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Grouping of *Salmonella enterica* serotype Montevideo strains by ribotyping and IS*200* profiling

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SUMMARY

One-hundred and twenty-one isolates of *Salmonella enterica* serotype Montevideo, representing different biotypes and incidents of infection detected in the UK between 1977 and 1995, were analysed by *Eco*RI ribotyping, *Pvu*II ribotyping and IS*200* fingerprinting. Among the isolates examined, 7 *Eco*RI ribotypes, 5 *Pvu*II ribotypes and 55 IS*200* profile types were recognized and 4 arbitrary groups defined. All 33 isolates of biotype 2d belonged to *Eco*RI/*Pvu*II ribotype 1/1 and IS*200* lineage A and comprised Group I. The other 88 isolates of biotype 10di and its variants were assigned to Groups II–IV. All 27 isolates in Group II were of *Eco*RI/*Pvu*II ribotype 2/2 and IS*200* lineage B. Among the 43 isolates in Group III, 42 of which were of *Eco*RI/*Pvu*II ribotype 3/3, IS*200* analysis identified 38 profiles in lineages C–I. Six *Eco*RI/*Pvu*II ribotypes and 8 IS*200* profiles, mostly in lineages C–E, were recognized among the 18 isolates in Group IV. The combined use of biotyping and ribotyping, and to some extent IS*200* profiling, has enhanced our understanding of the clonal structure of serotype Montevideo and provides a basis for further study.

INTRODUCTION

Salmonella enterica serotype Montevideo has long been recognized as an important pathogen of sheep in which it is a major cause of ovine abortion [1]. In the UK, serotype Montevideo is also found in other food animals such as chickens and, to a lesser extent, cattle [2–4; W. J. Reilly, personal communication]. By contrast, Montevideo is responsible for relatively few cases of human infection in the UK, with approx. 100–120 isolations each year in England and Wales [5] and an average of 20 isolations per year (range 13–27) in Scotland in the period 1988–97 (W. J. Reilly, personal communication). Transmission to man probably occurs by consumption of contaminated foods, particularly poultry and poultry products. Thus, for example, approx. 61% of Montevideo isolations from contaminated foods in a 5-year survey were made from these latter products [6].

Because there have been few studies of serotype Montevideo, the epidemiology of its infection in man and animals is still unclear. However, in an important study in which 622 Montevideo isolates from diverse sources in the UK were examined, 27 different biotypes were identified [7]. The discrimination index [8] provided by biotyping was 0·71 (D. C. Old, unpublished data). Of two major biogroups identified [7], one (biogroup '2d') was responsible for almost all infections in man, cattle and poultry in England and Wales but for only 25% of human infections in Scotland; the other (biogroup '10di') was predominant in all animals in Scotland but only in sheep in England and Wales [2].

Molecular-based methods have helped to clarify the epidemiology and clonal structure of other uncom-

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mon serotypes of salmonella such as Eimsbuettel, Livingstone and Thompson [9–11]. Representative strains of different biogroups of serotype Montevideo from the large series already biotyped [7] were analysed by ribotyping and IS*200* profiling to establish if a multiple typing approach would indicate further subgroups and so reveal whether any of them was associated with a particular animal host.

MATERIALS AND METHODS

Bacteria

Of the 121 isolates of *Salmonella* Montevideo examined in this study, 105 were from the series of 622 previously biotyped [7] and they represented different biotypes and sources in the years 1977–83. A further 16 isolates, representative of those present in Scotland in the years 1991–5, were obtained from Mr D. S. Munro, Scottish Salmonella Reference Laboratory, Stobhill NHS Trust, Glasgow, Scotland. In all, there were 93 isolates from Scotland and 28 from England and Wales and their sources included man (40), sheep (26), cattle (18), animal feeds (11), poultry (6), the environment (5), unspecified animals (4), meals (4), other birds (2) and pig, dog, mink, terrapin and human food (1 each). They represented all of the major, and some of the minor, biotypes previously reported [7], as follows: 10di (55 isolates), 2d (33), 10diz (12), 10bdi (10), 10difuc$^-$ (5), 10dix (3), 10bdifuc$^-$, 10bgi and 10dgi (1 each).

