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Molecular Method for Detection of Total Coliforms in Drinking Water Samples

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This work demonstrates the ability of a bacterial concentration and recovery procedure combined with three different PCR assays targeting the *lacZ*, *wecG*, and 16S rRNA genes, respectively, to detect the presence of total coliforms in 100-ml samples of potable water (presence/absence test). PCR assays were first compared to the culture-based Colilert and MI agar methods to determine their ability to detect 147 coliform strains representing 76 species of *Enterobacteriaceae* encountered in fecal and environmental settings. Results showed that 86 (58.5%) and 109 (74.1%) strains yielded a positive signal with Colilert and MI agar methods, respectively, whereas the *lacZ*, *wecG*, and 16S rRNA PCR assays detected 133 (90.5%), 111 (75.5%), and 146 (99.3%) of the 147 total coliform strains tested. These assays were then assessed by testing 122 well water samples collected in the Québec City region of Canada. Results showed that 97 (79.5%) of the samples tested by culture-based methods and 95 (77.9%), 82 (67.2%), and 98 (80.3%) of samples tested using PCR-based methods contained total coliforms, respectively. Consequently, despite the high genetic variability of the total coliform group, this study demonstrated that it is possible to use molecular assays to detect total coliforms in potable water: the 16S rRNA molecular assay was shown to be as efficient as recommended culture-based methods. This assay might be used in combination with an *Escherichia coli* molecular assay to assess drinking water quality.

Individual-pathogen monitoring in water is technically achievable but currently unfeasible due to the costs involved and the number of possible pathogens. Since numerous pathogens occur in feces, water is monitored for microbial contamination using indicator organisms such as total coliforms and *Escherichia coli* (1). Total coliforms are a group of bacteria commonly found in the aquatic environment in soil and vegetation, as well as in the intestines of mammals, including humans (2). Despite reservations about their usefulness as indicators of fecal contamination, the total coliform group remains a water quality indicator in many countries and continues to be used to some extent as a regulatory parameter (3, 4). Indeed, even in situations in which fecal contamination is present, total coliforms are more numerous than *E. coli*, thereby representing a more sensitive indicator (5). Furthermore, some members of the total coliform group are considerably more resistant to disinfection than *E. coli* and are better indicators of poor disinfection (3, 5). The presence of total coliforms in a water distribution system can also indicate a lack of system integrity (6). Thus, total coliform bacteria are commonly used to evaluate the general sanitary quality of water (3, 5).

The use of coliform organisms as indicators of water quality dates back to the early 20th century, when MacConkey described the presence of lactose-fermenting organisms in feces (7). In 1914, the U.S. Public Health Service determined that the total coliform group included all aerobic and facultative anaerobic, Gram-negative, non-spore-forming bacilli that, when incubated at 35 to 37°C, ferment lactose and produce acid and gas (CO_2) within 48 h (8). Since then, there have been developments in the methodologies used to detect coliform organisms, but for many years the group was defined by procedures that relied upon their relative resistance to the presence of bile salts and their ability to ferment lactose with the production of acid and gas (5). However, in the past 15 years, significant changes have occurred in terms of the definition and taxonomy of the coliform group and the methods used for their detection (5).

In 1994, regulatory authorities in the United Kingdom published the sixth edition of *the Bacteriological Examination of Drinking Water Supplies* (9), wherein it was acknowledged that a substantial proportion of coliform organisms did not produce gas or did so irregularly during the fermentation of lactose. Thus, the definition of the coliform group changed to include anaerogenic lactose-fermenting organisms. This broadened the number of genera termed total coliforms regularly found in water. The more narrow definition included primarily four genera—*Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*—while the inclusion of anaeorgenic lactose fermenters added *Kluyvera*, *Yersinia*, *Serratia*, *Hafnia*, and *Pantoea* (10). Additional changes in microbial taxonomy and the use of methods that recover coliforms based on the detection of β-D-galactosidase further increased the number of genera to include *Cedecea*, *Yokenella*, *Ewingella*, *Leclercia*, *Buttiauxella*, *Rahnella*, and *Moellerella*. Continuing improvements in microbial taxonomy will certainly result in more genera being included in the group known as total coliforms (5). Since the definition of the coliform group evolved in recent years, previous

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total-coliform-specific PCR assays as well as culture-based chromogenic methods must be evaluated in accordance with the new definition for specificity and ubiquity. In this study, all enterobacterial species included in the total coliform group were considered total coliforms (Table 1).

Since the total coliform group of organisms defined by the water industry is a diverse group containing many genera and excluding some that are closely related, the application of PCR technology to the detection of this group of organisms is much more difficult. Indeed, the primers used must be capable of detecting all coliform members but not noncoliform bacteria. Furthermore, the application of molecular testing to the microbiological quality of potable water is far from commonplace due to the scarcity of simple technological solutions for tackling the major task of efficiently concentrating and recovering as little as one microbial particle (indicator and/or pathogen) from a water sample (11).

Primers to detect total coliforms in water based on the *lacZ* gene, encoding the β-galactosidase protein, were first described by Bej et al. (12). These primers were further used, tested, and/or validated (13–19). However, the definition of the total coliform group has changed since 1990. Indeed, the total coliform group now includes anaerogenic lactose-fermenting and β-D-galactosidase-producing organisms (9, 10).

In this study, three PCR primer sets designed to detect total coliform or enterobacterial species from water samples were compared for their respective abilities to detect total coliform strains using genomic DNA extracted from an extensive panel of strains (147 total coliform strains representing 76 species belonging to the *Enterobacteriaceae*). The concentration and recovery procedure developed by Maheux et al. (20) was then applied to these three molecular assays to evaluate the detection of total coliforms in potable groundwater samples. Results obtained were compared to those obtained by the Colilert and MI agar (MI) culture-based methods in terms of ubiquity (ability to detect total coliform strains), specificity, sensitivity, time to result, ease of use, and affordability.

