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European Journal of Experimental Biology, 2013, 3(6):148-152

ISSN: 2248 –9215
CODEN (USA): EJEBAU

# Detection of *opr*L gene and antibiotic resistance of *Pseudomonas aeruginosa* from aquaculture environment

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**ABSTRACT**

*Pseudomonas aeruginosa is a gram-negative rod shape bacterium belonging to the family Pseudomonadaceae. The species is highly adaptable opportunistic pathogen, capable of surviving in a variety of environment, including aquaculture environment. Antibiotics are used in the aquaculture environment, and their improper usage poses a risk of potential transfer of resistance from aquaculture bacteria to human and animal pathogens. This study was conducted to isolate P. aeruginosa from fish, prawn and water samples, followed by PCR detection of oprL gene locus. The antibiotic resistance pattern of the isolates was also determined. Based on the results from PCR analysis performed, 13 isolates of P. aeruginosawere isolated. All of the isolates tested were resistance to at least one antibiotic. Highest level of resistance was observed against ampicillin and erythromycin while the lowest was observed against gentamicin, norfloxacin and nalidixic acid. This study suggested that the presence of the bacteria in the aquaculture environment may pose the risk of antibiotic resistance to those who are exposed to the aquaculture environment.Based on the results of this study, it can be said that gentamicin, norflaxin and nalidixic acid can be effective agents for the treatment of P. aeruginosa.*

**Keywords**: *Pseudomonas aeruginosa*, oprL, aquaculture environment, antibiotic resistance

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## INTRODUCTION

Fish culture industry is one of the most important industries as fish and fishproducts are the most important source of protein. It is estimated that more than 30% of fish for human consumption comes from aquaculture (Hasteine*t al*., 2006). Fishery products are also an important product of international trade and foreign exchange earnerfor 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.

Bacterial infections are major threats in both wild and culture fishes. *Pseudomonas aeruginosa* is a Gram-negative bacterium present in soil and aquaculture environment (Spiers*et al*., 2000) and is among major pathogens of fish responsible for heavy mortalities and spoilage in fish and fish product. Antibiotics are the most common method nowadays to treat bacterial infection inaquaculture industries. However, emergence of antibiotic resistance is the main concernamong researchers as bacterial resistance towards antibiotics may affect the consumers

*P. aeruginosa* is able to develop resistance to a wide variety of antimicrobial agents, frequently including 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. *P. aeruginosa*is characterized by the biofilm mode of growth, which protects the bacteria against antibiotics and the innate and adoptive defense mechanism (Anwar *et al*., 1992; Fux*et al*., 2005; Høiby, 2002a). A major reason for its prominence as a pathogen is its high intrinsic resistance to antibiotics, such that even for the most recent antibiotics, a modest change in susceptibility can thwart their effectiveness (Hancock and Speert, 2000). Development ofresistance to antibiotics makes infection difficult to treat efficiently (Høiby, 2002b).The aim of this study was to isolate and detect *P. aeruginosa* using *opr*L gene and to determine the antibiotic resistance pattern among the species at Sampadi River.

## MATERIALS AND METHODS

### *Sample*

The study was based on forty-six specimens screening for*P. aeruginosa* taken from , prawn, fish and water sampled from Sampadi River. Experiment was conducted at Microbiology Laboratory, Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak (UNIMAS). Samplings were done twice and were transported immediately to the laboratory in ice. All samples wereserially diluted in sterile test tube containing 8% saline solution. 100µl of 10dilutions 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.

### *Isolation and identification of Pseudomonas aeruginosa*

The isolation and identification of *P. aeruginosa*as outlined by Valentina and Lalitha (1989) was conducted. Identification analysis involves; Gram staining, Sulphide-indole-motility tests (SIM), Citrate test, growth on agar test were carried out.

### *DNA extraction*

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.

### 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 oprLf(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 distill water.PCR reactions were performed under the conditions as shown in Table 1.

