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Application of Random Amplified Polymorphic DNA Analysis To Differentiate Strains of *Salmonella enteritidis*

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A random amplified polymorphic DNA (RAPD) fingerprinting method has been developed to differentiate *Salmonella enteritidis* isolates. A total of 65 arbitrary primers were screened with *S. enteritidis* isolates of different phage types. This allowed selection of a panel of primers capable of detecting DNA polymorphisms among *S. enteritidis* isolates. This panel was used to examine a panel of 29 isolates of *S. enteritidis* which had been previously characterized by other subtyping methods, including phage typing (PT) ($n = 7$), ribotyping (RT) ($n = 13$), and pulsed-field gel electrophoresis (PFGE). Applied collectively, these three methods resolved the collection into 20 different subtypes. However, by the RAPD fingerprinting method alone, 14 RAPD subtypes were revealed. Eight isolates of *S. enteritidis* phage type 8 that failed to be discriminated by other typing methods (PT, RT, and PFGE) were resolved into three different subtypes by RAPD analysis. In contrast, isolates that were derived from the same sources were not differentiated by any of the subtyping methods employed, including PT, RT, PFGE, and RAPD analysis. This RAPD approach to *S. enteritidis* subtyping provided more discriminatory power than did any of several other subtyping methods applied individually. Once the challenging step of primer identification was accomplished, determinations of the appropriate concentrations of arbitrary primer, DNA template, and Mg^{2+} ion were also necessary for optimal discriminatory power. The bacterial DNA used in this RAPD protocol was obtained by boiling the bacterial sample. This simple procedure yielded DNA that produced fingerprint patterns as consistent as those obtained from phenol-chloroform-extracted DNA. Clearly, when appropriately constituted primer sets are identified and employed, RAPD analysis provides a simple, rapid, and powerful subtyping method for *S. enteritidis*.

The techniques that have been used to classify bacteria fall into two major categories: phenotypic methods and molecular methods. While long-established morphological, physiological, and biochemical markers in conjunction with traditional serology are still widely employed for typing, the last decade has seen the introduction and increased use of monoclonal antibodies, restriction enzymes, DNA probes, and PCR. Each of these tools has provided increased power and resolution. However, the development of approaches with the ability to reliably discriminate differences in isolates of the same species has posed a major challenge.

This is particularly true in the case of *Salmonella enteritidis*, now the most frequently isolated of the food-borne salmonellae in the United States (2, 14, 23, 25). Phage typing (9, 10, 24) is the most widely employed system for subtyping isolates of *S. enteritidis*, and in the United States phage type 8 is the most common. *S. enteritidis* is a highly clonal organism, particularly within phage type 8, and the ability to distinguish between strains within a phage type is important for epidemiologic investigations but has proven to be a formidable task. For example, using a combination of six subtyping methods, Stubbs et al. (30) were able to show differences among only 7 of 30 unrelated phage type 8 isolates. In the United Kingdom phage type 4 is the predominant phage type associated with *S. enteritidis* isolates (16, 22, 27). In a study conducted in the United Kingdom, Powell et al. (20) found the majority of phage type 4 isolates to have a single pulsed-field gel electrophoresis (PFGE) profile.

Although phage typing is the subtyping approach with which the *Salmonella* community has the widest experience, its requirement for specialized phage collections and the bacterial strains for their propagation has made it a technique that is routinely practiced only in a few reference laboratories. More important than issues of convenience, phage typing, in common with all taxonomic approaches that depend upon phenotypic characteristics, is inherently lacking in discriminatory power. In many cases the genetic differences between isolates will not encode differences in the particular phenotypic marker (phage type, antigen, enzyme or isozyme, antimicrobial susceptibility, or metabolic profile) that such typing systems target. Consequently, such phenotypic markers have been of limited utility for subtyping isolates of *S. enteritidis*. On the other hand, because subtypes must, by definition, differ at the DNA level, subtyping approaches that address this level offer the potential for the greatest discriminatory power (13).

