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Development and Validation of an Oligonucleotide Microarray for Detection of Multiple Virulence and Antimicrobial Resistance Genes in *Escherichia coli*†

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An oligonucleotide microarray detecting 189 *Escherichia coli* virulence genes or markers and 30 antimicrobial resistance genes was designed and validated using DNA from known reference strains. This microarray was confirmed to be a powerful diagnostic tool for monitoring emerging *E. coli* pathotypes and antimicrobial resistance, as well as for environmental, epidemiological, and phylogenetic studies including the evaluation of genome plasticity.

Escherichia coli, which is commonly found in the environment as well as in the intestinal tract of common animal species, including humans, is the causative agent of several diseases worldwide. Depending on their virulence properties and the types of clinical infection elicited, *E. coli* strains are classified into various pathotypes (17). Generally, strains belonging to the same pathotype possess the same virulence determinants, but some virulence factors can be associated with several pathotypes. Therefore, establishing the virulence gene content found within an *E. coli* isolate is critical in determining its pathogenic potential. As many virulence genes are located on mobile elements, such as plasmids, phages, or transposons (24), determining the virulence gene profile of a given *E. coli* isolate would help also in monitoring gene transfer between strains and would be consequently of great value in epidemiological and phylogenetic studies. Like virulence genes, many antimicrobial resistance genes can be acquired by horizontal transfer. Therefore, antimicrobial resistance profiling of a given *E. coli* isolate would be useful for antimicrobial resistance surveillance programs. It could also be of great diagnostic value and should be indispensable for designing effective antibiotic policies.

Although various molecular methods can be used to identify either virulence or antimicrobial resistance genes harbored by *E. coli* strains (11, 19, 25, 29, 31), there is still a lack of practical and cost-effective methods able to detect rapidly and simultaneously all these genes in a given isolate. Microarray technology offers a powerful alternative for determining simultaneously the presence of a wide diversity of genes within a given *E. coli* strain. DNA microarrays have been used successfully in various studies, involving taxonomy (9), genotyping of microbial strains (13), detection of environmentally important genes (28, 34), and, recently, detection of *E. coli* antimicrobial resistance genes (6, 16, 35) or virulence genes (1, 8, 18, 30). In this study, a DNA microarray was developed combining oligonucleotides designed to detect a complete set of virulence genes representative of all *E. coli* pathotypes and antimicrobial resistance genes representative of different antimicrobial families characteristically found in pathogenic *E. coli* strains (20, 22). The capacity of this microarray to detect all virulence and antimicrobial resistance genes in reference and clinical *E. coli* isolates was investigated, and the results were validated with other molecular techniques.

The microarray prototype was designed from a previous amplicon-based microarray developed in our laboratory (1). Oligonucleotides were preferred to longer double-stranded DNA amplicons due to the potential of the latter for cross-hybridization, while oligonucleotide probes, due to their short length, are generally considered to be more specific. Apart from the amplicon-to-oligonucleotide redesign, the prototype was also updated by adding oligonucleotides specific for recently characterized and new putative *E. coli* virulence genes and by coupling the determination of the virulence gene content with the detection of antimicrobial resistance genes. The newly designed microarray prototype was thus composed of 348 70-mer oligonucleotides that were designed either using the OligoPicker software program (32) or from published PCR primers (20) which were lengthened to 70 bases. Two hundred sixty-three of them correspond to 189 virulence genes or markers from all known *E. coli* pathotypes as well as, within some particular genes, to their genetic variants (see Table S1 in the supplemental material). Among these oligonucleotides, three were specific for the phylogenetic markers *chuA*, *yjaA*, and *tspE4.C2* used in the PCR-based method described by Clermont et al. for the determination of the main *E. coli* phyloge-

