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Screening and characterisation of two strains of *Pseudomonas aeruginosa* from aquaculture and water environment

Seng Chiew Toh[1], Samuel Lihan[1*], Khar Mun Soh[1], Natalia Uyub[1], Lay Ching Chai[2], Moritz Müller[3]

[1]Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.
[2]Institute of Biological Sciences, Faculty of Science, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.
[3]Faculty of Engineering, Computing and Science; Swinburne University of Technology, Jalan Simpang Tiga, 93300 Kuching, Sarawak, Malaysia.
Email: lsamuel@unimas.my

ABSTRACT

Aims: *Pseudomonas* has been associated with diseases occurring in people with weakened or compromised immune system after exposure to contaminated water. The diseases are commonly treated with antibiotics. However, the bacteria had developed resistances to commonly used antibiotics making treatment a difficult task. Therefore, the continuous surveillance of susceptibility of *Pseudomonas* especially for the human pathogen *P. aeruginosa* to commonly clinical and aquaculture farming used antibiotics is important to ensure that serious infections remain susceptible to those antibiotics.
Methodology and results: In this study, the bacteria were screened from water, sediment and fish from rivers and aquaculture farms around Kuching, Sarawak. A total number of 38 presumptive *P. aeruginosa* were isolated using CHROMagar™ *Pseudomonas* and subjected to a series of biochemical tests. Out of all the isolates tested, only two isolates designated as AS-R10(S) and BK2-OLT2(S) fulfilled the biochemical characteristics of *P. aeruginosa*. 16S rRNA gene sequencing further confirmed these two isolates as *P. aeruginosa* based on their 100% similarity with *P. aeruginosa* strain GD1 and *P. aeruginosa* strain PA1201 in NCBI database. These two isolates were tested for their susceptibilities against nine common antibiotics used in both clinical and aquaculture farming nowadays: imipenem, piperacillin, meropenem, amikacin, gentamicin, ciprofloxacin, ceftazidime, tobramycin and norfloxacin according to CLSI standard using disk diffusion method.
Conclusion, significance and impact of study: The two isolates exhibited total susceptibility to all the antibiotics analysed, suggesting the effectiveness of the antimicrobial agents towards *P. aeruginosa* isolated from aquaculture and water environment in the study area.

Keywords: P. aeruginosa, antibiotic susceptibilities, 16S rRNA gene sequencing, aquaculture

INTRODUCTION

The aquaculture industry has been started in Malaysia since 1920 and nowadays, this sector has developed into one of the most important economic activities which contributed about 1.73% to Gross Domestic Product (GDP) of the country (Hashim, 2008). Despite the contribution of aquaculture industry in international trade and foreign exchange, it also functions to provide a significant source of animal protein (Hashim, 2008). However, the increase in demand for aquaculture related products and the failure of captured fisheries to produce sufficient fish products thus make aquaculture becoming a major focus industry nowadays (Ling *et al.*, 2011). The use of antibiotics in the aquaculture farming in Sarawak may pose potential risk that susceptibility to antimicrobial agents will decrease in the water as well as in cultured organisms and surrounding environments including rivers through the waste disposal from the aquaculture activities.

Pseudomonas aeruginosa is a Gram-negative rod-shaped and non-fastidious bacillus that can cause disease in animal and human. Its simple nutritional requirement contributes to its high adaptability and survival in various environments including soil and water (Sylvia *et al*., 1974, Mena and Gerba, 2009). It is an opportunistic human pathogen which has been associated with several diseases such as severe pneumonia with a high fatality rate in people with weakened immune status; in patients underlying with cystic fibrosis, it triggers persistent inflammation that leads to the problem of the respiratory system (Iglewski, 1996; Pier and Ramphal, 2004). Besides, the bacterium is highly resistant to many different drugs making the

*Corresponding author

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treatment of the diseases a more difficult task (Gales *et al.*, 2001; Lister *et al.,* 2009; Abdullahi *et al.*, 2013).

