Antibiogram Of *Vibrio* sp. Isolated from Semi-Closed System Farm Asian Clam, *Corbicula fluminea*

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DECLARATION

I declare that this thesis entitled "Antibiogram of *Vibrio* sp. isolated from semi-closed system farmed Asian clam, *Corbicula fluminea*" is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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˚C	Degree Celsius
G	Gram
µg	Micro gram
%	Percentage

List of Abbreviation

APW	Alkaline peptone water
ATP	Adenosine Trisphosphate
CFU	Colony Forming Unit
GO	Graphene Oxide
IS	Intermediate Sensitive
LB	Lactose Broth
MAR	Multiple Antibiotic Resistant
MPN	Most Probably Number
NH	No inhibition
R	Resistance
S	Sensitive
TCBS	Thiosulphate Citrate Bile Salt Sucrose
TNTC	Too numerous to count
TSA	Trypticase soy agar
TSB	Tryptic soy broth
˚C	Degree Celsius
µg	Micro gram
G	Gram
%	Percentage

Antibiogram *Vibrio* sp. dari kerang Asia, *Corbicua fluminea* ladang system separuh tertutup

ABSTRAK

Vibrio sp. merupakan bakteria gram negatif yang mempunyai sifat fakultatif. Bakteria tersebut bukan sahaja memain peranan sebagai agen penyebab *vibriosis* dalam peternakan kerang tetapi juga membawa penyakit berjangkit terhadap manusia. Di Kelantan, *Corbicula fluminea* juga dikenali sabagai 'etok' merupakan makanan popular dalam kalangan rakyat tempatan. Objektif kajian ini adalah untuk memantau tahap keselamatan *Vibrio* yang tercemar melalui analisis *Most Probably Method* (MPN) dan antibiotik yang dapat mengelak petumbuhan *Vibrio* sp. secara berkesan. Sepanjang kajian ini, analisis MPN mendapati kerang tersebut tidak selamat untuk dimakan secara mentah. 100 koloni *Vibrio* yang berjaya cultiva dalam agar *Thiosulfate-citrate-bile-salt-sucrose* (TCBS) dan telah bertakluk kepada 18-disk antibiotik dalam kajian ini. Antara antibiotik yang dikajikan, asid nalidixic, asid oxolinik, *oxytetracycline* dan *tetracycline* didapati paling sensitive diikuti oleh *florfenicol* (90%) dan fluminik (70%) dalam mengawal *Vibrio* sp. yang dicultiva dari sampel air dan tisu kerang dipelihara di ladang sistem separuh tertutup. Tambahan pula, bakteria juga dikenal pasti dengan BD BBL crystal enteric/nonfermenter kit berjaya mengesah kewujudan *V. Cholerae* dari sample ekperimen ini. Oleh itu, antibiotik yang dinyatakan boleh diguna sebagai profilaktik dan rawatan untuk peternakan *Corbicula fluminea* manakala kenal pasti kerang tersebut dimasak dengan suhu sesuai untuk mengelak keracunan makanan.

Kata Kunci: *Corbicula fluminea, Vibrio sp., most probable number (MPN), antibiotik, BBL crystal enteric/ nonfermenter kit*

Antibiogram of *Vibrio* sp. Isolated from semi-closed system farm Asian clam, *Corbicula fluminea*

ABSTRACT

Vibrio sp. is gram-negative bacterium with facultative properties. They not only play as causative agent of vibriosis in clam farming but also lead to several disease for harming human health. Due to feeding feeder behaviour of Asian clam, the level of the *Vibrio* contamination on Asian clam tissues was studied. In Kelantan, Asian clam is known as 'etok' are popular among citizen and use to serve as snack or meal. This research provides an experimental study on *Vibrio* sp. isolated from Asian clam or also known as *Corbicula fluminea*. The objective of the study was to monitor the safety level of *Vibrio* contaminated via most probably number (MPN) analysis and antibiogram characterise of live Asian clam, *Corbicula fluminea* in semi-closed farm. Throughout the study, MPN test suggested it is not safe to be consume raw Asian clam. *Vibrio* colonies were successfully isolated in Thiosulfate-citrate-bile salts-sucrose (TCBS) agar and were subjected to 18 antibiotic discs for antibiogram test. Among all the antibiotics, nalidixic acid, oxolinic acid, oxytetracycline and tetracycline were found most sensitive followed by florfenicol (90%) and flumequine (70%) in controlling *Vibrio* sp. cultivated from water and tissue sample of Asian clam. Furthermore, the isolated bacteria identified by using BBL crystal enteric/nonfermenter kit confirmed the presence of *V. Cholerae* from the samples. Hence, mentioned antibiotics was advised to be used as prophylactic and treatment for clam farming while suitable treatment by applying appropriate temperature on clam cooking must conducted for safety consuming or preventing food poisonous.

Keywords: *Corbicula fluminea, Vibrio sp., Most Probably Number (MPN), Antibiogram, BBL crystal enteric/ nonfermenter kit*

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Asian clam or also known as *Corbicula fluminea* belongs to Corbiculidae family and a hermaphrodite species that can be found in fresh water or blackish environment. However, unlike other bivalve molluscs, *Corbicula fluminea* is proto-oogamous and oogenesis before spermatogenesis (Fernando et al., 2013). Based on the list of The international Union for Conservation of Nature (IUCN), (2017), Asian clam is natively from southern and eastern Asian but soon introduced to other region such as Africa, North America, throughout Europe and the Mediterranean. In Malaysia, Asian clam gain their popularity especially Kelantanese as it uses to serve as snack or meal. Since Asian clam feed by filtering plankton and water, bacteria have high chance of remaining in clams' tissues. So, improper treatment on clam where eaten raw or half cooked allow bacteria to continue reproducing after consumed by human. Generally, Asian clam can live two to three years and reproduce once or twice in a year. They are extremely sensitive to low oxygen hence flowing water with high oxygen level is preferred (University of Wisconsin Sea Grant Institute, 2013). Based on Fernando et al., (2013), *Corbicula fluminea* consist

high nutritional and medical values. Consuming Asian clam also have the curative effect on some disease such as anaemia, measles and nyctalopia. At the same time, since this species responsible for filter feeder under the water, it plays an important role inmaintaining hydro ecological balance of their habitats. However, due to environment issues happened nowadays, water pollution, overfishing and habitat destruction might greatly affect this habitat (Fernando et al., 2013).

Vibrio is a type of gram negative, highly motive bacteria with a comma-shaped where flagella appear at their end. The size of curved rods bacteria is approximately in 0.5µm and in between 1.5 to 3.0 µm long with either single or strung together in spirals. *Vibrio* sp. is facultative anaerobes microorganism which means they can synthesis adenosine trisphosphate (ATP) with the present or absent of oxygen. *Vibrio* sp. from the family of Vibrionaceae and consider as aquatic microorganisms. They can be found where free living in water, sediments, plankton and nearly most flora and fauna in coastal environment (Elbashir et al., 2018). Among the harmful bacteria which cause serious diseases, Vibrio species commonly found are *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* which contribute a significant threat to human health. Bacteria carrier in seafood is unseen, no smell or even taste bad while consuming raw and warm temperature of human body is the best condition for bacteria reproduce. The only way to prevent Vibrio infection is to cook the clam or seafood properly in order to kill harmful bacteria ("Vibrio and Oysters | Vibrio Illness (Vibriosis) | CDC," 2016).

1.2 PROBLEM STATEMENT

According to World Wildlife Fund (WWF), 2018, there are approximately three billion people in the world that rely on both wild-caught and farmed seafood as there are primary source of protein. However, due to issues such as water pollution, overfishing and habitat destruction in recent year, natural resources of *Corbicula fluminea* reported sharply declined in lake as other water source (Chang and Wang, 2015). At the same time, as the growing popularity of sushi trends nowadays, in order to consuming raw sea-food, it must free from contamination of any pathogen. Although clam was reported lowest in contaminations, the facts that Asian clam still filter feeder that feed on phytoplankton or microscopic organism. They have the ability of accumulate chemical and toxic from the contaminated water which will certainly harmful for human health after consuming raw or improper treatment on Asian clam. Therefore, studies and treatment for semi-closed farm Asian clam is needed.

1.3 OBJECTIVES

The objective of the present study is:

1. To monitor the safety level of *Vibrio* contamination in live Asian Clam, *Corbicula fluminea* in semi- closed farm via Most Probably Number (MPN) analysis.

2. To characterise antibiogram of *Vibrio* sp. isolated from Asian Clam, *Corbicula fluminea.*

1.4 SCOPE OF STUDY

The scope of the study is monitoring health of Asian clam from semi-closed system farming which will soon contribute to human as food and microbiology study on overcoming *Vibrio* sp. by using potential antibiotic.

1.5 SIGNIFICANCE OF STUDY

In this research, it is important to identify and monitor a suitable environment for the semi closed farm Asian clam. Due to lack of research on the relationship on water which affect and lead to contamination of clam, examined of semi-closed farm water ensured the whether the source is free from potential bacteria and prevent bacteria carried in the clam through filter feeding. To prevent any contamination of the Asian clam, having quality analysis on Asian clam in this research can be informative and as a guideline for clam's farmer for producing shellfish that is safe to consume.

Not all bacteria are harmful however they can be potential pathogen which cause infectious in human health, *Vibrio* spp. commonly give rise of harming human's health and yet it is not much studies on which chemical reagent can be effectively against it. So,

it is important to understand and able to identify the type of bacteria in this research including effective chemical against it. This research also provides informative suggestion on resistancy of antibiogram test by proving which selected antibiotic are sensitive or effective towards *Vibrio* sp. In aquaculture, antibiogram also used as prophylactic and treatment purposes since there are not many antibiotics specifically designed for aquaculture.

