DETECTION OF *Salmonella enterica* subsp. *enterica* serovar Typhi FROM CHOLECYSTECTOMY SAMPLES BY CONVENTIONAL, SEROLOGICAL AND MOLECULAR METHODS IN HOSPITAL USM

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celcius
%	Percent
µg	Microgram
µM	Micromolar
µl	Microliter
ATCC	American Type Culture Collection
bp	Base pair
CFU	Colony forming unit
DCA	Deoxycholate citrate agar
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
H_2O	Water
HCl	Hydrogen chloride
HE	Hektoen enteric agar
IAC	Internal Amplification Control
L	Liter
$MgCl_2$	Magnesium chloride
ml	Mililiter
mg	Milligram
mg/ml	Milligram per mililiter
MR-VP	Methyl Red / Vogas-Proskauer
NA	Nutrient agar

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
NA	Not available
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
SIM	Sulfide indole motility
TAE	Tris-acetate-EDTA
TSI	Triple Sugar Iron
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization
XLD	Xylose lysine deoxycholate

PENGESANAN *Salmonella enterica* subsp. *enterica* serovar Typhi DARIPADA SAMPEL KOLESISTEKTOMI MELALUI KONVENSIONAL, SEROLOGI DAN KAEDAH MOLEKUL DI HOSPITAL USM

ABSTRAK

Salmonella enterica subsp. *enterica* serovars Typhi (*S.* Typhi) adalah agen tifoid kepada manusia. Tifoid telah menyebabkan 11-20 juta orang sakit dan dianggarkan angka kematian mencecah antara 128 000 hingga 161 000 setiap tahun. Bakteria ini disebarkan melalui laluan mulut ke usus oleh makanan atau minuman yang tercemar. Infeksi kebiasaannya berlaku pada pundi hempedu, hati, ileum, limpa dan sumsum tulang. Sehingga kini, pembuangan hempedu secara pembedahan melalui prosedur kolesistektomi merupakan pilihan efektif untuk pesakit pembawa tifoid yang mempunyai batu hempedu. Dalam kajian ini, lapan puluh (80) pesakit yang mendaftar untuk kolesistektomi dan mempunyai penyakit kehepatohempeduan adalah dipilih sebagai sampel. Satu kajian mudah telah dijalankan untuk memencilkan *S.* Typhi melalui empat teknik kultur; iaitu kultur terus (teknik 1), pengumpulan lapisan atas (teknik 2), vorteks (teknik 3) dan pengasingan supernatan dan pelet (teknik 4) sebelum melakukan PCR dan ujian serologi menggunakan ujian Typhidot-C. Daripada jumlah 80 sampel, lapan sampel (Gb9, Gb10, Gb11, Gb15, Gb43, Gb50, Gb64 and Gb74) menunjukkan kehadiran koloni yang disyaki spesis *Salmonella* di agar-agar HE dan agar-agar XLD dengan menggunakan teknik pengasingan supernatant dan pelet. Kesemua lapan sampel tersebut telah diuji dengan ujian biokimia yang terdiri daripada ujian triple sugar iron (TSI), ujian urease, ujian sitrat, ujian indol dan ujian metil merah (MR). Keputusan

menunjukkan kesemua lapan sampel tersebut adalah negatif *Salmonella*. Ujian 'Electronic RapID Compendium (ERIC^{TM}) telah dilakukan dan keputusan menunjukkan *Citrobacter freundii* telah dikesan dengan kebarangkalian 99 peratus. Daripada lapan sampel tersebut, enam sampel menunjukkan pengesanan negatif *Salmonella* oleh PCR, manakala dua sampel positif oleh PCR tetapi ujian biokimia menunjukkan kedua-dua sampel adalah negatif *Salmonella*. Ujian serologi menggunakan Typhidot-C telah dijalankan dan keputusan menunjukkan kesemua 37 sampel darah adalah negatif pembawa *S.* Typhi. Sebagai kesimpulan, kajian ini tidak dapat memencilkan dan menghubungkan kehadiran *S.* Typhi daripada pesakit kehepatohempeduan di HUSM berbanding dengan negara yang endemik tifoid. Cadangan di masa akan datang, kriteria dalaman hendaklah diperhaluskan seperti merangkumi pesakit yang mempunyai sejarah tifoid, memperbesarkan saiz sampel dan bekerjasama dengan lebih banyak hospital dalam usaha mengumpul jumlah sampel.

