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CHARACTERIZATION AND VIRULENCE STUDIES OF MOTILE AEROMONADS ISOLATED FROM *CLARIAS BATRACHUS* AND *C. GARIEPINUS* AND THEIR IMMUNIZATION POTENTIAL

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of thesis submitted to the Senate of Universiti Pertanian Malaysia in partial fulfilment of the requirement for the degree of Master of Science.

CHARACTERIZATION AND VIRULENCE STUDIES OF MOTILE *Aeromonas* ISOLATED FROM *Clarias batrachus* AND *Clarias gariepinus* AND THEIR IMMUNIZATION POTENTIAL

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Morphological, physico-chemical, biochemical and virulence characteristics of 30 motile *Aeromonas* isolates were examined. Selected isolate were investigated for their ability to immunise fish.

Four *Aeromonas hydrophila*, ten *Aeromonas hydrophila*-like and sixteen unspecified *Aeromonas* spp. were isolated from *C. batrachus* and *C. gariepinus*. These two fish species were collected from fish farms at Bogor, Depok, Sukabumi and Bekasi, West Java.

It appears that the virulency of motile aeromonads was associated with the type and amount of extracellular substances produced.

Four isolates were selected for the determination of LD_{50} based on enzymatic activity, extracellular products, and preliminary screening of pathogenicity. The LD_{50} studies indicated that all the four *Aeromonas* spp. isolates tested were virulent to the target fish *C. gariepinus* ($LD_{50}=10^4$ cells/ml), two isolates were weakly virulent to *A. batrachus* ($LD_{50}=10^5$ cells/ml) while the other two isolates were avirulent to *A. batrachus* (LD_{50} $>10^7$ cells/ml).

All thirty isolates of motile aeromonads were screened for sensitivity to three antibiotics; oxytetracycline hydrochloride, enrofloxacin and flumequin. The results reveal that eleven isolates (36.7%) were very sensitive to oxytetracycline hydrochloride, two (6.7%) were fairly sensitive, eight (26.7%) were moderately resistant and nine (30%) were very resistant. None of 30 isolates were resistant to enrofloxacin; 19 isolates (63.3%) were sensitive and 11 (36.7%) were moderately sensitive. Twenty one isolates (70%) were resistant to flumequin, seven (23.3%) were moderately sensitive and two (6.7%) were sensitive.

Three types of vaccine, composed of whole cell bacterin, broth culture and supernatant of *A. hydrophila*

were tested. *Clarias batrachus* were immersed in the vaccine for variable periods (5, 10 and 15 minutes), using a single dose. After a standard time the fish were tested serologically for their ability to produce agglutinating antibody. The results showed that there was a significant difference ($P<0.05$) in antibody response between fish exposed to broth culture vaccine for 15 minutes and unvaccinated fish at two weeks post-vaccination. The highest level of protection (90%) was detected at the fourth week post-vaccination. However, there was no significant difference ($P<0.05$) between different types of vaccines used and also between different immersion times. The level of protection then decreased after six weeks.

These studies suggest that fish exposed to *Aeromonas* culture broth provided effective protection over a limited period of about 2 months.

Abstrak tesis yang dikemukakan kepada Senat Universiti Pertanian Malaysia, sebagai memenuhi sebahagian daripada keperluan untuk mendapat Ijazah Master Sains

KAJIAN MENGENAI PENCIRIAN DAN VIRULEN DARIPADA AEROMONADS MOTIL DARI *C. BATRACHUS* DAN *C. GARIEPINUS* SERTA POTENSI IMMUNISASI

oleh

Hambali Supriyadi

Mei 1991

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Suatu kajian mengenai pencirian morfologi, fiziko-kimia dan biokimia dan virulen bagi 30 pencilan bakteria aeromonads motil telah dijalankan. Pencilan terpilih dikaji untuk menentukan kemampuannya untuk mengimunisasikan ikan.

Empat pencilan *Aeromonas hydrophila*, sebelas berupa *A. hydrophila* dan enam belas *Aeromonas* spp. yang belum pasti spesiesnya telah dipencilkan dari ikan keli *C. batrachus* dan *C. gariepinus*. Kedua-dua spesies ikan ini diperolehi dari ternakan ikan keli di Bogor, Depok, Sukabumi dan Bekasi, Jawa Barat.

Kajian menunjukkan virulensi aeromonad motil adalah berkait dengan jenis dan jumlah bahan ekstraselular yang

dikeluarkan. Berdasar pada kajian aktiviti enzimatik, bahan ekstraselular dan tapisan awal empat pencilan dipilih untuk kajian LD_{50}. Hasil kajian mengenai LD_{50} dari empat pencilan bakteri aeromonad motil mendapati bahawa keempat-empat pencilan adalah virulen kepada *C. gariepinus* ($LD_{50}=10^4$ sel/ml); dua pencilan virulen separa lemah ($LD_{50}=10^5$ sel/ml) dan dua lagi pencilan tidak virulen (LD_{50} $>10^7$ sel/ml).

