Epidemiol. Infect. (1999), **123**, 37–46. Printed in the United Kingdom

Application of ribotyping and IS*200* fingerprinting to distinguish the five *Salmonella* serotype O6,7:c:1,5 groups: Choleraesuis *sensu stricto*, Choleraesuis var. Kunzendorf, Choleraesuis var. Decatur, Paratyphi C, and Typhisuis

S. UZZAU*, M. HOVI AND B. A. D. STOCKER

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305, USA

(*Accepted 24 March 1999*)

SUMMARY

Sixty-seven strains of the five described *Salmonella* serotypes having antigens 6,7:c:1,5, that is *S. enterica* serotype Choleraesuis *sensu stricto*, Choleraesuis var. Kunzendorf, Choleraesuis var. Decatur, Paratyphi C, and Typhisuis, were examined for 16S *rrn* profile ribotype, presence of IS*200* and phenotypic characters, including rate of change of flagellar-antigen phase and nutritional character. Choleraesuis *sensu stricto* and its Kunzendorf variant had related but distinct ribotypes. Therefore, ribotyping appears to be a suitable method for differentiating Choleraesuis non-Kunzendorf from Choleraesuis var. Kunzendorf. Some strains of Paratyphi C had 16S profiles that resembled that of Choleraesuis non-Kunzendorf, while others resembled that of Choleraesuis var. Kunzendorf. The Typhisuis profiles were like those of Choleraesuis non-Kunzendorf, while the Choleraesuis var. Decatur profiles were unlike those of any of the other four groups. Furthermore, IS*200* fingerprinting discriminated between Choleraesuis var. Decatur and the other strains with antigenic formula O6,7:c:1,5, and comparison of IS*200* patterns showed a high degree of genetic divergence within Choleraesuis var. Decatur. Our findings show that ribotyping and IS*200* fingerprinting, combined with classical microbiological methods, distinguish the groups Choleraesuis non-Kunzendorf, Choleraesuis var. Kunzendorf, Choleraesuis var. Decatur, Paratyphi C and Typhisuis.

INTRODUCTION

Salmonella strains with the same or closely related O (LPS) and H (flagellar) antigens have usually been assigned to the same species or, in current terminology, serotype. However, *Salmonella* strains with O antigen 6,7, phase-1 flagellar antigen c and phase-2 flagellar antigen 1,5 have been given several different names [1]. One group, Paratyphi C, is distinguished by possession of Vi antigen (or of the *viaB* gene responsible for its production, in laboratory strains which no longer produce Vi antigen) and by its ability to cause enteric fever in man. The current version of the Kauffmann–White table lists only Paratyphi C, Choleraesuis and Typhisuis as serotypes of group C1 (that is with O antigen 6,7) with flagellar antigens c and 1,5 [2]. In earlier work [3], Choleraesuis var. Kunzendorf (hereafter, for brevity, Cs-Kunz) was distinguished from standard Choleraesuis ('*sensu stricto*' or 'classical', for brevity, Cs-non-K) by its abundant production of H_2S and by apparent monophasic character, but was later merged with Choleraesuis non-Kunzendorf, as also was Choleraesuis var. Decatur [2]. However, Le Minor and his colleagues [4] examined strains of 4 of the 5 above mentioned groups (174 strains of Paratyphi C, 136 Choleraesuis var. Kunzendorf, 44 Choleraesuis non-Kunzendorf and 4 Choleraesuis var. Decatur) and found each of these 4 groups to differ from each of the others by biochemical characters or by the

* Author for correspondence: Dipartimento di Scienze Biomediche, Sezione di Microbiologia Sperimentale e Clinica. Università degli Studi di Sassari, Viale S. Pietro, 43B-07100-Sassari, Italy.

presence of minor epitopes of flagellar antigen c. Typhisuis was not included in the investigation because only one strain was available for testing, but was noted as being clearly distinguishable from the other four groups by its auxotrophy and relatively poor growth even on rich media.

Because of our involvement in a search for polymorphic characters in Choleraesuis strains isolated from swine in Iowa [5], we had occasion to examine many such strains and to make use of 2 techniques of molecular biology which have been found useful as providing epidemiological markers within *Salmonella* serotypes, that is the presence of the nearly *Salmonella*-specific insertion element IS*200*, and 'ribotyping', that is the restriction fragment length polymorphism (RFLP) around the 16S rRNA gene (*rrn*) loci. Our main purpose was to investigate to what extent these techniques could be used as epidemiological markers within O6,7:c:1,5 *Salmonella* strains.

METHODS

Bacterial strains

Forty-one strains of Choleraesuis (either Cs-Kunz or Cs-non-K), 9 of Paratyphi C, 6 of Typhisuis, and 9 of Choleraesuis var. Decatur were collected from various sources (Table 1). The strains examined were either standard strains from reference laboratories or collections, or fairly recent isolates from human or animal sources. Some of these strains, indicated with the suffix K in Table 1, are from the Kauffmann collection and may be considered the 'type' strains of the group concerned. We examined all the strains of antigenic formula O6,7:c:1,5 included in the SARB collection [6]. This collection includes, for many serotypes, one strain of the electrotype most commonly encountered in that group and one representative of one or more of the less common electrotypes in the serotype.

