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Identification and Characterization of *Salmonella* Isolates by Automated Ribotyping†

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ABSTRACT

A study was conducted with the RiboPrinter, an automated ribotyping system, to evaluate its ability to identify and characterize isolates of *Salmonella* from broiler operations. Isolates of *Salmonella* obtained from a local broiler company were serotyped by a reference laboratory and ribotyped using the RiboPrinter. The RiboPrinter generated ribotype patterns by probing EcoRI digests of *Salmonella* DNA with an *E. coli* DNA probe to the ribosomal RNA operon. The RiboPrinter identified isolates by band matching of their ribotype patterns to ribotype patterns in its database. In addition, the RiboPrinter characterized isolates by sorting them into ribotypes on the basis of the similarity of their ribotype patterns. Of 117 isolates, the RiboPrinter identified 34 (29%) at the serotype level, 11 (9%) at the strain level, 46 (39%) at the genus level, and 26 (22%) were not identified. Thus, only 38% of the isolates were identified at or below the serotype level, indicating that the RiboPrinter was limited in its ability to identify *Salmonella* isolates by band matching. In contrast, the RiboPrinter was very effective at characterizing *Salmonella* isolates. Out of 108 isolates, the RiboPrinter detected 31 ribotypes, compared to serotyping which only detected 22 types of *Salmonella*. Thus, automated ribotyping was more discriminatory than serotyping. However, when results of both typing methods were combined, 40 types of *Salmonella* were detected, indicating that the best discrimination was obtained when automated ribotyping and serotyping were used together.

The application of hazard analysis of critical control points (HACCP) programs to the farm to table continuum has significant potential for improving the microbiological safety of poultry products. A key component of a microbial-based HACCP plan is the identification and monitoring of critical control points in the process. Timely monitoring of critical control points as part of a HACCP plan requires rapid and sensitive methods for pathogen identification and characterization.

The classical method for identifying *Salmonella* isolates has been serotyping with antibodies that detect somatic and flagellar antigens. Over 2,000 serotypes of *Salmonella* have been identified by this method. However, serotyping is labor-intensive, time-consuming, and requires use of specialized antibodies that are not readily available *(8)*. In addition, expression of cell-surface antigens is influenced by environmental and cultural conditions, which may limit the reproducibility of serotyping results between laboratories. Consequently, it is best to have isolates serotyped by a reference laboratory. However, turnaround time of samples by a reference laboratory is a likely limitation of the use of serotyping as a method for the routine identification and monitoring of *Salmonella* isolates as part of a HACCP program. In addition, identification of critical control points may require a typing method that is more discriminatory than serotyping *(6)*.

Recently, a number of molecular typing methods (i.e., ribotyping, IS-200 typing, pulsed-field gel electrophoresis (PFGE), and polymerase chain reaction (PCR)-based typing methods) have been developed that are more discriminatory than serotyping *(8, 15)*. In addition, molecular typing methods based on genomic DNA are stable and use reagents that are readily available. However, like serotyping, molecular typing methods are labor-intensive and time-consuming. In addition, some (i.e., PFGE and PCR-based methods) generate hazardous wastes, such as ethidium bromide, that are costly to dispose of *(15)*.

One way to make typing methods less labor-intensive and time-consuming is to automate them with computer-driven robotics. Recently, such an automated system for ribotyping, the RiboPrinter, became commercially available. The RiboPrinter is capable of rapidly identifying and characterizing isolates of *Salmonella, Listeria monocytogenes, Staphylococcus aureus,* and pathogenic *Escherichia coli.* The RiboPrinter uses a standardized ribotyping procedure to generate ribotype patterns for each of these foodborne pathogens. Genomic DNA of the pathogen is extracted, digested with the restriction endonuclease *Eco*RI, separated, immobilized, and probed with an *E. coli* DNA probe for the ribosomal RNA operon. Identification of isolates is accomplished by band matching of their ribotype

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pattern to a ribotype pattern of strains in the RiboPrinter database, whereas characterization is accomplished by sorting isolates into ribotypes on the basis of the similarity of their ribotype patterns.