Isolates were maintained on Dorset's egg slopes at room temperature (*c.* 20 °C) and, for molecular analyses, were cultured for single colonies on blood agar (A & O Laboratories, Perth, Scotland) incubated overnight in air at 37 °C.

Digoxygenin labelled DNA probes

Gene probes of *Escherichia coli* ribosomal RNA and the IS*200* sequence of *Salmonella* Typhimurium LT2 were prepared as detailed elsewhere [9, 12, 13] and labelled with digoxygenin (DIG) by the High-Prime method as described by the manufacturer (Boehringer–Mannheim).

Ribotyping and IS*200* fingerprinting

For each isolate tested, a single colony from blood agar was cultured in 10 ml of L broth [14], shaken overnight (100 rpm) at 37 °C. Cellular DNA was extracted as detailed previously [15]. Briefly, bacterial pellets were recovered by centrifugation (3000 ***g***, 20 min) and suspended in 650 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8·0). SDS (10%) 40 μl and proteinase K (20 mg/ml) 4 μl were added; the suspension was shaken and incubated at 55 °C until it became clear. NaCl (5 M) 100 μl and hexadecyl trimethylammonium bromide (CTAB) solution (10% CTAB in 0·7 M NaCl) 100 μl were added and the suspension was mixed and incubated at 65 °C for 20 min. Two extractions with chloroform :isoamylalcohol (24:1) 1 ml were each followed by centrifugation at 6000 ***g*** for 10 min and transfer of the upper aqueous layer to a new tube. DNA was precipitated with cold (−20 °C) isopropanol (0·6 vols) and washed by resuspension in 1 ml of 70% ethanol and centrifugation (12000 ***g***, 5 min). DNA was dried at 37 °C, dissolved in 100–200 μl of TE buffer, pH 8·0, and stored at −20 °C. Aliquots (2 μg) of extracted DNA were digested to completion as directed by the manufacturer (Promega, Southampton, UK) with *Eco*RI or *Pvu*II (the latter has recognition sites outwith the IS*200* sequence). Restriction fragments were separated by electrophoresis through 0·8% w/v agarose gels (Ultra Pure agarose from Life Technologies, Paisley, UK) containing ethidium bromide (0·5 μg/ml) in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2 mM EDTA) at 3 V/cm and transferred by vacuum blotting on TransDNA Express (Appligene-Oncor, Birtley, Chester-le-Street, UK) for 75 min at $5{\cdot}5 \times 10^3$ Pa to positively charged nylon membrane (Appligene-Oncor). DNA was fixed to membranes by exposure to UV light (312 nm for 5 min).

Prehybridization of *Eco*RI- and *Pvu*II-digested DNA blots was performed in standard hybridization buffer (Boehringer–Mannheim) with added denatured, sheared salmon sperm DNA (50 μg/ml) for 2 h at 65 °C. Hybridization was carried out overnight at 65 °C in that same buffer containing 25 ng/ml of DIG-labelled ribosomal RNA gene probe. DIG-labelled hybrids were revealed with anti-DIG-alkaline phosphatase and the chemiluminescent substrate CSPD® as directed by the manufacturer (Boehringer–Mannheim), after exposure of membranes to Hyperfilm MP (Amersham) for up to 30 min. Molecular sizes of hybridized fragments were assessed with reference to DIG-labelled molecular-weight marker III (Boehringer–Mannheim). Sizes were estimated from six gels.

Before hybridization of *Pvu*II-digested DNA blots with the IS*200* probe, membranes were washed twice

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(15 min each) in stripping solution (0·2 M NaOH, 0·1% w/v SDS) preheated to 37 °C. Subsequent prehybridizations and hybridizations were performed as described above.

