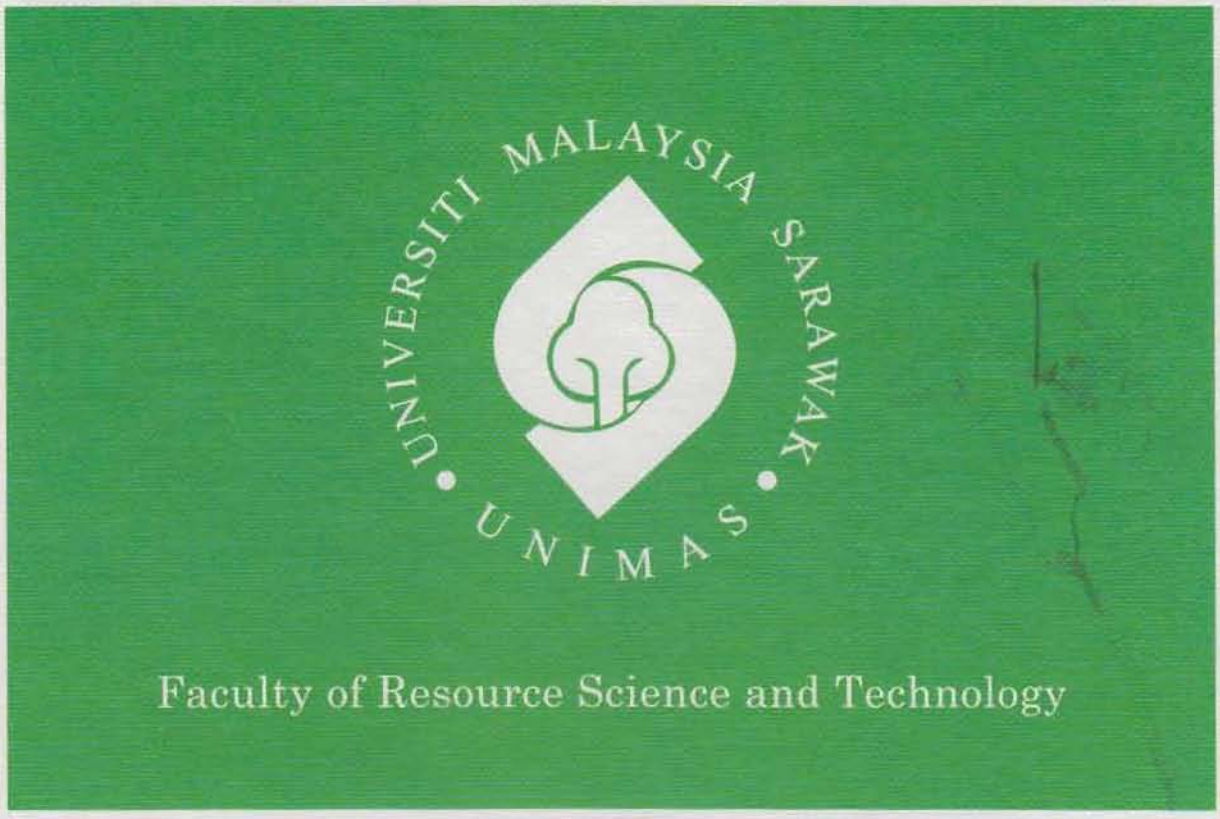

IDENTIFICATION OF *VIBRIO PARAHAEMOLYTICUS* FROM FOOD SAMPLES

Vivil Anak Bakas
(49382)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

IDENTIFICATION OF *VIBRIO PARAHAEMOLYTICUS* FROM FOOD SAMPLES

VIVIL ANAK BAKAS (49382)

A Thesis Submitted in Partial Fulfilment of the Requirement for the Degree of Bachelor of Science with Honours

