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Use of Diagnostic Microarrays for Determination of Virulence Gene Patterns of *Escherichia coli* K1, a Major Cause of Neonatal Meningitis

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Forty *Escherichia coli* strains isolated primarily from neonatal meningitis, urinary tract infections and feces were screened for the presence of virulence genes with a newly developed microarray on the array tube format. A total of 32 gene probes specific for extraintestinal as well as intestinal *E. coli* pathotypes were included. Eighty-eight percent of the analyzed strains were positive for the K1-specific probe on the microarray and could be confirmed with a specific antiserum against the K1 capsular polysaccharide. The gene for the hemin receptor ChuA was predominantly found in 95% of strains. Other virulence genes associated with K1 and related strains were P, S, and F1C fimbriae specific for extraintestinal *E. coli*, the genes for aerobactin, the α-hemolysin and the cytotoxic necrotizing factor. In two strains, the O157-specific catalase gene and the gene for the low-molecular-weight heat-stable toxin AstA were detected, respectively. A total of 19 different virulence gene patterns were observed. No correlation was observed between specific virulence gene patterns and a clinical outcome. The data indicate that virulence genes typical of extraintestinal *E. coli* are predominantly present in K1 strains. Nevertheless, some of them can carry virulence genes known to be characteristic of intestinal *E. coli*. The distribution and combination of virulence genes show that K1 isolates constitute a heterogeneous group of *E. coli*.

Escherichia coli is the most common causative agent of gram-negative neonatal bacterial meningitis and sepsis. Bacterial meningitis is a devastating disease and the major cause of high neonatal mortality and morbidity (40). More than half of the survivors develop long-term neurological sequelae, including seizure disorders, hydrocephalus, physical disability, developmental delay, and hearing loss (41). Most infections occur in the first month of life with a frequency of 0.22 to 2.66 per 1,000 live births worldwide (13, 34).

The development of sepsis and meningitis in the neonate depends on several risk factors in both the infant and the mother, as well as on the virulence of the pathogen. Prematurity, prolonged rupture of membranes, and low birth weight but also perinatal, intrauterine infections and maternal urinary tract infections are strongly associated with neonatal meningitis. The mode of infection of the neonate may be either hematogenous (transplacental) or directly through aspiration or inhalation of the pathogen (34). An early onset of neonatal bacterial meningitis (within the first week of life) indicates vertical transmission, whereas later onset is mainly caused by nosocomial infection.

It is known that strains possessing the K1 capsular polysaccharide are responsible for approximately 80% of *E. coli* neonatal bacterial meningitis cases and are strongly associated with infections occurring in the first 3 weeks of life rather than in older infants (16, 22, 36). Moreover, *E. coli* K1 strains belong to the normal flora and are detected in 20 to 40% of rectal swab cultures from healthy infants, children, and adult women as well as in vaginal swabs (27, 36).

The K1 encapsulated pathogen is important for bacteremia and possesses the ability to cross the blood-brain barrier (19, 20, 35). The molecular pathophysiology of meningitis is complex and not completely understood. Pathogenic *E. coli* is defined by a number of major virulence factors, including attachment functions, host cell surface-modifying factors, invasins, and different toxins and secretion systems (for a recent review, see reference 17). Based on the virulence mechanism, epidemiology, and clinical presentation, *E. coli* strains can be divided into two major categories, intestinal and extraintestinal pathogens. The first category comprises various pathotypes, including enterotoxigenic *E. coli*, enteropathogenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, and Shiga toxin-producing *E. coli*, comprising the subgroup of enterohemorrhagic *E. coli*. Extraintestinal pathogenic *E. coli* has been categorized mainly into uropathogenic *E. coli* and neonatal meningitis-causing *E. coli*.

The genome of *E. coli* shows a high plasticity, which enables it to gain or lose genes or modify their loci on the genome at a relatively high frequency (10, 31). Contributing to that are virulence plasmids and the chromosomal pathogenicity islands, which are mobile genetic elements (6, 12). This particular plasticity allows *E. coli* strains to share virulence genes within the species at a high rate, a process called lateral gene transfer. This allows the formation of new pathotypes with new virulence combinations (38).

Numerous phenotypic and genotypic methods have been

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employed to detect, classify, and type pathogenic *E. coli* strains for clinical diagnosis, epidemiological investigations, or routine surveillance. Serotyping is the most common method used for identification of virulent *E. coli* clones. Specific serogroups can be associated reproducibly with certain clinical syndromes, as is the case with K1 strains. However, with serotyping, pathogenic strains are only indirectly identified, and it does not always correlate to virulence. The PCR technique helps to determine the presence of certain genes, but its high specificity makes it impossible to detect variants of genes. Moreover, the throughput of PCR methods is relatively low since only a few genes can be detected in a single reaction.

