BACTERIAL PATHOGENICITY AND CHARACTERISATION

Distribution of insertion sequence IS200 among different clonal lines of the related *Salmonella* serotypes Livingstone and Emsbuettel

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The copy number and genetic location of IS200 have provided evidence of strain relatedness in many serotypes of *Salmonella*. In this study, 100 isolates of the related serotypes Livingstone (6,7:d:l,w) and Emsbuettel (6,7,14:d:l,w), representing 10 ribotype/biotype (RT/BT) groups isolated from human and non-human sources in seven countries over a 26-year period, were examined for their IS200 profiles. The distribution of IS200 in strains of these serotypes was limited, being present in all 53 isolates of ribotype 1 (RT1) and its variant type RT6, in one of five isolates of RT5 but in none of 42 isolates of RTs 2, 3 or 4. Although the seven IS200 profiles identified in RT1 isolates were of little value for further discrimination within different biotype groups, they were extremely valuable for confirming serotype: isolates of RT1/BT8/IS200 profile A (or its variants) and those of RT1/BT3/IS200 profile B (or its variants) were almost invariably associated with serotypes Livingstone and Emsbuettel, respectively.

Introduction

The mobile genetic element IS200 is widely distributed on conserved loci on the chromosome of many, but not all, serotypes of *Salmonella* [1–3]. The copy number and location of IS200 may change over time, and identity among strains in the number and position of IS200 provides evidence of their common genetic ancestry [4, 5]. Thus, the use of IS200 fingerprinting has become increasingly popular for discrimination of strains and identification of clones in many *Salmonella* serotypes [reviewed in 4, 5].

Because the prevalence of human infection by the serologically similar serotypes Livingstone (antigenic formula 6,7:d:l,w) and Emsbuettel (6,7,14:d:l,w) is generally low in the UK, serotype-specific phage-typing schemes have never been developed for either serotype. Hence, when faced with major outbreaks of human infection in Scotland by Emsbuettel [6] and Livingstone [7], a multiple typing approach was used – with biotyping, plasmid profiling and ribotyping – to identify the epidemic clones involved [8, 9].

This report presents the findings of a study on

the distribution of IS200 among the strains of Livingstone and Emsbuettel of different ribotype/biotype (RT/BT) groups and provides further evidence of the genetic relatedness of strains of these two serologically similar serotypes.

Materials and methods

Bacteria

Of the 166 isolates of Livingstone reported previously [8], 56 were selected for IS200 analysis. The properties of these isolates, which include representatives of seven different ribotype/biotype (RT/BT) groups isolated at different times and from various sources in six countries, are shown in Table 1. Of the 100 isolates of Emsbuettel reported previously [9], 44 were selected for IS200 analysis, including representatives of eight different RT/BT groups isolated at different times and from various sources in seven countries (Table 2).

All isolates were serotyped at the Scottish Salmonella Reference Laboratory, Glasgow. In this report, those with the antigenic formula 6,7:d:l,w are designated serotype Livingstone and those with the antigenic formula 6,7,c14:d:l,w as serotype Emsbuettel, as is the practice in both UK Salmonella Reference Laboratories.

Table 1. Details of 56 isolates of *Salmonella* serotype Livingstone examined

Country of origin	Years of isolation	RT/BT group	Source (number of isolates)
Scotland	1985–1993	1/8	Man (13), poultry (4), water (1)
England and Wales	1984–1992	1/3	Poultry (2), man (1)
		1/8	Poultry (3), man (1)
		2/1	Man (5), egg (3), poultry (2)
		3/5	Poultry (1)
		4/5	Man (1), poultry (1)
Canada	Not known	3/5	Poultry (2), animal feed supplement (1)
France	1990–1991	1/3	Egg (1), man (1)
		1/8	Delicatessen (1), man (1), water (1)
		2/1	Egg (1)
		1/3	Unspecified (1)
Israel	1968–1992	1/7	Animal feed (1)
		2/1	Feed meal (1)
		3/5	Man (1)
		5/3	Man (2), beef (1), pet meal (1), unspecified (1)