Numerical index of discrimination

Indices of discrimination (*D*) obtained after ribotyping and IS*200* fingerprinting of 121 Montevideo isolates were calculated as detailed elsewhere [8].

Genetic clustering of isolates

Ribotypes and IS*200* profiles of Montevideo isolates were compared visually and numbered in sequence according to their apparent relatedness, as assessed by the number and molecular sizes of bands. In addition, for an analysis of IS*200* profiles, Dice similarity coefficients [16] were calculated and cluster analysis was performed with the unweighted pair group mathematical average (UPGMA) algorithm in the Multivariate Statistical Package, version 3.1 (Kovach Computing Services, Pentraeth, Anglesey, Wales).

RESULTS

Ribotypes of *Salmonella* Montevideo

All 33 Montevideo isolates of biotype 2d were of *Eco*RI ribotype 1 which comprised 14 strongly hybridized bands of *c*. 19, 13, 11, 8, 6, 5·3, 2·8, 2·5, 2·4, 2·3, 2·1, 1·9, 1·7 and 1·4 kb (Fig. 1, lane 1). They also belonged to *Pvu*II ribotype 1, defined on the basis of strongly hybridized bands of *c*. 19, 16, 8, 5·5, 3·5, 2·1 and 2·0 kb (Fig. 2, lane 1), though other hybridized bands were sometimes present. Thus, *Eco*RI/*Pvu*II ribotype 1/1 was common to the 33 isolates of Group I (Table 1) which had been recovered from a wide variety of sources in different areas in the UK between 1979 and 1991 (Table 1). Two of the human isolates were from patients with a recent history of foreign travel.

*Eco*RI ribotype 2 (Fig. 1, lane 2) differed from *Eco*RI ribotype 1 by the presence or absence of hybridized bands at *c*. 11, 4·3, 2·4 and 1·9 kb. This ribotype was present in 27 Montevideo isolates belonging to biotypes 10di, 10bdi, 10difuc$^-$ and 10diz (Group II, Table 1). Although all 27 isolates were obtained from sources in Scotland between 1977 and 1995, they included five from Scottish patients who had travelled recently to Cyprus, Egypt, Israel, Malta and Romania in 1978–83. These same isolates were of *Pvu*II ribotype 2 (Fig. 2, lane 2) which, in addition to bands of *c*. 16, 8, 3·5 and 2·0 kb shared with *Pvu*II ribotype 1, possessed hybridized bands of *c*. 6 and 4·3 kb. Thus, *Pvu*II ribotyping confirmed the genetic relatedness of Group II strains indicated by *Eco*RI ribotyping.

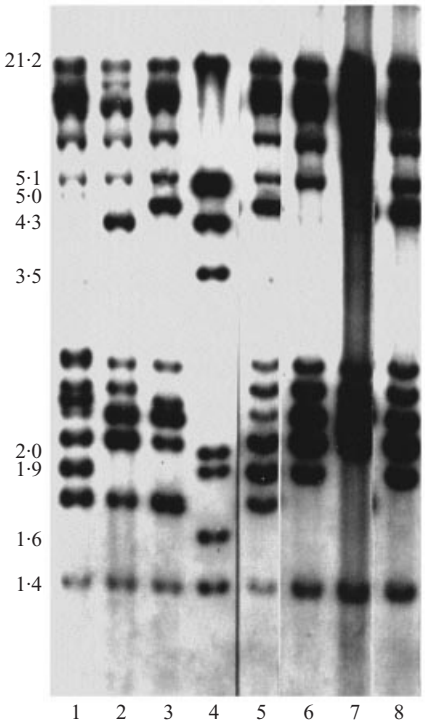

Fig. 1. *Eco*RI ribotypes of *Salmonella* Montevideo. Lanes 1–3, ribotypes 1–3, respectively; lane 4, molecular size markers (kb); lanes 5–8, ribotypes 4–7, respectively.

