Molecular Characterization of *Salmonella enterica* subsp. *enterica* Serovar *choleraesuis* Field Isolates and Differentiation from Homologous Live Vaccine Strains Suisaloral and SC-54

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Four independent molecular methods were used to characterize the *Salmonella enterica* subsp. *enterica* serovar *choleraesuis* live vaccine strains SC-54 and Suisaloral and to differentiate them from *S. choleraesuis* field isolates. Plasmid analysis revealed the presence of seven plasmid profiles. A virulence plasmid of 52-kbp was identified by hybridization with an *spvB-spvC* gene probe in each of the *S. choleraesuis* field isolates and in the Suisaloral vaccine strain, but not in the SC-54 vaccine strain. Ribotyping, performed with a gene probe that recognized 23S, 16S, and 5S rRNA genes, resulted in three closely related hybridization patterns. IS200 elements were not detected in the field isolates or in the two *S. choleraesuis* live vaccine strains. Macrorestriction analysis with the enzymes *Xba*I, *Spe*I, *Not*I, and *Sfi*I differentiated the 29 *S. choleraesuis* strains included in this study into 10, 13, 8, and 13 different fragment patterns, respectively. While the Suisaloral vaccine strain showed a unique *Xba*I macrorestriction pattern, the fragment patterns of the SC-54 strain obtained with the different enzymes were shared by 2 to 18 *S. choleraesuis* field strains. A combination of plasmid analysis and macrorestriction analysis proved to be most suitable for the molecular typing of *S. choleraesuis* and the differentiation of both live vaccine strains from field isolates of this serovar.

Isolates of *Salmonella enterica* subsp. *enterica* serovar *choleraesuis* cause economically important diseases in pigs of all ages (28, 32). Septicemic forms with mortality rates of almost 100% are seen most commonly in pigs of up to 4 months of age. Acute or chronic enteric forms of the disease are characterized by diarrhea and persistent or intermittent fever (28, 32). Clinically asymptomatic carriers may carry *S. choleraesuis* in their gut mucosa or in their mesenteric lymph nodes and contaminate their environment with infectious feces (28). Thus, other pigs are infected when penned with infected pigs or when exposed to their infectious feces during transport, on the farm, or in markets. These infected animals, their products, and their manure represent a health hazard not only for other pigs but also for humans who take care of these animals or consume their meat (28). To prevent salmonellosis in pigs caused by *S. choleraesuis*, avirulent *S. choleraesuis* live vaccines have been developed in several countries, including the United States and Germany. The vaccines Suisaloral (Impfstoffwerk Dessau-Tornau GmbH, Rosslau, Germany) (23) and SC-54 (NOBL Laboratories Inc., Sioux Center, Iowa) (11, 21) have been approved for use in pigs in Germany and in the United States, respectively.

In this study, molecular methods were used to characterize these two *S. choleraesuis* live vaccine strains and to differentiate them from *S. choleraesuis* field isolates of porcine origin. Furthermore, the methods applied were compared for their suitability to serve as tools for the epidemiological typing of *S. choleraesuis* isolates.

MATERIALS AND METHODS

Bacterial isolates. Twenty-six epidemiologically unrelated *S. choleraesuis* field isolates were isolated from pigs at Iowa State University, Ames. *S. choleraesuis* live vaccine strain Suisaloral and its prototype strain were kindly provided by H.-J. Selbitz, Impfstoffwerk Dessau-Tornau; *S. choleraesuis* live vaccine strain SC-54 was obtained from NOBL Laboratories Inc. Biochemical confirmation of the *S. choleraesuis* isolates followed standard techniques (2, 12); serotyping was performed with commercially available antisera (Behring Werke, Marburg, Germany). The *S. choleraesuis* isolates were cultivated overnight at 37°C on Luria-Bertani (LB) agar plates; glycerin stock cultures were kept at –70°C.

