*Epidemiol. Infect.* (1995), **114**, 25–40



# The distribution of serotype-specific plasmids among different subgroups of strains of *Salmonella enterica* serotype Enteritidis: characterization of molecular variants by restriction enzyme fragmentation patterns

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(*Accepted 3 October 1994*)

SUMMARY

Four hundred and thirty-four isolates of *Salmonella enterica* serotype Enteritidis were studied. They were grouped into five subsets defined by either the collection criteria or the parameter which formed the basis for subsequent analysis. Seventy-seven per cent harboured the serotype-specific plasmid (SSP). In 55% of the isolates this was the sole plasmid. Molecular variation in the SSP was detected in 17 (5%) of the isolates on the basis of restriction enzyme fragmentation pattern (REFP) analysis using *Pst* I and *Sma* I. The SSP variants were further characterized using additional restriction enzymes chosen to optimize the information content and analysed using a coefficient of similarity.

A variant SSP designated pOG690 showed greater resemblance to the SSP of *Salmonella enterica* serotype Typhimurium than Enteritidis; 89% and 68% respectively for *Pst* I and 79% and 55% respectively for *Sma* I. In respect of the *Pst* I data pOG690 shared at least 55 kb of DNA with the Typhimurium SSP and 37 kb with the SSP of Enteritidis. This variant was associated with poultry (duck, goose, chicken) and all isolates belonged to phage type 9b. Other variants were associated with phage types 4, 6, 6a, 9a, 11, 15 and 24. The epidemiological implications of these results are discussed.

## INTRODUCTION

The prevalence of individual serotypes of salmonellae associated with human infections varies with time, geographical isolation and a wide range of additional parameters. Nevertheless certain serotypes of *Salmonella enterica* tend to dominate over long periods (e.g. Typhimurium and Enteritidis), some remain notable by their persistence at low incidence such as Infantis whereas a third group, typified in the UK by Virchow, Hadar and Agona have each achieved temporary prominence within a 5–10 year period and then declined. The mechanisms that underlie these patterns of incidence remain largely unknown although the

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consistently dominant serotypes harbour plasmids that enhance virulence in animal models and are serotype-specific (SSP) [1–4].

Enteritidis has been consistently among the ten most prevalent serotypes isolated from humans in Scotland for 20 years and the dominant serotype since 1987 [5]. Before 1987 phage types 4 and 8 were isolated in similar numbers but since then phage type 4 has predominated. This has been attributed to clonal expansion and corresponds to the increase in egg-associated infections in both the UK and USA [6–8].

The value of plasmid profile analysis and restriction enzyme fingerprinting in epidemiological investigation is well established and has been extensively applied in the investigation of salmonellosis [9–15]. The strategic application of restriction enzyme fragmentation pattern (REFP) analysis [16] has demonstrated both overall conservation [17] and divergence [18] of the SSP of Typhimurium and this has also been shown in selected type strains of Enteritidis.

The aims of the study were to determine (a) the distribution of the Enteritidis SSP in defined subsets, (b) the extent of diversity which exists between homogeneous and heterogeneous populations, (c) whether molecular variants analogous to those reported by Brown and colleagues [19] were detectable and (d) to evaluate the combined results in terms of strain diversity based on the molecular characterization of Enteritidis plasmids.

## METHODS

### *Experimental design*

Realization of the aims and objectives of the investigation required that a large collection of Enteritidis be examined. The strains available were diverse in terms of origin, but varied considerably in respect of the availability of epidemiological information and could not justifiably be described overall as a single representative collection. They were therefore grouped into a series of sub-sets defined by either the collection criteria or the parameter which formed the basis of subsequent correlation. Several sub-sets included sporadic isolates which may have belonged to either episodes or outbreaks and were unrecognized on epidemiological grounds. However, a single strain was included where outbreaks were defined and the isolated strains were shown to be consistent with expansion of a single clone. All of the isolates were phage typed and this is referred to where necessary. Enteritidis GRI 16485, pOG674 was used as a reference strain for comparison of plasmid fingerprints as previously described [18].

Subset 1 contained isolates from Scotland during the period 1986–9. The isolates were heterogeneous in that they were from independent outbreaks or episodes and did not belong to any particular phage type. Subset 2 contained only phage type 4 isolates from both human and veterinary sources and all were isolated in 1990. Subset 3 contained 75 isolates from 1987–9 that were selected to represent phage types and include phage types isolated infrequently. Multiple isolates of selected phage types are included but where possible none of these were multiple isolates from the same outbreak. Subset 4 contained isolates from the United States of America from both a poultry and egg associated outbreak and a selection of

random veterinary isolates. This subset is further subdivided into three categories: (1) isolates were recovered from poultry; (2) isolates were egg associated; (3) isolates were collected from diverse geographic locations: 14 different states. Subset 5 contained isolates from Scotland and from the National Collection of Type Cultures (NCTC) all of which pre-dated 1979.

