Epidemiol. Infect. (1995), **114**, 237–248

Diversity and molecular variation among plasmids in *Salmonella enterica* serotype Dublin based on restriction enzyme fragmentation pattern analysis

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(*Accepted 28 November 1994*)

SUMMARY

Molecular variation within and between plasmids of *Salmonella enterica* serotype Dublin was analysed. Such variation has been demonstrated in the serotype-specific plasmids (SSP's) of Typhimurium and Enteritidis. The two aims of this study were to determine the plasmid diversity in a host-adapted serotype and also the incidence of molecular variation in the SSP among strains of Dublin using restriction endonuclease fragmentation pattern (REFP) analysis with *Pst*1, *Sma*1 and *Eco*RV. Sixty-five strains were examined from seven countries. Plasmid profile and REFP analysis showed that none of the strains was plasmid-free. Seventy-seven percent of the strains possessed the 72 kb SSP either alone or in combination with another plasmid; 23% harboured plasmids which were molecular variants of the SSP. Four of the variants were more closely related to each other than to the reference SSP and were harboured by Dublin isolated from both the USA and Europe. A further three were shown to be cointegrate plasmids and were similarly distributed. Thirty-two percent of strains possessed the SSP alone. None of the UK strains was resistant to any of the antimicrobial agents tested whereas 74% of the remaining strains were resistant to between one and five antimicrobial agents. This study corroborates previous findings concerning the high degree of stability of the SSP and confirmed the clonal nature of Dublin. Co-resident plasmids provided evidence of sub-clones within localized geographical areas.

INTRODUCTION

The association of particular plasmids with certain serotypes of *Salmonella enterica* is well recognized [1–5]. Hybridization and other studies [4–8] indicate that these plasmids are related and differ in size with respect to host serotype; the restriction enzyme fragmentation pattern (REFP) of each plasmid being

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Table 1. *Characteristics of* Salmonella enterica *serotype Dublin strains studied*

Country of origin	Strain designation	Plasmid profile (kb)	Antibiotic Resistance‡
UK	GR34285	72 (pOG675)	ND
	GR10290, GR10490, S/921039	72	
	S/921096, S/921209, S/921857,		
	S/924489, S/924957, S/925323,		
	S/925455, S/925824		
	S/923907	72* (pOG688):40	
	S/920682	72* (pOG689)	
	GR10190, GR10390	72* (pOG682):45	
	S/921207, S/925167, S/920507	72:3·3	
	S/920386, S/920038, GR14792	[72:70 (pOG647)]	
	S/923782, S/924759		
	S/922378	[72:70]†:3·3	
	S/921941	70 (pOG647):30	
	S/922442	70 (pOG647):40	
USA	GR9890	72	ND
	GR9690	72	Ap
	GR9990, GR10090	72	Ap Tc Km Gm Sm
	GR1590, GR1690	72* (pOG683)	ND
	GR2290	72* (pOG640):65	Ap Km
	GR9490	72:65	Ap Km
	GR9590	82* (pOG685)	Ap Km
	GR9790	72* (pOG686):60	Ap Km Su
	GR1890	72:65	Ap Tc Km
	GR1990	72:65	Ap Tc Km
	GR2090	72:65	Ap Tc Km Sm
	GR1790	125* (pOG650)	Ap
	GR2190	125* (pOG649)	Ap
Canada	GR8290	72:60:6	Ap Cm
	GR8390	72:60:6	Ap Tc Su Cm
	GR8490	72:60:6	Ap Sm Su
	GR8590	72:60:6	Ap Tc Cm Km Sm
Denmark	GR9090, GR9190	72	ND
	GR9290	72:3·8	ND
	GR9390	72:30:3·3	ND
France	GR8790	72	Tc Su Cm
	GR8690, GR8890	72* (pOG687):40	ND
	GR8990	72:4·3:3·3	ND
Germany	GR8090	72	Tc Su Cm
	GR7890	125* (pOG648)	Ap
	GR7990	72:3·3	Cm Su
	GR8190	72:9	Ap Cm Su
Holland	GR2490	72	Tc Su Cm
	GR2690	72	Km Su Sm Cm
	GR2590	72* (pOG684)	Ap Tc Km Su Cm
	GR2390	72:12	Ap Tc Su Cm
	GR2790	72:3·3	Nal Tc Su Cm
	GR2899	72:3·3	Tc Su Sm
	GR2990	72:3·3	Su Cm
	GR3090	72:3·3	Tc Su Cm

*, Indicates a variant plasmid on basis of REFP analysis.

