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Rapid and Specific Detection of the *pap*, *afa*, and *sfa* Adhesin-Encoding Operons in Uropathogenic *Escherichia coli* Strains by Polymerase Chain Reaction

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Adhesin-encoding operons (*pap*, *sfa/foc*, and *afa*) have been shown to be prevalent in *Escherichia coli* strains associated with urinary tract infections. A quick and sensitive assay to identify these operons was developed by using the polymerase chain reaction (PCR). Three pairs of 25-mer primers were defined from the sequences of the DNA fragments used as probes in hybridization studies to identify each of the three operons, and the six primers were used together in a single reaction of amplification. To validate the PCR approach for detection of adhesin-encoding operons among clinical isolates, we investigated a collection of 97 *E. coli* isolates with the following characteristics: all isolates originated from the urine of patients with pyelonephritis, and the adhesin responsible for specific binding of the isolates to uroepithelial cells was previously characterized by phenotypic assays, as well as genotypic tests based on hybridization. There was a perfect correlation between the results obtained with the PCR approach and those previously obtained by using DNA probes. These results indicate that the PCR method, which is highly specific and easier to perform than the hybridization method, is a powerful genotypic assay for detection of adhesin-encoding operons. Thus, this assay can be recommended for clinical use to detect virulent urinary *E. coli* strains, as well as for epidemiological studies.

Urinary tract infections (UTI), of which *Escherichia coli* is the major causative agent (12), are among the most common human infections (18). At least 10 to 20% of women experience an acute symptomatic UTI at some point during their lives (16). The severity of the UTI depends both on the virulence of the infecting bacteria and on the susceptibility of the host. Urinary infection most commonly occurs in patients with anatomically and functionally normal urinary tracts and involves spontaneous ascent of bacteria from the urethra to the bladder and (in a minority of patients) to the kidneys and bloodstream (8). Among the factors of pathogenicity commonly expressed by these ascending uropathogenic *E. coli* strains, adherence to uroepithelial cells seems to be important in the pathogenesis of the disease (17, 37, 40). In contrast, patients with underlying medical illness or defects of urine flow caused by urinary tract abnormalities or medical intervention are more susceptible to colonization of the urinary tract by conventional *E. coli*, as are immunocompromised adults (15, 21). Most studies evaluating the significance of impaired host defenses in adults with UTI document a decreased requirement for determinants known to promote adhesion in strains from patients with one or more compromising conditions (21, 24; for a review, see reference 15). These observations indicate that the absence of an adhesion system in a clinical isolate may be a good indicator of the existence of urological abnormalities in a patient and might be used as a diagnostic tool.

Specific adhesion is mediated by bacterial proteins designated adhesins which may or may not be associated with fimbriae; they can be differentiated on the basis of their binding receptor specificity. In the past 10 years, the determinants responsible for adhesion of *E. coli* strains to uroepithelial cells have been cloned from the chromosomes of various clinical isolates and analyzed at the molecular level. The genes involved in the biosynthesis of fimbriae and adhesins of uropathogenic strains were found to belong to phylogenetically independent clusters of genes organized as operons.

The important role of PAP adhesins in the pathophysiology of pyelonephritis caused by *E. coli* has been reported in several studies (10, 24, 27, 39). In a previous work (1), we confirmed the prevalence of PAP adhesins among uropathogenic *E. coli* strains (74.7% of the pyelonephritis-causing strains and 44.1% of the strains associated with cystitis) and reported that the SFA/F1C (13, 33) and AFA (20) adhesins (also designated the Dr hemagglutinin [38], the F1845 adhesin [3], or the O75X adhesin [41]) were also found associated with these pathogenic strains, although less frequently (19 and 10%, respectively). We also showed that adhesins may be multiple within a single strain (PAP plus SFA/F1C or PAP plus AFA) and that the presence of two operons was observed only in uropathogenic strains. This multiplicity of adhesins, necessary for the recognition of various receptors along the urinary tract, seems to be an important factor in the development of urinary tract infection and may help to increase the pathogenicity of a given *E. coli* strain.

As an alternative to the identification of an adhesin on the basis of hemagglutination pattern or identification of the specific receptor, we previously developed a genetic approach based on colony hybridization which appeared to be more reliable (2). However, this technique requires lengthy manipulations to prepare the probes and is too time-consuming for testing of individual strains. In this report, we describe the use of the polymerase chain reaction (PCR) protocol to detect and identify three adhesion systems in a

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single reaction of amplification to obtain a more practical and faster method than colony hybridization.

