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Screening antibiotic-resistant *Escherichia coli* in wastewater and river water using a novel simple phenotypic antibiotic-susceptibility testing method

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ABSTRACT

Recently, wastewater treatment plants have been identified as potential reservoirs of antibiotic-resistant bacteria. Hence, it is important to monitor antibiotic-resistant bacteria in wastewater treatment plants. Currently available methods are laborious, time-consuming, and costly. In this study, a novel simple phenotypic antibiotic-susceptibility testing method for *Escherichia coli* was developed. A growth curve for *E. coli* in the presence of an antibiotic was established by monitoring the fluorescence intensity of a fluorogenic substrate specific to *E. coli* using a microplate reader. The antibiotic-resistant *E. coli* to total *E. coli* ratios for wastewater and river water samples were determined using fluorescence intensity measurements. The lethal concentrations of antibiotics to *E. coli* could be roughly estimated using the method. The lethal concentrations were dependent on the antibiotic type rather than differences between the wastewater treatment plants and were higher for samples from primary clarifiers than secondary clarifiers in wastewater treatment plants. The lethal concentrations for river water decreased as the distance from the wastewater treatment plant discharge outlet increased. The novel simple phenotypic antibiotic-susceptibility testing method allows antibiotic-resistant *E. coli* concentrations in wastewater and river water to be determined rapidly with a high throughput and will allow effective and timely decisions to be made to control antibiotic-resistant *E. coli*.

Keywords

β-D-Glucuronidase; Microplate reader; Culture-based method; Logarithmic (log) growth phase; Lethal concentration; Fate of antibiotic-resistant *Escherichia coli*.

Synopsis

48 Antibiotic-resistant *Escherichia coli* in sewage wastewater and river water were quantified

49 using a fluorogenic substrate.

50

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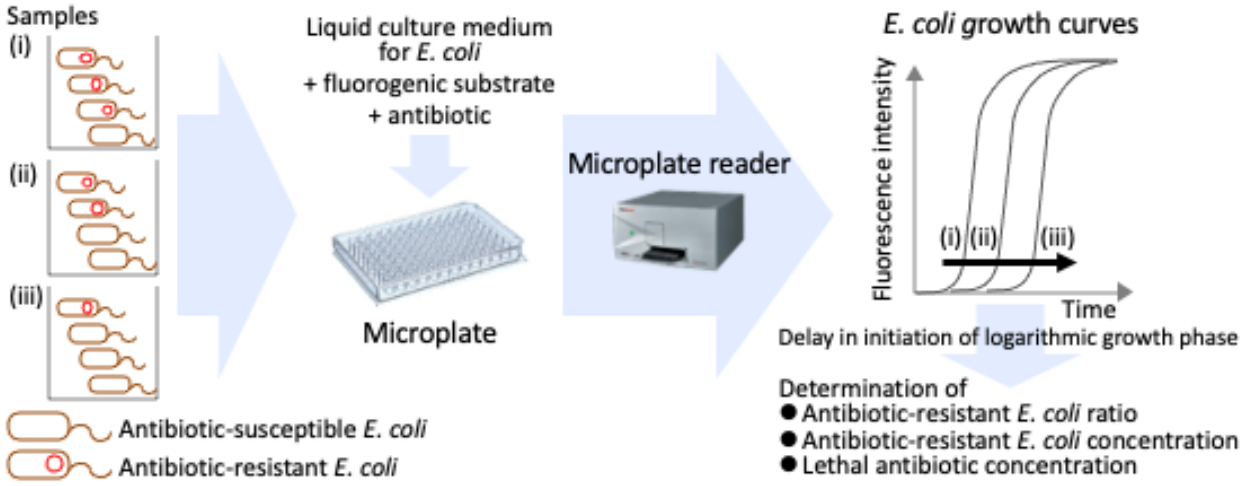

1. Introduction

Indiscriminate and inappropriate antibiotic use to combat bacterial infections has promoted the incidence, dissemination, and accumulation of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) in hospitals.[1] Antibiotic resistance (AR) is increasingly recognized as an important threat to human health around the world.[2] AR is not restricted to bacteria in hospitals but also occurs in aquatic environments around the world.[3] Amarasiri et al. recently reviewed the health risks posed by ARB and ARGs in aquatic environments to humans.[3] Wastewater (WW) treatment plants (TPs) are hotspots for the development and spread of AR because WW is a nutrient-rich environment at the optimal temperature for microbial growth and has high microbe concentrations, meaning horizontal transfer will be promoted because the conditions are optimal for genes to be transferred between bacteria. WW treatment processes cannot completely remove ARB and ARGs, so WWTP effluents containing ARB and ARGs are released into aquatic environments. Release of ARB in clinical and communal WWTP effluents into water bodies has been reviewed and investigated in several studies.[4–6] Water bodies receiving WWTP effluent, and particularly river and lake sediment, are hotspots for the introduction and spread of AR in the environment because of pollution with ARB, ARGs, and antibiotics caused by human activities and high bacterial densities and activities that promote horizontal gene transfer.[3] Water bodies downstream of WWTPs are often used for recreation and as sources of water for irrigating agricultural land and producing drinking water. Microbial risk assessment models have predicted that antibiotics, pathogens, ARB, and ARGs could pose health risks to exposed humans.[7]

80 Various methods, including culture-based and molecular-based methods, have been used to
81 investigate the presence, transportation, and fates of ARB in aquatic environments.[8] ARB
82 concentrations in WW and river water (RW) samples have been determined using methods
83 involving membrane filtration, plate counting, and quantitative polymerase chain reaction
84 (PCR) analysis.[6,8–10] However, culture-dependent methods involve laborious preparation
85 procedures, long incubation periods (≥18 h) for growing *Escherichia coli*, and large volumes
86 of culture media, meaning such methods are expensive. Molecular-based methods, such as
87 quantitative PCR methods, have high specificities, are rapid (<24 h), and have low
88 measurement errors. However, molecular-based methods alone cannot be used to identify
89 ARB with unknown ARGs and are limited to already identified genes. Determining a single
90 ARG may give limited information about ARB resistant to a specific antibiotic in a sample.
91 Analyzing only ARGs may mean that ARB that do not possess ARGs are overlooked.
92 Currently available methods are therefore incapable of determining ARB concentrations in
93 WW and RW simply and quickly. This makes it difficult to make effective and timely
94 decisions aimed at controlling ARB.
95
96 The need for a simple, rapid, low-cost, and high-throughput assay for screening ARB
97 in the aquatic environment led us to develop a novel simple phenotypic antibiotic
98 susceptibility testing (AST) method for *E. coli* inspired by real-time PCR analysis. Therefore,
99 we developed a simple AST method as a rapid and high-throughput assay for determining AR
100 *E. coli* (AREc) in WW and RW in this study. First, the validation of the method has been
101 performed for ciprofloxacin and tetracycline by comparing the results with the results of three
102 conventional AST methods. Then the AREc concentrations, the AREc to total *E. coli*
103 concentration ratios, and the lethal concentrations (LCs) of eight antibiotics to *E. coli* in WW
104 and RW were determined. The LCs of the antibiotics to *E. coli* from the secondary clarifiers

