Antibiotic resistance in environmental *Escherichia coli* – a simple screening method for simultaneous typing and resistance determination

Patricia Colque Navarro, Heriberto Fernandez, Roland Möllby, Laura Otth, Madeleine Tiodolf, Myra Wilson and Inger Kühn

ABSTRACT

We describe a simple and standardised screening system (AREB) for surveillance of antibiotic resistant bacteria in the environment. The system consists of 96 well microplates containing eight sets of breakpoint amounts of 10 different antibiotics. The incubated microplates are read by a desktop scanner and the plate images are analysed by special software that automatically presents the resistance data. The AREB method is combined with a rapid typing method, the PhenePlate system, which yields information on the diversity of the bacteria in the studied samples, and on the possible prevalence of resistant clones. In order to demonstrate the usage of AREB, a comparative study on the resistance situation among 970 *Escherichia coli* isolates from sewage and recipient water in Sweden, Norway and Chile, was performed. Resistance rates to all antibiotics were markedly higher in hospital sewage than in other samples. Our data indicate that the AREB system is useful for comparing resistance rates among *E. coli* and other environmental indicator bacteria in different countries/regions. Simple handling and automatic data evaluation, combined with low cost, facilitate large studies involving several thousands of isolates.

Key words | antibiotic resistance, bacteria, *Escherichia coli*, screening method, sewage, typing

Patricia Colque Navarro
Roland Möllby
Madeleine Tiodolf
Inger Kühn (corresponding author)
Department of Microbiology,
Tumor, and Cell Biology,
Karolinska Institutet,
SE 17177 Stockholm,
Sweden
E-mail: *inger.kuhn@gmail.com*

Heriberto Fernandez
Laura Otth
Myra Wilson
Institute for Clinical Microbiology,
Universidad Austral de Chile,
Valdivia,
Chile

INTRODUCTION

The normal microflora of man and animals act as a reservoir of many different resistant bacteria and resistance genes that may be transmitted further to pathogenic bacteria. The development of resistance against antibiotics could thus be detected in the intestinal flora before it appears in clinical infections. Intestinal bacteria like enterococci and *Escherichia coli* are easy to cultivate and identify and are therefore suitable indicators of faecal pollution of the environment. These bacteria are also useful indicators of the ecological impact of antibiotic usage in different populations. Surveillance studies on the occurrence of antibiotic-resistant bacteria in the microflora of humans and animals are usually performed by testing the susceptibility of bacteria isolated from a large number of clinical or faecal samples, e.g., DANMAP in Denmark (http://www.danmap.org), ECO-SENS (Kahlmeter 2003) in Europe, or studies on cattle in Chile (San Martin *et al.* 2005). An alternative method would be to isolate indicator bacteria from untreated urban sewage, and to test many bacteria per sample for antibiotic resistance using a simple screening method. Previous studies have shown that bacteria in sewage samples are good representatives of the indigenous flora of the individuals contributing to the sewage (Kühn *et al.* 2003; Blanch *et al.* 2006), and that resistance rates to many antibiotics correspond well to resistance rates among isolates from humans (Reinthaler *et al.* 2013). Some attractive features of this approach are that sewage samples are easy to obtain (in contrast to faecal samples from healthy adults) and that cultivation of one single sample may yield intestinal bacteria originating from hundreds or even thousands of individuals. By applying this approach, the resistance situation in defined populations, such as the population

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contributing to the sewage in a community sewage treatment plant (STP) or the patients and staff contributing to hospital sewage, could easily be monitored.

Several standardised methods for resistance determination have been developed: for example, based on disc diffusion, agar or broth dilution, antibiotic gradient discs, or automated instrument systems (Jorgensen & Ferraro 2009). However, these methods have been developed mainly for clinical isolates, and the methods are often too expensive and laborious to be used for large number of isolates from sewage or other environmental samples, where the aim is not an exact determination of the susceptibility of individual isolates in order to determine the best treatment option for the patient, but merely to be able to screen for resistance among many isolates in a population. We have therefore developed a simple screening assay for determination of resistance – antibiotic resistance in environmental bacteria (AREB), which is performed in 96-well microplates containing breakpoint concentrations of antibiotics, with scanner assisted measurements of plates and automated resistance calculations. Furthermore, we have combined the AREB method with a rapid typing method, the PhenePlate (PhP) system (biochemical fingerprinting in microplates), which simultaneously yields important information on the quality of the studied samples, and on the possible prevalence of resistant clones in the studied samples. We also demonstrate how a combination of AREB and PhP can be used to compare resistance rates among *E. coli* isolates in samples from different origins.

MATERIALS AND METHODS

AREB and PhP plates for simultaneous resistance screening and rapid typing of *E. coli*

The AREB plates consist of 8×12-well U-shaped microplates containing eight sets of 10 different dehydrated antibiotics. An example of antibiotic combination that was used in the present study of *E. coli* is presented in Figure 1. The wells of the first column in the microplate are empty and are used to make suspensions of the bacteria to be studied. The wells of the last column are also empty and serve as growth control for each studied bacterium.

