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IDENTIFICATION OF VIBRIO SPP. FROM CULTURED SEABASS LATES CALCARIFER AND THEIR ANTIBIOGRAM RELATIONSHIP WITH PLASMID PROFILES

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LIST OF ABBREVIATIONS

General - Non-scientific

etc.	et cetera
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
NCCLS	National Committee for Clinical Laboratory Standards
RM	Ringgit Malaysia
USA	United States of America

General - Scientific

0/129	2,4-diamino-6,7-di-isopropyl pteridine
AIDS	Acquired Immune Deficiency Syndrome
AP	Alkaline phosphatase
AST	Antibiotic Sensitivity Test
BKD	Bacterial kidney disease
BSA	Bovine serum albumin
CFU	Colony forming units
CO_2	Carbon dioxide
DDW	Double distilled water
DNA	Deoxyribonucleic acid
ELISA	enzyme-linked-immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incommplete adjuvant
Ig	immunoglobulin
MIC	Minimal inhibitory concentration
N_2	Reduction of nitrates to nitrogen gas
NO_2	Reduction of nitrates to nitrites
OF	Oxidative-Fermentative Test (Hugh and Leifson, 1953)
pABA	para-amino-benzoic acid
PBS	Phosphate buffer saline
pH	hydrogen ion concentration
pNNP	p-Nitrophenyl phosphate
RNA	Ribonucleic acid
TCBS	Thiosulphate Citrate Bilesalt Sucrose Agar
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron Agar
Tween®20	Polyoxyethylensorbitanmonolaurat

Chemical Names

EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloride acid
KCl	Potassium chloride
KH_2PO_4	Potassium di-hydrogen phosphate
$MgCl_2.6H_2O$	Magnesium chloride
Na_2CO_3	Sodium carbonate
$Na_2EDTA.2H_2O$	EDTA di-sodium salt
Na_2HPO_4	di-Sodium hydrogen-phosphate
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
Tris-Cl	Tris-chloride

Scientific Measurement

%	percent
µg	microgram
µl	microliter
µm	micrometer
g	gravity
g	gram
iu	international units
kb	kilobase
kg	kilogram
M	Molarity
m^2	meter square
min.	minutes
ml	milliliter
mm	millimeter
mM	millimolar
N	Normality
nm	nanometer
ppt	parts per thousand
rpm	revolution per minute
vol	volume
wt	weight

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IDENTIFICATION OF *VIBRIO* spp. FROM CULTURED SEABASS *LATES CALCARIFER* AND THEIR ANTIBIOGRAM RELATIONSHIP WITH PLASMID PROFILES

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Thirty eight bacteria isolates were recovered from seabass with vibriosis. These isolates were identified using the conventional biochemical tests and the API-20E system. Most isolates were cream in colour, round, convex and entire. They were grouped into the genus *Vibrio* with the following traits: Gram-negative short rods, motile, positive catalase and oxidase reactions, fermentative metabolism towards Hugh and Leifson glucose medium and ferments D-glucose by producing acid but not gas. Yellow colonies (identified as *Vibrio alginolyticus*) and green colonies (identified as *Vibrio parahaemolyticus*) were obtained on TCBS agar. The API-20E test strips identified fifteen isolates as *V. parahaemolyticus* and twenty three isolates as *V. alginolyticus*. Comparative studies between

the conventional method and the API-20E System revealed that the LDC, ADH, H_2S, IND, GLU, INO and OX tests gave the most reproducible, consistent and accurate results.

A polyclonal antibody against *V. alginolyticus* was developed for application in rapid identification. Serum containing antibodies obtained at 52 days after exposure displayed the best titer development with end dilution detection values (OD_{405} ≥ 0.286) of 1:12,800. The polyclonal antibody detected the antigen at 10^6 CFU/ml. The polyclonal antibody generated was specific to the genus level.

Antibiotic sensitivity patterns of the isolates were investigated. Antibiotic-resistance occured in 97.4% of the isolates tested. Resistance to gentamicin (7.7%), kanamycin (20.5%), neomycin (2.6%), streptomycin (56.4%), amoxycillin (92.3%), carbenicillin (89.7%), doxycycline (5.1%), polymyxin B (38.5%), rifampicin (33.3%) and the potentiated sulphonamide (2.6%) was observed. Multiple drug-resistance was common (94.8%), and was higher in the *V. alginolyticus* (58.9%) than the *V. parahaemolyticus* (35.9%) isolates. All (100%) isolates were susceptible to norfloxacin, ofloxacin and oxolinic acid. Isolates sensitive to pipemidic acid (97.4%), nalidixic acid

(94.9%), doxycycline (89.7%), ciprofloxacin (89.7%), tetracycline (71.8%) and sulphamethoxazole/trimethoprim (84.6%) were detected at high frequencies.