DETECTION OF *Salmonella enterica* subsp. *enterica* serovar Typhi FROM CHOLECYSTECTOMY SAMPLES BY CONVENTIONAL, SEROLOGICAL AND MOLECULAR METHODS IN HOSPITAL USM

ABSTRACT

Salmonella enterica subsp. *enterica* serovar Typhi (*S.* Typhi) is an agent of typhoid in human. Typhoid has been reported causing 11-20 million people illnesses and estimated 128 000 to 161 000 deaths every year. The bacteria were spread by fecal-oral route through infected food or water. The most common sites of infection are the gallbladder, liver, ileum, spleen and bone marrow. At the moment, removal of the gallbladder through cholecystectomy procedure remains the effective option for typhoid carriers with gallstones. In this study, eighty (80) patients that enrolled for cholecystectomy and having hepatobiliary disease were chosen as sample. A convenience study has been performed to isolate *S.* Typhi by using four culture techniques; which are direct incubation (technique 1), upper layer collection (technique 2), vortex (technique 3) and supernatant and pellet separation (technique 4), then proceed to conventional polymerase chain reaction (PCR) test and serology test using Typhidot-C. Out of 80 samples, eight samples (Gb9, Gb10, Gb11, Gb15, Gb43, Gb50, Gb64 and Gb74) showed the presence of suspected colonies of *Salmonella* species on the Hektoen Enteric (HE) agar and Xylose Lysine Deoxycholate (XLD) agar by using supernatant and pellet separation technique. All eight sample of suspected colonies were tested with biochemical test which included triple sugar iron (TSI) test, urease test, citrate test, indole test and methyl red (MR) test. The result showed that all suspected colonies were negative for detection of *Salmonella* species. Electronic RapID Compendium (ERIC^{TM}) test was done and

Citrobacter freundii was detected with probability of 99 per cent. From the eight samples mentioned, six samples were shown negative detection of *Salmonella* species by PCR, meanwhile, two samples were positive by PCR but both samples were negative for *Salmonella* by biochemical test. Serology test by using Typhidot-C were done and the result showed all 37 blood samples were negative for *S.* Typhi carrier. As a conclusion, this study not able to isolate and correlate the presence of the *S.* Typhi in HUSM patients with hepatobiliary diseases in comparison to other typhoid endemic countries. As for recommendation, there is a need to refine the inclusion criteria such as includes the patients that have typhoid history, increase the sample size and collaborate with many hospitals in collecting the samples.

CHAPTER 1

INTRODUCTION

1.1 Research background

Typhoid is a serious life-threatening worldwide public health problem caused by the *Salmonella enterica* subsp. *enterica* serovar Typhi (*S.* Typhi) that usually spread through contaminated food or water. Typhoid has been reported causing 11-20 million people illnesses and estimated 128 000 to 161 000 deaths every year (WHO, 2018). Typhoid causes significant mortality and morbidity especially in Asia, Africa, Middle East and Latin America (Ajibola *et al.*, 2018). The incidence of typhoid differs within Asian continent examples India and Pakistan that have been reported to have high incidence rate of typhoid. (Ochiai *et al.*, 2008).

It has been estimated that around 2-5% of individuals who have been infected with *S.* Typhi has a possibility of becoming a chronic carrier without showing any symptoms after one year of infection. These chronic carriers are very infective due to the excretion of the *S.* Typhi in stool or urine, which thus helping to maintain the endemicity of the disease (Levine *et al.*, 1982; Shpargel *et al.*, 1985). Therefore, there is a need to detect the *S.* Typhi carrier in order to eliminate or reduce the typhoid burden. So far, there is no carriers detection available in the market and few are still at the evaluation stage. At the moment, isolation of the *S.* Typhi from the stool or urine are the most method applied to identify the carriers (World Health Organization, 2003) However, isolation of the *S.* Typhi from the stool of carriers is difficult due to the intermittently low number of shedded bacteria (Baker *et al.*, 2010).

In 2003, the five-year Food and Water Borne Disease (FWBN) Plan of Action was launched which aim to reduce the incidence rate of typhoid in Kelantan. Typhoid patients were monitored with stool clearance at regular interval. By 2008, the incidence rate of typhoid in Kelantan was successfully reduced to 3.29/100,000 population and further to 2.8/100,000 in 2010 (Hamzah *et al.*, 2011). However, the case of typhoid is still ongoing. A study from Farooqui and colleague in 2009 have shown that untreated drinking water has increased the risk of infection since many people in Kelantan still used well water for domestic activities as the main water source. Moreover, an inadequate sanitary condition, especially in the rural area, also contributed to the increased rate of typhoid in Kelantan. This situation becomes worse with the chronic carriers are still intermittently release the *S.* Typhi.

It has been reported that the development of chronic carriage often correlates with the biliary disease associated to the abnormalities of the gallbladder especially in the presence of gallstones (Lai *et al.*, 1992). A study from Sharma and colleague in 2007 showed that chronic typhoid carriers in the endemic region represent around 10% of patients with cholelithiasis and 30% of gallbladder carcinoma (Sharma *et al.*, 2007). In HUSM, cholecystectomy which is surgical removal of the gallbladder is an effective option for hepatobiliary patients, especially with gallstones. By taking this opportunity, this study was conducted to isolate *S.* Typhi from the cholecystectomy patients related hepatobiliary diseases in HUSM for carrier detection.