Supervisor: Dr Lesley Maurice Bilung

Resource Biotechnology programme

Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

2017

Declaration

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LIST OF ABBREVIATION

APW	Alkaline Peptone Water
DNA	Deoxyribonucleic acid
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
sp.	Species
TCBS	Thiosulphate-Citrate-Bile-Salt Sucrose
tlh	Thermolabile hemolysin
trh	Thermolstable related Haemolysin gene
µl	Microlitre
UV	Ultraviolet
°C	Degree Celcius

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Identification of *Vibrio parahaemolyticus* from food samples

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ABSTRACT

The study was conducted to detect the presence of *Vibrio parahaemolyticus* from collected food samples obtained from Limbang, Sarawak district. The cultural based methods were employed for the identification and detection of *V. parahaemolyticus*. The Thiosulphate-Citrate-Bile Salt-sucrose (TCBS) agar was used to grow the *V. parahaemolyticus* cultures. The samples were revived in Luria Burtani broth with 3% of NaCl and incubate overnight at room temperature. Overnight cultures were streaked on TCBS agar plate to obtain the greenish colonies. The findings showed that 62 % or sixteen out of twenty six (16/26) of the samples appeared as green colonies on TCBS agar which is positive to *V. parahaemolyticus*. 38 % appeared as yellow colonies and some of the examined cultures were not growing. The positive samples were seafood and meat. Gram-staining test were conducted to confirm the presence of *V. parahaemolytics* in sixteen food samples suspected positive to *V.parahaemolyticus*. The results showed only three out of sixteen samples (3/16) were positive *V. parahaemolyticus* and thirteen out of sixteen (13/16) samples were other *Vibrio* sp. and some samples contaminated with other microorganism. The significant of the study is important for surveillance programme for *V. parahaemolyticus* to minimize foodborne disease due to *V. parahaemolyticus*.

Keywords: Thiosulphate-Citrate Bile Salt-sucrose (TCBS) agar, Green colony, *Vibrio parahaemolyticus*

ABSTRAK

Kajian ini dilakukan untuk mengenalpasti kehadiran V. parahaemolyticus dalam dua puluh enam sampel makanan dari daerah Limbang, Sarawak. Untuk mengesan V. parahaemolyticus dalam sampel makanan tersebut kaedah kultur telah digunaan. Agar TCBS telah digunakan untuk membiak V. parahaemolyticus. Sampel telah dipulihkan dalam Luria Burtani dengan 3% sodium chlorida dan dibiarkan selama satu malam dalam suhu bilik. Kultur akan disapu atas agar untuk mendapatkan koloni warna hijau. Hasil kajian menunjukkaN 62 % iaitu enam belas daripada dua puluh enam (16/26) sampel mengandungi bakteria V. parahaemolyticus. 38 % sampel yang lain menunjukkan koloni warna kuning atas agar. Terdapat beberapa sampel yang tidak tumbuh atas agar. Antara sampel yang positif atas agar TCBS ialah makanan laut dan juga daging. Seterusnya,ujian gram-staining dijalankan atas enam belas sampel yang dipercayai mengandungi bakteria V. parahaemolyticus untuk memastikan kehadiran V. parahaemolyticus dalam sampel makanan wujud. Hasil ujian menunjukkan hanya tiga daripada enam belas sampel (3/16) yang positif V. parahaemolyticus.dan tiga belas daripada enema belas sampel ialah Vibrio spesis lain dan juga sampel yang telah di cemari mikroorganisma yang lain.

***Kata Kunci**: Agar Garam hempedu Thiosulphate-Citrate- sukrosa (TCBS) , koloni hijau, V. parahaemolyticus*

CHAPTER 1

INTRODUCTION

1.1 Introduction

Vibrio parahaemolyticus is a Gram-negative halophilic, non-spore forming and curve rod-shaped bacterium that mainly found in marine environment. Several *Vibrio* species are serious pathogens to human that cause acute gastroenteritis due to the consumption of raw, undercooked or mishandled of seafood (Vengadesh *et al.*, 2015). Some of foods are naturally contaminated by foodborne pathogen that present in marine environment as the aquatic habitat are their natural niche (Lesley *et al.*, 2015). Due to contamination in harvest area may also cause the *V. parahaemolyticus* to propagate in seafood products.

