I	I
RDNC	VIII	VI	XI	VIII	XVI	XI	ND

ND, not done.

* Roman numbers assigned arbitrarily to each pattern demonstrated. The pattern of the type strain only is given but, if type strains were not included, all observed patterns of other strains of PT are listed.

† Of additional strains tested, ≥ 1 had patterns different from that of type strain by stated method.

‡ An extra, plasmid-carried copy of IS200 was present in the type strain of this PT.

HB101¹⁸ with pBR322 or pGEM3 as vectors. Five DNA fragments, fSE4 (1.7 kb), fSE5 (6 kb), fSE6 (2.1 kb), fSE9 (2.5 kb) and fSE10 (5 kb) were propagated, digested with *Eco*RI/*Hind*III, and separated from the vector by AGE. Fragments were purified and labelled as described above.

Pulsed-field gel electrophoresis

Strains were grown overnight on LB agar at 37°C and three or four colonies were inoculated into LB broth and incubated at 37°C until an OD₆₀₀ value of c. 0.1 was reached. Genomic DNA was prepared in low-gelling point agarose (BioRad) by a procedure developed at the Centers for Disease Control, Atlanta, GA USA (Dr D. N. Cameron, personal communication). Briefly, cells were embedded in agarose, lysed with lysozyme (Boehringer Mannheim) 0.1 mg/ml, sarcosyl (Sigma) 0.05% w/v and sodium deoxycholate (Sigma) 0.02% w/v, treated with proteinase K

(Boehringer Mannheim), rinsed with dH₂O and treated twice with TE containing 15 µl/ml of a solution of phenyl-methyl-sulphonyl fluoride (Sigma) 17 mg/ml in isopropanol (Merck). After rinsing with TE, the agarose plug was ready for use in restriction analysis. Slices of agarose blocks containing DNA were digested for 4 h with 20 U of *Not*I (Boehringer Mannheim or Amersham).

DNA fragments were subjected to AGE in agarose (Litex LSL) 1% w/v in 0.5 × TBE buffer¹⁸ in a contour-clamped homogeneous electric field (CHEF) system (Pulsaphor Plus, Pharmacia LKB). The running conditions were 12 V/cm at 14°C for 22 h. Pulse times were increased by stepping, as follows: 2 s in 5 h, 5 s in 6 h, 9 s in 6 h and 12 s in 5 h. Polymerised phage-λ DNA (Pharmacia LKB) was used as molecular-size marker. After electrophoresis, the gels were stained in aqueous ethidium bromide (Sigma) 2 µg/ml for 15 min, destained in distilled water for 15 min and photographed under 254-nm UV-light.

Results

Probing with IS200

Total DNA preparations from type strains of 27 different PTs of Enteritidis and strains belonging to additional PTs were digested with *Pvu*II and *Pst*I and

Fig. 2. *Sma*I ribotypes observed among Enteritidis strains: lane 1, type I (PT 1); 2, type 2 (PT 2); 3, type 3 (PT 3); 4, type IV (PT 14); 5, type VII (PT 16); 6, type VIII (RDNC); 7, type V (PT 14b); 8, type VI (PT 14b).

Fig. 3. PFGE patterns observed among Enteritidis strains after DNA digestion with *Not*I. Lane 1, multimers of phage λ (48.5 kb) as molecular-size marker; PFGE patterns are: lane 2, I (PT 3); 3, II (PT 5); 4, III (PT 9); 5, IV (PT 9b); 6, V (PT 11); 7, VI (PT 12); 8, VII (PT 14); 9, VIII (PT 14b); 10, IX (PT 14b); 11, X (PT 16, which is not cut by *Not*I); 12, XI (RDNC strain). Only bands larger than indicated by arrow were used to group strains according to PFGE.

Fig. 4. RFLP patterns observed among *Pst*I-digested DNA of type strains of Enteritidis after probing with five random cloned fragments of Enteritidis chromosome. RFLP patterns are: lane 1, I (PT 13); 2, III (PT 14); 3, I (PT 15); 4, IV (PT 16); 5 and 6, pattern I (PTs 17 and 18); 7, II (PT 19). RFLP pattern V demonstrated in a strain of PT RDNC is not shown.

probed with IS200; as shown in fig. 1, eight and six different restriction patterns, respectively, were demonstrated with these two restriction enzymes. The patterns observed with strains from different PTs are listed in table II. Strains from 17 of the PTs belonged to the most common type when both enzymes were used for grouping; two Enteritidis strains of PT14b tested had different patterns. Enteritidis strains were usually found to contain two copies of IS200 whereas the type strain of PT 6a contained three copies; the extra copy, which by Southern analysis was shown to be inserted into a plasmid, was not present in another strain of PT 6a tested. The type strain of PT 16 had at least 15 copies in the genome, just as the Enteritidis strain of PT RDNC contained at least eight copies (fig. 1).