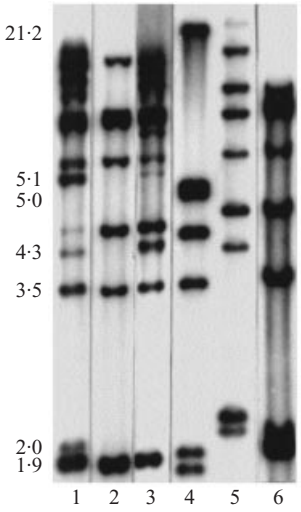

Fig. 2. *Pvu*II ribotypes of *Salmonella* Montevideo. Lanes 1–3, ribotypes 1–3, respectively; lane 4, molecular size markers (kb); lanes 5 and 6, ribotypes 4 and 5, respectively.

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Table 1. *Isolates of* Salmonella *serotype Montevideo from different sources grouped by biotyping, ribotyping and IS200 profiling*

Group	Country (*n*)	Sources of isolates (*n*)	Biotypes (*n*)	Ribotypes E/P* (*n*)	IS*200* lineages (*n*)
I	Scotland (15) England/Wales (18)	Man (11), cattle (5), poultry (2), sheep (1), other animals (5), feeds/meals (7), environment (2)	2d (33)	1/1 (33)	A (33)
II	Scotland (27)	Man (12), cattle (6), sheep (4), poultry (1), feeds/meals (3), human food (1)	10di (22), 10bdi (2), 10difuc$^-$ (2), 10diz (1)	2/2 (27)	B (27)
III	Scotland (38) England/Wales (5)	Sheep (21), cattle (6), man (6), poultry (2), other birds (2), other animals (2), environment (3), feeds (1)	10di (19), 10diz (9), 10bdi (8), 10difuc$^-$ (3), 10dix (3), 10bdifuc$^-$ (1)	3/3 (42), 3/4 (1)	C (8), D (10), E (10), F (2), G (3), H (1), I (2), M† (6), 0 (1)
IV	Scotland (13) England/Wales (5)	Man (11), cattle (1), poultry (1), other animals (1), feeds/meals (4)	10di (14), 10diz (2), 10bgi (1), 10dgi (1)	4/2 (6), 4/3 (4), 5/2 (1), 5/3 (3), 6/5 (1), 7/3 (3)	B (1), C (3), D (9), E (5)

* E/P, ribotypes after digestion of cellular DNA with *Eco*RI (E) and *Pvu*II (P).
† M, see text.

Each of the 43 Montevideo isolates of Group III belonged to *Eco*RI ribotype 3 (Fig. 1, lane 3) which was related to, but readily distinguishable from, *Eco*RI ribotypes 1 and 2. These 43 isolates had been recovered between 1977 and 1991 and belonged to biotypes 10di, 10diz, 10bdi, 10difuc$^-$, 10dix and 10bdifuc$^-$ (Table 1). Forty-two of the isolates belonged to *Pvu*II ribotype 3 (Fig. 2, lane 3) which was similar to *Pvu*II ribotype 1 except that it lacked a hybridized band at 2·1 kb. The remaining isolate, from a bird imported from Canada and of the unique biotype 10bdifuc$^-$, belonged to *Pvu*II ribotype 4 (Fig. 2, lane 5) which showed hybridized bands at 4·7 and 2·3 kb not detected in any other *Pvu*II ribotype.

The 18 Montevideo isolates in Group IV belonged to biotypes 10di, 10diz, 10bgi and 10dgi (Table 1); seven were from patients with a recent history of foreign travel (in Malta, Nigeria, Spain and the USA) and one was from poultry imported from Eire. They belonged to the related *Eco*RI ribotypes 4–7 (Fig. 1, lanes 5–8) and *Pvu*II ribotypes 2, 3 and 5; the latter shared hybridized bands but were, nevertheless, easily distinguishable (Fig. 2, lanes 2, 3 and 6). The isolate with the unique *Eco*RI/*Pvu*II ribotype 6/5 was from the patient who had travelled in Nigeria.