Recently, the very promising method of DNA array analysis was introduced to provide information about the presence of virulence genes in the *E. coli* genome. This technology has been applied with membrane arrays as well as glass arrays in various formats ranging from low density with few genes to higher density arrays including hundreds of probes (4, 10, 23, 42). Usually, the use of those techniques is labor intensive, time consuming, and quite expensive, which make them useful only for research laboratories and almost nonapplicable for routine diagnostic purposes.

An appropriate and fast detection of the virulence genes present in *E. coli* would help in defining the pathotypes of bacteria and could improve diagnostics, prevention, and surveillance. For this aim we have established a prototype diagnostic microarray test including a set of known *E. coli* virulence genes. The technique was established with reference strains and evaluated for diagnostics by screening a series of *E. coli* K1 strains to investigate the virulence gene pattern of such isolates.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and genomic DNA extraction. A total of 40 *E. coli* strains isolated from clinical cases (predominantly from newborn babies with meningitis) or previously typed as K1 isolates from sepsis, urinary tract infection, or feces were analyzed (Table 1). Strains previously used for the development of a membrane (23) or glass-slide based array system (42) and described there were included for validating the new microarray (Table 3). In addition, the following reference strains were used: bovine enterotoxigenic *E. coli* JF2762 (B44; fhu^+ fim^+ stI^+ K99$^+$), porcine enterotoxigenic *E. coli* JF1264 ($eltI^+$ cfa/II^+ hly^+ K88$^+$), human enterotoxigenic *E. coli* DS15-1 ($eltI^+$ fhu^+ cfa/II^+ fim^+ stI^+ ast^+ cof^+ lng^+), human Shiga toxin-producing *E. coli* N2611-99 (stx_1^+ fhu^+ cnf^+ fim^+ iuc^+ ast^+ cdt^+), and enterohemorrhagic *E. coli* O157:H7 (EDL933; stx_1^+ stx_2^+ fhu^+ eae^+ fim^+ ehx^+ ast^+ chu^+ $katP^+$). The *E. coli* K-12 strain XL1 Blue (Stratagene, Amsterdam, The Netherlands) was used as a nonpathogenic negative control.

For probe construction of the K88 and K99 fimbrial genes, strains JF1264 and JF2762 were used, respectively. All strains were grown aerobically at 37°C overnight on trypticase soy agar plates containing 5% sheep blood (Oxoid, Wesel, Germany). *E. coli* DH5α was used as a host strain for cloning and propagation of plasmids. Genomic DNA was isolated with the E.Z.N.A bacterial DNA kit (PEQLAB Biotechnologie GMBH, Erlangen, Germany) or by the method described by Pitcher et al. (33). All DNA samples were treated with RNase.

Agglutination. Overnight cultures of *E. coli* were serotyped by agglutination with a K1-specific horse antiserum (29).

Construction of an *E. coli* virulence gene array. The array was generated with a total of 32 probes. Twenty-eight of the probes have been previously described (23, 42). The remaining four probes contain parts of additional *E. coli* virulence factors, the cytolethal distending toxin gene (*cdtA*), the catalase-peroxidase gene (*katP*), the K88 pilin gene, and the K99 pilin gene, and were constructed in this study. The fragments of genes were amplified by PCR with genomic DNA. The primers used for amplification are listed in Table 2. Details of probe construction are given in the Results section. The primers were selected on the basis of available DNA sequences. Amplicons were purified with the High Pure PCR

TABLE 1. *E. coli* isolates analyzed in the study

Strain	Origin or clinical setting[a]	K1 agglutination	Pattern no.[b]
JF2840	NBM	+	12
JF2841	NBM	+	13
JF2842	NBM	+	15
JF2983	NBM	+	5
JF2984	NBM	+	7
JF2985	NBM	+	11
JF2986	NBM	+	17
JF2987	NBM	−	20
JF2988	NBM	+	7
JF2989	NBM	−	23
JF2990	NBM	−	20
JF2991	NBM	−	21
JF3051	NBM	+	7
JF2843	Sepsis	+	11
JF2993	Sepsis	−	22
JF2994	Sepsis	+	18
JF2844	UTI	+	13
JF2845	UTI	+	2
JF3038	UTI	+	7
JF3049	UTI	+	10
JF3034	Appendicitis	+	11
JF2837	Feces	+	5
JF2838	Feces	+	1
JF2839	Feces	+	16
JF3035	Feces	+	14
JF3036	Feces	+	14
JF3037	Feces	+	7
JF3039	Feces	+	7
JF3041	Feces	+	7
JF3046	Feces	+	7
JF3053	Feces	+	19
JF3044	Animal	+	3
JF3048	Animal	+	13
JF3052	Animal	+	7
JF3054	Animal	+	3
JF3040	Clinical isolate	+	8
JF3042	Clinical isolate	+	3
JF3043	Clinical isolate	+	4
JF3045	Clinical isolate	+	9
JF3050	Clinical isolate	+	6

[a] NBM, newborn bacterial meningitis; UTI, urinary tract infection.
[b] Corresponding to the patterns described in Table 4.