Pseudomonas aeruginosa that produced Metallo-Beta-Lactamase (MBL) was first reported in 1991 in Japan (Manoharan *et al.,* 2010). The resistance pattern of *P. aeruginosa* against β-lactams arose due to several developed mechanisms such as AmpC β-lactamase synthesis, extended-spectrum β-lactamases, a hindrance to enter the outer membrane, and efflux mechanisms (Manoharan *et al.*, 2010). Other mechanisms of resistant in *P. aeruginosa* from clinical and environment have been reported by researchers elsewhere (Philip *et al*., 2009; Abdullahi *et al.*, 2013). Besides, the ability of the bacterium to develop biofilm has also contributed to the antibiotic resistance of *P. aeruginosa* (Gales *et al.*, 2001). These resistant bacteria constitute a direct threat to those associated with the farms and its surrounding environment through potential transfer of resistance to human and animal pathogens. The presence of antibiotic resistant bacteria in aquaculture product will compromise the quality of its export markets. Therefore, surveillance on the level of susceptibilities of bacteria towards certain antibiotics commonly used in the aquaculture and its surrounding environment is important.

The objective of this study was to obtain a preliminary indication of the extent of antibiotic susceptibilities of *P. aeruginosa* from aquaculture farms around Kuching, Sarawak and their surrounding environments.

MATERIALS AND METHODS

Sample collection

Surface water were collected from rivers from selected locations around Kuching area such as at Permai river, Sungai Sarawak Kiri (Bintawa industrial zone and Muara Tuang area) and Sungai Sarawak Kanan (Siniawan and Waterfront area) using sterile falcon tubes. Surface water, sediment and fish (catfish, *Clarias gariepinus*) were collected from four aquaculture farms located at Sampadi, Bako and Kampung Haji Baki within Lundu and Kuching area in Sarawak, Malaysia. All samples were collected using sterile tubes or bags and then the samples were transported to the university laboratory in icebox-containing ice for analysis. Table 1 shows the details of the sampling locations, sample types and number of samples collected from each location.

Isolation of *P. aeruginosa*

Water and sediment samples collected were homogenize and 50 µL of each water and sediment samples were directly spread plated onto CHROMagar™ *Pseudomonas* to isolate the *P. aeruginosa*. Meanwhile, the skin surface, mouth, intestine and anus of the catfish were swabbed using sterile cotton bud and spread onto CHROMagar™ *Pseudomonas*. The samples were also spread plated onto tryptic soy agar (TSA) to enumerate bacteria colonies formed and expressed in colony forming units (CFU). All cultures were incubated in an incubator set at 37 °C for duration of 24 h. Colonies grown on the CHROMagar™ were randomly selected and sub-cultured onto TSA agar slant for further analysis. A total of 38 bacteria were isolated and further characterized through series of conventional biochemical tests.

DNA extraction using boiled cell method

Genomic DNA was extracted by boiling method as reported by Freschi *et al.* (2005) with slight modification. Briefly, 2.0 mL of culture was centrifuged at 10,000 rpm for 5 min. The supernatant was decanted, and another 2.0 mL of bacterial culture was added to the same tube for recollection. The tubes were centrifuged again at 10,000 rpm for 5 min. Supernatant was decanted and the pellet collected was re-suspended with 500 µL of sterile distilled water. The pellet was homogenized by vortex until no clump was observed and the cell was boiled at 100 °C for 10 min followed by immediate cooling in ice for 5 min. Lastly, the mixture was centrifuged at 10,000 rpm for 10 min and the supernatant was transferred into new tubes and stored at 4 °C for PCR analysis.