Kesemua tiga puluh pencilan aeromonads motil ditapis untuk kepekaan terhadap tiga jenis antibiotik iaitu oxytetracycline hydrochloride, enrofloxacin dan flumequin telah digunakan dalam kajian sensitiviti kepada 30 pencilan bakteria *Aeromonas* motil. Hasil kajian mendapati bahawa sebelas pencilan (36.7%) adalah sangat sensitif terhadap oxytetracycline hydrochloride, dua pencilan (6.7%) sensitif, lapan pencilan (26.7%) tahan dan sembilan pencilan (30%) sangat tahan. Tidak ada pencilan yang tahan terhadap enrofloxacin; sembilan belas pencilan (63.3%) adalah sensitif dan sebelas pencilan (36.7%) adalah separa sensitif. Dua puluh satu pencilan (70%) tahan terhadap flumequin, tujuh pencilan (23.3%) separa tahan, dan dua pencilan (6.7%) tahan.

Tiga jenis vaksin terdiri dari bakterin seluruh sel (whole cell bacterin), kultur kaldu, dan supernatan kultur *A. hydrophila* dikaji. *Clarias batrachus* telah dimandikan dalam vaksin dengan tiga peringkat waktu yang berbeza iaitu 5, 10 dan 15 minit dengan menggunakan satu dos. Selepas waktu yang ditetapkan, ikan tersebut telah diuji secara serologi untuk menentukan kemampuannya menghasilkan antibodi aglutinan. Hasil kajian mendapati bahawa ada perbezaan yang bererti ($P<0.05$) di antara ikan yang diberi vaksin kaldu selama 15 minit dan yang tidak pada dua minggu pasca vaksinasi. Paras ketahanan yang tertinggi (90%) didapati pada minggu ke empat setelah ikan diberi vaksin. Namun demikian tidak ada perbezaan yang bererti ($P<0.05$) antara ketiga-tiga jenis vaksin yang digunakan, dan juga ketiga peringkat masa perendaman. Paras ketahanan didapati menurun pada enam minggu setelah ikan diberi vaksin.

Kajian ini menunjukkan bahawa ikan yang dimandikan dalam bakterin *Aeromonas* seluruh sel memberikan ketahanan yang berkesan selama dua bulan.

CHAPTER I

GENERAL INTRODUCTION

Background

Fish is the primary source of animal protein in Indonesia. The majority of fish consumed originate from marine and freshwater capture fisheries. The contribution of fish through aquaculture production accounted for 12.71% of the total production in 1985 (Rahardjo, 1987) but in recent times the availability of marine and freshwater fishes has declined due to overfishing. The only means of increasing fish production is through intensification of aquaculture. For example, intensification of common carp through the use of a running water system in ponds began in 1971 (Supriyadi, 1986). Intensive fish culture in cages has also been operated intensively in reservoirs and lakes.

Catfish is the other commonly cultured freshwater fish species besides common carp. Intensive culture of walking catfish (*Clarias batrachus*) in Indonesia started only recently. In 1985, catfish production was estimated to be 694 tons, which is equivalent to 1.093 million Indonesian Rupiah (US$ 1 = 1800 Rupiah) (Rahardjo, 1987).

Intensification of catfish culture is practised by means of increasing the stocking rate and food supplement. Fish farmers are encouraged to culture catfish because it is highly profitable.

African catfish (*Clarias gariepinus*) was recently introduced into Indonesia and is now being cultured extensively. This species is bigger in size as compared with *Clarias batrachus*. *Clarias gariepinus* grows faster, is easier to handle and therefore preferred by farmers for culture.

Intensification of fish culture has resulted in increased outbreaks of fish diseases. The first record in Indonesia of bacterial disease in fish culture conditions was in 1980. Great damage to fish production occurred and losses of common carp broodstock was estimated at two million U.S. dollars (Dana, 1987).

The development of catfish culture in recent years has been hampered by the frequent occurrence of bacterial diseases. *Aeromonas hydrophila*, *Pseudomonas fluorescens* and *Flexibacter columnaris* have been isolated from ulcerative lesions of both cultured and wild catfish in Indonesia (Supriyadi, 1988). *Aeromonas hydrophila* is the bacterium most frequently and consistently isolated from epizootic ulcerative syndrome (EUS)-positive fish (Torres *et al.*, 1990). Hazen *et al.* (1978) and Snieszko (1974)

also stated that strains of *A. hydrophila* are widely distributed in the aquatic environment and are also abundant in sewage and waters that are contaminated with sewage.

