Identification and Detection of *Shigella* species from Wildlife Using Multiplex Polymerase Chain Reaction (mPCR)

Wong Ngah Kiat (20260)

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Supervisor: Prof. Madya Dr. Kasing Apun

Co-supervisor: Dr. Samuel Lihan

Programme: Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and technology

Universiti Malaysia Sarawak

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleotide acid
XLD	Xylose Lysine Deoxycholate
TSI	Triple Sugar Iron
S.I.M	Sulphide – Indole – Motility
HE	Hektoen Enteric
ml	Mililiter
µl	Microliter
mM	Milimolar
µM	Micromolar
s	Second
min	Minute
°C	Degree Celcius
mPCR	Multiplex Polymerase Chain Reaction
rpm	Revolution per minute
ddH_2O	Sterile distilled water
dNTPs	Deoxynucleotidetriphosphates
E.coli	*Escherichia coli*

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Wong Ngah Kiat

Resource Biotechnology Programme
Department of Molecular Biology
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Shigella spp. is Gram-negative bacteria that causes shigellosis in both developing and developed countries. *Shigella* spp. was reported to be the third common bacterial agent responsible for childhood diarrhoea in Malaysia. Most of the previous research on *Shigella* were focused on human. Hence, this study aimed to detect the occurrence of *Shigella* spp. from wildlife (rats, squirrels, birds, and bats) using biochemical tests and multiplex PCR assay. Biochemical tests were used to pre-identify the occurrence of *Shigella* spp. isolated from wildlife. Then, mPCR assay was used for simultaneous detection of virulence genes (*setl A, setl B, ial* and *ipaH*) in *Shigella* spp. Four isolates from a total of fifty swab samples (8%) collected from the wildlife were confirmed to be *Shigella* spp. using mPCR assay. The invasion plasmid antigen H (*ipaH*) gene was detected in all of the four isolates with two isolates from squirrels, one isolates from bat and one isolate from wild bird. On the other hand, invasion-associated locus (*ial*) gene was only detected in isolate from the squirrel. Both *ial* and *ipaH* genes are responsible for directing epithelial cell penetration by *Shigella*. This study therefore has successfully detected the occurrence of *Shigella* spp. from wildlife and eventually shows that animals (e.g. birds, rodents) can be vectors for *Shigella* spp. and have the potential to cause zoonotic infection.

Keywords: *Shigella* spp., wildlife, biochemical tests, mPCR

ABSTRAK

Shigella spp. ialah Gram-negatif bacteria yang mengakibatkan penyakit shigellosis di negara-negara yang sedang membangun dan telah membangun. *Shigella* spp. dilapor di Malaysia sebagai agen ke-tiga umum yang menyebabkan cirit-birit di kalangan kanak-kanak. kebanyakan kajian yang lalu tentang *Shigella* adalah berkaitan dengan manusia. Oleh itu, matlamat kajian ini adalah untuk mencari sama ada *Shigella* spp. wujud dalam binatang liar (tikus, tupai, burung, dan kelawar) dengan mengunakan ujian biokimia dan kaedah mPCR. Ujian biokimia telah digunakan untuk pegecaman awal kewujudan *Shigella* spp. dipencil dari binatang liar. Kemudian, kaedah mPCR telah digunakan untuk mencari gen virulen (*setl A, setl B, ial* and *ipaH*) dalam *Shigella* spp. sekaligus. Empat pencilan daripada lima puluh sampel (8%) yang dikumpul dari binatang liar dibukti sebagai *Shigella* spp. dengan menggunakan kaedah mPCR. Gen *ipaH* (invasion plasmid antigen H) dapat dicari dalam keempat-empat pencilan yang dua pencilan daripada tupai, satu pencilan daripada kelawar dan satu pencilan daripada burung liar. Manakala, gen *ial* (invasion-associated locus) hanya dapat dicari dalam pencilan daripada tupai. Kedua-dua gen *ial* dan *ipaH* adalah berfungsi untuk menghala *Shigella* memasuki sel epithelia. Secara keseluruhan, kajian ini Berjaya mencari *Shigella* spp. daripada binatang liar yang seterusnya menunjukkan bahawa binatang (seperti burung dan tupai) ialah vektor yang berpotensi menyebarkan penyakit zoonotik.

