JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1995, p. 987–989 Vol. 33, No. 4
0095-1137/95/$04.00+0

NOTES

Analysis of *Salmonella enteritidis* Isolates by Arbitrarily Primed PCR

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Received 26 August 1994/Returned for modification 3 November 1994/Accepted 5 January 1995

An arbitrarily primed PCR (AP-PCR) was developed to analyze the genomic DNAs of *Salmonella enteritidis* isolates from human outbreaks and from avian sources. The AP-PCR generated seven distinct randomly amplified DNA patterns among the *S. enteritidis* isolates studied. Differences in the DNA patterns among isolates of *S. enteritidis* phage types 13a and 8 as well as among *S. enteritidis* phage type 14b were observed. The AP-PCR analysis can be used to determine the differences among isolates within the same phage types and may be useful for tracing back the source of *S. enteritidis* outbreaks in humans more precisely.

Egg-borne *Salmonella enteritidis* infection has emerged as a major public health problem in the United States as well as several European countries during the past decade (2, 10, 12, 16). Although strong epidemiologic evidence links human *S. enteritidis* infections to egg consumption, some observers remain skeptical that infections of layer flocks are responsible for the increasing numbers of infections in humans (4, 18). In recent years researchers have identified differences in strains of *S. enteritidis* through the use of various phenotypic and genotypic techniques (5–7, 11, 14, 15, 20, 21). All of these procedures are very helpful in differentiating *S. enteritidis* isolates in epidemiologic studies, but they are laborious and time-consuming. Polymorphism assays (1) that are based on PCR require target DNA sequence information for the design of amplification primers, but this information is not available for *S. enteritidis.*

The most recently introduced assay for typing bacterial DNA is random amplified polymorphic DNA analysis (22), which has also been called arbitrarily primed PCR (AP-PCR). This method is faster, relatively simpler, and more economical than other genomic typing methods. AP-PCR is based on the amplification of genomic DNA with a single primer selected from an arbitrary nucleotide sequence. The multiple products resulting from AP-PCR are then separated according to size by conventional agarose gel electrophoresis, and the DNA banding patterns of different isolates can then be compared. In the study described here we developed an AP-PCR assay to analyze *S. enteritidis* isolates from various egg-borne human outbreaks as well as from epidemiologically unrelated isolates from avian sources.

Organisms and growth conditions. The *S. enteritidis* isolates of various egg-borne outbreaks in humans as well as from epidemiologically unrelated isolates from avian sources and other *Salmonella* species used in the study are listed in Table 1. All bacteria were grown in Luria broth medium (13). Single colonies of the *Salmonella* species listed in Table 1 were subcultured three times on solid medium by a previously described method (17) and were then serotyped by both the State Department of Health, Hartford, Conn., and the National Veterinary Science Laboratories, Ames, Iowa. All cultures were grown at 37°C for 24 h.

DNA extraction. Chromosomal DNAs from all *Salmonella* species listed in Table 1 were isolated as described previously (13). Briefly, after incubating at 37°C for 24 h in 5 ml of Luria broth medium in loose-top culture tubes by aeration at 90 rpm in a shaker incubator (Orbit shaker bath; Lab-Line Instruments Inc., Melrose Park, Ill.), the *Salmonella* organisms were pelleted by centrifugation at 8,000 × *g* for 10 min, washed two times with 10 ml of phosphate-buffered saline, and resuspended in 2 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.6]). The bacteria were lysed with sodium dodecyl sulfate at a final concentration of 1% and were incubated at 37°C for 1 h. The mixture was then treated with proteinase K (20 μg/ml) and was incubated at 37°C for 1 h. The chromosomal DNA was then extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The DNA was precipitated with 1/10th volume of 3 M sodium acetate and 2 volumes of absolute ethanol, and it was incubated overnight at −20°C. The precipitated DNA was pelleted by centrifugation at 8,000 × *g* for 10 min and was resuspended in 1 ml of TE buffer. The concentration of DNA was estimated from the A_{260} as described previously (13), and the DNA was stored at −20°C until use.

