



Faculty of Resource Science and Technology

CHARACTERISATION OF *Serratia* SPP. FROM AQUACULTURE ENVIRONMENT AND RIVER WATER

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Characterisation of *Serratia* spp. from Aquaculture Environment and River Water

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This project is submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

I declare that this thesis entitled “Characterization of *Serratia* spp. from aquaculture environment and river water” is my original work. I have not copied from any other students’ work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.



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LIST OF ABBREVIATIONS

AK	Amikacin
AMP	Ampicillin
CAZ	Ceftazidime
CRO	Ceftriaxone
KF	Cephalothin
C	Chloramphenicol
CIP	Ciprofloxacin
DO	Doxycycline
CN	Gentamicin
IPM	Imipenem
K	Kanamycin
NA	Nalidixic acid
NOR	Nitrofurantoin
F	Norfloxacin
PRL	Piperacillin
S	Streptomycin
SXT	Sulfamethoxazole trimethoprim
TE	Tetracyclin
TOB	Tobramycin
%	Percentage
°C	Degree Celcius
μl	Microlitre

DNA

Deoxyribonucleotide acid

kb

Kilo base pairs

MAR

Multiple Antibiotic Resistance

MHA

Mueller Hinton Agar

Min

Minutes

V

Volt

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Characterization of *Serratia* spp. from Aquaculture Environment and River water

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ABSTRACT

Serratia spp. is one of the significant human pathogen. Some *Serratia* spp. especially *Serratia marcescens* contain a lot of human clinical infection history as well as interesting taxonomic, military experimentation and medical experiment. Currently, *Serratia marcescens* and *Serratia liquefaciens* have been isolated as causative agents of numerous outbreaks and opportunistic infections. As the species appear to be common environmental organisms, large number of nosocomial infections due to these bacteria can be explained. Thus, molecular characterization of several *Serratia* spp. become the main objective of this research as an approach to gain better identification and characterisation of various of *Serratia* spp. In this study, antibiotic susceptibility and genetic diversity of *Serratia* spp. isolated from aquaculture environment and river water of Rayu river were determined. The levels of resistance of the *Serratia* isolates towards nineteen antibiotics were tested. These isolates displayed resistance towards most of the antibiotics tested but the level of resistance vary among the species. Genetic characterization of *Serratia* spp. were carried out by using GTG₅-PCR which clustered the *Serratia* isolates into three groups based on DNA fingerprinting by using Paleontologi statistic software. The results of this study suggest that the *Serratia* isolates from aquaculture environment and river water derived a mixture of sensitive and resistant strains with diverse genetic contents.

Keywords: *Serratia* spp., nosocomial infection, antibiotic susceptibility, GTG₅-PCR, DNA fingerprinting.

ABSTRAK

Serratia merupakan salah satu daripada patogen manusia yang penting. Beberapa spesies *Serratia* terutamanya *Serratia marcescens* mengandungi banyak sejarah jangkitan klinikal manusia serta taksonomi menarik dan kajian dalam bidang perubatan. Sejak kebelakangan ini, *Serratia marcescens* dan *Serratia liquefaciens* telah dikenalpasti sebagai agen penyebab pelbagai wabak dan jangkitan oportunistik. Disebabkan persamaan spesies ini dengan organism di sekeliling menjadi semakin ketara, sebilangan besar jangkitan nosocomial yang dibawa oleh bacteria ini dapat dijelaskan. Oleh itu, pencirian molekul beberapa spesies *Serratia* menjadi objektif utama kajian ini sebagai pendekatan untuk mendapatkan pengenalan yang lebih baik dan pencirian tentang pelbagai spesies *Serratia*. Dalam kajian ini, kerintangan antibiotik dan kepelbagaian genetik spesies *Serratia* yang diasingkan daripada akuakultur dan air sungai Sungai Rayu telah dikenalpasti. *Serratia* mempunyai rintangan terhadap antibiotik yang diuji tetapi tahap rintangan antara spesies tersebut adalah berbeza. Pencirian genetik spesies *Serratia* telah dijalankan dengan menggunakan GTG₅-PCR. GTG₅-PCR telah mengkategorikan spesies *Serratia* kepada tiga kumpulan berdasarkan cap jari DNA dengan menggunakan perisian. Keputusan kajian ini menunjukkan bahawa *Serratia* yang diasingkan daripada persekitaran akuakultur dan air sungai Sungai Rayu diperolehi daripada campuran strain jenis sensitif dan rintangan dengan kandungan genetik yang pelbagai.

