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# Anti–biogram and resistogram profiling of *Aeromonas* species isolated from Malaysian aquatic sources

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PEER REVIEW

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**Comments**
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ABSTRACT

**Objective:** To investigate antibiotics and heavy metals resistance profile of *Aeromonas* species isolated from Kuala Gula and Kuala Sepetang, Perak.
**Methods:** Isolated *Aeromonas* species were subjected to 12 antibiotics: penicillin G 10 units, tetracycline 30 µg, novobiocin 5 µg, nitrofurantoin 100 µg, ampicillin 10 µg, chloramphenicol 10 µg, fluconazole 25 µg, gentamicin 10 µg, streptomycin 10 µg and 25 µg, kanamycin 30 µg, and vancomycin 30 µg and 5 heavy metals: $MnSO_4 \cdot H_2O$, $Cr(NO_3) \cdot 9H_2O$, $CuSO_4$, $Pb(NO_3)_2$, $HgCl_2$.
**Results:** Results from this study revealed that isolates from the two sources–displayed varied resistance. Isolates from Kuala Sepetang showed 91%, 66%, 61%, and 52% resistance to vancomycin, Novobiocin, streptomycin and gentamycin while Kuala Gula isolates showed 76%, 93%, 28% and 14% respectively. High rate of resistance was shown to be against lead (Pb), Chromium (Cr), copper (Cu) and Manganese (Mn) with minimum inhibitory concentration ranging from 2 560 µg/mL to >5 120 µg/mL. Low resistance to mercury was observed among isolates from Kuala Gula.
**Conclusions:** This study showed that there was widespread of both heavy metals and antibiotics resistance among *Aeromonas* isolates from the two marine sources with isolates from Kuala Sepetang showing more resistance thereby posing danger to both aquatic animals and human health.

KEYWORDS
Virulence, Antibiotics profile, Resistance, Marine sources

## 1. Introduction

The bacteria family Aeromonadaceae comprises of a group of bacteria found everywhere. Species of this genus have been isolated from river water, bottled water, chlorinated and unchlorinated water, vegetables, pasteurized and unpasteurized milk, chicken[1–3], sea food, meat and meat products[4], individuals with compromised immunity, fresh water[5], drinking water[6], estuary[7], catfish and tilapia fish[8,9], cold and warm blooded animals such as bird and crocodile[10]. Biochemically, aeromonads are oxidase and catalase positive, facultatively anaerobes, Gram negative and mostly prevalent in aquatic sources. Presence of aeromonads in environmental, aquatic and clinical sources made it possible for other forms of life including humans to easily come in contact and become infected with *Aeromonas* species. These infections however, depend on either strain type of virulence factors inherent in the specie.

Virulence factors such as aerolysin, protease, DNase, heamolysin, amylase, lipase have been identified[11–13]. Aeromonads infections include gastroenteristrics[14], bacteraemia[15,16], septicaemia[3], diarrhoea[17], respiratory

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tract infections and urinary tract infection[18,19]. Aeromonads have also been implicated in food and water borne disease outbreaks in different parts of the world especially developing countries where personal hygiene and quality water are compromised. These bacteria are generally grouped into motile and non-motile species. Till date, over 25 species and 7 sub species of *Aeromonas* have been isolated and identified globally with at least one new species being added to the list yearly for the past four years[20].

Previously, this group of bacteria was placed in Vibrionaceae family. However, due to molecular phylogenetic analysis in recent years, the bacteria have now been assigned to Aeromonadaceae family separating from the initial family[14]. Discharge of animals and human wastes coupled with indiscriminate use of antibiotics in relation to horizontal transfer of resistance genes are major factors contributing to the resistance of bacteria from aquatic sources to antibiotics. Like discharge of industrial effluent into body of water have resulted into bioaccumulation of heavy metals therein. This study therefore aimed to investigate antibiotics and heavy metal resistance pattern of *Aeromonas* species isolated from different aquatic sources in Malaysia.

## 2. Materials and methods

### 2.1. Samples and sampling location

Water samples were collected in sterile 500 mL Schott bottle from both Kuala Gula and Kuala Sepetang. The samples were transported into the laboratory for processing. A volumn of 100 mL of collected sea water was filtered using 0.45 μm nitrocellulose Millipore membrane filter (Millipore Corporation, Bed Ford). The filter was then placed on *Aeromonas* agar base (Difco) prepared plate according to manufacturer's instruction. A volumn of 100 μL of collected water sample was also directly plated in duplicate on *Aeromonas* agar base using spread plate method. All plates were incubated at 30 °C for 24 h. Green colonies (0.5–1.5 mm diameter) with dark centres were then selected as presumptive *Aeromonas* species. Selected colonies were also streaked on modified Rimler Shott–mRS agar[7]. Yellow, round shape, flat elevation colonies with entire margin were further purified by streaking on freshly prepared *Aeromonas* agar base and incubated as stated earlier. All presumptive isolates were confirmed to genus level by subjection to oxidase, catalase and resistance to vibrio static agent 0129 (150 μg–Oxoid). The following biochemical tests–lysine decarboxylase, arginine decarboxylase, ornithine decarboxylase, acid production from–glucose, sucrose, mannitol, dextrose, sucrose, citrate utilization, esculine, urea and API 20 NE kit (BioMerieux®, USA) were used to confirm the species.