† [72:70], Indicates comigration of plasmids of similar size; distinguished only after REFP analysis. pOG675, *S.* Dublin reference SSP [15].

characteristic of the serotype [9]. The plasmids range in size from 90 kb in Typhimurium to 50 kb in Choleraesuis [1, 3]. These plasmids are non-conjugative, have been termed serotype-specific [9], are stable and highly conserved with respect to REFP in each serotype. The analogous plasmid in Dublin is 72 kb. These plasmids contribute to the virulence of the organisms in the mouse model [2–4, 10–13].

As part of a survey of salmonella plasmids in 1985 strains of Dublin were all found to harbour the serotype-specific plasmid (SSP) alone [14]. This contrasts with the situation in both Typhimurium and Enteritidis where a small but significant proportion of strains was plasmid free [9]. Molecular variation among SSPs has been demonstrated previously in Typhimurium [15] and Enteritidis [16], the incidence of which was about 5% in both serotypes. Neither of these serotypes is host-adapted and the first aim of this study was to examine overall plasmid diversity in a host-adapted serotype. The second aim was to determine both the incidence of molecular variation in, and the degree of variant relatedness between strains of Dublin using REFP analysis.

MATERIALS AND METHODS

Bacterial strains

The strains studied comprised part of the international collection held by the Central Veterinary Laboratory (Weybridge). Additional strains were kindly provided by Dr P. Jones (Institute of Animal Health, Compton) and a wide range of Scottish isolates (Table 1). Confirmation of serotype was carried out by standard methods [17]. GR34285, pOG675 was used throughout as a reference strain of Dublin for the comparison of REFPs [15]. Where epidemiological information indicated that multiple isolates were from a single outbreak or incident and molecular data were consistent with this conclusion a single isolate was included. However, all isolates epidemiologically defined as sporadic were included.

Media

Cystine lactose electrolyte deficient (CLED: Mast DM110) was used for maintenance of the cultures. Long-term storage was on Dorset's egg slopes at room temperature. Nutrient Agar (CM3) was used for bacterial growth for plasmid extraction. Plasmid purification for REFP analysis was carried out using cultures grown in Brain Heart Infusion broth (BHI: Oxoid CM225). Antimicrobial sensitivity testing was by disk diffusion assay on isosensitive agar using antibiotic disks (Oxoid) impregnated with individual antimicrobial agents: ampicillin (Ap; 10 μg), carbenicillin (Cb; 100 μg), cephazolin (Kz; 20 μg), tetracyline (Tc; 10 μg), kanamycin (Km; 30 μg), streptomycin (Sm; 10 μg), chloramphenicol (Cm; 10 μg), sulphamethoxazole (Su; 25 μg), trimethoprim (Tp; 1·25 μg), nalidixic acid (Nal; 30 μg), cephamandole (Ma; 30 μg), colistin sulphate (Ct; 25 μg), amikacin (Ak; 10 μg), tobramycin (Tb; 10 μg), gentamicin (Gm; 10 μg) and rifampicin (Rif; 50 μg) [17].

‡. Ap, ampicillin; Tc, tetracycline; Sm, streptomycin; Km, kanamycin; Su, sulphonamide; Cm, chloramphenicol; Nal, nalidixic acid. ND, No resistance detected.

Plasmid analysis

Plasmid profile (PP) and REFP analyses were performed as previously described [15]. Plasmid profiles were determined using a crude lysate method followed by vertical gel electrophoresis (200 V for 4 h). Plasmid visualization was carried out after staining the gel in ethidium bromide (6 μg/ml) for 15 min, viewed by ultra-violet light at 365 nm and photographed on type 665 film; size was determined by comparison with plasmids of known molecular weight.

