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Occurrence of Tetracycline Resistant Bacteria and *tet*(M) Gene in Seawater from Korean Coast

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Abstract—South coast area of Korea is used for chemical industry and aquaculture. Various chemical contaminants may therefore be released to sea and active as selective pressures for microbial community in this area. Since oxytetracycline (OTC) has been used in aquaculture for long-time, bacteria resistant to OTC (OTC^r) was suspected to occur in this area. Among the OTC^r determinants, *tet*(M) is one of most widely distributed tetracycline resistance determinants. However, result of monitoring study in November, 2010, showed that the OTC^r rate was lower less than 1% of total culturable number.

The concentration of OTC in seawater was below detection limit (0.1 μg/L), although one site (KWD-2) showed 0.18 ± 0.01 μg/L, indicating that drug use was negligible at the moment. Cross-resistance pattern of OTC^r isolates showed that ampicillin and erythromycin resistance were highly combined to OTC^r. We found nine types of resistance patterns in our study. The highest resistance patterns along with OTC^r were OTC-ABPC-SMX/TS-EM, following by OTC-EM and OTC-ABPC. Our finding suggested that OTC^r is linked to various drug-resistance mechanisms.

In this study, 30 strains out of 35 OTC^r isolates were positive for *tet*(M). Concentration of OTC in seawater was not detected in this study. However, *tet*(M) gene persisted in culturable OTC^r bacteria. In this study, the samples were collected in winter season. Further monitoring is needed in various seasons at which aquaculture is actively performed.

Keywords: antibiotics, oxytetracycline, resistant bacteria, *tet*(M), cross-resistance of antibiotics, coastal environments

INTRODUCTION

Antimicrobial agents have been extensively applied in aquaculture to prevent and control disease. Antibiotics can be metabolized after administration; but up to 80% of antibiotics administered are excreted through urine or feces without complete decomposition. Some antibiotics, such as oxytetracycline, are detected in the sediment, fish farms (Jacobsen and Berglind, 1988; Douglas *et al*., 1996). Residual antibiotics in an aquatic environment might impact on microbes to acquire drug resistance (Knapp *et al*., 2008). Therefore, the development of

multi-drug resistant (MDR) bacteria is suspected in aquatic environment are of great concern because the bacteria could be transported back to anthropogenic source through water utilization and fisheries products.

Oxytetracycline (OTC) has been extensively used in aquaculture and oxytetracycline-resistant (OTC^r) bacteria were reported in fish pathogens and environmental bacteria (Nonaka *et al*., 2007). The OTC^r genes can be transferred among bacterial community, which increased OTC^r bacteria in environments (Chopra and Roberts, 2001). OTC^r genes are often associated with mobile genetic elements, such as conjugative plasmid and transposons, facilitated their rapid spread across species and genus borders (Schmidt *et al*., 2001; Roberts, 2005). Presently, more than 40 different tetracycline resistance determinants have been reported (Roberts, 2005). Most previous studies have focused on the occurrence of OTC^r bacteria in aquatic habitats and their relationship to population composition of the microbial community (Furunshita *et al*., 2003; Kim *et al*., 2004; Nonaka *et al*., 2007; Rahman *et al*., 2008; Suzuki *et al*., 2008).

Among the tetracycline resistance genes, the *tet*(M) is one of the most widely distributed tetracycline resistance determinants (Zhang *et al*., 2009). The host range for the *tet*(M) is 42 genera and this gene continues to have the widest host range of any *tet* genes (Roberts, 2005). Previous report showed that the *tet*(M) is distributed in coastal aquaculture areas and sediments in Mekong River, Vietnam (Suzuki *et al*., 2008). The *tet*(M) is known to associate with mobile genetic elements such as plasmids, transposons, conjugative transposons, and integrons (Chopra and Roberts, 2001; Agersø *et al*., 2004). The *tet*(M) enable to move from bacterial species to other bacterial species thus, may result in its wide distribution among numerous bacteria in the environment.