The RiboPrinter database used in this study contained *Eco*RI ribotype patterns for 94 strains of *Salmonella.* In addition, it contained *Eco*RI ribotype patterns for 13 similarity groups of *Salmonella.* Similarity groups are clusters of strains that share a common *Eco*RI ribotype pattern. In essence, when the RiboPrinter identifies an isolate of *Salmonella* as belonging to a similarity group, it is identifying the *Salmonella* isolate at the level of the genus. From an epidemiological point of view this identification is not desirable, as it represents a situation where automated ribotyping is less informative than serotyping. In addition, when one considers that there are over 2,000 serotypes and many more strains of *Salmonella* and currently only 94 *Eco*RI ribotype patterns for *Salmonella* in the RiboPrinter database, it seems feasible that the RiboPrinter may not always identify *Salmonella* by band matching. The frequency with which the RiboPrinter identifies *Salmonella* at the genus level or fails to identify isolates of *Salmonella* is unknown. Consequently, the present study was undertaken to evaluate the RiboPrinter for its ability to identify isolates of *Salmonella* at or below the serotype level by band matching of ribotype patterns. In addition, the ability of the RiboPrinter to characterize isolates of *Salmonella* was evaluated by comparing automated ribotyping and serotyping for their ability to discriminate between isolates of *Salmonella.*

MATERIALS AND METHODS

Source of *Salmonella* isolates. One hundred and twenty-two isolates of *Salmonella* were obtained from the microbiology laboratory of a local broiler-chicken processor. Isolates were obtained at a rate of about six per week between 28 March and 26 August 1996. Salmonellae were isolated from samples collected at hatcheries, feed mills, growout houses, and processing plants. The sample types included: hatchery swabs ($n = 6$), eggshells ($n = 4$), litter drag swabs ($n = 18$), feed ($n = 22$), feeding equipment swabs ($n = 3$), flies ($n = 2$), beetles ($n = 3$), well water ($n = 1$), sludge ($n = 3$), ceca ($n = 43$), whole bird feather rinses ($n = 11$), and carcass rinses ($n = 8$).

Isolation of *Salmonella.* All collections and isolations were performed by the broiler company. Their procedure for isolation involved preenrichment of samples for *Salmonella* cells by incubation in buffered peptone water (18 to 24 h at 37°C) followed by selective enrichment in tetrathionate (18 to 24 h at 42°C) and selenite-cystine (18 to 24 h at 37°C) broths. The enrichment cultures were streaked onto xylose-lysine tergitol-4 (XLT-4) agar and a well-isolated colony on XLT-4 was picked and streaked onto brain heart infusion (BHI) agar for ribotyping.

Serotyping and ribotyping of *Salmonella.* On the morning of ribotyping, isolates which had been grown at 37°C to confluence overnight on BHI agar were transported from the broiler company's microbiology laboratory in 20 min to our laboratory where the ribotyping was done. Stock cultures of each isolate were prepared by growing isolates for 24 h at 30°C in BHI broth and then adding 0.1 ml of the stationary-phase culture to 0.9 ml of BHI broth plus 15% glycerol. These stock cultures were stored at −70°C. One vial of each isolate was taken to the *Salmonella* Reference Center at the University of Pennsylvania (Kennet Square, PA) for serotyping.

Ribotyping was accomplished with the RiboPrinter (Qualicon, Wilmington, DE). The details of the ribotyping procedure of the RiboPrinter have been reported *(4, 17).* All of the reagents and materials for using the RiboPrinter come preprepared and prepackaged. Thus, the only manual steps involved in ribotyping isolates with the RiboPrinter are the initial steps of sample preparation. Here, a small circle of cells is harvested from the BHI agar plate and the cells are suspended in sample buffer by vortexing. An aliquot of the cell suspension is then transferred to one well of an eight-well sample carrier. Once the sample carrier has been completely loaded, it is inserted into the preprogrammed heat-treatment station. After heat treatment to inactivate the cells, lysing agents A and B are added to each sample well. The sample carrier is then loaded into the microbial characterization unit and from this point on the procedure is completely automated.

