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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

Antibiotic-resistant *Escherichia coli* in sewage wastewater and river water were quantified using a fluorogenic substrate.

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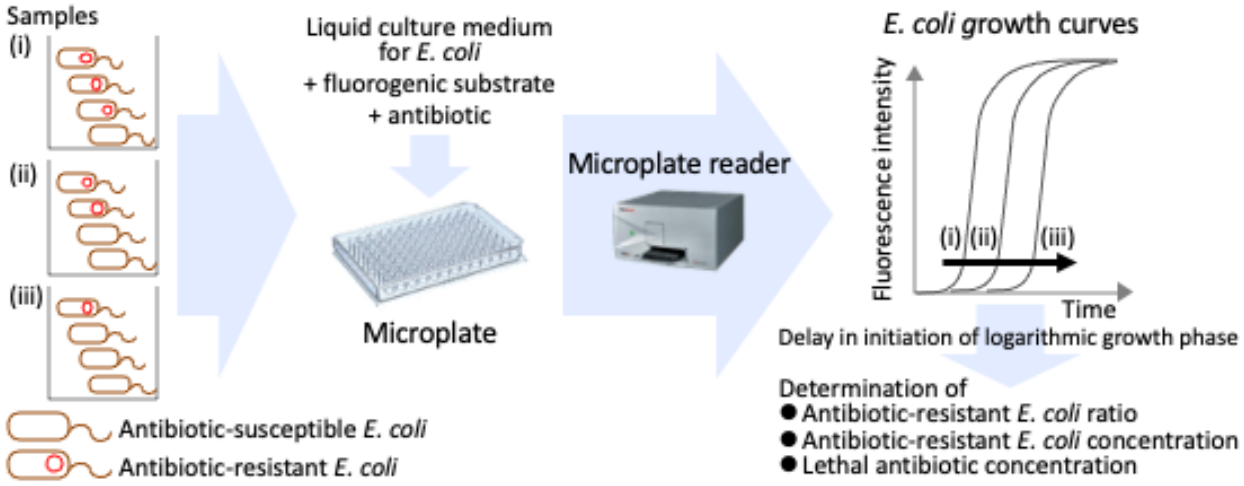

1. Introduction

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]

Various methods, including culture-based and molecular-based methods, have been used to investigate the presence, transportation, and fates of ARB in aquatic environments.[8] ARB concentrations in WW and river water (RW) samples have been determined using methods involving membrane filtration, plate counting, and quantitative polymerase chain reaction (PCR) analysis.[6,8–10] However, culture-dependent methods involve laborious preparation procedures, long incubation periods (≥18 h) for growing *Escherichia coli*, and large volumes of culture media, meaning such methods are expensive. Molecular-based methods, such as quantitative PCR methods, have high specificities, are rapid (<24 h), and have low measurement errors. However, molecular-based methods alone cannot be used to identify ARB with unknown ARGs and are limited to already identified genes. Determining a single ARG may give limited information about ARB resistant to a specific antibiotic in a sample. Analyzing only ARGs may mean that ARB that do not possess ARGs are overlooked. Currently available methods are therefore incapable of determining ARB concentrations in WW and RW simply and quickly. This makes it difficult to make effective and timely decisions aimed at controlling ARB.

The need for a simple, rapid, low-cost, and high-throughput assay for screening ARB in the aquatic environment led us to develop a novel simple phenotypic antibiotic susceptibility testing (AST) method for *E. coli* inspired by real-time PCR analysis. Therefore, we developed a simple AST method as a rapid and high-throughput assay for determining AR *E. coli* (AREc) in WW and RW in this study. First, the validation of the method has been performed for ciprofloxacin and tetracycline by comparing the results with the results of three conventional AST methods. Then the AREc concentrations, the AREc to total *E. coli* concentration ratios, and the lethal concentrations (LCs) of eight antibiotics to *E. coli* in WW and RW were determined. The LCs of the antibiotics to *E. coli* from the secondary clarifiers

of three WWTPs in Sapporo City were determined and the fate of AREc discharged from a WWTP into a river was investigated. Finally, raw and treated WW samples from five WWTPs around Japan were analyzed to confirm the versatility of the new method.

2. Materials and Methods

2.1. Study site and sampling

We collected 116 WW and RW samples in 65 sampling campaigns performed between January 2018 and January 2020. Most of the samples were collected in northern Sapporo in Hokkaido Prefecture, Japan, but some were collected outside Hokkaido Prefecture. The main sampling area is shown in Figure 1.

