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Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam

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ABSTRACT

The ubiquitous application and release of antibiotics to the environment can result in bacterial antibiotic resistance, which in turn can be a serious risk to humans and other animals. Southeast Asian countries commonly apply an integrated recycling farm system called VAC (Vegetable, Aquaculture and Caged animal). In the VAC environment, antibiotics are released from animal and human origins, which would cause antibiotic-resistant bacteria (ARB). This study evaluated occurrence of ARB in the VAC environment in northern Vietnam, with quantitative analysis of antibiotic pollution. We found that sulfonamides were commonly detected at all sites. In dry season, while sulfamethazine was a major contaminant in pig farm pond (475–6662 ng/l) and less common in city canal and aquaculture sites, sulfamethoxazole was a major one in city canal (612–4330 ng/l). Erythromycin (154–2246 ng/l) and clarithromycin (2.8–778 ng/ml) were the common macrolides in city canal, but very low concentrations in pig farm pond and aquaculture sites. High frequencies of sulfamethoxazole-resistant bacteria (2.14–94.44%) were found whereas the occurrence rates of erythromycin-resistant bacteria were lower (<0.01–38.8%). A positive correlation was found between sulfamethoxazole concentration and occurrence of sulfamethoxazole-resistant bacteria in dry season. The sulfamethoxazole-resistant isolates were found to belong to 25 genera. *Acinetobacter* and *Aeromonas* were the major genera. Twenty three of 25 genera contained *sul* genes. This study showed specific contamination patterns in city and VAC environments and concluded that ARB occurred not only within contaminated sites but also those less contaminated. Various species can obtain resistance in VAC environment, which would be reservoir of drug resistance genes. Occurrence of ARB is suggested to relate with rainfall condition and horizontal gene transfer in diverse microbial community.

1. Introduction

It is known that antibiotics cause antibiotic-resistant bacteria (ARB) in hospital-inquired infection. In recent years, antibiotics contamination is recognized as an emerging environmental pollution in aquatic environments, because of their potential adverse effects on the ecosystem and human health (Huang et al., 2001; Kümmerer, 2009). Majority of antibiotics used for human, plants and animals are excreted into the environment as intact or decomposed form via various pathways, including wastewater effluent discharge, runoff from land to which agricultural or human waste has been applied, and leaching (Zhang et al., 2009). Antibiotic residues in the environment impose selective pressure on bacterial populations, which results prevalence of resistant bacteria even at sub-inhibitory low concentrations. Other pollutants are also known as selective agents (Stepanauskas et al., 2005, 2006). Additionally, the raw wastewater contaminated by antibiotics released into aquatic environments often carries human and animal pathogenic bacteria, in addition to commensal bacteria, and many of these organisms harbor antibiotic-resistance genes. Therefore, water constitutes a way of dissemination of not only antibiotic-resistant bacteria, but also the resistance genes, which genetically change in natural bacterial ecosystems (Baquero et al., 2008; Rosenblatt-Farrell, 2009). The ARB has been found in various aquatic environments (Kümmerer, 2004; Kim and Aga, 2007; Schluter et al., 2007; Watkinson et al., 2007;Caplin et al., 2008; Vanneste et al., 2008). In particular, our previous studies showed that aquatic environment is potential reservoirs of ARB (Nonaka et al., 2000, 2007; Kim et al., 2003, 2004; Hoa et al., 2008) even in pristine conditions (Kobayashi et al., 2007; Rahman et al., 2008). On the other hand, a variety of antibiotics have been detected in the aquatic environments (Hirsch et al., 1999; Göbel et al., 2005; Zhang et al., 2009), from ng/l to μg/l levels, which are lower than

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therapeutic levels (Göbel et al., 2005; Managaki et al., 2007, Zhang et al., 2009). In some cases, high concentration with mg/l order was found (Le and Munekage, 2004; Le et al., 2005). At the sub-therapeutic concentrations of antibiotics detected in the aquatic environments, the question is whether antibiotics could have an impact on bacterial populations (Kümmerer, 2009). Very few studies have investigated the relationship between antibiotic contamination and antibiotic resistance in aquatic environments relating to human and agricultural activities. Furthermore the diversity of ARB within integrated recycling farm VAC (Vegetable, Aquaculture and Caged animal) systems is virtually unknown.

From these background, we hypothesized that Asian integrated agriculture environment should be polluted with various antibiotics derived from animal and human origins, which selects ARB in the environment. To clarify this, we conducted monitoring of concentrations of residual antibiotics and ARB in the VAC environments. Antibiotic residues were often detected in the human-impacted aquatic environments of Southeast Asian countries and China (Le and Munekage, 2004; Richardson et al., 2005; Managaki et al., 2007). In Vietnam, agriculture and aquaculture are the major economic activities, and excessive and unregulated use of antibiotics was commonly found in human medicine, management of livestock, and aquaculture (Le et al., 2005; Managaki et al., 2007; Duong et al., 2008). The Red River delta of northern Vietnam is an appropriate study site because of following conditions. This area is one of the largest deltas in Southeast Asia, including 9 provinces along with 2 municipalities, the capital city of Hanoi, and the main seaport of Haiphong. The delta is an agriculturally rich area, which is densely populated by 11,000,000 people (Berg et al., 2001). The heavy and unregulated use of antibiotics along with the discharge of untreated wastewater into the aquatic environments might cause significant contamination of both antibiotic residues and ARB. On the basis of the information obtained during our pre-study onsite interview-based survey, we identified sulfonamides, trimethoprim, and macrolides as the target antibiotics.