105 of three WWTPs in Sapporo City were determined and the fate of AREc discharged from a
106 WWTP into a river was investigated. Finally, raw and treated WW samples from five
107 WWTPs around Japan were analyzed to confirm the versatility of the new method.
108
109 **2. Materials and Methods**
110 *2.1. Study site and sampling*
111 We collected 116 WW and RW samples in 65 sampling campaigns performed between
112 January 2018 and January 2020. Most of the samples were collected in northern Sapporo in
113 Hokkaido Prefecture, Japan, but some were collected outside Hokkaido Prefecture. The main
114 sampling area is shown in Figure 1.
115
116 Sapporo is a city on the alluvial fan of the Toyohira River. The population and main
117 industries of Sapporo were described in a previous publication.[11] Veterinary activities occur
118 around points 3 and 4 in Figure 1 but not upstream of point 2. WW samples were collected
119 from the secondary clarifiers of three WWTPs (WWTP-A, WWTP-B, and WWTP-C) in
120 Sapporo City. The sizes, physicochemical characteristics, and operating conditions of
121 WWTP-A, WWTP-B, and WWTP-C were described in previous publications.[12,13] WWTP-A
122 is connected to a fully combined sewer. During heavy rain events, some of the primary
123 clarifier effluent from WWTP-A is disinfected by chlorination and discharged into the Sosei
124 River from the WWTP-A discharge outlet. RW samples were collected from ~100 m
125 downstream of the WWTP-A discharge outlet (point 1), ~5 km downstream of the WWTP-A
126 discharge outlet (point 2), and at two sampling sites (points 3 and 4) where no treated or
127 untreated municipal WW is discharged. All three WWTPs discharged effluent after secondary
128 treatment without disinfection during the sampling period. Each RW sample was collected in
129 a sterile 1 L polypropylene container. WW samples (40 mL each) from the primary and

secondary clarifiers of the three WWTPs in Sapporo City were collected in sterile 50 mL Falcon tubes. Each sample was processed within 60 min of collection.

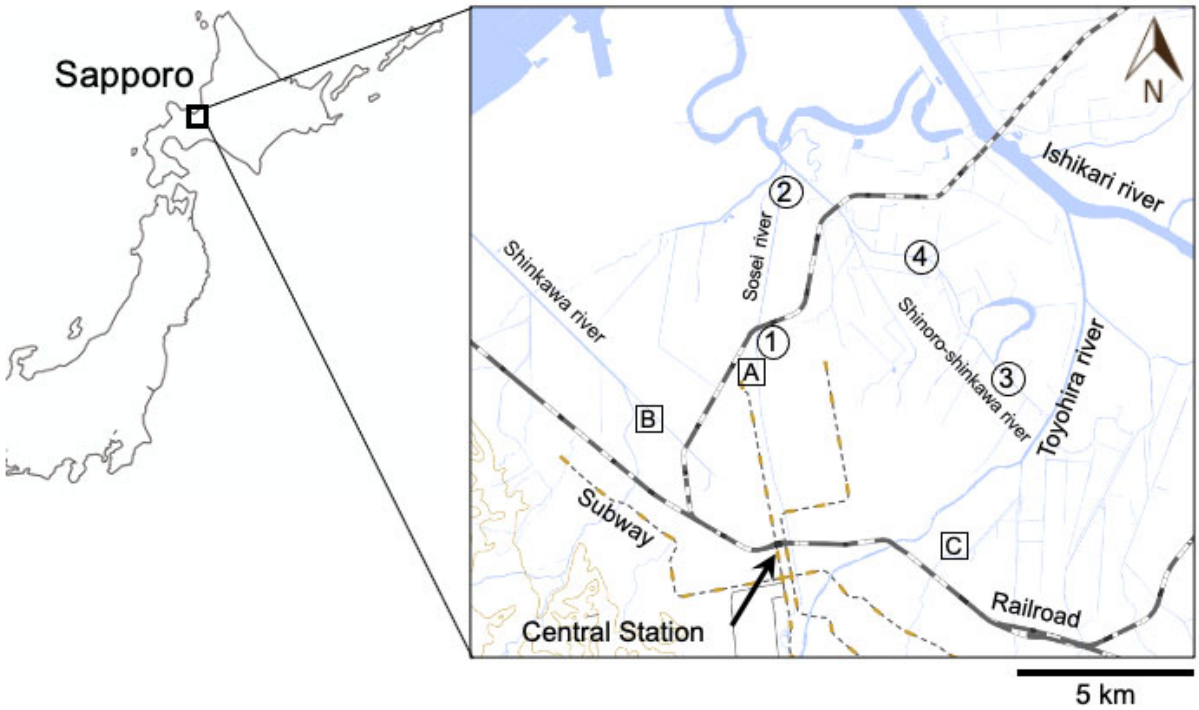

Figure 1. Map of the study area. The squares and circles indicate wastewater treatment plants (WWTPs) and sampling points, respectively. A = WWTP-A, B = WWTP-B, C = WWTP-C. Wastewater samples were collected from WWTP-A, WWTP-B, and WWTP-C. River water samples were collected from ~100 m downstream of the WWTP-A discharge outlet (1), ~5 km downstream of the WWTP-A discharge outlet (2), and at two sampling sites (3 and 4) unaffected by discharges of treated or untreated municipal wastewater.

In addition, 40 mL WW samples were collected in sterile 50-mL Falcon tubes from the primary and secondary clarifiers of five WWTPs (labeled WWTP-O, WWTP-H, WWTP-N, WWTP-K, and WWTP-KK) outside Hokkaido Prefecture, Japan. The samples were kept at 4 °C and were transferred to the laboratory in Sapporo City within 3 d of being collected.

145 The samples were not fed or aerated. The samples were analyzed as soon as they arrived in
146 the laboratory. Changes in the structures and functions of the microbial communities in the
147 samples during transportation were not assessed.
148
149 *2.2. Principles involved in the novel simple phenotypic AST method for detecting AREc*
150 The new method was inspired by the principles involved in real-time PCR analysis.
151 Amplification of the target DNA molecule during the PCR process is monitored using a
152 fluorescent molecule in real time, not only at the end of the PCR process. The target DNA
153 molecule, fluorescent dye, DNA polymerase, and other chemicals required for the PCR
154 process are mixed and subjected to the PCR process in a thermal cycler with a fluorescence
155 detector to amplify the target DNA molecules. The PCR process involves repeated cycles of a
156 set of (normally) three-step temperature changes. The number of DNA molecules
157 (corresponding to the fluorescence intensity for the mixture) will theoretically have doubled
158 at the end of each cycle. The fluorescence intensity will increase exponentially because 2^n
159 times the initial number of copies of the DNA fragment will theoretically be present after n
160 cycles. A fluorescence intensity threshold reliably higher than the background fluorescence
161 intensity is set for detecting the target DNA molecules. The number of cycles required for the
162 fluorescence intensity to exceed the threshold is called the threshold cycle. A standard curve
163 is established using the threshold cycle for samples containing known quantities of the target
164 DNA molecules each diluted by a factor of 10, then target DNA molecules in unknown
165 samples are quantified by comparing the threshold cycles for the samples to the standard
166 curve.
167
168 If the medium and conditions are appropriate, *E. coli* cells will multiply exponentially,
169 like DNA molecules during the PCR process. The concentration of *E. coli* cells can be

