DEVELOPMENT OF A MULTIPLEX PCR-LATERAL FLOW ASSAY FOR THE DETECTION OF *Salmonella* Typhi AND *Salmonella* Paratyphi A

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
ºC	Degree celcius
1 X	One time
bp	Base pair
BSA	Bovine serum albumin
CFU/ml	Colony forming unit per mililitre
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenyl
dNTPs	Deoxyribonucleotide triphosphate
dsDNA	Double stranded DNA
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyanate
g	Gram
HCl	Hydrogen Chloride
IAC	Internal amplification control
IMViC	Indole, methyl red, voges proskauer, citrate
kDa	Kilo Dalton
LFA	Lateral flow assay
LFIA	Lateral flow immunoassay
LoD	Limit of detection
mA	milliAmpere
mg/ml	Milligram per mililiter
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	milliMolar
mm	millimeter
mmol/L	Milimole per litre
mPCR	Multiplex PCR
mPCR-AGE	Multiplex PCR-agarose gel electrophoresis
mPCR-LFA	Multiplex PCR-lateral flow assay
NA	Nutrient agar
NAAT	Nucleic Acid Amplification Technology
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient broth
ng	Nanogram
ng/µl	Nanogram per microlitre
nm	nanometer
NPV	Negative predictive value
OD	Optical density
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PPV	Positive predictive value
RH	Relative humidity
RNA	Ribonucleic acid
Ser.	Serovars
SS agar	Salmonella Shigella agar
ssDNA	Single stranded DNA
Subsp.	Subspecies
Ta	Annealing temperature
TAE	Tris-Acetate-EDTA
TSI	Triple sugar iron
μg/ml	Microgram per milliliter
μl	microlitre
μm	Micrometer
UV	Ultra violet
V	Voltan
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organization

PEMBANGUNAN ASAI ALIRAN LATERAL PCR MULTIPLEKS UNTUK PENGESANAN *Salmonella* Typhi DAN *Salmonella* Paratyphi A

ABSTRAK

Salmonella enterica serovar Typhi dan *Salmonella enterica* serovar Paratyphi A merupakan ejen penyebab demam enterik dan kebanyakannya berlaku setiap tahun di negara-negara kurang membangun dan sedang membangun. Demam enterik terdapat di dunia akibat kehadiran pembawa kronik yang membantu pembiakan bakteria di dalam hempedu dan ditularkan melalui air dan makanan yang tercemar. Kaedah kultur tinja merupakan piawai "gold standard" untuk pengesanan pembawa tifoid dan paratifoid tetapi kaedah ini memakan masa dan memerlukan kakitangan yang sangat terlatih. Justeru, tindak balas rantaian polimerasi (PCR) adalah alternatif yang lebih baik kerana ia adalah cepat, sangat sensitif dan spesifik, dan membenarkan pengesanan bakteria patogenik secara serentak. Kaedah elektroforesis gel agarosa dikuti dengan transilluminator UV merupakan kaedah umum untuk pengesanan produk PCR, tetapi kaedah ini juga memakan masa yang lama, memerlukan banyak prosedur, peralatan yang spesifik dan kakitangan yang sangat terlatih, dan penggunanya terdedah kepada etidium bromida dan cahaya ultra ungu. Oleh sebab itu, objektif kajian ini adalah untuk membangunkan PCR multipleks yang digabungkan dengan asai aliran lateral (mPCR-LFA) untuk pengesanan *Salmonella enterica* ser. Typhi dan *Salmonella enterica* ser. Paratyphi A dengan kehadiran dua kawalan; i) pan-*Salmonella* sebagai kawalan gen sasaran dan ii) kawalan amplifikasi dalaman (IAC). Empat set primer direka secara manual berdasarkan kawasan spesifik gen sasaran; gen *stgA* untuk *S. enterica* ser. Typhi, "intergenic region" (SSPAI)

antara SSPA1723a dan SSPA1724 untuk *S. enterica* ser. Paratyphi A, gen *ompC* untuk *Salmonella* serovars dan gen *hemM* untuk *Vibrio cholerae* sebagai IAC. Gen sasaran dan jujukan yang serupa diperolehi daripada program "BLASTn" dijajar menggunakan "ClustalW Multiple Alignment" (perisian bioEdit). Primer "forward" ditandakan dengan FITC, Texas Red, DNP dan DIG pada bahagian hujung 5' dan primer "reverse" ditandakan dengan biotin pada bahagian hujung 5' bertujuan untuk pengesanan menggunakan asai aliran lateral. Selepas amplifikasi, produk PCR yang dilabel telah dikesan menggunakan asai aliran lateral melalui pengikatan "capture reagent" pada membran (anti-FITC, anti-Texas Red, anti-DNP and anti-DIG). Pengumpulan zarah nano emas terkonjugat dengan streptavidin (40 nm) pada kawasan tertentu menghasilkan keputusan positif sebagai titik merah yang kelihatan dalam masa 15 minit. Sensitiviti analitik mPCR-LFA berasaskan aras DNA untuk *S. enterica* ser. Typhi dan *S. enterica* ser. Paratyphi A adalah 0.16 ng dan 0.08 ng, manakala mPCR-elektroforesis gel agarosa adalah sebanyak 0.63 ng untuk kedua-duanya. Penilaian kesahihan mPCR-LFA sebagai bukti kepada konsep menggunakan 100 bakteria isolat terdiri daripada 25 *S. enterica* ser. Typhi, 25 *S. enterica* ser. Paratyphi A, 25 *Salmonella* serovar dan 25 bakteria lain menunjukkan sensitiviti dan spesifisiti 100% mencadangkan bahawa primer-primer yang diperolehi dari gen-gen sasaran adalah spesifik untuk *S. enterica* ser. Typhi, *S. enterica* ser. Paratyphi A dan *Salmonella* serovar tanpa kereaktifan-balas dengan DNA dari bakteria lain. Penilaian kesahihan mPCR-LFA menggunakan sampel tinja menunjukan ujian boleh mengesan sehingga 10^1 CFU/ml *S. enterica* ser. Typhi dan 10^2 CFU/ml *S. enterica* ser. Paratyphi A. mPCR-LFA juga menunjukkan 100% sensitiviti dan spesifisiti apabila diuji menggunakan sampel tinja yang dicampurkan dengan 100 isolat bakteria mencadangkan ujian berjaya dibangunkan tanpa sebarang perencat. Apabila

sampel tinja daripada pengendali makanan dan pembawa yang disyaki digunakan dalam mPCR-LFA ini, lima daripada 60 sampel adalah positif dan keputusan ini adalah sama seperti kaedah kultur. Sebagai kesimpulan, mPCR-LFA merupakan kaedah alternatif yang mudah, cepat, sensitif dan spesifik untuk mengesan kehadiran pembawa tifoid dan paratifoid berbanding dengan kaedah pengesanan menggunakan elektroforesis gel agarosa. Pembangunan mPCR-LFA adalah salah satu langkah untuk mengenalpasti aksesibiliti ujian diagnostik bagi negara-negara kurang membangun dan sedang membangun.