Sapporo is a city on the alluvial fan of the Toyohira River. The population and main industries of Sapporo were described in a previous publication.[11] Veterinary activities occur around points 3 and 4 in Figure 1 but not upstream of point 2. WW samples were collected from the secondary clarifiers of three WWTPs (WWTP-A, WWTP-B, and WWTP-C) in Sapporo City. The sizes, physicochemical characteristics, and operating conditions of WWTP-A, WWTP-B, and WWTP-C were described in previous publications.[12,13] WWTP-A is connected to a fully combined sewer. During heavy rain events, some of the primary clarifier effluent from WWTP-A is disinfected by chlorination and discharged into the Sosei River from the WWTP-A discharge outlet. RW samples were collected from ~100 m downstream of the WWTP-A discharge outlet (point 1), ~5 km downstream of the WWTP-A discharge outlet (point 2), and at two sampling sites (points 3 and 4) where no treated or untreated municipal WW is discharged. All three WWTPs discharged effluent after secondary treatment without disinfection during the sampling period. Each RW sample was collected in 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.

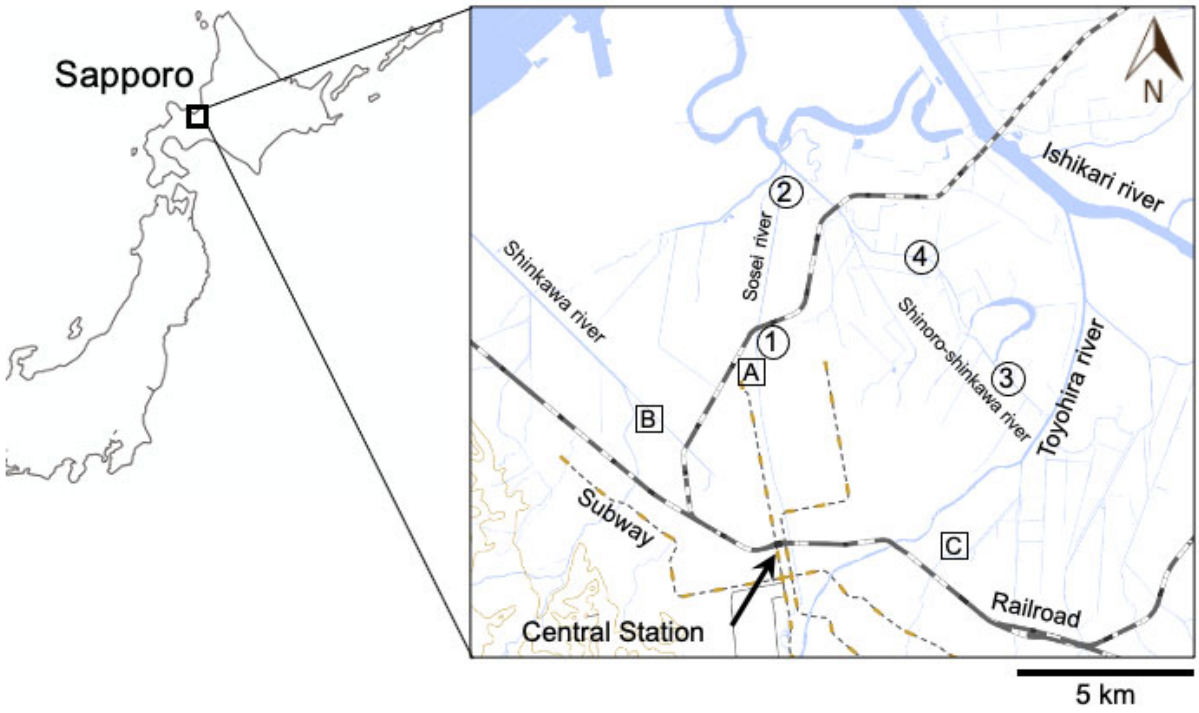

Figure 1. Map of the study area. The squares and circles indicate wastewater treatment plants (WWTPs) and sampling points, respectively. A = WWTP-A, B = WWTP-B, C = WWTP-C. Wastewater samples were collected from WWTP-A, WWTP-B, and WWTP-C. River water samples were collected from ~100 m downstream of 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.

The samples were not fed or aerated. The samples were analyzed as soon as they arrived in the laboratory. Changes in the structures and functions of the microbial communities in the samples during transportation were not assessed.

