Detection of *Salmonella enterica* serovar Typhi by nested PCR using different procedures of nucleic acid extraction.

A DISSERTATION SUBMITTED TO THE BRAC UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOTECHNOLOGY

Submitted by

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DEDICATED

TO

MY BELOVED PARENTS

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CERTIFICATION OF ORIGINALITY OF THE WORK

This is to certify that, the Thesis entitled 'Detection of *Salmonella enterica* serovar Typhi by nested PCR using different procedures of nucleic acid extraction., a requirement for the degree of Master of Science (MS) in Biotechnology under the Dept of Mathematics and Natural Sciences, BRAC University was carried out in the Immunology Laboratory of the Laboratory Sciences Division of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B), Dhaka during the period of October 2008 to January 2010, under our joint supervision.

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List of Abbreviation

PBS – Phosphate buffered saline

LSD – Laboratory Sciences Division

S. Typhi – *Salmonella* Typhi

dH_2O – Deionized water

µl – Micro liter

rpm – Rotation per minute

ICDDR,B – International Centre for Diarrheal Disease Research, Bangladesh

ORF – Open reading frame

Vi antigen – Virulence antigen

M cells – Microfold cells

TNF α – Tumor necrosis factor α

LPS – Lipopolysaccharide

PCR – Polymerase chain reaction

DNA – Deoxyribo nucleic acid

MDR – Multy drug resistant

LA – Luria agar

$MgCl_2$ - Magnesium chloride

Taq - Thermophillus aquaticus

dNTPs – Deoxyribo nucleotide tri phosphates

TBE – Tris borate EDTA

Abstract

Abstract

Typhoid fever is one of the major health problems in Bangladesh. It is caused by the bacterium *Salmonella enterica* serover Typhi (*S.* Typhi). The infection rate of *S.* Typhi is higher in children. The conventional diagnostic methods of typhoid have limitations. Most commonly used Widal test gives a high rate of false positive results. As such PCR method was tried to diagnose typhoid fever in some selected patients of Bangladesh. Fifty three (53) patients were enrolled in this study from Dhaka hospital of ICDDR, B and Kamalapur field site. Among them 27 (51 %) persons are male. Blood was collected from all patients. Three different methods namely, a method by Haque et al, direct boiling method and commercial Qiagen kit method were tried to extract *Salmonella* Typhi DNA from blood and invitro samples. To optimize the DNA extraction from blood invitro grown *S.* Typhi bacteria was spiked into blood. From the three different methods, commercial Qiagen kit method did well comparably to extract DNA from spiked blood than the other methods. However, none of these methods did appear promising to extract DNA from patient's blood. Because low percentage of PCR positive were found from patient's blood culture positive samples. So another method was tried which was described earlier by Boom's et al. [1990] for detection of *S.* Typhi from blood of suspected typhoid fever patient. This method (Boom's et al.[1990]) showed comparably satisfactory results than the other methods to deal with patient's blood. In this method the percentage of PCR positive was 35.7 % among the blood culture confirmed patients. DNA extraction was also done in blood culture negative samples by this method. Here 30.8% positive for PCR was found among the culture negative samples. However there are some drawbacks in DNA extraction procedures especially in low concentrated samples. In the method by Haque et al. [2001] unexpected band was found and same phenomenon was observed in direct boiling method. This might be due to contamination with *S.* Typhi. It seems that, *S.* Typhi can be detected from patient's blood specimen by PCR method for diagnostic purpose. However, more study is needed to evaluate the efficacy of PCR method with some modifications for detecting *S.* Typhi at low concentration in blood.

Chapter One

Introduction

Introduction

1.1 Background

Typhoid fever is caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) which is an important health problem in many developing countries [1]. *S.* Typhi is a highly virulent host restricted invasive pathogen that affects only humans [2]. Most of this burden occurs among people of low-income regions, particularly in Asia, Africa, Latin America, the Caribbean, and Oceania. About 80% of cases come from Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and Vietnam [3]. It has been estimated that, 16-33 million annual cases resulting in 216,000 deaths in endemic areas and its incidence is highest in children and young adults between 5 and 19 years old [4]. Although young children are also susceptible. In nonendemic areas, disease outbreak may occur from a contaminated food or through carriers [9, 10].

The disease is characterized by the onset of prolonged high fever, severe headache, malaise and abdominal pain [5]. The illness often causes diarrhea, especially in younger children, whereas constipation is common in older children and adults. Serious complications occur in up to 10% of typhoid fever patients, especially those who have been ill for more than two weeks and have not received proper treatment [5]. Almost half of the treated patients continue to excrete the pathogen for about one month after the symptoms have been disappeared and 5% still continue upto five months [6, 7]. Approximately 3% become chronic carriers and continue to excrete the organism lifelong [8]. The encounters of *S.* Typhi to humans are generally caused through fecal-oral route from infected individuals to healthy ones [11].

1.2 Structure & Comparisn of the *S.* Typhi Genome

The genus *Salmonella* has 3 species, *Salmonella enterica*, *Salmonella bongori* and *Salmonella subterranean* [15]. *S. enterica* has seven subspecies consistently delineated by sequence variation. The majority of diseases causing serovars are from subspecies, Typhimurium, Typhi and Paratyphi [12].

Complete genome sequence data provides the genetic characterization of pathogens and their hosts. The *S.* Typhi genome consists of 479 kb encoding around 4000 genes where over 200 genes are functionally inactive. Comparison of *S.* Typhi isolates from around the world indicates that, they are highly related (clonal) and they emerged from a single point of origin, around 30,000 – 50,000 years ago. Evidence suggests that *S.* Typhi undergoing gene degradation and it has also recently acquired genes, such as those encoding the Vi antigen by horizontal gene transfer [17].

