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Antibiotic resistance monitoring in heterotrophic bacteria from anthropogenic-polluted seawater and the intestines of oyster *Crassostrea hongkongensis*

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ABSTRACT

A total of 1,050 strains of heterotrophic bacteria isolated from farming seawater and the intestines of oyster species *Crassostrea hongkongensis* were tested for resistance to 10 antibiotics by the Kirby–Bauer diffusion method. The resistant rates of seawater-derived bacteria to chloramphenicol, enrofloxacin, and ciprofloxacin were low (less than 20%), whereas the bacteria obtained from oysters showed low resistance to chloramphenicol and enrofloxacin. Many strains showed high resistant rates (more than 40%) to furazolidone, penicillin G, and rifampin. A total of 285 strains from farming seawater and oysters were resistant to more than three antibiotics. Several strains showed resistance to more than nine antibiotics. Furthermore, the peak resistant rates of the seawater-derived strains to multiple antibiotics overlapped in April, June, September, and November, and those of oyster-derived strains overlapped during April, July, and September. The multi-resistant rate patterns of strains from farming seawater and oyster intestines were similar.

1. Introduction

Antibiotics have been used extensively for preventing or treating infections and for promoting animal growth in aquaculture and farming (Cabello, 2006; Sarmah et al., 2006). Approximately 2.1×10^5 t of antibiotics are produced in China each year, and approximately 9.7×10^4 t (46% of the total) are used in livestock production (including aquaculture). Antibiotics are only partially metabolized by humans and animals after administration at a metabolic rate of approximately 30% (Kümmerer and Henninger, 2003). Hence, large amounts of antibiotics and metabolites are potentially released into the environment; this phenomenon is considered the most important factor for the evolution and selection of antibiotic resistance in bacterial pathogens (Allen et al., 2010). Previous studies confirmed that antibiotic resistance can be induced and spread rapidly among bacterial species (Wang and Schaffner, 2013). The prevalence and persistence of antibiotic resistance in bacterial pathogens are a threat to public health and a source of considerable concern (Andersson and Hughes, 2010). Moreover, the majority of pathogenic bacteria are heterotrophic.

Crassostrea hongkongensis is a marine invertebrate that belongs to the family Ostreidae (Mollusca, Bivalvia); this oyster has a worldwide distribution and is the most abundantly harvested shellfish in the world (FAO, 1999; Guo et al., 2008). This oyster is also among the most important oyster species cultured in China. *Crassostrea hongkongensis* farms are widely distributed from the Li-zi River (Oyster River) on the border with Korea in the north (Zhang and Lou, 1956) to Bei-hai near the border with Vietnam (Guo et al., 1999; Xia et al., 2009). The application of extensive practices in the farming of aquatic animals has led to problems. Because aquatic products can easily become infected due to large stocking densities; to avoid disease and reduce losses, antibiotic overuse frequently occurs in artificial breeding (Zheng et al.,2012). Most oyster farms are near estuaries, such as Hai-ling Bay, and these water bodies are closely linked to polluted residential areas. All of these factors may lead to bacterial resistance. Thus, a total of 1,050 heterotrophic bacteria were isolated from oysters (582 strains) and farming seawater (468 strains) in this area. These bacteria were then tested for resistance to 10 antibiotics in

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different months. This study is the first report on the antibiotic resistance of bacteria obtained from one of the most important mollusks during different months in China. The results of this study will serve as reference data for monitoring antibiotics used in aquaculture.

2. Materials and methods

2.1. Materials

Tested strains: 1,050 strains of heterotrophic bacteria were isolated from farming seawater and the intestines of oyster species *C. hongkongensis* in Hai-ling Bay (N 21 21N 21stra E 111°45.291, Guangdong province, China) in 2011. Seawater samples were collected from sampling station where the depth was 3 m.The seawater was tenfold diluted with sterile seawater and smeared on Zobell2216E marine agar medium plates and thiosulfate citrate bile saccharose (TCBS) agar plates (three proper gradients and three parallel groups for every gradient, respectively). Then all the plates were layed up in biochemistry incubators at 28 °C for 96 h. The temperature, pH, and salinity of samples were measured with a Water Tester (YSI, USA). Five oysters were disserted every month and the alimentary tract (0.5 g) was triturated in 1 mL normal saline (0.85%) and then diluted with normal saline and smeared on nutrient agar medium and TCBS agar plates for three proper gradients, three parallel groups for every gradient. All the plates were layed up in biochemistry incubators at 28 °C for 96 h.

After incubation for 96 h, the number of the bacteria clones was counted and recorded (reportable numbers were limited between 30 and 300). Then 30–35 bacterial strains for every sample were selected randomly from the bacterial colonies for purification and identification by 16 S rDNA gene sequencing analysis and the isolates were stored at −80 °C in 0.85% saline solution supplemented with 20% glycerol (sterile,100 kPa 20 min). The identified strains were mainly *Vibrio*, *Pseudoalteromonas*, *Bacillus*, *Shewanella*, and *Alteromonas* by 16 S rDNA analysis.

Strains used for quality control: *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923.

Medium for antibiotics susceptibility tests: Muller Hinton agar (MHA).