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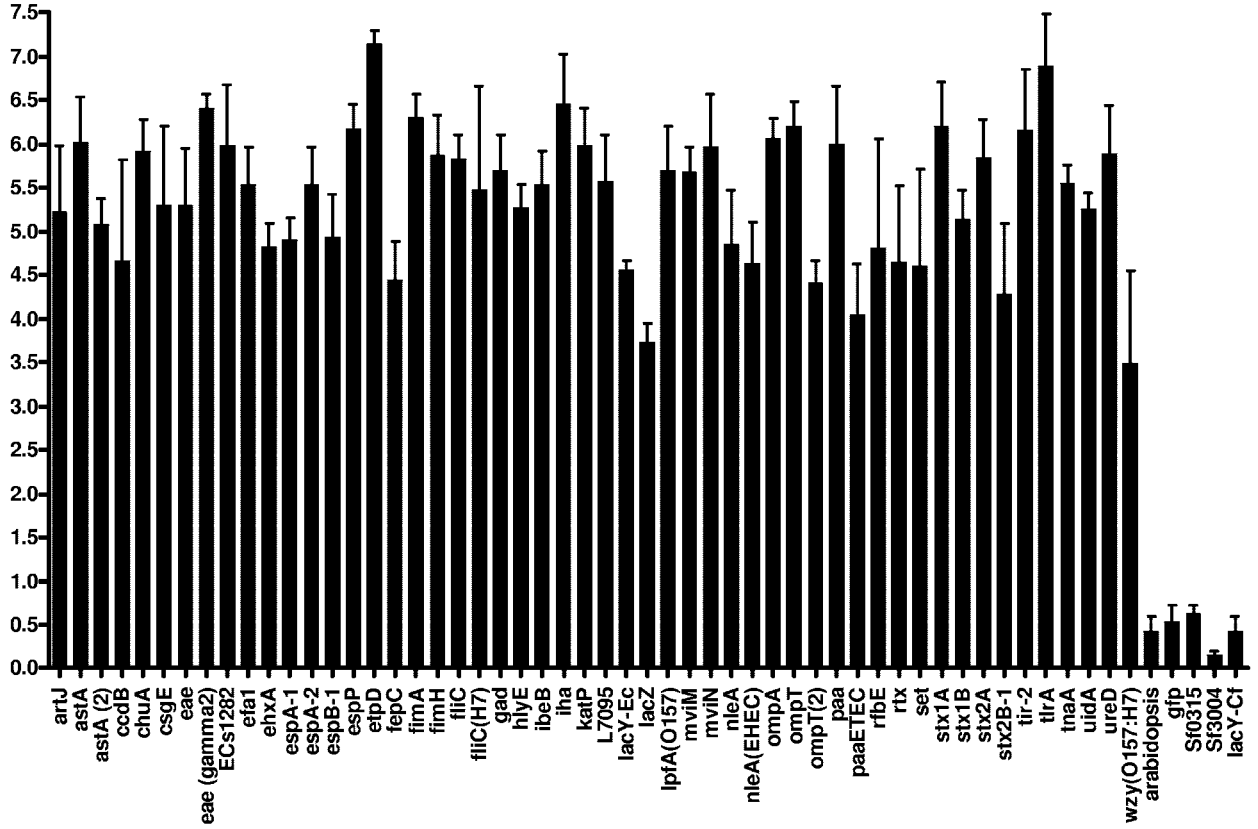

FIG. 1. Signal-to-noise fluorescence ratios obtained for the *E. coli* reference strain EDL933. The signal-to-noise ratios ($\log_2$) presented in this graph are the means of the ratios obtained by three independent replicate hybridizations performed with DNA from strain EDL933. All oligonucleotides which had a signal-to-noise fluorescence ratio of greater than 2.0 ($\log_2 > 1$) were considered positive. Oligonucleotides with a signal-to-noise ratio less than 2.0 ($\log_2 < 1$) were considered negative. For the negative results, only those for oligonucleotides specific to the green fluorescent protein (GFP) gene, *Arabidopsis* spp., *Shigella flexneri*, and *C. freundii* are shown.

netic groups (10). Thirty-three other oligonucleotides were designed to target 30 antimicrobial resistance genes conferring resistance to six well-known gram-negative antimicrobial families and the class 1 integron (see Table S2 in the supplemental material). The selectivity of each oligonucleotide sequence was individually verified through BLAST searches in GenBank and then simultaneously in the public BLAST server Goldorak (http://www.bioneq.qc.ca; BioneQ, Montréal, Québec) for a final global BLAST analysis. A complete array was composed of four subarrays, in which each oligonucleotide was printed in triplicate, as previously described (1), on Corning Ultra GAPS slides (Corning Canada, Whitby, Ontario). Positive and negative controls (see Table S1 in the supplemental material) as well as three printing buffer spots were added in each subarray (see Fig. S1 in the supplemental material). Three complete independent arrays were printed on the same slide, thus minimizing variations resulting from fluctuations in external parameters.

