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Abundance of Sulfonamide-resistant Bacteria and Their Resistance Genes in Integrated Aquaculture-agriculture Ponds, North Vietnam

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Abstract—The use of antibiotics in livestock production is a potential source of antibiotic contamination, which may increase the presence of antibiotic-resistant bacteria in the aquatic environment. Antibiotic resistance genes can be spread via the food web and through hydrologic processes to human pathogens. We examined the abundance of sulfonamide-resistant (SR) bacteria and their resistance genes in water samples from integrated aquaculture-agriculture (VAC) sites. Among the VAC sites investigated, all were contaminated by sulfonamides with varying concentrations and all showed the presence of sulfamethoxazole resistant bacteria (SMX^r). Farms rearing pig and duck (pig+duck VAC) showed high contamination with sulfamethazine. SMX^r in other sites were less than that in the pig+duck VAC. The SR genes, *sul1*, *sul2* and *sul3*, were detected from SR isolates with 45–95%. The prevalence of each gene was *sul1* (50%), *sul2* (21.17%) and *sul3* (4.17%). SR isolates possessing plural *sul* genes belonged to eight genera (*Bacillus*, *Terrabacter*, *Agrococcus*, *Enterobacter*, *Escherichia*, *Acinetobacter*, *Dietzia* and *Shigella*). The present study revealed that SR bacteria were distributed in various bacterial groups, including potential human pathogens. This report suggests that the aquatic environment is a reservoir of *sul* genes, in which the genes may be transferred among not only aquatic bacteria but also human related bacteria.

Keywords: sulfonamide, drug resistance, *sul* gene, *Acinetobacter*

INTRODUCTION

In Vietnam as well as other Southeast Asian countries, a combined agriculture system of vegetables, aquaculture and cage (VAC) is commonly used as an

economical system of farming. Intensive pig farming within this VAC system in Vietnam uses commercial feed supplemented with antibiotics for promoting growth and in therapeutic treatment. Over-use of antibiotics in this type of system often occurs, causing antibiotic contamination (Petersen *et al.*, 2002; Petersen and Dalsgaard, 2003; Hoa *et al.*, 2008a, b) and resultant antibiotic resistance in bacteria in the surrounding environment. Furthermore, the antibiotic resistance genes may be transferred through the food web and through hydrologic processes into human society. Previously we examined the contamination status of VAC sites during the dry season in Vietnam (Hoa *et al.*, 2008a, b), with results suggesting that wastewater from VAC sites are "hot spots" of *sul* genes. Bacterial resistance to sulfonamides occurs through mutations in the chromosomal DHPS gene (*folP*) or through acquisition of an alternative DHPS gene (*sul*), whose product has a low affinity for sulfonamides (Petersen and Dalsgaard, 2003). Of the two pathways, *sul* genes are the most prevalent mechanism of sulfonamide resistance (Enne *et al.*, 2002; Petersen and Dalsgaard, 2003). The abundance of the *sul* genes was from high to low, *sul1*, *sul2* and *sul3* (Hoa *et al.*, 2008a). Because hydrodynamics such as the amount and direction of water flow may influence various properties of antibiotics and their interactions with bacteria, in this study we examined the contamination status and presence of *sul*-possessing bacteria from VAC sites during the rainy season of Vietnam. To do this we examined the abundance of sulfonamide-resistant (SR) bacteria and their resistance genes in water samples from VAC sites. Water samples were collected from five VAC sites, two Red River sites and one natural lake site during the rainy season of northern Vietnam (July, 2007).

MATERIALS AND METHODS

Study sites and sample collection

Water samples were collected from a total of eight sites (Fig. 1) in the Red River delta, consisting of five VAC sites, two sites from the Red River in Hatay, and one control site (a lake with no presence of animal cages or no apparent inputs from domesticated animals) in Hanoi. Of the VAC sites chosen for study, two were from Hanoi, one consisting of duck cages with thousands of ducks placed along the edge of a freshwater fish pond, and another system consisting of a pig and duck farm (pig+duck-VAC), where one pig cage contained approximately 30 pigs and one duck cage contained thousands of ducks. From Hatay, three pig farms (pig-VAC1, 2, and 3) were investigated. For comparison among VAC cases, two water samples were collected from the Red River and the lake without domesticated or farmed animals. The samples were collected in July, 2007; the sampling procedure is described in Managaki *et al.* (2007) and Hoa *et al.* (2008a). A total of 20 ml of water was sampled and filtered, with 1 ml of water subsampled and stored at –20°C; all samples were transported from Vietnam to Japan for further analyses.