1.6 LIMITATION OF STUDY

The limitation of the current study is not many reports which study about bacteria found on Asian clam, if there is, some of the research data is too old to be use as references since the published period have quite some period. As for the study of the bacteria which is *Vibrio* sp., according to Osunla and Okoh, (2017), even until today, *Vibrio* spp. infections still a hot issues on public health concern. Regarding to this, more research on antibiogram test used as prophylaxis and prevention have to be done and create awareness among Asian clam's consumer.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction of Asian clam, *Corbicula fluminea*

Asian clam or *Corbicula fluminea* is an aquatic bivalve mollusc belongs to family Cyrenidae. It can burrow into the bottom of sediments of stream or lakes and feed on water and the substrate. It has siphon at the site of one end responsible to filter feed suspended particles. Similar to other bivalve, Asian clam is important filter feeder which feed on microscopic plants and bacteria in the water or in the sediment ground especially phytoplankton and zooplankton organism. The nutrient that have been process will use efficient for their growth and reproduction (MacNeill, 2012). Based on study by Bullard and Hershey, (2013), *Corbicula fluminea* have higher possibility for direct and indirect effects on its habitat while reducing of phytoplankton by filter feeder lead to nutrient burial through bio deposition. This also supported by few investigators that *Corbicula fluminea* meet their nutrition requirement by using both filters feeding and deposit feeding

(Yeager and Cherry, 1994; Hakenkamp and Palmer, 1999; Hakenkamp, 2001; Vaughn and Hakenkamp, 2001; Cummings and Graf 2010; Bullard & Hershey, 2013). As mentioned by Rosa, Ward and Shumway, (2018) the gills responsible for removing, retaining and sorting of particles from the surrounding water, filtered material is sorted at several points including gills, labial palps, and stomach, not all material able to remove from suspension is ingested but the rejected material and excreted material or faces will have deposited in sediments and this bio deposition may substantially accelerate natural seston deposition. It also has potentially process large volume of water up to 1 to 2 litter/hr per individual clam. Hence this clam is important in removal of suspended particulate matter in estuarine system.

Lauritsen, (2012) has study about the relation between filter rates with temperature and food concentration. Different temperature was studied such as in 8°C, 20°C and 31°C. In 8°C which is commonly present in winter period, clam appear to have low filtration rates due to inactive metabolism and as for 20°C and 31°C, filter rate is not significantly different and maximum filter rates is found at 24°C which is about 800 ml/hr for 22 mm clam. As for food concentration, it has significant effect on filtration rate. Filtration rates per individual decreased with the increasing of food concentration although volume of algae ingested increase. This result obtains believes that bivalve do not normally modify filtration rate in response to variations in food concentrations.

2.2 INTRODUCTION OF *Vibrio* sp.

Based on research carried out by Slayton, Newton, Depoala, Jones and Mahon, (2010) about Clam-associated vibriosis, survey carried out on contribution of clam to foodborne vibriosis and indicates comprehensive programme to prevent *vibrio*. *V. vulnificus* infection cause highest rate of mortality and severe illness, especially person who underlying health condition. *Vibrio* is abundance in environment and seafood, peak when summer period as water temperatures are warmest. This information is crucial as Malaysia is one of the tropical countries which have favourable condition for the breed of *Vibrio* sp. Surveys carried out time by time by collecting all the information is reported to Cholera and Other *Vibrio* Illness Surveillance system (COVIS) by state and local health officials to prevent any unforeseen incidents from occurring. Information includes demographic, isolation of bacteria, clinical, risk expose and seafood traceback information. Specific information received such as symptom onset date, duration of illness, medication used within 30 days, hospitalization and mortality also recorded. In USA, the Interstate Shellfish Sanitation Conference (ISSC) establishes policy for molluscan shellfish safety by implementing appropriate time and suitable temperature requirement and educate consumer to take address to the risk of *V. vulnificus* infection from shellfish consumption in person with existing conditions such as liver disease and alcoholism. This research also draw attention to risk associated with clam consumption, especially raw clams which are important source of vibriosis, especially, *V. parahaemolyticus* infection. The result obtained in this research shows that one third of patient claim that they consume only clam were hospitalized and the plausible explanation would

be consumer may ingest higher dose of *Vibrio* with clam than with other seafood. This occur if clam concentrate *vibrio* more than other seafood and *vibrio* replicates more rapidly in post-harvest clam and been consume those raw clams. Another study has examined the growth rates of *vibrio* in post-harvest oyster have shown variability in virulence of subpopulation (Gooch, 2002).

Vibrio sp. is type of halophilic bacteria found in aquatic environments. Most of them are pathogenic to human and animal. Species such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are common contributed to most cases of seafood-related sickness (Yutaka, Kaoru, Masataka, Isao, Masatoshi & Dusit Aue-umneoy, 2014). *Vibrio. cholerae* serogroup of O1 and O139 lead to epidemics cholerae while rest of serogroup usually do not associate with epidemics (FDA & WHO, 2005). *Vibrio. parahaemolyticus* infection leading bacterial cause of seafood-associated gastroenteritis. Similar to primary septicaemia causes by *Vibrio vulnificus* whenever consuming of infected seafood. Additionally, it might be lethal without proper treatment of antibiotic (Oliver & Kaper, 1997).

2.3 ISOLATION AND IDENTIFICATION OF *Vibrio* sp.

According to Yaashikaa, Saravanan and Senthil, (2016), bacterial disease is one of major problem which affect production, progress and growth of aquaculture. *Vibrio* sp. which studied in the present study are also one of bacteria species commonly widespread in marine and estuarine environment. *Vibrio* sp. contributes to *Vibrio* infections outbreak by consuming of food and water which contaminated by human

faces or discharge of sewage, exposure of skin lesion with the aquatic environment or marine animals. In research done by Yaashikaa et al., (2016), *Vibrio* sp. was studied by isolation and confirmed with biochemical methods. The growth rate of *Vibrio* sp. varies with few operating parameters. Isolation were done by using enrichment and selective planting methods. The objective of this research journal was *Vibrio* sp. isolation from prawn seafood. There are few methods mentioned and able to use as identify and isolate of *Vibrio* sp. such as gram staining, motility test by hanging drop method, biochemical characterization method by starch hydrolysis, casein hydrolysis or gelatine hydrolysis, fermentation reaction such as sugar fermentation, oxidation fermentation test, indole production, Methyl red test, voges- proskauer test and citrate utilization. As for respiratory reaction, several tests such as catalase test, oxidase test, urease test, nitrate reduction test. To successfully incubate of *Vibrio* sp., there are information on factors influence the growth and survival of *vibrio* sp. in seafood stated in this research such as temperature, salinity, moisture content salt and concentration of carbohydrate, presence of organic matter and bacterial flora as key factors in determining time for survival in different food stuff.

2.4 MOST PROBABLY NUMBER (MPN) ANALYSES

In this present research, Most Probably Number test used for coliform bacteria determine in *Corbicula fluminea*. It is a common, statistical, multiple assay consisting of presumptive, confirmation and completed test. The detection of coliform can be use as indicator of water salinity or general indicator for sanitation in food-processing

condition (Feng, Weagent, Grant and Ma, 2002). Presumptive test is the first step where the sample is added to lactose broth consisting Durham's tube and divided to three part which are 10ml, 1ml, and 0.1ml (Ruhil, Njiah, Nadirah, Lee and Aliuddin, 2008). The using of lactose broth is to enrich the growth of gram-negative bacteria. Turbidity and gas production will be observed after certain time of incubation. Lactose broth is a type of pre-enrichment medium providing nutrient for the growth of gram-negative bacteria due to presence of lauryl sulphate, as it turns turbidity, this means that there is the present of bacteria colony which consume the nutrient. However, studies also stated that it might consist false positive by Gram positive bacteria (Shrestha & Sujakhu, 2014). As in confirmed test, the positive tubes from the previous step which is presumptive step, will streak on selective agar depend on the bacteria which selected for further studies and incubate for certain time. Following with completed test, the selective colonies will be inoculated with hockey stick and transfer to lactose broth filled with Durham's tube once again. After incubation, the positive tube with gas bubble is streaked on Trypticase Soy agar (TSA) for the identification. Ruhil, (2008) the culture identified by Gram's staining, motility, colony formation on selective media, several biochemical tests including lysine and ornithine decarboxylase, acid production, indole production, citrate utilization, oxidation and fermentation. Hydrogen sulphide production, methyl red, oxidase, catalase and urea test were done. As mentioned in the research that have been done, MPN is done to detection of coliform as an indicator of water sanitation or as indicator of sanitary condition for food-processing condition. The result obtained in this research found that more than 2400 MPN/g coliform bacteria presence were found in short necked clams. This led to finalised that raw short necked clam meat harvested

at East Coast Malaysia were unsafe for consumer consumption if eaten either in raw or half-cooked due to present of *E. coli*, *Salmonella* spp. and *Klebsiella* spp.

2.5 ANTIBIOTIC SUSCEPTIBILITY TEST

Horvat, (2010). Initially, the development and presentation of an antibiogram are generate by the clinical microbiology laboratory with the collaboration from physicians, pharmacists, and infection control personnel. It used to determine the effectiveness of antimicrobial agents against clinical bacterial isolates. Antibiogram test allow standardization of producing reproducible, qualitative and quantitative result. Quantitative results are interpreted the result into susceptible (S), intermediate Sensitive (IS), or resistant (R) while qualitative results are derived from the measurement of inhibition zone recorded as millimetre (mm). Advantages of using disc diffusion method also been stated which is more flexibility when choosing the antibiogram agents used to tested against each isolate. Despite of advantages of using disk diffusion method, it also has some challenge as producing a concise antibiotic on annual basic in microbiology laboratory, precise and accurate data and document is needed. The development of sophisticated computer programs and improvement in laboratory information system have aid in this method. Antibiogram test has evolved over 60 years and in the future, it will continue to evolve as new antibiotics and procedure are adopted.

