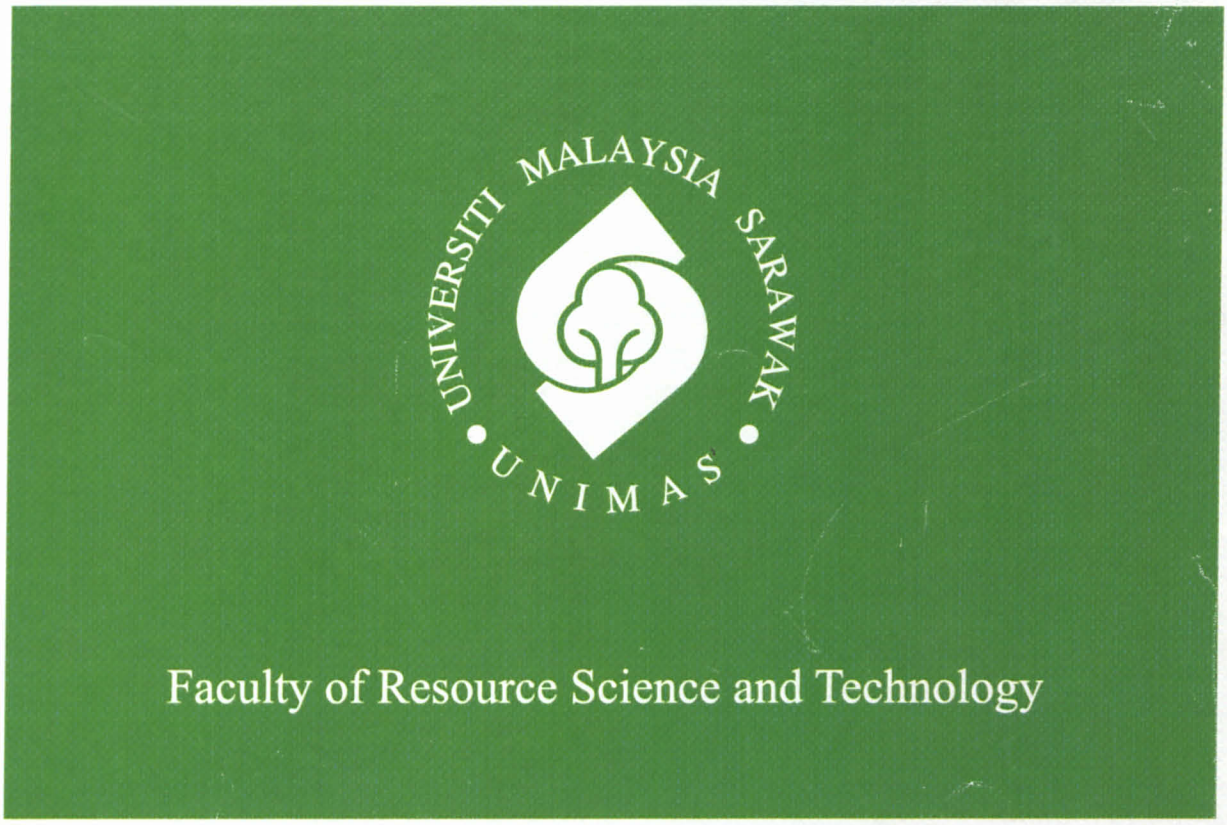

CHARACTERIZATION OF ENTEROBACTERIACEAE FROM WILD ANIMALS AND WATER IN CONSERVATION AREA IN SAREMAS PLANTATION

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(35658)

Bachelor of Science with Honours
(Resource Biotechnology)
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Characterization of Enterobacteriaceae from Wild Animals and Water in Conservation Area in Saremas Plantation

Chai Shin Nei (35658)

This project is submitted in fulfillment of the requirement for the Degree of Bachelor of Science with honours
(Resource Biotechnology)

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List of Abbreviation

ADH	Arginine DiHydolase
API 20E System	Analytical Profile Index 20E System
CIT	Citrate Utilization
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate
EDTA	Ethylene Diamine Tetraacetic Acid
EMB	Eosin Methylene Blue
GEL	Gelatin Hydrolysis
H_2S	Hydrogen Sulfide
LDC	Lysine Decarboxylase
LPS	Lipopolysaccharide
$MgCl_2$	Magnesium Chloride
NA	Nutrient Agar
NB	Nutrient Broth
ODC	Ornithinine Decarboxylase
PCR	Polymerase Chain Reaction
SDW	Sterile Distilled Water

