Chapter I

Introduction

In Thailand, ulcer disease in catfish caused by Aeromonas hydrophila has been known since 1976[1]. The outbreak of the disease was occurred sporadically around the year even when effecient husbandry techniques were in used. The infection speeded very rapidly throughout the fish population. The mortality rate was varied. The fish might be almost 100% dead within 3-15 days[2].

Various chemotherapeutics eg. sulfa-drugs, chloramphenicol and tetracycline has been used for cultured fish as feed additive in many country, unlike livestock and chickens, the growth promotion effect of chemotherapeutics has not been observed in fish[3,4,5]. A number of years ago it was discovered that chickens being given certain antibiotics (eg. tetracyclines or a sulfa drug) in their drinking water as a disease prophylaxis gained weight more rapidly and reached marketable size much sooner than those that were unmedicated. Soon it became standard practice in poultry farming. Apparently, weight gain in poultry is due to the depression of the normal intestinal bacterial flora, leaving more nutrient available for absorption by bird. Since fish are notoriously lacking in normal intestinal bacteria, no appreciable nutrient saving is accomplished, thus there is no appreciable weight-gaining advantage to feeding antibiotics to fish as part of their mormal diet[6].

In Japan it was found that, the use of these drugs had caused a mark in the frequency of drug-resistant strains. A majority of these drug-resistant bacteria was found to carry transferable, multi-drug-resistant R-factors[3,4].

Chlortetracycline was widlely tested in fishes, and in contrast to the result achieved in warm blooded animals, its use in fishes was notably without benefit and was, infact, sometimes accompanied by reduced growth. Rather curiously, oxytetracycline was found to be very effective for a number of systemic infections caused by gram-negative organisms[7,8].

In Thailand, tetracycline has been widely used to prevent and treat various infections of cultured fishes that may lead to increase some problems, for example, the tetracycline resistant bacteria, the tetracycline residues in fishes. And no one proves that tetracycline can prevent the infection of Aeromonas hydrophila causing ulcers in fishes.

For these reasons, it is important to investigate for solving these problems. The catfish (Clarias batrachus) were selected for studying.

The objectives of this research is to study the following items :

1. To study the pharmacokinetics of tetracycline when treat the catfish intraperitoneally, intramuscularly and orally.

2. To investigate the prophylaxis of tetracycline against the bacterial infection caused by Aeromonas hydrophila in catfish

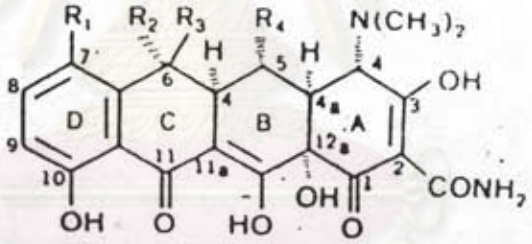
(Clarias batrachus).

3. To determine the Minimal Inhibitory Concentration (MIC) of tetracycline hydrochloride to Aeromonas hydrophila strains isolated from fishes, water, soil (in fish culturing ponds), and received from Japan Collection of Microorganisms.

I. Tetracycline

The tetracycline are broad spectrum antibacterial agents, extracted from species of Streptomyces or produced by chemical modification of the naturally occurring compounds. There are several derivatives eg. chlortetracycline, oxytetracycline (Fig. I). All members of this class are closely related chemically. In general, patterns of bacterial susceptibility and resistance to tetracyclines are similar, but there are some differences in the degree of activity among the various analogues. The newer tetracyclines, minocycline and doxycycline, are more active than the parent compound[9].

Figure 1 Structure of Tetracyclines



	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Chlortetracycline	Cl	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Demeclocycline	Cl	H	OH	H
Methacycline	H	CH_2	-	OH
Doxycycline	H	H	CH_3	OH
Minocycline	$N(CH_3)_2$	H	H	H

History[10]

The development of the tetracycline antibiotics was the result of a systematic screening of soil specimens collected from many parts of the world for antibiotic producing microorganisms. The first of these compounds, chlortetracycline, was introduced in 1948. Two years later,oxytetracycline became available. Elucidation of the chemical structure of these agents confirmed their similarity and furnished the basis for the production of a third member of this group, tetracycline, in 1952. In 1957, a new family of tetracyclines was developed, chlaracterized chemically by the absence of the attached CH_3 group present in the others. One of these, demethylchlortetracycline, subsequently, given the official name,demeclocycline, became available for general use in 1959. Methacycline, a derivative of oxytetracycline, was introduced in 1961; doxycycline became available in 1966; and minocycline in 1972.

Solubility and stability[11,12]

1 in 10 of water and 1 in 100 ml of alcohol, soluble in methyl alcohol and in aqueous solutions of alkali hydroxides and carbonates, practically insoluble in acetone, chloroform and ether. A 1% solution in water has a pH of 1.8 to 2.8. Solutions in water become turbid on standing owing to hydrolysis and precipitation of tetracycline. Solutions for injection are prepared aseptically. Tetracycline HCl darkens in moist air when exposed to strong sunlight. Its potency is reduced in solutions having a pH below 2 and it is slowly destroyed in solutions at pH 7 and above. All the tetracyclines are more antibacterially active in weakly acidic than in alkali media[11].