A variety of DNA-based typing methods have been applied to identify *Salmonella* species, including plasmid profile (6–8, 24), biotyping (3, 16, 30), ribotyping (19), IS*200* profile (19), PFGE (19), and multilocus enzyme analysis (28). Each of these approaches has provided useful insights into evolutionary and epidemiological relationships of several *Salmonella* serovars. However, while a variety of molecular subtyping approaches are available, from a practical standpoint, when several isolates are to be compared over the course of a few days, the most general procedure for the comparison of genomes is random amplified polymorphic DNA (RAPD) analysis (5). Based on the work of Welsh and McClelland (33) and of Williams et al. (34), RAPD analysis produces reproducible, and often distinctive, sets of DNA fragments by subjecting genomic DNA to PCR primed by short (10- to 25-base) oligonucleotide primers

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TABLE 1. Results of RAPD fingerprinting of *S. enteritidis* isolates using six arbitrary primers

Isolate		Phage type	Ribotype		PFGE type			RAPD profile (23, 17, 4, 1254, 6, 15)[a]
Designation	State or country		*Acc*I	*Sph*I	*Xba*I	*Avr*II	*Apa*I	
3759	California	8	1	2	2	2	1	C, D, A, B, C, B
1166	Colorado	8	3	2	1			C, C, A, B, C, B
3490	New Jersey	8	1	6	4	3	1	C, C, A, B, A, B
3773	Indiana	8	1	7	1	3	1	C, D, A, B, C, B
3984	Colorado	8	1	5	3	3	1	C, E, B, B, C, B
5098	Virginia	8	1	1	1	1	1	C, C, A, B, C, B
0799	Pennsylvania	8	1	11	1			C, C, A, B, A, B
0800	Pennsylvania	8	1	11	1			C, C, A, B, A, B
4902	Washington	8	1	3	1	3	1	C, B, A, B, C, B
5637	Maryland	8	1	2	1	3	1	C, C, A, B, A, B
8380	Alabama	8	1	11	1			C, C, A, B, C, B
8382	Delaware	8	1	11	1			C, C, A, B, A, B
8383	Delaware	8	1	11	1			C, C, A, B, A, B
8386	Delaware	8	1	11	1			C, C, A, B, A, B
8395	Indiana	8	1	11	1			C, D, A, B, C, B
8396	Indiana	8	1	11	1			C, D, A, B, C, B
1162	Colorado	8	3	2	1			C, C, A, B, C, B
3786	Utah	13a	1	3	1	3	1	C, C, A, B, A, B
6187	South Carolina	13a	1	9	6	5	1	B, C, A, B, C, B
8391	Michigan	13a	1	8	9			C, C, D, B, C, B
8387	Michigan	13a	1	12	1			E, C, A, B, C, B
8392	Michigan	13	1	12	1			D, D, C, B, C, B
3888	Rhode Island	14b	1	4	1	1	1	A, C, C, B, A, A
4823	New Jersey	14b	1	4	1	3	1	A, C, C, B, A, B
4839	Colorado	4	1	4	5	4	1	C, A, A, A, C, B
0237	Germany	4	7	10	7			C, A, A, A, B, B
1286	New York	2	1	1	1			C, C, A, B, A, B
3443	Pennsylvania	34	1	1	1	1	1	C, C, A, C, C, B
5733	Ohio	34	1	8	1	1	1	C, B, A, C, C, B

[a] 23, 23L; 17, OPB-17; 4, OPA-4; 1254, P1254; 6, OPB-6, 15, OPB-15.

of arbitrary sequences. This approach has been applied to detect genomic diversity among plants (21, 35), animals (26), parasites (18), and microbial organisms (1, 11, 12, 15, 36). The attractiveness of this method is that no knowledge of the sequence of the target organism is required and a very large number of arbitrary primers can be tested to identify those that might be suited to a particular application.

The application of RAPD fingerprinting to isolate subtyping requires two essential elements. First, oligonucleotide primers that are capable of recognizing DNA polymorphisms among isolates must be identified. Unfortunately, there is no way to predict which oligonucleotide sequences will be useful. Consequently, one must identify useful primers by testing large collections of oligonucleotides. Second, a well-characterized panel of *S. enteritidis* isolates is necessary in order to determine which primers are indeed useful for discriminating DNA polymorphisms. To satisfy this requirement, we employed a 29-member collection of *S. enteritidis* isolates that had been phage typed, ribotyped, and examined by PFGE. This allowed us to identify a panel of six oligonucleotides that can be used as primers in a RAPD fingerprinting method that allows one to distinguish genetic differences among isolates of *S. enteritidis.*

MATERIALS AND METHODS

Bacterial strains. Twenty-nine individually isolated *S. enteritidis* specimens of known origin, phage type, ribotype, and PFGE type were obtained from the collection of one of us (T. J. Barrett) at the Centers for Disease Control and Prevention (Table 1). The characterization of the tabulated isolates for phage type was performed as originally described by Ward et al. (32). Ribotyping was carried out according to methods previously described by Usera et al. (31), using cDNAs of 16S and 23S rRNAs from *Escherichia coli* as probes. PFGE was performed using methods previously described for *E. coli* O157:H7 (4). Briefly, agarose-embedded DNA was digested with one of three restriction enzymes (*Xba*I, *Avr*II, or *Apa*I), and the resulting DNA fragments were separated with a CHEF DR-II system (Bio-Rad Laboratories, Hercules, Calif.) with a linearly ramped pulse time of 5 to 50 s.