Ribotyping

When *Sma*I-digested DNA was probed with 16S and 23S cDNA, eight different ribotypes were observed (fig. 2). As seen in table II, strains from 19 of the PTs tested had the same *Sma*I ribotype. In one Enteritidis strain of PT 6, two strains of PT 7 and one strain of PT 21, the observed ribotype did not correspond to that of the type strain. Two strains of Enteritidis PT 14b had different ribotypes.

Pulsed-field gel electrophoresis (PFGE)

As shown in table II, the result of typing by PFGE was scored in two ways. If all bands were considered, 15 different restriction patterns could be distinguished. DNA from Enteritidis PT 16 was not cut by *Not*I. To

Fig. 5. Grouping of strains from 33 PTs of Enteritidis according to four different chromosomal typing methods: A, IS200 typing; B, ribotyping; C, PFGE and RFLP; D, all four typing methods together. PTs for which all strains showed the same characters are enclosed in a circle. RFLP separation of strains is shown by a dotted line in part C. Throughout the figure, the strains are listed by PT and "clonal line" (SECL)¹⁰. NB, individual PTs in most cases are in the same positions in all four parts of the figure. The RDNC strain examined has been omitted from the figure but, by all four criteria for grouping, would have formed its own group.

make PFGE comparable with other typing methods, the possible interference of plasmid bands was avoided by scoring only those bands > 125 kb. The different types observed among Enteritidis strains by this

method are shown (fig. 3). A total of 10 different patterns, in addition to the uncut DNA from Enteritidis PT 16, was observed by this method, and the most common pattern was demonstrated with

strains from 22 of the PTs (table II). For one strain each of Enteritidis PTs 1, 6, and 7, PFGE patterns differed from that of the type strain. Two Enteritidis strains of PT 14b had different PFGE patterns.

Random cloned chromosomal probes (RFLP)

As seen in fig. 4, four different restriction patterns were observed when five chromosomal fragments were hybridised to *Pst*I-digested DNA from strains representing 31 of the 33 PTs. The patterns observed with strains of different PTs are listed (table II). Strains from 27 of the 31 PTs tested showed the same pattern.

Grouping based on all four typing methods

Grouping of Enteritidis strains of different PTs by each method used alone or in combination is shown (fig. 5). Two groups of strains of eight and seven PTs were formed: (i) Enteritidis strains of PTs 4, 4a, 6, 6a, 7, 21, 25 and 31; and (ii) PTs 2, 8, 13, 13a, 22, 23 and 24. Another four minor groups were also formed: Enteritidis strains of PTs 1, 20a and 32; PTs 11 and 20; PTs 3 and 19; and PTs 12 and 17. The remaining PTs could not be allocated to any of these groups based on reactions from all four typing methods.

Discussion

Based on RFLP with insertion element IS200, Enteritidis¹⁰⁻¹² and other *Salmonella* serotypes²⁴⁻²⁶ have been grouped into "clonal lines". A similar RFLP method, based on insertion element IS6110, has been used with *Mycobacterium tuberculosis*.²⁷

The IS200 patterns of the type strains of 21 different Enteritidis PTs, belonging to Enteritidis clonal lines (SECL) I and II,¹⁰ were confirmed in the present investigation. Like Stanley *et al.*¹⁰ we detected three copies of IS200 in the type strain of Enteritidis PT 6a. The extra copy, which is carried on a plasmid,¹⁰ (and this investigation), was absent from an additional strain of PT 6a tested. Based on the remaining bands, which were the same in both strains of that PT, strains of Enteritidis PT 6a were allocated to IS200-SECLI, not SECLII as described by Stanley *et al.*¹⁰

Strains from several PTs not previously typed by IS200 were included in the present investigation. Of these, Enteritidis strains of PTs 20a, 31 and 32 belonged to IS200-SECLI and of PT 13a belonged to IS200-SECLII. IS200-SECLIII (see table I) in our study contained three different patterns; the type strains of the PTs included in SECLIII were also shown to be diverse by other genomic typing methods. In our opinion, IS200-SECLIII of Stanley *et al.*¹⁰ cannot be regarded as a "clonal line".

