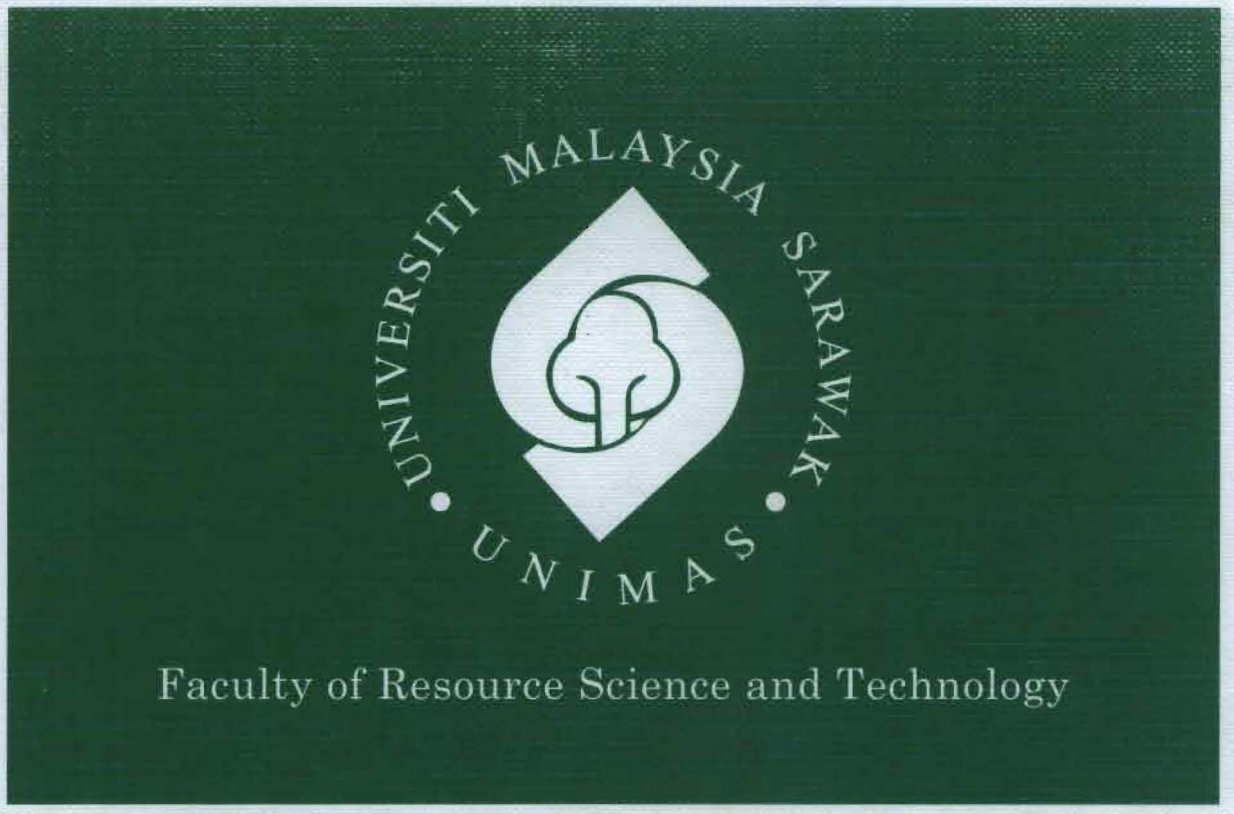


# CHARACTERIZATION OF *SERRATIA* SPP. FROM AQUACULTURE ENVIRONMENT AND RIVER OF KUBAH NATIONAL PARK

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| API | - Analytical Profile Index |
| CS | - Camp Site |
| $ddH_20$ | - distilled water |
| dNTPs | - dinucleotide |
| DNA | - Deoxyribonucleotide acid |
| EMB agar | - Eosin Methylene Blue agar |
| ERIC | - Enterobacterial Repetitive Intergenic Consensus |
| $MgCl_2$ | - Magnesium Chloride |
| MUCS | - Most Upper Camp Site |
| MR | - Methyl Red |
| NaCl | - Sodium chloride |
| LB broth | - Luria Bertani broth |
| PCR | - Polymerase Chain Reaction |
| RAPD | - Random Amplified Polymorphic DNA |
| SIM | - Sulfide Indole Motality |
| UCS | - Upper Camp Site |
| rpm | - Rotations Per Minute |
| Spp. | - Species |
| TBE | - Tris Borate EDTA |
| VP | - Voges-Proskauer |
| $^oC$ | - Degree Celsius |
| bp | - base pair |
| % | - Percentage |
| µl | -microlitre |

## LIST OF TABLES AND FIGURES

# Characterization of *Serratia* spp. from Aquaculture Environment and River of Kubah National Park

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## ABSTRACT

*Serratia* spp. is an opportunistic gram negative bacterium under the family of Enterobacteriaceae. Some species such as *Serratia marcescens* is related with the significant outbreak of nosocomial infections of severities in patients. In this study, *Serratia* spp. was isolated from aquaculture environment and the river. Biochemical tests comprised of IMViC and string test were conducted for the identification of bacteria isolates from the river water of Kubah National Park. The further identification of the isolates were carried out using API 20E kit. Genetic characterization of the isolates was carried out by ERIC-PCR to analyse their genetic diversity. Phylogenetic tree was constructed based on DNA fingerprinting using RAPD software to illustrate the relationship among the bacteria isolates from these two environments. Based on the phylogenetic tree dendrogram, all the isolates can be categorised into 3 major clusters of similarity. The phylogenetic tree created for DNA fingerprinting technique indicated that the population of the isolates in both environments are greatly diverse.