Kata kunci: spesies *Serratia*, jangkitan nosocomial, kerintangan antibiotik, GTG₅-PCR, cap jari DNA.

1.0 INTRODUCTION

Serratia spp. is a Gram negative, bacillus shaped bacteria that belongs to the family Enterobacteriaceae, named after the Italian physicist Serafino Serrati. The genus *Serratia* is comprised of a group of bacteria that are phenotypically related by DNA sequence. Some species and biotypes of *Serratia* produce prodigiosin which is a nondiffusible red pigment (William and Qadri, 1980). In the first edition of the Prokaryotes, Grimont and Grimont (1981) mentioned five species of *Serratia*. Few years later, seven species of *Serratia* were mentioned in Bergeys Manual of Systematic Bacteriology (Grimont & Grimont, 1984). Presently, there are ten species that are known to belong to the genus *Serratia* (Grimont & Grimont, 2006).

Serratia spp. are ubiquitous inhabitants of soil, air and water. They are commonly associated with food raw materials and give many implications in the spoilage of various foods of plants and animal origin. In percent, foodborne health hazard is highly associated with this species. The occurrence of outbreak of *S. marcescens* involving 4 childrens were detected in the neonatal intensive care unit (NICU) of the University Hospital of Zurich. It was revealed that the outbreaks was caused by three genetically unrelated strains of *S. marcescens* in contaminated milk (Fleisch *et al.*, 2002).

Nowadays, many researches conducted by researchers to familiarize with the outbreak of *S. marcescens* in order to minimize the outbreak to the patients. The virulence-associated properties and the increased number of documented infections caused by *Serratia* strains become the main concern of this research. The difficulties in identification of these bacteria by commercial systems also began to urge for further characterization of these species to get

more detail investigation regarding their molecular characteristics, antibiotic susceptibility, and DNA fingerprinting of this genus.

In this study, *Serratia* spp. that were isolated from aquaculture environment and Rayu river that kept in the Microbiology Culture Collection, Unimas, were characterized by antibiotic susceptibility testing by using Disc diffusion method. Further molecular method was carried out by using GTG₅-PCR.

Objectives

1. To characterize *Serratia* spp. from aquaculture environment and river water by using GTG₅-PCR.
2. To determine the antimicrobial resistance patterns among the *Serratia* spp.

2.0 LITERATURE REVIEW

2.1 Rayu River, Kubah National Park

Rayu River is also known as Sungai Rayu. It is a stream in the region of Sarawak with an average elevation of 1 meter above sea level (CWA, 2011). The area is mildly densely populated with 308 people per km². The Rayu river's water is clear and is known as rich in a variety of fish fauna (Doi *et al.*, 2001). Rayu River trail run through two famous recreation parks in Sarawak which are Kubah National Park and Matang Wildlife Centre. Kubah National Park is a tropical rainforest national park in the Kuching Division of Sarawak which covers an area of 2230 hectares and is dominated by a sandstone plateau that juts out to a height of 450 meters. Among the peaks here are Mount Serapi, Mount Selang and Mount Sendok. Matang Wildlife Centre is a wildlife centre at the western corner of Kubah National Park. It covers an area of 179 hectares of lowland forest. The forest promotes breeding and rehabilitation of uncommon animals such as Red-Haired Apes (orang utan) originating from Borneo and Sumatra. There is also a long house known as *Rumah Juqad* situated along Rayu River bank. Human activities, bathing and washing are carried out at or near the Rayu River stream.

2.2 Description of species

2.2.1 Family Enterobacteriaceae

Enterobacteriaceae species are among the most common nosocomial pathogens that caused serious infections in various organs and tissues. They are often referred as “enteric”. This family consists of a large number diverse organism. Members of family Entrobacteriaceae are gram negative, non-spore forming, and facultative anaerobic bacilli (Health Protection Agency, 2010). They appeared as coccobacilli or straight rods on gram-stained smear. The members of enterobacterial are normally found in soil, water, gastrointestinal tracts of human and animals. Several genera are grouped into this family which included *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Yersinia*, *Erwinia* and *Serratia* (Tortora *et al.*, 2007).