Salmonella Typhi CT18 has a large circular chromosome consisting of 4.8 Mb and two plasmids are pHCM1 and pHCM2, which are 218 kb and 106 kb respectively. *S.* Typhi CT18 has 4646 genes and 204 pseudogenes, nine of which resemble intact genes in strain Ty2. Along with the 195 pseudogenes common with CT18, strain Ty2 has also 11 unique ones. *S.* Typhi Ty2 consists one large chromosome that is 4.7 Mb with an average G+C content of 52.05% [16].

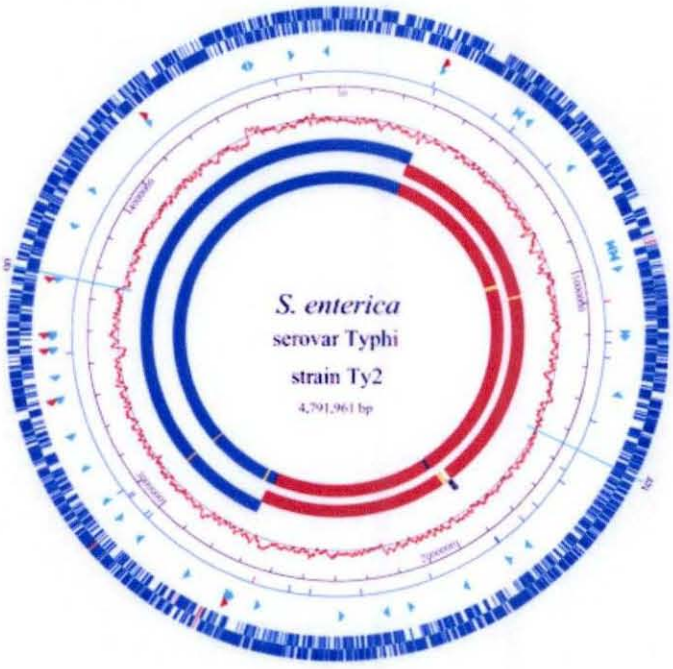

Fig 2: Circular genome map of *S.* Typhi [18]

The Ty2 genome has 4,545 ORFs and pseudogenes, where 4,516 are shared with CT18 (outer circle, blue) and 29 of which are unique (pink). The second circle shows the locations and orientations of rRNA operons (red) and tRNAs (turquoise). The third circle shows insertion element distributions. The fourth circle shows the scale in base pairs. The fifth circle shows the G/C skew, calculated for each sliding window of 10 kb along the genome. The sixth and seventh (innermost) circles show the CT18 and Ty2 genome comparison where, blue indicates collinear regions, red indicates inverted regions, green indicates a region that is translocated and inverted again within the half-genome inversion region, and yellow indicates unique regions [18]. More discrepancies are found when looking at prophages. Four prophages are located in identical parts of the genome relative to the adjacent nonphage genes, and both strains contain parts of prophages that are unique to one another [16].

1.3 Antigenic types of *Salmonella* Typhi

Salmonella is a genus of rod-shaped, Gram-negative, non-spore forming, predominantly motile bacteria belonging to the family of *Enterobacteriaceae* [12], with more than 2501 serotypes [13]. Its diameter is around 0.7 to 1.5 μm and lengths from 2 to 5 μm. The bacteria contains flagella which projected along with all directions [12].

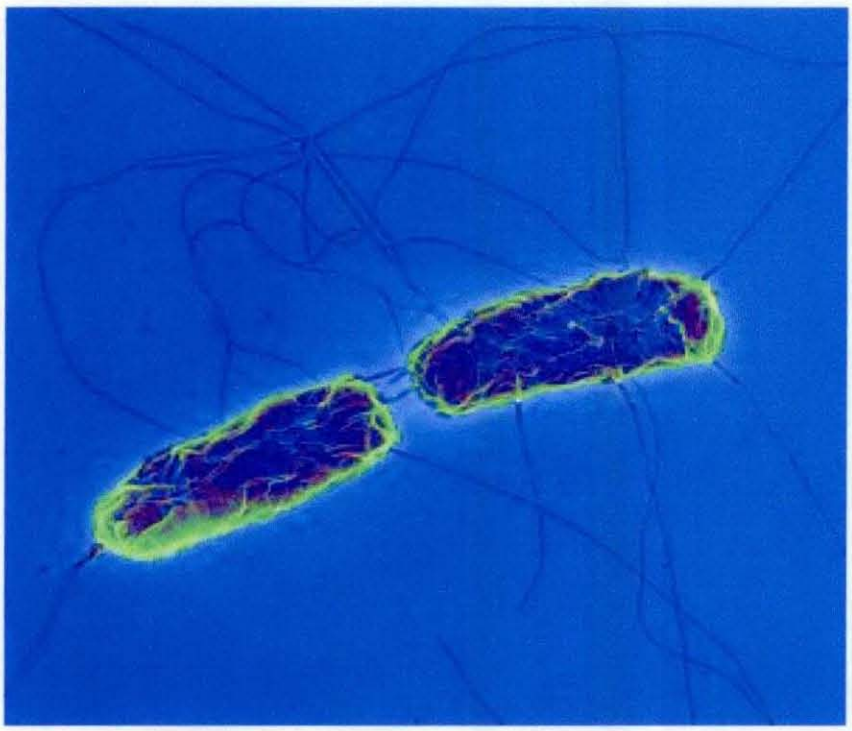

Figure 1: Structure of *Salmonella* Typhi [67].