*Media*

Cystine-lactose-electrolyte-deficient (CLED) agar (Mast) was used for growth and maintenance of organisms and isosensitest agar and broth (Oxoid) for sensitivity tests. Nutrient agar (NA; Oxoid) and brain heart infusion broth (BHI; Oxoid) were used for growth of organisms for DNA extraction. BHI was used for broth conjugation.

*Construction of transconjugants*

Transconjugants were constructed as described previously [18].

*Plasmid profiles and restriction fingerprints*

Plasmid DNA was examined in crude lysates by a modification of the method of Platt and Sommerville [20]. The molecular weight of plasmids was determined by reference to plasmids of known size (kb); Rts 1 (180), RA-1 (127), R1 (93), R702 (69) and RP4 (54). Supercoiled ladder (Life Technologies, Paisley, UK) was used for the molecular weight estimation of small plasmids (< 16 kb). Plasmid size values incorporated into plasmid profiles were determined on a minimum of two occasions.

Restriction fingerprinting was carried out as previously described in detail [16]. Plasmid DNA was extracted and purified from clinical isolates and transconjugants by an alkaline lysis, phenol extraction and ethanol precipitation method. Restriction enzymes were obtained from Life Technologies and used according to the manufacturer's instructions. Restriction fingerprints were compared using a coefficient of similarity [21] calculated from the formula,

$$S_D(\%) = \frac{2m}{a+b} \times 100,$$

where 'm' was the number of restriction fragments common to two plasmids (A and B) and 'a' and 'b' were the total number of fragments generated from each plasmid respectively after digestion by the same restriction enzyme.

*Computer-aided analysis of restriction fragments*

Restriction fragment mobility in ethidium bromide-stained agarose gels was recorded on Polaroid type 665 film and input to a Viglen 486 microcomputer via a digitizer (Summagraphics). Each gel was calibrated using restriction fragments from a *Pst* I digest of bacteriophage lambda either alone or in combination with a *Sma* I or *Hin*d III digest of the same phage. The molecular weight of these fragments was fitted to a robust modified hyperbola [22] from which the size of restriction fragments in adjacent tracks was estimated. Numerical values were stored for subsequent graphical output which was on a logarithmic scale. Experimental variation in fragment size did not exceed 5%.

Table 1. *Source, distribution and plasmid profile of* Salmonella enterica *serotype Enteritidis isolates studied*

| Source (No. of isolates) | Plasmid profile (kb) | Frequency | Phage type |
|---|---|---|---|
| 1. Scotland 1986–9 (75) | | | |
| | 54 | 50 | — |
| | ND | 10 | — |
| | 54:7·0 | 3 | — |
| | *80 | 2 | — |
| | 54:2·2 | 1 | — |
| | 54:2·7 | 1 | — |
| | 54:3·4 | 1 | — |
| | 54:5·1 | 1 | — |
| | 90:54 | 1 | — |
| | 100:54 | 1 | — |
| | 110:54 | 1 | — |
| | 110:54 | 1 | — |
| | 130:54 | 1 | — |
| | 110:100:54 | 1 | — |
| 2. Scotland 1990 (131) | | | |
| | 54 | 108 | — |
| | ND | 5 | — |
| | 54:1·3 | 9 | — |
| | 54:2·0:1·3 | 5 | — |
| | 54:4·0 | 1 | — |
| | 54:4·5 | 1 | — |
| | 54:30:8·0 | 1 | — |
| | 60:54:2·0 | 1 | — |
| 3. Scotland 1987–9 (75) | | | |
| | 54 | 4 | 1 |
| | ND | 1 | 2 |
| | 54:5·0 | 1 | 5 |
| | 54 | 1 | 5a |
| | 54 | 1 | 6 |
| | 54:8·0 | 1 | 6 |
| | 54:10 | 2 | 6 |
| | 54:14 | 1 | 6 |
| | 54:50 | 6 | 6 |
| | 54:20:9·0 | 1 | 6 |
| | 90 :4·0 | 1 | 6 |
| | ND | 1 | 6a |
| | 54:8·0 | 1 | 6a |
| | 54:40 | 2 | 6a |
| | 90 :7·0 | 1 | 6a |
| | 90:54 | 4 | 6a |
| | 130 | 1 | 6a |
| | 54 | 4 | 7 |
| | *80 | 1 | 11 |
| | 54:2·5 | 1 | 12 |
| | 54 | 4 | 13a |
| | 7·0 | 1 | 14b |
| | *54 | 1 | 15 |
| | 54:9·0 | 1 | 21 |