2.2.2. Genus *Serratia*

The most common species of genus *Serratia* is *Serratia marcescen*. This species is a bacterial species that is able to be distinguished by its production of red pigment. This species can be found on catherers, in saline irrigation solutions, and in other supposedly sterile solutions in hospital. The contaminations cause many urinary tract and respiratory tract infections in hospital (Tortora *et al.*, 2007).

2.2.3 Common species of genus *Serratia*

There are about 42 species that are related to genus *Serratia*. The most common species comprised of *S. marcescens*, *S. liquifaciens*, *S. proteamaculans*, *S. grimesii*, *S. plymuthica*, *S. rubidaea*, *S. odorifera*, *S. ficaria*, *S. entomophila* and *S. fonticola* (Grimont & Grimont, 2006). All the species have been frequently isolated from clinical samples except *S. entomophila*. Among all the species, *S. marcescens* is recognized as an opportunistic pathogen that act as an important nosocomial pathogen that capable of causing several diseases such as pneumonia, intravenous cathetera associated infections, urinary tract infections, osteomyelitis and endocarditis.

2.2.4 *Serratia* spp. in aquaculture environment and river water

Serratia spp. is one of the inhabitants in aquaculture environment and river water. Some of the *Serratia* spp. were recognized as fish pathogens that cause disease to the fishes. In the research by McIntosh & Austin (1990), *S. liquifacien* was recognized as one of the potential fish pathogen of atlantic salmon. The infection caused histopathological changes in the organ tissues of diseased fish. Another species that can be found in aquaculture environment is *S. plymuthica*. According to Nieto *et al.*, (1990), this species have been isolated from fresh water and fish may be a potential opportunistic pathogen for animals and humans.

2.3 Antibiotic susceptibility

Antibiotics is the substances that destroy or inhibit the growth of microorganism, particularly disease producing bacteria. Antibiotic susceptibility is the test that performed by the growth of an isolate in the presence of a given antibiotic. This test is a very important issue in dealing with infections particularly when there is a rapid spread of disease in community. There are several methods available for antimicrobial susceptibility testing. The most common methods included disc diffusion test and minimum inhibitory concentration method. Disc diffusion method as proposed by Kirby-Bauer and Stokes (1977) as mention in article by Lalitha (2004) were used to test the antibiotic susceptibility of the *Serratia* spp. This is the most common method that used for antimicrobial testing for pathogenic enterobacteriaceae. The strains of the bacteria will be cultured in Mueller-Hinton agar to check for their resistance against the antibiotic disc. Several antibiotics namely ampicillin, gentamicin, nalidixic acid, norfloxacin, nitrofurantoin, pefloxacin, cotrimoxazole, ciprofloxacin and chloramphenicol are used to test susceptibility of enterbacteriaceae.

In the previous study by Sethuraman *et al.* (2011), the result of the study shown proved that ciprofloxacin is an effective antibiotic to treat the *S. marcescens* infected patients. A total of 222 *S. marcescens* isolate strains were used for testing antibiotic sensitivity using ampicillin, gentamicin, cefotaxime, chloramphenicol, amikacin, aztreonam, ceftazidime, cephalothin, and ciprofloxacin. Through the antibiotic disc diffusion method, the sensitivity was analyzed in the presence of zon of inhibition around the antibiotic disc. In the result, 222 strains of *S. marcescens* gave 100% of susceptible to ciprofloxacin and it gave different resistant spectrum to the other eight antibiotics.

2.4 Characterization of *serratia* spp. by molecular method.

2.4.1 (GTG)₅-PCR

(GTG)₅-PCR is one of the Rep-PCR. It is a molecular biology based method that is very suitable for rapid grouping and tentatively identification of microorganisms. DNA of eukaryotic and prokaryotic contains repetitive DNA elements distributed randomly over the genome. In rep-PCR primers that anneals to these repetitive elements are used. The primer GTG₅ (5'GTG GTG GTG GTG GTG 3') are very suitable for grouping microorganism at the species level (Mohapatra *et al.*, 2007). The PCR-products are separated using agarose gel electrophoresis and a species specific pattern is obtained. Cluster analysis of isolates with similar patterns will cluster together. Full identification can be achieved by sequencing a limited number of isolates from each group within the cluster. In the previous study by Martin *et al.* (2012), thirteen bacterial genera (*Arthrobacter*, *Bacillus*, *Hafnia*, *Lysinibacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Streptomyces*, *Rhizobium*, *Rummeliibacillus*, *Enterobacter* and *Microbacterium*) and the distinct species belonging to these genera were identified and characterized by using Rep-PCR.