DEVELOPMENT OF A MULTIPLEX PCR-LATERAL FLOW ASSAY FOR THE DETECTION OF *Salmonella* Typhi AND *Salmonella* Paratyphi A

ABSTRACT

Salmonella enterica serovar Typhi and *Salmonella enterica* serovar Paratyphi A are causative agents of enteric fever and occur each year mostly in underdeveloped and developing countries. The persistence of enteric fever is due to the existence of chronic carriers who harbor the bacteria inside their gall bladder and transmitted the disease through contaminated water and food. Stool culture is the gold standard for the detection of typhoid and paratyphoid carriers but this method is time consuming and requires highly skilled personnel. Therefore, polymerase chain reaction (PCR) assay is a better alternative since it is rapid, highly sensitive and specific, and allows for simultaneous detection of pathogens. Agarose gel electrophoresis followed by UV transilluminator is the common method for the detection of PCR amplicons, but this method is also time consuming, tedious, requires special equipment and highly skilled personnel, and it exposes users to ethidium bromide and ultra violet light. Thus, this study aims to develop a multiplex PCR coupled with lateral flow assay (mPCR-LFA) for the detection of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A in the presence of two controls; i) pan-*Salmonella* as a target gene control and ii) internal amplification control (IAC). Four sets of primers were manually designed based on specific regions of the target genes; *stgA* gene of *S. enterica* ser. Typhi, intergenic region (SSPAI) between SSPA1723a and SSPA1724 gene of *S. enterica* ser. Paratyphi A, *ompC* gene of *Salmonella* serovars and *hemM* gene of *Vibrio cholerae* as an IAC. The target genes and the similar sequences obtained from

BLASTn program were aligned using ClustalW Multiple Alignment (BioEdit software). Forward primers were labeled with FITC, Texas Red, DNP and DIG at the 5'end, while reverse primers were labeled with biotin at the 5'end for the purpose of detection using lateral flow assay. After amplification, labeled PCR amplicons were applied onto the lateral flow strip and bound to their respective capture reagents immobilized on the membrane (anti-FITC, anti-Texas Red, anti-DNP and anti-DIG). The accumulation of streptavidin-colloidal gold conjugate (40 nm) at respective areas produced positive results, seen as red dots within 15 minutes. The analytical sensitivity at DNA level of mPCR-LFA for *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A were 0.16 ng and 0.08 ng, respectively, while mPCR-agarose gel electrophoresis were 0.63 ng for both. Validation of the mPCR-LFA using 100 bacterial isolates comprising of 25 *S. enterica* ser. Typhi, 25 *S. enterica* ser. Paratyphi A, 25 *Salmonella* serovars and 25 other bacteria as a proof of concept showed 100% sensitivity and specificity suggesting that the primers of the targeted genes were specific for *S. enterica* ser. Typhi, *S. enterica* ser. Paratyphi A and *Salmonella* serovars without cross-reactivity with DNA from other bacteria. Validation of the mPCR-LFA using spiked stool samples showed that the test can detect as low as 10^1 CFU/ml *S. enterica* ser. Typhi and 10^2 CFU/ml *S. enterica* ser. Paratyphi A. The mPCR-LFA also showed 100% sensitivity and specificity when validated using stool samples spiked with 100 bacteria isolates suggesting that the test was successfully developed without any inhibition. When stool samples from food handlers and suspected carriers were used in this mPCR-LFA, five out of 60 samples were positive and these results correspond to the culture results. As a conclusion, mPCR-LFA is a simple, rapid, sensitive and specific alternative method for the detection of typhoid and paratyphoid carriers compared to detection method

by agarose gel electrophoresis. The development of mPCR-LFA is one of the ways in making the diagnostic tests accessible to developing and underdeveloped countries.

CHAPTER 1

General Introduction

1.1 General background on *Salmonella*

Salmonella is named after an American bacteriologist, D.E. Salmon who first isolated *Salmonella Choleraesuis* from porcine intestine in 1884 (Black, 2005). It is a gram-negative bacterium, rod shaped bacillus, facultative anaerobic, motile and belongs to the family *Enterobacteriaceae* (Black, 2005, Hagren, 2009). Many of them are pathogenic and cause food borne illness that infect human and animals (Tirado and Schmidt, 2001, EFSA, 2009). *Salmonella* has a complex nomenclature and taxonomy. Under Kauffmann-White scheme, each serotype of *Salmonella* was considered as separate species based on serological identification of O (somatic) and H (flagella) antigen and 2463 species of *Salmonella* was found under this scheme (Brenner *et al.,*2000).

Other nomenclature was proposed by Crosa *et al.* (1973), demonstrated by DNA-DNA hybridization where all subspecies I (*S. enterica* subsp. *enterica*), II (*S. enterica* subsp. *salamae*) and IV (*S. enterica* subsp. *houtenae*) were related at the species level, *Salmonella enterica* and subspecies V is classified under species *Salmonella bongori* (Crosa *et al.*, 1973). Then, Minor and Popoff (1987) proposed seven subspecies of *Salmonella* subspecies I, II, IIIa, IIIb, IV, V and VI (*S. enterica* subsp. *indica*). The subspecies IIIa (*S. enterica* subsp. *arizonae*) and IIIb (*S. enterica* subsp. *diarizonae*) was differentiated by genomic relatedness and biochemical reaction (Brenner *et al.*, 2000, Popoff and Minor, 2007).

Based on the Kauffman-White Scheme, 1454 out of 2463 *Salmonella* serotypes belong to *S. enterica* subsp. e*nterica* that infect humans and warm-blooded animals (Popoff and Minor, 2007). The other *S. enterica* subsp. is usually isolated from cold-blooded animals and the environment (Farmer *et al.*, 1984).

