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Use of *Shigella flexneri ipaC* and *ipaH* Gene Sequences for the General Identification of *Shigella* spp. and Enteroinvasive *Escherichia coli*

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The products of the *ipaB*, *ipaC*, and *ipaD* genes are involved in the expression of the invasive phenotype in all species of *Shigella* and enteroinvasive *Escherichia coli* (EIEC). DNA probes derived from these genes are accurate indicators of the invasive phenotype (M. Venkatesan, J. M. Buysse, E. V. Vandendries, and D. J. Kopecko, J. Clin. Microbiol. 26:261–266, 1988); however, spontaneous loss of the invasion plasmid or selective deletion of invasion-associated genes may restrict the usefulness of such probes as general diagnostic tools. In this study, we report that laboratory-passaged strains of *Shigella* spp. and EIEC that were invasion and Sereny test negative were unable to hybridize to the *ipaC* DNA probe. However, a second DNA probe, derived from the *Shigella flexneri ipaH* gene, a multiple-copy element found on the chromosome and invasion plasmid that encodes a 60-kilodalton antigen, was more sensitive in its ability to detect virulent as well as avirulent shigellae and EIEC. Analysis of colony blots and stool blots from pediatric patients with diarrhea indicated that the *ipaH* probe was more effective in detecting shigellae and EIEC than was either the *ipaC* or 17-kilobase *Eco*RI fragment probe.

We have previously shown that the invasion plasmid antigen (*ipa*) genes *ipaB*, *ipaC*, and *ipaD* (2) can be used as specific nucleic acid probes for the identification of *Shigella* spp. and enteroinvasive *Escherichia coli* (EIEC) (16). These genes, which synthesize major surface immunogens detected with human convalescent-phase sera, are clustered on an 8-kilobase (kb) region of the large (180- to 210-kb) invasion plasmid that is present in all virulent *Shigella* spp. and EIEC. Several genetic determinants located on this large plasmid have been shown to be necessary for triggering the invasion of (i.e., endocytosis by) colonic epithelial cells (4, 6, 9). The *ipa* gene cluster constitutes one of these invasion-essential loci and resides near a region of the plasmid that is a hot spot for spontaneous deletions (10). Loss of the invasion plasmid or deletion of the small region encompassing the *ipa* genes, independent events which occur at a relatively high frequency (1 in 10^3 to 10^4 cells), renders the bacteria noninvasive and therefore avirulent (8, 16). Therefore, in native bacterial strains, the presence of the *ipa* genes correlates with, but is not an absolute indicator of, virulence. In this report, we demonstrate that the well-characterized *ipaBCD* gene sequences, which are unique to *Shigella* spp. and EIEC, add a novel level of specificity for the identification of invasive (and thus, in most cases, virulent) bacterial strains, especially in the analysis of laboratory-passaged EIEC.

In a previous paper (2), we described a 60-kilodalton antigen-coding gene, *ipaH*, which produces a polypeptide similar in size to the IpaB protein but distinct both immunologically and genetically. More recently, we have determined that *ipaH* is present in multiple copies on both the plasmid and the chromosome of *Shigella* spp. (M. M. Venkatesan, A. Hartman, and J. M. Buysse, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B92, p. 46; manuscripts in preparation) and is not a part of the *ipaBCDA* gene cluster. Because of its increased dosage and presence on the *Shigella* chromosome, a DNA probe derived from *ipaH* might be less compromised by plasmid loss and/or selective deletion events of the invasion plasmid and perhaps be more sensitive as a diagnostic tool than either the *ipaB*, *ipaC*, and *ipaD* genes or the 17-kb *Eco*RI fragment probe previously reported for *Shigella* spp. (1, 7, 13, 14). In this report, we describe our preliminary results with an *ipaH* gene segment as a DNA probe and show its utility, advantages, and specificity in identifying *Shigella* spp. and EIEC in colony and stool blots.

MATERIALS AND METHODS

Invasion plasmid DNAs from various shigella strains were isolated as described previously (2, 16). Chromosomal DNA was prepared from bacterial spheroplasts obtained after digestion with lysozyme (11). Restriction enzyme digestions of plasmid and chromosomal DNAs, agarose gel electrophoresis, and transfer of DNA fragments to nitrocellulose were carried out essentially as described earlier (2, 16). Colony blots of invasive, noninvasive, and other enteric bacteria have been described previously (16).