Keywords: *Serratia* spp., IMViC tests, API 20E kit, ERIC-PCR, DNA fingerprinting.

## ABSTRAK

*Serratia spp. adalah oportunis bakteria gram negatif di bawah keluarga Enterobacteriaceae. Beberapa spesies seperti Serratia marcescens berkait dengan wabak yang seperti nosocomial dalam kalangan pesakit. Dalam kajian ini, Serratia spp. diasingkan daripada persekitaran akuakultur dan sungai. Ujian biokimia yang terdiri daripada IMViC dan ujian String telah dijalankan untuk mengenal pasti bakteria yang akan diasingkan dari air sungai Taman Negara Kubah. Pengenalan pastian dan pengesahan lebih lanjut isolat bakteria telah dijalankan menggunakan API 20E kit. Pencirian genetik telah dijalankan menggunakan ERIC-PCR untuk menganalisis kepelbagaian genetic masing masing. Pokok filogenetik telah dibina berdasarkan cap jari DNA menggunakan perisian RAPD untuk menggambarkan hubungan antara strain bakteria daripada kedua-dua persekitaran. Berdasarkan dendrogram pokok filogenetik, semua pencilan boleh dikategorikan kepada 3 kelompok utama persamaan. Pokok filogenetik yang dicipta untuk teknik cap jari DNA menunjukkan bahawa populasi kedua-dua persekitaran adalah sangat pelbagai.*

*Kata kunci: Serratia spp., ujian IMViC, API 2E kit, ERIC-PCR, cap jari DNA*

# 1.0 INTRODUCTION

*Serratia* spp. is a gram negative, facultative anaerobic rod belonging to the family of Enterobacteriaceae in the tribe of *Klebsiella. Serratia* spp. comprised of approximately 13 described DNA related species. The most common studied of *Serratia* spp. is *Serratia marcescens.* According to the studies done by Grimont and Grimont (1984), they claimed that *Serratia* spp. showed negative result on Indole test and consequently brought into lecithinase, lipase production and DNAse positive.

S*erratia* spp. can be found in environment, rodent gut and in the human intestinal tracts (Grimont & Grimont, 1984). *Serratia marcescens* is described as nosocomial and opportunist pathogens that can cause outbreak to the patients. The earliest outbreak of the nosocomial infection by *S. marcescens* was identified at Stanford University Hospital claiming that 11 cases had occurred in 6 months period (Wheat *et al.*, 1951).