The microbial characterization unit consists of four modules: a DNA preparation module, a separation and transfer module, a membrane-processing module, and a detection module. All processes in the microbial characterization unit are carried out automatically by the liquid dispensing and dilution (LDD) pipette and the membrane-processing (MP) pipette. The prepackaged ribotyping reagents and materials are loaded into the RiboPrinter before the run is begun. They include the DNA preparation carrier, the gel cassette and nylon membrane, and the membrane-processing brick. Because there are two bays in the separation and transfer module and two bays in the membrane-processing module, runs can be stagger-started to permit the ribotyping of up to 32 isolates in a 24-h period.

Operations in the DNA preparation module use the DNA preparation carrier and are performed by the LDD pipette. The LDD pipette adds lysing agents and *Eco*RI from the DNA preparation carrier to each sample. After extraction and digestion of the DNA, the LDD pipette adds electrophoresis sample buffer and then transfers each sample to a predetermined lane in the gel cassette. The fragmented DNA is then separated by size through an agarose gel in the separation and transfer module and the fragments are transferred onto a nylon membrane. The nylon membrane migrates across the bottom of the gel during electrophoresis. Once transfer of the DNA fragments is complete, the MP pipette captures the nylon membrane and transfers it to the membrane-processing module. In the membrane-processing module the DNA fragments on the nylon membrane are hybridized to a DNA probe for the ribosomal RNA operon of *E. coli* and to chemiluminescent reagents. After hybridization, the MP pipette transfers the nylon membrane to the detection module.

The detection module contains a low-light camera which visualizes the chemiluminescing bands on the membrane. The image captured by the camera is digitized and sent to the computer workstation for analysis. The computer workstation converts the digitized image into a ribotype pattern. The ribotype pattern can be displayed in bar code, waveform, or both (Figure 1). Isolates are identified by band matching of their ribotype pattern to an existing ribotype pattern in the RiboPrinter database. If a close match (i.e., ≥ 0.85 similarity) is found, a positive identification is made. The RiboPrinter also characterizes isolates by sorting them into ribotypes on the basis of the similarity of their ribotype patterns.