PCR amplification

Ribosomal DNA was amplified in PCR as described by Kathleen *et al.* (2014) using primer sets 27F and 519R primers in 50 µL of PCR reaction master mix containing 10 µL of 5× Green GoTaq ® Flexi buffer, 6 µL of 25mM $MgCl_2$, 3 µL of 10mM dNTPs, 8 µL of sterile distilled H_2O, 20 µL of DNA sample, 1 µL of GoTaq ® Flexi DNA polymerase, 1 µL of each forward primer and reverse primer. The temperature cycling conditions consisted of an initial denaturation at 95 °C for 10 min, 26 amplification cycles of 94 °C for 30 sec, 55 °C for 1 min, 72 °C for 1 min 30 sec and a final polymerization step of 72 °C for 10 min. PCR product was run on agarose gel (1%, w/v) electrophoresis and further staining with ethidium bromide. The agarose gel was electrophoresed at 90 V with 200 mA for 30 min before it was visualised under UV transilluminator.

16S rRNA gene sequencing

DNA fragments were excised from the agarose gel on the UV transilluminator by using a clean and sterile scalpel. DNA was purified using Qiagen QIAquick ® Gel Extraction Kit. Purified product was sent to First Base Malaysia for sequencing procedure and sequences obtained were analysed with BLAST system. The similarities of the nucleotide sequence were determined with NCBI (National Center for Biotechnology Information) databases.

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Table 1: Location, sample type and number of samples collected from aquaculture farms and rivers.

Environment	Location	Sample type	Number of samples collected	Number of strains isolated and fulfil characteristics as *P. aeruginosa*
Aquaculture	Sampadi, Sarawak	Surface water	10	1 positive, 3 negative
		Sediment	8	1 negative
	Bako farm 1, Sarawak	Surface water	9	7 negative
		Sediment	6	1 negative
	Bako farm 2, Sarawak	Surface water	6	1 positive, 1 negative
		Sediment	5	2 negative
	Haji Baki Village, Sarawak	Surface water	13	7 negative
		Fish	6	5 negative
Water	Bintawa, Sg. Sarawak Kiri	Surface water	2	2 negative
	Muara Tuang, Sg. Sarawak Kiri	Surface water	2	2 negative
	Waterfront, Sg. Sarawak Kanan	Surface water	2	1 negative
	Siniawang, Sg. Sarawak Kanan	Surface water	2	2 negative
	Permai river, Sarawak	Surface water	4	2 negative
Total			74	38 isolates, 2 positive, 36 negative

Note: Sg. is short form for sungai (river)

Antibiotic susceptibility testing

The susceptibility of the two strains towards commonly used antibiotics was carried out following the disc diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI, 2012) on Mueller Hinton agar (MHA). The discs impregnated with different concentration of antibiotics (Oxoid, England) were used in this testing and *Escherichia coli* ATCC 25922 was used as the positive control. The antibiotics used were piperacillin (75 µg), ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), ciprofloxacin (5 µg), tobramycin (10 µg), amikacin (30 µg) and norfloxacin (10 µg). Briefly, a sterile cotton swab was dipped into the bacteria suspension and used to inoculate the surface of MHA plate evenly. The agar plates were allowed to dry before the antibiotic discs were placed on the surface of the MHA plate. The plates were incubated at 30 °C for 24 h. The diameter of inhibition zone was interpreted as susceptible (S) or resistance (R) based on the Clinical and Laboratory Standards Institute (CLSI, 2012).

RESULTS AND DISCUSSION

A total number of 38 water, 19 sediments and 6 fish samples were collected from the aquaculture farms in Lundu and Kuching area in Sarawak. Meanwhile, 12 water samples were collected from rivers around Kuching, Sarawak. In this study, fish and sediment were not collected from the river because there was no intention to compare samples from aquaculture and the river. The location of sampling and type of samples collected are as shown in Table 1. Two of the ponds located at Sampadi, Lundu and Bako, Kuching sampled in this study are covered with net to prevent feeding of cultured prawns by birds. In addition, individuals enter the farms must wear gloves, boots, and take the vehicle provided to ensure proper disinfection protocol. Before entering ponds, gloves are disinfected with 70% ethanol whereas boots are disinfected by dipping into potassium permanganate to reduce the number of bacteria and pathogens (Yanong and Erlacher-Reid, 2012). The transport provided by the farm also undergoes disinfection process by driving through an area with disinfectant located at the entrance and exit of the farm.

