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Genetic relationships among strains of *Salmonella enteritidis* in a national epidemic in Switzerland

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SUMMARY

A collection of *Salmonella enteritidis* strains isolated in Switzerland (1965–90) was characterized. The phage type and plasmid profile of isolates were compared with the copy number and insertion loci of the DNA insertion element IS*200*. Three clonal lines of *S. enteritidis* were identified by IS*200* profile; the various phage types were subtypes reproducibly associated with one of these lines. All human and poultry isolates contained a 38 Mda plasmid which hybridized with a mouse virulence-associated gene probe. In *S. enteritidis*, the IS*200* profile is a race-specific molecular marker of the chromosome, and may be particularly applicable for studying the epidemiology of less common serovars.

INTRODUCTION

In recent years *Salmonella enteritidis* has been isolated at greatly increased rates in many countries throughout the world. For example, this serovar has exhibited an almost 19-fold increase in the UK alone between the years 1981 and 1990 [1]. The question has therefore been raised whether this trend constitutes a new pandemic [2]. The increase of *S. enteritidis* has been linked epidemiologically both to contaminated poultry meat [3, 4] and to shell eggs [1, 5, 6]. Important details of the changing epidemiology of this pathogen have nonetheless remained unexplored and it would be desirable to investigate the situation as precisely as possible in affected countries.

In Switzerland the number of *S. enteritidis* isolations remained constant from 1965 to 1981. Neither strains nor data were available for 1982, but in 1983 the isolation rate increased by 61% relative to the previous years [7]. It is interesting to note that this dramatic increase occurred in 1983 despite the fact that in nearby Eastern European countries, *S. enteritidis* had been the most prevalent serovar since at least 1979 [2]. Approximately one third of eggs imported to Switzerland came from the former Eastern bloc countries such as Hungary and Poland [8], where pre-1980 isolation rates for *S. enteritidis* were very high [2]. Eggs are

Table 1. S. enteritidis *isolates from Switzerland: phage type, plasmid profile and evolutionary line of isolates*

Strain number*	Source	Epidemiology	Year of isolation	PT†	Plasmid profile‡	Clonal lineage§
UB1	human, stool	sporadic	1965	8	38‖	*Se*CLII
UB2	human, stool	sporadic	1965	5	38‖	*Se*CLI
UB10	human, stool	sporadic	1966	7	50, 38‖	*Se*CLI
UB67	human, stool	sporadic	1967	NT	38‖	*Se*CLIII
UB111	human, stool	sporadic	1967	15	38‖	*Se*CLIII
UB142	human, stool	sporadic	1968	8	38‖	*Se*CLII
UB146	human, stool	sporadic	1968	8	38‖	*Se*CLII
UB184	human, stool	sporadic	1969	8	38‖	*Se*CLII
UB652	human, stool	sporadic	1973	4	38‖	*Se*CLI
UB690	human, stool	sporadic	1973	8	38‖	*Se*CLII
UB716	human, stool	sporadic	1973	8	38‖	*Se*CLII
UB3	human, stool	sporadic	1983	4	38‖, 2	*Se*CLI
UB3789	egg pasta	production control	1983	4	38‖	*Se*CLI
UB4328	poultry	carcass	1983	1	38‖	*Se*CLI
UB2501	poultry	carcass	1984	8	38‖	*Se*CLII
UB2584	human, stool	sporadic	1984	4a	38‖	*Se*CLI
UB2825	human, stool	sporadic	1985	NT	38‖	*Se*CLI
UB2955	past. egg	production control	1985	4	38‖	*Se*CLI
UB1717	human, stool	outbreak senior residence	1988	4	38‖	*Se*CLI
UB5903	human, stool	sporadic	1989	1	38‖, 27	*Se*CLI
UB6363	human, stool	sporadic	1989	NT	38‖, 30	*Se*CLI
UB2833	poultry	flock control	1990	4	38‖	*Se*CLI
UB893	human, stool	ate egg dish: tiramisu	1991	4	38‖	*Se*CLI
UB895	human, stool	sporadic	1991	4	38‖	*Se*CLI
UB2786	human, blood	sporadic	1990	8	38‖	*Se*CLII
UB2702	turkey	chick imported W. Germany	1990	8	38‖	*Se*CLII
UB6625	human, stool	patient 1 ate tiramisu with UB6633	1989	4	50, 38‖	*Se*CLI
UB6626	human, stool	patient 2 ate tiramisu with UB6633	1989	4	38‖	*Se*CLI
UB6633	egg/tiramisu	source for UB6625 and UB6626	1989	4	38‖	*Se*CLI
UB6636	egg/tiramisu	source for UB6639	1989	8	38‖	*Se*CLII
UB6639	human, stool	patient 1 ate tiramisu with UB6636	1989	8	38‖	*Se*CLII
UB6640	human, stool	patient 2 ate tiramisu with UB6636	1989	8	38‖	*Se*CLII
UB6647	human, stool	canteen outbreak	1989	4	38‖	*Se*CLI
UB6651	chicken	served in canteen with UB6647	1989	RDNC	38‖	*Se*CLI
UB4612	hedgehog	septicaemic disease	1990	11	59, 30	*Se*CLIII

* Reading down the column corresponds to track numbers as shown (l–r) in Figs. 1, 2, and 4.

