Applied and Environmental Microbiology, Apr. 2007, p. 2373–2377 Vol. 73, No. 7
0099-2240/07/$08.00+0 doi:10.1128/AEM.02341-06

Detection and Identification by PCR of a Highly Virulent Phylogenetic Subgroup among Extraintestinal Pathogenic *Escherichia coli* B2 Strains▽

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Received 4 October 2006/Accepted 27 January 2007

Closely related *Escherichia coli* B2 strains O1:K1, O2:K1, O18:K1, and O45:K1 constitute a major subgroup causing extraintestinal infections. A DNA pathoarray analysis was used to develop a PCR specific for this subgroup that was included in the multiplex phylogenetic-grouping PCR method. Our PCR may serve to identify this virulent subgroup among different ecological niches.

Escherichia coli is the main bacterial constituent of mammalian and avian gut aerobic microflora and a major cause of extra-intestinal infections. *E. coli* strains causing extraintestinal infections (extraintestinal pathogenic *E. coli* [ExPEC]) belong mainly to the phylogenetic group B2 (4, 5, 17). Among the B2 ExPEC group, strains harboring serotypes O2:K1, O18:K1, and O1:K1 have been shown to predominate (2, 21, 22) and to be closely related, according to several methods, including, recently, multilocus sequence typing (MLST) (1, 32), and they therefore represent a major human ExPEC phylogenetic subgroup. This subgroup includes, notably, the worldwide O18:K1:H7 strains causing cystitis and neonatal meningitis (18). We have previously shown that B2 strains belonging to these serotypes share the same ribotype that we designated $B2_1$ (7). The $B2_1$ ribotype also contains the neonatal meningitis O45:K1:H7 serotype strains that predominate in France and that are also present elsewhere in Europe (14, 29). Finally, analyzing several clinical collections of *E. coli* strains, we found that $B2_1$ was the ribotype most frequently encountered among urosepsis strains infecting non-host-compromised adults and young infants as well as among meningitis strains (5, 9).

Serogroup strains O2:K1, O18:K1, and O1:K1, similar to those of human ExPEC, also cause invasive diseases in animals, notably fatal avian septicemia (27, 33). Moreover, O45:K1:H7 strains resembling human neonatal meningitis strains have been sporadically described in cases of severe avian dermatitis (29). Thus, it has been suggested that poultry may be a vehicle for human ExPEC infection (27, 29).

All these observations imply that ribotype $B2_1$ strains might provide a key to understanding the pathogenetic mechanisms of invasive *E. coli* infections. However, ribotyping and MLST are costly and time consuming and are not suited to large-scale studies of the prevalence of this subgroup among human and animal ExPEC or to its detection within complex microflora.

In this study, we first applied MLST to published collections of $B2_1$ strains (O1:K1, O2:K1, O18:K1, and O45:K1) in order to determine the sequence type(s) (ST) to which ribotype $B2_1$ corresponds. We then used subtractive DNA pathoarray analysis to develop a PCR-based tool for the rapid identification of this highly virulent clonal group. Finally, we tested the capacity of this PCR method to detect these strains directly in human stools.

Highly virulent strains harboring ribotype $B2_1$ share a unique ST. To establish the correspondence between ribotype $B2_1$ and MLST data, we selected 23 and 16 *E. coli* strains of ribotype $B2_1$ and non-$B2_1$, respectively, from clinical and reference collections (Table 1) (2, 6, 7, 9, 24, 31). MLST was performed as described by Whittam et al. at the EcMLST website, using seven housekeeping genes (http://www.shigatox.net) (26).

All $B2_1$ strains had identical DNA sequences in the seven genes studied, corresponding to ST 29 of the EcMLST database, an ST previously attributed to only two strains, including the *E. coli* O18:K1:H7 neonatal meningitis (ECNM) strain RS218. This showed that the "O1, O2, O18:K1" clonal group (3, 30, 32) also encompasses a fourth serotype, 045:K1. This clonal group is designated $B2_1$/ST29 throughout this report.