According to Lesley *et al.* (2015) consumption of raw seafood such as Sushi as a favourite style may cause many cases of foodborne disease by *V. parahaemolyticus*. In addition, the risk to infect by disease cause by *V. parahaemolyticus* is high as the ways to handle foods were not appropriate and the pathogens could multiply rapidly under favourable conditions. Most of seafood caught from its wild environment, which is marine environment then handle and processed without used any of chemical additives and distributed immediately to the market on the same day by freezing in order to maintain the condition of product means to preserve the food. Thus, these condition cause the growth of *V. parahaemolyticus* getting faster especially in raw seafood or any meat products.

In Malaysi,a *V. parahaemolyticus* is one of the most known bacterium that lead to foodborne disease (Zulkifli *et al.*, 2009). Since seafood is one of the important sources of nutrient in this country, food safety becomes a concern among the consumer. Besides that, seafood products are one of the important economic activities for many developing

countries including Malaysia. To make sure the quality of product is good and safe, it is important to meet the bacterialogical of importing and failure to do so may impact economic and loss of income. Thus, several studies have been conducted in order to detect, identify and also molecular characterize of *V. parahaemolyticus* from food samples. Recently, *V. parahaemolyticus* has been isolated and identified from coastal seawater in Peninsular Malaysia. The study conducted due to concerned on *V. parahaemolyticus* outbreak caused by the consuming of contaminated seafood. The findings of study showed that, several parameters such as temperature, dissolved oxygen, and salinity played important role in distribution of *V. parahaemolyticus* which can be used and possibly to prevent *V. parahaemolyticus* outbreaks (Lesley *et al.*, 2014).

Due to concern on the food safety, the present study was carried out to detect and identified *V. parahaemolyticus* from food samples obtained from Limbang, Sarawak Malaysia. Detection of *V. parahaemolyticus* from food samples were by streak plate-Briefly, isolated *V. parahaemolyticus* from food samples were revived in Luria Burtani Broth with 3 % NaCl and incubated at room temperature overnight. Overnight and diluted cultures were streak on TCBS agar. The plates were then incubated overnight at 37 °C. Positive samples on TCBS agar were then subjected to gram-staining test. The main objectives of the study are to identify the presence *V. parahaemolyticus* using TCBS agar and gram-staining test. Thus, the study provides information on the presence of *V. parahaemolyticus* in food samples and risk to human.

Objectives of study are:

1) To identify the presence and morphological of *V. parahaemolyticus* in food samples using TCBS agar.
2) To further confirm the presence of *V. parahaemolyticus* using gram-staining test.

CHAPTER 2

Literature Review

2.1 Family Vibrionaceae

The first person that proposed Vibrionaceae is Veron in 1965 when they were fermentative bacteria and found that the bacteria have polar flagella and a positive oxidase reaction for the purpose to differentiate between Vibrionaceae from Enterobacteriaceae. Species of this family can be pathogenic or non-pathogenic. Pathogenic species may cause serious infection that may cause fatal to an individual.

In addition, the family of Vibrionaceae has many of important organisms. The genus for the family is *Vibrio* and the examples of species are *Vibrio parahaemolyticus*, *Vibrio cholera*, *V. mimicus* and many other *vibrio* species. This organism especially *V. pararahaemolyticus* and *V. cholera* has caused death to millions of people during the epidemics infection that terrorized most parts of the world. The infection is gastrointestinal infections that may occur in both human and animal. Many species of Vibrionaceae are widely distributed in the environment. The importance of family Vibrionaceae is the species under this family has been widely used in the study of biochemical, molecular biology and pathogenicity (Farmer., 2010).