SIM	Sulfide-Indole-Motility
TBE	Tris-Borate-EDTA
TSA	Tryptic Soy Agar
TSI agar	Triple Sugar Iron Agar
URE	Ureaase
UTIs	Urinary Tract Infections
VP	Acetoin Production (Voges Proskauer)
µm	Micrometer
µl	Microlitre

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Characterization of Enterobacteriaceae from Wild Animals and Water in Conservation Area in Saremas Plantation

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ABSTRACT

The Enterobacteriaceae are gram-negative straight rod microorganism with capabilities of causing certain diseases, such as urinary tract infections (UTIs), diarrhoea and often fatal complication will occur if the Enterobacteriaceae penetrate into bloodstream and spread throughout whole body. Thus, this creates a critical public health concern. In this study, Enterobacteriaceae from wild animals and water in conservation area in Saremas Plantation, Miri, Sarawak were characterized using conventional biochemical tests, API 20E System and $(GTG)_5$–PCR fingerprinting. Series of conventional biochemical tests were conducted, including Gram staining, Sulfide Indole Motility (SIM) medium, Kligler Iron Agar (KIA), Triple Sugar Iron (TSI) agar and oxidase test. Bacteria were grouped into two major clusters and various sub-clusters through $(GTG)_5$–PCR analysis and dendrogram. There was a chance for bacteria distributed from wild animals into water and causes public health hazard to community within conservation area in Saremas Plantation. API 20E system revealed thirteen (54.2%) isolates with good identification level and above. The identification of the bacterial isolates using API 20E system via APIweb™ revealed seven bacterial species, including *Enterobacter cloacae, Serratia marcescens, Serratia rubidaea, Citrobacter freundii, Rahnella aquatilis, Pantoea* spp. 1 and *Pantoea* spp. 4.

Keywords: Enterobacteriaceae, API 20E system, $(GTG)_5$ –PCR fingerprinting

ABSTRAK

Enterobacteriaceae adalah gram-negatif lurus rod mikroorganisma yang mampu mengakibatkan penyakit tertentu, seperti jangkitan saluran kencing (UTIs), cirit-birit dan komplikasi sering membawa maut akan berlaku jika Enterobacteriaceae menembusi ke dalam aliran darah dan merebak ke seluruh badan. Oleh itu, masalah tertentang kesihatan awam yang kritikal tertimbul. Dalam kajian ini, Enterobacteriaceae terdapat daripada haiwan liar dan air dalam kawasan pemuliharaan di Saremas Plantation, Miri, Sarawak telah dicirikan dengan menggunakan ujian biokimia konvensional, API Sistem 20E dan $(GTG)_5$–PCR fingerprinting. Siri ujian biokimia konvensional telah dijalankan, termasuklah Pewarnaan gram, Sulfida indole motilitas (SIM), Kligler Besi Agar (KIA), Tiga Gula Besi (TSI) agar dan oxidase ujian. Bakteria dikumpulkan ke dalam dua kelompok utama dan pelbagai sub-kelompok melalui analisis (GTG) 5-PCR dan dendrogram.Bakteria dapat diedarkan daripada haiwan liar ke dalam air dan membahayakan kesihatan orang ramai di dalam kawasan Saremas Plantation. API 20E mendedahkan tiga belas (54.2%) backteria diasingkan dengan tahap pengenalan yang baik atau lebih baik. Pengenalpastian isolat bakteria dengan menggunakan API Sistem 20E melalui APIweb™ telah mendedahkan tujuh spesies bakteria, termasuklah Enterobacter cloacae, Serratia marcescens, Serratia rubidaea, Citrobacter freundii, Rahnella aquatilis, Pantoea spp. 1dan *Pantoea* spp. 4.

Kata kunci: Enterobacteriaceae, API Sistem 20E, $(GTG)_5$–PCR fingerprinting.

1.0 Introduction

The Enterobacteriaceae are a large family of gram-negative straight rod bacteria (Ryan & Ray, 2004). These microorganisms are facultative anaerobic which means they have the capability to grow under aerobic as well as anaerobic environment. These microorganisms can exist in various environments such as soil, water, sewage, plants, and food. Besides, most of them can be found in the intestine of human and animals (Hesta *et al.*, 2013).