The three reference strains EDL933, CFT073, and MG1655, for which the genomes have been completely sequenced (4, 26, 33), and 18 well-characterized strains coming from a previous study performed in our laboratory (1) and representing most of the *E. coli* pathotypes were used to validate the specificity of the virulence oligonucleotides. A collection of 55 *E. coli* strains coming from studies performed by Maynard et al. (20, 22) was used for the validation of the antimicrobial resistance oligonucleotides. *Citrobacter freundii* strain ATCC 8090 was used as a negative control.

To validate the microarray prototype, three independent hybridizations were performed for all strains described above. A 2-μl sample of a lysate from an *E. coli* overnight culture in Luria-Bertani broth grown at 37°C under agitation was labeled with a simple random-priming protocol based on Invitrogen's Bioprime DNA labeling system (Invitrogen Life Technologies, Burlington, Ontario). In a total volume of 50 μl, 20 μl of a 2.5× random primer solution (from the kit) and 1 μl of high-concentration Klenow polymerase (40 U/μl) were added to 5 μl of a deoxynucleoside triphosphate mix (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0] and 1 mM EDTA). Two microliters of 1 mM Cy5-dCTP were added to fluorescently label the DNA. The reaction was incubated in the dark at 37°C for 2 h, and the labeled samples were then purified on QIAquick columns (QIAGEN Inc., Mississauga, Ontario) according to the manufacturer's protocol. Microarrays were hybridized overnight at 50°C in a slide hybridization chamber (Corning Canada) with 500 ng of labeled DNA previously resuspended in 6 μl of prewarmed (37°C) DIG Easy Hyb buffer (Roche Diagnostics, Laval, Quebec) and denatured by heating for 5 min at 95°C. Stringency washes, three in 0.1× SSC (15 mM NaCl and 1.5 mM tri-

TABLE 1. Determination of the virulence gene content of *E. coli* reference strains and strains representing most *E. coli* pathotypes