E. coli, *Staphylococcus aureus*, *Hafnia alvei*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Salmonella pullorum*, *Salmonella dublin*, *Salmonella typhimurium*, *Salmonella montevideo*, *Salmonella heidelberg*, and *Salmonella arizonae* were obtained from the American Type Culture Collection.

Bacterial growth. *Salmonella* cultures were streaked on brilliant green agar plates to isolate single colonies. Other non-*Salmonella* bacterial cultures were streaked on nutrient agar plates.

DNA preparation. A single colony of each isolate from an agar plate was picked and resuspended in 150 μl of distilled H_2O. The suspension was boiled for 5 min, and the supernatant was collected after spinning for 2 min in a microcentrifuge. The DNA concentration of boiled extracts was determined with a DNA Dipstick kit (Invitrogen, San Diego, Calif.). The usual yield of bacterial DNA from a single colony was approximately 4 μg. Phenol-chloroform-extracted DNAs were prepared as described by Silhavy et al. (29).

Primers. Primers used in this project were either purchased from Operon Inc. (Alameda, Calif.) or synthesized by DNA Integrated Inc. (Coralville, Iowa) or Amitof Biotechnology, Inc. (Boston, Mass.).

RAPD fingerprinting. PCR was conducted under a layer of mineral oil in a 20-μl volume containing 40 ng of total *S. enteritidis* DNA, 3.5 mM $MgCl_2$, 50 pmol of primer, 0.5 U of *Taq* DNA polymerase (GIBCO), and 200 mM (each) dATP, dCTP, dGTP, and dTTP (Promega) in 20 mM Tris · HCl (pH 8.4)–50 mM KCl. A Perkin-Elmer TC480 thermal cycler or MJ Minicycler (MJ Research, Cambridge, Mass.) was used for amplification. The cycling program was 4 cycles of 94°C for 4 min, 35°C for 4 min, and 72°C for 4 min; 30 cycles of 94°C for 30 s, 35°C for 1 min, and 72°C for 2 min; and 1 cycle of 72°C for 5 min. After PCR, 10 μl of each amplified DNA product was loaded on 2% SeaKem agarose gels (FMC, Rockland, Maine) containing 0.5 μg of ethidium bromide per ml in the gel and electrophoresed in 1× Tris-acetate running buffer. To analyze the derivation of RAPD results on long-format polyacrylamide gels, 0.5 μl of [α-^{35}S]dATP (600 Ci/mmol) (Amersham, Arlington Heights, Ill.) was added to the PCR mixture (in place of 0.5 μl of distilled H_2O). After the PCR, 2 μl of each radioactively labeled PCR product was mixed with 2 μl of sample buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) and loaded on urea-polyacrylamide gels. The gels were electrophoresed in 0.6× Tris-borate buffer at 40 W for 2 h. After drying, the gels were exposed to X-ray