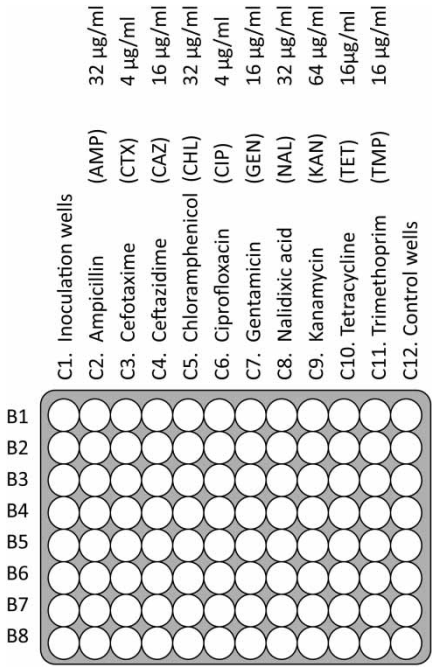

Figure 1 | AREB-EC plate configuration. Each plate contains 10 antibiotics in columns C2–C11. Eight different bacterial isolates (B1–B8) are inoculated into rows 1–8. Column C1 is used to make bacterial suspensions, and column C12 contains no antibiotic and is used as negative control for each bacterial isolate. The final concentration in 100 µL of medium is given for each antibiotic.

Columns 2–11 contain 10 different dehydrated antibiotics. The amount of the antibiotic added to each well yielded an antibiotic concentration corresponding to the breakpoints recommended by the Clinical and Laboratory Standards Institute when 100 µL of medium is added to the wells (http://www.clsi.org/). The plates with dehydrated antibiotics can be stored in plastic bags at $+4\ ^{\circ}C$ for at least a month, or at $-20\ ^{\circ}C$ for several months.

PhP-RE (rapid screening of *E. coli*) plates of the PhenePlate system (PhPlate microplate techniques AB; www.phplate.se) consist of 8×12 well flat bottomed microplates with eight rows of 11 substrates (carbohydrates and amino acids) (Kühn & Möllby 1993). The first well of each row is used to prepare bacterial suspensions.

Combined phenotyping and antibiotic resistance determination using PhP and AREB plates

Pure *E. coli* colonies are picked from agar plates using sterile tooth sticks (Figure 2(a)) and each colony is dispensed