As with all *Enterobacteriaceae,* the genus *Salmonella* has three kinds of major antigens with identifying characteristics [14] -

a) Somatic or O antigens (bacterial endotoxins): These are heat stable and alcohol resistant. Cross-absorption studies individualize a large number of antigenic factors, 67 of which are used for serological identification.

b) H antigen: This is a protein structure associated with the flagella. Flagellar antigens are heat-labile proteins. By mixing colonies of *Salmonella* with flagella-specific antisera gives a characteristic pattern of agglutination.

c) Surface Vi (for virulence) antigen: This is a polysaccharide on the exterior of the cell wall. The surface antigens (Vi) in *Salmonella* may mask O antigens, and the bacteria will not be agglutinated with 'O' antigen specific antisera [14].

1.4 Etiology

Most current understanding of *S.* Typhi is from observing the disease in humans, volunteer research and animal models. *S.*Typhimurium in the murine model is the best characterization of typhoid fever in humans [20]. In murine model during first exposure, the *S.* Typhi is ingested and it enters into the small intestine through the microfold cells (M cells) of the Peyer's patches. After being endocytosed by M cells, the bacterium is able to migrate to the mesenteric lymph nodes and then multiply [20]. Afterwards the bacteria are released into blood stream and circulate to cause a systemic infection [21]. *S.* Typhi is then taken up from the blood by antigen presenting cells such as the macrophages that line over the liver, spleen, and bone marrow sinusoids. The bacteria is able to replicate and stay in the macrophages [22]. The macrophages can lose the ability to kill intracellular bacteria. The clinical symptoms like fever, nausea, constipation and diarrhoea are observed when the bacteria reenters into the circulation. *S.* Typhi is then removed from the blood via the gall bladder to the small intestine.

are not sufficiently sensitive and specific [50]. Widal test has a presumptive diagnostic value in non endemic areas but in endemic areas it's use is controversial [51].

1.7.1 Widal test

The Widal test is used to demonstrate rising titres of antibodies to flagellar (H) and somatic (O) antigens in typhoid and paratyphoid fever [52, 53]. An increased O antibody level signifies acute infection, whilst an increased H antibody level may indicate the serotype of the infecting organism. Widal test has limited use, because H and O antibody levels may rise non-specifically due to cross reactions with other *enterobacteriaceae.*

1.8 Diagnosis of typhoid by nested Polymerase Chain Reaction (PCR)

The diagnosis of typhoid fever can be possible by Polymerase Chain Reaction (PCR) using blood and stool as sole source of template DNA of *Salmonella* Typhi [54]. The flagellin gene of *S.* Typhi can be detected by the Polymerase chain reaction (PCR). Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce the contamination in products due to non specific primer binding. In conventional PCR a commonly occurring problem is primers binding to incorrect regions of the DNA, giving unexpected products (non specific bands). Nested polymerase chain reaction involves two sets of primers, used in two successive runs of polymerase chain reaction, the second set intended to amplify a secondary target within the first run product [55].

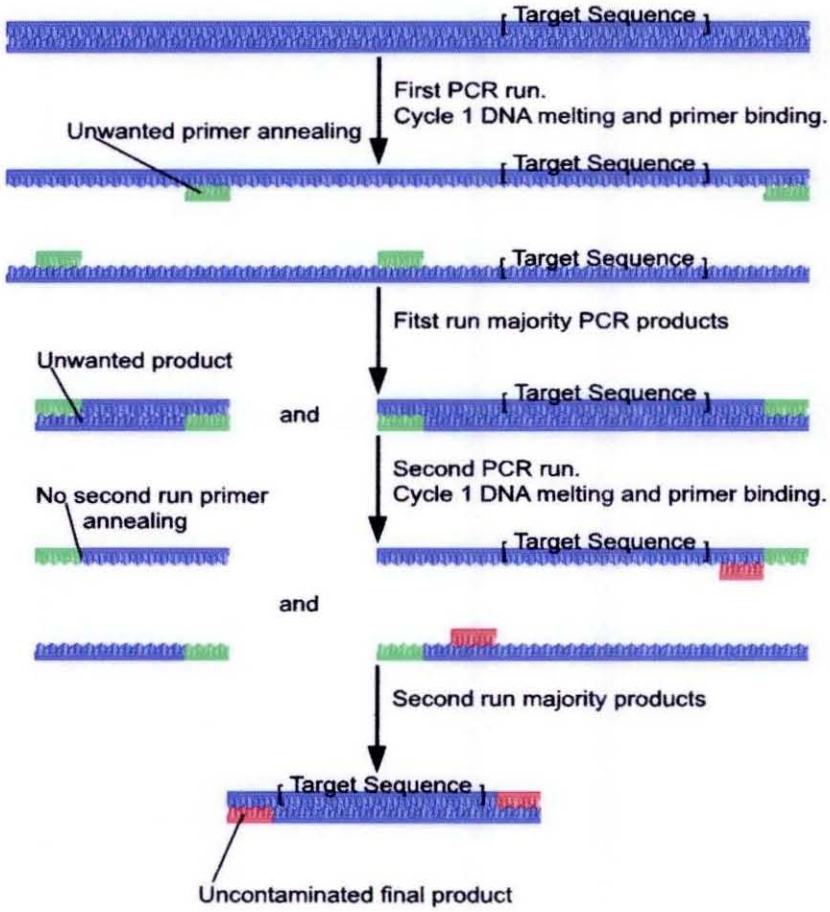

Figure 4: A schematic diagram of nested PCR [55].

