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# Evaluation and Comparison of Molecular Techniques for Epidemiological Typing of *Salmonella enterica* subsp. *enterica* Serovar *dublin*

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**A total of 28 unrelated isolates of the *Salmonella enterica* subsp. *enterica* serovar *dublin* (*S. dublin*) collected during a 6-year period, as well as four samples of the *S. dublin* live vaccine strain Bovisaloral and its prototype strain *S. dublin* 442/039, were investigated by different molecular typing methods for the following reasons: (i) to find the most discriminatory method for the epidemiological typing of isolates belonging to this *Salmonella* serovar and (ii) to evaluate these methods for their capacity to discriminate among the live vaccine strain Bovisaloral, its prototype strain *S. dublin* 442/039, and field isolates of the serovar *dublin*. Five different plasmid profiles were observed; a virulence plasmid of 76 kbp as identified by hybridization with an *spvB-spvC* gene probe was present in all isolates. The detection of 16S rRNA genes and that of IS*200* elements proved to be unsuitable for the epidemiological typing of *S. dublin*; only one hybridization pattern could be observed with each of these methods. The results obtained from macrorestriction analysis strongly depended on the choice of restriction enzyme. While the enzyme *Not*I yielded the lowest discriminatory index among all enzymes tested, it was the only enzyme that allowed discrimination between the Bovisaloral vaccine strain and its prototype strain. In contrast to the enzymes *Xba*I and *Spe*I, which only differentiated among the *S. dublin* field isolates, *Xho*I as well as *Avr*II also produced restriction fragment patterns of the Bovisaloral strain and of its prototype strain that were not shared by any of the *S. dublin* field isolates. Macrorestriction analysis proved to be the most discriminatory method not only for the epidemiological typing of *S. dublin* field isolates but also for the identification of the *S. dublin* live vaccine strain Bovisaloral.**

*Salmonella* infections in animals represent a threat to human health, particularly if the infected animals serve as food products. Among the various *Salmonella* serovars, only a few are of zoonotic importance. These include the *Salmonella enterica* subsp. *enterica* serovars *typhimurium* (*S. typhimurium*) (1, 6), *enteritidis* (*S. enteritidis*) (1, 6), and *dublin* (*S. dublin*) (5, 9, 20, 45). *S. dublin* has been reported to be the most common *Salmonella* serotype in cattle (2). While adult cattle infected with *S. dublin* most frequently do not show the clinical signs of an infection, fatal *S. dublin* infections in calves of 4 to 6 weeks of age are commonly observed and are characterized by fever, diarrhea, and circulatory collapses (12). Cattle surviving an *S. dublin* infection will spread these organisms for several months (12) and therefore be a source of infection not only for other animals but also for humans. Meat, unpasteurized milk, and milk products are considered vehicles of *S. dublin* infections in humans (5). Although *S. dublin* is considered a cattle-adapted pathogen, it may also cause severe systemic infections in humans (5, 9, 20, 41, 45).

To introduce immunity against *Salmonella* infections in cattle, two different live vaccine strains have been admitted to veterinary use in Germany. One of them, the attenuated and auxotrophic Zoosaloral R strain (Impfstoffwerk Dessau-Tornau GmbH, Roßlau, Germany), is identical to the Zoosaloral H strain, which has previously been characterized and can easily be differentiated from *S. typhimurium* field strains by molecular techniques (33, 34). The other one is the attenuated, auxotrophic *S. dublin* strain Bovisaloral (Impfstoffwerk Dessau-Tornau). In contrast to data on the Zoosaloral H strain, little is known about the genetic relationship of this attenuated live vaccine strain to field isolates of serovar *dublin* from cattle. However, the widespread use of the Bovisaloral live vaccine strain in cattle requires tools for the exact identification of this live vaccine strain and its differentiation from field isolates of the same serovar.

In this study, we investigated the *S. dublin* live vaccine strain Bovisaloral, its prototype strain *S. dublin* 442/039, and a representative pool of *S. dublin* field isolates collected during a 6-year period by different epidemiological typing methods. These included plasmid analysis and the detection of restriction fragment length polymorphisms (RFLPs) of 16S rRNA genes and IS*200* elements as well as macrorestriction analyses. All of these techniques were evaluated for their capacities to discriminate among isolates of serovar *dublin* in general and also for their ability to identify the live vaccine strain Bovisaloral and to differentiate it from field isolates and its prototype strain.

## MATERIALS AND METHODS

**Bacterial isolates and growth conditions.** Of the 33 *S. dublin* isolates used in this study, 28 were isolated from epidemiologically unrelated cattle specimens collected between 1988 and 1993 in Germany and Denmark. The remaining five isolates included four different samples of the *S. dublin* live vaccine strain Bovisaloral (Impfstoffwerk Dessau-Tornau) as well as the Bovisaloral prototype strain *S. dublin* 442/039. Biochemical characterization of the *S. dublin* isolates followed standard techniques; serotyping was performed with commercially available antisera (Behring Werke, Marburg, Germany). The *S. dublin* isolates were cultivated overnight on LB agar plates; glycerine stock cultures were kept at −70°C.

