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Water quality and microbiological risk associated with multiple antibiotic resistance (MAR) bacteria in water of fish facility

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Abstract

Fish can live healthier in aquarium with good water quality than they do in the wild. Maintaining the quality of the water in fish facility is needed to avoid fluctuation of physicochemical parameter values and contamination with pathogenic microorganisms that may cause serious illness or even death among the fish. Contamination of the water, especially with animal pathogens which are also pathogenic to human may pose health risk to those who are handling or in direct contact with the water and fish in the facility. Therefore, there is a need to assess the water quality and the risk associated with microorganisms in the water and the cultured animals. The aim of this study was to determine the water quality with regard to the physicochemical and microbiological parameters as well as the risk associated with bacteria in the water of the fish facility. Samples of water from the water source and also from aquariums in the fish facility were collected and analyzed. The water samples were plated on nutrient agar for bacterial enumeration then bacterial colonies growing on the agar plates were randomly picked and purified. $(GTG)_5$-PCR analysis was carried out to analyse the heterogeneity of the genome of the bacterial isolated and a dendrogram was constructed from the $(GTG)_5$-PCR profile to determine the genotypic group of the bacterial isolates. The risk associated with the bacteria from the water was analyzed with respect to their antibiotic resitance. The result of this study revealed that the $(GTG)_5$-PCR analysis was able to group the bacteria into 2 main genotypic clusters which were further grouped into several sub-clusters. From the dengrogram, 12 representative isolates were selected and identified using 16S rRNA sequencing. The identification confirmed the presence of *Aeromonas veronii* (8 isolates), *Aeromonas jandaei* (2 isolates), *Plesiomonas shigelloides* (1 isolate) and *Pseudomonas alcaligene* (1 isolate) from the water samples. All of the isolates exhibited resistant towards ampicillin, penicillin and gentamicin. This study revealed that the water from the fish facility harboured genetically diverse antibiotic resistance bacteria which may pose health risk to the fish and also to those who are in direct contact with the contaminated water and fish in the facility. Therefore, water in fish facility should be monitored regularly and handled with caution.

Introduction

Monitoring the water quality of aquariums in fish facility is crucial to ensure the healthy growth of the fish used for research purpose. Among the important parameters that need to be maintained at certain values are the pH, chlorine and chloramine, nitrite, nitrate, and temperature. Rapid changes in any one of the parameters is detrimental to the fish in the aquarium. It is also significant to maintain the quality of the water because the contamination of the water with pathogenic microorganisms may cause serious illness or even death among the cultured organisms in the water (Liguori *et al*., 2010). The more frequent we monitor the water quality, the better the prevention of the contamination problems.

Three major types of pollutants that can affect the water quality are nutrients, dirt and microorganisms. Nutrients such as nitrogen and phosphorus are the primary cause of water pollution. The excessive nutrients in the water can cause the overgrowth of algae which will use up the oxygen in the water and lead to the death of other organisms. Apart from that, the presence of dirt in the water may clog the gills of the fish and consequently result in the suffocation of the fish. Microorganisms are contaminants that play important role in the degradation of organic matter in the water. Some microorganisms are helpful but some are pathogenic to the fish and some may pose health risk to human. The fish in the aquarium may die due to fish pathogens infection.

The apparent increase of the occurrence of multiple antibiotic resistance among bacteria from various areas including aquaculture has become

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a global problem during the past years and lead to the surveillance of various sources for antibiotic resistance bacteria. While there are numerous researches that have been conducted on antibiotic resistant bacteria from the environmental water and aquaculture environment (Kathleen *et al.*, 2014; Samuel *et al*., 2011), not many work have been done on water from fish facility. The aim of this study was to determine the water quality with regard to the physicochemical and microbiological parameters as well as the risk associated with antibiotic resistance bacteria in the water of the fish facility.

Materials and Methods

Collection of water samples

Water samples were collected using sterile 50 ml Falcon tubes from the aquariums in the fish facility of the Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak in April 2012. Six water samples were collected from the fish facility at a time: four from four different aquariums hosted with zebrafish (Falcon tube A, B, C and D) and one each from the tap water supplying the water (inlet) for the aquariums (Falcon tube E) and water from the filtered water (outlet) from the filtration system (Falcon tube F). The water samples were analyzed for physicochemical parameters such as pH, dissolved oxygen, nitrate and ammonia content and microbiological properties.

Sample processing for microbiological analysis

Approximately 0.1 ml of the water samples were aseptically plated on nutrient agar (NA) (Oxoid, England) with sterile hockey stick shaped glass rod. The water samples were spread over the surface of the agar plates and incubated at 30°C for 18 to 24 h. After incubation, the colonies on the plates were counted.