170 monitored by measuring the β-D-glucuronidase activity in real time using a fluorogenic
171 substrate (e.g., 4-methylumbelliferyl-β-D-glucuronide, MUG) using a microplate reader.[12] We
172 hypothesized that the *E. coli* concentrations in unknown samples could be quantified by
173 incubating samples containing known and unknown *E. coli* concentrations on a microplate
174 reader, monitoring the fluorescence intensities of the samples, and defining a fluorescence
175 intensity threshold from blank samples. The incubation time at which the fluorescence
176 intensity exceeds the threshold will be related to the initial *E. coli* concentration.
177
178 *2.2. Quantification of* E. coli *and AREc*
179 Conventional methods for counting *E. coli* in WW and RW were performed using a
180 Colilert and Quanti-Tray/2,000 system (IDEXX Laboratories, Westbrook, ME, USA) and
181 Chromocult Coliform Agar ES (enhanced selectivity) medium (Merck, Darmstadt, Germany)
182 following the instructions provided by the manufacturers. In the colony counting method, 0.1
183 mL of a sample was added to an agar medium plate. If the *E. coli* concentration in a sample
184 was >1000 cfu mL^{-1}, the sample was diluted by a factor of 10 with sterile physiological NaCl
185 solution (0.9% NaCl) in a 100-mL sterile bottle before analysis. If the *E. coli* concentration in
186 a sample was <300 cfu mL^{-1}, the *E. coli* concentration was determined using the membrane
187 filtration technique and the colony counting method. The required volume of a sample was
188 passed through a sterile mixed cellulose ester filter with 0.45-μm pores (ADVANTEC,
189 Tokyo, Japan). The filter was then placed on a Chromocult Coliform Agar ES medium plate.
190 Each sample was tested in triplicate.
191
192 The AREc concentrations in the WW and RW samples were determined using four
193 methods, a colony counting method using Chromocult Coliform Agar ES, the Colilert
194 method, the ETEST method, and the new method described here. Tests were performed using

195 eight antibiotics, which were all purchased from Merck. The antibiotics were ampicillin
196 (AMP), ciprofloxacin (CIP, as ciprofloxacin hydrochloride monohydrate), clindamycin (CLI,
197 as clindamycin hydrochloride), erythromycin (ERY), gentamicin (GEN, as gentamicin sulfate
198 salt), methicillin (MET, as methicillin sodium), tetracycline (TET, as tetracycline
199 hydrochloride), and vancomycin (VAN, as vancomycin hydrochloride). In the colony
200 counting method, an aliquot of a sample was added to each of a series of agar medium plates
201 that each contained an antibiotic. Tests were performed at four concentrations (10, 1.0, 0.1,
202 and 0.01 μg mL^{-1}) of each antibiotic of interest. Tests were also performed using agar
203 medium plates without any antibiotics added (the controls). Each test was performed in
204 triplicate. The plates were incubated at 37 °C for 24 h. Violet colonies were counted as *E.*
205 *coli*. *E. coli* that formed colonies on the agar medium at the selected antibiotic concentration
206 were defined as AREc. The AREc to *E. coli* concentration ratio was calculated by dividing
207 the concentration of *E. coli* growing on the medium containing an antibiotic by the
208 concentration of *E. coli* growing on the medium that did not contain the antibiotic. In the
209 Colilert method, the AREc ratio was calculated using a method similar to that used for the
210 colony counting method described above. The AREc and *E. coli* concentrations were
211 determined using a Colilert and Quanti-Tray/2000 system (IDEXX Laboratories) using media
212 containing and not containing an antibiotic. The AREc to *E. coli* concentration ratio was
213 calculated by dividing the *E. coli* concentration in the medium containing an antibiotic by the
214 *E. coli* concentration in the medium not containing the antibiotic. In the new method, the
215 AREc and *E. coli* concentrations were determined using media containing and not containing
216 an antibiotic and, as described above, the AREc to *E. coli* concentration ratio was calculated
217 by dividing the *E. coli* concentration in the medium containing an antibiotic by the *E. coli*
218 concentration in the medium not containing the antibiotic. For the ETEST method
219 (bioMérieux Japan, Tokyo, Japan), 30 *E. coli* colonies on a Chromocult Coliform Agar ES

220 medium sample were selected at random and isolated. The minimum inhibitory concentration
221 of the antibiotic of interest for each isolated colony was then determined using an ETEST
222 strip and Chromocult Coliform Agar ES medium, following the instructions provided by the
223 manufacturer.
224
225 The lethal concentration (LC) of an antibiotic to *E. coli* was estimated from the
226 relationship between the antibiotic concentration and AREc ratio (see Figure 2) by
227 performing probit analysis.[14] Probit analysis is a specialized form of regression analysis that
228 can be applied to binomial response variables. The procedure transforms a concentration–
229 response curve into a straight line that can then be analyzed by either least-squares or
230 maximum-likelihood regression analysis.
231
232 **3. Results and Discussion**
233 *3.1. Determining the AREc concentration using the new method*
234 Typical temporal changes in the 4-methylumbelliferone (MU) fluorescence intensity
235 when incubating a WW sample from the secondary clarifier in WWTP-A in the *E. coli*
236 medium using the new method are shown in Figure S1. The sample was incubated in 10 wells
237 on a microplate, and the MU fluorescence intensity for each cell was measured every 10 min.
238 The fluorescence intensity of the medium–WW mixture did not increase until after 6.0 h of
239 incubation, then the fluorescence intensity increased exponentially, doubling every 10 h. The
240 fluorescence intensity reached a plateau at ~8000, probably because MUG had been
241 completely degraded into MU. The fluorescence intensity profile indicated that the *E. coli*
242 growth curve in the batch culture consisted of a lag phase, a logarithmic growth phase, and a
243 stationary phase. This would have been because the MU fluorescence intensity was related to
244 MUG degradation by *E. coli*. The incubation time at which the fluorescence intensity

245 exceeded the threshold was defined as the logarithmic growth phase initiation time (Ti),
246 similar to the threshold cycle for real-time PCR (Figure S1). The threshold was set as the
247 mean plus 10 times the standard deviation of the fluorescence intensities of the blank samples
248 (n=10). An aliquot of WW was diluted by a factor of three and another aliquot of WW was
249 diluted by a factor of nine with 0.9% saline, then temporal changes in the MU fluorescence
250 intensities of the samples were monitored. The results are shown in Figure S2. The Ti values
251 were determined using the procedure described above from the fluorescence intensity
252 profiles, and the mean Ti was calculated. Diluting the WW decreased the initial *E. coli*
253 concentration and therefore delayed initiation of the logarithmic growth phase (i.e., the Ti
254 increased). A calibration curve (Ti plotted against the initial *E. coli* concentration) was
255 established from the results. The calibration curve is shown in Figure S3. If an antibiotic was
256 added to a WW sample and the fluorescence intensity was monitored, the Ti was delayed
257 compared with the Ti for the WW incubated without an antibiotic added, as shown in Figure
258 S4. This was because *E. coli* growth would have been inhibited by the antibiotic in the
259 medium. We hypothesized that *E. coli* susceptible to the antibiotic did not grow in the
260 presence of the antibiotic but that the AREc growth rate was unaffected by the antibiotic. This
261 allowed the concentration of *E. coli* resistant to the antibiotic at the concentration in the
262 medium (i.e., AREc) in the WW to be estimated using the calibration curve, as shown in
263 Figure S5. The AREc to total *E. coli* concentration ratio for a sample was calculated by
264 dividing the AREc concentration by the *E. coli* concentration in the sample without the
265 antibiotic added (i.e., the total *E. coli* concentration).
266
267 *3.2. Determining the AREc to* E. coli *concentration ratios using four methods*
268 The AREc to *E. coli* concentration ratios for the WW samples from the secondary
269 clarifier of WWTP-A at four concentrations (10, 1.0, 0.1, and 0.01 μg mL^{-1}) of the antibiotic