Our preliminary observations indicate that one month-old catfish are usually more susceptible to bacterial disease. Bacterial infection is usually observed soon after the fingerlings are released into the ponds. Observations also revealed that catfish that are less than one month old are seldom infected by bacterial disease. However, ectoparasites are commonly found at this stage.

Supriyadi (1986) reported that walking catfish is most susceptible to *A. hydrophila* infection as compared with giant gouramy *(Osphronemus gouramy)* and common carp (*Cyprinus carpio*). Since catfish is more susceptible to bacterial disease, many catfish farmers in Indonesia experienced economic losses due to the frequent occurrence of bacterial infection which caused 90 - 100% mortality.

Objectives

Studies on the identification and characterization of *Aeromonas* spp. have been made by several workers (Shotts and Bullock, 1975; Popoff and Veron, 1976; Popoff and Lallier, 1984).

Aeromonas spp. isolated in Indonesia have been previously reported (Anon, 1980; Sumawidjaja *et al.*, 1981; Supriyadi, 1988). These studies identified the isolates to the genus level only. The aims of the present study were to characterize motile *Aeromonas* spp. isolated from two species of diseased and healthy catfish, *C. batrachus* and *C. gariepinus*, and to evaluate the immunization potential of a selected isolate. The specific objectives of this study were:

1. To isolate *Aeromonas* spp. from health fish.
2. To conduct morphologically, physicochemically and biochemically studies on these isolates.
3. To determine the virulence of selected *Aeromonas* spp. by plate assay technique and LD_{50}.
4. To evaluate the sensitivity of *Aeromonas* spp. against selected antibiotics and
5. To assess the immunization potential of *A. hydrophila* vaccine.

CHAPTER II

REVIEW OF LITERATURE

Taxonomy

Aeromonas hydrophila is phenotypically, serologically, and genetically a heterogenous taxon (Newman, 1983). Strains of *A. hydrophila* have been responsible for massive mortalities of cultured and feral fish populations.

Snieszko (1957) in the 7th edition of Bergey's Manual of Determinative Bacteriology described three species of motile Aeromonads : *Aeromonas liquefaciens*, *A. punctata* and *A. hydrophila*.

Popoff (1984) has grouped the fish-pathogenic aeromonads into two discrete groups, namely the non-motile psychrophilic species and the motile mesophilic species. The genus is, at present, classified in the family Vibrionaceae. However, on the basis of newly acquired evidence resulting from molecular genetic studies Colwell *et al.* (1986) have proposed that *Aeromonas* be removed from the Vibrionaceae and placed into a new family Aeromonaceae.

Classification of the species under the genus *Aeromonas* in the 8th edition of Bergey's Manual of Determinative Bacteriology (Schubert, 1974) differentiated three species within Aeromonads:

Species : *Aeromonas hydrophila*

Subspecies : - *A. hydrophila* subsp. *hydrophila*
- *A. hydrophila* subsp. *anaerogenes*
- *A. hydrophila* subsp. *proteolytica*

Species : *Aeromonas punctata*

Subspecies : - *A. punctata* subsp. *punctata*
- *A. punctata* subsp. *caviae*

Species : *Aeromonas salmonicida*

Subspecies : - *A. salmonicida* subsp. *salmonicida*
- *A. salmonicida* subsp. *achromogenes*
- *A. salmonicida* subsp. *masoucida*

Popoff (1984) classified four species under the genus *Aeromonas* :

1. *Aeromonas hydrophila*
2. *Aeromonas caviae*
3. *Aeromonas sobria*
4. *Aeromonas salmonicida* with three subspecies :
   - *A. salmonicida* subsp. *salmonicida*
   - *A. salmonicida* subsp. *achromongenes*
   - *A. salmonicida* subsp. *masoucida*

Pathogenicity

Aeromonas hydrophila has been isolated from a variety of environmental sources, including water, sediments and from diseased and healthy animals. Strains of *A. hydrophila* have been associated with a wide range of infections affecting cold and warm blooded animals (Davis, *et al.*, 1978).

De Figueiredo and Plumb (1977) examined the virulence of nine *A. hydrophila* strains isolated from diseased fish and shrimp from freshwater ponds and from channel catfish (*Ictalurus punctatus*) fingerlings. Significant differences in the ability to kill catfish were observed between strains isolated from water compared to strains from diseased fish. They concluded that the source of the isolate may be related to its ability to produce disease. Lallier *et al.* (1980) confirmed these observations when they found that strains of *A. hydrophila* isolated from healthy and diseased fish were more virulent for rainbow trout than an *A. sobria* strain isolated from healthy fish. *Aeromonas hydrophila* have been recognized as the causative agent of "red " disease in amphibians and reptiles (Shotts *et al.*, 1972), snails and cow (Popoff and Lallier, 1984) and man (Davis *et al.*, 1978).

Aeromonas hydrophila may posses virulence factors such as proteases, enterotoxin and hemolysins but their