MATERIALS AND METHODS

Bacterial strains and growth. A collection of 97 *E. coli* strains isolated from urine specimens of patients (children and adults) with pyelonephritis was used to validate the PCR approach. These strains were previously described, and their adhesion properties were determined by phenotypic and genotypic approaches (2). *E. coli* K-12 strain HB101 (5) was used as a negative control (absence of adhesin). *E. coli* J96 carrying at least three separate adhesin-encoding operons (*pap*, *prs* [*pap*-related sequences], and *foc*) (14, 23, 38a), *E. coli* KS52 carrying an *afa* operon (20), and *E. coli* K-12 strain HB101 carrying recombinant plasmid pANN801-13 (13), which contains the entire *sfa* gene cluster, were used as positive controls for detection of adhesins. Bacteria were grown in Luria broth medium without glucose (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter [pH 7.0]) for 18 h at 37°C.

Preparation of bacterial DNA. DNA to be amplified was released from whole organisms by boiling. Bacteria were harvested from 1 ml of an overnight broth culture, suspended in 200 μl of sterile water, and incubated at 100°C for 10 min. Following centrifugation of the lysate, a 150-μl sample of the supernatant was stored at −20°C as a template DNA stock.

Primer sequence determination for PCR. Oligonucleotides used as primers were deduced from the sequences of the DNA fragments previously used as probes in colony hybridization (see Fig. 1). The fragments were cloned in both orientations into M13mp18 (42); single-stranded DNA templates were prepared by the polyethylene glycol method (34), and the nucleotide sequence was determined by dideoxynucleotide chain termination (35) using a Sequenase kit (United States Biochemical Corp.).

Amplification procedure. PCR was done in a total volume of 50 μl containing 10 μl of the template DNA, each of the primers at 0.45 μM, the four deoxynucleoside triphosphates (each at 200 μM), 10 mM Tris hydrochloride (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, and 1.2 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.). The reaction mixture was overlaid with 3 drops of mineral oil. PCR amplifications consisted of 25 cycles of denaturation at 94°C for 2 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min in a Thermal Cycler (Perkin Elmer Cetus). Ten microliters of the reaction mixture was then analyzed by electrophoresis on 2% agarose gels, and the reaction products were visualized by staining with ethidium bromide. A reagent blank, which contained all components of the reaction mixture, except the template DNA, was included in every PCR procedure.

RESULTS

Nucleotide sequences of DNA fragments to be targeted by PCR. The knowledge of the nucleotide sequences of the fragments previously used as specific probes in colony hybridization (Fig. 1) was a prerequisite for development of a PCR test. Since the nucleotide sequence of the fragment specifying the *pap* operon had already been published (25), only the nucleotide sequences of both ends of the fragments specifying the *afa* and the *sfa* operons were determined (Fig. 2).

Selection of oligonucleotides used as primers and specificity.