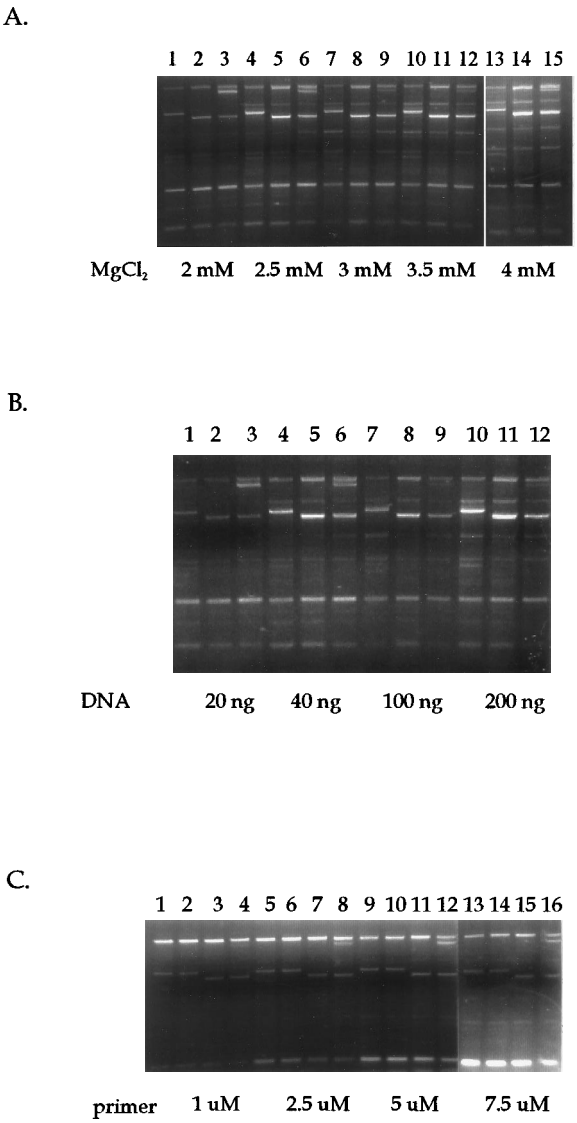

FIG. 1. Optimization of RAPD PCR conditions. All reactions were conducted at an annealing temperature of 35°C. (A) Identical concentrations of primer (2.5 μM) and template DNA (40 ng) were used throughout. The $MgCl_2$ concentration varied as follows: lanes 1 to 3, 2 μM; lanes 4 to 6, 2.5 μM; lanes 7 to 9, 3 μM; lanes 10 to 12, 3.5 μM; lanes 13 to 15, 4 μM. Lanes 1, 4, 7, 10, and 13, isolate 3443; lanes 2, 5, 8, 11, and 14, isolate 3888; lanes 3, 6, 9, 12, and 15, isolate 0237. (B) Identical concentrations of $MgCl_2$ (3.5 μM) and primer (2.5 μM) were used throughout. The template DNA amount varied as follows: lanes 1 to 3, 20 ng; lanes 4 to 6, 40 ng; lanes 7 to 9, 100 ng, lanes 10 to 12, 200 ng. Lanes 1, 4, 7, and 10, isolate 3443; lanes 2, 5, 8, and 11, isolate 3888; lanes 3, 6, 9, and 12, isolate 0237. (C) Identical concentrations of $MgCl_2$ (3.5 μM) and template DNA (40 ng) were used throughout. The primer concentration varied as follows: lanes 1 to 4, 1 μM; lanes 5 to 8, 2.5 μM; lanes 9 to 12, 5 μM; lanes 13 to 16, 7.5 μM. Lanes 1, 5, 9, and 13, isolate 3443; lanes 2, 6, 10, and 14, isolate 3759; lanes 3, 7, 11, and 15, isolate 3786; lanes 4, 8, 12, and 16, isolate 0237. RAPD PCR profiles were not affected by the concentrations of $MgCl_2$, primer, and template DNA examined in this study.

film for 48 h before development. The agarose gels were photographed under UV light. Photos and films were scanned with a Hewlett-Packard ScanJet IICX scanner driven by the Deskscan 4.0 program and were printed with the aid of a Tektronix Phaser IISDX color printer.

RESULTS

Optimization of concentrations of arbitrary primer, DNA template, and Mg^{2+} ion for ultimate discriminatory power. To optimize the discriminating power of this RAPD fingerprinting technique, the optimal concentrations of arbitrary oligonucleotide, DNA template, and $MgCl_2$ used in PCR were first determined (7, 8, 17) (Fig. 1). While a range of 2 to 4 mM $MgCl_2$ produced the same banding pattern, higher concentrations of $MgCl_2$ yielded some artificial background, and lower concentrations of $MgCl_2$ resulted in poor amplification. A concentration of 3.5 mM $MgCl_2$ was chosen for the RAPD reaction. Likewise, low levels of DNA template (<40 ng) were found to result in relatively poor amplification. The RAPD patterns, however, did not vary when amounts of 40 to 200 ng of total DNA were used. Consequently, the slight difference in DNA concentration obtained from preparation to preparation should not affect the RAPD patterns. Primer at a final concentration of 2.5 μM was used for most of the reactions. RAPD patterns remained the same when the concentration of primer was raised to 7.5 μM, while lower concentrations of some primers resulted in relatively poor amplification. Because of the utilization of primers with arbitrary sequences and low T_ds [$T_d = 4(G+C) + 2(A+T)$], a low annealing temperature of 35°C was used for all the PCR cycles (17).