REFP analysis was performed as detailed by Platt and colleagues [15] and involved plasmid DNA extraction after alkaline SDS lysis, purification with phenol-chloroform, precipitation with isopropanol; RNA'ase treatment and ethanol precipitation. The choice of enzymes for plasmid analysis was designed to maximize the information content of REFPs with neither too many nor too few fragments generated. For this reason *Pst*1, *Sma*1 and *Eco*RV were used. Additional enzymes *Hae*III, *Alu*I and *Hinc*II were used to compare small plasmids co-resident in some strains. Restriction enzymes were supplied by Life Technologies and used according to the manufacturer's instructions. The numerical designation of plasmid profiles was after analysis of REFP data and a requirement for inclusion was that both sources of data be consistent.

Incompatibility testing

Putative cointegrate plasmids were transferred to *Escherichia coli* K12 J53-2 with selection for ampicillin and rifampicin resistance. Thereafter confirmed transconjugants were introduced into Dublin GR34285 and the loss of pOG675 confirmed by agarose gel electrophoresis as previously described in detail [15].

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 computer using a digitizer and commercially available software (Molmatch, UVP). Each gel was calibrated using restriction fragments from both *Pst*1 and *Kpn*1 digests of bacteriophage lambda. The molecular weight of these fragments was fitted to a robust modified hyperbola [18] from which fragment sizes in adjacent tracks were estimated by interpolation. Numerical values were stored for subsequent calculation of similarity coefficients [19] and graphical output; the latter was on a logarithmic scale.

Definition of molecular variants: interpretation of REFPs

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

(1) To establish that an observed REFP represented a variant of pOG675 demanded that its recognition was initially in a strain of Dublin in which it was the sole plasmid.

(2) Alternatively, presumptive recognition of variants in clinical or veterinary isolates that contained additional plasmids was accepted if the additional plasmid was substantially different in copy number [20] or if the variant had lost at least one fragment.

(3) If the difference between the observed variant fingerprint and pOG675 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. Furthermore where there was a significant increase in plasmid size the sum of the molecular weight of the additional restriction fragments must correspond with the total size increase.

RESULTS

A total of 65 distinct isolates of Dublin were examined. They were geographically diverse; UK (26), USA (15), Holland (8), Canada (4), Denmark (4), France (4) and Germany (4). Strain designation, antimicrobial resistance pattern and plasmid profile are shown in Table 1. The majority were bovine isolates with the exception of 5 strains (2 human; 1 ovine; 1 reindeer; 1 environmental) and were collected between 1982 and 1992.

Plasmid analysis

None of the isolates was plasmid free; all except 4 harboured a 72 kb plasmid and 31 (48%) possessed 1 or more additional plasmids. Fifteen different strains were distinguished on the basis of PPA: 82 kb (1), 72 kb (30), 72:65 kb (5), 72:60 kb (1), 72:45 (2), 72:40 (4), 72:30 (1), 72:60:6 (4), 72:30:3·3 (1), 72:3·8 (1), 72:3·3 (9), 72:9 (1), 72:4·3:3·3 (1), 72:12 (1), 125 (3). The 15 recognizable plasmid profile types were further subdivided on the basis of REFP analysis into 28 strains.

Of the 30 isolates which possessed a single 72 kb plasmid from PPA data, 5 were shown to harbour 2 co-migrating plasmids after REFP analysis and the additional plasmid designated pOG647. The latter strain was found only among UK isolates and was associated with both outbreak and sporadic isolation.

The two human strains isolated in 1992 both possessed two plasmids (70:40 kb) and (70:30 kb). The 70 kb plasmid was identical in both strains. It was, however, unrelated to the SSP, and had the same fragmentation pattern as pOG647. However, REFP analysis showed no *Pst*1 or *Sma*1 restriction site in the 30 kb plasmid and a single restriction site for both enzymes in the 40 kb plasmid. The single ovine and environmental isolates each contained the SSP alone.