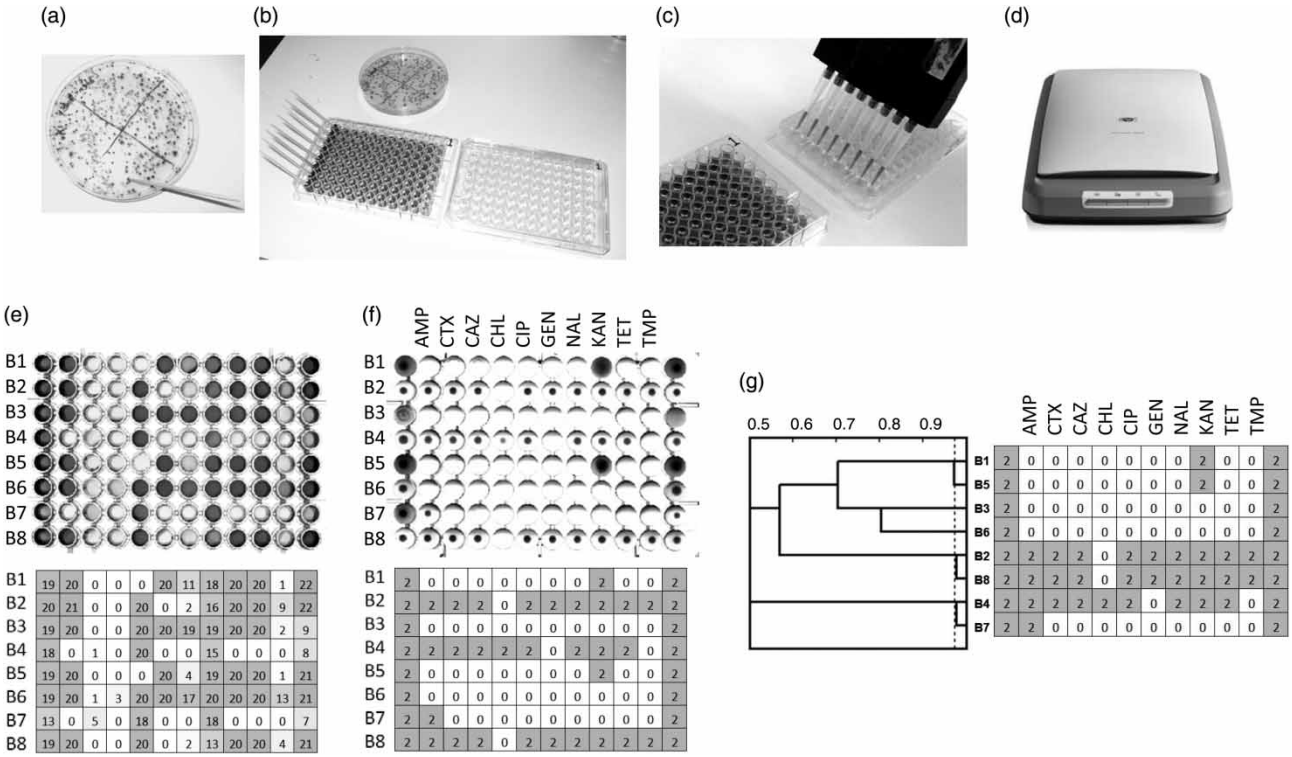

Figure 2 | Simultaneous typing and resistance determination of eight *E. coli* isolates using PhP and AREB. (a) Eight pure colonies are picked from an agar plate using a sterile tooth stick. (b) The colonies are suspended in the first column of a PhP plate that has been filled with PhP medium. (c) The bacterial suspensions are transferred to all wells of the same row in the PhP plate and mixed with iso-Sensitest broth in the first column of the AREB plate, from where the bacterial suspensions are transferred to the other wells in the AREB plate. (d) After incubation the plates are scanned with a desktop scanner. (e) Plate image and corresponding PhP typing data obtained from scanned PhP plate. (f) Plate image and corresponding resistance data obtained from scanned AREB plate. (g) Dendrogram derived from clustering of PhP typing data with corresponding resistance data added. Two common PhP types (clones?) with common resistance patterns can be seen (B1–B5 and B2–B8). In the third common PhP type (B4–B7), the isolates have different resistance profiles, and could represent a clone that has lost a resistance gene in B7.

into one well of the first column of PhP-RE plates that have previously been filled with 200 μL of PhP suspending medium (Figure 2(b)). Ten μL of the bacterial suspensions in column 1 are then transferred to all other columns in the PhP-RE plate that have previously been filled with 150 μL of PhP suspending medium. Thereafter, 20 μL of the suspension is dispensed into column 1 (the inoculation wells) of AREB plates that have been filled with 200 μL of iso-Sensitest broth (Oxoid), then 10 μL of the suspensions are dispensed into each of columns 2–12 in the AREB plate that each contain 100 μL of iso-Sensitest broth (Figure 2(c)). The final concentration of bacteria in the AREB plate will be about 10^6 cfu/mL in column 1 and in the colums 2–11 (with antibiotics) and column 12 (growth controls) approximately 10^5 cfu/mL. The top side of the corner of each plate is marked with the name or number of the plate and the plates are then placed in an incubator at 37 °C.

Reading and analysis of PhP and AREB plates

Microplate data are normally measured by microplate readers that measure the absorbance of each of the 96 wells. We have found it more useful to make images of the plates, using a cheap office flatbed scanner, and have developed a software that converts the images to 96-well data.

After 16–18 h incubation the plates are read using a desktop scanner. We used a HP G 4050 scanner from Hewlett Packard (Figure 2(d)), but any scanner supplied with an adaptor that makes it suitable to read transmissible originals in the size of a microplate can be used. The plate images produced by the scanner (Figures 2(e), (f)) are analysed using the

Microplate Analyser software (www.phplate.se), a specially developed software that can measure the colour of each well in the PhP plates and the relative amount of bacterial growth (expressed as density and size of the pellets produced in the round-bottomed microplates, see Figure 2(f)) in each well of the AREB plates. The software compares the amount of bacterial growth in each well of the AREB plate to that of the control well for the same bacterial isolate (column 12) and prints out resistance rates as 0 (no growth, sensitive), 1 (weak growth compared to that in the control well, intermediate), and 2 (similar amount of growth as in the control well, resistant). Furthermore, the software of the PhP system can cluster the data obtained from the PhP plates, and print out a dendrogram (Figure 2(g)) as well as calculate the diversity for each sample. After the software analysis, important isolates representing different resistant phenotypes (e.g., B1, B2, B4, B7 in the example in Figure 2(g)) can be picked from the control well in column 12 in the AREB plate and saved for further studies.

Comparisons between resistance rates obtained by AREB and standard techniques

For comparisons to standard methods, resistance determinations were also performed by the disc diffusion method (www.oxoid.se) on 36 isolates. Twenty-three of the isolates were *E. coli* from Swedish sewage, which had showed resistance to at least one antibiotic in the AREB test, and the remaining 13 isolates were culture collection strains belonging to various species. Another 49 *E. coli* isolates were subject to parallel resistance determination by AREB and the E-test method (http://www.biomerieux-usa.com). All these isolates were from incoming sewage in Valdivia, Chile, and had showed resistance to at least one antibiotic in the AREB test.

Analysis of samples

In order to demonstrate the usage of PhP and AREB, a comparative study on the resistance situation among 970 *E. coli* isolates from 39 sewage and recipient water samples in Stockholm (Sweden), Oslo (Norway) and Valdivia (Chile), was performed (Table 1). The samples were subject to serial dilution and cultivated on m-FC agar (Difco, BD Diagnostic Systems) for isolation of coliform bacteria. Samples were incubated at 44 °C in order to suppress all non-thermotolerant bacteria. After incubation, 24 presumed *E. coli* colonies from each sample (when available) were picked from the agar plates using sterile tooth sticks and inoculated into three PhP-RE and three AREB plates as described above.

