Ecotoxicology and Environmental Safety 143 (2017) 38–45

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Occurrence of antimicrobial resistance bacteria in the Yodo River basin, Japan and determination of beta-lactamases producing bacteria

Naoyuki Yamashita*, Yohei Katakawa, Hiroaki Tanaka

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

ARTICLE INFO

Keywords:
Antimicrobial resistance
Beta-lactamase
River basin
Wastewater effluent
Escherichia coli

ABSTRACT

Antimicrobial resistant bacteria are widespread in aquatic environments. The aim of the present study was to obtain information on the occurrence of bacteria with antimicrobial resistance and their multiple antimicrobial resistance (MAR) patterns in a river basin in Japan. In addition, the occurrence of fecal bacteria producing extended-spectrum beta-lactamases (ESBLs) and metallo-beta-lactamase (MBL) in the aquatic environment was determined. Among the *Escherichia coli* isolates recovered from river samples upstream, 55% isolates were resistant to at least one antimicrobial and 18% were MAR. Among the *E. coli* isolates recovered from wastewater treatment plant (WWTP) effluent samples, 74% isolates were resistant to at least one antimicrobial and 46% were MAR. These findings suggest that the presence of WWTP effluent will increase the degree of contamination with MAR in the aquatic environment. Among the ampicillin-resistant isolates recovered from river samples, 21% isolates were judged as ESBL-producing and none (0%) was judged as MBL-producing. Among the ampicillin-resistant isolates recovered from WWTP effluent samples, 21% were judged as ESBL-producing and 1% was judged as MBL-producing. As for the hospital wastewater samples, 48% were judged as ESBL-producing and 3% were judged as MBL-producing. The percentage of ESBLs and MBL production was highest in hospital wastewater samples. All of the ESBL-producing isolates detected had resistance to ampicillin, cephazolin, and cefpodoxime and many ESBL-producers had resistance not only to beta-lactams but also to other kinds of antimicrobials such as aminoglycosides and quinolones. The frequency of detection of MBL-producers was much lower than that of ESBL-producers and MBL-producers were not detected in the river samples. However, the detection in WWTP effluent samples indicated that bacteria with MBL were present downstream of the WWTP at low concentrations. Thus, ESBLs and MBL have already been spread around aquatic environments.

1. Introduction

The remarkable success of infectious disease therapy through the creation of new antimicrobials over the past half century has simultaneously led to the emergence of antimicrobial resistance in bacteria. Most currently used antimicrobials are chemically semisynthetic modifications of various natural compounds (Hamilton-Miller, 2008). These include, for example, the beta-lactams, which consist of the penicillins, the monobactams, the cephalosporins (the cephems), and the carbapenems. The beta-lactams have been used widely because of their strongly selective activity and few side effects. On the other hand, the emergence of pathogens producing beta-lactamases, which are enzymes that hydrolyze the basic skeletons of the beta-lactams, has made some of these antimicrobials ineffective (Bush et al., 1995). In the 1980s, a new group of enzymes, the extended-spectrum beta-lactamases (ESBLs), was first detected in Europe (Knothe et al., 1983). ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins (Bush et al., 1995), which include the third-generation cephalosporins such as cefotaxime and ceftazidime. Subsequently, bacteria producing metallo-beta-lactamase (MBL), which hydrolyzes not only the third-generation cephalosporins but also imipenem and others, were detected (Minami et al., 1996). Recently, a new-type MBL named NDM-1 (New Delhi metallo-beta-lactamase) was detected in *Escherichia coli* and *Klebsiella pneumoniae* isolated from a Swedish patient returned from India (Walsh et al., 2011). The most common bacteria in which NDM-1 is detected are the enterobacteria, including *E. coli* and *K. pneumoniae*, NDM-1 producers have now been isolated internationally from patients (Göttig et al., 2010; Livermore et al., 2011; Johnson et al., 2013). Most of the previously identified NDM-1 producers have been resistant not only to the beta-lactams but also to aminoglycoside and fluoroquinolone antimicrobials (Poirel et al., 2010, 2011). Therefore, there are fears that pathogens that cause diseases such as blood poisoning, pneumonia, and urinary-tract infections are developing resistance to a wide range of antimicrobials. Although the problems posed by this bacterial resis-

