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Antibiotic resistance profiling and phenotyping of *Aeromonas* species isolated from aquatic sources



Olumide A. Odeyemi^{a,b,*}, Asmat Ahmad^b

^a Ecology and Biodiversity, Institute of Marine and Antarctic Studies (IMAS), University of Tasmania, Launceston, Australia

^b School of Biosciences and Biotechnology, National University of Malaysia, Malaysia

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Abstract This study aimed to investigate antibiotics resistance pattern and phenotyping of *Aeromonas* species isolated from different aquatic sources in Melaka, Malaysia. A total of 53 *Aeromonas* species were isolated from the following sources: sediment ($n = 13$), bivalve ($n = 10$), sea cucumber ($n = 16$) and sea water ($n = 14$) and resistance to 12 antibiotics – Tetracycline (30 μg), Kanamycin (30 μg), Oxytetracycline (30 μg), Ampicillin (10 μg), Streptomycin (10 μg), Gentamicin (10 μg), Sulphamethoxazole (25 μg), Nalixidic acid (30 μg), Trimethoprim (1.25 μg), Novobiocin (5 μg), Penicillin (10 μg) and Chloramphenicol (10 μg) was tested. The results obtained from this study reveal multi drug resistance pattern among the isolates. All the isolates were completely resistant to Ampicillin, Novobiocin, Sulphamethoxazole and Trimethoprim, respectively but susceptible to Tetracycline (100%), Kanamycin (5.7%), Gentamicin (5.7%) and Oxytetracycline (24.5%). Antibiotics phenotyping of the bacteria revealed 21 different phenotypes among the isolates.

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1. Introduction

Aeromonadaceae is an ubiquitous bacterial family due to the fact that members of this family have been isolated from diverse sources such as seafood, river water, bottled water,

chlorinated and unchlorinated water, vegetables, pasteurized and unpasteurized milk, individuals with compromised immunity, fresh water (Abulhamd 2010), drinking water (Pablos et al., 2011), (Odeyemi et al., 2012), catfish and tilapia fish (Ashiru et al., 2011; Suhel et al., 2011), cold and warm blooded animals such as birds (Roh et al., 2011), meat products (Dallal et al., 2012), crocodiles (Tel and Keskin, 2012) and chicken (Kashhedikar and Chhabra 2009; Papadakis et al., 2012).

Most common biochemical characteristics of members of this family are Gram negative, oxidase positive, facultatively anaerobes, and catalase positive and prevalent in aquatic environment. Due to the ubiquitous nature of members of Aeromonadaceae, humans easily come in contact and become infected with the pathogenic species. However, infections are mostly determined by type of strain and type of virulence factors (Odeyemi and Ahmad 2014). Among virulence factors

* Corresponding author at: Ecology and Biodiversity, Institute of Marine and Antarctic Studies (IMAS), University of Tasmania, Launceston, Australia.

E-mail addresses: oluodeyemi@gmail.com (O.A. Odeyemi), drasmat@gmail.com (A. Ahmad).

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Table 1 Antibiogram profile.

Antibiotics	Number of resistance of isolates				
	Bivalve (<i>n</i> = 10)	Sea Cucumber (<i>n</i> = 16)	Sea water (<i>n</i> = 14)	Sediment (<i>n</i> = 13)	Total
Tetracycline	–	–	–	–	–
Kanamycin	–	3	–	–	3 (5.7%)
Oxytetracycline	–	1	–	12	13 (24.5%)
Ampicillin	10	16	14	13	53 (100%)
Streptomycin	4	5	8	8	25 (47.2%)
Gentamicin	–	3	–	–	3 (5.7%)
Sulphamethoxazole	10	16	14	13	53 (100%)
Nalixidic acid	1	2	9	12	24 (45.3%)
Trimethoprim	10	16	14	13	53 (100%)
Novobiocin	10	16	14	13	53 (100%)
Penicilin	9	13	14	13	49 (92.5%)
Chloramphenicol	2	3	4	2	11 (20.8%)

associated with *Aeromonas* species are aerolysin, lipase, protease, DNase, hemolysin and amylase (Aberoum and Jooyandeh 2010; Sharma et al., 2010). Infections such as bacteremia (Hochedez et al., 2010), respiratory tract infections (Issa and Napolitano 2011), gastroenteritis (Igbinsosa et al., 2012), septicemia (Papadakis et al., 2012), urinary tract infection and diarrhea (Senderovich et al., 2012) have been associated with *Aeromonas*. According to Odeyemi and Ahmad (2014), *Aeromonas* species have also been linked to both food and water-borne diseases in different parts of the world especially developing countries due to poor personal hygiene and lack of quality water. These bacteria are either motile or non-motile species. Currently, more than 26 species and 8 sub-species of *Aeromonas* have been identified (Figueras et al., 2011).