In summary, therefore, 7 *Eco*RI ribotypes sharing hybridized bands of *c*. 19, 13, 8, 6, 2·8, 2·3 and 1·4 kb were observed among the 121 Montevideo isolates examined: ribotype 1 (33 isolates), 2 (27), 3 (43), 4 (10), 5 (4), 6 (1) and 7 (3). *Pvu*II ribotyping of these same isolates identified five *Pvu*II ribotypes: ribotype 1 (33 isolates), 2 (34), 3 (52), 4 and 5 (1 each). Considered together, *Eco*RI/*Pvu*II ribotyping identified 10 distinct E/P ribotypes and provided a division of the 121 isolates into Groups I–IV (Table 1).

Ribotyping provided moderately good discrimination of the 121 Montevideo isolates with discrimination indices (*D*) as follows: *Eco*RI, 0·75; *Pvu*II, 0·67; and *Eco*RI/*Pvu*II, 0·76.

IS*200* profiles of *Salmonella* Montevideo

Of the 121 isolates examined, 120 contained IS*200* elements and 54 different profiles were identified. These profiles have been detailed elsewhere [17] and so only some of the data will be presented here. All but 1 of the 33 isolates of biotype 2d examined had 2 copies of IS*200*. By contrast, the great majority of isolates of biotype 10di and its variants (i.e. biogroup '10di') had 3–6 copies. However, 6 isolates of biogroup '10di' in Group III – from sheep (4), poultry

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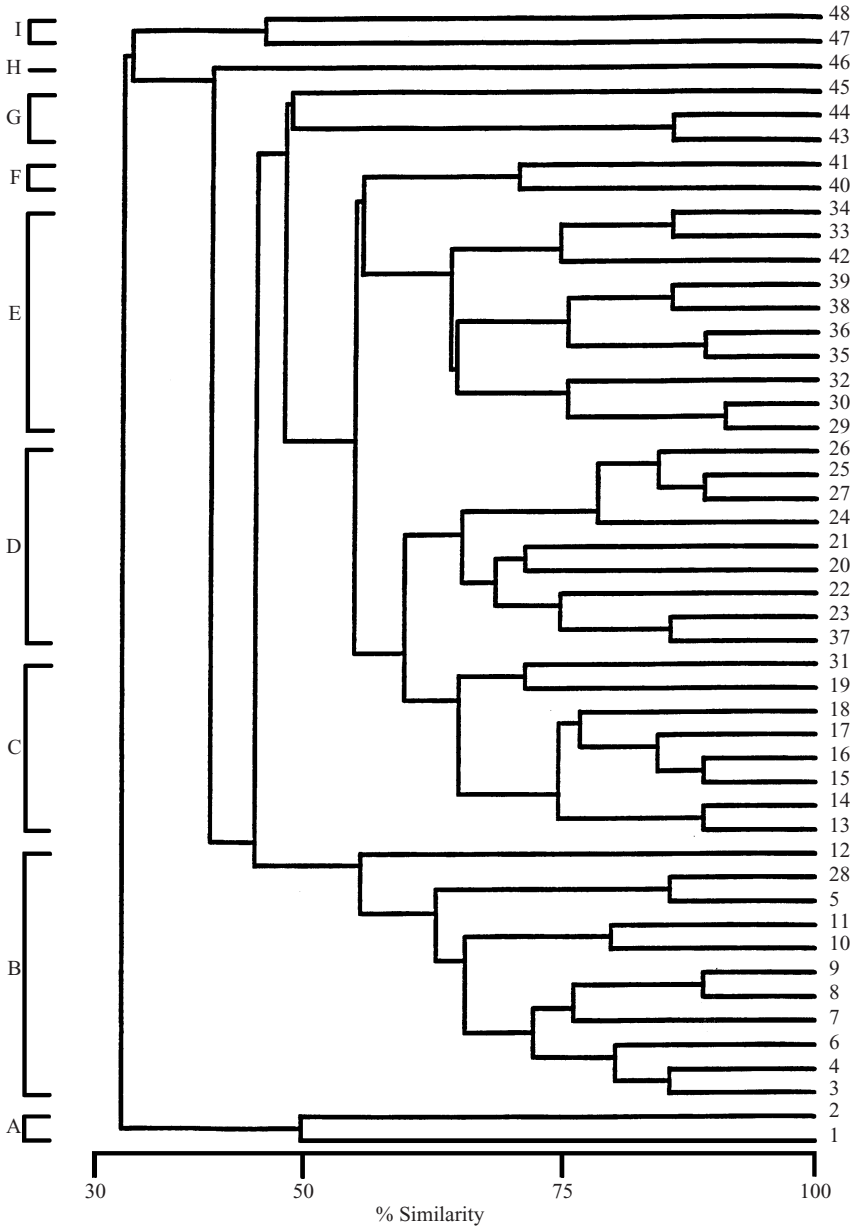