Antisera and serological methods

Commercial (Difco) anti-O6,7 and a monoclonal anti-O7 antibody, provided by Dr P. Duffy, of the California State Laboratory, Berkeley, were used to test O antigen character by slide agglutination; a single-factor anti-c antiserum, an anti-1,2 serum and a single-factor anti-5 (Salmonella H) serum were likewise provided by Dr P. Duffy. H antigen constitution was determined by test of antibody inhibition of spread in semisolid medium. To test bacterial ability to switch flagellar-antigen phase, anti-c serum or anti-1,2 (or anti-5) serum was included in the semisolid medium. Any strain which spread, uninhibited, in the presence of both anti-c and anti-1,2 (or anti-5) antibody was inferred to express a flagellar antigen other than c or 1,5.

Semisolid medium and motility test

The medium used for testing motility, or for antibody inhibition test of swarming, or for ability to switch phase, was Oxoid Nutrient Broth CM67 with Difco Gelatin, 80 g/l, and Difco Agar, 2 g/l, prepared by mixing, just before pouring, broth, broth with gelatin (160 g/l), and broth with agar (6 g/l), in ratios to give the final concentrations indicated, together with anti-H serum or sera if appropriate. Five cm plates of semisolid medium were inoculated at one point near the edge, incubated at 37 °C and examined at intervals to detect spreading growth.

Test of biochemical activities

Bacteria were tested for ability to ferment arabinose or trehalose by incubation at 37 °C of streak-inoculated plates of McConkey Base agar with 0·5% of the sugar. For some strains fermentation of these or other sugars was also tested in Phenol Red broth (Difco) with sugar at 0·5%. Production of H_2S was tested on triple sugar iron slants (TSI), incubated at 37 °C for several days.

The minimal medium used to determine nutritional character was an inorganic salts mixture (KH_2PO_4, 8 g, K_2HPO_4, 28 g, $(NH_4)_2SO_4$, 4 g, $MgSO_4(7\,H_2O)$, 0·4 g, water, 11), with glycerol, 4 g/l, and trisodium citrate, 1·7 g/l, added as carbon and energy sources, solidified with agar, 12 g/l.

DNA preparation and Southern blot

Chromosomal DNA was extracted from all the isolates shown in Table 1, as previously described [7]. DNA (20–40 μg) was then digested overnight with an excess of *Pvu*II or *Pst*I restriction enzyme. Digests were subjected to electrophoresis in 0·8% agarose gel at 70 mV/cm for 8 h and then transferred to Hybond N membranes (Amersham) by the method of Southern [8].

Probes and hybridization

IS*200* and 16S *rrn* probes were generated by PCR amplification, using *S. enterica* serotype Typhimurium SL1344 chromosomal DNA as template [9]. Primers utilized for amplification of IS*200* sequence were identical to those designed by Bisercic and Ochman (forward 5′-CAGATGCGCCTATAAGGCT-3′ and reverse, 5′-CTAGGCTGGGGGTTCCGGAA-3′) [10]. An internal fragment of the 16S *rrn* gene was amplified by use of primers 5′-GCAACGCGAAGAACCTTACC-3′ and 5′-GGTTACCTTGTTACGACTT-3′ [11]. The expected amplicon sizes for the two pairs of primers were 660 bp and 550 bp respectively; both lack *Pvu*II and *Pst*I restriction sites. All amplification reactions yielded a single product with the correct size; these were later biotinylated by use of the BioNick Labelling System (Gibco–BRL), according to the manufacturer instructions. Hybridization was performed with high stringency and detected by using Photogene Version 2.0 (Gibco–BRL) according to the manufacturers instructions.

RESULTS

Screening of strains and assignment to groups

All 67 strains received at Choleraesuis (either Cs-Kunz, Cs-nonK, or not specified), Paratyphi C, Typhisuis or Choleraesuis var. Decatur, were screened to assess if they satisfied the criteria for inclusion in our study, which was the presence of O antigen 6,7 and H antigens c and 1,5. Two strains of the SARB collection were excluded, SARB 7 by presence of H antigen(s) other than c and 1,5 and SARB 50 by absence of any phase-2 antigen. The results of tests for biochemical activities, 16S *rrn* profile, rate of phase change and presence of IS*200* are recorded in Table 1, in which strains are placed in groups according to our results. These indicated reassignment of two SARB strains to serotypes other than those shown [6].

Biochemical activities of each group

Of the 41 Choleraesuis strains, 26 fell in the Cs-Kunz group. None of them fermented arabinose or trehalose (Table 1). Twenty-four produced abundant H_2S, as indicated by complete blackening of TSI slants, after overnight incubation. The two H_2S-negative strains caused no blackening of TSI slants, even after several days incubation at 37 °C, but otherwise behaved as typical Cs-Kunz. To test the possibility that they were H_2S-negative Cs-Kunz we submitted them to the WHO Salmonella Research and Reference Laboratory, Institut Pasteur, Paris. These two strains gave the reactions with adsorbed anti-c sera characteristic of Cs-Kunz, not those of Cs-non K [4] (Dr M. Y. Popoff, personal communication). All 26 Cs-Kunz strains gave moderate to heavy growth after 1, or at most 2 days incubation of simple defined medium with a heavy streak inoculum. Growth of some strains was irregular in that there was little or no visible growth on parts of the inoculated area but obvious growth on other parts. We did not obtain consistent results in attempting to score this effect and for this reason all these strains are shown as 'Irreg' in Table 1. As described below, all these Cs-Kunz strains changed H antigen very infrequently.