MATERIALS AND METHODS

Analytical comparison of bacterial strains. The ability of culture-based and PCR-based methods to detect total coliform strains was verified using 147 different strains representing 76 species of the total coliform group (see first column of Table 1) as well as 23 non-total-coliform strains (representing 17 species; see first column of Table 2). Species identification was reconfirmed using an automated MicroScan Autoscan-4 system (Siemens Healthcare Diagnostic Inc., Newark, DE) or a Vitek-2 system (bioMérieux SA, Marcy l'Étoile, France). Bacterial strains were grown from frozen stocks kept at −80°C in a brucella medium (Becton, Dickinson and Company, Mississauga, Ontario, Canada) containing 10% glycerol and cultured on brain heart infusion (BHI) or sheep blood agar. Three passages were performed prior to analysis of each strain with each culture-based method.

Culture-based methods. (i) Preparation of the bacterial cell suspension. Bacterial colonies were suspended in BHI broth and adjusted to a 0.5 McFarland standard (Fisher Scientific Company, Ottawa, Ontario, Canada) before being serially diluted 10-fold in phosphate-buffered saline (PBS; 137 mM NaCl, 6.4 mM Na_2HPO_4, 2.7 mM KCl, 0.88 mM KH_2PO_4 [pH 7.4]). An aliquot (50 μl) of the 10^{-5} dilution was spiked in sterile reverse-osmosis water (resistivity of 18 MΩ · cm per min at 25°C) to produce suspensions containing approximately 50 CFU per 100 ml of water. Bacterial counts were verified by filtering 100 ml of each spiked water sample through a Millipore membrane filter (47-mm diameter and 0.45-μm pore size; Millipore Corporation, Billerica, MA) with a standard platform manifold (Millipore Corporation), followed by incubation on BHI or sheep blood agar for 24 ± 2 h at 35.0 ± 0.5°C. Tests to confirm the sterility of filter membranes and the buffer used for rinsing the filtration apparatus were also performed.

(ii) Membrane filtration method. Membrane filtration was performed according to the method of Maheux et al. (21). A 100-ml volume was filtered on Millipore filters with a standard platform manifold. The filter was incubated on MI agar (MI; BD, Franklin Lakes, NJ) for 24 ± 2 h at 35.0 ± 0.5°C before determination of colony counts and fluorescence. Each preparation of MI plates was tested for performance using positive- and negative-control strains (*Enterobacter aerogenes* ATCC 13048, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853), as recommended by the manufacturer's labeled instructions and the U.S. Environmental Protection Agency (USEPA) microbiology methods manual (22). Sterility controls of membrane filters and buffer were also included (8).

(iii) Liquid culture method. For the detection of total coliform strains with Colilert (IDEXX Laboratories Canada Corp., Toronto, Ontario, Canada), all steps involved in preparation, validation, storage, and handling were performed according to the manufacturer's instructions. Briefly, one snap pack containing the Colilert reagent was dissolved in 100 ml of a spiked water sample. The solution was then added to a Quanti-Tray and sealed and incubated at 35.0 ± 0.5°C for 24 ± 2 h prior to the identification of total-coliform-positive samples presenting yellow coloration.

Molecular methods. (i) Preparation of the DNA extract. PCR amplifications were performed using a bacterial suspension adjusted to a 0.5 McFarland standard (Fisher Scientific Company, Ottawa, Ontario, Canada). The cells were lysed using the BD Diagnostics-GeneOhm rapid lysis kit as recommended by the manufacturer (BD Diagnostics-GeneOhm, Québec City, Québec, Canada).

(ii) PCR primers. The sequences of the PCR primers evaluated in this study to detect total coliforms are presented in Table 3. The PCR primer set targeting the *wecG* gene, encoding the enterobacterial common antigen (ECA), was developed as follows. First, *wecG* gene sequences available from public databases were analyzed with GCG programs (version 8.0; Accelrys, Madison, WI). The PCR primers were designed from highly conserved regions of the *wecG* gene based on a multiple sequence alignment and the primer analysis software Oligo (version 5.0; National Biosciences, Plymouth, MN). The *Enterobacteriaceae*-specific PCR primers chosen included WecGEnbG118 (5′-ACGYTGGTIGCIATIAAYGCIG-3′) and WecGEnbG490 (5′-GAICCCATIGCIACRGTIAC-3′), which generated specific amplicons of 372 bp. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA).

(iii) PCR amplification. Briefly, 1 μl of the standardized lysed bacterial suspension was transferred directly to a 19-μl PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, a 0.4 mM concentration of each primer, 200 mM (each) deoxyribonucleoside triphosphate (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, Québec, Canada), 3.3 mg per ml of bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), 0.06 μg/μl of methoxsalen (Sigma-Aldrich Canada Ltd.), 0.5 U of *Taq* DNA polymerase (Promega, Madison, WI), and TaqStart antibody (Clontech Laboratories, Mountain View, CA). Decontamination of the PCR mixtures prior to PCR was achieved using the UV cross-linker Spectrolinker model XL-1000 (Spectronics Corporation, Westbury, NY) (23). For each experiment, 1 μl of sterile water was added to the PCR mixture as a negative control. The PCR mixtures were subjected to thermal cycling (3 min at 95°C and then 40 cycles of 1 s at 95°C, 30 s at 57°C, 60°C, or 65°C [Table 2], and 30 s at 72°C, with a 5-min final extension step at 72°C) with a PCT-200 DNA engine thermocycler (Bio-Rad Laboratories, Hercules, CA). An agarose gel analysis of the amplified PCR products was performed as previously described (24).