1.2 *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A

Salmonella enterica subsp. *enterica* ser. Typhi (referred as *S. enterica* ser.Typhi) and *Salmonella enterica* subsp. *enterica* ser. Paratyphi A (referred as *S. enterica* ser. Paratyphi A) belong to *Salmonella enterica* subsp. *enterica* (Brenner *et al.*, 2000). They are gram negative bacteria, rod shaped with 2-3 µm in length and 0.4-0.6 µm in diameter (Le Monor, 1981). Both serovars have peritrichous flagella that are uniformly projected in all directions on the surface and the flagella are used for motility. These bacteria are facultative anaerobic and undergo aerobic metabolism when oxygen is present, but when oxygen is absent they shift to anaerobic metabolism (Black, 2005). Both *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A are non-spore forming bacteria. Therefore, they could not survive in the environment in a long period of time and thus require humans as a host (Black, 2005). *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A are transmitted by fecal-oral route where the bacteria are excreted in feces and entered new host through oral route via consumption of contaminated food or water.

1.3 Enteric fever

Enteric fever is a systemic infection caused by *S. enterica* ser. Typhi (Typhoid fever) and *S. enterica* ser. Paratyphi A, B and C (Paratyphoid fever) (WHO, 2003). Enteric fever infects particularly among children and adolescents in developing and under-

developed countries with poor sanitation, limited access to clean drinking water and improper food preparation (Mirza *et al*., 2000, Morin *et al.,* 2004, Crump and Mintz, 2010). A person was diagnosed as a typhoid patient after having high fever (more than 38°C) for at least 3 days and culture positive for *S. enterica* ser. Typhi (WHO, 2003). The symptoms of typhoid fever are prolonged fever, headache, loss of appetite, malaise, stomach pains and appearance of rose-colored spots on the chest (WHO, 2003). On the third week of infection, serious complications such as intestinal hemorrhage or perforation, sudden rise in pulse rate, hypotension, abdominal tenderness and white blood cell count elevation may occur (Glynn *et al*., 1995). Paratyphoid fever was less severe compared to typhoid fever (Crum, 2003). The symptoms and severity of enteric fever are different in each individual and depend on the duration of the illness before the initiation of therapy. Other influential factors are the choice of antimicrobial treatment, age, previous exposure, vaccination history, the virulence of bacterial strain, the quantity of inoculums ingested, host factors and whether the individual was taking other medications such as antacids (WHO, 2003).

The pathogenesis of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A are similar (Bhan *et al.*, 2005). Both bacteria invade the human body through the ingestion of contaminated water or food (Bhan *et al.*, 2005). They pass through the pylorus and go to the small intestine. Then, they penetrate the mucosa of the terminal ileum through specialized antigen-sampling cells, M-cells and eventually become invasive infections (Talaro, 2008). The bacteria lipopolysaccharide induced the secretion of cytokines from epithelial cells and leads to infiltration of peripheral blood leucocytes into the lamina propria (Bhan *et al*., 2005). The bacteria were taken

up by the macrophages without killing and draining them into mesenteric nodes, thoracic duct and general circulation before releasing them into the bloodstream (WHO, 2003). Within 24 hours of their ingestion, these bacteria can reach the reticuloendothelial system (liver, spleen, bone marrow) and start to replicate (House *et al.*, 2008). When the bacteria are shed back into the bloodstream, the infected person will show the symptoms of typhoid or paratyphoid fever. The incubation period of enteric fever can be varied from six to 30 days depending on quantity of inoculum and host factors (Moudgil and Narang, 1985, Glynn *et al*., 1995, WHO, 2003).

1.4 Typhoid and paratyphoid carriers

Typhoid and paratyphoid carriers carried the *S. enterica* ser. Typhi or *S. enterica* ser. Paratyphi in their gall bladder and also other organs in the body such as liver and bone marrow without showing any clinical signs and symptoms (WHO, 2003). Chronic carrier means a recovered patient but still continuously excreting *S. enterica* ser. Typhi or *S. enterica* ser. Paratyphi intermittently through feces after a year of infection without showing any symptoms (Parry *et al.,* 2002, WHO, 2003). Approximately one to five percent of typhoid patients become carriers (WHO, 2003). Transient carrier means a person who excretes *S. enterica* ser. Typhi or *S. enterica* ser. Paratyphi through feces less than one year of infection or having no history of infection without showing any symptoms (Chua *et al.* 2012). Since carriers harbor the bacteria inside their gall bladder or liver, they play an important role in persistence of enteric fever among the community (Chua *et al.*, 2012, Shah *et al.*, 2012). According to WHO (2003), typhoid carrier can be characterized as a person who had typhoid infection previously, is currently asymptomatic, carried *S. enterica*

ser. Typhi in gall bladder and shown a positive stool or rectal swab culture after a year of infection.

1.5 Epidemiology of enteric fever

Enteric fever commonly occurs in developing and underdeveloped countries. According to Crump *et al.* (2004), the incidence of typhoid fever is over 22 million cases with 220,000 deaths every year worldwide. South-central and South-eastern Asia especially India, Pakistan and Bangladesh are the major contributors of typhoid fever (Figure 1.1) (Crump *et al.,* 2004). The incidence of paratyphoid fever is 5.4 million cases every year and commonly occurred in China, India and Pakistan (Crump *et al*., 2004, Ochiai *et al*., 2005). *S. enterica* ser. Paratyphi A is a major contributor for the increasing number of paratyphoid cases especially in Asia (Crump and Mintz, 2010). This is supported by the finding of a previous study that showed one to 33.8% of the enteric fever cases in Nepal, India and Philippines were contributed by *S. enterica* ser. Paratyphi A (Maskey *et al*., 2006).

In Malaysia, the incidence of typhoid fever was 201 to 1085 cases in the year 1995 until 2010 with the incidence rate below 5 per 100 000 population as shown in Figure 1.2 (MOH, 2007, DOSM, 2011). Since Malaysia has less than 10 cases per 100 000 population per year, the country is classified as a low endemic region for typhoid fever (Crump *et al*., 2004). In comparison with other states in Malaysia, Kelantan showed the highest number of typhoid cases followed by Sabah as shown in Table 1.1 (MOH, 2007).

In Kelantan, the incidence rate of typhoid fever showed a fluctuated pattern in the year 1995-2012. There was a huge increment in the year 2005 with 58.9 per 100 000 population due to typhoid outbreak (Figure 1.3). Shah *et al.* (2012) reported that flood risk areas are the major contributors of typhoid outbreak in Kelantan. Majority of the rural communities in Kelantan depend on well water supply. During the flood season most of the wells would be contaminated with sewage overflow and further contribute to the increasing number of cases (Aziah, 2009). In addition, food handlers with poor hygiene and practice and the presence of transient and chronic carriers also contributed to this typhoid outbreak (Shah *et al*., 2012).

