# Novel *Escherichia coli* genetic markers for tracking domestic wastewater contamination in environmental waters

Ryota Gomi[a,*], Eiji Haramoto[b], Hiroyuki Wada[c], Yoshinori Sugie[c], Chih-Yu Ma[c], Sunayana Raya[d], Bikash Malla[b], Fumitake Nishimura[c], Hiroaki Tanaka[c], Masaru Ihara[c, e, *]

[a]Department of Environmental Engineering, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, 615-8540, Kyoto, Japan

[b]Interdisciplinary Center for River Basin Environment, University of Yamanashi, 4-3-11 Takeda, Kofu, 400-8511, Yamanashi, Japan

[c]Research Center for Environmental Quality Management, Graduate School of Engineering, Kyoto University, 1-2 Yumihama, Otsu, 520-0811, Shiga, Japan

[d]Department of Engineering, University of Yamanashi, Kofu, 400-8511, Yamanashi, Japan

[e]Faculty of Agriculture and Marine Science, Kochi University, Nankoku, 783-8502, Kochi, Japan

*Corresponding authors

E-mail address: gomi.ryota.34v@kyoto-u.jp; Tel: +81-75-383-3354; Fax: +81-75-383-3358

E-mail address: ihara.masaru@kochi-u.ac.jp; Tel: +81-88-864-5163

**ABSTRACT**

*Escherichia coli* has been used as an indicator of fecal pollution in environmental waters. However, its presence in environmental waters does not provide information on the source of water pollution. Identifying the source of water pollution is paramount to be able to effectively prevent contamination. The present study aimed to identify *E. coli* microbial source tracking (MST) markers that can be used to identify domestic wastewater contamination in environmental waters. We first analyzed wastewater *E. coli* genomes sequenced in this study (n = 50) and RefSeq animal *E. coli* genomes of fecal origin (n = 82), and identified 144 candidate wastewater-specific marker genes. The sensitivity and specificity of the candidate marker genes were then assessed by screening the genes in 335 RefSeq wastewater *E. coli* genomes and 3,318 RefSeq animal *E. coli* genomes. We finally identified two MST markers, namely W_nqrC and W_clsA_2, which could be used for tracking wastewater contamination. These two markers showed higher performance than the previously developed human wastewater-associated *E. coli* markers H8 and H12. PCR assays to detect W_nqrC and W_clsA_2 were also developed and validated. The developed PCR assays are potentially useful for detecting *E. coli* isolates of wastewater origin in environmental waters. However, further studies are needed to assess the applicability of the developed markers to a culture-independent approach.

## 1. Introduction

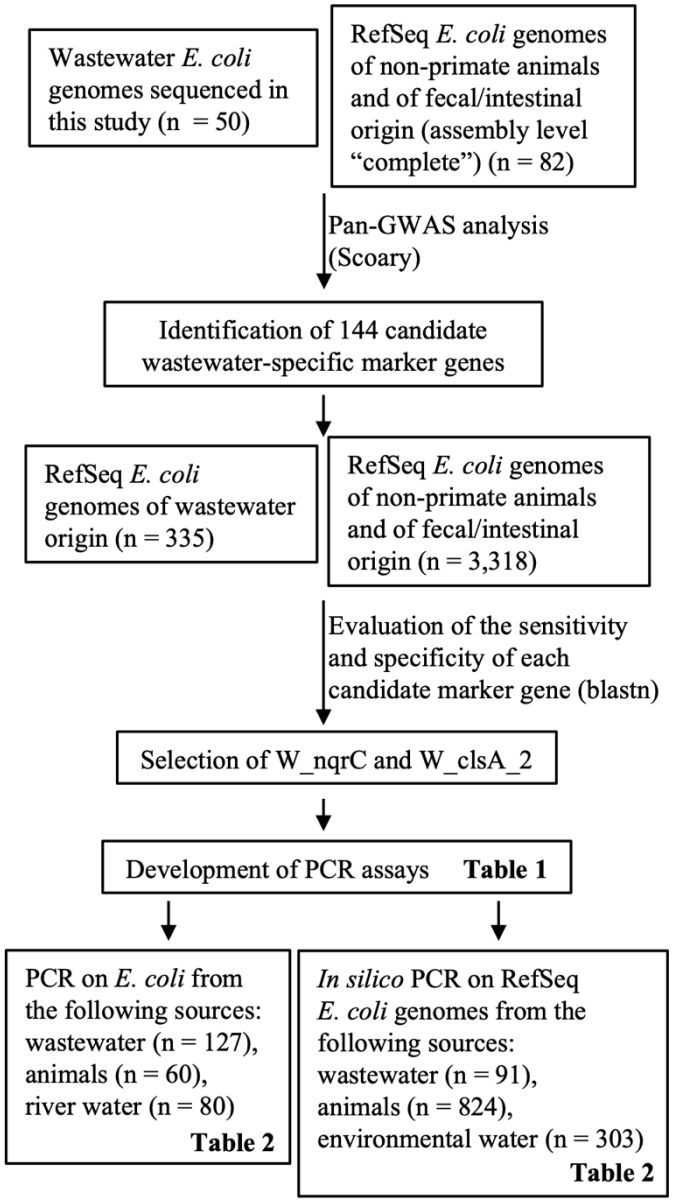
Surface waters contaminated by wastewater can increase human health risks because untreated and insufficiently-treated wastewater contains various pathogens (Castro-Hermida et al., 2008; Naidoo and Olaniran, 2013; Zhi et al., 2020). Humans can be exposed to such pathogens through activities such as swimming and the consumption of foods irrigated with poor-quality water (Boehm et al., 2018; Steele and Odumeru, 2004). Because it is impractical to test for all possible pathogens in surface waters, fecal indicator bacteria (FIB), such as total coliforms, fecal coliforms, and *Escherichia coli*, are monitored as proxies for pathogens (Field and Samadpour, 2007; Naidoo and Olaniran, 2013). However, the presence of FIB does not necessarily indicate that the water is contaminated by human pathogens for several reasons (e.g., the presence of naturalized FIB (Ishii et al., 2006)). The major limitation of FIB is that, due to their cosmopolitan nature, their presence does not provide information on the possible origin of contamination (Warish et al., 2015). FIB can originate from many sources, such as community sewage (containing human-specific pathogens and zoonotic pathogens) and diffuse pollution from animal farms and wildlife (containing zoonotic pathogens). It is known that human health risks can vary according to the source of fecal pollution, and human health risks from recreational waters impacted by human sources are generally considered to be higher than those impacted by non-human sources (Soller et al., 2010). Identifying the origin of water