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Table 1. (*cont.*)

| Source (*No. of isolates*) | Plasmid profile (kb) | Frequency | Phage type |
|---|---|---|---|
| | 54 | 1 | 23 |
| | 54 | 1 | 24 |
| | 54:45 | 17 | 24 |
| | 45 | 5 | 24 |
| | *100 | 1 | 24 |
| | 54 | 1 | 25 |
| | 54 | 1 | 26 |
| | 80:54 | 1 | 27 |
| | 10 | 1 | 29 |
| | ND | 1 | 32 |
| 4. USA (126) | | | |
| (1) | 54 | 19 | — |
| | ND | 21 | — |
| | 54:5·0 | 1 | — |
| | 54:6·0 | 1 | — |
| | 54:7·0 | 4 | — |
| | 54:10 | 1 | — |
| (2) | 54 | 59 | — |
| | ND | 1 | — |
| (3) | 54 | 10 | — |
| | *80 | 4 | — |
| | *54:8·0 | 1 | — |
| | 54:8·0 | 1 | — |
| | 70 | 1 | — |
| | 160:54:3·5 | 1 | — |
| | 60:*54 | 1 | — |
| 5. Scotland 1977–8 (27) | | | |
| | 54 | 11 | — |
| | *54 | 2 | — |
| | ND | 1 | — |
| | 100 | 1 | — |
| | *80 | 1 | — |
| | 65:54 | 1 | — |
| | 54:4·[a] | 2 | — |
| | 54:4·3 | 1 | — |
| | 54:4·5 | 1 | — |
| | 54:54 | 1 | — |
| | 3·5 | 1 | — |
| National Collection of Type Cultures | | | |
| NCTC 618 | *70: 7·0 | 1 | — |
| NCTC 3045 | 54 | 1 | — |
| NCTC 5188 | 70:*54 | 1 | — |
| NCTC 8515 | *80 | 1 | — |

[a] Designated sub-set.
* Denotes variant plasmid.

*Interpretation of restriction fingerprints*

The following general rules were applied to the interpretation of plasmid fingerprints:

(1) To establish that an observed fingerprint represented a variant of pOG674

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demanded that its recognition was initially in a strain of Enteritidis in which it was the sole plasmid or was similarly present in an *Escherichia coli* transconjugant. In the latter situation each of the fragments detected must have been present in the original isolate of Enteritidis.

(2) If the difference between the observed variant fingerprint and pOG674 was solely due to an additional fragment or fragments the same result must have been obtained when the plasmid was digested with twice the standard amount of restriction enzyme to exclude the presence of the products of partial digestion.

(3) Presumptive recognition of variants in clinical or veterinary isolates that contained additional plasmids was accepted either if the additional plasmid was substantially different in copy number or the variant had lost at least one fragment, or the additional plasmid(s) were conjugative and when each fragment in the transconjugant fingerprint was matched with the donor one or more additional fragments was seen compared to those from pOG674.

## RESULTS

A total of 434 isolates of Enteritidis were studied. They were grouped into five subsets. Overall 362 (83%) harboured the SSP and in 275 (63%) isolates this was the sole plasmid. Additional plasmids > 20 kb were present in 39 isolates, two of which co-migrated with the SSP; co-resident small plasmids < 20 kb were detected in 49 isolates. Plasmids other than, or in the absence of the SSP were harboured by 16 (4%) isolates: 41 (9%) isolates were plasmid free. Molecular variants of the SSP were detected in 17 (4%) of the isolates and were represented in four of the five subsets (Table 2 and Figs 1 and 2).

*Subset 1.* Among 75 current Scottish isolates, 65 (87%) possessed plasmids and 10 (13%) possessed none. Of these 65 isolates, 50 (67%) possessed the 54 kb SSP alone whereas all except two (3%) others harboured the SSP together with one or more additional plasmids. The 80 kb plasmid, in the two isolates that possessed a single plasmid of 80 kb, was found by REFP analysis to be an SSP variant (designated pOG691: Table 2). Of 13 (17%) isolates that harboured more than one plasmid all included an SSP; seven possessed an additional smaller plasmid of molecular weight less than 10 kb and in six isolates the additional plasmids ranged between 90 and 130 kb.

*Subset 2.* Among 131 Enteritidis PT4 isolates from 1990, 108 (82%) harboured the SSP alone, five (4%) were plasmid free and 18 (14%) possessed the SSP together with one or more additional plasmids. No variant plasmids were found in this subset.

*Subset 3.* Initially 20 isolates each of which belonged to a different phage type were examined for the presence of plasmids. Of these only five (25%) possessed the SSP alone, two (10%) were plasmid free, two (10%) harboured a small plasmid but no SSP and in a further two (10%), variant plasmids were detected. One of the variants was identical to pOG691 (subset 1). The other was very similar but not identical to pOG691 and differed as described in detail below and was designated pOG701. Nine isolates were found to possess the SSP together with one additional plasmid.