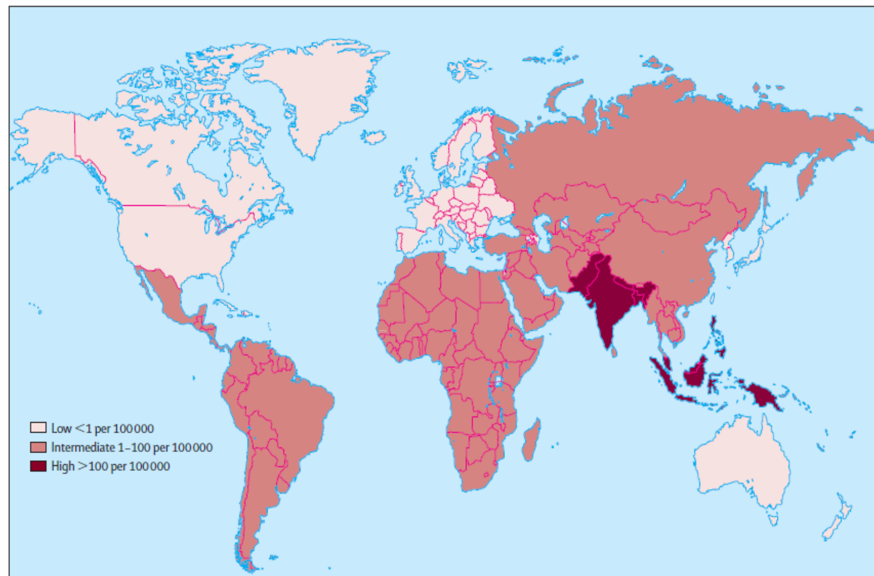

Figure 1.1: Global distribution of enteric fever

(Adapted from Connor and Schwartz, 2005)

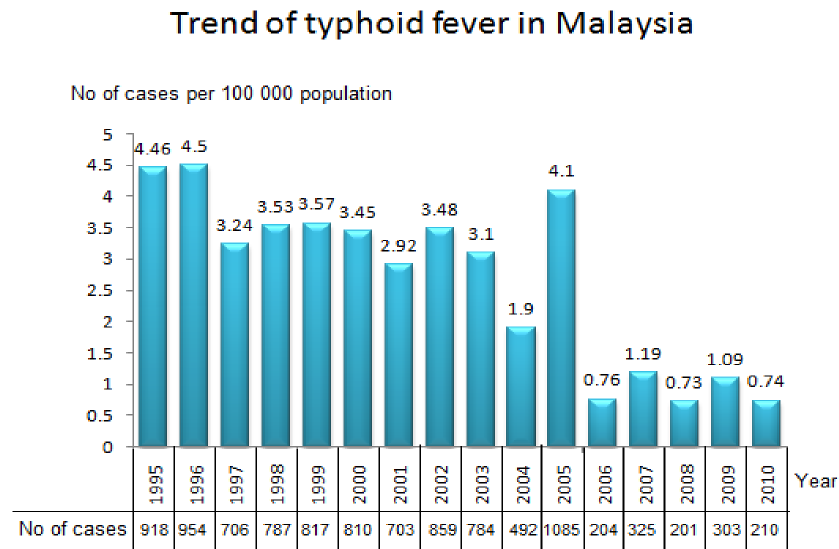

Figure 1.2: Trend of typhoid fever in Malaysia

(Data from MOH, 2007, DOE, 2010, DOSM, 2011)

Table 1.1: Incidence of typhoid/paratyphoid (per 100 000 population) in Malaysia by states, 2003-2007

States	Year				
	2003	2004	2005	2006	2007
Kelantan	24.8	7.5	58.9	4.9	8.7
Sabah	6.3	5.5	2.1	1.3	2.2
Johor	0.7	0.5	0.4	0.3	1.4
Pahang	1.5	0.6	0.8	0.1	1.2
P.Pinang	0.2	0.4	0.6	0.2	1.1
Melaka	0.3	0.3	0.1	0.3	1.0
Perak	1.3	1.1	0.8	0.6	0.9
Kedah	1.8	1.2	1.0	1.0	0.5
Selangor	0.6	1.1	1.0	1.0	0.4
W.P	0.7	0.8	0.1	0.1	0.3
Terengganu	7.3	3.2	0.8	0.9	0.3
Sarawak	1.1	1.5	0.7	0.5	0.2
N.Sembilan	0.4	0.4	0.7	0.0	0.1
Perlis	0.5	1.4	0.0	0.4	0.0
Malaysia	3.1	1.9	4.1	0.8	1.2

*W.P = Wilayah Persekutuan, N. = Negeri
(MOH, 2007)

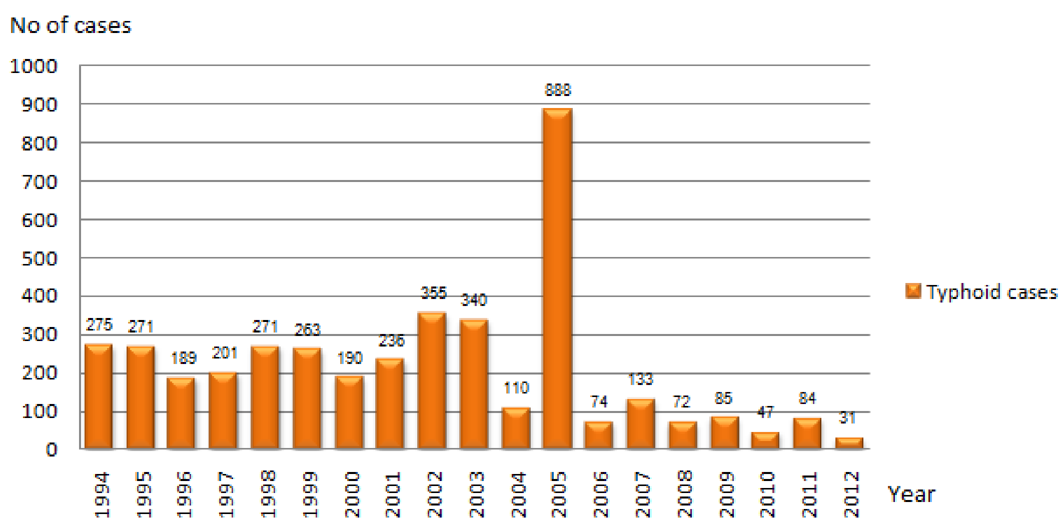

Figure 1.3: Trend of typhoid fever in Kelantan

(Adapted from Kelantan State Health Department, 2012)

1.6 Contribution factor for increased cases of enteric fever

Nowadays, enteric fever has become one of the major world health problems with increasing numbers of typhoid as well as paratyphoid fever. The contributing factors of this increment were due to the following; i) emergence of antimicrobial resistant strains of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi, ii) limited availability and efficacy of vaccines and iii) limitation of diagnostic tests (Parry *et al.*, 2002, Maskey *et al.*, 2006, Maurice, 2012). By knowing these factors, an improvement of the antibiotics, vaccines and diagnostic tests can be done to reduce the number of enteric fever cases.