**DNA preparation, Southern blot hybridization, and gene probes.** Plasmid

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TABLE 1. Characteristics of *S. dublin* isolates used in this study

| Isolate no. | Source[a] | Plasmid profile (kbp) | No. of macrorestriction patterns | | | | | Genomic group |
|---|---|---|---|---|---|---|---|---|
| | | | *Xba*I | *Spe*I | *Not*I | *Xho*I | *Avr*II | |
| 1 | Bovisaloral strain[b] | 76, 40 | 1 | 1 | 1 | 1 | 1 | I |
| 2 | Prototype strain[c] | 76, 40 | 1 | 1 | 4 | 1 | 1 | II |
| 3 | GER, 1988; diarrhea | 76, 52 | 1 | 1 | 1 | 2 | 2 | III |
| 4 | GER, 1988; diarrhea | 76, 52 | 1 | 1 | 1 | 2 | 2 | III |
| 5 | GER, 1988; septicemia | 76, 52 | 1 | 1 | 1 | 2 | 2 | III |
| 6 | GER, 1988; pneumonia | 76, 52 | 1 | 1 | 1 | 2 | 2 | III |
| 7 | GER, 1988; diarrhea | 76, 52 | 1 | 1 | 1 | 2 | 2 | III |
| 8 | GER, 1988; septicemia | 76 | 1 | 1 | 1 | 2 | 2 | IV |
| 9 | GER, 1988; diarrhea | 76 | 1 | 1 | 1 | 2 | 2 | IV |
| 10 | GER, 1988; diarrhea | 76 | 1 | 1 | 1 | 2 | 2 | IV |
| 11 | GER, 1989; pneumonia | 76 | 2 | 2 | 1 | 4 | 5 | V |
| 12 | GER, 1989; diarrhea | 76 | 3 | 1 | 2 | 4 | 3 | VI |
| 13 | GER, 1988; pneumonia | 76 | 1 | 1 | 1 | 3 | 3 | VII |
| 14 | GER, 1988; pneumonia | 76 | 1 | 1 | 1 | 3 | 3 | VII |
| 15 | GER, 1989; neuroparalysis | 76, 52 | 1 | 1 | 1 | 3 | 4 | VIII |
| 16 | GER, 1989; diarrhea | 76 | 4 | 3 | 1 | 4 | 6 | IX |
| 17 | GER, 1989; diarrhea | 76 | 1 | 1 | 1 | 3 | 2 | X |
| 18 | GER, 1988; diarrhea | 76 | 5 | 1 | 1 | 2 | 10 | XI |
| 19 | GER, 1989; pneumonia | 76, 52 | 1 | 4 | 3 | 2 | 7 | XII |
| 20 | GER, 1988; septicemia | 76, 52 | 1 | 4 | 1 | 2 | 7 | XIII |
| 21 | GER, 1988; diarrhea | 76, 52 | 1 | 4 | 1 | 2 | 7 | XIV |
| 22 | GER, 1989; diarrhea | 76, 40 | 6 | 1 | 1 | 3 | 2 | XIV |
| 23 | DK, 1990; diarrhea | 76, 4.4 | 1 | 1 | 1 | 4 | 5 | XV |
| 24 | DK, 1992; diarrhea | 76, 4.4 | 1 | 1 | 1 | 4 | 5 | XV |
| 25 | DK, 1992; septicemia | 76, 5.0 | 7 | 5 | 1 | 3 | 8 | XVI |
| 26 | DK, 1991; pneumonia | 76, 5.0 | 7 | 5 | 1 | 3 | 9 | XVII |
| 27 | DK, 1993; diarrhea | 76 | 1 | 1 | 1 | 2 | 5 | XVIII |
| 28 | DK, 1992; septicemia | 76, 4.4 | 1 | 1 | 1 | 2 | 5 | XIX |
| 29 | DK, 1993; diarrhea | 76 | 1 | 6 | 1 | 3 | 3 | XX |
| 30 | GER, 1993; septicemia | 76 | 1 | 7 | 1 | 3 | 3 | XXI |

[a] All *S. dublin* field isolates were obtained from cattle in Germany (GER) or Denmark (DK) between 1988 and 1993; clinical signs of the animals are indicated.
[b] Bovisaloral live vaccine strains from four different production charges were tested and yielded identical results with all methods applied.
[c] *S. dublin* 442/039.

profiles of the *S. dublin* isolates were prepared according to Kado and Liu (18). The plasmids of *Escherichia coli* V517 (23) as well as the *S. typhimurium* virulence plasmid pRQ51 (25) served as size standards for the determination of plasmid sizes in the *S. dublin* isolates. Plasmids suitable for restriction endonuclease analyses were obtained by the method of Olsen (26). Whole cellular DNA of the *S. dublin* isolates was extracted as described previously (34). Restriction analysis of plasmid DNA and whole cellular DNA and the separation of the respective fragments by agarose gel electrophoresis followed previously described protocols (32, 34). The 1-kbp ladder and λ DNA-*Hin*dIII fragments (both from Gibco-BRL, Paisley, Scotland) served as DNA size standards.