Comparison using well water samples. (i) Sample collection. During the summer of 2012, 122 1-liter raw well water samples from individual

TABLE 1 Abilities of Colilert and MI agar culture-based methods as well as *lacZ*, *wecG*, and 16S rRNA PCR assays to detect total coliforms

Organism (total no. of coliforms = 147)	Strain	Result of test method[a]				
		Colilert	MI agar	*lacZ* PCR	*wecG* PCR	16S rRNA PCR
Budvicia aquatica	ATCC 35567	−	−	−	−	+
Buttiauxella agretis	ATCC 33320	+	+	+	+	+
Cedeca davisae	ATCC 33431	−	−	+	+	+
Cedeca lapagei	ATCC 33432	−	+	+	+	+
Cedeca neteri	ATCC 33855	−	+	+	−	+
Citrobacter amalonaticus	ATCC 25405	−	+	+	+	+
Citrobacter braakii	ATCC 43162	+	+	+	−	+
Citrobacter farmeri	ATCC 51112	−	+	+	+	+
Citrobacter freundii	ATCC 6879	+	+	+	−	+
	ATCC 8454	+	+	+	−	+
	ATCC 8090	+	+	+	−	+
	CCRI[b]-14799	+	+	+	−	+
	CCRI-14827	−	+	+	−	+
	CCRI-14856	+	+	+	−	+
Citrobacter gillenii	ATCC 51117	+	+	+	+	+
Citrobacter koseri	ATCC 27028	−	+	+	+	+
	ATCC 27156	+	+	+	+	+
	ATCC 29225	+	+	+	+	+
Citrobacter murliniae	ATCC 51641	+	+	+	+	+
Citrobacter sedlakii	ATCC 51115	+	+	+	+	+
	ATCC 51493	+	+	+	−	+
Citrobacter werkmanii	ATCC 51114	+	+	+	−	+
Citrobacter youngae	ATCC 29935	+	+	+	+	+
Cronobacter sakazakii	ATCC 29004	+	−	+	+	+
	ATCC 29544	+	−	+	+	+
	ATCC 51329	+	+	+	−	+
	CCRI-17037	+	+	+	+	+
Enterobacter aerogenes	ATCC 13048	+	+	+	−	+
	ATCC 35029	+	+	+	+	+
	ATCC 51342	+	+	+	+	+
Enterobacter amnigenus	ATCC 33072	−	+	+	+	+
Enterobacter asburiae	ATCC 35954	+	+	+	+	+
	ATCC 35956	+	+	+	+	+
Enterobacter cloacae	ATCC 13047	+	+	+	+	+
	ATCC 23355	+	+	+	+	+
	ATCC 35588	+	+	+	+	+
	CCRI-17108	+	+	+	+	+
Enterobacter dissolvens	ATCC 23373	+	+	+	+	+
Enterobacter enterogenes	ATCC 33241	+	+	+	+	+
	ATCC 35317	+	+	+	+	+
	ATCC 49817	+	+	+	+	+
Enterobacter gergoviae	ATCC 33028	−	+	+	+	+
	ATCC 33426	−	+	+	+	+
	ATCC 33428	−	+	+	+	+
Enterobacter hormaechei	ATCC 49162	+	−	+	+	+
	ATCC 49163	−	+	−	+	+

(Continued on following page)

TABLE 1 (Continued)

Organism (total no. of coliforms = 147)	Strain	Result of test method[a]				
		Colilert	MI agar	*lacZ* PCR	*wecG* PCR	16S rRNA PCR
Enterobacter intermedius	ATCC 33110	−	+	+	+	+
Enterobacter pyrinus	ATCC 49851	−	+	+	+	+
Erwinia amylovora	ATCC 14976	−	−	−	+	+
Escherichia blattae	ATCC 29907	−	−	+	+	+
Escherichia coli	ATCC 11775	+	+	+	+	+
	ATCC 23511	+	+	+	+	+
	ATCC 35401	+	+	+	+	+
	ATCC 43886	+	+	+	+	+
	ATCC 43890	+	+	+	+	+
	ATCC 43894	+	+	+	+	+
	ATCC 43895	+	+	+	+	+
	ATCC 43896	+	+	+	+	+
	LSPQ 2086	+	+	+	+	+
	LSPQ 2092	+	+	+	+	+
	LSPQ 2113	−	+	+	+	+
	LSPQ 2115	+	+	+	+	+
	LSPQ 2117	+	−	+	+	+
	LSPQ 2118	+	+	+	+	+
	LSPQ 2125	+	+	+	+	+
	LSPQ 2127	+	+	+	+	+
	LSPQ 3760	+	+	+	+	+
	LSPQ 3761	+	+	+	+	+
	LSPQ 3762	+	+	+	+	+
Escherichia fergusonii	ATCC 35469	−	+	+	+	+
Escherichia hermanii	ATCC 33650	−	−	+	+	+
Escherichia vulneris	ATCC 29943	+	+	+	+	+
	ATCC 33821	−	+	+	+	+
	ATCC 33832	+	+	+	+	+
Ewingella americana	ATCC 33852	−	−	−	+	+
	ATCC 33854	−	−	−	+	+
Hafnia alvei	ATCC 13337	−	−	+	−	+
	ATCC 25927	−	−	+	−	+
	ATCC 51873	−	+	+	−	+
	CCRI-16651	−	−	+	−	+
Klebsiella oxytoca	ATCC 13182	+	+	−	+	+
	ATCC 33496	+	+	−	+	+
	ATCC 41931	+	+	+	+	+
Klebsiella pneumoniae	ATCC 27736	+	+	+	+	+
	CCRI-17014	+	+	+	+	+
	CCRI-17064	−	−	+	+	+
	CCRI-17074	+	+	+	+	+
Kluyvera ascorbata	ATCC 33433	+	+	+	+	+
	ATCC 33434	+	−	+	+	+
Kluyvera cryocrescens	ATCC 14239	+	+	+	+	+
	ATCC 33435	+	−	+	+	+
Kluyvera georgina	ATCC 51603	+	+	+	+	+
	ATCC 51702	+	+	+	+	+
Leclercia adecarboxylata	ATCC 23216	+	+	+	−	+
	ATCC 27984	+	+	+	+	+