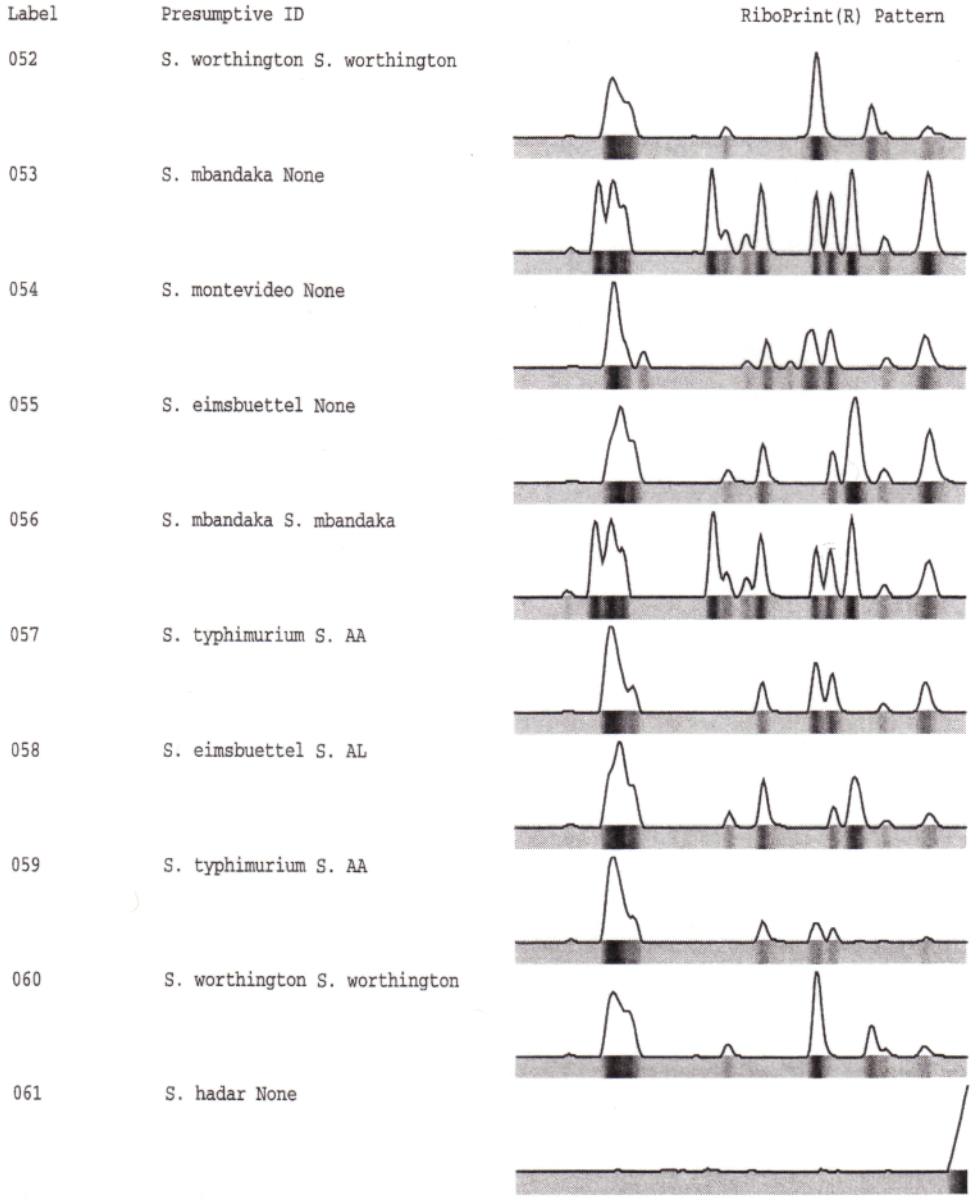

FIGURE 1. *Examples of ribotype patterns of* Salmonella *isolates from broiler operations. The label is the isolate number and the presumptive ID is the serotype followed by the identification result provided by the RiboPrinter. Isolates identified by the RiboPrinter as belonging to a similarity group are designated S. followed by double capital letters.*

RESULTS

For evaluating the identification capabilities of the RiboPrinter, complete serotyping and ribotyping results were obtained for 117 isolates. Twenty-two serotypes were identified by the reference laboratory (Table 1). The RiboPrinter assigned a serotype name to 54 isolates. However, nine of these serotype names disagreed with the serotyping results. Eleven isolates of *S. senftenberg* were correctly identified at the strain level. Assuming that the serotyping results were completely accurate, these results indicated that the RiboPrinter correctly identified 45 of 117 (38%) isolates of *Salmonella* at or below the serotype level.

Some serotypes of *Salmonella* produce *Eco*RI ribotype patterns that are similar. The RiboPrinter identifies these serotypes as belonging to one of thirteen similarity groups for *Salmonella* in its database. In essence, the RiboPrinter identifies these isolates at the genus level. In the present study, 37 of 117 isolates of *Salmonella* were assigned to similarity groups (Table 1). In addition, the nine isolates that were assigned incorrect serotype names by the RiboPrinter were considered to have been identified at the genus level. Thus, the RiboPrinter identified 46 of 117 or 39% of the *Salmonella* isolates at the genus level. Most notable, all 15 isolates of *S. typhimurium,* a common isolate from broilers in the U.S., were assigned to a similarity group.