Stool specimens were collected from children (under 5 years of age) with diarrhea at the outpatient department of Children's Hospital in Bangkok, Thailand. Stools were cultured microbiologically for the identification of shigellae and EIEC, and stool blots were prepared for probe hybridization as described previously (7, 13, 14). A random collection of culture-positive and culture-negative stool blots was sent to the Walter Reed Army Institute of Research to be hybridized with the *ipa* gene probes. Hybridization with the 17-kb *Eco*RI DNA sequence was carried out at the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand. Control bacterial strains *Shigella flexneri* serotype 5 M9OT-W, *E. coli* 2 (O:143), and *E. coli* Xac were from the Walter Reed Army Institute of Research collection.

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An *ipaC* DNA sequence was used in these studies as representative of the *ipaBCD* gene family, since in our earlier report we had shown that all three *ipa* gene sequences were equivalent in their ability to detect shigella and EIEC strains (16). The *ipaC* gene probe (pWR22) is a 1,750-base-pair segment of the *S. flexneri* serotype 5 invasion plasmid (2) cloned into the pUC8 plasmid vector. pWR22 directs the synthesis of a truncated IpaC protein, as determined on Western blots (immunoblots) of sodium dodecyl sulfate lysates of *E. coli* HB101(pWR22) developed with convalescent-phase monkey antisera. The 1,750-base-pair fragment probe was separated from vector DNA by digestion with *Eco*RI and two sequential rounds of agarose gel electrophoresis and electroelution. The *ipaH* probes were obtained in a similar manner from plasmid pWR390 and λ gt11 *ipaH* recombinant clone W7 (2). pWR390 contains a 2.9-kb fragment of the invasion plasmid cloned into the *Eco*RI site of pBR322; the *ipaH* structural gene is a 1.6-kb coding sequence within this 2.9-kb fragment. A 0.9-kb *Pvu*II-*Sal*I fragment, part of the 2.9-kb insert of pWR390, was separated from the rest of the insert by a series of restriction enzyme digestions, agarose gel electrophoresis, and electroelutions. W7 makes a Φ(*lacZ-ipaH*) fusion protein and contains 950 base pairs of the *ipaH* structural gene, which was separated from the vector by *Eco*RI digestion and electrophoresis. This fragment comprises two-thirds of the structural gene of the *ipaH* protein-coding region beginning at the carboxy-terminal end. The *ipaC* and *ipaH* fragments were radiolabeled with [α-^{32}P]dCTP to a specific activity of 10^8 cpm/mg. Approximately 5×10^6 cpm/ml was used in the DNA filter hybridization assays. DNA hybridization and further processing of the filters were done as described earlier (16). The 17-kb *Eco*RI fragment probe was prepared in a similar manner, as outlined previously (7, 11, 13).

RESULTS

Comparative analysis of laboratory-passaged EIEC strains by hybridization to the *ipaC* and 17-kb *Eco*RI DNA probes. Several strains of EIEC which, after repeated subculturing on Congo red agar, had become Congo red negative were found to be negative in the Sereny test. Table 1 compares the *ipaC* and 17-kb *Eco*RI probe hybridization patterns of these avirulent EIEC strains with those of negative and positive control strains. Most of the strains tested in Table 1 were obtained from a study described earlier (7), with the exceptions of strain J53(pSP1), an *E. coli* K-12 strain carrying the invasion plasmid, and strain EI-145-1-SL, a lactose-negative *E. coli* strain isolated from a child with diarrhea. With only one exception, EIEC strains that were noninvasive in HeLa cells and negative in the Sereny test did not hybridize to the *ipaC* probe, even though they contained a large plasmid and were positive with the 17-kb probe. Strain 2/33 hybridized to both of the DNA probes. These results confirm earlier observations that repeated subculturing of virulent shigellae or EIEC generates deletions of the *ipa* gene region on the invasion plasmid, resulting in avirulence. Thus, laboratory workers trying to keep strains in a virulent form would be better served to streak their bacteria on Congo red plates and then test the Congo red-positive strains with the *ipa* gene probes before undertaking the more expensive and time-consuming Sereny test. Strains that are *ipaBCD* gene probe negative would invariably be Sereny negative and avirulent.