The discovery of antibiotics decades ago, has helped to overcome microbial infections and diseases affecting humans and animals. However, bacteria that are initially susceptible to commonly used antibiotics are becoming resistant. Among these antibiotic resistant bacteria are some species of *Aeromonas* especially clinical and environmental isolates. According to Maria José (2012), there is an emerging public health related problem due to antibiotic resistance of *Aeromonas* spp. to commercial antibiotics. Water bodies receive human and animal waste especially wastewater discharges mostly believed to contain antimicrobial agents that can make natural flora in these water bodies to become resistant (Goñi-Urriza et al., 2000). Antibiotic resistance has been observed in environmental *Aeromonas* isolates isolated from heavily polluted waters (Huddlestone et al., 2006; Aravena-Román et al., 2012).

In Malaysia, most of the studies on antibiotic resistance of *Aeromonas* species have been focused mainly on clinical, seawater and sediment isolates. There is a dearth of information on other aquatic sources as potential reservoir. This study therefore aimed to investigate antibiotics resistance pattern and phenotyping of *Aeromonas* species isolated from different aquatic sources in Malaysia.

2. Materials and methods

2.1. Sample collection and isolation of *Aeromonas* spp. from seawater

Water samples were obtained from Melaka using sterile 500 mL, stored in ice box and transported to the laboratory for microbiological analysis. 1 mL of both seaweed and sea

grass that was cut into pieces using sterile scissors was weighed aseptically into sterile 9 mL normal saline water for subsequent serial dilution up to seven replicas. 0.1 mL of samples labeled 10^1 , 10^3 and 10^5 was plated on modified Rimler Shott (mRS) agar (Odeyemi et al., 2012) for isolation of presumptive *Aeromonas* spp. Master plates and stock culture of selected presumptive isolates were prepared. The isolates were then phenotypically characterized.

2.2. Sample collection and isolation of *Aeromonas* spp. from sediment, rinsed sample water from bivalve and sea cucumber

Samples of sediment, bivalve and sea cucumber were collected from Mussel farm Sebatu, Melaka. The samples were transported in ice box to the laboratory for processing. Serial dilution of the sediment sample was carried out using sterile universal bottles. One gram (1 g) of the sediment was introduced into sterile 9 mL 0.65% saline water and further diluted up to seven replicas. 0.1 mL of samples labeled 10^1 , 10^3 and 10^5 was seeded on modified RimlerShott (mRS) agar for isolation of presumptive *Aeromonas* spp.

Bivalves and sea cucumber were rinsed with 100 mL of sterile seawater (SSW). The first rinse was kept for further processing while the bivalves (11 samples) and sea cucumber (2 samples) were further washed twice to keep them free of any adherent particles. 1 mL of rinsed sample water (RSW) was serially diluted from 10^1 to 10^5 using SSW as diluents. Thereafter, 10 μ L of dilutions 10^1 , 10^3 and 10^5 was then inoculated on mRS medium.

2.3. Isolation of *Aeromonas* spp. from bivalves and sea cucumber

Bivalves were dissected after removing the shell. 5 g of bivalve muscle was weighed into sterile universal bottle containing SSW and vortexed for 2 min to dislodge the bacteria. The sample was further processed as above. Sea cucumber was also dissected while 10 g of the intestine and 10 g of the body tissue were weighed into sterile universal bottle, vortexed for 2 min to dislodge the bacteria and then processed as described above.

2.4. Biochemical characterization of presumptive isolates

All presumptive *Aeromonas* isolates were inoculated on *Aeromonas* medium with the following composition L-lysine 5 g/L,

L-ornithine 6.5 g/L, maltose 3.5 g/L, sodium thiosulphate 6.8 g/L, L-cystein HCL 0.3 g/L, ferric ammonium citrate 0.8 g/L, bile salt 5 g/L, yeast extract 3 g/L, bromothymol blue 0.03 g/L, NaCl 5.0 g/L, Bacto agar 13 g/L and sterile sea water. This medium bears similarity with colony morphology of the isolates which was compared with that of mRS agar. This was carried out in triplicates. Plates were then incubated at 30 °C for 24 h. After, aseptically weighing out the aforementioned components of the medium in a sterile conical flask, the mixture was then dissolved by stirring while the pH was adjusted to 7.8 using freshly prepared 6 M sodium hydroxide (6 M NaOH). This was then boiled for a minute for complete dissolution of the solutes.