All the remaining strains harboured the SSP either alone, in addition to one or more plasmids or as a molecular variant.

Eighteen (28%) strains harboured small plasmids (< 20 kb) in addition to the SSP. Five different small plasmids were identified. Three individual strains harboured 12, 9 and 3·8 kb plasmids. The four Canadian strains each possessed a 6 kb plasmid. The predominant small plasmid was 3·3 kb and was found in 11 (61%) of strains that harboured small plasmids. One of these strains also harboured a 4·3 kb plasmid. No small plasmids were found in strains from the USA.

The 3·3 kb plasmid contained a single *Pst*1 restriction site and no *Sma*1 restriction sites. *Hae*III digestion produced a doublet of 1·1 kb and a 0·9 kb fragment. *Alu*1 digestion generated three fragments (a doublet of 1, 0·8, 0·5 kb). All plasmids of this size were indistinguishable.

One strain (GR8990) in addition to this plasmid, possessed a plasmid of 4·3 kb which had no *Sma*1 restriction sites and a single *Pst*1 site. The 3·8 kb plasmid was found in a single strain. It contained both a single *Sma*1 and *Hinc*II restriction site and no *Pst*1 restriction sites. *Hae*III digestion generated three fragments, 1·6, 1·3, 0·9 kb. The 6 kb plasmids were identical and contained a single *Sma*1 restriction site. *Pst*1 digestion generated three fragments 2·9, 2·2, 0·9 kb.

The 45 kb plasmid present in strains GR10190 and GR10390 (pOG682) produced a single fragment after digestion with *Pst*1, and two fragments of 40 and 1·74 kb after digestion with *Sma*1.

The 65 kb plasmids present in five USA strains were all closely related to each other; three were identical and there were only minor differences in the remaining two.

Both *Pst*1 and *Sma*1 fragments from these plasmids showed remarkable similarity with some of the fragments in the REFPs of the 125 kb plasmids pOG649 and pOG650. This suggests that the 65 kb plasmids may have been co-integrate precursors or arisen by dissociation of the co-integrate plasmid. This hypothesis was strengthened by the behaviour of the co-integrates during conjugative transfer. If the selection pressure was not maintained then the resulting transconjugants contained two plasmids of 72 and 65 kb consistent with co-integrate dissociation.

The 60 kb plasmids seen in the four Canadian strains also exhibited a high degree of relatedness. GR8290, 8390 and 8490 were identical (*Sma*1 generated fragments of 40, 5·3, 4·5, 4·0 and 3·3) whereas in GR8590 which was isolated from a reindeer, the 3·3 kb fragment was replaced by fragments of 2·3 and 1·4 kb. In *Pst*1 digests the four plasmids were indistinguishable.

Molecular variants of the SSP

Fifteen strains harboured plasmids which were molecular variants of the reference SSP pOG675 – and comprised 12 distinct patterns (Figs 1 and 2).

Plasmid pOG689 was recognised as a variant plasmid only after digestion with *Sma*1 (REFPs after digestion with *Pst*1 and *Eco*RV were identical to pOG675). *Sma*1 digestion showed the 1·94 kb SSP fragment to be absent. No additional fragments were observed.

Plasmid pOG684 contained all the SSP fragments when digested with *Sma*1 together with an additional fragment of 7·5 kb. However, this plasmid was recognized as a variant SSP after digestion with *Pst*1. It lacked the 1·1 kb SSP fragment and three additional fragments were detected which totalled 7·8 kb. *Eco*RV digestion revealed loss of the 2·5 and 2·3 kb SSP fragments with one additional fragment generated of 4 kb. REFP analysis with these three enzymes indicates a net gain of 5 kb DNA. (This was not evident in a plasmid profile.) *Sma*1 digestion of pOG685 showed the loss of the 42·8 kb SSP fragment together with additional fragments 29·4, 14·7 and 7·5 kb. *Pst*1 digestion revealed the loss of the 3·1 kb SSP fragment with five additional fragments generated that totalled 19·4 kb. *Eco*RV digestion revealed the loss of the 2·3 kb SSP fragment with two additional fragments of 6·8 and 3·1 kb generated. The net result of these REFP data was an overall DNA gain of 10·8 kb DNA. The *Sma* digest of plasmid pOG640 contained all the SSP fragments together with four additional fragments that