Kata kunci: *Shigella* spp., binatang liar, ujian biokimia, mPCR

1.0 INTRODUCTION

Shigella is Gram-negative bacteria that can cause shigellosis (Theron *et al.* 2000), a major diarrhoea disease that causes morbidity and mortality of children in developing countries (Chiou *et al*. 2001). Shigellosis doesn't cause severe infection in normal healthy adult, but can be fatal to infants and young children (Centres for Disease Control and Prevention, 2008).

There was an estimated annual infection of 160 million infected individuals, with 1.1 million deaths and majority were children under 5 years old (Kotloff *et al*. 1999). A study had been done by Lee *et al*. (2002) shown that *Shigella* spp. was the third commonest bacterial agent responsible for childhood diarrhoeal in Malaysia.

Shigella is a bacteria species closely related to *Escherichia coli* and *Salmonella spp*. and is predominantly transmitted by faecal-oral contact (Clemens *et al*. 1999). According to Chein *et al*. (2000), there are four serogroups of *Shigella* spp. including *S. dysenteriae, S. flexneri, S. boydii,* and *S. sonnei* with the three major disease causing species are *S. dysenteriae*, *S. flexneri* and, *S. sonnei*.

It was reported that humans and higher primates are normally the reservoirs for *Shigella* spp. (Tanner, 2003). Also, according to King (1998), shigellosis is primarily human disease and thus majority of the research on *Shigella* had been focused on human and published in the medical literature and hence, there are not much information of *Shigella* spp. in animals is readily available. But, animals (e.g. birds, rodents) can be the vectors that are capable of transmitting *Shigella* to human through their body surfaces or the intestinal tracts (King, 1998).

In this project, swab samples from wildlife (rats, squirrels, birds, and bats) were collected from two locations of Nanga Merit, Kapit area. The occurrence of *Shigella* spp. in wildlife was then identified through different types of biochemical tests including Gram-staining, oxidase test, Triple Sugar Iron (TSI) test, motility test, Hydrogen Sulphide formation test, and Indole formation test. Next, multiplex PCR (mPCR) assay was used to detect the virulence genes (*setl A, setl B, ial* and *ipaH*) of the *Shigella* species. *ial* and *ipaH* genes are responsible for directing epithelial cell penetration by *Shigella* and for modification of the host response to infection, respectively (Thong *et al*. 2005).

The detected *Shigella* spp. was further sent for sequencing at First BASE Laboratories Sdn. Bhd. The DNA sequences were then analyzed using Nucleotide Basic Local Alignment Search Tool (BLASTN 2.2.23+) of National Center for Biotechnology Information (NCBI) to compare with the sequences from the database. The isolated *Shigella* was assumed to be certain species that has high degree of similarity with the *Shigella* spp. from the database.

The objectives of this study are to:-

1. determine the occurrence of *Shigella* spp. in wildlife such as rats, squirrels, birds, and bats using biochemical tests.
2. detect the virulence genes of *Shigella* spp. (*ipaH*, *ial, setI A*, and *setI B*) using multiplex PCR method.

2.0 LITERATURE REVIEW

2.1 *Shigella* species

According to a report from Centers for Disease Control and Prevention (2008), *Shigella* is a type of Gram-negative bacteria that was discovered over 100 years ago by a Japanese microbiologist named Shiga. *Shigella* is non-spore forming, rod-shaped, non-motile, non-lactose fermenting, facultative anaerobes and should be urea hydrolysis negative. It was known that *Shigella* is closely related to *Salmonella* spp. and *Escherichia coli* (Clemens *et al*. 1999).

Shigella was recognized as the agent that causes bacillary dysentery in 1890s with the genus adopted in 1950s and subdivided into four species (Nie *et al*. 2006). The species of *Shigella* are *S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei* and represented by serogroup A, B, C, and D, respectively (Chein *et al*., 2000). These Serogroups were further divided into 45 serotypes based on the presence of O-antigens. Besides, *Shigella dysenteriae* type 1 (Sd1) is known to cause large dysentery epidemics in developing countries mainly in South Asia because of the ability to produce Shiga toxin and contain multidrug resistant plasmid which resistant to commonly used antimicrobial drugs (Bercion *et al*. 2006).