Fig. 3. *Salmonella* Montevideo IS*200* profiles. The dendrogram was constructed with the UPGMA algorithm in the Multivariate Statistical Package, version 3.1.

(1) and stream water (1) – gave complex profiles (M1–M6, Table 1; data not shown) that contained multiple bands (including many of high molecular weight which were difficult to resolve). Resolution was not improved by the use of different restriction enzymes (*Hin*cII and *Sma*I) and further analysis of these profiles was not attempted. The only isolate lacking IS*200* elements was one from a bird that had been imported from Canada (see Group III above). The number of isolates (of 114) found in each of the resolvable profiles (48) was, as follows: profile 1 (32 isolates), 4 (16), 24 (9), 39 (5), 13 (3), 5, 8, 25, 27, 31, 33 (2 each), and 37 others (1 each). The overall discrimination index achieved with IS*200* profiling was high ($D = 0{\cdot}91$).

It was not possible to assess relatedness among strains solely by visual matching of bands in IS*200* profiles. Hence, cluster analysis was performed on the 48 profiles; 9 clusters (A–I) are apparent in the dendrogram (Fig. 3). Within each of the clusters labelled C, D, E and F, the constituent IS*200* profiles were linked at similarities of not less than 64% and these clusters were related to each other at not less than 55% similarity (Fig. 3). Clusters B and G were

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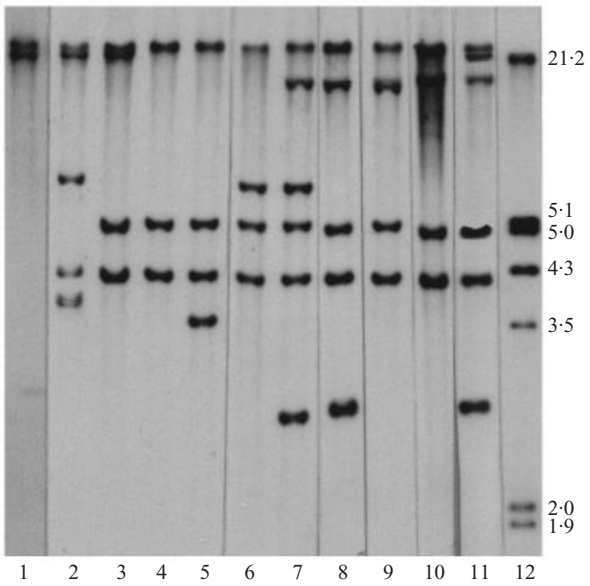

Fig. 4. IS*200* profiles of *Salmonella* Montevideo. Lanes 1–11, IS*200* profiles 1–11, respectively; lane 12, molecular size markers (kb).

linked to clusters C–F at levels of 46% and 48% similarity, respectively. The outliers A, H and I were linked to clusters B–G at 33% similarity.