Strain identification	Pathotype[a]	Virulence genes for which a signal-to-noise fluorescence ratio of greater than 2.0 was obtained[b]	Associated phylogenetic group[c]	Reference
EDL933[d]	EHEC (O157H7)	*stx1A*, *stx1B*, *stx2A*, *stx2B-1*, *eae*, *eae(gamma2)*, *espA1*, *espA2*, *espB1*, *tir2*, *ehxA*, *nleA*, *paa*, *wzy(O157H7)*, *artJ*, *astA*, *ccdB*, *chuA*, *csgE*, *Ecs1282*, *efa1*, *espP*, *etpD*, *fepC*, *fimA*, *fimH*, *fliC*, *fliC(H7)*, *gad*, *hlyE*, *ibeB*, *iha*, *katP*, *l7095*, *lacY-Ec*, *lacZ*, *lpfA(O157)*, *mviM*, *mviN*, *ompA*, *ompT*, *rfbE*, *rtx*, *set*, *tlrA*, *tnaA*, *uidA*, *ureD*	D	26
CFT073[d]	UPEC	*papA(7-1)*, *papA(7-2)*, *papC*, *papGII*, *chuA*, *fepC*, *irp1*, *irp2*, *fyuA*, *iroN*, *usp*, *agn43*, *artJ*, *astA*, *b1121*, *b1432*, *ce1a*, *csgE*, *fimA*, *fimH*, *fliC*, *focA*, *focG*, *gad*, *hlyA*, *hlyE*, *ibeB*, *iha*, *iss*, *iucD*, *kpsM-II*, *lacY-Ec*, *lacZ*, *malX*, *mchB*, *mviM*, *mviN*, *ompA*, *ompT*, *pic*, *sat*, *sfaD*, *tnaA*, *tspE4.C2*, *uidA*, *wzx(O6)*, *yjaA*	B2	33
MG1655[d]	K12	*agn43*, *artJ*, *b1121*, *csgE*, *fimA*, *fimH*, *fliC*, *gad*, *hlyE*, *ibeB*, *lacY-Ec*, *lacZ*, *mviM*, *mviN*, *ompA*, *ompT*, *tnaA*, *uidA*, *yjaA*	A	4
E2348/69	EPEC	*eae*, *eae(alpha)*, *espA1*, *espB2*, *tir3*, *bfpA*, *bfpA(alpha)*, *artJ*, *astA*, *b1121*, *ccdB*, *chuA*, *csgE*, *eaf*, *efa1*, *espC*, *fepC*, *fimA*, *fimH*, *fliC*, *gad*, *hlyE*, *ibeB*, *lacY-Ec*, *lacZ*, *malX*, *mviM*, *mviN*, *ompA*, *ompT*, *set*, *tnaA*, *traT*, *uidA*, *yjaA*	B2	14
H-10407	ETEC	*esta1*, *st*, *toxA*, *toxB*, *tia*, *tibA*, *leoA*, *agn43*, *artJ*, *astA*, *b1121*, *cfaB*, *csgE*, *fimA*, *fimH*, *flmA54*, *fyuA*, *gad*, *hlyE*, *ibeB*, *irp1*, *irp2*, *lacY-Ec*, *lacZ*, *mviM*, *mviN*, *ompA*, *tnaA*, *uidA*, *yjaA*	A	23
17.2	EAEC	*capU*, *shf*, *virK*, *aap*, *aggA*, *agn43*, *artJ*, *astA*, *b1121*, *ccdB*, *csgE*, *fimA*, *fimH*, *fyuA*, *gad*, *hlyA*, *hlyE*, *ibeB*, *iha*, *irp1*, *irp2*, *iss*, *iucD*, *kpsMII*, *lacY-Ec*, *lacZ*, *mviM*, *mviN*, *ompA*, *papA(16)*, *papC*, *papGII*, *sat*, *tnaA*, *uidA*, *yjaA*	A	27
J96	UPEC	*papA(13)*, *papC*, *papGI*, *papGIII*, *chuA*, *fepC*, *irp1*, *irp2*, *fyuA*, *iroN*, *usp*, *agn43*, *artJ*, *b1121*, *b1432*, *ce1a*, *cnf1*, *csgE*, *fimA*, *fimH*, *fliC*, *focA*, *focG*, *gad*, *hlyA*, *hlyE*, *hra1*, *ibeB*, *iss*, *kpsMIII*, *lacY-Ec*, *lacZ*, *malX*, *mchB*, *mviM*, *mviN*, *ompA*, *ompT*, *rfc*, *sfaD*, *tnaA*, *uidA*, *yjaA*	B2	5
2787	DAEC	*aidaI*, *agn43*, *artJ*, *astA*, *b1121*, *ccdB*, *csgE*, *fimA*, *fimH*, *flmA54*, *fyuA*, *gad*, *hlyE*, *ibeB*, *irp1*, *irp2*, *lacY-Ec*, *lacZ*, *mviM*, *mviN*, *ompA*, *tnaA*, *traT*, *uidA*, *yjaA*	A	2
31A	ExPEC (septicemia)	*papA(11)*, *papC*, *irp1*, *irp2*, *fyuA*, *iucD*, *iutA*, *agn43*, *artJ*, *b1121*, *clpG*, *csgE*, *espP*, *f165(1)A*, *F17cA*, *fimA*, *fimH*, *fliC*, *gad*, *gafD*, *hlyE*, *hra1*, *ibeB*, *iss*, *lacY-Ec*, *lacZ*, *lpfA(O113)*, *lpfA*, *mviM*, *mviN*, *ompA*, *ompT*, *tnaA*, *traT*, *uidA*, *yjaA*	A	3

[a] A pathotype is attributed to clinical strains according to their set of virulence genes or markers: EHEC (Shiga-like toxin-encoding genes, genes from the LEE, *ehxA*), EPEC (genes from the LEE, *bfpA*), ETEC (heat-stable and heat-labile toxin-encoding genes, F4 and F18 fimbria-encoding genes), EAEC (*capU*, *shf*, *virK*, aggregative adherence fimbria-encoding genes), DAEC (*aidaI*), UPEC (P pili-encoding genes, *chuA*, *fepC*, *irp1*, *irp2*, *fyuA*, *iroN*, *usp*).

[b] All virulence genes targeted in this study are described in an exhaustive list in Table S1 in the supplemental material.

[c] As previously described, according to the presence or absence of the three phylogenetic markers *chuA*, *yjaA*, and *tspE4.C2* (10).

[d] Reference strains for which the genome has been entirely sequenced (4, 26, 33).

sodium citrate, pH 7.0) with 0.1% sodium dodecyl sulfate and one in 0.1× SSC, were then performed at 37°C for 5 min under agitation. Data acquisition and analysis were performed as previously described (21).