2.2 Vibrio parahaemolyticus

2.2.1 Morphology and culture

Vibrio parahaemolyticus is a bacterium from family *Vibrionaceae.* This halophilic gram-negative bacterium has curved comma rod shape or straight rod and non-spore forming with size 0.5-0.8 μm in width and 1.4-2.6 μm in length that normally found in marine environment as their natural niche (Lesley *et al.,* 2015). *V. parahaemolyticus* can survive well in marine environment due to its ability to endure halophilic condition. High concentrations of this bacteria may presence during summer when salinity is higher in the aquatic environment (Zulkifli *et al.*, 2009). This means, the *Vibrios* species are particularly resistant to high salt concentration and sensitive to salinity due to the fact that *V. parahaemolyticus* is halophile. According to Hassan *et al.* (2012), these facultative anaerobes bacteria are motile, have one or more polar flagella, and are inhibited by the vibriostatic compound.

V. parahaemolyticus is able to grow well in alkaline condition with high pH value and detected in pH 6.47 (Lesley *et al.*, 2015). *V. parahaemolyticus* also sensitive to acids therefore it can grow better at pH 7.5 to 8.5. For the temperature, *V. parahaemolyticus* can multiply rapidly between 20 °C and 40 °C but its optimum temperature is at 37 °C . Zulkifli *et al.* (2009) also mentioned that high marine temperature between 25 °C and 35 °C resulted in the distribuition of *V. parahaemolyticus.* Previous study by Chai *et al.* (2014) reported that, occurrence of *V. parahaemolyticus* in the food samples were attributed to the favourable environmental temperature (26 to 27 °C). In unfavourable condition, *V. parahaemolyticus* can turn into viable but non-culturable state (Ling, 2009). Environment stress such as starvation, cold temperature and suboptimal pH can induce viable but non-culturable state. The bacteria are still alive as their metabolisms are still

going but they do not form colonies on nutrient media. Wang and Wong (2004) also state that, *V. parahaemolyticus* can enter viable state but non-culturable state. They can be revived within 3 days after the temperature are optimal for their growth.

2.2.2Vibrio parahaemolyticus in seafood

Seafood is known as one of the important sources of nutrients and balance diet to consumer around the world which needed to make a person healthy. Nevertheless, there is health risks by consumption of seafood (Lesley *et al.*, 2015) and one of the major risks involved consumption of undercooked seafood that already contaminated by foodborne pathogens that present in marine environment may cause infection. According to Sakata *et al.* (2012) and Micky *et al.* (2014) when ingested food that has been infected by pathogenic *V. parahaemolyticus*, a person may experience diarrhae, often the abdominal cramp, nausea, and vomiting fever because this bacteria produce toxigenic gene. Toxigenic gene mean an organism contain pathogenic gene that can produce toxin which may cause serious illness once an individual infected. For pathogenic gene, there are two common gene that are widely used in detecting of *V.parahaemolyticus* which are *trh* and *tlh* gene. Both genes have been widely applied to identify the presence of pathogenicity of *V. parahaemolyticus* that usually cause food poisoning to the consumer.

2.2.3 Epidemiology

Globally, *V. parahaemolyticus* is common cause of foodborne disease and there have been numerous reports of *V.parahaemolyticus* outbreaks in last few decades. In United States, *V. parahaemolyticus* was reported that caused the outbreaks in 1998 involving residents of Connecticut, New Jersey and New York due to consumption of contaminated

oysters and clams harvested from Long Island (Vincent *et al.*, 2015). Next, the largest reported outbreak in North America involving *V.parahaemolyticus* infection occurred when 209 individuals were admitted after eating raw oyster harvested from California, Oregon and Washington in 1997. In Japan and Taiwan, *V. parahaemolyticus* outbreaks have been reported due to consumptions of contaminated Sashimi and Sushi (Vincent *et al.*, 2015).

In Malaysia, seafood and meat are the most important sources of nutrient and balance diet but this kind of foods are one of the leading causes of foodborne outbreaks (Chai *et al.*, 2014). Serious infection includes septicaemia that threatens the life of immunodeficient groups and prolonged steroid users (Chai *et al.*, 2014). Increasing outbreaks of *V. parahaemolyticus* had resulted in serious economic loss to Malaysia , besides has raised consumer concern to food safety. In addition, infection may transmit through drinking water and open wounded skin. A person may infect as they swim in sea water that already contaminated with *Vibrio* especially in warmer months (Lesley *et al.*, 2014) and in higher concentration during summer when salinity is higher in the aquatic environment (Zulkifli *et al.*, 2009) and also naturally found in coastal marine waters throughout the world (Mickey *et al.*, 2014).

