APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1992, p. 3095–3100
0099-2240/92/093095-06$02.00/0

Vol. 58, No. 9

Detection of Virulence Factors in Culturable *Escherichia coli* Isolates from Water Samples by DNA Probes and Recovery of Toxin-Bearing Strains in Minimal *o*-Nitrophenol-β-D-Galactopyranoside–4-Methylumbelliferyl-β-D-Glucuronide Media

M. T. MARTINS,[1] I. G. RIVERA,[2] D. L. CLARK,[2] AND B. H. OLSON[2]*

Department of Microbiology, University of Sao Paulo, Sao Paulo, Brazil CEP 05508,[1] and School of Social Ecology, University of California, Irvine, California 92717[2]

Received 13 January 1992/Accepted 24 April 1992

A total of 449 *Escherichia coli* isolates in treated and raw water sources were submitted to DNA-DNA hybridization using seven different DNA probes to detect homology to sequences that code for Shiga-like toxins I and II; heat-stabile and heat-labile toxins, adherence factors EAF and *eae*, and the fimbrial antigen of enterohemorrhagic *E. coli*. Fifty-nine (13%) of the isolates demonstrated homology with one or more specific DNA probes. More than 50% of the isolates in treated water were not recovered in MMO-4-methylumbelliferyl-β-D-glucuronide media designed for detection of this indicator.

Between 1971 and 1983, according to the U.S. Environmental Protection Agency, there were 427 reported waterborne outbreaks of disease with over 100,000 individual cases of diarrhea in the United States. However, this number is considered by the Environmental Protection Agency to be an underestimation because most outbreaks and cases are not reported (13). Four outbreaks related to diarrheagenic *Escherichia coli* were reported in the United States during the period 1961 to 1970, with 188 cases of illness. One outbreak with 1,000 cases was reported during the period 1971 to 1988 (6). Recently, enterohemorrhagic *E. coli* (EHEC) O157:H7 was identified as the possible cause of a waterborne outbreak which occurred in Cabool, Mo., in 1989 and resulted in 240 illnesses and three deaths (33).

Within the *E. coli* species, there are four major categories of diarrheagenic *E. coli*: (i) enterotoxigenic *E. coli* (ETEC), a major cause of traveller's and infant diarrhea in less developed countries; (ii) enteroinvasive *E. coli*, a cause of dysentery; (iii) enteropathogenic *E. coli* (EPEC), a cause of infantile diarrhea and once a major cause of nursery outbreaks in industrialized countries; and (iv) EHEC, an important emerging cause of diarrhea and hemolytic-uremic syndrome (22).

The common approach for studying pathogenic *E. coli* is serological and biological tests. These techniques are expensive and laborious (3, 9, 28, 35) and are also limited in that they do not permit identification of a pathogenic strain outside a known pathogenic serogroup or of strains that do not fit previously identified phenotypic patterns (15). The DNA-DNA hybridization technique is a useful alternative in the study of virulence factors, especially for screening purposes and epidemiological studies (1, 3, 4, 9–11, 28). The filter hybridization technique has also been demonstrated to be 10^4 times more sensitive than the biological methods for detecting ETEC in water (8).

Many DNA probes for detection of virulence features of *E. coli* have been described. For ETEC, the probes commonly used are for heat-labile enterotoxin (LT) and toxins STh and STp (26). An *eae* probe has been used to detect genes that codify the production of attaching-effacing lesions caused by EPEC and EHEC (19). An EPEC adherence factor (EAF) probe has been proposed to detect homology with the genes responsible for the EAF (18, 29). A specific probe for detection of homology with sequences of the plasmid responsible for the adherence of EHEC to cell cultures has also been obtained (23). Probes that detect homology for bacteriophage-encoded Shiga-like toxin I (SLTI) and SLTII are frequently used (30). These probes have been used to detect virulence features of *E. coli* isolates from human clinical specimens, animals, and food samples and have shown good sensitivity and specificity (9–11, 23, 25, 26, 28, 30, 35). Although water is reported as one of the routes of dissemination of pathogenic *E. coli* strains (6, 13), research on their distribution in the aquatic environment has not been extensive (37) and few studies using molecular techniques for pathogenic *E. coli* detection in water samples have been reported (8–11).

The purpose of this study was to assess the genetic occurrence of virulence-producing factors in *E. coli* isolates from treated and raw water and relate these data to those obtained in a previous study for evaluation of the performance of minimal *o*-nitrophenol-β-D-galactopyranoside (MMO)–4-methylumbelliferyl-β-D-glucuronide (MUG) media (5) in the detection of natural isolates of toxin-bearing *E. coli*.

