Rapid molecular identification of fecal origin-colonies growing on *Enterococcus* spp.-specific culture methods

Andrée F. Maheux, Sébastien Bouchard, Ève Bérubé and Michel G. Bergeron

ABSTRACT

The mEI, Chromocult® enterococci, and m-*Enterococcus* culture-based methods used to assess water quality by the detection of *Enterococcus* spp. were first compared in terms of sensitivity using (1) 41 different type strains of *Enterococcus* spp. and (2) environmental colonies identified by 16S rRNA sequencing. Then, two specific-rtPCR assays targeting *Enterococcus* spp. and *Enterococcus faecalis/faecium* were tested for their ability to confirm the identity of putative enterococcal colonies. The mEI, Chromocult® enterococci, and m-*Enterococcus* methods detected β-glucosidase activity for 28 (68.3%), 32 (78.0%), and 12 (29.3%) of the 41 reference enterococcal strains tested, respectively. Analysis with environmental colonies showed that mEI and Chromocult® enterococci media had false positive rates of 4.3% and 5.0%, respectively. Finally, the two rtPCR assays showed a specificity of 100%. Only two (2/19) colonies of *E. faecium* isolated from mEI agar were not detected by the *Enterococcus faecium* rtPCR assay, for a sensitivity of 89.5%. Our results showed that Chromocult® enterococci medium recovered more *E. faecalis/faecium* cells than the two other methods. Thus, the use of Chromocult® enterococci combined with the *Enterococcus faecalis/faecium* rtPCR assay showed the best combination to decrease the high false-positive rate obtained when the entire *Enterococcus* genus is targeted.

Key words | Chromocult® enterococci agar, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus* spp., mEI agar, m-*Enterococcus* agar

Andrée F. Maheux (corresponding author)
Sébastien Bouchard
AFM Water Consulting,
1249 d'Édimbourg,
Québec City (Québec),
Canada G3J 1C8
E-mail: *afmwaterconsulting@outlook.com*

Ève Bérubé
Michel G. Bergeron
Centre de recherche en infectiologie de l'Université Laval, Axe Maladies infectieuses et immunitaires,
Centre de recherche du CHU de Québec-Université Laval,
Québec City (Québec),
Canada

Michel G. Bergeron
Département de microbiologie-infectiologie et d'immunologie, Faculté de médecine,
Université Laval,
Québec City (Québec),
Canada

INTRODUCTION

In the Province of Québec (Canada), the *Programme d'accréditation des laboratoires d'analyse* ('Accreditation program of analytic laboratories'; PALA) is administered by the *Centre d'expertise en analyse environnementale du Québec* (CEAEQ) which accredits private, municipal and institutional laboratories. The Regulation respecting the quality of drinking water (RRQDW) states that when the water supplied by a distribution system comes in whole or in part from nondisinfected and vulnerable groundwater, the person in charge of the distribution system is bound to control the presence of *Escherichia coli*, enterococci, and coliphage viruses (Government of Québec 2013). Consequently, a water sample is considered contaminated by fecal pollution if at least one colony of enterococci bacteria is detected. The presence of enterococci in water is also considered by the United States Environmental Protection Agency (USEPA) as an indication of fecal pollution and of the possible presence of enteric pathogens, although some enterococcal species are naturally found in the environment and not necessarily related to fecal pollution (Kjellander 1960; Cabelli *et al.* 1982; Franz *et al.* 1999; USEPA 2005).

In fact, detecting *Enterococcus* spp. is of limited significance for determining the source of contamination in water since the broad spectrum of species cannot be used

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to distinguish non-fecal (environmental) from fecal contamination (Bonds *et al.* 2006; Converse *et al.* 2009). Indeed, there are many possible sources of *Enterococcus* sp. in water including animal waste (Devriese & De Plesmaecker 1987; Devriese *et al.* 1991; Sinton *et al.* 1993; Harwood *et al.* 2001), soil (Fujioka *et al.* 1999), invertebrates (Martin & Mundt 1972; Svec *et al.* 2002), and plants (Müller *et al.* 2001).