**Table 1: 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 |

### Antibiotic susceptibility test

Antibiotic susceptibility test was performed by disc diffusion method on MuellerHinton agar based on Bauer *et al.*, (1966), using commercially available antibiotic disc *E. coli* ATCC 25922 as reference strain with standard *P. aeruginosa* obtained from Microbiology Laboratory, UNIMAS used as a positive control. Isolates were tested using eight differentantibiotics, which were chloramphenicol (30µg), nalidixic acid (30µg), nitrofurantoin(300µg),

gentamycin (30µg), ampicillin (10µg), erythromycin (15µg), norfloxacin (10µg), and carbenicillin (100µg).The diameters of the zone of inhibition were measured to the nearest whole millimeter using a ruler.

## RESULTS AND DISCUSSION

***Isolation rate***

Fourty six isolates suspected of *Pseudomonas* spp. were sampled from water, fish and prawn from Sampadi River. Samples were analyzed for the presence of *Pseudomonas* spp. by plating on PABagar. Figure 1 shows colonies of Pseudomonas spp. on Pseudomonas Agar Base.

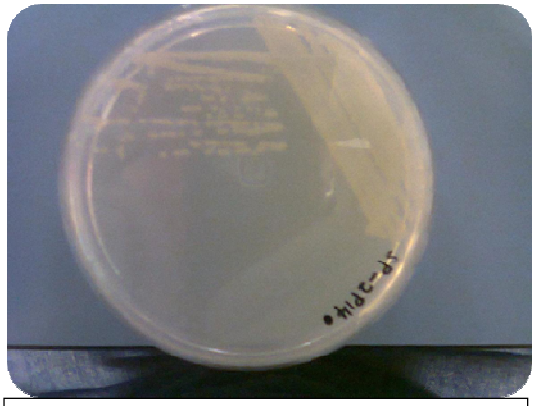

**Figure 1: Colonies of *P. aeruginosa* growing on PAB**

Out of the 46 isolates 36 isolates (78.26%) showed positive morphology and gram-stain characteristic of *Pseudomonas* spp. After performing SIM test, only 29 (63.04%) expressed positive result while, 24 isolates (52.17%) showed positive results under citrate test.

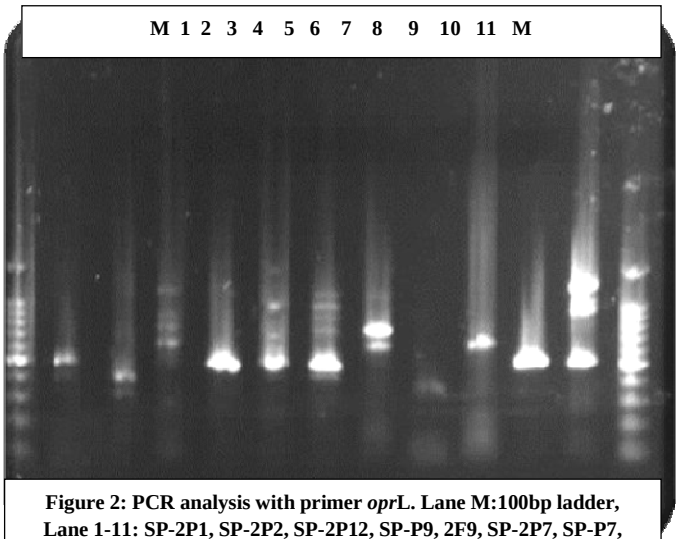


**Figure 2: PCR analysis with primer *opr*L. Lane M:100bp ladder, Lane 1-11: SP-2P1, SP-2P2, SP-2P12, SP-P9, 2F9, SP-2P7, SP-P7, 3F1/2C, SP-2P3, SP-P23, SP-P17**

***PCR analysis***

The PCR results showed that the primer used successfully detected the *opr*L gene locus for most of the isolates tested. The results of the PCR are shown in Figure 2. The size of amplicon for the gene of interest was 504bp.

Based on the results from PCR analysis performed, 13 isolates of *P. aeruginosa*were isolated and examined for their antibiotic susceptibility against eight different antibiotics, chloramphenicol (30µg), nalidixic acid (30µg), nitrofurantoin (300µg), gentamycin (30µg), ampicillin (10µg), erythromycin (15µg), norfloxacin (10µg) and carbenicillin (100µg).