MATERIALS AND METHODS

***E. coli* isolates.** A total of 212 isolates obtained from water samples collected from a treated water reservoir and a total of 237 isolates recovered from raw surface water were analyzed by DNA-DNA hybridization with genes that code for virulence features in *E. coli*. These 449 strains were isolated in a previous study to compare MMO-MUG media Colilert (CL) and Coliquik (CQ) with the standard membrane filtration method for fecal coliforms. The *E. coli* isolates were identified by using API 20E (Analytab Products, Plain-

* Corresponding author.

view, N.Y.) and reinoculated in MUG-containing medium. The results of sorbitol fermentation obtained in API 20E were also recorded. Descriptions of the water source, quality, and treatment and the method of bacterial isolation are presented elsewhere (5, 32).

Positive and negative controls. Nine *E. coli* strains obtained from the American Type Culture Collection (ATCC; Rockville, Md.) were used as control strains. *E. coli* ATCC 43886, serotype O25:K98:NM (CDC E2539-C1), produces LT; ATCC 43896, serotype O78:K80:H12 (CDC TX 1), produces heat-stable enterotoxin (ST); ATCC 35401, serotype O78:H11, produces ST and LT; ATCC 43890, serotype O157:H7 (CDC 3526-87) EHEC, produces cytotoxin SLTI; ATCC 43888, serotype O157:H7 (CDC B6914-MS 1), does not produce SLTI and SLTII; ATCC 43889, serotype O157:H7 (CDC B1409-C1) EHEC, produces cytotoxin SLTII; ATCC 43895, serotype O157:H7 (CDC EDL 933), produces SLTI and SLTII; ATCC 43887, serotype O111 (CDC B170), exhibits localized adherence to the mucosal surface of HeLa cells and the other hosts. Strain ATCC 43651 was used as a negative control (14).

Assessment of β-D-glucuronidase (GUR) in reference strains of pathogenic *E. coli*. The ATCC strains tested for growth in MUG-containing media to detect GUR activity were inoculated in EC medium with MUG (Difco) and incubated at 35 and 44.5°C for 24 h and in CL medium (Access Analytical, Branford, Conn.) at 35°C for 24 h. GUR activity was detected by the fluorescence of the medium when illuminated with a 6-W long-wavelength (366-nm) UV source.

Strains screened for the presence of the *uidA* gene. All natural isolates that had homology to any of the virulence feature sequences and five ATCC strains (43890, 43888, 43889, 43895, and 43887) were assayed for the presence of the *uidA* gene.

Plasmids used for probe preparation. For detection of homologies with virulence factors of *E. coli*, DNA probes were obtained from the following plasmids.

pCVD419 is a recombinant of the 3.4-kb segment of the 90-kb EHEC fimbrial-ecoding plasmid from O157:H7 strain 933 cloned into pBR325 (23). This was not found in a GenBank search.

pJN37-19 consists of a 1,142-bp fragment located between the *Taq*I restriction site and the final *Hin*cII restriction site of the SLTI-encoding gene cloned in pUC19. This fragment encodes 98% of the A subunit and all of the B subunit of SLTI (16, 30). The GenBank accession number is M19473.

pNN111-19 is a recombinant plasmid containing an 842-bp fragment present in the A subunit of the SLTII-encoding gene. This fragment encodes 95% of the SLTII A subunit and was cloned into pUC19 (16, 30). The GenBank accession number is X07865.

pJPN16 consists of a 1-kb *Sal*I-*Bam*HI fragment of pMAR2, the EAF plasmid that encodes localized adherence to HEp-2 cells, cloned into pCVD315 (18). This was not found in a GenBank search.

pDAS100 consists of insertion of a 215-bp *Hpa*II fragment from the ST-encoding gene of pSLM004 into the *Acc*I site of pUC8 containing the ST of ETEC (27). The GenBank accession number is M34916.

pDAS102 consists of insertion of a *Hin*dIII fragment of approximately 850 bp from the LT-encoding region of plasmid pEWD299 (7) into pUC8 (25a). This was not found in a GenBank search.

pCVD434 is a recombinant of the 1-kb *Sal*I-*Stu*I fragment from the *eae* structural gene cloned into the *Sal*I-*Sma*I site of pUC19 involved in attaching-effacing activity (19). The GenBank accession number is M58154 or M34051.

For the *uidA* gene, plasmid pRAJ255 (Clontech Laboratories, Inc., Palo Alto, Calif.) was used. This plasmid contains the 1.87-kb *Pst*I-*Eco*RI *uidA* fragment inserted into the polylinker site of pEMBL 9 (17). The GenBank accession number is M14641.

