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Genetic and Physiological Characterization of Oxytetracycline-Resistant Bacteria from Giant Prawn Farms

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Four hundred and thirteen oxytetracycline-resistant bacteria were recovered from six freshwater giant prawn farms with a history of oxytetracycline use. Most oxytetracycline-resistant isolates were Gram-negative bacteria. Six groups of oxytetracycline-resistant bacteria were classified using cluster analysis based on a comparison of levels of oxytetracycline resistance. Complex fingerprint patterns were obtained for 71 isolates studied. In general, the band patterns of isolates from different ponds were very similar, and the data indicated that the isolates were closely related. The exploration for cross-resistance found that most of the 71 oxytetracycline-resistant isolates were also resistant to tetracycline and chlortetracycline, but had a relatively low resistance to doxycycline. Many isolates showed higher chlortetracycline resistance than oxytetracycline resistance. Additionally, the oxytetracycline-resistant isolates were examined for the presence of tetracycline resistance (*tet*) genes. Fifty percent of the isolates carried one of the 14 known *tet* genes examined. The most common determinants were TetA and TetD. However, TetB, TetC, TetE, TetK, TetL, and TetM were also found with various frequencies.

Keywords: Oxytetracycline-resistant bacteria, freshwater giant prawn, antibiotics

The significant increase in global demand for shrimp has encouraged many developing countries to enter into the practice of shrimp farming. This has made Thailand the world's leader in shrimp exports. Thai shrimp farming has rapidly developed during the 1980s. Since 1993, Thai shrimp farmers produced 235,000–275,000 tons of cultured shrimps annually [10]. From shrimp exports, Thailand has earned more than 2 billion USD annually, which corresponds to 3–4% of the country's total export value in 2000 and 2001 [2–4].

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Like all food production sectors, aquaculture requires external inputs for successful production, including chemicals and antibiotics. Antibiotics are extensively used in shrimp aquaculture to treat bacterial infections. Some of the antibiotics widely used in Thailand include erythromycin, nitrofurans (furacin, furanace), oxytetracycline, sulfamonomethoxine (Dimeton), and oxolinic acid [25]. They are applied through feed additions or by simple addition to the water. Most of the unused antibiotics end up in sediments, where they are either degraded or slowly leached back into the surrounding water.

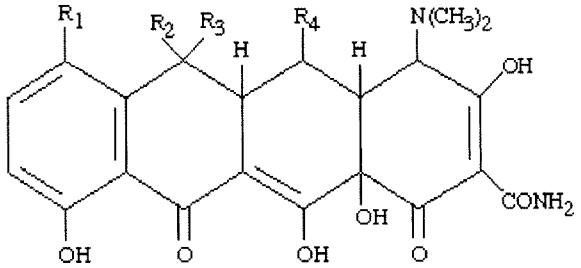
Antibiotics and other chemicals used in aquaculture may be toxic not only to the target pathogen, but also to nontarget populations such as the cultured species, wild flora and fauna, and human consumers. Many antibiotics mixed with feed tend not to be absorbed by fish, and many studies have reported that about 60–85% of antibiotics can be excreted through feces in an unchanged form [1, 22, 26]. In addition, a great deal of the antibiotic-treated feed falls, uneaten, to pond beds, where it accumulates in the sediments.

Antibiotics vary in their persistence in sediments, which can range from a day to 1.5 years. The most commonly used antibiotics, oxytetracycline (OTC) and oxolinic acid, can persist in sediments for 6 months [26]. Jacobsen and Berglind [15] studied the persistence of oxytetracycline in fish farms. They indicated that OTC is relatively persistent in anoxic sediments. OTC concentrations found in sediments can vary from 0.1 to 4.9 mg/kg dry matter. OTC may remain in concentrations capable of causing antibacterial effects for 12 weeks within the sediments after the cessation of treatment. Coyne *et al.* [8] investigated the concentration of OTC in the sediment of two cages at a fish farm site, and found half-lives of 16 and 13 days. Hektoen *et al.* [13] found that oxytetracycline, oxolinic acid, flumequine, and sarafloxacine were very persistent in sediments. In the deeper layer of the sediment, hardly any degradation occurred after 180 days with a calculated half-life of more than 300 days. However, antibiotic residues in the top layer of the sediment rapidly disappeared. The removal of these substances

from the sediment is most probably due to leaching and redistribution rather than degradation.

