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OCCURRENCE OF ANTIBIOTIC RESISTANT BACTERIA IN SOME SHRIMP FARMS OF BANGLADESH

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Abstract

Tetracycline (TC) and ampicillin (AMP) resistant bacteria were identified from both fresh and brackish water shrimp farming system, in Bangladesh. Among 78 isolates from freshwater samples, 14.10, 17.95 and 23.8% were found resistant to TC, AMP and TC plus AMP, respectively. On the other hand, isolates from the brackish water samples, the percentages of TC, AMP and TC plus AMP resistant isolates were 10.25, 12.82 and 15.38, respectively. In freshwater samples, the highest percentage of resistant bacteria was *Bacillus* sp. (38.9), *Pseudomonas* sp. (22.2), *Staphylococcus* sp. (16.7), *Acinetobacter* sp. (11.1), *Brevibacillus* sp. (5.5) and *Enterobacter* sp. (5.5). While in brackish water samples dominant resistant bacteria was *Bacillus* sp. (50) followed by *Pseudomonas* sp. (16.7), *Acinetobacter* sp. (16.7), *Enterobacter* sp. (8.3) and *Microvirgula* sp. (8.3).

Antibiotic is an important weapon for the treatment of bacterial diseases in human, animals and plants. However, the increasing development of antibiotic resistant bacteria in the environment is a serious issue in recent years (Kummerer 2004). It was reported that aquatic environment can be a potential reservoir for drug resistant bacteria that could make to transfer from bacteria to the animal and human (Chee-sanford *et al*. 2001).

Modern farming of shrimp is based on the use of antibiotic which add residue and subsequently help to develop antibiotic resistant bacteria (Neela *et al*. 2007). In Bangladesh, shrimp production and the use of antibiotics in the farming system have been increasing significantly but the information related to this is scanty (Khan *et al*. 2007). Therefore, much attention should be paid to monitoring the antibiotic resistance in the aquaculture industries of Bangladesh. In this study, tetracycline and ampicillin resistant bacteria of fresh and brackish water shrimp farms have been investigated.

Water samples were collected using a sterile bottle from the freshwater shrimp farms of Pabna (24.01º N and 89.18º E) and brackish water shrimp farms of Satkhira (22.35º N and 89.08º E). From each site four stations were sampled. All the samples were transported to the laboratory in an ice box and analyzed. To enumerate viable bacteria, water samples were mixed with sterile phosphate buffered saline (PBS) and a ten-fold serial dilution was made. Plate counts were performed in duplicate on nutrient agar. The plates were incubated at 25°C for 3 days.

The minimum inhibitory concentration (MIC) was determined by agar dilution method (Neela *et al*. 2007). A bacterial cell suspension was prepared in PBS and cell density was adjusted to Macfarland No. 1.0. Ten microliters of bacterial cell suspension was spotted onto nutrient agar medium containing two-fold dilution of TC and AMP concentration 0, 0.5, 1.0, 2, 4, 16, 32, 64, 128 and 256 µg/ml. Plates were incubated at 25°C for 24 hrs. Resistance to an antibiotic was defined as $\geq$ 32 µg/ml (Walsh 2003). Antibiotic resistant bacteria were cultured in nutrient broth at 25°C for 24 hrs with shaking. Cells were harvested by centrifugation at 3000 × g for 10 min and then DNA was extracted (Rahman *et al*. 2008).

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Resistant bacteria were classified by 16S rRNA gene analysis. PCR amplification of 16S rRNA gene was performed using the set of primers of f10 (5'-AGTTTGATCCTGGCTCAG -3'), and r907 (5'-CCGTCAATTCCTTTRAGTTT-3'). The PCR amplification was performed (Rahman *et al*. 2008). The PCR products of 16S rRNA was purified by ethanol precipitation and sequencing was performed for both strands with a BigDye Terminater version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) by a 3130× AB1 PRISM DNA sequencer (Applied Biosystems, Foster City, CA, USA). Online similarity searching was performed using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information web site (NCBI, http://www.ncbi.nlm.nih.gov/). Plasmid DNA was extracted using a QIAGEN plasmid kit (Qiagen, USA).