The aim of this study was the presence of OTC^r bacteria in nine Korean costal sites and to confirm the existence of *tet*(M) in culturable OTC^r bacteria. Furthermore, cross-resistance patterns of OTC^r isolates to other antibiotics was examined.

MATERIALS AND METHODS

Sampling and sampling procedure

Sampling sites are shown in Fig. 1. Sampling was performed between 8 to 12, November, 2010 in south coast of Korea. Samples were collected at 9 sites, which condition is summarized in Table 1. Nine sites are places affected by aquaculture (KYS-1, KGJ-1 and KWD-3), agriculture (KWD-2), chemical industries (KYS-3 and KGJ-3) and port (KYS-2, KGJ-2 and KWD-1). The seawater samples were taken using a stainless bucket from the water surface at all sites. The samples were pre-filtered through 50 μm nylon plankton net to remove large particles. Within a few hours, collected samples were kept in 4°C till transferred to the laboratory. The water temperature, pH and conductivity were measured with a pH/conductivity meter (D54, Horiba, Kyoto, Japan) immediately after sampling.

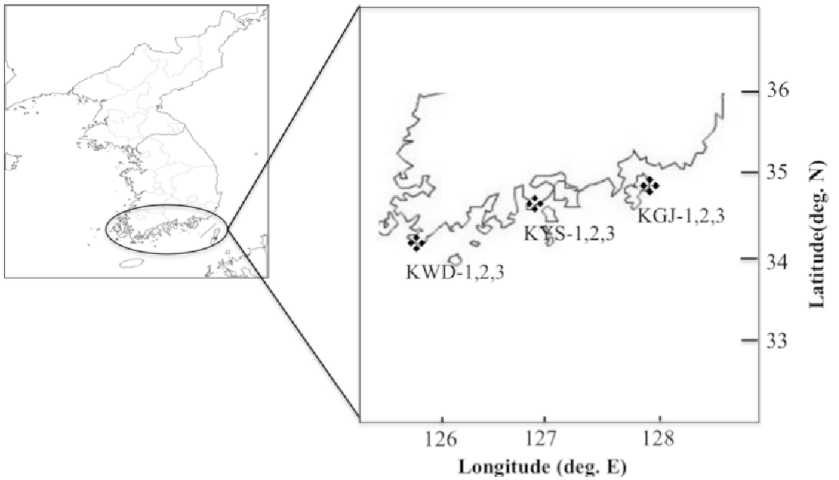

Fig. 1. Sampling sites in this study.

Total bacterial number

The total bacterial cell count was performed by epifluorescence microscopy. A 1 ml sample was filtered through a 0.22 μm black polycarbonate filter (Nucleopore, Whatman), and the filter was stained with 4′-6-diamidino-2-phenylindole (DAPI) (final concentration 0.1 μg/L) for 7 min. More than 300 cells were counted for each sample under an epifluorescence microscope (Olympus BX51, Japan).

Culturable bacterial number

The colony-forming units (CFUs) were measured using the plate-spread methods. Seawater samples of 0.5 mL were mixed in 4.5 mL of phosphate buffered saline (PBS) and serial 10 fold dilutions were prepared. The dilutions were spread on nutrient broth (Difco, UK) plus 1.5% bacto agar (Difco, UK) and 2% NaCl. The colony number was counted after incubation at 20°C for 4 days. For counting of OTC^r bacteria, oxytetracycline (Nacalai tesque, Japan, final concentration 60 μg/mL) was added to above media. Bacteria growing on this media were defined as OTC^r.

Antibiotic susceptibility test

Susceptibility to other antibiotics than OTC of the OTC^r strains was examined Etest strips (AB Biomeriúx, Sweden) for Ampicillin (ABPC), Ciprofloxacin (CI), Tetracycline (TC), Erythromycin (EM), Sulfamethoxazol/Trimethoprim (SMX/TS) and Streptomycin (SM). A bacterial cell suspension was prepared in PBS and the cell density was adjusted to McFarland No. 0.5. The suspension was spread on a Mueller Hinton agar (BD, USA) plates supplemented with 2.0%

Table 1. General information of sampling sites.

