Molecular typing of *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates

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A collection of 31 epidemiologically unrelated *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) isolates obtained during a 12-year period was characterised by different molecular typing methods. Plasmid profile analysis, the detection of plasmid-encoded virulence genes and ribotyping allowed little or no further differentiation amongst these isolates. Two different hybridisation patterns were observed by IS200-typing of the *S. Enteritidis* isolates. However, pulsed-field gel electrophoretic separation of restriction endonuclease-digested whole-cell DNA provided a high level of discrimination amongst the 31 *S. Enteritidis* isolates. This could be increased by the comparative use of the three suitable restriction endonucleases *Xba*I, *Spe*I and *Not*I. Thus, pulsed-field gel electrophoresis proved to be superior in its discriminatory value to other molecular methods such as plasmid analysis, ribotyping or IS200-typing and represents a most helpful tool for the epidemiological typing of *S. Enteritidis* isolates.

Introduction

Isolates of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (*S. Enteritidis*) are often involved in gastrointestinal diseases in man [1]. In 1992–1993, *S. Enteritidis* isolates represented more than two-thirds of all salmonellae isolated from human cases in Germany [2] and in England and Wales [3]. While other *S. enterica* serovars, such as *S. Typhimurium*, are predominant in cattle and pigs [3], strains of *S. Enteritidis* have been the most frequent isolations from poultry [1, 3]. Among the *S. Enteritidis* isolates, those assigned to phage type (PT) 4 have been isolated predominantly from poultry, and are also the most frequent cause of salmonellosis in man [1–3]. Poultry represent a major reservoir for zoonotic *S. Enteritidis* isolates and spread occurs through eggs [4] and poultry meat [5] to man [4–6].

Different epidemiological typing methods have been established to detect chains of salmonella infections and to identify their sources. Among them, molecular techniques that analysed plasmids and chromosomal DNA fingerprinting were considered useful for typing several *Salmonella* serovars [7–10]. However, isolates of *S. Enteritidis* from poultry have been shown to be difficult to discriminate, even by molecular techniques [6, 11, 12]. Nevertheless, exact strain identification is

of particular importance to relate *S. Enteritidis*-associated outbreaks of salmonellosis in man to the presence of this serovar in poultry or poultry products.

In this study, *S. Enteritidis* isolates from poultry were investigated by four independent molecular typing systems. These included the detection of restriction fragment length polymorphisms (RFLP) of 16S rRNA genes as well as *Salmonella*-specific insertion elements in the chromosomal DNA, the characterisation of plasmids by plasmid profile analysis, restriction endonuclease digestion and demonstration of plasmid-encoded virulence genes, and finally the analysis of whole-cell DNA by pulsed-field gel electrophoresis (PFGE). These techniques were compared for their discriminatory value in the molecular typing of *S. Enteritidis*.

Materials and methods

Bacterial isolates, antibiotic resistance testing and phage typing

Of the 31 *S. Enteritidis* isolates used in this study, 21 were isolated from epidemiologically unrelated poultry specimens collected between 1982 and 1994 in Northern Germany. The remaining 10 isolates, obtained from unrelated cases of salmonellosis in man (five), pigs (two), cattle (two) and a dog, were included in this study for comparative purposes. Biochemical characterisation of the *S. Enteritidis* isolates followed standard techniques; serotyping was performed with

commercially available antisera (Behring Werke, Marburg, Germany). All *S. Enteritidis* isolates were grown overnight on LB-agar plates; glycerine stock cultures were kept at -70°C . The isolates were checked for purity on sheep blood agar plates (Blood Agar Base, Oxoid, supplemented with sheep blood 7.5%). Antibiotic susceptibility testing was performed by the agar diffusion method [13] with disks containing ampicillin 10 μg , chloramphenicol 30 μg , fusidic acid 10 μg , gentamicin 10 μg , kanamycin 30 μg , minocycline 30 μg , nalidixic acid 30 μg , neomycin 30 μg , nitrofurantoin 300 μg , penicillin G 10 I.E. and tetracycline 30 μg . Phage typing was performed at the Statens Veterinære Serumlaboratorium, Copenhagen, Denmark, according to the method of Ward *et al.* [14] by the phage typing scheme developed at the Central Public Health Laboratory, Colindale Avenue, London.