(Continued on following page)

TABLE 1 (Continued)

Organism (total no. of coliforms = 147)	Strain	Result of test method[a]				
		Colilert	MI agar	*lacZ* PCR	*wecG* PCR	16S rRNA PCR
Moellerella wisconsensis	ATCC 35017	+	+	+	−	+
Pantoea agglomerans	ATCC 27155	−	−	+	−	+
Pantoea dispersa	ATCC 14589	−	−	+	−	+
Rahnella aquatilis	ATCC 33071	+	+	+	+	+
Raoutella ornithinolytica	ATCC 31898	+	+	+	+	+
Raoutella planticola	ATCC 33531	+	+	+	+	+
Raoutella terrigena	ATCC 33257	−	+	+	−	+
Salmonella bongori	ATCC 43975	+	+	+	+	+
Salmonella enterica subsp. *houtenae*	ATCC 43974	−	−	−	+	+
Salmonella enterica subsp. *salamae*	ATCC 43972	−	−	+	+	+
Salmonella enterica serovar Typhimurium	ATCC 14028	+	−	+	+	+
Serratia entomophila	ATCC 43705	−	+	+	+	+
Serratia ficaria	ATCC 33105	−	+	−	+	+
Serratia fonticola	ATCC 29844	−	+	−	+	+
Serratia grimesii	ATCC 14460	+	+	+	−	+
Serratia liquefaciens	ATCC 27592	+	+	+	+	+
	ATCC 25641	−	+	+	+	+
Serratia marcescens	ATCC 8100	−	+	−	−	+
	ATCC 29021	+	+	+	−	+
	ATCC 43862	+	+	+	−	+
Serratia odorifera	ATCC 33077	+	+	+	−	+
	ATCC 33132	+	+	+	+	+
	ATCC 33133	+	+	+	+	+
Serratia plymuthica	ATCC 183	+	+	−	+	+
Serratia proteomaculans	ATCC 33765	+	+	−	−	−
Serratia rubidaea	ATCC 27593	+	+	+	+	+
Serratia rubidaea	ATCC 29023	+	+	+	+	+
Shigella boydii	ATCC 9207	−	−	+	+	+
Shigella dysenteriae	ATCC 11835	−	−	+	+	+
Shigella flexneri	ATCC 12022	−	−	+	+	+
Trabulsiella guamensis	ATCC 49490	+	+	+	−	+
Yersinia aldovae	ATCC 35236	−	−	+	+	+
	ATCC 35237	−	−	+	+	+
Yersinia bercovieri	ATCC 43970	−	+	+	+	+
Yersinia enterocolitica	ATCC 9610	−	−	−	+	+
Yersinia frederiksenii	ATCC 29912	−	+	+	−	+
	ATCC 33641	+	+	+	+	+
Yersinia intermedia	ATCC 29909	−	−	+	+	+
	ATCC 33647	−	+	+	+	+
	ATCC 33648	−	+	+	+	+
Yersinia kristensenii	ATCC 33638	−	−	+	−	+
Yersinia mollaretii	ATCC 43939	−	−	+	+	+
Yersinia pseudotuberculosis	ATCC 13979	−	−	+	+	+
	ATCC 27802	−	−	+	+	+
	ATCC 29833	−	−	+	+	+
Yersinia rohdei	ATCC 43380	−	−	+	−	+
	ATCC 43871	−	−	+	−	+
	ATCC 43873	−	+	+	−	+
Yersinia ruckeri	ATCC 29473	−	−	+	+	+

(Continued on following page)

TABLE 1 (Continued)

Organism (total no. of coliforms = 147)	Strain	Result of test method[a]				
		Colilert	MI agar	*lacZ* PCR	*wecG* PCR	16S rRNA PCR
Yokenella regensburgei	ATCC 35313	−	+	+	−	+
	ATCC 43001	−	−	+	+	+
	ATCC 43001	−	−	+	+	+
	ATCC 43003	−	+	+	−	+
Total		86/147 (58.5%)	109/147 (74.1%)	133/147 (90.5%)	111/147 (75.5%)	146/147 (99.3%)

[a] +, positive; −, negative.
[b] CCRI, Centre de recherche en Infectiologie.

households were collected in the Québec City region. For all well water samples, a process control consisting of approximately 60 *Bacillus atrophaeus* subsp. *globigii* spores per 100 ml was added prior the filtration. Spores were prepared according to the method of Picard et al. (25). Then, each well water sample was divided into 100-ml subsamples for simultaneous testing by standard microbiological methods consisting of MI agar and Colilert (see "PCR primers" and "PCR amplification" above). One 100-ml subsample was used for molecular detection, and the microbial DNA preparation obtained was used to perform the three total coliform PCR assays as well as the *B. atrophaeus* subsp. *globigii* PCR assay (25).

(ii) Water sample treatment for molecular detection. The filtration of each 100-ml sample of well water was completed using a 3-place manifold, according to the method of Maheux et al. (21). Following filtration, the membrane was aseptically removed from the filtration manifold, transferred to a 15-ml polypropylene tube (Sarstedt, Newton, NC), exposed for 10 s to 8.5 ml of high-performance liquid chromatography (HPLC)-grade methanol (Sigma-Aldrich, St. Louis, MO), and vortexed for 10 s. The reaction tube and its contents were then centrifuged for 3 min at 2,100 × *g*. The supernatant was removed and 1 ml of histological-grade acetone (EMD Chemicals, San Diego, CA) was added to the pellet. Complete dissolution was achieved by vortexing. The resulting clear acetone solution was transferred to a 2-ml tube containing a mixture of glass beads (150 to 212 μm and 710 to 1180 μm; Sigma-Aldrich) and centrifuged for 3 min at 15,800 × *g*, and the supernatant was removed.