Transfer of the DNA fragments from agarose gels to nitrocellulose membranes (Hybond N; Amersham-Buchler, Braunschweig, Germany) was achieved by the capillary blot procedure (32). The following gene probes were used: the 3.6-kbp *Hin*dIII fragment of plasmid pRQ51 (25) served for the detection of *spvB-spvC* virulence genes, the 2.3-kbp *Hin*dIII fragment of plasmid pBA2 represented the 16S rRNA probe (17), and the 0.6-kbp *Pvu*II fragment of plasmid pIZ45 (10) was used as the IS*200* probe. All gene probes were labelled by the nonradioactive ECL system (Amersham-Buchler). Hybridization and signal detection were carried out strictly according to the manufacturer's recommendations with the solutions included in the ECL kit (34).

**Macrorestriction analysis.** Preparation of whole cellular DNA for pulsed-field gel electrophoresis experiments followed the protocol of Olsen et al. (30). Slices of DNA-containing agarose plugs were incubated for 4 h in the presence of 20 U of *Xba*I (Boehringer), *Not*I (BioLabs), *Spe*I (BioLabs), *Xho*I (Boehringer), or *Avr*II (Amersham-Buchler). The respective DNA fragments were separated by agarose gel electrophoresis (SeaKem GTG; 1%, wt/vol; FMC BioProducts) in a CHEF DR II system (Bio-Rad) at 15 V/cm with 0.5× Tris-borate-EDTA as running buffer. The pulse times for *Xba*I and *Spe*I digests were increased from 7 to 12 s during the first 11 h and subsequently from 20 to 40 s during the next 13 h; those for *Not*I digests were increased from 2 to 5 s during the first 11 h and from 9 to 12 s for another 11 h. Pulse times for *Xho*I digests were increased from 2 to 7 s for the first 13 h and from 9 to 25 s for the following 11 h, while those for the *Avr*II digests were increased from 7 to 12 s for the first 11 h and from 20 to 65 s for the following 13 h. Polymerized phage λ DNA (Pharmacia LKB) served as a size standard. The gel was stained with ethidium bromide (2 μg/ml; Sigma) for 15 min, destained in distilled water for 15 min, and photographed under UV illumination.

Dice coefficients of similarity were calculated to compare the macrorestriction patterns (7). Clustering was based on the unweighted pair group average method (UPGMA) (37) and was performed with ALMO statistics software (14).

**Calculation of discriminatory indices.** The discriminatory value for each typing method was calculated as an index of discrimination (*D*) according to Hunter and Gaston (16). It served for the comparison of the different methods and for the selection of the most discriminatory system for the molecular differentiation of *S. dublin* isolates. Only one of the four Bovisaloral live vaccine strains was included in the calculation of *D* values.

## RESULTS

**Plasmid analysis.** Plasmid analysis revealed the presence of five different plasmid profiles. This corresponded to a discriminatory index, *D*, of 0.722. A 76-kbp plasmid was detected in all 33 *S. dublin* isolates. It was present as the only plasmid in 13 field isolates, whereas another 9 field isolates also carried a plasmid of 52 kbp. The Bovisaloral live vaccine strains, their prototype strain, and one *S. dublin* field isolate harbored an additional plasmid of 40 kbp. Small plasmids of 5.0 and 4.4 kbp were found in two and three *S. dublin* field isolates, respectively (Table 1). The 76-kbp plasmid was identified as a virulence plasmid by hybridization with an *spvB-spvC* gene probe. This gene probe hybridized to a 3.6-kbp *Hin*dIII fragment in all cases. Comparative restriction endonuclease analysis of the Bovisaloral vaccine strains, the prototype strain, and the one field isolate that exhibited the same plasmid profile did not reveal any differences in the plasmid fragment patterns ob-

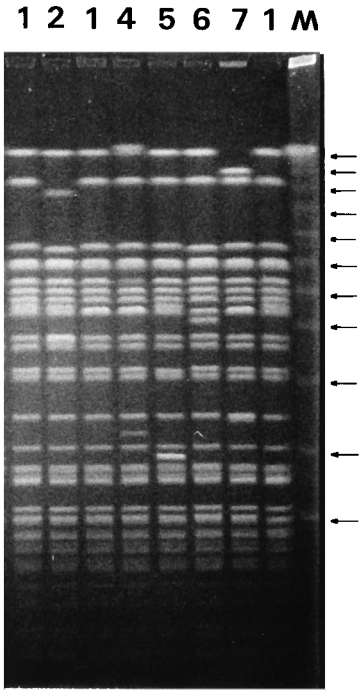


FIG. 1. *Spe*I macrorestriction patterns observed among the *S. dublin* isolates; numbering refers to the patterns listed in Table 1. Arrows on the right-hand side indicate the sizes of the marker DNA fragments (from the bottom): 48.5, 97, 145.5, 194, 242.5, 291, 339.5, 388, 436.5, 485, and 533.5 kbp.