DNA preparation, agarose gel electrophoresis and restriction endonuclease analysis

The preparation of whole-cell DNA of the *S. Enteritidis* isolates followed a *Salmonella*-specific modification [15] of the method of Jordens and Hall [16]. Plasmid profiles of the *S. Enteritidis* isolates were prepared according to Kado and Liu [17]. The plasmids of *Escherichia coli* V517 [18] as well as the *S. Typhimurium* virulence plasmid pRQ51 [19] served as standards for the determination of plasmid sizes in the *S. Enteritidis* isolates. Plasmids suitable for subsequent restriction endonuclease analyses were obtained by the method of Olsen [20]. Uncleaved plasmids as well as their restriction fragments were separated electrophoretically in agarose 0.8% w/v gels [21], stained with an aqueous solution of ethidium bromide (Sigma) 10 $\mu\text{g}/\text{ml}$ and photographed under UV illumination. *S. Enteritidis* plasmids were digested for 2 h with 10 U of *Hind*III (Boehringer Mannheim, Germany) according to the manufacturer's recommendations.

Approximately 1 μg of whole-cell DNA was digested at 37°C for 4 h in the presence of 30 U of the enzymes *Sma*I, *Pvu*II or *Pst*I [21]. The resulting DNA fragments were separated in agarose 0.8% w/v gels. A 1-kb ladder and λ -DNA/*Hind*III fragments (both from Gibco-BRL, Paisley) served as DNA size standards.

Southern blot hybridisation and preparation of gene probes

The DNA fragments were transferred from agarose gels to nitrocellulose membranes (Hybond N, Amersham-Buchler, Braunschweig, Germany) by the capillary blot procedure [21]. The 2.3-kb *Hind*III fragment of plasmid pBA2 [22] was used as a 16S rRNA probe and the 0.6-kb *Pvu*II fragment of plasmid pIZ45 [23] served for the detection of IS200 elements. A 3.6-kb *Hind*III fragment of plasmid pRQ51 [19] — which comprised the virulence genes *spvB* and *spvC* within the common

region of virulence plasmids in *S. enterica* serovars Typhimurium, Enteritidis, Dublin and Choleraesuis [23–26] — served as a virulence gene probe. The non-radioactive ECL system (Amersham-Buchler) was used to label all gene probes. Hybridisation was performed overnight at 42°C in a shaking water bath (80 rpm) with the hybridisation buffer included in the kit. The membranes were washed twice for 20 min at 42°C in a buffer consisting of 6 M urea SDS 0.4% w/v and $0.1 \times \text{SSC}$ (1.5 mM sodium citrate, 15 mM sodium chloride, pH 7.0) and twice for 5 min at room temperature in $2 \times \text{SSC}$. The detection solutions included in the ECL kit were used for the detection of hybridisation signals.

Pulsed-field gel electrophoresis (PFGE)

Whole-cell DNA for PFGE experiments was prepared as described previously [11, 27]. DNA containing slices of the agarose plugs were digested for 4 h with 20 U of *Xba*I (Boehringer), 20 U of *Not*I or 20 U of *Spe*I (BioLabs). The resulting DNA fragments were separated by agarose gel electrophoresis with SeaKem GTG (FMC Bio Products) 1% w/v in a CHEF DR II system (BioRad) at 15 V/cm with $0.5 \times \text{TBE}$ as running buffer. The pulse times for *Xba*I and *Spe*I digests were increased from 7 s to 12 s during the first 11 h and finally from 20 s to 40 s during the next 13 h; for *Not*I digests, the pulse times were increased from 2 s to 5 s during the first 11 h and from 9 s to 12 s for further 11 h. Polymerised phage λ DNA (Pharmacia LKB) served as size standard. The gel was stained with ethidium bromide, (Sigma) 2 $\mu\text{g}/\text{ml}$ for 15 min, destained in distilled water for 15 min and photographed under UV illumination. To determine the possible interference of virulence plasmid bands on the PFGE fragment patterns, the DNA fragments were transferred to nitrocellulose membranes, fixed by baking and hybridised with the *spvB/spvC* gene probe.