Product Purification kit (Roche Diagnostic, Mannheim, Germany), digested with the appropriate enzyme (Roche), and cloned into plasmid pBluescript II SK (−) (Stratagene).

Ultrapure PCR fragments used for array printing were produced as described previously (23). In short, fragments were cut out from the plasmid by corresponding restriction enzymes. The fragments were purified twice over agarose gels in order to eliminate plasmid contamination that would result in nonspecific hybridization signals. These doubly purified inserts were then used for PCR amplification of fragments in amounts suitable for spotting. The PCR products were purified and array printing as well as assembly of the array tubes containing the *E. coli* specific virulence genes was done by Clondiag Chip Technologies GmbH, Jena, Germany (www.clondiag.com).

Sequencing. For quality control, all clones were further verified by sequencing with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystem, Foster City, Calif.) according to the supplier's guidelines with either the T3 or T7 vector matching primers on an automated ABI Prism 3100 genetic analyzer (Applied Biosystems). Comparisons of DNA sequences in the EMBL and Gen-Bank databases were performed with BLAST (3).

DNA labeling. For the random amplification and labeling of total genomic DNA, an adapted method of Bohlander et al. (7) modified by DeRisi Laboratory was followed (www.microarrays.org/pdfs/Round_A_B_C.pdf). The whole procedure consisted of three rounds of enzymatic reactions. In a first round approximately 0.1 to 0.5 μg of genomic DNA was mixed with 0.5 μl of 5× Sequenase reaction buffer (supplied with Sequenase; USB, Cleveland, Ohio) and 0.5 μl of

TABLE 2. Primers used for amplification of virulence gene probes

Plasmid	Gene	Primer	Sequence	Length (nt)	Reference	Accession no.
pJFFECCDT	cdtA	ECCDT-L	GCGGAATTCTCAAGTAGAGGGAGGACCA	598	15	AJ508930
		ECCDT-R	GCGGAATTCTGGCTTAACAATAGTGGC			
pJFFECKAT	katP	ECKAT-L	GCGGAATTCACATTTCGTGTGACTGATT	699	9	X89017
		ECKAT-R	GCGGAATTCATCCGAGGCATATACTTCT			
pJFFECK88	K88	ECK88-L2	TGCTCTAGAATCGGTGGTAGTATCACTGC	595	11	M25302
		ECK88-R2	CCGCTCGAGAACCTGCGACGTCAACAAGA			
pJFFECK99	K99	ECK99-L	GCGGAATTCTGCGACTACCAATGCTTCTG	450	11	M35282
		ECK99-R	GCGGAATTCTATCCACCATTAGACGGAGC			

the random primer A (5′-GTT CCC AGT CAC GAC TCN NNN NNN NN) (40 μM), where N is any nucleotide. The solution was denatured for 2 min at 94°C with a thermal cycler (GeneAmp PCR System) and rapidly cooled down to 10°C and held for 5 min. At this point 1.25 μl of Sequenase mix was added. The Sequenase mix was prepared for four reactions and consisted of 1 μl of 5× Sequenase reaction buffer, 1.5 μl of 3 mM deoxynucleoside triphosphates, 0.75 μl of 0.1 M dithiothreitol, 1.5 μl of bovine serum albumin (500 μg/μl), and 0.4 μl of Sequenase version.2 DNA polymerase (13 U/μl). The DNA was randomly amplified at temperatures raising from 10°C to 37°C for 8 min and then kept at 37°C for 8 min before it was rapidly heated to 94°C for 2 min. The mixture was cooled down again to 10°C and kept at this temperature for 5 min while adding 0.3 μl of Sequenase diluted 1:4 in Sequenase enzyme dilution buffer (supplied with Sequenase). The reaction was repeated with temperatures rising from 10°C to 37°C for 8 min and kept at 37°C for 8 min. Finally, 11 μl of diluted water was added to each sample.

In the second round, randomly obtained fragments were amplified with specific primer B (5′-GTT TCC CAG TCA CGA TC). The reaction was performed as follows: 1× PCR buffer (supplied with Taq DNA polymerase), 1 mM deoxynucleoside triphosphate, 2.5 U of Taq DNA polymerase (Roche), 40 pmol of primer B, 7.5 μl of template from the first round, and deionized water up to a final volume of 50 μl. PCR conditions were as follows: 25 cycles at 94°C for 30 s, 40°C for 30s, 50°C for 30 s, and 72°C for 2 min. For quality control, 5.0 μl of the PCR product was run on a 1% agarose gel. In the last step, 3.5 μl of this PCR product was used for the labeling reaction. PCR was run under the same conditions as in the previous step, with 1× PCR buffer, 100 μM each dATP, dCTP, and dGTP, 65 μM dTTP, 60 μM biotine-16-dUTP (Roche), 2.5 U of Taq DNA polymerase, 40 pmol of primer B, and deionized water up to 25 μl.