270 CIP determined using the four methods mentioned above (Chromocult Coliform Agar ES, the
271 Colilert method, the ETEST method, and the new method) are shown in Figure 2. The ratios
272 for *E. coli* resistant to CIP at a concentration of 10 μg mL^{-1} were <3% for all four AST
273 methods. For the range 10–0.01 μg mL^{-1}, the results of the new AST method were
274 comparable to those of the colony counting method, the mean AREc ratios being <7%
275 different. Lower AREc ratios were obtained using the Colilert method than the new method
276 and the colony counting method when the CIP concentrations were 0.1 and 0.01 μg mL^{-1}. A
277 higher AREc ratio was given by the ETEST method than by the other methods at a CIP
278 concentration of 0.01 μg mL^{-1}, probably because it was difficult to read the intersection of
279 the ellipse using the minimum inhibitory concentration scale on the strip. The standard
280 deviation of the AREc ratio at each CIP concentration was higher for the new method (<23%)
281 than the other methods (<3%), indicating that the new method was less accurate than the
282 other methods. This may have been because only 0.18 mL of sample was used in the new
283 method, whereas >10 mL of sample was used in each of the other methods. The new method
284 could therefore be used to semiquantitatively screen for AREc.
285

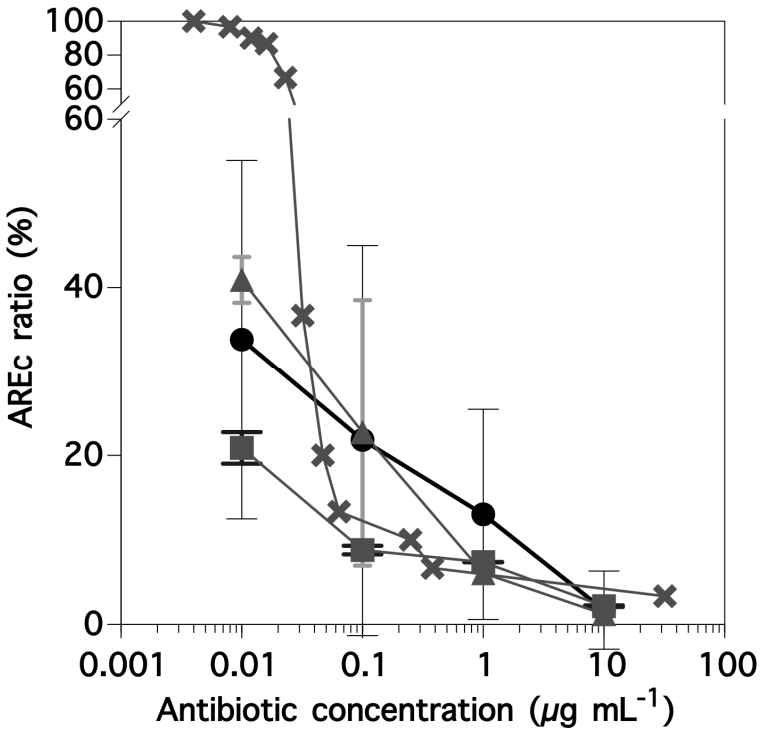

Figure 2. Ciprofloxacin-resistant *E. coli* (AREc) to total *E. coli* ratios plotted against the antibiotic concentration. The ratios were determined using the new method (●), colony counting method (▲), Colilert method (■), and ETEST method (×). The error bars indicate the standard deviations (n=10 for the new method and n=3 for the colony counting and Colilert methods).

The AREc ratios for the WW samples at four antibiotic (TET) concentrations (10, 1.0, 0.1, and 0.01 μg mL^{-1}) were also determined using the four methods (Figure S6). The AREc ratios for the new method and Colilert method were similar, but the AREc ratios were higher for the colony counting and ETEST methods than for the new method and Colilert method.

Furthermore, the ratios of AREc to other 6 antibiotics (AMP, CLI, ERY, GEN, MET, and VAN) were determined using the new method (Figure S7). Some antibiotics (AMP, ERY and GEN) inhibited >50% of *E. coli* at 0.01 μg mL^{-1} while >50% of *E. coli* survived in the presence of other ones (CLI, MET and VAN) at a concentration of 1.0 μg mL^{-1}. Since the

302 LCs of these 6 antibiotics for *E. coli* fluctuated greatly, we analyzed AREc ratios and LCs of
303 antibiotics using CIP and TET.
304
305 *3.3. Determining LCs of the antibiotics for* E. coli *using the new method*
306 We performed the AST method using CIP and TET again and determined the LCs of
307 antibiotics for *E. coli* in the WW samples from the secondary clarifier of WWTP-A. The
308 results are shown in Table 1. The LC90 (Table 1A) and LC80 (Table 1B) antibiotic
309 concentrations are the concentrations at which 10% and 20%, respectively, of the *E. coli*
310 survive (i.e., the AREc ratios are 10% and 20%, respectively). It can be seen from Table 1B
311 that 20% of the *E. coli* in the WW from the secondary clarifier in WWTP-A survived in the
312 presence of CIP at a concentration of ~0.1 μg mL^{-1} and of TET at a concentration of ~1.0 μg
313 mL^{-1}. The differences between the LC80s determined using the new method and the other
314 three methods were <4.4 μg mL^{-1} and were lower than the differences for the LC90s (>10 μg
315 mL^{-1}) (Table 1A) and LC50s (by one order of magnitude or more, data not shown). We
316 therefore concluded that the new method could be used as a simple AST method and that the
317 LC80 (i.e., the antibiotic concentration at which the AREc ratio is 20%) is a more reliable
318 index than other LCs for comparing AREc ratios for WWs.
319
320 **Table 1.** The (A) 90% lethal concentrations (LC90s) and (B) 80% lethal concentrations
321 (LC80s) of ciprofloxacin (CIP) and tetracycline (TET) for *E. coli* in the wastewater samples
322 from the secondary clarifier of WWTP-A. Two experiments were performed for each
323 antibiotic and the number in parenthesis indicates the number of the experiment. The LC90s
324 and LC80s for CIP (1) and TET (1) were calculated from the results shown in Figures 2 and
325 S6, respectively.
326 **Table 1A.** LC90s (μg mL^{-1}) of CIP and TET for *E. coli* in the wastewater samples

Method	CIP (1)	CIP (2)	TET (1)	TET (2)
New method	1.41	2.15	4.34	3.30
Colilert method	0.15	0.25	>10.0	>10.0

Colony counting method	0.42	N.D.	9.89	0.92
ETEST method	0.26	3.37	>10.0	>10.0

N.D.: not determined

Table 1B. LC80s (μg mL^{-1}) of CIP and TET for *E. coli* in the wastewater samples

Method	CIP (1)	CIP (2)	TET (1)	TET (2)
New method	0.15	0.14	1.61	0.37
Colilert method	0.01	0.01	1.21	0.84
Colony counting method	0.10	N.D.	6.00	0.64
ETEST method	0.05	0.10	2.43	1.56

N.D.: not determined.