Calculation of the discriminatory indices for the different typing methods

An index of discrimination (*D*) for each typing method was calculated according to Simpson's index of diversity as described by Hunter and Gaston [28]. It is based on the probability that two unrelated strains randomly sampled from the test population will be assigned to different typing groups. This index was used to compare the different typing methods and select the most discriminatory system.

Results

Resistance testing, phage typing and plasmid analysis

None of the 31 *S. Enteritidis* isolates exhibited resistance to any of the antibiotics tested. Phage typing

Table 1. Characteristics of the 31 *S. Enteritidis* isolates

Isolate (Source*)	Phage type	Plasmid profile (kb)	Ribotype	IS200 type	PFGE patterns			Genomic group
					<i>Xba</i> I	<i>Spe</i> I	<i>Not</i> I	
1 (P)	RDNC†	55	1	1	1	1	4	I
2 (P)	8	55	1	2	1	2	2	II
3 (P)	8	55	1	2	1	2	2	
4 (P)	8	55	1	2	1	2	2	
5 (P)	8	55	1	2	1	2	2	
6 (P)	8	55	1	2	1	2	2	
7 (H)	8	55	1	2	1	2	2	
8 (P)	RDNC	55	1	2	1	2	2	
9 (P)	13	55	1	2	1	2	5	III
10 (P)	4	55	1	1	2	3	2	IV
11 (P)	4	55	1	1	2	3	2	
12 (P)	4	55	1	1	2	3	2	
13 (P)	4	55	1	1	2	3	2	
14 (P)	4	55	1	1	2	3	2	
15 (P)	7	55	1	1	2	3	2	
16 (P)	RDNC	55	1	1	2	3	2	
17 (D)	4	55	1	1	2	3	2	V
18 (P)	RDNC	55	1	1	3	3	6	
19 (P)	1	55	1	1	4	2	1	
20 (P)	1	55	1	1	4	2	1	
21 (P)	1	55	1	1	4	2	1	
22 (P)	1	55	1	1	4	2	1	
23 (S)	1	55	1	1	4	2	1	
24 (H)	1	55	1	1	4	2	1	VI
25 (H)	1	55	1	1	4	2	1	
26 (C)	1	55	1	1	4	2	1	
27 (C)	1	55	1	1	4	2	1	
28 (P)	6	55; 23	1	1	2	4	3	VII
29 (H)	6	55; 23	1	1	2	4	3	
30 (S)	1	55; 3	1	1	2	5	2	VIII
31 (H)	1	55	1	1	2	2	2	IX

*P, poultry; H, human; C, cattle; S, swine; D, dog.

showed that six isolates each belonged to the PTs 4 or 8, 11 isolates could be assigned to PT1 and two to PT6, whereas single isolates corresponded to phage types 7 and 13, respectively. Another four *S. Enteritidis* isolates reacted with the typing phages, but their lytic pattern did not correspond to any recognised phage types; these isolates were classified as RDNC (Table 1). Phage typing resulted in a discriminatory index (*D*) of 0.755. Twenty-eight of the 31 isolates carried a single plasmid of *c.* 55 kb, and additional plasmids of either 23 kb or 3 kb were detected in the remaining three *S. Enteritidis* isolates (Table 1). This corresponded to a discriminatory index of 0.187 for plasmid profile analysis. Hybridisation of these plasmids with a virulence gene probe identified the 55-kb plasmid in each isolate as a virulence plasmid. Restriction endonuclease analyses of these virulence plasmids revealed no differences in the fragment patterns. Moreover, hybridisation experiments

demonstrated that the virulence genes were located on fragments of 3.6 kb in each of the 31 virulence plasmids (Fig. 1).