Meanwhile, serological tests have often been proposed as possible test for chronic carrier of *S.* Typhi. It because this tests are mostly cost effective, easy to handle and are free from the limitations of culture and other available methods since

the serum shows the status of a longer period (Baker *et al.*, 2010). A carrier screening test, Typhidot-C which used for detection of Immunoglobulin A (IgA) and Immunoglobulin G (IgG) antibodies toward the *S.* Typhi outer membrane protein. A study from Chua *et al.* (2015) have successfully detected all four chronic carrier and ten suspected carriers from food handler's population by using Typhidot-C test.

1.2 Rationale of study

S. Typhi has developed mechanisms to survive and grow in the bile-rich environment (Lovane *et al.*, 2016). A study from Freedman and Goldenberg (1962) has found that disease of the hepatobiliary system mostly associated with common bile duct obstruction closely related to bacterial infection. The bacteria can establish an infection in the human by colonizing the gallbladder and continue to survive with biofilm formation (Crawford *et al.*, 2010). Meanwhile, the asymptomatic typhoid carrier in human showed no symptom and up to 5% of them shed the organisms for years. Identification of the *S.* Typhi through stool culture remains a gold standard method, however finding the pathogen is difficult since the shedding of the pathogen is typically at the low level and intermittent especially for carriers (Crawford *et al.*, 2010). Besides, their adaptation in various environments makes the way harder to find them. Therefore, there is a need to find an alternative method to identify the carriers.

A study from Dongol and colleague (2012) at Patan Hospital, Kathmandu, Nepal have found that *S.* Typhi can be isolated from bile samples in gallbladders of patients undergoing cholecystectomy. Mansour and colleague (2012) also successfully isolated the *S.* Typhi from Egyptian patients which diagnosed with chronic cholecystitis and acute chronic cholecystitis. However, previous study by

Monalis *et. al* (2008) suggested that toxicity of bile salts will affect the recovery of the organism in bile containing cultures. Here, modification of the gallbladder culture techniques is required in order to enhance the success rate of isolate *S.* Typhi from the gallbladder. Conventional ways to isolate the bacteria is by culture the specimen into the enriched media and incubated at 37°C with shaking condition.

S. Typhi has been shown to be associated with hepatobiliary disease and in Hospital USM, cholecystectomy is the most common method for handling patients with hepatobiliary diseases with two to three patients were continuously doing cholecystectomy every week in the surgery department. With a strong justification and statistic on Kelantan has the highest incidence of typhoid in 2015, this study hypothesized that patients with hepatobiliary disease-carrying of the *S.* Typhi are high. In the recent technique, only stool culture and polymerase chain reaction (PCR) were done to isolate and detect *S.* Typhi. However, isolation of *S.* Typhi is difficult due to the intermittently shedding of the organism. Since gallbladder was reported to become a niche for *S.* Typhi, a modification method is needed to culture bile, tissue and stones from the gallbladder (Gonzalez-Escobedo and Gunn, 2013). Therefore, this study was focusing on isolating and identification of *S.* Typhi from the hepatobiliary patients' gallbladder using PCR, serology test by using patient's sera and gallbladder culture for typhoid carrier identification.

1.3 Objectives

1. To identify the presence of *S.* Typhi from gallbladder culture using four different techniques of optimization; direct incubation, upper layer collection, vortex and supernatant and pellet separation.
2. To detect *S.* Typhi from the patient with hepatobiliary disease using Polymerase Chain Reaction (PCR).
3. To determine the identification of typhoid carrier from patients' sera using Typhidot-C test.

CHAPTER 2

LITERATURE REVIEW

2.1 Typhoid fever

Typhoid is a systemic infection characterized by a fever caused by the *S.* Typhi (Waddington *et al.*, 2014). Typhoid is transmitted by a fecal-oral route through infected food or water. The pathogen penetrates the gastrointestinal mucosa, duplicates within macrophage, and spread via the bloodstream to the gallbladder, bone marrow, intestinal lymph nodes, liver and spleen (Charles *et al.*, 2013). The most common sites of infection are the gallbladder, liver, ileum, spleen and bone marrow (Gonzalez-Escobedo *et al.*, 2011). Furthermore, this pathogen can survive for days in a normal environment such as in a well, and months in infected eggs and frozen oysters (Bhan *et al.*, 2005). They may also survive in acid foods and resist dehydration. This means that it is difficult to eradicate the bacteria.

2.2 General background on *Salmonella*

Salmonella is gram-negative bacteria that cause enteric disease in animals and human. *Salmonella* was early discovered and isolated by Theobald Smith in 1855, from intestines of pigs which infected by swine fever (Eng *et al.*, 2015). Later, French bacteriologist Joseph Leon Marcel Lignieres proposed that the group of bacteria represented by the swine-cholera should be named as '*Salmonella*' as credited to an American pathologist, Dr. Danial Elmer Salmon (Eng *et al.*, 2015).

The nomenclature of *Salmonella* is complicated and still evolves from initial one serotype-on species concept proposed by Kauffmann (1966) which based on the

serologic identification of O (somatic) antigen and H (flagellar) antigen. Currently, the Centers for Disease Control and Prevention (CDC) use the nomenclature system of *Salmonella* recommended by the World Health Organization (WHO) Collaborating Centre (Popoff *et al.*, 2003).