Aquaculture is rapid growing industry and fish is considered as important protein used for human consumption, due to the outbreak of disease, the use of antibiograms has become a practice use as prophylaxis and treatment in aquaculture management

(Lucia & Fernando, 2017). In the research of Yataka et al., (2014), antibiogram susceptibility is done on *Vibrio* sp. isolated from shrimp. In most shrimp farm, antibiogram that often choose for therapeutic and prophylactic against bacteria are tetracycline and quinolone. However, overdose of antibiotic amount has led to serious antibiotic resistancy among bacteria in those environments. In clinical, antibiotics are used to treat *Vibros* infection in some dehydrated patients and especially for septicaemia patient infected by *Vibrio. vulnificus*. Some other research where *Vibrio* sp. discovered and isolates from shrimps cultured showed resistant toward common commercial antibiotic (Tendecia & Pena, 2001; Vaseeharan, Ramasamy, Murugan, and Chen, 2005). In Yutaka et al., 2014 research, selected antibiogram were ampicillin, ampicillin/sulbactam, oxytetracycline and nalixic acid and some more were placed on TCBS and cultivate. All the antibiogram were tested on shrimp samples and overall with 140 isolates of *Vibrio. cholerae*, 70 for *Vibrio. parahaemolyticus* and 25 for *Vibrio. Vulnificus*. In *Vibrio. cholerae* case, 90% of the isolates were inhibited were 2 and 8 g/ml ampicillin, respectively, 8% of the isolates were resistant to ampicillin and 2% were resistance to oxytetracycline. This indicate that *Vibrio. Cholerae* showed susceptible to remaining antibiogram. *Vibrio. parahaemolyticus* also susceptible to all antimicrobial beside from ampicillin and oxytetracycline. As for *Vibrio. vulnificus* isolates, beside from nalidixic acid, the rest were susceptible to the rest of antibiogram. Bacteria that are found in 'intermediate' should not be included as susceptible to be accurate. Monitoring of antibiotic resistance is still important as microbial pathogen continuously development antibiotic resistance (Horvat, 2010). In conclusion, impropriate use of antibiogram might disseminate resistance genes through aquatic environment. While taken account that the aquaculture uses to consume freely, they will be a mode of

disseminate potential pathogens and resistance towards most awareness that have been done by public. Management plans and efforts to reduce the risk of the bacteria are needed in aquatic farm before the product are shipped.

Yoshika et. al, (2016), conducted an experiment to test on resistant and sensitivity of *Vibrio* species isolated in prawn (Penaeus monodon), few antibiogram used are Penicillin G, Clindamycin, Piperacilin and Co-Trimoxazole. The result obtained from 2 isolates was completely resistance (100%) toward Penicillin G, Clindamycin and Peperacillin, while 100% sensitive to Co-Trimoxazole. As the isolates was resistance to antibiotic, it is quite threatening to public health as antibiotic resistant determinacies may be transferred to other bacteria of clinical significance. At the same time rapid developing of antibiotic resistance in bacteria or exposure of drug-resistant microbial disease in aquaculture industries might lead to huge issues to many fields including environment, economic and management and creating human health hazards. So, with the accurate use of the data and guidelines enable to track antibiotic resistance and assisting physician in making empiric antibiotic selections (Horvat, 2010).

CHAPTER 3

METHODOLOGY

3.1 MATERIALS AND EQUIPMENT

3.1.1 CHEMICALS AND REAGENTS

Corbicula fluminea, semi-closed farmed water, plastic zipper bag, Thiosulphate Citrate Salts Sucrose (TCBS) (Oxoid Ltd., England), Tryptic soy agar (TSA) (Himedia, India), Tryptic soy broth (TSB)(Oxoid Ltd, England), Lactose broth (LB)(Himedia, India), Graphene Oxide (GO)(GO Advanced Solution Sdn Bhd, Malaysia), antibiotics disc, BBL commercial Crystal kit (Becton, USA), alcohol (HmbG, Malaysia), parafilm, aluminium foil.

3.1.2 EQUIPMENT

Scalper, ruler, maker pen, cotton bud, face mask, hand gloves, petri dish (Labmart GQ, Malaysia), Scott bottle (Bomex), measuring cylinder beaker (HmbG, Malaysia), Durham tube, inoculate wire loop, test tubes, bijou bottle, vortex machine, pipette, oven, Lamina flow cabinet (ESCO), autoclave machine, electronic balance (Shimadzu, Malaysia), Bunsen burner, incubator (Antel, India)

3.2 METHODS

3.2.1 SAMPLE COLLECTION AND PREPARATION

Approximately 1 to 2 cm wide of semi-closed farmed Asian clam (*Corbicula fluminea*) were collected from aquarium filled with Graphene Oxide (GO) as bio-filter and without GO bio-filter. The clams were opened with disinfected scalper. The flesh had been scraped out while moisture content been collected in a clean plastic zipper bag and homogenized evenly. Water samples also collected from both aquarium and covered with aluminium foil to prevent contamination.

3.2.2 MOST PROBABLY NUMBER TEST

The water sample and *Corbicula fluminea* sample were undergo 3 steps of MPN test which is presumptive test, confirmed test and completed test.

Presumptive test: in each sample, 15 test tubes were prepared with Durham tubes inserted upside down inside. 10 ml of Lactose broth (Himedia, India) was filled inside every test tube. 5 of the test tubes were pipetted with 10 ml, 1.0 ml and 0.1 ml of sample respectively. All the test tube was incubated in incubator (Antel, India) on 27˚C for a day.

Confirmed test: the test tube filled with bubbled gas was chosen and streak on Thiosulphate Citrate Bile Salt (TCBS) (Merek, Germany) medium and incubated for other 24 hours in incubator (Antel, India).

Completed test: Single colony of bacteria was chosen and inoculated with inoculation loop and inserted into lactose broth (Himedia, India) filled with Durham tube inserted upside down. Incubated overnight and the bubble gas appeared was recorded and referred to the MPN index table (United States Department of Agriculture [USDA], 2014). Further identification of bacteria by using BBL Crystal ™ Enteric/Nonfermenter (E/NF) ID kit (Becton, USA).

3.2.3 BACTERIA IDENTIFIED BY USING BD BBL CRYSTAL™ ENTERIC/NONFERMENTER ID KIT (BECTON, USA)

Bijou bottle filled with suspended colonies were poured to the target area of the kit's base. Inoculum fluid was rolled gently until all wells were filled and excess fluid rolled back to the target area. Labelled and aligned the lid on top of the target area of the base. The panel was inoculated overnight in the incubator. The colour changes within the well were recorded by referred of the colour reaction chart in the manual book. The BBL Crystal profile number were calculated and analysed the bacteria present through official software.

3.2.4 ISOLATION AND IDENTIFICATION OF *Vibrio* sp.

Ten-fold serial dilution of water sample and *Corbicula fluminea* was prepared and 1 ml of each dilution was added to test tube filled with 9 ml of saline solution. Next, 1 µl of each dilution was pipetted and spread plate on TCBS medium (Merck, Germany) and Tryptic Soy Agar (TSA) (Himedia, India) medium. All media were incubated in incubator (Antel, India) for 24 h.

3.2.5 TOTAL PLATE COUNT AND COLONY FORMING UNIT (CFU) CALCULATION

The single and pure colony of bacteria grew were marked. The accepted countable colonies on standard plate is between 25 to 250 for most bacteria. More than 250 colonies were recorded as TNTC (Scott Sutton, 2011). The counted bacteria colony was applied in Colony Forming Unit (CFU) calculation with the following formula (Munmun and Sirshendu, 2015):

$$\text{CFU/ml} = \frac{CFU\ per\ plate \times dilution\ factor}{Volume\ of\ sample\ taken\ (ml)}$$

3.2.6 SELECTION OF TYPICAL SINGLE COLONY

Single colony bacteria were subculture into 10 Tryptic Soy Broth (TSB) (Oxiod, England) for each sample. Incubated for 24 h.

3.2.7 ANTIBIOGRAM SUSCEPTIBILITY TEST

The cotton bud was submerged in TSB (Oxiod, England) and swabs aseptically on the TSA (Himedia, India) medium. Kirby-Bauer disk diffusion method was conducted. Antibiotics tested included amoxicillin (25 µg) AML 25; ampicillin (10 µg) AMP 10; chloramphenicol (30 µg) C 30, doxycycline (30 µg) DO 30; erythromycin (15 µg) E 15;

florfenicol (30 µg) FFC 30; flumequine (30 µg) UB 30; fosfomycin (30 µg) FOS 30; kanamycin (30 µg) K 30; lincomycin (15 µg) MY 15; nalidixic acid (30 µg) NA 30; novobiocin (30 µg) NV 30; oleandomycin (15 µg) OL 15; oxolinic acid (2 µg) OA 2; oxytetracycline (30 µg) OT 30; sulfamethoxazole (25 µg) RL 25; spiramycin (100 µg) SP 100 and tetracycline (30 µg) TE 30. Disc diffusion method was subjected to Graphene Oxide (GO) sensitivity and placed on the TSA (Himedia, India) and incubated overnight.

3.2.8 MULTIPLE ANTIBIOTIC RESISTANCE (MAR) INDEX

The results of inhibition zone were interpreted according to standard measurement of inhibitory zones in millimetres (mm) categories as sensitive (S), intermediate sensitive (IS) and resistance (R) and Antibiotic resistance index (MAR) calculated with the formula below (Nandi et al., 2016):

$$\text{MAR}= \frac{Total\ antibiotic\ resistance\ cese}{(Number\ of\ total\ antibiotics\ used \times Total\ isolate) Total\ resistance\ case}$$

Once the MAR index value equals or less than 0.2 indicates that the antibiotics is effective but seldom or never been used on animals for treatment purposes, whereas index higher than 0.2 indicates higher risk exposure to the antibiotics tested.

CHAPTER 4

RESULT AND DISCUSSION

4. 1 Most Probably Number (MPN) of Bacteria Coliform

Generally, MPN test is microbial examination used to detect coliform in water salinity or sanitary condition in food or food-processing conditional. As water is the essential medium for the living aquatic animal, it is important to test on water where Asian clam feed through filter feeding which also lead to high potential to change seston quality and concentration of water (Bullard & Hershey, 2013). To be accurate, three trial were conducted in each sample while each result was obtained from presumptive test, confirmed test and complete test. The samples include semi-closed farmed water and Asian clam, *Corbicula fluminea* with the use of graphene oxide (GO) and without the use of graphene oxide as treatment.

In some research, graphene oxide (GO) which consisting nanoparticles begun to apply in some surgical implant surface modification. However, mechanism regarding the antibacterial activity of GO nanosheets still remain as an undeveloped. So, in this study, GO bio-filter was tested not effective against *Vibrio* sp.

In presumptive test, with the use of GO as treatment, the MPN index obtained in water sample was range 240, 79 and 280 respectively while 1600, 170 and 170respectively for Asian clam (Table 4.1), *Corbicula fluminea* sample. With the use of GO as treatment the MPN index data obtain was 110, 33 and 240 respectively for water sample while 130, 110 and 70 for Asian clam sample (Table 4.1).