Step One: The DNA target template is bound by the first set of primers shown in blue. The primers may bind to other, similar binding sites which give multiple products. However only one of these PCR products give the intended sequence (multiple products not shown).

Step Two: PCR products from the first reaction are subjected to a second PCR run with a second new set of primers shown in red [55].

1.9 Treatment of typhoid fever

1.9.1 General management

Supportive measures are important in the management of typhoid fever, such as oral or intravenous hydration, the use of antipyretics, appropriate nutrition and blood transfusions if indicated. More than 90% of patients can be managed at home with oral antibiotics, reliable care and close medical follow-up [57]. However, patients with persistent vomiting, severe diarrhea and abdominal distension may require hospitalization and parenteral antibiotic therapy.

1.9.2 Antimicrobial therapy

In areas of endemic disease typhoid fever is managed at home with antibiotics and bed rest. Effective antibiotic treatment for typhoid was developed in 1948, but even with modern drugs typhoid still takes the life of about one in every hundred people [58]. Typhoid fever in most cases is not fatal. Antibiotics, such as ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, amoxicillin and ciprofloxacin, have been commonly used to treat typhoid fever in developed countries. Prompt treatment of the disease with antibiotics reduces the case-fatality rate to approximately 1%. When untreated, typhoid fever persists for three weeks to a month. Death occurs in between 10% and 30% of untreated cases [59]. Since the appearance of multidrug resistant strain, sensitivity test is very important for selecting drug.

1.9.3 Resistance

Resistance to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole and streptomycin are now common, and these agents have not been used as first line treatment since almost 20 years. Typhoid that is resistant to these agents is known as multidrug-resistant typhoid (MDR typhoid). Ciprofloxacin resistance is an increasing problem especially in the Indian subcontinent and Southeast Asia. For these patients, the recommended first line of treatment is ceftriaxone. It has also been suggested that azithromycin is better at treating typhoid in resistant populations than both fluoroquinolone and ceftriaxone drugs [60].

1.10 Prevention of typhoid fever

Sanitation and hygiene are the measures that can be taken to prevent typhoid. Careful food preparation and washing of hands are therefore crucial for preventing typhoid. Health education is of paramount importance to raise public awareness and induce behaviour change [59].

There are two vaccines currently recommended by the World Health Organization for the prevention of typhoid, these are the live, oral Ty21a vaccine and the injectable Typhoid polysaccharide vaccine. Both are between 50% to 80% protective and are recommended for travelers to areas where typhoid is endemic [61].

1.10.1 Vaccination

Two types of typhoid vaccines are currently by WHO to preventing typhoid fever, (1) An oral live-attenuated vaccine, (2) A Vi polysaccharide vaccine for parenteral use.

1.10.2 An oral live-attenuated vaccine

The oral vaccine containing live attenuated *Salmonella* Typhi Ty21a strains in enteric coated capsules, taken every two days, a total of 3 doses. The capsule must be refrigerated but not frozen to achieve maximum efficacy. The vaccination should be completed within a week and the booster dose should be given every 5 years. The vaccine recipient should not be younger than 6 years [62]. The commercial vaccine is Vivotif Berna.

1.10.3 Vi polysaccharide vaccine

The vaccine is composed of purified Vi ("virulence") capsular polysaccharide antigen of *Salmonella* Typhi isolated from blood cultures. This capsular polysaccharide of *S.* Typhi is conjugated with nontoxic recombinant *Pseudomonas aeruginosa* exotoxin A and enhances immunity. It is given by the parenteral route once a time. This vaccine must be kept at 4°C temperature. Children below two years of age are not given this vaccine. A booster dose is given every 2 years to maintain protection [63, 64]. The commercial vaccine is Typherix.

Aim of the study

To optimize the DNA isolation of *Salmonella* Typhi from blood of patients with enteric fever which leads to rapid, sensitive and specific detection of *Salmonella* Typhi as well as typhoid fever using nested PCR procedure.

Chapter Two

Materials and Methods

Materials and Methods

The study was carried out at the Immunology Laboratory of the Laboratory Sciences Division of the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B).

2.1 Study Subjects

Patients with high fever (≥ 39°C) for 3-7 days with or without diarrhea who came to the ICDDR, B hospital or from the Kamalapur field site in Dhaka were enrolled for this study. Written consent was taken before taking blood from them.

2.2 Sample collection

One ml blood sample was collected from each suspected typhoid fever patient for DNA isolation and 3 ml blood was collected from five healthy adults in EDTA containing tube for spiking experiments.

Fig 5: Collection of blood in EDTA tube

2.3 DNA extraction from invitro grown *Salmonella* Typhi pure culture:

Salmonella enterica serovar Typhi, ST-004 (stored at -70°C) was streaked on MacConkey agar plate and incubated over night at 37°C.

A loop of *S.* Typhi bacteria from the MacConkey agar plate was taken and suspended into an eppendorf tube containing 100 µl of Phosphate Buffered Saline (PBS, 10 mM. pH 7.4). The suspension was boiled at 100°C in water bath for 10 minutes. The tube was then transferred on ice and kept for 1 minute. After that the suspension was centrifuged at 16099.2 x g for 10 minutes. The supernatant was collected and used as template and positive control for PCR (Positive control).

2.4 Preparation of *S.* Typhi bacteria for spiking in blood:

1. *S.* Typhi control strain (laboratory strain st-004) was streaked on Luria agar plate (LA) and incubated overnight at 37°C.
2. From the overnight grown plate 2 colonies were inoculated into eppendorf tube containing 1 ml PBS. This was then vortexed for 15 seconds.