† PT, phage type. Strains were phage typed according to the scheme of Ward and colleagues [14]. NT indicates an isolate which was non-reactive with typing phages, and thus non-typable. RDNC indicates an isolate which 'reacts with the typing phages but does not conform' to a designated type.

‡ Molecular weight given in Mda.

§ Clonal lines were derived from IS*200* probe hybridization to *Pst* I-digested total DNA, as described by Stanely and colleagues [13].

‖ Plasmid hybridizing with *vag*A (virulence associated gene) probe.

imported to Switzerland from these countries [8], and at least half of the broiler chicks are also imported from other neighbouring European countries [8]. Therefore the consumption of eggs and poultry meat in Switzerland reflects the situation in central Europe. The *S. enteritidis* isolation rate in Switzerland increased by 610% between 1979 and 1987 [7], the second highest increase reported for Europe [2]. A few *S. enteritidis* isolates from Switzerland have already been included in studies [9, 10] and outbreak-associated strains have been phage-typed by the UK Division of Enteric Pathogens or the US Centers for Disease Control. However, to date no systematic molecular study has been made of strains of *S. enteritidis* in Switzerland.

DNA insertion elements, associated with spontaneous polar mutations in *Escherichia coli*, are 'selfish DNAs' which encode their own transposition and its regulation [11]. The 700 bp element IS*200* is the smallest DNA insertion sequence so far characterized [12]. Molecular characterization of the IS*200* profile has been described [13] with respect to the phage-type strains of *S. enteritidis* constituting the phage-typing scheme of Ward and colleagues [14]. The present study analyses a collection of *S. enteritidis* strains chosen to reflect the pre-epidemic and current (epidemic) situation in Switzerland. Our objective was twofold; to place IS*200* profile data within the context of phage and plasmid typing of a diverse group of *S. enteritidis* and to provide a better understanding of the evolving epidemiology of this serovar in Switzerland.

METHODS

Bacterial strains

Isolates for the years 1965–73 were from sporadic human cases in the pre-epidemic period, which had been kept lyophilized. They were a gift of Dr A. Roupas, Geneva and have been employed in another study [9]. The remaining strains were chosen to reflect the current epidemic in Switzerland. They included human (faecal and blood) isolates from sporadic cases, as well as outbreak-related isolates. Poultry and egg-related food isolates were included to reflect the established sources of human infection by this serovar [4–6]. One hedgehog isolate was also included because these animals have been known for some time to carry *S. enteritidis*.

DNA preparation and hybridization experiments

Plasmid DNA was analysed by the method of Kado and Liu [15]. Genomic DNA was prepared from all isolates according to the method described for *S. enteritidis* [13], digested with the restriction enzyme *Pst* I, and vacuum-blotted (LKB Vacu-Gene apparatus) onto Hybond N nylon membrane (Amersham). The 300 bp internal *Eco*R I–*Hind* III fragment of IS*200* was prepared from pIZ45 [16] by polymerase chain reaction, and labelled with [^{35}S]ATP (Amersham) as previously described [13]. All hybridizations were made according standard methods [17, 18]. Membrane filters were washed in 5 × SSC (0·6 M sodium chloride, 0·06 M sodium citrate, pH 7), 0·1% sodium dodecyl sulphate. They were then exposed for 24 h to Fuji RX X-ray film.