Ribotype/IS*200* relationships

Thirty-two of the 33 isolates in ribotype Group I had IS*200* profile 1, containing two copies of IS*200* on hybridized *Pvu*II fragments of > 21·2 kb (Fig. 4, lane 1). The remaining isolate had the variant IS*200* profile 2, with an additional four copies of IS*200* on fragments of *c*. 5·5, 4·0, 3·7 and 3·6 kb (Fig. 4, lane 2). These profiles formed cluster A (Fig. 3).

The IS*200* profiles 3–11 (Fig. 4, lanes 3–11) that were identified among 26 of the 27 Group II Montevideo isolates included: profile 4 (16 isolates); profiles 5 and 8 (2 each); and profiles 3, 6, 7, 9, 10 and 11 (1 each). These profiles shared hybridized bands of > 21·2, 4·8 and 4·0 kb (Fig. 4). IS*200* profile 12 (> 21·2, 4·0 and 2·2 kb) of the remaining isolate in this group lacked the 4·8-kb band of the other IS*200* profiles in lineage B (Fig. 4) but, nevertheless, was linked to them at 55% similarity (Fig. 3).

Among the 61 isolates in Groups III and IV, there were 43 different IS*200* profiles which generally had more copies of IS*200* than, and contained the common 4·0-kb band of, profiles 3–12 of Group II isolates (data not shown). Isolates assigned by ribotyping to Groups III and IV had 38 and 8 different IS*200* profiles, respectively, of which 3 were common to both groups. IS*200* profiles in clusters C, D and E were found in isolates of both Groups III and IV; those in clusters F–I were found in Group III isolates only. Among the IS*200* profiles of Groups III and IV isolates, only profile 28 (> 21·2, 4·0 and 3·4 kb) belonged to lineage B in which it was linked at 86% similarity to profile 5 (Fig. 3). Profile 28 was found in the isolate of unique *Eco*RI/*Pvu*II ribotype 6/5 from the patient who had travelled recently in Nigeria (Group III, see above).

DISCUSSION

In recent years, the application of multiple genotyping methods has clarifed sources and routes of human and animal infection with particular salmonella serotypes and has revealed associations between certain strains and their animal hosts [10, 12, 18–20]. Biotyping of Montevideo had previously shown that 93% of isolates of biogroup '2d' belonged to biotype 2d, with only four minor biotypes representing the other 7% [7]. Hence, there was little phenotypic variation in this group. The finding that all 33 representative isolates of biotype 2d from diverse sources throughout the UK (and two of which may have been acquired abroad) belonged to *Eco*RI/*Pvu*II ribotype 1/1 and IS*200* lineage A confirmed this homogeneity; the likelihood, therefore, is that they were members of a remarkably stable clone.

By contrast, 95% of Montevideo isolates of biogroup '10di' belonged to 5 biotypes (10di, 10bdi, 10difuc$^-$, 10dix and 10diz) and the other 5% belonged to 13 other minor biotypes [7]. That biotype diversity has been confirmed in this study by ribotyping and IS*200* profiling, which allowed a broad division of isolates of biogroup '10di' into Groups II, III and IV. Group II isolates of biogroup '10di' belonged to *Eco*RI/*Pvu*II ribotype 2/2 and had related IS*200* fingerprints, all of which were in lineage B. Since some isolates were from Scottish patients who may have acquired their infections in countries in the Mediterranean region, Eastern Europe or West Africa, Group II may well represent a second, widely distributed Montevideo clone. The observation that most Group II isolates from non-human sources in Scotland were from cattle and cattle feeds might indicate a bovine source of human infection. However, given the low incidence of Montevideo in cattle [4; S. C. Rankin, personal communication], that proposition is unlikely. Analysis of additional animal isolates from diverse sources in the UK, and else-

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where, will be needed to identify the reservoir of this putative clone.