Fig 6: *S.* Typhi colonies cultured on Luria Agar medium

3. Bacterial suspension was then serially diluted down thirty times by 10 fold dilution each time.
4. A 100µl of bacterial suspension from each dilution was spread on LA plate for determining bacterial count.
5. Plates were incubated overnight at 37°C and colonies were counted.
6. Rest of the bacterial suspensions from each dilution were centrifuged for 8 minutes at 16099.2 X g and the supernatent was discarded.
7. A100µl of blood (from healthy subjects) was added on the bacterial pellet into each eppendorf tube and mixed by pulse vortexing for 5 seconds.

2.5.1 DNA Extraction procedure from spiked blood by Haque et al. [66]:-

1. *S.* Typhi spiked blood samples containing serially diluted bacteria were taken into eppendorf tubes serially.
2. The tubes were centrifuged for 5 minutes at 11180 x g.
3. The pellet was mixed with lysis buffer (1 ml in each tube) and centrifuged for 6 minutes at 11180 x g . The supernatent was discarded.
4. Steps 2 & 3 were repeated.
5. The pellet was mixed with water (1 ml in each tube), and centrifuged for 1 minute at 11180 x g. The supernatent was discarded.
6. Deionized water was added (30 µl in each tube) to the pellet and boiled for 20 minutes.
7. The samples were kept at 4°C before use.

2.5.2 DNA Extraction from spiked blood by direct Boiling :- (Here 100 µl deionized water was added into the serially diluted bacteria not blood)

1. *S.* Typhi spiked blood samples containing serially diluted bacteria were taken into eppendorf tubes serially.
2. The tubes were boiled at 100°C in water bath for 10 minutes and then transferred on ice and kept for a minute.
3. The tubes were then centrifuged at 16099.2 x g for 10 minutes.
4. The supernatant was collected as the source of DNA.

2.5.3 DNA Extraction from spiked blood by using commercial Qiagen Kit :-

1. *S.* Typhi spiked blood samples containing serially diluted bacteria were taken into eppendorf tubes serially.
2. Then 20 µl of Proteinase k and 100 µl of PBS were added into each of the tubes.
3. 200 µl AL buffer was added there and vortexed properly.
4. The tubes were heated at 56°C for 10 minutes in water bath.
5. 200 µl ethanol was added into each tube and then vortexed.
6. The samples were transferred into DNeasy Mini spin column in a 2 ml collection tube.
7. The columns were centrifuged at 7155.2 x g for 1 minute and the tubes were placed in a new 2 ml collection.
8. 500 µl of AW1 buffer was added into each tube, centrifuged at 7155.2 x g for 1 minute, and the flows through with the collection tube were discarded.
9. The columns were placed in a new collection tube and 500 µl of AW2 buffer was added into each column.

10. The columns were centrifuged for 3 minutes at 21912.8 x g to dry the DNeasy membrane and the flow through was discarded.
11. The columns were transferred to new eppendorf tubes and 50 µl of AE buffer were added into each column.
12. Then the tubes were incubated for 5 minutes at room temperature and centrifuged for 1 minute at 11180 x g .
13. Finally the flow through fluids were collected as extracted DNA.

2.6.1 DNA extraction from patient's blood specimens by using commercial Qiagen Kit :-

1. Blood sample was collected (100 µl) from patients in each labeled eppendorf tube.
2. Proteinase k (20 µl) and PBS (100 µl) were added into each of the tubes.
3. AL buffer (200 µl) was added and vortexed properly.
4. The tubes were heated at 56°C for 10 minutes in water bath.
5. Ethanol was added (200 µl) into each tube and then vortexed.
6. The samples were transferred into DNeasy Mini spin column in a 2 ml collection tube (provided by the reagent company).
7. The columns were centrifuged at 7155.2 x g for 1 minute and the tubes were placed in a new 2 ml collection tube (provided in the kit).
8. AW1 buffer was added (500 µl) into each tube, centrifuged at 7155.2 x g for 1 minute, and the flows through with the collection tube were discarded.
9. The columns were placed in a new collection tube (provided by the reagent company) and AW2 buffer was added (500 µl) into each column.
10. The columns were centrifuged for 3 minutes at 21912.8 x g to dry the DNeasy membrane and the flow through were discarded.
11. The columns were transferred to new eppendorf tubes and AE buffer were added (50 µl) into each column.
12. Then the tubes were incubated for 5 minutes at room temperature and centrifuged for 1 minute at 11180 x g .
13. Finally the flow through were collected as extracted DNA.

2.6.2 DNA extraction from blood of suspected typhoid fever patient by using a procedure of R. Boom et al [65].

1. Fresh blood (100µl) was collected and mixed with 900 µl L6 lysis buffer and then centrifuged for 10 minutes at 16099.2 x g .
2. After that, 100 µl supernatant was collected and added 25 µl of diatom suspension into it.
3. The samples were vortexed for 5-10 seconds and vigorously shaked at RT (Room Temperature) for 15 minutes using Vary-max Thermolyne shaker at full speed.
4. The samples were then vortexed again for 5 seconds and centrifuged for 15 seconds at 18894.2 x g. The supernatant was discarded.
5. The pellets were washed twice with 500 µl L2 extraction buffer, twice with 500 µl of 70% ethanol and once with 500 µl of acetone. The tubes were centrifuged for 15 seconds at 18894.2 x g after each wash. The supernatant was collected for disposal.
6. Acetone was removed from the tubes and the tubes were placed at 56°C in a dry heating block/water bath with lids open for 5-10 minutes.
7. 60µl TE buffer was added to the pellet and vortexed for 10 seconds and then the DNA eluted by incubation at 56°C in a heating block/water bath for 10 minutes.
8. The tubes were centrifuged at 18894.2 x g for 2 minutes and the supernatant containing the extracted nucleic acids were collected.