Antimicrobial resistance (AMR) strain is an organism that can survive and multiply in the presence of antimicrobial agents which was previously observed to be sensitive (WHO, 2011). This is due to the over usage of antibiotics such as fluoroquinolone, ceftriaxone and chloramphenicol to treat an organism or the organism itself mutates or acquires a resistant gene (Parry *et al.,* 2002, WHO, 2011). Chloramphenicol and flouroquinolones have been used for the treatment of typhoid fever since they are highly effective in reducing the typhoid fatality rates from 25% to 1% (Maurice, 2012). However, the AMR strains of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi spread rapidly nowadays (Maurice, 2012).

The multi drug resistant (MDR) strains of *S. enterica* ser. Typhi were reported against the first-line drugs such as chloramphenicol, amoxicillin and trimethoprim-sulfamethoxazole (TMP-SMZ) (Parry *et al.*, 2002). Based on previous studies, 50% of typhoid patients were infected with MDR strains and these case was higher in India, Pakistan and Vietnam (Maskey *et al.*, 2006, Maurice, 2012). While, the MDR

strains of *S. enterica* ser. Paratyphi are still at lower rates (Gupta *et al.*, 2006, Parry and Threlfall, 2008). The second-line drug, flouroquinolones such as ofloxacin and ciprofloxacin were used to replace the first-line drug (Parry *et al.*, 2002, MOH, 2008). Fluoroquinolone resistant strains were reported among *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A due to chromosomal mutation and plasmid mediated resistance at the region of *gryA* gene (Maskey *et al.*, 2006, Chuang *et al.,* 2009). The third line drug cephalosporin such as ceftriaxone and gatifloxacin were used as an alternative for the previous drugs (MOH, 2008). Nonetheless, the third line drug is costly and less suitable for treatment in developing and under developed countries (Crump and Mintz, 2010).

In Malaysia, the majority of *S. enterica* ser. Typhi strains are still sensitive to the first line drug such as chloramphenicol or ampicillin and this drugs are in use till now as a treatment for typhoid patients and suspected carriers (MOH, 2007). Typhoid patients are treated with perfloxacin (400mg bd orally) for five to seven days or by ampicillin or chloramphenicol (500mg or 75mg/kg/days for children for every six hours) for 14 days. Chronic carriers are treated with amoxicillin (1g for every 8 hours) for 14 days and a dose is reduced to 750mg for every 8 hours and prolonged treatment for 3 months. Another alternative treatment is ciprofloxacine (500mg bd) for 4 weeks, and children are treated with ampicillin (100mg/kg/day for every 6 hours) for 3 months. During treatment, chronic carrier is forbidden to cook or sell food and water in order to prevent spreading of *S. enterica* ser. Typhi or *S. enterica* ser. Paratyphi A. In some cases, chronic carriers are advisable to do surgical removal of their gallbladder (cholecystectomy) because *S.* Typhi was able to form a biofilm on cholesterol

gallstone surface, thus, make it resistance to humoral immune host responses and antibiotic regimens (Crawford *et al.,* 2010).

Limited availability and less efficacy of vaccine are factors that have also contributed to the increasing numbers of enteric fever cases. There are two types of typhoid vaccines available in the market, Ty21a and Typhim Vi vaccine (Vivotif Berna, Typhim Vi Pasteur Merieux). Ty21a vaccine is a live, attenuated *S. enterica* ser. Typhi strain and is administered orally. The protection efficacy of Ty21a for five to nine years old and 10 to 14 years old children were reported as 71% and 63%, respectively (Engels *et al.*, 1998). Typhim Vi vaccine is a purified Vi antigen and is administered via injection and the protection efficacy of Typhim Vi vaccine was reported as 74% in endemic areas such as Nepal (Acharya *et al*., 1987). As a summary, both vaccines are unable to provide full protection to the people.

As an alternative, a conjugated typhoid vaccine was developed to give high protection against *S. enterica* ser. Typhi. Peda Typh^{TM} is developed using Vi antigen conjugated with tetanus toxoid (BioMed Pvt. Ltd). It showed more than 90% protective efficacy when tested amongst the Indian population (Garg, 2009; BioMed Pvt.Ltd). Other conjugated vaccines include Vi-rEPA, (Vi antigen conjugated with *Pseudomonas aeruginosa* exotoxin A). The Vi-rEPA vaccine showed 91.5% protection efficacy when tested amongst two to five years old Vietnamese children (Lin *et al*., 2001). However, both vaccines need to be tested in other endemic countries involving infants, children, adults and adolescent in order to get a real percentage of protection efficacies.

Currently, there is no licensed vaccine available to protect against *S. enterica* ser. Paratyphi A. However, researchers have found several potential *S. enterica* ser. Paratyphi A strains for the development of a live oral vaccine (Roland, *et al.,* 2010, Gat *et al*., 2011). A group from Novartis Vaccine Institute for Global Health, Italy has developed a conjugate vaccine using the O antigen (O:2) conjugated to the carrier protein, CRM_{197}. This O:2-CRM_{197} vaccine was safe in laboratory trial when tested with mice and showed great potential as a paratyphoid vaccine (Micoli *et al*., 2012). However, further clinical trials are required to determine the efficacy of this vaccine among people in different geographical areas.

Another factor that has contributed to the increasing numbers of enteric fever is limitation of diagnostic tests for typhoid and paratyphoid carriers. Since carriers play a role in the persistence of *S. enterica* ser. Typhi and increasing number of *S. enterica* ser. Paratyphi A, a sensitive and specific diagnostic test is required for the detection of carriers to help in reducing the number of cases and identify the source of infection. There are several methods used for the detection of enteric fever and carriers such as culture method, Widal test, IDL Tubex, Typhidot, Tubex-PA, Vi-ELISA, Typhidot-C and PCR assay (Levine *et al.,* 1978, Ismail *et al.,* 1991, Lim *et al.,* 1998, Song *et al.,* 1993, Choo *et al.,* 1999, Hatta *et al.,* 2002, Hirose *et al.,* 2002).