IS200-type has usually been stable in strains within phage types of Enteritidis;^{10,11} in the present investigation, however, that was not true for Enteritidis strains of PTs 6a and 14b. In PT 6a,

divergence was due to a plasmid-encoded band in the type strain; two strains of PT 14b were different by IS200-, ribo- and PFGE typing, and it seems likely that this PT contains strains of more than one "clonal line". Thus, all typing methods could be used to subdivide strains of PT 14b.

In serotype Heidelberg, one "IS200 clonal line" is mainly associated with human infections whilst another is dominant among chicken isolates.²⁵ A corresponding relationship between IS200 patterns and "clonal lines" has not yet been demonstrated for Enteritidis. However, other genotypic typing methods, such as ribotyping, PFGE and RFLP, may be used to support results obtained by IS200-typing.

In this study, only *Sma*I was used for ribotyping. In comparison with other enzymes, *Sma*I has been preferred previously for subdivision of *Salmonella* serotypes Berta and Enteritidis,^{20,28} whilst it was of medium discriminatory power in Typhi.²⁹ Other enzymes may be more discriminatory; for example, *Hind*III has been preferred for Gallinarum¹⁹ and *Clal* for Typhi.³⁰

Ribotyping gave much the same degree of discrimination as IS200, but, as shown in fig. 5, it grouped Enteritidis strains differently. For Enteritidis strains of PTs 6, 7, 14b and 21, more than one ribotype was observed, and for epidemiological purposes, *Sma*I ribotypes may be used to discriminate further among strains of these PTs. The present data do not disclose whether PT or ribotype is the best evolutionary indicator; however, the fact that strains of several PTs have the same ribotype makes the latter more likely. It seems also that PT is subject to convergence by external factors, as the introduction of *incN* plasmids has been shown to change the lytic pattern of a number of Enteritidis PTs.^{31,32}

In a number of cases, such as enterohaemorrhagic *E. coli* and pathogenic *Leptospira*, PFGE has subdivided the bacteria into biologically meaningful subgroups,^{33,34} and so the method was included in this investigation. PFGE was the most discriminatory of the genomic typing method for Enteritidis, when all restriction fragments were used to group strains. As has been reported for a number of other bacteria, e.g., *Staphylococcus* spp.,³⁵ *Legionella pneumophila*³⁶ and *Campylobacter hyointestinalis*,³⁷ PFGE is of potential use for outbreak investigations, used alone or together with less stable markers such as plasmid profiles and antibiotic resistance.

To exclude restriction polymorphism due to plasmid DNA, only DNA fragments > 125 kb were used for grouping of strains of PTs. Thus, strains within groups shared a minimum of five to eight large bands, but sometimes differed in smaller bands, some of which may have been of plasmid origin.

In essence, PFGE with this scoring method united strains from 22 of the 33 PTs in a single cluster. Of the PTs in IS200-SECLI and SECLII,¹⁰ only strains from PTs 5, 9, 12 and 17 had PFGE patterns different from the main cluster. By IS200-, ribo- and RFLP-typing,

however, strains of these phage types grouped in the main cluster(s) and we suspect that, until relatively recently in evolutionary terms, they belonged to the main PFGE group. Unique PFGE patterns were observed in additional Enteritidis strains of PTs 1, 6 and 7 and, even when only bands > 125 kb are scored, PFGE may be used to discriminate among these phage types. For unknown reasons, *NotI* was found not to cut DNA from Enteritidis PT 16. The same DNA preparations could be digested with *SmaI* or *XbaI* (data not shown); it may be that strains of PT 16 contain a modification system protecting against *NotI*.

Like PFGE, RFLP does not cover a particular part of the chromosome. Tompkins *et al.*¹⁶ described RFLP as a potential epidemiological tool, since most unrelated strains of Typhimurium showed unique patterns when probed with random cloned fragments. However, the five fragments used in the present investigation seem to cover conserved parts of the chromosome, as all but four of the Enteritidis strains tested showed the same pattern. Of these, strains of PTs 3 and 19 belonged to the main IS200-, ribotype and PFGE groups, and in all methods showed the same patterns. The RFLP result underlines the close genomic relationship between Enteritidis strains of PTs 3 and 19 and, based on the results in other typing methods, the two PTs should still be considered close to the main group of Enteritidis.