Sample ID	Location	Date	Latitude	Longitude	Temperature (°C)		pH	Salinity	Conductivity
					Air	Surface water			(S/m)
KYS-1	Oyster farm	Nov 9 2010	N 34°38′14.6″	E 127°48′24.5″	14.6	12	7.98	33	4.89
KYS-2	Port	Nov 9 2010	N 34°43′13.6″	E 127°42′14.5″	12.8	12	8.08	34	4.87
KYS-3	Chemical industry	Nov 9 2010	N 34°51′33.5″	E 127°43′14.6″	15	14.4	7.89	35	4.77
KGJ-1	Aquaculture	Nov 10 2010	N 34°54′33.6″	E 128°30′49.2″	15	14.7	8.02	31	4.82
KGJ-2	Port	Nov 10 2010	N 34°54′14.1″	E 128°34′34.4″	15	14.5	8.0	32	4.83
KGJ-3	Shipyard	Nov 10 2010	N 34°53′48.3″	E 128°36′51.2″	15	15	7.92	30	4.65
KWD-1	Port	Nov 11 2010	N 34°19′32.7″	E 126°45′0.7″	15	14.5	8.08	34	5.00
KWD-2	Agriculture and aquaculture	Nov 11 2010	N 34°20′24.2″	E 126°44′17.4″	15	14.7	7.88	34	5.02
KWD-3	Aquaculture	Nov 11 2010	N 34°20′28.5″	E 126°47′1.2″	15	14.4	8.04	34	5.02

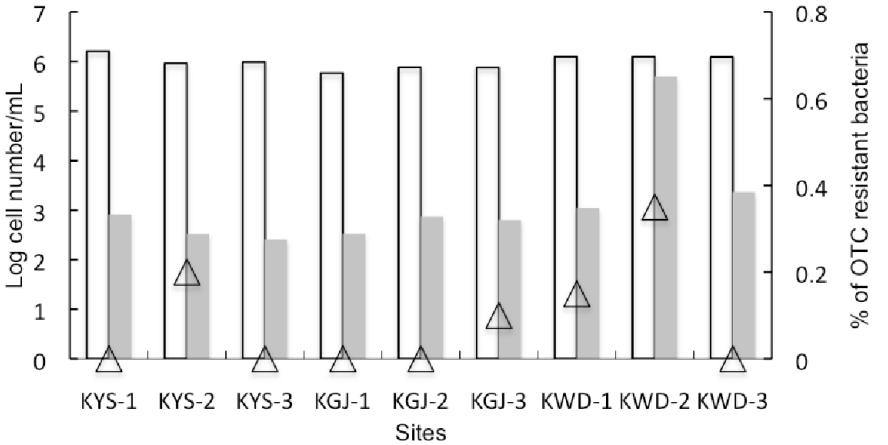

Fig. 2. Total cell count by DAPI staining (open bar, unit is left axis), viable count (closed bar, unit is left axis) and occurrence rate (%) of OTC^r bacteria (triangle, unit is right axis).

NaCl. Etest strips were then placed on the agar plate and incubated at 20°C for 24 h. The minimum inhibitory concentration (MIC) values were determined by the concentration on strip scale. In case of OTC, since the Etest would not give a reliable result (Nonaka *et al*., 2000). An agar dilution method (Nonaka *et al*., 2000) was performed. Two μL of bacterial cell suspension with a density of McFarland No. 0.5 was spotted on Mueller Hinton agar medium supplemented with 2% NaCl containing 60 μg/mL of OTC.