The remaining 15 Choleraesuis strains, likewise arabinose- and trehalose-negative, were classified as Cs-non-K, since they failed to blacken the TSI slants, even after several days incubation, and changed phase at normal frequency. When tested on the simple defined-medium agar, these strains gave either virtually no growth or showed the same pattern of irregular growth as the Cs-Kunz strains but to a greater extent; they were all recorded 'Irreg' in Table 1.

Of the 10 strains received as Paratyphi C, 3 were from the SARB collection; 1 of them, strain SARB 50, was excluded from our analysis since it could not be shown to have any phase-2 H antigen. The nine accepted Paratyphi C strains gave biochemical reactions corresponding to expectation for this serotype. All strains blackened TSI slants. The results of tests of the ability of these strains to ferment arabinose or trehalose were found to depend, for some strains, on the method of testing. Thus, only 6 of the 9 strains produced a red streak of growth with a surrounding zone of precipitation on McConkey Base agar with arabinose, but 8 of the 9 gave a positive result when tested in Phenol Red broth with the sugar after overnight incubation, or after 2 days for the strains which scored arabinose-negative on the solid medium. Similarly, only 1 of the 9 strains was scored trehalose-positive on the McConkey-trehalose plate but the other 8 gave acid in Phenol Red broth with trehalose after overnight at 37 °C. Since sugar reactions are commonly tested in liquid medium, we entered the Phenol Red broth results in Table 1. All isolates were able to grow on simple defined-medium agar. Only three of the Paratyphi C strains scored positive for the Vi antigen by slide agglutination (data not shown).

Table 1. *Source and characters of* Salmonella *serotype O6,7:c:1,5 strains studied*

Strain	Source* and original I.D.	H_2S†	Treh‡	Arab§	Rate of phase change‖	Prototrophy¶	IS*200***	Ribotype††
Choleraesuis var. Kunzendorf								
SL2839	(a) Swine 117	+	−	−	S	Irreg	−	Ia
SL2840	(a) Swine 110	+	−	−	S	Irreg	−	Ia
SL2841	(a) Swine 7	+	−	−	S	Irreg	−	Ia
SL2842	(a) Swine 113	+	−	−	S	Irreg	−	Ib
SL2843	(a) Swine 46	+	−	−	S	Irreg	−	Ia
SL2844	(a) Swine 126	+	−	−	S	Irreg	−	Ia
SL2805	(a) 38	+	−	−	S	Irreg	−	Ic
SARB4	(b) SARB 4	+	−	−	S	Irreg	−	Ia
991/94	(c) TK363	+	−	−	S	Irreg	−	Ia
992/94	(c) TK366	+	−	−	S	Irreg	−	Ia
993/94	(c) TK478	+	−	−	S	Irreg	−	Ia
994/94	(c) TK479	+	−	−	S	Irreg	−	Ia
995/94	(c) TK554	+	−	−	S	Irreg	−	Ib
1000/94	(d) 2467	+	−	−	S	Irreg	−	Ia
1007/94	(c) TK557	−	−	−	S	Irreg	−	Ia
1008/94	(c) TK558	−	−	−	S	Irreg	−	Ia
980/94	(a) 46168	+	−	−	S	Irreg	−	Ia
981/94	(a) 46208	+	−	−	S	Irreg	−	Ia
982/94	(a) 46277	+	−	−	S	Irreg	−	Ia
983/94	(a) 46283	+	−	−	S	Irreg	−	Ia
984/94	(a) 46385	+	−	−	S	Irreg	−	Ia
1042/94	(e) 36K	+	−	−	S	Irreg	−	Ia
1043/94	(e) 37K	+	−	−	S	Irreg	−	Ia
1044/94	(e) 7128/89	+	−	−	S	Irreg	−	Ia
1045/94	(e) 6447/88	+	−	−	S	Irreg	−	Ia
1046/94	(e) 1-84	+	−	−	S	Irreg	−	Ia
Choleraesuis								
SARB 6	(b) SARB 6	−	−	−	R	Irreg	−	IIa
1001/94	(d) 4640	−	−	−	R	Irreg	−	IIb
998/94	(d) 6558	−	−	−	R	Irreg	−	IIa
999/94	(d) 7665	−	−	−	R	Irreg	−	IIa
SL7528	(f) BS 2181	−	−	−	R	Irreg	−	IIa
1038/94	(e) 34K	−	−	−	R	Irreg	−	IIa
1039/94	(e) 6631/88	−	−	−	R	Irreg	−	IIa
1040/94	(e) 10-74	−	−	−	R	Irreg	−	IIa
1041/94	(e) 2-72	−	−	−	R	Irreg	−	IIa
1055/94	(g) 87	−	−	−	R	Irreg	−	IIa
1056/94	(g) 15230	−	−	−	R	Irreg	−	IId
1057/94	(g) 20939	−	−	−	R	Irreg	−	IIa
1058/94	(g) 157118/87	−	−	−	R	Irreg	−	IId
1059/94	(g) 171164	−	−	−	R	Irreg	−	IIa
1060/94	(g) 19991	−	−	−	R	Irreg	−	IIa
Paratyphi C								
SARB 48	(b) SARB 48	+	−	+	R	Yes	−	Ia
SARB 49	(b) SARB 49	+	−	−	R	Yes	−	IIe
1004/94	(d) 10485/11/76	+	−	+	R	Yes	−	Ia
1032/94	(e) 32K	+	−	−	NM	Yes	−	IIg
1033/94	(e) 4827/84	+	−	+	R	Yes	−	Ia
1034/94	(e) 13-87	+	−	+	R	Yes	−	Ia
1035/94	(e) 1535K	+	−	+	NM	Yes	−	Ia
1036/94	(e) 33K	+	−	+	R	Yes	−	Ia
1037/94	(e) 7972/93	+	−	+	R	Yes	−	IIe