Thus, water quality assessment should more focus on a group of *Enterococcus* sp. that is associated with sources of fecal pollution rather than relying on the entire *Enterococcus* genus. *Enterococcus faecalis* and *Enterococcus faecium* are the predominant species of the *Enterococcus* genus found in human feces (Ruoff *et al.* 1990). All mammals carry these microorganisms in the colon (Noble 1978). Consequently, *E. faecalis* and *E. faecium* are potentially good fecal species as they have been consistently identified as predominant enterococcal species in warm-blooded animal feces and sewage, but not from environmental sources (Chenoweth & Schaberg 1990; Ruoff *et al.* 1990; Manero *et al.* 2002; Gelsomino *et al.* 2003). Furthermore, since *Escherichia coli* is 100 to 1,000 times more concentrated than *Enterococcus* spp. in feces, the probability of detecting non-*faecalis*/*faecium Enterococcus* species in water without any detection of *E. coli* nor *E. faecalis*/*E. faecium* is highly improbable (Slanetz & Bartley 1957; Layton *et al.* 2010). Thus, a detection method that would allow the specific detection of *E. faecalis*/*E. faecium* cells rather than all *Enterococcus* spp. should be more appropriate to assess water quality by the detection of fecal contamination.

Currently, enterococci in water are detected by different chromogenic culture-based methods comprising USEPA method 1600 on mEI agar (USEPA 2005), Chromocult® enterococci agar, and method 9230C of the *Standard Methods for the Examination of Water and Wastewater* manual (membrane filtration on m-*Enterococcus* agar; American Public Health Association/American Water Works Association/Water Environment Federation (APHA/AWWA/WEF 2012)). However, these methods do not discriminate between enterococci of environmental origin and enterococci of fecal origin.

In this study, we first compared mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar, in terms of sensitivity, using a panel of different types of strains of enterococci and harvested environmental colonies identified by 16S rRNA sequencing. Secondly, two specific real-time polymerase chain reaction (rtPCR) assays targeting *Enterococcus* spp. and *Enterococcus faecalis*/*faecium* were tested for their ability to confirm, in less than 2 hours, the identity of putative enterococcal colonies grown on chromogenic culture-based agar in order to decrease the high false-positive rate obtained when the entire *Enterococcus* genus is targeted.

MATERIAL AND METHODS

Analytical comparison

Bacterial strains

The ability of mEI, Chromocult® enterococci, and m-*Enterococcus* agar as well as *Enterococcus* spp.- and *Enterococcus faecalis*/*faecium*-specific rtPCR assays to detect enterococci strains was verified using 41 (for culture-based methods evaluation) and 55 (for rtPCR validation) different strains of *Enterococcus* spp. (Tables 1 and 2). Species identification was reconfirmed using an automated MicroScan Autoscan-4 system (Siemens Healthcare Diagnostic Inc., Newark, DE, USA) or a Vitek 2 system (bioMérieux SA, Marcy l'Étoile, France). Bacterial strains were grown from frozen stock kept at −80 °C in Brucella medium (Beckton Dickinson, Mississauga, Ontario, Canada), containing 10% glycerol. The strains were cultured on Brain Heart Infusion (BHI) agar. Three passages were performed prior to analysis of each strain with each culture-based method.

Preparation of the bacterial cell suspension for analytical analysis

Colonies obtained from frozen stocks 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). For each strain, an aliquot of the 10^{-5} dilution was spiked in sterile reverse

Table 1 | Ability of the mEI, Chromocult® enterococci, and m-*Enterococcus* agar methods to detect enterococci strains

Tested species ($n = 41$)	Origin	No. Reference	mEI	Chromocult® enterococci	m-*Enterococcus*
E. aquimarinus	E	CCRI-15963	−	−	−
E. avium	C	ATCC 14025	−	−	−
E. caccae	C	ATCC BAA1240	+	+	−
E. canintestini	A	CCUG 37857	+	+	−
E. canis	A	CCUG 46666	+/−	+	−
E. casseliflavus	E	ATCC 25788	+	+	+
E. casseliflavus	N/A	ATCC 51328	+	+	+
E. casseliflavus	C	ATCC 12819	+	+	+
E. cecorum	A	ATCC 43198	−	+	−
E. columbae	A	ATCC 51263	+	−	−
E. devriesi	A	CCUG 37865	−	+	−
E. dispar	C	ATCC 51266	+	−	−
E. durans	C	ATCC 19432	+	+	−
E. faecalis	C	ATCC 19433	+	+	+
E. faecalis	C	ATCC 29212	+	+	+
E. faecalis	C	ATCC 51299	−	+	+
E. faecium	C	ATCC 19434	+	+	−
E. faecium	C	ATCC 700221	−	+	−
E. faecium	E	CCRI-16518	+	+	+
E. gallinarum	C	CCRI-1433	+	+	+
E. gallinarum	C	LSPQ 3364	+	+	+
E. gallinarum	C	LSPQ 5375	+	+	+
E. gilvus	C	ATCC BAA350	+/−	+	−
E. haemoperoxidus	W	CCUG 45916	+	+	−
E. hirae	C	ATCC 8043	+	+	+
E. hirae	C	CCUG 37829	+	+	+
E. italicus	F	CCUG 50447	−	−	−
E. malodoratus	F	ATCC 43197	−	+	−
E. mundtii	E	ATCC 43186	+	+	−
E. pallens	C	ATCC BAA351	+/−	+	−
E. phoeniculicoli	A	CCUG 48923	+	+	−
E. pseudoavium	A	ATCC 49372	−	−	−
E. quebecensis	W	CCRI-16985	+	+	−
E. raffinosus	C	ATCC 49427	−	−	−
E. ratti	A	ATCC 700914	−	−	−
E. sileciacus	W	CCUG 53830	+	+	−
E. saccharolyticus	C	ATCC 43076	−	+	−
E. sulfureus	E	ATCC 49903	+	−	−
E. termitis	A	CCUG 53831	+	+	−
E. ureasiticus	W	CCRI-16620	+	+	−
E. villorum	A	CCRI-8858	−	+	−
Total:			28/41 (68.3%)	32/41 (78.0%)	12/41 (29.3%)