56 pollution is paramount in order to assess the associated health risks and determine the actions
57 needed to prevent contamination (Scott et al., 2002).
58 Microbial source tracking (MST) is an approach used to determine the sources of fecal
59 contamination in environmental waters (Harwood et al., 2014). MST methods can be divided
60 into culture-dependent methods and culture-independent methods. Culture-dependent methods
61 require the growing of environmental isolates from water, while in culture-independent
62 methods genetic markers are assayed directly from a water sample or from DNA extracted from
63 a water sample (Field and Samadpour, 2007). Markers such as the 16S rRNA gene of
64 *Bacteroides* and pepper mild mottle virus (PMMoV) have been used to track the sources of
65 fecal contamination in MST in a culture-independent manner (Gonzalez-Fernandez et al., 2021;
66 Mathai et al., 2020). However, routine water quality assessment has focused primarily on
67 traditional FIB, and alternative indicators such as *Bacteroides* are not usually monitored for
68 this purpose (Senkbeil et al., 2019; Teixeira et al., 2020). Because *E. coli* is still a widely-used
69 FIB, MST markers targeting *E. coli* would be useful if included in the course of routine
70 monitoring. Even a culture-dependent approach would be useful for *E. coli*, because users do
71 not have to collect isolates exclusively for MST purposes but can use *E. coli* colonies obtained
72 in routine water quality assessments.
73 Although little success has been achieved in developing MST markers targeting *E. coli*, we
74 have previously identified a set of human wastewater, cattle, pig, and chicken feces-associated

75 *E. coli* MST markers (Gomi et al., 2014). The following studies reported two of the proposed
76 markers, namely H8 and H12, showed relatively high sensitivity and specificity and could be
77 useful as human wastewater-associated markers (Nopprapun et al., 2020; Senkbeil et al., 2019;
78 Warish et al., 2015). However, the original genetic markers were developed by analyzing a
79 small number of *E. coli* genomes (n = 22). Since then, a growing number of *E. coli* genomes
80 have become available in public databases. These *E. coli* genomes include those from various
81 isolation sources and geographically diverse hosts, which might allow the development of
82 reliable MST markers that can be used globally. Here, we analyzed *E. coli* genomes in the
83 database of the National Center for Biotechnology Information (NCBI) as well as genomes
84 sequenced in the present study to identify more reliable *E. coli* genetic markers for tracking
85 domestic wastewater contamination in environmental waters. Our aim in the present study was
86 to identify culture-dependent MST markers, but applicability to a culture-independent
87 approach was also discussed. The sensitivity and specificity of the previously developed human
88 wastewater-associated markers H8 and H12 were also re-evaluated.
89
90
91 **2. Materials and methods**
92 **2.1. *E. coli* isolation from wastewater and genome sequencing.**

93 The flowchart of the study design is shown in **Figure 1**. We obtained wastewater *E. coli* isolates
94 from municipal wastewater treatment plants (WWTPs) in Shiga Prefecture, Japan. Briefly,
95 biologically treated wastewater samples before/after chlorination were collected from three
96 different WWTPs (WWTP A, WWTP B, and WWTP C) between December 2016 and
97 December 2017. The chlorinated samples were treated with sodium thiosulfate immediately
98 after collection to neutralize the chlorine. Samples were collected using sterile sampling bags
99 or sterile sampling bottles, stored at 4 °C, and processed within 24 hours. *E. coli* were cultured
100 using the pour plate method with XM-G agar (Nissui, Tokyo, Japan) at 37 °C for 18h, and
101 colonies were randomly picked from the plates. Colonies suspected to be mixed were sub-
102 cultured with fresh XM-G agar plates to obtain pure isolates. A total of 50 isolates (29 isolates
103 from WWTP A, nine isolates from WWTP B, and 12 isolates from WWTP C) were obtained
104 for genome sequencing (see **Table S1** for information on the collected *E. coli*).
105 DNA was extracted from each isolate using a DNeasy blood and tissue kit (Qiagen, Hilden,
106 Germany) following the manufacturer's instructions, and the concentration of extracted DNA
107 was measured using a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, United
108 States). Extracted DNA was stored at -30°C for up to a week until sample preparation for
109 genome sequencing. The extracted DNA was processed using a Nextera XT DNA sample
110 preparation kit (Illumina, San Diego, CA) and sequenced on an Illumina MiSeq instrument for
111 600 cycles (300-bp paired-end sequencing).

112




113

114 **Figure 1**. Overview of the strategy to identify and test the wastewater-specific *E. coli* genetic

115 markers. RefSeq *E. coli* genomes used for blastn analysis (335 wastewater *E. coli* genomes and

116 3,318 animal *E. coli* genomes) and *in silico* PCR analysis (91 wastewater *E. coli* genomes, 824

117 animal *E. coli* genomes, and 303 environmental *E. coli* genomes) included both complete and

118 draft genome assemblies. There was no overlap between the genomes used for blastn analysis

119 and *in silico* PCR analysis.

120

121 **2.2. Retrieving complete *E. coli* genome sequences from the NCBI database.**

122 The Reference Sequence (RefSeq) *E. coli* genomes with an assembly level of "complete

123 genome" (n = 1,278) were downloaded in May 2021. Metadata information, such as strain

124 name, host, and isolation source, was extracted from the GenBank files using in-house Python

125 scripts. *E. coli* genomes of non-primate animals and of fecal/intestinal origin (n = 82) were

126 kept for further analysis (*E. coli* genomes from birds were also included here. See **Table S2** for

127 information on these animal *E. coli* genomes).