The viable bacterial number ranged from 1.1×10^6 - 2.2×10^6 cfu/ml, and 1.6×10^5 - 1.3×10^6 cfu/ml in freshwater and brackish water shrimp farms, respectively (Table 1). Of the randomly selected 78 strains from freshwater, 14.10, 17.95 and 23.08% of them showed resistance to TC, AMP and TC plus AMP, respectively. In brackish water samples, the percentage of TC, AMP and TC plus AMP resistant isolates were 10.25, 12.82 and 15.38, respectively (Table 1). There are many reports showing that quite a high number of antibiotic resistant bacteria distributed in different aquatic environments associated with aquaculture (DePaola *et al*. 1988, Nonaka *et al*. 2007).

Table 1. Total viable bacteria and resistant bacteria to different antibiotics from two sites.

Study sites	Total viable bacteia (cfu/ml)		Resistant bacteria (%)		
	Minimum	Maximum	TC	AMP	PC + AMP
Freshwater	1.1 - 106	2.2 - 106	14.10	17.95	23.08
Brackish water	1.6 - 105	1.3 - 106	10.25	12.82	15.38

The MIC investigation on 78 isolates from both the freshwater and brackish water habitats revealed 14.10, 7.69, 7.69 and 17.95, 7.69, 5.12% isolates showed resistant to 32, 64 and 128 µg/ml of TC and AMP in freshwater, respectively (Table 2). In the case of brackish water 10.25, 5.12, 3.85 and 12.82, 6.41, 6.41% was found resistant to 32, 64 and 128 µg/ml of TC and AMP, respectively (Table 2). Earlier reports (Walsh 2003, Neela *et al*. 2007) indicated that bacteria from environmental samples can be considered as resistance, which are resistant to at least 32 µg/ml. A high number of isolates were found resistant to 32 and 128 µg/ml of TC and AMP, respectively. Beta-lactamase resistance has been reported commonly in *Aeromonas* spp. from aquaculture sites (Alderman and Hastings 1998). On the contrary, maximum number of tetracycline resistant bacterial flora were isolated from shrimp cultured ponds (Tendencia and Peña 2001). In the present study, the highest and lowest incidence of resistance to AMP and TC, respectively was found to be similar to Vaseeharan *et al.* (2005).

To classify the resistant bacteria, 18 strains from freshwater and 12 from brackish water were considered. The 16S rRNA analysis revealed that *Bacillus* sp. (38.9%) was dominant in freshwater followed by the species of *Pseudomonas*, *Staphylococcus*, *Acinetobacter*, *Brevibacillus* and *Enterobacter* (Table 3). Similarly, in brackish water *Bacillus* (50%) was dominant followed by *Pseudomonas*, *Acinetobacter*, *Enterobacter* and *Microvirgula* (Table 3). *Bacillus* sp. was isolated from necrotic muscular lesions in catfish in a commercial pond in Alabama, USA (Goodwin *et al*. 1994). The tetracycline resistance determinant *Tet* 39 was reported in TC resistant *Acinetobacter* spp. isolated from integrated fish farms in Thailand (Agerso and Petersen 2007). *Staphylococcus* sp. is a clinically important pathogen, as it is isolated from freshwater samples of shrimp farms. Furushita *et al*. (2003) noted that tetracycline resistant bacteria isolated from fish farm were

identical to clinical isolates suggesting a contamination of hospital waste. In the present study, *Microvirgula* sp. was isolated from water samples in shrimp farms. *Microvirgula* sp. is a Gram-negative bacterium exhibiting simultaneous removal of nitrogen and phosphorus as aquatic contaminant by marine bacteria isolated from shrimp aquaculture ponds, Japan (Atureau *et al*., 1998). Recently, this organism has been isolated as causative agent in clinical infection (Murphy *et al.* 2012).

Table 2. Number of bacterial strains showed resistant to different concentration of tetracycline and ampicillin in the two sites.

Study sites	Antibiotics	Concentrations									
		0	1	2	4	8	16	32	64	128	256
Freshwater	Tetracycline	78	52	28	22	17	14^S	11	6^R	6	0
	Ampicycline	78	56	55	24	21	17	14	6	4	0
Brackish water	Tetracycline	78	63	52	29	23	10	8	4	3	0
	Ampicycline	78	56	55	51	40	30	10	5	5	0

R and S represent resistant and sensitive respectively.

Table 3. Classification of TC and AMP resistant bacteria from two sites.

Study sites	Closest relations to	Similarity (%)	Presence of plasmid
Freshwater	*Acinetobacter* sp.	95 - 100	-
	Bacillus sp.	99 - 100	+
	Brevibaciilus sp.	99	-
	Enterobacter sp.	97	+
	Pseudomonas sp.	98 - 100	+
	Staphylococcus sp.	100	-
Brackish water	*Acinetobacter* sp.	95 - 99	-
	Bacillus sp.	97 - 100	-
	Enterobacter sp.	99	-
	Microvirgula sp.	97	-
	Peudomonas sp.	97 - 99	-

+ and - represent presence and absence, respectively.