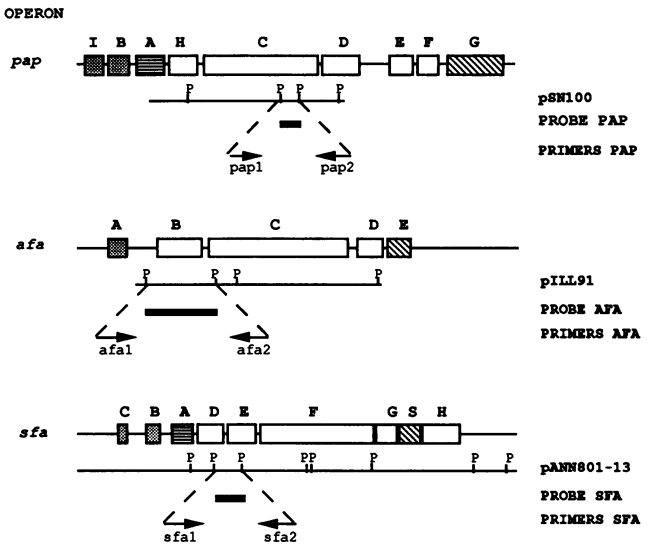

FIG. 1. Primers used in PCR. Diagram of the genetic organization of the *pap*, *afa*, and *sfa* operons. The products of the various cistrons (their names are indicated by letters) are drawn. Symbols: ▤, ▨, and ▥, fimbrial structural proteins, adhesin proteins, and proteins involved in regulation of expression, respectively. The *Pst*I (P) restriction fragments used as probes in colony hybridization are indicated. These fragments, specifying the *pap*, *afa*, and *sfa* operons, originated from the following *Pst*I-digested hybrid plasmids: pSN100 (26); pILL91, consisting of the 4-kb *Sau*3A restriction fragment of pILL61 (19) cloned into pBR322 (4); and pANN801-13 (13), respectively. The PAP probe consists of a 0.3-kb DNA fragment internal to the *papC* gene described by O'Hanley et al. (28); it detects both the *pap* (14) and *prs* (23) operons. The AFA probe consists of a 1.1-kb DNA fragment overlapping the *afaB* and *afaC* genes described by Labigne-Roussel and Falkow (19); it detects all operons related to the *afaI* operon (20). The SFA probe consists of a 0.8-kb DNA fragment, designated the F probe by Ott et al. (29); it recognizes the *sfa* and *foc* operons, which are highly homologous (33). The positions of the three sets of primers used in PCR assays are indicated.

Three sets of 25-mer primers were chosen from the sequences of the DNA restriction fragments specifying the *pap*, *afa*, and *sfa*/*foc* operons. To use the six oligonucleotides together in a single assay, we selected them on the basis of percent G+C%, the degree of homology of their sequences, and the sizes of the segments that might be amplified by the three pairs of primers.

Primer set 1 was derived from the published sequence of the *papC* gene (25). Oligonucleotides pap1 and pap2 flanked the 313-bp *Pst*I internal fragment of *papC* and targeted a 328-bp DNA segment (Fig. 1). Their sequences were as follows: pap1, 5′-GACGGCTGTACTGCAGGGTGTGGCG-3′; pap2, 5′-ATATCCTTTCTGCAGGGATGCAATA-3′.

Primer set 2 was derived from the sequences of the ends of the 1.1-kb *Pst*I DNA fragment specifying the *afa* operon (Fig. 1 and 2A). Oligonucleotides afa1 and afa2 flanked a 750-bp DNA segment, and their sequences were as follows: afa1, 5′-GCTGGGCAGCAAACTGATAACTCTC-3′; afa2, 5′-CATCAAGCTGTTTGTTCGTCCGCCG-3′.

Primer set 3 was derived from the sequences of the ends of the 0.8-kb *Pst*I fragment specifying the *sfa* operon (Fig. 1 and 2B). These sequences showed minor disagreements (three base pairs) with those recently published by Schmoll et al. (36). Oligonucleotides sfa1 and sfa2 delineated a 410-bp segment, and their sequences were as follows: sfa1, 5′-