2.3 Selective agar for *Vibrio* species detection

Currently, the standard method for the detection and identification of *V. parahaemolyticus* is by using microbial media (Vincent *et al.*, 2015) such as Alkaline Peptone Water (APW), Thiosulphate-Citrate-Bile Salts-sucrose (TCBS) agar and a range biochemical test like gram-staining test. TCBS were widely used as the colour of colony on agar can be observed. This procedure has advantage as the cost are low but also has the disadvantages whereby the procedure not only lengthy and their reproducibilities are also low. This is because to prepare the media and incubation, extra time is needed which may cause contaminations to the cultures (Micky *et al.*, 2014).

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strain

The bacterial strains used in this study are listed in Table 1. The strain listed identified as known *Vibrio* species isolated from food samples at Limbang Sarawak, Malaysia. Twenty six of isolate food samples as listed in Table 1 were revived in Luria Burtani broth with 3% of NaCl and incubated in room temperature overnight.

Table 1: List of sample type examine in the study

SAMPLE	SAMPLE TYPE
VCO1A	*Gerai No. 26*
VCO1B	*Isi ayam*
VCO1C	*Sotong*
VCO2A	*Gerai no. 21*
VCO2B	*sotong*
VCO2C	*lokan*
VCO3A	*Gerai no.19*
VCO3B	*Insang ikan sultan*
VCO4A	*Gerai no.21*
VCO4B	*Insang ikan kembung*
VCO4C	*Lokan*
VCO5A	*Gerai no.15*
VCO5B	*Sayap ayam*
VCO5C	*Ketam*
VCO6A	*Gerai no. 11*
VCO6B	*Sayap ayam*
VCO6C	*Kepala ayam*
VCO7A	*Gerai no.10*
VCO7B	*Lokan*
VCO7C	*Daging Babi*
VCO8B	*Tekoyong siyak*
VCO8C	*Siput Sedut*
VCO9A	*Gerai no .6*
VCO9B	*Berungun*
VC10A	*Gerai no 3 dan 4*
VC10B	*Ketam*

3.2 Streaking plate

The overnight and diluted cultures in Luria Burtani Broth with 3 % NaCl were streaked on TCBS agar plate to confirm the presence of *Vibrio parahaemolyticus*. Firstly, the inoculating loop used to streak was heat by flaming before used in order to minimize the contamination towards the cultures. The mouth of the cultures tubes were opened and briefly flame. Next, some of the culture growth were picked up with the sterile loop and transferred to fresh selective medium TCBS agar. Finally, the plates were seal and incubated in room temperature for 24-28 hours.

3.3 Gram staining

The smear prepared and fixed from the cultures to slide surface. The slides were dried and heat near flame for two to eight times. The slides were then let to cool down before staining. Briefly, the slides were place on staining rack and flood the slides with the crystal violet and stain for 20 to 30 seconds. Excess crystal violet washed with tap water and drain off excess water. Next, the slide flood with iodine and stain for 20 to 30 seconds then washed again. After that, the smear tilt and decolourized with 70% ethanol for 20 to 30 seconds until colourless alcohol draining from the slide appeared. The slides were washed again with tap water. The smear was counterstain with the safranin for 20 to 30 seconds and washed then dry the excess with Bibulous paper. Finally, the slides were examined under light microscope to determine whether the samples were gram-negative or gram-positive bacterium. If gram-negative it appeared pink and purple if gram-positive. Bacterial isolates were preserved in Luria Burtani broth with 3% NaCl containing 30% glycerol stock in 4° C freezer

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

Seafood samples such as VCO1C (*sotong*), VCO4C, (*lokan*) (Figure 1), VCO9B (*berungun*) and VCOC5C (*ketam*) showed presence of *V.parahaemolyticus* (Table 2). Meat samples also showed the presence of *V. parahaemolytius* such as chicken and pig meat (Table 2). However, there was no detection of *V. parahaemolyticus* recorded in 36 % of samples (10 out of 26). The samples were VCO3B (*Insang ikan sultan*), VCO4A (*Gerai No. 21*), VCO6A (*Gerai No. 11*), VCO6B (*sayap ayam*) and six more food samples (Table 2).