128

129 **2.3. Genome assembly and annotation.**

130 Raw sequence reads from wastewater *E. coli* were trimmed using fastp (v0.20.0) (Chen et al.,

131 2018), and the trimmed reads were assembled using Unicycler (v0.4.8) with the --no_correct

132 option (Wick et al., 2017). For the complete RefSeq genomes, we generated simulated paired-

133 end reads using wgsim (v1.12) (https://github.com/lh3/wgsim). Simulated reads were trimmed

134 using fastp and assembled using Unicycler with the same parameters as those used for

135 assembling the wastewater *E. coli* genomes. We did not use the complete RefSeq genomes but

136 used these draft assemblies in the following analysis. This was to remove any biases introduced

137 by differences in assembly status (i.e., complete assemblies vs. draft assemblies) in the pan-
138 genome-wide association study (pan-GWAS). Assembled genomes were annotated using
139 Prokka (v1.14.6) (Seemann, 2014).
140
141 **2.4. Identification of candidate wastewater-specific marker genes using a pan-GWAS**
142 **approach.**
143 The pan-genome of 132 *E. coli* genomes (50 wastewater *E. coli* genomes + 82 animal *E. coli*
144 genomes) was constructed by running Roary (v3.13.0) on GFF3 files generated by Prokka
145 (Page et al., 2015). The -s option was used to turn off paralog splitting. The pan-GWAS
146 approach implemented by Scoary was used to find genes that were enriched in wastewater *E.*
147 *coli* genomes (Brynildsrud et al., 2016). Scoary (v 1.6.16) was run on Roary's gene
148 presence/absence file. The --collapse option was used to collapse genes that had identical
149 distribution patterns among the genomes. Because our aim was to identify genes that were
150 enriched in wastewater *E. coli* genomes and not to infer causal association, the --no_pairwise
151 option was used as recommended by the developers (https://github.com/AdmiralenOla/Scoary).
152 Genes with Benjamini-Hochberg adjusted p-values of lower than 0.05 and with a specificity
153 exceeding 95% were defined as candidate wastewater-specific marker genes. We set the
154 specificity threshold so as to reduce the number of false positives, considering the future
155 application to DNA directly extracted from environmental samples (this type of DNA can

156 contain DNA from multiple *E. coli* isolates and thus is sensitive to false positives). When a set
157 of collapsed genes met the p-value and specificity criteria, one gene was randomly selected as
158 a candidate marker.
159
160 **2.5. Retrieving genome sequences from the NCBI database for sensitivity and specificity**
161 **analysis.**
162 The RefSeq *E. coli* genomes (n = 20,237), including both complete and draft assemblies, were
163 downloaded in June 2021. Metadata information was extracted from the GenBank files using
164 in-house Python scripts. Genomes of wastewater origin (n = 335) and genomes of non-primate
165 animals and of fecal/intestinal origin (n = 3,318) were kept for sensitivity and specificity
166 analysis (see **Table S3** for information on the wastewater *E. coli* genomes and **Table S4** for
167 information on the animal *E. coli* genomes).
168
169 **2.6. Evaluating the sensitivity and specificity of candidate marker genes.**
170 The candidate marker genes were detected in 3,653 genome assemblies (335 RefSeq
171 wastewater *E. coli* genomes + 3,318 RefSeq animal *E. coli* genomes) using the blastn program
172 with the default parameters. Thresholds of a minimum percent identity of 80% and a minimum
173 DNA coverage of 50% were used to define the presence of a gene. For each candidate marker
174 gene, the following metrics were calculated: true positive (TP) (the number of wastewater *E.*

175 *coli* genomes positive for the candidate marker), true negative (TN) (the number of animal *E.*
176 *coli* genomes negative for the candidate marker), false positive (FP) (the number of animal *E.*
177 *coli* genomes positive for the candidate marker), and false negative (FN) (the number of
178 wastewater *E. coli* genomes negative for the candidate marker). Sensitivity [TP/(TP + FN)] and
179 specificity [TN/(TN + FP)] were then calculated for each candidate marker gene (note that
180 sensitivity and specificity were reported as %). The above blastn analysis and calculation were
181 also performed for H8 and H12. Note that H8 corresponded to one of the candidate marker
182 genes.
183
184 **2.7. Development of PCR assays.**
185 Primers that yield desired product sizes were designed for the identified marker genes as shown
186 in **Table 1**. For each target gene, the validity of the primers was checked with Primer 3 software
187 using the default settings (Untergasser et al., 2012). Moreover, using Primer-BLAST with the
188 default settings, we confirmed that an amplicon of the desired size was produced from each
189 wastewater genome with the target gene that had been sequenced in **2.1** (Ye et al., 2012).
190 To further validate the developed PCR primers, we performed PCR on *E. coli* isolates collected
191 from the following sources: wastewater influent (n = 78), wastewater effluent (after biological
192 treatment and chlorination) (n = 49), deer (n = 30), and cattle (n = 30). Wastewater influent
193 isolates (n = 48) and effluent isolates (n = 49) were newly collected in 2021 from WWTP A in

194 Shiga Prefecture, Japan. Other wastewater influent isolates (n = 30) were collected in 2018
195 from a WWTP (WWTP D) in Yamanashi Prefecture, Japan. We used the same sampling
196 protocol and *E. coli* isolation procedure as described in **2.1**, except that we used Chromocult
197 coliform agar (Merck, Darmstadt, Germany) or CHROMagar ECC (CHROMagar, Paris,
198 France) and incubated the plates for ~24 hours at 37 °C to isolate *E. coli* from the influent
199 samples collected from WWTP D. Animal *E. coli* isolates were obtained from animal feces.
200 Briefly, eight deer fecal samples were collected in Yamanashi Prefecture, and two deer fecal
201 samples and 10 cattle fecal samples were collected in Shiga Prefecture. Deer fecal samples
202 were collected using sterile sampling bags, stored at 4 °C, and processed within 24-48 hours.
203 The deer fecal samples collected in Yamanashi Prefecture were stored at 4 °C and transported
204 to the laboratory in Shiga Prefecture, where the samples were processed. Cattle fecal samples
205 were collected using sterile centrifuge tubes, stored at 4 °C, and processed within 24 hours.
206 Animal feces were suspended and diluted with PBS and processed using the pour plate method
207 with XM-G agar, except for two deer fecal samples for which CHROMagar ECC was used.
208 The plates were incubated for 18 hours at 37 °C. Sub-culturing was performed to obtain pure
209 colonies. Up to three isolates were obtained from a single individual (Dombek et al., 2000).
210 DNA was extracted from *E. coli* obtained from WWTP A and animal feces using a DNeasy
211 blood and tissue kit. The concentration of extracted DNA was measured using a Qubit
212 fluorometer. The extracted DNA was stored at -30°C for up to one year until the PCR

