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A Virulence and Antimicrobial Resistance DNA Microarray Detects a High Frequency of Virulence Genes in *Escherichia coli* Isolates from Great Lakes Recreational Waters

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Escherichia coli **is generally described as a commensal species with occasional pathogenic strains. Due to technological limitations, there is currently little information concerning the prevalence of pathogenic *E. coli* strains in the environment. For the first time, using a DNA microarray capable of detecting all currently described virulence genes and commonly found antimicrobial resistance genes, a survey of environmental *E. coli* isolates from recreational waters was carried out. A high proportion (29%) of 308 isolates from a beach site in the Great Lakes carried a pathotype set of virulence-related genes, and 14% carried antimicrobial resistance genes, findings consistent with a potential risk for public health. The results also showed that another 8% of the isolates had unusual virulence gene combinations that would be missed by conventional screening. This new application of a DNA microarray to environmental waters will likely have an important impact on public health, epidemiology, and microbial ecology in the future.**

Until fairly recently, there was a common perception that pathogenicity traits in *Escherichia coli* are more the exception than the rule, and *E. coli* was generally regarded as part of the normal lower intestinal flora (1, 14, 36, 39). However, an increasing number of categories of pathogenic *E. coli* isolates have been identified over the past few decades, which has led to the current situation in which there are now at least 11 recognized pathotypes of *E. coli* in humans (19). Pathogenic *E. coli* strains are divided into pathotypes on the basis of their distinct virulence properties and the clinical symptoms of the host (26). Three main types of clinical syndrome can result from infection with one of these pathotypes: enteric and diarrheal diseases, urinary tract infections, and sepsis/meningitis. The *E. coli* pathotypes responsible for intestinal infections include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, diffusely adherent *E. coli*, necrotoxic *E. coli*, and cell-detaching *E. coli*. Three additional *E. coli* pathotypes, collectively called ExPEC (34), are responsible for extraintestinal infections. ExPEC is composed of uropathogenic *E. coli* (UPEC) isolates that cause urinary tract infections, neonatal meningitis-associated *E. coli* (MNEC), and *E. coli* strains that cause septicemia (2, 19). These pathotypes are defined by the presence of combinations of virulence and virulence-related genes; conversely, the pathotype of an uncharacterized strain can be inferred from its virulence gene profile (19).

Even though pathogenic *E. coli* is primarily associated with food-borne diseases, contamination of drinking or recreational waters with some pathotypes has resulted in waterborne disease outbreaks and associated mortality. Recent examples in the Great Lakes area include pathogenic *E. coli* O157:H7 isolates that contributed to a drinking water outbreak in Walkerton, Ontario, in 2000 that resulted in 2,300 illnesses and seven deaths (15) and to a recreational water outbreak in 2001 at a beach in Montreal, Quebec, that resulted in the hospitalization of four children (4).

However, there have been few studies (6, 21, 22, 26, 27) in which the proportion of pathogenic or potentially pathogenic *E. coli* isolates in the environment has been determined. Furthermore, the scope of the studies that have dealt with virulence and virulence-related gene content has been limited, and workers have generally looked for several characteristic genes that define a few of the known pathotypes of *E. coli*, using classical detection methods such as DNA hybridization or PCR. When PCR is used, the search is inherently restricted to small specific pathotype gene subsets, such as *hlyA*, *sfa*, *iroN*, *pap*, and *cnf1* for UPEC (20, 25, 26, 42). Consequently, numerous other virulence genes can be overlooked.

With the advent of DNA microarrays, this technological limitation can be overcome. The oligonucleotide microarray used in the present study carries more than 300 probes representing 189 virulence and virulence-related genes and 30 anti-

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microbial resistance genes (3), and it provides a much more complete picture of the virulence and antimicrobial resistance gene profiles present and is not limited by an a priori selection of PCR targets. *E. coli* strains were isolated from cultures of Lake Ontario recreational water samples and were analyzed using the *E. coli* pathotyping and antimicrobial gene typing microarray mentioned above. The results showed that a substantial proportion (29%) of *E. coli* isolates from these recreational waters contained sets of virulence or virulence-related genes found in one of the pathotypes and that a subset of these strains contained antimicrobial resistance genes. This important representation of potential pathogens may have serious implications for public health.