According to the system, the genus *Salmonella* is classified into two species, *Salmonella enterica* (type species) and *Salmonella bongori*, based on its differences in their 16S rRNA sequence analysis. *S. enterica* can be further categorized into six subspecies which denoted with Roman numerals; l, *S. enterica* subsp. *enterica*; ll, *S. enterica* subsp. *salamae;* llla, *S. enterica* subsp. *arizonae*; lllb, *S. enterica* subsp. *diarizonae*; lV, *S. enterica* subsp. *hountenae*; Vl, *S. enterica* subsp. *indica.* Almost 99% of *Salmonella* found in human and warm-blooded animals are from group l *S. enterica* subsp. *enterica*. While other subspecies and *S. bongori* rarely found in human but often found in the environment and cold-blooded animals (Eng *et al.*, 2015).

The species *Salmonella enterica* comprises over 2 500 serovars, which are classified by the flagellar and lipopolysaccharide (LPS) antigens, and it includes both typhoidal and non-typhoidal *Salmonella* strain. *Salmonella enterica* subsp. *enterica* serovars Typhi (*S.* Typhi) together with other salmonella serovars such as Paratyphi are restricted human pathogens that cause the systemic disease and abdominal pain (LaRock *et al.*, 2015).

Diarrhea is a more common symptom in children infected with *Salmonella*, while for the people with immunosuppression is likely to develop constipation

(Thielman and Guerrant, 2004). Typhoid shows a specific pattern of fever with initial low-grade fever and slowly develops to high-grade fever in the second week. If untreated, the fever can carry on up to a month and more (Patel *et al.*, 2010). The typhoid incidence is highest in regions that have poor sanitation and a further increase during the dry and hot season due to the high concentration of the bacteria in a limited amount of water (Crum, 2003). Study results from the International Vaccine Institute in Korea have shown variation in the distribution of typhoid fever from sites in five Asian Countries by using standardized surveillance, clinical and microbiological procedures as shown in Table 1 from August 2002 till July 2004. Consumption of water at labor site (Luby *et al.*, 1998), drinking from contaminated tap water (King *et al.*, 1989) and using non-boiled untreated spring water (Swaddiwudhipong, 2001) can lead to typhoid infection.

Table 2.1 Surveillance data of typhoid fever from sites in five Asian countries from August 2002 till July 2004.

	Site	**The incidence of typhoid fever**
China	Urban and rural	15.3 cases per 100 000 per year in people aged 5-60 years old
Vietnam	Urban	24.2 cases per 100 000 per year in people aged 6-18 years old
Indonesia	Urban slum	81.7 cases per 100 000 per year (all years)
Pakistan	Urban slum	451.7 cases per 100 000 per year in children aged 2-15 years
India	Urban slum	493.5 cases per 100 000 per year (all ages)

(Adapted from John Wain *et al*. (2015)).

2.3 Typhoid in Malaysia

In Malaysia, typhoid is one of the major infectious diseases related to food and waterborne disease. Figure 2.1 showed the geographical distribution of typhoid in Malaysia with high incident rate of more than 100 per 100 000 per population. In 2008, 201 cases of typhoid were reported and before that, in 2005, 735 cases and 2 deaths have been reported occurred in Kelantan (MOH, 2008). Table 2.2 showed Kelantan and Selangor are the most infected state in Peninsular, while Sabah remains reported the highest case in Malaysia in 2010 (MOH, 2011).

Kelantan has been reported endemic for typhoid (Goay *et al.*, 2013). Malik and Malik (2001) reported from the Ministry of Health Malaysia that the 'highest number of typhoid cases was in 1998 and 1999 which was from Kelantan and the majority were children'. Meanwhile, Choo *et al.* (1988) reported that patients admitted to Hospital Universiti Sains Malaysia (HUSM) for typhoid with the average age was 7.3 years old, which is comparable to Malik and Malik (2001) who stated 7.5 years old from the same hospital. However, these data contrasted with the study by Levine *et al* (1982) which stated that in Santiago, Chile, most of the infected patients were forty years old and above.

From 2011, the incidence rate of typhoid fever in Malaysia shown decreases number from 1.71 per 100,000 population to 0.70 per 100,000 population in 2014 (Figure 2.2). However, in 2015 the incidence has increased to 1.42 per 100,000 population. In comparison with other states, Kelantan has been reported to have a higher incidence of typhoid cases compared to the other states in Malaysia (Baddam *et al.*, 2012). In 2015, the cases of typhoid in Kelantan have increased to 35 cases

when compared to year 2014. Meanwhile, the incidence rate of typhoid in Kelantan has increased up to 10.6 per 100 000 population, which was 10 times above national level and median five years incidence rate (Akhir *et al.*, 2018).