Shigella invasion plasmid and chromosome *ipaH* hybridization patterns. The multicopy nature of the *ipaH* gene is demonstrated in Fig. 1, in which invasion plasmid DNA isolated from *S. flexneri* serotype 5 was digested with several restriction enzymes and probed with radiolabeled *ipaH* (Fig. 1A) and *ipaC* (Fig. 1B) fragments. The *ipaH* probe hybridized to more than one band in each set of digestions, whereas the *ipaC* probe recognized a single band in each case. Since there are no *Hin*dIII sites within the *ipaH* DNA sequence (manuscript in preparation), the five bands detected in the *Hin*dIII digest of the plasmid (Fig. 1A, lane 3) reflected five copies of the *ipaH* gene or portions of the gene.

TABLE 1. Comparison of the *ipaC* DNA probe with other tests for invasiveness in 13 EIEC strains that hybridized to the 17-kb *Eco*RI probe

Strain	Sereny test result	DNA probe result	
		ipaC	17 kb
Control			
S. flexneri serotype 5 strain M9OT-W	+	+	+
E. coli 2 (O:143)	+	+	+
E. coli Xac	−	−	−
Test			
J53(pSP1)	−	−	+
EI-145-1-SL	−	−	+
1457-75/31	−	−	+
2/33	−	+	+
34/1	−	−	+
34/2	−	−	+
34/3	−	−	+
1457-75/29/a	−	−	+
1457-75/28	−	−	+
1457-75/5	−	−	+
1457-75/3	−	−	+
1457-75/30	−	−	+
280-83FAV/6	−	−	+

The presence of the *ipaH* gene on the shigella chromosome is shown in Fig. 1C. Plasmid DNA isolated from *S. sonnei* 53GI and chromosomal DNA from a plasmid-cured derivative of the strain (53GII) were digested with *Hin*dIII and hybridized to nick-translated *ipaH* DNA. Multiple bands were detected in the plasmid and chromosomal DNAs (Fig. 1C). Analogous heterogeneous patterns have been documented in the genomic DNA of several shigella and EIEC strains probed with the pWR390 *ipaH* insert (manuscript in preparation).

Analysis of colony blots hybridized to the *ipaH* sequence. In an earlier report (16), we had demonstrated that on colony blots of isolated bacteria, the *ipaBCD* gene sequences were specific for HeLa cell invasion-positive shigellae and EIEC and did not hybridize to numerous noninvasive strains. Some of these *ipaBCD*-negative noninvasive strains were cured of the invasion plasmid, as in *S. sonnei* 53GII, while others had selective deletions of the *ipa* gene region, as in *S. flexneri* serotype 5 strain M9OT-A3. The *ipaBCD* genes also did not hybridize to several hundred other enteric and nonenteric bacterial strains (16). Duplicate colony blots of the same samples were hybridized to the *ipaH* probe. Unlike the *ipaBCD* gene sequences, *ipaH* hybridized to all shigella and EIEC strains irrespective of their invasive behavior (Table 2). Thus, both *S. sonnei* 53GII and *S. flexneri* M9OT-A3 tested positive with the *ipaH* probe. Like the *ipaBCD* genes, however, *ipaH* did not hybridize to any of the 200 control enteric and nonenteric bacteria tested, dem-