2.2. Principles involved in the novel simple phenotypic AST method for detecting AREc

The new method was inspired by the principles involved in real-time PCR analysis. Amplification of the target DNA molecule during the PCR process is monitored using a fluorescent molecule in real time, not only at the end of the PCR process. The target DNA molecule, fluorescent dye, DNA polymerase, and other chemicals required for the PCR process are mixed and subjected to the PCR process in a thermal cycler with a fluorescence detector to amplify the target DNA molecules. The PCR process involves repeated cycles of a set of (normally) three-step temperature changes. The number of DNA molecules (corresponding to the fluorescence intensity for the mixture) will theoretically have doubled at the end of each cycle. The fluorescence intensity will increase exponentially because 2^n times the initial number of copies of the DNA fragment will theoretically be present after n cycles. A fluorescence intensity threshold reliably higher than the background fluorescence intensity is set for detecting the target DNA molecules. The number of cycles required for the fluorescence intensity to exceed the threshold is called the threshold cycle. A standard curve is established using the threshold cycle for samples containing known quantities of the target DNA molecules each diluted by a factor of 10, then target DNA molecules in unknown samples are quantified by comparing the threshold cycles for the samples to the standard curve.

If the medium and conditions are appropriate, *E. coli* cells will multiply exponentially, like DNA molecules during the PCR process. The concentration of *E. coli* cells can be

monitored by measuring the β-D-glucuronidase activity in real time using a fluorogenic substrate (e.g., 4-methylumbelliferyl-β-D-glucuronide, MUG) using a microplate reader.[12] We hypothesized that the *E. coli* concentrations in unknown samples could be quantified by incubating samples containing known and unknown *E. coli* concentrations on a microplate reader, monitoring the fluorescence intensities of the samples, and defining a fluorescence intensity threshold from blank samples. The incubation time at which the fluorescence intensity exceeds the threshold will be related to the initial *E. coli* concentration.

2.2. Quantification of E. coli *and AREc*

Conventional methods for counting *E. coli* in WW and RW were performed using a Colilert and Quanti-Tray/2,000 system (IDEXX Laboratories, Westbrook, ME, USA) and Chromocult Coliform Agar ES (enhanced selectivity) medium (Merck, Darmstadt, Germany) following the instructions provided by the manufacturers. In the colony counting method, 0.1 mL of a sample was added to an agar medium plate. If the *E. coli* concentration in a sample was >1000 cfu mL^{-1}, the sample was diluted by a factor of 10 with sterile physiological NaCl solution (0.9% NaCl) in a 100-mL sterile bottle before analysis. If the *E. coli* concentration in a sample was <300 cfu mL^{-1}, the *E. coli* concentration was determined using the membrane filtration technique and the colony counting method. The required volume of a sample was passed through a sterile mixed cellulose ester filter with 0.45-μm pores (ADVANTEC, Tokyo, Japan). The filter was then placed on a Chromocult Coliform Agar ES medium plate. Each sample was tested in triplicate.

The AREc concentrations in the WW and RW samples were determined using four methods, a colony counting method using Chromocult Coliform Agar ES, the Colilert method, the ETEST method, and the new method described here. Tests were performed using

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

medium sample were selected at random and isolated. The minimum inhibitory concentration of the antibiotic of interest for each isolated colony was then determined using an ETEST strip and Chromocult Coliform Agar ES medium, following the instructions provided by the manufacturer.

The lethal concentration (LC) of an antibiotic to *E. coli* was estimated from the relationship between the antibiotic concentration and AREc ratio (see Figure 2) by performing probit analysis.[14] Probit analysis is a specialized form of regression analysis that can be applied to binomial response variables. The procedure transforms a concentration–response curve into a straight line that can then be analyzed by either least-squares or maximum-likelihood regression analysis.

3. Results and Discussion

3.1. Determining the AREc concentration using the new method

Typical temporal changes in the 4-methylumbelliferone (MU) fluorescence intensity when incubating a WW sample from the secondary clarifier in WWTP-A in the *E. coli* medium using the new method are shown in Figure S1. The sample was incubated in 10 wells on a microplate, and the MU fluorescence intensity for each cell was measured every 10 min. The fluorescence intensity of the medium–WW mixture did not increase until after 6.0 h of incubation, then the fluorescence intensity increased exponentially, doubling every 10 h. The fluorescence intensity reached a plateau at ~8000, probably because MUG had been completely degraded into MU. The fluorescence intensity profile indicated that the *E. coli* growth curve in the batch culture consisted of a lag phase, a logarithmic growth phase, and a stationary phase. This would have been because the MU fluorescence intensity was related to MUG degradation by *E. coli*. The incubation time at which the fluorescence intensity