Culture method is an antigen based detection method which detects the presence of organism inside human host and it is a confirmatory test. In the diagnosis of enteric fever, several samples were used such as blood (collected at 1st week of infection), stool (collected at 3rd weeks of infection), rectal swab, urine and bone marrow. The sensitivity of culture method was varies from 45% to 95% depending on types of

samples used. Based on previous study, the sensitivity of culture method can achieved 95% when culture was performed using all types of samples (Wain and Hosoglu, 2008). In the detection of carriers, stool culture was used since the bacteria are shed intermittently via stool. However, the stool culture has an isolation rate of 25% for typhoid fever hence making it less sensitive for the detection of carriers (Braddick, *et al.*, 1991). There are three steps in performing culture method, i) the enriched bacterial culture was streaked onto the agar plates (Table 1.2), ii) a single colony of bacteria from the agar plate was used to perform biochemical tests (Table 1.3) and iii) serotyping method was performed through agglutination with specific antisera for *S. enterica* ser. Typhi or *S. enterica* ser. Paratyphi A (Table 1.4). Since it involved with many steps, approximately two to seven days are required to produce results, thus, make this method is time consuming and tedious. In addition, culture method also required highly skilled personnel in performing this method to pick up the right colonies (Osek, 2002).

Table 1.2: Characteristics of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A colonies on agar plate media

(WHO, 2003)

Media	***S. enterica* ser. Typhi**	***S. enterica* ser. Paratyphi A**
Blood agar	Non-haemolytic smooth white colonies.	Non-haemolytic smooth white colonies.
MacConkey agar	Lactose non-fermenting smooth colonies.	Lactose non-fermenting smooth colonies.
SS agar	Lactose non-fermenting with black centered colonies.	Lactose non-fermenting without the black centered colonies.
Desoxycholate agar	Lactose non-fermenting with black centered colonies.	Lactose non-fermenting without the black centered colonies.
Xylose-lysine-desoxycholate agar	Transparent red colonies with black centers.	Transparent red colonies and without the black centers.
Hektoen enteric agar	Transparent green colonies with black centers.	Transparent green colonies without the black centers.
Bismuth sulfite agar	Black colonies.	Black colonies.

Table 1.3: Biochemical tests for the differentiation of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi A

(WHO, 2003)

Organism	**Triple sugar iron agar**				**Motility**	**Indole**	**Urease**	**Citrate**
	Slant	**Butt**	**H_2S**	**Gas**				
S. enterica ser. Typhi	Alkali	Acid	weak	-	+	-	-	-
S. enterica ser. Paratyphi A	Alkali	Acid	-	+	+	-	-	-
Other *Salmonella* serovars	Alkali	Acid	V	V	+	-	-	V

H_2S = Hydrogen sulfide, V = variable result, '+' = positive result and '–' = negative result

Table 1.4: Antigenic composition of *S. enterica* ser. Typhi and *S. enterica* ser. Paratyphi

(WHO, 2003)

Serotype	**O antigen**	**H antigen**	**Serogroup (Phase 1:2)**
S. enterica ser. Typhi	9, 12, Vi	d	Group D1
S. enterica ser. Paratyphi A	1, 2, 12	a: 1, 5	Group A
S. enterica ser. Paratyphi B	1, 4, 5, 12	b: 1, 2	Group B
S. enterica ser. Paratyphi C	6, 7, Vi	c: 1, 5	Group C1

Instead of culture method, the serological method was developed which detects the presence of antibody that release after infection by an organism and it is a suggestive of enteric fever and carriers. There are several methods available for diagnosis of i) enteric fever such as Widal test, IDL TUBEX, TUBEX-PA, Typhidot® and Typhidot-M® and ii) typhoid carriers such as Typhidot-C® and Vi-ELISA (Losonsky *et al.,* 1987, Chua *et al.,* 2012). The Widal test is used for the diagnosis of typhoid patient based on agglutination of antibodies from patient sera against O and H antigens. However, Widal test is less sensitive since it failed to detect the presence of *S. enterica* ser. Typhi in 30% of positive culture samples due to antibiotic treatment which reduce the antibody response. It also tends to produce false-positive results since O and H antigens also presence in other *Enterobacteriaceae* (Olopoenia and King, 2000). Besides, it is difficult to interpret Widal test result in typhoid endemic area and where the antibody titres of the normal population are often not known (Onyekwere *et al.,* 2007). This could lead to false-positive result in diagnosis of enteric fever.

Another method used was IDL TUBEX or TUBEX®TF (TUBEX™, IDL Biotech, Sweden) which detect anti-*Salmonella* O9 (both IgM and IgG) antibodies from a patient's serum against the *S. enterica* ser. Typhi O9 lipopolysaccharide (LPS) antigen (Lim *et al.,* 1998). It is based on inhibition binding assay. The anti-O9 IgM conjugated to colored latex particles and *S. enterica* ser. Typhi LPS conjugated to magnetic latex particles. Absence of antibody will cause the color of test change from blue to red due to cosendimentation of the colored latex particle with magnetic latex particle. While, in a presence of antibody will prevent the change of color (Tam and Lim, 2003). The sensitivity and specificity of IDL TUBEX were 78% and 89%,

respectively. However, this method has disadvantages such as difficult to interpret a result from haemolysed samples, tends to produce false positive results in person with *S*. Enteritidis infection, (Olsen *et al.,* 2004, Oracz *et al.,* 2003, Lim *et al.,* 1998). For diagnosis of paratyphoid A fever, TUBEX-PA was developed based on the principle of TUBEX-TF. The TUBEX-PA detect anti-*Salmonella* O2 antibodies from a patient's serum against the *S. enterica* ser. Paratyphi A O2 lipopolisaccharide (LPS) antigen (Tam *et al.,* 2008). Previous study showed the sensitivity and specificity of the TUBEX-PA were 81% and 98.1%, respectively. However, when tested with 14 positive typhoid serum, TUBEX-PA showed 7 positive results and this lead to false-positive result (Tam *et al.,* 2008).

The other test available in the market is the dot enzyme immunoassay known as Typhidot® (Reszon Diagnostics International Sdn. Bhd., Malaysia) test which used for the detection of IgM and IgG antibodies against a 50 kDa protein of *S. enterica* ser. Typhi. The 50 kDa protein is a specific protein on the outer membrane of *S. enterica* ser. Typhi (Ismail *et al.,* 1991). The Typhidot® showed 78% sensitivity and 89% specificity (Choo *et al.,* 1994). However, the Typhidot® has a limitation which the IgG result cannot differentiate between convalescence and chronic carriers of typhoid infection since the IgG antibody were higher in both cases (Choo *et al.,* 1994, Chua *et al.,* 2012). To overcome this limitation, the Typhidot-M® was developed to detect the presence of IgM alone since IgM is used for the detection of acute infection. Previous study showed the Typhidot-M® able to increase the sensitivity and specificity to 92% and 100%, respectively (Choo *et al.,* 1994). Another dot enzyme immunoassay test available was Typhidot-C® which used for the detection of typhoid carriers. The Typhidot-C® is used for the detection of IgG

and IgA antibodies against 50kDa protein from *S. enterica* ser. Typhi. This assay provided a rapid and high sensitivity of 100% when tested with 14 serum samples collected from carries (Chua *et al.,* 2012).