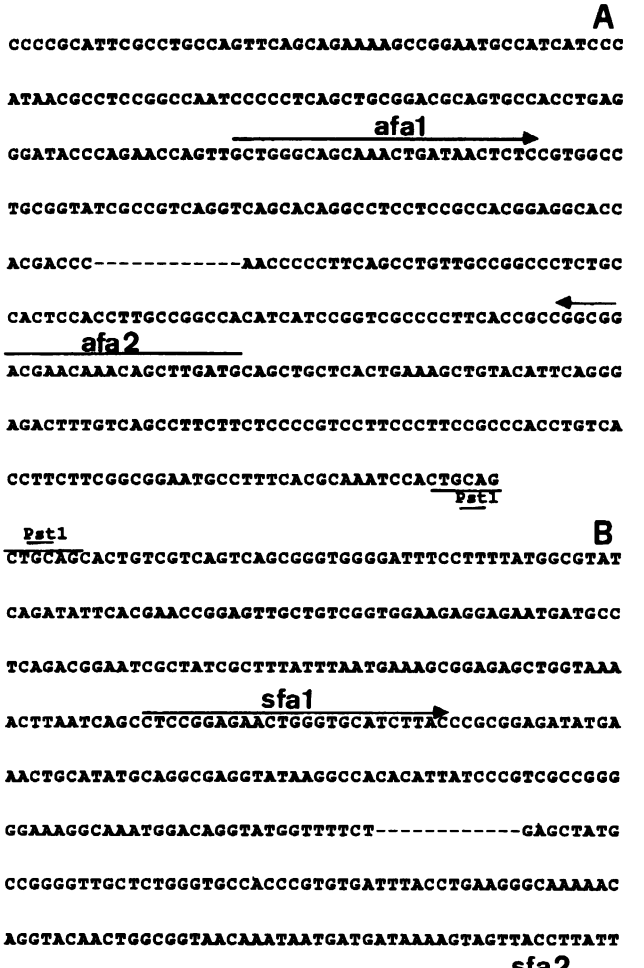

FIG. 2. DNA sequences of the fragments specifying the *pap* and *afa* operons and of the amplification primers. (A) DNA sequence internal to the 1.1-kb *Pst*I fragment specifying the *afa* operon; the sequence begins approximately 60 bp from an upstream *Pst*I site. (B) DNA sequence of the 0.8-kb *Pst*I fragment specifying the *sfa* operon. The sequences of the primers (afa1, afa2, sfa1, and sfa2) are identified by arrows. Regions for which the nucleotide sequence was not determined are symbolized by dashed lines.

CTCCGGAGAACTGGGTGCATCTTAC-3'; sfa2, 5'-CGG AGGAGTAATTACAAACCTGGCA-3'.

To evaluate the specificity of the three sets of primers, they were used independently or together in a single amplification reaction to detect the presence of the corresponding adhesion operons among representative *E. coli* strains: J96, KS52, HB101(pANN801-13), and HB101. The distribution and sizes (i.e., 328 bp for the *pap* primers, 750 bp for the *afa* primers, and 410 bp for the *sfa/foc* primers) of the amplified products were as predicted.

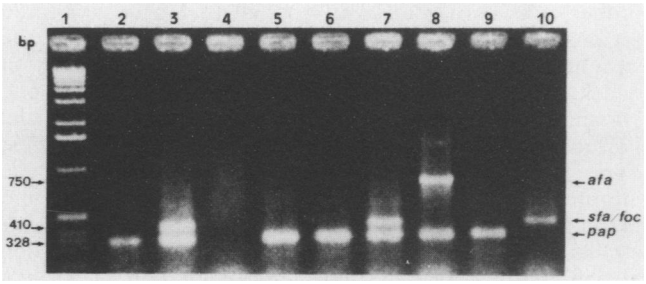

FIG. 3. Detection of the *pap*, *afa*, and *sfa/foc* operons among *E. coli* strains associated with pyelonephritis. The sizes of the amplification products are shown on the left. PCR products are indicated on the right. Lanes: 1, 1-kb DNA ladder used as molecular weight markers; 2, 5, 6, and 9, *E. coli* strains carrying a *pap* operon; 3 and 7, *E. coli* strains carrying *pap* and *sfa/foc* operons; 8, an *E. coli* strain carrying *pap* and *afa* operons; 10, an *E. coli* strain carrying an *sfa/foc* operon; 3, an *E. coli* strain devoid of *pap*, *afa*, and *sfa/foc* operons.

Detection of the *pap*, *afa*, and *sfa/foc* operons among 97 clinical isolates. To validate the PCR approach for detection of adhesins among clinical isolates, well-characterized strains associated with pyelonephritis were investigated. The adhesins expressed by these strains were previously identified with a colony hybridization assay (2). The results of the PCR investigation of 97 *E. coli* strains associated with pyelonephritis genotypically identified 50 *pap*, 4 *sfa/foc*, 3 *afa*, 18 *pap sfa/foc*, and 9 *pap afa* strains; no operons were detected in 13 strains. Representative PCR results are presented in Fig. 3. A total of 84 bacterial strains shared DNA sequences related to one or two operons encoding a PAP, AFA, or SFA/F1C adhesin. It was found that 77, 12, and 22 of the bacterial strains exhibited the *pap*, *afa*, and *sfa/foc* genotypes, respectively. A total of 27 strains carried two adhesin-encoding operons. Thus, 100% correlation was observed between the results obtained with the PCR approach and those determined previously by using the colony hybridization technique (2).