After overnight incubation, the plates were scanned and analysed with the PhP software. Resistance rates for each antibiotic were calculated as resistance percentages of all analysed isolates. Total antibiotic resistance in a population was measured using the multiple antibiotic resistance (MAR) index (Krumperman 1983; Zhang *et al.* 2013), calculated as $\text{sumA}/(2 \times n \times N)$, where sumA is the sum of resistance codes (as 0, 1 or 2) to all tested antibiotics for all tested isolates, n is the number of antibiotics tested, and N is the number of isolates analysed from the sample. The maximum possible MAR-value is 1.00, obtained when all isolates are resistant to all antibiotics tested.

RESULTS

Comparisons between resistance rates obtained by AREB and other techniques

Disc diffusion

Altogether 360 bacteria–antibiotic combinations were tested in parallel with disc diffusion and AREB. Only in seven cases (1.9%) a disagreement between the results were obtained for the two methods. In three cases, an isolate was sensitive with AREB but resistant with disc diffusion, whereas in four cases, isolates were resistant with AREB and sensitive with disc diffusion. In another 18 cases (5%), the result was intermediate with one method and sensitive or resistant with the other method, whereas in 93.1% of all cases, a total agreement between the two methods was obtained.

E-test

For E-test 283 bacteria–antibiotic combinations were evaluated. Also with the E-test, a high agreement (95.4%) was obtained when compared to the AREB test. The only noteworthy discrepancy was for ampicillin, to which four isolates were sensitive in the E-test but resistant in AREB.

Table 1 | Samples studied, resistance rate, MAR index and diversity of each sample

Sample[a]	Source	No. of isolates	% resistant isolates[b]	MAR index	Diversity index (Di)	Comment
NO-01	Incoming sewage	24	67	0.15	0.98	
NO-02	Incoming sewage	22	23	0.04	0.95	
NO-03	Incoming sewage	24	29	0.04	0.97	
NO-04	Incoming sewage	24	17	0.03	1.00	
NO-05	Outgoing sewage	22	64	0.17	0.98	
NO-06	Outgoing sewage	23	39	0.09	0.98	
NO-07	Outgoing sewage	27	48	0.12	0.96	
NO-08	Outgoing sewage	21	38	0.08	0.96	
NO-09	Hospital sewage	18	67	0.17	0.83	Low Di
NO-10	Hospital sewage	21	67	0.24	0.87	Low Di high MAR
NO-11	Hospital sewage	21	100	0.34	0.76	Low Di high MAR
NO-12	Hospital sewage	22	23	0.06	0.60	Low Di
CH-01	Incoming sewage	21	33	0.07	0.96	
CH-02	Incoming sewage	22	50	0.13	0.94	
CH-03	Incoming sewage	22	41	0.13	0.97	
CH-04	Incoming sewage	22	27	0.07	0.97	
CH-05	River water	21	5	0.00	0.98	
CH-06	River water	22	5	0.01	0.97	
CH-07	River water	22	9	0.02	0.97	
CH-08	River water	54	19	0.04	0.98	
CH-09	Hospital sewage	22	45	0.23	0.94	High MAR
CH-10	Hospital sewage	20	90	0.32	0.93	High MAR
CH-11	Hospital sewage	22	32	0.07	0.89	Low Di
CH-12	Hospital sewage	22	50	0.16	0.92	
CH-13	Hospital sewage	22	50	0.14	0.87	Low Di
CH-14	Slaughterhouse sewage	22	41	0.04	0.90	Low Di
CH-15	Slaughterhouse sewage	22	32	0.03	0.92	
SE-01	Incoming sewage	48	17	0.03	0.97	
SE-02	Incoming sewage	24	29	0.04	0.95	
SE-03	Lake water	26	35	0.07	0.97	
SE-04	Lake water	13	31	0.05	0.90	Low Di
SE-05	Lake water	27	48	0.11	0.97	
SE-06	Lake water	27	0	0.00	0.82	Low Di
SE-07	Lake water	26	12	0.02	0.89	Low Di
SE-08	Lake water	16	0	0.00	0.77	Low Di
SE-09	Lake water	25	8	0.02	0.74	Low Di
SE-10	Hospital sewage	54	80	0.13	0.81	Low Di
SE-11	Hospital sewage	30	53	0.19	0.98	
SE-12	Hospital sewage	27	37	0.04	0.92	
All isolates	970	37	0.09	0.98		

[a]NO = Norway; CH = Chile; SE = Sweden.
[b]Resistant towards at least one antibiotic.