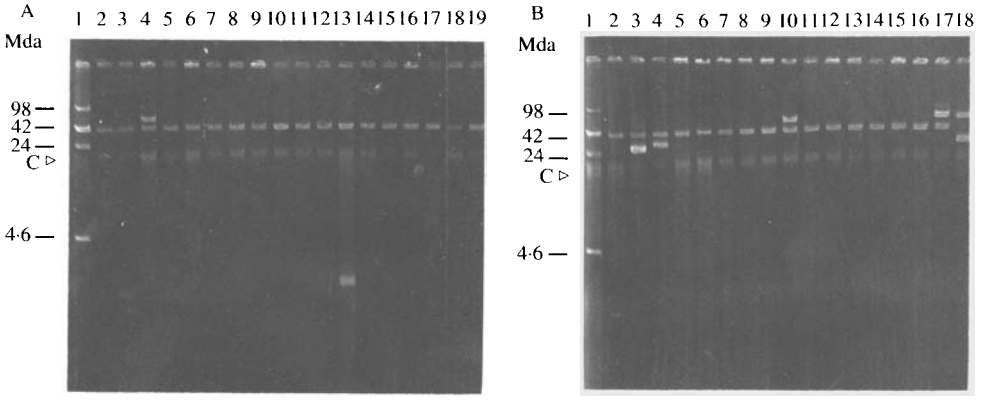

Fig. 1. Plasmid profiles of Swiss *S. enteritidis* isolates. A: Track 1 contained strain 39R861, with molecular weight marker plasmids of 98, 42, 24 and 4·6 Mda. Tracks 2–19 contained, respectively: track 2, UB1; 3, UB2; 4, UB10; 5, UB67; 6, UB111; 7, UB142; 8, UB146; 9, UB184; 10, UB642; 11, UB690; 12, UB716; 13, UB3; 14, UB3789; 15, UB4328; 16, UB2501; 17, UB2584; 18, UB2825; 19, UB2955. B: Track 1 contained strain 39R861. Tracks 2–18 contained, respectively: track 2, UB1717; 3, UB5903; 4, UB6363; 5, UB2833; 6, UB893; 7, UB895; 8, UB2786; 9, UB2702; 10, UB6625; 11, UB6626; 12, UB6633; 13, UB6636; 14, UB6639; 15, UB6640; 16, UB6647; 17, UB6651; 18, UB4612.

RESULTS

Phage type of isolates from the pre-epidemic and epidemic periods

Selected (stool) isolates from sporadic human salmonellosis from the years 1965–73 were phage-typed in accordance with the scheme of Ward and colleagues [14]. A variety of phage types (PTs) were identified, including PT5, PT7, and PT15. Only one of this group of pre-1980s isolates belonged to PT4, whilst PT8 was predominant. Twenty-three isolates were analysed for the years 1983–91; all but one were from poultry, egg or meat products, or from cases of human salmonellosis. Two sets (three isolates each) from food-poisoning outbreaks for which the sources of infection were the egg-based dish tiramisu, were epidemiologically related by phage typing. These six isolates belonged either to PT4 or to PT8. Apart from two non-typable and one PT4a isolate, all isolates in the set belonged to PT4, PT8, and PT1 in that order of prevalence. The single isolate from septicaemic disease of hedgehog belonged to PT11. These data are summarized in Table 1.

Plasmid profiles and presence of mouse-virulence genes

The isolates were analysed for the presence of plasmids by the method of Kado and Liu [15] and with the exception of the hedgehog strain UB4612 were seen to contain a plasmid of 38 Mda (Fig. 1). UB4612 carried two plasmids sized at approximately 60 and 30 Mda. This gel was Southern-blotted [18] and hybridized with a probe for the virulence locus, the *vag*A gene [19] cloned from the 60 Mda serovar-specific mouse virulence plasmid of *S. typhimurium*. Results are shown in Fig. 2 and summarized in Table 1. The probe hybridized strongly to the 38 Mda plasmid in all isolates except UB4612 (PT11). In this strain it hybridized to a plasmid of 59 Mda.