Primer selection and synthesis. A 15-mer oligonucleotide TGAGCATAGACCTCA was selected from the sequence data for a *Salmonella*-specific PCR obtained in our laboratory (9). This 15-mer oligonucleotide primer was synthesized on a model 380B DNA synthesizer (Applied Biosystem Inc., Foster City, Calif.) with the assistance of the University of Connecticut Biotechnology Center. The primer was desalted through a Sephadex G-25 column (Pharmacia, Inc., Piscataway, N.J.). The concentration of the primer was measured by spectrophotometry, and the primer was divided into 50-μl volumes and stored at −20°C.

PCR amplification and detection of PCR products. Amplification reactions were carried out in a volume of 100 μl of a PCR mixture containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 0.9 μM primer, 2.5 U of AmpliTaq DNA polymerase, and 2.5 mM

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TABLE 1. Results of AP-PCR banding patterns

Source and *Salmonella* isolate	Phage type	AP-PCR banding pattern[a]	Source
Avian sources			
S. enteritidis 203	8	B	Ovary of hen (15)
S. enteritidis 205	8	B	Liver of hen (15)
S. enteritidis 29	8	A	Chicken liver and heart pool (15)
S. enteritidis 16	13a	B	Poultry house manure (15)
S. enteritidis 27	13a	C	Hen ovary (15)
S. enteritidis 28	13a	A	Hen ovary (15)
S. enteritidis 30	13a	A	Chicken small intestine (15)
S. enteritidis 44	13a	B	Cat feces in poultry house (15)
S. enteritidis 85	13a	E	Poultry house manure (15)
S. enteritidis 11	14b	B	Chicken liver and heart pool (15)
S. enteritidis 12	14b	B	Chicken liver (15)
S. enteritidis 13	14b	F	Poultry house manure (15)
S. enteritidis 17	14b	B	Hen ovary and oviduct pool (15)
S. enteritidis 25	14b	F	Poultry house manure (15)
S. enteritidis 38	14b	F	Chicken eggs (15)
S. enteritidis 39	14b	F	Chicken eggs (15)
S. enteritidis 40	14b	F	Chicken ovary (15)
S. enteritidis 42	14b	B	Chicken egg follicle (15)
S. enteritidis 43	14b	C	Poultry manure (15)
S. enteritidis 118	14b	B	Mouse feces in chicken house (15)
Human infections			
S. enteritidis Y8-P2	8	D	Human, New York
S. enteritidis 8567	8	B	Human, Pennsylvania
S. enteritidis 2036	8	B	Human, Kansas
S. enteritidis 2037	8	B	Human, Kansas
S. enteritidis B6996	13a	A	Human isolate, CDC[b] (15)
S. enteritidis 357S	13a	E	Human isolate, CDC (15)
S. enteritidis 354KT2	13a	F	Human isolate, CDC (15)
S. enteritidis 354MJ	13a	A	Human isolate, CDC (15)
S. enteritidis 354M4	13a	G	Human isolate, CDC (15)
S. enteritidis 4272	UT	B	Human, South Carolina
S. enteritidis 4257	UT	B	Human, South Carolina
S. enteritidis 4269	UT	E	Human, South Carolina
Other *Salmonella* spp.			
Salmonella typhi			University of Connecticut
Salmonella pullorum			University of Connecticut
Salmonella typhimurium			University of Connecticut

[a] AP-PCR DNA patterns: A, contains bands of 0.73 and 1.15 kbp; B, contains bands of 0.65, 0.73, 1.15, 1.3, 1.5, and 1.7 kbp; C, contains bands of 0.73, 1.15, 1.3, and 1.7 kbp; D, contains bands of 0.73, 1.0, and 1.15 kbp; E, contains bands of 0.73, 1.15, 1.3, 1.5, and 1.7 kbp; F, contains bands of 0.73, 1.0, 1.15, 1.5, and 1.7 kbp; G, contains bands of 0.65, 0.73, 1.0, 1.15, and 1.7 kbp; UT, untypeable.
[b] CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