*Serratia* spp. has developed a significant concern in causing chronic diseases due to its wide distribution in the environment. *S. marcescens* is regarded as an aetiological agent that can cause infections in the respiratory tract, urinary tract, septicaemia, meningitis and wound infection to the patients (Hejazi and Falkiner, 1997). It has also reported that *S. marcescens* can cause infective endocarditis to the patients in the hospital. The cause of outbreak on the contaminated blood products of patient by *Serratia* spp. was due to the contaminated fluid and devices.

Hence, the objectives of this study are:

1) To isolate *Serratia* spp. from water and sediment samples from Sungai Rayu, Kubah National Park.

2) To identify the isolates of *Serratia* spp. by performing biochemical tests and API 20E kit.

3) To characterize the isolated strains by using DNA fingerprinting.

# 2.0 LITERATURE REVIEW

## 2.1 Taxonomy of Genus *Serratia* spp.

The Genus *Serratia* spp. is categorised under the family of Enterobacteriaceae, in the tribe *Klebsiella* which comprised of a group of bacteria that associated with DNA sequence. *Serratia* spp. is unique with the production of three hydrolytic enzymes namely lipase, gelatinase, and DNAse. The generic name *Serratia* were used since 1920s by the physicist named Serafino Serrati.

There are about 42 species related with the genus *Serratia* spp. The most familiar *Serratia* spp. consisted of 10 genus namely *S. marcescens, S. liquefaciens, S. plymuthica, S. orodifera, S. ficara, S. rubidaea. S. grimesii, S. proteomaeulans, S. entomophilia* and *S.fanticola.* The prominent feature to distinguish *Serratia* spp. and the rest of bacteria is by the resistance against colistin and cephalothin. *S. marcescens* is the central member of *Serratia* spp. associated with a variety of human nosocomial infection (Koneman *et al.*, 1997). *S. marcescens* was devised by Bartolomeo Bizio who gave the name *'marcescens'* which was associated with the case of food contamination (Sehdev, 1999).

**2. 2 *Serratia* spp. in environment.**

*Serratia* spp. is widely distributed in the water, soil, plants, animals and human intestinal tract. They are opportunist human pathogen that was isolated from human infections. The probability of *Serratia* spp. disease's outbreak and the parasite manifestation can be found in the aquaculture environment with the rise in fish production. The existence of *S. marcescens* in soil was due to the role in biological cycle of metals in mineralizing organic iron and dissolving gold and copper. The mineralization was ascribed to cold tolerant of *Serratia* spp. interrelated to the low-moor (Bassalik, 1963).

According to the studies done by Agbalika and the coworkers, they have concluded that *S .marcescens* attributed to the higher percentage of 150 strains of *Serratia* spp. in the river. In the studies, they have isolated *Serratia* spp. in river water and concluded that 75% were accounted for *S. liquefaciens* ( 11%), *S. proteamaculans* ( 8%), *S. grimesii* (5%) and *S. plymuthica* ( 1% ). The reports on the outbreak were caused by the sources of contaminated parenteral infusions and injections, environmental sources and the usage of contaminated faucets, air conditioner and respiratory equipment on the species (Laupland and Church, 2005).

## 2. 3 Morphology and Identification of *Serratia* spp.

The appearance of *Serratia* spp. is straight rod ranging from the size of 0.5-0.8 um X 0.9-2.0 um. *S. marcescens* produced a red pigment that was insoluble in water called prodigiosin. The pigmented bacterium can be found in various ecological niches including soil, water, air plant and animal. According to the survey of respiratory tract isolates done by Hornick *et al.* (1991), they stated that approximately 17% of the *Serratia* spp. were belonged to type 3. Majority of the *Serratia* spp. were non-capsulated and the appearance of *S. plymuthica* was appeared in mucoid colonies which differ them from the the rest of the *Serratia* spp.