Table 2: The susceptibility of strains of *P. aeruginosa* AS-R10(S) towards different antimicrobial agents.

Antimicrobial agent	Disk content (µg)	Diameter of inhibition zone (to the nearest whole mm)	Zone diameter interpretive criteria
Piperacillin	75	22	Susceptible
Ceftazidime	30	18	Susceptible
Imipenem	10	30	Susceptible
Meropenem	10	26	Susceptible
Gentamicin	10	24	Susceptible
Tobramycin	10	21	Susceptible
Amikacin	30	25	Susceptible
Ciprofloxacin	5	24	Susceptible
Norfloxacin	10	24	Susceptible

Water, fish and sediment samples were processed and then plated directly onto CHROMagar™ *Pseudomonas* for the isolation of *P. aeruginosa*. From the total number of 76 samples collected, 38 presumptive isolates were successfully isolated. Although CHROMagar™ *Pseudomonas* agar was used for

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preliminary detection of *Pseudomonas* spp. meanwhile inhibit other microorganisms such as *Staphylococcus* spp. and *Proteus* spp., some multi-resistant Gram-negative bacteria may turned out as false positive result. Hence, a series of biochemical confirmation tests and molecular identification was conducted to further identify the *Pseudomonas* spp. (Rambach, 2013). The thirty-eight isolates of presumptive *P. aeruginosa* were subjected to Gram staining and other biochemical tests such as triple sugar iron agar test, oxidase test, citrate utilization test, catalase test, starch hydrolysis and nitrate reduction test. Although *P. aeruginosa* is a rod-shaped bacterium, this bacterium can also be observed in bacillus-shaped (Koneman *et al.,* 1997). This is because the bacteria may change its morphology due to the selective pressure from environmental clues and physiological mechanisms. As a result, the changing in the length of these isolates can be devoted by forces like nutrient, attachment of surface, and passive dispersal (Young, 2007). However, only two isolates, AS-R10(S) and BK2-OLT(S) were able to fulfil the characteristics of *Pseudomonas* spp. during biochemical test. The isolates that did not fulfil the characteristics of *Pseudomonas* spp. after the biochemical test were discarded.

Table 3: The susceptibility of strains of *P. aeruginosa* BK2-OLT(S) towards different antimicrobial agents.

Antimicrobial agent	Disk content (µg)	Diameter of inhibition zone (to the nearest whole mm)	Zone diameter interpretive criteria
Piperacillin	75	23	Susceptible
Ceftazidime	30	20	Susceptible
Imipenem	10	25	Susceptible
Meropenem	10	31	Susceptible
Gentamicin	10	17	Susceptible
Tobramycin	10	17	Susceptible
Amikacin	30	21	Susceptible
Ciprofloxacin	5	33	Susceptible
Norfloxacin	10	29	Susceptible

Two isolates; AS-R10(S) isolated from an aquaculture farm at Sampadi, Lundu and BK2-OLT2(S) isolated from an aquaculture farm at Bako, Kuching were selected for further identification through molecular technique using 16S rRNA PCR and DNA sequencing. PCR was carried out with the parameters which had been optimized for 16S rRNA sequencing using primers 27F and 519R. These primers which hybridize in opposite orientation of highly conserved region of 16S rRNA gene of virtually all organisms in the domain bacteria (Sipos *et al.*, 2007; Winsley *et al.*, 2012) which involved large gene size of 1542 base pair that ease informatics purpose (Clarridge, 2004; Janda and Abbott, 2007; Kathleen *et al.*, 2014). The gel picture of the agarose gel electrophoresis viewed by using UV transilluminator is shown in Figure 1. The extracted DNAs were sent to First BASE Laboratories Sdn. Bhd. Malaysia for sequencing. After the DNA sequencing, the DNA sequences were analysed using Basic Local Alignment Search Tool (BLAST) service provided by NCBI website. The DNA sequences of *P. aeruginosa* from database showed high similarity (100%) to the DNA sequence of the two isolated bacteria, confirming those two isolates as *P. aeruginosa.*