$MgCl_2$. Different concentrations of DNA were then added to the mixture. The reaction mixture was overlaid with 50 μl of mineral oil. Amplification was performed in a DNA thermal cycler (Perkin-Emer Cetus Corporation, Norwalk, Conn.). Following preliminary trials with different annealing temperatures and times and with various concentrations of *Salmonella* DNA, the thermal cycler was programmed for optimum conditions. Initially, the reaction mixture was heated at 94°C for 7 min. Then the PCR was run for 45 cycles at a melting temperature of 94°C for 1 min, an annealing temperature of 40°C for 1 min, and an extension temperature of 72°C for 2 min. The sample was then heated at 72°C for 5 min for the final extension reaction.

Gel electrophoresis was used to detect amplified DNA products. A volume of 8 μl of amplified PCR products was subjected to electrophoresis at 60 V in horizontal gels containing 0.8% agarose (Ultrapure; Bethesda Research Laboratories) with Tris-borate buffer (45 mM Tris borate, 1 mM EDTA). The gel was stained with ethidium bromide, exposed to UV light to visualize the amplified products, and photographed.

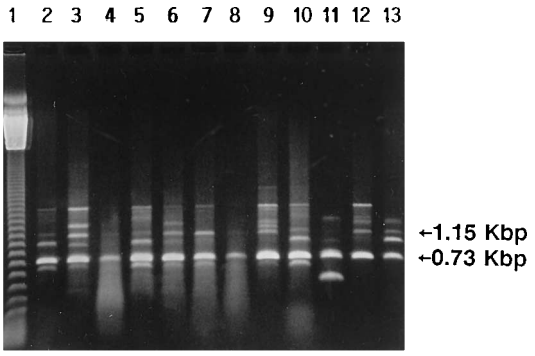

FIG. 1. AP-PCR amplification of *Salmonella* DNA. Lanes: 1, molecular size marker (123-bp DNA ladder); 2, human SE-354M4 (phage 13a); 3, avian SE-118 (phage 14b); 4, human SE-B6996 (phage 13a); 5, human SE-354KT2 (phage 13a); 6, human SE-8567 (phage 8); 7, avian SE-27 (phage 13a); 8, avian SE-29 (phage 8); 9, avian SE-17 (phage 14b); 10, avian SE-25 (phage 14b); 11, *Salmonella typhi*; 12, *Salmonella typhimurium*; 13, *Salmonella pullorum.*

Twelve *S. enteritidis* isolates from egg-borne infections in humans, 21 *S. enteritidis* isolates from avian sources, and 3 isolates of other *Salmonella* spp. (Table 1) were analyzed by AP-PCR. Four different primers with G+C contents ranging from 30 to 70% were used. These were MK21 (GCCAGG CTCCAG; G+C content, 70%), MK22 (TGAGCATAGAC CTCA; G+C content, 47%), MK23 (GACTCGTAC; G+C content, 55%), and MK24 (TGCAATGTAT; G+C content, 30%). MK22, the primer containing a 47% G+C content, gave the best results, and therefore, it was used throughout the study.

Amplification of genomic DNAs from the *S. enteritidis* isolates with the MK22 primer resulted in AP-PCR DNA patterns consisting of three to seven distinct DNA fragments ranging from approximately 0.65 to 1.7 kbp (Table 1). During the study, seven different AP-PCR DNA patterns designated A to G, which are shown in Fig. 1 and 2 and described in Table 1, were found among the 33 *S. enteritidis* isolates. Random genomic DNAs from various *S. enteritidis* isolates were subjected to AP-PCR with the MK22 primer to check the reproducibility of the test three times at intervals of different days; the resulting AP-PCR DNA patterns were found to be similar.