exceeded the threshold was defined as the logarithmic growth phase initiation time (Ti), similar to the threshold cycle for real-time PCR (Figure S1). The threshold was set as the mean plus 10 times the standard deviation of the fluorescence intensities of the blank samples (n=10). An aliquot of WW was diluted by a factor of three and another aliquot of WW was diluted by a factor of nine with 0.9% saline, then temporal changes in the MU fluorescence intensities of the samples were monitored. The results are shown in Figure S2. The Ti values were determined using the procedure described above from the fluorescence intensity profiles, and the mean Ti was calculated. Diluting the WW decreased the initial *E. coli* concentration and therefore delayed initiation of the logarithmic growth phase (i.e., the Ti increased). A calibration curve (Ti plotted against the initial *E. coli* concentration) was established from the results. The calibration curve is shown in Figure S3. If an antibiotic was added to a WW sample and the fluorescence intensity was monitored, the Ti was delayed compared with the Ti for the WW incubated without an antibiotic added, as shown in Figure S4. This was because *E. coli* growth would have been inhibited by the antibiotic in the medium. We hypothesized that *E. coli* susceptible to the antibiotic did not grow in the presence of the antibiotic but that the AREc growth rate was unaffected by the antibiotic. This allowed the concentration of *E. coli* resistant to the antibiotic at the concentration in the medium (i.e., AREc) in the WW to be estimated using the calibration curve, as shown in Figure S5. The AREc to total *E. coli* concentration ratio for a sample was calculated by dividing the AREc concentration by the *E. coli* concentration in the sample without the antibiotic added (i.e., the total *E. coli* concentration).

3.2. Determining the AREc to E. coli *concentration ratios using four methods*

The AREc to *E. coli* concentration ratios for the WW samples from the secondary clarifier of WWTP-A at four concentrations (10, 1.0, 0.1, and 0.01 μg mL^{-1}) of the antibiotic

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

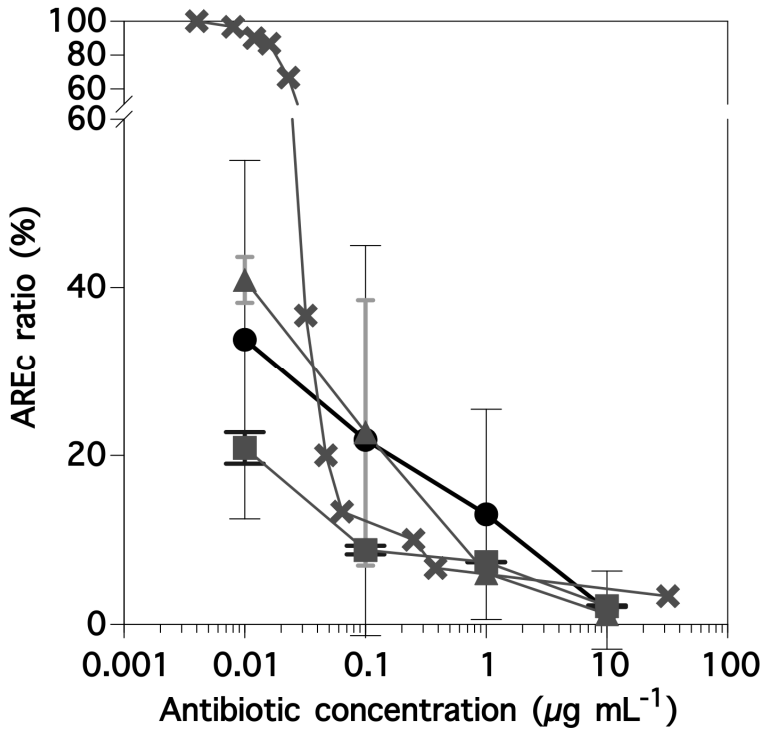

Figure 2. Ciprofloxacin-resistant *E. coli* (AREc) to total *E. coli* ratios plotted against the antibiotic concentration. The ratios were determined using the new method (●), colony counting method (▲), Colilert method (■), and ETEST method (×). The error bars indicate the standard deviations (n=10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

LCs of these 6 antibiotics for *E. coli* fluctuated greatly, we analyzed AREc ratios and LCs of antibiotics using CIP and TET.

3.3. Determining LCs of the antibiotics for E. coli *using the new method*

We performed the AST method using CIP and TET again and determined the LCs of antibiotics for *E. coli* in the WW samples from the secondary clarifier of WWTP-A. The results are shown in Table 1. The LC90 (Table 1A) and LC80 (Table 1B) antibiotic concentrations are the concentrations at which 10% and 20%, respectively, of the *E. coli* survive (i.e., the AREc ratios are 10% and 20%, respectively). It can be seen from Table 1B that 20% of the *E. coli* in the WW from the secondary clarifier in WWTP-A survived in the presence of CIP at a concentration of ~0.1 μg mL^{-1} and of TET at a concentration of ~1.0 μg mL^{-1}. The differences between the LC80s determined using the new method and the other three methods were <4.4 μg mL^{-1} and were lower than the differences for the LC90s (>10 μg mL^{-1}) (Table 1A) and LC50s (by one order of magnitude or more, data not shown). We therefore concluded that the new method could be used as a simple AST method and that the LC80 (i.e., the antibiotic concentration at which the AREc ratio is 20%) is a more reliable index than other LCs for comparing AREc ratios for WWs.

