Faculty of Resource Science and Technology

Detection of *opr*L Gene and Antibiotic Resistance of *Pseudomonas aeruginosa* From Aquaculture Environment

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LISTS OF ABBREVIATION

ºC	Degree celcius
%	Percentage
DNA	Deoxyribonucleic acid
g/L	Gram/litre
µl	Microlitre
mm	Millimeter
min	Minutes
g	Gram
ml	Milimitre
rpm	Revolution per time

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ABSTRACT

Pseudomonas aeruginosa is a gram-negative rod shape bacterium belonging to family Pseudomonadaceae. The species is a highly adaptable opportunistic pathogen capable of surviving in a variety of environment, including aquaculture environment. Antibiotics are used in the aquaculture environment, and their usage poses a risk of potential transfer of resistance to human and animal pathogens. This study was conducted to isolate *P. aeruginosa* and identify them based on the PCR detection of *opr*L gene locus. These species were isolated from fish, prawn and water. Antibiotic resistance pattern was determined by conducting antibiotic susceptibility tests on the isolates. Thirteen isolates of *P. aeruginosa* were isolated and all of the isolates show resistance to at least one antibiotic. Highest level of resistance was observed against ampicillin and erythromycin and the lowest group was against gentamicin, norflaxin and nalidixic acid.

Keywords : *Pseudomonas aeruginosa, opr*L, aquaculture environment, antibiotic susceptibility test

ABSTRAK

Pseudomonas aeruginosa merupakan bakteria negative berbentuk rod tergolong dalam keluarga Pseudomonadaceae. Spesis tersebut merupakan bacteria berbahaya yang dapat menyesuaikan diri dalam pelbagai persekitaran, termasuklah persekitaran akuakultur. Antibiotik digunakan di dalam persekitaran akuakultur dan penggunaannya berpotensi menyebabkan risiko penyebaran daya tahan kepada patogen manusia dan haiwan. Kajian ini dijalankan untuk mengasingkan P. aeruginosa berdasarkan pengesanan reaksi rantai polimerase gen oprL dan spesis tersebut dipencilkan daripada ikan, udang dan air. Corak ketahanan antibiotik telah ditentukan dengan melaksanakan ujian kepekaan terhadap antibiotik ke atas semua bahan yang dipencilkan. Tiga belas P. aeruginosa telah berjaya dipencilkan dan semua menunjukkan daya ketahanan terhadap sekurang-kurangnya salah satu antibiotik. Daya tahan paling tinggi telah dikesan terhadap penggunaan ampisilin dan eritromisin dan daya tahan paling rendah dikesan terhadap penggunaan gentamicin,norflaksin dan acid nalidisik.

Kata kunci : Pseudomonas aeruginosa, oprL, persekitaran akuakultur, ujian kepekaan terhadap antibiotik

1.0 Introduction

Fish culture industry is one of the most important industries as fish and fish products are the most important source of protein. It is estimated that more than 30% of fish for human consumption comes from aquaculture (Hastein *et al.*, 2006). Fishery products are also an important product of international trade and foreign exchange earner for a number of countries in the world (Yagoub, 2009). Consumption of raw fish or insufficiently processed fish and fish products may pose risks to human health as fish functions as carriers of several microbial and other health hazards and due to this, maintenance of quality of production and trade of fishery products are important.

Pseudomonas aeruginosa is a Gram-negative bacterium present in soil and aquaculture environment (Spiers *et al*., 2000). The bacterium is considered as a highly adaptable opportunistic pathogen highly capable of growing on a variety of substances and may alter its properties in accordance to changes in the environment (Lambert, 2002). The species is tolerant to a variety of physical conditions, needing only simple nutritional requirement and is able to develop resistance to a wide variety of antimicrobial agents, frequently including multiple classes of multiple classes of antimicrobial agent (Kiska, 1999). Due to this, the species is considered a problematic pathogens and its ability to develop mutational resistance made it hard to treat infections. Its progressive antimicrobial resistance to most classes of antibiotics has made treatment of infection caused by this bacterium particularly difficult, even with combination therapy (Sevillano *et al*., 2006). The bacterium has also been reported to infect aquaculture industry and may infect fishery products (Yagoub, 2009).

In this project, *Pseudomonas* spp. from cultured fish are isolated and detected by various means such as isolation via selective agar and biochemical tests. PCR detection is

used to isolate and identify *Pseudomonas aeruginosa*. The species are then be subjected to antibiotic susceptibility tests and their antibiotic resistance pattern are analyzed.