213 experiments. For *E. coli* obtained from WWTP D, we used cell suspensions as the inputs in the
214 PCR experiments. Cell suspensions were prepared by either of the following two methods: (i)
215 a single *E. coli* colony grown on the XM-G plate was picked up using a clean tip, suspended
216 in 500 μL of ultrapure water, and then vortexed; or (ii) a glycerol stock (*E. coli* stored in 25%
217 glycerol) was diluted 10 times using ultrapure water. PCR reactions were performed on the
218 extracted DNA or cell suspension using QuantiFast SYBR Green PCR Kit (Qiagen, Hilden,
219 Germany) with PCR conditions as described below. PCR mixture (25 μL) was composed of
220 12.5 μL of 2× QuantiFast SYBR Green PCR Master Mix, 2.5 μL each of forward and reverse
221 primers (5 μM), 5 μL of template DNA or cell suspension, and 2.5 μL of PCR grade water or
222 ultrapure water. The reactions were carried out by incubation at 95 °C for 5 min, followed by
223 25 to 30 cycles (depending on the template concentrations) consisting of 95 °C (denaturation)
224 for 10 s and 60 °C (combined annealing/extension) for 30 s. A melting curve analysis was
225 performed after the amplification. In addition to the primers for W_nqrC and W_clsA_2, we
226 also used previously published primers for amplifying H8, H12, and also *uidA* (primers UAL
227 and UAR): we included *uidA* primers to confirm the *E. coli* identification (Gomi et al., 2014;
228 Maheux et al., 2009). All PCR reactions were performed on a Thermal Cycler Dice Real Time
229 System III (TaKaRa Bio, Inc.). Previously sequenced *E. coli* isolates, namely KOr026
230 (positive: W_clsA_2 and H8, negative: W_nqrC and H12, BioSample accession:
231 SAMD00053091) and KMi027 (positive: W_nqrC and H12, negative: W_clsA_2 and H8,

232 BioSample accession: SAMD00053165), were used as positive/negative controls (Gomi et al.,
233 2017b). We also included no template controls. The correct PCR products were confirmed by
234 comparison with the melting curves of the positive controls, which showed peaks at melting
235 temperatures of 82.5 °C, 89.0 °C, 91.6 °C, 85.7 °C, and 84.4 °C for W_nqrC, W_clsA_2, H8,
236 H12, and *uidA*, respectively. We performed replicate reactions for a subset of samples (50
237 samples, including 42 for extracted DNA and 8 for cell suspensions) and confirmed the results
238 to be consistent between all technical replicates.
239 We also performed *in silico* PCR using the developed primers on RefSeq *E. coli* genomes that
240 were made public after June 2021 (i.e., genomes that were not used for blastn analysis). This
241 is to test the developed PCR primers using *E. coli* genomes of diverse origins and from various
242 geographical locations. *In silico* PCR was previously shown to be highly concordant with *in*
243 *vitro* PCR when used for gene detection in *E. coli* (Beghain et al., 2018; Lindsey et al., 2017).
244 Briefly, RefSeq *E. coli* genomes, including both complete and draft assemblies, were
245 downloaded in August 2022. Among the downloaded genomes, those used for blastn analysis
246 were removed, leaving 6,399 genomes. Metadata information was extracted from the GenBank
247 files, and the genomes of wastewater origin (n = 91) and those of non-primate animals and of
248 fecal/intestinal origin (n = 824) were retrieved. *In silico* PCR was performed on the Primer-
249 BLAST website using primer sequences of W_nqrC, W_clsA_2, H8, and H12 with the default
250 parameters, except that we used 500 bp as max target amplicon size (Ye et al., 2012).

251

252 **Table 1**. Primers designed in this study.

| Target | Sequence (5′-3′) | Product size (bp) |
|---|---|---|
| W_nqrC | F: CATGCGTTTCTTGCTCTGGG | 105 |
| | R: GTTGGCGTCTTCCACCCTAG | |
| W_clsA_2 | F: ACCGATGATTGGTGCGATAC | 165 |
| | R: GCTCAAAGCGCCCATGAAG | |

253

254 **2.8. Detection of MST markers in *E. coli* isolates from environmental waters.**

255 *E. coli* isolates were obtained in January 2022 from a river in Kyoto Prefecture in Japan. The
256 sampling points in the river were downstream of two wastewater treatment plants and known
257 to be heavily affected by treated wastewater (**Figure S1**). A total of eight water samples were
258 collected from the river using sterile sampling bags, stored at 4 °C, and processed within 24
259 hours. Ten *E. coli* colonies were obtained from each sample using the membrane filter method
260 with XM-G agar plates. The plates were incubated for 18 hours at 37 °C, and colonies were
261 sub-cultured with fresh XM-G agar plates to obtain pure isolates. PCR reactions were
262 performed as described above to test for the presence of W_nqrC and W_clsA_2 in the obtained
263 *E. coli* isolates.

264 We also performed *in silico* PCR on RefSeq *E. coli* genomes of environmental water origin.
265 Environmental *E. coli* genomes were extracted from the RefSeq *E. coli* genomes downloaded
266 in August 2022 based on the metadata information. *In silico* PCR was performed using the
267 retrieved genomes (n = 303) on the Primer-BLAST website as described above.