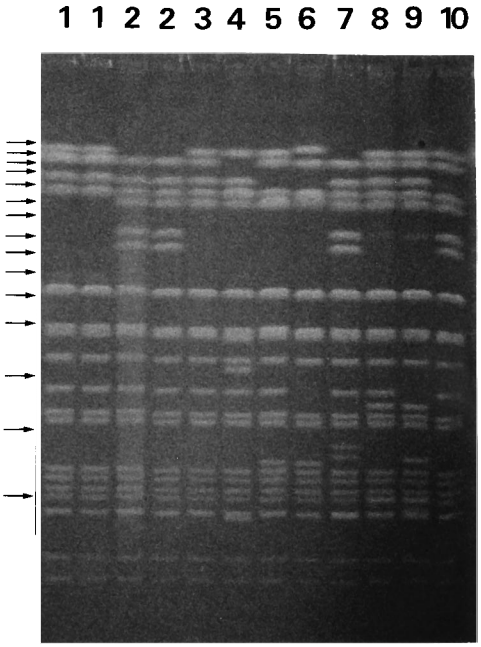


FIG. 2. Comparative analysis of the 10 *Avr*II macrorestriction patterns observed among the *S. dublin* isolates; numbering refers to the patterns listed in Table 1. Positions of the DNA marker fragments are indicated by arrows on the left-hand side (from the bottom): 48.5, 97, 145.5, 194, 242.5, 291, 339.5, 388, 436.5, 485, 533.5, 582, 630.5, 679, and 727.5 kbp.

tained with the enzymes *Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Dra*I, *Eco*RI, *Hae*III, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, and *Xho*I.

**RFLPs of 16S rRNA genes and IS*200* elements.** All 33 *S. dublin* isolates were typeable by the detection of RFLPs of 16S rRNA genes and IS*200* elements. However, with the 16S rRNA-containing fragments in *Pvu*II-digested whole cellular DNA, the same hybridization pattern was seen in all 33 *S. dublin* isolates. This pattern consisted of six hybridizing fragments of 9.7, 9.5, 9.3, 6.5, 4.7, and 3.4 kbp in size (data not shown). All 33 *S. dublin* isolates carried IS*200* copies in their chromosomal DNA. However, no differences in the IS*200* hybridization patterns could be observed among the *S. dublin* isolates. The IS*200*-specific gene probe recognized two *Pst*I fragments of 4.5 and 3.9 kbp in each of the 33 isolates (data not shown). Consequently, the discriminatory indices for both the detection of 16S ribotypes and IS*200* hybridization patterns were calculated to be $D = 0.0$.

**Macrorestriction analysis.** Whole cellular DNA of all *S. dublin* isolates was digested with rare-cutting restriction endonucleases, and the resulting restriction fragments were separated in pulsed-field gel electrophoresis to detect RFLPs. Five different restriction enzymes were tested for their discriminatory capacities among the *S. dublin* isolates.

*Xba*I digestion resulted in seven different fragment patterns with 14 to 17 fragments in the range of 40 to 533 kbp. The majority of the field isolates as well as the Bovisaloral live vaccine strains and their prototype strain exhibited pattern XT1; patterns XT2 to XT6 were represented by one field isolate each, whereas two field isolates exhibited pattern XT7. Thus, the discriminatory index for *Xba*I macrorestriction analysis was $D = 0.416$.

*Spe*I digestion also yielded seven fragment patterns consisting of 18 to 20 fragments (Fig. 1) within the same range as reported for *Xba*I. Pattern ST1 was shared by 19 field isolates, the Bovisaloral strains, and the prototype strain. Patterns ST4 and ST5 were represented by three and two field isolates, respectively, while only single isolates exhibited patterns ST2, -3, -6, and -7. The discriminatory index for *Spe*I macrorestriction analysis was slightly higher than that for *Xba*I at $D = 0.508$.

*Not*I digestion produced a larger number of smaller fragments. Four different patterns were observed. Although 24 to 25 fragments in the range of 40 to 291 kbp were detected, differences became obvious only in fragments larger than 97 kbp. Patterns NT2 and NT3 were represented by one field isolate each. Pattern NT1 encompassed the remaining 26 *S. dublin* field isolates and all four Bovisaloral strains. The prototype strain, however, exhibited its own pattern, NT4, which was characterized by the lack of a fragment of approximately 160 kbp. The discriminatory index for *Not*I macrorestriction analysis was low at $D = 0.193$.

*Xho*I digestion also resulted in four different fragment patterns. These consisted of 19 or 20 fragments in the range of 50 to 340 kbp. An abundance of similar-sized fragments was found in the range of 97 to 160 kbp as well as in the low-molecular-mass area of less than 50 kbp. The Bovisaloral strain and its prototype strain exhibited a unique fragment pattern, XHT1. The *S. dublin* field isolates exhibited three *Xho*I patterns with 14, 9, or 5 isolates representing the respective patterns (Table 1). This distribution resulted in a discriminatory index of $D = 0.683$ for *Xho*I macrorestriction analysis.