Woodward *et al*. (2005) reported that the infection of *Shigella* spp. is very easy because small inoculum (10 – 100 organisms) is sufficient to cause shigellosis. The infection is typically via ingestion through faecal-oral route that is through consumption of water or food sources which have been contaminated by the diarrheal stools contained *Shigella* spp.

Shigellosis is endemic in Malaysia (Hoe *et al*. 2005). Based on Lee & Puthucheary (2002), from 1978 to 1997, a total of 26, 320 stool specimens were collected by the University of Malaya Medical Center (UMMC) from children below 16 years old with clinical diarrhoea and 386 isolates tested were positive for *Shigella* species. However, the actual incidence of this disease could be higher in Malaysia, as the disease is self-limiting in adults (Hoe *et al*. 2005).

Hoe *et al*. (2005) had used plasmid profiling method to characterize 219 strains of *Shigella* species isolated in Malaysia during the period 1994 – 2000. The study showed that there was a correlation between plasmid patterns and serotypes of *S. flexneri*, *S. dysenteriae*, and *S. sonnei*. Five common small plasmids (>20.0 kb) were observed in *S. flexneri* 1b and 2a, while 4.40kb plasmid was found in serotype 3a. Besides, 9.00 kb plasmid was observed in all *S. dysenteriae* type 2, whereas 2.10 kb plasmid was found in *S. sonnei*.

Tanner (2003) reported that humans and higher primates are the common reservoir for *Shigella*. According to Enurah *et al*. (1986), an adult male captive chimpanzee (*Pan troglodytes*) from the Zoological Garden at Jos, Nigeria, was died by the infection of *Shigella dysenteriae* after a brief recovery from the illness at least 3 weeks. On postmoderm, *S. dysenteriae* was isolated from intestinal contents, lung, liver and spleen.

Based on King (1998), most of the research regarding *Shigella* are normally focused on human and rarely focused on animals. Hence, there are limited informations of *Shigella* spp. on wildlife except primates. However, animals such as birds and rodents may serve as mechanical carriers or vectors for *Shigella* and transmitted to other life form including human (King, 1998). In addition, it was reported that houseflies (*Musca*

domestica) also can serve as mechanical vectors for *Shigella* transmission (Levine *et al*. 1991).

Although studies on *Shigella* in wildlife are limited, there were some research on *Shigella* had been carried out using animals as experimental models in laboratories. Linberg *et al*. (1992) had done a research on the auxotrophic live oral *Shigella flexneri* vaccines protects monkey against challenge with *S. flexneri* of different serotypes. It was found that the *S. flexneri* vaccine strain SFL 114, auxotrophic for PABA when given to *Macaca fascicularis* monkeys, has the properties desirable as vaccine strain.

Other than that, Chakrabarti *et al*. (2003) had used two animal models including rabbit and guinea pig in the research on protective efficacy of oral immunization with heat-killed *Shigella flexneri* 2a in animal models to study the cross protection, immune response and antigenic recognition. It was demonstrated that rabbits and guinea pigs that were immunized with heat-killed *S. flexneri* 2a had conferred 100% protection. So, this proved that immunization with heat-killed *S. flexneri* 2a can give serotype specific protection.

2.2 Multiplex Polymerase Chain Reaction (mPCR)

According to Shen *et al*. (2010), multiplex polymerase chain reaction (PCR) was defined as the simultaneous amplification of a multiple regions of a DNA template or multiple DNA templates through the use of multiple primer sets in a single tube. It is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction (Henegariu *et al*. 1997).