Bacterial isolation

About 5 single colonies growing on a nutrient agar were randomly selected. The isolates were labelled according to the labelling system as described by Hutter *et al.* (2003) as shown by the alphabetical letter sequence below:

Xyn,

Where;
X = sources of water samples (A, B, C, D, E, and F)
y = no. of replicates of agar plate (1 and 2)
n = no. of pure culture obtain from the agar plate (a, b, c, d, and e)

Storage and preservation of bacteria

The bacterial isolates were stored in slant agar as working stock and kept at 4ºC for short term preservation. Another set of the bacterial culture was stored in 20% (v/v) glycerol stock in -20ºC freezer for long term preservation.

Biochemical tests

Several biochemical tests were carried out for the preliminary identification of the bacteria. These tests include gram staining, Voges-proskauer, methyl red, Simmon's citrate, hydrogen sulfide, indole and motility test (Mac Faddin, 1980).

DNA extraction

The boiling-centrifugation method was used to extract DNA from bacteria as described by Soumet *et al*. (1994) with minor modifications where bacterial cells were boiled instead of direct boiling of samples containing bacteria.

$(GTG)_5$-PCR fingerprinting

The $(GTG)_5$-PCR analysis was carried out in a volume of 25 µl reaction consisting 5 µl of 10x PCR buffer, 3 µl of 25 mM $MgCl_2$, 0.8 µl of 10mM dNTP's, 1 µl of 25 µM (GTG)5 primer (5'-GTG GTG GTG GTG GTG-3'), 9.9 µl of sterile distilled water, 0.3 µl of 5U/µl Taq DNA polymerase and 5 µl of about 20-40 ng template DNA (Matsheka *et al*., 1994). Amplification was performed in a Bioer Little Genius thermal-cycler (Bioer, China) with a temperature cycling program consisting of the initial denaturation at 95°C for 7 min, 4 cycles of denaturation at 95°C for 2 min, annealing at 55°C for 2 min, polymerization at 72°C for 2 min followed by another 34 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and polymerization at 72°C for 1 min. An additional cycle of final elongation at 72°C for 5 min was also included. The DNA bands were analysed by agarose electrophoresis in a 1.0% (w/v) agarose in 1.0x TBE (0.1 M Tris, 0.1 M boric acid, 0.1 mM EDTA) at 80 V for 45 min. A 1kb DNA ladder (Promega, USA) was used as a reference DNA size marker. Gels were stained in ethidium bromide solution and the DNA bands were visualized with UV transilluminator (Bio-rad).

Identification of bacteria by 16S rRNA sequencing

The identification of bacteria was carried out by 16S rRNA gene amplification using PCR (Hutter *et al.*, 2003) with the primer 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 519R: 5'-GWATTACCGCGGCKGCTG-3'. The expected size of the PCR product was 500 bp. The PCR was

performed in a 50 µl volume reaction containing 10 µl of 10x PCR buffer, 6 µl of 25 mM $MgCl_2$, 3 µl of 10mM dNTP's, 1 µl of 25 pmol each primer, 8 µl of sterile distilled water, 1 µl of 5U/µl Taq DNA polymerase and 20 µl of 20-40 ng template DNA. Amplification was performed in a Bioer Little Genius thermal-cycler (Bioer, China) with a temperature program consisting of the initial denaturation at 95°C for 10 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min and polymerization at 72°C for 1.5 min. A final elongation cycle at 72°C for 10 min was also included. The amplification products were analyzed by electrophoresis in a 1.0% (w/v) agarose in 1.0x TBE (0.1 M Tris, 0.1 M boric acid, 0.1 mM EDTA) at 90 V for 40 min. A 1kb DNA ladder (Promega, USA) was used as a DNA size marker. Gels were stained with ethidium bromide and the amplified fragments were visualized with UV transilluminator (Bio-rad).

Purification of PCR product

The DNA was purified using the QIAquick gel extraction kit (Qiagen, Germany). After DNA purification, the purified products were run again on agarose (1% w/v) to make sure that the DNA recovered was sufficient for the DNA sequencing.