High performance liquid chromatography (HPLC) analysis of oxytetracycline from seawater

Seawater samples of 100 mL were filtrated through 47 mm diameter GF/F filters (Whatman). The extract was loaded onto solid-phase extraction columns (Sep-Pak Plus-2 C18, Waters) after conditioning with 10 mL of methanol and 5 mL of EDTA. After loading the samples, the column was washed with 10 mL of Milli-Q water, and the absorbed antibiotics were eluted with methanol. The extract was dried under as N_2 stream and dissolved in 1 mL of 1.36% KH_2PO_4. The sample solution was injected into an HPLC instrument with a fluorescence detector (Hitachi Elite LaChrom; excitation and emission wavelengths: 380 and 520 nm, respectively). The mobile phase solution consisted of 8% methanol in imidazole buffer (1.0 M imidazole, 1.0 mM EDTA, 0.08 M Mg-acetate pH 7.2), and the flow rate was 1.2 mL/min. The extracted antibiotics were separated on a C18 column (Bridge C18, Waters; 4.6 mm diameter, 150 mm length, 5 μm pore size) at 40°C.

Extracted environmental DNA from seawater samples

Total environmental DNA was also collected on filter. Seawater samples (10

Table 2. Cross-resistance patterns of OTC^r strains in sampling sites.

Resistance to	Occupation number (%) (Total number = 35)
OTC	7 (20%)
OTC-ABPC-SMX/TS-EM	8 (23%)
OTC-ABPC	6 (17.1%)
OTC-EM	6 (17.1%)
OTC-ABPC-EM	2 (5.7%)
OTC-EM-SMX/TS	2 (5.7%)
OTC-EM-SM	1 (2.85%)
OTC-ABPC-SMX/TS	1 (2.85%)
OTC-ABPC-SMX/TS-EM-CI	1 (2.85%)
OTC-ABPC-SMX/TS-EM-CI-SM	1 (2.85%)

ABPC: ampicillin; CI: ciprofloxacin; OTC: oxytetracycline; SM: streptomycin; SMX/TS: sulfamethoxazol/trimethoprim; EM: erythromycin.

mL to 20 mL) were filtered through 47 mm diameter polycarbonate filters (0.2 μm pore size, Millipore), which were stored at –20°C until use. The extraction of DNA from the filter was carried out according to a modified version of the cethyltrimethylammonium bromide (CTAB)-method (Wilson, 1987). This DNA was examined for PCR.

Detection of tet(M) by PCR

For PCR of *tet*(M) genomic DNA was extracted from the isolated OTC^r strains according to Kim *et al.*, (2003). The extracted DNA was precipitated using ethanol. Purified DNA was resuspended in 30 μL of TE buffer and stored at –20°C.

To detect *tet*(M), PCR was performed according to Aarestrup *et al.*, (2000). Primers were *tet*(M)-1 (5′-GTTAAATAGTGTTCTTGGAG-3′) and *tet*(M)-2 (5′-CTAAGATATGGCTCTAACAA-3′), which generated a 657 bp amplicon (Aarestrup *et al.*, 2000). PCR products on 1.5% agarose gel were stained with ethidium bromide and visualized on an Epi-Light UV FA1100 system with a Luminous Imager version 2.0 (Aisin Cosmos R&D, Aichi, Japan).

RESULT AND DISCUSSIONS

Seawater samples were collected from coastal seawater in November, 2010 in Korea. This season was not active season for aquaculture. As shown Fig. 2, total cell counts (DAPI count) were 9.17×10^5 to 1.67×10^6 cells/mL in the seawater, whereas colony count was $0.26 \pm 0.14 \times 10^3$ to $5.00 \pm 2.8 \times 10^5$ CFU/mL. OTC^r bacteria at KYS-2 and KWD-2 sites were accounted for 0.2–0.35% of the total CFU, while other sites were under the 0.2%. The tetracycline-resistant (TC^r) bacterial rate in the sediment under the fish cages were 2.7–60.7% and 4.7–64.8% in seawater, in aquaculture site (Neela *et al.*, 2007) and the occurrence rate

of TC^r bacteria against 60 μg/mL of tetracycline was 0.79–14.7% and against 120 μg/mL of tetracycline was 0.0–4.4% in open ocean sediment (Rahman *et al*., 2008). Compare to those, present data showed lower rate. In Korea, low contamination possibly did not induce OTC^r bacteria.