Preparation of DNA probes. Plasmid extraction was performed by the alkaline lysis procedure of Birnboim and Doly (2) modified by purification of nucleic acids in a Qiagen column (Qiagen, Inc., Chatsworth, Calif.). Plasmids were digested with appropriate restriction endonucleases (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) under conditions specified by the manufacturer. Isolation and purification of the fragments after electrophoresis in 1% agarose were performed with Geneclean II (Bio 101, Inc., La Jolla, Calif.). The DNA probes were radiolabeled with [α^{32}P]dCTP by random priming to specific activities ranging from 1.0×10^9 to 3.6×10^9 cpm/μg of DNA (ICN Biomedical, Inc., Costa Mesa, Calif.) and purified in Bio-Spin columns (Bio-Rad, Life Sciences Research Products, Richmond, Calif.).

Processing of cells for DNA hybridization assays. Overnight cultures grown in L broth (36) were washed twice in phosphate-buffered saline (PBS) and adjusted to a cell concentration of $\sim 5.0 \times 10^6$ CFU/200 μl of PBS. Before filtration, nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.) were exposed to filter paper (Schleicher & Schuell) saturated in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) until completely wet. They were then used to filter 100 μl of 20× SSC under a gentle vacuum. A 200 μl volume of a cell suspension was then added per well of a slot blot apparatus (Minifold; Schleicher & Schuell), filtered under a gentle vacuum, and washed with 200 μl of PBS. The filter was removed from the apparatus and placed cell side up on filter paper (Whatmann 3MM) saturated with 0.5 M NaOH for 10 min. This was followed by transfer to neutralizing filter paper saturated with buffer I (0.6 M NaCl, 1.0 M Tris-HCl, pH 6.8) for 5 min. The filter was then transferred to a filter paper saturated with buffer II (1.5 M NaCl, 0.5 M Tris-HCl, pH 6.8) for 5 min. Filters were air dried and baked at 80°C for 2 h under a vacuum (National Appliance Co., Portland, Oreg.).

Hybridization. Filters were incubated overnight at 42°C in a prehybridization solution (50% formamide, 5× Denhardt's solution, 5× SSPE, 0.1% sodium dodecyl sulfate [SDS], 100 μg of denaturated salmon sperm DNA per ml). A DNA template was then added, and the combination was incubated overnight at 42°C. Two stringency washes were performed to study the virulence factors. The low-stringency wash consisted of two steps in 1.0× SSC–0.1% SDS; the first step was 15 min at 50°C, and the second was 30 min at 50°C. For high stringency, two washes of 15 min each were performed at 65°C in 0.1× SSC–0.1% SDS (20). The study of the *uidA* gene was carried out at high stringency only, and the wash temperature was 68°C (36). For detection of autoradiographic signals, filters were exposed overnight at −70°C to X-Omat AR X-ray film (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen (Fisher Scientific, Pittsburgh, Pa.) and the films were developed in accordance with the manufacturer's instructions. Filters were stripped to remove the radioactive label and stained with 1% methylene green to confirm cell lysis.

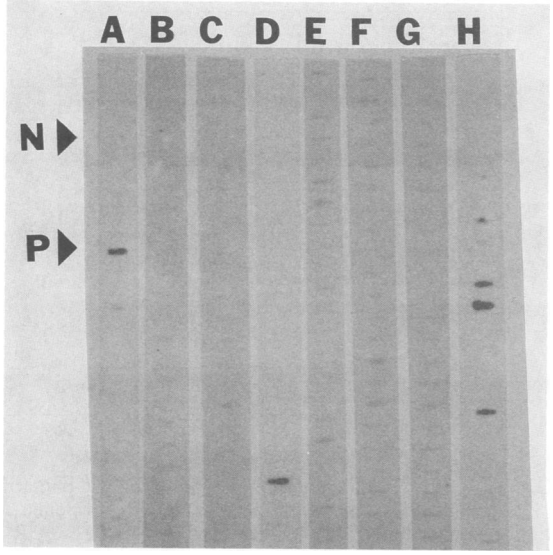

FIG. 1. Autoradiographic detection of virulence factor genes in *E. coli* isolated from raw and treated drinking water. Lanes: A, positive (P) and negative (N) controls; B, template probed with fimbrial antigen DNA for EHEC; C, template probed with localized adherence to HEp-2 cells (EAF) DNA for EPEC; D, template probed with attaching and effacing lesion DNA (*eae*) for EPEC and EHEC; E, template probed with SLTI DNA; F, template probed with SLTII DNA; G, template probed with ST DNA; H, template probed with LT DNA.

RESULTS AND DISCUSSION

For results obtained with *E. coli* isolates that reacted with probes specific for virulence features under the two stringency conditions used, see Tables 1 (treated water) and 2 (raw water). For the phenotypic and genotypic characteristics of these strains in isolation media for *E. coli*, see Fig. 2 and 3. For results obtained in the study of reference pathogenic *E. coli* strains under conditions for isolation from water, see Table 3. One example of the results obtained in the hybridization assays is presented in Fig. 1.