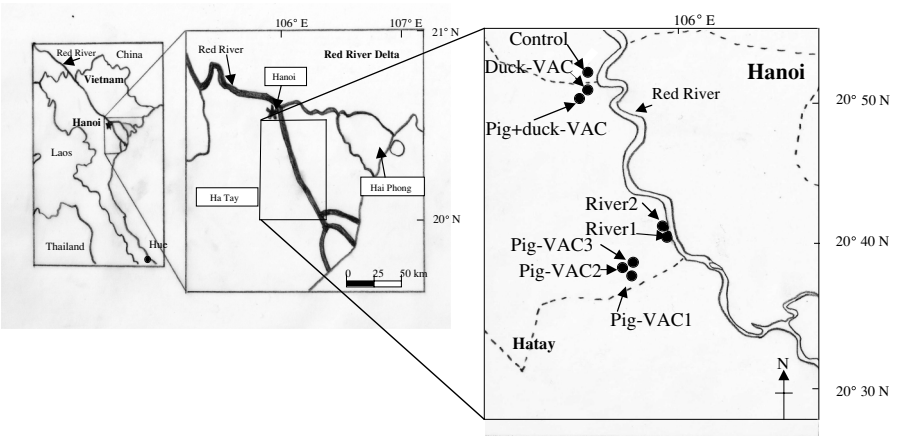

Fig. 1. Study area and sampling sites.

Antibiotic concentration analysis

Analyses of macrolides (azithromycin, erythromycin, clarithromycin, and roxithromycin), sulfonamides (sulfapyridine, sulfamethoxazole, sulfathiazole, sulfamerazine, sulfamethizole, sulfamethazine, and sulfadimethoxine) and trimethoprim were performed as previously described (Managaki *et al*., 2007).

Enumeration of sulfamethoxazole-resistant bacteria (SR)

The colony forming units (CFU) were enumerated by the plate spread method (Nonaka *et al*., 2000); a detailed description of this procedure is provided in Hoa *et al*. (2008a). In this study, selection of the sulfamethoxazole (SMX) was based on bacterial colony growth on selected media, which were nutrient broth (Difco, Detroit, MD, USA) plus 1.5% agar that were supplemented with 60 μg/ml or 120 μg/ml of SMX ("SMX-resistant" is abbreviated as SMX^r hereafter). Bacterial colony numbers were enumerated after five days of incubation at 30°C. These experiments were conducted in duplicate.

Isolation of the selected antibiotic-resistant bacteria

To investigate the prevalence of *sul* genes, from each site we randomly selected 10–25 SMX^r colonies from the plate containing 60 μg/ml SMX. From this we obtained total 120 SMX^r isolates.

DNA extraction and sul gene detection

DNA extraction from SMX^r isolates was performed by using the protocol described previously (Hoa *et al*., 2008a). Detection of the *sul1*, *sul2*, and *sul3* genes was carried out by PCR (Hoa *et al*., 2008a).

Identification of SR bacteria carrying plural sul genes

All SR isolates possessing two or more *sul* genes were identified by 16S rRNA gene (primers F984GC, R1378) by the method of Heuer *et al.* (1997). A detailed description of this procedure is in the previous study by Hoa *et al.* (2008a).