The presence of plasmids was examined using the modified Kado and Liu (1981) method. No plasmid bands were detected, indicating that the antibiotic-resistance of the isolates was not plasmid-mediated.

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IDENTIFIKASI *VIBRIO* spp. DARIPADA IKAN SIAKAP *LATES CALCARIFER* DAN PERHUBUNGAN DI ANTARA ANTIBIOGRAM DENGAN PROFIL PLASMID

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Tiga puluh lapan isolat bakteria diperolehi daripada ikan siakap yang dijangkiti oleh vibriosis. Isolat-isolat ini dikenalpasti dengan menggunakan ujian biokimia dan sistem API-20E. Kebanyakan isolat berwarna krim, adalah bulat, berbentuk cembung dengan sisi lengkap. Mereka dikumpulkan ke dalam genus *Vibrio* dengan ciri-ciri berikut: rod pendek Gram-negatif, motil, tindak balas katalase dan oksidase positif, metabolisma fermentatif terhadap medium glukosa Hugh dan Leifson dengan menghasilkan asid tanpa gas. Koloni kuning (dikenalpasti sebagai *Vibrio alginolyticus*) dan koloni hijau (dikenalpasti sebagai *Vibrio parahaemolyticus*) diperolehi di atas agar TCBS. Jalur ujian API-20E mengenalpasti lima belas isolat sebagai *V. parahaemolyticus* dan dua puluh tiga isolat sebagai *V. alginolyticus*. Kajian perbandingan

di antara kaedah lama dengan Sistem API-20E menunjukkan yang ujian-ujian LDC, ADH, H_2S, IND, GLU, INO dan OX memberikan keputusan yang paling berulang, seragam dan tepat.

Suatu antibodi poliklonal terhadap *V. alginolyticus* telah dihasilkan untuk digunakan dalam proses kenalpasti pantas. Serum yang mengandungi antibodi yang diperolehi pada 52 hari selepas suntikan memberikan pembentukan titer yang paling baik dengan nilai kesan pencairan akhir (OD_{405} ≥ 0.286) 1:12,800. Antibodi poliklonal dapat mengesan antigen pada kepekatan 10^6 CFU/ml. Poliklonal antibodi yang terjana adalah khusus sehingga peringkat genus.

Corak kesensitifan antibiotik isolat-isolat dikaji. Rintang-antibiotik didapati dalam 97.4% isolat-isolat yang dikaji. Rintang terhadap gentamisin (7.7%), kanamisin (20.5%), neomisin (2.6%), streptomisin (56.4%), amoksisilin (92.3%), karbenisilin (89.7%), doksisiklin (5.1%), polimisin B (38.5%), rifampisin (33.3%) dan sulfonamid terpotensi (2.6%) didapati. Rintang-antibiotik beraneka adalah biasa (94.8%), dan adalah lebih tinggi dalam isolat-isolat *V. alginolyticus* (58.9%) daripada *V. parahaemolyticus* (35.9%). Kesemua (100%) isolat adalah peka terhadap norfloksasin, oflosasin dan asid oksolinik. Isolat-isolat yang sensitif terhadap asid pipemidik

(97.4%), asid nalidisik (94.9%), doksisiklin (89.7%), ciprofloksasin (89.7%), tetrasiklin (71.8%) dan sulfametoxasol/trimetoprim (84.6%) dikesan pada frekuensi tinggi.

Kehadiran plasmid dikaji dengan menggunakan kaedah Kado dan Liu (1981) yang diubahsuai. Tiada jalur plasmid dikesan, menunjukkan yang rintang-antibiotik isolat-isolat bukanlah disebabkan oleh plasmid.