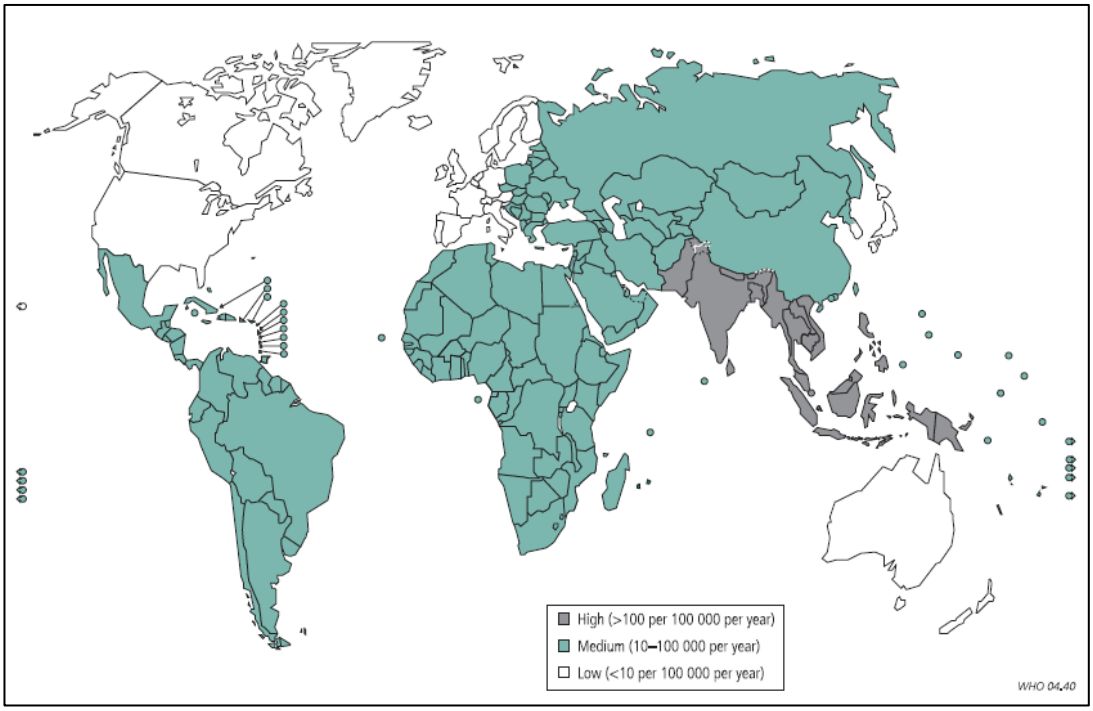

Figure 2.1: Map above shows the geographical distribution of typhoid. Malaysia is shown to have a higher incident rate of typhoid with more than 100 per 100 000 per population. Adapted from Crump *et. al* (2004).

Table. 2.2 Incidence rate per 100,000 population and the number of typhoid cases by states in Malaysia, year 2010.

State	Incidence rate (per 100 000)	Number of cases
Kedah	0.1	2
Pulau Pinang	0.1	2
Negeri Sembilan	0.2	2
Pahang	0.2	3
Perak	0.2	5
Sarawak	0.2	5
Johor	0.4	13
Terengganu	0.4	4
Selangor	0.6	33
Kuala Lumpur	0.7	12
Perlis	0.9	2
Sabah	2.5	80
Kelantan	3.0	46

(Adapted from Ministry of Health, Malaysia (2011)).

