

## Genotype analysis of faecal and blood isolates of *Salmonella dublin* from humans in England and Wales

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### SUMMARY

An analysis of genotype was made for representative strains of *Salmonella dublin*. The collection consisted primarily of strains isolated from humans in England and Wales, and were of both intestinal and extra-intestinal origin. Three genetic elements were characterized by DNA hybridization. They were the *sprBC* genes, extrachromosomal virulence determinants, the salmonella-specific insertion sequence IS200, and the 16S ribosomal RNA genes, a phylogenetic marker. Two clones of *S. dublin* (*SdRI* and *SdRII*) which shared an identical IS200 profile, were identified on the basis of restriction fragment length polymorphism at the 16S rRNA locus. With one exception, all strains harboured a 52 MDa plasmid which contained a conserved 3·7 kbp *Hind* III fragment homologous to the *sprBC* mouse-virulence genes of *S. typhimurium*. However, a single plasmid-free strain of *SdRI*, isolated from a patient with septicaemia exhibited no *spr* homology. In *SdRI* there was no observable genotype distinction between strains causing gastroenteritis or bacteraemia. In contrast, none of the strains of *SdRII* were from cases of bacteraemia, and all human isolates of this clone were from cases of gastroenteritis.

### INTRODUCTION

*Salmonella dublin* is a Group D serotype which is a pathogen of both cattle and man. It is the most common serotype in adult cattle [1] and can be transmitted to humans via meat products, unpasteurized milk and milk products. Although not a major causative agent of human salmonellosis in England and Wales, extra-intestinal spread is an important clinical feature of many infections, and from 1981 to 1990, 25% of isolates of this serotype were from blood cultures [2].

Phenotypic subtyping with bacteriophages has provided highly discriminatory schemes for salmonella serovars of epidemiological importance such as *S. enteritidis*, *S. typhimurium*, and *S. typhi*. However, in England and Wales the relatively low incidence of *S. dublin* in humans has not justified the development of such a scheme for this serovar. Plasmid profile typing is also inappropriate for *S. dublin*, since most isolates contain a single large serotype-specific plasmid of 52 megadaltons (MDa) [3] implicated in virulence of the organism for BALB c mice. A

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physical genetic map has been described for this plasmid [4] and *Salmonella* plasmid virulence genes termed *spvR*, A, B, C, D, E, have been identified therein by transposon mutagenesis and DNA sequencing [4–7]. Plasmids of three other sizes have been reported in some isolates of *S. dublin*, but no significant difference was noted between plasmid profiles for bovine and human isolates [8].

We have previously shown that the mobile DNA element IS200, which is probably specific to salmonella [9, 10] can be used to identify three chromosomal genotypes in the Group D serovar *S. enteritidis*. Three genotypes could be distinguished by the presence of one conserved and one variable IS200 band, carried on *Pst* I fragments of 4.5 kbp (conserved band) and 5.2, 7.0 or 3.9 kbp (variable band) [11, 12]. Copy numbers and locations of many enterobacterial insertion sequences such as IS1 or IS5 can change rapidly. Identical number and position of these mobile elements between strains provides important evidence of common genetic ancestry [13]. However, IS200 transposes rarely in the laboratory [14]. It was not observed to have changed rapidly in *S. enteritidis* [11] and this stability enhances its value as a phylogenetic marker. The 16S ribosomal RNA gene is a conserved and stable chromosomal marker, whose sequence and organization are of phylogenetic significance [15, 16]. The purpose of the present investigation was twofold. Firstly we wished to employ these different types of genetic elements to elucidate genotype relationships within *S. dublin* from England and Wales. Secondly we wished to establish what, if any, genetic differences might exist between isolates causing gastroenteritis and bacteraemia respectively.