Representative AP-PCR DNA patterns from an experiment

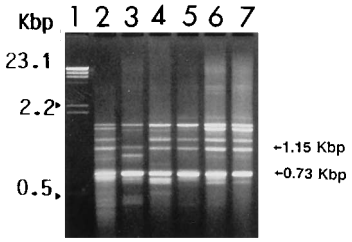

FIG. 2. AP-PCR amplification of *S. enteritidis* DNAs showing variations among similar phage types. Lanes: 1, molecular size markers (bacteriophage lambda *Hin*dIII-digested DNA); 2, human SE-357S (phage 13a); 3, human SE-354KT2 (phage 13a); 4, avian SE-16 (phage 13a); 5, avian SE-27 (phage 13a); 6, avian SE-44 (phage 13); 7, avian SE-17 (phage 14b).

in which the primer MK22 was used to amplify fragments of genomic DNA from 10 *S. enteritidis* isolates and 3 other *Salmonella* spp. are shown in Fig. 1. Figure 1 shows that while most of these amplified DNA fragments were common to all *S. enteritidis* isolates, some amplified DNA fragments were unique to some isolates. Interestingly, it was noticed that two DNA fragments of 0.73 and 1.15 bp, respectively, were common to all *S. enteritidis* isolates and were also present in *Salmonella* spp. (Fig. 1 and 2).

The AP-PCR DNA analysis enabled us to detect a DNA fragment of approximately 1.0 kbp, as shown in lanes 2 and 3 of Fig. 2, from an *S. enteritidis* isolate of phage type 13a derived from an egg-borne infection, in a human. This DNA fragment was missing from an *S. enteritidis* isolate from an avian source which also belonged to phage type 13a (Fig. 2, lanes 4 and 5). Similar observations were also made among *S. enteritidis* isolates of phage type 8 as well as phage type 14b and are described in detail in Table 1.

The AP-PCR analysis in which the MK22 oligonucleotide sequence was used as a primer in the present study demonstrated differences among *S. enteritidis* isolates. Analogous results have been reported with randomly cloned DNA probes by Southern blot analysis (5, 20).

The use of the AP-PCR DNA analysis developed in the present study enabled us to recognize differences in DNA patterns among isolates of *S. enteritidis* of similar phage types. The differences in amplified DNA fragments within *Salmonella* isolates of the same phage types found in the present study confirmed similar findings reported by Threlfall et al. (19). It was suggested that one phage type of *S. enteritidis* may be the progenitor of another phage type (19). Therefore, the use of phage typing alone as an epidemiologic marker in the area where one phage type is common could be misleading for tracing egg-borne infections in humans. To investigate this possibility, we are conducting a study using AP-PCR analysis to compare *S. enteritidis* organisms of known phage types before inoculation into the birds with the *S. enteritidis* organisms obtained after reisolation from the inoculated birds.

The selection of an appropriate primer and optimization of PCR conditions are of great importance for maximization of the discriminatory power of AP-PCR analysis (3). Primers that work for some bacteria may not work well for others. We tested four primers with G+C contents varying from 30 to 70%. The primer MK22 (47% G+C content) was found to be superior in defining differences among *S. enteritidis* isolates. The percent C+G content of MK22 was in the optimal range for obtaining reproducible amplified DNA patterns, as described by others (24).

It was suggested that the discriminatory power of AP-PCR analysis could be increased by using two primers (8, 23). In the present study, however, we achieved good results by using a single primer, and the results obtained by the AP-PCR analysis were found to be reproducible.

In conclusion, the AP-PCR test system developed here can be used to establish whether *S. enteritidis* isolates of the same phage type have identical DNA banding patterns. Thus, the sources of egg-borne *S. enteritidis* infections in humans can be traced more accurately.

We are grateful to H. P. Opitz for supplying the *S. enteritidis* isolates, and we thank Antonio Garmendia for critical reading of the manuscript.

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