Oxytetracycline, furazolidone, erythromycin, and kanamycin have been found to be health hazards associated with digestive disorders and allergies [23]. Lutzhøft *et al.* [18] found that cyanobacteria have a greater sensitivity towards antibacterial agents compared with crustaceans and fish. Moreover, widespread antibiotic applications have the potential to cause the development of drug resistance among pathogens. Antibiotic resistance has been identified in strains of *Aeromonas salmonicida*, the bacteria responsible for furunculosis [5, 12]. Antibiotic resistance has also been reported in natural sediment bacteria from antibiotics accumulating below net pens [7, 14, 17, 21]. The presence of antibiotics in the bottom sediments may affect the natural bacterial composition and activity, and thereby change the ecological structure of benthic microbial communities. The accumulation of antibiotics in pond sediment can also lead to decreased or inhibited microbial activity in the sediment.

Antibiotic-resistant bacteria isolated from animal farms or aquaculture are raising concerns that antibiotics used in agriculture may play an important role in selecting for antibiotic resistance among foodborne bacteria. The environmental fate of veterinary drugs and the factors that influence the persistence and biodegradation of antibiotics used in agriculture is not yet well understood. Metabolites resulting from the biotransformation of these drugs may have either enhanced or reduced biological activity compared with the parent compound, and may affect the microbial ecology of these systems.



Antibiotics	R1	R2	R3	R4
Tetracycline	H	CH_3	OH	H
Chlortetracycline	Cl	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Doxycycline	H	CH_3	H	OH

Fig. 1. The chemical structure of the compounds used in this study.