For the confirmed test, the positive test in presumptive test were swap on the Thiosulphate citrate bile sucrose (TCBS) agar and incubated overnight. Among the all sample, result obtained were yellow colony was spotted on the TCBS agar.

The final test of MPN which is completed test was conducted by again transferring of the single colony to the lactose broth and incubated. The results retrieved where all the tubes were presence with bubble gas. Another completed test conducted by using BD BBL Crystal kit confirmed that the presence of bacteria was *Vibrio* sp. which is *Vibrio Cholerae* (Fig 4.5).

In the present study, the MPN index obtained from semi-closed farm's water test was in ranged of 79 MPN/ml to 280 MPN/ml. In Malaysia, recommended and acceptable value of raw water quality for total coliform is in range of 5000 MPN/ml while 0 MPN/ml for drinking water ("Drinking Water Quality Surveillance Programme - Ministry of Health," 2010). Another guidance published by Food and Agriculture Organization of the United Nation (FAO) mentioned that for food safety purposed, do not exceed 100 Most Probable Number (MPN)/g in any shellfish harvested (FAO, 2016). The MPN index obtained in Asian clam's tissue were 70 to 1600 MPN/ml, which indicate that it might not safe for raw consumption and treatment might applied for monitoring or consumption. There are also studies stated that since presumptive test in MPN test considered as preliminary test where the use of lactose broth is for enrichment of the coliform, the

presence of bubble gas cannot confirm the presence coliform as some Gram-positive bacteria like *Bacillus*, *Streptococcus*, *Clostridium* and *Lactobacillus* also give false positive result (Shrestha & Sujakhu, 2014). Hence further identify in a must carried out. In current study, further identify were applied on Eosin Methylene Blue (EMB) and TCBS for selective coliform while BBL crystal enteric/nonfermenter kit were also used for final identified. The study by Hayati Hamdanusa et al., 2008, found that the presence of coliform bacteria in short neck clams harvested at East Coast Malaysia were more than 2400 MPN/g which also suggested not safe for consumer consumption particular eaten raw or half-cooked due to the present of different bacteria. In the study of Yano et al., 2014, *Vibrio cholerae* were ranged highest isolated in shrimp, among other *Vibrio* spp. where 15 out of 16 shrimp (94 %) found were range from 62 to 252000 MPN/g, while *Vibrio parahaemolyticus* isolated 6 of 16 shrimp (38 %) ranged from 370 to 6300000 MPN/g and *Vibrio vulnificus* were ranged 16 to 1300 as reported low in culture environment in Malaysia.

Table 4.1: Results of Most Probable Number (MPN) for Semi-closed farm water with treatment of Graphene Oxide (GO)

	Test	Observation											
	No of Trial	1				2				3			
		Number of positive tubes				Number of positive tubes				Number of positive tubes			
1	Presumptive No. of positive tubes	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index
		5	5	0	240	5	3	0	79	5	4	3	280
2	Confirmed test	Yellow colony present on the Thiosulphate citrate bile salt sucrose (TCBS)											
3	Completed test	Bubble gas was produced. Presence of *Vibrio Cholerae*											

Table 4.2: Results of Most Probable Number (MPN) for Semi-closed farm water without treatment of Graphene Oxide (GO)

	Test	Observation											
	No of Trial	1				2				3			
		Number of positive tubes				Number of positive tubes				Number of positive tubes			
1	Presumptive No. of positive tubes	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index
		5	3	1	110	5	1	0	33	5	5	0	240
2	Confirmed test	Yellow colony present on the Thiosulphate citrate bile salt sucrose (TCBS)											
3	Completed test	Bubble gas was produced. Presence of *Vibrio Cholerae*											

Table 4.3: Results of Most Probable Number (MPN) for Asian clam sample with treatment of Graphene Oxide (GO)

	Test	Observation											
	No of Trial	1				2				3			
		Number of positive tubes				Number of positive tubes				Number of positive tubes			
1	Presumptive No. of positive tubes	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index
		5	5	4	1600	5	4	1	170	5	4	1	170
2	Confirmed test	Yellow colony present on the Thiosulphate citrate bile salt sucrose (TCBS)											
3	Completed test	Bubble gas was produced. Presence of *Vibrio Cholerae*											

Table 4.4: Results of Most Probable Number (MPN) for Asian clam sample without treatment of Graphene Oxide (GO)

	Test	Observation											
	No of Trial	1				2				3			
		Number of positive tubes				Number of positive tubes				Number of positive tubes			
1	Presumptive No. of positive tubes	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index	10 mL	1 mL	0.1 mL	MPN index
		5	4	0	130	5	3	1	110	5	2	1	70
2	Confirmed test	Yellow colony present on the Thiosulphate citrate bile salt sucrose (TCBS)											
3	Completed test	Bubble gas was produced. Presence of *Vibrio Cholerae*											

4.2 ANTIBIOTIC SENSITIVITY AND MULTIPLE ANTIBIOTIC RESISTANT (MAR) TEST

The total plate count of *Vibrio* sp. of water sample from Asian clam farm was 0 colony forming unit (CFU)/g. In current study, most of the bacterial isolates present (exceeding 70 %) were found to be sensitive to chloramphenicol, doxycycline, florfenicol, flumequine, nalidixic acid, oxolinic acid, oxytetracycline and tetracycline (Fig 4.1). However, the percentages of bacterial isolates present which were sensitive to amoxicillin, ampicillin, erythromycin, fosfomycin, kanamycin, lincomycin, novobiocin, oleandomycin, sulphamethxazole and spiramycin were ranged from 0 % to 60 %. All the bacterial isolates were found to be resistant to amoxicillin, ampicillin, novobiocin, oleandomycin, sulphamethoxazole and spiramycin. Overall, the sensitive category accounted for 38.9 % (74 cases), whereas 53.1 % (101 cases) and 15 cases or 7.9 % were reported resistant and intermediary sensitive category. The MAR value of current study was 0.532 which is higher than 0.2, this indicates that the Asian clam is high risk contamination.

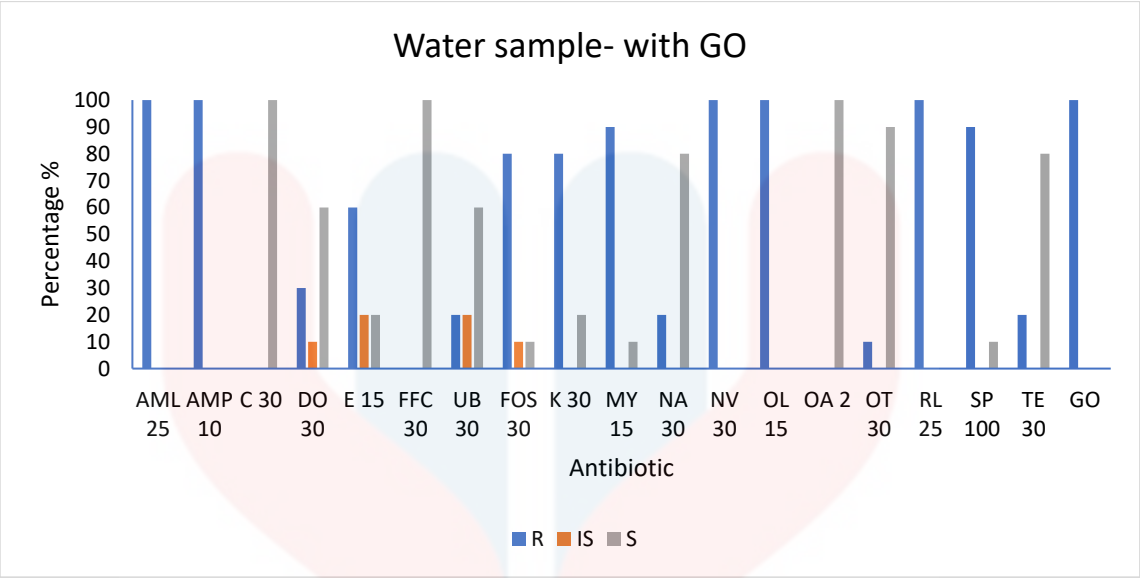

Figure 4.1: Graph of antibiotic sensitivity in semi-closed water sample with graphene oxide

As for the water sample without the use of Graphene oxide as antibiotic treatment, the total plate count of *Vibrio* sp. was 0 colony forming unit (CFU/g). Antibiotic discs that found to be sensitive to most bacterial isolates including chloramphenicol, florfenicol, doxycycline, flumequine, nalidixic acid, oxolinic acid, oxytetracycline and tetracycline. Nevertheless, the percentages of bacterial isolates which were sensitive to amoxycillin, ampicillin, erythromycin, Fosfomycin, kanamycin, lincomycin, novobiocin, oleandomycin, sulfamethoxazole and spiramycin were ranged from 0 % to 60 % (Fig 4.2). All bacterial isolates present were found to be resistant to amoxycillin, ampicillin, erythromycin, Fosfomycin, kanamycin, lincomycin, novobiocin, oleandomycin, sulfamethoxazole and spiramycin. Among all, the sensitivity category accounted for 45.2 % (86 cases) whereas 51.1 % (97 cases) and 3.7 % (7cases) respectively were reported as resistant and intermediary sensitive category. The MAR value for this study was 0.510 which is higher than 0.2, this show that the Asian clam is highly contaminated.