DISCUSSION

The ability to adhere to epithelial surfaces has been shown to be a prerequisite for *E. coli* strains to colonize the urinary tract, i.e., to cause UTI in the absence of urological abnormalities. Hitherto, two approaches, one phenotypic and one genotypic (by colony hybridization), have been used to detect and identify the presence of adhesin-encoding operons. We previously showed that the genotypic assay was more sensitive and reliable than the phenotypic test (2). Indeed, the latter is dependent upon expression of the adhesin, which is prone to phase variation (30, 31) and is regulated by environmental conditions (11, 22) and by variability in binding specificity within a family of related operons (19, 23, 29, 33). The purpose of this work was to evaluate a PCR assay rather than DNA probes for detection of adhesin-encoding operons. We concentrated on establishing an easy, quick, and inexpensive method that, in contrast to colony hybridization, would facilitate the testing of individual *E. coli* strains.

DNA probes were previously defined for adhesin-encoding operons *pap* (and *prs*), *afa*, and *sfa/foc*, which were the most frequently found in uropathogenic *E. coli* (1, 2). These

probes consist of sequences internal to highly conserved regions of the different operon families but distinct from the structural genes coding for pilin or adhesin production which, belonging to the same family, may show genetic variability. In this study, PCR was used to test for the presence of these three adhesin-encoding operon families. Type 1 adhesin was not characterized, since the genetic information responsible for synthesis of this adhesin is found in the genome of intestinal, as well as extraintestinal, *E. coli* strains (6) and has been reported to be consistently associated with uropathogenic strains (28). In addition, we did not search for the *bma* operon (32), as it is rarely found in strains isolated from cases of pyelonephritis (2).

Three sets of primers were selected such that sequences of each of the three DNA probes would be amplified. The results demonstrate that PCR can be used to detect specific genes of *E. coli* isolates and that three determinants could be detected simultaneously. Multigene amplification of three *E. coli* virulence genes has been reported (9), but the amplification products could be detected only with corresponding labeled probes. Our findings have shown that a standard agarose gel electrophoresis procedure was sufficient for routine detection of the adhesion systems. By comparing the sizes of the amplified products to those of standard molecular weight markers, it was possible to identify the adhesin-encoding operon accurately.

The study showed that detection of the adhesion systems by the PCR method appears highly specific and is as reliable as the hybridization method. *pap* operons were found in 79.4% of the pyelonephritis strains, either alone (51.5% of the isolates) or in association with either the *afa* or *sfa/foc* operon (27.9%), whereas in 12.4 and 22.7% of the isolates the adhesive properties were associated with the presence of *afa* or *sfa/foc* operons, respectively. The simultaneous presence of *pap*, *afa*, and *sfa/foc* operons was never detected. These observations, which confirm the high prevalence of *pap* operons among *E. coli* strains associated with pyelonephritis, also indicate that the *afa* and *sfa/foc* operons represent uropathogenic determinants per se. The possibility that the frequency of these minor groups might increase if a vaccine directed solely against the PAP adhesins were used should be taken into consideration.

The PCR assay was more rapid than colony hybridization, since less than 4 h was necessary to obtain a result independently of the number of strains tested (up to 48).

E. coli is the most common cause of acute pyelonephritis, which is the most serious form of UTI, particularly harmful to newborns and small children (17). Surveys of patients with acute pyelonephritis have suggested that urinary tract abnormalities or medical intervention may allow nonpathogenic organisms access to the kidneys (15). Because of this, some researchers have proposed that the absence of adhesive properties in a pyelonephritis isolate could be used as an indicator of a possible underlying anatomic abnormality or medical illness, warranting further investigation of a patient (7, 21, 24). However, this approach is not currently used, nor is it well documented. Moreover, recurrent urinary infections are frequent and raise the question of the persistence of pathogenic bacteria within an area of the urinary tract. Hence, another possible application of the identification of the virulence factors of an *E. coli* isolate is as an epidemiological marker. Among the virulence factors of uropathogenic *E. coli*, the adhesive properties are easy to test for. The development of the PCR assay should facilitate detection of the adhesion determinants, which may be of use to clinicians as an indirect means of diagnosing underlying urinary disease. This assay is easy to perform, does not require special methods of DNA extraction and purification, and is inexpensive because it detects three common adhesin-encoding operons in a single reaction. Therefore, it should be useful for large-scale studies as well as for sporadic analysis of clinical strains of interest.

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