Antibiotics: enrofloxacin (ENX, 10 μg), kanamycin (KNA, 30 μg), gentamicin (GM, 10 μg), chloramphenicol (C, 30 μg), trimethoprim/sulfamethoxazole (TMP/SMZ, 23.75 μg/1.25 μg), rifampin (RA, 5 μg), furazolidone (F, 300 μg), penicillin G (P, 10 μg), ciprofloxacin (CIP, 5 μg), and tetracycline (TE, 30 μg).

2.2. Antimicrobial sensitivity assay

Selected isolates of different bacteria were tested for antimicrobial sensitivity by using the disk diffusion method with MHA culture plates (Wang et al., 2008; Singh et al., 2011a, 2011b). All procedures and corresponding results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006, 2009; NCCLS, 2010). Isolates that showed resistance to three or more drugs were classified as multidrug-resistant type (Magiorakos et al., 2012). The quality control range and the standard determined for the results were in accordance with the data provided in Table 1 and the references (CLSI, 2006, 2009; NCCLS, 2010).

2.3. Statistical analysis

Data on all bacterial strains isolated from farming seawater and oysters, including their responses to 10 different antibiotics in resistant assays (%), were entered in an MS Excel worksheet. The formula used was as follows: percentage of resistance of strains to the single antibiotic = quantity of resistant strains to the single antibiotic/total strains for the test × 100%.

3. Results

3.1. Resistance of 1,050 strains isolated from seawater and oysters to 10 different antibiotics

The total percentage of strains from farming seawater and oysters with resistances to the 10 antibiotics are presented in Fig. 1. The data showed minor differences between strains from farming seawater and oysters in their responses to the same antibiotics. Correlation analysis indicated that a significant and positive correlation existed between the seawater-derived strains and the oyster-derived strains ($r = 0.929$).

Table 1
Diameter limits for antimicrobial susceptibility testing of heterotrophic bacteria by using the Kirby–Bauer diffusion method.

Antibacterial	*Content in the script/μg*	*E. coli ATCC25922/mm*	*S. aureus ATCC25923/mm*
ENX	10	28–36	22–28
CIP	5	30–40	22–30
GM	10	19–26	19–27
KNA	30	17–25	19–26
TMP/SMZ	1.25/23.75	23–29	24–32
F	300	–	–
RA	5	8–10	26–34
P	10 U	–	26–37
TE	30	18–25	24–30
C	30	21–27	19–26

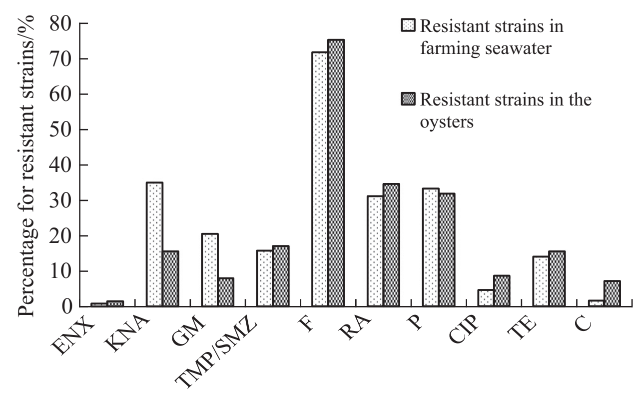

Fig. 1. Percentage of resistance of strains to 10 antibiotics.

3.2. Variation in resistance rates of 1,050 strains to every antibiotic in different months

The water temperatures ranged from 18.7 °C to 32.8 °C, with the highest temperatures in June. Salinity ranged from 0.2% to 2.76%. The pH ranged from 7.3 to 8.39. DO ranged from 2.75 to 7.67 mg/L.

The resistance rates of bacterial strains isolated from seawater and oysters at different months are presented in Figs. 2 and 3, respectively. The rates fluctuated at regular intervals and resembled zigzags on the graph. The peaks of the resistance rates of seawater-derived strains to multiple antibiotics mostly overlapped in April, June, September, and November, whereas those of oyster-derived strains overlapped in April, July, and September. The resistant rate to F every month was obviously higher than the resistance to all other tested antibiotics (Figs. 2 and 3). The maximum resistance values to F were 93.3% in April for seawater-derived strains and 97.1% in July for oyster-derived strains, respectively. The resistance rates of seawater-derived bacteria to C, ENX, and CIP were low (less than 20%). Furthermore, the resistance rates of oyster-derived bacteria to C and ENX were low. Many strains showed resistance to F (at resistance rates higher than 40%), P, and RA. The total resistance rate to F, which is a banned drug in aquaculture, was much higher than the resistance rates of other tested antibiotics (Fig. 1). The maximum resistance values were 71.8% for seawater-derived strains and 75.3% for oyster-derived strains, respectively.

The trends of multi-resistance rates for seawater- and oyster-derived strains were similar (Fig. 4). In April and May, during the early development stages of the oysters, the multidrug resistance rate of oyster-derived strains were much lower than the multidrug resistance rate of seawater-derived strains. However, after June, the multidrug resistance rates of oyster-derived bacterial strains