Figure 1: Green colonies of *V. parahaemolyticus* on TCBS agar from sample of VCO2C, *lokan*

About 19 % (5 out of 26) of samples appeared yellow colonies on TCBS agar such as VCO4A (*Gerai No. 21*), VCO6B (*Sayap ayam*) (Figure 2) and VCO8B (*Tekoyong siyak*)(Table 2).

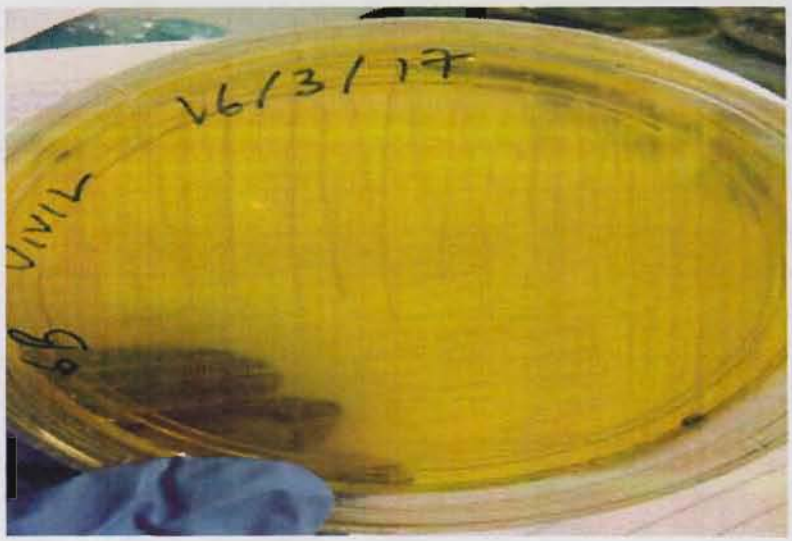

Figure 2: Yellow colonies of other *Vibrios* sp. on TCBS agar from food sample VCO6B, *sayap ayam*

Table 2: Detection of *Vibrio parahaemolyticus* isolated from food samples based on cultural method

SAMPLE	SAMPLE TYPE	Sample positive on TCBS (greenish colony)
VCO1A	*Gerai No. 26*	+
VCO1B	*Isi ayam*	+
VCO1C	*Sotong*	+
VCO2A	*Gerai no. 21*	+
VCO2B	*sotong*	+
VCO2C	*lokan*	+
VCO3A	*Gerai no.19*	+
VCO3B	*Insang ikan sultan*	-
VCO4A	*Gerai no.21*	Yellow
VCO4B	*Insang ikan kembung*	+
VCO4C	*Lokan*	+
VCO5A	*Gerai no.15*	+
VCO5B	*Sayap ayam*	+
VCO5C	*Ketam*	+
VCO6A	*Gerai no. 11*	-
VCO6B	*Sayap ayam*	Yellow
VCO6C	*Kepala ayam*	+
VCO7A	*Gerai no.10*	+
VCO7B	*Lokan*	-
VCO7C	*Daging Babi*	+
VCO8B	*Tekoyong siyak*	Yellow
VCO8C	*Siput Sedut*	Yellow
VCO9A	*Gerai no .6*	-
VCO9B	*Berungun*	+
VC10A	*Gerai no 3 dan 4*	-
VC10B	*Ketam*	Yellow

Based on the observation under 100x light microscope, only three samples out of sixteen (3/16) positive *V. parahaemolyticus*. 18 % (3/16) positive samples were *Isi ayam, sotong* and food samples obtained from *Gerai No. 15.*