DNA pathoarray-based identification of an *svg* open reading frame specific for the $B2_1$/ST29 clonal group. Open reading frames (ORFs) specific for O18:K1, without homologs on the *E. coli* K-12 MG1655 chromosome, were amplified by PCR with primers based on the incomplete chromosomal sequence of the O18:K1 ECNM strain RS218 (www.genome.wisc.edu) (2, 6, 28), because the UTI89 (uropathogenic *E. coli* isolate O18:K1:H7) sequence (11) was not yet available. DNA fragments specific for strain RS218 were sought in silico by using sequences of clones (GenBank accession numbers AF222070 to AF222307) generated by subtractive hybridization between O18:K1:H7 ECNM and nonpathogenic *E. coli* strains (10). Specific ORFs were amplified in sections of about 500 bp. The amplicons of 300 known or putative ORFs generated by PCR

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▽ Published ahead of print on 9 February 2007.

TABLE 1. *E. coli* strains belonging to ribotype $B2_1$ or to other ribotypes used for MLST and DNA pathoarray analyses

Ribotype and strain category[a]	Source[b]	Country	Phylogenetic group	Serotype	Ribotype/sequence type[c]
Ribotype $B2_1$					
S14	NM	Finland	B2	O18:K1	$B2_1$/ST29
C5	NM	United States	B2	O18:K1	$B2_1$/ST29
S26	NM	United States	B2	O18:K1	$B2_1$/ST29
S67	NM	France	B2	O18:K1	$B2_1$/ST29
S69	NM	France	B2	O18:K1	$B2_1$/ST29
RS218	NM	United States	B2	O18:K1	$B2_1$/ST29
S111	NM	United States	B2	O18:K1	$B2_1$/ST29
S126	NM	France	B2	O18:K1	$B2_1$/ST29
S32	NM	France	B2	O45:K1	$B2_1$/ST29
S50	NM	France	B2	O45:K1	$B2_1$/ST29
S53	NM	France	B2	O45:K1	$B2_1$/ST29
S72	NM	France	B2	O45:K1	$B2_1$/ST29
S88	NM	France	B2	O45:K1	$B2_1$/ST29
S132	NM	France	B2	O45:K1	$B2_1$/ST29
S174	NM	France	B2	O45:K1	$B2_1$/ST29
S136	NM	France	B2	O1:K1	$B2_1$/ST29
S158	NM	France	B2	O1:K1	$B2_1$/ST29
ECOR62	ECOR, P		B2	O2:K1	$B2_1$/ST29
HN7	P	France	B2	O2:K1	$B2_1$/ST29
HN30	P	France	B2	O2:K1	$B2_1$/ST29
HN50	P	France	B2	O2:K1	$B2_1$/ST29
NNC28	NC	France	B2	O2:K1	$B2_1$/ST29
NNC59	NC	France	B2	O2:K1	$B2_1$/ST29
Ribotype non-$B2_1$					
S114	NM	United States	A	O12:K1	A_{01}/ST171
S122	NM	United States	A	O12:K1	A_{01}/ST171
S82	NM	France	A	ONT:K1	A_{01}/ST171
K-12 (MG1655)		United States	A	O16:K^-	A_{01}/ST171
ECOR4	ECOR		A	ONT	A_{01}/ST167
ECOR15	ECOR		A	O25	A_{01}/STND
CFT073	P	United States	B2	O6:K2	$B2_6$/ST27
S107	NM	United States	B2	O16:K1	$B2_7$/ST304
S108	NM	United States	B2	O16:K1	$B2_8$/ST304
ECOR56	ECOR		B2	O6	$B2_6$/STND
ECOR59	ECOR		B2	O4	$B2_{J96}$/STND
ECOR35	ECOR		D	O1	D_{10}/STND
ECOR41	ECOR		D	O7	D_{04}/STND
S16	NM	United States	D	O7:K1	D_{04}/ST301
S39	NM	France	D	O7:K1	D_{04}/ST301
S18	NM	United States	D	O7:K1	D_{08}/ST301

[a] From ST7 of EcMLST. Origin and ribotype as previously described (2, 5, 6, 7, 9, 24, 31).
[b] NM, neonatal meningitis; P, pyelonephritis; NC, neonatal colonization; ECOR, *E. coli* reference collection.
[c] STND, ST not determined.

from chromosomal DNA were spotted in duplicate by a robot (Eurogentec, Belgium) on nylon membranes which were then hybridized and analyzed as previously described (25).