Reproducibility of RAPD fingerprinting. The reproducibility of the RAPD fingerprinting technique was confirmed by comparing the reproducibility of the fingerprint patterns obtained from duplicate runs of RAPD analysis of several different bacterial species (Fig. 2a) and closely related *Salmonella* serovars (data not shown). A single primer, DM152, was used to discriminate six bacterial species, including *E. coli*, *S. aureus*, *H. alvei*, *K. pneumoniae*, *C. freundii*, and *S. enteritidis*. The experiments were carried out twice using two different brands of thermal cyclers, and the results were resolved on the same long-format polyacrylamide gel to evaluate the reproducibility. Figure 2a shows the high reproducibility of this RAPD analysis. The technique was then employed to examine a panel of 29 individually isolated *S. enteritidis* strains which had been previously characterized by a variety of typing methods, including phage typing ($n = 7$), ribotyping ($n = 13$), and PFGE. The reproducibility of DNA polymorphisms was confirmed in more than five experiments (data not shown). To simplify this RAPD fingerprinting technique, a rapid boiling procedure was employed for obtaining preparations of bacterial DNA. The RAPD results derived by using boiled DNA preparations present the same reproducible results as those from phenol-extracted DNA preparations (Fig. 2b).

Discriminatory power of RAPD applied to *S. enteritidis* isolates. To select suitable candidate primers for subtyping *S. enteritidis* isolates, 65 arbitrary primers were first tested with four isolates of *S. enteritidis* (isolates 3443, 3759, 3786, and 3888 in Table 1), each of which is of a different phage type. Primers that produced at least two polymorphic DNA patterns among these four isolates were tested with the full panel of 29 *S. enteritidis* isolates. This approach allowed identification, from the 65 primers tested, of 6 primers (Table 2) which (each)

TABLE 2. Primers used in the RAPD fingerprinting of *S. enteritidis* strains

Primer	Sequence	G+C content (%)
23L	5′-CCGAAGCTGC	70
OPB-17	5′-AGGGAACGAG	60
OPA-4	5′-AATCGGGCTG	60
OPB-6	5′-TGCTCTGCCC	70
P1254	5′-CCGCAGCCAA	70
OPB-15	5′-GGAGGGTGTT	60