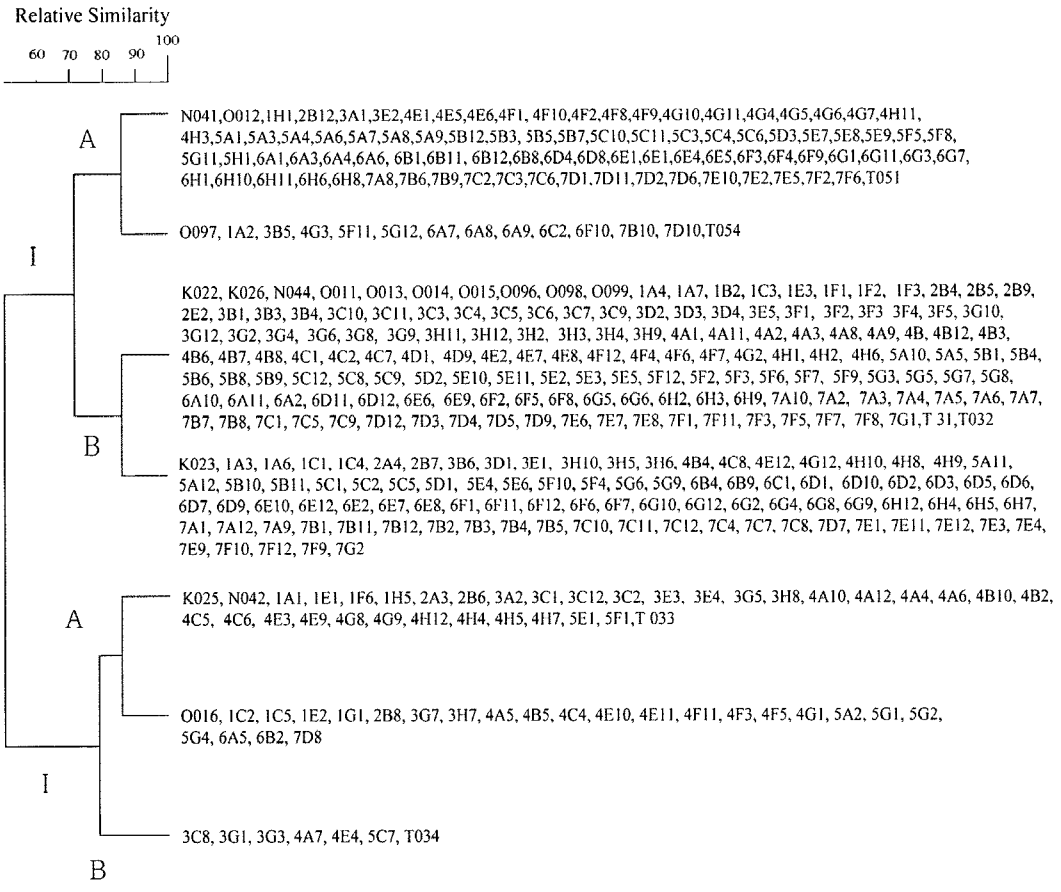


Fig. 2. Dendrogram prepared from the comparison of level of oxytetracycline resistance.

To better understand the fate and effect of antibiotics in the aquaculture system, oxytetracycline was chosen for this study. Oxytetracycline is not only one of the most commonly used antibacterials in aquaculture, but is also used in animal farms. Therefore, the main purpose of this study was to examine resistance profiles of bacteria in an aquaculture system.

MATERIALS AND METHODS

Chemicals

The tetracycline class antibiotics used in this study, oxytetracycline, chlortetracycline, doxycycline, and tetracycline, were obtained from Sigma (St. Louis, MO, U.S.A.). The chemical structures of these compounds are shown in Fig. 1.

Freshwater Giant Prawn Farms and Sampling Sites

This study was initiated with soil, sediment, and water samples obtained from six freshwater giant prawn (*Macrobrachium rosenbergii*) ponds, on farms located in Nakhon Pathom Province in Thailand, which is approximately 80 km northwest of Bangkok. The ponds were chosen based on their histories of oxytetracycline applications. The sizes of the ponds varied between 0.3 and 1.6 ha.

Isolation of Oxytetracycline-Resistant Bacteria

Soil and sediment samples were diluted 10-fold on 0.85% NaCl and agitated using a vortex mixer. Extracts were serially diluted 10-fold in 0.85% NaCl, and 0.1-ml aliquots were plated on Mueller-Hinton agar supplemented with 10 μg/ml of oxytetracycline. Medium without antibiotic was used as a positive control. The plates were incubated at 30°C for 24 to 48 h. Total and oxytetracycline-resistant colony counts were acquired. Oxytetracycline resistant bacteria were purified and stored in 96-well microtiter plates containing freezing medium [27] and kept at -80°C until used for later study.

Identification and Characterization

The resistance profiles of oxytetracycline-resistant bacteria were determined for each of the isolates. Strains were grown at 28°C for 18–36 h in Mueller-Hinton (MH) broth with six concentrations of oxytetracycline (10, 20, 40, 60, 100, and 120 μg/ml) using 96-well microtiter plates. Resistance was defined as showing bacterial growth that was 70% of OD_{600} compared with growth of the same strain in the control MH broth without oxytetracycline. Strains were categorized as being resistant or sensitive to each concentration of oxytetracycline, and assigned a value of 1 or 0, respectively. The resistance profiles of 413 oxytetracycline-resistant isolates were used for dendrogram analyses. The dendrogram was produced using Jaccard similarity coefficients.

HFERP DNA Fingerprinting

Seventy-one isolates were further characterized by DNA fingerprint analysis. Isolates were chosen based on profiles of resistance to the tested antibiotics. DNA fingerprints were obtained by using the horizontal, fluorophore-enhanced, rep-PCR (HFERP) method as described by Johnson *et al.* [16]. Fingerprint data were normalized and analyzed using BioNumerics v.3.5 software (Applied Maths, Sint-Martens-Latem, Belgium). DNA fingerprint similarities were calculated by using Pearson's product-moment correlation coefficient, with 1% optimization. A binary band-matching character table was generated by using the HFERP-derived PCR DNA fingerprint data, and results were analyzed, accounting for the covariance structure, by using the multidimensional scaling (MDS) and multivariate analysis of variance (MANOVA), forms of discriminant analysis, subroutines of the Bionumerics software.