Distribution of *E. coli* bacteria containing sequences for virulence genes in water. (i) Treated water. Two hundred twelve *E. coli* isolates from fully treated water were screened by using the seven selected probes that detect virulence features of *E. coli*. Thirty-eight (17.9%) reacted with one or more DNA probes specific for diarrheagenic *E. coli*. The data obtained are displayed in Table 1. *E. coli* strains that produced positive results with the probe containing sequences of the gene that encodes the ST of ETEC were predominant, with a frequency of 9.7% (20 of 212), followed by *E. coli* strains that contained a fraction of the nucleotide sequence with homology to the *eae* gene of EPEC (4.2%; 9 of 212). *E. coli* strains with homology to one or both of the sequences related to SLT production had a frequency of 4.2% (9 of 212); three of these strains reacted with more than one probe. Of these three that reacted with the SLTI and SLTII probes, one also reacted with the EAF probe and another reacted with the EAF probe and the *eae* locus probe.

TABLE 1. Distribution of *E. coli* isolates[a] in treated water that reacted with DNA probes specific for virulence features of diarrheagenic *E. coli* under low- and high-stringency conditions

DNA probe(s)	No. of strains positive, negative	
	Low stringency	High stringency
STh	20, 192	19, 193
eae	9, 203	9, 203
SLTI	1, 211	1, 211
SLTII	5, 207	3, 209
SLTI + SLTII	1, 211	0, 212
SLTI + SLTII + EAF	1, 211	1, 211
SLTI + SLTII + EAF + *eae*	1, 211	1, 211
Total	38, 174	34, 178

[a] n = 212 strains.

TABLE 2. Distribution of *E. coli* isolates[a] from raw surface water that reacted with DNA probes specific for virulence features of diarrheagenic *E. coli* under low- and high-stringency conditions

DNA probe	No. of strains positive, negative	
	Low stringency	High stringency
STh	6, 231	5, 232
LT	1, 236	1, 236
eae	3, 234	3, 234
SLTI	8, 229	2, 235
EAF	3, 234	1, 236
Total	21, 216	12, 225

[a] n = 237 strains.

None of the isolates tested reacted with the probe containing the sequences of the EHEC plasmid or the probe that detects the sequences for LT. These last results could be explained by the temperature used for primary isolation of the strains in the previous study. The method used for fecal coliform detection in membrane fecal coliform (M-FC) medium requires incubation at 44.5°C, and this temperature is reported to cause plasmid loss in *E. coli* strains (8).

(ii) Raw surface water. Two hundred thirty-seven *E. coli* isolates from raw water were submitted to the same probes used to test the isolates from treated water. Twenty-one (8.9%) of these isolates reacted with one of five probes (Table 2). Negative results were obtained with all the isolates with the probes for SLTII and the fimbrial antigen of EHEC. *E. coli* strains with sequences homologous to the probe containing the genes that encode SLTI were predominant, with a frequency of 3.4% (8 of 237), followed by the strains homologous to the probe for the ST of ETEC (2.5%; 6 of 237). One *E. coli* isolate had a sequence homologous to that of the LT of ETEC. Three isolates reacted with the *eae* probe, and three reacted with the EAF probe.

As the raw water was fully treated and postdisinfected with chloramines before leaving the treatment plant, the bacterial content of the reservoir was not related to that of raw water. The open nature of the reservoir allows introduction of bacteria from a variety of sources. As this is the first research using several probes for virulence, only a few studies exist in the literature for comparison. Echeverria et al. studied the distribution of ETEC in water from villages in Thailand by using LT, STp, STh, and STII probes (8–11). They reported that 9% of the total number of water samples (31 of 350) contained bacteria with DNA homologous to enterotoxin gene-encoding probes (8) and 20% of water samples collected from homes of patients with ETEC-associated diarrhea contained such bacteria. In homes of patients

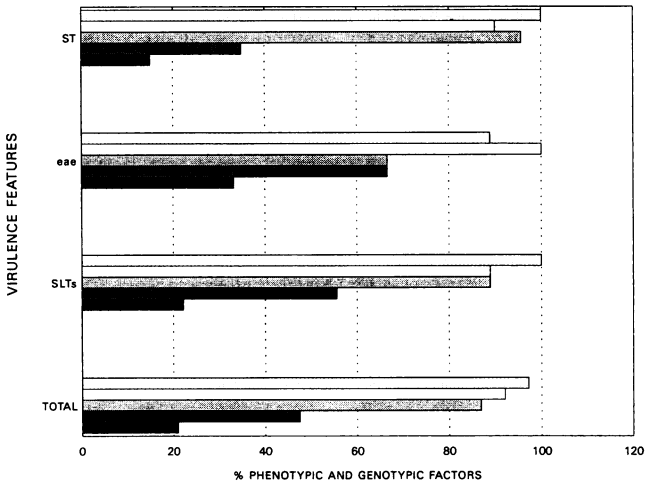

FIG. 2. Phenotypic and genotypic factors of *E. coli* bacteria that were isolated from treated water and reacted with virulence factor DNA probes. SLTI, SLTII, SLTI-SLTII, SLTI-SLTII-EAF, and SLTI-SLTII-EAF-*eae* were included. Symbols: ▨, *uidA*; □, sorbitol; ▨, GUR after reinoculation; ■, CQ; ■, CL.