## METHODS

### *Bacterial strains and culture conditions*

Strains of *S. dublin* used in this study were from the culture collection of the Laboratory of Enteric Pathogens and are listed in Table 1. *S. typhimurium* LT2 was used as a control in hybridization studies. Stock cultures were maintained on Dorset's egg slopes at 20 °C. Strains were grown in nutrient broth/agar for DNA isolation, and purity was checked on blood agar plates. Antibiotic resistances were determined in accordance with the scheme of Anderson and Threlfall [17].

### *DNA preparation and hybridization*

Plasmid DNA of *S. dublin* was isolated by the method of Kado and Liu [18]. Plasmid pIZ45 was isolated by alkaline lysis and purified in a CsCl/ethidium bromide gradient according to standard methods [19]. Genomic DNA was extracted from *S. dublin* by the method of Wilson [19], and 10 µg quantities were digested with restriction enzymes. Genomic restriction digests or whole plasmid DNAs were electrophoresed in 0.7% agarose, and vacuum-blotted (LKB Vacu-gene apparatus) onto Hybond N nylon membrane (Amersham Intl.). The *spvBC* virulence gene probe was a 2.6 kbp *Kpn* I fragment of the plasmid pIP1367 [21].

Membranes were baked at 80 °C for 2 h, wetted with 5 × SSC, prehybridized (4 h), and hybridized (16 h) at 45 °C in small volumes (2 ml). Prehybridization mix consisted of 5 × SSC (standard saline citrate, 150 mmol/l sodium chloride, 15 mmol/l sodium citrate) containing 50% (v/v) formamide, 25 mM sodium phosphate buffer, 0.1% (w/v) ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v)

Table 1. *S. dublin isolates and their genotypes*

Strain no.	Source	Isolation from/year	Plasmid content (MDa)	IS200* profile	16S <i>rrn</i> profile
NCTC 9676	Bovine	1955	52†	3·9, 4·5	R1
S77681	Human	Pus /1988	52†, 6·0, 1·8	3·9, 4·5	R1
S77682	Human	Faeces/1988	52†	3·9, 4·5	R1
S78071	Human	Faeces/1988	52†	3·9, 4·5	R1
S77923	Human	Blood /1988	52†, 3·0	3·9, 4·5	R1
S77843	Human	Faeces/1988	52†	3·9, 4·5	R1
S78152	Human	Blood /1988	52†	3·9, 4·5	R1
S78489	Human	Faeces/1988	52†	3·9, 4·5	R1
S80021	Human	Faeces/1988	52†	3·9, 4·5	R1
S79345	Human	Blood /1988	—	3·9, 4·5	R1
S78500	Human	Blood /1988	52†	3·9, 4·5	R1
S82750	Human	Faeces/1988	52†, 1·8	3·9, 4·5	R1
S79292	Human	Blood /1988	52†	3·9, 4·5	R1
S82344	Human	Faeces/1988	52†	3·9, 4·5	R1
S82150‡	Human	Faeces/1988	52†	3·9, 4·5	R1
S77727	Human	Faeces/1988	52†	3·9, 4·5	R1
S82550	Human	Blood /1988	52†	3·9, 4·5	R1
S78993	Bovine	1988	52†	3·9, 4·5	R1
S116204	Human	Faeces/1990	52†	3·9, 4·5	R11
S116303	Human	Faeces/1990	52†	3·9, 4·5	R11
S116354	Cheese	1990	52†	3·9, 4·5	R11
S116379	Bovine	1990	52†	3·9, 4·5	R1
S116543§	Human	Faeces/1990	52†	3·9, 4·5	R11
S116750	Human	Faeces/1990	52†	3·9, 4·5	R1
S116802	Human	Faeces/1990	52†	3·9, 4·5	R1

\* Sizes of homologous *Pst* I fragments (kbp).

† Positive hybridization with *speBC*.

‡ Chromosomally-integrated resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, sulphonamides, tetracyclines, and furazolidone.