For all hybridizations performed with *E. coli* DNA, positive results (signal-to-noise fluorescence ratio of greater than 2.0 [21] [Fig. 1]) were obtained for all the oligonucleotides used as positive controls, and no fluorescence (signal-to-noise fluorescence ratio of less than 2.0 [21] [Fig. 1]) was observed for those used as negative controls. Expected results were also obtained for the *C. freundii* negative control strain. The microarray results were shown to be reproducible since, for each of the 76 *E. coli* strains tested, no missing spot was observed between the three independent replicate hybridizations. As shown in the example in Fig. 1, where the microarray results obtained for strain EDL933 are presented, oligonucleotides giving positive results always showed a signal-to-noise ratio clearly greater than 2.0 and, on the other hand, oligonucleotides giving negative results always showed a signal-to-noise ratio of less than 2.0. Expected results were obtained for the three reference strains MG1655, EDL933, and CFT073 and for the collection of 18 well-characterized *E. coli* isolates from the study of Bekal et al. (1). Among the 263 virulence-specific oligonucleotides, only the *astA*-specific probes gave unexpected positive results. BLAST searches in GenBank revealed the presence of a short truncation (24 nucleotides) in the *astA* sequences of various *E. coli* strains which cannot be differentiated from complete sequences with a 70-mer oligonucleotide probe. Consequently, positive results obtained with *astA*-specific oligonucleotides have to be confirmed by PCR with primers described previously (1). For all the other virulence-specific oligonucleotides, a perfect concordance was observed between microarray results and (i) BLAST searches against the sequenced genomes of the three reference strains and (ii) previous characterization of the 18 *E. coli* strains with an amplicon-based virulence microarray (1). The virulence gene content and the phylogenetic groups determined by our microarray of nine *E. coli* reference strains are presented in Table 1. For the 55 clinical *E. coli* isolates coming from the study of Maynard et al. (22), some discrepancies were observed comparing both microarray results and membrane hybridizations previously performed (22) (Table 2). Contrary to membrane hybridizations, where

TABLE 2. Determination of the antimicrobial resistance gene content of clinical *E. coli* extraintestinal isolates by microarray and comparison with membrane hybridizations

Strain identification	Origin	Microarray result(s)[a,b]	Membrane hybridizations[a,b]
01-8344-0611	Caribou	bla_{TEM}, *aadA1*[d], *tet*(A), *tet*(B), *dhfrI*, *sulI*, *sulII*, class 1 integron	bla_{TEM}, *tet*(A), *tet*(B), ***tet*(C)**, *dhfrI*, ***dhfrXV***, *sulI*, *sulII*, class 1 integron
EcL1329 (01-D913)[c]	Pig	bla_{TEM}, *aadA1*[d], *aphA1*, *tet*(A), *dhfrV*, *sulII*	bla_{TEM}, *aphA1*, *tet*(A), ***tet*(C)**, *dhfrV*, *sulII*
EcL10038 (02-779-175)[c]	Pig	bla_{TEM}, *aadA1*[d], *tet*(A), *sulI*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(C)**, *sulI*, class 1 integron
EcL10040 (02-839)[c]	Pig	*aadA1*[d], *tet*(A), *sulI*, class 1 integron	*tet*(A), ***tet*(C)**, *sulI*, class 1 integron
EcL10044 (02-1273-175)[c]	Cattle	bla_{TEM}, *tet*(A)	bla_{TEM}, *tet*(A), ***tet*(C)**
EcL1335 (02-1902-2)[c]	Pig	bla_{TEM}, *aadA1*[d], *aphA1*, *tet*(A), *dhfrV*, *sulI*, *sulII*, class 1 integron	bla_{TEM}, *aphA1*, *tet*(A), ***tet*(C)**, *dhfrV*, *sulI*, *sulII*, class 1 integron
02-1926	Avian	bla_{TEM}, *aadA1*[d], *tet*(A), *sulI*, *sulII*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(C)**, *sulI*, *sulII*, class 1 integron
EcL10106 (02-1940-1)[c]	Pig	bla_{TEM}, *aadA1*[d], *aphA1*, *tet*(A), *dhfrV*, *sulI*, *sulII*, class 1 integron	bla_{TEM}, *aphA1*, *tet*(A), ***tet*(C)**, *dhfrV*, *sulI*, *sulII*, class 1 integron
02-6386	Cattle	bla_{TEM}, *tet*(A), *sulII*	bla_{TEM}, *tet*(A), ***tet*(C)**, *sulII*
1182768	Human	bla_{TEM}, *aadA1*[d], *tet*(A), *sulI*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(C)**, *sulI*, class 1 integron
67101013-1	Human	bla_{TEM}, *aadA1*[d], *tet*(A), *catI*, *sulI*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(C)**, *catI*, *sulI*, class 1 integron
67170713-3	Human	*aadA1*[d], *tet*(A), *sulI*, class 1 integron	*tet*(A), ***tet*(C)**, *sulI*, class 1 integron
67170751-1	Human	bla_{TEM}, *aadA1*[d], *aadB1*[d], *aphA1*, *tet*(A), *sulI*, *sulII*, class 1 integron	bla_{TEM}, *aphA1*, *tet*(A), ***tet*(C)**, *sulI*, *sulII*, class 1 integron
67300471-1	Human	bla_{TEM}, *aadA1*[d], *tet*(A), *dhfrI*, *sulI*, *sulII*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(B)**, ***tet*(C)**, *dhfrI*, ***dhfrXV***, *sulI*, *sulII*, class 1 integron
67310973-1	Human	*aadA1*[d], *tet*(A), *sulI*, class 1 integron	*tet*(A), ***tet*(C)**, *sulI*, class 1 integron
68030679-1	Human	bla_{TEM}, *tet*(A), *sulI*	bla_{TEM}, *tet*(A), ***tet*(C)**, *sulI*
68070861-1	Human	bla_{TEM}, *tet*(A), *tet*(B), *catI*, *sulII*	bla_{TEM}, $\boldsymbol{bla_{SHV}}$, *tet*(A), *tet*(B), ***tet*(C)**, *catI*, *sulII*
EcL8134 (98-2453)[c]	Dog	*tet*(A)	*tet*(A), ***tet*(B)**, ***tet*(C)**
EcL1333 (P02-014-1)[c]	Pig	bla_{TEM}, *aadA1*[d], *tet*(A), *sulI*, class 1 integron	bla_{TEM}, *tet*(A), ***tet*(B)**, ***tet*(C)**, *sulI*, class 1 integron