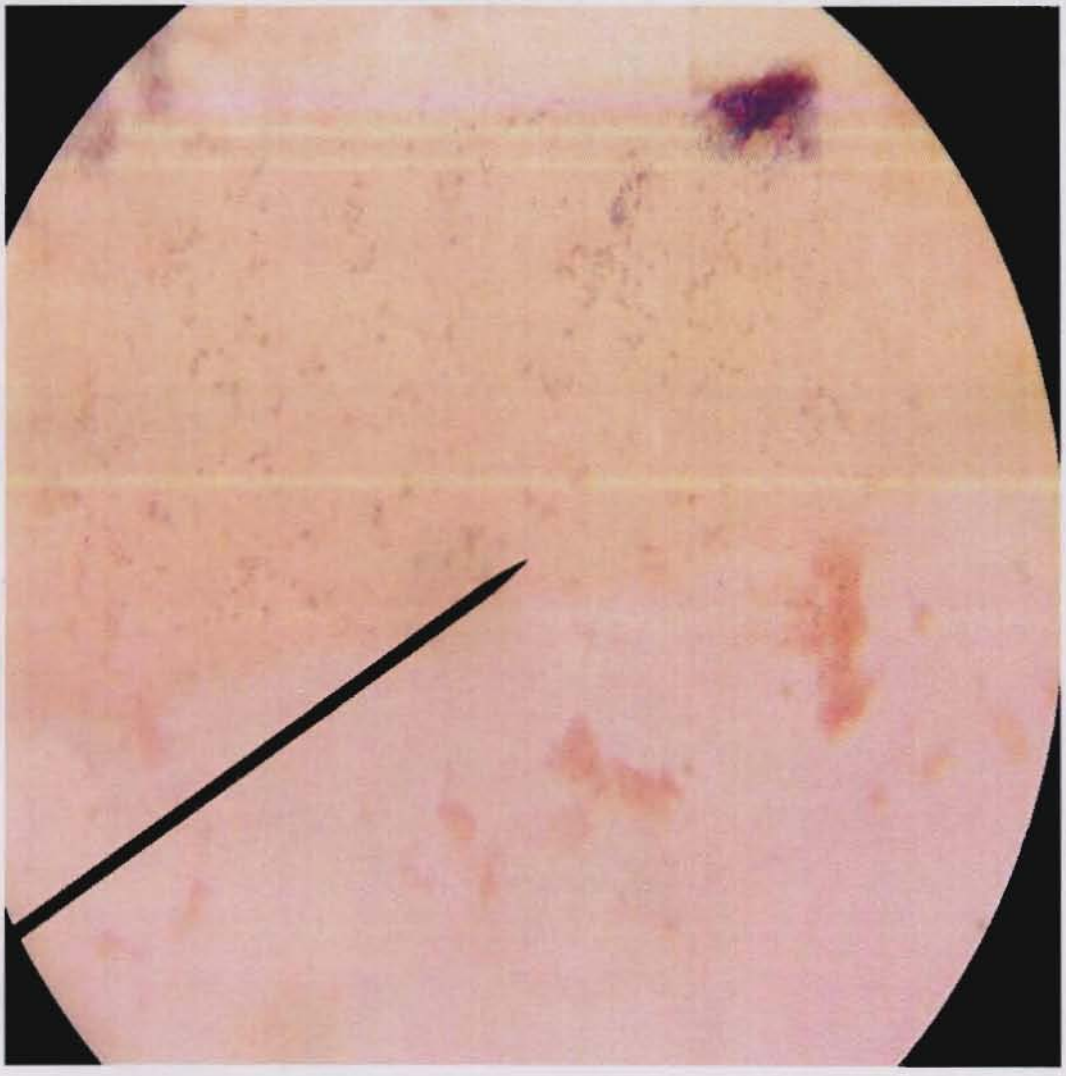

Figure 3: Gram-staining of positive samples (VCO1C: *Sotong*) on TCBS agar under 100X light microscope

Four out of sixteen samples (4/16) were positive to *V. parahaemolyticus* based on the colonies colour on TCBS agar but negative *V.parahaemolyticus* after subjected to gram-staining test. The food samples were negative *V. parahaemolyticus* as the cultures were contaminated by other microorganisms. The samples were observed under microscope and there is contamination presence (Figure 2). Some of the food samples were negative *V. parahaemolyticus* as the other vibrio species present. Figure 2 showed the results of gram-staining test for negative result, contaminated cultures.