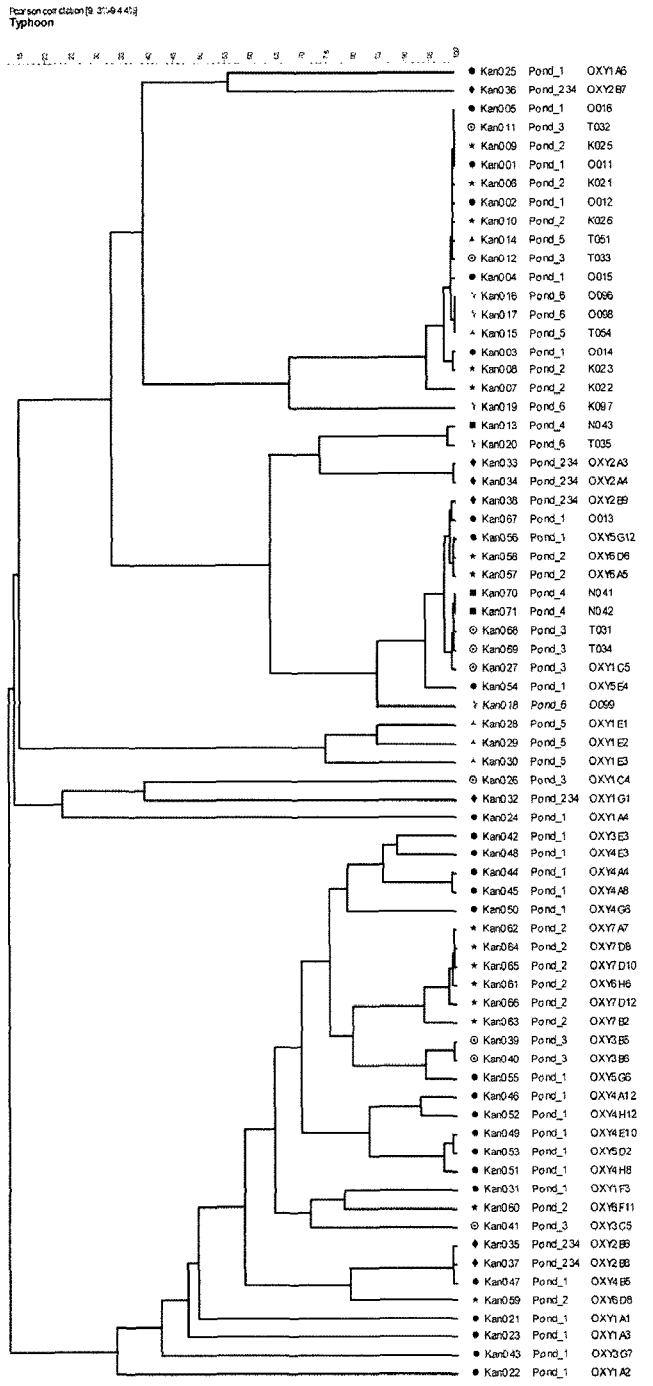


Fig. 3. Dendrogram showing the oxytetracycline resistance isolates obtained from soil, sediment, or water from six prawn ponds and irrigation system.

Determination for Cross-resistance to Antibiotic of Tetracycline Class

Seventy-one oxytetracycline-resistant bacterial isolates were examined for cross-resistance to chlortetracycline, doxycycline, or tetracycline using 96-well microtiter plates as described above.

