Antibiotic Residues in Tiger Shrimp *(Penaeus monodon)*

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Abstract

Tiger shrimp (*Penaeus monodon* Fabricius) from Bangkok, Thailand, were examined for antibiotic residues. Samples were purchased monthly from open markets between October 1990 and October 1991.

The standard four plate test was used for antibiotic detection during October 1990 through April 1991. Later on, *Bacillus subtilis* in peptone free media, and *B. stearothermophilus* were included. Overall, 8.4 per cent of the 1,461 samples tested were positive. The incidence rate of antibiotic residues each month varied from 0 to 37 per cent.

Identification of antibiotics from 21 positive samples carried out using HPLC showed that oxolinic acid (OA) and oxytetracycline (OTC) were present in 12 and 9 samples, respectively. The concentrations of the drugs were 0.02-2.25 mg·l⁻¹ for OA and 0.06-0.42 mg·l⁻¹ for OTC.

Introduction

Over the past few years, marine shrimp culture has expanded rapidly in Thailand, changing from extensive to intensive systems. Production of tiger shrimp (*Penaeus monodon* Fabricus) rose steadily from 15,841 t worth US$ 53.9 million in 1985 to 80,000 t worth US$ 400 million in 1989. The total area for shrimp farming in 1989 was about 78,201 ha.

The increasing intensification of culture systems has created many environmental problems. There have been heavy mortalities of shrimp either in growout farms or in hatcheries. Bacterial infection caused by *Vibrio* spp. is one

of the major problems. Antibiotics and other chemical agents for prevention and treatment of the infections are used excessively.

There are several reports concerning antibiotic residues in pigs (Panichkriangkrai and Davitiyananda 1986; Saitanu and Kondo 1990), and chickens and eggs (Saisorn et al. 1981; Vongbuddhapitak and Atisook 1984). However, reports on drug residues in shrimp are limited. Recently, the Japanese authorities claimed that frozen tiger shrimp imported from Thailand contained oxolinic acid. This has created many trading problems.

This study was carried out to elucidate the situation of the antibiotic residue problem in tiger shrimp in Thailand.

Materials and Methods

Samples

A total of 1,461 samples of cultured *P. monodon* of different sizes, 30-60 shrimp/kg, were purchased monthly from open markets in Bangkok from October 1990 to October 1991. They were kept in a freezer (-15°C) and tested within one week.

Detection of Antibiotic Residues

The four plate test (FPT) (Smither et al. 1980) was used to test 845 samples. In addition to FPT, *Bacillus subtilis* seeded on Minimal medium (MM) (Spizizen 1958) and *B. stearothermophilus* var. *calidolactis* in assay medium (AM) were used to test 616 samples.

Preparation of Assay Plates

B. subtilis ATCC 6633 and *Micrococcus luteus* ATCC 9341 on nutrient agar and *B. stearothermophilus* var *carlidolactis* C593 NIZO on assay medium (AM) were kept in the refrigerator. Spores of *B. subtilis* were prepared as in Smither et al. (1980) and Medium 1 (Arret et al. 1971) was used.

Muller Hinton Agar (MHA, Difco) was prepared and adjusted with 0.1 N HCl or 0.1 N NaOH to pH 6.0; pH 7.2 with 0.06 $\mu g \cdot ml^{-1}$ trimethoprim (TMP); and pH 8.0 (for *B. subtilis*) and pH 8.0 (for *M. luteus*). The spore suspension of *B. subtilis* was added to the MHA and minimal medium to give 10^6 colony forming units (CFU)/ml. Fresh culture of *M. luteus* in nutrient broth was added to the MHA pH 8.0 at a concentration of 0.5%. The suspension of *B. stearothermophilus* was added to AM to obtain 10^5 CFU/ml. The composition of minimal medium (Spizizen 1958) was as follows: dipotassium phosphate 1.4 g, potassium diphosphate 0.6 g, ammonium sulfate 0.2 g, sodium hydrogen citrate 0.1 g, magnesium sulfate (MgSO4.7H20) 0.22 g, glucose 0.5 g, bacto agar 1.5 g and distilled water 100 ml, pH 6.8. The assay medium consisted of: yeast ex-

tract 0.25 g, tryptone 0.5 g, glucose 0.1 g, bacto agar 1.5 g and distilled water 100 ml, pH 7.0. The plates were kept at 4°C and used within 3 days.