268

269 **2.9. Sequence Data Accession Number(s).**

270 The raw sequencing data obtained in the present study were deposited in the NCBI SRA under

271 BioProject accession number PRJNA765288.

272

273

274 **3. Results and discussion**

275 **3.1. Basic characteristics of wastewater *E. coli* genomes sequenced in this study.**

276 The 50 wastewater *E. coli* genomes sequenced in this study were highly diverse, comprising

277 44 different sequence types (STs). These draft assemblies had a median of 140 contigs (range

278 49 contigs to 346 contigs), a median genome size of 4,875,061 bp (range 4,576,995 bp to

279 5,375,884 bp), and a median N50 of 179,046 bp (range 63,435 bp to 737,274 bp) after removing

280 contigs shorter than 100 bp (**Table S1**). All 50 assemblies met the quality control criteria of (i)

281 contig numbers: ≤ 800; (ii) genome size: 3.7 Mbp to 6.4 Mbp; and (iii) N50: >20 kb, as

282 recommended by EnteroBase (Zhou et al., 2020).

283

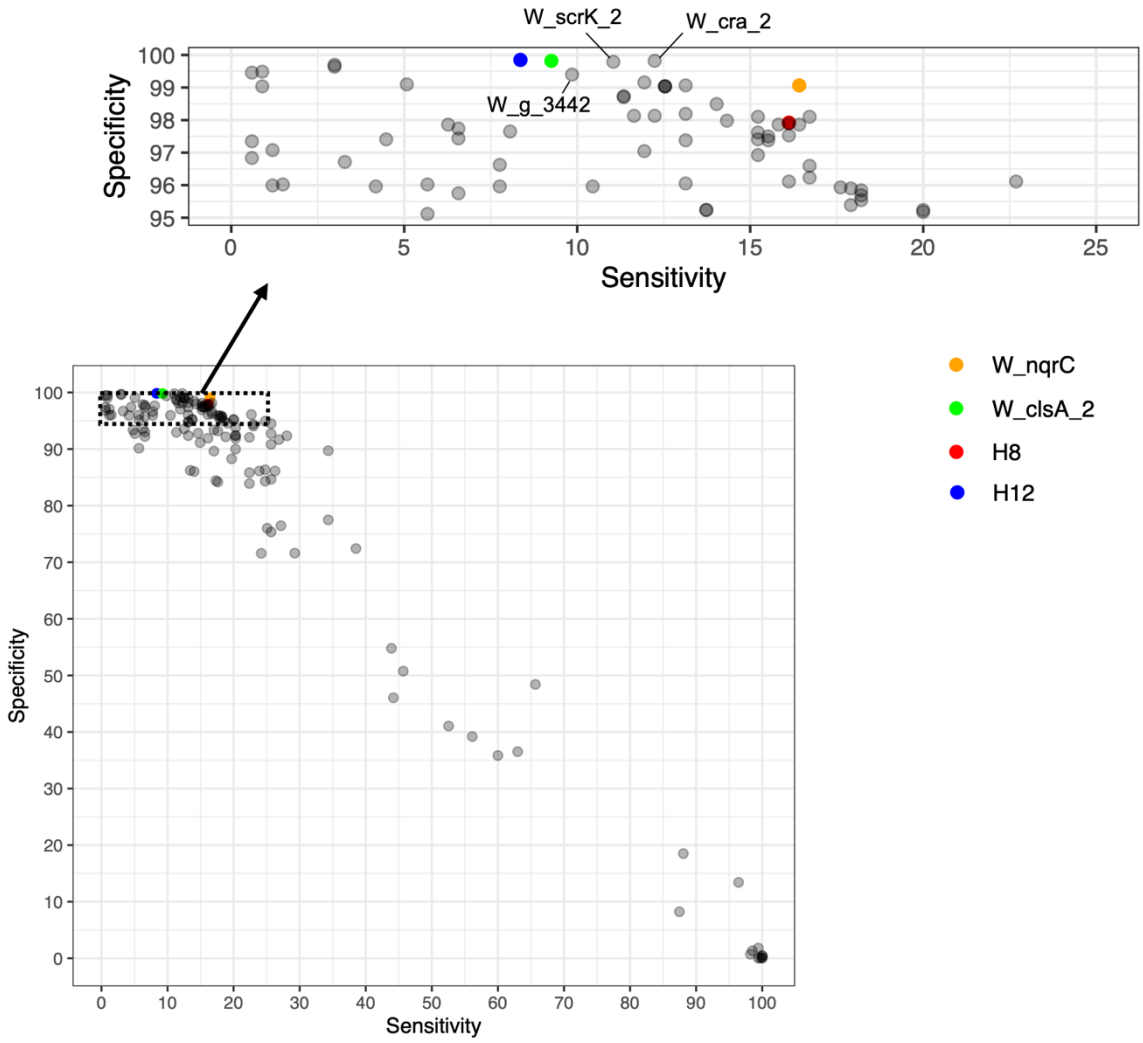
284 **3.2. Identification of candidate wastewater-specific marker genes.**

285 A pan-genome was constructed with Roary using 50 wastewater *E. coli* genomes sequenced in

286 this study and 82 RefSeq animal *E. coli* genomes. Roary identified a total of 25,343 genes,

287 including 2,925 core genes (genes shared among ≥ 99% of strains) and 22,418 accessory genes
288 (genes observed among < 99% of strains). The pan-GWAS approach was employed to identify
289 candidate wastewater-specific genes among the accessory genes. A total of 144 genes met the
290 criteria of Benjamini-Hochberg adjusted p-values of less than 0.05 and specificity higher than
291 95%, and thus were defined as candidate marker genes (see **Table S5** for details of the
292 identified genes). These 144 genes were subjected to sensitivity and specificity analysis by
293 blastn using a larger number of RefSeq *E. coli* genomes.
294
295 **3.3. Sensitivity and specificity of candidate marker genes determined by blastn analysis.**
296 The sensitivity and specificity of 144 candidate marker genes were assessed by screening these
297 genes in 335 RefSeq wastewater *E. coli* genomes and 3,318 RefSeq animal *E. coli* genomes.
298 **Figure 2** shows the distribution of sensitivity and specificity of each candidate marker gene.
299 Most candidate markers achieved high specificity, which is congruent with the fact that we
300 used a threshold of 95% specificity for identifying candidate markers in the Scoary analysis.
301 However, there were no genes that simultaneously showed high sensitivity and high specificity,
302 which may be partially due to the presence of *E. coli* isolates that can colonize multiple host
303 species (Johnson and Clabots, 2006; Stenske et al., 2009). In fact, among the genes showing
304 >70% specificity, no genes showed >40% sensitivity. It should be noted that there were genes
305 with almost 100% sensitivity and 0% specificity (i.e., detected in almost all the tested isolates)