MATERIALS AND METHODS

Water sampling and *E. coli* isolation. Water samples were collected in sterile 250-ml polypropylene bottles from Hamilton Harbor, Ontario, Canada, between 10 May and 21 June 2004 (spring) and between 5 July and 23 August 2004 (summer). Hamilton Harbor on Lake Ontario is an active recreational environment with beaches and offshore areas for windsurfing and boating, although beaches have frequently been closed in recent years as a result of high *E. coli* counts (29). While there is little agricultural influence in this urban area (population in 2001, 640,000), four municipal sewage treatment plants discharge into the harbor. Birds such as ring-billed gulls and Canada geese are also frequently present in beach areas. Two replicate water samples were collected weekly at three water depths: (i) ankle depth at BayFront Park Beach, (ii) offshore at BayFront Park Beach where the water depth is 6 m (samples obtained 1 m below the surface), and (iii) further offshore in the middle of Hamilton Harbor where the water depth is 24 m (samples obtained 1 m below the surface). Water samples were filtered through 0.45-μm membrane filters, and the filters were incubated overnight at 44.5°C on differential coliform agar supplemented with cefsulodin (Oxoid Inc.). Ten colonies from each water sample were randomly picked with a sterile toothpick and streak plated onto MacConkey agar (Difco Inc.) for overnight growth at 37°C. Putative *E. coli* isolates were tested for glucuronidase activity by growth and fluorescence in EC-MUG (Difco Inc.) and for indole production by growth in 1% (wt/vol) tryptone (Difco Inc.) and reaction with Kovac's reagent (Oxoid Inc.). Isolates that were positive in both tests were stored at −80°C in tryptic soy broth and 15% (vol/vol) glycerol. *E. coli* ATCC 29194 and *Klebsiella* strain ATCC 33495 were used as positive and negative controls, respectively, during confirmation tests.

DNA extraction. *E. coli* isolates were grown overnight in 5 ml of tryptic soy broth at 37°C. One-milliliter portions of the cell suspensions were transferred to 1.5-ml centrifuge tubes and spun at 15,500 × *g* for 2 min. Each supernatant was removed, and the cell pellet was resuspended in 200 μl of sterile water with vortexing. The suspension was boiled for 10 min and centrifuged as described above, and 150 μl of the supernatant containing DNA was removed for testing.

***E. coli* DNA labeling.** Bacterial DNA was labeled using the Bioprime DNA labeling system (Invitrogen Life Technologies, Burlington, Ontario, Canada). Briefly, to remove any contaminating RNA, 2 μl of RNase A (10 mg/ml; USB, Cleveland, OH) was incubated with a genomic DNA sample for 2 min at 24°C, and this was followed by centrifugation (15,500 × *g*, 2 min, 24°C). Four microliters of the supernatant was added to a 50-μl (final volume) mixture containing 20 μl of a random primer solution, 1 μl of high-concentration DNA polymerase (40 U/μl; Klenow fragment), 5 μl of a deoxynucleoside triphosphate mixture (1.2 mM dATP, 1.2 mM dGTP, 1.2 mM dTTP, and 0.6 mM dCTP in 10 mM Tris [pH 8.0]–1 mM EDTA), and 2 μl of 1 mM Cy5-labeled dCTP. Labeling reactions were performed in the dark at 37°C for 3.5 h and were stopped by addition of 5 μl of 0.5 M Na_2EDTA, pH 8.0. After 2.5 μl of 3 M sodium acetate (pH 5.2) was added, the labeled samples were purified with a PureLink PCR purification kit (Invitrogen Life Technologies) used according to the manufacturer's protocol. The amount of incorporated fluorescent Cy5 dye was then quantified by scanning the DNA samples at wavelengths from 200 to 700 nm and subsequently inputting the data into the Internet-based Percent Incorporation Calculator found at http://www.pangloss.com/seidel/Protocols/percent_inc.html.

