Chapter II

Materials and Methods

Materials

1. Micro-organisms

Bacillus cereus ATCC 11778

Aeromonas hydrophila : 57 strains

Fourty-four, ten and three strains were received from Microbiology Division (Department of Veterinary Pathology, Faculty of Veterinary Sciences, Chulalongkorn University, Bangkok, Thailand), Dr. Sommanee Sukrungoung (Mahidol University, Thailand), and Japan Collection of Microorganisms (JCM) repectively.

2. Experimental Fish

Each healthy catfish (Clarias batrachus) weighing about 100-300 gm were used, after bringing from fish culturing ponds at least ten days. Before beginning any experiments using catfish, they must be samplinged for testing to make sure that there was no tetracycline in serum, muscle and liver.

3. Media

- Antibiotic Medium I
- Antibiotic Medium II

- Mueller-Hinton Agar
- Nutrient Broth
- Blood Agar
- Plate Count Agar

The ingredients and preparation are demonstrated in appendix I.

4. Chemicals

- Tetracycline hydrochloride : Batch no PXP 82057, Mfg. date (Sept, 1982), Exp. date (Oct, 1986), China National Chemicals Import & Export Corporation, Shanghai, Branch, The People's Republic of China.
- Potassium phosphate, monobasic (KH_2PO_4) (Mallickredt Inc)
- Sodium Chloride (BDH Chemicals Ltd)
- Phosphoric acid

5. Equipments and Instruments

- Slide & Cover glass
- Beaker
- Volumetric flask 10, 50, 100, 200, 250 ml
- Glass Centrifuge tube 15 ml
- Glass bead
- Roux bottle
- Test tubes
- Syringe 2 ml
- Petri-dish, 90 mm diameter
- Pasteur pipet
- Erlenmeyer flask
- Homoginizer (Blender) glass 10 ml, 100 ml

- Plastic centrifuge tube (50 ml) : 50 PA tube & Cap (C_2-PP)
- Oral needles no 18 (3.5" long) with an un-sharp-round terminal
- Cork borer no 3 (6 mm diameter)
- Analytical balance : Mettler H 35 AR
- Two-pan balance
- Autoclave : Hirayama MFG corp.
- Hot air oven : Heracus
- Colony counter : New Brunswick Scientific, USA
- Deep Freeze Refrigertor : Continental
- Hot plates : Chromalox, USA
- Lyophilizer : Thermovac
- Refrigerator : Marco
- Centrifuge : Labofuge, Siam & Co company Ltd
- Automatic High Speed Refrigerated Centrifuge : Hitachi (20 PR-52 D) & Rotor (RPR 20-2)
- Incubator : Memmert
- Spectrophotometer : Spectronic 710
- Vertex Cyclomixer : Clay adams, USA
- Water bath : Precision Scientific Co, USA
- Homoginizer
- Millipore filter set (0.22 μ)
- Micropipet & tip (50 μl) : Finland
- Replicator with 28 hole-plate
- Vacuum pump
- Million Scale Automatic Dial Scale : Fuji
- Cylindrical Fish tank (diameter x high = 120 x 60 cm.)
- Scientific Calculator : Sharp EL 5100

Methods

1. Pharmacokinetic Study

1.1 Intraperitoneal administration

The 65 catfish were injected with 5 mg tetracycline HCl/ kg catfish body weight intraperitoneally (using 1 ml syringe and no. 27 needle). After the injection 0.5, 1, 2, 4, 6, 9, 12, 24, 48 and 96 h, 5 catfish per period were subjected for testing. Draw blood from the heart of each catfish about 1-3 ml with a 2 ml syringe and a no. 21 needle rinsed with normal saline solution, after that the catfish were killed immediately. Let the blood at room temperature for about 0.5-1 h and then separate serum from red blood cells by centrifuging at 3,000 rpm. Keep the serum at -20°C until assay.

Separate catfish liver and muscle from each other. Cut the liver into small pieces with operating scissors. Mince the catfish muscle. Keep both of them at -20°C until performing the extraction.