306 even though we selected genes with specificity higher than 95% in the Scoary analysis. This
307 can be because we used blastp with a minimum percent identity of 95% for clustering
308 sequences in the Roary analysis but we used blastn with a minimum percent identify of 80%
309 for gene screening. In other words, genes assigned to different clusters by Roary can share
310 >80% nucleotide sequence identity and thus can be detected in the same blastn analysis.
311 Because the species composition of the hosts of RefSeq animal *E. coli* genomes was biased
312 (some host species were more prevalent than others), the specificity of candidate markers was
313 separately calculated for different host groups, namely, "Cattle", "Swine", "Chicken", "Wild
314 boar (*Sus scrofa*)", "Canine", "Sheep and goat", and "Others" (**Figure S2**). We also considered
315 the specificity calculated for these host groups when selecting MST markers from the
316 candidates.
317




318

319 **Figure 2**. Sensitivity and specificity of candidate wastewater-specific marker genes as

320 determined by blastn analysis. The distribution of sensitivity and specificity of 144 candidate

321 marker genes and previously identified marker genes, H8 and H12, are plotted (H8 in fact

322 corresponded to one of the 144 candidate marker genes). The positions of markers mentioned

323 in the main text (W_scrK_2, W_cra_2, and W_g_3442) are also indicated.

324

325 **3.4. Selection of W_nqrC and W_clsA_2.**

326 As noted in the Materials and Methods section, selecting a marker with high specificity is
327 required to reduce the number of false positives, especially when the marker is applied to DNA
328 directly extracted from environmental samples. We therefore selected W_nqrC from the 144
329 candidates, as this marker showed high specificity (99.1%) and relatively high sensitivity
330 (16.4%) (**Figure 2**). Moreover, W_nqrC showed the highest sensitivity among the candidate
331 markers which showed >95% specificity for all host groups (**Figure S2**). We then tried to select
332 another marker that was prevalent among the RefSeq wastewater *E. coli* genomes without
333 W_nqrC. This was to increase the sensitivity when both markers are used in combination.
334 Among the candidate markers showing >99% specificity, W_cra_2, W_scrK_2, W_clsA_2,
335 and W_g_3442 showed different marker distribution patterns (the red bars in **Figure S3**) from
336 W_nqrC and also showed relatively high sensitivities (**Figure S3**). Although W_cra_2 and
337 W_scrK_2 showed high specificity (>99.7%) and relatively high sensitivity (12.2% and 11.0%,
338 respectively), similar sequences with 60%–75% nucleotide sequence identity were prevalent
339 among animal *E. coli* isolates. We therefore selected W_clsA_2 as the second marker because
340 there were no similar sequences prevalent among animal *E. coli*, and this marker showed higher
341 specificity than W_g_3442 (99.8% vs. 99.4%). Moreover, the specificity of W_clsA_2 was
342 100% for all host groups except "Others" (only six *E. coli* genomes from silver gulls were
343 positive for W_clsA_2) (**Figure S2)**. The sequences of W_nqrC and W_clsA_2 are shown in
344 **Figure S4**.

345 Prokka annotation indicated W_nqrC encodes Na(+)-translocating NADH-quinone reductase
346 subunit C, and W_clsA_2 encodes major cardiolipin synthase ClsA. The BLASTN searches
347 against the nucleotide collection (nr/nt) database revealed that W_nqrC is mostly present on
348 plasmids, while W_clsA_2 is equally present on chromosomes and plasmids. Among the
349 RefSeq wastewater *E. coli* genomes positive for W_nqrC (n = 55), almost half of the genomes
350 (n = 27) were assigned to ST131, which was previously reported to be present among
351 wastewater *E. coli* (Dolejska et al., 2011; Gomi et al., 2017a; Sghaier et al., 2019) and also is
352 known for its association with extraintestinal infections (Nicolas-Chanoine et al., 2014; Riley,
353 2014; Stoesser et al., 2016). The remaining W_nqrC-positive genomes were assigned to various
354 STs, including ST69 (n = 4), ST95 (n = 4), and ST10 (n = 3). The W_nqrC-positive wastewater
355 *E. coli* genomes sequenced in this study (n = 9) belonged to diverse STs, including ST73 (n =
356 2), ST95 (n = 2), and ST357 (n = 2) (**Table S6**). Among the RefSeq wastewater *E. coli* genomes
357 positive for W_clsA_2 (n = 31), more than half of the genomes (n = 17) were assigned to ST635,
358 which was previously reported to be prevalent among wastewater *E. coli* isolates in Canada
359 and hospital sink *E. coli* isolates in the UK (Constantinides et al., 2020; Zhi et al., 2019). The
360 remaining W_clsA_2-positive genomes belonged to various STs, including ST399 (n = 3),
361 ST401 (n = 2), ST607 (n = 2), and ST3168 (n = 2). The W_clsA_2-positive wastewater *E. coli*
362 genomes sequenced in this study (n = 5) belonged to five different STs (ST399, ST472, ST607,
363 ST635, and ST5295) (**Table S7**).