2.7 Nested Polymerase Chain Reaction

2.7.1 Primer sets :

Sl. no	Primers:		Sequences
1	Primer ST1	Forward	(5'-ACT GCT AAA ACC ACT ACT-3')
2	Primer ST2	Reverse	(5'-TTA ACG CAG TAA AGA GAG -3')
3	Primer ST3	Forward	(5'-AGA TGG TAC TGG CGT TGC TC -3')
4	Primer ST4	Reverse	(5'-TGG AGA CTT CGG TCG CGT AG -3')

Table 1 : Primer sets

2.7.2 Master mixture preparation for first round PCR

No	Reagent	Amount per reaction (conc)	Amount per reaction (volume)
1.	PCR buffer (with $MgCl_2$)		2.5 µl
2.	dNTP mix	200µM (each)	0.5 µl
3.	Primer ST1mixtur	25 pmol	0.25 µl
4.	Primer ST2 mixture	25 pmol	0.25 µl
5.	$MgCl_2$	25mM	0.5 µl
6.	*Taq* DNA polymerase	0.625 U	0.125 µl
7.	Distilled water		10.875µl
Total volume of Master Mix			15 µl
Extracted DNA template			10 µl
Final Volume per reaction			25µl

Table 2 : Master mixture preparation for first round of PCR

2.7.3 Thermal cycle for first round of PCR:

First step	95°C for 5 minutes (initial denaturation)
Second step	94°C for denaturation-----1 min
Third step	57°C for prime annealing-----1 min
Fourth step	72°C for elongation-----1 min

Then second step is repeated fourty times

Fifth step	72°C for 7 minutes (final extension step)

2.7.4 Master mixture preparation for 2nd round PCR

No	Reagent	Amount per reaction (conc)	Amount per reaction (volume)
1.	PCR buffer (with $MgCl_2$)		2.5 µl
2.	dNTP mix	200µM (each)	0.5 µl
3.	Primer ST1mixtur	25 pmol	0.25 µl
4.	Primer ST2 mixture	25 pmol	0.25 µl
5.	$MgCl_2$	25mM	0.5 µl
6.	*Taq* DNA polymerase	0.625 U	0.125 µl
7.	Distilled water		15.875µl
Total volume of Master Mix			20 µl
Extracted DNA template			5 µl
Final Volume per reaction			25µl

Table 3 : Master mixture preparation for second round of PCR

2.7.5 Thermal cycle for 2nd round of PCR:

First step	95°C for 5 minutes (initial denaturation)
Second step	94°C for denaturation-----1 min
Third step	57°C for prime annealing-----1 min
Fourth step	72°C for elongation-----1 min

Then second step is repeated fourty times

Fifth step	72°C for 7 minutes (final extension step)

2.7.6 Agarose Gel electrophoresis :

Reagents: Agarose, Tris-Borate-EDTA (TBE) buffer, Ethidium bromide, 100bp ladder.

Preparation of 1.5% Agarose gel

1. TBE buffer was taken (100 ml) in a conical flask and added 1.5 gm Agarose powder into it.
2. The flask was heated for 3 minutes in microwave oven.
3. Ethidium Bromide was added (4 µl) and mixed properly.
4. The mixture was layed on gel tray for cooling.
5. PCR samples were stained with loading dye. (4 µl dye with 10µl PCR product).
6. Electrophoresis was performed for 90 minutes at 80V.
7. The gel was observed under UV light in gel-doc machine.

Chapter Three

Results

Results

3.1 Detection of *Salmonella enterica* serovar Typhi by nested PCR using different nucleic acid extraction methods.

Demographic Data :

Parameter	Number
Patient enrolled	53
Blood collected	From 53 patients
Gender	Percentage
Male	27 (51%)
Female	26 (49%)

Fifty three (53) patients were enrolled in this study from Dhaka hospital of ICDDR, B and Kamalapur field site. Among them 27 (51 %) persons were male. Blood was collected from all patients studied here.

3.2 Optimization of DNA extraction procedure :

For the evaluation of efficient DNA extraction procedure nested PCR was performed using DNA samples extracted from spiked blood by using different extraction procedures.

3.2.1 Nested PCR results of DNA samples (only even number) extracted by a procedure described by Haque et al. [66] showed successful detection of bacteria at 1.9×10^2 cfu/ml of spiked blood (Figure 6). However a nonspecific band was also observed in a negative control in similar dilutions with no bacteria (Lane 8).