Tetracycline extraction from catfish muscle

Weigh 10 gm of minced catfish muscle in a 100 ml-blender flask, blend with 20 ml of 0.1 M phosphate buffer, pH 4.5 about 4 minutes, add 20 ml of the phosphate buffer and blend again about 3 minutes. Transfer the suspension into centrifuge tubes and wash the blender flask with 30 ml of the phosphate buffer. Centrifuge the suspension with a refrigerated centrifuge at 6,000 rpm, 4°C for 10 minutes to separate the extracting solvent (0.1 M phosphate buffer, pH 4.5) from the extracted sample. Transfer the supernatant into

a 100 ml volumetric flask. Extract the fish muscle again,twice with 20 ml and 10 ml of the phosphate buffer respectively, centrifuge with the refrigerated centrifuge at 6,000 rpm, 4°C for 10 minutes each, and transfer the supernatant into the 100 ml volumetric flask. Adjust to 100 ml volume with the phosphate buffer, if necessary.

Transfer 50.0 ml of the 100 ml adjusted extracting solvent into a 250 ml volumetric flaks and bring it to be lyophilized. Add 5.0 ml sterile distilled water to the lyophilized residue, mix completely and centrifuge if necessary. Keep the specimen at -20°C until assay.

Tetracycline extraction from cat-fish liver

Weigh accurately 1.0 ± 0.1 g of the liver into a 10 ml blender flask, blend with 2 ml of 0.1 M phosphate buffer, pH 4.5 about 3 minutes, add 2 ml of the phosphate buffer and blend again about 3 minutes. Transfer the suspension into a centrifuge tube, and wash the blender flask with 4 ml of the phosphate buffer centrifuge the suspension with a centrifuge at 3,000 rpm for 10 minutes to separate the extracting solvent (0.1 M phosphate buffer, pH 4.5) from the extracted sample. The supernatant was transferred into a 50 ml volumetric flask. Extract the fish liver again 2 times by using each 1.0 ml of the phosphate buffer, centrifuge at 3,000 rpm for 10 minutes, and transfer the supernatant into the 50 ml volumetric flask. Bring the 50 ml volumetric flask containing the extracting solvents to be lyophilized. Add 1.0 ml of sterile distilled water to the lyophilized residue, mix completely, and centrifuge if necessary.

1.2 Intramuscular administration

The procedure in the same as intraperitoneal administration (1.1) except the route of tetracycline administration and the sampling time. The 55 catfish were injected intramuscularly, at the right site of their bodies, after the injection 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72, 96 and 20 h, they were samplinged. The muscle from the left site of the fish was used for determining the drug levels.

1.3 Oral administration

The procedure is the same as intraperitoneal administration (1.1) except the route of tetracycline administration, the sampling time, and the dose. The 51 catfish were fed 50 mg/kg catfish body weight orally. The no. 18 oral needle and 1 ml syringe were used. The tetracycline HCl solution was given by inserting the oral needle into fish stomach. The sampling time was 0.5, 1, 2, 4, 6, 9, 12, 24, 48, 72 and 96 h.

1.4 Determination of the % recoveries of Tetraycline HCl in muscle and liver

Add various amount of tetracycline HCl into antibiotic-free catfish muscle and antibiotic-free catfish liver (see table 4). Carry out the procedure with these sample in parallel with the unknown being tested for determining the efficiency of the extraction method.

Table 4 Tetracycline HCl added in muscle & liver

muscle or liver \ drug	Tetracycline HCl added (mcg)								
10 gm (muscle)	3.0	5.1	6.4	8.0	1.0	12.5	15.6	30.0	50.0
1.0 ± 0.1 gm (liver)	0.5		1.0		1.5	3.0	6.0		12.0

1.5 Microbiological assay of tetracycline HCl[44]

The method was adapted from Kramer et al (1974).[44] The material and methods were set out as follows.