The plasmid of *Bacillus*, *Pseudomonas* and *Enterbacter* were detected in the present study (Table 3). Antibiotic resistance is often accompanied, such as plasmids and transposons, such association contributing to the wide distribution of this gene in bacterial genera obtained from different environments by horizontal gene transfer (Chopra and Roberts 2001). This suggests that resistant bacteria can be increased in the wide area of aquatic environments in Bangladesh. Presence of plasmid in *Bacillus* sp., *Enterbacter* sp. and *Pseudomonas* sp. indicated that the resistance gene can be transferred from resistant bacteria to other non-resistant bacteria creating serious health hazards to the public.

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References

Atureau D, Godon JJ and Dabert P 1998. *Microvirgula aerodenitrificans* gen. nov., sp. nov., a new Gram-negative bacterium exhibiting co-respiration of oxygen and nitrogen oxides up to oxygen-saturated conditions. Int. J. Syst. Evol. Microbiol. **48**: 775-782.

Agerso Y and Petersen A 2007. The tetracycline resistance determinant *Tet* 39 and the sulphonamide resistance gene sulII are common among resistant *Acinetobacter* spp. isolated from integrated fish farms in Thailand. J Antimicrob. Chemother. **59**: 23-27.

Alderman J and Hastings TS 1998. Antibiotic use in aquaculture; development of antibiotic resistance - potential for consumer health risks. Int. J. Food Sci. Technol. **33**: 139-155.

Chee-sanford JC, Aminov RI, Krapac IJ, Garrigues-Jean N and Mackie RI 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. **67**: 1494-1502.

Chopra I and Roberts M 2001. Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. **65**: 232-260.

DePaola A, Flynn PA, McPhearson RM and Levy SB 1988. Phenotypic and genotypic characterization of tetracycline and oxytetracycline-resistant *Aeromonas hydrophila* from cultured channel catfish (*Ictalurus punctatus*) and their environments. Appl. Environ. Microbiol. **54**: 1861-1863.

Furushita M, Shiba T and Maeda T 2003. Similarity of tetracycline resistance genes isolated from fish farm bacteria to those from clinical isolates. Appl. Environ. Microbiol. **69**: 5336-5342

Guardabassi L, Dijkshoorn L, Collard JM, Olsen JE and Dalsgaard A 2000. Distribution and *in vitro* transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. J. Med. Microbiol. **49**: 929-936.

Goodwin AE and Roy JS 1994. *Bacillus mycoides* - a bacterial pathogen of channel catfish. Dis. Aquatic Org. **18**: 173-179.

Kummerer K 2004. Resistance in the environments. J Antimicrobial. Chemother. **54**:311-320.

Khan AW, Hossain SJ and Uddin SN 2007. Isolation, identification and determination of antibiotic susceptibility of *Vivrio parahaemolyticus* from shrimp at Khulna region of Bangladesh. Res. J. Microbiol. **2**: 216-227.

Murphy ME, Goodson A, Malnick H, Shah J, Neelamkavil R and Devil R 2012. Recurrent *Microvirgula aerodenitrificans* bacteraemia; a first clinical case report. J. Clinc. Microbiol. doi: 10.1128/JCM.00392-12

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

Nonaka L, Ikeno K and Suzuki S 2007. Distribution of tetracycline resistance gene, *te t*(M), in Gram-positive and Gram-negative bacteria isolated from sediment and seawater at a coastal aquaculture site in Japan. Microbes Environ. **22**: 355-364.

Rahman MH, Nonaka L, Tago R and Suzuki S 2008. Occurence of two genotypes of tetracycline (TC) resistance gene *tet*(M) in the TC-resistant bacteria in marine sediments of Japan. Environ. Sci. Technol. **42**:5055-5061.

Tendencia EA and Peña LD 2001. Antibiotic resistance of bacteria from shrimp ponds. Aquaculture **195**: 193-204.

Vaseeharan B, Ramasamy P, Murugan T and Chen JC 2005. *In vitro* susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. Int. J. Antimicrobial Agents **26**: 285-291.

Walsh C 2003. Natural and producer immunity versus acquired resistance. *In:* Antibiotics: action, origin, resistance. ASM press, Washington DC 2003. p. 91-105.

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