Comparisons of diversities and resistance rates among *E. coli* isolates from different environments

We analysed resistance to 10 antibiotics at breakpoint concentrations in 970 *E. coli* isolates from recipient water, municipal sewage and hospital sewage in Sweden, Norway and Chile. Some samples from recipient water in Chile and Sweden were also analysed. Figure 3 shows resistance rates towards individual antibiotics in different countries and Figure 4 shows the total resistance as MAR indices for different *E. coli* populations. As expected, hospital sewage clearly showed the highest resistance rates to all antibiotics in all three countries. Hospital sewage receives bacteria from hospitalised patients and hospital staff, and probably the resistance rates in those samples to a large extent reflect the resistance situation in the respective hospital. Lower resistance rates and MAR indices in all three countries were detected among bacteria in urban sewage. The *E. coli* population in urban sewage is mainly derived from the faecal flora in the urban population, and resistance rates among bacteria from urban sewage therefore probably reflect resistance rates among bacteria in the normal human population in these areas. The lowest resistance rates were found among bacteria in samples from recipient water (river, lake) in Chile whereas in Sweden the resistance rates were higher for some antibiotics in recipient water than in sewage. Available data from other studies also have shown lower proportions of antibiotic resistant bacteria in surface water than in sewage (Bouki *et al.* 2013). Possibly these samples contain a mixture of *E. coli* from humans and from wild/domestic animals, and the contribution of bacteria from animals has decreased the resistance rates among the *E. coli* in surface water.

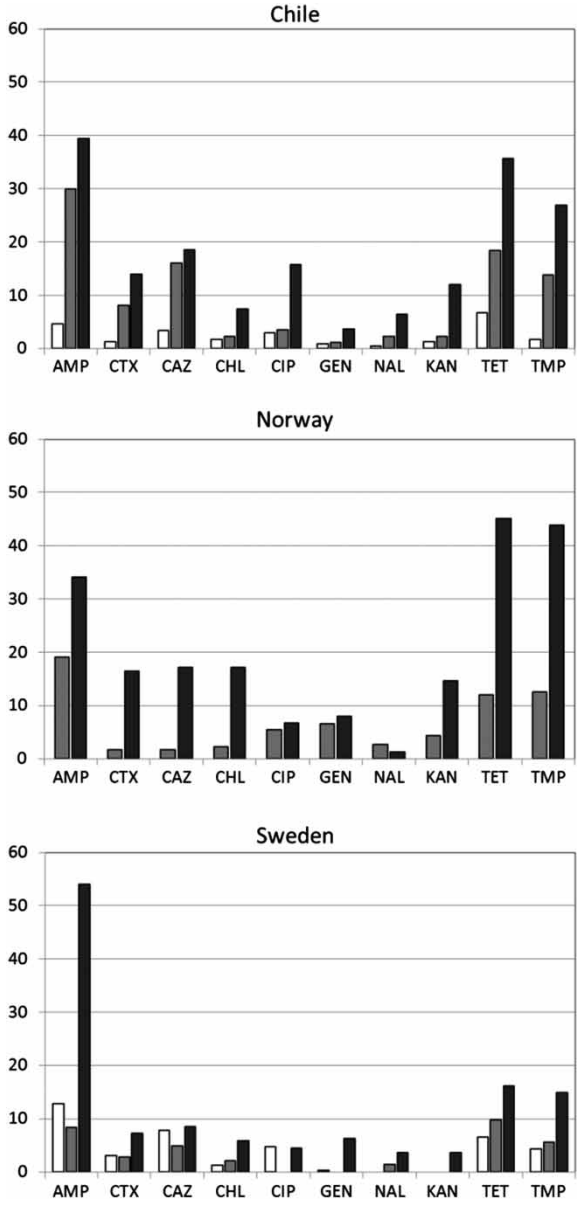

Figure 3 | Resistance rates among 970 *E. coli* isolates from sewage and surface water in Chile, Norway and Sweden. White columns = surface water; grey columns = urban sewage; black columns = hospital sewage. (No samples from surface water were available from Norway.)

DISCUSSION

In the present study we present a simple screening method for determination of antibiotic resistance among indicator bacteria from sewage and surface water, and some examples of results obtained from a study on *E. coli* isolates from different kinds of samples and different countries. It should be noted that these data are from a preliminary study, involving too few isolates per sampling area to form the basis for any conclusions regarding antibiotic resistance rates in the studied areas. For a 'real' study, more samples and more isolates per sample should be evaluated. Large studies, involving assays of more than a hundred isolates per day, can easily be performed by one person with the methods described here. Final results from the assay are obtained 2 days after sampling the sewage. The material cost for analysis of 100 isolates from one sewage sample is

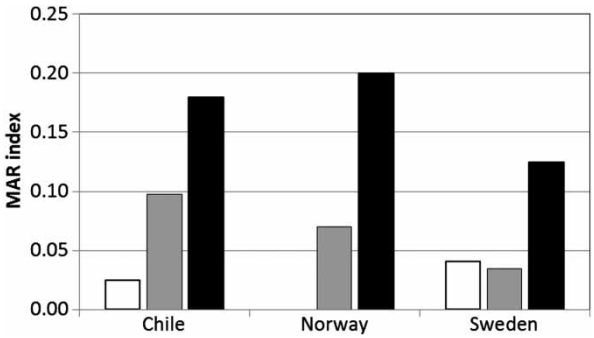

Figure 4 | MAR indices among 970 *E. coli* isolates from sewage and surface water in Chile, Norway and Sweden. White columns = surface water; grey columns = urban sewage; black columns = hospital sewage. (No samples from surface water were available from Norway.)

less than US$ 500, including costs for disposables, agar plates for isolation, media, and pre-prepared plates from PhPlate AB for phenotyping and resistance determination.