DNA microarray. The microarray used in this study was based on previously published work (2), with the addition of more recently identified virulence-related genes and of the most common antimicrobial resistance genes found in gram-negative bacteria (23). The current version carries 312 oligonucleotides that are 70 bases long targeting 189 virulence or virulence-related genes and 30 antimicrobial resistance genes. The microarray also carries four positive controls for *E. coli* derived from the sequences of a tryptophanase-encoding gene (*tnaA*), a beta-glucuronidase-encoding gene (*uidA*), a lactose permease-encoding gene (*lacY*) and a beta-galactosidase-encoding gene (*lacZ*). The negative controls added to this microarray consist of oligonucleotides derived from the gene sequences for the green fluorescent protein of *Aequoria victoria* and the chlorophyll synthase from *Arabidopsis thaliana*. Validation and the details of the construction of the microarray were described recently (3).

Hybridization of labeled DNA. Microarrays were prehybridized at 50°C for 1 h under a Hybri-slip (22 by 60 mm; Sigma Chemical Co., St. Louis, MO) in a slide hybridization chamber (Corning Canada, Whitby, Ontario, Canada), with 30 μl of prewarmed digoxigenin (DIG) Easy Hyb buffer (Roche Diagnostics, Laval, Quebec, Canada) containing 5% (vol/vol) bovine serum albumin (1 mg/ml; New England Biolabs Inc., Beverly, MA). After prehybridization, the Hybri-slip was removed by dipping the slide in 0.1× SSC (15 mM NaCl plus 1.5 mM trisodium citrate, pH 7.0), and the slide was dried by centrifugation at 800 × *g* for 5 min. Before hybridization, 1 μg of labeled DNA was resuspended in 6 μl of prewarmed DIG Easy Hyb buffer (Roche Diagnostics) and denatured by heating 5 min at 95°C. Microarrays were then hybridized overnight at 50°C under Hybri-slips (11 by 11 mm) and in a slide hybridization chamber. After hybridization, the Hybri-slips were removed by dipping the slide in 0.1× SSC–0.1% (wt/vol) sodium dodecyl sulfate (pH 7.2), and four stringency washes (three in 0.1× SSC–0.1% [wt/vol] sodium dodecyl sulfate and one in 0.1× SSC) were performed at 37°C for 5 min with agitation. The slide was then air dried and scanned at a resolution of 10 μm at 85% laser power with a ScanArray Lite fluorescent microarray analysis system (Perkin-Elmer, Missasauga, Ontario, Canada). Acquisition of fluorescent spots and quantification of fluorescent spot intensities were performed using the ScanArray Express software, version 2.1 (Perkin-Elmer, Foster City, CA). The data were normalized by subtracting the local background intensity from the recorded spot intensities from one subarray. For each subarray, the median value for each set of triplicate spotted oligonucleotides was compared to the median value for all of the subarray. Oligonucleotides with a signal-to-noise fluorescence ratio greater than 2.0 were considered positive.

Statistical analysis. The likelihood ratio test (18) was used to assess the significance of comparative results with the binomial and multinomial models.

RESULTS

Water sampling and *E. coli* isolation. A total of 308 *E. coli* isolates were obtained from water samples collected weekly in the spring (10 May to 21 June) and in the summer (5 July to 23 August) at three sites in Hamilton Harbor (Lake Ontario). Prominent fecal pollution sources in Hamilton Harbor include municipal wastewater and birds such as gulls and geese. Although there is little agricultural influence in this urban area (population in 2001, 640,000), four municipal sewage treatment plants discharge into the harbor area. Hamilton Harbor supports an active recreational environment with beaches and offshore areas for windsurfing and boating. One of the Hamilton Harbor beaches, BayFront Park Beach, has frequently been closed in recent years as a result of high *E. coli* counts (29). Previous studies of BayFront Park Beach suggested that the source of *E. coli* was more likely the fecal droppings of the many ring-billed gulls and Canada geese on the beach than wastewater (9). The three sampling sites were (i) ankle depth water at BayFront Park Beach (110 isolates), (ii) surface water where the depth was 6 m offshore from the beach (99 isolates), and (iii) surface water where the depth was 24 m further offshore in the middle of Hamilton Harbor (99 isolates). These sites represent different recreational waters for activities ranging from children bathing to adults windsurfing and boating.