The medium was allowed to cool to 45 °C before pouring in 100 mm × 50 mm sterile petri dishes in Biohazard (Germany) Laminar flow. Colonies with yellow coloration were picked and further cultured in freshly prepared Trypton Soy Agar supplemented with 0.65% yeast extract (Difco) – TSA-YE. Isolates were morphologically identified via colony morphology, Gram staining and motility test. However, the following biochemical tests were used to further identify and classify the isolates into species: triple iron was carried out using triple iron agar, Bile Esculine (Bile Esculine agar), protease production, lysine decarboxylase, arginine decarboxylase, ornithine decarboxylase, catalase, oxidase, methyl red, acid production from inositol, mannitol, dulcitol, glucose, starch hydrolysis, citrate utilization, raffinose, cellulose, xylose, salicin, arabinose, mannose, sucrose and lactose.

The following commercial media purchased were used for decarboxylase, ornithine, ureas, dextrose, lactose, maltose, sucrose and Simmon citrate agar. Methyl Red Proskauer was carried out as follows: The MR VP broth (Oxoid) was prepared according to the manufacturer's instruction. The prepared broth was divided into two parts. 0.1 g/100 mL of methyl red dye was weighed and dissolved in 95% absolute alcohol and added to 250 mL sterile distilled water before adding it to the first part of the broth. This was dispensed in test tubes before autoclaving the tubes at 121 °C for 15 min. After autoclaving, the tubes were incubated with fresh bacteria culture and then incubated at 30 °C. The second portion of the broth was used for VP test. All the tubes for VP test were also autoclaved and inoculated with bacteria and then incubated accordingly. After 24 h incubation, VP reagent (alpha-naphthol 5% in absolute alcohol) was added in drops. The tubes were shaken and kept at 37 °C for 10 min before observing color change for positive and crimson red for negative result.

All morphological and biochemically identified isolates were differentiated from *Vibrio* species using 10 g and 150 g of 0/129 vibriostatic agent. Stock cultures and master plates of *Aeromonas* spp. were prepared and stored at – 4 °C for further study. API 20 NE was further used to confirm the isolates as *Aeromonas* species. Master plates of the stock cultures of each isolate were prepared and preserved in agar slants overlaid with 20% sterile glycerol for further study.

2.5. Antibiotics resistant test (AST)

The antibiotic resistant pattern of isolated *Aeromonas* species was tested against 12 Oxoid made antibiotics. It was carried out on Brain Heart Infusion Agar (BHIA, Sharlau). The

medium was prepared according to the manufacturer's instruction autoclaved at 121 °C for 15 min and poured into 100 mm × 50 mm petri dishes in a laminar flow (Biohazard, Germany) to avoid cross contamination. The plates were allowed to solidify. Thereafter, each isolate was then streaked on the plate with the aid of sterile swabs. Antibiotics to be tested were further placed on the surface of the solidified agar.

The plates were kept for 10–15 min for antibiotics diffusion purpose and then incubated at 30 °C overnight. Antibiotics used are Tetracycline (30 µg), Kanamycin (30 µg), Oxytetracycline (30 µg), Ampicillin (10 µg), Streptomycin (10 µg), Gentamicin (10 µg), Sulphamethoxazole (25 µg), Nalixidic acid (30 µg), Trimethoprim (1.25 µg), Novobiocin (5 µg), Penicillin (10 µg) and chloramphenicol (10 µg). The size of the zone of inhibition was measured in millimeters (mm) after 24 h and interpreted as sensitive (S) – inhibition zone ≥ 18 mm, intermediate (I) – inhibition zone 13–17 mm and resistance (R) – inhibition zone < 13 mm (Okonko et al., 2009). Resistance to more than 3 antibiotics (≥ 3) was noted as multi antibiotic resistance (MAR). The results obtained were also used to calculate the MAR index of the isolates as described by Odeyemi et al. (2012).