Multiplex PCR is an essential cost-saving technique (Rachlin *et al*., 2008) used widely in environmental microbiology studies (Oggioni *et al*. 2002). In addition, it is useful for large scale genotyping which benefits in whole genome sequencing, forensic analysis, and facilitate un the diagnosis of infectious diseases (Rachlin *et al.* 2008)

A study by Thong *et al*. (2005) had used mPCR method to detect the prevalence of four virulence genes in Malaysian *Shigella* species. The research found that the *ipaH* sequence was present in all 110 *Shigella* strains tested, while each of the *setI A*, *setI B* and *ial* genes was present in 40% of the strain tested. None of the non-*Shigella* pathogens tested in this study was amplified. Consequently, this research proved that mPCR is a reproducible and sensitive method that able to identify pathogenic strains of *Shigella* species. Besides, it showed that mPCR can be easily performed with high throughput to give a presumptive identification of the causal pathogen.

A research by Vasconcelos *et al*., (2008) on serotyping *Listeria monocytogenes* isolated from human clinical specimens had also involved the used of mPCR. The main aim of the research was to detect *L. monocytogenes* in the cerebrospinal fluid sample of premature newborns and to characterize this sample using biotyping, serotyping and molecular typing method. After analysed the strain isolated using mPCR technique, the isolate was identified as the 4b serovar by the presence of a unique 691 bp band.

Other than that, a research using mPCR method had been carried out by Kaylan Kumar *et al*. (2009) because of the advantage of mPCR to rapidly detect a few different pathogens simultaneously compared to monoplex PCR which can only detect single organism in one time. Also, this rapidly detection eventually helps in cost saving for the study. The research was to simultaneous detected pathogenic *B. cereus*, *S. aureus* and *L. monocytogenes* (the major food-borne pathogens) by multiplex PCR. The results showed that, all the three organisms were identified individually or in combination without non-specificity. Also, the study found that as low as 10 – 100 organisms per ml of enriched spiked food samples and their presence in naturally contaminated samples were able to be detected by mPCR assay.

In addition, Mahony *et al*. (2009) reported about the use of multiplex PCR to test the appearance of pandemic influenza viruses including H1N1 swine influenza. In the test, the presence of non-seasonal (non-H1, non-H3) influenza in all the patients infected with H1N1 swine flu virus was able to be detected by mPCR. Also, mPCR was able to detect H5N1 avian influenza virus that propagated in South East Asia in year 2003, as well as all influenza A viruses representing 11 HA subtypes isolated from birds, swine, and horses that not yet seen in human population. Thus, this scientific evidence shows that mPCR can perform an important role as a sentinel test to detect novel non-seasonal influenza A viruses in patients. Subsequently, it may acts as an early warning system for the detection of future pandemic influenza threats.

3.0 MATERIALS AND METHODS

3.1 Sampling and Collection

A total of fifty swab samples (**Appendix 8.1 & 8.2**) were provided by Miss Chen Yik Ming (MSc candidate) from microbiology lab of department of Molecular Biology. The animal models were different species of wildlife such as rats, squirrels, birds and bats that were caught using nets setup in two sites of Nanga Merit area, Kapit, Sarawak (**Figure 1**), on 15th – 25th June 2009. Sampling site 1 is near a village of Nanga Merit area, while sampling site 2 is a forest area of Nanga Merit. Swab samples collected were cultured in Phosphate Buffer Saline in cryotubes and kept at 4°C.