[a] All antimicrobial resistance genes targeted in this study are described in an exhaustive list in Table S2 in the supplemental material.
[b] Discrepancies are indicated in boldface. For these genes, PCR and DNA sequencing performed with primers previously described (22) validated the microarray results.
[c] Previous nomenclature.
[d] Gene not tested by membrane hybridization.

cross-hybridizations between *tet*(A) and *tet*(C) probes were observed (data not shown), the absence of such ambiguity with the microarray, as confirmed by PCR, underscores the high level of specificity. In the few other cases of discrepancies, PCR analysis and DNA sequencing performed as previously described (22) confirmed all the microarray results. Expected results were also obtained for the 29 positive control strains as well as the negative control reference strain MG1655, which lacks all the antimicrobial resistance genes targeted by the microarray.

In comparison with previous microarray-based studies (6–8, 16, 18, 30), we have developed a powerful molecular tool by coupling the detection of an exhaustive set of virulence genes and the detection of numerous antimicrobial resistance genes. This oligonucleotide microarray is a valuable tool not only for the assessment of the pathotype and the determination of the pathogenic potential of *E. coli* strains, but also for monitoring the transfer of virulence genes between strains (15). It should thus facilitate the identification of emerging pathotypes as well as the evaluation of genome plasticity by investigating the capacity of a strain to acquire virulence genes from other pathotypes, as shown in previous studies (1, 12, 18). This microarray also represents a valuable tool for diagnostic-based studies, surveillance programs of antimicrobial resistance, and monitoring of resistance gene dissemination between *E. coli* isolates. The presence in the microarray of oligonucleotides specific for the three phylogenetic markers used by Clermont et al. (10) for the determination of the main *E. coli* phylogenetic groups is of great help in epidemiological and phylogenetic studies (K. Hamelin et al., unpublished data). Finally, this microarray should not only be applicable in veterinary and medical diagnosis but should also find widespread use in microbial quality control of food and water and in environmental studies (K. Hamelin et al., unpublished data).

Work is under way to further improve the performance of this microarray (i) by coupling microarray technology with bioinformatics software to automate pathotype determination from an isolate's virulence gene content directly from the hybridization image and (ii) through array updating by adding oligonucleotides specific for newly recognized virulence genes.

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