Preparation of Samples for Bioassay

Shrimp samples were thawed and immersed in boiling water for 10-20 seconds to decontaminate the shells. The shells were peeled and the shrimp meat was immersed again as above, then cut transversely into pieces about 0.5 cm in length. One piece was placed on each of the four or six seeded assay plates. The samples were pressed gently to allow firm attachment to the agar. The plates were kept at 4°C for 1 hour before incubation. *B. subtilis* plates were incubated at 30°C, the *M. luteus* plate was incubated at 37°C and the *B. stearothermophilus* plate was incubated at 62°C for 18-24 hours. Positive results were recorded when a clear zone, not less than 2 mm wide, of bacterial inhibition formed around the sample.

Identification of the Antibiotic Residues

Twenty-one pooled samples of positively testing shrimp were further examined for verification and identification of antibiotics.

Extraction Method

Pooled 5-g positive samples from the screening test were homogenized in 0.01 M di-Na EDTA McIlvaine buffer (pH 4.0) and centrifuged. Hexane was added to the supernatant and the mixture was centrifuged. The aqueous layer was mixed well with chloroform and centrifuged again. The chloroform layer was evaporated to dryness and the residue was redissolved in phosphate buffer (pH 8.0) to prepare fraction A. The aqueous layer was passed through a Sep-Pak C_{18} cartridge column. The Sep-Pak C_{18} cartridge column was then washed with water, and absorbed antibacterial agents were eluted with methanol. The eluate was evaporated to dryness and the residue was redissolved in phosphate buffer (pH 4.5) to prepare fraction B.

HPLC Analysis

(a) Liquid chromatography: The apparatus consisted of a Model 6000 A pump, a U6K septumless injection system, a Model 481 variable-wavelength UV detector (Waters Associates, Inc., Milford, MA 01757), and a Chromatopac R1B (Shimazdu Seisaku Co. Ltd., Kyoto, Japan) as an integrator. Chromotographic conditions were as follows: flowrate 1.0-2.5 ml·min^{-1}, at room temperature, detection, 230 nm, 254 nm or 360 nm, 0.002 AUFS.

(b) HPLC column: 4.6 x 150 mm, prepacked with a 10 μm particle size Puresil C18 column (Waters Associates).

(c) Standard solutions and mobile phase: The following standard solutions of drugs and mobile phases were used for each antimicrobial agent group:

(1) *Penicillins (Sigma)*. Penicillin-G and ampicillin were used as standard solutions; the mobile phase was a solution of acetonitrile: methanol:0.01 M KH_2PO_4 (19:11:70, v/v/v), pH 7.1.

(2) *Tetracyclines (Sigma)*. Oxytetracycline, chlortetracycline, tetracycline and minocycline were used as standard solutions; the mobile phase was water 760 ml, acetonitrile 240 ml, N,N'-dimethylformamide 60 ml, ethanolamine 5 ml, $NaH_2PO_4 \cdot 2H_2O$ 2.5 g (pH 2.5).

(3) *Nalidixic acid group (Sigma)*. Nalidixic acid and oxolinic acid were used as standard solutions; the mobile phase was methanol:0.1 M citric acid: acetonitrile (6:7:1, v/v).

(4) *Sulfonamides (Sigma)*. Five sulfonamides: sulfadimethoxine, sulfamethizole, sulfamethazine, sulfamethoxazole and sulfadiazine, were used as standard solutions. The mobile phase was acetonitrile : acetic acid : water (70:0.1:30, v/v/v).