Table 2. Details of 44 isolates of *Salmonella* serotype Eimsbuettel examined

Country of origin	Years of isolation	RT/BT group	Source (number of isolates)
Scotland	1977–1990	1/3	Man (9), poultry (5), environment (1), pig (1), thermometer (1), water (1)
		2/1	Man (5), poultry (1)
		3/5	Man (2), poultry (1)
		6/3	Man (1)
England and Wales	1984–1992	1/3	Poultry (1)
		2/1	Animal feed (1), meat/bone meal (1)
		2/3	Poultry (1)
		3/5	Meat/bone meal (1)
		4/5	Man (1)
Australia	1991	3/5	Cotton seed meal (1)
Canada	1989–1990	3/6	Bone meal (1)
		3/5	Poultry (1)
		3/6	Animal feed (1), bone meal (1)
France	1990–1991	4/5	Cat (1)
		1/8	Poultry (1)
Israel	1972	3/5	Unspecified animal (1), fish (1)
		3/5	Unspecified (1)

Cultures were stored on Dorset's egg slopes at room temperature (18–20°C) until they were subcultured on MacConkey Agar (Oxoid).

DNA extraction

DNA was extracted by a modification of the method described previously [10]. One colony from a 24-h culture on MacConkey agar was inoculated into L broth [11] (10 ml) in a 20-ml screw-capped plastic bottle and incubated with shaking (100 rpm) at 37°C for 18 h. The culture, cooled on ice, was centrifuged at 3000 *g* for 20 min; the cell pellet was resuspended in 650 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and transferred to a 2-ml microfuge tube to which 40 µl of SDS 10% w/v and 4 µl of proteinase K 20 mg/ml were added. After incubation at 50°C for 1 h, 100 µl of 5 M NaCl and 100 µl of CTAB solution (CTAB 10% w/v in 0.7 M NaCl) were added and the tube was shaken to mix the contents before incubation at 65°C for 20 min. Chloroform:isoamyl alcohol (24:1) (1 ml) was added and the tube was shaken thoroughly and centrifuged at 6000 *g* for 10 min. Supernate was transferred gently to a 1.5-ml microfuge tube. After repeating the chloroform-extraction step, 0.6 volumes

of cold (–20°C) isopropanol were added and the tube was inverted several times. Precipitated DNA was transferred gently to a tube containing 1 ml of ethanol 70% v/v. The tube was centrifuged at 12 000 *g* for 5 min and the supernate was discarded. The DNA pellet was dried at 37°C in the open tube and dissolved in ≥ 100 µl of TE buffer (pH 8.0).

DNA digestion and transfer to nylon membranes

DNA (*c.* 2 µg) was digested to completion with *Pst*I (Promega, Southampton), a restriction endonuclease with no recognition site within the IS200 sequence [3, 12]. Digests were electrophoresed at 2 V/cm in TBE buffer (0.089 M Tris-HCl, 0.089 M boric acid, 0.002 M EDTA), along with digoxigenin (DIG)-labelled lambda DNA digested with *Eco*RI and *Hind*III (Boehringer Mannheim) through agarose (Oxoid Type 1: Low EEO from Unipath, Basingstoke, Hants) 0.8% w/v, containing ethidium bromide 0.5 µg/ml. DNA fragments were transferred to Positive Membrane (Appligene Oncor, Birtley, Chester-le-Street, Co. Durham) on TransDNA Express (Appligene) for 75 min at 55 mbar with 20 × SSC buffer [13] and fixed by UV light (312 nm) for 5 min.

Hybridisation and detection by chemiluminescence

A PCR-generated 692-bp DNA probe of IS200 [12, 14] was labelled with digoxigenin-11-dUTP by random priming (Boehringer Mannheim) and a 'mock' hybridisation was performed to optimise the amount of DIG-labelled probe per ml of prehybridisation solution. Prehybridisation, hybridisation and detection of hybrids with anti-DIG alkaline phosphatase and CSPD[®] were performed as recommended by the manufacturer (Boehringer Mannheim) except that boiled, sheared salmon sperm DNA 50 µg/ml was included in prehybridisation and hybridisation solutions.