* Corresponding author.
E-mail address: yamashita@biwa.eqc.kyoto-u.ac.jp (N. Yamashita).

http://dx.doi.org/10.1016/j.ecoenv.2017.04.053
Received 28 November 2016; Received in revised form 21 April 2017; Accepted 26 April 2017
Available online 09 May 2017

tance have received broad attention in the field of medicine, the prevalence of multiple antimicrobial resistance (MAR) among bacteria in aquatic environments has received much less attention.

Enterobacteria expelled as feces by humans and animals enter aquatic environments directly or through sewerage systems. These bacteria could develop tolerance to some antimicrobials. In addition, they could pass their resistant genes to other bacteria through transferable plasmids (Goldstein et al., 1983; Watanabe et al., 1991; Giakkoupi et al., 2006). The presence of pathogenic bacteria with antimicrobial resistance in aquatic environments could be a source of emerging infections, particularly in water-based recreation areas and fishing locations. For this reason, monitoring of antimicrobial resistance and MAR among the bacteria in aquatic environments is important in terms of sanitary water management. Antimicrobial resistance has already been found in fecal bacteria from a variety of water samples (Ham et al., 2012; Korzeniewska et al., 2013; Zhang et al., 2014; Titilawo et al., 2015; Luo et al., 2015; Honda et al., 2016). However, few studies of MAR in aquatic environments have focused on cephalosporin resistance caused, for example, by ESBLs, which have emerged as a serious problem in medicine and livestock production.

Escherichia coli is considered to be the important microorganism in the aquatic environment as bacteria originating from humans and animals and is an indicator of fecal contamination. Then, investigation of antimicrobial resistant *E. coli* will lead to an understanding of the contamination by antimicrobial resistant bacteria originating from humans and animals feces in the river basin. There are many kinds of antimicrobials and effects of the antimicrobials onto bacteria are different depending on bacterial types. Since the susceptibility of *E. coli* onto different antimicrobials is well-known (Stelling et al., 2005), investigation of *E. coli* will be advantageous to understand the occurrence of antimicrobial resistant bacteria in the river basin. Additionally, one of the problems by antimicrobial resistant bacteria is horizontal gene transfer of antimicrobial resistant genes by bacterial transmission. The horizontal gene transfer from *E. coli* to other bacteria has been reported (Hayashi et al., 2001). Therefore, pathogenic bacteria other than *E. coli* could have antimicrobial resistance by the horizontal gene transfer if *E. coli* has antimicrobial resistant genes.

Our first objective was to obtain information on the occurrence of bacteria with antimicrobial resistance and their resistance patterns by characterizing the MAR of fecal bacteria in water samples in the Yodo River basin in Japan. This basin is located in western Japan; percentage of sewered population in the area is about 95%. Water quality of the Yodo River is considerably affected by wastewater treatment plant (WWTP) effluent. Our second objective was to determine the occurrence and quantity of fecal bacteria with MAR producing ESBLs or MBL in the aquatic environment. We assessed antimicrobial resistance by using the disk diffusion method usually used in medical field. By this method, bacterial resistance to a number of antimicrobials is tested phenotypically.