Other detection method for typhoid carriers is Vi-ELISA. This enzyme linked immunosorbent assay (ELISA) is used to measure the binding of IgG and IgA antibodies against Vi capsular polysaccharide antigen from *S. enterica* ser. Typhi. The Vi-ELISA showed 86% sensitivity and 95% specificity in screening of chronic carriers (Losonsky *et al*., 1987). However, Vi-ELISA is less suitable in endemic areas since the widely usage of Vi-vaccine in the areas that would contribute to high background levels of anti-Vi antibody. This situation interfered with the results and led to misinterpretation of the test (Gupta *et al*., 2006).

Due to the limitation of culture and serological methods, a rapid and highly sensitive and specific molecular method known as nucleic acid amplification technology (NAAT) is developed to detect the presence of the bacteria inside host through amplification of DNA or RNA (Levy *et al*., 2008, Ngan, *et al.*, 2010). Based on previous studies, the PCR assay was successfully developed for the detection of *S. enterica* ser. Typhi, *S. enterica* ser. Paratyphi A, *S. enterica* ser. Paratyphi B and other *Salmonella* serovars from bacteria culture and blood samples with 100% sensitivity and specificity (Hirose *et al.,* 2001, Levy *et al.,* 2008, Lim and Thong, 2009). Unfortunately, there is no commercially available PCR assay for the detection of typhoid and paratyphoid carriers. All the developed kits are meant for the detection of *Salmonella* serovars in food and water samples together with other bacteria such as *E.* coli and coliform.

1.7 Nucleic Acid Amplification Technology (NAAT)

Nucleic acid amplification technology (NAAT) is a platform to amplify and detect small amounts of nucleic acids. This technology focuses on an antigen detection method that detects the presence of microorganism in the human body (Lee *et al.,* 1997). NAAT involves three main steps which are sample preparation, DNA amplification and detection of the amplified products (McPherson and Meller, 2006). Sample preparation aims to extract DNA from samples with the additional steps to reduce inhibitory substances presence in samples and increase the concentration of the target organism via enrichment in suitable media (Radstrom *et al.*, 2004). The enrichment step will enhance the growth of bacteria to a detectable level (Stone *et al.*, 1994). After enrichment, the extraction of DNA from bacterial cells can be performed using the boiling method, commercial kit and/or the available automated DNA preparation.

1.7.1 DNA amplification

DNA amplification is based on the three steps; denaturation, annealing and extension including the enzymatic amplification parts (Vaneechoutte and Eldere, 1997). This amplification occurs in the presence of a pair of primers targeted a specific part of the genome and DNA polymerase enzyme to synthesis a new DNA strand. Polymerase chain reaction (PCR) is a conventional method used in the amplification of DNA. PCR is an *in-vitro* process to make large copy numbers of a specific DNA fragment (McPherson and Meller, 2006). It works via the ability of DNA polymerase and deoxyribonucleotide triphosphate (dNTPs) to synthesis a new DNA stands based on target DNA strands that initiate by a pair of primers through repeated cycles of denaturation, annealing and extension steps. In each cycle, the double stranded target

DNA is separated (denaturation) to allow binding of the primers to the target DNA (annealing) and attachment of DNA polymerase to synthesis new strands that are complementary to the targeted DNA (extension). Then, the cycles are continuously repeated from denaturation until extension step to produce billion copies of DNA. Thus, the total number of DNA fragments produced is measured as 2n, where n is the number of cycles (McPherson and Meller, 2006).

The main strength of PCR assay is the ability to multiplex that enabled simultaneous detection of several targets within a tube. This innovation has shown promising use in a clinical diagnostic perspective since it allows detection of multiple pathogens from a clinical specimen (Yager *et al.*, 2008). Previous studies showed the successfulness of multiplex PCR in the detection of *S. enterica* ser. Typhi, *S. enterica* ser. Paratyphi A, *S. enterica* ser. Paratyphi B and *Salmonella* serovars using various target genes (Hirose *et al*., 2002, Banavandi *et al.*, 2005, Levy *et al*., 2008). However, these multiplex PCR assay lacked an internal amplification control (IAC) which is important for validation of negative results in PCR assay.

IAC is a non-target DNA sequence present in the same reaction with the target sequence. IAC has the ability to differentiate between true negative results and false negatives due to the presence of PCR inhibitors such as incorrect PCR mixture, malfunction of thermal cycler or the presence of inhibitory substances in the sample (Hoorfar *et al*., 2003). Due to the importance of IAC, The European Standardization Committee in collaboration with International Standard Organization has proposed a guideline that the incorporation of IAC in a PCR reaction is a mandatory for the validation of the PCR assay (Hoorfar *et al*., 2003).

1.7.2 Detection and visualization of PCR amplicons

The amplified product can be detected and visualized using several methods such as agarose gel electrophoresis, realtime-PCR, enzyme-linked immunosorbent assay (ELISA), mass spectrometry and lateral flow assay.

1.7.2 (a) Agarose gel electrophoresis

Agarose gel electrophoresis is a common method for detection of PCR amplicons. It is based on charge separation where a negative charge of phosphate backbone of DNA migrates toward a positive charge (anode) of the system (Karcher, 1995). During the agarose gel electrophoresis, small fragments of DNA move faster compared to larger fragments of DNA since small molecule can easily pass through the gel pores (Reece, 2004). After the separation of DNA is completed, the DNA band is visualized under ultraviolet (UV) transilluminator in the presence of ethidium bromide (EtBr). The agarose gel is stained with the EtBr using two methods which are i) pre-staining, where the EtBr is added during preparation of the agarose gel and ii) post-staining, where the EtBr is added after the DNA separation process finished.