2.4.2 RAPD-PCR

RAPD is a powerful technique used for genetic studies. The random amplified polymorphic DNA (RAPD) fingerprinting method based on PCR is a technique that uses a single oligonucleotide of arbitrary chosen sequences to prime DNA synthesis from pairs of sites to which it is partially matched. This technique has the advantage whereby the molecular preliminary information of the species studied is not necessary (Welsh & McClelland, 1990). The RAPD method is a useful technique that has been reported as a reproducible tool for monitoring complex microbial community. In the previous studies, RAPD-PCR has been successfully used for genetic fingerprinting and molecular typing for many species, including fingerprinting of *Serratia marcescens* (Hajezi *et al.*, 1997), *Lactococcus lactis* and *Lactobacillus* species such as *L. plantarum* and *L. paracasei* (Martín-Platero *et al.*, 2009).

2.4.3 ERIC-PCR

ERIC elements are repetitive sequence elements in bacterial genomic DNA. This technique is not arbitrary because the primer was designed to known target sequence. The primers used in this method will be complementary to the repetitive sequence that is highly conserved in the genome. There are several repetitive sequences that have been reported in bacterial genome. These sequences include enterobacterial repetitive intergenic consensus (ERIC) sequences, repetitive extragenic palindromic (REP) sequences, and BOX elements. The enterobacterial repetitive intergenic consensus (ERIC) sequences are present in many copies in the genomes of *Escherichia coli*, *Salmonella typhimurium*, and other enterobacterial species such as *Yersinia pseudotuberculosis*, *Klebsiella pneumonia* and *Vibrio cholera* (Hulton *et al.*, 1991). These elements are 126 bp long and highly conserved at the nucleotide level. A central core inverted repeat is also included. The position of ERIC elements in the genome of enterobacterial varies between different species. In previous studies, these elements has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic *et al.*, 1991).

3.0 MATERIALS AND METHOD

3.1 Revival of isolates

Isolates of *Serratia* spp. from aquaculture environment and river water were revived from the previous study done in Microbiology Laboratory Culture Collection, Unimas. A total of 31 isolates of *Serratia* spp. (Appendix A) were used in this study. Eight of the isolates were isolated from aquaculture environment from Samariang while 23 isolates were isolated from river water of Rayu River, Kubah National Park. About 100 µl of each isolates from glycerol stocks were pipetted into Luria bertani (LB) broth and incubated at 30°C for 24 hours.

3.2 Storage and preservation of *Serratia* spp.

Each of the bacteria culture was transferred onto a Nutrient Agar plate by streaking method. The plates were incubated at 30°C for 24 hours. One pure single colony was obtained from each incubated Nutrient Agar plate and streaked onto Nutrient slant agar. The nutrient slant agars with bacteria culture were stored at 4°C and were used as the working stocks. Before conducting any test, bacteria from slant agar were streaked on NA and incubated overnight at 30°C to obtained single colony. After that, the selected single colony was inoculated in LB broth. The broth culture were incubated overnight at 28°C and tests were carried out by using the samples from the broths.