A, Animal; C, Clinical; E, Environmental; F, Food; W, Water; N/A, Non applicable; CCRI, Centre de recherche en infectiologie strain collection; ATCC, American Type Culture Collection; CCUG, Culture Collection (University of Gothenburg); LSPQ, Laboratoire de santé publique du Québec.

Table 2 | Ability of the *Enterococcus* spp.-specific rtPCR assay and the multiplex *Enterococcus faecalis/faecium*-specific rtPCR assay to detect enterococci strains

			***Enterococcus*-specific rtPCR assays**		
Tested species ($n = 55$)	**Origin**	**No. Reference**	***Enterococcus* spp.[a]**	***E. faecalis*[b]**	***E. faecium*[b]**
E. aquimarinus	E	CCRI-15963	+	−	−
E. avium	C	ATCC 14025	+	−	−
E. caccae	C	ATCC BAA1240	+	−	−
E. canintestini	A	CCUG 37857	+	−	−
E. canis	A	CCUG 46666	+	−	−
E. casseliflavus	E	ATCC 25788	+	−	−
E. casseliflavus	N/A	ATCC 51328	+	−	−
E. casseliflavus	C	ATCC 12819	+	−	−
E. cecorum	A	ATCC 43198	+	−	−
E. columbae	A	ATCC 51263	+	−	−
E. devriesi	A	CCUG 37865	+	−	−
E. dispar	C	ATCC 51266	+	−	−
E. durans	C	ATCC 19432	+	−	−
E. faecalis	C	ATCC 19433	+	+	−
E. faecalis	C	ATCC 23241	+	+	−
E. faecalis	C	ATCC 29212	+	+	−
E. faecalis	C	ATCC 49533	+	+	−
E. faecalis	C	ATCC 51299	+	+	−
E. faecalis	E	CCRI-16012	+	+	−
E. faecalis	N/A	LSPQ 5192	+	+	−
E. faecalis	N/A	LSPQ 5378	+	+	−
E. faecalis	N/A	LSPQ 5548	+	+	−
E. faecalis	N/A	LSPQ 5570	+	+	−
E. faecalis	N/A	LSPQ 5638	+	+	−
E. faecalis	N/A	LSPQ 5660	+	+	−
E. faecium	C	ATCC 19434	+	−	+
E. faecium	C	ATCC 700221	+	−	+
E. faecium	E	CCRI-16518	+	−	+
E. faecium	E	CCRI-19447	+	−	+
E. faecium	E	CCRI-19448	+	−	+
E. flavescens	C	ATCC 49996	+	−	−
E. flavescens	C	ATCC 49997	+	−	−
E. gallinarum	C	CCRI-1433	+	−	−
E. gallinarum	C	LSPQ 3364	+	−	−
E. gallinarum	C	LSPQ 5375	+	−	−
E. gilvus	C	ATCC BAA350	+	−	−
E. haemoperoxidus	W	CCUG 45916	+	−	−
E. hirae	C	ATCC 8043	+	−	−

(*continued*)