3.4. Analysis of AREc in the WWTP samples

The LC of each antibiotic for *E. coli* in the secondary clarifiers of WWTP-A, WWTP-B, and WWTP-C (in Sapporo City) were determined using the new method, and the results are shown in Table 2. *E. coli* was more susceptible to ERY than the other antibiotics. Growth of 80% of *E. coli* in the samples from all three plants was inhibited at an ERY concentration of <0.1 μg mL^{-1}. Growth of 80% of *E. coli* in the samples from WWTP-A and WWTP-C (i.e., not in the sample from WWTP-B) was also inhibited at a TET concentration of <0.1 μg mL^{-1} and an AMP concentration of <1.0 μg mL^{-1}. Growth of *E. coli* was moderately (50%) inhibited by MET. Growth of 30% of *E. coli* was inhibited by CLI and VAN at concentrations of >1.0 μg mL^{-1} and >10 μg mL^{-1}, respectively. This indicated that the AREc ratios for CLI and VAN for WW from the secondary clarifiers were >70% even at CLI and VAN concentrations of >1.0 μg mL^{-1} and >10 μg mL^{-1}, respectively.

Table 2. Lethal concentrations (LCs) of six antibiotics for *E. coli* in wastewater samples from the secondary clarifiers of WWTP-A, WWTP-B, and WWTP-C in Sapporo City. The LCs were determined using results acquired using the new method.

WWTP	ERY LC80 (μg mL^{-1})	TET LC80 (μg mL^{-1})	AMP LC80 (μg mL^{-1})	MET LC50 (μg mL^{-1})	CLI LC30 (μg mL^{-1})	VAN LC30 (μg mL^{-1})
A	0.02	0.02	0.47	0.09	6.5	>10
B	0.05	3.4	0.03	1.4	1.3	>10
C	0.05	0.07	0.38	0.63	N.D.	>10

ERY: erythromycin, TET: tetracycline, AMP: ampicillin, MET: methicillin, CLI: clindamycin, VAN: vancomycin N.D.: not determined.

The macrolide antibiotic ERY has a bacteriostatic effect (inhibits growth of bacteria), particularly at high concentrations. ERY irreversibly binds to the 50s subunit of the bacterial rRNA complex and therefore inhibits protein synthesis and subsequent structural and functional processes that are critical to life or replication. It has previously been found that ERY has a good antibiotic effect against *E. coli*, with a minimum inhibitory concentration of 10 μg mL^{-1}.[15] TET inhibits protein synthesis by preventing aminoacyl-tRNA becoming attached to the ribosomal acceptor (A) site.[16] TET is a broad-spectrum agent that is active against a wide range of gram-positive and gram-negative bacteria. AMP is a semi-synthetic β-lactam antibiotic that can penetrate gram-positive and some gram-negative bacteria. AMP is widely used to treat *E. coli* infections in humans and livestock.[17] CLI, MET, and VAN inhibit synthesis of the peptidoglycan layer in the bacterial cell wall[18,19] or synthesis of proteins by inhibiting the peptidyltransferase reaction on the 50S ribosomal subunit,[20] so are only effective against gram-positive bacteria. These characteristics explained the results shown in Table 2 well. The LCs for *E. coli* were higher for CLI, MET, and VAN than for AMP, ERY, and TET because the target bacteria in this study were *E. coli*.

3.5. Fate of AREc in the environment

The fate of AREc discharged from WWTP-A into the receiving river was investigated, and the results are shown in Table 3. The LCs of CIP, MET, and TET for *E. coli* in WW from

370 the secondary clarifier in WWTP-A were 0.15, 0.09, and 1.0 μg mL^{-1}, respectively. The LC
371 of CIP for *E. coli* in RW collected at point 1 was comparable to the LC for the WW from the
372 secondary clarifier. The LC of TET for *E. coli* in RW collected at point 1 was an order of
373 magnitude lower than the LC for the WW from the secondary clarifier. This indicated that
374 different types of AREc survived in different ways. The LCs of CIP, MET, and TET for *E.*
375 *coli* in RW collected at point 2 were one order of magnitude lower than the LCs for RW
376 collected at point 1. The total *E. coli* concentration was 34% lower at point 2 than at point 1.
377 The LCs for the RW collected at point 2 (i.e., 5 km downstream of the WWTP discharge
378 point) were one order of magnitude higher than the LCs for RW collected from the river that
379 did not receive municipal WW effluent (points 3 and 4). Possible sources of AREc at point 2
380 were treated and untreated wastewater discharged from WWTP-A and diffusive sources in the
381 watershed. The AREc ratios in the RW may have decreased because of dilution, dispersion,
382 and sedimentation of AREc,[5] decreases in the AR of AREc caused by exposure to ultraviolet
383 light,[21] and/or plasmid loss in AREc.[22] Selective inactivation of AREc in preference to
384 antibiotic-susceptible *E. coli* in RW was found to be unlikely in a study performed by Wang
385 et al.[23]
386

387 **Table 3.** Lethal concentrations (LCs) of antibiotics for *E. coli* in wastewater collected from
388 the secondary clarifier of wastewater treatment plant WWTP-A, river water from 0.1 and 5
389 km downstream of the effluent discharge point (points 1 and 2, respectively), and water from
390 a river that did not receive wastewater effluent (points 3 and 4). The locations of the sampling
391 sites are shown in Figure 1. The LCs were determined using results acquired using the new
392 method.

	CIP LC80 (μg mL^{-1})	TET LC80 (μg mL^{-1})	MET LC50 (μg mL^{-1})	Total *E. coli* concentration (cfu mL^{-1})
Secondary clarifier	0.15	1.0	0.09	43±14
point 1	0.17	0.13	N.D.	25±10
point 2	0.08	0.01	N.D.	8.5±0.2
point 3	N.D.	N.D.	0.001	0.07±0.02

point 4	0.001	N.D.	0.002	2.8±0.3

CIP: ciprofloxacin, TET: tetracycline, MET: methicillin, N.D.: not determined.

Treated effluents released from municipal WWTPs are important sources of ARB to aquatic ecosystems.[6,8] ARB in treated effluent will readily and rapidly spread through an aquatic ecosystem. ARB can pose direct risks to humans and animals exposed to contaminated water through drinking, recreation, or irrigation.[7] *E. coli* is an important indicator of pathogen contamination of an aquatic ecosystem. Most *E. coli* strains are not pathogens, but some strains acquire genes making them virulent and able to cause various clinical symptoms such as intestinal and extraintestinal infections, respiratory tract infections, meningitis, and sepsis.[6] It is therefore necessary to understand the transport and fate of AREc in treated effluent discharged into aquatic ecosystems to develop strategies to control the spread of AREc in the environment.

3.6. Versatility of the new method

To confirm whether the new method could be applied to WW from other WWTPs, the method was used to analyze WW samples from five WWTPs (WWTP-O, WWTP-H, WWTP-N, WWTP-K, and WWTP-KK), then the TET and CIP LC80s for *E. coli* were estimated. The results are shown in Table 4. The TET LC80s for the samples from all five WWTPs were comparable (0.29–0.69 μg mL^{-1}) and were also comparable to the TET LC80s for the samples from the secondary clarifier in WWTP-A (Table 1B). In contrast, the CIP LC80s were one order of magnitude higher for *E. coli* in the samples from the primary clarifiers in WWTP-O and WWTP-K than for *E. coli* in the samples from the secondary clarifiers in WWTP-H1, WWTP-N1, WWTP-N2, and WWTP-KK. This implies that AREc could be selectively removed and/or inactivated more effectively than antibiotic-susceptible *E. coli* in aeration tanks and/or secondary clarifiers.[23] The CIP LC80s for *E. coli* in the

418 secondary clarifiers in WWTP-H1 and WWTP-N1 were comparable to the CIP LC80s for *E.*
419 *coli* in the secondary clarifier in WWTP-A (Tables 1B and 3) and one order of magnitude
420 higher than the CIP LC80s for *E. coli* in the secondary clarifiers in WWTP-N2 and WWTP-
421 KK. The AREc ratios for CIP varied more than the AREc ratios for TET for the WWTPs.
422 These results indicated that the behaviors of the different types of AREc were different under
423 the same environmental conditions.
424

425 **Table 4.** Lethal concentrations (LCs) of tetracycline (TET) and ciprofloxacin (CIP) for *E. coli*
426 in wastewater from the primary and secondary clarifiers of WWTP-O, WWTP-H, WWTP-N,
427 WWTP-K, and WWTP-KK. The LCs were determined using the results acquired using the
428 new method.