Store in airtight containers. It needed for injection, the containers should be sterile and sealed to exclude microorganism. Protect from light.[11]

Sometimes it may be desirable to maintain antibiotic preparations for extended periods of time. No loss in tetracycline activity has been observed when serum or urine specimens were stored frozen for periods as long as 6 months. It should be necessary to freeze a sample which was initially dissolved in diluted acid, an intermediate dilution using 0.1 M phosphate buffer, pH 4.5, should be made before freezing. It is important that the entire contents of the frozen vessel are melted and thoroughly mixed before any portion is withdrawn, because some local concentration may occur during freezing.[12]

Solutions of the tetracyclines cannot be sterilized by heat. Adsorption may cause serious losses in activity when solutions of the tetracycline are sterilized by filtration. Membrane type filter are least likely to cause problems. Ultrafine sintered glass filters may also be used, but the solution being filtered should be at the highest practical concentration and the first portion of the solution passing through the filter should be discarded. It is not advisable to use Seitz or Mandler filters with solutions of the tetracyclines.[12]

There are no practical enzyme systems capable of inactivating the tetracyclines. While there are chemical treatments which effectively destroy the activity of tetracyclines, they are not satisfactory if the objective is the quantitative recovery of viable microorganisms originally present in a preparation. When such a solution is passed through a 0.4 μ membrane filter, followed by three separate 200 ml

rinsed with sterile broth, microorganisms retained on the filter can be demonstrated if the center portion only of the filter is planted on a appropriate culture medium. The activity of relatively small amount of tetracycline antibiotics present in clinical specimen can be partially reversed and any microorganisms present grown if the material being subjected to test is inoculated into media containing relatively large amounts of iron or magnesium salts. Supplementing the medium with 0.05% $FeCl_2.4H_2O$ or 1% $MgSO_4.7H_2O$ in the media was recommended. Alternative procedure is to add the specimen to a volume of culture medium sufficiently large to dilute the antibiotics activity below the level likely to inhibit growth and to incubate for periods longer than normal.[12]

Unit[11]

One unit of tetracycline is contained in 0.00101833 mg of the second International Standard Preparation (1970) of Tetracycline HCl which contains 982 unit per mg.[11]

Assay[12,13,14,15,16,17,18,19,20,21]

In biological material, spectrophotometric method is insensitive and the interference from other material cannot always be excluded. High pressure liquid chromatography (HPLC), fluorometry, microbiological assay and radioimmunoassay (RIA) are sensitive (especially RIA, the sensitivity is 1 ng/ml). HPLC, fluorometry and RIA are more specific than microbiological assay, but they require special equipments. Microbiological assay is very simple, but lacks specificity.

For microbiological assay, because pH is such a critical factor in the activity of the tetracyclines,it is important that samples and standards within a single assay have the same pH during the incubation period. In most instance this is accomplished when the solutions are diluted to the desired antibiotic level with buffer. The microbiological assay methods in general use today are not specific for any one of the tetracycline analogs and the same microorganisms and media may be used to measure the activity in preparations containing any of them. While the same methods may be used for all, the tetracycline do differ in their activities relative to each other and this may vary from system to system. For this reason it is important that the reference antibiotic against which the activity in a test preparation is measured is the same tetracycline analog as that in the specimen. When several different tetracycline are present in samples the total activity may be expressed in terms of "equivalents" relative to one of them, but it should be remembered that the activity relationship may be different in another test system.[12]

Mechanism of Action[6,10,22]

Tetracycline, the bacteriostatic agent does not have a lethal effect on bacteria. They merely act to inhibit protein synthesis in susceptible organisms which is essential to their ability to multiply and reproduce. It is thus neccessary for the defense mechanism of the host to be operative in order to eliminate the infective agent.[6]

The site of action of tetracycline is the bacterial ribosome, but at least two processes appear to be required for these antibiotics to gain access to the ribosome of gram-negative bacteria.[10]

The first is passive diffusion through hydrophilic pores in the outer cell membrane. These structures have been specifically located within protein IA, one of three proteins in the envelope. Minocycline and perhaps doxycycline are more lipophilic than the other congeners and pass directly through the lipid layer.

The second, process involves an energy-dependent active transport system that pumps all tetracyclines through the inner cytoplasmic protein carrier.(10)

Such transport may require a periplasmic protein carrier. Although permeation of these drugs into gram-positive bacteria is less well understood, it too requires an energy-dependent system. Once the tetracyclines gain access to the bacterial cell, they inhibit protein synthesis and, like the aminoglycosides, bind specifically to 30S ribosomes. They appear to prevent access of aminoacyl t-RNA to the acceptor site on the mRNA ribosome complex. This prevents the addition of amino acids to the growing peptide again. Only a small portion of the drug is irreversibly bound, and the inhibitory effect of the tetracyclines can be reversed by washing. Therefore, it is probable that the reversibly bound antibiotic is responsible for the anti-bacterial action. These compounds also impair protein synthesis in mammalian cells at high concentrations, however, the host cells lack the active transport system found in bacteria.(10)

Therefore, the increased accumulation of drug by bacterial cells partially explains the greater susceptibility of bacteria to inhibition of protein synthesis. However,active transport does not account for the high sensitivity of various intracellular microorganisms(eg. rickettsia, chlamydia) to tetracycline, and other factors

appear to be involved.[10]