The OTC concentration in water was below detection limit (0.1 μg/L), although one site (KWD-2) showed 0.18 ± 0.01 μg/L indicating that drug use was negligible at the moment.

Among the OTC^r colonies, we randomly isolated 35 strains. OTC^r were defined when MIC value is greater than 60 μg/mL. To know the cross-resistance patterns of OTC^r isolates, the susceptibility of the 35 OTC-resistant strains against other antibiotics was examined. Eight strains out of 35 (23%) showed resistance to 3 drugs, ABPC, SMX/TS and EM (Table 2), followed by cross-resistance to EM and ABPC. Neela *et al*. (2007) showed that in Japanese coastal aquaculture site showed that water column isolates of OTC^r were cross-resistant to ABPC-mecillinam (26%) and ABPC-EM-mecillinam (22%), whereas sediment isolates were cross-resistant to ABPC-EM (42%). Furthermore, it is found that the seawater strains showed low occurrence rates to ABPC-EM (Neela *et al*., 2007). Present result in Korea showed similar trend. The OTC^r determinants might be linked to ABPC and EM resistance determinants, but not to SM and CI in examined area. Correlation between OTC^r and resistance to ABPC-analogous β-lactams and macrolides were known to be occurred by multi-drug efflux system (Putman *et al*., 2000). We found nine types of resistance patterns in our study. The highest resistance patterns along with OTC^r were OTC-ABPC-SMX/TS-EM, followed by OTC-EM and OTC-ABPC. Sayah *et al*. (2005) reported that the occurrence rate of β-lactam (ABPC and MPC) resistance was high (86%) in seawater strains. These findings suggest that OTC^r is linked to various drug-resistance mechanisms, but a specific trend of pattern would be present which depends on regional difference. Single drug exposure can lead the cross-resistance to other unrelated drugs (George, 1996). Our result indicated presence of OTC^r and multidrug-resistant bacteria in OTC-free environment, suggesting that OTC^r bacteria possess various resistance mechanisms to antibiotics, especially to ABPC-EM combination.

The *tet*(M) is a well studied ribosomal protection protein (RPP) gene, which is known to distribute widely in terrestrial bacteria and various aquatic bacteria (Kim *et al*., 2004; Kobayashi *et al*., 2007). In this study, 30 strains out of 35 isolates were positive for *tet*(M), suggesting that this gene was reserved in culturable bacteria in seawater. The *tet*(M) was detected in bacteria isolated from healthy fish and seawater samples in Korea and Japan (Kim *et al*., 2004). The *tet*(M) can be reserved in bacterial species in fish, water and sediment, although it is not abundant. The *tet*(M) possessing bacteria would include many non-culturable ones. These would be increased in on-season of aquaculture, at which OTC is used. Further monitoring is needed in various seasons when aquaculture is actively performed.

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REFERENCES

Aarestrup, F. M., Y. Agersø, P. Gerner-Smidt, M. Madsen and L. B. Jensen (2000): Comparison of antimicrobial resistance phenotypes and resistance genes in *Enterococcus faecalis* and *Enterococcus faecilum* from humans in the community, broilers, and pigs in Denmark. *Diagn Microbiol Infect Dis*., **37**, 127–137.

Agersø, Y. L., G. Sengeløv and L. B. Jensen (2004): Rapid method for direct detection of specific genes from farm soil, used for detection of *tet*(M) gene in Danish farmland. *Environ. Int*., **30**, 117–122.

Chopra, I. and M. C. Roberts (2001): Tetracycline antibiotics: mode of action, applications, molecular biology and epidemiology of bacterial resistance. *Microbiol. Mol. Rev*., **65**, 232–260.

Douglas, G. C., D. P. Weston, V. Miller and C. Shoemaker (1996): Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. *Aquaculture*,**145**, 55–75.

Furushita, M., T. Shiba, M. Maeda, A. Tahata, Y. Kenekoa, Y. Takahashi, T. T. Hesegawa and M. Ohta (2003): Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. *Appl. Environ. Microbiol*., **69**, 5336–5342.