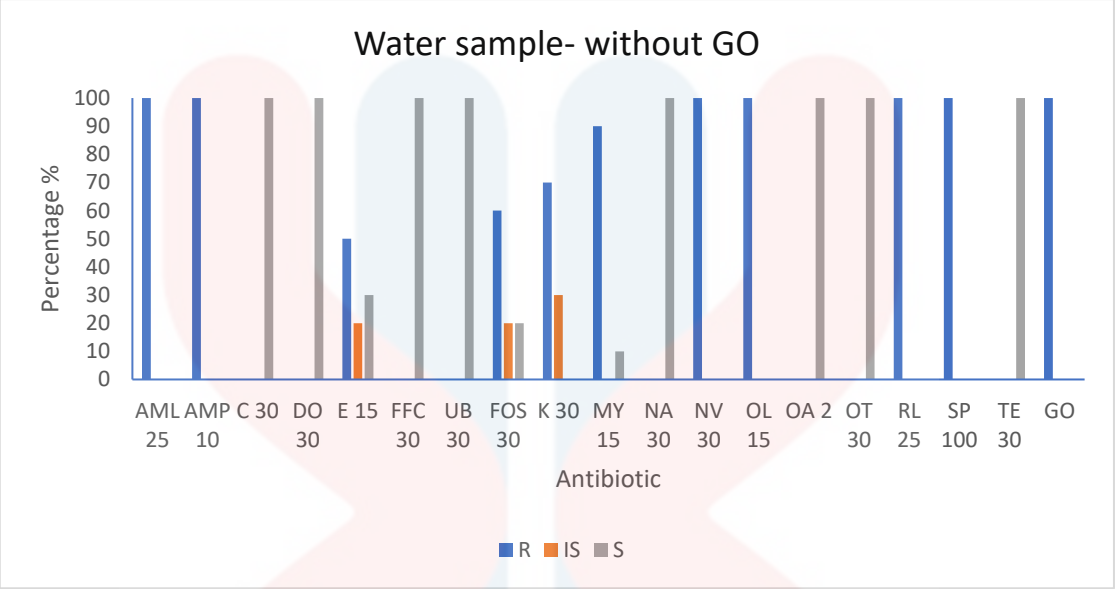

Figure 4.2: Graph of antibiotic sensitivity in semi-closed water sample without graphene oxide

On the other hand, total plate count of *Vibrio* sp. isolated from tissue sample of Asian clam was 3.75×10^3 colony forming unit (CFU)/g. Based on the result, most of the bacterial isolates present (exceeding 70 %) of bacterial isolates present were found sensitive to chloramphenicol, doxycycline, florfenicol, nalidixic acid, oxolinic acid, oxytetracycline and tetracycline (Fig 4.3). But, the percentage of bacterial isolates present which were sensitive to amoxicillin, ampicillin, erythromycin, flumequine, fosfomycin, kanamycin, lincomycin, novobiocin, oleandomycin, sulphamethoxazole and spiramycin were ranged from 0 % to 60 %. All the bacterial isolates present were found to be resistant to amoxicillin, ampicillin, lincomycin, oleandomycin, sulphamethoxazole, spiramycin and graphene oxide. Overall, the sensitive category accounted for 45.3 % (86 cases), whereas 52.1 % (99 cases) and 2.6 % (5 cases) were reported as resistant and intermediary

sensitive categories. Based on the present finding, since the MAR index show 0.521 which is higher than 0.2, it means the Asian clam is having high risk contamination.

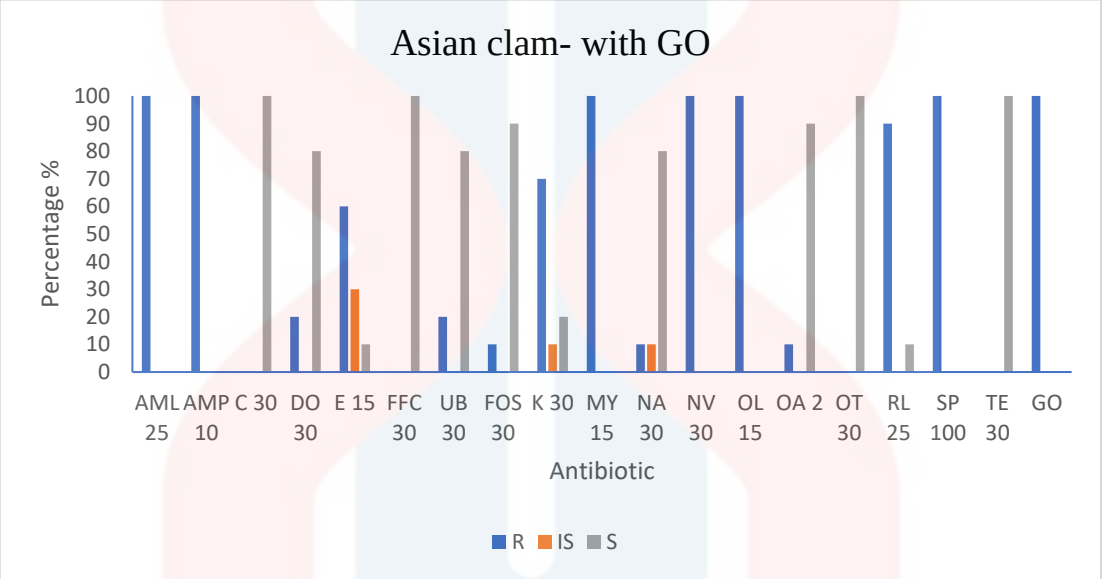

Figure 4.3: Graph of antibiotic sensitivity in Asian clam sample with graphene oxide

In this finding, total plate count of *Vibrio* sp. isolated from tissue sample without using Graphene oxide as antibiotic treatment was 4.7×10^3 colony forming unit (CFU)/g. Based on the result, most of the bacterial isolates present (exceeding 70 %) of bacterial isolates present were found sensitive to chloramphenicol, florfenicol, doxycycline, flumequine, nalidixic acid, oxolinic acid, oxytetracycline and tetracycline. The percentage of bacterial isolates present which were sensitive to amoxycillin, ampicillin, erythromycin, Fosfomycin, kanamycin, lincomycin, novobiocin, oleandomycin, sulfamethoxazole and spiramycin were ranged from 0 % to 60 % (Fig 4.4). All the

bacterial isolates present were found to be resistant to amoxycillin, ampicillin, kanamycin, lincomycin, oleandomycin, sulfamethoxazole, spiramycin and graphene oxide. Overall, the sensitive category accounted for 42.6 % (81 cases), whereas 54.7 % (104 cases) and 2.63 % (5 cases) respectively were reported as resistant and intermediary sensitive categories. The MAR index shows 0.547 which is higher than 0.2, it means the Asian clam is not suitable to intake raw as it is high risk contamination.

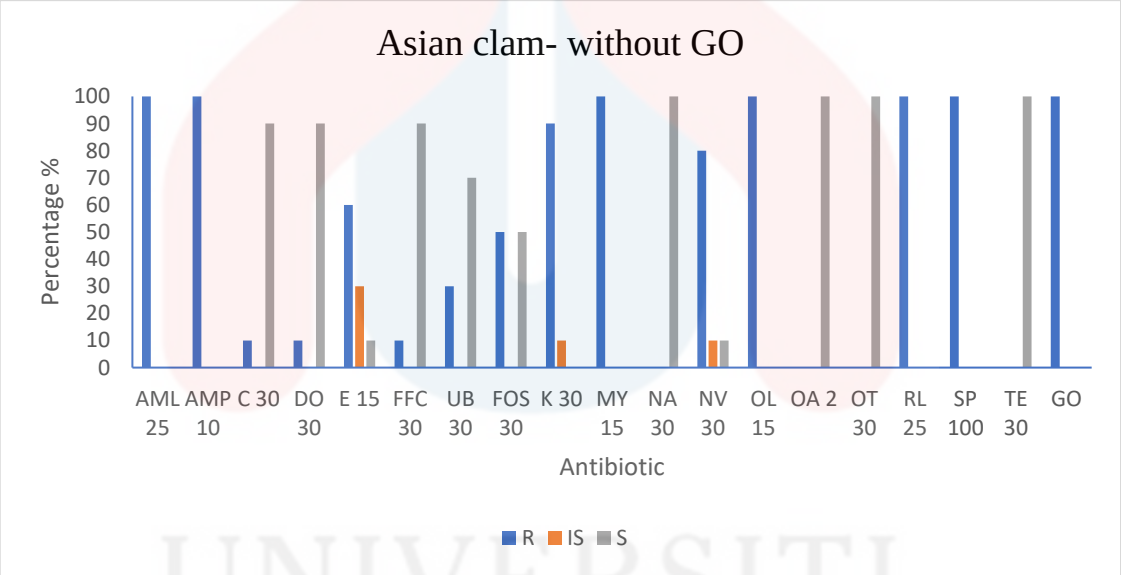

Figure 4.4: Graph of antibiotic sensitivity in Asian clam sample without graphene oxide

Based on Slayton et al., (2010), *Vibrio* spp. present abundance in the aquatic environment and seafood especially peak during summer periods as water temperatures are warmest (between 17°C to 35°C) (Cervino et al., 2004; Slayton et al., 2014). This indicates that tropical country such as Malaysia with average temperature 27°C is favour for *Vibrio* sp. to survive in the *Corbicula fluminea* farm. There is also proof of *Corbicula fluminea* to be highly applicable in biomonitoring for bacterial pollutants of freshwater habitats contaminated with human harmful microorganism (Gracyk et al., 2002), the fact of *Vibrio* sp. concentrating in the clams is undeniable.

In the present research, *Corbicula fluminea* are used because it is a newly high potential of aquaculture species that can form dense population with relatively small. Poor hygienic practice tends to allow water contaminate by *Vibrio* sp.. Infected human and animal, sewage, soil, and aquaculture can be sources of water bacterial contamination and may resulted death or disease due to enteric pathogens in aquatic population by release bacteria into the water (Momtaz & Dehkordi et al., 2013). Since the condition applied in current study was from fresh water, there was some situation faced during selection of *Vibrio* sp. colony on TCBS medium. As mentioned in results that the colonies obtained were 0 colony forming unit (CFU)/g. Hence, further time consumed needed for cultivation of *Vibrio* sp. for proceeding of antibiogram test. Few researches suggested that for specimen, which contain relatively fewer *Vibrio* and *Vibrio*-like organism, usually do not require dilution before culturing or incubation. In contrast, specimen which grow well with *Vibrio* sp. and other microorganism, increment of 10-fold dilution to 10^{-3} can reduce the numbers of competing microorganism. (Examination of Food and Environmental Samples, Centers for Disease Control and Prevention, n.d.). Another method to cultivate *Vibrio* sp. after detection can be done by enrich of sample on alkaline peptone water (APW) for further isolation of bacteria colony (Shrestha & Sujakhu, 2014).