Statistical analyses

A Fisher's exact test was used to test for differences in the *sul* gene distribution in 120 SR isolates. A p-value of <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Macrolide, sulfonamide and trimethoprim contamination status

The site-specific status of antibiotic concentrations are summarized in Table 1. Among 12 antibiotics, five (sulfamethoxazole, sulfamethazine, trimethoprim, erythromycin and clarithromycin) were detected from almost all VAC farms sampled, whereas no antibiotic residue was detected in the Red River sites and the control lake site. Among the antibiotics detected, sulfamethoxazole was the most common with a range from 3.22 to 326 ng/l from all VAC sites, which comprised 11.76% of the total concentration of antibiotics. Sulfamethazine was found from four VAC sites with a range of 6.78–1966 ng/l, which was 86.5% of the total concentration. Trimethoprim and erythromycin were found to be 26.0–26.4 ng/l and 0.59–12.8 ng/l, respectively, which were a small proportion (1.29% and 0.42% respectively) of the total antibiotics detected. These results showed that VAC sites were contaminated by sulfonamides and macrolides, whereas antibiotic concentrations at the control and river sites were below detection limits. The concentrations of antibiotics detected in the rainy season were as high as those detected in the dry season at the VAC sites.

Sulfonamide-resistant bacteria

Total cultivable colonies ranged from 3×10^4 to 1.7×10^6 CFU/ml, whereas cultivable SR colonies were from 3×10^2 to 4.1×10^4 CFU/ml (data not shown). The frequency of SR occurrence is shown in Fig. 2. Overall, antibiotic-resistant bacteria were found at all sites investigated. SMX^r was found to be highest at pig+duck-VAC (42.6% and 30.5% for SMX^r_{60} and SMX^r_{120} respectively), followed by river sites (19.56–27.66% for SMX^r_{60} and 20.8–30% for SMX^r_{120}), pig-VAC sites (15.30–23.8% for SMX^r_{60} and 12–19.3% for SMX^r_{120}), and duck-VAC site (12.8 and 14.3% for SMX^r_{60} and SMX^r_{120}, respectively), with the control lake site being lowest (4.4 and 3.5% for SMX^r_{60} and SMX^r_{120}, respectively) (Fig. 2). The frequency of SR bacteria detected in the rainy season was not statistically different from that of our results in the dry season (Hoa *et al.*, 2008a).

Table 1. Antibiotic concentrations (ng/l).

Site	Sulfapyridine	Sulfamethoxazole	Sulfathiazole	Sulfamerazine	Sulfathiazole	Sulfamethazine	Sulfadimetoxine
duck-VAC	nd	6.14	nd	nd	nd	nd	na
pig+duck-VAC	nd	3.22	nd	nd	nd	1966	na
pig-VAC1	nd	326	nd	nd	nd	851	na
pig-VAC2	nd	68.2	nd	nd	nd	658	na
pig-VAC3	nd	69.9	nd	nd	nd	6.78	na
River 1	nd	nd	nd	nd	nd	nd	na
River 2	nd	nd	nd	nd	nd	nd	na
Control lake	nd	nd	nd	nd	nd	nd	na
Sum	0	474	0	0	0	3482	0
%	0	11.8	0	0	0	86.5	0

Site	Trimethoprim	Azithromycin	Erythromycin	Clarithromycin	Roxithromycin	Reference
duck-VAC	nd	nd	nd	nd	nd	This study
pig+duck-VAC	nd	nd	3.70	0.04	nd	This study
pig-VAC1	26.4	nd	12.8	nd	nd	Hoa *et al*., 2008b
pig-VAC2	26.0	nd	0.59	0.01	nd	Hoa *et al*., 2008b
pig-VAC3	nd	nd	nd	nd	nd	Hoa *et al*., 2008b
River 1	nd	nd	nd	nd	nd	This study
River 2	nd	nd	nd	nd	nd	This study
Control lake	nd	nd	nd	nd	nd	This study
Sum	52	0	17	0.05	0	
%	1.29	0	0.42	0	0	

nd, not detected; na, not available due to overlapped interfering peak.