George, A. M. (1996): Multidrug resistance in enteric and other Gram-negative bacteria. *FEMS Microbiol. Lett*., **139**, 1–10.

Jacobsen, P. and L. Berglind (1988): Persistence of oxytetracycline in sediment from fish farms. *Aquaculture*, **70**, 365–370

Kim, S. R., L. Nonaka, M. J. Oh, C. R. Lavilla-Pitogo and S. Suzuki (2003): Distribution of an oxytetracycline resistance determinant *tet*(34) among marine bacteria isolates of a Vibrio species. *Microbes Environ*., **18**, 74–81.

Kim, S. R., L. Nonaka and S. Suzuki (2004): Occurrence of tetracycline resistance genes *tet*(M) and *tet*(S) in bacteria from marine aquaculture sites. *FEMS Microbiol. Lett*., **237**, 147–156.

Knapp, C. W., C. A. Engemann, M. L. Hanson, P. L. Keen, K. J. Hall and D. W. Graham (2008): Indirect evidence of transposon-mediate selection of antibiotic resistance genes in aquaculture systems at low-level oxytetracycline exposures. *Environ. Sci. Technol*., **42**, 5348–5353.

Kobayashi, T., F. Suehiro, B. C. Tuyen and S. Suzuki (2007): Distribution and diversity of tetracycline resistance genes encoding ribosomal protection protein in Mekong river sediments in Vietnam. *FEMS Microbiol. Ecol*., **59**, 729–737.

Neela, F. A., L. Nonaka and S. Suzuki (2007): The diversity of multi-drug resistance profiles in tetracycline-resistant *Vibrio* species isolated from coastal sediments and seawater. *J. Microbiol*., **45**, 64–68.

Nonaka, L., T. Isshiki and S. Suzuki (2000): The occurrence of oxytetracycline resistant bacteria in the fish intestine and the seawater environment. *Microbes Environ*., **15**, 223–228.

Nonaka, L., K. Ikeno and S. Suzuki (2007): Distribution of tetracycline resistance gene, *tet*(M), in Gram-positive and Gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture sites in Japan. *Microbes Environ*., **22**, 335–364.

Putman, M., H. van Veen and W. Knonings (2000): Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev*., **64**, 672–693.

Rahman, M. H., K. O. Sakamoto, L. Nonaka and S. Suzuki (2008): Occurrence and diversity of the tetracycline resistance gene *tet*(M) in enteric bacteria of Antartic Adélie penguins. *J. Antimicrob. Chemother*., **62**, 627–628.

Roberts, M. C. (2005): Update on acquired tetracycline resistance genes. *FEMS Microbiol. Lett*., **245**, 195–203.

Sayah, R. S., J. B. Kaneene, Y. Johnson and R. A. Miller (2005): Patterns of antimicrobial resistance observed in *Escherichia coli* isolates obtained from domestic- and wild-animal fecal samples, human septage, and surface water. *Appl. Environ. Microbiol*., **71**, 1394–1404.

Schmidt, A. S., M. S. Bruun, I. Dalsgaard and J. L. Larsen (2001): Incidence, distribution, and spread of tetracycline resistance determinants and integron-associated antibiotic resistance genes

among motile aeromonad from a fish farming environment. *Appl. Environ. Microbiol.*, **67**, 5675–5682.

Suzuki, S., T. Kobayashi, F. Suehiro, B. C. Tuyen and T. S. Tana (2008): High occurrence rate of tetracycline (TC)-resistant bacteria and TC resistance genes relates to microbial diversity in sediment of Mekong river main waterway. *Microbes Environ.*, **23**, 149–152.

Wilson, K. (1987): *Preparation of Genomic DNA from Bacteria: Current Protocols in Molecular Biology*. John Willey & Sons, New York, U.S.A., 2.4.1–2.4.5 pp.

Zhang, X. X., T. Zhang and H. H. Fang (2009): Antibiotic resistance genes in water environment. *Appl. Microbiol. Biotechol.*, **82**, 397–414.

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