Results

When the 100 isolates of *Salmonella* of serotypes Livingstone and Emsbuettel were analysed in Southern hybridisation with the 692-bp IS200 probe, 54 were found to contain IS200. Thus, 33 (60%) of 56 isolates of Livingstone and 21 (48%) of 44 isolates of Emsbuettel contained between two and seven copies of IS200 (Figs. 1 and 2). In all, eight different IS200 hybridisation profiles were detected (Fig. 2), each

Fig. 1. Southern blot analysis: lanes 1, IS200 profile A; 2, IS200 profile B; 3, mol. size markers.

Fig. 2. Line drawing representing the eight IS200 profiles detected among 100 isolates of *Salmonella* serotypes Livingstone and Emsbuettel. Lane 1, mol. size markers; 2–9, profiles A, B, C, D, E, F, H and L. Recorded mol. sizes of IS200-containing *Pst*I DNA fragments are the average of those obtained from up to eight separate gel runs.

containing a conserved band of 6.7 kb. However, two types, designated profile A (19 isolates) and profile B (23), accounted for most (78%) of the 54 IS200-positive isolates. The molecular sizes of the IS200-containing *Pst*I-generated DNA fragments were calculated from the average of those obtained from up to eight different gel runs. Profile A comprised bands of *c.* 2.0, 4.8, 6.7, 10 and ≥ 21 kb; profile B comprised bands of *c.* 5.0, 6.7, 7.3, 17 and ≥ 21 kb. These two major IS200 profiles are compared in Fig. 1.

IS200 profile C, lacking the 2.0-kb fragment (4.8, 6.7, 10 and ≥ 21 kb), and profile E, with an additional fragment of *c.* 3.7 kb (2.0, 3.7, 4.8, 6.7, 10 and ≥ 21 kb), were considered to be variants of IS200 profile A. IS200 profile D, with an additional 8.0-kb fragment (5.0, 6.7, 7.3, 8.0, 17 and ≥ 21 kb), profile F, with an additional 5.5-kb fragment (5.0, 5.5, 6.7, 7.3, 17 and ≥ 21 kb) and profile H, with two additional fragments of 3.8 and 5.5 kb (3.8, 5.0, 5.5, 6.7, 7.3, 17 and ≥ 21 kb), were considered to be variants of IS200 profile B.

The eighth IS200 profile, L, which was only distantly related to the other profiles observed (Fig. 2), had only two copies of IS200 in *Pst*I-generated fragments of *c.* 5.2 and 6.7 kb. Representations of the eight IS200 profiles are shown in Fig. 2.

IS200 in Livingstone strains

The distribution of *IS200* among Livingstone isolates from the different epidemic strains (clones) delineated by RT/BT grouping is shown in Table 3.

All 25 Livingstone isolates of the major clonal group RT1/BT8 (18 from Scotland, four from England and Wales and three from France) were *IS200*-positive. Fifteen of the 18 Scottish isolates of RT1/BT8, whether or not they carried the 6.5-kb plasmid characteristic of the Scottish epidemic strain [8], showed *IS200* profile A. Another three Scottish isolates of RT1/BT8, isolated from patients recently returned from Spain or with a plasmid profile different from that of the Scottish epidemic strain, were also *IS200*-positive but their profiles were type C (two isolates) or type E (one). Thus, all 18 Scottish isolates of RT1/BT8, isolated over the 9-year period 1985–1993 and regardless of source or plasmid profile, had *IS200* profile A or its variants C or E.

Four Livingstone isolates of RT1/BT8 that were isolated from human or poultry sources in England and Wales during 1984–1991 also had *IS200* profile A (one isolate) or its variant C (three). A further three Livingstone isolates from diverse sources (human, water, delicatessen) in France and obtained during 1990–1991 also had *IS200* profile A which was found commonly among Scottish isolates of RT1/BT8 (Table 3). Biotype 7 differs from biotype 8 in only one biotyping property [see 8]. Thus, it was of interest to find that the only isolate of RT1/BT7, from an animal-feed source in Israel, also had *IS200* profile C, uncommonly found among isolates of RT1/BT8 (Table 3). Thus, all 26 Livingstone isolates of the RT1/BT8 or related RT1/BT7 groups were *IS200*-positive and regardless of source or country of origin had *IS200* profile A (19 isolates) or its recognised variants C (6) or E (1) (Table 3).