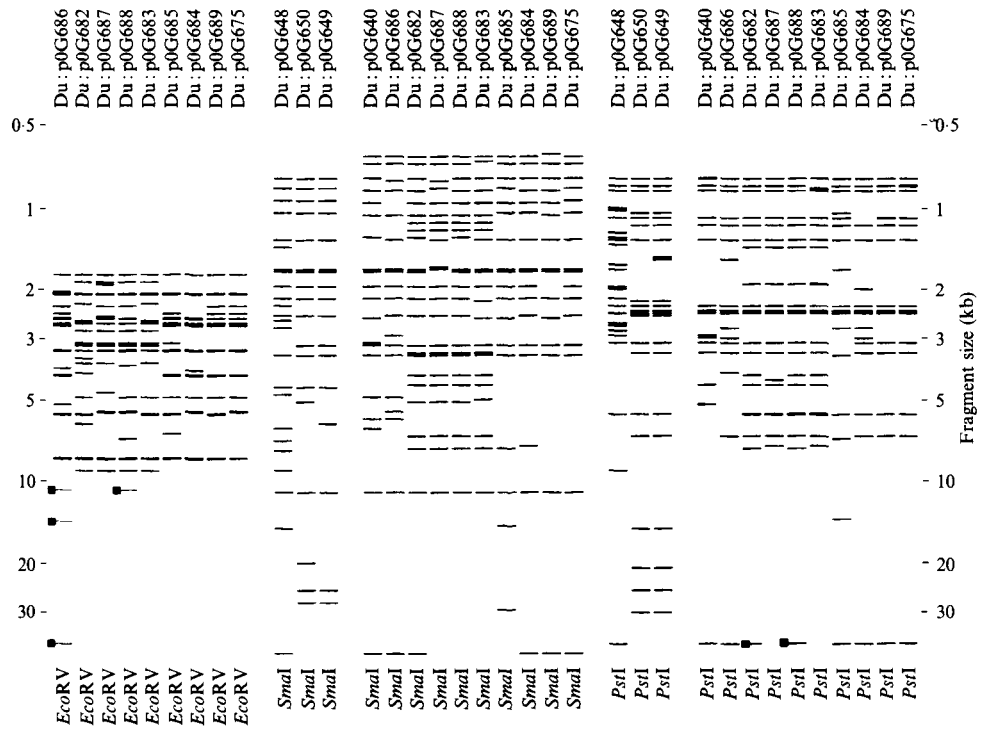

Fig. 1. Computer-generated REFPs of *S. enterica* serotype Dublin plasmids using *Pst*1, *Sma*1 and *Eco*RV. ■, Fragments derived from coresident plasmid.

totalled 20·5 kb. *Pst*1 digestion revealed the absence of both the 6·8 and 5·7 kb SSP fragments with four additional fragments generated that totalled 15·6 kb. This strain died before completion of the investigation. The presence of an additional 65 kb plasmid further complicated the analysis but the loss of two *Pst*1 fragments unequivocally indicated the SSP to be a variant.