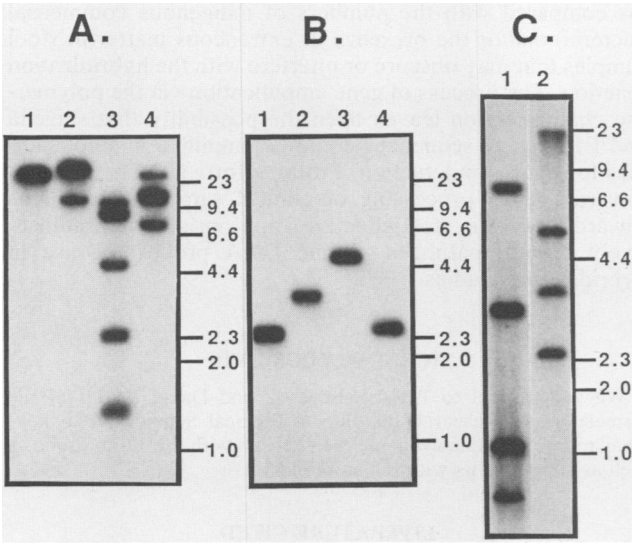

FIG. 1. Hybridization of *ipaH* to invasion plasmid and chromosomal DNAs. Invasion plasmid DNA from *S. flexneri* serotype 5 strain M9OT-W (A and B) was digested with restriction enzymes *Eco*RI (lane 1), *Bgl*II (lane 2), *Hin*dIII (lane 3), and *Pst*I (lane 4), and the DNA fragments were separated on an 0.8% agarose gel. After electrophoresis, the nucleic acid fragments were transferred to nitrocellulose filters and hybridized either to the *ipaH* sequence obtained from clone W7 (A) or to *ipaC* (B). Invasion plasmid DNA from *S. sonnei* 53GI (C, lane 1) and chromosomal DNA from a plasmid-cured derivative, 53GII (C, lane 2) were digested with *Hin*dIII and separated on an 0.8% agarose gel. After being blotted onto nitrocellulose filters, they were hybridized to *ipaH* obtained from pWR390 as described in Materials and Methods. Numbers to the right of the gels are in kilobases.

onstrating that *ipaH* also is a shigella- and EIEC-specific DNA probe.

Analysis of stool blots from children with diarrhea. Having shown that both of the *ipa* probes were shigella and EIEC specific, we wanted to compare the ability of several defined probes to detect shigellae and EIEC in clinical samples. Fifty-nine stool blots which were prepared from patients with diarrhea and which had been proven positive for shigellae and EIEC both by bacteriologic assays and with the 17-kb *Eco*RI probe, 3 stool blots which were culture positive but 17-kb-probe negative, and 77 culture-negative samples which were 17-kb-probe negative were sent to the Walter Reed Army Institute of Research for testing with the *ipaC* and *ipaH* probes. Fifty-eight of the 59 culture-positive, 17-kb-probe-positive stool blots reacted with the *ipaC* probe (Table 3). Stool blots which were culture positive but 17-kb-probe negative were also negative with the *ipaC* probe (Table 3), suggesting that the bacteria in these samples had probably lost the corresponding *ipaC* and 17-kb *Eco*RI fragment regions through plasmid curing or spontaneous deletion. One stool blot from a patient infected with *S. sonnei* reacted with the 17-kb probe but not the *ipaC* probe, indicating a selective deletion of the *ipaBCD* region noted above (Table 1) and observed previously in other strains (e.g., *S. flexneri* serotype 5 strain M9OT-A3) (16).

In contrast to the exclusively plasmid-carried sequences represented by the *ipaC* and 17-kb probes, the *ipaH* probe hybridized to all 62 culture-positive stool samples (Table 3). Furthermore, the *ipaH* probe did not hybridize to the 77 culture-negative stool samples (Table 3).

TABLE 2. Comparison of *ipaC* and *ipaH* probes in the detection of invasive and noninvasive *shigella* and EIEC strains

Bacteria	Response to probe:	
	ipaC	*ipaH*
EIEC and invasive shigellae		
S. flexneri		
Serotype 1b strain M25-8	+	+
Serotype 2a strain 2457T	+	+
Serotype 3 strain J17B	+	+
Serotype 4 strain M76-39	+	+
Serotype 5 strain M90T-W	+	+
Serotype 6 strain 2924-71	+	+
S. dysenteriae (n = 11)	+	+
S. boydii (n = 8)	+	+
S. sonnei form I strain 53GI (n = 4)	+	+
EIEC (n = 4)	+	+
Noninvasive shigellae		
S. flexneri (n = 9)		
Serotype 1b	−	+
Serotype 1a strain M25-8A	−	+
Serotype 2 strain 384	−	+
Serotype 2b	−	+
Serotype 3c	−	+
Serotype 4b	−	+
Serotype 5	−	+
Serotype 5 strain M90T-A3	−	+
Serotype 6	−	+
S. sonnei 53GII	−	+
S. dysenteriae (n = 2)	−	+
Other bacteria: 200 enteric strains (nonshigellae)[a]	−	−

[a] The bacteria tested are listed in reference 16.