Antibiotic susceptibility testing

The antibiotic susceptibility of the isolates was carried out by disc diffusion method (Wan *et al*., 2003). The bacterial isolates were tested with eleven different types of antibiotics (Oxoid, England) which were ampicillin (10 µg), norfloxacin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), nitrofurantoin (300 µg), gentamicin (10 µg), chloramphenicol (30 µg), penicillin (10 µg), tetracycline (30 µg) and trimethoprim-sulfamethoxazole (23.75 µg). Briefly, a sterile cotton bud was dipped into the bacterial broth culture and swabbed onto the dried surface of Mueller-Hinton agar (MHA) (Oxoid, England). A control strain *E. coli* ATCC 25922 was used as positive control in this study. The MHA plates were incubated at 30°C for 18 to 24 h. After the incubation period, the clear zone around the antibiotic discs was observed on the agar surface. The size of inhibition zone was measured to the nearest whole millimeters by using a ruler.

Chemical analysis of water

Certain parameters of the biochemical in the water were measured. The pH of water was measured using pH meter, dissolved oxygen was measured using electrometric method, nitrate and ammonia content were measured following standard methods [American Public Health Association (APHA) (2005)].

Table 1. The plate count of water samples taken from fish facility

Water samples	Number of cells (colony-forming units) per ml of water sample
A(1)	31
A(2)	39
B(1)	42
B(2)	74
C(1)	130
C(2)	98
D(1)	276
D(2)	394
E(1)	0
E(2)	0
F(1)	9
F(2)	24

Note: *A, B, C and D are water samples from four different aquariums hosted with zebrafish, E is the tap water (water inlet), and F is the filtered water from the filtration system (water outlet).

Results and Discussion

Water quality in aquariums of fish facility

Monitoring the water quality in the aquariums of a research fish facility is important in order to have healthy fish for the continuous supply of fish used for research purpose. Besides, good quality of water reduced the risk of infections by fish and human pathogens through handling the fish and contacts with the water. In this study, chemical analysis of the water in the facility hosting zebra fish revealed that the average pH value for different aquariums ranged from pH 7.52 to 7.92, dissolved oxygen ranged from 5.97-6.83 mg/l, nitrate content ranged from 0.38 to 0.56 mg/l and the ammonia content ranged from 0.01 to 0.07 mg/l. All the physical and biochemical parameters were still within acceptable level.

The result of the plate count of the bacteria in the water samples is shown in Table 1. The bacterial count ranged from 0 to 394 cfu per ml of water. Samples of water collected from the aquariums hosted with zebrafish and the water from the outlet of the aquariums showed the presence of bacteria. The water in the aquarium may have been contaminated from unknown sources and the bacteria may have been propagated in the water (Monks, n.d). Some beneficial bacteria are required to keep an ideal condition for the growth of the fish such as nitrosomonas and nitrobacter (Petco Animal Suppies, 2004). Nitrosomonas are group of aerobic

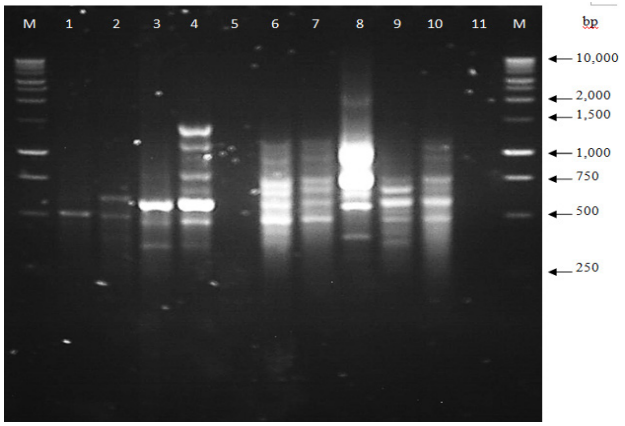

Figure 1. Representative profile of agarose [1.5% (w/v)] gel electrophoresis of $(GTG)_5$-PCR of the bacterial isolates from water of the fish facility. Lane M: 1kb ladder, Lane 1: A1d, Lane 2: F1a, Lane 3: F1b, Lane 4: F1c, Lane 5: F1d (the final profile not shown here), Lane 6: F2a, Lane 7: F2b, Lane 8: F2c, Lane 9: F2d Lane 10: F2e, Lane 11: Negative control, Lane M: 1kb ladder

bacteria that can convert deadly ammonia generated from fish waste and decaying food into another toxic substance such as nitrites. The harmful nitrites will be converted into less harmful nitrates by nitrobacter. However, the presence of these bacteria must be at low level because they may become harmful to the fish when they are in excessive number in the water.