Prevalence of *E. coli* isolates possessing virulence genes. Several isolates contained partial sets of virulence genes, particularly genes for the ExPEC pathotypes. It has been shown that ExPEC isolates can exist as commensals in the guts of healthy animals and humans (16), where they may gain or lose

TABLE 1. Pathotypes and antimicrobial resistance of all *E. coli* isolates

Pathotype[a]	No. of isolates (%)	
	Total	With antimicrobial resistance[b]
ExPEC		
UPEC	28 (9.1)	4 (1.3)
MNEC	6 (2.0)	2 (0.7)
Incomplete ExPEC	47 (15.3)	11 (3.6)
Subtotal	81 (26.4)	17 (5.6)
Enteric *E. coli*		
EAEC	5 (1.6)	3 (1.0)
ETEC	1 (0.3)	1 (0.3)
Atypical EPEC	1 (0.3)	0 (0.0)
Subtotal	7 (2.2)	4 (1.3)
Nonpathogenic	220 (71.4)	22 (7.1)
Total	308 (100.0)	43 (14.0)

[a] Pathotypes were attributed to *E. coli* samples based on their sets of virulence genes or markers, as follows: for EAEC, *capU*, *shf*, *virK*, and aggregative adherence fimbria-encoding genes; for ETEC, heat-stable and heat-labile toxin-encoding genes and F4 and F18 fimbria-encoding genes; for atypical EPEC, *espA*, *espB*, *tir*, *eae* and variants, and absence of *bfpA*; for UPEC, P pilus-encoding genes, *hlyA*, S fimbria-encoding genes, *chuA*, *fepC*, *cnf1*, *irp1*, *irp2*, *fyuA*, *iroN*, and *usp*; for MNEC, *ibeA*, *neuA*, and *neuC*; and for incomplete ExPEC, *kpsM*, *iutA*, *iucD*, *traT*, *malX*, *irp1*, *irp2*, *fyuA*, *chuA*, *fepC*, *iss*, and *kfiB*. Isolates which did not possess any virulence genes or had a few scattered virulence-related genes were considered nonpathogenic.

[b] *E. coli* isolates carrying at least one antimicrobial resistance gene.

virulence genes through genetic exchange, either individually or as pathogenicity islands (PAIs) (12, 24, 30). For the purposes of this study, we defined incomplete ExPEC as *E. coli* isolates carrying ExPEC related-genes that could not be assigned to one of the three defined ExPEC classes (UPEC, MNEC, and *E. coli* strains that cause septicemia). This category includes commensal *E. coli* isolates possessing virulence genes which presumably were acquired through genetic exchange (16) and UPEC with incomplete pathogenic profiles due to the absence of some genes associated with PAIs. Indeed, it has been shown that some UPEC genes located on PAIs are lost as the PAIs become unstable at temperatures lower than 21°C (24).

Our results revealed relatively high numbers and relatively high diversity of virulence and virulence-related genes in *E. coli* isolates from recreational waters (Table 1). Indeed, microarray hybridizations demonstrated that 29% of the *E. coli* isolates possessed virulence genes related to a pathotype. Pathotypes were attributed to *E. coli* samples on the basis of their sets of virulence genes or markers, as follows: for EAEC, *capU*, *shf*, *virK*, and aggregative adherence fimbria-encoding genes; for ETEC, heat-stable and heat-labile toxin-encoding genes and F4 and F18 fimbria-encoding genes; for atypical EPEC, *espA*, *espB*, *tir*, *eae* and variants, and absence of *bfpA*; for UPEC, P pilus-encoding genes, *hlyA*, S fimbria-encoding genes, *chuA*, *fepC*, *cnf1*, *irp1*, *irp2*, *fyuA*, *iroN*, and *usp*; for MNEC, *ibeA*, *neuA*, and *neuC*; and for incomplete ExPEC, *kpsM*, *iutA*, *iucD*, *traT*, *malX*, *irp1*, *irp2*, *fyuA*, *chuA*, *fepC*, *iss*, and *kfiB*. In our study, most isolates possessing virulence genes were classified as ExPEC (26.4% of the *E. coli* isolates); in contrast, the proportion of *E. coli* isolates that were classified as enteric pathotypes was low (2.2%). Within the ExPEC group, most isolates were classified as UPEC, a pathotype associated with urinary tract infections. Since the microarray carries all known virulence factors, numerous incomplete ExPEC which would normally be missed in a PCR-based assay were found. Thus, various unusual gene combinations were discovered, such as ExPEC pathogenic profiles with assorted ETEC genes like the toxin exporter gene *leoA* or the invasion protein gene *tia*. These unusual combinations provide evidence of genetic exchange between the various pathotypes (2). It is also surprising that we found almost as many isolates of MNEC (2.0%) as isolates of enteric pathotypes (2.2%), as MNEC is not a relatively common pathotype.