Table 1 (*cont.*)

Strain	Source* and original I.D.	H_2S†	Treh‡	Arab§	Rate of phase change‖	Prototrophy¶	IS*200***	Ribotype††
Typhisuis								
1005/94	(c) TK555	−	+	+	R	No	−	IIe
1006/94	(c) TK556	−	+	+	R	No	−	IIa
SARB 69	(b) SARB 69	−	+	+	R	No	−	IIf
1062/94	(h) 2	−	+	+	R	No	−	IIf
1063/94	(h) 3	−	+	+	R	No	−	IIe
1064/94	(h) 4	−	+	+	R	No	−	IIe
Choleraesuis var. Decatur								
SARB 5	(b) SARB 5	+	+	+	R	Yes	+	IIIa
SARB 70	(b) SARB 70	+	+	+	R	Yes	+	IIIb
SARB 8	(b) SARB 8	+	+	+	R	Yes	+	IIIb
1002/94	(d) 831	+	+	+	R	Yes	+	IIIa
1003/94	(d) 1562	+	+	+	R	Yes	+	IIIa
1047/94	(e) 631K	+	+	+	R	Yes	+	IIIb
1048/94	(e) CM 2-84	+	+	+	R	Yes	+	IIIa
1049/94	(e) CM 1-74	+	+	+	R	Yes	+	IIIc
1050/94	(e) CM 1-70	+	+	+	R	Yes	+	IIIa

* (*a*) Dr R. Griffith, Dept. of Veterinary Microbiology, Iowa State University, Ames, Iowa, IO, USA; (*b*) *Salmonella* reference collection B (SARB); (*c*) Prof T. T. Kramer, Veterinary Medicine Research Institute, Iowa State University, Ames, Iowa, IO, USA; (*d*) Sharon Abbott, Microbial Disease Laboratory Section, State of California, Department of Health Services, CA, USA; (*e*) Dr M. Y. Popoff, WHO Collaborating Center for Reference and Research on Salmonella, Institut Pasteur, France; (*f*) Dr G. Leori. Istituto Zooprofilattico della Sardegna, Sassari, Italy; (*g*) Dr P. K. Chau, Department of Microbiology, University of Hong Kong, Republic of China; (*h*) Dr D. C. Hirsh, Department of Veterinary Microbiology, University of California at Davis, CA, USA;

† (+), all or much of the TSI slant blackened after overnight at 37 °C; (−), no or only a small part of TSI slant blackened after overnight at 37 °C;

‡ Fermentation of Trehalose, Treh, or § Arabinose, Arab. See text for test procedure;

‖ Rate of flagellar phase change was tested on semisolid media with antiserum directed against antigen *c* (phase 1 flagellar antigen) or antigen *1,5* (phase 2 flagellar antigen): R, rapid; S, slow; NM, non motile

¶ Phototrophy was recorded after 2 days incubation on simple defined medium heavily inoculated by streaking of growth from rich medium: Yes, continuous growth over the whole inoculated area; Irreg, growth only over part of the inoculation area; No, no or hardly visible growth.

** Presence or absence of IS*200* in chromosome. See text for test procedure.

†† Classification of ribotypes was based on patterns observed after *Pvu*II digestion. See text for test procedure.

Seven strains of Typhisuis were available for testing. Strain SARB 70, shown as Typhisuis [6], gave a reaction incompatible with this assignment, including strong production of hydrogen sulphide, good growth on simple defined medium, a ribotype allied to those of Decatur and presence of IS*200* insertions (see below). We reclassified this strain as Choleraesuis var. Decatur. The six accepted strains gave reactions characteristic of this serotype as described by Ewing [12], including weak or delayed production of hydrogen sulphide as tested in TSI slants. They also failed to ferment mannitol (tested in Phenol Red broth) and scored negative for lysine decarboxylase activity when tested on the medium of Falkow [12], with oil overlay (data not shown). Two representative strains from each of the other four groups, were, as expected, positive in the same test. None of the 6 Typhisuis strains grew on simple defined-medium agar; supplementing it with pools of amino acids or vitamins, or provisions of glucose as main energy source instead of glycerol did not produce responses allowing recognition of any specific biosynthetic defects (data not shown).