The tap water supplying the water for the aquarium was free of bacteria. This was probably because of the chemicals such as chlorine or chloramines which are usually added to the water to remove harmful organisms (Wooding, n.d.). Chlorine is the most well-known chemical used to decrease bacterial growth in the water. Nowadays, the use of chloramines in water treatment is increasing as it is much more stable than chlorine and does not dissipate from the water easily. However, the use of chloramines has to be regulated as it may convert the organic materials into some carcinogenic substances, such as chloroform and carbon tetrachloride (Dunnick and Melnick, 1993).

Isolation and identification of bacteria from water of fish facility

Isolation and identification of big number of bacterial from water can be very costly and time consuming. In this study, DNA fingerprinting using $(GTG)_5$-PCR was utilized to group the isolates before representative isolates can be chosen for identification to avoid repeated strains identification (Samuel *et al*., 2014; Kathleen *et al*., 2014). (GTG)5 PCR was performed on 24 bacterial isolates to assess their DNA banding patterns that can assist in the observation of genetic similarity among the isolates. The dendrogram shows the existence of two main groups (group 1 and 2) in which group 2 were further

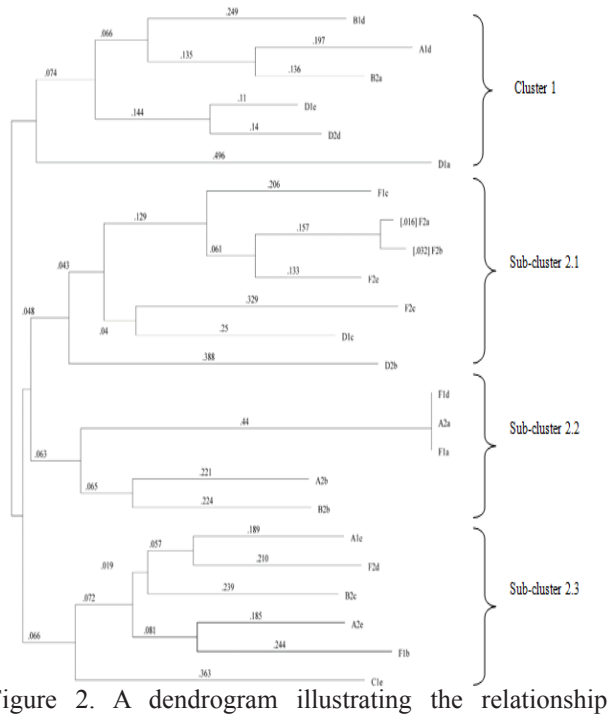

Figure 2. A dendrogram illustrating the relationship between the bacterial isolates analysed by repetitive sequence-based PCR using $(GTG)_5$ primer

grouped into three sub-groups (sub-group 2.1, 2.2, 2.3). Figure 1 shows the representative gel photo of the PCR product of the $(GTG)_5$-PCR analysis. It shows a high banding complexity and reproducibility, so it is suitable to be applied for the grouping of bacterial species (Gevers, 2001).

The information from the dendrogram in Figure 2 was used to select representative bacteria for DNA sequencing. The 16S rRNA gene was commonly used for the identification of species of bacteria (Kathleen *et al*., 2014). Table 2 shows the isolation code, identity and their antibiotic resistance. In this study, 16S rRNA gene identification confirmed the presence of *Aeromonas veronii* (8 isolates), *Aeromonas jandaei* (2 isolates) and *Plesiomonas shigelloides* (1 isolate) and *Pseudomonas alcaligene* (1 isolate). *A. veronii* is a Gram negative, rod-shaped bacterium found in freshwater and is usually associated with animals. *A. veronii* can cause mortality to the cultured organisms if it is present in high numbers. It can be a pathogen of humans and beneficial symbiont of leeches. In human, it can cause diarrhea, wound infections and cholecystitis (Buller, 2004; Hickman-Brenner *et al*., 1987).

A. jandaei is a Gram negative, rod shaped bacterium which is a human pathogen. The diseases caused by *A. jandaei* is almost similar to those that caused by *A. veronii*. Isolates C1e and F1b were found to have high similarity with *P. shigelloides* and *P. alcaligenes*, respectively. *P. shigelloides* is a

Table 2. Antibiotic resistance patterns and MAR indexes of the bacterial isolates

Isolate Code	Bacteria Species	Antibiotic Resistance*	Antibiotic Resistance Patterns	MAR Index
A2b	*Aeromonas veronii*	AmpNorKNaSCnPTe	1	0.727
B1d	*Aeromonas jandaei*	AmpKNaSCnPTe	2	0.636
B2a	*Aeromonas veronii*	AmpNorKNaSCnPTe	1	0.727
D1a	*Aeromonas veronii*	AmpKNaSCnPTe	2	0.636
D1c	*Aeromonas jandaei*	AmpNorKNaSCnPTe	1	0.727
F1b	*Pseudomonas alcaligenes*	AmpFCnCP	3	0.455
F1d	*Aeromonas veronii*	AmpNorKNaSCnPTe	1	0.727
F2d	*Aeromonas veronii*	AmpKNaSCnPTe	2	0.636