*Avr*II digestion produced 10 different patterns of 16 to 20 fragments in the range of 48 to 630 kbp (Fig. 2). As previously seen with *Xho*I, a unique restriction fragment pattern of the Bovisaloral strain and its prototype strain could be observed after cleavage with *Avr*II. As expected from the large number of fragment patterns, the discriminatory index for *Avr*II macrorestriction analysis was high at $D = 0.871$. The genomic relationships of the 33 *S. dublin* isolates as determined by *Avr*II

macrorestriction analyses are presented in a dendrogram in Fig. 3. Although *Avr*II macrorestriction analyses represented the most discriminatory method, the dendrogram confirmed the close relationship among these *S. dublin* isolates; cluster analysis of the *Avr*II macrorestriction patterns showed that all isolates were similar at an 86.7% level (Fig. 3).

Comparative analysis of plasmid profiles and macrorestriction analysis subdivided the 33 *S. dublin* isolates used in this study into 21 genomic groups (Table 1). This corresponded to a discriminatory index of $D = 0.968$. The observation that only one to five *S. dublin* field isolates could be assigned to each genomic group confirmed the usefulness of these molecular techniques for the epidemiological typing of *S. dublin*. Moreover, the different samples of the Bovisaloral vaccine strain exhibited identical results with each method applied. This not only indicated the genetic uniformity of these vaccine strains but also might confirm the effectiveness of these molecular techniques for monitoring the genetic stability of the vaccine strain.

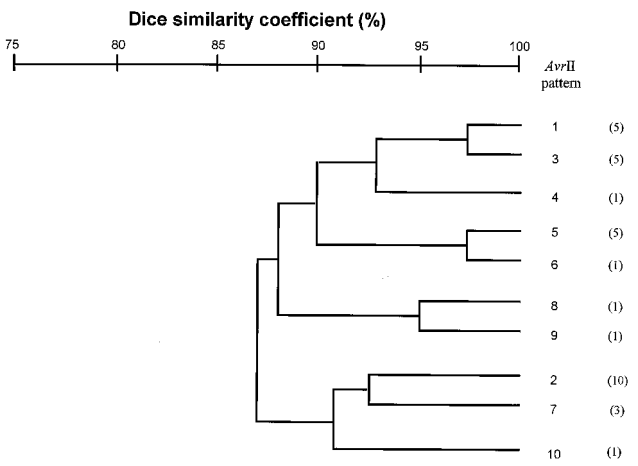


FIG. 3. Dendrogram obtained from cluster analysis of *Avr*II macrorestriction patterns of the 33 *S. dublin* isolates. The numbers of *S. dublin* isolates exhibiting the respective *Avr*II pattern, as given in Table 1, are indicated in parentheses.

## DISCUSSION

To reduce *Salmonella* infections in livestock, live vaccine strains have been introduced into veterinary use (6, 13, 15, 35, 36). While vaccination of rearing poultry units against *Salmonella* infections has been prescribed by law in 1994 in Germany, vaccination of cattle is still voluntary. For this, the auxotrophic attenuated *S. dublin* live vaccine strain Bovisaloral has been successfully applied to cattle in the former German Democratic Republic and other Eastern European countries for years (22); since 1994, it has been used in reunified Germany. Although other mutagenized *S. dublin* strains have been successfully used as live vaccine strains in other countries (13, 15, 35, 36), the *S. dublin* strain Bovisaloral represents the only *S. dublin* live vaccine strain admitted for use in cattle in Germany so far. The Bovisaloral strain was obtained from the prototype strain by growth in the presence of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (22). This chemical mutagenesis resulted in two auxotrophic markers (*ade* and *thi*) which reduce its survival not only in the environment but also in cells of the host defense system (3, 22). Up to now, identification of the Bovisaloral live vaccine strain has been based on its inability to grow in an adenine- and thiamine-deficient diagnostic medium. In contrast to other *S. dublin* live vaccine strains which carried mutations in the aromatic gene *aroA* (13, 15, 35) or in the purine utilization genes *purA* and *purE* (36) that also led to auxotrophy and attenuation, little is known about the nature of the mutations in the Bovisaloral strain. Since neither the area(s) nor the extent(s) of the mutations has been identified (3, 22), it may be possible that mutations also affected restriction endonuclease cleavage sites in the plasmid or genomic DNA. Such newly generated or destroyed recognition sequences for restriction endonucleases might account for RFLPs which could be used for the identification of this vaccine strain and for its differentiation from field isolates of the same serovar.

Previous studies identified the detection of RFLPs of 16S rRNA genes, IS*200* elements, and plasmid-encoded *spvB-spvC* genes to be a suitable method for the differentiation of *Salmonella* isolates of the same serovar (5, 8, 21, 24, 27–30, 34, 38–40, 44). Moreover, differences in the macrorestriction patterns as monitored by pulsed-field gel electrophoresis allowed a high level of discrimination among isolates of *S. typhimurium* (33) and *S. enteritidis* (21, 30, 31, 43). While each of these molecular techniques differentiated the *S. typhimurium* live vaccine strain Zoosaloral H from *S. typhimurium* field strains (33, 34), most of the methods failed in the differentiation of the Bovisaloral live vaccine strain from *S. dublin* field isolates.