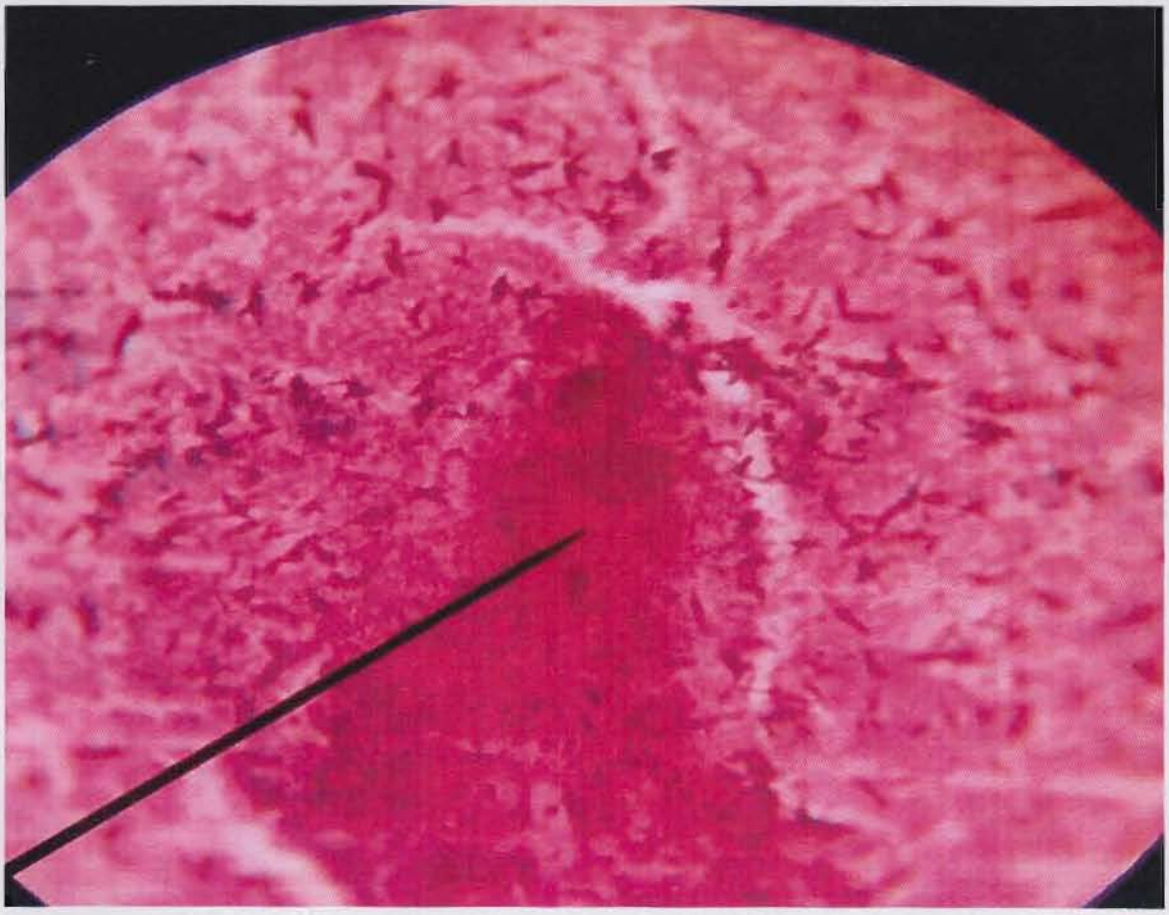

Figure 4: Food sample (VCO2A: *isi ayam*) that showed negative presence of *V. parahaemolyticus* based on Gram-staining test.