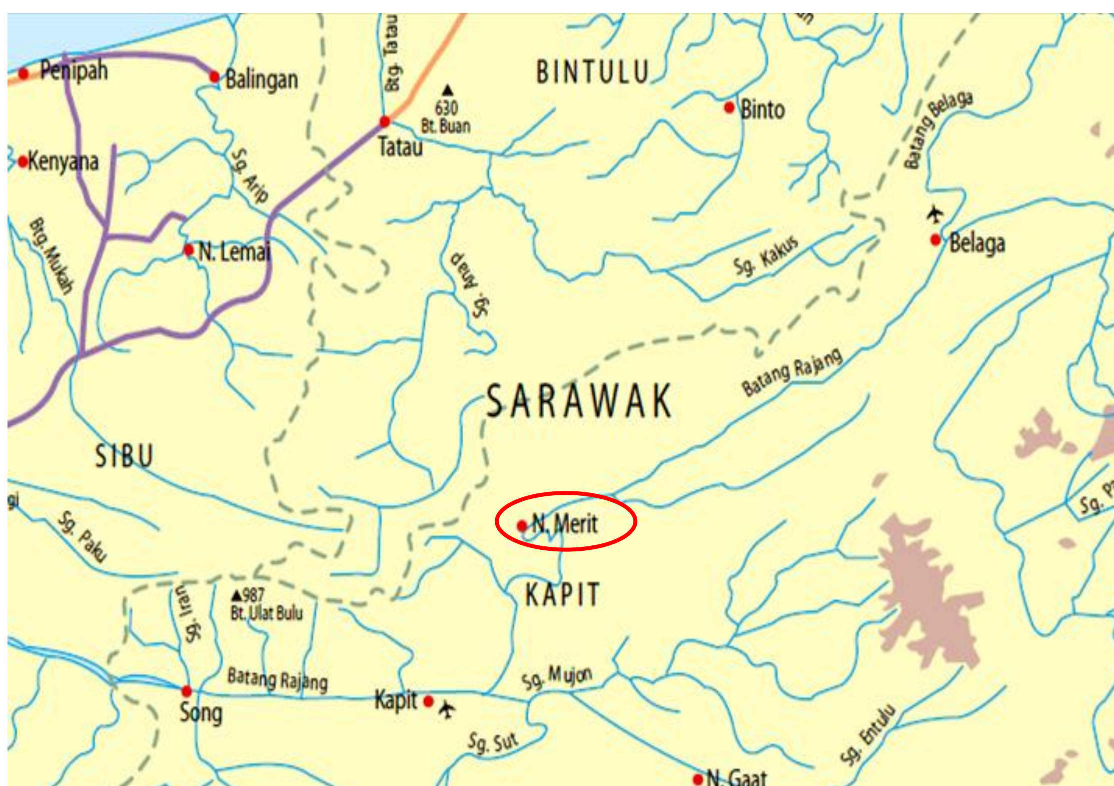

Figure 1: Location of Nanga Merit, Kapit, Sarawak

(Source: http://www.tourismmalaysia.or.jp/map/pdf/map_Sarawak.pdf)

3.2 Enrichment and Isolation of *Shigella* from Selective Plating Media

The method of isolation was used based on the protocol provided by Clemens *et al*. (1999) from the Department of Vaccines and Biologicals of World Health Organization.

First, 100 µl of sample from Phosphate Saline Buffer was pipetted and enriched in 1mL of Gram-negative (GN) broth (MERCK) and incubated at 35°C ± 2°C for 6 to 8 hours. Then, the enriched samples were streaked on Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric (HE) agar and incubated at 35-37°C for 18-24h. Growth of red colonies with no black centres and green, moist raised colonies were chosen from XLD and HE agar respectively. Colonies chosen were subcultured onto new selective agar plates for pure cultures.

Shigella flexneri (ATCC 12022) was also enriched and cultured on the selective media as a reference for its appearance on the selective media.

3.3 Presumptive Identification of *Shigella* spp. with Biochemical Tests

Biochemical tests were carried out based on the protocol provided by Clemens *et al*. (1999) from the Department of Vaccines and Biologicals of World Health Organization.

Colonies from subcultured were Gram-stained. Firstly, bacterial smears were prepared before Gram-staining. Cultures from XLD agar and HE agar were transferred and emulsified with distilled water on the centre of the slides. The slides were then rapidly passed over the flame of Bunsen burner for a few times until the water dried up. Then, the dried bacterial smears were stained in the sequence of crystal violet, Gram's Iodine, and safranin dye. Lastly, the slides were blot dried gently with soft tissue and the Gram-stained

smears were observed under a microscope. The strains with pink colour represent Gram-negative bacteria and violet colour represent Gram-positive bacteria.

Subsequently, oxidase test was done for the pure cultures on filter paper. The reactions were observed within 2 minutes. If the colour changes from colourless to purple-blue colour, it showed a positive reaction, while if the colour remains unchanged, it showed a negative reaction.

Triple Sugar Iron test was done by stabbing a pure culture colony into the butt and streak a pure culture on slant surface of TSI agar and incubated for 18-24h at 37°C. Colour changes on the butt and surface was observed. On the other hand, motility, hydrogen sulphide (H_2S) formation and indole formation tests were perform by stabbing a pure culture into centre of the Sulphide-Indole-Motility (S.I.M) and incubated for 18-24h at 37°C. After inoculation, motility and H_2S production were observed. Afterwards, a few drops of Kovacs' reagent were added and indole production was observed.