EtBr or 3,8-diamino-5-ethyl-6-phenyl phenanthridinium bromide is an intercalating agent that attached to DNA by inserting itself into spaces between base pairs of double stranded DNA (dsDNA) shown in Figure 1.4 (Nafisi *et al.*, 2006). The contact of the hydrophobic ring of EtBr with the base pairs of dsDNA produced Van der Walls force which can distort the double helix of DNA as shown in Figure 1.5 (Reha *et al.*, 2002, Yuhzaki and Hamaguchi, 2004). When exposed to the ultraviolet light, EtBr is absorbed and then transmitted energy as visible orange light to make the band visible (Reha *et al.,* 2002). However, EtBr is carcinogenic because it can

cause distortion of DNA. It can also interfere with DNA replication, transcription and DNA repair that can cause cancer if it is not properly handled.

Due to the drawback of the EtBr, other dyes such as SYBR green, Gel Star®, silver staining, acridine orange and methylene blue dyes are used for the agarose gel staining. Furthermore, these dyes are more sensitive compared to the EtBr in detecting DNA since SYBR green dye is able to detect < 20 pg of dsDNA and Gel Star ® and silver staining is able to detect 4 to 16 fold more DNA (Sambrook and Russell, 2001, Cambrex BioScience). For this method, the UV light is still required for visualization of DNA band. UV light is harmful to humans because excessive exposures can alter human DNA and cause cancer (WHO, 2009). Silver is an alternative dye for agarose gel staining since the DNA band can be visualized directly by the naked eye. However, silver staining produced high background results due to non-specific deposition of silver ions (Willoughby and Lambert, 1983). This problem can be eliminated by treating the agarose gel with 10% acetic acid and developed in 3% NaOH containing 37% formaldehyde (Prieto and Leonardelli, 1997). The treatment process however makes the preparation of agarose gels become more tedious.

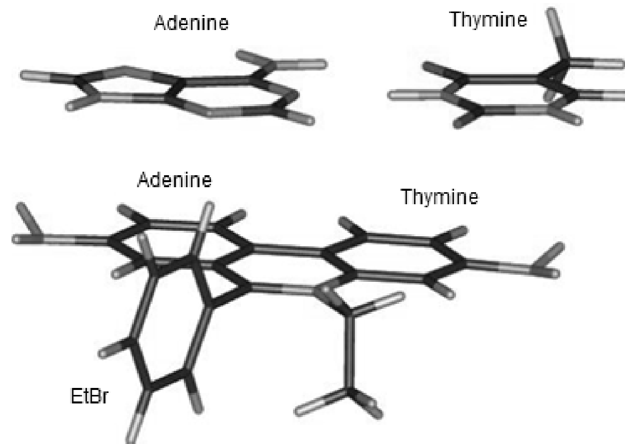

Figure 1.4: Structure of intercalation of EtBr between adenine and thymine (Reha *et al*., 2002)

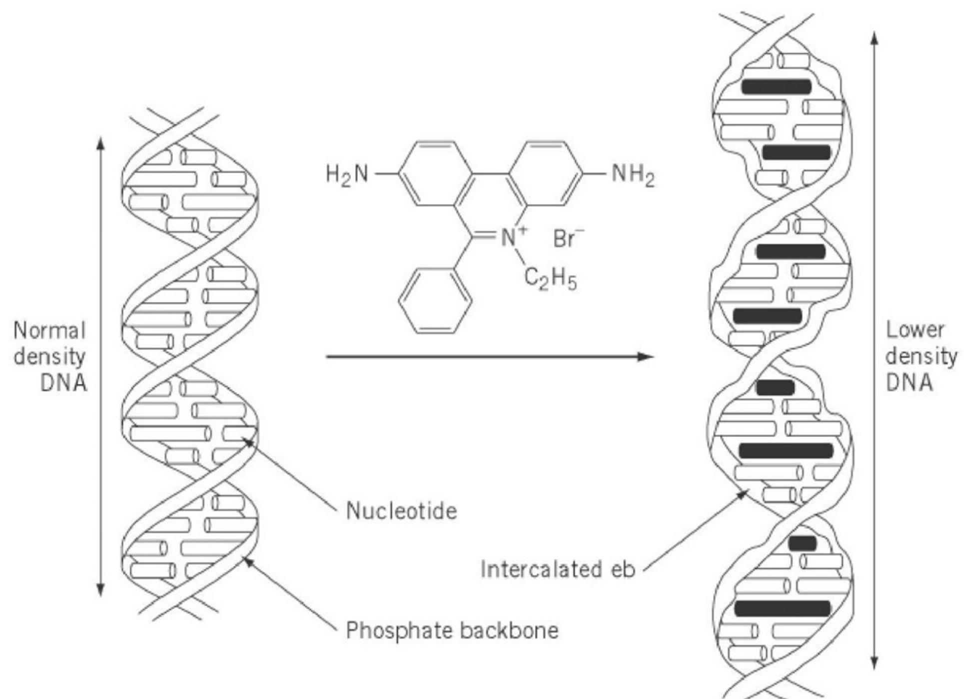

Figure 1.5: Distortion of double helix stranded DNA due to intercalated EtBr ("Ethidium bromide", n.d.)

1.7.2 (b) Real Time PCR

Real-Time PCR is an improvement of the conventional PCR which quantitatively measure the amount of the DNA in real time. A target region of amplicons is bound with dyes or probed to produce fluorescence signal and detected by real-time machine. Emission of light can be produced through intercalation of dyes between dsDNA such as SYBR green dye or depends on Fluorescence Resonance Energy Transfer (FRET) such as TaqMan Probes ("Real-Time PCR Vs. Traditional PCR", n.d). The intercalation of SYBR green and DNA occurs between positively charged thiazole group of aromatic ring of SYBR green and negatively charged phosphate bone of the DNA and the SYBR green is excite at 485 nm to emit the fluorescence light at 490-600 nm (Figure 1.6). (Dragan *et al*., 2012). However, the SYBR green can cause overestimation of the target DNA since it binds to any dsDNA including non-specific products and primer-dimers (Ririe *et al.*, 1997, Reece, 2004).

The Fluorescence Resonance Energy Transfer (FRET) involves a fluorescence dye (R) attached to 5'-end and a quencher moiety (Q) coupled to 3'-end of the PCR product. Several fluorescence dyes and quenchers are commercially available such as FAM, TET, Texas red, ROX, TAMRA, BHQ, ECLIPSE and DABCYL (Jena Bioscience, 2012). The TaqMan probe is an oligonucleotide sequence that has the fluorescence dye at 5' end and the quencher at 3' end. This probe is bound to the respective target sequence. During amplification, *Taq* Polymerase cleaves the probe and decouples the fluorescence dye with quencher to emit the fluorescence signal (Figure 1.7). Although, real-time PCR can detect the presence of the target DNA during amplification, it requires a special equipment to monitor the amplification