On our DNA array, only one ORF, of unknown function and coding for a hypothetical protein 277 amino acids long with no significant homology in the databases (now corresponding to GenBank accession number ABE08649 for *E. coli* strain UTI89) (11), hybridized with all the $B2_1$ strains and with none of the strains belonging to other groups and subgroups. We then assessed the specificity of this ORF, which we designated *s*pecific for *v*irulent subgroup (*svg*) ORF, by using a PCR method. A pair of primers (svg.1, 5′-TCCGGCTGATTACAA ACCAAC-3′; and svg.2, 5′-CTGCACGAGGTTGTAGTCCT G-3′) were designed to amplify a 434-bp fragment of the *svg* ORF. This PCR was then included, together with *uidA* (a β-glucuronidase gene) as a control amplification (16), in our triplex PCR method for phylogenetic group affiliation (12), with a modified protocol. Briefly, PCR was carried out in a 50-μl volume with 25 μl of 2× QIAGEN Multiple PCR Master Mix (QIAGEN, Courtaboeuf, France), 5 μl of 5× Q-solution, 1 μM of each primer, and 5 μl of bacterial lysate. PCR was performed as follows: DNA denaturation and polymerase activation for 15 min at 95°C; 30 cycles of 30 s at 94°C, 90 s at 55°C, and 90 s at 72°C; and a final extension step for 10 min at 72°C. Samples were electrophoresed as previously described (8) (Fig. 1A). This PCR screening test was then applied to 340 *E. coli* strains (including 97 strains of ribotype $B2_1$) belonging to previously published collections (5, 7, 9) (Table 2). In addition, 13 avian pathogenic *E. coli* (APEC) strains were assayed by PCR. Eight of them were B2 strains belonging to ribotype $B2_1$: one O1:K1 strain (LDA 6042253), three O2:K1 strains (LDA 5063391, LDA 5067912, and LDA 6081105), and four O45:K1 strains (BEN1068, BEN1082, BEN1090, and BEN1354). The five other APEC strains were group D strains belonging to