In this study, the method used to isolate *E. coli* strains from water was based on a high incubation temperature (44.5°C) and selection for β-glucuronidase activity. However, many studies have shown that isolates belonging to the O157:H7 serotype do not grow at this temperature (8, 32, 40) and are also known to be glucuronidase negative. For this reason, the frequency of *E. coli* isolates possessing virulence genes that we found is a minimum estimate. Although the prevalence of *E. coli* O157:H7 in surface waters has been found to be low (10, 17), it is quite possible that the proportion of pathogenic *E. coli* in our water samples could have been higher if our culturing methods had not excluded detection of O157:H7 and possibly other pathogenic *E. coli* strains.

Another interesting finding of our microarray study is the high genetic diversity among the *E. coli* isolates. We observed a very low frequency of identical microarray gene profiles for a given sampling date and even for the total set of *E. coli* isolates, except for one day. On that day, all 10 *E. coli* isolates were ExPEC, but they had only four different virulence gene profiles. For one of these, five isolates had identical profiles and thus may have represented one clone.

Pathotype distribution according to location and season. Table 2 shows the pathotype distributions for the three sampling locations (ankle depth, 6-m depth, and midharbor [depth, 24 m]) and the two seasons when samples were obtained (spring and summer). We found no significant difference (as determined using a binomial likelihood ratio test) in the prevalence of *E. coli* isolates possessing virulence genes at the three locations as approximately similar numbers were obtained for ankle depth and depths of 6 m and 24 m. However, enteric *E. coli* pathotypes were found only in deeper waters.

The most striking observation involved the temporal difference between spring and summer. As shown in Table 2, the proportion of spring *E. coli* isolates carrying virulence genes (21% of the *E. coli* isolates) was significantly greater than the proportion of summer isolates carrying virulence genes (8%) ($P < 0.0001$). The basis for this seasonal difference is unclear. Since some local sewage treatment plants do not start chlorinating final effluents until mid-May, it is possible that nonchlorinated effluents contributed to the higher frequency and diversity of *E. coli* pathotypes in offshore harbor water in the spring.

During the spring, the diversity of the pathotypes of the harbor (24-m) isolates was greater than the diversity of the pathotypes of the isolates obtained from the 6-m-deep water, and the isolates obtained from ankle depth water exhibited the lowest diversity (Table 2). This might reflect more diversity of

TABLE 2. Pathotypes and presence of antimicrobial resistance genes in *E. coli* isolates at different sampling locations during the spring and summer

Pathotype[a]	No. of isolates from indicated depth					
	Ankle depth		6 m		Harbor (24 m)	
	Total	With antimicrobial resistance	Total	With antimicrobial resistance	Total	With antimicrobial resistance
Spring						
ExPEC						
UPEC	4	0	7	1	11	2
MNEC	3	0	2	1	1	1
Incomplete ExPEC	9	0	9	3	11	6
Subtotal	16	0	18	5	23	9
Enteric *E. coli*						
EAEC	0	0	3	2	2	1
ETEC	0	0	0	0	1	1
Atypical EPEC	0	0	0	0	0	0
Subtotal	0	0	3	2	3	2
Nonpathogenic	49	3	38	4	34	7
Total	65	3	59	11	60	18
Summer						
ExPEC						
UPEC	2	0	1	1	3	0
MNEC	0	0	0	0	0	0
Incomplete ExPEC	11	0	3	1	4	1
Subtotal	13	0	4	2	7	1
Enteric *E. coli*						
EAEC	0	0	0	0	0	0
ETEC	0	0	0	0	0	0
Atypical EPEC	0	0	0	0	1	0
Subtotal	0	0	0	0	1	0
Nonpathogenic	32	1	36	2	31	5
Total	45	1	40	4	39	6