Nine strains which we considered to be Choleraesuis var. Decatur were tested, comprising seven described as such by the source laboratory. Two further strains, SARB 5 and SARB 70, listed, respectively, as Choleraesuis and Typhisuis [6] gave results indicating their re-assignment as Choleraesuis var. Decatur. All nine accepted strains gave reactions identical to those of

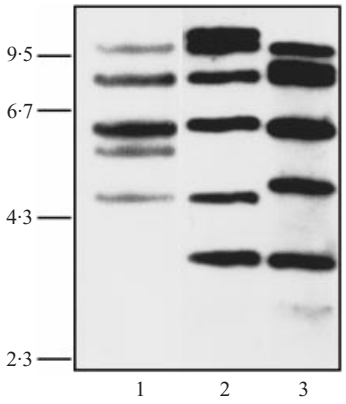

Fig. 1. Southern hybridization analysis of genomic DNA from Cs-Kunz SL2840 (lane 1), Cs-non-K 999/94 (lane 2), and Choleraesuis var. Decatur SARB 5 (lane 3) digested with *Pvu*II and probed by the PCR-generated 550 bp internal fragment of the 16S *rrn* gene. These most common ribotypes were termed Ia, IIa, and IIIa, respectively.

the first-described isolate [13], including fermentation of trehalose and arabinose, abundant production of H_2S and ability to grow on simple defined medium.

Alternative expression of phase-1 and phase-2 flagellar antigens

Each strain was inoculated into two plates of semisolid agar medium, one containing antiserum against antigen c (phase-1), the other with anti-5 or anti-1,2 (phase-2) serum, each at a concentration sufficient to inhibit spread by bacteria expressing the revelant H antigen. Strains showing spreading growth in each of the two plates after 2 h incubation were scored as regularly diphasic, that is with frequent change of phase. As shown in Table 1, all the Cs-non-K, Typhisuis and Choleraesuis var. Decatur strains showed normal frequency of change of phase by this test, as also did the seven strains of Paratyphi C found motile on first test and the motile mutants of the 2 strains initially found non-motile. None of the Cs-Kunz strains gave spreading growth on short incubation in each of the 2 serum plates. Of the 26 strains 22 swarmed in semisolid agar with antiserum for phase-2 antigen only after 10–36 h; the other 4 strains did not spread in the presence of anti-phase-1 serum until after 10–36 h. Single-colony reisolates from the late-developing spreading growth in anti-1,5 plates were inoculated in semisolid medium with anti-c serum; spreading growth was again seen only after prolonged incubation. Similar results were obtained with late-developing spread in presence of anti-c serum.

Ribotyping

*Pvu*II yielded the clearest resolution of 16S *rrn* bands and the most characteristic profiles. Therefore, this enzyme was used for all strains listed in Table 1. The ribotypes obtained could be grouped in three main profiles (Fig. 1). None of the *Pvu*II fragments were ubiquitous, whereas bands of 3·6, 4·7, 6·4 and 8·4 kbp were present among otherwise diverse patterns (Fig. 2). All the 26 Cs-Kunz strains and 6 Paratyphi C belonged to the group I (ribotype I). A 5·7 *Pvu*II fragment is specific for this ribotype that, on the other hand, lacks the 3·6 band. A second group (ribotype II) includes the Cs-non K, 3 Paratyphi C and all 6 Typhisuis strains tested. The profiles of this group share the 3·6, 4·7 and the 6·4 fragments, with the exception of subtype IIe and IIc. Finally, the Choleraesuis var. Decatur strains gave a rather different, though homogeneous, group of profiles (ribotype III) sharing only the 3·6 band with the ribotype II (Fig. 1).

The commonest Cs-Kunz profile, consisting of 5 bands, was called Ia (Fig. 1); 23 out of 26 strains possessed this ribotype. Two similar patterns, Ib and Ic, were observed in other three strains. These profiles showed a sixth additional band of 19 kbp and 2·2 kbp, respectively (Fig. 2).

The IIa ribotype was the most prevalent among Cs-non-K strains, being shared by 12 out of 15 isolates; it consisted of 6 bands (Fig. 1). Cs-non-K strain 1001/94 showed an additional band (Fig. 2, IIb). Two Hong Kong Cs-non-K isolates, recorded as 1056/94 and 1058/94, showed peculiar ribotype patterns (Fig. 2, IId and IIc, respectively). Strain 1056/94 gave a unique profile of 8 bands. Interestingly, the profile of strain 1058/94, allied to the group II pattern, lacks the 3·6 band while possessing the 5·7 fragment typical of ribotype group I.

Six of the 9 Paratyphi C strains exhibited the Ia profile whereas the other 3 belonged to subtypes IIe or IIg (Table 1, Fig. 2).

All Typhisuis isolates showed ribotypes of group II (Table 1, Fig. 2). Three strains had the subtype IIe, shared also by two of the Paratyphi C analysed.

The Choleraesuis var. Decatur ribotypes were very homogeneous; all the strains analysed belonged to the ribotype group III (Table 1, Fig. 2). Isolates SARB 5 and SARB 70, whose ribotypes were allied to this group, also displayed biochemical activities that allowed them to be identified as Choleraesuis var. Decatur strains (see above).