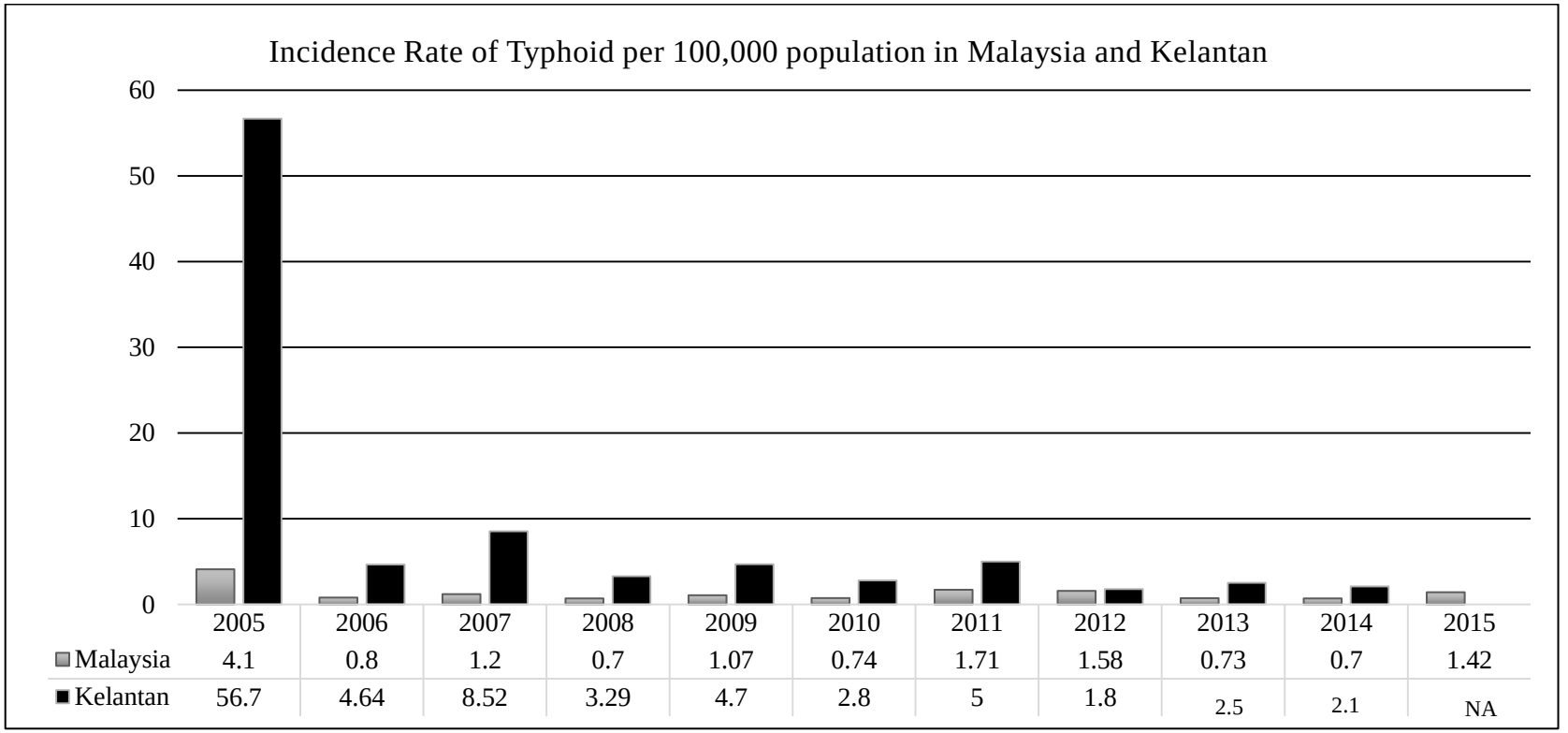

Figure 2.2 : The incidence rate of typhoid per 100,000 population in Malaysia and Kelantan (MOH, 2016).

2.4 Detection of *S.* Typhi for acute and carriers

There are several methods to isolate and detect the *S.* Typhi: bacterial culture; serological test; and polymerase chain reaction (PCR) (Wain and Hosoglu, 2008; Chua *et al.*, 2015). Culture remains the most effective method in diagnosing the typhoid fever. However, it may lack sensitivity and speed due to the culture results will produce within 2-7 days. As for the negative culture, the result will easily interpret with no colonies growth or nonsuspected colonies growth after overnight cultured on the agar plate (Ismail, 2000a).

2.4.1 Bacterial culture

2.4.1(a) Blood culture

Sensitivity of blood culture is variable between 40% and 60%, in contrast with the sensitivity of bone marrow aspirate cultures which is more than 80% (Gilman *et al.*, 1975; Baker *et al.*, 2010). However, in countries with limited resources, diagnosis for blood cultures or bone marrow aspirate for typhoid fever could not be done due to the limited skill personnel and high expense (Farooqui *et al.*, 1991).

2.4.1(b) Stool Culture

In low-resource setting area, stool culture is commonly used in most diagnostic laboratories (Ajibola *et al.*, 2018). Stool sample should be collected in sterile wide-mouthed containers and inoculated within two hours of collection or stored at 4°C until ready to inoculate. Even though stool culture is the gold standard for diagnosing typhoid fever, some of the challenges in isolating *S.* Typhi such as time consuming, low sensitivity, lack of infrastructure and insufficient skilled manpower (Ajibola *et al.*, 2018).

2.4.2 Molecular Detection

Development of molecular tests for typhoid diagnosis involves genetic markers that are specific and sensitive for detection of bacterial DNA (Goay *et al.,* 2016). Nucleic acid amplification tests, including conventional polymerase chain reaction (PCR), multiplex, nested and real-time PCR, has been established for the detection of *S.* Typhi in blood (Song *et al.,* 1993; Wain *et al.,* 1998; Ali *et al.,* 2009; Baker *et al.,* 2010).

PCR is used to diagnose typhoid fever by using the flagellin gene because its hypervariable region Vi is unique for *S.* Typhi and its amplification provides 100% specificity (Song *et al.,* 1993; Frankel, 1994). However, application of molecular techniques in clinical settings has technical limitations because of the few number of bacteria in blood, approximately 0.5 CFU/ml (Wain *et al.*, 1998). Previous study has shown to overcome the low sensitivities of samples, PCR test has been developed with some pre-enrichment step in culture in order to improve sensitivity and to reduce PCR inhibitors (Chiu and Ou, 1996). However, these test still be influenced by the adequate concentration of DNA within the detection limit being presented in a sample specimen tested (Chua *et al.,* 2015).