Table 1. The (A) 90% lethal concentrations (LC90s) and (B) 80% lethal concentrations (LC80s) of ciprofloxacin (CIP) and tetracycline (TET) for *E. coli* in the wastewater samples from the secondary clarifier of WWTP-A. Two experiments were performed for each antibiotic and the number in parenthesis indicates the number of the experiment. The LC90s and LC80s for CIP (1) and TET (1) were calculated from the results shown in Figures 2 and S6, respectively.

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

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

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

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

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

3.7. Advantages and disadvantages of the new method

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

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**

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

and one blank sample with 10 replicates per sample means that tests at five different antibiotic concentrations can be performed simultaneously.

5. Conclusions and outlook

We developed a novel phenotypic AST method for semiquantitatively screening AREc in WW and RW samples. The method has several advantages over previously available methods, including being simple to perform, being rapid, having a high throughput, and having low running costs. The method can be used as a rapid and simple AST method for determining AREc in WW and RW samples as an alternative to established methods to ensure that effective and timely measures are taken to decrease the prevalence and spread of ARB in the environment. We are trying to determine antibiotic-resistant coliforms using the new method and to develop a fluorogenic substrate specific to pathogenic bacteria such as *Vibrio* spp., *Salmonella* spp., and *Shigella* spp.

Supporting Information

These materials are available free of charge via the internet at http://pubs.acs.org.

- The procedure for creating a calibration curve for antibiotic-resistant *Escherichia coli*
- The ratios of antibiotic-resistant *Escherichia coli* to the total *Escherichia coli* concentrations at various antibiotic concentrations

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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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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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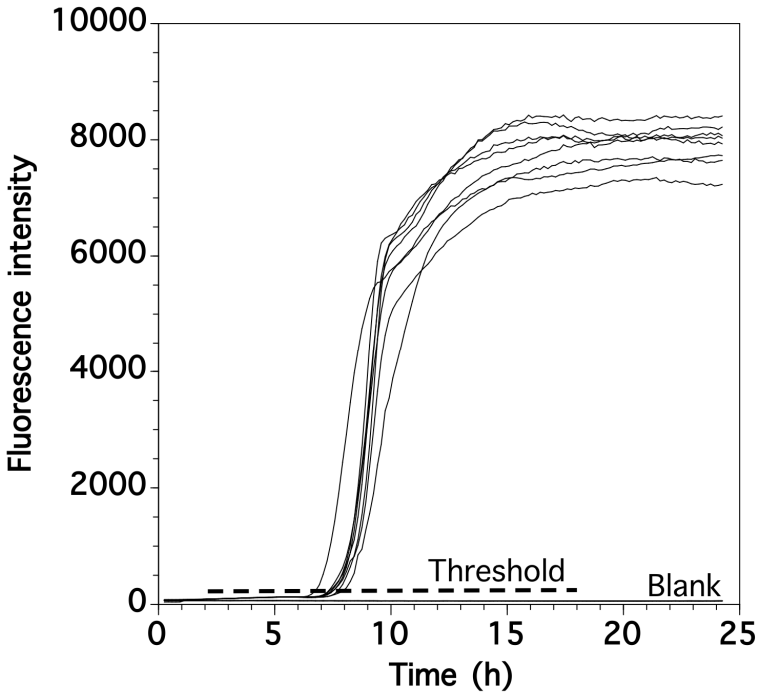

Figure S1. Typical temporal changes in MU fluorescence intensities during incubation of a wastewater sample from the secondary clarifier in WWTP-A with an *E. coli* concentration of 9.4 ± 4.6 most probable number (MPN) mL^{-1} in the medium. The sample was incubated in 10 wells on a microplate (n=10).