1.5.1 The preparation of plates

The test organism (Bacillus cereus ATCC 11778) which is grown at 30°C and maintained on slants of Antibiotic Medium No I was transferred the growth from a fresh slant with 2-3 ml of sterile normal saline solution to a roux bottle containing 300 ml of the Medium I and incubate at 30°C for 1 week. Harvest the growth with 20-30 ml of sterile normal saline solution (NSS) and heat 30 minutes at 65°-70°C. Wash the culture 3 times with sterile NSS (centrifuging and decanting). Heat for 30 minutes at 65°-70°C and resuspend in 30 ml of sterile NSS. Keep this spore suspension in refrigerator. The spore suspension may be used indefinitely if protected from evaporation and contamination.

Sterile uniform glass petridishes of 90 mm diameter were used. Seven ml of Bacillus cereus spores (1.15×10^{10} viable cells/ml) were added to each 1,000 ml of the Antibiotic Medium 2 which had been melted and cooled at about 50°C. The flask was swirled to obtain a homogeneous suspension, 15 ml of the seeded medium were added to each sterile petridish, and allowed to become hardened on flat level surface. The sterile cork borer no 3 with 6 mm diameter was used to press upon the hardened agar to make 6 sharp circles and to give holes after removing the agar with vaccuum pump. Keep these plates in refrigerator until assay. The prepared plates must be used within 12 hours.

1.5.2 Preparation of the standard solution

1.5.2.1 For assaying tetracycline in serum

Prepare 500 mcg/ml of tetracycline HCl in sterile distilled water. Dilute the tetracycline HCl solution with antibiotic-free catfish serum to the following concentrations : 0.11, 0.21, 0.33, 0.51, 0.64, 0.80, 1.0, 1.25 and 1.56 mcg/ml. Use 0.51 mcg/ml tetracycline HCl solution as the intermediate reference standard concentration.

1.5.2.2 For assaying tetracycline in muscle and liver

Prepare 500 mcg/ml of tetracycline HCl in 0.1 M phosphate buffer, pH 4.5. Dilute the tetracycline HCl solution with 0.1 M phosphate buffer, pH 4.5 to the following concentrations: 0.11, 0.21, 0.33, 0.51, 0.64. 0.80, 1.0, 1.25 and 1.56 mcg/ml. Use 0.51 mcg/ml tetracycline HCl as the intermediate reference standard concentration.

1.5.3 Preparation of samples

1.5.3.1 For assaying tetracycline HCl in serum

The concentration of tetracycline in serum should not be higher than the maximum standard concentration (1.56 mcg/ml). If the drug concentration in serum is higher than the standard preparation, it should be diluted with the antibiotic-free catfish serum to give an appropriate concentration (about 0.51 mcg/ml).

1.5.3.2 For assaying tetracycline HCl in muscle and liver

The tetracycline concentration in the extraction solvent should not be higher than the maximum standard concentration (1.56 mcg/ml). If the tetracycline in muscle or liver is higher than the standard preparation. It should be diluted with the 0.1 M phosphate buffer pH 4.5 to give an appropriate concentration (about 0.51 mcg/ml).

1.5.4 Assay procedure

Each concentration of samples and standards, which should be assayed at least in triplicate, must be set at the same day.

Every inoculated agar plate from 1.5.1 had 6 holes, 3 of them were filled with 50 µl of a standard solution (or sample solution) and the other 3 holes with 50 µl of the intermediate reference standard solution using alternative holes.

The plates were incubated at room temperature (28-32°C) for 12 -18 h. The diameters of the inhibition zones (clear zones) were examined under a colony-counter reader and then were measured with a vernier caliper.

1.5.5 Satandard curve determination and calculation of the sample Concentrations

The corrected standard values were plotted on two cycle semilogarithmic paper, using the concentration as the ordinate (logarithmic scale) and the diameter (in mm) of the inhibition zones as the abscissa. The straight line obtained from linear regression line equation was used as the standard curve for calculating the sample concentration. The average zone diameters of the sample solution and the intermediate reference standard solution were compared and then read the sample concentration from the standard curve or calculate the sample concentration from established linear regression line equation.