DNA preparation and manipulation. Preparation of plasmids was carried out according to the methods of Kado and Liu (10). The plasmids of *Escherichia coli* V517 (15) as well as the *Salmonella enterica* subsp. *enterica* serovar *typhimurium* virulence plasmid pRQ28 (17) served as standards for the determination of plasmid sizes. Extraction of whole-cell DNA suitable for ribotyping, IS200 typing, restriction analysis, and agarose gel electrophoresis followed previously described protocols (25). Transfer of the DNA fragments from agarose gels to nitrocellulose membranes (Hybond N; Amersham Buchler, Braunschweig, Germany) was achieved by the capillary blot procedure (22). The 7.4-kbp *Bam*HI fragment of plasmid MM290 (3) served as the 16S-23S-5S rRNA gene probe and the 0.6-kbp *Pvu*II fragment of plasmid pIZ45 (7) served as the IS200 probe. Both gene probes were labelled by the nonradioactive enhanced chemiluminescence system (Amersham-Buchler). Macrorestriction analyses using the enzymes *Xba*I (Boehringer), *Spe*I (BioLabs), *Not*I (BioLabs), and *Sfi*I (BioLabs) followed previously described protocols (14, 22). The pulse times for *Xba*I and *Spe*I digests were increased from 7 to 12 s during the first 11 h and from 20 to 40 s in the next 13 h; those for *Not*I and *Sfi*I digests were increased from 2 to 5 s during the first 11 h and from 9 to 12 s for another 11 h. Polymerized phage λ DNA (Pharmacia LKB) served as a size standard. The DNA fragments were separated in a CHEF DR II system (Bio-Rad) at 15 V/cm with 0.5× Tris-borate-EDTA as the running buffer.

Calculation of discriminatory indices. The discriminatory value for each typing method was calculated as an index of discrimination (*D*) according to the method of Hunter and Gaston (9).

RESULTS

Plasmid analysis. Seven different plasmid profiles were obtained (Fig. 1). Plasmids of approximately 52 kbp were identified as virulence plasmids by hybridization with an *spvB-spvC* gene probe in all isolates except the SC-54 vaccine strain. Small plasmids in the range of 6 kbp were present in most of the *S. choleraesuis* isolates. Occasionally, additional plasmids larger

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FIG. 1. Agarose gel electrophoretic demonstration of the seven different plasmid profiles (lanes 1 to 7) observed in the *S. choleraesuis* isolates. The plasmid profiles of *E. coli* V517 and *S. typhimurium* are shown in lanes C and T, respectively; the sizes of these size marker plasmids are in kilobase pairs, chr, chromosomal-DNA band.

than 52 kbp were detected (Fig. 1). The SC-54 vaccine strain exhibited a unique plasmid profile, consisting of only the 6-kbp plasmid, whereas the Suisaloral vaccine strain and its parental strain carried only the 52-kbp virulence plasmid. The plasmid profiles of the vaccine strains were not shared by any of the *S. choleraesuis* field isolates. Plasmid profile analysis had a *D* value of 0.596.

Ribotyping and IS200 typing. Three different hybridization patterns were observed when *Pvu*II-digested whole-cell DNA of the *S. choleraesuis* strains was hybridized with a 23S-16S-5S rRNA gene probe. Twenty-three field isolates as well as the Suisaloral vaccine and its prototype strain exhibited identical hybridization patterns consisting of 11 hybridizing bands with sizes of 1.7, 2, 3, 3.5, 4.1, 4.3, 5.5, 6, 8, 10, and 14 kbp. Two field isolates showed a hybridization pattern in which the 4.3-kbp fragment was missing. The SC-54 vaccine strain and one of the field isolates showed a third pattern, in which the 4.1-kbp fragment was not detectable. The *D* value of *Pvu*II-directed ribotyping was calculated to be 0.256. No differences in the hybridization patterns were found by using the same rRNA gene probe with *Sma*I instead of *Pvu*II for digestion of the whole-cell DNA.

Hybridization signals were not detected by using the IS200-specific gene probe and *Pst*I-digested whole-cell DNA, either in the *S. choleraesuis* field isolates or in the two live vaccine strains. This observation suggested that none of the *S. choleraesuis* isolates included in this study carried an insertion sequence of the IS200 type.

Macrorestriction analysis. Four different restriction enzymes, *Xba*I, *Spe*I, *Not*I, and *Sfi*I, were used to cleave whole-cell DNA of *S. choleraesuis*. The resulting fragments were separated by pulsed-field gel electrophoresis.

*Xba*I digestion yielded 10 different patterns consisting of 10 to 12 fragments between 48.5 and 533.5 kbp (Fig. 2). The Suisaloral vaccine strain exhibited a unique fragment pattern, while that of the SC-54 vaccine strain was shared by four field isolates. *Xba*I macrorestriction analysis had a *D* value of 0.714. *Spe*I digestion resulted in 13 patterns of 16 to 19 fragments in the same range as that of the *Xba*I macrorestriction analysis (Fig. 3). No fragment patterns specific for any of the vaccine strains could be detected. The *D* value of *Spe*I macrorestriction analysis was calculated to be 0.929. *Not*I digestion produced eight patterns composed of 16 to 19 fragments in the low-molecular-size range (between 48.5 and 242.5 kbp). *Not*I macrorestriction analysis had a *D* value of 0.571. *Sfi*I digestion produced a large number of smaller fragments in the same range as that of *Not*I. Thirteen different patterns consisting of

FIG. 2. *Xba*I macrorestriction patterns observed among the *S. choleraesuis* isolates. Lane numbers correspond to pattern numbers in Table 1. The sizes of the DNA marker fragments (M) in kilobase pairs are on the right.