The Enterobacteriaceaeferment glucose to produces lactic acid and other end products. In addition, these bacteria also reduces nitrate to nitrite(Ryan & Ray, 2004). Mostly they are motile by means of peritrichous flagella except for *Shigella, Tatumella, Klebsiella* and some other immotile members of this family (Baylis *et al.*, 2001). These kinds of bacteria usually are non-capsulated except *Klebsiella.*

This family includes a number of members which are opportunistic pathogens and potentially cause disease, such as gastroenteritis, diarrhea, vomit, urinary tract infections (UITs) and inflammatory bowel disease (Hetsa *et al.*, 2013). *Yersinia enterocolitica, Salmonella spp., Shigella spp.* and pathogenic *Escherichia coli* including *E.coli* O157:H7 are treated as vital foodborne pathogens and able to arouse disease in the intestines (Baylis *et al.*, 2001). Apart from that, some of these bacteria also associated with food spoilage and thus generally have a negative impact on the economy especially for agricultural and food industries (Baylis *et al.*, 2001).

There are two main methods for characterizing the Enterobacteriaceae which are phenotype and genotype analysis. Phenotype analysis involves morphological analysis and chemotaxonomy whereas DNA sequencing and gel electrophoresis are grouped in the genotype analysis (Teramoto, 2010). Furthermore, the advantages by utilizing PCR-based

fingerprinting for detection and characterization of these microorganisms are rapid, highly sensitive, and shorter time required for bacterial identification.

According to Teramoto (2010), there is a need for rapid characterization of these bacteria, especially in certain fields, such as clinical diagnosis, food inspection and environmental energy since they are extremely active and possess high survival rate. Treatment of bacterial infection and trace-back of disease outbreaks also have greatly impelled the development of various characterization methods. Moreover, this study seeks to create general public awareness toward the health problems raised from the pathogenic members of Enterobacteriaceae to community within Saremas Plantation.

The objectives of this study were to:

1. Characterize the Enterobacteriaceae isolated using conventional biochemical tests and API 20E system.
2. Investigate the genetic relatedness among the bacteria isolates from the conservation area within Saremas Plantation.

2.0 Literature Review

2.1 Characteristic of Enterobacteriaceae

The Enterobacteriaceae members are small gram-negative filamentous rods microorganisms with 2 to 4 μm in length and 0.4 to 0.6 μm in width (Ryan & Ray, 2004). Most of them have morphologically similar components, including cell wall, cell membrane, pili and some other internal structures. O antigen is the outer membrane lipopolysaccharide (LPS) whereas K antigen is known as the cell surface polysaccharides that may form capsule or a slime layer (Ryan & Ray, 2004). In addition, H antigen is termed as the peritrichous flagella which extend well beyond the cell wall and causes them to be motile (Ryan & Ray, 2004). Most of the member of Enterobacteriaceae are motile and posses the peritrichous flagella. However, some species are non-motile such as *Shigella* and *Klebsiella* species (Sanders & Fryer, 1988).

Most of them grow readily at 35 °C except *Yersinia* which grows well at 25 °C to 30 °C. They can be either inhabit the intestine of humankind and other wild animals in order to gain the nutrients and minerals or may also free living. However, *Shigella* and *Salmonella typhi* are categorized as a small number of the members which have to be adapted strictly to living in human (Ryan & Ray, 2004).

Basically, Enterobacteriaceae are oxidase negative but show positive result to catalase test. *Pseudomonas* and Enterobacteriaceae become distinguishable through this oxidase test which is a biochemical test that checks up the presence of cytochrome oxidase. They are able to reduce nitrates to nitrites except some strains of *Erwinia* (Kumar, 2012).

2.2 Taxonomy of Enterobacteriaceae

Vasanthakumari (2007) found that the Enterobacteriaceae are a large family of gram negative microorganisms. The order is known as Enterobacteriales and class is Gamma proteobacteriam. *Escherichia, Edwardsiella, Erwinia, Klebsiella, Shigella, Salmonella, Enterobacter, Proteus, Yersinia* and *Serratia* are some examples of genera that belong to the Enterobacteriaceae (Vasanthakumari, 2007). The biochemical and antigenic characteristics are useful for the nomenclature and classification of these microorganisms. Some amendments have been made in classification among Enterobacteriaceae and the finding of new genera and species are due to the emergence of new technologies nowadays (Kumar, 2012).