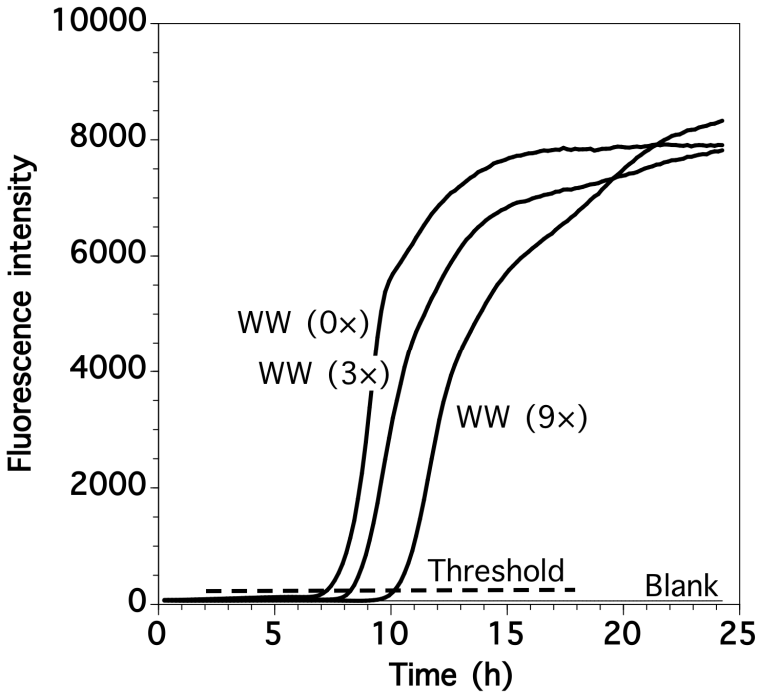

Figure S2. Temporal changes in the averaged MU fluorescence intensities during incubation of the WW sample used to give the data shown in Figure S1 and the sample diluted by factors of three and nine using the medium. The data (n=10) were averaged at each measurement time (every 10 min).

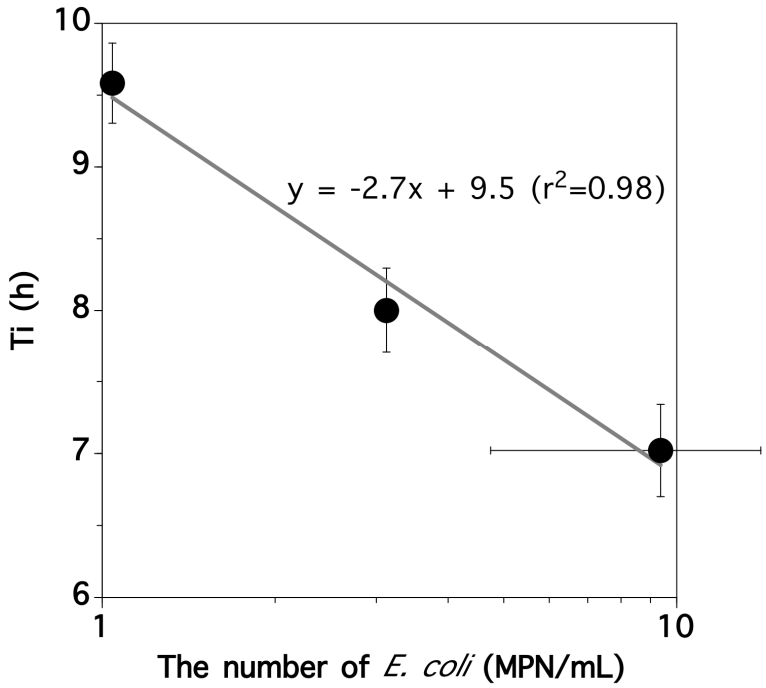

Figure S3. Calibration curve, Ti plotted against the initial *E. coli* concentration. The error bars indicate the standard deviations (n=10 for Ti and n=3 for the *E. coli* concentration).