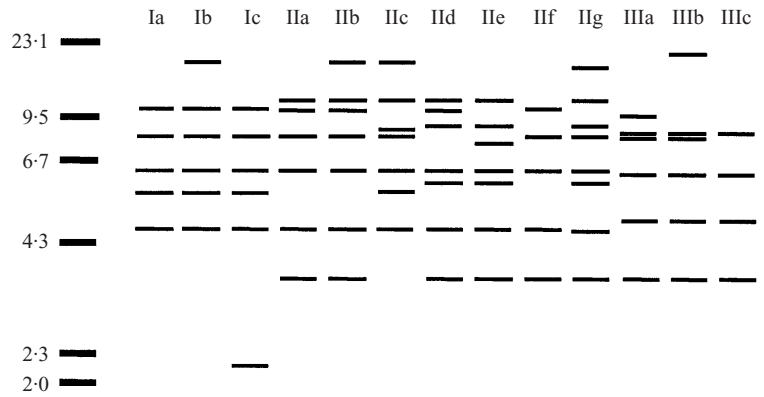

Fig. 2. Ribotypes of the five O6,7:c:1,5 *Salmonella* groups. Computer generated lane map (Image Master 1D, Pharmacia) of the 16*rrn* gene profiles observed in *Pvu*II digests among the 67 strains analysed.

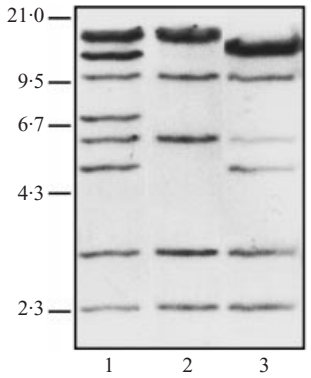

Fig. 3. Examples of IS*200* profiles observed among Choleraesuis var. Decatur strains with ribotype IIIa. Southern hybridization of genomic DNA digested with *Pvu*II and probed by the PCR-generated 660 bp internal fragment of IS*200*. Lane 1: 1050/94; lane 2: SARB 5; lane 3: 1048/94.

IS*200* insertion analysis

We performed a preliminary screening by PCR to detect IS*200* insertion(s). Each positive strain gave a band of the expected size on 1% agarose gel. Chromosomal DNA insertions were further defined by Southern hybridization. None of the Cs-non-K, Cs-Kunz, Typhisuis, and Paratyphi C strains were positive in this assay. All the strains received as Choleraesuis var. Decatur, as well as SARB 5 and 70, contained at least three copies of the transposon (Fig. 3). The variability of the IS*200* fingerprints was remarkable, even among strains of identical ribotype. Only two strains, 1002/94 and 1003/94, had identical profiles, suggesting epidemiological relatedness.

DISCUSSION

Boyd and colleagues [6] employed multilocus enzyme electrophoresis (MLEE) to establish a *Salmonella* reference collection B (SARB) of strains representing 37 serovars of *S. enterica* subspecies I, including Choleraesuis, Paratyphi C, Typhisuis, and Choleraesuis var. Decatur. However, they did not explore the validity of Choleraesuis var. Kunzendorf as a separate group. Our ribotype analysis of 41 Choleraesuis strains showed that there are two groups of 16S *rrn* profiles, ribotype I and ribotype II, distinctive respectively for Choleraesuis var. Kunzendorf (Cs-Kunz) and Choleraesuis *sensu stricto* (Cs-non-K). Nearly all the isolates in each group belong to a single predominant subtype, Ia for Cs-Kunz or IIa for Cs-non-K.

According to Selander and colleagues [6], Choleraesuis is a polyphyletic serovar comprising a predominant, widely distributed clone, of electrotype Cs1 (represented by SARB 4), and 12 other, much less common electrotypes. These authors included four Choleraesuis isolates, SARB 4, 5, 6 and 7 in the SARB collection. Our observations that SARB 4, of the predominant Cs1 electrotype, is a Cs-Kunz strain (group I) and that SARB 6, of electrotype Cs11, is a Cs-non-K strain (group II), is in agreement with the greater frequency of the Cs-Kunz group (Table 1). We propose to reassign SARB 5, of electrotype Cs6, as Choleraesuis var. Decatur, since it fermented arabinose, showed IS*200* insertions and group III ribotype (Table 1). Furthermore, the electrotype profile of SARB 5 is very similar to that of the predominant Choleraesuis var. Decatur electrotype [6]. Most serotypes of the SARB collection comprise only a single electrotype; therefore, any strain misidentified, despite the precautions taken to avoid this [6], may have been included in the SARB set and would represent a second, third, etc., electrotype. Correction of misassignments eliminates much of the

heterogeneity of electrotype within the O6,7:c:1,5 groups included in the SARB set. The last of the Choleraesuis strains in the SARB set, strain SARB 7, of electrotype Cs13, was excluded from our study by apparent presence of a flagellar antigen other than *c* and *1,5*; furthermore, its ability to ferment arabinose and trehalose are incompatible with its being Choleraesuis of either variety.

Choleraesuis does not have a strict host specificity for swine and it is reported to cause enteric and other diseases in humans [14–17]. In the first decades of this century Choleraesuis infection in man was much commoner in the US than now. Human infections were well known for severity, with 10–40% case mortality and the majority of isolates from non-intestinal sites (i.e. blood-stream, bones, joints). Most of the reports do not mention the isolates as hydrogen sulphide producers or the reverse (i.e. var. Kunzendorf or non-Kunzendorf). In south-east Asia Choleraesuis seems to have a higher incidence in man than in the rest of the world [14,16]. This may be due to the large consumption of porcine products, together with some culinary customs and, perhaps, a high frequency of Choleraesuis infection in the local swine. Among the strains that we tested two, both from Hong Kong, showed diverse and unique 16S *rrn* patterns. The profile of Cs-non-K strain 1058/94 (IIc) seems to provide a link between the Cs-non-K (ribotype II) and Cs-Kunz (ribotype I) profiles.