### 2.2. Antibiotics resistance test

Resistance to antibiotics was carried out using Mueller Hinton Agar (Oxoid). Medium was prepared following manufacturer's instruction and incubated at 121 °C for 15 min. The medium was allowed to cool to 50 °C before pouring into sterile petri (plates 50 mm×100 mm) in a laminar flow (Biohazard, Germany). Thereafter, sterile swabs were used to evenly streak 18–24 h isolates from prepared blood agar +5% sheep blood (Isolab, Malaysia). The following Oxoid made antibiotics–penicillin G 10 units, tetracycline 30 μg, novobiocin 5 μg, nitrofurantoin 100 μg, ampicillin 10 μg, chloramphenicol 10 μg, fluconazole 25 μg, gentamicin 10 μg, Streptomycin 10 μg and 25 μg, Kanamycin 30 μg, and vancomycin 30 μg were then placed on the plates and allowed to be absorbed for 10 minutes before incubating at 30 °C.

### 2.3. Determination of heavy metal resistance and minimum inhibitory concentration (MIC)

The salts of the following five heavy metals $MnSO_4 \cdot H_2O$ (Chemar); $Cr(NO_3) \cdot 9H_2O$ (R&M chemicals); $CuSO_4$ (Merck); $Pb(NO_3)_2$ (Oxoid) and $Hg(HgCl_2)$ (Oxoid) were weighed and dissolved in freshly prepared autoclaved TSA before pouring into sterile plates (90 mm×150 mm) and allowed to solidify. The following concentrations of each salt were used for the assay 160 μg/mL, 320 μg/mL, 640 μg/mL, 1 280 μg/mL, 2 560 μg/mL and 5 120 μg/mL as described with modifications[7,21]. Fresh cultures of the tested isolates were streaked along a straight line on the agar containing varied concentrations of the metal salts. Inoculated plates were incubated at 30 °C for 48 h and observed for any visible growth. The MIC was also determined as the lowest concentration that inhibited growth of the tested bacteria.

## 3. Results

### 3.1. Isolation of Aeromonas

From the results of this study, a total of 73 *Aeromonas* (Kuala Gula–29, Kuala Sepetang–44) isolates were obtained–and are predominantly *Aeromonas hydrophila* (*A. hydrophila*) based on the biochemical characterization methods of previous study[8,20,22,23]. Kuala Sepetang has the highest number of the isolates. Similar results were reported by Havelaar AH, *et al*.[24] who found *A. hydrophila* more frequently distributed in Netherland rivers compared to other species.

### 3.2. Antibiogram–antibiotics resistance test

Result of this study showed that multi drug resistance was observed among the isolates as seen in Figure 1. Isolates from Kuala Sepetang were generally found to be more resistant to antibiotics than isolates from Kuala Gula although the cause of this is subject to further research. Isolates from Kuala Sepetang showed 91% tovancomycin, 66% novobiocin, 61% streptomycin and 52 % gentamicin. This was similar to the results obtained by Hatha M, *et al*.[25] who investigated antibiotics resistance of *Aeromonas* isolated from fish raised from fresh water in India. Their study revealed high resistance of *A. hydrophila* to novobiocin. In 2007 and 2012 some researchers also observed high resistance of *Aeromonas* strains from eastern Mediterranean Sea in Turkey to streptomycin[26,27]. Least resistance of these isolates to chloramphenicol (23%) and tetracycline (16%) was observed. In a recent study by Matyar F, *et al.*[28] low resistance to chloramphenicol was observed among *Aeromonas* strains isolated from Iskenderun Bay, a northeast Mediterranean Sea in Turkey. *Aeromonas* isolates from Kuala Gula isolates showed resistance of 76%, 93% and 28% against vancomycin, novobiocin and streptomycin, respectively. Meanwhile, low resistance to gentamicin (14%) and tetracycline (7%) was observed among the Kuala Gula isolates respectively. All the isolates were resistant (100%) to both penicillin and nitrofurantoin but showed 93% (Kuala Sepetang) and 82% (Kuala Gula) to ampicillin as shown in Figure 1. Ashiru and others in 2011 in their study stated that *Aeromonas* species isolated from catfish in Lagos, Nigeria were resistant to nitrofurantoin[8]. Likewise in 2009 Awan Mohammad Bashir, *et al.*[29] obtained similar result in Dubai among food originated *Aeromonas*.