Plasmid pOG686, when digested with *Sma*1, revealed the 0·95 kb SSP fragment to be absent with four additional fragments totalling 19·4 kb generated. *Pst*1 digestion revealed the absence of the 5·7 kb SSP fragment with four additional fragments generated that totalled 11·3 kb. *Eco*RV digestion revealed loss of the 4·9 kb SSP fragments with three additional fragments of 5·6, 5·2 and 3·9 kb, together with three fainter fragments (40, 14·1, 10·7 kb) which is consistent with their derivation from a lower copy number (60 kb) plasmid. Plasmid pOG683 lacked the 42·9 kb SSP fragment after digestion with *Sma*1; eight additional fragments were generated which totalled around 34 kb. *Pst*1 digestion revealed loss of the 39·7 kb SSP fragment with five additional fragments generated which totalled 19·4 kb. *Eco*RV digestion revealed the 4·06, 2·71 and 2·46 kb SSP fragments. Additional fragments of 9·1, 3·7, 3·2, 3·1 and 2·8 kb were generated. Overall, the three enzymes indicate a DNA loss of 5·5 kb. When pOG688 was digested with *Sma*1 the REFP was the same as that of pOG683 but for the additional 40 kb plasmid which lacked *Sma*1 sites. After *Pst*1 digestion the REFP of pOG688 was also identical to that of pOG683. However, the additional 40 kb plasmid had a single *Pst*1 restriction site and contributed a single 40 kb fragment.

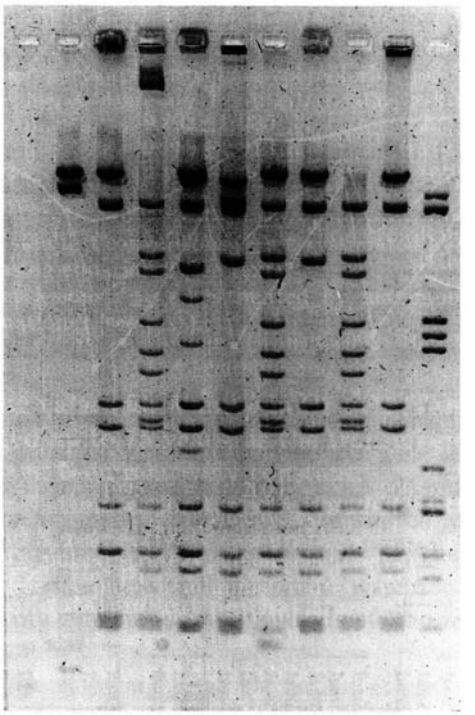

Fig. 2. REFP of *S. enterica* serotype Dublin variant SSPs digested with *Sma*1: left to right, Lambda Kpn, pOG689, pOG688, pOG686, pOG685, pOG687, pOG684, pOG683, pOG675, Lambda Pst.

*Eco*RV digestion was the same as that of pOG683 with two additional doublets of 10·7 and 6·98 which totalled 35 kb and probably comprised the coresident plasmid.

Plasmid pOG687 when digested with *Sma*1 revealed loss of the 42·9 and 1·74 kb SSP fragments; additional fragments matched those in pOG683. *Pst*1 digestion produced the same REFP as that of pOG683 with the exception of the 4·2 kb fragment of pOG687 which was paralleled in pOG683 and pOG688 by an additional 4·1 kb fragment. *Eco*RV digestion matched that of pOG683 together with two additional fragments of 2·48 and 1·92 kb.

Plasmid pOG682 was shown to be a variant plasmid only after digestion with *Eco*RV. *Pst*1 digestion matched that of pOG688 with the co-resident plasmid linearised at 43 kb. *Sma*1 digestion matched pOG688 with the co-resident plasmid also linearised. *Eco*RV digestion contained all the fragments of pOG683 together with three additional of 6·1, 4·06 (also present in the pOG675) and 3·4 kb.

The three 125 kb plasmids all contain the majority of SSP fragments. pOG648 lacked the 6·84, 3·41, 2·44 and 1·32 kb SSP fragments when digested with *Pst*1; *Sma*1 digestion showed the loss of the 3·2 kb fragment. pOG649 and pOG650 after *Pst*1 digestion showed the loss of the 39·7 kb fragment. Eight common additional fragments were generated and pOG649 contained a further two additional fragments of 1·5 and 1·45 kb. The *Sma*1 digests showed both plasmids to lack the 42 kb fragment; three common additional fragments were seen and pOG649 produced one unique fragment and pOG648 two unique fragments. Each of these plasmids was conjugative, incompatible with pOG675, and were thus co-integrate