It has been suggested that Paratyphi C and Choleraesuis share a common ancestor [18]. In our study, as in that of Selander and colleagues [6], Paratyphi C appeared closely related to Choleraesuis. In particular, the commonest ribotype, Ia, among Cs-Kunz is shared by 6 of the 9 Paratyphi C strains we tested. This predominant type would correspond to the electrotype Pt1 of the SARB collection, represented by strain SARB 48. The other Paratyphi C strains, including SARB 49, had 16S *rrn* profiles very similar (but not identical) to those of Cs-non-K. A third 'Paratyphi C' strain of the SARB collection, SARB 50, whose electrotype was reported as quite distant from that of the other isolates, has a ribotype that is not related to those of any of the other Paratyphi C tested, possesses IS*200* element insertions (data not shown), and does not show any phase-2 antigen, and for this last reason was excluded from our analysis.

All the seven isolates of Typhisuis had similar ribotypes, suggesting that the group is monophyletic. Selander and colleagues previously reported a strain (SARB 70, of electrotype Ts 3) with significant allelic differences from those of the other Typhisuis isolates and related to that of Choleraesuis var. Decatur, Dt 1 [6]. We now propose the reassessment of SARB 70 as Choleraesuis var. Decatur on the basis of its ribotype, biochemical characters, prototrophy, and IS*200* insertions. Since none of the Typhisuis strains analysed had a ribotype I pattern, and none of them produced H_2S, one may speculate that this group evolved from Cs-non-K after the latter diverged from Cs-Kunz.

IS*200* offers highly defined molecular fingerprinting of the *Salmonella* chromosome and has been used as epidemiological marker within groups of closely related strains [19–21]. Since no insertions of this element were found in the Choleraesuis, Typhisuis and Paratyphi C strains analysed, whereas IS*200* was identified in all Choleraesuis var. Decatur examined (9 clones from different areas), it can be proposed as a further character for Choleraesuis var. Decatur identification. Although this serotype is not frequently isolated, it can affect human hosts [3, 13]. On the basis of its 16S *rrn* profiles, Choleraesuis var. Decatur appears to be a monophyletic group and the least related to the other groups analysed. The IS*200* profiles of this group were remarkably diverse even among strains displaying an identical ribotype. Only two Cholerasuis var. Decatur strains, 1002/94 and 1003/94, had an identical IS*200* profile, which suggested that these two strains might be epidemiologically related. Inquiry to the California State Laboratory (Sharon Abbott) found that the strains had been submitted by the same County laboratory a few weeks apart, both being isolates from children, though with different family names.

Our finding that in Cs-Kunz prolonged incubation is needed before the onset of naked-eye-visible spreading growth in the presence of antibody against either one or the other of the two flagellar antigens, c and 1,5 (potentially expressed by all the strains examined), corresponds to the earlier description of Cs-Kunz as monophasic, with most isolates expressing the phase-2 antigen 1,5 and a minority expressing, instead, the phase-1 antigen c. Both observations are explicable by the hypothesis of very infrequent change of phase, in each direction, occurring by the inversion of the *hin* sequence. The preponderance of isolates in phase-2 indicates that the rate of change from the phase-1 orientation to the other is higher than the rate of the opposite change; this is the reverse of the situation in (normally diphasic) Typhimurium [22].

Our observation that the product of PCR amplification of the *hin* locus was of the expected size for both Cs-Kunz and Cs-non-K (data not shown) excludes gross deletions or rearrangement as an explanation of the infrequent change of phase, in either direction, in Cs-Kunz. Our results suggest that observation of the time required before the onset of visible spreading in the presence of antibody against the expressed flagellar antigen is a convenient method for detection of infrequent change of phase.

In a recent study Weide-Botjes and colleagues [21] characterized many field isolates of Choleraesuis and a live vaccine strain of this species by four independent molecular methods (i.e. plasmid content, IS*200* element detection, ribotyping, and macrorestriction analysis). Ribotyping did not differentiate the Choleraesuis species (probably all Cs-Kunz), whereas macrorestriction analysis showed a high discriminatory power. Macrorestriction analysis is therefore a suitable method for differentiation of strains within a serotype, just as ribotyping is applicable to distinguish between the five groups with antigenic formula O6,7:c:1,5.

We have undertaken a study of molecular fingerprinting of *Salmonella* divergent clonal lineages possessing an identical antigenic profile (O6,7:c:1,5). The genetic diversity within this group can theoretically be accounted for by the retention of antigen profiles in otherwise divergent lineages. This hypothesis seems plausible for Choleraesuis, Typhisuis, and Paratyphi C, whose ribotype patterns are related. However, for Choleraesuis var. Decatur, the conservation of ancestral antigens seems less probable, in view of the greater differences observed. Rather, horizontal genetic transfer of loci for the somatic O and/or flagellar H antigens might have occurred. This possibility and the location of all genes determining O or H antigens within a few short segments of the *Salmonella* chromosome, has been noted [23, 24]. We have also devised and applied a procedure for semi-quantitative estimation of rate of change of flagellar antigenic phase and found that Cs-Kunz strains differed from those of the other four groups by infrequent change, in either direction. Finally, our results indicate that two strains in the *Salmonella* reference set B (SARB) are wrongly assigned to species or serotype.