Plasmid analysis showed that all *S. dublin* isolates included in this study carried a virulence plasmid of approximately 76 kbp; the *spvB-spvC* region was located on a 3.6-kbp *Hin*dIII fragment in the vaccine strains, the prototype strain, and the field strains. A common *spv* gene region had been reported to be present in the virulence plasmids of *S. typhimurium*, *S. enteritidis*, *S. cholerae-suis*, and *S. dublin* (4, 11, 19, 25). No additional markers, such as IS*200* elements, were found on this virulence plasmid. This observation was in accordance with previous data on *S. dublin* virulence plasmids (4, 19). Since the vaccine strains also harbored a cryptic plasmid of 40 kbp, it was impossible to compare the virulence plasmid present in the vaccine strain directly with those present in the majority of the field isolates by restriction endonuclease digestion. However, the combination of a 76-kbp virulence plasmid and a cryptic 40-kbp plasmid was also detected in one field isolate. Comparative restriction analyses of these two plasmids with those of the Bovisaloral strains and the prototype strain showed no differences when 13 different endonucleases were used. Although plasmid analyses yielded a relatively high discriminatory value for the epidemiological typing of *S. dublin*, one must be aware that plasmids represent mobile extrachromosomal elements that do not code for essential properties and therefore may be lost from or acquired by *S. dublin* cells. Thus, additional typing systems based on the detection of more stable chromosomally encoded markers were included in this study.

The detection of RFLPs of 16S rRNA genes and IS*200* elements proved to be helpful for the epidemiological typing of certain *Salmonella* serovars, such as *typhimurium* (24, 34, 38) and *berta* (27, 40). For other *Salmonella* serovars, such as *enteritidis* (21, 24, 39) and *dublin* (5, 29), this technique did not represent the method of choice. Although all *S. dublin* isolates used in this study were typeable with this technique, all isolates exhibited the same 16S ribotypes as well as IS*200* types. The ribotype observed in this study corresponded exactly to the respective hybridization pattern previously described to occur in most *S. dublin* isolates from humans and cattle in England and Wales (5). Moreover, the two *Pst*I fragments to which the IS*200* gene probe hybridized were the same size as those found in the reference strain *S. dublin* NSC65 as well as in field isolates of *S. dublin* from humans, cattle, and horses in England, Wales, France, Denmark, Tanzania, and Sweden (5, 29).

Macrorestriction analysis has been described as the method of choice for the discrimination of certain *Salmonella* serovars (28). So far, this method has been used successfully for the differentiation of isolates belonging to serovars *typhimurium* (33), *typhi* (42), *enteritidis* (21, 30, 31, 43), and *dublin* (29). Previous studies showed that the use of one restriction enzyme alone provided only limited information for strain characterization. The discriminatory value of macrorestriction analysis could be distinctly increased by the comparative use of several suitable enzymes (21, 33). Up to now, only *Not*I macrorestriction patterns have been described for *S. dublin* (29). A small number of fragment patterns could be identified with most of the strains exhibiting a single *Not*I pattern (29). This observation corresponded closely to our data. Among all enzymes tested for macrorestriction analysis, *Not*I yielded the lowest discriminatory index. Nevertheless, *Not*I was the only enzyme that allowed a differentiation between the Bovisaloral live vaccine strains and their prototype strain. Macrorestriction analyses with *Xba*I and *Spe*I resulted in higher discriminatory indices; again, one predominant pattern was observed with these two enzymes. No matter whether *Not*I, *Xba*I, or *Spe*I was used for cleavage of the whole cellular DNA, Bovisaloral live vaccine strains exhibited the same fragment pattern as the majority of the *S. dublin* field isolates. However, a differentiation between Bovisaloral vaccine strains and their prototype strain on the one hand and *S. dublin* field isolates on the other hand was achieved by using the enzymes *Xho*I and *Avr*II. Although *Xho*I analysis resulted in only four different fragment patterns, we observed a more heterogeneous distribution of the field isolates among the three remaining patterns. However, the majority of fragments obtained from *Xho*I digestion were of less than 160 kbp. Although differences between the patterns could be observed, the presence of a number of similar-sized fragments in the same range rendered the evaluation of the patterns more difficult. Among all restriction enzymes used for macrorestriction analysis, *Avr*II yielded the clearest resolution of fragments as well as the highest discriminatory index, $D = 0.871$. This meant that two *S. dublin* isolates randomly selected from the test population could be assigned to different *Avr*II patterns with a probability of 87%. Furthermore, the high discriminatory power of *Xho*I and *Avr*II macrorestriction analyses could be increased by the comparative use of these two restriction enzymes. Thus, isolates which represented the same *Xho*I type, e.g., XHT2, could be assigned to different *Avr*II types, such as AT2, AT5, AT7, or AT10. In return, AT5 isolates exhibited either pattern XHT2 or pattern XHT4 (Table 1).

The most effective discrimination, however, was achieved by the comparative use of plasmid analysis and macrorestriction analysis. Thus, isolates representing the genomic groups III and IV, as well as XVIII and XIX, were indistinguishable by macrorestriction analysis but differed in their plasmid profiles (Table 1).