The objectives of the study are:

- to isolate *Pseudomonas* spp. from aquaculture industry
- to detect *Pseudomonas aeruginosa* using *opr*L gene
- to determine the antibiotic resistance pattern among the species

2.0 Literature Review

2.1 Fish Culture Industry

Fish and fish products are the most important source of protein and constitute an important part of international trade, currently worth more than US$ 50 billion, indicating increasing consumer interest in the commodity (Abraham, 2011). More than 80% of global aquaculture products are produced in freshwater (Hastein *et al*., 2006). Fish however, are highly perishable and are prone to variations in quality due to differences in species, environmental habitats and feeding habits. Additionally, cultured fish may also function as carriers of several microbial and health hazards. Fish and fish products have long been considered a vehicle of food-borne bacteria and parasitic infections leading to human illnesses (Hastein *et al.,* 2006). In aquaculture setting, Pseudomonads, *P. aeruginosa and P. flourescens* in particular, are considered pathogenic species causing disease when the host is subjected to some type of stress (Shiose *et al.*, 1974; Angelini and Seigneur, 1988; Fan, 2001). Infection of *Pseudomonas* spp. from fish samples is of high importance as the bacterium plays a considerable role as potential pathogenic bacteria (Yagoub, 2009). Also, the presence of bacterium may act as indicator for food quality as spoilage organism (Jeyasekaran *et al*., 2006; Koutsomanis and Nychas, 2000).

2.2 *Pseudomonas* Taxonomy and Characteristics

The term *Pseudomonas* means false single unit, with both the term originating from Greek words pseudo- (false) and monas (single unit). The species name is a Latin word, meaning copper rust. It is a member of the Gamma Proteobacteria class of Bacteria. The species are rod-shaped bacteria belonging to family Pseudomonadaceae. *Pseudomonas aeruginosa* is the type species of its group, which contains 12 other members. Pseudomonas is a rod bacterium measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm.

P. aeruginosa is an aerobic, motile, gram-negative, rod, and normally inhabits soil, marshes, costal marine habitats, plants and mammalian tissue (Stover *et al.,* 2000; Mathee *et al.,* 2008). Its metabolism is respiratory, never fermentative and the bacteria are free-living bacteria. Moreover, Pseudomonas bacterium occurs regularly on surfaces of plants and occasionally on animal surface as they are also normal skin flora. The bacteria can also thrive in hypoxic environment and even resist multiple antibiotics subjected to it. The bacterium are also considered opportunistic human and plant pathogens. The species are facultative anaerobes, able to asexually reproduce in condition of partial or total oxygen depletion.

Due to its simple nutrition requirements, it is often observed "growing in distilled water". This is evident that the bacterium has minimal nutritional needs. It also possesses metabolic versatility for which it can use more than seventy-five organic compounds for growth. The bacteria also are able to grow at temperature as high as 42ºC, while it's optimal growth temperature is at 37ºC. *P. aeruginosa* favours moist environment for growth, which is probably a reflection of its natural existence in soil and water.

P. aeruginosa are true pathogens of plants and are increasingly recognized as emerging opportunistic pathogens of clinical relevance. Its antibiotic resistance is increasing in clinical isolates. It is an opportunistic pathogen, and will exploit some break in the host defences to initiate an infection and capable of employing both tactical and defensive strategies during infections in hospitalized-patients.

P. aeruginosa can also produce severe infections in immunocompromised hosts (Quinn, 1998). The bacterium is also the predominant cause of chronic lung infection in cystic fibrosis patients (Frederiksen *et al.,* 1997).

2.3 Mechanism of Antimicrobial Resistance

P. aeruginosa is a highly adaptable organism which can grow on a wide variety of substrates and alter its properties in response to changes to the environment (Lambert, 2002). The species is inherent to many drug classes and is able to acquire resistance, through mutation, to all treatments of drugs. *P. aeruginosa* has an inducible AmpC β-lactamase and is inherently resistant to those of β-lactams, eg. cephalotin and ampicillin, that induce this enzyme and are hydrolyzed by it (Livermore, 1995). Due to the cell's impermeability, many antibiotics are excluded from the cell. However, the evidence of the cell's impermeability was very little and was proved to be difficult to reconcile when it was discovered that *P. aeruginosa* copiously manufactures OprF porin that forms large outer membrane pores (Benz and Hancock, 1981). Then in early 1990s, it was discovered that this "impermeability mediated resistance" was actually reflected efflux by MexAB-OprM. The MexAB-OprM system removes a wide range of antibiotic groups such as β-lactams, chloraphenicol, flouroquinolones, macrolides, novobiocin, sulfanomides, tetracycline, and trimethaphorim (Poole, 2001). Amphipathic permeants could disorganize cytoplasmic membranes, thus, the role of MexAB-OprM is to remove those permeants.