One of 12 isolates which belonged to phage type 6 possessed the SSP alone. Six

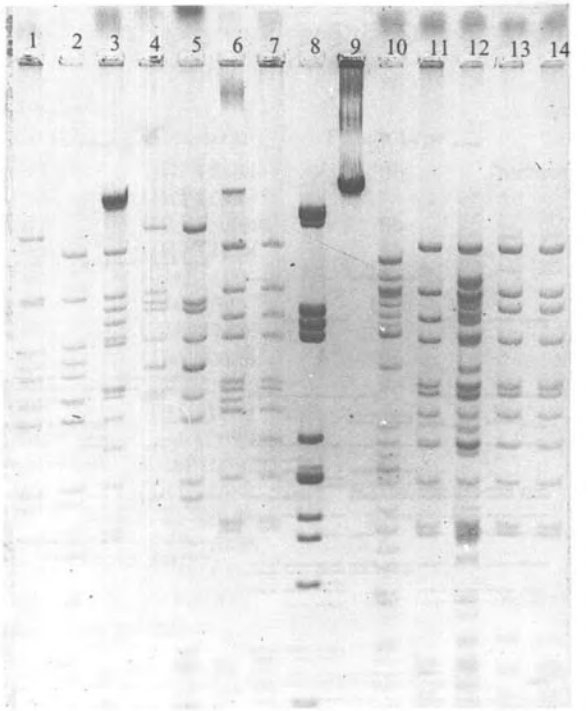


Fig. 1. *Pst* I REFPs of Enteritidis plasmids (left to right). Lanes 1, pOG660; 2, pOG690; 3, pOG703; 4, pOG691; 5, pOG701; 6, pOG704; 7, pOG674; 8, lambda phage DNA *Pst* I; 9, lambda phage DNA undigested; 10, undesignated; 11, pOG674; 12, pOG674 together with one other plasmid; 13, pOG700; 14, pOG700.

olates showed the plasmid profile 54:50 kb and fingerprints very similar to two hage type 6a isolates with a plasmid profile 54:40 kb. The 50 kb plasmid from hage type 6 strains and the 40 kb plasmid from phage type 6a strains were ansferred to *E. coli* K12 to simplify analysis. The REFP showed interesting atures which were nevertheless difficult to interpret. The 40 and 50 kb plasmids en in these isolates were related. *Eco*R I and *Pst* I REFP's show this and from ıe data it is assumed that the 40 kb plasmid is a derivative of one of the 50 kb lasmids (results not shown).

Among the remaining six phage type six isolates, five contained an SSP together ith one or more plasmid(s) and one isolate had the plasmid profile 90:4·0 kb. one of the nine phage type 6a isolates contained the SSP alone, six contained the SP and one other plasmid (two of which were described above) one had the lasmid profile 90:7·0 kb and one harboured a single plasmid of 130 kb.

Twenty-four isolates representative of phage type 24 showed one containing the SP alone, five isolates harboured a single 45 kb plasmid one had a 100 kb plasmid nd the remaining 17 had the plasmid profile 54:45 kb. The 45 kb plasmid, after onjugative transfer from the 17 isolates which also contained the SSP, was found ɔ be identical on the basis of REFPs to the 45 kb plasmid from the five strains ı which this plasmid was present alone. The 100 kb plasmid had the same REFP ith four enzymes as those 17 isolates which contained both the 54 kb SSP and

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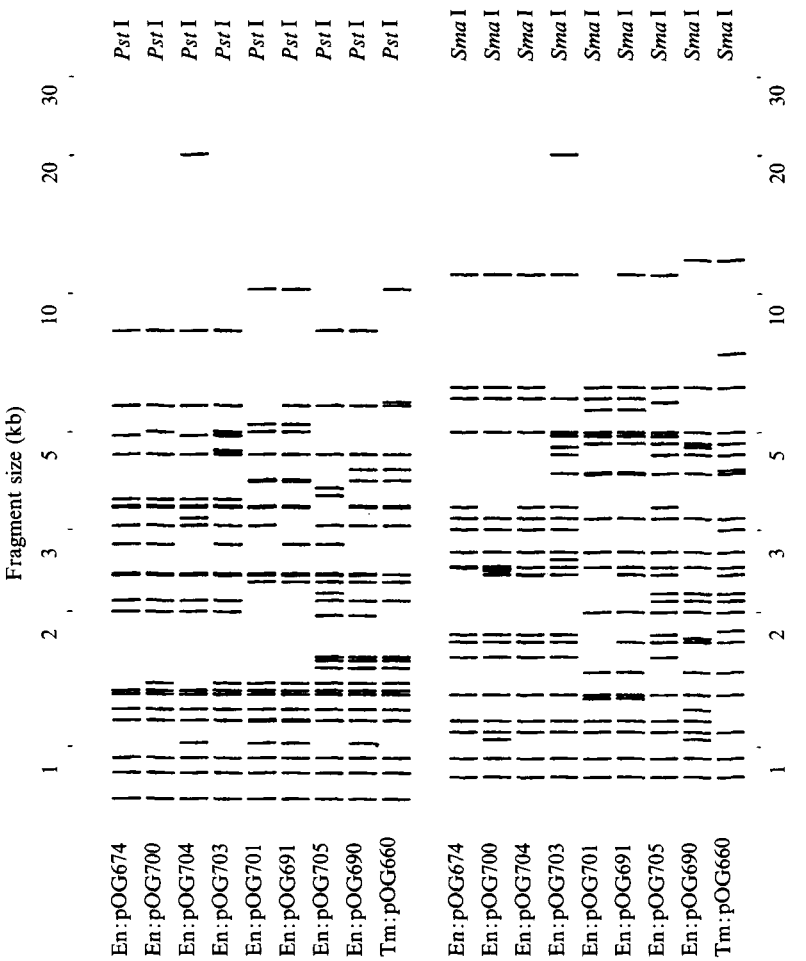


Fig. 2. Graphical output of digitized images from *Pst* I and *Sma* I REFPs of variant SSPs. Lanes 1 and 10. pOG674; 2 and 11, pOG700; 3 and 12, pOG704; 4 and 13, pOG703; 5 and 14, pOG701; 6 and 15, pOG691; 7 and 16, pOG705; 8 and 17, pOG690; 9 and 18 pOG660. Plasmids were ordered to optimize recognition of variant fragments.