WWTP sample	TET LC80 ($\mu g\ mL^{-1}$)	CIP LC80 ($\mu g\ mL^{-1}$)	Total *E. coli* concentration ($cfu\ mL^{-1}$)	Sampling site
O	0.50	6.6	3320 ± 1270	Primary clarifier
H1	0.58	0.24	180 ± 72	Secondary clarifier
N1	0.29	0.12	17 ± 9	Secondary clarifier
K	0.54	4.8	3790 ± 1990	Primary clarifier
N2	0.69	0.03	40 ± 27	Secondary clarifier
KK	0.30	0.03	12 ± 2	Secondary clarifier

429 N.D.: Not determined; samples N1 and N2 were collected from WWTP-N on different days.
430

431 *3.7. Advantages and disadvantages of the new method*

432 The new method has several advantages over traditional methods, including having a
433 simple protocol, giving a high throughput, and being inexpensive (Table 5).[12] The new
434 method only requires a water sample to be mixed with a liquid medium with or without
435 antibiotic added in a microplate. No pretreatment (e.g., filtration and/or purification) is
436 required and no chemical reagents need to be added. Determining the fluorescence intensity
437 using a microplate reader eliminates subjective bias and human errors. In contrast, the plate
438 counting method involves laborious media preparation procedures and serially diluting
439 samples that have high *E. coli* concentrations. Up to 96 samples can be analyzed
440 simultaneously using the new method, so a large number of replicates (n=10 in this study)
441 could be used to improve the accuracy of the method. The small sample volume required (0.2
442 mL per sample) and lack of need for reagents (e.g., cell lysis reagents or fluorescence
443 enhancers) other than the culture medium and fluorogenic substrate mean that the new
444 method has much lower running costs (USD 0.02 per sample) than the other methods. The
445 quantitative PCR method is particularly costly because of the use of DNA polymerase.
446

447 **Table 5.** Comparison of the characteristics of the new method and other methods

Method	Running cost per sample	Detection time	Sample size per assay	Quantitativeness
This study [12]	**0.02 USD**	**12 h**	**96**	Semiquantitative
Colque Navarro et al. [24]	5 USD	2 days	**96**	Screening
Membrane filtration [25]	3 USD	24 h	1	**Quantitative**
Colilert method [25]	**9 USD**	18 h	1	**Quantitative**

448

449 The new method has some limitations. First, we assumed that *E. coli* susceptible to
450 antibiotics would never grow in the presence of antibiotics but *E. coli* resistant to antibiotics
451 would have the same growth rate in the presence or absence of antibiotics. This will not
452 always be true. Basra et al. investigated natural variations in 39 extraintestinal clinical
453 isolates of AREc to assess trade-offs between growth rates and resistance to the antibiotics
454 fluoroquinolone and cephalosporin.[26] They found evidence for a negative correlation between
455 the growth rate and antibiotic resistance. Second, the method requires a fluorogenic substrate.
456 Only the fluorogenic substrates β-D-glucuronidase for *E. coli* and β-D-galactosidase for total
457 coliforms are currently commercially available. Third, the new method requires a calibration
458 curve (i.e., an equation for the correlation between Ti and the *E. coli* concentration) to be
459 established for each sample. No universal calibration for the method could be established.
460 Fourth, the results indicated that the method gives higher standard deviations than can be
461 achieved using the other methods, meaning the new method should be limited to
462 semiquantitatively screening for AREc. Fifth, microplate readers are not cheap. However, the
463 aim of the new method was not to determine the minimum inhibitory concentrations for
464 different *E. coli* isolates in aquatic samples but to roughly estimate AREc concentrations and
465 ratios for as many antibiotics and samples as possible to acquire data to allow measures to be
466 taken to decrease the risks of AREc infection and the spread of ARGs in aquatic ecosystems.
467 Using a 96-well microplate for three samples to establish the calibration curve, five for AST,

468 and one blank sample with 10 replicates per sample means that tests at five different
469 antibiotic concentrations can be performed simultaneously.
470
471 **5. Conclusions and outlook**
472 We developed a novel phenotypic AST method for semiquantitatively screening
473 AREc in WW and RW samples. The method has several advantages over previously available
474 methods, including being simple to perform, being rapid, having a high throughput, and
475 having low running costs. The method can be used as a rapid and simple AST method for
476 determining AREc in WW and RW samples as an alternative to established methods to ensure
477 that effective and timely measures are taken to decrease the prevalence and spread of ARB in
478 the environment. We are trying to determine antibiotic-resistant coliforms using the new
479 method and to develop a fluorogenic substrate specific to pathogenic bacteria such as *Vibrio*
480 spp., *Salmonella* spp., and *Shigella* spp.
481
482 Supporting Information
483 These materials are available free of charge via the internet at http://pubs.acs.org.
484 - The procedure for creating a calibration curve for antibiotic-resistant *Escherichia coli*
485 - The ratios of antibiotic-resistant *Escherichia coli* to the total *Escherichia coli*
486 concentrations at various antibiotic concentrations
487
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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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608

Supplemental Information for:

Screening antibiotic-resistant *Escherichia coli* in wastewater and river water using a novel simple phenotypic antibiotic-susceptibility testing method

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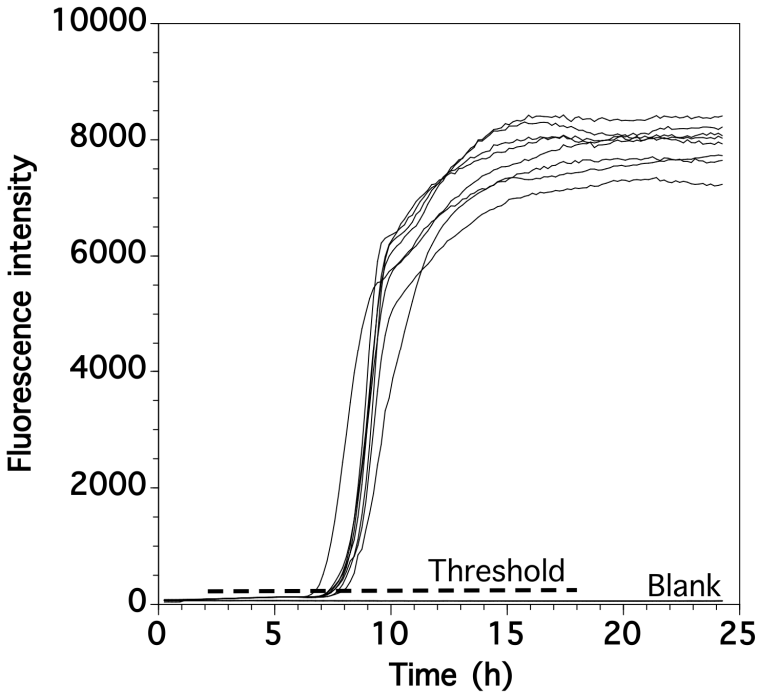

Figure S1. Typical temporal changes in MU fluorescence intensities during incubation of a wastewater sample from the secondary clarifier in WWTP-A with an *E. coli* concentration of 9.4 ± 4.6 most probable number (MPN) mL^{-1} in the medium. The sample was incubated in 10 wells on a microplate (n=10).