3.4 Boiling Method for DNA Extraction

DNA was extracted using boiled-cell method based on journal prepared by Theron *et al*. (2000) with slight modification with the bacteria colonies were first enriched in 1000 µl of LB broth and the cells were pelleted by centrifugation at 14,000 rpm for 5 min (**Appendix 8.3**). DNA of *S. flexneri* (ATCC 12022), *S. sonnei* (ATCC 25931), *S. boydii* (ATCC 9027) and RCPA *S. dysenteriae* were also extracted as positive controls in mPCR assay.

The isolates were first streaked on XLD agar from NA work cultures and incubated at 37°C for 18-24h. Then, a single colony of each isolates from XLD agar and positive controls from glycerol stocks were enriched and recovered in Luria Bertani (LB) broth (Miller, Himedia Lab), and incubated in incubator shaker for 18-24h at 37°C.

A 1000 µl of the broth from the turbid tube cultured strain was centrifuged at 14,000 rpm for 5 min in order to pellet the bacteria cells. The pellet was then resuspended in 1mL sterile distilled water, and boiled for 10 min followed by freezing on ice for 5 min. It was then centrifuged at 10,000 rpm for 5 min and the supernatant was transferred into a new microcentrifuge tube and stored at -20°C as DNA template for use in mPCR.

3.5 Multiplex Polymerase Chain Reaction (mPCR) and DNA Extraction

Multiplex PCR for detection of virulence genes in *Shigella* species was done based on method by Thong *et al*. (2005) with some modifications, which involved changing the volume of the DNA template from 2 µl to 8 µl, and the final concentration of each ShET1B primer, Shig 1 and 2 primers were changed from 3 µM to 5 µM (**Table 3.1**).

Table 3.1: Parameter of multiplex PCR (mPCR)

Stock	Final Concentration	Volume, with DNA template	Volume, without DNA template
PCR buffer, 10X	1.8X	4.5 µl	4.5 µl
$MgCl_2$, 50 mM	4 mM	2 µl	2 µl
dNTPs, 10 mM	130 µM	0.325 µl	0.325 µl
ShET1B (Fw), 25 µM	0.5 µM	0.5 µl	0.5 µl
ShET1B (Rv), 25 µM	0.5 µM	0.5 µl	0.5 µl
ShET1A (Fw), 25 µM	0.5 µM	0.5 µl	0.5 µl
ShET1A (Rv), 25 µM	0.5 µM	0.5 µl	0.5 µl
Shig 1, 25 µM	0.5 µM	0.5 µl	0.5 µl
Shig 2, 25 µM	0.5 µM	0.5 µl	0.5 µl
***ial* (Fw), 25 µM**	0.5 µM	0.5 µl	0.5 µl
***ial* (Rv), 25 µM**	0.5 µM	0.5 µl	0.5 µl
***Taq* polymerase, 1U**	1U	1 µl	1 µl
DNA Template		8 µl	
ddH_2O		5.175 µl	13.175 µl
Total Volume		**25 µl**	**25 µl**

The simultaneous gene amplifications were performed in a reaction volume of 25 µl consisting of 1.8X PCR buffer (Invitrogen), 4 mM $MgCl_2$, 130 µM of dNTPs, 0.5 µM of each ShET1B primer, Shig 1 and 2 primers, each ShET1A primer and *ial* primers (**Table**

3.2), 1U of DNA *Taq* polymerase (Invitrogen), 8 µl of DNA template and sterile distilled water.

Table 3.2: Primers used to identify various virulence-associated genes of *Shigella* spp.