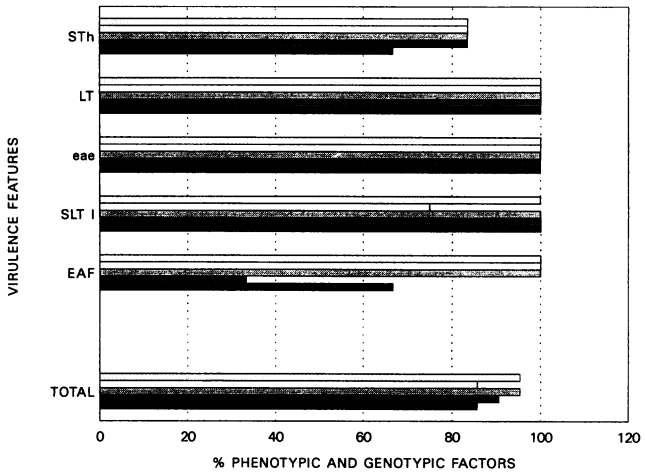

FIG. 3. Phenotypic and genotypic factors of *E. coli* bacteria that were isolated from raw water and reacted with virulence factor DNA probes. Symbols: ▨, *uidA*; □, sorbitol; ▨, GUR after reinoculation; ■, CQ; ■, CL.

with non-ETEC-associated diarrhea, bacteria with DNA homologous to enterotoxin gene-encoding probes were also found in water but in smaller proportions. Those researchers detected *E. coli* strains with sequences homologous to the ST and LT probes with similar frequency (9, 11).

In this study, we detected a predominance of *E. coli* strains with sequences homologous to the ST-encoding gene. LT probably was not detected because our isolates were obtained from M-FC medium at 44.5°C and the plasmid that encodes this toxin might be lost at this temperature (8). Sato et al. (37), in Brazil, studied the distribution of ETEC in water and sewage by using the standard method of Y-1 cell cultures for LT and suckling mice for ST and found low incidences of ETEC in sewage (1.9%) and polluted water (2.4%). The disagreement between these results and those of our study could be explained by the low sensitivity of the earlier biological methods. One of the advantages of direct use of nucleic acid probes is that it allows identification of strains that can have virulence properties and could be missed if only strains that have phenotypic patterns of pathogenic *E. coli* were tested (15). Thus, there is a need to determine whether *E. coli* strains that contain virulence features produce disease symptoms to develop a true comparison between our data and previously reported data.

McGowan et al. (24) reported the isolation of O157:H7 EHEC from a water reservoir in Philadelphia, Pa. As this reservoir had no evidence of human fecal pollution, they suggested that wildlife, mainly deer, could be the source of this pathogen. We concluded that this serotype was not present in our study, as no isolates reacted with the DNA probe obtained from pCVD419 (23). None presented the basic phenotypic features described for preliminary identification of O157:H7 EHEC (MUG negative, sorbitol negative [21]). The isolates in this study fermented sorbitol, as evidenced in API 20E (92.1%), and when reinoculated in medium containing MUG were GUR positive (86.9%) (Fig. 2). A similar pattern was observed in raw water isolates (Fig. 3). It is important to note that strains with fractional nucleotide sequence similarity to SLTI and SLTII, the toxins of EHEC, were detected. This can be explained because other serotypes of *E. coli*, including the ones not previously reported as pathogenic, that produce SLTI, SLTII, or both are being isolated and are now described as EHEC (1, 3, 4, 25, 30).

The absence of a response for O157:H7 is not surprising, as this serotype is reported as unable to grow at the temperature (44.5°C) normally used in the *Standard Methods* procedure for fecal coliform detection (34).

In this study, strains that were positive for either ST or LT from ETEC and either SLTI or SLTII from EHEC were identified. These findings are consistent with reports in the literature that ETEC, instead of producing both ST and LT, can produce either toxin and be pathogenic (28). This is also valid for SLT (1, 4, 31).

In our study, *E. coli* strains were found more frequently to have homology to only one of these virulence factor probes but several *E. coli* strains had homology to both SLTs and also contained either the *eae* or the EAF gene or both. Many researchers have reported EPEC strains that produce verocytotoxins or SLTs (1, 4), so our results are consistent with the pathogenic potential of *E. coli*. Also, the strains that produced positive results with probes for virulence and adherence can be considered a major risk to human health (8).