Note: *Isolates were tested for ampicillin (Amp), norfloxacin (Nor), kanamycin (K), nalidixic acid (Na), streptomycin (S), nitrofurantoin (F), gentamicin (Cn), chloramphenicol (C), penicillin (P), tetracycline (Te) and trimethoprim-sulfamethoxazole (Sxt).

facultative anaerobic, Gram negative, rod shaped, oxidase-positive and motile bacterium, which is grouped under the *Vibrionaceae* (Jagger, 2000). It is phenotypically related to the genus *Aeromonas*. It can be isolated from freshwater, freshwater fish and other animals such as cattle, goats, swine, cats and dogs. The infections caused by *P. shigelloides* are gastroenteritis, diarrhea, nausea, vomiting and septicemia for immune deficient patients (Ingram *et al.*, 1997). *P. alcaligenes* is a Gram negative, aerobic bacterium. The occurrence of *P. alcaligenes* as a pathogen is very rare.

Antibiotic resistance of the bacterial isolates

Seven isolates of *Aeromonas* spp. and one isolate of *P. alcaligenes* isolated from the water samples were tested for their antibiotic resistance towards eleven different types of antibiotics: ampicillin (10µg), norfloxacin (10µg), kanamycin (30µg), nalidixic acid (30µg), streptomycin (10µg), nitrofurantoin (300µg), gentamicin (10µg), chloramphenicol (30µg), penicillin (10µg), tetracycline (30µg) and trimethoprim-sulfamethoxazole (23.75µg). The antibiotic susceptibility was conducted on Mueller Hinton agar and a positive control strain (*E. coli* ATCC 25922) was included with the bacterial testing. The result of antibiotic resistant testing shows that all (100%) of the isolates were resistant towards ampicillin, gentamicin, and penicillin. The multiple antibiotic resistance (MAR) index of the isolates ranged from 0.455 to 0.727. Three different patterns of antibiotic resistance were observed among all the isolates. Antibiotics are chemicals extensively used for the treatment of fish disease and also for growth promoter for fisheries products (Yang *et al.*, 2004). The use of antibiotics in the fish feeds has led to the emergence of antibiotic resistant strains that can be pathogenic and cause infection in human.

According to Awan *et al.* (2009), most strains of *Aeromonas* tested were resistant to penicillin, sulfamethoxazole, trimethoprim and microlides, but susceptible to tetracycline, chloramphenicol, nitrofurantoin, aminoglycosides, cephalosporins, quinolone, colistin sulphate and trimethoprim-sulfamethoxazole. *Aeromonas* is one of the fish and human pathogens. The infection associated with *Aeromonas* will be no longer can be treated with these antibiotics. Continuous effort to search for new antimicrobial agents has been carried out to combat bacterial pathogens (Samuel *et al*., 2014). MAR index has been used in examining the antibiotic resistance and evaluating health risk (Riaz *et al*., 2011). MAR index for the isolates ranged from 0.455 to 0.727. It has been reported that MAR indexes for the isolates greater than 0.2 indicate that the source of samples is highly exposed to the contamination where antibiotics are widely used, for example area for treatment purpose and disease control (Paul *et al*., 1997). The analysis of MAR index of all isolates showed that all of the eight isolates were having MAR index greater than 0.2. This result suggests the risk with handling contaminated water in a fish facility. Besides that, three different patterns of antibiotics resistance were observed from the antibiotic susceptibility test suggesting the complexity of antibiotic resistance management associated with bacterial infection from the water source.

Conclusion

This study reported the physicochemical and microbiological aspect of water from the fish facility. The physicochemical parameter values of water samples were within the tolerable limit. Bacterial strains isolated from the water samples exhibits genetic sequence heterogeneity and multiple antibiotic resistance. The 16S rRNA sequencing revealed that Aeromonas veronii was abundantly found in the fish facility together with *Aeromonas jandaei, Pseudomonas alcaligenes* and *Plesiomonas*

shigelloides. This study suggests the risk associated with multiple antibiotic resistance bacteria in the water of fish facility and therefore, such facility should be monitored routinely to ensure the safety of the fish and the caretaker of the facility. Proper management of waste water from fish facility is important to minimise the distribution of MAR bacteria into the surrounding environment.

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