The data presented in this study confirmed that macrorestriction analysis is a most suitable tool for the epidemiological typing of *S. dublin* isolates. Its discriminatory power, however, strongly depends on the choice of the restriction enzyme used for cleavage of the whole cellular DNA. Enzymes such as *Xho*I and *Avr*II not only exhibited the highest discriminatory indices but also produced fragment patterns that differentiated the Bovisaloral live vaccine strain and its prototype strain from a representative pool of *S. dublin* field isolates. Moreover, macrorestriction analysis as performed with a number of different restriction enzymes proved to be a suitable method for monitoring the genetic relationships among *S. dublin* field isolates as well as the genetic stability of single strains, such as the live vaccine strain Bovisaloral.

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## REFERENCES

1. **Anonymous.** 1993. PHLS-SVS: update on *Salmonella* infection, vol. 16. Public Health Laboratory Service-State Veterinary Service, Colindale, United Kingdom.
2. **Anonymous.** 1991. Animal salmonellosis 1990. Ministry of Agriculture, Fisheries and Food, Welsh Office Agriculture Department, Department of Agriculture and Fisheries for Scotland.
3. **Beyer, W., and L. Geue.** 1992. Characterization of the virulence gene regions in the plasmids of the three live *Salmonella* vaccines. Zentralbl. Bakteriol. **277:**10–21.
4. **Browning, L. M., C. Wray, and D. J. Platt.** 1995. Diversity and molecular variation among plasmids in *Salmonella enterica* serotype Dublin based on restriction enzyme fragmentation pattern analysis. Epidemiol. Infect. **114:** 237–248.
5. **Chowdry, N., E. J. Threlfall, B. Rowe, and J. Stanley.** 1993. Genotype analysis of faecal and blood isolates of *Salmonella dublin* from humans in England and Wales. Epidemiol. Infect. **110:**217–225.
6. **Cooper, G. L.** 1994. Salmonellosis—infections in man and the chicken: pathogenesis and the development of live vaccines—a review. Vet. Bull. **64:**123–143.
7. **Dice, L. R.** 1945. Measures of the amount of ecologic association between species. Ecology **26:**297–302.
8. **Esteban, E., K. Snipes, D. Hird, R. Kasten, and H. Kinde.** 1993. Use of ribotyping for characterization of *Salmonella* serotypes. J. Clin. Microbiol. **31:**233–237.
9. **Fang, F. F., and J. Fierer.** 1991. Human infections with *Salmonella dublin*. Medicine (Baltimore) **70:**198–207.
10. **Gibert, I., J. Barbe, and J. Casadesus.** 1990. Distribution of insertion sequence IS*200* in *Salmonella* and *Shigella*. J. Gen. Microbiol. **136:**2555–2560.
11. **Gulig, P. A.** 1990. Virulence plasmids of *Salmonella typhimurium* and other salmonellae. Microb. Pathog. **8:**3–11.
12. **Hirsh, D. C.** 1990. *Salmonella*, p. 110–115. *In* E. L. Biberstein and Y. C. Zee (ed.), Review of veterinary microbiology. Blackwell Scientific Publications, Inc., Boston.
13. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
14. **Holm, K.** 1993. ALMO Statistik System. Institut für Soziologie, University of Linz, Linz, Austria.
15. **Hormaeche, C. E., H. S. Joysey, L. Desilva, M. Izhar, and B. A. D. Stocker.** 1991. Immunity conferred by aro-*Salmonella* live vaccines. Microb. Pathog. **10:**149–158.
16. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's Index of Diversity. J. Clin. Microbiol. **26:**2465–2466.
17. **Iglesias, A., P. Ceglowski, and T. A. Trautner.** 1983. Plasmid transformation in *Bacillus subtilis*. Effects of the insertion of *Bacillus subtilis* rRNA genes into plasmids. Mol. Gen. Genet. **192:**149–155.
18. **Kado, C. I., and S. T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
19. **Lax, A. J., G. D. Pullinger, G. D. Baird, and C. M. Williamson.** 1990. The virulence plasmid of *Salmonella dublin*: detailed restriction map and analysis of transposon mutagenesis. J. Gen. Microbiol. **136:**1117–1123.
20. **Lester, A., N. H. R. Eriksen, H. Nielsen, P. B. Nielsen, A. Friis-Moller, B. Bruun, J. Scheibel, K. Gaarslev, and H. J. Kolmos.** 1991. Non-typhoid *Salmonella* bacteremia in Greater Copenhagen 1984–1988. Eur. J. Clin. Microbiol. Infect. Dis. **10:**486–490.
21. **Liebisch, B., and S. Schwarz.** Molecular typing of *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates. J. Med. Microbiol., in press.
22. **Linde, K.** 1980. Herstellung von stabilen *Salmonella*-Impfstämmen durch Kopplung von zwei unabhängig voneinander nichtvermehrungsbegrenzenden attenuierenden Markern. Arch. Exp. Veterinaermed. **34:**19–32.
23. **Macrina, F. L., D. J. Kopecko, K. R. Jones, D. J. Ayers, and S. M. McCowan.** 1978. A multiple plasmid-containing *Escherichia coli* strain: convenient source of size reference plasmid molecules. Plasmid **1:**417–420.
24. **Millemann, Y., M.-C. Lesage, E. Chaslus-Dancla, and J.-P. Lafont.** 1995.