Tetracycline-Resistant Gene Determination

The 71 isolates were examined further using a multiplex PCR for the presence of the 14 tetracycline resistance genes: *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX* [20]. Pairs of primers were multiplexed in groups as described by Ng *et al.* [20]: Group I: *tetB*, *tetC*, *tetD*; Group II: *tetA*, *tetE*, *tetG*; Group III: *tetK*, *tetL*, *tetM*, *tetO*, *tetS*; Group IV: *tetA(P)*, *tetQ*, *tetX*. PCR reactions were prepared as described by Bryan *et al.* [6]. Single-colony isolates were streaked onto MH agar supplemented with 10 μg/ml oxytetracycline and picked using sterile loops and suspended in 50 μl of sterile H_2O. One μl of the standardized cell suspension served as a template DNA for colony-based multiplex PCR. The primers used for PCR amplification of the 14 tetracycline resistance genes were as described by Ng *et al.* [20]. The primers were aliquoted into four groups: group I contained primers for *tetB*, *tetC*, and *tetD*; group II contained primers for *tetA*, *tetE*, and *tetG*; group III contained primers for *tetK*, *tetL*, *tetM*, *tetO*, and *tetS*; and group IV contained primers for *tetA(P)*, *tetQ*, and *tetX*. PCR was performed with an MJ Research (Waltham, MA, U.S.A.) model PTC100 thermocycler, by using the following conditions as described previously: 5 min of initial denaturation at 94°C, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. The PCR products were separated by gel electrophoresis in 1% (w/v) agarose gels in 1× Tris-acetate-EDTA buffer, stained with ethidium bromide, and visualized under UV illumination. The validity of multiplex PCRs and product sizes was ascertained by using the following positive control plasmids: pSL18, pRT11, pBR322, pSL106, pSL1504, pJA8122, pAT102, pVB.A15, pJ13, pUOA1, pAT451, pJIR39, pNFD13-2, and pBS5, for the genes *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetK*, *tetL*, *tetM*, *tetO*, *tetS*, *tetA(P)*, *tetQ*, and *tetX*, respectively. The sizes of the PCR products were determined by comparison with the migration of a GeneRuller 100-bp ladder (MBI Fermentas).

Results and Discussion

Isolation, Identification, and Characterization of Oxytetracycline-Resistant Bacteria

A total of 413 bacterial colonies were isolated from six freshwater giant prawn ponds and the percentage of oxytetracycline-resistant isolates was different from each pond (data not shown). These isolates were further purified and examined for oxytetracycline resistance and dendrogram analysis (Fig. 2). Of the 413 isolates, 37.0% (153 isolates), 22.3% (92), 21.3% (88), 3.4% (14), 5.8% (24), and 8.5% (35) were resistant to >120, 100, 60, 40, 20, and 10 μg/ml oxytetracycline, respectively. However, 1.7% (7) were not resistant to 10 μg/ml oxytetracycline, and not used in further studies.

Dendrogram analysis (Fig. 2) indicated that the strains could be divided into two major subgroups, I and II, which diverged at a similarity value of 50%. The group I isolates contained the majority of strains, and could be further divided into two subgroups (A and B) that diverged at a similarity value of 70%. Each of these subgroups could be further divided into two subsubgroups (1 and 2). Overall strains in subgroups A1 and A2, and B1 and B2, were related to each other, with similarity values of 85%. In contrast, Group II consisted of far fewer strains. There was no apparent relationship between isolation pond and subgroup status.

The HFERP DNA fingerprinting technique was conducted to differentiate oxytetracycline-resistant isolates. Complex fingerprint patterns were obtained for the 71 isolates studied.