Whereas the majority of Livingstone isolates belonging to RT1 were of BT8, six belonged to RT1/BT3, a group more commonly associated with Eimsbuettel strains [9]. Like the majority, all six isolates of RT1/BT3 were *IS200*-positive but had *IS200* profile B (five isolates) and D (one) (Table 3). The only other Livingstone isolate that gave a positive hybridisation

result with the *IS200* probe was one of RT5/BT3 from Israel, isolated originally from pig-feed supplement in the USA. Its *IS200* profile, L, was unique, with only two copies of *IS200* in *Pst*I fragments of c. 5.2 and 6.7 kb (Fig. 2).

None of the 19 Livingstone isolates of ribotypes 2, 3 or 4, regardless of biotype, source or country of origin, contained *IS200* elements (Table 3).

IS200 in Eimsbuettel strains

The predominant Eimsbuettel clone present among isolates obtained from diverse sources in the UK during 1986–1991 was RT1/BT3, all 19 isolates of which were *IS200*-positive with *IS200* profile B (18 isolates) or its variant profile F (one). Whereas the majority of Eimsbuettel isolates belonging to RT1 were of BT3, one belonged to RT1/BT8, the major group associated with Livingstone strains [8]. This Eimsbuettel isolate, from poultry in France in 1991, had *IS200* profile C (Table 4), associated with a minority of Livingstone isolates of RT1/BT8 (Table 3) but not those from France that had *IS200* profile A.

The only Eimsbuettel isolate of the uncommon RT/BT group RT6/BT3 present in this large series of 100 isolates [9] was one isolated from a baby at the outset of the Glasgow hospital outbreak [6]. RT6 is known to be closely related to RT1 [9]. This isolate of RT6/BT3 had the unique *IS200* profile, H, which is related to profiles F and B (Fig. 2). None of the 23 Eimsbuettel isolates of RTs 2, 3, or 4, isolated from

Table 4. Distribution of *IS200* among isolates of different ribotype/biotype (RT/BT) groups of *Salmonella* Eimsbuettel

RT/BT group	Number of isolates	Number of <i>IS200</i> -positive isolates with <i>IS200</i> profile			
		B	F	H	C
1/3	19	18	1	0	0
1/8	1	0	0	0	1
6/3	1	0	0	1	0
2/1	8	0	0	0	0
2/3	1	0	0	0	0
3/5	9	0	0	0	0
3/6	3	0	0	0	0
4/5	2	0	0	0	0

Table 3. Distribution of *IS200* among isolates of different ribotype/biotype (RT/BT) groups of *Salmonella* Livingstone

RT/BT group	Number of isolates	Number of <i>IS200</i> -positive isolates with <i>IS200</i> profile					
		A	C	E	B	D	L
1/8	25	19	5	1	0	0	0
1/7	1	0	1	0	0	0	0
1/3	6	0	0	0	5	1	0
2/1	12	0	0	0	0	0	0
3/5	5	0	0	0	0	0	0
4/5	2	0	0	0	0	0	0
5/3	5	0	0	0	0	0	1

various sources in different countries during 1972–1996 (Table 2) was IS200-positive (Table 4). In summary, all 53 isolates of the related ribotypes RT1/RT6 were IS200-positive, whereas all but one of 47 isolates of ribotypes RT2–5 were IS200-negative (Table 5).

Discussion

The only antigenic difference between the closely related *Salmonella* serotypes Livingstone and Eimbsbuettel is that the latter produces a cell-wall antigen O14 which is present in Livingstone cultures lysogenised by phage 14 [15]. Deletion of serotype names previously accorded to lysogenised variants of extant serotypes was recommended in the Kauffmann-White scheme [16]; however, that practice is not followed in the UK where both names, e.g., Livingstone and Eimbsbuettel, are used [17].