Array hybridization. The array tubes were placed in a thermomixer (Eppendorf, Hamburg, Germany), washed three times with 500 μl of deionized water and once with 500 μl of hybridization buffer (Clondiag Chip Technologies), each time at 55°C for 5 min and 550 rpm. Usually, 10 μl of labeled genomic DNA was diluted in 100 μl of hybridization buffer (Clondiag Chip Technologies), denatured at 94°C for 5 min, cooled down on ice for 2 min, and added to the array tubes. The hybridization was carried out at 55°C overnight by gentle shaking in a hybridization oven. After hybridization the array tubes were washed with 500 μl of 2× sodium chloride-sodium citrate (SSC)--0.2% sodium dodecyl sulfate (SDS) at 30°C for 5 min in the thermomixer at 550 rpm, followed by 500 μl of 2× SSC at 20°C for 5 min at 550 rpm and finally with 0.2× SSC at 20°C for 5 min at 550 rpm. A blocking step was carried out with 2% (wt/vol) milk powder in 100 μl of 6X SSPE–0.005% Triton at 30°C for 15 min. Poly-horseradish peroxidase-streptavidin conjugate (100 pg/μl) was added in 100 μl of 6× SSPE--0.005% Triton and incubated at 30°C for 15 min at 550 rpm. The previous washing procedure was repeated after blocking with 2× SSC--0.01% Triton, 2× SSC, and 0.2× SSC. Array tubes were kept in the last buffer at 20°C until further processing.

Visualization of hybridization was achieved by adding 100 μl of peroxidase substrate (Clondiag Chip Technologies) to the array tubes, and detection of signals was done in the array tube reader (ATR01, Clondiag Chip Technologies). Signals were recorded at 25°C for 15 min. The data were then analyzed with the IconoClust version 2.2 software (Clondiag Chip Technologies).

RESULTS

Probe construction. In addition to the 28 virulence gene probes developed earlier (23, 42), four new probes were constructed for the array tube in order to broaden the spectrum of detectable virulence genes. They include cdtA and katP, found in the highly pathogenic enterohemorrhagic E. coli strains, and probes for the K88 and K99 fimbrial genes of two important animal pathogens. Plasmid pJFFECCDT, which contains the cdtA gene, specific for the cytolethal distending toxin of enterohemorrhagic E. coli strains (15), was generated by cloning a 600-bp fragment amplified from enterohemorrhagic E. coli N2110_01 with the primers ECCDT-L and ECCDT-R (Table 2). The PCR fragment was digested with EcoRI and cloned into the corresponding site of pBluescript. The gene probe was then amplified from the purified EcoRI insert of plasmid pJFFECCDT with primers ECCDT-L and ECCDT-R.

The plasmid pJFFECKAT was constructed by amplifying a 700-bp fragment of the katP gene with primers ECKAT-L and ECKAT-R (Table 2) from strain N2110_01. The fragment specific for strains of the O157 serotype (9) was digested with EcoRI and cloned accordingly. Preparation of the specific katP probe was achieved by cutting out the insert with EcoRI and amplifying the purified fragment with primers ECKAT-L and ECKAT-R. Plasmid pJFFECK88 contains an insert specific for porcine enterotoxigenic E. coli K88 strains, causing diarrhea and edema disease in piglets (11). It contains the 600-bp amplicon generated by PCR with strain JF1264 and primers ECK88-L and ECK88-R (Table 2), digestion with XbaI and XhoI, and cloning into pBluescript. The probe was then generated by excision with XbaI and XhoI followed by PCR on the purified insert with primers ECK88-L and ECK88-R. Finally, pJFFECK99, specific for K99 fimbriae of bovine enterotoxigenic E. coli strains (11), was generated with primers ECK99-L and ECK99-R (Table 2) and DNA from strain JF2762. The resulting PCR product was cloned into the EcoRI site of pBluescript. After digestion with EcoRI and purification of the insert, the probe was amplified with primers ECK99-L and ECK99-R.

Evaluation of the diagnostic microarray. The performance of the array tube system was tested with a series of reference strains (Table 3). Analysis of the data with the IconoClust software allowed the definition of threshold values for positive signals. Every spot showing an intensity higher than the determined local background was counted as a positive signal. All the probes spotted on the array tube showed up (Table 3). For the gene of the heat-labile toxin II, no reference strain containing this gene was available.