Table 2. *Comparison of REFPs of molecular variants of the* S, enterica *serotype Dublin SSP using Dice coefficients of similarity. Upper matrix shows mean and lower matrix the individual values obtained with each of three restriction enzymes; Pst*1 (*bold*), *Sma*1 (*italic*) *and Eco*RV (*roman*). *ND = Not done*

Plasmid	pOG675	pOG683	pOG640	pOG684	pOG685	pOG686	pOG687	pOG688	pOG689	pOG682
pOG675	—	74	84	90	86	83	72	74	99	75
	81									
pOG683	*74*	—	69	70	66	62	94	97	73	96
	67									
	80	**71**								
pOG640	*88*	*67*	—	80	72	80	70	71	82	71
	ND	ND								
	87	**71**	**75**							
	97	*78*	*85*	—	84	80	66	70	89	71
	87	62	ND							
	84	**69**	**67**	**79**						
pOG685	*87*	*65*	*77*	*90*	—	73	68	69	85	71
	88	64	ND	83						
	84	**69**	**79**	**85**	**77**					
pOG686	*77*	*63*	*80*	*75*	*67*	—	63	63	81	62
	87	55	ND	80	74					
	81	**94**	**71**	**71**	**69**	**69**				
pOG687	*74*	*100*	*68*	*78*	*76*	*83*	—	91	71	93
	62	88	ND	50	60	58				
	85	**97**	**74**	**74**	**72**	**72**	**92**			
pOG688	*74*	*100*	*68*	*78*	*76*	*58*	*100*	—	73	95
	63	94	ND	57	60	60	83			
	100	**81**	**80**	**87**	**84**	**84**	**81**	**85**		
pOG689	*96*	*71*	*84*	*93*	*83*	*72*	*71*	*71*	—	74
	100	67	ND	87	88	85	62	62		
	85	**97**	**74**	**74**	**72**	**72**	**92**	**100**	**85**	
pOG682	*74*	*100*	*68*	*78*	*76*	*58*	*100*	*100*	*71*	—
	67	91	ND	62	65	56	86	86	67	

plasmids. pOG649 and pOG650 were closely related (S_D values > 90% for both *Pst*1 and *Sma*1). pOG648 was not closely related to either except in regard to the fragments derived from the SSP. This result is not surprising since pOG648 originated in Germany and pOG649 and 650 were both from American strains of Dublin.

It is clear from *Pst*1 and *Sma*1 digest results that plasmids pOG683, pOG687 and pOG688 are almost identical. *Eco*RV digestion supported these findings: all three were shown to lack the 4·06 kb SSP fragment with the same additional fragments generated – with the exception of pOG687 which had two fragments of 2·02 and 2·57 kb not present in pOG683 and pOG688. pOG685 was about 10 kb larger than the SSP as indicated by PP and confirmed by REFP analysis.

Relationships between variant plasmids

The similarily matrices (Table 2) show that each of the three enzymes corroborated the relationships between variant plasmids. In comparisons with pOG675 all variants were ⩾ 80% similar (*Pst*1) ⩾ 74% (*Sma*1) and ⩾ 62% (*Eco*RV).

The highest degree of relatedness (> 90% with each of three enzymes) between

the variant plasmids was seen in comparisons of pOG682, 683, 687 and 688. In contrast each of these plasmids was < 75% similar to pOG675. Thus, these four strains represent either parallel or progressive divergence from a single variant.

Antimicrobial resistance

None of the UK strains was resistant to any of the antimicrobials tested. Seventy-four percent of all Dublin isolates obtained from outwith the UK were resistant to between 1 and 5 antimicrobial agents.

There was no overall correlation between antimicrobial resistance and plasmid presence as some strains which possessed the typical SSP alone were also resistant to 2–5 antimicrobials and indicate a chromosomal location for the resistance determinants.

DISCUSSION

The combination of plasmid profile and REFP analysis has confirmed the international dispersion of common clones of Dublin and also demonstrated distinct strains to be common within a locality and restricted to it. Strains that harbour the 72 kb plasmid alone are particularly common and their demonstration confers no epidemiological specificity unless REFP analysis indicates molecular variation.