§ Patient consumed imported cheese from which S116354 was separately isolated.

bovine serum albumen, 0·5% (w/v) sodium dodecyl sulphate (SDS) and 500 µg/ml denatured herring sperm DNA. Hybridization mix, in addition, contained 5% (w/v) dextran sulphate. Hybridized filters were washed twice with each of the following: 2 × SSC/0·1% SDS for 5 min at 20 °C, 0·2 × SSC/0·1% SDS for 5 min at 20 °C and 0·16 × SSC/0·1% SDS for 30 min at 60 °C. Hybridization reactions were visualized colorimetrically with the BluGENE (Gibco-BRL Ltd) non-radioactive nucleic acid detection system.

#### *Polymerase chain reaction*

The cloned insert fragment was amplified from a 0·6 kbp *Pvu* II fragment of pIZ45 by the polymerase chain reaction (PCR) employing forward and reverse sequencing primers. 16.dUTP-biotin was incorporated during PCR or subsequently by random priming (Boehringer Mannheim kit). The biotinylated product was subjected to centrifugal ultrafiltration (Millipore Ultrafree-MC: 30000 NMWL unit) before use as a non-radioactive probe.

An intragenic 16S *rrn* gene probe was prepared from genomic DNA of NCTC 9676, employing as primers the sequences 5'-GCAACGCGAAGAACCCTTACC-3' and 5'-GGTTACCTTGTTACCGACTT-3' which represent respectively nucleotides

966–985 and 1492–1510 of the *E. coli* 16S *rrnB* sequence [22]. A single PCR product of approximately 550 bp was generated from the target genome according to standard methods and verified on a NuSeive (agarose) gel. The product was separated from primers by GeneClean according to manufacturer's instructions (BIO101, Inc), and labelled by random priming (Boehringer-Mannheim kit).

## RESULTS

### *Chromosomal IS200 profiles*

Genomic DNA of all strains was digested with enzymes which lack any site within the sequence of the IS200 element (*Pst* I, *Bgl* II, *Hinc* II). Gels were blotted and hybridized with the internal probe for IS200 prepared by PCR as described in Methods, in order to avoid sequences extraneous to the internal *Hind* III-*Eco*R I fragment of IS200. In digests with any of the above enzymes all strains carried only two homologous fragments. *Pst* I fragments were sized at 3.9 and 4.5 kbp (Fig. 1A); *Hinc* II fragments of 3.5 kbp (doublet) and *Bgl* II fragments of 1.7 and 6.1 kbp were similarly found to be common to all strains (data not shown). The copy number of two for IS200 was therefore uniform and no inter-strain polymorphisms were observed with the different enzymes. No homology of IS200 with plasmid DNA was observed (see below).