Table 2 | continued

			Enterococcus-specific rtPCR assays		
Tested species ($n = 55$)	**Origin**	**No. Reference**	***Enterococcus* spp.**[a]	***E. faecalis***[b]	***E. faecium***[b]
E. hirae	C	CCUG 37829	+	−	−
E. italicus	F	CCUG 50447	+	−	−
E. malodoratus	F	ATCC 43197	+	−	−
E. moraviensis	W	CCUG 45913	+	−	−
E. mundtii	E	ATCC 43186	+	−	−
E. pallens	C	ATCC BAA351	+	−	−
E. phoeniculicoli	A	CCUG 48923	+	−	−
E. pseudoavium	A	ATCC 49372	+	−	−
E. quebecensis	W	CCRI-16985	+	−	−
E. raffinosus	C	ATCC 49427	+	−	−
E. ratti	A	ATCC 700914	+	−	−
E. sileciacus	W	CCUG 53830	+	−	−
E. saccharolyticus	C	ATCC 43076	+	−	−
E. sulfureus	E	ATCC 49903	+	−	−
E. termitis	A	CCUG 53831	+	−	−
E. ureasiticus	W	CCRI-16620	+	−	−
E. villorum	A	CCRI-8858	+	−	−

[a]Frahm & Obst (2003).
[b]Maheux *et al.* (2011).
A, Animal; C, Clinical; E, Environmental; F, Food; W, Water; N/A, Non applicable; CCRI, Centre de recherche en infectiologie strain collection; ATCC, American Type Culture Collection; CCUG, Culture Collection (University of Gothenburg); LSPQ, Laboratoire de santé publique du Québec.

osmosis-purified water (resistivity of 18 MΩ-cm min at 25 °C) to produce suspensions containing approximately 10^2 colony forming units (CFU) per 100 mL of water. Bacterial counts were verified by filtering three 100 mL volumes of each spiked water sample through Millipore membrane filters (47 mm diameter, 0.45 μm pore size; Millipore Corporation, Billerica, MA, USA) with a standard platform manifold (Millipore Corporation) followed by an incubation on BHI agar for 24 ± 2 h at 35.0 ± 0.5 °C. Tests to confirm the sterility of filter membranes and buffer used for rinsing the filtration apparatus were also performed.

Membrane filtration method

Membrane filtration was performed according to Maheux *et al.* (2009). Volumes (300 mL) spiked with reference enterococcal bacteria were split into three 100 mL volumes and filtered on Millipore filters with a standard platform manifold. The first filter was incubated on mEI agar (BD, Franklin Lakes, NJ, USA), the second filter on Chromocult® enterococci agar (Merk KGaA, Darmstadt, Germany) and both were incubated for 24 ± 2 h at 35.0 ± 0.5 °C. The third filter was incubated on m-*Enterococcus* agar plates (BD Company, Franklin Lakes, NJ, USA) for 48 ± 3 h at 35.0 ± 0.5 °C before determining colony counts and color (Table 1). Each preparation of mEI, Chromocult® enterococci, and m-*Enterococcus* was tested for performance using pure cultures of target and non-target microorganisms, as recommended by the USEPA microbiology methods manual. Tests were also performed to confirm the sterility of the filter membranes and buffer used for rinsing the filtration apparatus (APHA/AWWA/WEF 2012).

Sample collection

The sewage water sample used in this study to test environmental colonies was harvested at the discharge of the grit chambers of the west wastewater treatment plant of

Québec City, in December 2014. Three 2 μL volumes of sewage water were spiked in 100 mL sterile water and filtered on Millipore filters with a standard platform manifold. The filters were then incubated on mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar as described in the above section 'Membrane filtration method'.

Molecular-based confirmation method

Preparation of bacterial suspensions

Each environmental colony recovered on mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar was touched with a sterile toothpick and resuspended in 100 μL of sterile reverse osmosis-purified water (resistivity of 18 MΩ-cm min at 25 °C). This suspension was used for rtPCR tests described below.

PCR primers

The identity of the environmental colonies isolated on mEI, Chromocult® enterococci, and m-*Enterococcus* plates was confirmed by nucleotide sequencing of 16S rRNA gene using amplification and sequencing primers, SSU27 and SSU534R, an adaptation of Sistek *et al.* (2012). The SSU534R PCR primer, designed for this study, was developed as follows. First, 16S rRNA gene sequences available from public databases were analyzed with GCG programs (version 8.0; Accelrys, Madison, WI, USA). Based on a multiple sequence alignment and the Oligo primer analysis software (version 5.0; National Biosciences, Plymouth, MN, USA), the SSU534R PCR primer was designed from highly conserved regions of the gene. Primers and probes for *Enterococcus* spp.- and *E. faecalis/faecium*-specific rtPCR assays were used as described by Maheux *et al.* (2011). Sequences of the PCR primers are presented in Table 3. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

PCR amplification

For sequencing of the 16S rRNA gene for genotypic identification, 1 μL of each bacterial suspension was transferred directly to 49 μL of PCR mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 2.5 mM MgCl2, 0.4 mM of each primer, 200 mM each of 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,