Occasionally the RiboPrinter cannot match a ribotype pattern of an isolate to a ribotype pattern in its database. In this situation, the RiboPrinter makes no identification. In the current study, 26 of 117 or 22% of the *Salmonella* isolates were assigned an identification of "none" by the RiboPrinter (Table 1). Actually, only 16 of these isolates had ribotype patterns that did not match a ribotype pattern in the

TABLE 1. *Identification of* Salmonella *isolates by serotyping and automated ribotyping*

Serotyping serotype (*n*)	Automated ribotyping		
	Serotype	Similarity group	None
S. agona (2)	0	2	0
S. alachua (4)	1	0	3
S. amsterdam (2)	0	2	0
S. bareilly (1)	0	1	0
S. binza (4)	0	1	3
S. brandenburg (2)	1	1	0
S. cerro (2)	0	1	1
S. drypool (1)	0	1	0
S. eimsbuettel (2)	0	1	1
S. enteritidis (8)	0	5	2
S. hadar (15)	12	0	3
S. harrdt (2)	2	0	0
S. heidelberg (1)	0	1	0
S. indiana (3)	3	0	0
S. mbandaka (11)	5	0	6
S. montevideo (2)	1	0	1
S. schwarzengrund (1)	0	1	0
S. senftenberg (14)	11	0	3
S. simsbury (3)	2	0	1
S. thompson (2)	2	0	0
S. typhimurium (15)	0	15	0
S. worthington (21)	14	5	2
Total (117)	54	37	26

RiboPrinter database. The other 10 isolates (Table 2) repeatedly did not yield ribotype patterns (see isolate 61, *S. hadar* in Figure 1 for an example) even after subculturing.

Subsequent manual ribotyping of isolates 10, 13, 15, 16, and 18 (Table 2) revealed that their DNA was resistant to *Eco*RI but could be digested by *Pvu*II. Interestingly, *Pvu*II produced two ribotype patterns: one that was common to isolates 10 and 18, which were both *S. binza,* and one that was common to isolates 13, 15, and 16, which were all *S. senftenberg*. In general, the non-cutting isolates were obtained in clusters. However, they were obtained from five sources and represented five serotypes. The most prevalent sources were ceca ($n = 4$) and hatchery equipment swabs ($n = 3$); *S. binza* ($n = 3$) and *S. senftenberg* ($n = 3$) were the most prevalent serotypes. Considering that the DNA of 11 other isolates of *S. senftenberg* and the DNA of 39 other isolates from ceca were successfully processed by the RiboPrinter, it does not appear that the occurrence of "non-cutters" is related to a specific source or serotype of *Salmonella.*

TABLE 2. *Isolates whose DNA was not processed by the RiboPrinter*

Isolate no.	Date of ribotyping	Source	Serotype
10	4 April 1996	Broiler ceca	*S. binza*
13	4 April 1996	Broiler ceca	*S. senftenberg*
15	11 April 1996	Broiler ceca	*S. senftenberg*
16	11 April 1996	Broiler ceca	*S. senftenberg*
18	11 April 1996	Broiler feed	*S. binza*
45	23 May 1996	Broiler house feedline swab	*S. binza*
61	6 June 1996	Hatchery equipment swab	*S. hadar*
65	13 June 1996	Hatchery equipment swab	*S. alachua*
66	13 June 1996	Hatchery eggshell	*S. alachua*
67	13 June 1996	Hatchery equipment swab	*S. simsbury*

In addition to identification, we evaluated the RiboPrinter for its ability to characterize isolates of *Salmonella.* We did this by comparing automated ribotyping and serotyping for their ability to discriminate between isolates of *Salmonella.* This comparison involved 108 isolates. The RiboPrinter characterized the isolates by sorting them into ribotypes on the basis of the similarity of their ribotype patterns. A total of 31 ribotypes of *Salmonella* were detected by the RiboPrinter (Table 3), compared to serotyping which only detected 22 serotypes. Thus, automated ribotyping was more discriminatory than serotyping.