Value of plasmid profiling, ribotyping, and detection of IS*200* for tracing avian isolates of *Salmonella typhimurium* and *S. enteritidis*. J. Clin. Microbiol. **33:**173–179.
25. **Montenegro, M. A., G. Morelli, and R. Helmuth.** 1991. Heteroduplex analysis of *Salmonella* virulence plasmids and their prevalence in isolates of defined sources. Microb. Pathog. **11:**391–397.
26. **Olsen, J. E.** 1990. An improved method for rapid isolation of plasmid DNA from wild-type Gram-negative bacteria for plasmid restriction profile analysis. Lett. Appl. Microbiol. **10:**209–212.
27. **Olsen, J. E., D. J. Brown, D. L. Baggesen, and M. Bisgaard.** 1992. Biochemical and molecular characterization of *Salmonella enterica* serotype Berta and comparison of methods for typing. Epidemiol. Infect. **108:**243–260.
28. **Olsen, J. E., D. J. Brown, M. N. Skov, and J. P. Christensen.** 1993. Bacterial typing methods suitable for epidemiological analysis. Applications in investigations of salmonellosis among livestock. Vet. Q. **15:**125–135.
29. **Olsen, J. E., and M. Skov.** Genomic lineage of *Salmonella enterica* serovar Dublin. Vet. Microbiol. **40:**271–282.
30. **Olsen, J. E, M. N. Skov, E. J. Threlfall, and D. J. Brown.** Clonal lines of *Salmonella enterica* serotype Enteritidis documented by IS*200*-, ribo-, pulsed-field gel electrophoresis and RFLP typing. J. Med. Microbiol. **40:**15–22.
31. **Powell, N. G., E. J. Threlfall, H. Chart, and B. Rowe.** 1994. Subdivision of *Salmonella enteritidis* PT-4 by pulsed-field gel electrophoresis: potential for epidemiologic surveillance. FEMS Microbiol. Lett. **119:**193–198.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **Schwarz, S., and B. Liebisch.** 1994. Pulsed-field gel electrophoretic identification of *Salmonella enterica* serovar Typhimurium live vaccine strain Zoosaloral H. Lett. Appl. Microbiol. **19:**469–472.
34. **Schwarz, S., and B. Liebisch.** 1994. Use of ribotyping, IS*200* typing and plasmid analysis for the identification of *Salmonella enterica* subsp. *enterica* serovar Typhimurium vaccine strain Zoosaloral H and its differentiation from wild type strains of the same serovar. Zentralbl. Bakteriol. **281:**442–450.
35. **Segall, T., and A. A. Lindberg.** 1991. *Salmonella dublin* experimental infection in calves: protection after oral immunization with an auxotrophic *aro*A live vaccine. J. Vet. Med. Ser. B **38:**142–160.
36. **Sigwart, D. F., B. A. D. Stocker, and D. Clement.** 1989. Effect of a *purA* mutation on efficacy of *Salmonella typhimurium* live vaccine vectors. Infect. Immun. **57:**1858–1861.
37. **Sneath, P. H. A., and R. R. Sokal.** 1973. Numerical taxonomy, p. 214–234. W. H. Freeman Co., San Francisco.
38. **Stanley, J., N. Baquar, and E. J. Threlfall.** 1993. Genotypes and phylogenetic relationships of *Salmonella typhimurium* are defined by molecular fingerprinting of IS*200* and 16S *rrn* loci. J. Gen. Microbiol. **139:**1133–1140.
39. **Stanley, J., A. P. Burnens, E. J. Threlfall, N. Chowdry, and M. Goldsworthy.** 1992. Genetic relationships among strains of *Salmonella enteritidis* in a national epidemic in Switzerland. Epidemiol. Infect. **108:**213–220.
40. **Stanley, J., N. Chowdry, N. Powell, and E. J. Threlfall.** 1992. Chromosomal genotypes (evolutionary lines) of *Salmonella berta*. FEMS Microbiol. Lett. **95:**247–252.
41. **Taylor, D. N., J. M. Bied, J. S. Munro, and R. A. Feldman.** 1982. *Salmonella dublin* infections in the United States, 1979–1980. J. Infect. Dis. **146:**322–327.
42. **Thong, K.-W., Y.-M. Cheong, S. Puthucheary, C.-L. Koh, and T. Pang.** 1994. Epidemiologic analysis of sporadic *Salmonella typhi* isolates and those from outbreaks by pulsed-field gel electrophoresis. J. Clin. Microbiol. **32:**1135–1141.
43. **Thong, K.-W., Y. Ngeow, M. Altwegg, P. Navaratnam, and T. Pang.** 1995. Molecular analysis of *Salmonella enteritidis* by pulsed-field gel electrophoresis and ribotyping. J. Clin. Microbiol. **33:**1070–1074.
44. **Threlfall, E. J., and J. A. Frost.** 1990. The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. J. Appl. Bacteriol. **68:**5–16.
45. **Threlfall, E. J., M. L. M. Hall, and B. Rowe.** 1992. *Salmonella* bacteremia in England and Wales, 1981–1990. J. Clin. Pathol. **35:**34–36.