Ribotyping

Independently of the enzyme used for digestion of the whole-cell DNA, ribotyping with the 16S rRNA probe resulted in identical hybridisation patterns for the 31 isolates. An extended hybridisation pattern consisting of six bands was obtained by probing *Pvu*II-digested DNA with the 16S rRNA gene probe (Fig. 2); only one hybridising fragment was detected by *Sma*I-digested DNA. The latter observation suggested that an internal fragment of the 16S rRNA gene was obtained by digestion with *Sma*I to which the gene probe hybridised. The discriminatory index for ribotyping was 0.

Fig. 1a, *Hind*III restriction patterns of the virulence plasmid found in all 31 *S. Enteritidis* strains (lane 1); **b,** corresponding hybridisation pattern obtained with an *spvB/spvC* virulence gene probe (lane 1); M, DNA length standard (1-kb ladder, Gibco-BRL). The arrows indicate the hybridising fragment of 3.6 kb.

IS200 typing

Hybridisation experiments with an IS200-specific gene probe confirmed that all 31 *S. Enteritidis* strains harboured insertion elements of this type. Two different hybridisation patterns were observed. Twenty-three *S. Enteritidis* isolates carried their IS200 copies on *Pst*I fragments of 4.5 kb and 6.7 kb (IS200 type 2); the remaining eight isolates showed hybridising *Pst*I fragments of 4.5 kb and 5.2 kb (IS200 type 1) (Fig. 3). Since hybridisation of the electrophoretically separated plasmids with the IS200-specific gene probe revealed no signals, all IS200 copies were considered to be located in the chromosome. IS200 typing yielded a *D* value of 0.396.

PFGE

PFGE analyses of restriction endonuclease-digested whole-cell DNA revealed the presence of four different fragment patterns with *Xba*I (Fig. 4a, lanes 6–9). *Spe*I

Fig. 2. Typical *Pvu*II-ribotyping pattern observed in all 31 *S. Enteritidis* isolates. Arrows indicate the positions of the DNA marker fragments (λ DNA *Hind*III-digested, Gibco-BRL) as they appeared in the respective agarose gels (from the top: 23130, 9416, 6557, 4361, 2322, 2027 bp).

digestion resulted in another five different fragment patterns (Fig. 4a, lanes 1–5) while six fragment patterns were observed by digestion with the enzyme *Not*I (Fig. 4b, lanes 1–6). Whereas *Xba*I and *Spe*I produced fragment patterns consisting of 13–16 bands in the range 48–533 kb, those obtained from *Not*I digestion resulted in 29–32 fragments of 48–339 kb. The indices of discrimination were 0.703 for the *Xba*I patterns and 0.591 for the *Spe*I and *Not*I patterns. The comparative use of the three restriction endonucleases in PFGE analyses significantly increased the discriminatory value of this technique to *D* = 0.815 as isolates that exhibited the same fragment pattern with one enzyme occasionally differed in their fragment patterns obtained with the other enzymes. Thus, nine different genomic groups could be differentiated among the 31 *S. Enteritidis* isolates (Table 1) on the basis of combined PFGE analyses.

Discussion

A specific strain identification is essential for monitoring the spread of salmonellae during outbreaks of infection. Traditional epidemiological studies included biochemical and serological characteristics of the

Fig. 3. IS200 patterns obtained by hybridising *Pst*I-digested chromosomal DNA with a gene probe specific for the insertion element IS200. The hybridisation patterns 1 and 2 represent the IS200 types 1 and 2 as

isolates as well as their antibiotic resistance properties and, if available, their assignment to certain phage types.

Genotypic analysis of salmonellae by molecular typing methods has proved to be helpful in the characterisation of strains from different *Salmonella* serovars, such as *S. Typhimurium* [6, 15, 27, 29], *S. Enteritidis* [6, 9–11, 30–33], *S. Dublin* [24, 34], *S. Virchow* [35], *S. Heidelberg* [36], *S. Berta* [37, 38], *S. Typhi* [39], *S. Paratyphi* [40] and *S. Java* [40]. The techniques included plasmid analysis, ribotyping, IS200 typing and PFGE analysis. Although these methods are universally applicable, they have different discriminatory values for isolates of the different *Salmonella* serovars.