CHAPTER I

GENERAL INTRODUCTION

Background

Aquaculture is a relatively young industry in Malaysia, which began in the early 1930s with freshwater fish culture. The total fish production from aquaculture was 114,113 metric tonnes in 1994, generating an income of RM 365 million (Annual Fisheries Statistics 1994). Marine floating fish-pens were first introduced to Malaysia in 1973 for rearing groupers, *Epinephelus tauvina*, in the Straits of Penang (Chua and Teng, 1977). After three years of experimentation, this fish culture method was proven to be technically feasible and commercially viable. Presently, there are 679,438 m^2 of floating net cages for marine finfish culture in the coastal waters of Malaysia (Annual Fisheries Statistics 1994). It is one of the major culture systems used in Malaysia. In terms of the number of cages used, Malaysia has the largest number of cages in the South East Asia

(Leong, 1990). The popular areas used for culturing marine finfish in floating net-cages are located in Johor, Penang and Selangor.

Pulau Ketam is the only place in Selangor where marine fish are reared commercially (Chin, 1994). There were only about seventeen floating fish farms in 1989 and today the figure has doubled to more than forty. Each farm has approximately 100 to 200 floating net-cages. He further mentioned that the number of floating cages was only 4,247 in 1989 compared to 7,031 in 1992. The rapid expansion of the marine fish culture industry in Pulau Ketam is due to the handsome returns from this activity. The culture fish species include seabass (*Lates calcarifer*), grouper (*Epinephalus tauvina*), snapper (*Lutianus argentimaculatus*) and red snapper (*L. malabaricus*). At present, the production of cultured marine fish in Pulau Ketam exceeds 50 metric tonnes per month (Chin, 1994), thus making it one of the main producers of marine fish in Malaysia. Among the marine and brackishwater cage products mentioned above, the seabass contributes the most economically, in terms of production and value.

Vibriosis in Cultured Seabass

Seabass, or locally known as *siakap,* is a popular food fish. It is a favourite among the Chinese for its delicious well-flavoured flesh. It is highly demanded in the market, fetching a good price of between RM 10.00 to RM 12.00 per kilogram. Seabass is an euryhaline fish, thus it is considered to be a hardy fish that is able to withstand salinity fluctuations in the floating cage-culture environment. However, as the culture of seabass intensified to meet the increasing demands of fish supply, many management problems have resulted. The practice of over-stocking has restricted the water circulation and the fish movement in the cages. Consequently, the environmental and physical stress have rendered the fish susceptible to diseases. Vibriosis caused by *Vibrio* spp. is the most common bacterial disease affecting cultured seabass (Wee and Leong, 1986).

Vibrio spp. are Gram-negative, non-sporing rods which are straight or have a single, rigid curve. They are 0.5-0.8 μm in width and 1.4-2.6 μm in length. These species are highly motile with a single polar flagellar. They are oxidase positive, catalase positive and ferment glucose producing acid but no gas. Members of this genus can be distinguished based on their colony morphology and pigmentation, growth conditions and nutrients, physiology and metabolism, genetics and plasmids, antigenic

structure and antibiotic sensitivity. In general, they can be found in aquatic habitats with a wide range of salinity.

Vibriosis spreads very rapidly, thus causing severe economic losses to fish farmers. It is a serious problem which can hamper the future development of the seabass culture industry. At the moment there is still insufficient knowledge on the diagnosis, prevention and control of vibriosis. It is, therefore, the aim of this study to contribute to these aspects of the disease.

In the first part of this study, attempt has been made to identify the causative agent(s) of vibriosis using the conventional method and the polyclonal antibody-based ELISA technique. Following diagnosis, some measures must be taken to curb the spread of vibriosis. For this reason, a study on the relationship of an antibiogram and the plasmid contents of the aetiological agents was carried out.

Polyclonal Antibody-Based ELISA

In this present age, antibodies have become important analytical tools. Antibodies are plasma proteins produced during an immune response of an animal to pathogenic organisms. They bind specifically to the antigen that stimulated their synthesis. Therefore, based

on this principle, they can be used for detecting aetiological agent(s) of a disease. For quick identification of pathogens, immunologically-based methods such as the enzyme-linked immunosorbent assay (ELISA) are employed. The advantage of ELISA is its simple handling and since it is a partially automated process, rapid results may be obtained within a few hours.

Relationship of Antibiogram and Plasmid Contents

A variety of antibiotics are used to treat vibriosis. Tetracycline is the common antibiotic of choice. However, the proper selection of alternative antibiotics is required if bacteria developed tetracycline-resistance due to the extensive use of the drug. For this purpose, the antibiotic sensitivity patterns or an antibiogram would be useful. Disease treatment and control may be based on an antibiogram. Thus, errors in choice due to clinical urgency can be avoided. Presently, there is a lack of information on the antibiotic sensitivity patterns of bacteria associated with vibriosis in seabass, therefore a study from this viewpoint is greatly needed. An antibiogram obtained from this study will be useful in the future to control vibriosis.