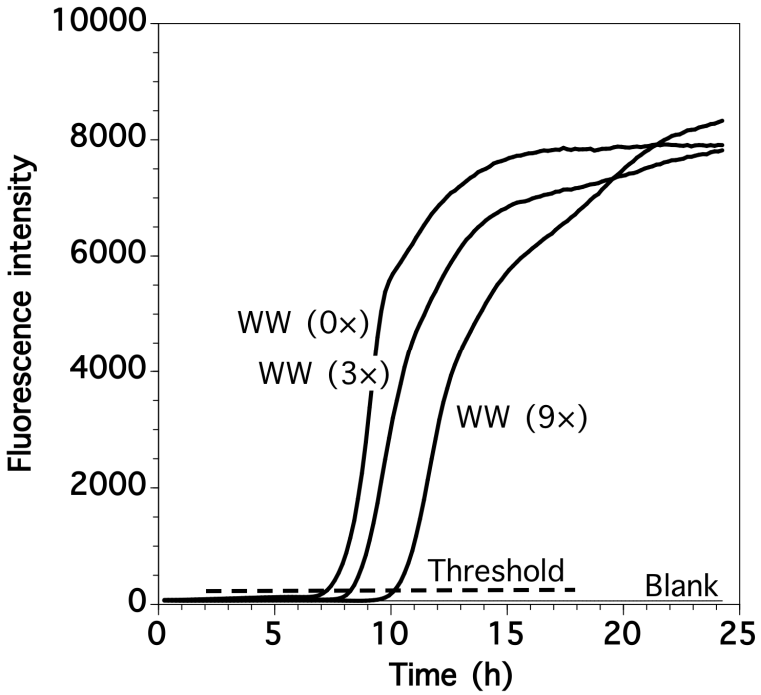

Figure S2. Temporal changes in the averaged MU fluorescence intensities during incubation of the WW sample used to give the data shown in Figure S1 and the sample diluted by factors of three and nine using the medium. The data (n=10) were averaged at each measurement time (every 10 min).

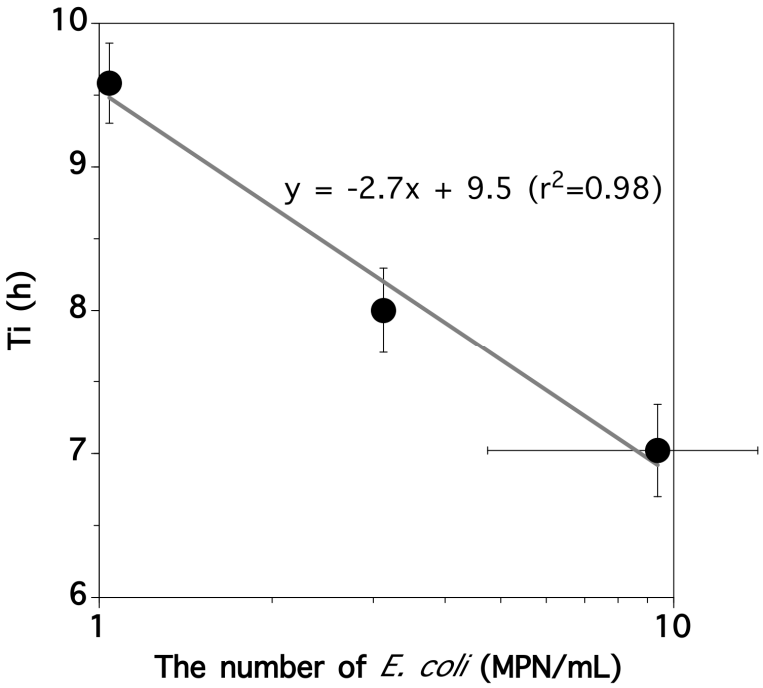

Figure S3. Calibration curve, Ti plotted against the initial *E. coli* concentration. The error bars indicate the standard deviations (n=10 for Ti and n=3 for the *E. coli* concentration).

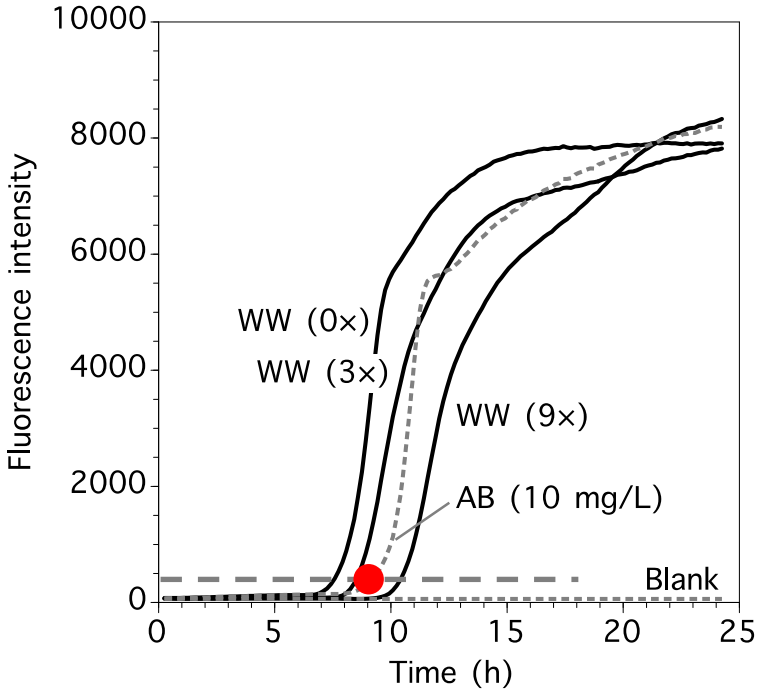

Figure S4. Temporal changes in the averaged MU fluorescence intensities during incubation of WW samples in the medium without an antibiotic added (as shown in Figure S2) and with an antibiotic added. In this case, the antibiotic was gentamicin. The red circle indicates Ti for the sample in the presence of the antibiotic.

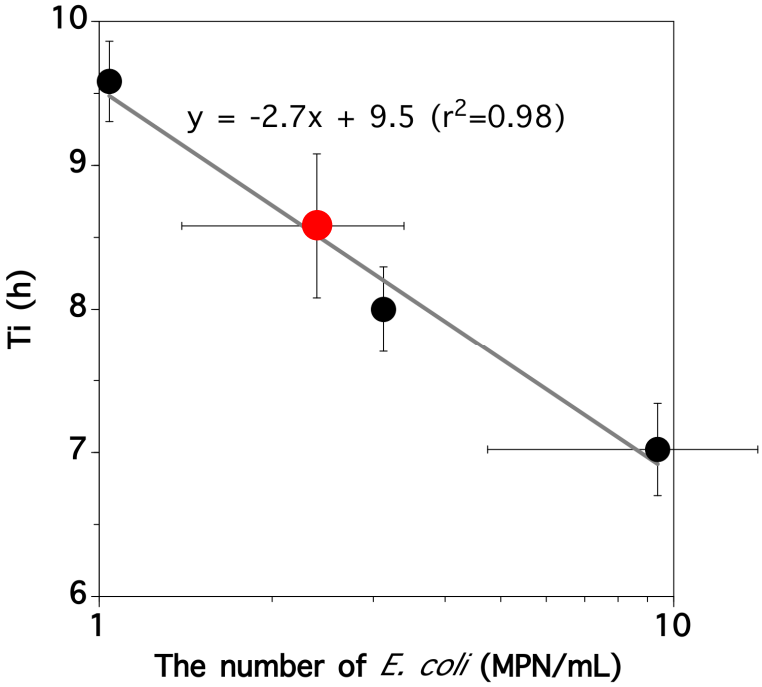

Figure S5. Calibration curve shown in Figure S3 with the average Ti for the sample with an antibiotic added shown as a red circle. The error bars indicate the standard deviations (n=10 for Ti and n=3 for the *E. coli* concentration).