19 to 21 fragments were observed. Again, no fragment pattern specific for either of the live vaccine strains could be obtained with this enzyme. The *D* value of *Sfi*I macrorestriction analysis was 0.921.

Although ribotyping did not contribute to the molecular differentiation of the *S. choleraesuis* isolates, comparative analysis of plasmid profiles and macrorestriction analysis allowed the assignment of the 29 *S. choleraesuis* isolates used in this study to 25 genomic groups (Table 1). A high *D* value, 0.988, was calculated, which confirmed the usefulness of these methods for the epidemiological analysis of *S. choleraesuis*.

DISCUSSION

Molecular methods have proven to be very useful, reliable, and highly discriminative tools (18, 31) for the differentiation of strains of several *S. enterica* subsp. *enterica* serovars. Some techniques, such as plasmid analysis, ribotyping, IS200 typing (14, 25), and macrorestriction analysis (14, 24), also enabled the differentiation of *Salmonella* live vaccine strains from field isolates of the respective serovars. However, previous studies

FIG. 3. *Spe*I macrorestriction patterns seen in the *S. choleraesuis* isolates. Lane numbers correspond to pattern numbers in Table 1. The sizes of the DNA marker fragments (M) in kilobase pairs are on the left.

TABLE 1. Characteristics of the *S. choleraesuis* isolates included in this study

Isolate ^a	Size(s) of plasmids (kbp) (plasmid profile pattern ^b)	Ribotype pattern	Macrorestriction pattern				Genomic group
			<i>Xba</i> I ^c	<i>Spe</i> I ^d	<i>Not</i> I	<i>Sfi</i> I	
1	52, 6 (1)	1	2	1	1	1	I
2	52, 6 (1)	1	3	2	2	2	II
3	>90, 52 (2)	1	3	3	2	3	III
4	>90, 52 (2)	1	4	4	2	4	IV
5	>90, 52 (2)	1	5	1	2	5	V
6	>90, 52 (2)	1	6	5	2	4	VI
7	52, 6 (1)	1	3	6	3	6	VII
8	52, 6 (1)	1	3	2	2	2	II
9	54, 52, 50, 6 (3)	1	3	7	4	7	VIII
10	52, 6 (1)	1	2	8	5	8	IX
11	90, 52, 6 (4)	1	3	9	2	3	X
12	>90, 52 (2)	1	7	1	2	4	XI
13	52, 6 (1)	1	3	9	2	3	XII
14	52, 6 (1)	1	3	3	2	9	XIII
15	52, 6 (1)	1	3	9	2	3	XII
16	52, 6 (1)	1	2	10	2	2	XIV
17	52, 6 (1)	1	8	11	2	2	XV
18	52, 6 (1)	2	9	12	6	10	XVI
19	52, 6 (1)	2	9	12	6	10	XVI
20	52, 6 (1)	1	2	13	7	11	XVII
21	>90, 52, 6 (5)	1	10	10	2	12	XVIII
22	52, 6 (1)	3	3	3	2	4	XIX
23	52, 6 (1)	1	3	2	2	2	II
24	52, 6 (1)	1	3	3	2	4	XX
25	52, 6 (1)	1	3	3	2	5	XXI
26	52, 6 (1)	1	3	13	7	13	XXII
SC-54	6 (6)	3	2	2	2	1	XXIII
Suisaloral	52 (7)	1	1	7	4	7	XXIV
Suisaloral	52 (7)	1	3	1	8	1	XXV
prototype strain							

^a All field isolates (isolates 1 to 26) were obtained from clinical cases of salmonellosis in pigs.

^b Pattern numbers correspond to lane numbers in Fig. 1.

^c Pattern numbers correspond to lane numbers in Fig. 2.

^d Pattern numbers correspond to lane numbers in Fig. 3.

have shown that the capacity of these molecular methods to discriminate between strains varies significantly with respect to the properties of the serovar. So far, very few data have been published on the applicability of molecular typing methods for the analysis of *S. choleraesuis* strains.