To maximize the recovery of filtered cells, the 15-ml polypropylene tube used in the step described above was rinsed briefly with 1.0 ml of

TABLE 2 Abilities of Colilert and MI agar culture-based methods as well as *lacZ*, *wecG*, and 16S rRNA PCR assays to detect non-total-coliform *Enterobacteriaceae* strains

Organsim (no. of non-total coliforms = 23)	Strain	Result of test method[a]				
		Colilert	MI agar	*lacZ* PCR	*wecG* PCR	16S rRNA PCR
Edwardsiella hoshinae	ATCC 33379	−	−	+	−	+
Edwardsiella ictaluri	ATCC 33202	−	−	−	+	+
Edwardsiella tarda	ATCC 15947	−	−	+	−	+
Leminorella richardii	ATCC 33998	−	−	+	−	+
	ATCC 33999	−	−	+	−	+
Morganella morganii subsp. *morganii*	ATCC 9237	−	−	+	−	+
	ATCC 27975	−	−	+	−	+
Morganella morganii subsp. *sibonii*	ATCC 51206	−	+	+	−	+
Obesumbacterium proteus	ATCC 12841	−	−	+	−	+
Photorhabdus asymbiotica	ATCC 43948	−	−	+	−	+
Plesiomonas shigelloides	ATCC 14029	−	−	+	+	+
Pragia fontium	ATCC 49100	−	−	+	+	+
Proteus mirabilis	ATCC 7002	−	−	−	−	−
	ATCC 25933	−	−	+	−	+
	ATCC 33583	−	−	+	−	−
	ATCC 35659	−	−	−	−	−
Providencia rettgeri	ATCC 9250	−	−	+	−	−
	ATCC 29944	−	−	+	+	−
Salmonella enterica serovar Choleraesuis	ATCC 7001	−	−	−	+	+
Salmonella enterica subsp. *arizonae*	ATCC 13314	+	+	+	+	+
Shigella sonnei	ATCC 29930	−	+	+	+	+
Tatumella ptyseos	ATCC 33301	−	−	−	−	+
Xenorhabdus nematophilus	ATCC 19061	−	−	−	−	+

[a] +, positive; −, negative. Untested enterobacterial genera included *Arenicola*, *Averyella*, *Biostraticola*, *Brenneria*, *Buchneria*, *Dickeya*, *Gibbsiella*, *Grimontella*, *Guhaiyinggella*, *Lonsdalea*, *Mangrovibacter*, *Margalefia*, *Pectobacterium*, *Phytobacter*, *Samsonia*, *Sodalis*, *Thorsellia*, *Tiedjeia*, and *Wigglesworthia*.

TABLE 3 PCR primes and real-time PCR primers and probes used in this study

Microorganism(s) tested	Genetic target	Primer or probe	Primer or probe sequence (5′→3′)[a]	Annealing temp (°C)	Amplicon size (bp)	Reference
Total coliforms	*lacZ*	ZL-1675	ATGAAAGCTGGCTACAGGAAGGCC	65	264	12
		ZR-2025	GGTTTATGCAGCAACGAGACGTCA			
	wecG	WecGEnbG118	ACGYTGGTIGCIATIAAYGCIG	57	372	This study
		WecGEnbG490	GAICCCATIGCIACRGTIAC			
	16S rRNA	ENT-F	GTTGTAAAGCACTTTGAGTGGTGAGGAAGG	60	424	30
		ENT-R	GCCTCAAGGGCACAACCTCCAAG			
Bacillus atrophaeus subsp. *globigii*	*atpD*	Abgl158	CACTTCATTTAGGCGACGATACT	60	210	25
		Abgl345a	TTGTCTGTGAATCGGATCTTTCTC			
		Abgl-T1–A1	FAM[b]-CGTCCCAATGTTACATTACCAACCGG CACT-(BHQ-1[c])-GAATAGG			

[a] Primer degeneracies: R, A or G; Y, C or T; I, inosine.
[b] FAM, 6-carboxyfluorescein (fluorescence quencher dye).
[c] BHQ-1, Black Hole Quencher-1, a fluorescence quencher dye.

histological-grade acetone, and the resulting mixture was transferred to the glass bead tube used previously. The tube was then centrifuged for 3 min at 15,800 × *g*. The resulting pellet was washed with 1.0 ml of TE buffer (100 mM Tris-HCl, 1 mM EDTA [pH 8.0]) and centrifuged for 3 min at 15,800 × *g*. After centrifugation of the washed filtrate-glass bead suspension in the presence of TE buffer, the supernatant was removed. The dead volume in the glass beads was estimated to be approximately 25 μl.

Forty microliters of Illustra GenomiPhi V2 sample buffer (part of the Illustra GenomiPhi DNA amplification kit; GE Healthcare, Montréal, Québec, Canada) was added to the 25-μl reaction mixture. The cells contained in the pellet were lysed mechanically by mixing at maximum speed on a vortex mixer for 5 min. The reaction tube containing the crude cell extract was then incubated for 3 min at 95°C and kept on ice for a minimum of 3 min. A mixture of 45 μl of GenomiPhi reaction buffer and 4 μl of ϕ29 DNA polymerase (GenomiPhi DNA amplification kit) was added to the extract and gently mixed by finger tapping before being briefly spun in a microcentrifuge. The whole-genome amplification (WGA) reaction mixture was incubated for 3 h at 30°C. The enzymatic reaction was then arrested by 10 min of incubation at 65°C. One microliter of WGA-amplified product was then used as a template for the total-coliform-specific *lacZ*, *wecG*, and 16S rRNA PCR, as well as *B. atrophaeus* subsp. *globigii* real-time PCR (rtPCR), amplification using the conditions described above. The detection of *B. atrophaeus* subsp. *globigii* served as a procedural control and to monitor for inhibition.