364 We note that W_nqrC/W_clsA_2-positive RefSeq wastewater *E. coli* genomes originated from
365 various countries, including the Czech Republic, Germany, Canada, the USA, and South Africa
366 (**Table S6**, **Table S7**). In fact, 43 (78%) of W_nqrC-positive RefSeq wastewater *E. coli*
367 genomes (n = 55) and 31 (100%) of W_clsA_2-positive RefSeq wastewater *E. coli* genomes
368 (n = 31) originated from countries other than Japan, which indicates that these markers could
369 be used globally.
370
371 **3.5. Sensitivity and specificity of H8 and H12.**
372 We also assessed the sensitivity and specificity of the two previously developed markers, H8
373 and H12, which were calculated based on the results of blastn analysis (**Figure 2**). Notably, H8
374 corresponded to one of the 144 candidate marker genes identified by Scoary. **Figure 2** shows
375 that both H8 and H12 achieved high specificity (97.9% and 99.8%, respectively), which is
376 consistent with previous studies (Senkbeil et al., 2019; Warish et al., 2015). However, the
377 markers proposed in the present study showed better performances. W_nqrC showed higher
378 specificity and sensitivity than H8 (99.1% vs. 97.9% and 16.4% vs. 16.1%, respectively), and
379 W_clsA_2 showed almost the same specificity (~99.8%) but higher sensitivity than H12 (9.3%
380 vs. 8.4%). Moreover, when used in combination, a combination of W_nqrC and W_clsA_2
381 showed higher specificity and sensitivity than a combination of H8 and H12 (98.9% vs. 97.8%
382 and 25.7% vs. 24.2%, respectively). These results suggest that although H8 and H12 could be

383 useful for tracking wastewater contamination, W_nqrC and W_clsA_2 may yield better
384 performances.
385
386 **3.6. Development of PCR assays targeting W_nqrC and W_clsA_2.**
387 We designed PCR primers for detection of W_nqrC and W_clsA_2. PCR assays on positive
388 controls yielded the expected DNA bands for both W_nqrC (105 bp) and W_clsA_2 (165 bp),
389 whereas no bands were observed for the negative controls (**Figure S5**). The developed PCR
390 assays were applied to detect the MST markers in *E. coli* isolates obtained from wastewater
391 and animal feces (deer and cattle feces) (**Table 2**). W_nqrC or W_clsA_2 was detected in 21
392 (26.9%) and 9 (18.4%) of wastewater influent and effluent isolates, respectively (**Table 2**).
393 Importantly, there was no overlap between the isolates positive for W_nqrC and W_clsA_2,
394 which suggests that the use of W_nqrC and W_clsA_2 in combination could increase the
395 sensitivity. On the other hand, no isolates from animal feces were positive for W_nqrC or
396 W_clsA_2. We also performed PCR to detect H8 and H12 using the same set of *E. coli* isolates.
397 H8 or H12 was detected in 11 (14.1%) and 7 (14.3%) of wastewater influent and effluent
398 isolates, respectively (**Table 2**). This indicates that a combination of W_nqrC and W_clsA_2
399 is more sensitive for detecting wastewater *E. coli* than a combination of H8 and H12 and
400 supports the findings of the above blastn analysis. As with W_nqrC and W_clsA_2, no isolates
401 from animal feces were positive for H8 or H12.

402 We also performed *in silico* PCR analysis using RefSeq *E. coli* genomes that were not used for

403 blastn analysis. Among the 91 wastewater *E. coli* genomes, 29 genomes (31.9%) were positive

404 for W_nqrC or W_clsA_2 (**Table 2**, **Table S8**). On the other hand, only 16 genomes (1.9%)

405 among the 824 animal *E. coli* genomes were positive for W_nqrC or W_clsA_2 (**Table 2**,

406 **Table S9**). These results highlight the high specificity of the developed MST markers. *In silico*

407 PCR analysis of H8 and H12 revealed 25 wastewater *E. coli* genomes (27.5%) to be positive

408 for H8 or H12, while 27 animal *E. coli* genomes (3.3%) were also positive for H8 or H12

409 (**Table 2**, **Table S8**, **Table S9**). This again highlights that a combination of W_nqrC and

410 W_clsA_2 can yield better performance than a combination of H8 and H12.

411

412 **Table 2**. Prevalence of W_nqrC, W_clsA_2, H8, and H12 in *E. coli* determined by (*in silico*)

413 PCR.

| Source | No. of *E. coli* isolates/ genomes tested | No. of *E. coli* isolates/ genomes positive for W_nqrC (%) | No. of *E. coli* isolates/ genomes positive for W_clsA _2 (%) | No. of *E. coli* isolates/ genomes positive for H8 (%) | No. of *E. coli* isolates/ genomes positive for H12 (%) | No. of *E. coli* isolates/ genomes positive for W_nqrC or W_clsA _2 (%) | No. of *E. coli* isolates/ genomes positive for H8 or H12 (%) |
|---|---|---|---|---|---|---|---|
| **PCR** | | | | | | | |
| Wastewater influent | 78 | 15 (19) | 6 (8) | 8 (10) | 5 (6) | 21 (27) | 11 (14)[a] |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Wastewater effluent | 49 | 5 (10) | 4 (8) | 6 (12) | 1 (2) | 9 (18) | 7 (14) |
| Deer | 30 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Cattle | 30 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| River water | 80 | 8 (10) | 6 (8) | -[b] | -[b] | 14 (18) | -[b] |
| ***In silico* PCR** | | | | | | | |
| Wastewater[c] | 91 | 26 (29) | 3 (3) | 5 (5) | 20 (22) | 29 (32) | 25 (27) |
| Pig (swine, *Sus scrofa domestica*) | 237 | 3 (1) | 0 (0) | 11 (5) | 0 (0) | 3 (1) | 11 (5) |
| Bird (other than chicken) | 199 | 4 (2) | 0 (0) | 3 (2) | 0 (0) | 4 (2) | 3 (2) |
| Cattle (bovine, cow, *Bos taurus*) | 178 | 6 (3) | 1 (1) | 7 (4) | 0 (0) | 7 (4) | 7 (4) |
| Chicken (*Gallus gallus*) | 100 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Goat/sheep | 19 | 0 (0) | 0 (0) | 2 (11) | 0 (0) | 0 (0) | 2 (11) |
| Dog (*Canis lupus familiaris*) | 12 | 2 (17) | 0 (0) | 0 (0) | 2 (17) | 2 (17) | 2 (17) |
| Red deer | 12 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Wild boar | 11 | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| (*Sus scrofa*) | | | | | | | |
| Other animals[d] | 56 | 0 (0) | 0 (0) | 2 (4) | 0 (0) | 0 (0) | 2 (4) |
| Environmental waters | 303 | 21 (7) | 1 (0.3) | 12 (4) | 8 (3) | 22 (7) | 20 (7) |

414 [a]Two isolates carried both H8 and H12.