Population genetic studies of Dublin [23] based on multilocus enzyme electrophoresis (MLEE) distinguished 4 clones, 3 of which were closely related (Du1, Du3 and Du4); Du1 had a world-wide distribution, Du3 was restricted to the UK and France and Du4 was unique to the USA. The distribution of plasmids within Du1 was limited to either the SSP alone or the SSP together with a single small (3 kb) plasmid whereas Du3 harboured no small plasmids and either the SSP alone, the SSP together with a plasmid of intermediate size (40–50 kb) or an intermediate sized plasmid alone. A survey of Danish isolates of Dublin [24] showed 9 plasmid profiles 4 of which corresponded to profiles typical of Du1 and Du3 and thus extends the range of Du3 to include Denmark.

The more detailed analysis of plasmids presented here indicates considerably greater diversity in which there is no direct correspondence between the plasmid profiles of many strains and Du1 or Du3. Nevertheless, by inference both clones were represented among UK, Danish or French strains. The 3·3 kb plasmid found among isolates from all European countries sampled was identical on the basis of REFP analysis with four restriction enzymes although no individual enzyme generated a fingerprint with an optimal information content. Thus strains with the profile 72:3·3 are widely distributed and equate with Du1 and indicate the stability of the small plasmid. One strain from France harboured an additional 4·3 kb plasmid but is presumably clonally related. Individual strains from Denmark (PP 72:30:3·3) and the UK (72:70:3·3), neither of which had acquired drug resistance, may belong to Du1 but the presence of additional plasmids of intermediate size also raises the possibility of association with Du3. Similarly the Canadian strain that harboured small (6 kb) and intermediate sized (60 kb) plasmids and the American isolates that possessed additional plasmids cannot be readily assimilated into the clonal framework proposed [23].

The demonstration of identical REFPs among the 72 kb SSPs from all seven

countries confirms the overall conservation of this plasmid. However, molecular variation in the SSP was detected in 15 (23%) of the isolates studied of which 3 were co-integrate plasmids. On the basis of REFP similarity and dissociative behaviour *in vitro* two of these were associated with the strains of plasmid profile 72:65. A further seven of the molecular variants were detected in strains that harboured additional plasmids of intermediate size. Five of these corresponded to clone Du3; pOG682 and 688 (UK) and pOG687 (France) were almost identical to each other and also to pOG683 (USA). The latter plasmid, present alone in two American isolates, provides circumstantial evidence that Du3 is also present in the USA. The three remaining molecular variants were each recognized in single strains from different countries and none had diverged greatly from the reference SSP pOG675. Together these data suggest the possibility that co-resident plasmids may have influenced the evolution of the SSP in Dublin and go some way to explain why the SSPs of Dublin and Enteritidis are more markedly divergent than the genomic DNA appears to be on the basis of MLEE [23] and IS200 fingerprinting [25].

One interesting feature of the study was the difference in antimicrobial resistance seen in isolates from different countries: the most notable observation being the lack of resistance markers in UK isolates, which markedly contrasts with the situation in cattle with respect to Typhimurium DT204c which have progressively acquired resistance determinants since their initial detection in 1979 [21]. Plasmid analysis suggests much antibiotic resistance to be chromosomally determined as strains with the SSP alone possessed resistance markers and have been previously reported [22].

Overall, this study has demonstrated considerable plasmid diversity within Dublin and evolutionary divergence of the Dublin SSP. These findings offer some scope for the application of plasmid analysis in epidemiological investigation but not where strains harbour the undiverged SSP alone. Further studies are required to clarify the association between plasmid variation and genotypic markers; comparative analysis of molecular variants of the SSPs from different serotypes will contribute to a better understanding of salmonella phylogeny.

ACKNOWLEDGEMENTS

We thank Dr P. Jones, Institute of Animal Health, Compton and Mr T. Patterson, Veterinary Investigation Centre, Dumfries for the supply of some of the strains.

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