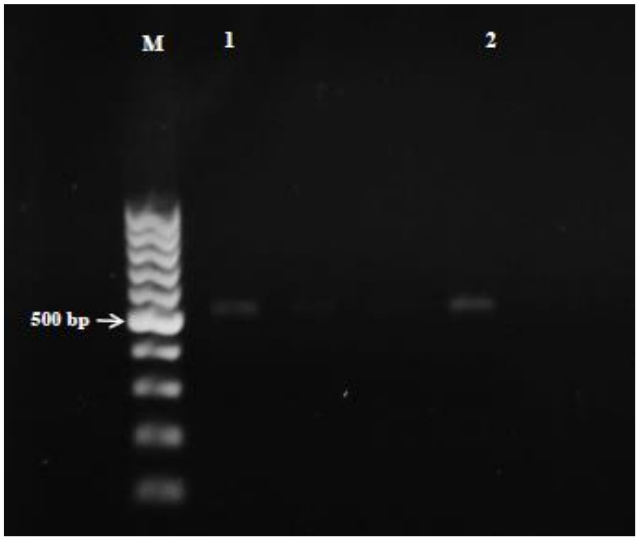

Figure 1: Agarose gel (1%) of purified DNA of *P. aeruginosa*. The targeted sequence for 16S rDNA conserved region is located approximately at 500 bp. Lane M: 100 bp DNA marker (Promega, USA); lane 1: AS-R10(S); lane 2: BK2-OLT(S).

Antibiotic susceptibility test was conducted to determine the susceptibility of the two *P. aeruginosa* isolated from aquaculture farms. The antibiotics were selected based on the antibiotics commonly used in clinical and aquaculture. The testing was conducted according to CLSI standard. The result of the susceptibility testing for strains AS-R10(S) and BK2-OLT(S) are shown in Table 2 and Table 3, respectively.

There are studies reported that at least 10% of the global isolates of *P. aeruginosa* were resistant to commonly used antimicrobial drugs and hence the emergence of multi-drug resistance should be continuously surveyed (Lister *et al.*, 2009; Nasreen *et al.*, 2015). The increasing trends in the development of resistances to antibiotics are known as a public health threat since this will increase the cost of medication, rate of mortality and morbidity (Gales *et al.*, 2001; Hill *et al.*, 2005). It has been reported that bacteria in the aquaculture environment are multiple resistant to antibiotics including *P. aeruginosa* (Samuel *et al*., 2011; Abdullahi *et al.*, 2013; Ng *et al.*, 2014). In contrast to those findings, this study showed that the two *P. aeruginosa* strains tested were susceptible towards all the antibiotics. This result suggested that those antibiotics are still effective against certain strains of *P. aeruginosa* including those two strains isolated in this study. *P. aeruginosa* is one of the most common fish and human pathogens present in the aquaculture. The antibiotic

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resistance patterns of *P. aeruginosa* can be developed from susceptible to resistance through horizontal gene acquiring and multiplying of resistance genes from bacteria that carry antibiotic resistance gene (Barker, 1999). This study provides information on the extent of antibiotic susceptibility among *P. aeruginosa* in aquaculture and water environments.

CONCLUSION

Two isolates of *P. aeruginosa* from aquaculture farms were confirmed through molecular identification using 16S rRNA sequencing. These two isolates showed total susceptibility to all the antimicrobial agents tested, suggesting the antimicrobial agents tested remain effective against certain environmental strains of *P. aeruginosa* including strains isolated in this study. Nevertheless, antibiotic resistance can be transferred through horizontal gene transfer from antibiotic resistant bacteria to susceptible strains in the water systems. Therefore, appropriate use of antibiotics and continuous surveillance of antibiotic susceptibility patterns in the aquaculture setting is essential to avoid rapid emergence of resistant strains in *P. aeruginosa* which will pose public health threat.

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