The tetracyclines are bacteriostatics at blood levels achieved with usual doses. High concentrations may be bactericidal in vitro. Tetracyclines, even in subinhibitory concentrations, have been shown to reduce the ability of E. coli to adhere to mamalian epithelial cells in vitro. Tetracyclines do not inhibit cell wall synthesis and thus are effective against cell wall-deficient organism (eg. Mycoplasma, Pneumoniae).[22]

Bacterial Resistance[3,4,22,23]

Several species of bacteria, especially E. coli, beta-hemolytic streptococci, S. pneumoniae, N. gonorrhoeae, A. liquefaciens (A. hydrophila), and strains of Bacteroides and Shigella, have become increasingly resistant to the tetracyclines.[22]

Resistance may occur through several mechanisms. Strains of mutant E. coli with tetracycline-resistant ribosomes have been isolated in the laboratory, and there is evidence that some bacteria may be induced to synthesize enzymes that degrade the antibiotic. The major mechanism of resistance, however, is to decrease uptake of tetracyclines by the bacterial cell. This results from alterations in the energy-dependent transport process. Certain strains of staphylococci (and possibly other organisms) may remain sensitive in vitro to minocycline and doxycycline, because these highly lipid-soluble derivatives may passively penetrate the resistant bacterial cell wall in sufficient concentrations to inhibit the drug-sensitive ribosome. The clinical importance of this observation is unclear. Resistance to one tetracycline

usually implies resistance to all, except for minocycline's activity against S. aureus and doxycycline's activity against B. fragilis.[22]

Resistance can be transferred from one organism to another by transfer of small plasmids (circular, self-replicating, extrachromosomal DNA) called R-factors that contain genetic information for the development of resistance. An R-factor often induces resistance to several antibiotics simultaneously.[22]

Transferable drug resistance factors (R-factors) have been detected in Aeromonas. In naturally occurring A. salmonicida strains, the non-motile, plasmids conferring resistance to streptomycin, chloramphenicol, tetracycline, and sulfathiazole are transferable to Escherichia coli. In motile Aeromonas species, the presence of R-factors has also been detected. These R factors have markers of resistance to sulfonamide, tetracycline, streptomycin and chloramphenicol.[23]

Antibiotics and other chemotherapeutics are now being used in many countries for treating infections of cultured fish. The high incidence of R-factor carrying bacteria in cultured fish and fish ponds is assumed to have resulted from the use of chemotherapeutics for fish culturing. It seems likely that R-factors in the bacteria in fish and fish pond water may have come from human or animal bacteria contaminating the fish ponds.[3,4]

Antimicrobial Action[11]

The group of tetracycline antibiotic have a broad spectrum of antimicrobial activity. They are active against a large number of gram-positive and gram-negative pathogenic bacteria, including some

which are resistant to penicillin, and are mainly bacteriostatic.[11]

The organisms which are sensitive to tetracyclines in concentrations usually achieved in the body during treatment include Bacillus anthracis, Bordetella spp., Brucella spp., Escherichia coli, Haemophilus spp., Klebsiella spp.,Staphylococci, Streptococci, Mycoplasma, Entamoeba histolytica, Trichomonas vaginalis, Treponema pallidum and other species, and certain rickettsias and larger viruses. Chlamydiae are also sensitive as is Coxiella burneti , Proteus vulgaris, Pseudomonas aeruginosa, Salmonella spp., Shigella spp, and Mycobacterium tuberculosis are less susceptible. With the exception of Actinomyces spp. which are sensitive, fungi and yeasts are resistant.[11]

Adverse effect[11]

The side-effects of tetracycline hydrochloride are common to all tetracyclines.

Gastro-intestinal effects including nausea, vomiting and diarrhoea are common especially with high doses and most are attributed to irritation of the mucosa.

Oral candidiasis, vulvovaginitis, and pruritus ani occur due to overgrowth with Candida albicans and there may be overgrowth of resistant coliform organisms, Pseudomonas spp. and Proteus spp., causing diarrhoea. The most serious supra-infection is by resistant staphylococci, causing a fulminating enteritis with dehydration and, occasionally, death; this complication is rare,except after abdominal surgery, especially gastrectomy.

Therapeutic doses given to patients with renal disease increase the severity of uremia with increased excretion of nitrogen and losses of sodium and development of anorexia, nausea, vomiting, and weakness, accompanied by acidosis and hyperphosphataemia. These effects are related to the dose and the severity of renal impairment and are probably due to the antianabolic effects of the tetracycline.

Severe and sometimes fatal hepatotoxicity associated with fatty changes in the liver and pancreatitis has been reported in pregnant women given tetracycline intravenously for pyelonephritis, and in other patients with renal impairment or those given high doses.

Haemolytic anaemia, eosinophilia, neutropenia, and thrombocytopenia have been reported rarely.

Vitamin deficiency may occur especially with prolonged dosage.

Tetracyclines are deposited in dediduous and permanent teeth causing discoloration, enamel hypoplasia, and reduced mineralisation. They are also deposited in calcifying areas in bone and the nails and when given in therapeutic doses to young infants or women during the late stages of pregnancy tetracyclines interfere with bone growth. An increase in intracranial pressure, which may be associated with a bulging fontanelle in infants, has been reported in patients given tetracyclines.

Allergic reactions to tetracycline and its analogs have been reported and cross-sensitisation is common; photosensitivity of the skin and nails has occurred, especially after demeclocycline,

and onycholysis may be associated with nail discoloration. Local irritation can occur when tetracyclines are given parenterally and thrombophlebitis may follow intravenous injections. A Herxheimer-like reaction has been reporting in patients with relapsing fever treated with tetracycline.