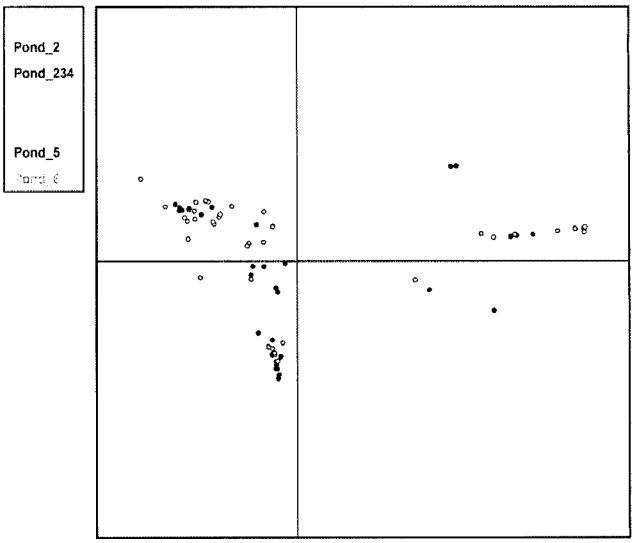


Fig. 4. Pearson's Correlation Coefficient Analysis (PCA) of rep-PCR genomic fingerprint of the 71 oxytetracycline resistance isolates obtained from soil, sediment, and water from six prawn ponds and irrigation system.
Note: Pond 234 is a site nearby ponds 2, 3, and 4.

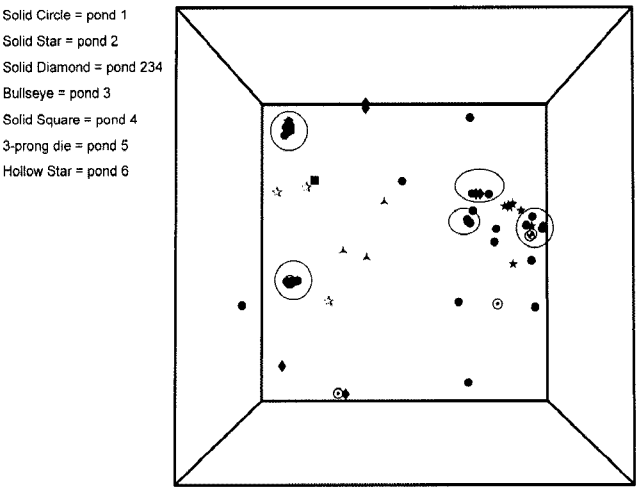


Fig. 5. Multidimensional scaling (MDS) of rep-PCR genomic fingerprint of the 71 oxytetracycline resistance isolates.
Note: Pond 234 is a site nearby ponds 2, 3, and 4.

There was a very high proportion of genetically identical clones found, shared in all of the ponds (Figs. 3 and 4). Almost half of the isolates present in the analysis were clones of some isolates present in another pond. We define a clone in HFERP as any isolate sharing 92% or more similarity based on Pearson's Correlation Coefficient (Fig. 4). This condition is usually found in areas of low genetic diversity in which all of the sampling points share a common source of contamination. Multidimensional scaling (MDS) was performed to visualize the large clusters of clones within the dataset. The MDS indicated that a large number of strains in different ponds were related and clustered together in MDS analysis (Fig. 5). Multivariate analysis of variance (MANOVA) was then performed to confirm that there were no significant variables affecting genetic similarity from site to site (Fig. 6). This indicates that there was little to no change in the genetic diversity between sites.

Additionally, all of these 71 isolates were further tested for Gram-stain reaction. The result showed that most oxytetracycline-resistant isolates were Gram-negative bacteria with rod shape. Only a few of them were Gram-positive bacteria.

Determination for Cross-resistance to Antibiotics of Tetracycline Class

The 71 isolates were randomly selected based on sample sites and classification using dendrogram analysis. These 71 isolates were determined for cross-resistance to antibiotics of tetracycline class. Isolates were resistant to oxytetracycline, tetracycline, chlortetracycline, and doxycline.