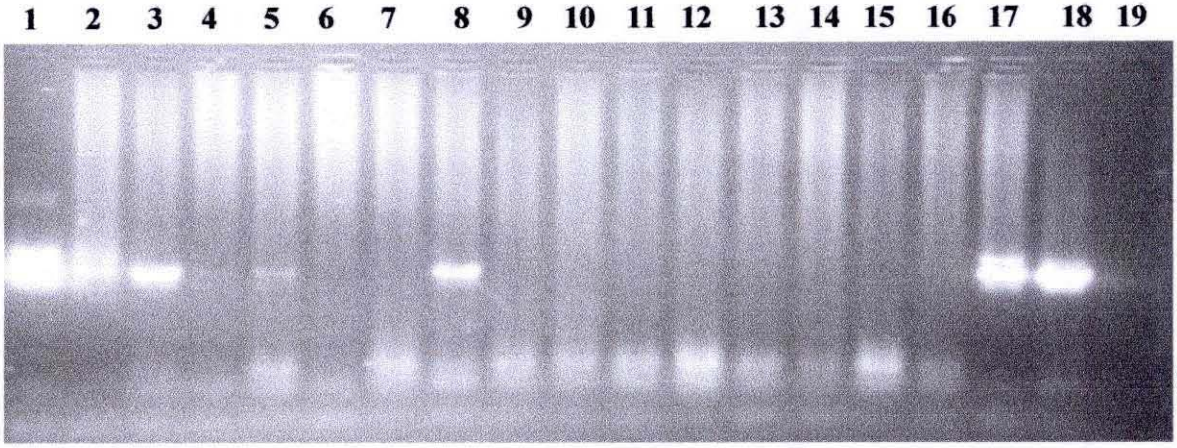

Fig 6: Electrophoresis of the PCR products performed by using 1.5% agarose gel. Lanes 1-5 indicate positive PCR bands for $1.9X10^{10}$,$1.9X10^{8}$,$1.9X10^{6}$, $1.9X10^{4}$ and $1.9X10^{2}$ cfu/ml respectevly. Lane 8 indicate non specific band in a dilution with no bacteria where, lanes 17 and 18 indicate positive (st-004) and 19 indicate negative control respectively.

3.2.2 Nested PCR results of DNA samples (only even number) extracted by direct boiling procedure showed successful detection of bacteria at $1.6X10^{2}$ cfu/ml of spiked blood (Figure 7). However two nonspecific bands were also observed in dilutions with no bacteria (Lanes 12 and 18).

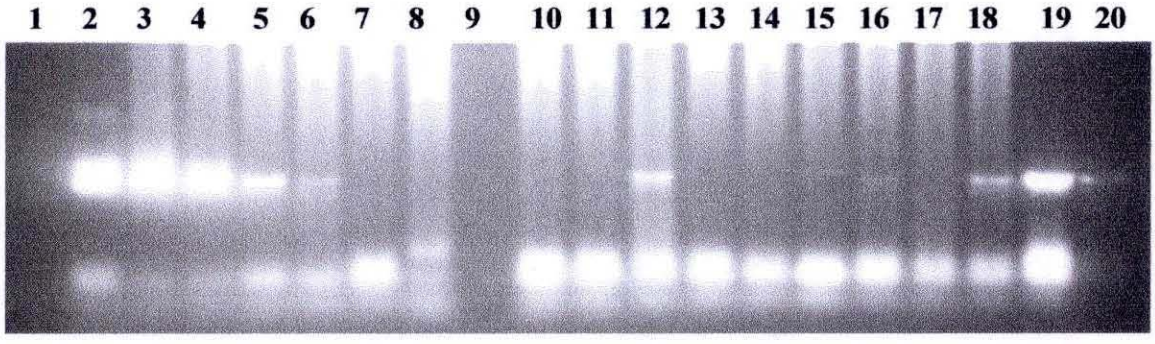

Fig 7: Electrophoresis of the PCR products performed by using 1.5% agarose gel. Lanes 2-6 indicate positive PCR bands for $1.6X10^{10}$, $1.6X10^{8}$, $1.6X10^{6}$, $1.6X10^{4}$ and $1.6X10^{2}$ cfu/ml respectevly. Lanes 12 and 18 indicate nonspecific bands in dilutions with no bacteria where, lane 19 and 20 indicate positive (st-004) and negative control respectively.

3.2.3 Nested PCR results of DNA samples extracted by Qiagen kit: It showed successful detection of bacteria at 1.5X10 cfu/ml of spiked blood (Figure 8). But here bands have been found also in dilutions with individually 1.5 and 0.15 cfu/ml of bacteria in lanes 5 and 6. Here lanes 9 and 10 indicate negative and positive (st-004) control respectively.

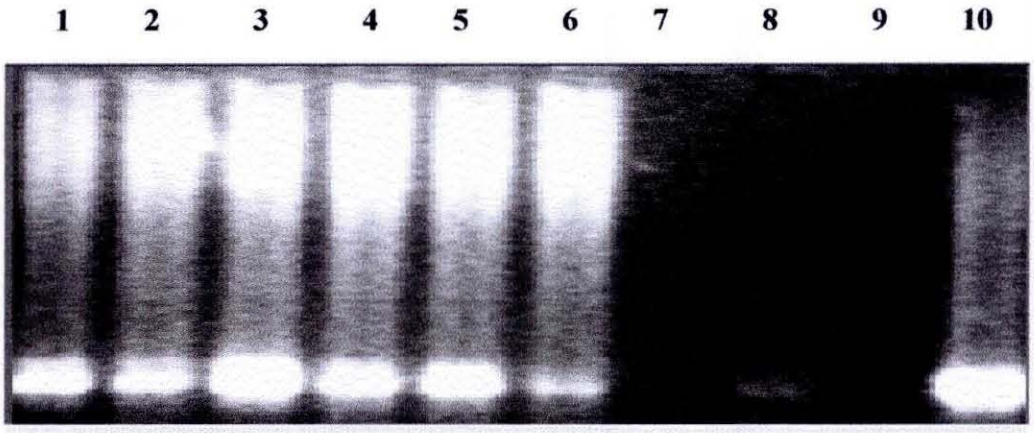

Fig 8: Electrophoresis of the PCR products performed by using 1.5% agarose gel. Lanes 1-4 indicate positive PCR bands for $1.5X10^{10}$, $1.5X10^{3}$, $1.5X10^{2}$ and 1.5X10 cfu/ml respectevly. But lanes 5 and 6 indicates positive bands in dilutions with individually 1.5 and 0.15 cfu/ml of bacteria. Here lanes 9 and 10 indicate negative and positive (st-004) control respectively.