As an example, the array tube hybridization with the reference strains enterohemorrhagic E. coli EDL933, uropathogenic E. coli J96, and K1 isolate JF3053 analyzed in this study is shown in Fig. 1. Hybridization results were further compared with the other methods established for broad-range virulence

TABLE 3. Virulence gene profiles of reference *E. coli* strains[a]

Gene probe	Enterotoxigenic								Enteropathogenic	Enteroinvasive	Enterohemorrhagic		Shiga toxin-producing	Enteroaggregative	Oropathogenic			Neonatal meningitis	K-12
	34344f	NZ3211-94	C9221a	IMI100	DS15-1	JF1264	ORG178-03	JF2762	NZ1743-95	NZ1679-94	EDL933	NZ4253-91	NZ2611-99	NZ1470-95	RZ475	J96	536	IHE3034	XL1-Blue
Shiga toxin 1 (stx_1)											•	•	•						
Shiga toxin 2 (stx_2)											•	•	•						
Heat-labile toxin I (*eltIA*)																			
Heat-labile toxin II (*eltIIA*)																			
Aggregative adhesion fimbria (*aafII*)														•					
Ferrichrome-iron receptor (*fhuA*)	•	•		•	•						•	•	•		•	•	•	•	
Bundle-forming pilus (*bfpA*)									•										
Colonization factor antigen I (*cfaI*)	•	•	•	•	•	•	•	•											
Cytotoxic necrotizing factor (*cnf*)				•					•										
Colonization factor antigen II (*cfaII*)	•	•	•	•				•											
Intimin (*eaeA*)									•		•	•	•						
FIC fimbria (*foc*)																			
Type 1 fimbria (*fimA*)	•	•		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Invasion plasmid antigen (*ipaH*)						•	•			•									
Aerobactin (*iucC*)										•	•		•	•					
K1 capsule (*neuC*)															•	•	•	•	
K5 capsule (*kfiB*)															•	•			
P fimbria (*papA*)															•	•	•	•	
S fimbria (*sfaS*)															•	•	•		
Heat-stable toxin (*stIA/B*)	•	•	•	•	•	•	•												
S fimbria (*sfaA*)															•	•	•	•	
α-Hemolysin (*hlyA*)											•	•				•	•		
Enterohemorrhagic hemolysin (*ehxA*)											•	•							
Heat-stable toxin (*astA*)		•		•	•														
Colonization factor antigen III (*cofA*)					•														
Longus filus (*lngA*)					•														
Hemin receptor (*chuA*)									•		•	•	•	•	•	•	•	•	
16S rRNA gene (*rrs*)	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
Catalase (*katP*)											•	•							
Cytolethal distending toxin (*cdtA*)						•							•	•					
K88 fimbria (K88)								•											
K99 fimbria (K99)																			

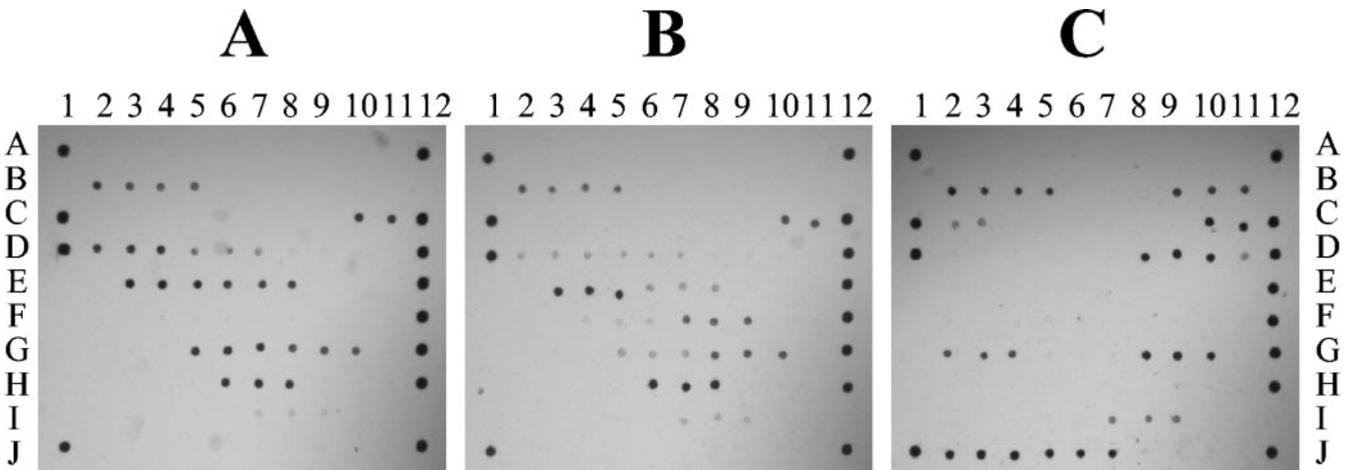[a] Positive signals are indicated by a dot.