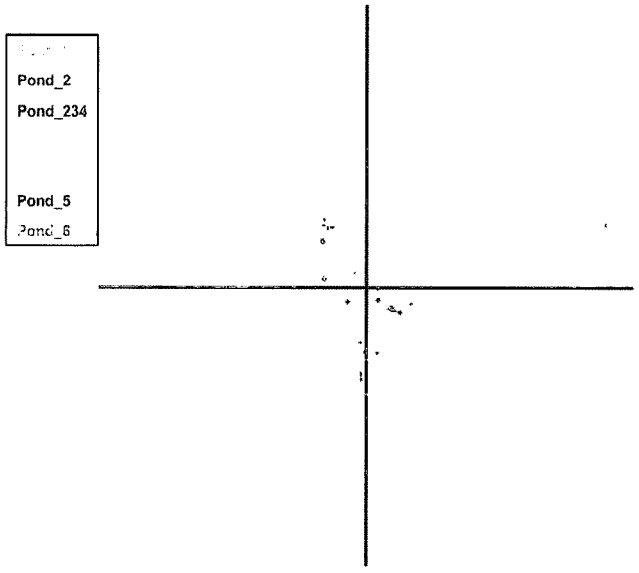


Fig. 6. Multivariate analysis of variance (MANOVA) of rep-PCR genomic fingerprint of the 71 oxytetracycline resistance isolates.
Note: Pond 234 is a site nearby ponds 2, 3, and 4.

Most of the isolates in the ponds had high resistance to three antibiotics (*i.e.*, oxytetracycline, tetracycline, and chlortetracycline), but relatively low resistant to doxycycline. Of all isolates, 72% were resistant to all four antibiotics, 16% were resistant to oxytetracycline, tetracycline, and chlortetracycline, 6% were resistant to only oxytetracycline, 3% were resistant to oxytetracycline and tetracycline, and 1.5% were resistant to oxytetracycline and chlortetracycline.