2.3 Medically Important Genera of the Family Enterobacteriaceae Members

2.3.1 *Citrobacter* Species

Citrobacter species are short rods microorganisms which have enlarged the genus into 11 genomespecies, such as genomespecies 10 as *Citrobacter gilleniis*p. nov. and genomespecies 11 as *Citrobacter murlinia* sp. nov. by utilizing DNA hybridization of which 10 have been recovered from clinical material (Brenner *et al.*, 1999). They are mostly found in the faeces of humans and animals and widely distributed in nature. In laboratory, they grow well on ordinary media, such as Tryptic Soy Agar (TSA) or Nutrient agar (NA). There is a difficulty in differentiating between *Citrobacter* species and *Salmonella* species because they may response in the same way towards several biochemical tests (Kumar, 2012). In general, *Citrobacter* species are positive for indole and catalase test, able to reduce nitrate to nitrite as well as permit gas formation (Kumar, 2012).

2.3.2 *Enterobacter* Species

Respiratory infection, urinary tract infections (UTIs), and sepsis are common diseases that caused by these species (Miyoshi-Akiyama *et al.*,2013). They are widely distributed and can be found from many sources such as sewage, water, soil and food. Furthermore, they may also inhabit the intestine of human and animals (Kumar, 2012). *Klebsiella* species can be distinguishable from *Enterobacter* species by performing motility and ornithine test, although they may share the same characteristics in some other conventional microbiology tests. *Enterobacter* species exhibit positive results in motility, catalase and ornithine tests but negative for cytochrome oxidase test due to lack of cytochrome c oxidase activity (Kumar, 2012).

2.3.3 *Serratia* Species

Recently, there are fourteen species and 2 sub-species of *Serratia*, such as *S. marcescens, S. rubidaea, S. liquefaciens* and *S. fonticola* have been recognized (Mahlen, 2011). *Serratia* species are facultative anaerobes, motile and gram-negative rod microorganisms (Kumar, 2012). These species are generally opportunistic pathogens causing human infections and able to generate virulence factors, such as fimbriae for adherence (Mahlen, 2011). They can be found from many sources, like faeces, blood, urine, wound and respiratory specimen. *Serratia* species resemble other members of the family Enterobacteriaceae which lack of lack of cytochrome c oxidase activity and hence lead to no color changes on the cytochrome oxidase test. However, they are positive for motility and nitrate tests (Kumar, 2012).

2.3.4 *Escherichia* Species

There are 5 species of *Escherichia* genus, which are *Escherichia coli, Escherichia blattae, Escherichia hermannii, Escherichia vulneris*and *Escherichia fergusonii*, all are well known to trigger human disease (Abbott *et al.*, 2003). Abbott *et al.* (2003) proposed *E. albertii* was considered as the sixth species to this genus in family Enterobacteriaceae in few years ago. Besides, *Escherichia coli* (*E. coli)* is the most commonly isolated from clinical material and one of the most frequent causes of many diseases. *Escherichia* species are gram-negative straight rod and facultative anaerobes. Additionally, they possess peritrichous flagella and grow well at 37 °C (Kumar, 2012). They are negative for oxidase test and hydrogen sulfide production but positive for motility, catalase and indole tests (Kumar, 2012).

2.4 Health Problems Caused by Enterobacteriaceae

Enterobacteriaceae are known as opportunistic pathogens. UTIs are the most common infection that caused by them, and a small number of them are vital etiologic agents of diarrhea (Ryan & Ray, 2004). For example, an epizootic which means an epidemic outbreak of disease that may harm humankind, caused by *Proteus rettgeri* in silver carp happened after the trauma of handling had allowed entrance of the bacteria (Sanders & Fryer, 1988). In Israel, the carp ponds are fertilized with domestic bird faeces that encourage the growth of *Proteus rettgeri,* motile bacteria of the Enterobacteriaceae with capability of causing urinary infections (Sanders & Fryer, 1988). Apart from that, Sanders and Fryer (1988) stated that *Edwardsiella tarda* is an opportunistic pathogen. The new bacteria that related to strains of *Edwardsiella tarda* had been isolated from chinook salmon (*oncorhynchus tshawytscha*) in Oregon's Rogue River (Sanders & Fryer, 1988).

