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Research article

Analysis of antibiotic multi-resistant bacteria and resistance genes in the effluent of an intensive shrimp farm (Long An, Vietnam)

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ABSTRACT

In Vietnam, intensive shrimp farms heavily rely on a wide variety of antibiotics (ABs) to treat animals or prevent disease outbreak. Potential for the emergence of multi-resistant bacteria is high, with the concomitant contamination of adjacent natural aquatic habitats used for irrigation and drinking water, impairing in turn human health system. In the present study, quantification of AB multi-resistant bacteria was carried out in water and sediment samples from effluent channels connecting a shrimp farming area to the Vam Co River (Long An Province, Vietnam). Bacterial strains, e.g. *Klebsiella pneumoniae* and *Aeromonas hydrophila*, showing multi-resistance traits were isolated. Molecular biology analysis showed that these strains possessed from four to seven different AB resistance genes (ARGs) (e.g. *sul*1, *sul*2, *qnr*A, *erm*B, *tet*A, aac(6)Ib, *dfr*A1, *dfr*12, *dfr*A5), conferring multidrug resistance capacity. Sequencing of plasmids present within these multi-resistant strains led to the identification of a total of forty-one resistance genes, targeting nine AB groups. qPCR analysis on the *sul*2 gene revealed the presence of high copy numbers in the effluent channel connecting to the Vam Co River. The results of the present study clearly indicated that multi-resistant bacteria present in intensive shrimp cultures may disseminate in the natural environment. This study offered a first insight in the impact of plasmid-born ARGs and the related pathogenic bacteria that could emerged due to inappropriate antibiotic utilization in South Vietnam.

1. Introduction

Production of farmed aquatic food increased rapidly since 30 years and has driven aquaculture to be one of the fastest-growing animal-food-producing sectors (Allison, 2011; Letchumanan et al., 2015). In Vietnam, shrimp farming (so-called “red-gold farming”) is a particularly profitable activity, targeting essentially the export market, with a recent booming in the income, passing from US$ 2.5 billion in 2013 to ca. US$ 3.1 billion in 2015. A large number of farmers converted their rice fields in shrimp breeding pools (Levasseur, 2015). In 2012, shrimp farms covered more than 640 million hectares and ca. 89% were localized in the delta of the Mekong River (Lan, 2013).

While providing economic benefits, aquaculture causes several environmental problems and affects sustainable use of natural resources, such as the loss of valuable coastal and wetland habitats (Nguyen et al., 2016), environmental degradation (Ottinger et al., 2016), as well as the dispersion of chemicals and nutrients into the environment (Lan, 2013). According to Anh et al. (2010), shrimp production during the past ten years has increased faster than the area of shrimp ponds, due to the shift from extensive-traditional to intensive systems. Intensive shrimp farms are typically inland-based freshwater or brackish water ponds that heavily rely on pesticides and antibiotics to avoid shrimp disease outbreak (Rico and Van den Brink, 2014). In practice, farmers gave access to and make use of a wide variety of antibiotics (ABs) to treat animals or prevent disease outbreak in their farms. In Vietnam, ABs are

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approved by the Ministry of Agriculture and Rural Development and include more than 30 compounds, including β-lactams, aminoglycosides, macrolides, tetracyclines, polymyxins, pleuromutilins, lincosamides, sulfonamides, diaminopyrimidine (trimethoprim) (Anh et al., 2010; Pham et al., 2015). Broad-spectrum ABs, such as chloramphenicol, are banned in the aquaculture industry (Binh et al., 2018). On average, each farm used three different ABs, with 10% of the farms using up to six different products (Binh et al., 2018). A recent report showed that ca. 700 g of ABs is used per ton of cultured fish in Vietnam, which is seven times more than other countries, resulting in a high prevalence of residual concentrations in aquaculture products and waste (Andrieu et al., 2015; Uchida et al., 2016). For instance, it is estimated that 12 tons of sulfamethoxazole is discharged annually from the Mekong River into the South China Sea (Shimizu et al., 2013).