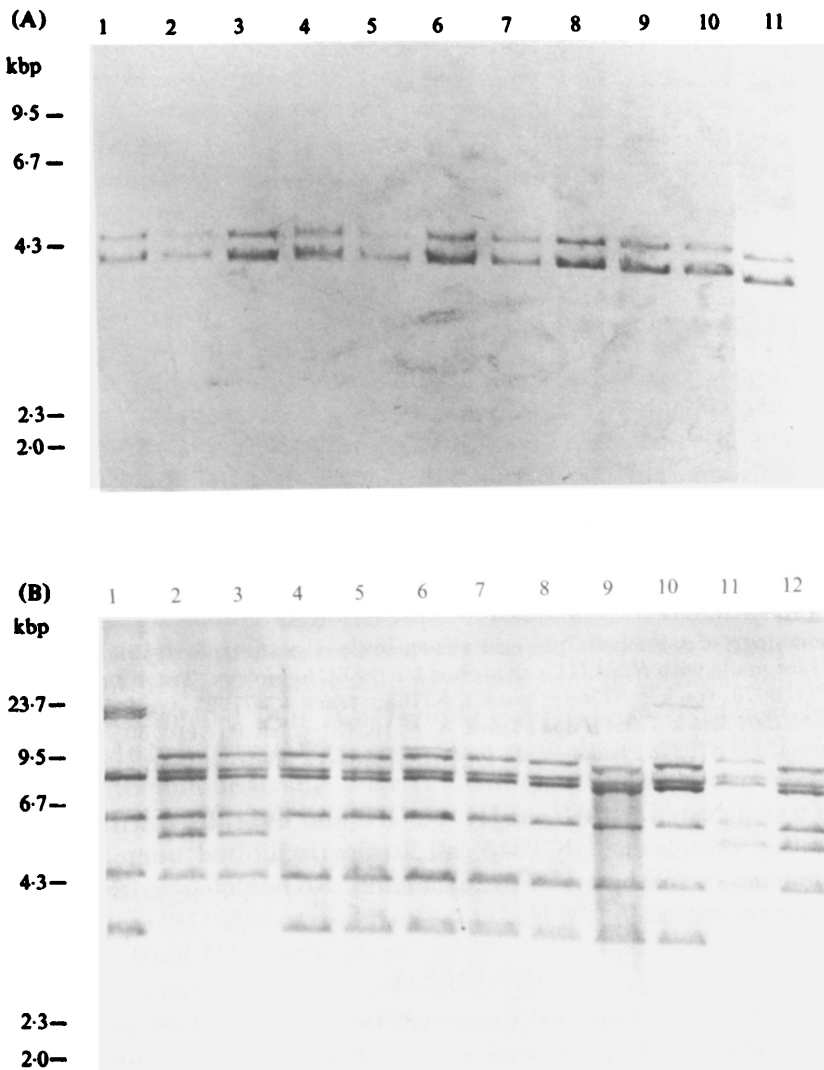
### *16S rrn gene profiles*

Genomic DNA of all strains was digested with two enzymes (*Pst* I, *Pvu* II) which lack sites within, and one enzyme (*Sma* I) which has a single site within the 16S *rrn* gene. Gels were blotted and hybridized with a 16S *rrn* gene probe generated by PCR from genomic DNA. This probe was a single fragment of approximately 550 bp corresponding to an intragenic sequence from nucleotide 966 to nucleotide 1510 of the 16S *rrn* gene. No interstrain polymorphisms were detected in *Pst* I or *Sma* I-cut DNAs. As shown in Figure 1B, two possible *Pvu* II polymorphisms (RFLPs) were observed. The first of these was shared by the great majority of strains, while the second, in which the homologous *Pvu* II fragment was replaced by one of 5.6 kbp was present in strains S116204, S116303, S116354 and S116543.

### *Plasmids and presence of mouse-virulence genes*

Isolates were analysed for plasmids by the method of Kado and Liu [18]. With one exception (S79345) all isolates harboured a 52 MDa plasmid. Strains S77681 and S82750 each carried additional small plasmids of 1.8 MDa, and S77681 had a second small plasmid of 6 MDa. One strain, S77923, carried an additional plasmid of 3 MDa. Strain S79345 was plasmid-free.

Plasmid DNA preparations from all strains were Southern-blotted and filters were hybridized with probes for the *spvBC* genes of the *S. typhimurium* virulence plasmid, and IS200. Whilst IS200 did not show homology with any plasmid DNA, the *spv* probe hybridized uniquely with the 52 MDa plasmid found in 24/25 isolates (data not shown). In order to elucidate more precisely the homologous plasmid locus and to establish whether either the complete plasmid or the *spv* genes themselves were chromosomally integrated in the plasmid-free S79345, total DNA



**Fig. 1. A:** IS200 profiles of *S. dublin*. Genomic Southern blot made with *Pst* I was hybridized with a PCR-generated 300 bp probe internal to the IS200 sequence. Track 1 contained the type strain NCTC 9676; track 2, S77681; track 3, S77682; track 4, S77923; track 5, S79345; track 6, S78993; track 7, S82750; track 8, S116354; track 9, S116543; track 10, S116750; track 11, S78152. Molecular weight markers in kilobase pairs are shown at left. All strains in the set exhibited this identical IS200 profile despite different origins, plasmid profiles or 16S *rrn* profile. **B:** 16S *rrn* gene profiles of *S. dublin*. Genomic Southern blot made with *Pvu*II was hybridized with a PCR-generated 550 bp probe internal to the 16S *rrn* gene sequence. Track 1 contained *S. typhimurium* LT2 control. Track 2 contained S116354; track 3, S116543; track 4, S79292; track 5, S82344; track 6, S77923; track 7, S77843; track 8, S78489; track 9, S78500; track 10, S82150; track 11, S116303; track 12, S116204.