The use of out-of-date tetracyclines has been associated with the development of a reversible Fanconi-type syndrome characterised by polyuria, glycosuria, acidosis, and aminoaciduria. These effects have been attributed to the prescence of degradation products : epitetracycline, epianhydrotetracycline, and anhydrotetracycline, resulting from exposure to acid, moisture, and heat.

Precautions[11]

The tetracycline with the exception of doxycycline are generally contra-indicated in patients with renal impairment. Care must be taken when liver impaired. Potentially hepatotoxic drugs should not be given with tetracyclines nor should compounds such as methoxyflurane that can be nephrotoxic.

The use of tetracycline in pregnancy should be avoided. When administered to women during the latter half of pregnancy, to nursing mothers, or during childhood up to the age of 12 years, permanent discoloration of the child's teeth may occur. Symptoms of myasthenia gravis may be exacerbated by tetracyclines. Absorption of tetracyclines is diminised by milk, alkali, aluminium, hydroxide, and the salts of other trivalent, and divalent cations including calcium, iron, and magnesium, when these are taken concomitantly.

Doses of anticoagulants may need to be reduced when patients are given tetracyclines.

Tetracycline should not be given to patients with known hypersensitivity of any of this group of antibiotics.

Because of possible antagonism of the action of the penicillins by predominantly bacteriostatic tetracyclines it has been recommended that the two types of antibiotic should not be given concomitantly, especially when a rapid bactericidal action is necessary.

Absorption and Fate[11,13,24,25]

The tetracyclines are incompletely and irregularly absorbed from gastro-intestinal tract. The degree of absorption is diminished by the soluble salts of divalent and trivalent metals, with which tetracyclines form stable complexes and to a variable degree by milk or food. It has been recommended that tetracyclines should be given before food. Sodium metaphosphate may enhance the absorption of tetracycline.[11]

As a rule, doses of 250 to 500 mg by mouth every 6 hours produce therapeutically effective plasma concentration of tetracycline ranging from 1 to 3 mcg per ml and 1.5 to 5 mcgper ml respectively. Intravenously injections of 250 to 500 mg produce plasma concentration of 15 to 20 mcg per ml at 0.5 hours falling to 4 to 10 mcg per ml at 1 to 2 hours, though at 12 hours 1 to 3 mcg per ml may still be present. Intramuscular doses of 100 mg yield plasma concentration of up to 2 mcg per ml and 250 mg-doses up to 3.6 mcg per ml at 3 to 4 hours.[11]

In the circulation, tetracycline are bound to plasma proteins in varying degrees, and figures have been reported ranging from 20 to 35% for oxytetracycline, from 24 to 65% for tetracycline, and from 41 to 90% for demeclocycline. They are widely distributed throughout the body tissue and fluids. Concentrations in cerebrospinal fluid are relatively low, but may be raised if the meninges are inflamed. Tetracyclines appear in the milk of nursing mothers where concentrations may be 60% or more of those in the plasma. They diffuse across the placenta and appear in the foetal circulation in concentrations of about 25 to 75% of those in the maternal blood. Only small amounts appear in saliva, tears, intra-ocular fluids. Tetracyclines are retained at sites of new bone formation and recent calcification, in developing teeth, and in some injured soft tissues.(11)

The biological half-life of tetracycline has been reported to be 8.5 hours; comparable figures reported for other tetracyclines are chlortetracycline 5.5 hours, demeclocycline 12 hours, doxycycline 15 to 17 hours, methacycline 15 hours, minocycline 17 to 19 hours, and oxytetracycline 9.5 hours.(11)

The tetracyclines are excreted in the urine and in the faeces. Renal clearance is by glomerular filtration and concentration in the urine of up to 300 mcg per ml of tetracycline may be reached 2 hours after a dose is taken and be maintained for about 6 to 12 hours. Up to 60% of an intravenous dose and rather less of a dose by by mouth is eliminated in the urine but only about 10 to 15% of dose of chlortetracycline is eliminated in the urine.

The tetracycline are excreted in the bile when concentrations 5 to 20 times those in plasma can occur. Since there is some

reabsorption, complete elimination is slow. Considerable quantities occur in the faeces after administration by mouth and lesser amounts after administration by injection.[11]

In cows and ewes, the biological half-life of tetracycline has been reported to be 5.73 ± 0.6 hours, comparable figures reported for other tetracyclines are oxytetracycline 4.10 ± 0.6 hours, chlortetracycline 4.20 ± 0.8 hours, demethylchlortetracycline 7.10 ± 1.2 hours, methacycline 9.15 ± 1.6 hours, doxycycline 9.24 ± 1.0 jours, minocycline 8.80 ± 1.4 hours, pyrrolidinomethyl-tetracycline 4.10 ± 0.5 hours, and tetracycline-L-methyl lysine 4.20 ± 0.8 hours.[24]

The biological half-life of oxytetracycline in dogs (6.02 ± 1.51 hours) was shorter than that found in cows (9.12 ± 1.50 hours) and in horse (10.50 ± 2.91 hours).[25] And the biological half life of tetracycline in dogs was also short (3-4 hours).[13]

Dose[26]- Oral, 250 mg to 1 gm 4 times a day; intramuscular, 200 to 500 mg a day in 2 to 3 divided doses; intravenous, 0.1 to 0.2 ml of a 0.5% solution or suspension or sufficient 1% ointment applied to eyelid or conjunctiva every 2 hours.