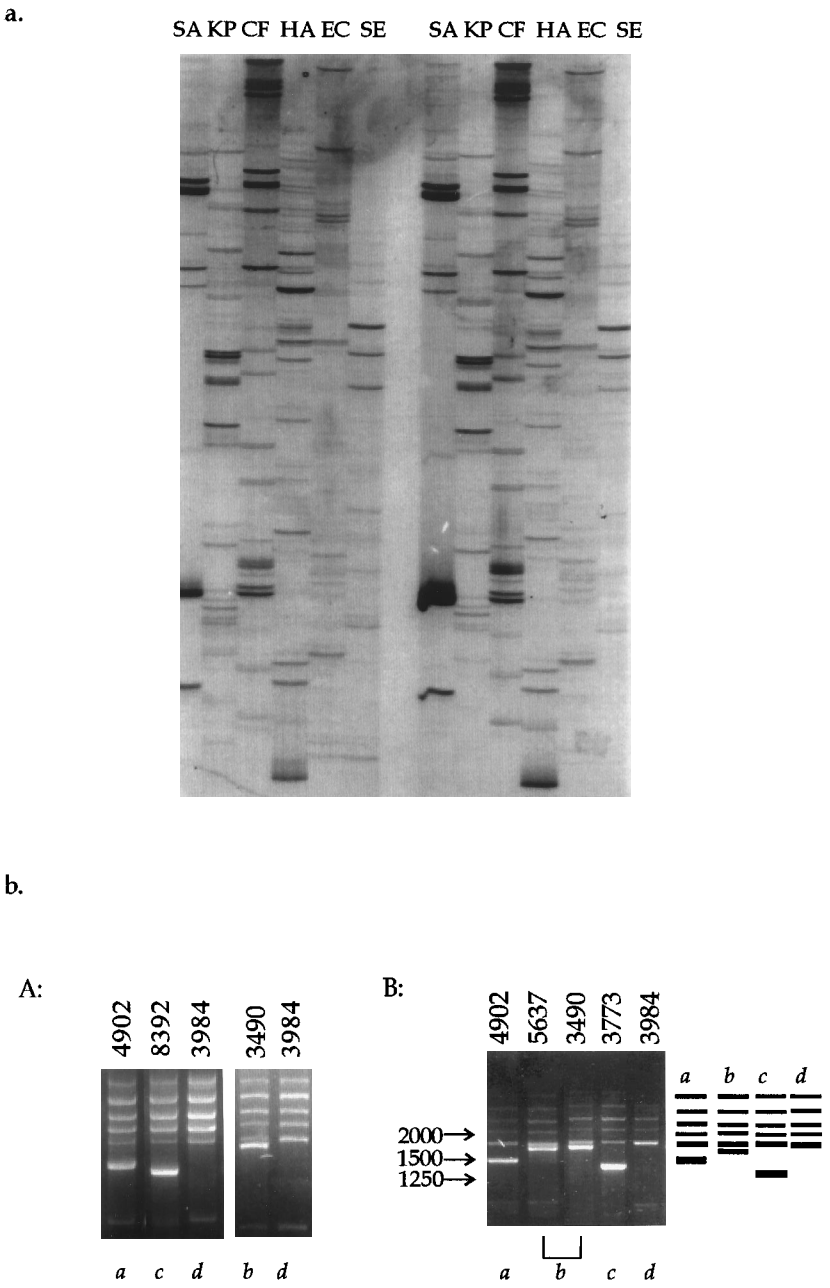

FIG. 2. (a) Reproducibility of RAPD fingerprinting profiles in RAPD PCR. RAPD PCRs were conducted twice separately using two different brands of thermal cycler (TC480 thermal cycler from Perkin-Elmer [left] and Minicycler from MJ Research [right]). EC, *E. coli*; KP, *K. pneumoniae*; SA, *S. aureus*; HA, *H. alvei*; CF, *C. freundii*; SE, *S. enteritidis*. Primer DM152 (5′-CATGTCAAATTTCACTGCTTCATCC) was used in both experiments. The ^{32}P-labeled RAPD PCR products were resolved on long-format polyacrylamide gels for maximum sensitivity and band resolution. (b) Comparison of RAPD PCR profiles obtained with DNAs prepared by the boiling method as described in the text (A) and by the traditional phenol-chloroform extraction method (33) (B). The primer used was 5′-GGGGGGGGGG-3′ (primer OPB-17). Molecular weights are indicated on the left in thousands.

produce three or more polymorphic patterns of DNA fingerprinting when used to examine a well-characterized panel of 29 isolates of *S. enteritidis*. A longer PCR cycle (35 cycles) was found to be required for those reactions in which the applied primer resulted in poor amplification (i.e., OPB-17 and OPB-15 reactions).

Different primers produced different numbers of patterns when employed with the 29-member defined panel of *S. enteritidis* isolates. Specifically, primers 23L and OPB-17 revealed five polymorphic patterns; OPA-04 yielded four polymorphic patterns; and OPB-15, OPB-06, and P1254 each produced three patterns (Fig. 3). Primer 1281, which exhibited great discriminatory power for other bacterial species (1, 12, 15), was not useful for the differentiation of strains of *S. enteritidis* (data not shown). Several primers were found to produce nonreproducible RAPD results and were therefore rejected. In sum, the panel of 29 *S. enteritidis* isolates, representing seven phage types and 13 ribotypes, was differentiated into 14 subtypes by combining the RAPD patterns by using these six primers. Significantly, eight *S. enteritidis* isolates of phage type 8 isolated in