To treat *P. aeruginosa* infections, antibiotics used must cross the cell wall to reach their targets. Antibiotics of class aminoglycosides must inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones on the other hand must bind to a subunit of DNA gyrase, where the DNA gyrase functions to maintain ordered structure of chromosome inside the cells. Other than that, β-lactams inhibits peptidoglycan-assembling transpeptidases located outside of the cytoplasmic membrane. Finally, polymyxins bind to phospholipids in the cytoplasmic membrane to destroy barrier function. Innate resistance of the species is generally attributed to the low permeability of its cell wall. Antibiotics failed to accumulate within organism because of combination of restricted permeability of

the outer membrane and the efficient removal of antibiotic molecules that do penetrate by action of efflux pumps (Lambert, 2002).

2.4 Mutations and Resistance

Inherent defences of *P. aeruginosa* can be overcome by various antibiotics such as penicillins, cephalosporins, monobactams, aminoglycosides, fluoroquinolones, and polymyxins and are active against most isolates (Livermore, 2002). However, all the antibiotics are prone to being compromised by mutational resistance. Due to poorer inherent susceptibility of *P. aeruginosa*, mutations to topoisomerase II and IV confer fluoroquinolone resistance more readily (Jalal and Wretlind, 1998). Susceptibility to penicillins and cephalosporins are reduced by derepression of chromosomal AmpC β-lactamase. Up-regulation of MexAB-OprM can compromise fluoroquinolones, penicillins, cephalosporins and meropenem and can also enhance resistance to many drugs that lack useful antipseudomonal activity. Moreover, the up-regulation of MexCD-OprJ and MexEF-OprN confers resistance to fluoroquinolones and β-lactams while up-regulation of MexXY-OprM affects aminoglycosides. Previous studies have shown that the most active agent to treat *P. aeruginosa* infection were amikacin and meropenem while highest rate of resistances were seen in the treatment agent of gentamicin and cefotaxime (Sevillano *et al*., 2006). Mutational resistance of *P. aeruginosa* can be summarized in Table 1.

Table 1: Mutational resistance in *P. aeruginosa*

Mechanism	Mutation site	Effect on strain, according to antipseudomonal drug								
		Fq	Carb-Tic	Pip-Azl	Czid-Atm	Cpm-Cpr	Imi	Mero	Agl	Pm
Reduced Affinity										
Of topoisomerase II	gyrA	r/R	-	-	-	-	-	-	-	-
Of toposiomerase IV	parC	r/R	-	-	-	-	-	-	-	-
Derepression of AmpC										
Partial	ampD	-	R	R	R	r	-	-	-	-
Total	ampD+other	-	R	R	R	R	-	-	-	-
Up-regulation										
Of MexAB-OprM	nalB at mexR;	R/R	R	r/R	r/R	r/R	-	r	-	-
Of MexCD-OprJ	nalC at other	r/R	r/R	r/R	r/R	R	-	r	-	-
Of MexEF-OprN	nfxB	r/R	r/R	r/R	r/R	r/R	r	r	-	-
Of MexXY-OprM	nfxC at mexT	rR	r/R	r/R	r/R	r/R	-	r	r/R	-
Reduced aminoglycoside transport		-	-	-	-	-	-	-	r/R	-
Loss of OprD	oprD;nfxC at mexT	-	-	-	-	-	R	r	-	-
Membrane changes		-	-	-	-	-	-	-	-	R

Note: Agl, aminoglycosides; Azl, azlocillin; Atm, aztreonam; Carb, carbenicillin; Czid, ceftazidime; Cpm, cefepime; Cpt, cefpirome; Fq, fluoroquinone; Imi, imipinem; Mero, meropenem; Pip, piperacillin; Pm, polymyxin; r, reduced susceptibility; R, frank resistance, which may vary in its distinction from "r", according to the breakpoints adopted; Tic, ticarcillin.