For children,15 to 25 mg/kg/day, parenterally, up to a maximum of 250 mg, in 2 to 3 divided doses or 25 to 50 mg/kg/day in 4 divided doses.

Veterinary Dose[26]- In general, intramuscular or intravenous, 1 to 5 mg/lb once or twice daily; oral, daily divided in 3 or 4 doses, large animals (nonruminants), 5 to 10 mg/lb and small animals,

15 to 50 mg/lb.

Administration of drugs in fish[6]

Chemotherapy of bacterial diseases affecting fish has been attempted by administering antibiotics and other chemical in the feed, by injection, and by incorporation of the agents into bath.[6,27] Oxytetracycline[28], chloramphenicol[29] and some sulfonamides[30] have been demonstrated as efficacious for treatment of systemic infections when incorporated into feed. However, when fish are anoretic due to disease, stress conditions or natural phenomena such as observed in adult salmon [31], medicated feeds are of no value. Those fish that are weaker or less aggressive receive little or no medication, while the healthy, aggressive ones probably obtain an overdose of the drug being applied.[6] It is generally conceded that feeding medications to fish is most useful as a prophylaxis rather than as a treatment, yet, as we have seen, this may be even more damaging in the long run. Still, there are occasions when it may be the only way to save any appreciable number of fish at all in a complement of valuable stock that is difficult to catch out of an area for treatment, i.e. when for some reason a pond cannot be drained or during an acute attack of bacterial hemorrhagic septicemia, where speed of administering a drug over an extensive area to an extensive population may be the only workable solution.[6] Dosage problems also can be encountered with medicated feeds because environmental temperature changes affect food consumption or because of leaching of the antibiotic from the feed in water.[27]

While parenteral administration of antimicrobial agents in fish assures the proper dosage to all individuals, it requires time consuming handling of the fish and is not economically feasible unless the fish are very valuable or only a few involved. Handling may also mechanically damage the skin or spread infections by contact contamination with handling equipment.(27)

Intravenous injection (I.V) affords the most rapid dispersal and the most effective route by which an antibiotic can be administered. Unfortunately, it can only be used on the largest of specimens, as a fish's body is composed primary of extensive capillary networks rather than major vessels. Two primary sites (the heart & the caudal artery) lend themselves to the introduction of drugs in the circulatory system.(6)

There are some drawbacks in intramuscular injection in fish. The skin of a fish apparently does not have a great deal of contractibility,and once a needle hole is punched into it, it does not seal itself as well as most animal tissues. Therefore, leakage of the medication back out of the injection site occurs frequently enough to be considered a significant problem. Many drugs injected intramusculary are not absorbed into the fish system rapidly enough to be effective, and the fish can die before the drug starts to take effect. This is the poikilothermic-nature of the fish and the metabolic activity of its muscles rather that to any fault of the drug. Still other drugs are not absorbed at all and become pockets of localized overdoses. This causes the cells in the vicinity of the infection site to die from antibiotic toxicity, resulting in sterile abscess. Injectable oxytetracycline and some of sulfonamides fall

into this category. On the other hand, gentamycin works best when injected intramascularly.[6]

The intraperitoneal method is quite simple. It is only to insert the needle into visceral cavity and squeeze the plunger. The drug must be highly absorbable and able to pass through either the intestinal wall or some other membrane and absorbed into the fish's system.[6]

Bath treatments with antibiotics have been used almost exclusively for control of superficial infection.[27] Absorption through the fish's gills, mucous membranes, or the integument for treatment in cases of internal bacterial disease is a rather inefficient method of treatment, but there are exceptions, eg. Euranac.[6]

Uses or Treatment in fish[6] (Tetracycline HCl or Oxytetracycline HCl) : gram-positive and most gram-negative bacteria, cold-water disease (Cytophaga psychrephila), bacterial hemorrhagic septicemia, columnaris (Chondrococcus columnaris), emphysematous putrefactive disease of channel catfish (Edwardsiella tarda), furunculosis,Pseudomonas, redmouth disease of trout, ulcer disease (Hemophilus piscium), vibriosis, fungi (Saprolegnia), protozoa (Ichthyopththirius)

Dosage

1. 3 mg/100-400 gm[6] or 0.5 mg/120 gm[32] of fish body weight as intraperitoneal injection

2. 50-75 mg/kg[6] of fish weight per day in food for 10 days or 750 mg/kg[32] of fish body weight per day in food for 14 days.

3. 10 ppm in water[6] (not effect against Saprolegnia)

4. 10-20 mg/l[6] as a long duration bath

5. 5-8 mg/l[6] as a long duration bath

Withdrawal time[6]

Probably the most widely used and recommended of all antibiotics used in the treatment for fish diseases, U.S. law require that its use must be discontinued for at least 21 days before fish are killed for human consumption.