2.4.3 Serological test

The Widal test was established by Georges Ferdinand Widal in 1896. This test helps to identify the presence of *Salmonella* antibodies in serum of patients by measures agglutinating antibodies against the O (somatic) antigen and H (flagellar) antigens of *S.* Typhi in people sera with suspected with typhoid fever. Agglutination reaction proposed a positive result, while an absence of agglutination proposed a negative

result. The Widal test is simple, cost effective and widely used in developing countries. However, these test only useful for diagnosis of acute typhoid fever and defective in endemic areas (Pang and Puthucheary, 1983).

The Vi capsular antigen of *S.* Typhi is used as a screening tool for typhoid carriers since they frequently produce higher levels of antibody compared to acute patients (Lanata *et al.*, 1983). However, the Vi capsule is known to be less immunogenic than other antigens since the importance of Vi antigen for immune evasion and invasion in various studies has been established (Raffatellu *et al.*, 2006).

Previous study has demonstrated that the 50 kDa of the outer membrane protein of *S.* Typhi antigenically specific for *S.* Typhi (Ismail *et al.*, 1991). A rapid dot enzyme immunosorbent assay (EIA) method was developed based on the 50 kDa which detects immunoglobin (Ig) M and IgG antibodies toward the 50 kDa antigen in human sera (Ismail *et al.*, 1991). Evaluation of the tests in clinical settings, have showed the dot EIA test (Typhidot) offers simplicity, specificity (75%), sensitivity (95%), speed (1-3 hours) and with high positive and negative predictive values (Choo *et al.*, 1994). However, the IgM detection only suitable for acute cases while IgG result cannot differentiate between acute and convalescent cases due to the IgG persist for more than two years in patients infected with typhoid fever (Choo *et al.*, 1997).

Typhidot-M is a modification test from Typhidot which demonstrated the inactivation of IgG and allow accessibility of the antigen to the specific IgM. The

detection of specific IgM within three hours would suggest acute typhoid infection (Ismail, 2000b).

2.5 Characteristics of *S.* Typhi

2.5.1 Morphological characteristics

Salmonella are rod-shaped bacteria with 2-3 µm long and 0.4-0.6 µm diameter. *S.* Typhi belongs to the Enterobacteriaceae family, Gram-negative bacteria that have flagellated bacilli and facultatively anaerobe (Khan *et al.*, 2008).

2.5.2 Culture characteristics

The common selective agar used are MacConkey, Hektoen enteric (HE), Xylose lysine deoxycholate (XLD), Deoxycholate citrate agar (DCA) and *Salmonella-Shigella* (SS) agar which incubated at 37°C for 18-24 hours (World Health Organization, 2003). *Salmonella* produce lactose non-fermenting colonies on lactose enriched media such as MacConkey agar, deoxycholate agar and SS agar.

On the HE agar, *Salmonella* produce transparent green colonies with a black dot in the centers. While on the XLD agar, *Salmonella* produce transparent red colonies with a black dot in the centers. The black dot in the centers represents the presence of hydrogen sulphide (H_2S) (World Health Organization, 2003). Table 2.3 shows the colonies characteristics of *Salmonella* serovars/ *S.* Typhi.

Table 2.3: Colonies characteristics of *Salmonella* serovars/ *S.* Typhi.

Media	***Salmonella* serovars/ *S. enterica* ser. e*nterica* Typhi**
MacConkey agar	Non-lactose fermenter with smooth colourless colonies
HE agar	Transparent green colonies with black dot in the centers
XLD agar	Transparent red colonies with black dot in the centers
DCA	Non-lactose fermenter with black dot in the centers
SS agar	Non-lactose fermenter with black dot in the centers
Blood agar	Non-haemolytic smooth white colonies

(Adapted from WHO, 2003).

2.5.3 Biochemical and serological characteristic

There are well-established confirmation and identification procedures for *Salmonella* spp. after preliminary identification on colony appearance on selective agar media. The colony will further analyse using classical biochemical and serological testing. Key biochemical tests are fermentation of glucose. As for *S.* Typhi, the bacteria produce hydrogen sulphide in triple-sugar (TSI) iron agar slant with negative reaction for urease, Simmon's citrate and indole test. Table 2.4 shows the result of biochemical identification of *Salmonella* serovars and *Enterobacteriaceae* family. Serological confirmation tests typically use polyvalent antisera for flagellar (H) and somatic (O) antigens. Isolates with the typical biochemical profile, which agglutinate with both H and O antisera are usually used to identify *Salmonella* spp.

Table 2.4: Biochemical identification among *Enterobacteriacae* family (WHO, 2003).

Organism	Triple-sugar iron (TSI)				Motility	Indole	Urea	Citrate
	Slant	Butt	H_2S	Gas				
1. *S. enterica* ser. *enterica* Typhi	Alk	Acid	Weak	-	+	-	-	-
2. *S. enterica* ser. *enterica* Parayphi A	Alk	Acid	-	+	+	-	-	-
3. *Salmonella* spp.	Alk	Acid	V	V	+	-	-	V
4. *Escherichia coli*	Acid	Acid	-	+	+	+	-	-
5. *Klebsiella* spp.	Acid	Acid	-	++	-	V	+	+
6. *Citrobacter* spp.	V	Acid	+++	+	+	V	-	+
7. *Proteus* spp.	Alk	Acid	+	+	+	V	++	V

The production of acid makes the agar turn to yellow. The slant section is for detection of lactose fermentation meanwhile butt section is for glucose fermentation.