According to Yaashikaa et al., (2016), bacteria disease is a serious problem affecting productivity, development and further growth of aquaculture. Few pathogenic species that commonly associated with *vibrio* sp. outbreak and mainly contributed to food poisonings were especially *Vibrio Cholerae* strain. The outbreak of *vibrio* infectious might due to consuming of food or contaminated water with human faces and waste water including exposure of skin lesion with aquatic environment. However, there are also research found out not every *Vibrio cholerae* is pathogenic, some are part of normal bacteria flora of estuaries and not directly harmful to human. Two cholerae strain which associated with establishment of diarrhoeal disease are from serogroup _O1 and _O139 (Cabral, 2010). Hence, further study should be done on those risky *vibrio* sp. such as Vibrio vulnificus, Vibrio parahaemolyticus and Cholerae serogroup O1 and O139.

In the present study, chloramphenicol (C 30), doxycycline (DO 30), florfenicol (FFC 30), flumequine (UB 30), nalidixic acid (NA 30), oxolinic acid (OA 2), oxytetracyline (OT 30) and tetracycline (TE 30) were found effective in controlling *vibrio sp*. cultivated from water and tissue samples of Asian clam. Thus, it is encouraged for clam farmer to use these antibiotics for prophylactic and treatment purposes in clam culture. The results from this research also showed that nalidixic acid, oxolinic acid, oxytetracycine and tetracycline could control almost 90% of all bacteria strain. In the research done by (Lee et al., 2009), the marketable size shellfish was found 100% sensitive against *vibrio sp*. were oxolinic acid, chloramphenicol, florfenicol and nalidixic acid. Furthermore, the current study, all bacteria isolates present were found to be resistant were amoxicillin (AML 25), ampicillin (AMP 10), lincomycin (MY 15), oleandomycin (OL 15), sulphamethoxazole (RL 25) and spiramycin (SP 100). In contrary with Hua & Apun, (2013) findings where ampicillin was reported to be 100% resistance to *vibrio sp*. in shrimp farming similar with nalidixic acid. Tetracycline is commonly used

as therapeutic and prophylactic antibiotic against most bacterial present in aquatic farm (Yano et al., 2014). Meanwhile another widely used antibiotic in aquaculture farm, oxytetracycline was found out to be 75% sensitive against *vibrio sp*. and successful in controlling vibriosis in shellfish culture (Thakur et al, 2003). Similarly, this research's finding also found both antibiotic to be sensitive against *Vibrio* spp. Since there are less reference for *Corbicula fluminea* to be used as sample in antibiogram resistance test as most research use shrimp as their sample, thus further study must be carried out to test which antibiotic can inhibit the growth of all strain of *Vibrio* spp. in *C. fluminea*.

Although few antibiotic can efficiency use as prophylactic agents, however, improper use such as widespread of antibiotic will lead to negative side-effect where increasing of antibiotic resistance among environment bacteria on the living organism (Chang, et al., 2015). Hence, management plans must be conducted strictly and making efforts such as introducing of antibiogram agents extract from natural ingredients or resources as described by research of Lee and Najiah, (2009) where *C. microcarpa* could be used to inhibit growth of *Vibrio*. *alginolyticus*. Not only that, by implementing stricter time for clam cooking, temperature requirement and educate consumer related the risk of *Vibrio vulnificus* infectious as established by the Interstate Shellfish Sanitation Conference (ISSC) in USA may reduce the occurrence of the bacteria-causing disease (Slayton et al., 2014b).

4.3 IDENTIFICATION OF BACTERIA WITH BBL CRYSTAL ENTERIC /NONFERMENTER KITS

In the present study, the bacterial identified by using BD BBL Crystal E/NF were *V. cholerae.* After 24 hours of incubation, among the reagent and biochemical substrates been activated, first 10 ingredients (4A-4J) were tested negatives of *Vibrio Cholerae* where the ingredients appeared in either orange or red colour as no gas produced from carbohydrates. Activation of following ingredients such as p-n-p-phosphate, p-n-p α-β-glucoside and p-n-p-β-galactoside indicates that enzyme hydrolysis of colourless aryl substituted glycoside or phosphate ester and releases of yellow p-nitrophenol. Positive test on location 2D which is proline nitroanilide turned yellow due to enzymatic hydrolysis of colourless amide substrate to yellow p-nitroaniline. P-n-p bis-phosphate, p-n-p-xyloside, p-n-p-α-arabinoside, p-n-p-phosphorylcholine, p-n-p-β-glucuronide and p-n-p-N-acetyl glucosaminide also turned up positively by hydrolysis of enzymatic through substituted glycoside or phosphate ester releases yellow p-nitrophenol. Reagent 1A, γ-L-glutamyl p-nitroanilide activated by hydrolyse amide group. Hydrolyse of esculin in 1B resulted in black precipitate with the presence of ferric ion. In p-nitro-DL-phenylalanine, phenylalanine group undergoes deamination oxidation when ferric ion presence. Another identification test commonly uses on *Vibrio* sp. test which is urea (1D) able to be hydrolyse by turn pH indicator from yellow or green to blue as ammonia present. Similar, *Vibrio* sp. able to reduce nitrate to nitrite through nitrification (Cabral, 2010). positive test on glycine showed blue precipitate as glycine degradation, resulting alkaline metabolites reaction and change colour of pH indicator. Citrate (1F) and Malonic acid (1G) will be utilized by metabolites of alkaline group causing the indicator turns to bromthymol blue colour as *Vibrio* sp. are from gram negative and alkaline condition is

preferred (Huq, Anwar, Haley, Bradd, Taviani, Elisa, Chen and Arlene, 2012). Triphenyl Tetrazolium chloride been activated to pink or red and sometimes turns invisible in different bacteria strain as tetrazolium compound undergoes reduction. Lastly, chemical ingredient such as arginine (1I) and lysine (1J) tested positively and turned to red or purple stain as anaerobic catabolism resulted rise of pH. As mentioned, *Vibrio* sp. are oxidase and catalase positive, the enzyme of *Vibrio* sp. will catalyse an oxidation-reduction reaction, especially one involve dioxygen as electron acceptor, donating hydrogen and resulting water and hydrogen peroxide as side product (Rattanachaikunsopon & Phumkhachorn, 2010).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

In conclusion, this research proved that *vibrio* sp. present in *Corbicula fluminea* hence without proper handling during cooking, it is not safe to be consumed as it might lead to food poisoning by bacteria as the MPN value for both samples is quite high. Few antibiotics were detected and able to use by the clam farmers as prophylactic and treatment properties on their aquaculture farm. Those antibiotics including nalidixic acid (NA 30), oxolinic acid (OA 2), Oxytetracycline (OT 30) and tetracycline (TE 30). However, the antibiotic should be applied according to the instruction to prevent antibiotic resistance on cultured species. Lastly, graphene oxide (GO) was found that not effective in controlling *Vibrio* sp. in this research.

5.2 RECOMMENDATION

The current study identified the *vibrio* sp. available in the water of semi-closed farmed and Asian clam sample. It is advising to carry out and focus on certain bacteria strain of *vibrio* sp. especially the harmful strain such as cholerae serogroup O1 and O139 which exactly bring harm to human. For the cases such as limited colonies cultivated, it should cultivate in enhancement broth for better survival of bacteria strain. Furthermore, more study on graphene oxide (GO) which carrying antibiotic properties must be further identify and study.

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Appendix A

Table A1: the raw data of the Colony Forming Unit (CFU) on Water sample in Thiosulphate Citrate bile Salt-Sucrose (TCBS) agar. Standard colonies for agar plate count is between 25 to 250 the most (Sutton, 2011)

	With Graphene Oxide (GO)				Without Graphene Oxide (GO)			
Dilution	1st Trial	Colony Forming Unit (CFU/ml)	2nd Trial	Colony Forming Unit (CFU/ml)	1st Trial	Colony Forming Unit (CFU/ml)	2nd Trial	Colony Forming Unit (CFU/ml)
10^{-1}	-		-		1		-	
10^{-2}	-		-		-		-	
10^{-3}	-		-		-		-	
10^{-4}	-		-		-		-	
10^{-5}	-		-		-		-	
10^{-6}	-		-		-		-	
10^{-7}	-		-		-		-	
10^{-8}	-		-		-		-	
10^{-9}	-		-		-		-	
10^{-10}	-		-		-		-	
Total		0		0		0		0

Table A2: the raw data of Colony Forming Unit (CFU) on Asian clam sample on Thiosulphate Citrate bile Salt-Sucrose (TCBS) agar.

	With Graphene Oxide (GO)				Without Graphene Oxide (GO)			
Dilution	1st Trial	Colony Forming Unit (CFU/ml)	2nd Trial	Colony Forming Unit (CFU/ml)	1st Trial	Colony Forming Unit (CFU/ml)	2nd Trial	Colony Forming Unit (CFU/ml)
10^{-1}	37	3700	38	3800	47	4700	24	
10^{-2}	13		19		6		1	
10^{-3}	2		-		4		-	
10^{-4}	-		-		-		-	
10^{-5}	-		-		-		-	
10^{-6}	-		-		-		-	
10^{-7}	-		-		-		-	
10^{-8}	-		-		-		-	
10^{-9}	-		-		-		-	
10^{-10}	-		-		-		-	
Total		3700		3800		4700		

Appendix B

Table B1: Raw data of categories of Antibiotic Sensitivity of Semi-closed water sample with the treatment of Graphene Oxide (GO). Sensitive (S) indicate more than 18mm, Intermediate sensitive (IS) indicate 16mm to 17mm, Resistance (R) indicates less than 15mm and No inhibition (NH)

Antibiotic	Sample									
	1	2	3	4	5	6	7	8	9	10
Amoxycillin (AML 25)	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP 10)	R	R	R	R	R	R	R	R	R	R
Chloramphenicol (C 30)	S	S	S	S	S	S	S	S	S	S
Doxycycline (DO 30)	IS	R	R	S	S	S	R	S	S	S
Erythromycin (E 15)	R	IS	R	S	IS	R	R	S	R	R
Florfenicol (FFC 30)	S	S	S	S	S	S	S	S	S	S
Flumequine (UB 30)	R	R	S	IS	IS	S	S	S	S	S
Fosfomycin (FOS 30)	IR	R	R	R	R	R	S	R	R	R
Kanamycin (K 30)	R	R	R	R	R	R	S	R	S	R
Lincomycin (MY 15)	R	R	R	R	R	R	R	R	S	R
Nalidixic Acid (NA 30)	S	S	S	S	S	S	R	S	S	R
Novobiocin (NV 30)	R	R	R	R	R	R	R	R	R	R
Oleandomycin (OL 15)	R	R	R	R	R	R	R	R	R	R
Oxolinic Acid (OA 2)	S	S	S	S	S	S	S	S	S	S
Oxytetracycline (OT 30)	S	S	R	S	S	S	S	S	S	S
Sulfamethoxazole (RL 25)	R	R	R	R	R	R	R	R	R	R
Spiramycin (SP 100)	R	S	R	R	R	R	R	R	R	R
Tetracycline (TE 30)	R	S	R	S	S	S	S	S	S	S
GO	NH	NH	NH	NH	NH	NH	NH	NH	NH	NH

Table B2: Raw data of Antibiotic Sensitivity express in percentage of Semi-closed water sample with the treatment of Graphene Oxide (GO).