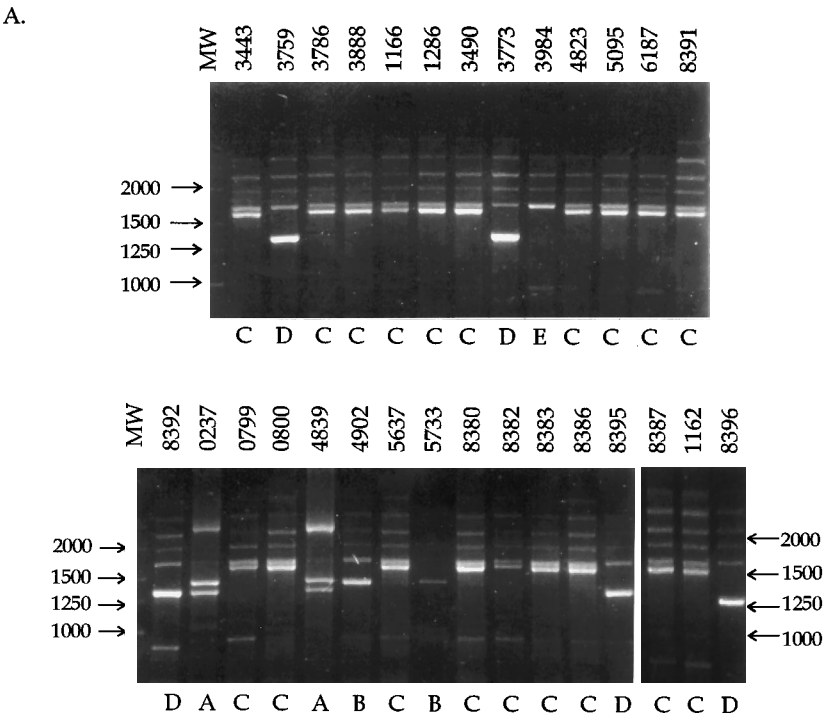

FIG. 3. Representative results of RAPD fingerprinting generated by RAPD PCR using six arbitrary primers. (A) RAPD fingerprinting profiles of a collection of 29 isolates of *S. enteritidis* obtained by RAPD PCR using primer OPB-17. (B) Representative RAPD fingerprinting profiles obtained with the indicated primers. Molecular weights (MW) are shown in thousands.

three different states (0799 and 0800 from Pennsylvania; 8380, 8382, 8383, and 8386 from Delaware; and 8395 and 8396 from Indiana) that were not differentiated by ribotyping or PFGE were differentiated into three subtypes by RAPD analysis. Clearly, not all isolates were differentiated from each other; isolates in one cluster, 8382, 8383, and 8386, were derived from the same source and were shown to be identical by all methods applied. Isolates in a second cluster, 8395 and 8396, which were also derived from the same source gave identical RAPD patterns.

DISCUSSION

The application of PCR-based techniques has had a revolutionary impact on the diagnosis of infectious disease. Because these techniques have the ability to detect or allow analysis of minute amounts of microbial DNA or RNA sequences, they have emerged as a highly sensitive and specific method for identifying pathogens. The PCR-based RAPD fingerprinting technique of utilizing arbitrary oligonucleotides to prime DNA synthesis at low annealing temperatures to divulge genomic diversity is a particularly powerful typing method. Unlike the traditional PCR analysis, which requires specific knowledge of DNA sequences and the application of target-specific sequences, RAPD does not require any specific knowledge of the DNA sequences of the target organism. This makes it a tool of great power and general applicability. In this paper we report the application of the RAPD technique to the differentiation of strains of *S. enteritidis*.

The most challenging aspect of a protocol for bacterial subtyping by RAPD analysis is the selection of suitable primers. In this study, we have used a panel of *S. enteritidis* isolates that have been well characterized by both phage type and a variety of molecular subtyping approaches to identify primers that are appropriate for *S. enteritidis* subtyping. By this approach, a library of 65 arbitrary primers was examined for suitability. Of these 65 primers, 6 proved to be useful reagents for the RAPD analysis of strains of *S. enteritidis*. Additionally, primer OPB-17 appears to be useful for subdivision of phage type 8 strains (the most predominant phage type in the United States). This RAPD analysis exhibits greater discriminatory power than the other subtyping methods applied by revealing 14 RAPD types among the collection of 29 *S. enteritidis* isolates and by successfully discriminating isolates that had failed to be subtyped previously (Table 1). The reliability of the RAPD analysis was confirmed by the fact that isolates that were derived from the same source and not differentiated by other typing methods were also not differentiated by this method.

As seen in Table 1, seven phage types are found among the collection of 29 *S. enteritidis* isolates. However, molecular subtyping methods reveal that the collection is significantly more genetically diverse than is revealed by phage typing. Thus, one finds 13 distinct ribotypes and 10 different patterns following restriction with an infrequently cutting restriction enzyme and subsequent PFGE analysis. When the RAPD fingerprinting method is used, 14 RAPD subtypes were found with the six primers found in this study to be effective as discriminators of *S. enteritidis* genetic diversity. As seen in Table 1, upon combining the results from phage typing, ribotyping, and PFGE, this collection of 29 isolates of *S. enteritidis* can be differentiated into 20 subtypes. The joining of the RAPD results to those obtained by the application of these other techniques further extended the discriminatory power, and 23 subtypes of *S. enteritidis* were resolved among the collection of 29 isolates of *S. enteritidis* when all four methods were applied collectively. Not at all unexpectedly, these results show that application of several molecular methods in combination with phage typing gives the highest discriminatory power. However, when one considers a combination of discriminatory power and ease of appli-