Some ribotypes of *Salmonella* contained more than one serotype (Table 3). Likewise, some serotypes were present in more than one ribotype, indicating the presence of different strains of these serotypes. The serotypes with the most ribotypes or strains were *S. worthington* ($n = 6$), *S. hadar* ($n = 4$), *S. mbandaka* ($n = 3$), and *S. senftenberg*

TABLE 3. *Characterization of* Salmonella *isolates by automated ribotyping*

Ribotype (*n*)	Serotype (*n*)
198-S-1 (7)	*S. worthington* (7)
198-S-5 (9)	*S. mbandaka* (8), *S. hadar* (1)
200-S-7 (1)	*S. worthington* (1)
201-S-1 (7)	*S. worthington* (7)
201-S-7 (3)	*S. worthington* (3)
202-S-1 (16)	*S. typhimurium* (13), *S. brandenburg* (1), *S. cerro* (1), *S. heidelberg* (1)
202-S-4 (1)	*S. binza* (1)
203-S-8 (1)	*S. bareilly* (1)
205-S-3 (9)	*S. senftenberg* (9)
205-S-5 (3)	*S. amsterdam* (2), *S. drypool* (1)
205-S-6 (2)	*S. thompson* (2)
205-S-8 (1)	*S. schwarzengrund* (1)
206-S-3 (4)	*S. indiana* (3), *S. brandenburg* (1)
207-S-3 (1)	*S. senftenberg* (1)
207-S-6 (1)	*S. cerro* (1)
208-S-3 (3)	*S. simsbury* (2), *S. senftenberg* (1)
208-S-7 (3)	*S. agona* (2), *S. montevideo* (1)
209-S-8 (2)	*S. eimsbuettel* (2)
211-S-6 (2)	*S. typhimurium* (2)
213-S-5 (1)	*S. alachua* (1)
214-S-4 (1)	*S. worthington* (1)
214-S-8 (13)	*S. hadar* (11), *S. mbandaka* (2)
219-S-5 (1)	*S. worthington* (1)
219-S-6 (1)	*S. hadar* (1)
220-S-2 (4)	*S. enteritidis* (4)
223-S-1 (1)	*S. hadar* (1)
223-S-2 (1)	*S. mbandaka* (1)
223-S-6 (1)	*S. alachua* (1)
224-S-8 (1)	*S. montevideo* (1)
225-S-7 (2)	*S. harrdt* (2)
227-S-1 (4)	*S. enteritidis* (4)

($n = 3$). When the serotype and ribotype information were combined, 40 types of *Salmonella* were identified among the 108 isolates. Thus, the best discrimination was obtained when results of serotyping and automated ribotyping were combined.

Thirteen isolates were ribotyped twice, which provided us with an opportunity to assess the repeatability of the ribotyping results. Repeatability was evaluated using both the identification and characterization capabilities of the RiboPrinter. The rates of repeatability were 85% (11 of 13) for identification, 92% (12 of 13) for characterization, and 77% (10 of 13) for identification and characterization combined. The mean similarity of ribotype patterns between runs was 0.95 (range 0.90 to 0.98). Thus, ribotype patterns were highly reproducible between runs but their interpretation was somewhat less repeatable.

DISCUSSION

As an alternative to serotyping, we evaluated automated ribotyping as a method for identifying isolates of *Salmonella*. The RiboPrinter identifies isolates by band matching of their ribotype patterns to ribotype patterns in its database. It was found that the RiboPrinter was limited in its ability to identify *Salmonella* isolates by band matching. Out of 117 isolates, the RiboPrinter correctly identified only 38% at or below the serotype level. Considering that there are over 2,000 serotypes and many more strains of *Salmonella* and that the RiboPrinter database used only contained ribotype patterns for 94 strains, it was perhaps not surprising that the RiboPrinter had such a low success rate for identification of isolates of *Salmonella*.

Although expansion of the RiboPrinter database to include additional ribotype patterns for other strains of *Salmonella* will improve its identification capabilities, I do not believe that expansion of the RiboPrinter database, no matter how extensive, will result in high rates (>95%) of identification at or below the serotype level. In fact, only 16 of 72 isolates not identified at or below the serotype level by the RiboPrinter in this study contained ribotype patterns not found in the database. Furthermore, of these 16 isolates, there were only six potentially unique (i.e., not shared by other strains in the database) ribotype patterns. The main reason an expanded database may not greatly enhance the RiboPrinter's ability to identify *Salmonella* at or below the serotype level is that the discriminatory power of ribotyping may not be sufficient to establish a database capable of discerning over 2,000 serotypes and many more strains of *Salmonella*.