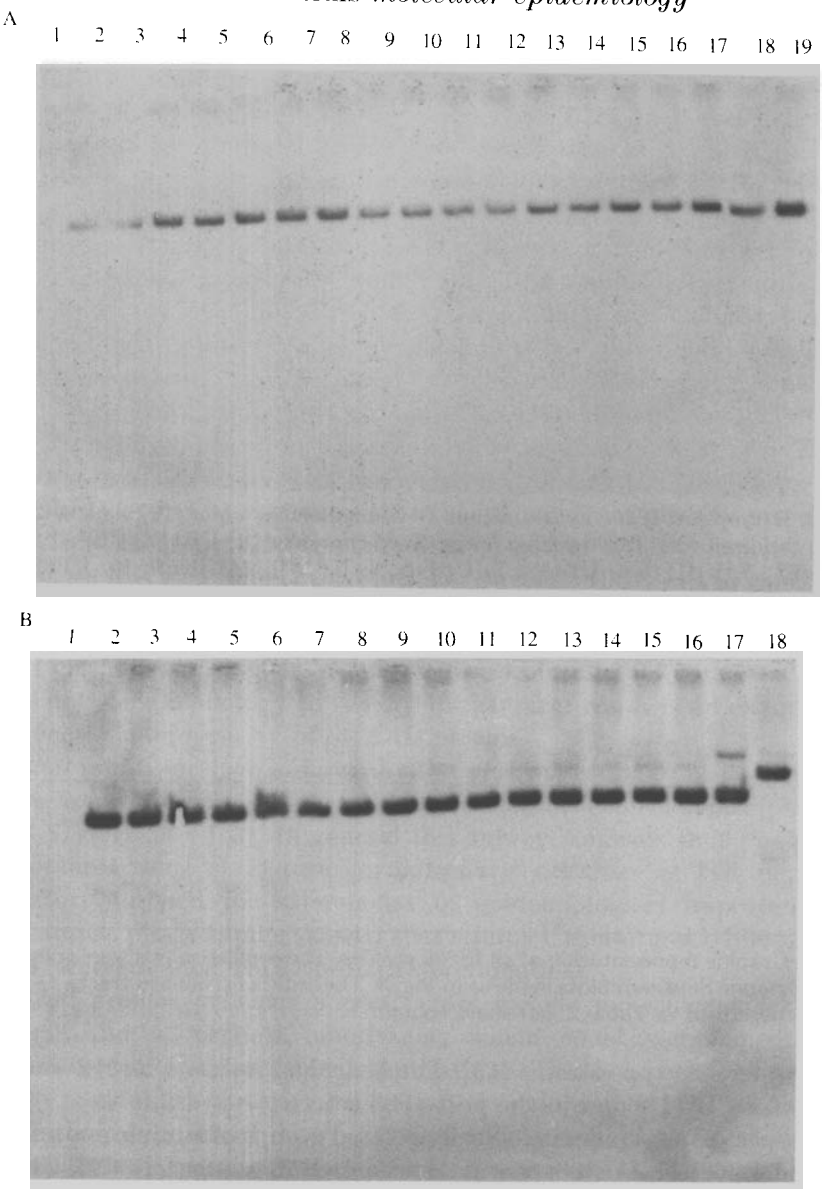

Fig. 2. Plasmid-linked virulence genes. Gels shown in Fig. 1 A, B were Southern blotted and hybridized with *vag*A. Tracks and strains were as for Fig. 1.

*IS*200 *profiles*

Genomic Southern blots made from digests with the enzyme *Pst* I were probed for the presence and copy number of IS*200*, with results exemplified by Fig. 3. Data for all strains were then represented diagramatically (Fig. 4). All the isolates generated IS*200* profiles which corresponded to one of three evolutionary lines of *S. enteritidis*. As summarized in Table 1, the correspondence between phage type and clonal line among these natural isolates precisely matched that previously

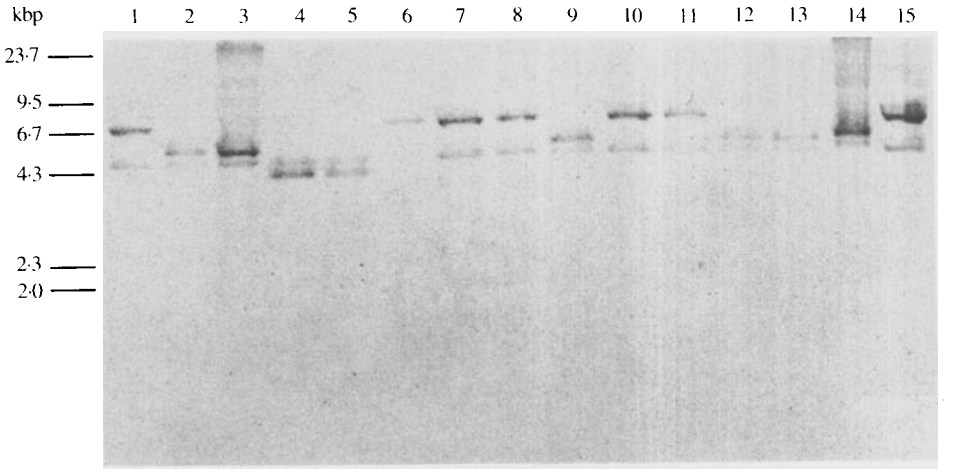

Fig. 3. Example of IS*200* hybridization. Genomic Southern blot [18] made with *Pst* I and hybridized with IS*200* probe. Tracks 1–15 contained: 1, UB1; 2, UB2; 3, UB10; 4, UB67; 5, UB111; 6, UB142; 7, UB146; 8, UB184; 9, UB652; 10, UB690; 11, UB716; 12, UB3; 13, UB3789; 14, UB4328; 15, UB2501.