2.5 Culture Transfer Technique in Microbiology Exercises and Experiments

Culture transfer technique is a technique used to move bacteria from one medium to another by subculturing (Pommerville, 2010). This aseptic technique is extremely crucial in maintaining pure cultures as well as other microbiological test procedures. Microorganisms exist everywhere and easily contaminate the work if appropriate precautions are not taken. Therefore, transfer of desired bacteria must be carried out utilizing proper aseptic techniques so that contamination by unwanted bacteria can be avoided.

An autoclave is considered as a moist heat method in aseptic techniques and known as a huge metal pressure cooker that utilizes stream under pressure to completely remove all microbial life (Engelkirk *et al.,* 2011). Autoclaving at 121 °C for 15 minutes at a pressure of 15 psi destroys vegetative bacteria, viruses and other contaminants since they are now not protected by feces or blood (Engelkirk *et al.,* 2011). There are some precautions while using autoclave machine such as bottles should not be covered tightly and pressure-sensitive autoclave tape is used to make sure the autoclave is functioning properly.

Furthermore, sterilize the inoculating loop by flaming the loop on the Bunsen burner until the loops turns red hot (Pommerville, 2010). Therefore, any remaining bacteria on the loop can be incinerated. The neck of the tube must be flamed before and after the transferring process. Some modern laboratories use disposable inoculating loops and electrical heating devices to remove unwanted microorganisms (Pommerville, 2010). This process is known as dry heat.

3.0 Materials and Methods

3.1 Bacteria Culture

Materials used are listed in Appendix 1. A total of thirty isolates from conservation area in Saremas Plantation were gained from UNIMAS Virology Lab. Isolates were aseptically streaked onto Tryptic Soy Agar (TSA) (Merck, Germany) medium by streak plate method and were incubated for 24 to 48 hours at 37 °C. Then, single colony was picked and streaked onto Eosin Methylene Blue (EMB) agar plate and incubated at 37°C for 24 to 36 hours for isolation of pure culture. After the pure cultures were obtained, they were stocked into NA slant and again incubated at 37°C for the growth of the purified isolates. After that, the NA slants containing purified isolates were stored in a refrigerator as stock culture for later use. These isolates code were recreated to avoid confusion.All the following tests, including Gram staining, SIM test, KIA test, TSI test, oxidase test, $(GTG)_5$-PCR fingerprinting and API 20E were done with the pure cultures.

3.2 Culture Characterization (Gram Staining)

EMB agar was made as instructed by manufacturer. The bacteria were inoculated from purified culture to EMB agar plate and incubated for 24 to 36 hours at 37 °C incubator. The morphology of colonies, including cell shape, size and color and ability of lactose fermentation were observed and recorded.

Microscopic identification was performed using gram staining method (Benson, 2002). The smears were heat fixed prior to staining so that the fresh bacteria can be fixed on the microscopic slides and more permeable to stains. A drop of crystal violet was added to the smear for 1 minute and then washed with water. After that, Gram's iodine was also added for 1 minute. The decolorizing agent, 95% ethanol was added after the Gram's

iodine was poured off. The slide was washed with water before safranin was added for 45 seconds. Subsequently, the slide was washed gently and blotted dry. The morphology of the bacteria was observed under 4×, 10×, and 40× light microscopes (Eclipse E100, Nikon, Japan). The gram stained slides were observed and evaluated utilizing the oil immersion lens (1000×, magnification) after addition of the immersion oil. Gram-negative bacteria appeared as pink in color whereas purple indicated gram-positive bacteria.

3.3 Biochemical Identification

3.3.1 Kligler Iron Agar (KIA) Test

After the KIA medium was solidified in slanted position in each test tube, a sterile inoculating needle was utilized for aseptically inoculating a single colony from fresh isolates into the tube. The needle containing the single colony was stabbed into the medium in the bottom of the tube, and followed by streaked back and forth along the surface of the agar slant. The cap was loosened for facilitating exchange of air inside the tube. Then, each tube was incubated at 37 °C for 18 to 24 hours. The results of cultural response in KIA test were recorded and tabulated.

3.3.2 Sulfur Indole Motility (SIM) Test

The fresh test organism was inoculated into a test tube containing SIM medium with stab motion using a sterile inoculating needle to avoid contamination which might affect the results. Each tube was then incubated with loose cap at 37 °C for 18 to 24 hours. After incubation, the tubes were examined for production of hydrogen sulfide which indicated by blackening of the medium and motility indicated by the cloudy of the medium. Moreover, indole production indicated by the formation of red colour after the addition of Kovac's reagent into the test tube. The results were then recorded.

