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Nucleic acid scanning-by-hybridization of enterohemorrhagic *Escherichia coli* isolates using oligodeoxynucleotide arrays

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ABSTRACT

Nucleic acid scanning by hybridization (NASBH) is a non-electrophoretic typing strategy that uses gridded oligonucleotides to reproducibly characterize arbitrarily amplified nucleic acid sequences. Membrane-bound arrays of terminally-degenerate oligonucleotides were hybridized to DNA amplification fingerprinting (DAF) products from enterohemorrhagic *Escherichia coli* O157:H7 isolates. Numerical and cluster analysis of 64 isolates, selected by DAF to represent a single dominant amplification type identified 14 hybridization types. Results show that NASBH is a powerful alternative for the identification of closely related bacteria, can be used successfully in epidemiological studies, and holds potential in general nucleic acid diagnostics.

Nucleic acid scanning techniques driven by arbitrary primers (1) have been used in bacterial identification (2–4) and have proven superior to multilocus-enzyme electrophoresis (MLEE) and pulsed field gel electrophoresis (PFGE) in their detection sensitivity (5). However, faster and more powerful nucleic acid scanning alternatives are needed to distinguish closely related bacteria. Here we introduce a non-electrophoretic method for nucleic acid analysis, nucleic acid scanning-by-hybridization (NASBH), a strategy that uses gridded oligonucleotide arrays to type arbitrarily amplified nucleic acid sequences. NASBH was applied to the characterization of regional enterohemorrhagic *Escherichia coli* (EHEC) isolates that were previously indistinguishable by conventional nucleic acid analysis providing information on relationship and number of bacterial types present in disease outbreaks.

The NASBH strategy followed a format II design, where biotin and digoxigenin-labeled amplification products generated by DNA amplification fingerprinting (DAF; 6) with at least one arbitrary primer were hybridized to an arbitrary array of nylon-bound terminally degenerate oligodeoxynucleotides. Total DNA was isolated from stationary phase-grown bacteria (7). DAF reactions, optimized for bacterial fingerprinting (2,7), contained 3 μM of standard and/or labeled primer(s), 0.3 U/μl Stoffel fragment DNA polymerase (Perkin-Elmer, Norwalk, CT), 200 μM of each deoxynucleoside triphosphate [sometimes including 10 μM digoxigenin-11-dUTP (alkali-labile or stable)], 6 mM $MgCl_2$ and buffer (10 mM KCl, 10 mM Tris–HCl; pH 8.3), and were amplified in 35 cycles of 30 s at 96°C, 30 s at 30°C and 30 s at 72°C. DAF products were directly hybridized to an arbitrary matrix of 100 oligodeoxynucleotides with sequence $N(X)_9N$ (X being any one nucleotide). Alternatively, digoxigenin-labeled amplification products were hybridized to the arrays (8), following an initial removal of biotinylated fragments with streptavidin-coated Dynabeads (Dynal AS, Oslo, Norway). Individual oligonucleotides (20 pmol) were attached to GeneScreen membranes (New England Nuclear) using a custom made replicator (3 × 3 cm) with pins that carry ~0.5 μl of liquid. Membranes were heated at 80°C for 60 min and UV-irradiated for 1 min. DAF products (20–40 nM) were hybridized to the oligonucleotide arrays (cf. 8,9) in 0.5 M Na_2HPO_4 (pH 7.2), 7% (w/v) sodium lauroyl sarcosine (pH 7.0) and 50 g/l polyethylene glycol for 2 h at 6°C.

Hybridization was stopped by washing the membranes three times in 6× SSC solution (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7), 5 min each at 6°C. The membranes were then incubated for 45 min in PBS–7% sodium lauroyl sarcosine containing 0.15 U/ml streptavidin–alkaline phosphatase conjugate. The unbound conjugate was removed by successive rinsing, the membrane treated with diethanolamine and Attophos™, and positive signals detected by fluorescence (8). The nature and integrity of amplification products was confirmed by denaturing polyacrylamide gel electrophoresis and silver staining (6).

NASBH produced reliable signatures from *E.coli*, *Streptococcus uberis* and *Rhizobium meliloti* bacteria. Reproducible hybridization patterns were obtained from DNA isolated independently from different colonies of the same isolate (Fig. 1A), or from independent amplification or hybridization reactions. Reproducible results were obtained from DAF profiles generated with different primers and labeled with either biotin or digoxigenin. Presently, degeneracy in oligonucleotide termini was necessary to insure reproducibility, probably by avoiding dangling-end stability and end fraying effects.

Potent Shiga-like cytotoxin-producing *E.coli* belonging to the O157:H7 serogroup are emergent human pathogens recognized as causing serious illness in the form of hemorrhagic colitis and hemolytic-uremic syndrome (HUS) throughout the world (10). These EHEC strains appear to have evolved only recently from an extant *E.coli* lineage (11,12), and are therefore difficult to distinguish by protein-based MLEE (12) and nucleic acid-based PFGE (13). MLEE analysis of protein polymorphisms within a group of 248 clinical EHEC isolates belonging to serogroup O157 and obtained from stool specimens collected from HUS patients (generally children of <3 years of age), showed that 213 of these isolates fell within a single MLEE type analogous to the common electrophoretic type 11 (clonal group 4) (12). The 213 O157:H7 isolates were further characterized using DAF with 8 octamer