If sewage is to be used as source of bacteria for surveillance studies, an important question is whether the resistance rates among the tested bacteria could have increased in the sewage through selection, presumably by antimicrobials in the sewage water, or by growth of resistant bacteria in the biofilm or in sediments. Antibiotic residues in amounts that could affect the bacteria have been detected in STPs and sewage effluents from hospitals (Kümmerer & Henninger 2003; Jarnheimer *et al.* 2004; Novais *et al.* 2005). However, if the samples are collected directly at the sewage outlets from hospitals, or at the inflow to the STP, the bacterial populations have probably not been affected by low amounts of antibiotics in the sewage. Thus, most probable antibiotic resistance in *E. coli* isolated from incoming sewage mainly reflect resistance in intestinal *E. coli* flora of the individuals contributing to the sewage and not an autonomous development of resistance among bacteria in the sewers.

PhP typing as quality control of sewage and surface water samples

For surveillance studies on antibiotic resistance, the quality of resistance data obtained from sewage depends on whether the analysed bacteria really represent the faecal flora of many independent individuals, and not just faeces from one individual or a single household. A population of *E. coli* derived from floras of many individuals consists of a mixture of many different types/clones. This fact yields a high diversity of the *E. coli* flora in each sample, which can be measured using the PhP typing. The diversity is calculated by the PhP software and expressed as Simpson's diversity index (Di) (Atlas 1984). This index describes how well the isolates investigated are distributed into different types. Di can vary between 0 (all isolates are identical, i.e., belong to the same type) and 1 (all isolates are unique, i.e., belong to different types). The higher the diversity among the bacteria in a sewage sample is, the higher is the probability that the sample contains bacteria from many independent individuals. The PhP-RE system has been used in many previous studies involving several thousands of isolates (Ahmed *et al.* 2005; Landgren *et al.* 2005; Blanch *et al.* 2006; Reyes *et al.* 2010; Anastasi *et al.* 2012) to type *E. coli* and as a tool to measure the diversities of the studied populations. Using this method it has been shown that populations of normal *E. coli* of diverse origins always show high Di-values (usually >0.95), whereas low values (<0.90) indicate high prevalence of certain clonal groups among the studied isolates. Di is also a very useful measure when analysing data from epidemiological studies (Reyes *et al.* 2010).

In our study, resistance determination with AREB plates was combined with diversity determination using PhP-RE typing for all 970 isolates in the 39 samples studied. In Table 1, it can be seen that the *E. coli* populations in some samples show diversity indices considerably below 0.95, indicating that some bacteria were related and derived from the same individual, as for example the *E. coli* in samples collected from hospital sewage in Norway (Table 1). For example, sample NO11 has the highest MAR index of all samples (0.34) which could raise the suspicion that this hospital releases high amounts of multiresistant bacteria. However, the low diversity (only 0.76) indicates that this sewage sample contains bacteria from a few individuals only, something that is further supported by the dendrogram constructed from the PhP data of sample NO11 (Figure 5). Two dominating phenotypes, each one with a different antibiotic resistance pattern, are clearly visible in the dendrogram. Probably the isolates belonging to these two phenotypes are not random bacteria from different individuals, but are derived mainly from faeces originating from one or two individuals.

Low diversity in a sample from sewage can be due to improper sampling methods. In our study, samples from

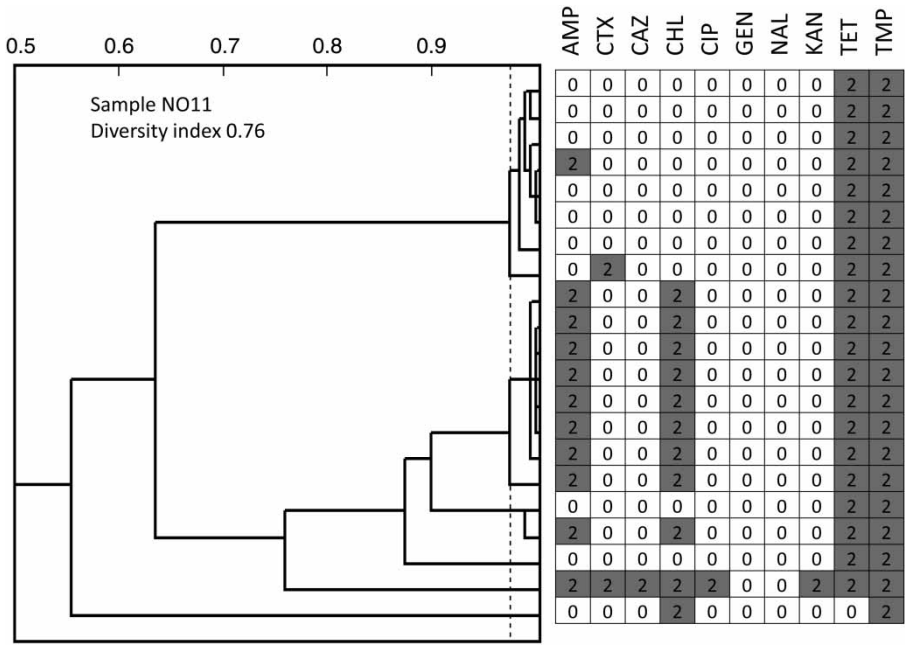

Figure 5 | Clustered PhP typing data and antibiotic resistance data added for 21 *E. coli* isolates from a sample from hospital sewage (NO11).