II Aeromonas hydrophila

Taxonomy & Characteristics

The genus Aeromonas including in the Family of Vibrionaceae was divided into 4 species, namely A. hydrophila, A. caviae, A. sobria and A. salmonicida.[23] (see table 1 & 2)

The definition of A. hydrophila given in Bergey's Manual of systemic Bacteriology (1984)[23] is as follows: gram negative straight rods (0.3-1.0 µm in diameter and 1.0-3.5 µm in length), motile by a single polar flagellum in liquid medium, peritrichus flagella may occur on solid media in young culture, not encapsulated, optimum growth temperature : 28°C, colonies on nutrient agar (white to buff, circular and convex with an entire margin), the ability to use L-histidine, L-arabinose and salicin as sole carbon sources, the ability to hydrolyse esculin, the ability to grow in KCN medium, the ability to ferment salicin, the ability to produce gas and acetoin from glucose, the ability to produce H_2S from cysteine, and the mol % G + C of the DNA ranging drom 58-62 (G = guanine, C = cytosine).

Characteristics common to all of the identification schemes proposed for A. hydrophila include : mannitol fermentation (+); inositol fermentation (-); ornithine decarboxylase (-); growth in 0% NaCl (+); H_2S production from TSI (-) (H_2S may be produced by some strains from cysteine), and motility (+). A multi-test medium was devised for presumptive identification of A. hydrophila, allowing determination of all of the above reactions in single tube medium.

Table 1 Differentiation between Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria, and Aeromonas salmonicida

Characteristics	1. A. hydrophila	2. A. caviae	3. A. sobria	4. A. salmonicida subsp.		
				salmonicida	achromogenes	masoucida
Motility	+	+	+	-	-	-
Monotrichous flagellation in liquid medium	+	+	+	-	-	-
Lophotrichous flagellation in liquid medium	-	-	-	-	-	-
Coccobacilli in pairs, chains and clumps	-	-	-	+	+	+
Rods in singles and pairs	+	+	+	-	-	-
Brown water-soluble pigment	-	-	-	+	-	-
Growth in nutrient broth at 37°C	+	+	+	-	-	-
Indole production in 1% peptone water	+	+	+	-	+	+
Esculin hydrolysis	+	+	-	+	-	+

Symbols; +, typically positive ; -, typically negative; d, differs among strains.

Table 1 Differentiation between Aeromonas hydrophila, Aeromonas caviae, Aeromonas sobria, and Aeromonas salminicida (continued)

Characteristics	1. A. hydrophila	2. A. caviae	3. A. sobria	4. A. salmonicida subsp.		
				salmonicida	achromogenas	masoucida
Growth in nutrient broth at 37°C	+	+	+	-	-	-
Indole production in 1% peptone water	+	+	+	-	+	+
Esculin hydrolysis	+	+	-	+	-	+
Growth in KCN broth (Møller technique)	+	+	-	-	-	-
L-Histidine and L-arginine utilization	+	+	-	-	-	-
L-Arabinose utilization	+	+	-	+	-	+
Fermentation of salicin	+	+	-	d	d	d
Fermentation of sucrose	+	+	+	-	+	+
Fermentation of mannitol	+	+	+	+	-	+
Breakdown of inositol	-	-	-	-	-	-
Acetoin from glucose (Voges-Proskauer)	+	-	d	-	-	+
Gas from glucose	+	-	+	+	-	+
H_2S from cysteine	+	-	+	-	-	+

Symbos; +, typically positive; -, typically negative; d, differs among strains.

Table 2 Other characteristics of motile Aeromonas species, (species 1-3) and Aeromonas salmonicida (species)[a]

Characteristics	Motile Aeromonas Species	A. salmonicida
Oxidase	+	+
NO_3^- reduced to NO_2^-	+	+
Lysine decarboxylase, Møller's medium	d	d
Ornithine decarboxylase, Møller's medium	-	-
Arginine dihydrolase, Møller's medium	+	+
Tryptophan ans phenylalanine deaminases	-	-
Urease	-[b]	-
Starch, gelatin, DNA and RNA hydrolysis	+	+
Tween 80 esterase	+	+
Citrate (Simmons')	d	-
Citrate (Christensen's)	d	-
Growth in peptone water without NaCl	+	+
ONPG test	+	d
Fermentation of maltose, galatose and trehalose	+[b]	+[b]
Fermentation of cellobiose, lactose ans sorbitol	d	-
Fermentation of dulcitol, rhamnose, inositol, xylose, raffinose and adonitol	-	-
Breakdown of malonate, mucate and D-tartrate	-	-
Fermentation of glycerol	d	d
Tetrathionate redutase	d	-

[a] For symbols see Table I.

[b] Aberrant strains occur.

With addition of the cytochrome oxidase (+), gelatinase (+) and 0/129 (2,4 diamino-6,7-diisopropylpteridine) sensitivity (-) tests the "minimum plexus" of unit characteristics for identification of A. hydrophila was obtained.[33]

Medium for the isolation of Aeromonas hydrophila

Bacterial species comprising microbial communities in aquatic ecosystems often may be of public health significance. Usually, selective and differential media are required for their detection and enumeration. The principles of such media are to eliminate species of secondary interest to select for specific microorganisms. Unfortunately, most media are not sufficiently selective for individual species, and additional biochemical tests must be applied after selection and purification of isolates before presumptive indentification is possible. Biochemical screenning on a large scale is laborious and time consuming.[34]

A. hydrophila has recieved increased attention both as an indicator of pollution and as pathogen. The organism has been reported to produce at least three virulence factors, including enterotoxin, cytotoxin, and hemolysin, and is recognised to be of public health significance when found in large numbers in the environment.[34]

Shotts and Rimler (1973)[35] developed a simple medium called Rimler-Shotts medium (RS medium) for the rapid isolation and identification A. hydrophila. This medium was effective in presumptive identification of the strains of A. hydrophila. However, strains of

Citrobacter which were hydrogen-sulfide-variable could not be separated from A. hydrophila.