FIG. 1. Microarray results of different clinical *Escherichia coli* strains with the array tube. Panel A: hybridization with the uropathogenic reference strain *E. coli* J96. Panel B: hybridization with JF3053, isolated from a case of neonatal meningitis. Panel C: hybridization with the enterohemorrhagic reference strain EDL933. The DNA probe arrangement on the array tube is as follows: A2-4, K88 fimbria (*K88*); A5-7, K99 fimbria (*K99*); B2 and C10-11, hemin receptor (*chuA*); B3-5, 16S rRNA gene (*rrs*); B6-8, cytolethal distending toxin (*cdtB*); B9-11, catalase-peroxidase (*katP*); C2-3, D11, heat-stable toxin (*astA*); C4-6, colonization factor antigen III (*cofA*); C7-9, longus pilus (*lngA*); D2-4, S fimbria (*sfaS*); D5-7, α-hemolysin (*hlyA*); D8-10, enterohemorrhagic *E. coli* hemolysin (*ehxA*); E2 and F10-11, K5 capsule (*kfiB*); E3-5, P fimbria (*papA*); E6-8, S fimbria (*sfaA*); E9-11, heat-stable toxin (*stIA/B*); F2-3 and G11, invasion plasmid antigen (*ipaH*); F4-6, aerobactin (*iucC*); F7-9, K1 capsule (*neuC*); G2-4, intimin (*eae*); G5-7, F1C fimbria (*foc*); G8-10, type 1 fimbria (*fimA*); H2 and I10-11, bundle-forming pilus (*bfpA*); H3-5, colonization factor antigen I (*cfa/I*); H6-8, cytotoxic necrotizing factor (*cnf*); H9-11, colonization factor antigen II (*cfa/II*); I2-3 and J11, heat-labile toxin II (*eltIIA*); I4-6, aggregative adhesion fimbria (*aaf/I*); I7-9, ferrichrome-iron receptor (*fhuA*); J2-4, Shiga toxin 1 (*stx$_1$*); J5-7, Shiga toxin 2 (*stx$_2$*); J8-10, heat-labile toxin I (*eltIA*); columns 1 and 12, biotin-modified DNA sequence dots.

gene detection. All spots expected to be positive from the membrane (23) and the glass-slide format (42) could be detected with the conditions optimized for the array tube. There was one discrepancy for the F1C probe signal in strain IHE 3034. Whereas there was a clear positive signal on the membrane (23) as well as the array tube, this gene was missed in this strain with the classical glass-slide microarray (42). However, PCR analysis with F1C gene-specific primers (23) confirmed that the gene is present in this strain. Similarly, the somewhat surprising positive signal for the aerobactin gene in strain N2611-99 could be confirmed by PCR.

Screening of clinical isolates. A total of 40 strains isolated from clinical cases or previously typed as K1 by agglutination were screened with the diagnostic microarray. The results are summarized in Table 4. All strains showed signals with the positive controls for 16S rRNA (*rrs*) and type 1 fimbriae (*fimA*). The *fhuA* gene encoding the ferrichrome iron receptor of the low-efficiency iron transport system was positive for 31 strains (78%). Thirty-five of the 40 strains showed a clearly positive signal with the K1-specific probe (88%). All these positive strains could be confirmed phenotypically by agglutination with a K1-specific antiserum. Five strains were negative for the K1 capsule genetically as well as by the agglutination assay. One of the non-K1 strains did not show any positive signal with the virulence probes on the microarray (pattern 23, Table 4).

TABLE 4. Virulence gene patterns of *E. coli* K1 and related strains analyzed in this study[a]

Gene probe	Pattern no.																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
K1 capsule (*neuC*)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
P fimbria (*pap*)				●	●	●	●	●	●	●	●			●	●	●	●	●	●	●	●	●	
S fimbria (*sfaA*)											●	●	●	●			●	●	●	●	●	●	
S fimbria (*sfaS*)											●	●	●	●			●	●	●	●	●	●	
FIC fimbria (*foc*)					●					●	●	●		●			●	●	●	●	●	●	
α-Hemolysin (*hlyA*)										●						●	●	●	●	●			
Cytotoxic necrotizing factor (*cnf*)																●	●	●	●	●			
Aerobactin (*iucC*)						●	●	●	●	●	●									●	●		
Hemin receptor (*chuA*)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	
K5 capsule (*kfiB*)																						●	
Heat-stable toxin (*astA*)																		●					
Catalase (*katP*)						●																	
16S rRNA gene (*rrs*)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Type 1 fimbria (*fimA*)	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Ferrichrom-iron receptor (*fhuA*)				●	●	●	●	●	●	●	●		●	●			●	●	●	●	●		

[a] Only probes hybridizing with one or more strains are listed. Negative probes are mentioned in the text. Positive signals are indicated by a dot.