3.3 Antibiotic Susceptibility test

The antibiotic susceptibility test was conducted by disc diffusion method with the use of the Mueller-Hinton Agar (MHA) as described by Kirby-Bauer and Stokes (1977) mentioned in article by Lalitha (2004). There were 19 types of antibiotics used in this study. These antibiotics include Sulfamethoxazole trimethoprim (SXT, 25 µg), Streptomycin (S, 10 µg), Chloramphenicol (C, 10 µg), Nalidixic acid (NA, 30 µg), Ciprofloxacin (CIP, 5 µg), Ceftazidime (CAZ, 30 µg), Doxycycline (DO, 30 µg), Amikacin (AK, 30 µg), Tobramycin (TOB, 10 µg), Norfloxacin (NOR, 10 µg), Gentamicin (CN, 10 µg), Ampicillin (AMP, 10 µg), Nitrofurantoin (F, 300 µg), Ceftriaxone (CRO, 30 µg), Cephalothin (KF, 30 µg), Tetracyclin (TE, 30 µg), Piperacillin (PRL, 75 µg), Kanamycin (K, 30 µg), and Imipenem (IPM, 10 µg). Well isolated colonies of the bacteria were selected from the nutrient agar plate. The colonies were transferred into a tube containing 4 ml of LB broth by using an inoculation loop. The broth cultures were incubated at room temperature for 24 hours until it achieved the turbidity of 0.5 McFarland standards. A sterile cotton swab was dipped into the broth culture. Then, it was swabbed onto the surface of MHA. After the swabbing steps, antimicrobial discs were dispensed onto the surface of the agar plate by using a sterile forcep. The antimicrobial disc were distributed evenly. Then, the agar plates were inverted and incubated at 30°C for 24 hours. After the incubation period, a clear zone around the antimicrobial disc was observed on the agar surface if the bacteria appeared to be susceptible towards the antibiotics. The clear zone or inhibition zone was measured by using a ruler. The diameter of the zone was measured to the nearest whole millimeter. The size of inhibition was interpreted into prefixed susceptible (S), intermediate (I) and resistant (R) categories by referring to the interpretation

table. *Escherichia coli* strain ATCC 25922 was used as a control in the antibiotic susceptibility test to validate the accuracy of the procedures.

3.3 DNA Extraction

Single colony of the bacteria isolates were transferred to 5 ml of LB broth and incubated at 30°C with shaking at 220 rpm for 18 to 24 hours. The bacteria DNA were extracted using a modified version of boiling-centrifugation method as described by Soumet *et al.* (1999). About 1.5 ml of bacterial culture was centrifuged for 5 minutes at 10,000 rpm and the supernatant was removed. The pellet was then reharvested with another 1.5 ml of the culture. It was then centrifuged at 10,000 rpm for 5 minutes. Next, supernatant was discarded. Four hundred microliters of sterile distilled water was added to the tube. The microcentrifuge tubes were then vortexed until the pellet was suspended throughout. It was boiled for 15 minutes and allowed to cool in ice for 10 minutes. Then, centrifugation was done in 10,000 for 10 minutes. The supernatants were then collected and put into a new tube before it was store at -20°C.

3.4 (GTG)₅- Polymerase Chain Reaction ((GTG)₅-PCR)

Genotypic characteristics of *Serratia* spp. was determined by using (GTG)₅-PCR which is a PCR amplification of repetitive bacterial DNA elements fingerprinting. (GTG)₅-PCR was conducted in order to detect the genetic diversity and genetic relatedness among the *Serratia* spp. that were collected from different sampling sites particularly aquaculture environment and river water. The primer used in this PCR was 5'-GTG GTG GTG GTG GTG-3'. This PCR was carried out as described by Versalovic *et al.* (1991), in a volume of 25 µl with 5 µl DNA template, 3.0 mM MgCl₂, 1.0 µl primer, 0.3 µl of Taq polymerase (Promega), 0.8 mM dNTPs and 5X PCR with a slightly modification as shown in Table 1. The PCR was carried out by using thermo-cycler (Eppendorf). The condirions for the (GTG)₅-PCR were as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 2 min, 1 min of annealing at 40°C, elongation for 2 min at 72°C and a final elongation for 10 min at 72°C. The parameter of (GTG)₅-PCR for amplification of bacteria isolate was summarized in Table 2.

Table 1: (GTG)₅- PCR mastermix

GTG- PCR Cocktails	Volume (μl)
Distilled water	9.9
Buffer	5.0
MgCl₂	3.0
dNTPs	0.8
Primer	1.0
Taq Polymerase	0.3
Template DNA	5.0
Total	25

Table 2: (GTG)₅-PCR parameter and condition

Parameter	Temperature (°C)	Time (min)	Number of cycle
Initial Denaturation	94	5	1
Denaturation	94	2	} 35
Annealing	40	1	
Extension	72	2	
Final Extension	72	10	1