hospital sewage were often collected manually by dipping a flask into the sewage ('random sampling technique'), and there is always a danger that such a sample will contain faecal material from only one or a few individuals. Thus, in real life, several of the samples collected here using random sampling techniques from hospital sewage would be excluded from the epidemiological analysis due to low diversity.

Municipal STPs are often equipped for a continuous collection of samples, and, when possible, sewage samples were collected from the incoming flow during 24 hours. This means that the probability that each isolate represents a single individual is high, something that is supported by the high diversity of the *E. coli* flora in these samples. It is thus of high importance to use adequate sampling techniques, and to measure the diversity of the bacteria in each studied sample, in order to ensure that the bacteria studied are truly representative for a large population of humans.

Usage of a desktop scanner to read PhP and AREB plates

There are several microplate readers available on the market which can be used for reading PhP and AREB data. However, a microplate reader is an expensive piece of equipment, which often costs 4,000 euro or more. Our group has been using cheap desktop scanners for several years for quantification of microbial growth in microplates (Gabrielson *et al*. 2002; Rahman *et al*. 2004), and for measuring the indicator colour changes in the PhP plates (Landgren *et al*. 2005) with great success. Using the scanner, pictures of the microplates, such as the AREB plates and the PhP plates, are created, and by using a special software the microplate images can be converted to absorbance data. There are several advantages by using this approach, compared to normal measurements using a microplate reader or visual recordings of bacterial growth. First of all, no subjective judgements of positive or negative reactions are needed. Second, the plate images created by the scanner can be stored in an Excel file, together with the absorbance data and the result of the final analysis, as shown in the example in Figure 2. In this way data can be controlled and analysed any time, and errors that might have occurred during the plate handling can easily be discovered. Third, since the scanner only reads the actual colour in the wells and not the amount of absorbance, readings of colour changes in the PhP plates will not be affected by bacterial growth on the bottom of the plates, as it will when reading absorbances with microplate readers. Other advantages

are that errors that might occur by visual readings and recordings of 96 reactions in a microplate are eliminated, and that all raw data, including plate images, are always available for later evaluation. Thus, our approach is suitable for comparative studies, involving several laboratories. The low price and low need for high tech equipment also makes it suitable for investigations in developing countries or less well-equipped laboratories. It also should be noted that even pictures produced by a digital camera (or mobile phone) can, in principle, be analysed by the software, which could facilitate field studies with this technique.

Rahman *et al.* (2004) used a similar scanner method to read MIC data in microplates and compared the data to those obtained by a conventional microdilution test. He obtained an overall essential MIC agreement (maximum ± one dilution step difference) of 96.6% for 2,712 antibiotic–bacteria combinations, a high value that is similar to our data. Thus our data and previous studies show that resistance data obtained by measurements by a scanner are reliable and similar to those obtained by conventional methods. It should be noted that scanner assisted measurements of microplates (and other kinds of plates) of course also can be performed for other kinds of assays, provided that a software that can transfer images to absorbance data is available (such as the Microplate Analyser software used here).

CONCLUSIONS

In conclusion, we have developed a simple and rapid screening method for surveillance of antibiotic resistance in bacteria from sewage and aquatic environments, combined with a rapid typing system that serves as quality control. We have also shown that this method could be useful for monitoring resistance rates among *E. coli* in sewage and surface water of different origins, and that resistance rates in sewage seem to give information representative of resistance rates in the population contributing to the sewage. Repeated analysis of samples from the same source might give useful information on the appearance and the spread of resistance in the population. For a more detailed analysis of resistance, only those isolates that are resistant according to AREB need to be further studied. Furthermore, by including the PhP typing system for quality control, the appearance of and changes in frequencies of resistant clones will be apparent. It is our belief that for surveillance studies of antibiotic resistance in various populations, like hospitals, care centres or a defined community, this method could be useful. The advantages of our method compared to existing methods based on analysis of a large number of faecal samples from sick and/or healthy individuals are, among others, the simplicity, the higher speed, the lower cost and the ability to analyse many isolates simultaneously.

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REFERENCES

Ahmed, W., Neller, R. & Katouli, M. 2005 Evidence of septic system failure determined by a bacterial biochemical fingerprinting method. *J. Appl. Microbiol.* **98** (4), 910–920.

Anastasi, E. M., Matthews, B., Stratton, H. M. & Katouli, M. 2012 Pathogenic *Escherichia coli* found in sewage treatment plants and environmental waters. *Appl. Environ. Microbiol.* **78** (16), 5536–5541.