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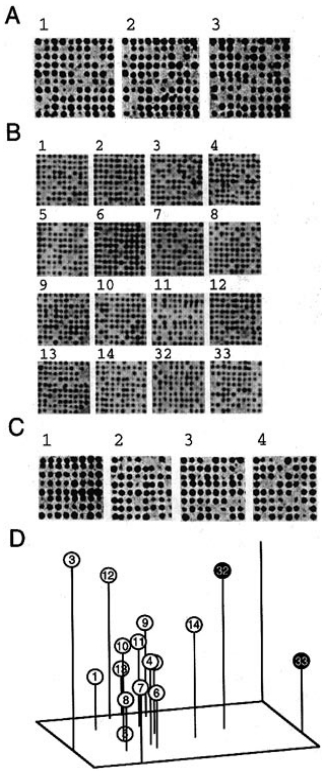

Figure 1. Nucleic acid scanning by hybridization (NASBH) analysis of enterohemorrhagic *Escherichia coli* (EHEC) bacteria. (**A**) NASBH analysis of a single EHEC isolate belonging to clonal group 4 (DEC4; cf. 12) using a 10× 10 arbitrary panel of 100 membrane-bound oligonucleotides of sequence $N(X)_9N$. The format II arrays were used to probe DAF amplification products generated with 5′ biotinylated CGAGCTG primer from DNA isolated independently from three different colonies of the isolate. (**B**) NASBH analysis of 64 EHEC isolates belonging to DEC4 and a single DAF amplification type using the oligonucleotide arrays of (A). DAF products generated by amplification with GTAACGCC and 5′ biotinylated GATCGCAG primers were enriched for single-stranded DNA by magnetic bead separation and hybridized to the undecamer arrays. Two O55:H7 group 5 isolates (32 and 33) were chosen as an outgroup. Fourteen hybridization types (labeled 1–14) were detected. (**C**) Validation of NASBH results using a format I array. The 64 amplified DAF fragments obtained in B were gridded in a 8× 8 format and hybridized with biotin-labeled GTAACGCC (1), NGGTAGCTAAN (2), NGCCCTCACCN (3) and NAAGCTGCTTN (4) probes as described (8). (**D**) Principal coordinate analysis (PCO) of the NASBH matrix. Genetic relationships between EHEC strains were inferred using the NTSYS-pc program (Exeter Software, Setauket,NY) using Dice similarity coefficients.

primers. The number of unique DAF patterns identified with each primer ranged from 10 to 26, depending on the primer used, and averaged 18.9 ± 5.8 (SE). In a previous study, the randomly amplified polymorphic DNA (RAPD) scanning technique detected 1 and 5 amplification types within this clonal group using 5 arbitrary decamers (4). Despite the relative large number of bacterial groups identified with DAF, a large proportion of isolates (71–74%) fell within one amplification type. In particular, a group of 151 isolates could not be distinguished from each other, 64 of which were selected for NASBH analysis. Strains were collected in three independent summer outbreak episodes, expressed EHEC pathogenicity determinants, and carried either one or both verotoxin *slt* genes following dot-blot hybridization. The oligonucleotide arrays identified 14 hybridization types within this clonal group (Fig. 1B), producing 81–92 hybridization signals [mean = 85.7 ± 3.0 (SE)]. The number of positive hybridization signals was higher than the 43 expected, probably due to mismatching during nucleic acid scanning (14) and the existence of amplification products undetected by silver staining (15). The panel of 64 DAF amplification products were also spotted on Nylon membranes and hybridized to individual oligonucleotides following a format I protocol (Fig. 1C). Hybridization to the biotinylated octamer originally used to generate amplification profiles produced signals from all target DNA, confirming their integrity. Three other terminally degenerate oligonucleotides were hybridized to the arrays of amplified DNA (Fig. 1C). Positive hybridization signals matched those observed when using the oligonucleotide arrays.

Genetic relationships established by numerical (PCO) (Fig. 1D) or cluster (UPGMA) analysis identified unequivocally the individual EHEC isolates, grouping them separately from an outlying group (outgroup). Interestingly, bacteria isolated in a first outbreak (summer 1993) expressed all hybridization types except types 12 and 14. In contrast, bacteria from two subsequent outbreaks (summer 1994) contained either type 14 or types 3 and 12. Hybridization types 3, 12 and 14 were genetically distant from the cluster of types observed in the first outbreak (Fig. 1D), and probably represent bacterial introductions to the region or new *E.coli* strains that have gained pathogenicity determinants. Genetic distances obtained by NASBH should be concordant with those obtained by nucleic acid scanning. A previously typed group of five *E.coli* (7) and seven *S.uberis* (2) isolates were similarly grouped by DAF and NASBH analysis using UPGMA algorithms (not shown). However, NASBH analysis of monomorphic amplification fingerprints identified isolates that were otherwise indistinguishable by DAF.

NASBH was unprecedented in its abilities to detect bacterial types within a clonal group of bacteria previously defined by MLEE and DAF analysis. Compared with prior reports (3,12,13,16), NASBH appears faster than and far superior to PFGE, established nucleic acid scanning techniques (RAPD and DAF) and MLEE in the analysis of closely related bacteria. NASBH constitutes a precursor of a new generation of nucleic acid scanning techniques capable of addressing nucleic acid sequence information through the use of oligonucleotide arrays. NASBH can be used to screen any nucleic acid, including RNA fingerprints generated by differential display of mRNA populations. Microfabrication of gridded arrays in a silicon based addressable system (17) or the use of alternative oligonucleotide array strategies (18,19), will facilitate the hybridization of nucleic acids.

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