For *B. atrophaeus* subsp. *globigii* detection, 1 ml of the standardized lysed bacterial suspension was transferred directly to a 24-μl PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM $MgCl_2$, 0.4 μM (each) primer, 0.2 μM *B. atrophaeus* subsp. *globigii* probe, 200 μM (each) deoxyribonucleoside triphosphate (GE Healthcare Bio-Sciences Inc., Baie d'Urfé, Québec, Canada), 3.3 μg per μl of bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada), 0.06 μg/μl of methoxsalen (Sigma-Aldrich Canada Ltd.), 0.5 U of *Taq* DNA polymerase (Promega, Madison, WI), and TaqStart antibody (Clontech Laboratories, Mountain View, CA). Decontamination of the PCR mixtures prior to PCR was achieved using the UV cross-linker Spectrolinker model XL-1000 (Spectronics Corporation, Westbury, NY) (23). The rtPCR mixture was subjected to thermal cycling (1 min at 95°C and then 45 cycles of 15 s at 95°C and 60 s at 60°C) with a Rotor-Gene thermocycler (Corbett Life Science, Sydney, Australia; now Qiagen).

Statistical analysis. All individual results were recorded using Microsoft Excel 2010 software (Microsoft Corporation; Redmond, WA), and statistical analysis was performed using SAS 9.3 (SAS Institute Inc., Cary, NC).

Species identification by the MicroScan AutoScan-4 or the Vitek-2 system was used as the reference to determine the ability of the molecular method to detect all or most of the total coliform strains used in the study (ubiquity). Agreement was calculated by dividing the number of strains detected by the test by the number of total coliform strains tested.

All water samples were recorded as positive or negative for total coliforms. The culture-based methods were used to determine the specificity and sensitivity of a particular PCR test for the detection of total coliforms.

McNemar's test was used to compare paired proportions with a 95% confidence interval. When the (two-sided) *P* value was less than 0.05, we concluded that there was a significant difference between the methods.

An overly conservative measure of agreement, Cohen's kappa coefficient, was also used to measure the interrater agreement. Fleiss's (26) magnitude guidelines were used to characterize the κ values (>0.75, excellent, 0.40 to 0.75, fair to good; and <0.40, poor).

Ubiquity is the ability of a method to detect all or most targeted strains. We used it in this study when we validated our assays against a panel of known microorganisms. Positive predictive values could also have been used in this study with Colilert and MI agar serving as "gold standards" when we compared results obtained with well water samples. However, since the ubiquity and specificity tests of reference methods were not perfect, the methods were compared using the index of agreement, Cohen's kappa coefficient, and McNemar's statistical test.

RESULTS

Abilities of MI agar, Colilert, and the three PCR assays to detect total coliform strains. One hundred forty-seven total coliform strains, representing 76 species isolated from fecal and environmental settings, were used to demonstrate the ability of the culture-based Colilert and MI agar (MI) methods as well as three different PCR assays targeting *lacZ*, *wecG*, and 16S rRNA genes, respectively, to detect total coliform strains (Table 1). Eighty-six (58.5%) and 109 (74.1%) strains yielded a β-galactosidase-positive signal with the Colilert and MI culture-based methods, while the *lacZ*, *wecG*, and 16S rRNA primer sets tested positive for 133 (90.5%), 111 (75.5%), and 146 (99.3%), respectively, out of 147 total coliform strains tested.

The specificity of both the culture-based methods and the three PCR assays was verified by testing 23 non-total-coliform strains from fecal and environmental settings (Table 2). Only one (4.3%) and three (13.0%) yielded a β-galactosidase-positive signal with the Colilert and MI culture-based methods, respectively, while the *lacZ*, *wecG*, and 16S rRNA primer sets tested positive with 17 (73.9%), 7 (30.4%), and 18 (78.3%) out of 23 non-total-coliform enterobacterial strains tested, respectively. For confirmatory pur-

poses, all strains that had negative results were tested a second time with a different lot of kits or media.

Detection of total coliforms in potable water samples. The microbiological quality of 122 well water samples collected in the Québec City region was assessed by both culture-based and molecular methods (sensitivity testing). Results showed that 97 (79.5%) and 97 (79.5%) samples using the culture-based methods (Colilert and MI) and 95 (77.9%), 82 (67.2%), and 98 (80.3%) samples using the PCR-based methods (*lacZ*, *wecG*, and 16S rRNA) contained total coliforms. Positive and negative controls performed as expected.

DISCUSSION

In the present study, Colilert and MI agar detected 58.5% and 74.1% of total coliform strains tested, respectively. The difference observed between the two culture-based methods (15.6%) is in accordance with the 15.0% difference observed by Maheux et al. (27) after testing a panel of 107 total coliform strains isolated from fecal and environmental settings. Interestingly, 27 (18.4%) of the 147 total coliform strains tested in this study with the Colilert method yielded a lighter yellow coloration than the comparator provided by the company after a 28-h incubation, leading to false-negative results.

It is well known in environmental microbiology that the total coliform group is not well defined based on phenotypic characteristics (28). Indeed, our results showed a lack of correlation between the two culture-based methods tested either within the same genera or within the same species. Thus, according to Maheux et al. (27), identification methods relying solely on the activity of a single enzyme are subject to a lack of robustness and may lead to misinterpretations since enzymatic activity can be transient and highly regulated by environmental factors. Consequently, since molecular assays are based on more stable genetic parameters, PCR assays should potentially offer greater sensitivity than culture-based methods.