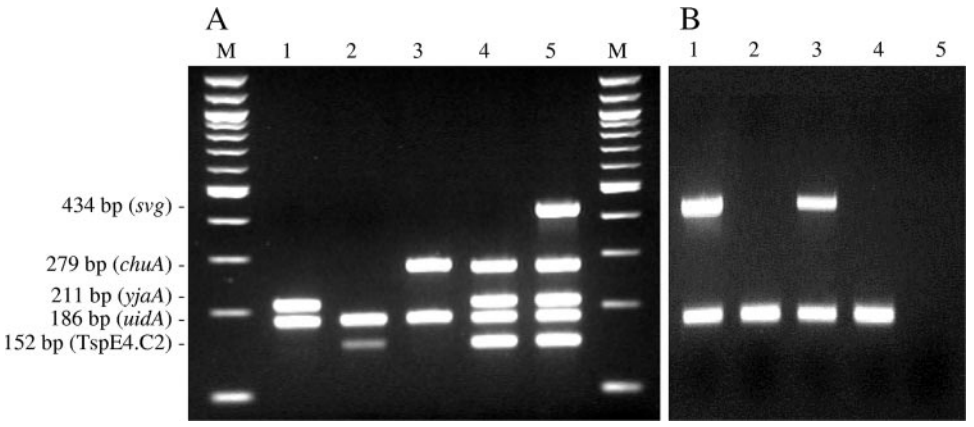

FIG. 1. (A) Pentaplex PCR combining the amplification of a 434-bp fragment of the *svg* ORF specific for highly virulent $B2_1$/ST29 *E. coli* strains; *uidA* (β-glucuronidase gene) was used as a control (16), and *chuA* (outer membrane hemin receptor gene) and *yjaA* (unknown function) and DNA fragment TspE4.C2 were used for phylogenetic group affiliation (12). M, molecular weight marker (100-bp DNA ladder; New England BioLabs); lanes 1, 2 and 3, strains belonging to phylogenetic groups A, B1, and D, respectively; lane 4, reference strain belonging to the phylogenetic group B2 but not to $B2_1$/ST29 (strain CFT073); lane 5, meningitis strain belonging to $B2_1$/ST29 (strain S88). (B) Biplex PCR combining the amplification of a 434-bp fragment of ORF *svg* (upper band) together with *uidA* (β-glucuronidase gene, lower band), used as a control (16), for the detection of highly virulent $B2_1$/ST29 *E. coli* strains directly in stool samples. Lanes 1 and 3, stool samples harboring $B2_1$/ST29 *E. coli* strains (*svg*-positive PCR); lane 2 and 4, stool samples harboring *E. coli* strains not belonging to $B2_1$/ST29 subgroup; lane 5, negative control.

serogroup O1 (LDA 6072791) and to serogroup O45 (BEN0058, BEN0214, BEN0289, and BEN0456). Strains "LDA" and strains "BEN" were kindly provided by Hervé Morvan (Laboratoire de développement et d'analyses des Côtes d'Armor, Ploufragan, France) and Maryvonne Moulin-Schouleur (INRA, Centre de Tours, UR1282 IASP, Pathogenie Bacterienne, Nouzilly, France), respectively. The results showed perfect specificity and sensitivity for the *svg* PCR for the identification of $B2_1$/ST29 strains (Table 2).

***svg* amplification for $B2_1$/ST29 strain identification in a complex microflora.** We then tested the capacity of the *svg* ORF simplex PCR to detect $B2_1$/ST29 strains in a complex microflora. Specificity was first tested with 52 strains belonging to different *Enterobacteriaceae* species and with 30 stool samples that were culture negative for *E. coli*, as described below (Table 2). None of these strains or stool samples was positive, suggesting that the *svg* ORF is restricted to *E. coli*. We then applied the new method to 92 stool samples from healthy children who were culture positive for *E. coli* on two different types of samples: (i) 10 colonies of *E. coli* strains obtained by stool cultured on Uriselect 4 chromogenic medium (Bio-Rad, Marnes-la-Coquette, France) and (ii) whole-stool samples, as previously described (23). Briefly, about 100 μg of stool was mixed with 9 ml of peptone water and incubated at 37°C for 4 h. After centrifugation (1500 rpm for 10 min), the supernatant was boiled and 5 μl was used as template DNA for the PCR. Amplification of the gene *uidA* was used to check for PCR inhibitors (Fig. 1B). The *svg* PCR was positive for 10% of cases (9/92) of the predominant isolates and 14% (13/92) of the stool samples. Four specimens were thus PCR positive only for stool samples, suggesting that $B2_1$/ST29 strains may be a subdominant *E. coli* population in the microflora of some healthy children.