The gene for the cytotoxic necrotizing factor was found in six strains (15%). The probe for F1C fimbriae (*foc*) showed positive signals in 19 strains (48%), the one for the P fimbriae in 26 (65%), and the S fimbrial genes *sfaS* and *sfaA* were concomitantly detected in 14 (35%) of the isolates. The α-hemolysin gene could be identified in nine (23%) strains. Genes indicating high-efficiency iron transport systems could be found in 20 (50%) strains in the case of the aerobactin gene *iucC* and in 38 (95%) strains in the case of the hemin receptor gene *chuA*. A single strain was positive for the *katP* gene, another one for the *astA* gene, and one of the non-K1 strains hybridized with the gene specific for the K5 capsule (*kfiB*). The positive signals for the probes *katP* and *astA*, specific for intestinal pathogens, detected in strains JF3050 and JF2994, respectively, were confirmed by PCR.

Probes included on the array tube that did not hybridize to any of the clinical isolates are the ones for the two Shiga toxins (*stx1* and *stx2*), the two heat-labile toxins (*eltI* and *eltII*), the heat-stable toxins (*stIA* and *stIB*), the aggregative adhesion fimbria (*aafI*), the bundle forming pilus (*bfpA*), the colonization factor antigens I, II, and III (*cfa*, CS3, *cof*), the intimin (*eaeA*), the invasion plasmid antigen (*ipaH*), the enterohemorrhagic *E. coli* hemolysin (*ehxA*), the longus pilus (*lng*), the cytolethal distending toxin (*cdt*), as well as the gene probes for the K88 and K99 fimbrial antigens.

DISCUSSION

We present the development of a diagnostic microarray test, its evaluation and validation as well as the application of this user-friendly tool for screening a series of *E. coli* strains associated with newborn meningitis. By applying the established array tube, we were able to carry out a comprehensive analysis of K1 and related strains concerning their virulence gene profile. Several studies have described the presence of specific genes in K1 strains, however, these were restricted to PCR detection of genes known to be present in such strains or focusing on virulence factors specific for extraintestinal pathotypes. This is the first time such strains were analyzed for the presence of multiple virulence genes specific for extraintestinal as well as intestinal pathotypes of *E. coli*. The array tube includes positive control probes, of which the probe for the 16S rRNA (*rrs*) is universal. The second control for type 1 fimbriae (*fimA*), which can be found on average in about 70% of *E. coli* strains, including nonpathogenic strains (21), was positive for all the isolates. The gene *fhuA*, indicating an iron transport system present in most *E. coli* strains, showed positive signals for only 78% of the isolates. The one strain which did not hybridize to any of the virulence genes was isolated from the cerebrospinal fluid of a neonate who presented bacterial meningitis with clinical sepsis. However, the absence of virulence genes in this strain suggests that the isolate might represent a contaminant from sampling.

Looking at specific virulence genes, we found the K1 determinant in 88% of our strains. In order to validate the microarray test for typing of K1, we agglutinated all the strains with a K1 antiserum. There was a perfect correlation of the K1 phenotype and genotype, indicating that the probe used on the array tube is highly specific for K1 strains.

A series of probes for fimbriae of uropathogenic *E. coli* were included on the array tube. Such fimbrial genes were found to be very common, since one or more of them could be detected in 85% of the strains. The prevalence of the P and S fimbrial genes was generally higher than described by phenotypic methods (22). In 65%, 48%, and 35% of strains, we found genes for P, F1C, and S fimbriae, respectively. This difference can be explained by the lack of expression of fimbriae in certain strains analyzed phenotypically. In fact, Bingen et al. (5), with PCR, showed a similar genetic prevalence, with *pap* being higher than *sfa*.

Several strains contained the two toxin genes *hlyA* and *cnf*. Hemolysin production was described by others in 25% of the strains (22), in agreement with the 23% of strains where we found the gene for the α-hemolysin. The cytotoxic necrotizing factor is known to contribute to the virulence of K1 strains, allowing penetration of the blood-brain barrier (18). We found *cnf* in six strains (15%), and one of them was a non-K1 isolate. This rather low prevalence of *cnf* questions its importance and indicates that other factors like the K1 capsule itself or the Ibe protein (not included on the array tube) contributes to the invasion of the central nervous system (20).

