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A new adsorption-elution technique for the concentration of aquatic extracellular antibiotic resistance genes from large volumes of water

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ABSTRACT

Extracellular antibiotic resistance genes (eARGs) that help in the transmission and spread of antibiotic-resistant bacteria are emerging environmental contaminants in water, and there is therefore a growing need to assess environmental levels and associated risks of eARGs. However, as they are present in low amounts, it is difficult to detect eARGs in water directly with PCR techniques. Here, we prepared a new type of nucleic acid adsorption particle (NAAP) with high capacity and developed an optimal adsorption-elution method to concentrate eARGs from large volumes of water. With this technique, we were able to achieve an eARG recovery rate of above 95% from 10 L of water samples. Moreover, combining this new method with quantitative real-time PCR (qPCR), the sensitivity of the eARG detection was 10^4 times that of single qPCR, with the detection limit lowered to 100 gene copies (GCs)/L. Our analyses showed that the eARG load, virus load and certain water characteristics such as pH, chemical oxygen demand (COD_{Mn}), and turbidity affected the eARGs recovery rate. However, high eARGs recovery rates always remained within the standard limits for natural surface water quality, while eARG levels in water were lower than the detection limits of single qPCR assays. The recovery rates were not affected by water temperature and heterotrophic plate counts (HPC). The eARGs whatever located in the plasmids or the short-length linear DNAs can be recovered from the water. Furthermore, the recovery rate was high even in the presence of high concentrations of plasmids in different natural water (Haihe river, well water, raw water for drinking water, Jinhe river, Tuanbo lake and the Yunqiao reservoir). By this technology, eARGs concentrations were found ranging from $(2.70 \pm 0.73) \times 10^2$ to $(4.58 \pm 0.47) \times 10^4$ GCs/L for the extracellular ampicillin resistance gene and $(5.43 \pm 0.41) \times 10^2$ to $(2.14 \pm 0.23) \times 10^4$ GCs/L for the extracellular gentamicin resistance gene in natural water for the first time, respectively. All these findings suggest that NAAPs have great potential for the monitoring of eARGs pollution in water.

1. Introduction

The development of antibiotic resistance is a serious problem globally (Allen et al., 2010; Cabello, 2006; WHO, 2012). A large number of antibiotic-resistant bacteria (ARB) and even multiple drug-resistant bacteria (MDRB) are found in water (Bessa et al., 2014; Moges et al., 2014; Mohanta and Goel, 2014), and the transfer and spread of antibiotic resistance genes (ARGs) in water is considered to play a key role in the dissemination of ARB (Pruden et al., 2012; Rahube and Yost, 2010; Rizzo et al., 2013).

Two forms of natural ARGs—intracellular ARGs (iARGs) and extracellular ARGs (eARGs)—are present in water(Nielsen et al., 2007; Zhang et al., 2013). iARGs are located in the bacterial compartment and promote ARB dissemination via conjugation and transduction (Mao et al., 2014). Increasing number of researchers focus on iARGs concentration in natural water bodies which is up to 6.7 × 106 GCs/L in natural water (Luo et al., 2010; Wang et al., 2012; Xu et al., 2015). eARGs, unlike iARGs, originate from the lysis of dead ARB or are secreted by live ARB. Thus, with eARGs, dissemination of drug resistance is possible via natural transformation wherein competent non-resistant bacteria acquire

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antibiotic resistance by taking up eARGs from the aquatic environment under particular (micro) environmental conditions such as biofilm formation and sedimentation(Li, 2001; Molin and Tolker, 2003; Pietramellara et al., 2009). eARGs with genetic mobile platforms such as plasmids, transposons and integrons in the water have been gained increasing attention for their potential contribution to the spread of antibiotic resistance among bacterial communities(Baur et al., 1996; Bennett, 2008; Han et al., 2012; Paget and Simonet, 1994; Stokes, 2011). eARGs are defined as an emerging environmental contaminant that can persist in the aquatic environment for an extended period of time, ranging up to months, with the help of soil colloids and sand particles(Borin et al., 2008; Cai et al., 2006; Pruden et al., 2006, Pietramellara et al., 2009); thus, eARGs in water may pose a serious threat to both human health and ecosystem (Pruden et al., 2006). Investigating the pollution caused by eARGs can not only help in assessing their environmental concentrations and associated risks, but also help to better understand and clarify their contribution to the dissemination of ARGs in water.

According to Zhang et al.(Zhang et al., 2013), the amount of eARGs in extracellular DNA extracts from sludge are usually two to three orders of magnitude lower than the amount of iARGs in the corresponding intracellular DNA extracts(Beebee, 2006; Deflaun, 1986; Zhanbei and Ann, 2013). However, very little is known about eARG pollution in water due to its limited concentration: eARGs are undetectable directly by PCR and qPCR as a result of their low concentration. It is therefore essential to concentrate eARGs from 1 L or more water prior to eARG detection. Nucleic acid precipitation with organic solvents such as ethanol and isopropanol is a widely used technique to purify or concentrate free nucleic acids in aqueous solution. The recovery rate with this procedure is quite high and the extracted nucleic acids have high purity (Deflaun, 1986; Tan, 2009). However, this procedure is rather complicated to perform, requires a lot of time, and causes environmental toxicity because it requires the use of a large amount of organic reagents. In addition, the recovery rate and purity of the nucleic acids obtained are easily affected by the quality of the water. Silica gel membrane adsorptive method, which utilizes the adsorption of spin column (silica gel) to nucleic acid, is another widely applied method in the forms of commercial kits such as E. Z.N.A. gel extraction kit (Omega Bio-Tek) and DNA Isolation kit (Roche). This method is rapid and sensitive, and it is suitable for small volumes of less than 1 mL(Kjeldsen et al., 2010; Metcalf and Weese, 2012). In recent years, filtration environmental DNA capture method has been developed to capture environmental DNA in the water(Jerde et al., 2011). However, only 16% recovery of the spiked extracellular DNA (eDNA) were reported from 200 mL water in the help of the mixed cellulose acetate and cellulose nitrate (MCE) membrane filter(Zhanbei and Ann, 2013). To the best of our knowledge, there are no reported techniques for recovering aquatic eARGs from large volumes of water (1 L or more).