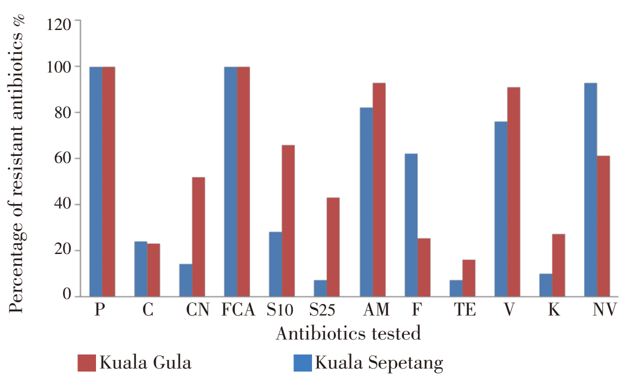


**Figure 1.** Percentage of antibiotics resistance observed among isolates from the two locations Kuala Sepetang and Kuala Gula.

P: Penicillin G 10 units, TE: Tetracycline 30 μg, NV: Novobiocin 5 μg, F:Nitrofurantoin 100 μg, AM: Ampicillin 10 μg, C: Chloramphenicol 10 μg, FCA: Fluconazole 25 μg, CN: Gentamicin 10 μg, S10: Streptomycin 10 μg, S25: Streptomycin 25 μg, K: Kanamycin 30 μg, V: Vancomycin 30 μg.

### *3.3. Resistogram–heavy metal resistance and MIC*

This present study to our best knowledge is the first to be carried on heavy metal resistance among *Aeromonas* isolates from Malaysian sea water samples. Resistance and tolerance of isolated bacteria to six heavy metals was carried out in this study. This was revealed as either growth or no growth on heavy metal supplemented agar plate as described by Mgbemena IC[30]. Variation in heavy metals resistance among the isolates from the two sources investigated was observed. The resistance pattern in Kuala Sepetang was Mn>Cr>Cu>Pb while that of strains from Kuala Gula was reverse as shown in Table 1. Mercury was found to inhibit growth of all the isolates with MIC 2 560 μg/mL unlike other metals that could not inhibit the bacteria except copper at 5120 μg/mL MIC. Matyar *et al.*[31] also obtained similar results from sea water Cd>Cu>Pb>Cr>Mn. Miranda and Castillo[32] observed the following resistance pattern Cd>Cu>Hg>Cr among *Aeromonas* strains in their study.

**Table 1**

Heavy metal resistance and MIC.

| Metals/isolates | Concentrations of heavy metals (μg/mL) | | | | | |
|---|---|---|---|---|---|---|
| | 160 | 320 | 640 | 1 280 | 2 560 | 5 120 |
| Mn | | | | | | |
| Kuala Gula (*n*=29) | 29 | 29 | 29 | 29 | 20 | 17 |
| Kuala Sepetang (*n*=44) | 44 | 44 | 44 | 44 | 23 | 6 |
| Cr | | | | | | |
| Kuala Gula (*n*=29) | 29 | 29 | 29 | 29 | 29 | 6 |
| Kuala Sepetang (*n*=44) | 44 | 44 | 44 | 44 | 43 | 7 |
| Cu | | | | | | |
| Kuala Gula (*n*=29) | 29 | 29 | 29 | 26 | 21 | – |
| Kuala Sepetang (*n*=44) | 44 | 44 | 44 | 44 | 4 | 1 |
| Pb | | | | | | |
| Kuala Gula (*n*=29) | 29 | 29 | 29 | 29 | 29 | 21 |
| Kuala Sepetang (*n*=44) | 44 | 44 | 44 | 44 | 44 | 4 |
| Hg | | | | | | |
| Kuala Gula (*n*=29) | 11 | 19 | 6 | 6 | – | – |
| Kuala Sepetang (*n*=44) | 42 | 40 | 36 | 32 | – | – |

–: No growth observed.

## 4. Discussion

*A. hydrophila* was the prevailing aeromonad isolated in this study. This was similar to the study of Matyar F, *et al*[28]. They reported that *A. hydrophila* was the prevailing *Aeromonas* in Iskenderun Bay, Turkey. This species of *Aeromonas* has been implicated in both human and marine animals like fish diseases. Its virulence is attributed to various virulence factors such as proteases, aerolysin, gelatinase, lipase and exhibited by this water loving bacterium[11–13]. Aeromonads–related infections also include gastroenteristrics[14], bacteraemia[15,16], septicaemia[3], diarrhoea[17], respiratory tract infections[18] and urinary tract infection[19]. It has also been isolated from marine sediment[7], sea foods such as cat and tilapia fish[8,9], cold blooded animals–crocodile[32] and warm blooded–bird[16].