[a] Pathotypes were attributed to *E. coli* samples based on their sets of virulence genes or markers, as follows: for EAEC, *capU*, *shf*, *virK*, and aggregative adherence fimbria-encoding genes; for ETEC, heat-stable and heat-labile toxin-encoding genes and F4 and F18 fimbria-encoding genes; for atypical EPEC, *espA*, *espB*, *tir*, *eae* and variants, and absence of *bfpA*; for UPEC, P pilus-encoding genes, *hlyA*, S fimbria-encoding genes, *chuA*, *fepC*, *cnf1*, *irp1*, *irp2*, *fyuA*, *iroN*, and *usp*; for MNEC, *ibeA*, *neuA*, and *neuC*; and for incomplete ExPEC, *kpsM*, *iutA*, *iucD*, *traT*, *malX*, *irp1*, *irp2*, *fyuA*, *chuA*, *fepC*, *iss*, and *kfiB*.

E. coli isolates carrying virulence genes in sewage treatment plant effluents entering the harbor waters than in bird feces at the beach. This situation was not seen during the summer, when the profile diversities were similar for the three locations, which may also have reflected a reduced influence of *E. coli* from sewage treatment plant effluents, most of which were chlorinated at this time.

Prevalence of antimicrobial resistance genes. A total of 43 isolates (nearly 14% of the *E. coli* isolates analyzed) possessed at least one antimicrobial resistance gene (Table 3). Whereas one-half of the isolates containing antimicrobial resistance genes belonged to the ExPEC group, interestingly, the other isolates were found to be distributed within the nonpathogenic group (*E. coli* isolates which do not possess any virulence gene or have only a few scattered virulence-related genes).

For the isolates carrying resistance genes, the genes that were found most frequently were *tet*(A) and *tet*(B) (5.2% and 2.6% of *E. coli* isolates, respectively), bla_{TEM} (4.9%), *aadA1* (4.9%), and *sulII* (4.2%), which code for resistance to the tetracycline, ampicillin, streptomycin, and sulfonamide families, respectively (Table 4). In another study the workers found a similar distribution of antimicrobial resistance genes in animal feces, an agriculture environment, and human sewage (37). In our study, many of the *E. coli* isolates that carried resistance genes had a class 1 integron (4% of the isolates) and

TABLE 3. Distribution of antimicrobial resistance genes in *E. coli* according to season and location

Season	No. (%) of *E. coli* isolates from indicated depth carrying at least one antimicrobial resistance gene[a]			
	Ankle depth	6 m	Harbor (24 m)	Total
Spring	3 (7)	11 (26)	18 (42)	32 (74)
Summer	1 (2)	4 (9)	6 (14)	11 (26)
Total	4 (9)	15 (35)	24 (56)	43 (100)

[a] The percentages are the percentages of all *E. coli* isolates carrying at least one antimicrobial resistance gene.