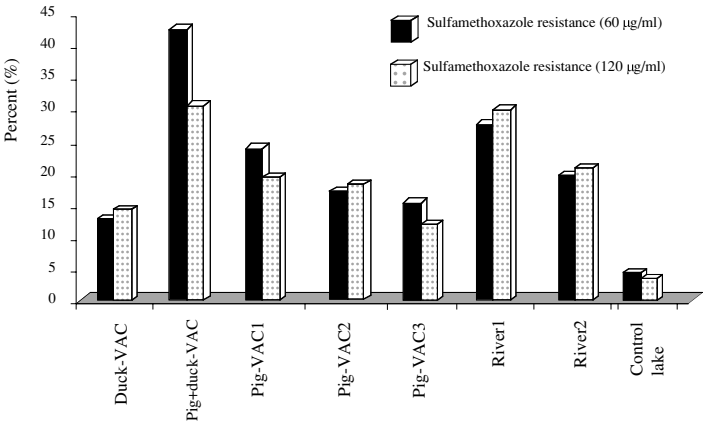

Fig. 2. Occurrence rate of sulfamethoxazole-resistant bacteria.

Detection of the sul genes

The *sul* genes were detected from all sites, although *sul3* was not found at 3 sites (Table 2). Among the *sul* genes, *sul1* was the most prevalent gene (50%), followed by the *sul2* (21.17%), and *sul3* (4.17%); these rates were statistically significant each other. This result is consistent with our previous study from the dry season (Hoa *et al*., 2008a), which is different from European cases (Binh *et al*., 2008). The highest proportion of the *sul1* was found from pig+duck-VAC (90%), whereas the *sul2* was highest at pig-VAC (55%) and the *sul3* at duck-VAC (15%). The highest sulfamethazine concentration (1966 ng/l) and the highest proportion of *sul* genes containing isolates were observed at pig+duck-VAC (95%). This observation mirrored our previous results in the dry season from VAC sites, and suggests that high concentrations of sulfonamide in the environment selects for *sul* genes.

Identification of SR bacteria with plural sul genes

The isolates were classified into eight genara (*Acinetobacter*, *Agrococcus*, *Bacillus*, *Dietzia*, *Enterobacter*, *Escherichia*, *Shigella* and *Terrabacter*) (Table 3). Results showed that SR isolates were distributed among various bacterial groups, including human environmental inhabitants. *Acinetobacter* was found to be a potential reservoir of *sul* genes. This genus is generally considered to be an important opportunistic pathogen in humans with multi-drug resistance. More attention is needed in terms of gene risk from the environment, particularly in aquatic habitats.

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Table 2. Detection rate of *sul* genes in SR isolates.

Sites	*sul1* (%)	*sul2* (%)	*sul3* (%)	% of isolates possessing any *sul* genes
duck-VAC	35.00	15.00	15.00	21.66
pig+duck-VAC	90.00	10.00	5.00	35.00
pig-VAC1	50.00	55.00	5.00	36.66
pig-VAC2	30.00	50.00	0.00	26.66
River 2	40.00	5.00	0.00	15.00
Control lake	55.00	10.00	0.00	21.66
Average	50.00	21.17	4.17	26.11
∑ isolates	120	120	120	

River 1 and pig-VAC3, not done.

Table 3. List of SR isolates possessing plural *sul* genes.

Strain	Closest species	GenBank # of the closest species	%ID	*sul* genes detected
T68	*Acinetobacter* sp. TS11	EU073077.1	99	1, 2
T74	*Acinetobacter* sp. TS11	EU073077.1	99	1, 2
T76	*Acinetobacter* sp. TS11	EU073077.1	99	1, 2
T48	*Agrococcus* sp. RCML-30	FJ005071.1	100	1, 2
T1	*Bacillus* sp. JSM 081037	FJ527422.1	98–100	1, 3
T30	*Bacillus* sp. JSM 081037	FJ527422.1	100	1, 3
T41	*Bacillus megaterium* strain JC3	EU704698.1	100	1, 2
T83	*Bacillus* sp. AK2641	FJ573193.1	99	1, 2, 3
T81	*Dietzia* sp. W5026	FJ468338.1	99	1, 2
T61	*Enterobacter* sp. BSRA3	FJ868807.1	99	1, 3
T73	*Escherichia coli* strain EC1	FJ434458.1	99	1, 2
T96	*Shigella* sp. MAC17169	DQ874994.1	97	1, 2
T20	*Terrabacter* sp. 211002/XP	AY278993.1	99	1, 2

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