2.5 PCR Analysis

Previous studies done by Xu *et al.*, (2004) suggested that there are two primers for detection of *P. aeruginosa.* The molecular detection of the gene loci targeted, namely exotoxin A (ETA) gene and outer membrane lipoprotein (*opr*L) gene. The detection of *opr*L locus is more sensitive than the ETA locus (Xu *et al*., 2004). ETA is produced to inhibit eukaryotic protein biosynthesis at the level of polypeptide chain elongation factor (Khan and Cerniglia, 1994). *opr*L is an outer membrane lipoprotein which has been implicated in efflux systems, as well as affecting cell permeability (De Vos *et al.*, 1997).

3.0 Materials and Methods

3.1 Preparation of Isolates and Samples

3.1.1 Revival of Isolates

Eight isolates were taken from Microbiology Collection, Microbiology Laboratory, Department of Molecular Biology, Faculty of Resource Science and Technology, UNIMAS. The samples were isolated by previous undergraduate students from cultured fish.

The isolates were inoculated on a selective media for *Pseudomonas* spp. which is the Pseudomonas Agar Base (PAB) agar. The agar is strongly selective for *Pseudomonas* spp. and greatly suppressing *Klebsiella, Proteus, and Providencia* spp. The agar will be incubated at 37°C for 48 hours. The bacterial colony on each agar was inoculated onto a slant agar and the slant agar was stored at 4ºC.

3.1.2 Sample Processing

Samples of shrimps were taken from Sampadi. Samplings were done twice and were transported immediately into the laboratory. Upon arrival in Microbiology Laboratory, Department of Molecular Biology, Faculty of Resource Science and Technology, UNIMAS, the samples were processed immediately. All samples were serially diluted in sterile test tube containing 8% saline solution. 100µl of 10^{-1}, 10^{-2}, 10^{-3} dilutions were spread onto PAB agar in duplicates. One microliter of the samples was diluted with saline before they were spread on the PAB agar. The plates were then incubated at 25-30ºC for 24 hours.

3.1.3 Isolation of *Pseudomonas* spp. from samples

Colonies which showed yellowish straw colour on the PAB agar were presumed as *Pseudomonas* spp. and 3 randomly selected colonies with appropriate morphological characteristics were subcultured on new PAB agar. The agars were incubated at 25-30ºC for 24 hours to obtain pure culture. The pure cultures were transferred into slant agar and stored at 4ºC for further usage.

3.2 Conformation

3.2.1 Gram-Staining

To confirm that the isolates are *Pseudomonas* spp., several more tests were conducted. Gram-staining is a method done for differentiation of bacterial into two classes, Gram-negative and Gram-positive. Isolates in the slant agar were restreaked onto a new PAB agar. The agar was incubated for 48 hours and the single colony on the agar was transferred and smeared onto a glass slides. The bacteria were then heat-fixed by heating briefly using a Bunsen burner. Then primary stain of crystal violet was applied to the slides. A trapping agent was added, usually iodine is used. Then, rapid decolorization was done using alcohol. Counterstaining procedure was done using safranin. The slides were observed under microscope for its morphology.

3.2.2 Sulphide-Indole-Motility (SIM test).

The isolates were inoculated into a semisolid agar (SIM medium) to test for hydrogen sulphide, indole and motility of the organism. The inoculation was done using the stab method and the media was incubated for 24 hours at 35ºC. H_2S and motility tests were interpreted first before addition of Kovak's reagent for indole test. Turbidity and fuzzy growth along the line of inoculation indicates motility. Negative result for motility

was represented by growth along the stab line. H_2S production was represented by the blackening of the medium, while absence of the black color indicated negative results of H_2S production. A few drops of Kovak's reagents were added to test for the production of indole. Color changes were observed as pink indicates positive results while yellow color indicates negative results for indole test.

3.2.3 Citrate Test

Citrate test was done to screen bacterial isolates for their ability to utilize citrate as their carbon and energy source. Single colony was inoculated on the slant. Since citrate utilization requires oxygen, the screw cap was placed loosely on the tube. Positive result will yield blue color while negative result will maintain the green color of the agar.

3.3 Crude DNA preparation by Boiled Cell Method

Boiled cell method was used for the extraction of DNA as described by Bilung *et al.*, (2005). Briefly, a colony was picked from the nutrient agar and inoculated into 5ml of LB broth. The colony was grown for 24 hours with shaking at 120 rpm at 37ºC. From the LB broth, 1.5 ml was transferred to a centrifuge tube and was spun at 10,000 rpm for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 1 ml sterile distilled water and was boiled for 10 minutes. The tube was placed immediately on ice for 10 minutes. Afterwards, the tube was spun again for 5 minutes at 10,000 rpm. The supernatant was transferred into a new tube.