Serotype strains O1:K1, O2:K1, and O18:K1 encountered in human extraintestinal infections were recently shown to cluster within a single sequence type (ST95), based on the MLST method described by Achtman et al. (www.mlst.net) (19, 30, 32). However, several other sets of MLST target genes have been published, and no reference set of genes has yet been established (15, 26). In our study, using the set of genes cited by Whittam et al., we confirmed that strains O1:K1, O2:K1, and O18:K1 (all ribotype $B2_1$ in our study) clustered in a single ST (EcMLST, ST29) and that this clonal group includes meningitis strains of serotype O45:K1. Considering that (i) ST29 corresponds perfectly to ribotype $B2_1$, the leading ribotype causing septicemia in non-host-compromised humans (5, 7, 9) and (ii) since serotype strains O1:K1, O2:K1, O18:K1, and O45:K1 are major causes of fatal bacteremia in birds (29, 33), it would be useful to be able to detect this highly virulent ExPEC subgroup both rapidly and simply. By using DNA array technology, we developed a PCR test for inclusion in our multiplex phylogenetic grouping PCR (12), one of the most widely used methods for determining the main phylogenetic groups (A, B1, B2, and D). The resulting pentaplex PCR test will be helpful for determining in a unique reaction both the main phylogenetic group of a strain and its affiliation with the highly virulent subgroup $B2_1$/ST29.

We also showed that an *svg* PCR applied to stool samples is more sensitive than culture for $B2_1$/ST29 *E. coli* strains. These results are consistent with previous reports suggesting that *E. coli* strains harboring virulence factors may represent a minor subpopulation of the fecal microflora that might not be detected by colony sampling (20). To our knowledge, this is the first PCR-based approach to permit the detection of *E. coli* strains belonging to a particular phylogenetic subgroup directly within a complex bacterial population. In addition to its value as an epidemiological tool, our *svg* PCR method may prove useful in several fields of human and veterinary medicine. For example, it could serve for early identification (and treatment) of human neonates colonized by such *E. coli* strains and to

TABLE 2. Results of *svg* ORF PCR specific for highly virulent $B2_1$/ST29 *E. coli* strains, applied to *E. coli* collections and other *Enterobacteriaceae* species

E. coli strain and phylogenetic group (subgroup) (reference)	No. of strains	*svg* ORF-positive PCR
ECOR strains ($n = 72$) (13)		
A	25	0
B1	16	0
B2 ($B2_1$)	2	2
B2 (other than $B2_1$)	13	0
D	12	0
E	4	0
Meningitis strains ($n = 132$) (7)		
A	11	0
B1	2	0
B2 ($B2_1$)	69	69
B2 (other than $B2_1$)	30	0
D	20	0
Young infant urinary tract infection strains ($n = 36$) (9)		
A	1	0
B1	1	0
B2 ($B2_1$)	7	7
B2 (other than $B2_1$)	25	0
D	2	0
Adult uropathogenic strains ($n = 100$) (5)		
A	11	0
B1	1	0
B2 ($B2_1$)	19	19
B2 (other than $B2_1$)	42	0
D	27	0
APEC strains ($n = 13$)		
B2 ($B2_1$)	8	8
D	5	0
Other *Enterobacteriaceae* species ($n = 52$)		
Shigella (*S. flexneri*, $n = 2$; *S. sonnei*, $n = 3$)	5	0
Salmonella enterica	6	0
Enterobacter (*E. cloacae*, $n = 5$; *E. aerogenes*, $n = 1$)	6	0
Escherichia (*E. hermanii*, $n = 1$; *E. vulneris*, $n = 1$)	2	0
Hafnia alvei	2	0
Yersinia (*Y. pseudotuberculosis*, $n = 3$; *Y. enterocolitica*, $n = 1$)	4	0
Serratia (*S. liquefaciens*, $n = 1$; *S. marcescens*, $n = 2$)	3	0
Proteus mirabilis	4	0
Providencia stuartii	1	0
Morganella morganii	1	0
Citrobacter (*C. freundii*, $n = 3$; *C. koseri*, $n = 3$; *C. youngae*, $n = 1$)	7	0
Klebsiella (*K. pneumoniae*, $n = 4$; *K. oxytoca*, $n = 3$)	7	0
Raoultella terrigena	1	0
Leclercia adecarboxylata	1	0
Pantoea agglomerans	2	0

screen avian hosts in industrial animal husbandry. Finally, this method could serve as a cost-effective first-line screening test in an *E. coli* serogrouping assay, as $B2_1$/ST29 strains belong mainly to only four serogroups.

We thank Colin Tinsley for helpful discussions.

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