Rimler-Shotts medium components

L-lysine-hydrochloride	5	gm.
L-ornithine hydrochloride	6.5	gm.
maltose	3.5	gm.
sodium thiosulfate	6.8	gm.
L-cysteine hydrochloride	0.3	gm.
bromothymol blue	0.03	gm.
ferric ammonium citrate	0.05	gm.
sodium deoxycholate	1.0	gm.
novobiocin	0.005	gm.
yeast extract	3.0	gm.
sodium chloride	5.0	gm.
agar	13.5	gm.
water qs.	1,000	ml

The components were dissolved by stirring; pH was adjusted to 7.0; and the mixture was boiled for 1 minute, cooled to 45°C and poured into plates. Prepared plates were refrigerated until needed.

The ingredients used in this medium were selected and compounded so as to achieve a maximal acidic (maltose fermentation) or basic reaction (decarboxylation of lysine or ornithine, or both). Hydrogen sulfide production was primarily dependent upon the utilization of sodium thiosulfate or L-cysteine hydrochloride, or both, with ferric ammonium citrate being utilized to help visualize this

reaction. The inhibitors, sodium deoxycholate, and novobiocin, were added to eliminate gram-positive organisms and vibrio spp. which may have caused false reactions. The use of novobiocin to inhibit the growth of Vibrio spp. minimizes the confusion after encountered in differentiation of these organism from anaerobic strains of A. hydrophila. The balance of the ingredients provide a nutrient base and chemophysical stability for the medium.

The colony-color of A. hydrophila strains in RS medium was yellow caused by maltose fermentation. It is important that the medium be incubated at 37°C to eliminate the possible growth of A. salmonicida which would occur at reduced temperature. This organism will produce a characteristic yellow colony on RS medium at reduced temperature. Since color changes occur due to pH reactions, colonial growth must be observed between the 20 th and 24 th for maximal accuracy.

The specific medium, discribed by Shotts and Rimler is highly selective and limited in application for natural environment. Strains of A. hydrophila which are novobiocin sensitive and lysine decarboxylase positive will not be detected by this medium. In addition, RS medium cannot be used to distinguish A. hydrophila from so called "group F" or EF 6 vibrio which are widely distributed in the marine and estuarine environment.[34]

Kaper et al (1979)[34] developed a new medium called A. hydrophila medium (AH medium) for the rapid presumptive identification of A. hydrophila. It also offered good differentiation of Klebsiella,

Proteus, and other enteric species. Mannitol fermentation, inositol fermentation, ornithine decarboxylation and deammination, indole production, motility, and H_2S production from sodium thiosulfate and cysteine could be recorded in a single tube of the medium. (see table 3.)

Table 3 Reactions of enteric bacteria in AH medium

Species	No.of strains tested	Reaction[a]				
		Top	Butt	Motility	H_2S	Indole
A. hydrophila	700	K	A	+	-	+
K. pneumoniae	21	A	A	-	-	-
K. oxytoca	9	A	A	-	-	+
E. coli	10	K	K or A[b]	+ or -	-	+
Salmonella spp.	10	K or A	K or A	+	+	-
Enterobacter spp.	6	K or A	K or A	+	-	-
Proteus spp.	4	R	K or A	+	+ or -	+[b]
Yersinia enterocolitica	2	K or N	K or N	-	-	+ or -
Citrobacter spp.	2	K	A or K	+	+	-
Serratia spp.	2	N or K	N or K	+	-	-

[a]Symbols; K, alkaline reaction; A, acid reaction; R, red; N, bleached neutral color due to the destruction indicator; +, 90% or more positive; -, 90% or more negative.

[b]The repsonse of individual strains of the species listed may vary as indicated, consistent with biochemical patterns compiled by Edwards and Ewing[36]

AH medium (in grams per liter) components

proteose peptone	5
yeast extract	3
tryptone	10
L-ornithine hydrochloride	5
mannitol	1
inositol	10
sodium thiosulfate	0.4
ferric ammonium citrate	0.5
bromocresol purple	0.02
agar	3

To prepare the medium, one must thoroughly mix the ingredients in 1 liter of distilled water and adjust the pH to 6.7. The medium is heated to boiling, dispensed in 5-ml quantities in tubes (13 by 100 mm), and autoclaved at 121°C for 12 min. Colonies of Aeromonas and enteric baeteria can be picked directly from isolation plates or membrane filters and inoculated into the medium by stabbing to the base of the tube with a straight needle. The inoculated tubes are incubated at 35°C for 18 to 24 h , after which reactions are recorded.

Variations in the basic formula of the AH medium are possible. For example, agar can be added to a final concentration of 15 gm/liter, and the medium can be prepared as a slant, rendering gas production obvious by the splitting of the agar. Lactose may be substituted for inositol, if lactose-negative organisms are sought.

For isolation and enumeration of A. hydrophila, MacConkey agar with trehalose substituted for lactose in the formulation was

used by Kaper et al (1981)[33], because RS agar was found to be too selective. Incubation was at 35°C for 24 h. Colonies were picked and the isolates presumptively identified, using a multitest screening medium (AH medium). Strains which yielded an alkaline surface and an acid butt and were motile were considered, presumptively, to be A. hydrophila and were tested further for cytochrome oxidase(+), gelatin hydrolysis (+) and sensitivity (-) to 0/129 (2,4-diamino-6,7-diisopropyl-pteridine).