Table 3 | Real-time PCR primers and probe used in this study

Microorganisms (Targeted gene)	Primer and probe	Primer and probe sequence (5′ → 3′)	Reference
Sequencing and genotypic identification (16S rRNA)	SSU27 SSU534R	AGAGTTTGATCMTGGCTCAG ATTACCGCGGCTGCTGG	Adapted from Sistek *et al.* (2012)
Enterococcus spp. (23S rRNA)	ECST784F ENC854R GPL813TQ	AGAAATTCCAAACGAACTTG CAGTGCTCTACCTCCATCATT FAM[a]-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-BHQ-1[b]	Frahm & Obst (2003)
Enterococcus faecalis (*mtlf*)	Mefs569 Mefs670 Mefs-TL1-A1	GAACAGAAGAAGCCAAAAAA GCAATCCCAAATAATACGGT FAM[a]-CA[L]GGAAT[L]CTGT[L]GTA[L]GTG[L]CAAG-BHQ-1[b]	Maheux *et al.* (2011)
Enterococcus faecium (*ddl*)	Defm273 Defm468 Defm-T1-F2	TGCTTTAGCAACAGCCTATCAG TAAACTTCTTCCGGCACTTCG CalFluorRed610[c]-CTCGAGCAATCGTTGAACAAGGAATTG-BHQ-2[d]	

[a]FAM, 6-carboxyfluorescein, fluorescence reporter dye.
[b]BHQ-1, Black Hole Quencher-1, fluorescence quencher dye.
[c]CalFluorRed610, fluorescence reporter dye.
[d]BHQ-2, Black Hole Quencher-1, fluorescence quencher dye.
[L]N: locked nucleic acid (LNA) analog of a nucleotide.

Canada), 0.06 μg/μL methoxalen (Sigma-Aldrich Canada Ltd), 0.5 enzyme unit (U) of *Taq* DNA polymerase (Promega, Madison, WI, USA) and TaqStart antibody (Clonetech Laboratories, Mountain View, CA, USA). Decontamination of the PCR mixtures prior to PCR was achieved using the UV crosslinker Spectrolinker™ model XL-1000 (Spectronics Corporation, Westbury, NY, USA; Maheux *et al.* 2008). 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 60 °C, and 30 s at 72 °C, with a 5-min final extension step at 72 °C) with a Mastercycler PRO (Eppendorf Canada Ltd, Mississauga, ON, Canada). An agarose gel analysis of the amplified PCR products was performed as previously described (Martineau *et al.* 1998).

Sequencing of the 16S rRNA gene was performed as described by Picard *et al.* (2004). Molecular analysis of sequences was conducted using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi, Standard Nucleotide BLAST optimized for highly similar sequence) and the Ribosomal database project (http://rdp.cme.msu.edu/, isolates with good quality sequence in Sequence Match search).

For the validation of the *Enterococcus* spp. and the *E. faecalis/faecium* rtPCR assays and or confirmation of colony identity, 1 μL of each 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 of *Enterococcus* spp. or *E. faecalis/faecium*, primers, 0.2 μM of *Enterococcus* spp. or *E. faecalis/faecium* 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), 0.025 enzyme unit (U) of *Taq* DNA polymerase (Promega, Madison, WI, USA), and TaqStart antibody (Clontech Laboratories, Mountain View, CA, USA). For each experiment, 1 μL of sterile water was added to the rtPCR mixture as negative control. The rtPCR mixtures were 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 for *Enterococcus* spp. rtPCR assay; 1 min at 95 °C and then 45 cycles of 15 s at 95 °C, 10 s at 60 °C and 20 s at 72 °C for *E. faecalis/faecium* rtPCR assay) with a Rotor-Gene Thermocycler (QIAGEN Inc., Mississauga, Ontario, Canada).

RESULTS AND DISCUSSION

Ability of mEI, Chromocult® enterococci, and m-*Enterococcus* agar methods as well as *Enterococcus* spp.- and *Enterococcus faecalis/faecium*-specific real-time PCR assays to detect enterococcal strains