A few data only showed the effects of these substances on shrimp pathogens and on the autochthonous bacterial communities in general. Prolonged usage of ABs induces continuous selective pressure on bacterial communities, even well below minimum inhibitory concentrations and increases horizontal gene transfer (Watts et al., 2017). Spreading of antibiotic resistance genes (ARGs) among pathogenic bacteria already has a significant effect on the culture of shrimp worldwide. Recently, it was reported that shrimp farming industry in Asia have lost approximately US$ 1 billion due to the acute hepatopancreatic necrosis syndrome (AHPND) caused by *Vibrio parahaemolyticus* (Global Aquaculture Alliance, 2016). Ca. 39,000 ha of shrimp ponds present in the Mekong delta were affected by the AHPND in 2011, with mortality rates of up to 100% (FAO, 2013). Presence of ARGs in shrimp pathogens increases with time worldwide, with observations made recently in India (Santhyia et al., 2015), Malaysia (Letchumanan et al., 2015) and Brazil (Albuquerque Costa et al., 2015), where productivity declined by one third in just four years. The risk of transmission of resistances to human pathogens is then increasingly present. It was reported for instance that a multi-resistant strain of *Vibrio cholerae* was the agent of an epidemic in Ecuador. This strain was linked to AB-resistant bacteria emerging and spreading from the heavy ABs use in the local shrimp industry (Angulo et al., 2004). Despite the very high variety of ABs used in the Vietnamese aquaculture industry, a limited number of studies only targeted the quantification and the characterization of bacterial multi-resistances in the aquaculture environment (Binh et al., 2018). As shrimps cultivated under non-regulated conditions are produced massively, multi-resistant bacterial pathogens are potentially impairing ABs effectiveness in the future. Negative consequences would affect inevitably our health system if this undiscerning usage were maintained. However, the scarcity of data on ABs consumption reduces the possibility of coordinated action (WHO, 2014). For instance, the Vietnamese National Action Plan against Drug Resistance does not include the monitoring of antibiotic residues in the environment (Binh et al., 2018). It is therefore urgent to understand better the impact of indiscriminate usage of ABs on natural ecosystems, and to find practical solutions to limit the dissemination of the induced resistances.

In this study, microbiological cultures and molecular analysis were carried out on water and sediment samples taken from the effluent channel of an intensive shrimp farming area, as well as the Vam Co River (Long An Province, Vietnam) in which the culture waste are being discharged. The goal of the study was i) to obtain information about the presence of multi-resistant bacteria among bacterial communities, ii) to identify multi-resistant bacterial strains and characterize their ARGs and finally iii) quantify the presence of a selected resistance gene, *sul2*, among the effluent of the culture basins and in the adjacent natural environment.

2. Materials and methods

2.1. Study sites and sample collection

To quantify the importance of bacterial multi-resistances, we selected a representative shrimp farming area (with intensive farming practices) located on the shore of the Vam Co River (Long An Province, Vietnam, 10°28′12.84″N; 106°33′18.60″ E). This facility covers about 10′000 m^2, with 15 shrimp ponds. Samples (including water and sediment) were taken from three effluent channels connecting the ponds to the receiving Vam Co River, and two sets of samples were collected from the river itself. Water samples were collected by hand from the water surface (ca. 20 cm depth) and stored in 5 L sterile plastic containers. Sediment samples (ca. 0–5 cm depth) were collected manually with a bucket and transferred in sterile containers. All samples were shipped to the laboratory within 8 h and stored at 4 °C until being processed.

2.2. Screening for AB multi-resistant bacteria

Culture of AB multi-resistant bacteria and isolation of pure cultures were carried out on Nutrient Agar plates (Himedia, India), with an additional 1% NaCl (purum, Himedia, India). Serial dilutions of the water and sediment samples were carried out in sterile physiological solution (0.9 g/L NaCl). Agar plates were supplemented with a combination of five ABs: sulfamethoxazol (152 μg/mL), trimethroprim (16 μg/mL), ciprofloxacin (4 μg/mL), enrofloxacin (4 μg/mL) (puriss, all Himedia, India) and erythromycin (8 μg/mL) (puriss, Calbiochem, USA). Plates were incubated at 35° C for 48 h before visual inspection. For DNA and plasmid isolation, multi-resistant bacterial strains were grown in LB liquid medium (Bio basic, Canada) supplemented with an identical combination of antibiotics at 35° C for 24 h. Cells were harvested by centrifugation (8′000 × g, 5 min) before DNA extraction.

2.3. DNA and plasmid extractions

Environmental DNA from water and sediment samples was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) according to the manufacturer instructions. DNA from the isolated bacterial strains was extracted using a SDS-based extraction method (Zhou et al., 1996). Plasmids were isolated with a method developed by Bimboim and Doly (1979). Both genomic DNA and plasmids were re-suspended in TE buffer and inspected on agarose gels (all Himedia, India).

2.4. PCR amplification

ARGs present within multi-resistant bacterial strains were screened by PCR with gene-specific primers (Table SI-1). The PCR amplification were carried out in 25 μL volume containing 100 ng template DNA, 1.25 U MyTaq DNA polymerase, 1 × MyTaq Reaction Buffer (all Bioline, UK) and 10 pmol of each primer (Microsynth, Switzerland). Amplification conditions were as follows: denaturing at 95° C for 3 min, 30 cycles including denaturing at 95° C for 45 s, annealing at 55° C for 45 s and elongation at 72° C for 45 s, followed by a final step at 72° C for 6 min. PCR was carried out in a MyCycler device (Bio-Rad, UK) and PCR products were analyzed by electrophoresis on 1.5% agarose gels (Peqlab, Germany). Sizing of the products was carried out by visual inspection and comparison with size reference ladders (Bioline, UK).

2.5. Real-time PCR amplification of the sul2 gene

The *sul*2 gene encodes a sulfonamide resistance trait, which is