Primers based on the *lacZ* gene, encoding the β-galactosidase protein, for the detection of total coliforms in water were first described by Bej et al. (12). To determine the specificity of coliform detection by their *lacZ* PCR-gene probe method, Bej et al. (12) tested the following bacterial strains: *E. coli* ATCC 11775, *E. coli* ATCC 10798, *E. coli* ATCC 15224, *E. coli* ATCC 25404, *Enterobacter cloacae* ATCC 13047, *Salmonella enterica* serovar Typhimurium ATCC 19585, *Citrobacter freundii* ATCC 33128, *Klebsiella pneumoniae* ATCC 13883, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 25931, *Pseudomonas putida* mt-2, *Lactococcus lactis* ATCC 19435, and 32 environmental isolates from m-Endo agar plates identified as *E. coli*. They detected *E. coli* and other coliform bacteria (including *Shigella* spp.) but not *Salmonella* spp. or noncoliform bacteria with this assay.

Fricker and Fricker (15) used the *lacZ* primers (12) for the identification of total coliform colonies isolated after overnight growth on MacConkey agar. They tested 441 different putative total coliform colonies randomly selected from those routinely isolated from water and identified using the ATB 32E system (bioMérieux, Marcy l'Étoile, France). More specifically, the total coliform panel was composed of *Buttiauxella agrestis* ($n = 4$), *Citrobacter freundii* ($n = 54$), *Enterobacter agglomerans* ($n = 63$), *Enterobacter amnigenus* ($n = 25$), *Enterobacter cloacae* ($n = 51$), *Enterobacter intermedius* ($n = 1$), *Enterobacter taylorae* ($n = 1$), *Escherichia coli* ($n = 122$), *Escherichia vulneris* ($n = 1$), *Hafnia alvei* ($n = 8$), *Klebsiella terrigena* ($n = 11$), *Klebsiella oxytoca* ($n = 52$), *Klebsiella ozaenae* ($n = 8$), *Klebsiella pneumoniae* ($n = 35$), *Leclercia adecarboxylata* ($n = 4$), and *Serratia rubidaea* ($n = 1$). Using this panel, Fricker and Fricker (15) found that the *lacZ* primer set correctly identified 79.4% (350/441) of the bacterial strains tested as coliforms.

In the present study, among the bacterial species tested by Fricker and Fricker, we tested *B. agrestis* ($n = 1$), *C. freundii* ($n = 6$), *Enterobacter amnigenus* ($n = 1$), *E. cloacae* ($n = 4$), 1 *E. intermedius* ($n = 1$), *E. coli* ($n = 19$), *E. vulneris* ($n = 3$), *H. alvei* ($n = 4$), *K. oxytoca* ($n = 3$), *K. pneumoniae* ($n = 4$), *L. adecarboxylata* ($n = 2$), and *S. rubidaea* ($n = 2$). *E. agglomerans*, *E. taylorae*, *K. terrigena*, and *K. ozaenae* were not tested. In this study, 48 (96%) out of the 50 total coliform strains tested were detected, contrary to the findings of Fricker and Fricker (15), who had noted that the *lacZ* primer set did not perform very well, since many total coliform strains tested did not amplify sufficiently to be detected on an agarose gel. While Fricker and Fricker (15) used a PCR amplification protocol of 25 cycles and an annealing temperature of 60°C, we modified the PCR amplification protocol, using 40 cycles and an annealing temperature step of 57°C. The detection problem observed by Fricker and Fricker (15) was not observed using this optimized protocol. Among our more extended panel composed of 147 total coliform strains representing 76 enterobacterial species isolated from fecal and environmental settings (Table 1), 133 strains yielded a *lacZ* PCR-positive signal, for a sensitivity of 90.5%. This lack of sensitivity can be explained by the fact that microbes are known to evolve rapidly. Thus, different strains of the same species could present significant genetic polymorphisms, and the design of a PCR primer pair enabling detection of all target strains becomes more difficult. Using conserved genes to design primer sets could be a good alternative since they present more conserved DNA regions. In order to develop a PCR assay with a higher sensitivity, other target genes were studied. Thus, a PCR assay targeting the *wecG* gene, encoding the enterobacterial common antigen, was developed and tested against our extended panel of total coliform strains. The *wecG* gene was chosen on the basis that the *Enterobacteriaceae* family encompasses approximately 20 genera, including *Escherichia* and all members of the coliform group. In addition, it includes the food-borne pathogens *Salmonella*, *Shigella*, and *Yersinia*. The family was also originally proposed as an alternative indicator to the coliform group because testing the entire family would be more inclusive for pathogenic bacteria. Collectively, they have greater resistance to the environment than coliforms (29). Consequently, *Enterobacteriaceae* may be superior to coliforms as indicators of sanitation. Unfortunately, in the present study, of the 147 total coliform strains tested, the *wecG* PCR assay yielded a positive signal for only 111 (75.5%) strains (Table 1).

The *Enterobacteriaceae*-specific PCR assay targeting the 16S rRNA designed by Nakano et al. (30) was also tested against our extended panel of total coliform strains. Of the 147 total coliform strains tested, the 16S rRNA PCR assay yielded a positive signal for 146 (99.3%) (Table 1). This is similar to the findings of Nakano et al. (30), who tested 72 different bacterial species representing 49 genera and found positive PCR results for every enterobacterial total coliform strain tested. Among all PCR primer sets tested, the *Enterobacteriaceae*-specific PCR assay targeting the 16S rRNA designed by Nakano et al. (30) presents the best sensitivity.