2.6. Phenotypic typing of isolates

A numerical code was created for the sole aim of phenotypic typing of the isolates based on resistance and susceptibility to antibiotics tested. The codes were obtained as follows: 3 – resistance, 2 – intermediate and 1 – susceptibility.

3. Results

A total of 53 *Aeromonas* species were isolated from all the sources. Sea cucumber had the highest number of isolates ($n = 16$), seawater ($n = 14$), sediment ($n = 13$), while bivalve has 10 *Aeromonas* spp. as seen in Table 1. Only yellow raised and round colonies on mRS agar and confirmed as on *Aeromonas* medium were considered presumptive *Aeromonas* isolates. These were further biochemically tested as oxidase and catalase positive.

Gram staining of the isolates also revealed Gram negative short rod bacteria. During the period of this study, *Aeromonas hydrophila* ATCC 7966 was used as positive control. All identification procedure was as described by previous workers (Abulhamd, 2010; Hochedez et al., 2010; Suhet et al., 2011; Ashiru et al., 2011). All the isolates were subjected to antibiotic resistant test against 12 commercially available antibiotics using modified method described by (Odeyemi et al. 2012). The obtained result revealed high level of multi antibiotic resistance among the isolates. All the isolates were completely (100%) resistant to Ampicillin, Novobiocin, Sulphamethoxazole and Trimethoprim, respectively. It was observed that 24 (45.3%) *Aeromonas* spp. were resistant to Nalixidic acid while 49 (92.5%) were resistant to Penicillin. Of all the isolates, 25 (47.2%) were resistant to Streptomycin. The least resistance was observed in Kanamycin and Gentamicin 3 (5.7%). However, all isolates 53 (100%), were susceptible to Tetracycline, Kanamycin (5.7%), Gentamicin (5.7%) and Oxytetracycline (24.5%).

Isolates from bivalve and sea water were completely susceptible to Kanamycin, Gentamicin, Oxytetracycline and

Table 2 Antibiotics susceptibility test (AST) typing of *Aeromonas* spp.

Isolates	AST profile	Phenotypic typing profile	MAR index
Bv.m 1	111121131333	A	0.33
Bv.m 2	111131132333	B	0.42
Bv.m 3	111131231333	C	0.42
Bv.m 4	111121131333	A	0.33
Bv.m 5	111131333333	D	0.58
Bv.m 6	111121331333	E	0.33
Bv.m 7	111121131333	A	0.33
Bv.m 8	111131231333	C	0.33
Bv.m 9	111121231333	F	0.33
Bv.m 10	111121131332	A	0.25
Sb.m 11	111131131333	G	0.42
Sb.m 12	131121332333	H	0.50
Sb.m 13	111331233333	I	0.58
Sb.m 14	111321131332	J	0.33
Sb.m 15	131323231332	K	0.50
Sb.m 16	113333131333	L	0.67
Sb.m 17	111211131333	M	0.33
Sb.m 18	111311131333	N	0.42
Sb.m 19	111121331333	O	0.42
Sd.m 20	111121331333	O	0.42
Sd.m 21	111321133333	P	0.50
Sd.m 22	121332133333	Q	0.58
Sd.m 23	121322133333	R	0.50
Sd.m 24	121332133333	Q	0.58
Sd.m 25	121332133333	Q	0.58
Sd.m 26	121332133333	Q	0.58
Sd.m 27	121321133333	S	0.50
Sd.m 28	121332133333	Q	0.58
Sd.m 29	121332133333	Q	0.58
Sd.m 30	121332133333	Q	0.58
Sd.m 31	111332133333	T	0.58
Sd.m 32	121322333333	U	0.58
Si.m 33	111121131333	A	0.33
Si.m 34	111131131333	G	0.42
Si.m 35	111121331333	E	0.33
Si.m 36	131323231332	K	0.50
Si.m 37	111121231333	F	0.33
Si.m 38	111331233333	I	0.58
Si.m 39	111121131333	A	0.33
Sw.m 40	111321133333	P	0.50
Sw.m 41	111331233333	I	0.58
Sw.m 42	121322333333	U	0.58
Sw.m 43	121321133333	S	0.50
Sw.m 44	111131231333	C	0.33
Sw.m 45	111131333333	D	0.58
Sw.m 46	111121131333	A	0.33
Sw.m 47	111131131333	G	0.42
Sw.m 48	121332133333	Q	0.58
Sw.m 49	121332133333	Q	0.58
Sw.m 50	111131333333	D	0.58
Sw.m 51	111332133333	T	0.58
Sw.m 52	111211131333	M	0.33
Sw.m 53	111121331333	O	0.42

Sw.m = sea weed and sea grass; Si.m = sea cucumber (intestine);

Sb.m = sea cucumber (body).