*S. marcescens* and *S. rubidaea* are the two bio group that will produce prodigiosins in 20 $^{0}$C to 35 $^{0}$C. The appearance of the colonies of the bacteria was red in color and produced fishy urinary smell. In the recent studies done by Ajithkumar *et al.* (2003), they claimed that the spore forming strains of *S. marcescens* was successfully isolated from the domestic wastewater. DNA homologies and many intense biochemical comparison studies were done with other groups on the proliferation of species within the genus.

## 2. 4. Epidemiology and Infections of *Serratia* spp.

The most common *Serratia spp.* that associated with the outbreak on human is *S. marcescens.* They are able to survive at the low temperature and abide by the plastic condition that provides a reservoir for the infection. *S. marcescens* can inhabit premature neonates although they not in an adult state (Christensen *et al.*, 1982). The application of the disinfectant cleansing solution can convey the spread of the disease. The spread can be directed through contact with hands although *Serratia* spp. was rarely identified on hands (Christensen *et al.,* 1982). This can clinch that patient to patient transmission can occur due to not sufficient hygienic practice. The presence of *S. phymuthica, S. ficaria and S. proteamaculans* were originated from the contamination from the soil and plants.

Several reports concerning the outbreaks of severe diseases and clinical experiences were reported to distinguish *Serratia* spp. on the prevalence nosocomial infections. According to the studies done by Laupland and Church (2005), they highlighted that the preeminent method to state the epidemiology of all infectious disease were the population based on the study the entire episode incurring in hospitalized and non-hospitalised residents. Thus, two recent studies in Canada and Australia were conducted to investigate the incidence of *Serratia* spp. infections at the population level to provide a new standpoint on the epidemiology. According to Newport *et al.* (1985), they stated the epidemiology typing methods for *Serratia* spp. were comprised of serotyping, bacitracin susceptibility, phage typing and biotyping, antibiograms. The further molecular technique of polymerase chain reaction of repetitive intergenic consensus sequences were also conducted (Lin *et al.,* 1994).

## 2. 5 Detection of *Serratia* spp. by biochemical tests

According to Grimont and Grimont, (1984), the biochemical test of Indole test on *Serratia* spp. showed negative result. Standard biochemical tests were performed on the *Serratia* spp. indicated the production of lecithinase, lipase and gelatinase and DNAse were positive (Grimont & Grimont, 1984). *Serratia* spp. are facultative anaerobes and can be grown on a variety of non-selective and selective agar media at temperature ranging from 25 $^{0}$C to 37 $^{0}$C.

According to the studies by Singh *et al.* (2011), they claimed that *Serratia* spp. have formed a white sticky colony and no spore was found. They appeared to be in a rod-shape, pH ranging from 6 to 8, catalase positive, amylase negative, protolytic positive, and citrate utilization test were positive. Methyl Red test (MR) reduction have shown a negative result but Voges Proskauer test (VP) shown the positive result. This can be proven by the colour changes of the medium after addition of reagent. Nitrate production showed a positive result, cellulose production showed positive and $H_2S$ production showed negative results. *Serratia* spp. was a weak lactose fermentor and were considered unique among all the Enterobacteriaceae (Laupland and Church, 2005).

The further identification of *Serratia* spp. can be done by API 20E kits that have grown on the nutrient agar. API 20E bacterial identification system has been applied to identify the members of the family Enterobacteriaceae. API 20E kits comprised 10 genera and 17 known species of bacteria include *Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Pantoea, Providencia, Pseudomonas, Raoultella and Serratia.* The 20 biochemical test reactions on the strip were converted into an octal profile number. Each profile number was then decoded by using the Analytical Profile Index for the identification of the isolates.

## 2. 6 Detection of *Serratia* spp. by molecular tests

Polymerase chain reaction (PCR) technique was applied to selectively replicate a particular DNA region to produce a large amount of DNA at a particular time (Lawrence, 2000). PCR-based methods combined reproducibility and discriminatory power with the speed and ease of PCR (Patton *et al.*, 2001). DNA fingerprinting strategy was conducted based on the PCR amplification of variable-length chromosomal sequences with a variety of primers.