A membrane filter method (mA) for the enumeration of A. hydrophila in natural water was developed by Rippey and Cabelli.[37] The complex, primary medium employs trehalose as a fermentable carbohydrate and ampicillin and ethanol as selective inhibitors. After 20 h of incubation at 37°C, an in situ mannitol fermentation test followed by an in situ oxidase test is used to further differentiate A. hydrophila from other aquatic and terrestrial microorganisms present in fresh-waters. The primary medium decreases background microbial growth by about two orders of magnitude.

The superiority of the mA method over RS medium, when the latter is used in a membrane filter procedure, for the recovery of A. hydrophila from several factors. First, the RS medium was not developed or recommended by the other for use with membrane filter, nor was it developed primarily for the quantitative examination of water samples. Second, the use of novobiocin in the RS medium eliminates those environmental A. hydrophila which are sensitive to that antibiotic. Third, the weakly lysine decarboxylase-positive A. hydrophila strains observed among our environmental isolates may not be counted on the RS medium.[37]

The incorporation of vibriostatic agent 0/129 in the mA medium may impove recovery of Aeromonas spp by the mA method when estuarine and marine samples are processed.[33]

Aeromonas infection[33,38,39]

Species of the genus Aeromonas are considered autochthonous inhabitants of aquatic environments. A. hydrophila has recieved particular attention because of its association with human disease and economic fish disease. It has been isolated from both polluted and unpolluted bodies of water throughout the world. In addition it is considered to comprise a portion of the normal flora of fishes, as well as other aquatic animals and plants. It can cause infections and epizootics in a variety of animals, including alligators, turtles, frogs, snails, snakes, lizards, reptile, birds, vampire bat, and fish.[33,39] A. hydrophila is not generally considered to be normal inhabitant of human G-I tract. In fact, results of most studies have shown that less than 1% of healthy adult carry aeromonads.[33]

Fatal and non fatal of A. hydrophila in human have been reported that are associated with a variety of clinical manifestations, including septicemia, meningitis, corneal ulcers, wound infections, peritonitis, and acute diarrhoeal disease.[33] Infections have been reported in both healthy and immunologically compromised hosts. The center for disease control documented an epidemic of A. hydrophila nasocomial infections, including two deaths. Aeromonas infection could occur as a result of contact with water and soil.[33]

From results of laboratory studies,aeromonads are now recognized to produce entertoxin, haemolysin, endotoxin, and cytotoxin.[33]

Some strains of Aeromonas induce fluid accumulation in ligated ileal loops of adult rabbits, comparable to toxigenic strains of Vibrio cholera.[33]

Formerly, Aeromonas spp. were considered opportunistic pathogens of low virulence often associated with polymicrobial infection. More recent literature, however, indicated that Aeromonas spp. might act as primary pathogens of serious consequence.[38]

Immunity[40,41]

Heat killed, and formalinized vaccines from A. hydrophila were prepared by Saitanu et al.[41] The antibodies were produced, after the injection of the different 2 typed-vaccines in cat-fish (Clarias batrachus). The agglutinating titer in the fish which were vaccinated by formalinized vaccine was higher and lasting longer than in the fish, vaccinated by the heat killed vaccine. They also proved that the immunity could prevent the infection of A. hydrophila.[41]

Environment effects to A. hydrophila and the infection in fish

Sihanonth et al (1983)[42] found that A. hydrophila F 588 could grow in the very wide range of environmental condition. A. hydrophila F 588 could grow in the wide range of pH, whereas the optimal pH for the all growth was between pH 5 to 11.5. The growth of A. hydrophila F 588 decreased at the pH 5, or higher than 11.5, morever the all growth was completely stopped at pH 4.0. Sodium chloride concentration from 0 to 3 percent had no effect on the growth of this organisms. The organism was severely inhibited when cultivated in a nutrient broth containing sodium chloride higher than 6 percent.

The optimal temperature for the growth of A. hydrophila was at 30°C. A. hydrophila could not grow at 8°C and 45°C.

Water temperature was found to affect significantly the population size of A. hydrophila in Chesapeake Bay. Counts were highest in the summer months and lowest in the winter. The majority of human infections caused by Aeromonas occurred in the spring and summer months.(33)

The effect of paraquat at various concentrations showed no different when the culture was incubated at 30°C in the absence or in the prescence of paraquat at the concentration levels from 0-10 ppm. However the growth of bacteria decreased at the concentration of paraquat higher than 10 ppm and completely stopped at 200 ppm.(42)

A. hydrophila strains are usually found in healthy fish, and infected fish. The weak fish were eventually infected by A. hydrophila.(43) The environment factors, for example, temperature changes, low dissolved oxygen, high ammonia content, waste product form fish and pollutant from factories caused fish weak and stress.(39)

Sihanonth et al (1985) found that casamino acid (the organic nitrogen which in a nutrient for A. hydrophila.) at 0.1% resulted in the total fish (Ophicephalus striatus) death within 2-4 days, 0.05% casamino acid plus the inoculation of A. hydrophila F 588 resulted in faster infection and higher fish death than the group without the inoculation and they found that paraquat was not predisposing cause for the infection of this fish disease.(43)