SLT synthesis has been strongly implicated as a factor contributing to the pathogenesis of EHEC. Production of SLTI and SLTII in some *E. coli* strains is associated with toxin-converting bacteriophages that represent potentially mobile elements. Production of SLTs has been demonstrated in other enterobacteria, so the full range of variability among the SLT family, as well as the evolutionary pressure that resulted in their widespread presence among diverse bacterial strains, remains to be determined (16). More studies of the distribution of SLT-converting phage in the aquatic environment and its possible transmission to other strains are need.

The *eae* locus can be present in either EPEC or EHEC (19). The probe for its detection was described recently, and our study is the first to examine its presence in *E. coli* strains from water samples.

Stringencies used in this study. Two stringencies were chosen to assess *E. coli* isolates for sequences homologous

to those related to virulence factors. Of the 38 strains detected in treated water, 34 (89.5%) gave positive results at both stringencies (Table 1). The strains isolated from raw water showed more genetic divergence, with 12 (57%) of the isolates producing positive results at high stringency, as shown by the results for SLTI and EAF (Table 2).

Most studies with probes are performed under high-stringency conditions using low salt concentrations and a temperature range of 60 to 68°C (4, 8, 28, 30, 35). Some researchers have reported the use of lower temperatures, in the range of 50 to 55°C, and thus their work was done under lower-stringency conditions (3, 27). SLT probes require high stringency for detection of more than 80% homology (31). The results of this study suggest that evolutionary divergence in virulence genes occurred, because only 34.2% (13 of 38) of the virulence factors were detected at lower stringency.

Detection of *E. coli* strains that show potential virulence in MMO-MUG preparations. The results obtained in the DNA-DNA hybridization assays were compared with those obtained with isolates collected from a previous study that evaluated the performance of MMO-MUG media (5). These results are shown in Fig. 2 and 3.

Treated water. The total data for treated water (Fig. 2) show that only 8 (21%) of the isolates were recovered in CL medium and only 18 (47.4%) were recovered in CQ medium. Thirty (79%) and 20 (52.6%) of these isolates would be missed, respectively, if CL or CQ were used alone for assessment of drinking water safety. The identification of these *E. coli* isolates (12) was good, as the API 20E profile codes were all classified as excellent (84.7%) or very good (15.3%), with a predominance of codes 5144572, 5044552, and 5144552. Most (97.2%) of the strains were homologous to a nucleotide template from the *uidA* gene that encodes GUR activity. When these isolates were reinoculated in MUG-containing medium, 86.9% were positive. A similar pattern was obtained for the different virulent strains (Fig. 2).

Raw water. It is noteworthy that in raw water, 85.7% of the virulence factor-bearing *E. coli* isolates were recovered in CL and 90.5% were recovered in CQ (Fig. 3). From these data, we can assume that the poor performance of the MMO-MUG preparation in treated water could be related to the physiological conditions of the bacteria. Also, the *uidA* gene and GUR activity after reinoculation were detected in 95.3% of the strains.

Assessment of GUR activity and presence of the *uidA* gene in reference ATCC strains of pathogenic *E. coli*. To verify that pathogenic *E. coli* strains behave differently in MUG-containing media, nine ATCC strains of *E. coli* were inoculated in CL and EC-MUC and incubated, respectively, at 35°C and 44.5°C for 24 h. Five of the strains were also tested for the presence of the *uidA* gene (Table 3). Only three of the nine strains produced GUR at 44.5°C. Three EHEC strains were also GUR negative at 35°C. These data confirm other reports that EHEC does not utilize MUG (21). Only one O157:H7 strain, ATCC 43888, reportedly isolated from human feces and not an SLT producer, was GUR positive. Five of the strains were tested with the probe for the *uidA* gene, and this sequence was detected in all of the strains. The data obtained in the study of pathogenic reference strains are qualitative, preliminary, and not intended to assess the performance of MMO-MUG preparations. They were included in this study for one possible explanation of the results gathered for natural isolates, and they also show that incubation temperature should be considered in the evaluation of new methods for examination of drinking water.

TABLE 3. GUR activity and presence of the *uidA* gene in ATCC strains of pathogenic *E. coli*

ATCC strain	Virulence features	Growth in MMO-MUG medium			*uidA* gene[a]
		CL, 35°C	EC-MUG		
			35°C	44.5°C	
43890[b]	SLTI (EHEC)	−	−	−	++
43888[b]	Absent	+	+	−	+
43889[b]	SLTII (EHEC)	−	−	−	+
43895[b]	SLTI + SLTII (EHEC)	−	−	−	+
43887	EAF (EPEC)	+	+	+	++
43886	LT (ETEC)	+	−	−	NT
43896	ST (ETEC)	+	+	+	NT
43651	Absent	+	+	−	NT
35401	LT + ST (ETEC)	+	+	+	NT

[a] NT, not tested.
[b] Strain belongs to serotype O157:H7.