2. Determination of Minimal Inhibitory Concentration (MIC)

The agar dilution method was used.[45] It was performed by incorporating the antimicrobial agent into Mueller Hinton agar just before it was poured into a petridish. Twenty-eight bacterial strains (A. hydrophila) were spot-inoculated simultaneously onto a series of petridishes containing many concentrations of tetracycline HCl, then the results were read.

Dissolve 400,000 IU of tetracycline HCl in sterile distilled water to make. 100 ml. This will obtain a 4,000 IU/ml solution. Filter

this solution through a millipore filter. Discard the first 10 ml filtrate.

Dilute the 4,000 IU/ml tetracycline HCl solution with sterile distilled water to make a 2,560 IU/ml solution. Then it was further diluted with sterile distilled water to make a series of two fold dilution of tetracycline HCl containing 1,280, 640, 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 IU/ml.

The Mueller Hinton agar was melted and allowed to cool to 45°C-50°C in a water bath. The diluted tetracycline HCl solutions were added to the melted and cooled medium in a ratio of one parts of tetracycline HCl solution to nine parts of the agar medium, and mix thoroughly but gently. The mixed medium was poured into the sterile 90 mm petridishes (20 ml/petridish) and allowed to be become hardened on a flat level surface. Two test plates were used for each concentration of tetracycline HCl.

The agar plates would give the final tetracycline HCl concentration of 0.0625, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0, 128.0 and 256.0 IU/ml.

At least one control plate,containing 18 ml Mueller Hinton agar without antimicrobial agents mixed with 2 ml sterile distilled water was prepared for every series of dilutions and different groups of the tested bacteria. The plates were used within 24 h after preparation.

All of the tested strains were purified on blood agar plates, incubated at 37°C, inoculate growth from 1-2 freshly isolated colonies into nutrient broth and incubate for 4-6 hours at 36-37°C.

Then standardize the inocula to match a turbidity of No I Mc. Farland Standard diluted with an equal volume of water. The standardized inocula were further diluted with normal saline in a 1:20 ratio.

The inoculum-replicating apparatus was used. About 28 standardized bacterial suspensions were transfered to the appropriated wells in each seed plate containing 28 resevoirs.

An aluminium replicating device (the hand-held multipoint plate inoculator) was dipped into the wells of inocula in the seed plate and then the inoculum suspensions were spotted onto previously dried surface of each antimicrobic containing plate, by touching the ends of the inoculators on the agar surface. The plates were incubated at 36-37°C for 16-20 h.

Two control plates without antimicrobial agents were inoculated as a control in every set of tests.

2.6 Reading of test results

All inoculated plates were examined for growth within 16-18 h incubation. First, the control plates without antimicrobial agents were checked to be sure that each test strain was capable for providing adequate growth. Then the remaining plates were examined to determine the minimal concentration of drug required for inhibition of growth. The end points were judged when there was a definite dense film of growth in the next plate of lesser concentration.

3. Prophylaxis testing of tetracycline HCl against A. hydrophila infection in catfish (Clarias batrachus)

A. hydrophila F 181 was cultured in Antibiotic Medium I and incubated at 37°C for 16-20 h. Harvest the growth with normal saline

solution and adjust the turbidity of the suspension yielding about 10^9 viable cells per ml with spectrophotometer at 520 nm. Plate count agar was used for counting viable cells of the suspension diluted by normal saline solution every time of the experiments.

About 100 catfish were fed at least 15 days after being brought from Bangkok Sa pan pla, the fish Market. Separate the catfish into 2 equal groups, the first group was injected with tetracycline HCl aqueous solution, 5 mg/kg catfish body weight intraperitoneally and the other was not treated being a control group. After the administration of the antibitiotic, 6 h, 1 d, 2 d, 3 d, 4 d, 6 d and 7 d, the catfish in each group 10, 10, 5, 5, 5, 5 and 5, were randomly challeged with 1 ml of the bacterial suspension per catfish at the right sites of the animals intramuscularly. The infected catfish were observed for ulcer development within 18-24 h. The control group must show positive result.