***Antibiotic susceptibility test and pattern***

All of the 13 isolates tested were resistant to ampicillin and erythromycin (100%) (Table 2). 12 isolates (92.30%) were resistant to nitrofurantoin, 10 isolates (76.92%) resistant to carbenicillin and 3 isolates (23.08%) were detected to be resistant to gentamicin.

**Table 2: Antibiotic susceptibility test**

| Isolates | Antibiotic Resistance* | MAR Index# | Patterns |
|---|---|---|---|
| **2F9** | Amp,E,F,Car | 0.5 | 1 |
| **5F1/1A** | Amp,E,F,Car | 0.5 | 1 |
| **SP-P9** | Amp,E,F,Car | 0.5 | 1 |
| **SP-2P1** | Amp, E,F,C,Car | 0.6 | 2 |
| **SP-P17** | Amp,E,F,C | 0.5 | 3 |
| **Fla** | Amp,E,F,Car | 0.5 | 1 |
| **6Fld** | Amp,E | 0.2 | 4 |
| **SP-2P9** | Amp,E,F,Car | 0.5 | 1 |
| **SP-P23** | Amp,E,F,Car | 0.5 | 1 |
| **SP-2P7** | Amp,E,F,Car | 0.5 | 1 |
| **4F1/1B** | Amp,E,F,Car | 0.5 | 1 |
| **SP-2P15** | Amp,E,F,C | 0.5 | 3 |
| **SP-P26** | Amp,E,F,Car | 0.5 | 1 |

*Note: * Tested for chloramphenicol (C), nalidixic acid (NA), nitrofurantoin (F), gentamicin (CN), ampicillin (Amp), erythromycin (E), norflaxin (Nor), and carbenicillin (Car).*

# MAR index: $\frac{Numbertibioticesistant}{Numberofantibioticsused}$

Multiple antibiotic resistant (MAR) index of the isolates ranges from 0.20 to 0.60 with 4 patterns of resistance among the 13 isolates. Results from this study showed that there are highest resistances towards two types of antibiotics, namely ampicillin and erythromycin, with all isolates showing resistance towards it. All isolates also showed that there are least resistance towards three antibiotics, namely gentamicin, norflaxin and nalidixic acid. The findings are consistent with previous study, whereGentamicin is considered by some authors suitable as one of aminoglycoside antibiotics for drug resistant *P.aeruginosa*. (Kettner *et al., 1*995; Jones *et al.,* 1997).The results of antibiotic susceptibility test showed four different antibiotic resistant pattern among the thirteen isolates. A clearer distinction of these isolates can be done via antibiotyping on the basis of their susceptibility towards chloramphenicol, nalidixic acid, nitrofurantoin, gentamicin, ampicillin, erythromycin, norflaxin, and carbenicillin. However, antibiotyping provide limited degree of discrimination and this may be due to small number of antibiotics used in this study and that antibiotyping is based on phenotyping and not genotyping (Radu *et al.*, 2000). According to previous studies, antibiotic resistance pattern of *P. aeruginosa* isolates also varied with geographical location (Tripathi *et al.*, 2011). It has also been shown that *P. aeruginosa* has the capacity to develop resistance rapidly during the course of antimicrobial therapy by several mechanisms (Fish *et al.*, 1995; Hancock, 1998).

## CONCLUSION

Based on the results of this study, it could be concluded that PCR detection was successfully conducted using *opr*L primer and thirteen isolates were successfully detected to be *P. aeruginosa*. The isolates were analyzed for resistant towards 8 different types of antibiotics, chloramphenicol (30µg), nalidixic acid (30µg), nitrofurantoin (300µg), gentamycin (30µg), ampicillin (10µg), erythromycin (15µg), norflaxin (10µg), and carbenicillin (100µg). The results

showed 4 different patterns of antibiotics resistance. Gentamicin, norfloxacin and nalidixic acid can be effective agents for the treatment of *P. aeruginosa* in aquaculture

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