Although plasmid analyses were not included, previous studies of *S. Enteritidis* that included the type strains of the different phage types, as well as small numbers of additional strains of selected phage types, have shown that these molecular typing methods were useful for the determination of evolutionary lines among *S. Enteritidis* strains from different phage types [11, 30].

However, these studies also revealed that IS200 typing did not allow discrimination between strains of the same phage type or among strains from different PTs, such as 1, 4, 6 or 7 [11, 30]. Similar observations were made for the discriminatory value of ribotyping [11, 41]. Another study of field isolates of *S. Enteritidis* showed that both methods — ribotyping and IS200 typing — failed to discriminate between 14 *S. Enteritidis* isolates [6]. PFGE analyses appeared to have the best discriminatory value of all molecular typing methods [11]. By using PFGE analysis with *Not*I-digested whole-cell DNA, *S. Enteritidis* isolates of the same phage type as shown for those belonging to PTs 1, 6 or 7 could be differentiated, whereas isolates of PT4 remained indistinguishable [11, 33]. However, Powell *et al.* subdivided 39 *S. Enteritidis* PT4 isolates obtained during a 25-year period into nine *Xba*I-PFGE patterns [12].

Based on these findings four independent molecular techniques were used to study 31 unrelated *S. Enteritidis* isolates. As previously demonstrated by others [6], ribotyping with *Sma*I and *Pvu*II failed to discriminate between the different *S. Enteritidis* isolates. Although *Sma*I had been reported to be helpful for ribotyping *Salmonella* serovars Enteritidis [41] and Berta [38] and *Pvu*II had been used successfully for serovars Typhimurium [15, 29], Paratyphi [40] and Java [40], neither enzyme revealed differences between the *S. Enteritidis* isolates used in this study.

Furthermore, plasmid analyses did not demonstrate striking differences between the *S. Enteritidis* isolates. Most carried a single plasmid of 55 kb that had been reported to be specific for serovar Enteritidis [42], whereas additional smaller plasmids could be detected in three isolates. This observation is in good agreement with other studies on plasmid profiles in *S. Enteritidis* [42, 43]. The 55-kb plasmid of each isolate was identified as a virulence plasmid by hybridisation with a specific gene probe. Moreover, all these virulence plasmids proved to be indistinguishable by their fragment patterns obtained after *Hind*III digestion. This enzyme had been used successfully to detect structural differences among virulence plasmids in *S. Typhimurium* [15] and *S. Enteritidis* [43]. In each case the virulence gene probe hybridised to a 3.6-kb *Hind*III fragment that had been reported to be an internal area of the common virulence gene region in plasmids of serovars Typhimurium, Enteritidis, Dublin and Choleraesuis [25]. Thus, plasmid analyses did not provide substantial strain-specific information suitable for tracing strains of *S. Enteritidis*.

The detection of IS200 copies has proved to be a useful tool for the epidemiological typing of many different *Salmonella* serovars. This method was particularly helpful for serovars that usually harboured a large number of IS200 copies such as Typhimurium

Fig. 4. Agarose gel electrophorogram of the different PFGE-patterns of *S. Enteritidis* isolates obtained with: **a**, the restriction endonucleases *SpeI*- (lanes 1–5) as well as *XbaI*- (6–9) or **b**, *NotI* (lanes 1–6); **0**, DNA size standard (polymerised phage λ DNA, Pharmacia LKB); arrows indicate the positions of the marker DNA fragments starting from the bottom at 48.5 kb.