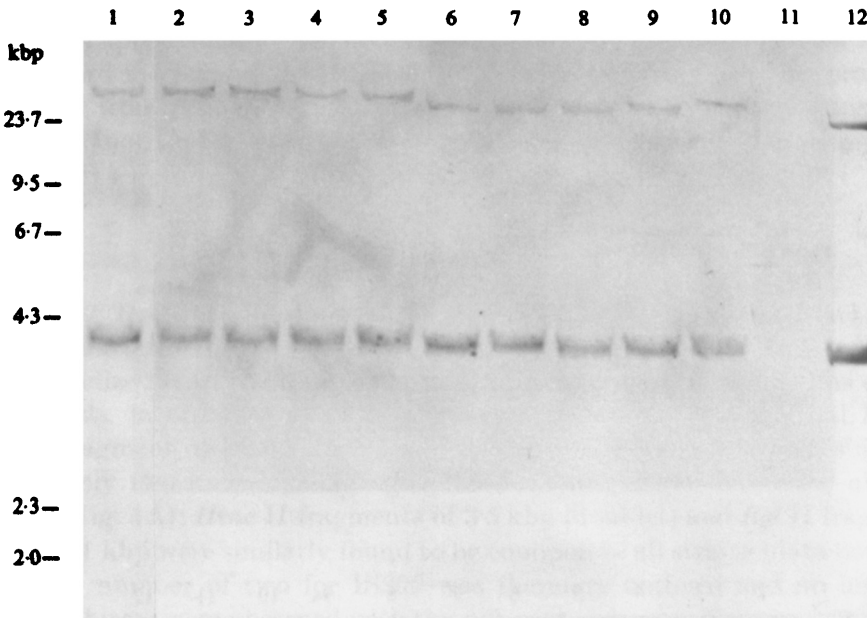


Fig. 2. Homology of salmonella plasmid virulence (*spv*) genes in *S. dublin*. Genomic Southern blot made with *Hind* III was probed for *spvBC* homology. Track 1 contained strain NCTC 9676; track 2, S77681; track 3, S77682; track 4, S77923; track 5, S78993; track 6, S82750; track 7, S116354; track 8, S116543; track 9, S116750; track 10, S78152; track 11, S79345; track 12, *S. typhimurium* LT2 control.

of all strains was digested with *Hind* III, blotted and hybridized with the *spvBC* probe. With the exception of S79345, all strains exhibited homology to two *Hind* III fragments of 25 kbp and 3.7 kbp (Fig. 2). No homology with the S79345 chromosome was detected.

#### DISCUSSION

In this report we have examined the genotype of strains of *Salmonella dublin* isolated from humans in England and Wales by combining the analysis of the mobile DNA element IS200 with rRNA gene restriction patterns, and with plasmid analysis. The latter two methods have previously been combined for a typing analysis of *S. enteritidis* [23] and plasmid typing has been used extensively for a variety of applications [24]. IS200 profiling has previously been combined with the analysis of restriction fragment length polymorphisms at several cloned chromosomal genes in a phylogenetic analysis of *S. enteritidis* [12].