Non-total-coliform enterobacterial strains were also tested

TABLE 4 Comparison of methods for detection of total coliform presence in well water samples ($n = 122$)

Method and result	No. of results by WGA-*lacZ* PCR		No. of results by WGA-*wecG* PCR		No. of results by WGA-16S rRNA PCR	
	+	−	+	−	+	−
MI						
+	90	7	82	15	95	2
−	5	20	0	25	3	22
Colilert						
+	90	7	82	15	95	2
−	5	20	0	25	3	22

(Table 2). Only one (4.3%) and three (13.0%) yielded a β-galactosidase-positive signal with the Colilert and MI culture-based methods, while the *lacZ*, *wecG*, and 16S rRNA primer sets tested positive with 17 (73.9%), 7 (30.4%), and 18 (78.3%) of 23 non-total-coliform enterobacterial strains, respectively. The impact of this nonspecific amplification was determined by testing real drinking water samples.

Guidelines of the United States Environmental Protection Agency (USEPA), Canada, and the World Health Organization (WHO) require that total coliforms not be detectable per 100 ml of water (3, 31, 32). Assessments of drinking water quality can be performed using quantitative or presence/absence tests (3). However, the use of rapid molecular testing is hampered by the lack of simple solutions for concentrating and recovering very low numbers of microbial particles (indicators and pathogens) present in a water sample. In this study, we found that culture-based methods identified 97 (79%) of potable water samples containing total coliforms, while PCR assays reported 77.9% (*lacZ*), 67.2% (*wecG*), and 80.3% (16S rRNA) of samples containing total coliforms (Table 4). A statistical analysis revealed that the WGA-*wecG* PCR method is statistically different from both the Colilert and MI agar methods, whereas WGA-*lacZ* PCR and WGA-16S rRNA PCR are statistically comparable (Table 5). However, with these 122 drinking water samples, the WGA-16S rRNA PCR presents the highest interrater agreement and paired proportions (Table 5).

Regarding ease of use, Colilert was the simplest method, and its unit-dosed packaging eliminates medium preparation. Furthermore, there is no repeat testing due to clogged filters. Finally, its use does not require well-trained employees. The MI method, on the other hand, provides ease of use comparable to those of all membrane filtration methods. The medium must be prepared and quality control led with each batch prepared. Employee training is also more important than for the Colilert method. However, employees already using the membrane filtration equipment can easily use this method. The CRENAME (concentration and recovery of microbial particles, extraction of nucleic acids, and molecular enrichment) WGA-rtPCR assay requires highly trained employees.

Regarding time to result, the Colilert and MI methods both required 24 h prior to obtaining results without a confirmation step, whereas the CRENAME WGA-rtPCR assay needed 5 h. Reduction of microbiological risk to an acceptable level remains a priority in drinking water treatment. Currently, culture-based methods are routinely used to determine the microbiological quality of raw water and check the compliance of treated water using indicator microorganisms. However, the relatively long time required to obtain results (≥24 h) and the poor prediction of the presence of more resistant pathogens (parasites and viruses) do not allow conventional methods to fully and reliably ensure the microbiological quality of the water before it is distributed (8, 33). CRENAME WGA-rtPCR assay could help to overcome the limitations of culture-based methods by providing results more quickly (5 h instead of 24 h) and by leading to better correlations with the presence of pathogens (20, 34, 35).

Of these methods, the MI agar method is the most affordable. The Colilert method is 6 to 10 times more expensive than MI agar per water sample. However, when no trained employee and/or membrane filtration equipment is available, the Colilert method is more advantageous. For example, in Nunavik, Canada, potable water is routinely tested for total coliform and *E. coli* contamination using the Colilert method because it is more user-friendly and less equipment-intensive than standard membrane filtration-based methods (36, 37). At this time, molecular technologies are expensive (around US$30 from water sample to result) and not fully automated. However, the day will come when assessing drinking water quality by molecular methods will be more cost-effective and rapid. Furthermore, automation will not require well-trained employees. Then, molecular technologies will compete advantageously with culture-based technologies.

Conclusion. Despite the high genetic variability of the total coliform group, this study showed that it is possible to use molecular assays to detect total coliforms in drinking water: the 16S rRNA molecular-based assay proved to be as sensitive as recommended, culture-based methods. Since the total coliform group is not well defined, perhaps the detection of all *Enterobacteriaceae* species should be considered to evaluate the microbiological quality of water by molecular technologies. This suggestion is supported in the present study by the fact that the detection of non-total-coliform *Enterobacteriaceae* species did not influence the results when real water samples were tested. Accordingly, this assay could be used in combination with an *Escherichia coli* molecular assay to assess potable water quality. However, larger-scale

TABLE 5 Statistical analysis of WGA-PCR assays versus MI agar and Colilert culture-based methods for the detection of total coliform presence in well water samples

Method	WGA-*lacZ* PCR				WGA-*wecG* PCR				WGA-16S rRNA PCR			
	Index of agreement	Cohen kappa	McNemar value	*P* value[a]	Index of agreement	Cohen kappa	McNemar value	*P* value[a]	Index of agreement	Cohen kappa	McNemar value	*P* value[a]
MI	0.90	0.89	0.33	0.7414	0.88	0.76	15	<0.0001	0.96	0.96	0.2	0.8414
Colilert	0.90	0.89	0.33	0.7414	0.88	0.76	15	<0.0001	0.06	0.96	0.2	0.8414

[a] A *P* value of <0.05 is necessary to establish a statistically significant difference.

validation studies will be required to demonstrate its usefulness and applicability.

Additionally, rapid molecular microbiology testing could provide useful tools for these purposes, since the monitoring of fecal contamination indicators might not suffice to assess the risk of waterborne diseases attributed to pathogens such as *Vibrio cholerae* and protozoan parasites (e.g., *Cryptosporidium*, *Giardia*, and/or *Toxoplasma*), whose presence is not well indicated by conventional index microorganisms.

The results obtained in the present study are applicable only to drinking water samples. Results could be different with other types of water.

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