45 kb plasmid as separate plasmids. However, after digestion with *Hinc* II comparison of the REFPs showed minor differences in fragmentation pattern between the 100 kb and the 54/45 kb combination consistent with the former plasmid being a co-integrate which was designated pOG706 (Fig. 3).

Four phage type 13a, 3 phage type 7 and 3 phage type 1 isolates harboured the SSP alone.

In summary, 22% possessed the SSP only, 4% possessed a variant SSP, 4% were plasmid free, 53% possessed the SSP plus one or more additional plasmids and 17% possessed plasmids other than and in the absence of the SSP.

*Subset 4.* Episode 1 isolates recovered from a poultry associated source in the USA showed a high percentage (45%) of plasmid free strains: 19 of the remaining 26 isolates (40%) possessed the SSP alone and 7 (15%) the SSP together with one additional small plasmid.

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Table 2. *Molecular variants of the serotype-specific plasmid of* Salmonella enterica *serotype Enteritidis*

| Plasmid designation | Molecular weight (kb) | Host strain | Phage type | Comment |
|---|---|---|---|---|
| pOG690 | 80 | GRI 12888 | 9b | Subset 4. Intermediate |
| | 80 | GRI 12988 | | — |
| | 80 | GRI 13088 | 9b | — |
| | 80 | GRI 13488 | 9b | — |
| pOG691 | 80 | SR881190 | 11 | Subset 1 |
| | 80 | SR882596 | 11 | Subset 2 |
| | 80 | SR893743 | 9 | Subset 1 |
| | 80 | GRI 10688 | 9a | Subset 5 |
| | 80 | NCTC 8515 | 9a | Subset 5 |
| pOG700 | 54 | GRI 11888 | 4 | Subset 5 |
| | 54 | GRI 12088 | 4 | — |
| pOG701 | 54 | SR881508 | 15 | Subset 2 |
| pOG702 | 54 | GRI 12588 | RDNC | Subset 4 |
| pOG703 | 54 | GRI 12388 | 24 | Subset 4 |
| pOG704 | 54 | NCTC 5188 | 6a | Subset 5 |
| pOG705 | 70 | NCTC 618 | 6 | Subset 5 |
| pOG706 | 100 | SR890702 | 24 | Subset 2. Co-integrate |

Episode 2 isolates recovered from an egg associated source (egg yolk or selenite washed shells) had 59 (98%) isolates which contained the SSP only and one plasmid free strain.

Nineteen random veterinary isolates collected in the USA over a 3-year period from diverse geographical locations (14 states) were studied. Ten (53%) possessed the 54 kb SSP alone, three others possessed the SSP in combination with one or more plasmids and one isolate had only a 70 kb plasmid. The isolate with plasmid profile 54:8·0 kb possessed a variant SSP designated pOG702. The isolate with plasmid profile 60:54 kb possessed two plasmids which, after REFP analysis showed the 54 kb plasmid to be a variant SSP and was designated pOG703. The 60 kb R-plasmid was transferred to *E. coli* K12 and showed no resemblance to the SSP with any of the enzymes used (Fig. 3). In four further strains an 80 kb SSP variant plasmid (designated pOG690) was found.

*Subset 5*. Four strains from the National Collection of Type Cultures (NCTC) showed one strain which possessed an SSP alone. NCTC 618, harboured a 90 kb SSP variant designated pOG705, profile 90:7·0 kb. NCTC 5188 showed the plasmid profile 70:54 kb. The 54 kb plasmid was a variant SSP, designated pOG704. The 80 kb plasmid, pOG691, was found in NCTC 8515. Twenty-three strains, from the collection held by the Scottish Salmonella Reference Laboratory, isolated between 1977–8 made up the remainder of this subset. Eleven isolates possessed the SSP alone, one contained a small 3·5 kb plasmid, another only a 100 kb plasmid and one was plasmid free. Six isolates possessed the SSP together with one other plasmid; one of these was found to contain two 54 kb plasmids (which co-migrated) neither of which were variant SSPs. Two other isolates harboured a 54 kb plasmid alone, which were variants based on REFP analysis. These two isolates were Enteritidis phage type 4, and no variant SSPs have been reported in this phage type until this time. This plasmid was designated pOG700.