Significance of the results for virulence factors. Echeverria et al. (8) performed epidemiological studies of ETEC in Thailand and concluded that since 10^8 ETEC bacteria are required to cause diarrhea in healthy volunteers and that colonization factors are presumably also required to elicit clinical illness, the widespread presence of low levels of ETEC in water may not pose a serious health hazard. Sato et al. (37) concluded that their findings of *E. coli* with virulence features in water were epidemiologically important and emphasized the necessity that drinking water be free from fecal coliforms.

This study showed a significant incidence of strains that have the genetic potential for production of virulence factors. Although it is thought that the colonization factors are required for a strain to be considered pathogenic, we conclude that the isolates found in this study can represent a health risk and support the requirement that no fecal coliforms be present in drinking water. Another important finding is the lack of recovery of many of these strains by MMO-MUG media.

Conclusions. The DNA-DNA hybridization technique provides a sensitive method for detecting organisms that carry genes that encode virulence factors in water samples. *E. coli* bacteria homologous to template DNA for virulence features of EPEC, ETEC, and EHEC were isolated in water samples.

The MMO-MUG preparations failed to detect these bacteria in more than 50% of the samples of treated water.

In the evaluation of new methods for drinking water assessment, not only the phenotype but also the genotype, incubation temperature, and trait expression must be considered.

ACKNOWLEDGMENTS

We are grateful to J. B. Kaper (University of Maryland), J. W. Newland (Walter Reed Army Institute), and S. L. Moseley (University of Washington) for kindly providing the plasmids utilized in this study.

REFERENCES

1. **Bettelheim, K. A., J. E. Brown, S. Lolekha, and P. Echeverria.** 1990. Serotypes of *Escherichia coli* that hybridized with DNA probes for genes encoding Shiga-like toxin I, Shiga-like toxin II, and serogroup O157 enterohemorrhagic *E. coli* fimbriae isolated

from adults with diarrhea in Thailand. J. Clin. Microbiol. **28**:293–295.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Bohnert, M. G., H. M. d'Hauteville, and P. J. Sansonetti.** 1988. Detection of enteric pathotypes of *Escherichia coli* by hybridization using six DNA probes. Ann. Inst. Pasteur/Microbiol. (Paris) **139**:189–202.
4. **Brown, J. E., P. Echeverria, D. N. Taylor, J. Seriwatana, V. Vanapruks, U. Lexomboon, R. N. Neill, and J. W. Newland.** 1989. Determination by DNA hybridization of Shiga-like-toxin-producing *Escherichia coli* in children with diarrhea in Thailand. J. Clin. Microbiol. **27**:291–294.
5. **Clark, D. L., B. B. Milner, M. H. Stewart, R. L. Wolfe, and B. H. Olson.** 1991. Comparative study of commercial 4-methylumbelliferyl-β-D-glucuronide preparations with the *Standard Methods* membrane filtration fecal coliform test for the detection of *Escherichia coli* in water samples. Appl. Environ. Microbiol. **57**:1528–1534.
6. **Craun, G. F.** 1990. Causes of waterborne outbreaks in the United States, p. 149–152. AWWA Water Quality Technology Proceedings. American Water Works Association, Denver.
7. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139**: 850–858.
8. **Echeverria, P., J. Seriwatana, U. Chityothin, W. Chaicumpa, and C. Tirapat.** 1982. Detection of enterotoxigenic *Escherichia coli* in water by filter hybridization with three enterotoxin gene probes. J. Clin. Microbiol. **16**:1086–1090.
9. **Echeverria, P., J. Seriwatana, U. Lecksomboon, C. Tirapat, W. Chaicumpa, and B. Rowe.** 1984. Identification by DNA hybridization of enterotoxigenic *Escherichia coli* in homes of children with diarrhea. Lancet **i**:63–66.
10. **Echeverria, P., J. Seriwatana, U. Patamaroj, S. L. Moseley, A. McFarland, O. Chityothin, and W. Chaicumpa.** 1984. Prevalence of heat-stable II enterotoxigenic *Escherichia coli* in pigs, water, and people at farms in Thailand as determined by DNA hybridization. J. Clin. Microbiol. **19**:489–491.
11. **Echeverria, P., J. Seriwatana, D. N. Taylor, C. Tirapat, W. Chaicumpa, and B. Rowe.** 1985. Identification by DNA hybridization of enterotoxigenic *Escherichia coli* in a longitudinal study of villages in Thailand. J. Infect. Dis. **151**:124–130.
12. **Edberg, S. C.** 1991. More about Colilert. J. Am. Water Works Assoc. **83**:5. (Letter.)
13. **Federal Register.** 1989. Drinking water; national primary drinking water regulation; total coliforms (including fecal coliforms and *E. coli*); final rule. Fed. Regist. **54**:27544–27568.
14. **Gherna, R., P. Pienta, and R. Cote (ed.).** 1989. American Type Culture Collection catalogue of bacteria and phages, 17th. American Type Culture Collection, Rockville, Md.
15. **Gomes, T. A. T., M. R. F. Toledo, L. R. Trabulsi, P. K. Wood, and J. G. Morris, Jr.** 1987. DNA probes for identification of enteroinvasive *Escherichia coli*. J. Clin. Microbiol. **25**:2025–2027.
16. **Jackson, M. P., R. J. Neill, A. D. O'Brien, R. K. Holmes, and J. W. Newland.** 1987. Nucleotide sequence analysis and comparison of the structural genes for Shiga-like toxin I and Shiga-like toxin II encoded by bacteriophages from *Escherichia coli* 933. FEMS Microbiol. Lett. **44**:109–114.
17. **Jefferson, R. A., S. M. Burgess, and D. Hirsh.** 1986. β-Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proc. Natl. Acad. Sci. USA **83**:8447–8451.
18. **Jerse, A. E., W. C. Martin, J. E. Galen, and J. B. Kaper.** 1990. Oligonucleotide probe for detection of the enteropathogenic *Escherichia coli* (EPEC) adherence factor of localized adherent EPEC. J. Clin. Microbiol. **28**:2842–2844.
19. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87**:7839–7843.
20. **Keller, G. H., and M. N. Manak.** 1989. DNA probes, p. 168–180. Stockton Press, New York.
21. **Krishnan, C., V. A. Fitzgerald, S. J. Dakin, and R. J. Behme.** 1987. Laboratory investigation of outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7. J. Clin. Microbiol. **25**:1043–1047.
22. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155**:377–389.
23. **Levine, M. M., J. Xu, J. B. Kaper, H. Lior, V. Prado, B. Tall, J. Nataro, H. Karch, and K. Wachsmuth.** 1987. A DNA probe to identify enterohemorrhagic *Escherichia coli* of O157:H7 and other serotypes that cause hemorrhagic colitis and hemolytic uremic syndrome. J. Infect. Dis. **156**:175–182.
24. **McGowan, K. L., E. Wickersham, and N. A. Strockbine.** 1989. *Escherichia coli* O157:H7 from water. Lancet **i**:967–968. (Letter.)
25. **Montenegro, M. A., M. Bülte, T. Trumpf, S. Aleksić, G. Reuter, E. Bulling, and R. Helmuth.** 1990. Detection and characterization of fecal verotoxin-producing *Escherichia coli* from healthy cattle. J. Clin. Microbiol. **28**:1417–1421.