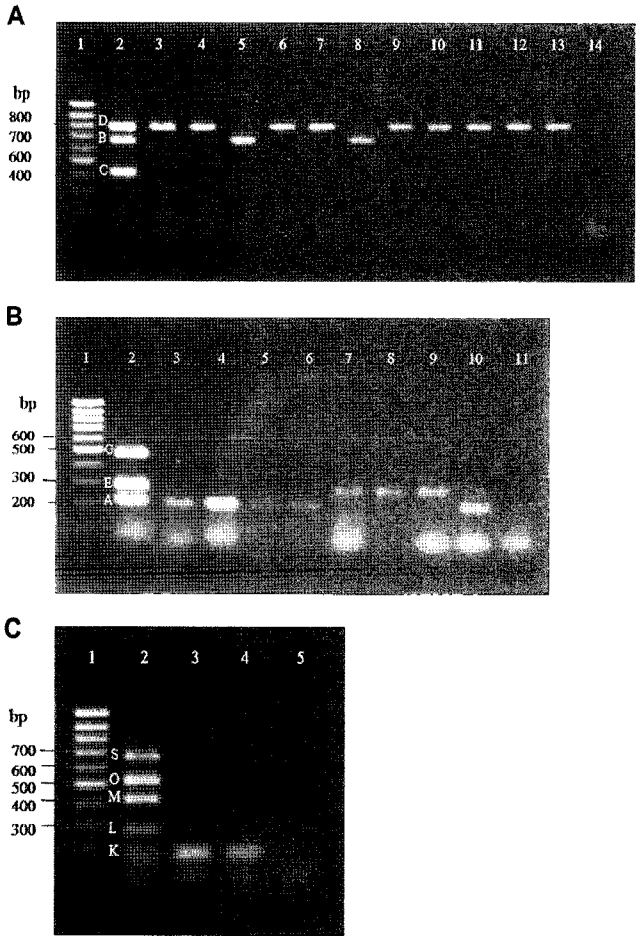


Fig. 7. A. Representative agarose gel of PCR products from oxytetracycline-resistant isolates, using primer group I, containing primers for *tetB*, *tetC*, and *tetD*. Lanes: 1, molecular weight markers (GeneRuller 100-bp ladder); 2, positive control; 3, O011; 4, O014; 5, O099; 6, 1A1; 7, 1A2; 8, 1A4; 9, 2A3; 10, 2A4; 11, 3B5; 12, 3B6; 13, 3C5; 14, no template control. The sizes of the amplicons in base pairs are indicated on the left. **B.** Representative agarose gel of PCR products from oxytetracycline-resistant isolates, using primer group II, containing primers for *tetA*, *tetE*, and *tetG*. Lanes: 1, molecular weight markers (GeneRuller 100-bp ladder); 2, positive control; 3, 3E3; 4, 4B5; 5, 4H8; 6, 5C4; 7, 3A2; 8, 5G6; 9, 5G12; 10, 1C4; 11, no template control. The sizes of the amplicons in base pairs are indicated on the left. **C.** Representative agarose gel of PCR products from oxytetracycline-resistant isolates, using primer group II, containing primers for *tetK*, *tetL*, *tetM*, *tetO*, and *tetS*. Lanes: 1, molecular weight markers (GeneRuller 100-bp ladder); 2, positive control; 3, N044; 4, O098; 5, no template control. The sizes of the amplicons in base pairs are indicated on the left.

Table 1. Tetracycline-resistant genes in oxytetracycline-resistant isolates.

Pond	Source	Isolation	Tetracycline-resistant genes			
			Group 1 (B, C, D)	Group 2 (A, E, G)	Group 3 (K, L,M, O, S)	Group 4 (A(P), Q, X)
1	Soil	3E3	-	A	-	-
		3G7	D	-	-	-
		4A4	D	-	-	-
		4A12	-	A	-	-
		4B5	-	A	-	-
		4E10	-	A	-	-
		4H8	-	A	-	-
		5C4	-	A	-	-
		5D2	D	-	-	-
		5E4	D	-	-	-
		5G6	D	E	-	-
		5G12	D	E	-	-
		O011	D	-	-	
		O013	-	E	-	-
		O014	D	-	-	
		O015	-	A	-	-
	Water	1A1	D	-	-	-
		1A2	D	-	-	-
		1A4	B	-	-	-
		1A6	-	A	-	-
2	Sediment	6F11	-	A	S	-
		6D6	-	A	-	-
		6D8	-	-	K	-
		6H6	B	-	-	-
		K022	-	A	-	-
		K026	-	A	-	-
3	Sediment	3A2	-	E	-	-
		3B5	D	-	-	-
		3B6	D	-	-	-
		3C5	D	-	-	-
		3C12	D	-	-	-
		T031	-	A	-	-
		T032	-	A	-	-
		T033	-	A	-	-
	Water	1C4	-	A	-	-
4	Sediment	N041	-	A, E	-	-
		N042	-	-	L	-
		N044	-	-	K	-
2, 3, 4	Drainage outlet	1G1	-	-	L	-
		2A3	D	-	-	-
		2A4	D	-	-	-
	Irregation system	2B6	D	-	-	-
5	Sediment	T051	-	E	-	-
		T054	-	E	-	-
6	Sediment	O096	-	A	-	-
		O097	-	A	-	-
		O098	-	-	K	-
		O099	B, C	-	M	-

Surprisingly, it was found that most of the isolates showed higher resistance to chlortetracycline than oxytetracycline. This is because the freshwater giant prawn farmers had been using oxytetracycline for diseases disinfection for long periods of time and only until recently has the government tried to control the use of oxytetracycline in the prawn farm. Then, the prawn farmers changed from using oxytetracycline to chlortetracycline.

Distribution of Tetracycline-Resistant Genes

Results of the distribution of the *tet* genes using PCR showed that 67.6% of the 71 isolates contained at least 1 of 14 tetracycline resistance genes. The most common determinants were TetA (26.8% of isolates) and TetD (23.9% of isolates) (Figs. 7A, 7B, and 7C and Table 1). However, TetB, TetC, TetE, TetK, TetL, and TetM were also found with various frequencies. The presence of *tetA* through *tetG* genes had previously been reported in bacteria isolated from freshwater fish farms [9, 11, 19, 24]. Moreover, TetK, TetL, and TetM were also found in this study. However, the presence of any tetracycline-resistant gene was not found in group 4 (*tetA*(P), *tetQ*, *tetX*).

Acknowledgments

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