The finding that the great majority (98%) of biogroup '10di' isolates from Group III belonged to *Eco*RI/*Pvu*II ribotype 3/3 showed that this group was also homogeneous and suggested that it too might represent a major clone. However, the IS*200* profiles of Group III isolates were extraordinarily diverse and scattered throughout lineages C–I. This degree of diversity revealed by IS*200* profiling was surprising in strains that were so homogeneous by *Eco*RI/*Pvu*II ribotype. Not only did IS*200* fingerprinting fail to establish clonality among the 43 Group III strains, it also afforded a discrimination that was so high ($D = 0{\cdot}99$) as to call into question its usefulness as a typing tool. The poor resolution of multiple IS*200* bands in the profiles of a small subset of Group III isolates presented an additional complication. We have no explanation for either the diversity or the multiplicity of bands observed, but it is noteworthy that similar problems were not encountered with the other groups. Transposition of IS*200* under laboratory conditions is unusual [21]; carriage of IS*200* elements by plasmids is also so rare [22] as to be an unlikely explanation for these complex profiles. An analysis of the plasmids of Montevideo strains will be reported elsewhere [S. C. Rankin, personal communication]. An exceptional Group III isolate did not carry IS*200* and belonged to *Eco*RI/*Pvu*II ribotype 3/4; it came from a bird imported from Canada. Whether this type is common there or is found only occasionally in the wild bird population will require examination of isolates from other countries.

Group IV isolates from a variety of sources in Scotland and England formed a genetically heterogeneous group (ribotypes *Eco*RI 4, 5, 6 and 7 and *Pvu*II 2, 3 and 5). Because many of the isolates were from patients who had travelled abroad, it was impossible to ascertain the sources of their infections. Isolates had been obtained from animal feed, cattle, poultry and a terrapin, observations suggesting that various animals may cause human infection.

Pulsed-field gel electrophoresis (PFGE) has been used for fine structure fingerprinting of DNA of various serotypes of *Salmonella* including Montevideo [6]. In North Tyneside in 1996, it successfully traced an outbreak of Montevideo infection in man to cooked chicken from a supermarket hot food outlet [6]. Profiles of 10 isolates from patients, left-over cooked chicken and the hot food area were indistinguishable; another 8 Montevideo isolates from North Tyneside in the 3 previous years (1993–5) gave 3 PFGE profiles, each of which differed from the outbreak profile [6]. Previous biotyping studies would suggest that these strains were likely to be of biogroup '2d' [2], a group shown to be genetically stable. By contrast, examination of 33 Montevideo isolates representing different incidents of infection in animals (mainly sheep) on farms in one small shire in the south of Scotland revealed eight PFGE profiles when only one or a few related types might have been expected; again, some of these isolates belonged to biogroup '10di' [S. C. Rankin, personal communication]. Thus, PFGE may help to highlight circumscribed Montevideo incidents caused by the stable biogroup '2d'; its role in the analysis of incidents associated with the more labile strains of biogroup '10di', especially those from sheep, remains unclear. The observed heterogeneity of PFGE profiles among the few Montevideo strains in these earlier studies did not persuade us that it would be useful in this investigation of the clonal structure of Montevideo strains isolated over two decades and from diverse sources.

It has been argued that biotyping is an unreliable means of typing bacteria because biochemical differences may reflect variable gene expression rather than true genetic differences [23]. However, this molecular study has confirmed that biotyping correctly identified the major groups of Montevideo. Although biotyping indicated that Montevideo of biogroup '10di' was endemic in sheep in the UK, no correlations between different '10di' variants and either severity of disease or geographical distribution were established [2]. Whether sheep are susceptible to all strains of Montevideo remains unknown. Confirmation of the existence of a virulent 'sheep strain' will need further study of Group III isolates from Scotland and elsewhere [1, 2].

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