The random-amplified polymorphic (RAPD) DNA assay was based on the application of simple arbitrary primers in a PCR of low stringency. The technique has shown an amplification of the segments of the genome and has been used successfully done for the typing of several bacterial species. On the other hand, Versalovic *et al.* (1991) had utilized consensus primers in the PCR to amplify DNA sequences located between successive repetitive elements such as the 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence in the gram-negative bacteria. This method has shown the potential for subtyping gram-negative enteric bacteria.

# 3.0 MATERIALS AND METHOD

## 3.1 Sample Collections

The water and sediment samplings were conducted in Sungai Rayu, Kubah National Park. Samples were collected from four different spots comprised of LCS (Lower Camping Site), CS (Camping Site), MUCS (Most Upper Camping Site) and UCS (Upper Camping Site). The samplings were done twice in one week. One hundred and twenty-two bacteria isolates were preserved from the Kubah sampling in Sungai Rayu. There were 66 isolates gained for the first sampling while 56 isolates for the second sampling.The samplings for the water and sediment samples were conducted in 2 locations on each spot of the Sungai Rayu. Samples were stored in ice box containing ice during transportation to the laboratory, FSTS, UNIMAS. Samples were transported within two hours after sampling time. Samples were then immediately processed upon reaching laboratory.

### 3.2 Revival of *Serratia* spp. from aquaculture environment

The samples of *Serratia* spp. from aquaculture environment were revived from the previous student experiment done from microbiology laboratory culture collection, Unimas. Eight samples were taken for aquaculture environment which were isolated from Samariang and the $7^{th}$ mile aquaculture ponds. The samples were taken from the glycerol stock and a loop of the bacteria was streaked on a nutrient agar and incubated for 24 hours at 37°C. A colony of the bacteria was then enriched on the LB broth for overnight at 37°C. The bacteria from the LB broth were then preceded with the DNA extraction.

### 3. 3 Sampling processing and Isolation of *Serratia* spp.

The sample processing was conducted by diluting in a 10-fold serial dilution and the sediment samples from $10^{-1}$ until $10^{-2}$. The water and sediment samples were collected in sterile 100 ml Schott's bottles. About 100 µl of each dilution were spread on Eosine Methylene Blue (EMB) agar and incubated for 24 hours at 37°C. The colonies grown on the EMBA were counted. The samples were labelled S and W for the sediment and water respectively. Eight bacteria colonies were isolated for each type of samples. The colonies were re-streaked on EMBA for few times until pure single colonies were obtained.

### 3.4 Storage and Preservation of *Serratia* spp.

The pure bacteria isolates were stored in the 20% glycerol stock and kept at -20°C. The bacteria that did not grow on the EMB agar during revival from the glycerol stock were enriched in the Luria Bertani (LB) broth. One hundred microliter of each of the bacteria isolate were pipetted into the LB broth and incubated at 37$^0$C for 24 hours. The overnight culture was streaked on nutrient agar until single colonies were obtained. After incubation for 24 hours at 37$^0$C, a colony of the pure isolate each were streaked onto the slant agar. Bacteria cultures on slant agar were used as the working stock. Before conducting further test, bacteria from slant agar were streaked on NA to obtained single colony.

### 3.5 Identification of *Serratia* spp.

#### 3.5.1 Biochemical tests (IMViC) for Kubah sampling

The biochemical tests that were conducted for the identification were the citrate utilization test, Methyl Red-Voges Proskeur (MRVP) test, SIM test and string test.

##### 3.5.1.1 Citrate Utilization Test

Simmon's Citrate agar media was used to test the ability of bacteria to utilise citrate. This media contained sodium citrate of carbon and nitrogen source as well as pH indicator of bromothymol blue. The citrate test was performed by inoculating slant from a colony of bacteria with inoculating loop. It is selective for bacteria to consume citrate as the sole source of carbon and ammonium as sole nitrogen source. It was then incubated at 37°C for 24 to 48 hours. The positive result of Citrate test is indicated by a royal blue colour at the end of the experiment.