The specificity of the mEI, Chromocult® enterococci, and m-*Enterococcus* agar methods was demonstrated by testing genomic DNA isolated from 41 enterococcal strains from different species and origin (Table 1). The mEI agar method detected 68.3% (28/41) of the 41 enterococcal strains tested in which only 75% (3/4) of *E. faecalis* and 66.7% (2/3) *E. faecium* tested were detected. A positive signal on mEI agar was also detected for *Enterococcus caccae*, *Enterococcus canintestini*, *Enterococcus canis*, *Enterococcus casseliflavus*, *Enterococcus columbae*, *Enterococcus dispar*, *Enterococcus durans*, *Enterococcus gallinarum*, *Enterococcus gilvus*, *Enterococcus haemoperoxidus*, *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus pallens*, *Enterococcus phoeniculicoli*, *Enterococcus quebecensis*, *Enterococcus sileciacus*, *Enterococcus sulfureus*, *Enterococcus termitis*, and *Enterococcus villorum*. These results showed that the recovery of *E. faecalis* and *E. faecium* cells could not be optimal with this medium. Furthermore, strictly environmental *Enterococcus* spp. (*E. mundtii* and *E. sulfureus* seem to be strictly associated to plants and soil (Müller *et al.* 2001)) are also detected by the mEI agar method suggesting that colonies obtained from water samples should be identified to confirm a contamination of fecal origin.

The Chromocult® enterococci agar method detected 78.0% (32/41) of the 41 enterococcal strains tested in which all (100%) of *E. faecalis* and *E. faecium* tested were detected. The only enterococcal species not detected by Chromocult® enterococci method were *E. aquimarinus*, *E. avium*, *E. columbae*, *E. dispar*, *E. italicus*, *E. pseudoavium*, *E. raffinosus*, *E. ratti*, and *E. sulfureus*. These results showed that the Chromocult® enterococci method could be a good choice to assess water quality since all *E. faecalis* and *E. faecium* tested were detected. However, as for the mEI agar method, strictly environmental *Enterococcus* spp. are also detected suggesting that colonies obtained from water samples should be identified to confirm a contamination of fecal origin.

Finally, the m-*Enterococcus* agar method detected only 29.3% (12/41) of the 41 enterococcal strains tested in which all (100%) of *E. faecalis* but only 33% (1/3) of *E. faecium* tested were detected. With the exception of *E. faecalis* and *E. faecium*, the spectrum of detection of m-*Enterococcus* agar method was only *E. casseliflavus*, *E. gallinarum*, and *E. hirae* when colonies were subjected to phenotypical confirmation tests (CEAEQ 2014). However, in order to bypass those phenotypic confirmations tests, colonies harvested on m-*Enterococcus* agar from sewage water described below were only subjected to confirmation using rtPCR assays. These results showed that the recovery of *E. faecium* cells could not be optimal with this medium.

In traditional water quality assessment, the definition of *Enterococcus* sp. is phenotypical. Indeed, an *Enterococcus* is defined as spherical bacterium, in pair or chain, Gram-positive, catalase-negative and facultatively anaerobic. It does not form endospores and some species demonstrate mobility. In addition, an *Enterococcus* hydrolyzes esculin in the presence of bile and has the ability to grow at 10 °C and 45 °C, pH 9.6 or in the presence of NaCl 6.5% (Mundt 1986; Knudtson & Hartman 1992; APHA/AWWA/WEF 2012). However, since the use of genotypic classification, it has been shown some species of the *Enterococcus* genus do not express β-glucosidase at 35 °C after 24–48 hours on chromogenic media (Maheux *et al.* 2008; Sistek *et al.* 2012). However, that does not mean that they do not express the enzyme at all. But using those culture conditions, the expression of β-glucosidase is not detected.

The ability of *Enterococcus* spp.- and *Enterococcus faecalis/faecium*-specific real-time PCR assays to detect enterococcal strains was demonstrated by testing genomic DNA isolated from 55 enterococcal strains including 12 *E. faecalis* and 5 *E. faecium* strains (Table 2). The *Enterococcus* sp.-specific rtPCR primers and probe efficiently amplified DNA from all 55 enterococcal strains tested whereas the multiplexed *E. faecalis/faecium* rtPCR assay efficiently amplified DNA from 12 of 12 (100%) *E. faecalis* and 5 of 5 (100%) *E. faecium* strains tested, respectively. Thus, against all enterococcal strains, the *Enterococcus* sp. rtPCR assay is 100% sensitive in its ability to detect all enterococcal strains, whereas the multiplex *E. faecalis/faecium* rtPCR assay is 100% sensitive for the detection of *E. faecalis* and *E. faecium*. Maheux *et al.* (2011) tested 150 closely related non-enterococcal species among the *Enterococcus* spp.-specific rtPCR assay and showed that there was no specific amplification of the 150 non-enterococcal bacterial species with the exception of *Tetragenococcus solitarius*. Phylogenetically, *T. solitarius* is very closely related to enterococci (Ke *et al.* 1999; Ennahar & Cai 2005) and controversy in its taxonomical classification persists.