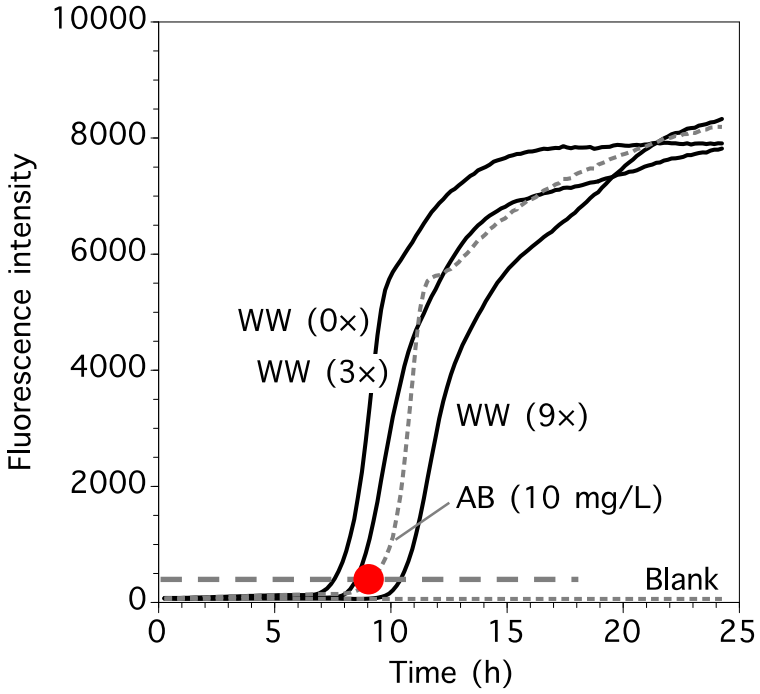

Figure S4. Temporal changes in the averaged MU fluorescence intensities during incubation of WW samples in the medium without an antibiotic added (as shown in Figure S2) and with an antibiotic added. In this case, the antibiotic was gentamicin. The red circle indicates Ti for the sample in the presence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

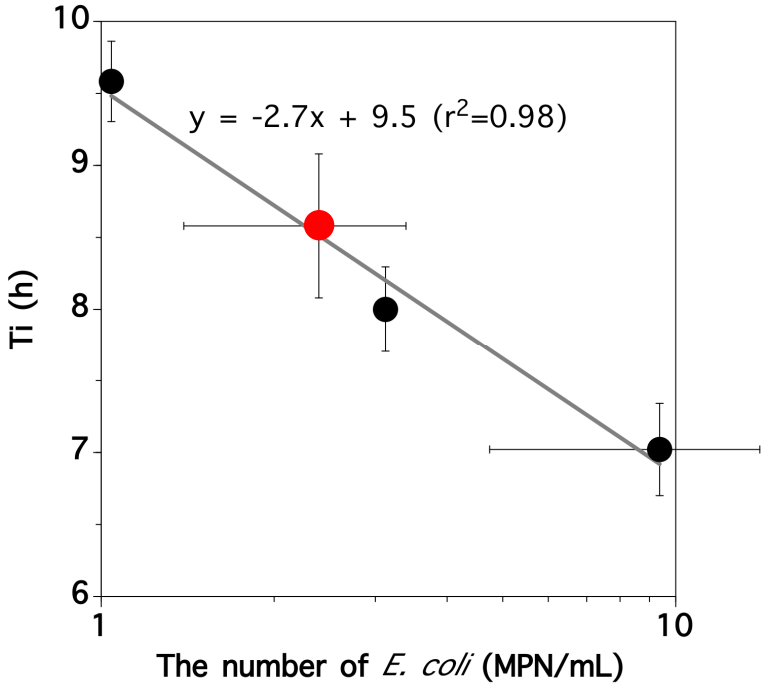

Figure S5. Calibration curve shown in Figure S3 with the average Ti for the sample with an antibiotic added shown as a red circle. The error bars indicate the standard deviations (n=10 for Ti and n=3 for the *E. coli* concentration).

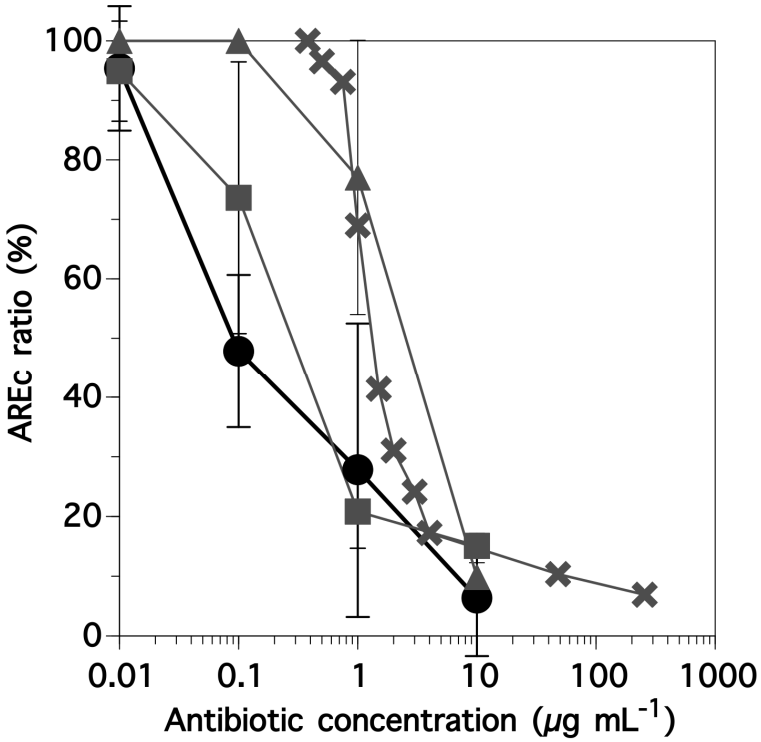

Figure S6. Antibiotic (TET) resistant *E. coli* (AREc) to total *E. coli* ratios at different antibiotic concentrations. The ratios were determined using the new method (●), the colony counting method (▲), the Colilert method (■), and the ETEST method (×). The error bars indicate the standard deviations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