### 3.5.1.2 Methyl Red- Voges–Proskauer (MRVP test)

Single colony of the each isolates was first cultured into the sterile LB broth. Methyl Red-Voges–Proskeur(M R/ VP) test were conducted by the autoclaved broth of MRVP powder and distilled water. A loop of isolate were inoculated in 10mL of each MRVP broth and left incubated in the shaker for overnight. The tests were separated into two parts which is 5 ml each for MR (Methyl Red) and 5 ml to VP (Voges Proskaeur) test respectively.

The Vogus Proskeur test was done based on the conversion of acetyl methoy carbinol to diacetyl through the act of potassium hydoxide and atmospheric oxygen. The production of acetoin and butylene glycol by glucose fermentation is an important biochemical property used for the identification of *Klebsiella, Enterobacter, and Serratia*. The VP tests required only 24 hours of incubation where MR required about 48 hours of the incubation period. The VP tests were done by adding 6 drops of Barritt's reagent A and 3 drops of Barritt's reagent B. It was then shake gradually for 30 seconds until formation of pink to red color is seen. The formation of the red colour indicated the positive result. Five drops of methyl red solution were added to MR and an immediate red reaction signified positive test of methyl red and the yellow colour signified negative results.

### 3.5.1.3 SIM test

The SIM medium tests consisted of sulphide- indole- motility medium. The medium was inoculated by stab method in the SIM tubes and incubated for about 24 hours. The motility test was done by the stabbing a straight hole through the medium using a straight wire with the bacteria on and left it for incubation for 24 hours. The migration of bacteria away from the stab line indicated the motility test was positive and presence of appearance of turbidity throughout the medium. The turbidity can be viewed by the diffusion along the stab line. The bacteria were considered to be motile when the medium has shown a fairly turbid throughout it. The bacteria were not motile when the appearance of the medium is clear.

The Indole portion of the test was performed by adding Kovac's reagent to the inoculated medium. The Indole test was performed on the bacteria isolate to check that the production of tryptophan in the enzyme tryptophanase to produce indole. The positive reaction of the Indole test was the formation of red colour after adding 3 drops of Kovac reagent and negative reaction will show no red colour changes after the addition of the reagent. Free indole detection was done by *p*- dimethylamino-benzaldehyde in which react with indole to formed red colured complex.

The $H_2S$ test was performed to determine the ability of the isolates to reduce sulfur containing compounds to sulfide during the metabolism process. The SIM medium for the $H_2S$ test contained sulfates served as the substrate for detecting sulfide production. The isolates reduced sulfur to hydrogen sulfide by the black precipitation in the medium indicated the positive test.

### 3.5.1.4 String test

The string test was performed on one hundred and twenty-two isolates to categorize the isolates into gram negative or gram positive. The isolates were streaked on the NA agar and left incubated for 18 to 24 hours at 37°C. A loop of single colony was picked and 10 µl of NaOH was mixed on the area of microscope slide. The mixing was done until a mucoid appearance was formed when the loop was slowly withdrawn from bacteria isolate indicated the positive result.

### 3.5.2 API 20E kit

The API 20E System was performed on the bacteria isolates. The API 20E kit comprised of plastic strips that contained 20 miniature tubes for the identification of bacteria. It has the widespread database of characteristic biochemical reactions of micro-organisms and standardized. The test was done by the preparation of suspension bacteria in the saline solution. The inoculation of a colony of pure bacteria is done into the 0.85% NaCl to homogenize the suspension. The bacteria suspension was inoculated into each well with sterile pipette. It was incubated in the chamber over the bottom position at 37° C for 18 to 24 hours. After the incubation, a few reagents were added to produce the changes of the kit according to the bacteria isolates. The regents used were a drop of TDA reagent, a drop of James Reagent for the Indole test, a drop of each VP1 and VP2 for the VP test and waited for 10 minutes for the color changes and lastly a drop of NIT 1 and NIT 2 drops each on the Glu tests. The results were recorded and analyzed manually or by the computer software.