Sd.m = sediment; Bv.m = bivalve; m = Melaka.

Tetracycline. All *Aeromonas* isolates from sediment samples were susceptible to Kanamycin, Gentamicin and Tetracycline unlike isolates from bivalve and seawater. It was observed that isolates from sea cucumber were only susceptible to Tetracycline. Antibiotics phenotyping of the isolates using numerical codes revealed 21 different phenotypes of the isolates. Additionally, the MAR index of all the *Aeromonas* isolates from the different aquatic sources ranged between 0.25 and 0.68 as seen in Table 2 which reveals high level of use of antibiotics indiscriminately. This has resulted in the development of bacterial resistance among the isolates. Isolates from sediment and seawater had the highest MAR index compared with other sources. The least values of MAR index were found in bivalve and sea cucumber isolates.

4. Discussion

Aeromonads especially isolates from clinical and environmental samples have recently been implicated in various infections such as gastroenteritis (Igbinosa et al., 2012), bacteremia (Hochedez et al., 2010), septicemia (Papadakis et al., 2012), diarrhea (Senderovich et al., 2012), respiratory tract infections (Issa and Napolitano, 2011) and urinary tract infection. It is therefore of importance to investigate various aquatic sources for presence of *Aeromonas* species and their resistance pattern to various commercial antibiotics. This is due to the fact that aquatic sources can serve as means of contacting any species of the bacteria to both human and other forms of lives including sea food. In a review by (Kümmerer, 2009), two sources of antibiotics resistance among bacteria isolates were stated. Indigenous or primary resistance is present naturally in microbes and spread across to progeny through cell division during growth. This was termed “vertical resistance transfer”. Secondly, (“horizontal resistance transfer”) antibiotics resistance can be acquired as a result of microbes coming in contact with antibiotics in their environment either naturally or through therapeutic use of antibiotics.

This type of resistance is often plasmid – extrachromosomal DNA mediated. Human and other animals could then acquire resistant bacteria through contact with form of water such drinking, sea water and/or waste water. It could also be through food, various sea foods like bivalve, sea cucumber, shrimps and prawns. Contact with soil and sediment from mangrove, rain forest or estuary (Odeyemi et al., 2012), live-stocks (Ceylan et al., 2009) and pet animals could also be sources of acquiring antibiotics resistant pathogenic bacteria. Results from this study reveal high level of multi drug resistance among the isolates tested. All the isolates 53 (100%) were resistant to Ampicillin and 49 (92.5%) were resistant to Penicillin respectively. This was similar to results obtained by other researchers. Jalal et al. (2010), investigated resistance of bacteria from mangrove sediments in Malaysia. The result of their study revealed that all *Aeromonas* isolates from the sediment samples were resistant to β -lactam antibiotics (Ampicillin and Penicillin). This was as a result of the presence of β -lactamase enzymes among the isolates (Jalal et al., 2010). Ramalivhana et al. (2010), in their study of antibiotics resistance among water and stool isolates of *Aeromonas* found that 100% of the tested isolates were also resistant to Ampicillin.

Aeromonas isolates in this current study was however susceptible to Gentamicin except 3 (5.7%) from sea cucumber that was resistant to the antibiotics. This was unlike the report of Jalal et al. (2010). Although, samples of sea cucumber were not considered in their study. Presence of antibiotics resistant *Aeromonas* spp. in aquatic environment especially seawater poses danger to seafood. Researchers have reported various studies on isolation of resistant *Aeromonas* spp. from catfish and tilapia (Ashiru et al., 2011; Huang et al., 2010).

5. Conclusion

In study, we were able to isolate *Aeromonas* from different aquatic sources. The results of this study confirms the presence of multi antibiotic resistant *Aeromonas* species from aquatic sources, reflect indiscriminate use of antibiotics and poses public health problems to both humans and aquatic animals. This corroborates with other studies on antibiotic resistance of Aeromonads. It is therefore imperative if a continuous monitoring surveillance or a study is carried out in riverine areas. Seafood and other marine life forms should also be investigated for possible presence of antibiotic resistant *Aeromonas* species. There is a need for further study on the antibiotic resistance genes and genetic relatedness of these isolates.

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