Ability of mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar to detect *Enterococcus* spp. from a sewage water sample

Three 2 μL volumes of a 1-L sewage water sample harvested at the discharge of the grit chambers of the west wastewater treatment plant of Québec City were used for testing by mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar methods to verify their comparative ability to detect *Enterococcus* spp. (Table 4). All the colonies were harvested and subjected to molecular identification using a 527 base pairs fragment of the 16S rRNA gene. On mEI agar, 23 blue (phenotype +) and 14 pink (phenotype −) colonies were harvested for a total of 37 colonies. On Chromocult®

Table 4 | Specificity[a], sensitivity[b], and predictive values of each method for the detection of *Enterococcus* spp. as compared to 16S rRNA gene identification

Method and results	16S rRNA identification		Specificity[a]	Sensitivity[b]	Positive predictive value[c]	Negative predictive value[d]
	+	−				
mEI agar						
+	22	1	92.3%	91.7%	95.6%	85.7%
−	2	12				
Chromocult® enterococci agar						
+	38	2	0%	100.0%	100.0%	N/C
−	0	0				
m-*Enterococcus* agar						
+	39	0	100%	100.0%	100.0%	N/C
−	0	0				

N/C, not calculable.
[a]No. of true negative results/(no. of true negative + false positive results).
[b]No. of true positive results/(no. of true positive + false negative results).
[c]No. of true positive results/(no. of true positive + false positive results).
[d]No. of true negative results/(no. of true negative + false negative results).

enterococci and m-*Enterococcus* agar, 40 pink (phenotype +) and 49 red/pink (phenotype +) colonies were harvested, respectively. All these colonies were harvested and subjected to molecular identification using the 16S rRNA gene. Molecular analysis showed that 1 blue (1/23) colony on mEI agar and 2 pink (2/40) colonies on Chromocult® enterococci agar were not enterococcal species for a false positive rate of 4.3% and 5.0%, respectively. No false positive results have been found on m-*Enterococcus* agar. On the contrary, 2 pink (2/12) colonies on mEI agar were enterococcal species for a false negative rate of 16.7%. By using 16S rRNA molecular identification as gold standard, m-*Enterococcus* agar method has presented the highest specificity (100%), sensitivity (100%), and positive predictive value (100%; Table 4) for the detection of *Enterococcus* spp. Finally, Chromocult® enterococci agar and m-*Enterococcus* agar detected 38 and 39 *Enterococcus* spp. colonies respectively, whereas mEI agar detected only 24 *Enterococcus* spp. colonies.

Population of *Enterococcus* per species detected by mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar isolated from the same sewage water sample

After colonies were identified using 16S rRNA analysis, the population of total coliforms enumerated by mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar, were classified per species (Table 5). Results showed that the population of enterococcal species detected was more extended for m-*Enterococcus* agar (7 different species) than mEI and Chromocult® coliform agar (only 3 and 4 different species, respectively; Table 5). Like the Maheux *et al.* (2009) study, the results of the present study showed the lack of correlation between test methods based on the same enzymatic principle to recognize a strain as *Enterococcus* spp. Indeed, our results showed that there is a weak correlation between the three methods tested within the same species. Since all colonies of the present study were isolated from the same water sample and treated in the same way (filtration, incubation, etc.), the difference observed in the population of strains detected by each method cannot just be attributed to environmental factors. The composition of each medium is also involved. As observed in the analytical sensitivity section above, the mEI agar method detected less *E. faecalis* than the two other methods. Furthermore, m-*Enterococcus* detected less *E. faecium* than the two other methods. Consequently, the Chromocult® enterococci method seems to be the best method among the three tested to recover *E. faecalis* and *E. faecium* cells.

Ability of *Enterococcus* spp.- and *Enterococcus faecalis/faecium*-specific rtPCR assays to detect colonies isolated on chromogenic culture-based methods from a sewage water sample

It is impossible to exclude that an *E. faecalis* or an *E. faecium* detected by a culture-based method was naturally found in the environment. However, some non-*faecalis* and non-*faecium* *Enterococcus* species are not present in feces, but are naturally and highly present in the environment. By detecting these non-fecal *Enterococcus* species,