Ribotyping and IS*200* insertion analysis are reliable and easy-to-perform typing methods, and can complement more sophisticated techniques such as MLEE. Particularly, we have provided evidence of their potential as epidemiological tools in the analysis of strains of antigenic formula O6,7:c:1,5.

ACKNOWLEDGEMENTS

We thank our colleagues named above for provision of strains, sera, monoclonal antibody, and information. S.U. was supported by the Consiglio Nazionale per le Ricerche (Italy).

REFERENCES

1. Kaufmann F. The bacteriology of *Enterobacteriaceae*. Copenhagen: Munksgaard, 1966.
2. Popoff MY, Le Minor L. Antigenic formulas of the *Salmonella* serovars, 7th revision. WHO Collaborating Center for Reference and Research on *Salmonella*. Institut Pasteur, Paris, 1997.
3. Bruner, DW, Edwards PR. Microorganisms of the *Salmonella choleraesuis* group isolated in the United States. Amer J Hyg 1939; **30**: 75–81.
4. Le Minor L, Beaud R, Laurent B, Monteil V. 1985. Etude des *Salmonella* possedant les facteurs antigeniques 6,7:c:1,5. Ann Inst Pasteur/Microbiol 1985; **136B**: 225–34.
5. Stocker BAD, Kramer TT. Unpublished data.
6. Boyd EF, Wang F, Beltran P, Plock SA, Nelson K, Selander RK. *Salmonella* reference collection B (SARB): strains of 37 serovars of subspecies I. J Gen Microbiol 1993; **139**: 1125–32.
7. Wilson K. Preparation of genomic DNA from bacteria. In: Ausubel FA, Brent R, Kingstone RE, et al, eds. Current protocols in molecular biology. New York: Greene Publishing and Wiley-Interscience, 1994: 2·4·1–2·4·5.
8. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975; **98**: 503.
9. Hoiseth SK, Stocker BA. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature 1981; **291**: 238–9.
10. Bisercic M, Ochman H. Natural populations of *Escherichia coli* and *Salmonella typhimurium* harbor the same classes of insertion sequences. Genetics 1993; **133**: 449–54.
11. Stanley J, Baquar N, Threfall EJ. Genotypes and phylogenetics relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS*200* and 16S *rrn* loci. J Gen Microbiol 1993; **139**: 1133–40.
12. Ewing WH. Edwards and Ewing's identification of Enterobacteriaceae, 4th ed. New York: Elsevier Science Publishing, Inc., 1986.
13. Kaufmann K, Edwards PR, McWhorther AC. A new *Salmonella* type, *Salmonella decatur* (6,7:c:1,5). Acta Path Microbiol Scand 1955; **36**: 568–70.
14. Allison MJ, Dalton HP, Escobar MR, Martin CJ. *Salmonella choleraesuis* in man: a report of 19 cases and a critical literature review. South Med J 1969; **62**: 593–6.

15. Blaser MJ, Feldman RA. *Salmonella* bacteremia reports to the Center for Diseases Control 1968–79. J Infect Dis 1981; **143**: 743–6.
16. Huang CT, Lo CB. Human infection with *Salmonella choleraesuis* in Hong Kong. J Hyg 1967; **65**: 149–63.
17. Klarke RC, Gyles CL. *Salmonella*. In: Gyles CL, Thoen CO, eds. Pathogenesis of bacterial infections in animals. Iowa City: State University Press, 1986: 95–109.
18. Selander RK, Beltran P, Smith NH, et al. Evolutionary genetic relationships of clones of *Salmonella* serovars that cause human typhoid and other enteric fevers. Infect Immun 1990; **58**: 2262–75.
19. Schiaffino A, Beuzon CR, Uzzau S, et al. Strain typing with IS*200* fingerprints in *Salmonella abortusovis*. Appl Envir Microbiol 1996; **62**: 2375–80.
20. Torre E, Threlfall EJ, Hampton MD, Ward LR, Gilbert I, Rowe B. Characterization of *Salmonella virchow* phage types by plasmid profile and IS*200* distribution. J Appl Bacteriol 1993; **75**: 435–40.
21. Weide-Botjes M, Liebisch B, Schwarz S, Watts JL. Molecular characterization of *Salmonella enterica* subsp. *enterica* serovar *choleraesuis* field isolates and differentiation from homologous live vaccine strain Suisaloral and SC-54. J Clin Microbiol 1996; **34**: 2460–3.
22. Stocker BAD. Measurement of rate of mutation of flagellar antigen phase in *Salmonella typhimurium*. J Hyg 1949; **47**: 398–413.
23. Beltran PJ, Musser M, Helmuth R, et al. Toward a population genetic analysis of *Salmonella*: genetic diversity and relationships among strains of serotypes *Choleraesuis*, *S. derby*, *S. dublin*, *S. enteritidis*, *S. heidelberg*, *S. infantis*, *S. newport*, and *S. typhimurium*. Proc Natl Acad Sci USA 1988; **85**: 7753–7.
24. Sanderson KE, Hurley JA. Linkage map of *Salmonella typhimurium*. In: Neidhardt FC, Ingraham JL, Low KB, Magasanik B, Schaechter M, Umbarger HE, eds. *Escherichia coli* and *Salmonella typhimurium*, cellular and molecular biology. Washington, DC: American Society for Microbiology, 1987: 877–918.