3.4 PCR Analysis

PCR was carried out for the detection of *P. aeruginosa* as described by Xu *et al.,* (2004) using sequence-specific target, the outer membrane protein (*opr*L) gene locus. The *opr*L f(5'-ATG GAA ATG CTG AAA TTC GGC-3') and *opr*L r(5'-CTT CTT CAG CTC GAC GCG ACG-3') were used for the analysis.

PCR was carried out in a volume of 25 µl containing 3µl of *P. aeruginosa* DNA template, 2 µl (50mM) $MgCl_2$, 1 µl (5mM) each of primer, 1 µl deoxynucleoside triphosphate mix, 0.5 µl Taq DNA polymerase and 13 µl dH_2O.

PCR reactions were performed under the conditions as shown in Table 2:

Table 2: Cycle profiles of PCR

Condition	**Temperature (ºC)**	**Time (minutes)**	**Cycles**
Initial denaturation	96	5	1
Denaturation	96	1	40
Annealing	55	1	40
Extension	72	1	40
Final extension	72	10	1

3.5 Agarose Gel preparation and Agarose Gel Electrophoresis

A 1% of the agarose gel was prepared by heating 0.5g of agarose powder in 50ml 1X TBE buffer in Erlenmeyer flask with 5µl Ethidium Bromide in a microwave oven for 40 seconds. The solution was allowed to cooled and poured into a gel mold with combs positioned in it. The gel was allowed to solidify and the comb was removed. The tank was filled with 1X TBE buffer until the gel was completely covered by the buffer.

Five microlitres of DNA template was mixed with 1µl of gel loading dye. Ten microlitres of PCR products were separated on a 1% agarose gel. The DNA ladder and the samples were loaded into appropriate well. Gel electrophoresis was performed at 85 volts for 45 minutes. The gel was visualized on the UV transilluminator.

3.6 Antibiotic Susceptibility Test

Antibiotic susceptibility test was performed by disc diffusion method on Mueller Hinton agar based on Bauer *et al.,* 1966. Isolates were tested using eight different antibiotics, which were chloramphenicol (30µg), nalidixic acid (30µg), nitrofurantoin (300µg), gentamycin (30µg), ampicillin (10µg), erythromycin (15µg), norflaxin (10µg), and carbenicillin (100µg).

Single colony of *P. aeruginosa* were picked up with a loop and transferred into the LB broth. The broth cultures were incubated at 35ºC for 24 hours. The inoculum was the inoculated on the Mueller Hinton agar surface using a sterile cotton swab. The procedure was repeated by streaking two more times while rotating the plates at 60º each times to ensure even distribution of inoculums.

The antimicrobial discs were dispensed on the inoculated agar plate using disc dispenser and the plates were inverted and placed in an incubator at 35ºC for 16 to 18 hours. The diameters of the zone of inhibition were measured. Zones were measured to the nearest whole millimeter using a ruler.

4.0 Results

4.1 Biochemical tests

Fish samples were analyzed for the presence of *Pseudomonas* spp. by plating on PAB agar. Forty six samples were suspected of *Pseudomonas* spp. and shows showing yellow colour on PAB agar. Figure 1 shows colonies of Pseudomonas spp. on Pseudomonas Agar Base. Out of 46 isolates, 36 isolates (78.26%) show positive morphology and Gram-stain characteristic of *Pseudomonas* spp. After performing SIM test, only 29 (63.04%) showed positive result. After performing Citrate test, 24 isolates (52.17%) showed positive results for *Pseudomonas* spp. Total *Pseudomonas* spp. isolates are shown in Table 3. The microscopic analyses and results of biochemical tests can be referred to Table 5 (Appendix 1, Results of Microscopic Analysis and Biochemical Tests of 46 Isolates).

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Table 3 shows the isolates and their sources used in this study. Five isolates were taken from the Microbiology Collection while sixteen isolates were isolated from aquaculture environment from Sampadi.

Table 3: Lists of *Pseudomonas* spp. Isolates

Isolates	**Source**
2F9	Microbiology Collection
3F1/1C	
3F1/2C	
4F1/1A	
4F1/1B	
5F1/1A	
6Fld	
Fla	
SP-P7	Sampadi
SP-P9	
SP-P17	
SP-P23	
SP-P26	
SP-2P1	
SP-2P2	
SP-2P3	
SP-2P4	
SP-2P6	
SP-2P7	
SP-2P9	
SP-2P10	
SP-2P12	
SP-2P14	
SP-2P15	