Table 5 | Population of *Enterococcus* per species detected by mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar from 2 µL of sewage water

mEI agar			Chromocult® enterococci agar			m-*Enterococcus* agar		
Species	**No.**	**%**	**Species**	**No.**	**%**	**Species**	**No.**	**%**
E. faecium	22	91.7	*E. faecium*	20	28.2	*E. faecalis*	15	38.5
E. faecalis	1	4.2	*E. faecalis*	9	25.6	*E. faecium*	13	33.3
E. casseliflavus	1	4.3	*E. hirae*	7	23.1	*E. hirae*	6	15.4
			E. casseliflavus	2		*E. casseliflavus*	2	5.1
						E. avium	1	2.6
						E. mundtii	1	2.6
						E. sulfureus	1	2.6
Total	**24**	**100.0**		**38**	**100.0**		**39**	**100.0**

we cause false-positive results that could be avoided by only detecting the *Enterococcus* species mostly found in feces rather than relying on the entire *Enterococcus* genus. Furthermore, since *Escherichia coli* is 100 to 1,000 times more concentrated than *Enterococcus* spp. in feces, the probability of detecting non-*E. faecalis*/*E. faecium* in water without any detection of *E. coli* nor *E. faecalis*/*E. faecium* is highly improbable (Slanetz & Bartley 1957; Layton *et al.* 2010). Thus, the sole detection of *E. faecalis* and *E. faecium* in water could decrease the false-positive rate obtained with the detection of the entire *Enterococcus* genus and by this, improve the water quality assessment.

In the present study, colonies harvested on mEI agar, Chromocult® enterococci agar, and m-*Enterococcus* agar were identified using two specific-rtPCR assays targeting *Enterococcus* spp. and *Enterococcus faecalis*/*faecium* to confirm, in less than 2 hours, their identity. A positive rtPCR signal obtained with the *Enterococcus faecalis*/*faecium*-specific rtPCR assay allows the discrimination between fecal and environmental contamination of water samples.

All colonies harvested, for the present study, on mEI, Chromocult® enterococci, and m-*Enterococcus* agar were subjected to *Enterococcus* spp.- and *Enterococcus faecalis*/*faecium*-specific rtPCR assays (Table 6). Both of the rtPCR

Table 6 | Specificity[a], sensitivity[b], and predictive values of each culture-based method combined to *Enterococcus* spp.-, *Enterococcus faecalis*-, and *Enterococcus faecium*-specific rtPCR assays for the identification of *Enterococcus* spp., *E. faecalis*, and *E. faecium*, respectively, as compared to 16 s rRNA genotypic identification

Culture-based methods[c]	PCR assays	16S rRNA identification +	16S rRNA identification −	Specificity[a]	Sensitivity[b]	Positive predictive value[d]	Negative predictive value[e]
mEI agar ($n = 22$)	*Enterococcus* spp.						
	+	21	0	100%	100%	100%	100%
	−	0	1				
	E. faecalis						
	+	1	0	100%	100%	100%	100%
	−	0	0				
	E. faecium						
	+	17	0	100%	89.5%	100%	0%
	−	2	0				
Chromocult® enterococci agar ($n = 40$)	*Enterococcus* spp.						
	+	38	0	100%	100%	100%	100%
	−	0	2				
	E. faecalis						
	+	9	0	100%	100%	100%	100%
	−	0	0				
	E. faecium						
	+	20	0	100%	100%	100%	100%
	−	0	0				
m-*Enterococcus* agar ($n = 39$)	*Enterococcus* spp.						
	+	39	0	100%	100%	100%	100%
	−	0	0				
	E. faecalis						
	+	15	0	100%	100%	100%	100%
	−	0	0				
	E. faecium						
	+	12	0	100%	100%	100%	100%
	−	0	0				

[a]No. of true negative results/(no. of true negative + false positive results).
[b]No. of true positive results/(no. of true positive + false negative results).
[c]Calculated on *Enterococcus* spp. colony phenotype.
[d]No. of true positive results/(no. of true positive + false positive results).
[e]No. of true negative results/(no. of true negative + false negative results).

assays showed a specificity of 100% on the three media tested. Only two (2/19) colonies of *E. faecium* were not detected by the *Enterococcus faecalis/faecium*-specific rtPCR assay on mEI agar for a sensitivity of 89.5%. These results showed that the *Enterococcus faecalis/faecium*-specific rtPCR assay could be used with success to discriminate between fecal and environmental contamination of water samples when testing on colonies isolated on mEI, Chromocult® enterococci, or m-*Enterococcus* agar.

The results obtained in the present study were obtained using sewage water samples. Results could differ with other types of water.

CONCLUSION

The sensitivity evaluation of the three culture-based methods tested using both reference strains and environmental strains identified by 16S rRNA gene sequencing, as well as the validation of the *Enterococcus* spp.- and *Enterococcus faecalis/faecium*-specific rtPCR assays showed that the detection and identification of enterococcal colonies on Chromocult® enterococci agar combined with *Enterococcus faecalis/faecium* rtPCR assay presents the best combination to decrease the high false-positive rate obtained when the entire *Enterococcus* genus is targeted.

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