The discovery of antibiotics decades ago was seen as permanent cure for microbial infections. However, an increased level of bacterial resistance to different antibiotics has been observed among both clinical and environmental microbial isolates. Application of antibiotics has been used in human and veterinary medicines respectively. Likewise the abuse of antibiotics globally cannot be overemphasized due to the fact that it poses treat to public and global health[14,31]. Results of various studies worldwide have identified couple of factors linked to increasing antibiotics resistance among which are plasmids[25,33]. Plasmids are extra chromosomal deoxyribonucleic acids capable of replicating independently within the cell. Genes encoding for resistant plasmids can hence be transfer to progeny of such antibiotics resistant bacteria. Resistance to antibiotics can also be acquired through horizontal gene transfer from the environment. Among the most common genes that can be environmentally acquired is β-lactamase enzyme which is capable of destroying antimicrobial agents which ever before can affect the bacterial cells. Some researchers opined that local select pressure also constitute bacterial resistance to antibiotics[33,34]. In this current study, antibiotics resistance and susceptibility to 12 commercial antibiotics were investigated among all the sea water isolates. Resistance of microorganisms to at least three antibiotics has been termed multi drug resistance[27].

In Malaysia, only few literatures could be found on resistance of bacteria to heavy metals. The few studies have been on heavy metals resistance of bacteria including *Aeromonas* isolated from American bullfrog by group of researchers in department of fisheries and aquaculture, University of Malaysia, Terengganu[35]. Resistance to heavy metals among the isolates in this study could be as a result of discharge of industrial effluents into the rivers. The result of this current study also reveal the most prevailing metal contamination in the aquatic environment due to anthropogenic activities such as discharge of toxic industrial effluents into water bodies. Pollution of water is a global occurrence which calls for actions due to the fact that water pollution is one of the causes of diseases and death in both human and aquatic animals' death. Heavy metal pollution in water has been attributed to industrialization and use of automobiles[30]. Although heavy metals are found naturally in mother earth, aquatic sources are more prone to the harmful effect of these metals. As man consumes some of these aquatic animals like fish, shrimps, prawn, etc, bioaccumulation of the metals become inherent in human body. It is to be noted that living organisms (cells) also require minute quantities of these metals for survival. However, macro amount of the metals can be toxic to the cells. Heavy metal resistance found in bacteria due to plasmids is similar to that of antibiotics resistance. However, heavy metal resistance plasmids are larger compared to antibiotics resistance plasmids[36]. The results of our study showed that there is widespread of both heavy metal and antibiotics resistance among *Aeromonas* isolates from the two marine sources considered in this study with isolates from Kuala Sepetang showing more resistance thereby posing danger to both aquatic animals and human health. Although this study focuses on sea water samples, further study is required on the antibiotics and heavy metal resistance of *Aeromonas* strains from aquatic animals found in the sampling area and a correlation study on effect of heavy metal resistance on antibiotics resistance of the strains. Likewise a further investigation into major source of toxic chemicals discharge into the area is also recommended.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgements

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## Comments

### *Background*

Infections involving aeromonads have been reported to be responsible for significant incidences of both food and water borne disease worldwide. Also, resistance to antibiotics has become a major concern in the treatment of diseases. A study of aeromonads' anti-biogram and resistogram in Malaysia waters is therefore important.

### *Research frontiers*

The authors conducted study on the antibiogram of important microorganism, aeromonads. The study also presented resistance of aeromonads to metals, reflecting a possible pollution from industrial sources. It also adds to the known few reports on bacterial resistance to metals in Malaysia.

### *Related reports*

The results obtained in this study are comparable to those obtained from water sources in Netherlands and from fish raised from fresh water in India.

### *Innovations and breakthroughs*

The paper has studied an important subject that reflects the sanity of waters in Malaysia and the level to which industrialization can affect water bodies. The study of resistance to metals is particularly interesting.

### *Applications*

Water bodies form a major reservoir and source of infection for aeromonads. A study into the species, antibiogram and resistogram will help in the knowledge database of the microorganism in Malaysia. It will also aid in the treatment of infection with the species identified in this study.

### *Peer review*

This is a valuable study that have characterized the susceptibility of aeromonads to antibiotics. The work done on the resistance to metals is also worthwhile and informative. This reflects the level of pollution in the Malaysia water bodies studied in this paper.

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