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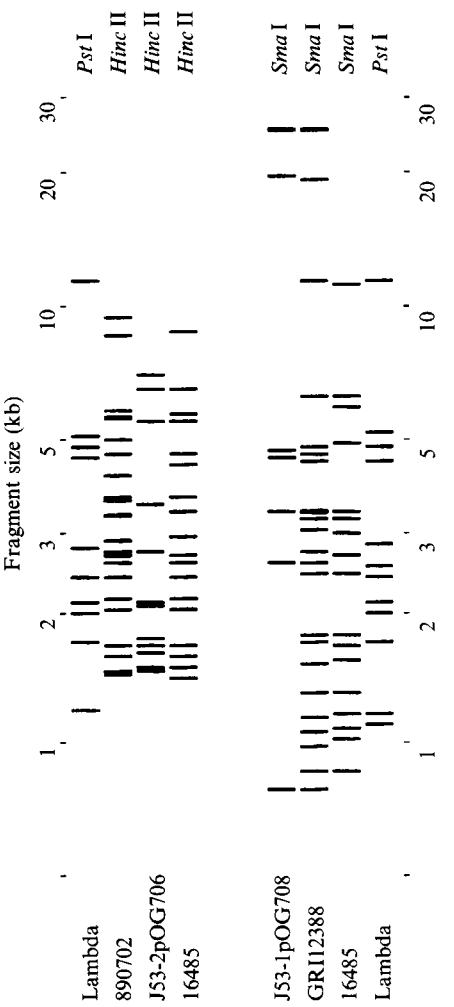


Fig. 3. Graphical output of digitized images from REFPs of plasmid DNA digested as follows. Lane 1, lambda DNA digested with *Pst* I; 2, SR890702 – 100 kb co-integrate, *Hinc* II; 3, *E. coli* K12 J53-2 pOG706, *Hinc* II; 4, GRI 16485, (pOG674), *Hinc* II; 5, J53-1 pOG708 *Sma* I; 6, GRI 12388, *Sma* I; 7, GRI 16485 (pOG674), *Sma* I; lambda DNA, *Pst* I.

The 80 kb plasmid found in this subset was indistinguishable from pOG691. In summary, 12 (44%) isolates possessed the SSP alone, one (3%) isolate was plasmid free, six (22%) harboured variant SSPs, six (22%) harboured the SSP together with one or more additional plasmids and three (9%) harboured plasmids other than the SSP.

*Analysis of variants*

The SSP variants are shown in Figure 2, in which those plasmids most similar to the Enteritidis reference plasmid pOG674 are juxtaposed and those showing a greater similarity to the Typhimurium SSP are grouped with the Typhimurium reference plasmid pOG660. The similarities seen with both enzymes indicate that

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Table 3. *Dice coefficients of similarity* ($S_D$) *from* Salmonella enterica *serotype Enteritidis plasmids on the basis of* Pst I (*upper*) *and* Sma I (*lower*) *REFPs*

| Plasmid designation (pOG) | 660 | 674 | 690 | 691 | 700 | 701 | 702 | 703 | 704 | 705 |
|---|---|---|---|---|---|---|---|---|---|---|
| pOG660 | — | 62 | 89 | 73 | 59 | 68 | 62 | 55 | 62 | 71 |
| pOG674 | 57 | — | 68 | 70 | 93 | 63 | 92 | 55 | 92 | 70 |
| pOG690 | 79 | 55 | — | 68 | 58 | 72 | 67 | 60 | 70 | 79 |
| pOG691 | 74 | 68 | 83 | — | 56 | 86 | NT* | 54 | 51 | 75 |
| pOG700 | 73 | 91 | 62 | 78 | — | 60 | NT | 90 | 86 | 73 |
| pOG701 | 73 | 55 | 78 | 90 | 60 | — | NT | 51 | 54 | 56 |
| pOG702 | 64 | 100 | 61 | NT | NT | NT | — | 90 | NT | NT |
| pOG703 | 59 | 78 | 65 | 68 | 81 | 58 | NT | — | 92 | 78 |
| pOG704 | 68 | 97 | 63 | 65 | 89 | 70 | NT | 86 | — | 63 |
| pOG705 | 81 | 75 | 74 | 81 | 71 | 74 | NT | 75 | 73 | — |

* NT denotes not tested.

a gradation of relatedness is evident. This is confirmed by the similarity coefficients shown in Table 3.

Plasmid pOG690 was detected in three American isolates of Enteritidis phage type 9b. Initial REFP analysis using *Pst* I, suggested a closer resemblance to the reference SSP from Typhimurium (pOG660) than the reference Enteritidis SSP (pOG674). Detailed comparison of the *Pst* I fingerprint of pOG690 with the SSP from both serotypes indicated that with the exception of a single restriction fragment (1·0 kb) each of 24 fragments between 0·7 and 10 kb was present in either the Typhimurium or the Enteritidis reference SSP. Both of these SSPs shared only 13 restriction fragments in common. This finding suggested that pOG690 was an evolutionary intermediate – *Sma I*, *Ava II*, *Hind III* and *EcoR I* fingerprints corroborated these findings. Coefficients of similarity [5] between the reference SSPs (Table 3) were 62% (*Pst* I) and 57% (*Sma* I). Comparisons of pOG690 with pOG674 gave $S_D$ values of 68% (*Pst* I) and 55% (*Sma* I) and with pOG660 the values were 89% (*Pst* I) and 79% (*Sma* I).