Although the prevalence of human infections by these serotypes in the UK is generally low, each has caused sizeable outbreaks in Scotland [6–9]. All of 114 Livingstone isolates from human and other sources in Scotland during 1985–1993 belonged to RT1, all but one were of BT8 and most carried a 6.5-kb plasmid characteristic of the Tayside outbreak strain [8].

Indeed, RT1 was the only ribotype present among Livingstone strains from Scotland (Table 6). Again, most (80%) of the 67 Eimbsbuettel isolates from diverse sources in Scotland in 1968–1993, and including the epidemic strain involved in the Glasgow outbreak [6], belonged to RT1 [9]; a minority (20%) of Eimbsbuettel isolates, particularly those from the East of Scotland, were of RT2 and RT3 [9]. Biotyping readily distinguished isolates of RT1 from Scotland [8, 9]: all isolates of Livingstone and Eimbsbuettel belonged to BT8 and BT3, respectively (Table 6). Together with plasmid profiling, biotyping also identified poultry as the likely source of human infections caused by Livingstone and Eimbsbuettel strains in Scotland [7–9].

The finding that all Livingstone and Eimbsbuettel isolates of RT1 from Scotland, regardless of biotype, plasmid profile, source or time of isolation, had IS200 profiles that were type A (or its variants) or type B (or its variants), respectively, confirmed the clonal differences that had already been suggested by biotyping (Table 6). Although both IS200 profiles A and B contained five bands, they were readily distinguishable, having only one common band. Thus, IS200 profiling provided little additional type discrimination of RT1 isolates, there being so few isolates of A variant or B variant profiles. Furthermore, the use of

Table 5. Distribution of IS200 among 100 isolates of *Salmonella* of the related serotypes Livingstone and Eimbsbuettel

RT/BT group	Number of isolates tested	Number of IS200-positive isolates with profile								Number of IS200+ isolates/number tested
		A	C*	E*	B	D†	F†	H†	L	
1/7	1	0	1	0	0	0	0	0	0	53/53
1/8	26	19	6	1	0	0	0	0	0	
1/3	25	0	0	0	23	1	1	0	0	
6/3	1	0	0	0	0	0	0	1	0	
2/1	20	0	0	0	0	0	0	0	0	1/47
2/3	1	0	0	0	0	0	0	0	0	
3/5	14	0	0	0	0	0	0	0	0	
3/6	3	0	0	0	0	0	0	0	0	
4/5	4	0	0	0	0	0	0	0	0	
5/3	5	0	0	0	0	0	0	0	1	

*C and E are variants of profile A.

†D, F and H are variants of profile B.

Table 6. Biotypes and IS200 profiles of 53 isolates of serotypes Livingstone and Eimbsbuettel of ribotype 1 (RT1)* from different countries

Serotype	Country	Number of isolates that were		
		tested	of RT/BT/IS200 type	in serotype
Livingstone	Scotland	18	1/8/A-var.†	26
	Other‡	8	1/8/A-var.	
		6	1/3/B-var.	
Eimbsbuettel	Scotland	19*	1/3/B-var.	20
	Other‡	1	1/3/B-var.	
		1	1/8/A-var.	

*Includes one isolate of RT6, a variant of RT1 [9].

†A-var. includes variants (C, E) of IS200 profile A; B-var. includes variants (D, F, H) of IS200 profile B.

‡See Tables 1 and 2.

IS200 profiling with, e.g., ribotyping, would not have linked poultry to human infections by RT1 isolates in Scotland.