In the present study, we first characterized the pattern of contamination by antibiotics and ABR in the rainy and dry seasons in the Red River delta area. To better understand whether the antibiotic residues in the aquatic environment is an important source in selecting and creating an increasingly resistant bacteria in the environments, the statistical correlation between the concentration of antibiotic residues and ARB occurrence was analyzed. A part of sulfonamide-resistant (SR) bacteria was isolated and classified in January when the relationship between sulfonamide and occurrence of SR bacteria was possibly observed. The possession of *sul* genes was also monitored. The variation and distribution of *sul* genes in manure were examined in Europe, and the molecular information for a method of detection is available (Heuer et al., 2009). Therefore *sul* genes were the priority to survey in this study.

2. Materials and methods

2.1. Sampling area and procedure

Specific integrated aquaculture-agriculture system is major in Vietnam, at which freshwater fishponds directly receive excreta from intensive pig farms (termed “pig farm/fish ponds”) (Hoa et al., 2008). Sampling was performed at 10 sites in the Red River delta of northern Vietnam, including 3 sites of a city canal in Hanoi (HNC-1–3), 3 sites at 3 pig farm/fish ponds in Hatay (HNP-1–3), and 4 sites at 4 coastal shrimp ponds of Haiphong (HNAQ-1–4) (Fig. 1). The HNC sites were upper-, middle- and down-stream of the main canal of Hanoi City, and the HNP and HNAQ sites were representative farms of animal and shrimp culture. The sampling sites were at least 3 km away from each other. These 3 habitats were selected because they were representative of the aquatic environments exposed to antibiotics. The city canal in Hanoi directly receives various types of untreated municipal wastewater, such as wastewater from households and hospital (Duong et al., 2008). According to the onsite interviews conducted during our field trips in both sampling periods, antimicrobials were rarely used for the freshwater fishponds in Hatay province, but very frequently used in the pig farms. The categories of the drugs and their amounts varied widely among the individual pig farms. We chose coastal shrimp ponds in Haiphong because this province is one of the centers of fish and shrimp cultivation, which is an industry that supplies seafood to domestic and foreign markets (Tran et al., 2006). The use of antibiotics in the Vietnamese shrimp industry has been reported by Le and Munekage (2004) and Le et al. (2005). The investigated shrimp ponds were located near the estuary mouths, and the water level of the ponds was somewhat influenced by tidal movement; the water level in the ponds was adjusted by using pumps. The collection of water samples was repeated twice, once in January (dry season) and then in July (rainy season) in 2007. A detailed description of the characteristics of water samples and the climate conditions of the sampling locations is provided in Tables S1 and 2. The sampling procedure was identical to that described in our previous studies (Managaki et al., 2007; Hoa et al., 2008). Then, the samples were preserved on ice and analyzed in the laboratory within 3–6 h after sampling.

2.2. Analysis of antibiotics

The concentrations of sulfonamides, trimethoprim, and macrolides were determined by tandem mass spectrometry equipped with high-performance liquid chromatography (LC–MS/MS), which was performed after solid-phase extraction. The detailed procedure has been described by Managaki et al. (2007), and the outline of the procedure is as follows. We extracted 50 ml (city canal samples), 20 ml (pig farm/fish pond samples), and 250 ml (shrimp pond samples) on 6-ml Oasis HLB sorbent cartridges (200 mg; Waters) (flow rate, <5 ml/min; pH, 4). After extraction, the cartridges were stored at −30 °C, transported to the laboratory in Tokyo, and defrosted before elution of the antibiotics. The cartridges were washed with 5 ml of water–methanol (75:25) and dried in a nitrogen flow for 30 min. The analytes were eluted with 2 × 1.5 ml of methanol–ethyl acetate (1:1) and 2 × 1.5 ml of methanol containing 1% (v/v) ammonia. A fixed amount 25 ng (500 pg/μl × 50 μl) of each antibiotic surrogate standard (sulfamethoxazole-d4, clarithromycin-d3, and roxithromycin-d9) was spiked to the sample extracts. The extracts were evaporated to dryness using a rotary dryer and dissolved in 1 ml of water–methanol (1:1), and the antibiotic contents were determined by separating the extracts by LC–MS/MS.

HPLC analyses were performed on a Hewlett-Packard G1310. The antibiotics were separated on a reverse-phase column (YMC Pro C18; 3 μm, 150 mm × 2 mm) that was operated at 30 °C at a flow rate of 0.15 ml/min. The mobile-phase solvents were water-acidified with 1% (v/v) formic acid (eluent A) and methanol-acidified with 1% (v/v) formic acid (eluent B) to pH 2.5 by using a gradient program. The antibiotics were detected using a triple-quadrupole mass spectrometer (TSQ Quantum 7000; Thermo Finnigan, Japan) equipped with electrospray ionization. The analyses were performed in the positive-ion mode. The detection was performed in the selected-reaction-monitoring mode (SRM) using the 2 most intense and specific fragment ions.

To compensate for matrix effects and experimental losses during sample treatment, we corrected the concentrations of the target antibiotics with the recovery values of the corresponding surrogates spiked in the same extracts. The following compounds were used as recovery surrogate standards: sulfamethoxazole-d4 for all sulfonamides and trimethoprim; clarithromycin-d3 for clarithromycin, erythromycin-H_2O, and azithromycin; and roxithromycin-d9 for roxithromycin. The analytical precision was examined by performing