*pOG691.* This plasmid was commonly seen in Enteritidis phage types 9a and 11. Dice coefficients of similarity indicated that this plasmid shows slightly more similarity with pOG660 than pOG674. However, this is in the order of a 2% difference with each of three enzymes.

*pOG700.* This plasmid was found in two isolates of Enteritidis phage type 4, from the Scottish collection of 1977–8. This plasmid showed an overall increase of 1·5 kb compared to pOG674. The *Pst* I digest showed the loss of a 4·9 kb fragment which is replaced by two additional fragments of 5·1 kb and 1·4 kb. The *Sma* I digest showed the loss of a 3·4 kb fragment which has been replaced with two new fragments of 2·45 and 2·4 kb, Figure 2.

*pOG701.* This plasmid was found in Enteritidis phage type 15 isolates. It closely resembled pOG691 and Dice coefficients showed 86% and 90% similarity with *Pst* I and *Sma* I respectively. The Dice coefficients of this plasmid with the SSPs of Enteritidis and Typhimurium indicates a more marked similarity to pOG660 than pOG674.

*pOG702.* Preliminary data with *Pst* I and *Sma* I showed the loss of a 4·9 kb fragment in *Pst* I digests however, no detectable differences were seen in the *Sma* I

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REFP. This plasmid was accepted as a variant SSP by previously defined criteria [22] for the interpretation of restriction fragments. No subsequent work was done on this plasmid as the organism has since died.

*pOG703*. *E. coli* K12 J53-1 transconjugants were characterized by plasmid profile and REFP in parallel with the parent strain, GRI 12388. This confirmed that the 54 kb plasmid present in the Enteritidis donor was a variant SSP. The *Pst* I showed an additional single fragment of 5·5 kb, whereas the *Sma* I showed the loss of a 5·9 kb fragment (Fig. 3).

*pOG704*. This plasmid was seen in NCTC 5188 which was isolated in 1953 and was phage typed as 6a. The *Pst* I digest showed an additional 3·3 kb fragment, concomitant with the loss of a 2·8 kb fragment. The *Sma* I digest showed an additional 2·4 kb fragment. The additional fragments seen in both digests were confirmed to have come from the 54 kb plasmid by comparison with transconjugants as described above for pOG703.

*pOG705*. This plasmid was seen in NCTC 618 which was isolated in 1920 and was phage typed as 6a. In comparison with pOG674 the *Pst* I digest showed the loss of 4·9, 3·5 and 3·4 kb fragments together with additional fragments of 3·7, 3·6, 2·3, 2·2, 1·95, 1·7, 1·6 and 1·4 kb. This indicates at least 10 kb of additional DNA together with several fragments of less than 1·2 kb. The *Sma* I digest showed an overall increase of about 20 kb which concurs with the *Pst* I data above.

## DISCUSSION

The results presented here demonstrate considerable diversity among the plasmids of *Salmonella enterica* serotype Enteritidis. However, the degree of diversity varies with the population studied. In contrast to the report of Brown and colleagues [19], a wider range of polymorphisms was demonstrated among 54 kb plasmids. This is no doubt related in part to the greater number and diversity of strains examined in this study. However, close comparison indicates further contrasts, for example, the polymorphism shown in pOG701 (from Enteritidis PT15) differs from that described [19] and suggests that rather than being unique within this phage type, this phage type may show a propensity for polymorphisms in the SSP. This may also be true of PT6a, in which two distinct plasmid fragmentation patterns were demonstrated (pOG704 and pOG705, Tables 2 and 3). With regard to the 80 kb plasmids, it is likely that at least some of these are analagous between the two studies, notably those harboured by strains of Enteritidis PT11; unfortunately, phage types 9a and 9b were not represented among strains studied by Brown and colleagues [19] and detailed comparison is further restricted by lack of *Sma* I fragmentation patterns.

Plasmid analysis has proved to be a useful tool in epidemiological investigations of Salmonellosis [9, 11, 12]. However, this study has shown that in Enteritidis it offers little discrimination and can lead to the assumption that plasmids of the same molecular weight are the same plasmids. Plasmid fingerprinting can resolve differences that remain undetected in PPA and demonstrates that plasmids of the same molecular weight can be unrelated and furthermore that plasmids of different molecular weights can be closely related. This study showed 45 different plasmid profiles when the isolates were examined by PPA alone but increased

to 65 when the plasmid profiles were interpreted in the light of REFP analysis. Many of these would therefore have gone undetected using PPA alone. A simple pre-defined strategy [16] of PPA together with REFP analysis can optimize the information content and increase the level of discrimination in investigations of the epidemiology of Enteritidis.