25a. **Moseley, S. L.** Personal communication.

26. **Moseley, S. L., P. Echeverria, J. Seriwatana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow.** 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. **145**:863–869.
27. **Moseley, S. L., J. W. Hardy, M. I. Huq, P. Echeverria, and S. Falkow.** 1983. Isolation and nucleotide sequence determination of a gene encoding a heat-stable enterotoxin of *Escherichia coli*. Infect. Immun. **39**:1167–1174.
28. **Moseley, S. L., I. Huq, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142**:892–898.
29. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152**:560–565.
30. **Newland, J. W., and R. J. Neill.** 1988. DNA probes for Shiga-like toxins I and II and for toxin-converting bacteriophages. J. Clin. Microbiol. **26**:1292–1297.
31. **Newland, J. W., N. A. Strockbine, and R. J. Neill.** 1987. Cloning of genes for production of *Escherichia coli* Shiga-like toxin type II. Infect. Immun. **55**:2675–2680.
32. **Olson, B. H., D. L. Clark, B. B. Milner, M. H. Stewart, and R. L. Wolfe.** 1991. Total coliform detection in drinking water: comparison of membrane filtration with Colilert and Coliquik. Appl. Environ. Microbiol. **57**:1535–1539.
33. **Parmelee, M. A.** 1990. Outbreak reveals vulnerability of water operations. Am. Water Works Assoc. MainStream **34**:1–9.
34. **Raghubeer, E. V., and J. R. Matches.** 1990. Temperature range for growth of *Escherichia coli* serotype O157:H7 and selected coliforms in *E. coli* medium. J. Clin. Microbiol. **28**:803–805.
35. **Samadpour, M., J. Liston, J. E. Ongerth, and P. I. Tarr.** 1990. Evaluation of DNA probes for detection of Shiga-like-toxin-producing *Escherichia coli* in food and calf fecal samples. Appl. Environ. Microbiol. **56**:1212–1215.
36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.48–9.50. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
37. **Sato, M. I., P. S. Sanchez, M. T. Martins, M. H. L. Reis, and L. R. Trabulsi.** 1983. Isolation of enterotoxigenic Escherichia coli in water and sewage in Sao Paulo, Brazil. Rev. Microbiol. Sao Paulo **14**:276–281.