To a large extent the ability of ribotyping to discriminate between isolates of *Salmonella* is dependent on the restriction enzyme used. A number of studies have demonstrated differences in the number of ribotypes obtained when different restriction enzymes are used to digest DNA from a variety of *Salmonella* serotypes, such as *S. enteritidis (9, 16), S. typhi (1, 11), S. gallinarum (5), S. pullorum (5), S. berta (12), S. brandenburg (3),* and *S. typhimurium (10).* In addition, some restriction enzymes are more effective with one serotype than another *(7, 10, 13).* Thus, it may be better to use one restriction enzyme for one serotype and another restriction enzyme for another serotype of *Salmonella.* The version of the RiboPrinter used in this study was only capable of using one restriction enzyme, *Eco*RI, for ribotyping *Salmonella* isolates. Consequently, part of the identification problems encountered may have arisen from *Eco*RI not being the best restriction enzyme for ribotyping some of the isolates. In fact, we had 10 isolates whose DNA was not processed by the RiboPrinter. Upon subsequent manual ribotyping of five of these isolates it became apparent that their DNA was resistant to *Eco*RI but was sensitive to digestion with *Pvu*II. The reason that the DNA from these isolates was resistant to *Eco*RI is not known, but it did not appear to be related to a particular sample type or serotype. It also is not without precedent. Altwegg et al. *(1)* reported that the DNA from an isolate of *S. typhi* was resistant, for an unknown reason, to *Eco*RI but could be digested by *Pst*I and *Sma*I.

Recently, Qualicon upgraded the RiboPrinter software to permit users to choose which restriction enzyme to use for ribotyping. This upgrade will make it possible to ribotype isolates, such as those encountered in this study, that are resistant to *Eco*RI. It may also allow further discrimination of isolates identified at the genus level, i.e., those assigned to similarity groups. These isolates could be ribotyped with a second restriction enzyme to determine their similarity to other isolates in the similarity group. In this way it may be possible to expand the RiboPrinter database to contain identifications based on the combination of two or more ribotype patterns, each generated with a different restriction enzyme. What rates of identification could be achieved by band matching ribotypes when using a combination of restriction enzymes remains to be determined. However, within a serotype, using a combination of restriction enzymes to ribotype isolates results in only modest increases in ribotyping sensitivity. For example, Christensen et al. *(5)* found that when *S. gallinarum* was ribotyped with a combination of three restriction enzymes the number of ribotypes increased from 13 to 15 and the number of *S. pullorum* ribotypes increased from 12 to 17. Likewise, Martinelli and Altwegg *(9)* reported an increase from 8 to 10 in the number of ribotypes when two restriction enzymes were used in combination for typing of *S. enteritidis.* Thus, it does not appear likely that having the capability of using multiple restriction enzymes in the RiboPrinter will result in high rates (>95%) of *Salmonella* identification.

Although the RiboPrinter was limited in its ability to identify *Salmonella* isolates at or below the serotype level in this study, it was effective at characterizing isolates into ribotypes. In addition, repeatability of ribotype patterns between runs was very good, with a mean similarity of 0.95. The RiboPrinter sorted our *Salmonella* isolates into 31 ribotypes, compared to serotyping, which only detected 22 types of *Salmonella.* Thus, automated ribotyping was more sensitive than serotyping at discriminating between isolates of *Salmonella.* However, when serotyping and ribotyping were used in combination, we were able to discern 40 types of *Salmonella* among our isolates. Thus, the greatest sensitiv-

ity for typing *Salmonella* was achieved when serotyping and automated ribotyping were applied together. Other investigators *(2, 10, 13, 14)* have reached similar conclusions, i.e., that it is best to use more than one typing method when conducting epidemiological investigations of *Salmonella* outbreaks.

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