3.3.3 Triple Sugar Iron (TSI) Test

The inoculating needle was sterilized by flaming on a bunsen burner until it became red hot and then allowed to cool down so that it would not accidentally destroy the isolates. The cap of the test tube was removed and neck of the tube was flamed before a sterile straight inoculation needle was carefully touched the center of an isolated colony on a TSA plate. The needle containing the single colony was stabbed into the medium in the butt of the tube, and followed by streaked back and forth along the surface of the agar slant. The neck of TSI tube was flamed again and capped loosely before the tube was incubated at 35 °C for 18 to 24 hours for carbohydrate fermentation, gas and hydrogen sulfide yield. The cap was loosened to permit the exchange of air took place and improve the alkaline condition on the slant.

3.3.4 Oxidase Test

This test was carried out with filter paper saturated with oxidase test reagent that had been mentioned by Benson (2002). A piece of filter paper was dipped into a beaker containing oxidase test reagent and subsequently put onto a clean Petri dish. Single colony was picked from TSA plate with the autoclaved toothpick and rubbed on the filter paper stripe. In positive reaction, a dark purple color was formed on the paper within 10 to 15 seconds but no changes were detected for negative results.

3.4 $(GTG)_5$-PCR Fingerprinting

3.4.1 Genomic DNA Extraction

Bacterial DNA was extracted using the boiling cell method as described by Kathleen *et al.* (2014). Approximately 1.5 ml of the overnight culture was centrifuged at 10,000 rpm for 5 minutes before the supernatant was discarded. This step was repeated twice. After that, the pellet was resuspended in 500 µl of sterile distilled water and heated in boiling water for 10 minutes. Subsequently, the boiled suspension was cooled in iced at -20°C for 5 minutes and followed by centrifugation at 10,000 rpm for another 10 minutes. The supernatants were utilized for GTG-PCR assay.

3.4.2 $(GTG)_5$-PCR Amplification

$(GTG)_5$-PCR was performed according to Kathleen *et al.* (2014). Bacterial DNA was amplified in PCR using $(GTG)_5$ (5' GTGGTGGTGGTGGTG-3') oligonucleotide primer. A total of 25 µl PCR mixture was prepared as shown in Table 3.0. Table 3.1 illustrates the step cycle, temperature and duration for the PCR amplification.

Table 3.0: PCR mixture used in this study for the PCR amplification

Components	**Volume (µl), 1X**
5X Green GoTaq® Flexi buffer, 5 u/ µl	5.0
$MgCl_2$, 25 mM	3.0
dNTPs, 10 mM	0.8
(GTG)5-PCR primer	1.0
ddH_2O	9.9
DNA	.5.0
GoTaq® Flexi DNA Polymerase, 5 u/ µl	0.3
Total Volume	25

Table 3.1: Step cycle, temperature and duration for $(GTG)_5$-PCR Amplification

Step Cycle	Temperature / Duration	
Initial Denaturation	95°C for 2 minutes	
Denaturation	95°C for 1 minutes	30 cycles
Annealing	50°C for 1 minutes	
Extension	72°C for 1 minutes	
Final Extension	72°C for 5 minutes	

3.4.3 Agarose Gel Electrophoresis and Construction of Dendrogram

3 µl of 1 kb DNA ladder mixed with 2 µl of DNA loading dye were utilized as the ladder. 5 µl of the PCR products were electrophoresed on 1.2% (w/v) agarose gel pre-stained with Ethidium bromide (EtBr) in 50 ml of Tris-borate-EDTA (TBE) buffer. The gel was visualized under the UV transilluminator after the agarose gel was electrophoresed at 400 mA with 100 V for 75 minutes. Construction of dendrogram or tree-structured graph was performed by scoring, in which the presence of bands was scored as "1" but scored as "0" for the absence of bands. The data was then keyed in into RAPDistance software and dendrogram was constructed.

3.5 API 20E System

The oxidase-negative isolates were continued for implementing this API 20E System as conducted by Benson (2002) with slight modification. One well-isolated colony was selected from the TSA plate and transferred to a tube containing 0.85% saline solution. After that, the tube was vortex with a Vortex mixer so that the bacteria can be dispersed evenly throughout the saline solution. An API 20E test strip from API 20E kit (BioMerieux,