415 [b]We could not determine the prevalence of H8 and H12 due to the loss of glycerol stocks.

416 [c]Influent and effluent were not distinguished for many of the RefSeq wastewater *E. coli*

417 genomes.

418 [d]Animal hosts with fewer than ten *E. coli* genomes were combined into this category.

419

420

421 **3.7. Prevalence of MST markers in *E. coli* isolates from environmental waters.**

422 We performed PCR to determine the prevalence of W_nqrC and W_clsA_2 in *E. coli* isolates

423 in river water. Of the 80 isolates tested, eight (10.0%) and six (7.5%) were positive for W_nqrC

424 and W_clsA_2, respectively (**Table 2**). The prevalence of W_nqrC and W_clsA_2 was lower

425 than but almost the same as that in *E. coli* isolates obtained from wastewater effluents (10.2%

426 and 8.2%), which is congruent with the fact that the sampling points were strongly affected by

427 wastewater.

428 We also performed *in silico* PCR on RefSeq environmental *E. coli* genomes. Of the 303

429 genomes, 21 genomes (6.9%), one genome (0.3%), 12 genomes (4.0%), and eight genomes

430 (2.6%) were positive for W_nqrC, W_clsA_2, H8, and H12, respectively (**Table 2**, **Table S10**).

431 The prevalence of MST markers was lower than in the RefSeq wastewater *E. coli* genomes:

432 this is consistent with the fact that not all environmental *E. coli* originate from wastewater.

433 We note that the two markers developed in the present study, especially W_nqrC, are

434 commonly found on plasmids and could be subjected to horizontal gene transfer (HGT) in the

435 environment. Previous studies suggested that HGT could occur, for example, in biofilms,

436 although HGT by conjugation rarely occurs between motile planktonic cells (Abe et al., 2020).

437 Though we assume the contribution of HGT to the spread of the developed markers to be low

438 in environmental waters, where water is constantly flowing, this should be kept in mind.

439

440 **3.8. Study limitations.**

441 This study has some limitations. First, when used in combination, the developed markers

442 showed quite high specificity but showed relatively low sensitivity, indicating that a relatively

443 large number of *E. coli* isolates would be needed in MST for each environmental sample. This

444 was due to the tradeoff between the sensitivity and specificity of genetic markers (**Figure 2**).

445 We assume this limitation to stem from using *E. coli* as an MST target organism rather than

446 the methodologies employed in our study. Actually, not a few *E. coli* strains appear to be able

447 to colonize multiple host species (Gomi et al., 2014; Tenaillon et al., 2010), which might have

448 hampered the development of high-sensitivity and high-specificity MST markers. However,

449 we note that the sensitivity calculated in this study is at the isolate level, and sample-level
450 sensitivity (i.e., sensitivity calculated for a wastewater sample, which can contain multiple *E.*
451 *coli* strains) should be higher (Senkbeil et al., 2019).
452 Second, the developed markers were tested at the isolate level (i.e., in a culture-dependent
453 manner) but not tested for DNA directly extracted from samples (i.e., in a culture-independent
454 manner). It should be noted that *in silico* PCR analysis using the nr database on the Primer-
455 BLAST website, which also includes the genomes of non-*E. coli* species, returned PCR
456 products from non-*E. coli* species for both W_nqrC (e.g., *Klebsiella pneumoniae* and
457 *Salmonella enterica*) and W_clsA_2 (e.g., *Pseudomonas aeruginosa* and *K. pneumoniae*). In
458 the case of W_clsA_2, only 29 (18%) of 157 products were from *E. coli*. However, in the case
459 of W_nqrC, 517 (93%) of 556 products were from *E. coli*, and only 39 (7%) were from other
460 species. Moreover, the hosts of those 39 genomes from other species were found to be mainly
461 from humans (*Homo sapiens*), while only two were from animals. (Note that the hosts of some
462 genomes could not be identified due to the lack of metadata information in the GenBank
463 database.) Interestingly, three of the 39 products were from plasmids of uncultured bacteria
464 (accession numbers: AJ851089.1, JX127248.1, and MF554641.1), all of which were from
465 WWTPs. These results indicate that W_nqrC could be used in a culture-independent method
466 (note that this does not preclude the applicability of W_clsA_2 to a culture-independent
467 method). We also observed H8 to be prevalent among non-*E. coli* bacteria (Gomi et al., 2014),

468 but it was proven in a subsequent study to be applicable in a culture-independent manner
469 (Senkbeil et al., 2019). These indicate that further studies are needed to test the sensitivity and
470 specificity of W_nqrC and W_clsA_2 using DNA directly extracted from wastewater and feces
471 from animal hosts to verify their applicability to culture-independent methods. Future studies
472 should also assess the applicability of these markers to DNA directly extracted from
473 environmental samples. Nonetheless, we believe the two markers to be useful, since culture-
474 dependent methods are used to detect *E. coli* in routine water quality assessments, and there is
475 a need to identify the sources of the detected *E. coli* colonies. Moreover, cell suspensions
476 prepared from *E. coli* collected in routine water quality assessments can be used directly for
477 PCR reactions without DNA extraction. We also found it possible to use colored *E. coli*
478 colonies on selective media, in our case XM-G agar, for PCR reactions.
479
480
481 **4. Conclusions**
482 In the present study we identified two *E. coli* MST markers, namely W_nqrC and W_clsA_2,
483 which can be used for tracking wastewater contamination in environmental waters. These two
484 markers showed better performance than the previously developed H8 and H12 markers at the
485 isolate level. Although further studies are needed to test the applicability of the developed
486 markers in a culture-independent manner, this study showed that the developed MST markers

could be useful for identifying the sources of *E. coli* (whether they originated from domestic wastewater or not) at least at the isolate level. We believe that the PCR assays developed in this study can be readily applied to identify the sources of *E. coli* isolates collected during routine water quality assessments.

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**Supporting information**

Supplementary information associated with this article can be found, in the online version, at _____.

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