The results obtained from plasmid analysis corresponded closely to previously published data on plasmids in *S. choleraesuis* (1, 8, 17). All field isolates, including the Suisaloral vaccine strain and its prototype strain, carried a virulence plasmid of approximately 52 kbp as determined by hybridization with an *spvB*-*spvC* gene probe. This virulence plasmid carried the common *spv* gene cluster present on virulence plasmids of *S. typhimurium*, *Salmonella enterica* subsp. *enterica* serovar *enteritidis*, and *Salmonella enterica* subsp. *enterica* serovar *dublin* (8, 17). In addition to the virulence plasmid, smaller or larger plasmids were present in all *S. choleraesuis* field isolates. Thus, the Suisaloral vaccine strain, which carried only the 52-kbp virulence plasmid, as well as the SC-54 vaccine strain, which harbored only a small plasmid, could easily be distinguished from one another and from the field isolates by their plasmid profiles. On the basis of the potential mobility of plasmids and the observation that plasmids can be acquired or lost under environmental conditions, plasmid profile analysis may not be the most reliable and stable molecular typing system.

Ribotyping, which implies the detection of restriction fragment length polymorphisms of rRNA genes, has been used for the differentiation of strains assigned to various *Salmonella* serovars (6). However, the discriminatory power of this method depends on the choice of the gene probe, the restriction enzyme used for digestion of the chromosomal DNA, and the *Salmonella* serovar. *S. typhimurium* isolates have been typed by this method (16, 25, 26), whereas little or no discrimination could be observed for strains of *S. enteritidis* (13, 16, 27), *S. dublin* (4, 14, 19), or *Salmonella enterica* subsp. *enterica* serovar *gallinarum* (5). Our results also indicated that ribotyping, even if performed with a gene probe that recognized the entire rRNA operon, is not the method of choice for the differentiation of *S. choleraesuis* isolates. When the enzyme *Sma*I was used, all isolates exhibited the same hybridization pattern; in *Pvu*II-digested DNA, at least three closely related hybridization patterns were observed, but the vast majority of isolates exhibited one pattern.

IS200 typing proved to be not applicable at all to *S. choleraesuis* isolates. Although initial studies of the prevalence of IS200 elements in various *Salmonella* serovars reported the presence of two IS200 copies in *S. choleraesuis* (7), no such insertion elements could be detected in any of the *S. choleraesuis* field isolates or in the two live vaccine strains included in this study. Even if IS200 elements occasionally occur in *S. choleraesuis*, our data suggest that IS200 typing should not be included in a basic set of molecular methods for typing *S. choleraesuis* isolates.

Macrorestriction analysis has been reported to be the most powerful tool for the discrimination of even closely related bacterial isolates (18, 29, 31). Moreover, this method showed not only the greatest discriminatory power but also the best reproducibility of results among all molecular methods currently used for the epidemiological typing of *Salmonella* isolates of various serovars (18). As shown in several studies, the discriminatory value of macrorestriction analysis can be significantly increased by the comparative use of several different enzymes (13, 14, 30). Thus, we have used the enzymes *Xba*I, *Spe*I, and *Sfi*I for analysis of the *S. choleraesuis* isolates. The fragment patterns obtained with these enzymes differed in both the numbers and the sizes of fragments. The best resolution of the fragments was seen with *Xba*I (Fig. 2). This enzyme also produced a fragment pattern for the Suisaloral vaccine strain that was not shared by any other isolate. *Spe*I macrorestriction analysis proved to be the most discriminative single method, as revealed by the high *D* value, 0.928. Even if no vaccine strain-specific patterns were seen, *Spe*I macrorestriction analysis might be a valuable tool for the epidemiological typing of *S. choleraesuis* isolates. As previously seen with *S. typhimurium* (25a), *S. enteritidis* (13, 20), and *S. dublin* (14, 19), *Not*I macrorestriction analysis resulted in a large number of similarly sized fragments, which rendered the evaluation of the fragment patterns difficult. Moreover, only a small number of patterns could be detected, which corresponded to a low discriminatory index, comparable to that of plasmid profile analysis. Nevertheless, the Suisaloral prototype strain exhibited a unique *Not*I fragment pattern. *Sfi*I macrorestriction analysis also produced a large number of fragments in the low-molecular-size range (up to 242.5 kbp); its discriminatory value corresponded to that of *Spe*I macrorestriction analysis. However, the better resolution of the fragments achieved with *Spe*I might favor the use of *Spe*I rather than *Sfi*I.

The combination of the data obtained from plasmid analysis with those obtained from macrorestriction analysis with four different enzymes subdivided the 29 *S. choleraesuis* isolates used in this study into 25 different genomic groups. The high

discriminatory index, 0.988, confirmed the usefulness of this set of methods for the epidemiological typing of *S. choleraesuis* isolates. Methods that allow the differentiation of strains to a very great extent, as shown in this study, might also represent valuable tools for the identification of sources of *S. choleraesuis* infections, for following the spread of a particular strain during an infection, and for epidemiological investigations of disease outbreaks. Moreover, this study also provided information on the two live vaccine strains, Suisaloral and SC-54, which might be useful for the identification of these strains and for monitoring their genetic stability either under field conditions or during production processes.

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