Iron acquisition systems are important for septicemic *E. coli* strains, in order to enable the strains to access iron under iron-limited conditions in the blood. Therefore it is not surprising that all strains except one have a high-efficiency iron acquisition system. The gene for the hemin receptor (*chuA*) is predominantly present in 95% of the strains. The siderophore aerobactin, represented by the *iucC* probe, is less prevalent and found in 50% of the strains. Interestingly, 19 out of 20 strains having the aerobactin system also contain the *chuA* gene. Negre et al. (26) hypothesized that the hemin transport system could act as a "backup" system for iron acquisition. This could explain the high prevalence of this gene and the presence of additional iron acquisition systems in some strains. All strains that contained the α-hemolysin also contained *chuA*, indicating a synergistic action of the hemolysin with the hemin receptor.

In one of the non-K1 strains we could detect the gene indicating the presence of a K5 capsule. This strain was otherwise very similar to the others, having uropathogenic *E. coli*-specific fimbrial genes as well as the *chuA* gene. This strain was isolated from a placental swab in a neonate who presented with culture-proven *E. coli* sepsis (without meningitis, and recovery from the infection after 21 days of therapy).

Virulence genes characteristic of intestinal *E. coli* were typically absent in the strains analyzed. Given the common habitat of the gut, a genetic exchange of such genes could be expected. It was therefore somewhat surprising that few of them were found in our strains. In fact, only two strains showed the presence of the O157 catalase gene and the gene for the small heat-stable toxin AstA, respectively. These positive hybridization signals were confirmed by PCR. Both genes are located on plasmids and therefore relatively easy transfer could be anticipated (9, 37). However, in the case of the O157 catalase, which is located on the pO157 plasmid, no other virulence genes found on this plasmid (e.g., the enterohemorrhagic *E. coli* hemolysin) were detected in this strain.

Six strains representing five different virulence gene patterns show linkage of *hlyA*, *pap*, and *cnf*. These genes are located on pathogenicity island PAI$_{II}$ of uropathogenic *E. coli* strain J96 (28). In fact there are indications of pathogenicity island in-

volvement in certain neonatal meningitis-causing *E. coli*, and for one strain the presence of a pathogenicity island similar to PAI$_{II}$ of J96 was postulated (8, 14). Our findings show that this pathogenicity island is distributed among K1 and related *E. coli* strains.

The observation of so many different virulence gene patterns for K1 strains is an indication that they constitute a rather heterogeneous group of *E. coli* strains. Similarly, Mühldorfer et al. (25), with colony hybridization with fewer gene probes, found 10 different virulence gene patterns in K1 strains isolated from the stools of healthy volunteers. This broad variety of *E. coli* K1 was also observed in a study of Achtman et al. (1) with several classical typing techniques. It is clear that many *E. coli* K1 clones have the potential of causing meningitis, which reflects the commensal nature of K1 strains (25). However, some clones seem to be less virulent than others, and the predominance of clonal groups may vary geographically (2). Up to 40% of healthy individuals are carriers of such strains that are recovered from the feces, urinary tract, and vagina (27, 36). Therefore, a broad variety of isolates could be expected, considering the ecological niches these strains live in and have been described by others, including form variation, serotype, genetic relationship, and distribution of extraintestinal virulence genes (16, 22, 30, 32, 39).

The array tube combines the advantage of the classical microarray technique, allowing parallel detection of large numbers of genes, with an easily manageable and cost-efficient system. The Eppendorf tube format allows easy pipetting, handling, and reading of the hybridization signals, for which conventional laboratory equipment can be used, in contrast to the glass slide format. The use of biotin-labeled nucleotides in the array tube assay is more sensitive than comparable Cy3-based detection used on glass slides (24). With the array tube, a single analysis of a strain can be achieved in less than 48 h, and several strains can be processed in parallel. The small amount of DNA used in labeling would even allow the testing of patient samples rather than overnight cultures. This in combination with an optimized hybridization protocol of less that 2 h (as they exist for oligo-based array tubes) will further cut down analysis time to a few hours (24).

Furthermore, there are several advantages of the array tube over PCR, including real-time PCR. The parallel detection of numerous genes can be achieved in a single experiment. In order to get the same coverage of the 32 *E. coli* virulence genes in triplicate as on the array tube, 96 PCR tests would have been necessary for analyzing a single strain. It is obvious that this is much more work and cost intensive than running a single array tube. Moreover, PCR is prone to contamination, which is almost impossible with the array tube system. Therefore, extreme precaution, including physical separation of PCR processes, is not needed with the tubes. Furthermore, the array tube allows the detection of variants of *E. coli* virulence genes by hybridization. A sequence variation in one or both PCR primers would lead to negative results in a PCR-based detection method.

In summary, the array tube detection system proved to be a helpful diagnostic tool for genetic typing of K1 strains as well as other pathogenic *E. coli* strains. Given the open platform, this prototype of an *E. coli* virulence gene detection system can be easily optimized, and new gene probes can be included in the future (e.g., newly described virulence or antibiotic resistance genes).

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