Primer	Virulence gene	Nucleotide Sequences (5'→3')	Size of amplicon (bp)
ShET1A	*setI A*	TCA CGC TAC CAT CAA AGA TAT CCC CCT TTG GTG GTA	309
ShET1B	*setI B*	GTG AAC CTG CTG CCG ATA TC ATT TGT GGA TAA AAA TGA CG	147
ial	*ial*	CTG GAT GGT ATG GTG AGG GGA GGC CAA CAA TTA TTT CC	320
Shig1 **Shig2**	*ipaH*	TGG AAA AAC TCA GTG CCT CT CCA GTC CGT AAA TTC ATT CT	423

The cycling conditions were an initial denaturation at 95°C for 5 min, template denaturation at 95°C for 50s, annealing at 55°C for 1.5 min, and extension at 72°C for 2 min for a total of 30 cycles, with a final extension at 72°C for 7 min.

S. flexneri (ATCC 12022), *S. sonnei* (ATCC 25931), *S. boydii* (ATCC 9027) and RCPA *S. dysenteriae* were chosen as positive controls while a negative control was performed by using sterile distilled water as template in this multiplex PCR assay. The DNA fragments were separated in 2% agarose gel using 90V for 50 min.

Isolates with detected virulence gene were further proceed to monoplex PCR assay (**Table 3.3**) with the thermal cycle profile is the same as mPCR assay but using only the primer which the specific virulence gene was detected. The total volume of reaction per isolate prepared was 100 µl. *S. flexneri* (ATCC 12022) was chosen as positive control

while sterile distilled water instead of template was used as negative control. The DNA fragments of the PCR products were separated in 2% agarose gel.

Table 3.3: Parameter of monoplex PCR

Stock	Final Concentration	Volume, with DNA template	Volume, without DNA template
PCR buffer, 10X	1.8X	4.5 µl	4.5 µl
$MgCl_2$, 50 mM	4 mM	2 µl	2 µl
dNTPs, 10 mM	130 µM	0.325 µl	0.325 µl
Shig 1, 25 µM	0.5 µM	0.5 µl	0.5 µl
Shig 2, 25 µM	0.5 µM	0.5 µl	0.5 µl
***Taq* polymerase, 1U**	1U	1 µl	1 µl
DNA Template		10 µl	
ddH_2O		6.175 µl	16.175 µl
Total Volume		**25 µl**	**25 µl**

DNAs of the isolates were then extracted from the agarose gel using Fermentas DNA Extraction Kit (**Appendix 8.7**) and sent for sequencing at First BASE Laboratories Sdn. Bhd.

The sequencing results were analyzed using Nucleotide Basic Local Alignment Search Tool (BLASTN 2.2.23+) of National Center for Biotechnology Information (NCBI). The DNA sequences of each sample were compared with the sequences from the database and identify the database sequences that resemble the query sequences with high degree of similarity.

4.0 RESULTS

4.1 *Shigella* spp. on Selective Plating Media

A total of fifty samples were cultivated in GN broth and streaked on Xylose Lysine Deoxycholate (XLD) agar and Hektoen Enteric (HE) agar. Growth of red colonies with no black centres and green, moist raised colonies from XLD and HE agar respectively (as shown in **Fig. 1**) were suspected to be *Shigella* spp. In total, there were twelve samples out of the fifty samples (24%) appeared in red colonies on XLD agar and green colonies on HE agar as shown in **Table 4.1**. *Shigella flexneri* (ATCC 12022) was enriched and cultured on the selective media as a reference for its appearance on the selective media.

Table 4.1: Colonies appearance of samples on XLD agar and HE agar

Sample	Appearance of Colonies on XLD Agar	Appearance of Colonies on HE Agar
***S. flexneri* (ATCC 12022)**	**Red**	**Green**
B 2961	Yellow	Orange
A 2741(C)	Yellow	Orange
A 2779 (F)	Yellow	Orange
A 2730	Yellow	Orange
A 2753 (F)	Yellow	Orange
A 2762 (C)	Yellow	Orange
U 2013	Yellow	Orange
C 03306 (A)	Yellow	Orange
U 2080 (F)	Yellow	Orange
U 2058	**Yellow**	**Green**
U 2031	Yellow	Orange
U 2055	**Red**	**Green**
U 2104 (A)	Yellow	Orange
U 2111 (I)	Yellow	Orange
U 2083 (I)	Yellow	Orange
U 2101 (I)	**Yellow**	**Green**
U 2100 (A)	Yellow	Orange
U 2082 (A)	Yellow	Orange
U 2099 (A)	**Red**	**Green**
U 2075 (A)	Yellow	Orange
U 2049 (A)	**Red**	**Green**
U 2050 (A)	Yellow	Orange
U 2078 (I)	Yellow	Orange
U 2113 (A)	Yellow	Orange
U 2112 (A)	Yellow	Orange