Alk = alkaline, V = variable result
'+' = positive result and '-' = negative result

2.6 Typhoid carrier

WHO defined a carrier who continues to excrete *S.* Typhi for more than one year after infected with typhoid. Approximately 2-5 % of typhoid patients fail to clear the infection hence become chronic carriers of *S.* Typhi (Levine *et al.*, 1982). These carriers are most difficult to diagnose and the condition is further complicated with carriers are asymptomatic (Mortimer, 1999) and almost 25% of carriers experience no clinical history of typhoid (Parry *et al.*, 2002). Therefore, their recognition and treatment constitute a serious public health problem due to the continuously spreading of the disease. Moreover, since *S.* Typhi is a human-specific pathogen, these carriers form a crucial reservoir for the spread of the disease by shedding the pathogen through urine and feces (Bhan *et al.*, 2005).

The most popular case of typhoid fever is reported as Typhoid Mary case. Mary Mallon, an immigrant cook, who first caused the spread of fever in New York. She was quarantined in a cottage at Riverside Hospital after had a positive result for *S.* Typhi (Soper, 1939). In 1910, she was released with the condition that she never becomes a cook. However, she broke the promise and worked as a cooker at Sloane Maternity in Manhattan. In three months, 25 people were identified infected with the *S.* Typhi and two of them died. She was placed back in North Brother Island and remained there until death.

2.6.1 Gallbladder disease

Cholecystitis is caused by obstruction of the biliary tract due to the gallstones existing in the gallbladder which causes bile stasis, distention, inflammation and infection of the gallbladder (Swidsinski and Lee, 2001).

Other study showed that approximately 90% of chronic carriers have gallstones in the gallbladder (Shioler *et al.*, 1983; Karaki, 1984) and *S.* Typhi more preferred to form the biofilm on cholesterol gallstones compared to pigmented stones (Crawford *et al.*, 2008).

2.6.2 Association of gallbladder disease with *S.* Typhi

A study from Dongol and colleague (2012) at Patan Hospital, Kathmandu, Nepal have found that *S.* Typhi can be isolated from bile samples in gallbladders of patients undergoing cholecystectomy. Out of 1,377 patients underwent cholecystectomy, 274 bile samples were Gram-negative organism isolated included *E. Coli*, *Klebseilla* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Enterobacter* spp., *Citrobacter freundii, Vibrio* spp. and *Serratia marcescens* and 24 of them were *S.* Typhi.

Out of 48 patients of *Salmonella* bile-positive, only seven patients had a memorable history of typhoid, which none of them were confirmed by microbiological culture. Other researchers such as Mansour and colleague (2012) also successfully isolated the *S.* Typhi from Egyptian patients which diagnosed with chronic cholecystitis and acute chronic cholecystitis. Out of 257 patients, *S.* Typhi was successfully isolated from 28 samples of gallstones, 12 samples of gallbladder epithelial tissues and 4 samples of bile. Meanwhile, from the 28 samples of gallstones, 21 of them were found had biofilm surrounded on the gallstones.

2.6.4 Adaptation of *Salmonella* to bile

Bile is a body fluid containing bile salts, fatty acids, cholesterol and variety of protein and electrolytes (Hernandez *et al.*, 2012). Bile helps in the breakdown of fats, removal of excess cholesterol in the liver and helps absorption of fat-soluble vitamins in intestines (Hoffman, 1998). Bile salts consist of 61% of the bile composition, followed by 12% fatty acids, 9% cholesterol, 7% proteins, 3% phospholipids and bilirubin, and 5% from inorganic salts such as potassium, sodium and bicarbonate (Kristiansen *et al.*, 2007).

Bile also acts as bactericidal agent following with hydrochloric acid and gastric secretions which found in the digestive system. Bile salts in bile have been found to protect against pathogenic bacteria besides helping in the digestion of fatty acid (Merritt and Donaldson, 2009). For example, a patient that having cirrhosis of the liver, bacterial overgrowth is detected in the small intestine due to the less bile is secreted (Ding *et al.*, 1993). Meanwhile, in the small intestine, only a few bacteria were harboured due to the contain of high amount of bile (Inagaki *et al.*, 2006).

Although bile containing high bile salts, some bacterial species are resistant to it activities (Begley *et al.*, 2005). *Salmonella enterica* is one of the examples of the bile-resistant pathogen. This pathogen colonizes the hepatobiliary tract throughout systemic infection and continues to live in gallbladder during chronic infection (Gonzales-Escobedo *et al.*, 2011). This bile resistance is caused by the presence of a glycolipid, the enterobacterial common antigen, found in the outer membrane of *Enterobacteriaceae* (Ramos-Morales et al., 2003).