Holmberg and colleagues [23] concluded that PPA appeared to be at least as specific as phage typing in the recognition of epidemiologically related isolates of Typhimurium. Similarly, Kapperud and colleagues [24] have stated that PPA and phage typing are valuable and convenient tools of considerable versatility in epidemiological tracing. Although useful, this approach is not without certain limitations and the results should be interpreted, at the very least, with caution.

Five of the isolates in this study (Table 2) were found to harbour 54 kb plasmids which were molecular variants of the Enteritidis SSP. The variation would have been undetected by PPA alone. Similarly in sub-set 5, one isolate was found to harbour two co-migrating 54 kb plasmids. The presence of two plasmids was recognized only by REFP analysis. Two Enteritidis phage type 4 isolates were shown to harbour molecular variants of the SSP and appears to be the first report of phage type 4 isolates which show SSP variation. However, most have been characterized on the basis of PPA and phage typing alone.

The importance of these findings in the interpretation of PPA in epidemiology are twofold. The widespread presence of the 54 kb plasmid among Enteritidis strains indicates that no epidemiological specificity is conferred by its recognition unless molecular variation is demonstrated by REFP analysis. Secondly the accumulation of additional plasmids by Enteritidis although relatively uncommon, when demonstrated, significantly increase strain specificity because the additional plasmids are diverse.

Popoff and colleagues [25] first suggested that the large plasmids of some serotypes of salmonella constituted a family of related plasmids and concluded that they may represent the divergence of an ancestral plasmid. This report describes molecular variants of the Enteritidis SSP in 17 of 434 isolates. Of 9 variant plasmids which showed different degrees of divergence from the SSP, pOG690 showed particularly interesting features. This plasmid showed a more marked similarity to the SSP of Typhimurium rather than Enteritidis. We can assume that the virulence genes are present in this plasmid based on the information that certain *Pst* I fragments have been sequenced in this region and that these fragments are present in all three plasmids [26]. Beninger and colleagues [27] concluded that despite differences in size and REFP of plasmids isolated from different Salmonella serotypes all these plasmids encode virulence functions by a common genetic mechanism. This mechanism may have arisen from a common progenitor which has proliferated and diverged to give rise to those plasmids which have been termed serotype-specific: especially those of Enteritidis and Typhimurium. The findings with Dublin discussed by Platt and colleagues [5] suggests that the maintenance of selection for virulence need not have co-selected for other determinants, thus explaining the lower level of fingerprint similarity of the Dublin SSP with pOG660. This explanation could be extended to the large plasmids of other Salmonella serotypes, many of which show similarity in the virulence region [3, 28].

Heteroduplex analysis has shown [29] that the plasmids of Salmonella serotypes Typhimurium, Enteritidis, Dublin and Choleraesuis show large areas of homology. It is stated that the Enteritidis plasmid is 99% homologous to the Typhimurium plasmid and from this data a common ancestor was also suggested. We currently consider that pOG690 is a direct evolutionary intermediate in the descent of virulence plasmids from Typhimurium to Enteritidis. Logically the molecular variation must have arisen by one of two general mechanisms. Given that the currently predominant REFP is widely distributed among different phage types (Table 1) and is also evident in NCTC 3045 isolated in 1929, variant plasmids with a significantly higher molecular weight have either recently acquired additional DNA, in which case additional restriction fragments may be expected to bear resemblance to SSPs from other serotypes, or alternatively the additional DNA represents an intermediate stage in the evolution of SSPs which currently range from 50 kb (Choleraesuis) to 90 kb (Typhimurium). The results presented favour the latter alternative. Nevertheless the question raised by Montenegro and co-workers [29] of whether SSPs have evolved by ascent from the smaller plasmids of Enteritidis and Choleraesuis to the larger plasmids of Typhimurium, or the converse, remains unanswered. Although the elegant scenario proposed [30] for the late evolution of the host adapted serotypes Gallinarum and Pullorum from an Enteritidis-like ancestor is internally consistent it seems neither to take adequate account of the non host adapted nature of Typhimurium or the size of its virulence plasmid.

The results revealed that those populations which were epidemiologically homogeneous retained this feature after PPA and REFP. There were however some surprising results. The overall data showed that variant SSPs occurred among approximately 4% of the isolates. This result is paralleled in subset 1 which contained a representative collection of current Scottish isolates. In subsets 4(3) and 5 variant SSPs occurred at 31 and 22% respectively. This level of variation is not unexpected and is principally a consequence of sample incomparibility. Its anticipation was the reason why the strains included were separated into sub-sets from the outset to minimize interpretative bias.

Although this report has focused on molecular variation in the SSP of Enteritidis this plasmid is nevertheless highly conserved on the basis of REFP analysis like the SSP of Typhimurium [5] and in marked contrast to resistance plasmids [31, 32]. Whereas the conservation of a virulence region might be expected and this has been shown to be about 8 kb, the conservation of a further 46 kb (Enteritidis) and 82 kb (Typhimurium) is more surprising.

ACKNOWLEDGEMENT

We wish to acknowledge the Scottish Salmonella Reference Laboratory, Stobhill General Hospital, Glasgow for the provision of strains, serotyping and phage typing.

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