Atlas, R. M. 1984 Use of Microbial Diversity Measurements to Assess Environmental Stress. In: *Current Perspectives in Microbial Ecology*. American Society of Microbiology, Washington, DC, pp. 540–545.

Blanch, A. R., Belanche-Muños, L., Bonjoch, X., Ebdon, J., Gantzer, C., Lucena, F., Ottoson, J., Kourtis, C., Iversen, A., Kühn, I., Mocé, L., Muniesa, M., Schwartzbrod, J., Skraber, S., Papageorgiou, G. T., Taylor, H., Wallis, J. & Jofre, J. 2006 Integrated analysis of established and novel microbial and chemical methods for microbial source tracking. *Appl. Environ. Microbiol.* **72** (9), 5915–5926.

Bouki, C., Venieri, D. & Diamadopoulos, E. 2013 Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: a review. *Ecotox. Environ. Safe.* **91**, 1–9.

Gabrielson, J., Hart, M., Jarelov, A., Kühn, I., McKenzie, D. & Möllby, R. 2002 Evaluation of redox indicators and the use of digital scanners and spectrophotometer for quantification of microbial growth in microplates. *J. Microbiol. Methods* **50**, 63–73.

Jarnheimer, P. A., Ottoson, J., Lindberg, R., Stenström, T.-A., Johansson, M., Tysklind, M., Winner, M. M. & Olsen, B. 2004 Fluoroquinolone antibiotics in a hospital sewage line; occurrence, distribution and impact on bacterial resistance. *Scand. J. Infect. Dis.* **36** (10), 752–755.

Jorgensen, J. H. & Ferraro, M. J. 2009 Antimicrobial susceptibility testing: a review of general principles and contemporary practices. *Clin. Infect. Dis.* **49**, 1749–1755.

Kahlmeter, G. 2003 An international survey of the antimicrobial susceptibility of pathogens from uncomplicated urinary tract infections: the ECO.SENS Project. *J. Antimicrob. Chemother.* **51** (1), 69–76.

Krumperman, P. H. 1983 Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol.* **46** (1), 165–170.

Kühn, I. & Möllby, R. 1993 The PhP RS system – A simple microplate method for studying coliform bacterial populations. *J. Microbiol. Methods* **17**, 255–259.

Kühn, I., Iversen, A., Burman, L. G., Olsson-Liljequist, B., Franklin, A., Finn, M., Aarestrup, F., Seyfarth, A.-M., Blanch, A. R., Vilanova, X., Taylor, H., Caplin, J., Moreno, M. A., Dominguez, L., Herrero, I. A. & Möllby, R. 2003 Comparison of enterococcal populations in animals, humans, and the environment – a European study. *Int. J. Food Microbiol.* **88** (2–3), 133–145.

Kümmerer, K. & Henninger, A. 2003 Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin. Microbiol. Infect.* **9** (12), 1203–1214.

Landgren, M., Odén, H., Kühn, I., Österlund, A. & Kahlmeter, G. 2005 Diversity among 2481 *Escherichia coli* from women with community acquired lower urinary tract infections in 17 countries. *J. Antimicrob. Chemother.* **55** (6), 928–937.

Novais, C., Coque, T. M., Ferreira, H., Sousa, J. C. & Peixe, L. 2005 Environmental contamination with vancomycin-resistant enterococci from hospital sewage in Portugal. *Appl. Environ. Microbiol.* **71** (6), 3364–3368.

Rahman, M., Kühn, I., Rahman, M., Olsson-Liljequist, B. & Möllby, R. 2004 Evaluation of a scanner-assisted colorimetric MIC method for susceptibility testing of gram-negative fermentative bacteria. *Appl. Environ. Microbiol.* **70** (4), 2398–2403.

Reinthaler, F. F., Galler, H., Feierl, G., Haas, D., Leitner, E., Mascher, F., Melkes, A., Posch, J., Pertschy, B., Winter, I., Himmel, W., Marth, E. & Zarfel, G. 2013 Resistance patterns of *Escherichia coli* isolated from sewage sludge in comparison with those isolated from human patients in 2000 and 2009. *J. Water Health* **11** (1), 13–20.

Reyes, D., Vilchez, S., Paniagua, M., Colque-Navarro, P., Weintraub, A., Möllby, R. & Kühn, I. 2010 Diarrheagenic *Escherichia coli* markers and phenotypes among fecal *E. coli* isolates collected from Nicaraguan infants. *J. Clin. Microbiol.* **48** (9), 3395–3396.

San Martin, B., Bravie, V. & Borie, C. 2005 Antimicrobial resistance monitoring in cattle in Chile using *E. coli* as the indicator bacteria. *Archivos de Medicina Veterinaria* **37**, 117–123.

Zhang, R. Q., Ying, G. G., Su, H. C., Zhou, L. J. & Liu, Y. S. 2013 Antibiotic resistance and genetic diversity of *Escherichia coli* isolates from traditional and integrated aquaculture in South China. *J. Environ. Sci. Health B* **48** (11), 999–1013.

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