DISCUSSION

Genetic analysis of virulence factors in shigellae and EIEC has shown that several regions on the invasion plasmid, including the *ipaBCD* genes and determinants within the 17-kb *Eco*RI fragment, are required for full expression of the virulence phenotype (2–5, 9, 15). The *ipaBCDA* region of all shigella and EIEC invasion plasmids encodes surface peptides that trigger uptake into intestinal epithelial cells; however, the exact function of each Ipa antigen in the invasive process is not yet clear. The critical role that the *ipa* gene products play in the invasive process is reflected in the conservation of the DNA sequences of these genes (9, 15). Thus, invasive shigella and EIEC laboratory strains, as well as clinical isolates from dysenteric patients, were found to hybridize to the *ipaC* probe. Since invasiveness is a prerequisite phenotype for virulence in shigellae, the presence of *ipaBCD* sequences correlates quite well with virulence. In this regard, the 17-kb *Eco*RI probe is more likely to give false-positives, i.e., to detect bacteria that are noninvasive. Neither probe, however, is an absolute indicator of shigella invasiveness, since chromosomal mutations or other plasmid mutations may abrogate this phenotype (3), as was found for EIEC strain 2/33 (Table 1). Colony variants such as *S. flexneri* serotype 2 strain 2457-0 (3), which contains an intact

TABLE 3. DNA probe analysis of stool samples from patients with diarrhea

Stool samples (*n*)	No. of samples that hybridized to the following probe:		
	ipaC	17 kb	*ipaH*
EIEC (7)	7	7	7
S. flexneri serotype 1 (4)	4	4	4
S. flexneri serotypes 2 and 3 (16)	14	14	16
S. flexneri serotype 4 (3)	3	3	3
S. sonnei (31)	29	30	31
S. boydii (1)	1	1	1
Culture negative (77)	0	0	0

invasion plasmid and hybridizes to both the *ipaC* and the 17-kb *Eco*RI probes (unpublished data), represent other examples of chromosomal mutations that render the bacteria noninvasive and avirulent.

During the isolation of *ipaBCDA* recombinants (2), a new gene, *ipaH*, was identified; it has recently been characterized in more detail (Venkatesan et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1989; manuscripts in preparation). This gene, of unknown function, is present in multiple copies on the plasmid and the chromosome. Thus, the hybridization of *ipaH* sequences to *S. sonnei* 53GII, a strain that has lost the entire invasion plasmid and is therefore both 17-kb- and *ipaC*-probe negative, reflects the presence of *ipaH* sequences on the bacterial chromosome. The *ipaH* gene sequence is not an indicator of the invasive phenotype but is specific for shigellae and EIEC. Since the loss of the whole plasmid and/or selective deletion of certain regions of the invasion plasmid is a fairly common occurrence, a shigella-specific DNA probe that is present in multiple copies on the shigella genome would have advantages over purely plasmid-specific, single-copy sequences. It is clear from these studies that the *ipaC* and *ipaH* genes are, at a minimum, comparable to the 17-kb DNA sequence in terms of specificity and sensitivity when used in clinical hybridization studies. The *ipaH* sequence, by virtue of its chromosomal as well as plasmid locations, is superior to both the *ipaC* and the 17-kb probes for the general detection of all shigellae.

Novel methods for the early detection of enteric pathogens, such as the use of radiolabeled nucleic acid probes, are being developed to supplement routine microbiological and biochemical analyses that sometimes take several days to provide a definitive diagnosis. With the characterization of several virulence-essential genes from different enteric bacteria such as *Salmonella* spp. or enterotoxigenic, enterohemorrhagic, and enteropathogenic *E. coli*, efforts are under way to use these sequences as DNA probes to identify pathogens directly in clinical samples (7a). With shigellae, for example, the 17-kb probe has been successfully used to identify dysentery bacilli in colony blots of stool samples grown on MacConkey agar (7, 13, 14). Similarly, a 2.5-kb *Hin*dIII fragment isolated from the invasion plasmid of EIEC 11 has also been shown to hybridize on colony blots specifically to all four virulent shigella species and to EIEC (12). However, the use of the 17-kb probe in the hybridization of filters processed from directly streaked stool samples allowed to grow overnight appears to identify only 40 to 60% of samples containing bacteriologically identified shigellae and only 70 to 80% of culture-proven EIEC-containing samples (13, 14). The low efficiency of shigella and EIEC detection in these direct stool blot assays may be due to the low number of pathogenic bacteria per unit weight of stool (as compared with the numbers of indigenous commensal bacteria) and/or the presence of extraneous matter in stool samples that may obscure or interfere with the hybridization reaction. The process of gene amplification via the polymerase chain reaction leaves open the possibility that shigella and EIEC gene sequences could be amplified in stool samples for optimum detection. Future efforts must be directed towards better processing of clinical samples as well as towards devising cost-effective, appropriate, nonradioactively tagged, pathogen-specific DNA probes for use in hybridization studies.

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