In this study, we described novel nucleic acid adsorption particles [NAAPs, silica gel coated with $Al(OH)_3$] with high capacity for eARGs and established a new adsorption-elution method to concentrate eARGs from large volumes of water with a high efficiency for the first time, which is based on the electrostatic adsorption of eARGs with NAAPs, followed by the interaction disruption of organic eluent which is consisted of 3× broth and 0.05 mol/L glycine (Gly), and then isopropanol precipitation. The effect of the conditions of the procedure, e.g., eluents, flow rate and water quality indicators e.g. pH value, turbidity and chemical oxygen demand (COD_{Mn}) on recovery rate were also investigated using antibiotic-resistant plasmids. Moreover, to validate its applicability and efficiency, various natural water samples were explored and eARGs concentrations in natural water were reported for the first time.

2. Materials and methods

2.1. Microorganisms

Escherichia coli (ATCC 25922), the bacteriophage MS2 and its host strain *E. coli* (ATCC 15597) were purchased from the American Type Culture Collection. *Pseudomonas aeruginosa PA14*, whose gentamicin resistant gene was on the chromosome (Gm^r), was provided by Nankai University (Choi and Schweizer, 2006). They were grown in lysogeny broth (LB) medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37 °C. The bacteriophage MS2 was prepared and detected by the double-layer plaque assay (Hornstra et al., 2011). They should be filtered with a PVDF filter (0.45 μm, Millipore, USA) to remove its host. *E. coli* K12 harboring the RP4 plasmid ($Ap^rKm^rTc^r$, 60099 bp) and *E. coli* HB101 harboring the pUC19 plasmid (Ap^r, 2686 bp) or pRI 909 plasmid (Km^r, 9168 bp) were reserved in our laboratory. *E. coli* K12 with RP4 was cultured in LB medium containing 100 μg/mL ampicillin, 50 μg/mL kanamycin, and 7.5 μg/mL tetracycline. *E. coli* HB101 with pUC19 or pRI 909 was cultured in LB medium containing 100 μg/mL ampicillin or 100 μg/mL kanamycin, respectively.

2.2. Quality of the water samples

The water samples were collected between March and May 2015 or in October 2015. The sites of collection were as follows: Haihe river water (39°6′58.36″N, 117°13′20.66″E), well water (38°58′21.37″N, 116°8′11.21″E), raw water for drinking water (38°54′39.06″N, 117°10′20.22″E), Jinhe river water (39°4′5.18″N, 117°6′12.20″E), Tuanbo lake (38°54′39.06″N, 117°10′20.22″E), and Yunqiao reservoir (40°3′49.64″N, 117°30′48.24″E). The parameters of water quality were measured and are summarized in Table 1. After the temperature and pH data were collected at the site, the water samples were delivered on ice to the laboratory within 2 h. According to the protocols described by the manufacturer, heterotrophic plate counts (HPC) and total thermotolerant coliforms (TTC) in all the water samples were assayed on Luria–Bertani agar and deoxycholate agar (BD Difco, USA). Turbidity was measured using a portable turbidimeter (Hash 2100Q, UK). COD_{Mn} was determined using the standard reflux titrimetric method (APHA) (Association et al., 2012).

2.3. Preparation of NAAPs

First, 25.2 g $AlCl_3$ was slowly dissolved in 4 L of deionized water to form a clear solution at room temperature; then, 180 mL of 2 mol/L Na_2CO_3 was added. The pH of the mixed solution was adjusted to 7.2 with 1 mol/L Na_2CO_3 (3–5 mL), and it was stirred well until it was homogeneous. After the solution was maintained at room temperature for 6–12 h, it was centrifuged at 1100 × *g* for 15 min in Allegra X-12R Centrifuge (Beckman Coulter, Inc., USA.) and the supernatant was discarded. The $Al(OH)_3$ sediment was resuspended in 1 L 0.14 mol/L NaCl and centrifuged at 1100 × g for 15 min again. Then, the supernatant was discarded and $Al(OH)_3$ sediment was resuspended in 1 L 0.14 mol/L NaCl and centrifuged once more as above. Al $(OH)_3$ sediment was suspended in 2 L of 0.14 mol/L NaCl and sterilized by autoclaving. The autoclave was maintained at 120 °C for 20 min and cooled to room temperature. Finally, the $AL(OH)_3$ solution (47.8%, V/V) was mixed well with 5–6% (g/mL) silica gel with a 60–100 mesh size (Kunhai Co., Shanghai, China) and dried at 60 °C for 36 h. The final drying silica gels coated with $Al(OH)_3$ were obtained as NAAPs.