2. Material and methods

2.1. Sample collection

Water samples were collected from the Yodo River basin in Japan, which is located in western Japan (Fig. 1). The Yodo River basin extends over 8240 km^2, and covers urbanized areas such as Kyoto and Osaka, and the water quality of the downstream is influenced by WWTP effluent upstream. The Entire population of Kyoto city, which is located at the upper reaches of the Yodo river basin, is about 1.4 million. WWTP effluent equivalent to about 0.5 million inhabitant is discharged into the Katsura river from WWTP effluent site (site no.2) shown in the Fig. 1. In addition, part of the water is recovered and used downstream site (in Osaka) as tap water. The Katsura River, one of the main tributaries, passes through Kyoto, and its flow continues for several kilometers through Osaka. Both Kyoto and Osaka are big cities with populations of more than 1 million. Effluent from several WWTPs flows into the Katsura River and accounts for about 40% of the water in that river (Kyoto City Waterworks and Sewerage Bureau, 2015). Samples collected were river water, WWTP effluent, and hospital wastewater. WWTP effluent was considered to be the main source of fecal bacteria from human feces in the Yodo River basin. Hospital wastewater was suspected to contain bacteria with MAR because of the heavy use of antibiotics in hospital.

River water and WWTP effluent were sampled in November and December 2011 and 2012 from the Katsura River in the Yodo River basin. River water samples were collected at the Kuze Bridge (site no.1) and the Miyamae Bridge (site no.3), which were located upstream and downstream, respectively, of the WWTP (Fig. 1). WWTP effluent samples were collected at the outlet of the WWTP (site no.2), located 1.8 km downstream from sampling site no.1 and 6.0 km upstream from sampling site no.3. Hospital wastewater was collected in November and December 2011 from a wastewater outlet of a hospital located in the Yodo watershed (site no.4, site is not shown in Fig. 1). This hospital wastewater eventually flows into the Yodo River through a main drainage canal. All water samples were collected in stainless-steel cans, kept at 4 °C in sterile bottles, and analyzed within 24 h. Sample collections were conducted at a stable condition of river water volume. Since we avoid the sample collections during rainy condition, water samples were not affected by rain water. Sample collections for *E. coli* measurement were conducted 4–6 times for each sampling site and antimicrobial susceptibility of *E. coli* was tested 3 times for each sampling site during sampling campaigns.

2.2. Enumeration and isolation of E. coli

E. coli was selected as the test microorganism for the following three reasons: (i) it is an indicator of fecal contamination originating from humans and animals; (ii) it has well-known susceptibility to different antimicrobials; (iii) it has the capacity for horizontal gene transfer by bacterial transmission, for example via transferable plasmids, transposons, or transduction.

The abundance of *E. coli* in the water samples taken from sites no.1, 2, and 3 was estimated by using the membrane filtration method. Chromocult Coliform agar (Merck KGaA, Darmstadt, Germany), the selective chromogenic medium specific for *E. coli*, was used to enumerate *E. coli*. Chromocult Coliform Agar enables the specific detection of *E. coli* in water samples and US-EPA has approved the Chromocult Coliform Agar as a method for detection of *E. coli* and total coliforms in water (US Environmental Protection Agency, 2002). Typical *E. coli* colonies appear blue color on Chromocult Coliform agar plates. Plates were incubated for 24 h at 37 °C and then the number of colonies was counted. Typical colonies from each water sample were randomly selected and subcultured on new Chromocult Coliform agar plates.

In the samples taken from sites no.2, 3, and 4, ampicillin-resistant *E. coli* was isolated. Ampicillin-resistant *E. coli* was isolated on Chromocult Coliform agar containing 32 mg/L ampicillin (Wako, Osaka, Japan). According to the Clinical and Laboratory Standard Institute (CLSI) standard (Clinical and Laboratory Standards Institute, 2014), *E. coli* strains that were able to colonize the plate at that ampicillin concentration were considered resistant to ampicillin. Plates were incubated for 24 h at 37 °C and then the number of colonies was counted. Typical colonies from each water sample were randomly selected and subcultured on new Chromocult Coliform agar plates.

2.3. Antimicrobial susceptibility testing

Antimicrobial resistance was determined by using the disk diffusion method as described by CLSI (Clinical and Laboratory Standards Institute, 2014). The resistance of the *E. coli* isolates and ampicillin-resistant *E. coli* isolates was tested against 10 and 12 antimicrobials,