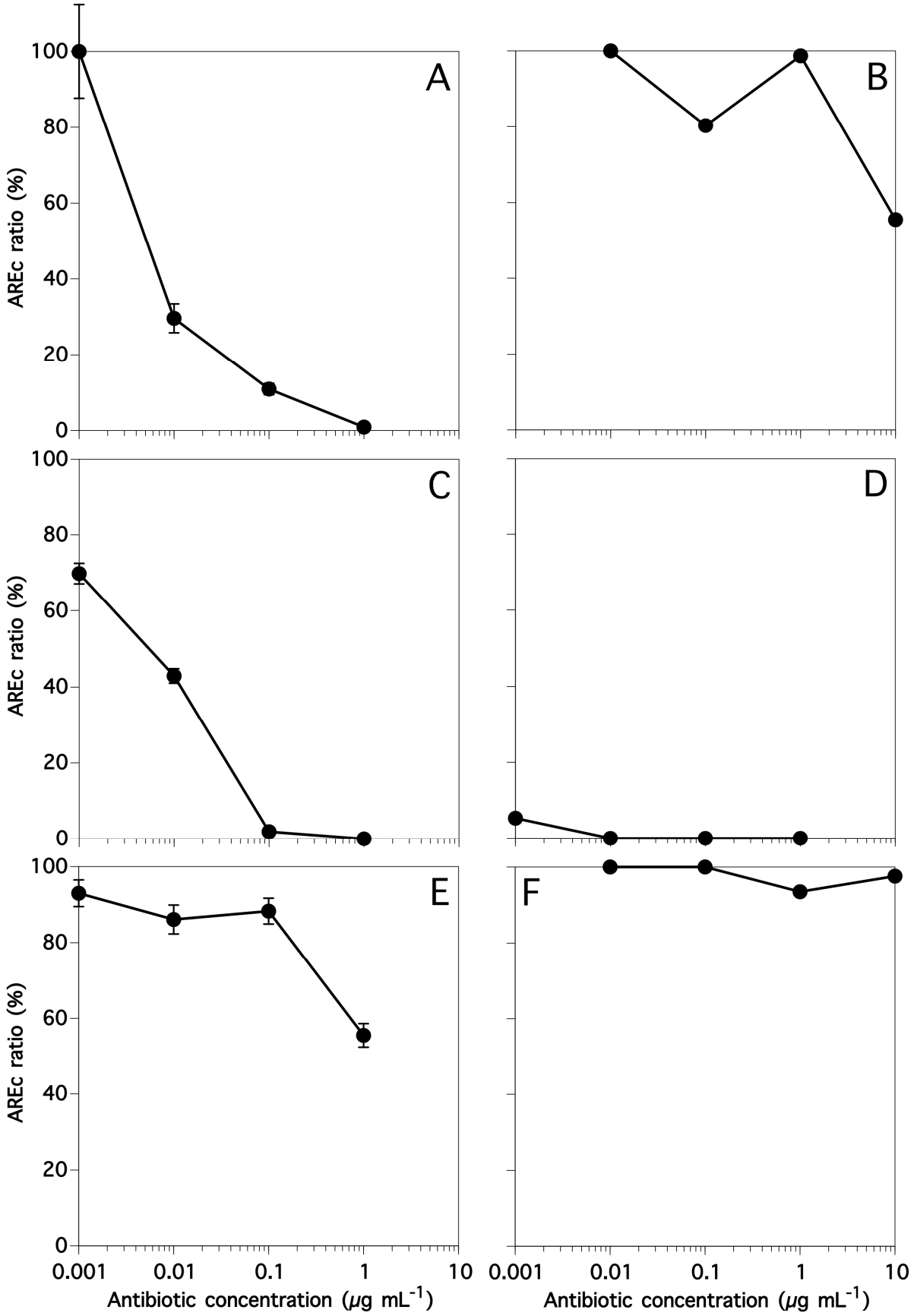
A
B
C
D
E
F
AREc ratio (%)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
Antibiotic concentration (μg mL-1)

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).