TABLE 4. Characterization of antimicrobial resistance genes present in *E. coli* isolates

Antimicrobial agent(s)	Antimicrobial resistance gene[a]	No. (%) of isolates carrying the resistance gene[b]
Ampicillin	bla_{TEM}	15 (4.9)
	bla_{OXA-1}	2 (0.6)
Tetracycline	*tet*(A)	16 (5.2)
	tet(B)	8 (2.6)
	tet(C)	5 (1.6)
	tet(D)	1 (0.3)
Streptomycin, spectinomycin	*ant*(3″)-*Ia*	15 (4.9)
Gentamicin	*aac*(3)-*IIa*	1 (0.3)
Kanamycin, neomycin	*aph*(3′)-*Ia*	3 (1.0)
Chloramphenicol	*catI*	4 (1.3)
Trimethoprim	*dhfrI*	5 (1.6)
	dhfrVII	2 (0.6)
Sulfonamide	*sulI*	6 (1.9)
	sulII	13 (4.2)

[a] After hybridization, all isolates exhibited negative results with probes specific for bla_{SHV}, bla_{OXA-7}, bla_{PSE-4}, bla_{CTX-M}, *tet*(E), *tet*(Y), *ant*(2″)-*Ia*, *aac*(3)-*IV*, *aph*(3′)-*IIa*, *catII*, *catIII*, *floR*, *dhfrV*, *dhfrIX*, *dhfrXIII*, and *dhfrXV*.

[b] Percentages were calculated as follows: number of isolates with resistance gene/total number of *E. coli* isolates × 100. The total number of *E. coli* isolates was 308.

also carried two or more antimicrobial resistance genes. More specifically, all multiresistant *E. coli* isolates (isolates carrying more than three antimicrobial resistance genes) and the majority of *E. coli* isolates with three resistance genes contained a class 1 integron. Integrons, which can carry different antimicrobial resistance gene cassettes (23), are known to be a very efficient genetic mechanism for the diffusion of antimicrobial resistance genes and for the dissemination of resistance among bacterial pathogens (5). Among the genes found in our isolates, streptomycin (*aadA1*), sulfonamide (*sulI*), chloramphenicol (*catI*), and trimethoprim (*dhfrI* and *dhfrVII*) resistance genes were the genes that were most frequently associated with a class 1 integron, which agrees with the results of other studies (23, 35, 38).

The occurrence of antimicrobial resistance genes in *E. coli* was significantly different ($P < 0.0001$) for different water depths. Indeed, more than 50% of *E. coli* isolates carrying resistance genes were found in the middle of the harbor; in contrast, 35% of the isolates were found in 6-m-deep water, and only 9% of the isolates were found in ankle depth water (Table 3). The lower percentage of antimicrobial resistance genes in *E. coli* isolates from ankle depth beach water is consistent with the hypothesis that these *E. coli* isolates originated from bird droppings on the beach (9).

There were also variations between the spring and summer seasons (Table 3). Seventy-four percent of the *E. coli* isolates carrying resistance genes were found during the spring, and only 26% of the *E. coli* isolates carrying resistance genes were found during the summer ($P < 0.001$), although the diversities of the resistance genes found in the two seasons were similar. This may also have reflected the reduced influence of *E. coli* from sewage treatment plant effluents in the summer and reinforced the notion that most of the *E. coli* isolates carrying resistance genes had human origins.

Avian fecal pollution on beach. In order to investigate the relationship between pollution by wild birds and the three sampling locations, the *tsh* gene was used as an indicator of *E. coli* isolates from avian sources (7, 41). We observed differences in temporal and spatial distributions among the 30 *E. coli* isolates that were positive for *tsh*. More *E. coli* isolates carrying the *tsh* gene were found during the spring (20 isolates) than during the summer (10 isolates), and most of them were found near the beach (ankle depth, 13 isolates; 6 m, 10 isolates) rather than in the middle of the harbor (7 isolates), although the level of significance is not high ($P = 0.072$). Microarray data for *tsh*, therefore, seem to be consistent with antibiotic resistance analysis source tracking data and with the observation that many birds and their fecal droppings were present on the beach (9), which indicated the importance of bird contamination rather than human contamination of the beach water.