Whilst IS200 profiling was unhelpful for type discrimination in this study, it was extremely valuable for establishing clonal groupings: among RT1 isolates from sources in Scotland, a perfect correlation was found for the phenotypic and genotypic markers tested; all Livingstone isolates were of RT1/BT8/IS200-A-var. (Table 6) and, hence, RT/BT/IS200 type was strongly predictive of serotype among RT1 isolates in Scotland. Consideration of another 16 isolates of RT1 collected during 1984–1992 [8, 9] from countries other than Scotland confirmed the association between RT/BT/IS200-type and serotype. In all, there were only six Livingstone isolates that belonged to RT1/BT3/IS200-B-var. (Table 6) and only one Eimsbuettel isolate of type RT1/BT8/IS200-A-var; gain or loss of phage 14 by occasional isolates of the subclones affords the most likely explanation for the finding of so few isolates of these 'rogue' serotypes. Thus, the only Eimsbuettel isolate of type RT1/BT8/IS200-A-var. was isolated from poultry in France in 1991 when Livingstone strains of the same RT/BT/IS200-type were present in diverse sources [8]; acquisition of phage 14 by any such Livingstone strain would have resulted in its phage-mediated conversion to Eimsbuettel. Similarly, the origin of the few Livingstone isolates of RT1/BT3/IS200-B-var. from Eimsbuettel strains of that same RT/BT/IS200 type is not difficult to imagine.

Most of the isolates of Livingstone and Eimsbuettel from countries other than Scotland, and a few Eimsbuettel isolates from Scotland, belonged to ribotypes RT2, RT3 and RT4. Within each ribotype, a few related biotypes were represented. Thus, 20 of 21 isolates of RT2 were of BT1 (one of the related BT3); and 21 isolates of the related ribotypes RT3 and RT4 [8, 9] were of the closely related biotypes BT5 (18 isolates) and BT6 (3). However, both serotypes were present in each RT/BT group. Further analysis of relatedness of Livingstone and Eimsbuettel clones of these ribotypes was not possible by IS200 profiling, because all isolates of RTs 2, 3 and 4 were IS200-negative.

All but one member of the clone designated RT5/BT3 were isolated in Israel during 1968–1971, carried plasmids and were IS200-negative. The remaining isolate, also donated by colleagues in Israel, but originally isolated in USA from pet meal in 1991, was plasmid-free and had the unique IS200 profile L, not detected in any other isolate examined in this study. Representatives of the RT5/BT3 group have been found in serotype Livingstone only [8, 9].

In the few serotypes of *Salmonella* of serogroup C examined so far, results have shown that the distribu-

tion of IS200 is limited [18, 19]. For example, the major clone of Bovismorbificans (representing 93% of strains examined) was IS200-negative, whereas two minor clones – very different from the major clone and from each other – were IS200-positive (one strain each), with no conserved bands in their profiles [18]. Again, in serotype Virchow only six of 57 phage-typed strains (not representing clonal groups) were IS200-positive and showed three different IS200 profiles containing low copy numbers of IS200 with no conserved bands [19]. However, among strains of serotype Infantis, 129 of 131 strains that represented five clonal lines found in Finland were IS200-positive; up to seven copies of IS200 were present in all strains, with four conserved, serotype-specific IS200 bands identified [20]. The findings of the present study show that the distribution of IS200 is also limited in strains of serotypes Livingstone and Eimsbuettel.

Our interest in serotypes Livingstone and Eimsbuettel arose because of their major contribution to human infections in Scotland during 1985–1993. Because the Scottish clone belonged to RT1, that ribotype may be somewhat over-represented in our collection compared with RTs 2–5 which, for the most part, were isolated in other countries. Nevertheless, the almost invariable association of IS200 of two quite different profiles with the two serotypes was impressive, suggesting that each subclone acquired its IS200 and antigenic markers at an early stage. Therefore, for the majority of IS200-positive strains of RT1 it was not possible to speculate if acquisition of antigenic characters preceded that of IS200 [5]. However, for the seven isolates whose serotype was unexpected for the RT/BT/IS200-type involved, it seems likely that the serotype-determining antigen O14 was acquired after the IS200 determinants. The findings that isolates of RT1 of both serotypes from sources outside Scotland were IS200-positive, and that isolates of RTs 2, 3 and 4 (regardless of their country of origin), were IS200-negative, suggests that it was not the Scottish milieu but the genotype of the strains that influenced IS200 status.

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