The most detailed picture of relationships among Enteritidis strains of different PTs is provided by the combined use of all four typing methods. As illustrated in fig. 5, IS200-SECLI of Stanley *et al.*¹⁰ could be divided into one group containing Enteritidis strains of eight PTs with PT 4 the most prominent member; a second containing strains of three PTs with PT 1 as a prominent member; another two groups each of two PTs; and two separate PTs. IS200-SECLII¹⁰ was intact with the exception of Enteritidis PTs 6a and 10. PT 13a was included in this clonal line, which then contains the predominant PTs among poultry in the USA and Canada.³⁸ The reason for the dominance in these countries of SECLII rather than the PTs of SECLI is unknown, but the fact that two different "clonal lines" are dominant on different continents may be considered to support the division of Enteritidis into "clonal lines" by our methods.

The relationships among strains of PTs in SECLI and SECLII and the remaining Enteritidis PTs (i.e., PTs 9b, 11, 14, 14b, 15, 16, 18 and 20) were less clear. The type strains of Enteritidis PTs 11 and 20 grouped together, and as with Enteritidis PT 15, they were linked to PTs 1, 5, 20a and 32 by ribotype. Enteritidis PT 18, which was grouped in the IS200-SECLIII by Stanley *et al.*,¹⁰ was related to PTs 12 and 17 by PFGE and to a number of PTs by the *SmaI* ribotype.

The only isolate of Enteritidis PT 9b tested was linked to the main group of Enteritidis by ribotype and RFLP, whilst strains of Enteritidis PTs 14, 14b and 16 had unique patterns by all, or all but one, of the methods used. This finding may indicate that strains of

these PTs diverged early from those of other Enteritidis PTs, or that they were created by larger mutational events. The latter explanation might be true for the Enteritidis strains of PT 16 and RDNC that contain numbers of IS200 elements higher than normal for Enteritidis.

The data presented do not disclose how the events that led to different IS200-, ribo-, PFGE- and RFLP-types relate to each other in time. One possible interpretation is that PFGE and RFLP, which identify similar groupings and put strains of most PTs into one cluster, reflect the most ancient picture of phage-type relationships. By mutations and IS200 insertions into these ancestral strains, a number of ribo-, IS200- and PTs have developed since; the more distant that two PT groups have become, the earlier they must have diverged.

There is only limited biological evidence to support the phylogenetic conclusion. Strains of Enteritidis PT 4 converts to PT 7 by loss of LPS;³⁹ these two PTs were grouped together by all four typing methods. It was noted, however, that strains of Enteritidis PT 7 had more than one ribo- and PFGE type, and the biological relationships between Enteritidis PTs 4 and 7 may not be valid for all strains. As mentioned, a number of Enteritidis PTs can converge by the introduction of *incN* plasmids: Enteritidis PTs 4 to 24, 7 to 23, 8 to 24, and 30 to untypeable, whilst PTs 13 and 13a do not change PT upon introduction of these plasmids.^{31,32} This convergence seems to occur between PTs which, in the present investigation, had the same *SmaI* ribotype, the same *NotI* PFGE pattern and the same *PstI* RFLP pattern, whereas, for example, strains of Enteritidis PTs 4 and 24, like PTs 7 and 23, belonged to two different IS200-SECLs. Unless the introduction of the *incN* plasmids provokes a rearrangement of IS200 elements, this observation calls into question the stability of the phage-typing system or of the IS200 pattern for epidemiological investigations, and there is a need to look more carefully into this matter.

The way in which IS200 and ribotyping cross-divided the PTs might mean that the chromosome behaves, not as one molecule, but as a number of subunits where evolution takes place at different rates. When Enteritidis strains of two PTs have the same IS200 pattern but different ribotype, or *vice versa*, the same IS200 insertion or the same ribotype mutation must have occurred independently in different clonal lines indicating that either IS200-insertion or mutations in rRNA genes, or both, are non-random events. Regardless, the observation of the same IS200 type in different ribotypes calls for the use of both typing methods in parallel when performing phylogenetic or epidemiological studies.

This study was supported by The National Veterinary and Agricultural Research Council (Grant Nos. 13-4322 and 13-4538-1). We are grateful to Gitte Christensen, Susanne Rasmussen and Pia R. Mortensen for skilful technical assistance. We thank Dr Dorte Lau Baggesen of the National Veterinary Laboratory for phage-typing control of the strains used in this investigation; Dr Ørjan

Olsvik, CDC, Atlanta, GA for help with setting up the pulsed-field gel electrophoresis system; Dr B. Rowe Central Public Health

Laboratory, for providing the Enteritidis phage-type strains, and Dr J. Casadesús for providing pIZ45 plasmid.

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