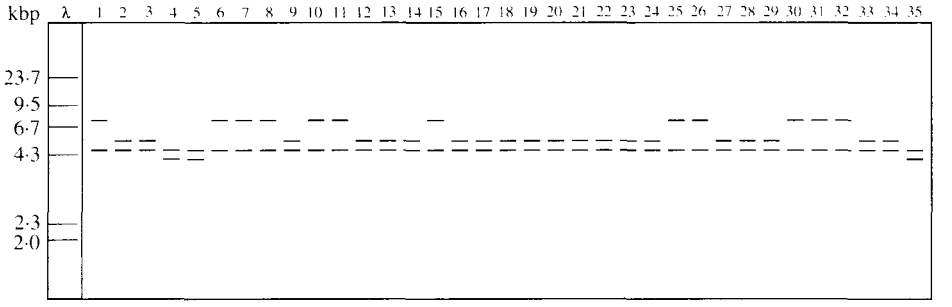

Fig. 4. Graphic representation of all IS*200* profiles. The profiles shown were transposed from genomic Southern blots made as in Fig. 3. The order of strains in tracks 1–35 (l–r) was as described in Table 2, left-hand column.

found for reference type strains [13]. The hedgehog isolate UB4612 was the only example of *Se*CLIII found in the post-1983 set.

With respect to the epidemiologically-related groups of strains, one a PT4 group (UB6636, UB6639 and UB6640) and the other a PT8 group (UB6636, UB6639 and UB6640), we observed that the IS*200* profile (Fig. 4) was able to differentiate their chromosomal genotypes, whilst the plasmid profiles (Fig. 3) were indistinguishable.

DISCUSSION

In the present report we have applied a novel approach to the analysis of a national epidemic, combining IS*200* profile typing with phage typing and plasmid profiling. Phage typing of the pre-1980s Swiss strains in the present investigation showed that a greater variety of phage types existed among the sporadic isolates from the 1960s and 1970s, and that PT8 was the most significant of these. At the same time, clonal line *Se*CLII was predominant, and *Se*CLIII was also well represented. Evidence exists that the latter is the most heterogeneous of these

three lines of *S. enteritidis* [13]. In the epidemic period *Se*CLI became the predominant line and its major phage type, PT4, became the predominant phage type. Among this sample of strains, *Se*CLIII had receded to background status in Switzerland (from 2/11 human isolates pre-1980, to 0/14 human isolates post-1980), and the single representative found was a 1990 hedgehog isolate belonging to PT11.

With one exception all isolates contained a plasmid of 38 Mda, which hybridized with a virulence-associated gene (*vag*) probe. This result concurs with previous data on the 38 Mda plasmid of *S. enteritidis* which is non-transferable *in vitro* [20–22]. The precise phenotype of this plasmid is best described as virulence for BALB/c mice, but its role in human infection is questionable, since strains associated with outbreaks of gastroenteritis have been characterized which lack any plasmid or chromosomal virulence-associated gene sequences [23]. The IS*200* chromosomal profiles can be most easily reconciled with the ubiquitous presence of the 38 Mda plasmid by assuming that this plasmid was already present in the *S. enteritidis* ancestor and that the IS*200* transposition which generated the three clonal lineages was a subsequent evolutionary event. All strains in this set which contained a second plasmid belonged to *Se*CLI; these varied in size from 2–50 Mda. The hedgehog isolate was unusual in that its candidate virulence-associated plasmid was sized at 59 Mda, whilst a plasmid of about 30 Mda in this strain did not show homology to *vag*A. This atypical result is an example of the relative genetic heterogeneity of *Se*CLIII strains.

The IS*200* profile method is shown by the present results to be as applicable to uncharacterized outbreak isolates as it was for established reference (phage type) strains of *S. enteritidis* [13]. In general this survey confirms that phage typing, which measures very short-term evolutionary distance, is the most highly discriminatory method for salmonellas of epidemiological importance. As a specific example, phage typing showed that strains UB6647 and UB6651 were not associated, whereas their IS*200* profiles were identical.

The IS*200* profiles provides a useful race-specific molecular marker of the chromosome and of vertical inheritance, which could complement plasmid profiling for certain epidemiological applications. This is especially true where it is necessary to establish chromosomal genotypes independently of plasmid profile. From a technical point of view, IS*200* profiling has distinct advantages of accessibility (there is no requirement for reference type strains and typing phages), and the potential to be used with non-radioactive labelling. Whilst phage typing is eminently suitable to analyse a high throughput of an epidemic serovar, IS*200* profiling could be used with advantage in low-throughput analyses of less common serovars, especially those for which phage typing does not presently exist.

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