[15, 29], Typhi [39], Paratyphi [40], Java [40] and Heidelberg [36]. In other serovars, such as Berta [37, 38] and Enteritidis [30–32], its use for epidemiological typing was limited due to the low number of IS200 copies in these strains. Nevertheless, IS200 typing revealed the presence of two distinct and stable hybridisation patterns among the strains investigated in this study. By using *PstI* for the digestion of whole-cell DNA IS200 types were obtained that corresponded very closely to the types 1 and 2, as reported previously [11, 32]. The data on IS200 typing strongly support the observation that certain *S. Enteritidis* phage types are identified by their IS200 hybridisation patterns. Thus, all isolates of PTs 8 and 13 corresponded to IS200 type 2 whereas isolates of PTs 1, 4, 6 and 7 exhibited IS200 pattern 1 (Table 1).

PFGE analysis has been shown to be highly effective for epidemiological studies of a wide variety of bacteria. Recently, this technique has also been used for the molecular typing of *Salmonella* serovars Enteritidis [11, 33] and Typhimurium [27]. Our data confirmed the observation of Olsen *et al.* [11] that

PFGE was the most discriminatory of the molecular typing methods applied to *S. Enteritidis*. As all *S. Enteritidis* isolates harboured large virulence plasmids, the PFGE fragment patterns were hybridised with a virulence gene probe to determine the possible interference of virulence plasmid bands on the fragment patterns observed. None of the DNA bands visible in ethidium bromide-stained agarose gels could be associated with the faint hybridisation signal obtained with the *spvB/spvC* gene probe. This observation led to the suggestion that large plasmid bands do not account for the restriction fragment length polymorphism detected in the PFGE experiments. Each of the three enzymes used for digestion of the whole-cell DNA of the *S. Enteritidis* isolates resulted in four-to-six different fragment patterns (Table 1). In general, isolates that exhibited the same *XbaI* pattern (e.g., pattern 2) could be further differentiated by their *SpeI* (e.g., patterns 2–5) or *NotI* patterns (e.g., patterns 2 and 3) and *vice versa*. Thus, the comparative PFGE analysis with three different suitable restriction endonucleases distinctly increased the discriminatory value of this method.

Moreover, the use of several enzymes provided a way to circumvent difficulties that might arise from the presence of protection systems in *S. Enteritidis* against cleavage by a particular restriction endonuclease. Protection against cleavage by *NotI* was reported to occur in *S. Enteritidis* PT16 [11]. As shown in Table 1, the combination of the results of the three PFGE experiments subdivided the 31 *S. Enteritidis* isolates into nine genomic groups that corresponded closely to the assignment of the isolates to different phage types. The observation that a PT7 strain was indistinguishable from strains of PT4 by IS200 typing and PFGE analysis confirmed the close genetic relationship between strains of these two phage types. This had been suggested by the observation that a *S. Enteritidis* PT4 strain could be converted into a PT7 strain by loss of lipopolysaccharide [44]. Isolates of PT1 could be subdivided into three genomic groups. This finding accorded with the data of Olsen *et al.* [11] and confirmed the usefulness of this molecular typing method for discrimination among PT1 strains. Although the *S. Enteritidis* isolates used in this study were isolated from different geographical areas in Northern Germany during a 12-year period, isolates of phage types other than PT1 could not be differentiated further by any of the molecular techniques applied. These observations support the assumption of a clonal relationship of *S. Enteritidis* isolates of certain phage types such as PT4 or PT8. Nevertheless, extended PFGE analyses resulted in a detailed strain characterisation that might be particularly helpful for the characterisation of *S. Enteritidis* isolates that were non-typable by phage typing. These molecular typing methods not only help to follow such non-phage-typable strains in epidemiological studies, they also show the genotypic relationship of these strains to *S. Enteritidis* strains of known phage types (Table 1).

Although these data were obtained from a relatively small number of unrelated *S. Enteritidis* field isolates, this study demonstrated that PFGE analysis might represent a valuable tool for the characterisation of *S. Enteritidis* isolates. Moreover, as the results from PFGE analysis matched closely those from phage typing, PFGE analysis might represent a useful molecular alternative to phage typing in epidemiological studies of *S. Enteritidis*.

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