Results

Table 1 shows the prevalence of residues in shrimp. Positive samples varied from 0% in February to 36.9% in June. Table 2 demonstrates the sensitivity of assay plates in detecting the residues. Six hundred and sixteen samples were compared with all 6 assay plates. It was found that the combination of 4 assay-plates, i.e., *B. subtilis* in MHA pH 6.0 and MM, *M. luteus* on MHA pH 8.0 and *B. stearothermophilus* in AM, could detect all positive samples. *B. subtilis* in MM was the most sensitive plate followed with *B. subtilis* in MHA pH 6.0, *B. stearothermophilus* in AM, *B. subtilis* in MHA pH 7.2 with trimethoprim, *B. subtilis* in MHA pH 8 and *M. luteus* in MHA pH 8. Oxytetracycline and oxolinic acid were identified in 9 and 12 samples, respectively. Table 3 shows the concentration of the drugs in the positive samples; these samples were within the range 0.06-0.42 $mg \cdot l^{-1}$ for OTC and 0.02-2.25 $mg \cdot l^{-1}$ for OA.

Table 1. Incidence of antibiotic residues in *P. monodon*.

Year and month	No. of samples	No. positive (%)
1990		
October	50	13 (26.0)
November	141	8 (5.7)
December	97	7 (7.2)
1991		
January	204	7 (3.4)
February	137	0
March	107	17 (15.9)
April	109	7 (6.5)
May	111	8 (7.2)
June	103	38 (36.9)
July	143	10 (7.0)
August	108	6 (5.6)
October	151	1 (0.7)
TOTAL	1,461	122 (8.4)

Table 2. The sensitivity of the 6 assay plates for detection of antibiotic residues in tiger shrimp.

No. test	No. positive	Sensitivity of assay plates	
		Assay plates	No. +sample (% positive)
616	63	pH 6, MM, ML, BS	63 (100.0)
		pH 6, MM, BS	60 (95.2)
		BS, MM	56 (88.9)
		MM	54 (85.7)
		pH 6	58 (82.5)
		BS	50 (79.4)
		pH 7.2T	28 (44.4)
		pH 8	26 (41.3)
		ML	18 (28.6)

pH 6	=	*B. subtilis* in Muller Hinton Agar (MHA) pH 6.0
pH 7.2T	=	*B. subtilis* in MHA pH 7.2 with 0.06 µg/ml trimethoprim
pH 8	=	*B. subtilis* in MHA pH 8
MM	=	*B. subtilis* in Minimal medium
ML	=	*Micrococcus luteus* in MHA pH 8.0
BS	=	*B. stearothermophilus* in assay medium

Table 3. The residue levels of oxytetracycline and oxolinic acid in tiger shrimp.

No. sample	Drug residues	Concentration (mg·l^{-1})
9	Oxytetracycline	0.42, 0.35, 0.32, 0.20, 0.18, 0.15, 0.10, 0.08, 0.06
12	Oxolinic acid	2.25, 2.15, 1.56, 1.21, 1.10, 1.06, 1.05, 0.93, 0.52, 0.23, 0.12, 0.02

Discussion

The incidence of antibiotics in shrimp during the study period was markedly high. The residues were verified as oxolinic acid and oxytetracycline, and the level of the residues was above the accepted level. For detection of the antibacterial residues, several assay plates were needed. However, in small laboratories, we recommend the use of at least two plates, *B. stearothermophilus* in AM, and *B. subtilis* in MM. These detected antibacterial agents in 88.9% of the samples.

The high rate of drug residues in cultured shrimp in the open markets of Thailand reflects the failure of shrimp farm management. There are no regulations for the application of drugs in animals or for drug residues in food. Therefore, various antibacterial agents, mostly oxolinic acid and oxytetracycline are used in shrimp culture without prescription. Animals for export used to be strictly examined for drug residues but not those for domestic consumption.

Drug residues in animal tissues are a potential human hazard. Various problems, including toxicity and allergies to antimicrobial drug residues, e.g.,

penicillin, chloramphenicol, tetracycline and others, have been recognized (Huber 1971; Anon 1984). To safeguard public health, we strongly recommend that the supplies and use of antibiotics in cultured shrimp be regulated.

Acknowledgements

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