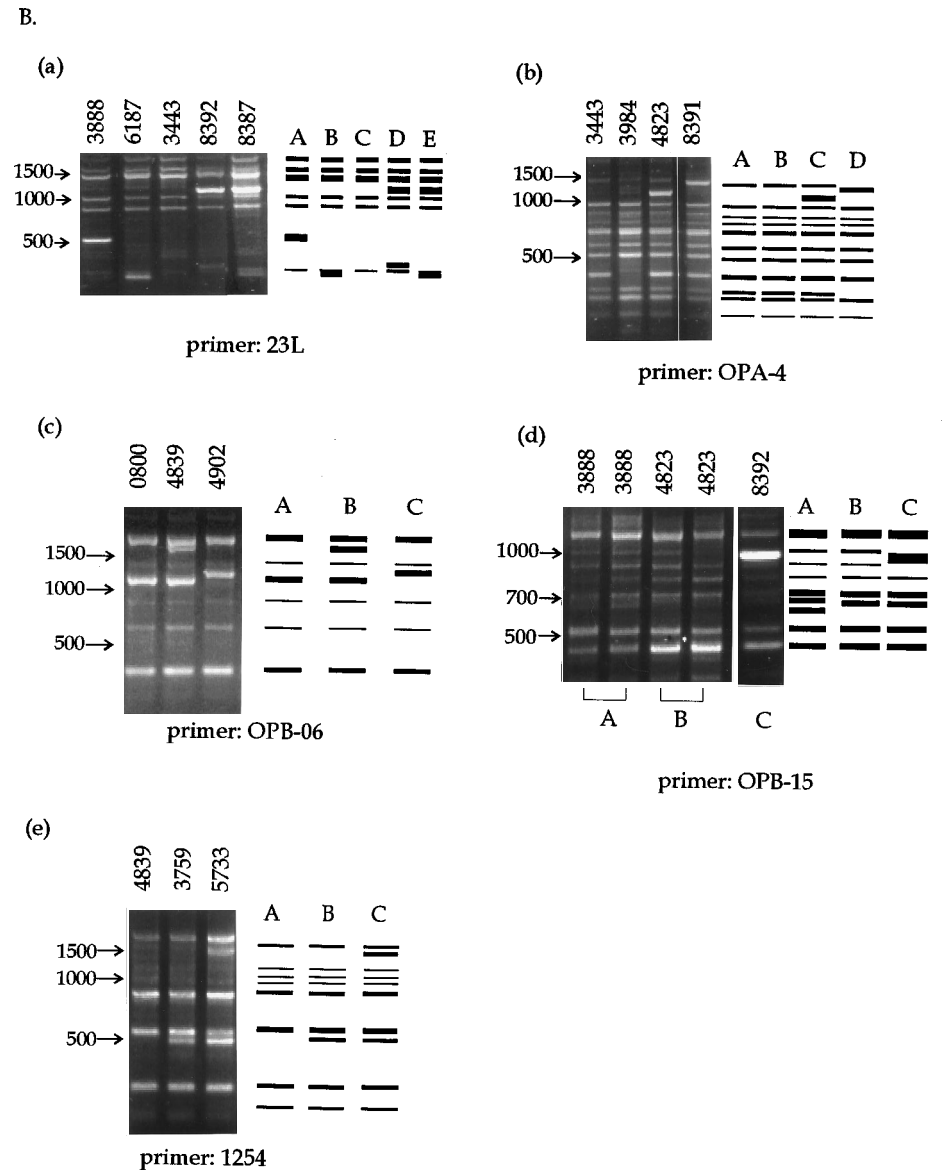

FIG. 3—*Continued*.

cation, RAPD emerges as a particularly attractive molecular technique. This is especially true if one employs the simplified RAPD technique which uses boiled bacterial DNA templates. This modification greatly reduces the amount of time and labor required for the performance of this molecular technique.

The results of this study establish that when an appropriately chosen set of primers is employed, RAPD analysis provides an alternative rapid, reproducible, and powerful genomic typing method for *S. enteritidis*.

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