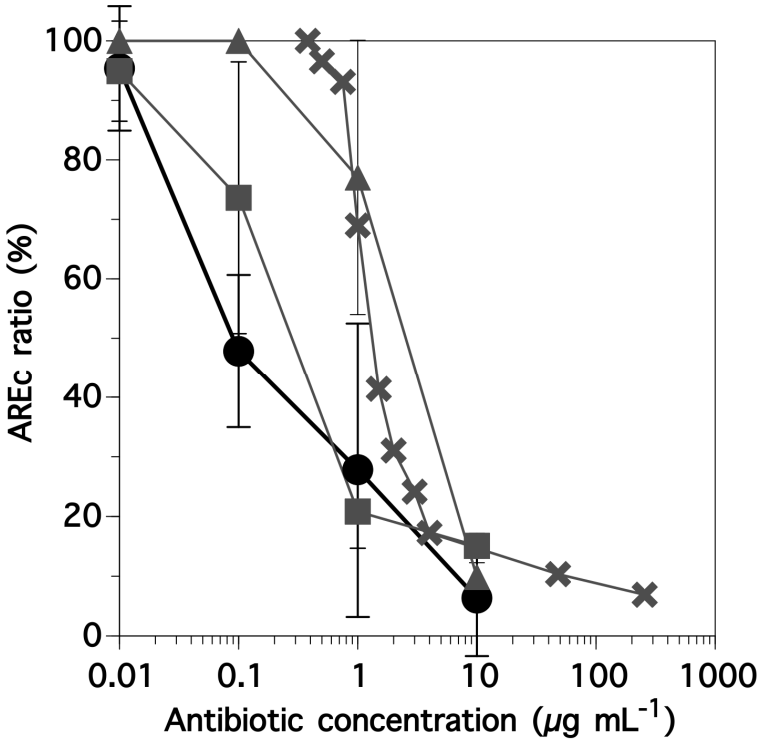

Figure S6. Antibiotic (TET) resistant *E. coli* (AREc) to total *E. coli* ratios at different antibiotic concentrations. The ratios were determined using the new method (●), the colony counting method (▲), the Colilert method (■), and the ETEST method (×). The error bars indicate the standard deviations (n=10 for the new method and n=3 for the colony counting and Colilert methods).

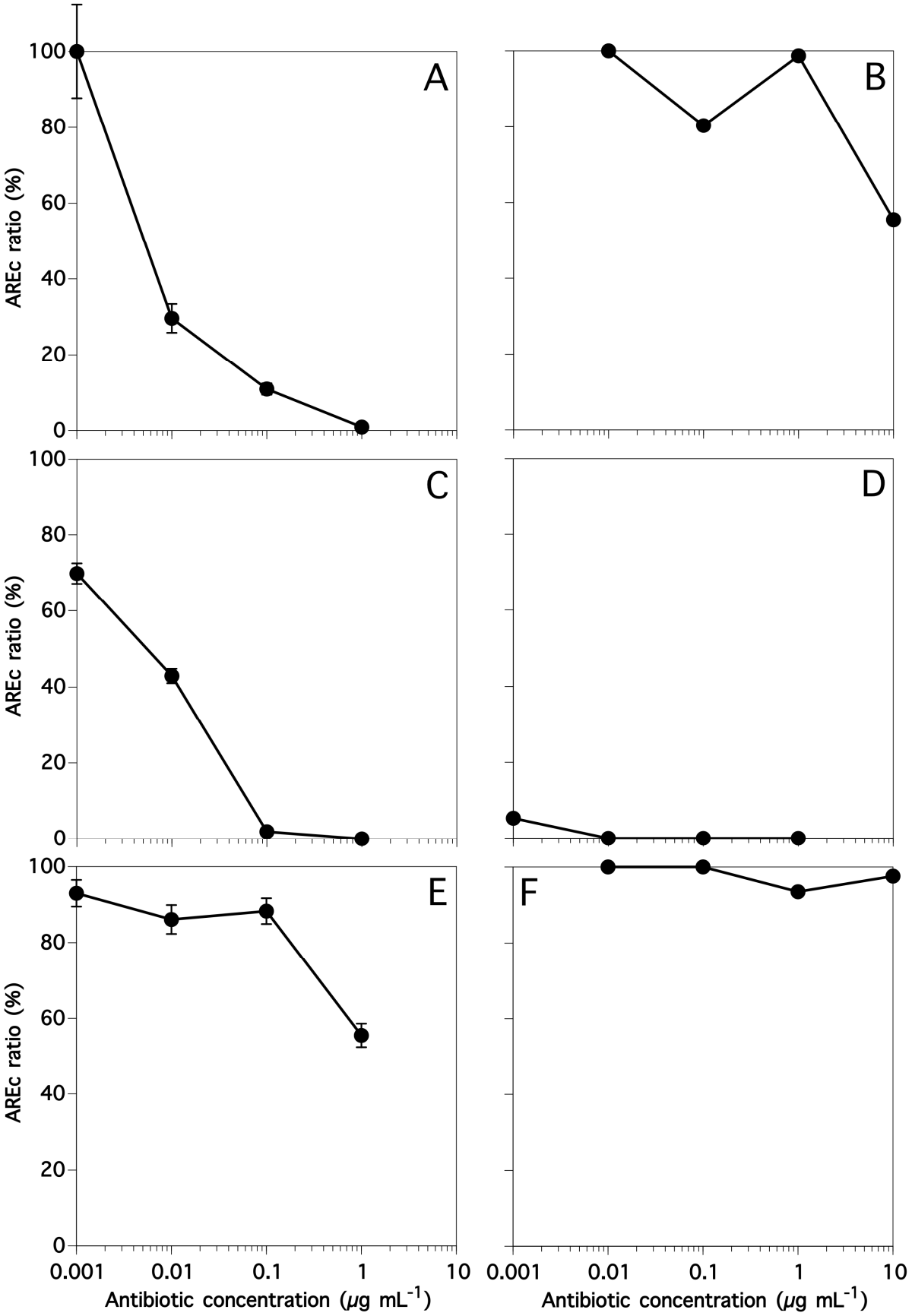
A
B
C
D
E
F
AREc ratio (%)
0
20
40
60
80
100
0.001
0.01
0.1
1
10
Antibiotic concentration (μg mL-1)

Figure S7. The AREc ratios for the WW samples to 6 antibiotics (A; AMP, B; CLI, C; ERY, D; GEN, E; MET, and F; VAN) plotted against the antibiotic concentration. The ratios were determined using the new method. The error bars indicate the standard deviations (n=10).