DISCUSSION

The parallel processing power of DNA microarrays allowed us to perform the first comprehensive assessment of the presence of virulence and antimicrobial resistance genes in waterborne *E. coli* isolates. Using an oligonucleotide microarray capable of detecting 189 virulence genes and markers as well as 30 commonly found antimicrobial resistance genes, a high percentage (29%) of *E. coli* isolates possessing virulence or virulence-related genes was found in recreational waters, along with a lower but significant number of isolates (14%) containing genes coding for antimicrobial resistance. Due to differences in methodology (PCR versus microarray), these numbers are difficult to compare with previously published results. In a previous PCR-based study done with recreational waters, a prevalence of pathogenic *E. coli* isolates of 0.9% was found (21). Two other studies, one performed with raw surface water and one performed with water from an agricultural waste lagoon, showed that the percentages of *E. coli* isolates possessing virulence genes were 10 and 7%, respectively (6, 22). In contrast, in two separate studies workers found higher numbers of *E. coli* isolates possessing virulence genes; however, the sampling was done in very different environments. The first study was conducted with water from a German river contaminated with communal sewage, and 41% of the *E. coli* isolates carried virulence markers; all these isolates were ExPEC (26). The second study was carried out with water from a highly polluted South African river (28), and 68% of the *E. coli* isolates were found to possess enteric virulence markers using PCR (27). Although both PCR and microarray methods are limited to determining genotypes and not phenotypes, these results nevertheless raise potential issues for public health. Additionally, it has been shown that the number of ExPEC virulence genes in an *E. coli* isolate is proportional to its pathogenic potential (31).

In spite of the large number of virulence gene-containing *E. coli* isolates, PCR studies are technologically constrained to focusing on a limited number of pathotypes. In contrast, by

virtue of their ability to detect all known virulence genes in parallel, DNA microarrays are inherently unbiased and are more able than conventional molecular techniques to detect unexpected combinations of virulence genes. Consequently, our DNA microarray is a powerful new molecular tool for (i) evaluation of genome plasticity by monitoring the transfer of virulence and antimicrobial resistance genes between *E. coli* strains and (ii) identification of new pathotypes. Indeed, for the most part virulence and antimicrobial resistance genes are on plasmids, bacteriophages, or pathogenicity islands. These genetic determinants contribute to the rapid evolution of *E. coli* strains and to the creation of new pathogenic variants since they are frequently subject to rearrangement, excision, and horizontal transfer. The situation is further complicated by the observation that pathogenicity islands are unstable and can be deleted from the genome in the environment (12, 24). The information obtained with this technique should be valuable in areas ranging from microbial ecology and population dynamics to epidemiology.

With our microarray, we found a high level of genetic diversity among the 308 environmental *E. coli* isolates tested, along with evidence of genetic exchanges. Furthermore, in addition to demonstrating that a high proportion of isolates carried a full pathotype set of virulence-related genes, the microarray also identified other isolates carrying unusual virulence gene combinations, and these unusual combinations could easily have been missed by conventional PCR tests. These isolates may have been commensal isolates which acquired virulence determinants in order to better survive in the host. This is in agreement with other studies which showed that nonpathogenic commensal and probiotic *E. coli* isolates could harbor many virulence-associated genes or PAI-localized genes, supporting their survival and successful colonization of the host (11, 13). By trading virulence or virulence-related genes to improve their chances of survival, these *E. coli* isolates also appear to be disseminating antimicrobial resistance genes since a relatively high number of the resistant isolates were characterized as nonpathogenic by our microarray analysis.

Pathotyping of *E. coli* isolates present in water sources used for drinking or recreation could be an important tool in the development of strategies to better protect public health. Duplication in other studies of the association of the presence of virulence and antimicrobial resistance genes with human wastewater found here could have an impact on the perceived need to achieve a high level of disinfection for wastewater treatment plant effluents. In terms of public health, it is also significant that a high percentage of ExPEC isolates was found; these pathogens, which can also be part of the human and animal intestinal flora (16), are responsible for an estimated 40,000 deaths and annual expenditures of at least $2.6 billion in the United States alone (33). Within the ExPEC group, we found an abundance of UPEC, the main cause of urinary tract infections (25), a frequent reason for consultation with a general practitioner. Therefore, the data clearly indicate that there is a need to better understand the public health implications of *E. coli* carrying virulence genes in recreational waters. In our laboratory another microarray study is in progress to investigate the occurrence of virulence and antibiotic resistance genes in *E. coli* from three other different locations (pristine water, water affected by agricultural wastes, and water affected by urban wastes).

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