Antibiotic (µg/disk)	Bacteria Isolated (Vibrio Cholera)		
	Sensitive (%)	Intermediate sensitive (%)	Resistance (%)
Amoxycillin (AML 25)	-	-	100
Ampicillin (AMP 10)	-	-	100
Chloramphenicol (C 30)	100	-	-
Doxycycline (DO 30)	60	10	30
Erythromycin (E 15)	20	20	60

Florfenicol (FFC 30)	100	-	-
Flumequine (UB 30)	60	20	20
Fosfomycin (FOS 30)	10	10	80
Kanamycin (K 30)	20	-	80
Lincomycin (MY 15)	10	-	90
Nalidixic Acid (NA 30)	80	-	20
Novobiocin (NV 30)	-	-	100
Oleandomycin (OL 15)	-	-	100
Oxolinic Acid (OA 2)	100	-	-
Oxytetracycline (OT 30)	90	-	10
Sulphamethoxazole (RL 25)		-	100
Spiramycin (SP 100)	10	-	90
Tetracycliae (TE 30)	80	-	20
GO	-	-	100

Table B3: Raw data of categories of Antibiotic Sensitivity of Semi-closed water sample without the treatment of Graphene Oxide (GO). Sensitive (S) indicate more than 18mm, Intermediate sensitive (IS) indicate 16mm to 17mm, Resistance (R) indicates less than 15mm and No inhibition (NH)

Antibiotic	Sample									
	1	2	3	4	5	6	7	8	9	10
Amoxycillin (AML 25)	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP 10)	R	R	R	R	R	R	R	R	R	R
Chloramphenicol (C 30)	S	S	S	S	S	S	S	S	S	S
Doxycycline (DO 30)	S	S	S	S	S	S	S	S	S	S
Erythromycin (E 15)	R	S	S	IS	S	R	R	IS	R	R
Florfenicol (FFC 30)	S	S	S	S	S	S	S	S	S	S
Flumequine (UB 30)	S	S	S	S	S	S	S	S	S	S
Fosfomycin (FOS 30)	R	R	R	R	IS	S	R	S	IS	R
Kanamycin (K 30)	IS	R	R	R	R	R	R	IS	R	IS
Lincomycin (MY 15)	R	R	R	R	R	R	R	R	S	R
Nalidixic Acid (NA 30)	S	S	S	S	S	S	S	S	S	S
Novobiocin (NV 30)	R	R	R	R	R	R	R	R	R	R
Oleandomycin (OL 15)	R	R	R	R	R	R	R	R	R	R
Oxolinic Acid (OA 2)	S	S	S	S	S	S	S	S	S	S
Oxytetracycline (OT 30)	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole (RL 25)	R	R	R	R	R	R	R	R	R	R
Spiramycin (SP 100)	R	R	R	R	R	R	R	R	R	R
Tetracycliae (TE 30)	S	S	S	S	S	S	S	S	S	S

GO	R	R	R	R	R	R	R	R	R	R

Table B4: Raw data of Antibiotic Sensitivity express in percentage of Semi-closed water sample without the treatment of Graphene Oxide (GO).

Antibiotic (µg/disk)	Bacteria Isolated (Vibrio Cholera)		
	Sensitive (%)	Intermediate sensitive (%)	Resistance (%)
Amoxycillin (AML 25)	-	-	100
Ampicillin (AMP 10)	-	-	100
Chloramphenicol (C 30)	100	-	-
Doxycycline (DO 30)	100	-	-
Erythromycin (E 15)	30	20	50
Florfenicol (FFC 30)	100	-	-
Flumequine (UB 30)	100	-	-
Fosfomycin (FOS 30)	20	20	60
Kanamycin (K 30)	-	30	70
Lincomycin (MY 15)	10	-	90
Nalidixic Acid (NA 30)	100	-	-
Novobiocin (NV 30)	-	-	100
Oleandomycin (OL 15)	-	-	100
Oxolinic Acid (OA 2)	100	-	-
Oxytetracycline (OT 30)	100	-	-
Sulphamethoxazole (RL 25)	-	-	100
Spiramycin (SP 100)	-	-	100
Tetracycliae (TE 30)	100	-	-
GO	-	-	100

Table B5: Raw data of categories of Antibiotic Sensitivity of Asian clam sample with the treatment of Graphene Oxide (GO). Sensitive (S) indicate more than 18mm, Intermediate sensitive (IS) indicate 16mm to 17mm, Resistance (R) indicates less than 15mm and No inhibition (NH)

Antibiotic	Sample									
	1	2	3	4	5	6	7	8	9	10
Amoxycillin (AML 25)	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP 10)	R	R	R	R	R	R	R	R	R	R
Chloramphenicol (C 30)	S	S	S	S	S	S	S	S	S	S
Doxycycline (DO 30)	S	S	R	S	S	S	S	S	R	S

Erythromycin (E 15)	R	R	R	R	IS	S	R	IS	IS	R
Florfenicol (FFC 30)	S	S	S	S	S	S	S	S	S	S
Flumequine (UB 30)	R	S	S	S	R	S	S	S	S	S
Fosfomycin (FOS 30)	S	S	S	S	R	S	S	S	S	S
Kanamycin (K 30)	S	R	R	R	S	R	IS	R	R	R
Lincomycin (MY 15)	R	R	R	R	R	R	R	R	R	R
Nalidixic Acid (NA 30)	S	S	S	S	R	S	S	S	IS	S
Novobiocin (NV 30)	R	R	R	R	R	R	R	R	R	R
Oleandomycin (OL 15)	R	R	R	R	R	R	R	R	R	R
Oxolinic Acid (OA 2)	S	S	S	S	R	S	S	S	S	S
Oxytetracycline (OT 30)	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole (RL 25)	R	R	R	S	R	R	R	R	R	R
Spiramycin (SP 100)	R	R	R	R	R	R	R	R	R	R
Tetracycliae (TE 30)	S	S	S	S	S	S	S	S	S	S
GO	R	R	R	R	R	R	R	R	R	R

Table B6: Raw data of Antibiotic Sensitivity express in percentage of Asian clam sample with the treatment of Graphene Oxide (GO).

Antibiotic (µg/disk)	Bacteria Isolated (Vibrio Cholera)		
	Sensitive (%)	Intermediate sensitive (%)	Resistance (%)
Amoxycillin (AML 25)	-	-	100
Ampicillin (AMP 10)	-	-	100
Chloramphenicol (C 30)	100	-	-
Doxycycline (DO 30)	80	-	20
Erythromycin (E 15)	10	30	60
Florfenicol (FFC 30)	100	-	-
Flumequine (UB 30)	80	-	20
Fosfomycin (FOS 30)	90	-	10
Kanamycin (K 30)	20	10	70
Lincomycin (MY 15)	-	-	100
Nalidixic Acid (NA 30)	80	10	10
Novobiocin (NV 30)	-	-	100
Oleandomycin (OL 15)	-	-	100
Oxolinic Acid (OA 2)	90	-	10
Oxytetracycline (OT 30)	100	-	-
Sulphamethoxazole (RL 25)	10	-	90
Spiramycin (SP 100)	-	-	100
Tetracycliae (TE 30)	100	-	-
GO	-	-	100

Table B7: Raw data of categories of Antibiotic Sensitivity of Asian clam sample without the treatment of Graphene Oxide (GO). Sensitive (S) indicate more than 18mm, Intermediate sensitive (IS) indicate 16mm to 17mm, Resistance (R) indicates less than 15mm and No inhibition (NH)

Antibiotic	Sample									
	1	2	3	4	5	6	7	8	9	10
Amoxycillin (AML 25)	R	R	R	R	R	R	R	R	R	R
Ampicillin (AMP 10)	R	R	R	R	R	R	R	R	R	R
Chloramphenicol (C 30)	S	S	S	S	S	R	S	S	S	S
Doxycycline (DO 30)	S	S	S	S	S	S	S	R	S	S
Erythromycin (E 15)	R	R	R	IS	R	R	S	R	IS	IS
Florfenicol (FFC 30)	S	S	S	S	S	R	S	S	S	S
Flumequine (UB 30)	S	S	S	R	S	S	R	R	S	S
Fosfomycin (FOS 30)	R	S	S	S	S	R	R	S	R	R
Kanamycin (K 30)	R	R	R	R	R	R	IS	R	R	R
Lincomycin (MY 15)	R	R	R	R	R	R	R	R	R	R
Nalidixic Acid (NA 30)	S	S	S	S	S	S	S	S	S	S
Novobiocin (NV 30)	R	R	IS	S	R	R	R	R	R	R
Oleandomycin (OL 15)	R	R	R	R	R	R	R	R	R	R
Oxolinic Acid (OA 2)	S	S	S	S	S	S	S	S	S	S
Oxytetracycline (OT 30)	S	S	S	S	S	S	S	S	S	S
Sulphamethoxazole (RL 25)	R	R	R	R	R	R	R	R	R	R
Spiramycin (SP 100)	R	R	R	R	R	R	R	R	R	R
Tetracycliae (TE 30)	S	S	S	S	S	S	S	S	S	S
GO	R	R	R	R	R	R	R	R	R	R

Table B8: Raw data of Antibiotic Sensitivity express in percentage of Asian clam sample without the treatment of Graphene Oxide (GO).