On the other hand, the indiscriminate use of antibiotics for treating vibriosis may result in the emergence and spread of antibiotic-resistant bacteria strains through plasmids. Plasmids are double-stranded DNA molecules which are generally circular and small in size. They are only one to two percent of the size of the chromosomal DNA. Plasmids are independent "replicons" which have their own genetic system that controls their own replication. They carry genes for a variety of enzymes that can degrade antibiotics and modify membrane transport system. These features give rise to antibiotic-resistance in a bacterium. In order to determine their role in antibiotic-resistance, the relationship of an antibiogram and the analysis of plasmid contents of bacteria associated with vibriosis is necessary.

Plasmids in a host bacterial cell can be detected by referring to a plasmid profile. A plasmid profile is obtained by isolating the plasmid DNA molecules from the host bacterial cell and separating them using agarose gel electrophoresis. Fragments of the plasmid DNA molecules are exhibited as bands at varying positions in the gel. These bands make up the plasmid profile of an individual bacterial cell.

In recent years, as fish production has increased at comparatively high costs, prevention and control of diseases have gained major importance. In Malaysia,

vibriosis affecting seabass production is significantly important. An urgent need exists for the rapid diagnosis of the aetiological agent(s) and subsequent control of the disease. The whole idea of rapid diagnostic tools is to bypass the lengthy procedure of testing using the conventional method of identification. The relationship of the antibiotic sensitivity patterns and the plasmid contents of *Vibrio* spp. needs to be elucidated. With this knowledge, it is hoped that vibriosis may be controlled effectively in the near future.

With all these needs in mind, the present study was designed to:

(1) identify *Vibrio* spp. isolated from diseased seabass (*Lates calcarifer*) using conventional methods.

(2) identify *Vibrio* spp. isolated from diseased seabass (*Lates calcarifer*) using a polyclonal antibody-based enzyme-linked immunosorbent assay (ELISA) technique.

(3) obtain the antibiotic sensitivity patterns of *Vibrio* spp.

(4) determine the relationship between the antibiotic sensitivity patterns and the plasmid profile of *Vibrio* spp.

CHAPTER II

LITERATURE REVIEW

Seabass

Seabass, *Lates calcarifer* Bloch, belongs to the Perciformes order and the Serranidae family. Seabass is a euryhaline species that occupies the coastal, estuarine and freshwater environments. It is widely distributed throughout the Indo-west Pacific region including India, Myanmar, Sri Lanka, Bangladesh, Malaysia, Indonesia, Philippines, Papua New Guinea, Northern Australia, Southern China and Taiwan. In nature, it is usually found at the river mouths, bays and lagoons with brackish waters. The adult fish is comfortable in salinity ranging from 15 to 32 ppt, while the frys can be seen upstream or near the river mouth where the salinity falls between 5 to 10 ppt. It is a carnivorous fish that feeds on small fish and prawns that can be found in the water column. It has a cannibalistic nature when food supply is limited.

Seabass Culture

Breeding, larval rearing and the culture of seabass in cages and ponds have been the subject of many studies. In 1973, the seabass culture industry in the Asia and Indo-Pacific Region was given a big boost following the first successful artificial propagation of seabass frys in Thailand (Wongsomnuk and Manevonk, 1973). The dependence on frys captured from the wild which is often insufficient and inconsistent, will no longer be a limiting factor to the development of seabass culture. The west coast of the Peninsular Malaysia is blessed with suitable sites for seabass culture in floating cages. Coastal areas, bays, straits, lagoons and esturines provide good growth condition for the euryhaline seabass. Therefore, anticipating the potential of seabass culture in the country, the Penang Fisheries Research Institute embarked on a programme of fry production in 1982. The Tanjong Demong Marine Fish/Prawn Hatchery in Trengganu followed suit and is currently the main supplier of seabass frys in Malaysia. However, the supply of frys is far from adequate and has to be supplemented by imports from Thailand.

Seabass needs about a year of culture to attain the size of 1.3 kg. However, 5 to 9 months-old seabass weighing between 500 to 600 g has already attained marketable size. Trash feed made up of small anchovy