The value of IS200 profiling relies on the fact that transposable genetic elements produce an evolving molecular 'fingerprint' of enterobacterial chromosomes. Sawyer and colleagues [13] showed that even in *E. coli* strains with 35/35 identical loci by multilocus enzyme electrophoresis, only 48% of IS bands were the same. Hence the present finding that all strains of *S. dublin* examined are identical with respect to IS200 provides evidence of clonality. In fact, these *S. dublin* isolates possess an identical IS200 profile to SECLIII, one of three clonal lineages of *S. enteritidis* [11]. Nonetheless at least two variants of *S. dublin* defined by polymorphism at the *rrn* loci were detected and one must therefore assume that

the DNA base substitution responsible for this variation is of relatively recent occurrence. Recent results in our laboratory [Stanley, Powell and Jones, in preparation] indicate that three group D<sub>1</sub> serotypes, clone *SEC*LIII of *S. enteritidis*, *S. dublin* clone SdRI and *S. rostock* (NCTC 5767), have identical IS200 and 16S *rrn* profiles. In this context it is noteworthy that Selander and colleagues [25] have recently shown that clones of *S. dublin* have a virtually uniform multilocus enzyme genotype (electrophoretic type or ET) as a globally predominant ET clone of *S. enteritidis*. They postulate that *S. dublin* is evolutionarily derived from an *S. enteritidis*-like ancestor, and our data concur with this hypothesis.

The restriction fragment length polymorphism (RFLP) in and around 16S ribosomal RNA genes was examined with respect to three enzymes, using a probe generated by PCR from the *S. dublin* chromosome. In principle, 'ribotyping' with a probe for 16+23S rRNA produces different patterns which correspond to significant DNA divergences within a species [15, 16]. In *S. enteritidis* such rDNA patterns did yield some intraserovar strain discrimination [23]. Similar discrimination was also found for the present collection of *S. dublin*. Strains S116204, S116303, S116354 and S116543 were thereby shown to represent a second clone. In this respect, it should be noted that S116354 and S116543 were known to be epidemiologically related since the former was isolated from a sample of imported cheese responsible for an outbreak of food-poisoning in England and Wales, whilst the latter was isolated from a patient known to have consumed this product. However, S116204 and S116303 were from sporadic cases in 1990. We therefore assume that these four strains are representatives of one of two clones prevalent in 1990.

With one exception, all strains of *S. dublin*, including two strains of bovine origin (S78993 and S116379), and one isolated in 1990 as a contaminant of imported cheese (S116354, see above), contained a 52 MDa plasmid, which was the origin of two *Hind* III fragments exhibiting homology with the *spr* gene probe. This result is in agreement with previous molecular genetic analyses of the large *S. dublin* plasmid [3-7]. The role of this mouse virulence plasmid in human disease remains unclear. For example, it is noteworthy that S79345 did not contain this plasmid, and chromosomal integration of either the plasmid or the *spr* genes themselves was excluded by lack of homology to *Hind* III-digested total DNA. Since S79345 was isolated from a patient with septicaemia, this result implies that the 52 MDa plasmid was either not required for bloodstream invasion or was subsequently lost on laboratory subculture. However, loss of this plasmid was not found in other strains examined and is not a general feature of *S. dublin* isolates [Threlfall, unpublished observations]. Furthermore, strains of *S. enteritidis* and *S. typhimurium* which lack large mouse-virulence plasmids have been identified among human salmonellosis isolates [26, Threlfall, unpublished]. The absence of chromosomally-integrated *spr* genes in S79345 suggests that these genes, responsible for the virulence of *S. dublin* for BALB/c mice [4-7], may have no direct functionality in human gastroenteric disease. Smaller plasmids of 1.8, 3.0 and 6.0 MDa found in three strains exhibited no *spr* homology. The latter results concur with the detection and frequency of small plasmids in *S. dublin*, as noted by other workers [8, 23].

Since *S. dublin* is a zoonotic serovar which produces an exceptionally high incidence of septicaemia in infected humans [2], a focus of this investigation was to elucidate the genotypes of strains recovered from patients with gastroenteritis or diverse bacteraemias. Evidence provided here for common chromosomal genetic origins indicates the existence in 1990 of two clones of *S. dublin* in humans in England and Wales which were distinguished by RFLP in and around the 16S *rrn* genes and have been here termed *SdRI* and *SdRII* (R indicates a distinct '16S ribopattern'). The prevalence of these clones should be evaluated for larger isolate numbers to validate trends of statistical significance.

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