Antibiotic (µg/disk)	Bacteria Isolated (Vibrio Cholera)		
	Sensitive (%)	Intermediate sensitive (%)	Resistance (%)
Amoxycillin (AML 25)	-	-	100
Ampicillin (AMP 10)	-	-	100
Chloramphenicol (C 30)	90	-	10
Doxycycline (DO 30)	90	-	10
Erythromycin (E 15)	10	30	60
Florfenicol (FFC 30)	90	-	10

Flumequine (UB 30)	70	-	30
Fosfomycin (FOS 30)	50	-	50
Kanamycin (K 30)	-	10	90
Lincomycin (MY 15)	-	-	100
Nalidixic Acid (NA 30)	100	-	-
Novobiocin (NV 30)	10	10	80
Oleandomycin (OL 15)	-	-	100
Oxolinic Acid (OA 2)	100	-	-
Oxytetracycline (OT 30)	100	-	-
Sulphamethoxazole (RL 25)	-	-	100
Spiramycin (SP 100)	-	-	100
Tetracycliae (TE 30)	100	-	-
GO	-	-	100

Appendix C

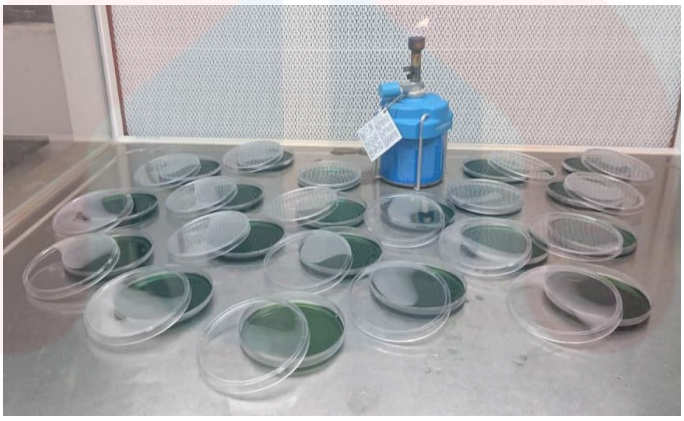


Figure C1: Preparation of TCBS agar

Figure C2: Sample of Asian clam, *Corbicula fluminea*

Figure C3: Homogenised the tissues sample evenly

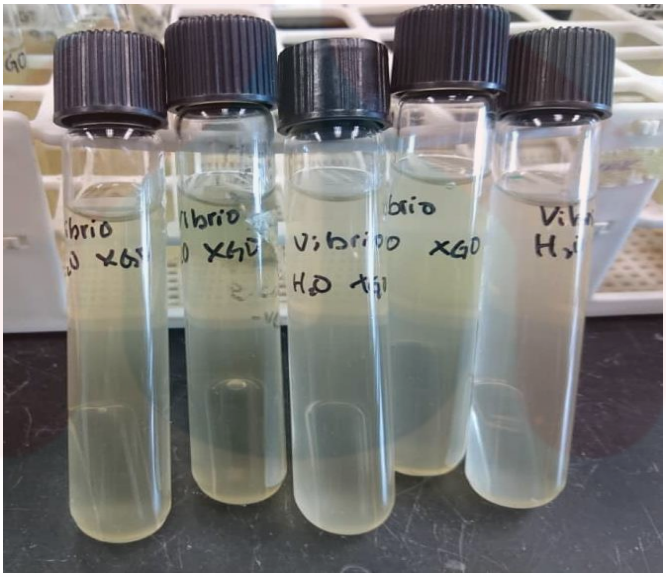


Figure C4: Positive results of presumptive test

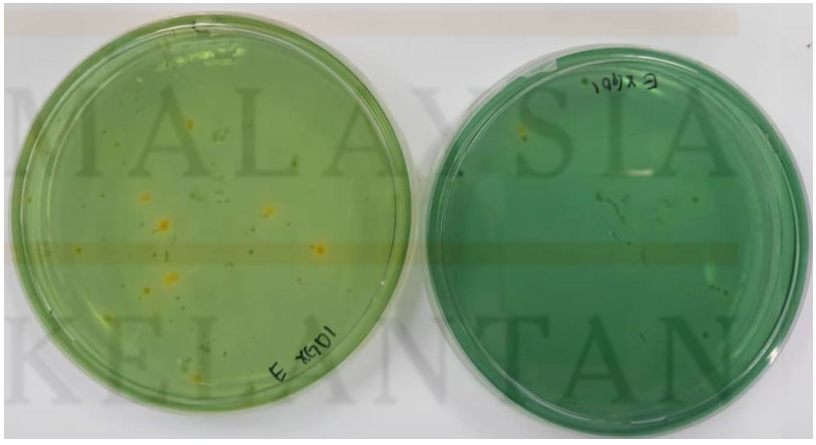


Figure C5: Yellow colony spotted on TCBS agar

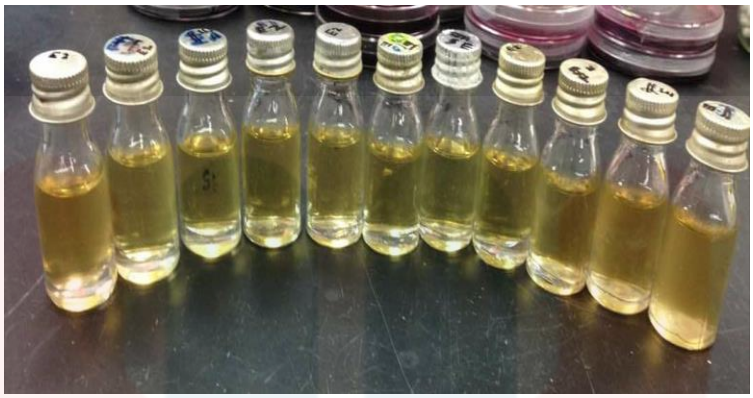


Figure C6: Preparation of TSB for subculture

C7: Swapping of subculture bacteria on TSA for antibiogram test

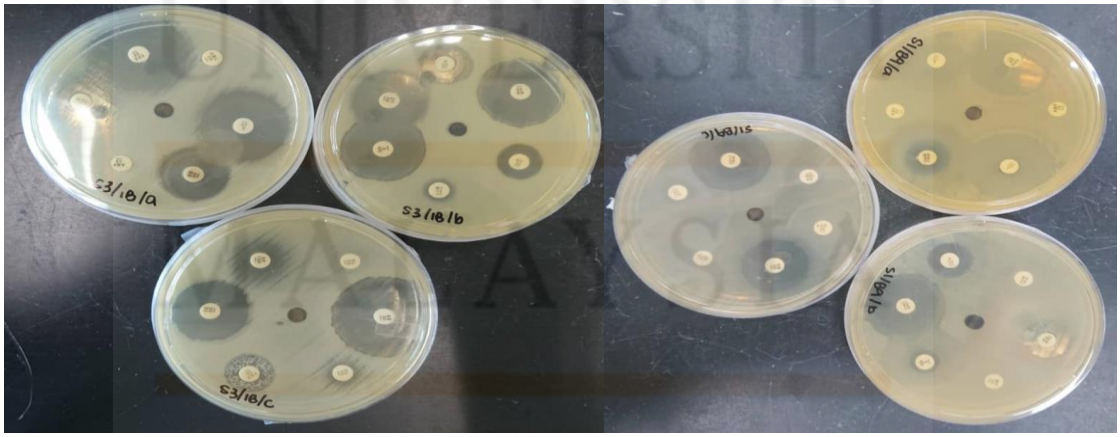


Figure C8: Inhibition zone of different antibiotic

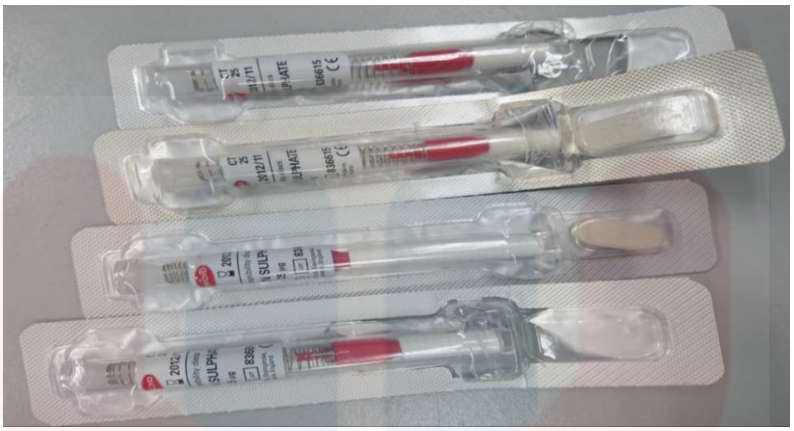
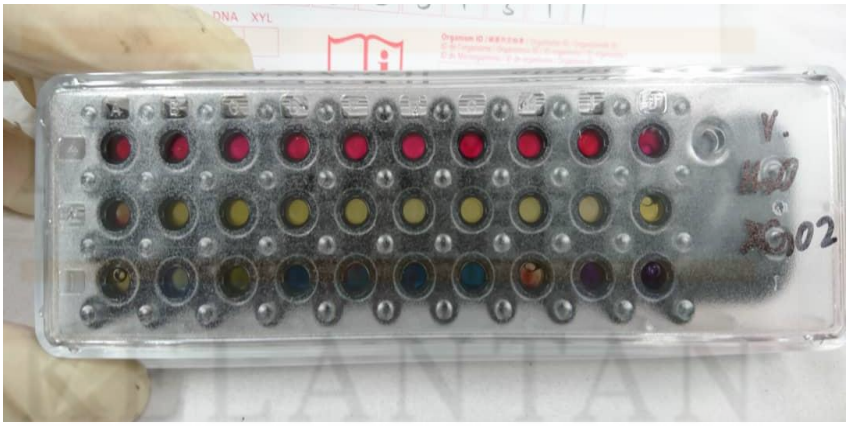


Figure C9: Sample of antibiotic discs

Figure C10: Bacteria identification by using BD BBL crystal kit and tabulated data on tabulation sheet



C11: Incubated BBL crystal enteric/nonfermenter kit