A 09008 (C)	Yellow	Orange
B 09703 (C)	**Red**	**Green**
A 09009 (C)	**Red**	**Green**
A 09027 (C)	Yellow	Orange
A 09041 (C)	**Red**	**Green**
A 09055 (C)	**Red**	**Green**
A 09085 (C)	**Red**	**Green**
B 09729 (C)	**Red**	**Green**
A 09095 (C)	Yellow	Orange
B 09714	Yellow	Orange
A 2790 (C)	Yellow	Orange
A 09061 (C)	Yellow	Orange
C 03379 (A)	Yellow	Orange
FNM 018 (A)	Yellow	Orange
FNM 037 (A)	Yellow	Orange
FNM 121 (A)	Yellow	Orange
FNM 136	Yellow	Orange
FNM 110 (I)	Yellow	Orange
FNM 145 (A)	Yellow	Orange
FNM 024	Yellow	Orange
FNM 090	Yellow	Orange
FNM 038	Yellow	Orange
FNM 151 (A)	Yellow	Orange
FNM 069	Yellow	Orange
FNM 126 (A)	Yellow	Orange
FNM 040 (A)	**Red**	**Green**
FNM 072 (I)	Yellow	Orange

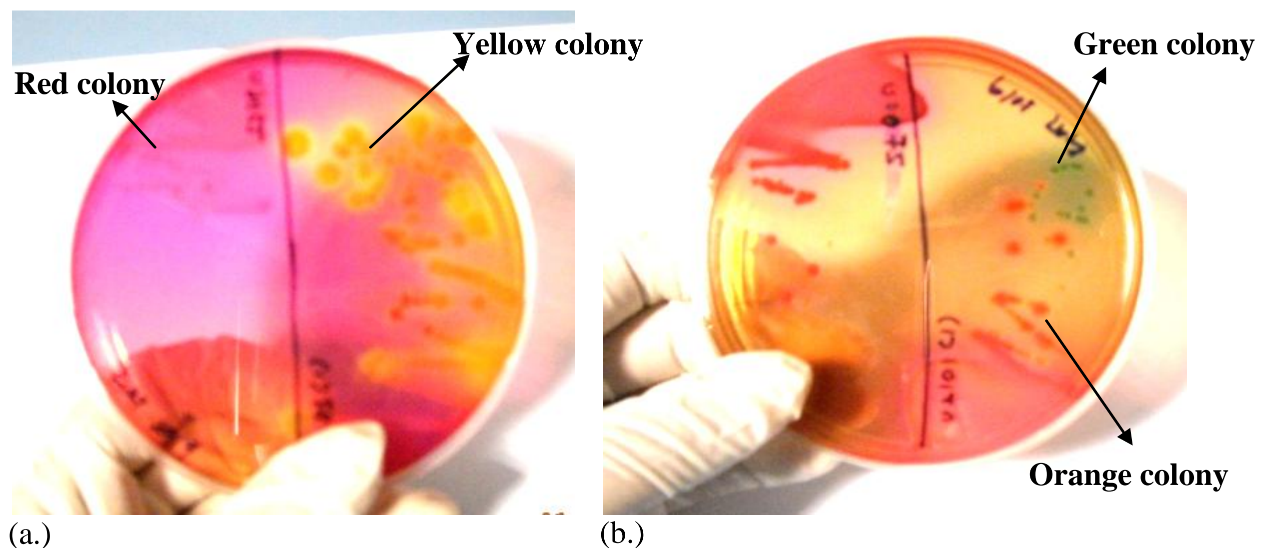

Figure 2: Colonies appearance on XLD and HE agar. (a.) appearance of red and yellow bacteria colonies on XLD agar, red colonies were suspected to be *Shigella* spp.; (b) appearance of green and orange bacteria colonies on HE agar, green colonies were suspected to be *Shigella* spp.