3.3 Detection of *S.* typhi in blood of patients with typhoid fever :

Out of fifty three (53) patients 14 (26.4%) were positive by blood culture .

3.3.1 DNA extraction from patient's blood specimens by using commercial Qiagen Kit.

Nested PCR was done for *S.* Typhi detection of four (4) patients of blood culture positive samples. Only One (25%) patient was positive in PCR method among 4 blood culture confirmed typhoid patients.

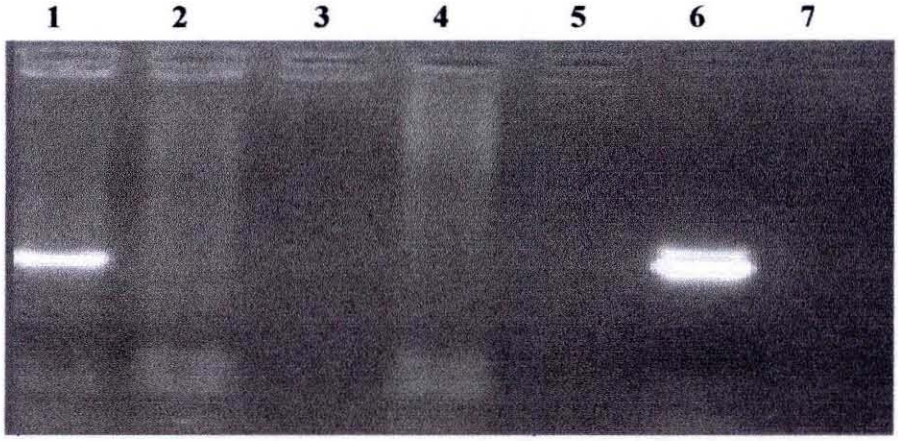

Fig 11 : Electrophoresis of the PCR products performed by using 1.5% agarose gel. Lanes 1-4 indicate patient's blood sample where lane 1 shows only one band. Lane 6 is positive control (st-004) and lane 7 indicates in negative control .

3.3.2 DNA extraction from patient's blood specimens by using a previously described procedure:

Nested PCR was done for typhi detection of all patients regardless of blood culture positive and negative using a procedure described earlier (R. Boom et al, [65]) . A total of 5 (35.7%) patients were positive in PCR method among 14 blood culture confirmed typhoid patients. 12 (30.8%) patients were also found positive by PCR among blood culture negative patients.

Parameters	Number
Total Blood Samples	53
Culture (+) ve	14
Culture (-) ve	39
PCR positive of Culture (+) ve	5
PCR positive of Culture (-) ve	12

Table 4 : Detection of Typhi by nested PCR method in blood culture (+)ve and (-)ve patients.

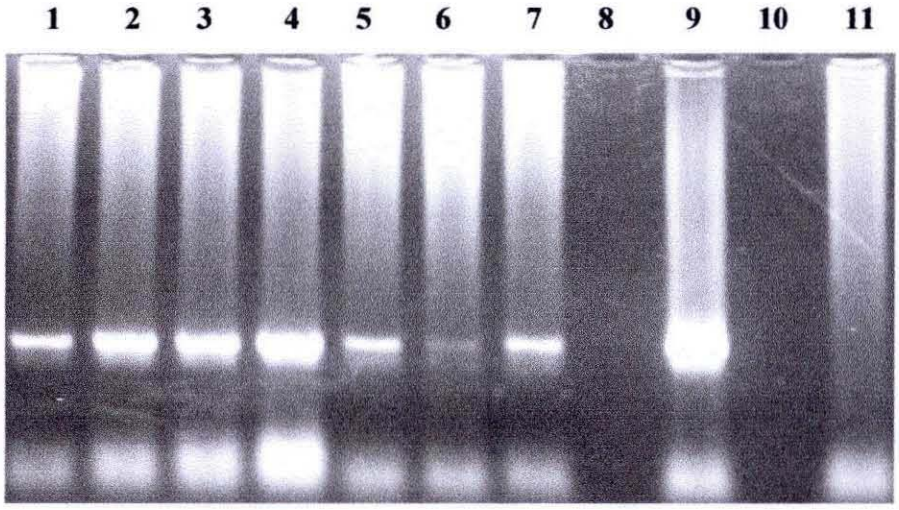

Fig 10 : Electrophoresis of the PCR products performed by using 1.5% agarose gel. Lanes 1-7 indicate blood specimen from patients. Lane 9 indicates a positive control (st-004) and, lane 11 is negative control where no sample was inlcluded in lane 8 and 10.

Chapter Four

Discussion

Discussion

In this study PCR method used by different scientists to diagnose typhoid fever patients in Bangladesh was evaluated for devising a better procedure to diagnose the disease at low bacterial titer.

Three different methods were tried for extracting *Salmonella* Typhi DNA from blood as well as laboratory control specimens. To optimize the DNA extraction procedure from blood, spiked invitro grown *S.* Typhi bacteria was added to blood. The blood was serially diluted to determine the minimum concentration of bacteria that could be extracted from the blood specimens.

A method previously described by Haque et al. [66] was sensitive upto $1.9x10^2$ cfu/ml in blood. So this method is not sensitive enough to use for typhoid fever blood specimens, because this is very high load of bacteria that might be necessary to be present in blood for detection of *S.*Typhi. In natural infection, the concentration of *S.* Typhi is much lower.

Another method that was tried, the direct boiling method which was sensitive up to $1.6x10^2$ cfu/ml in blood. So this method was also not sensitive enough for diagnostic purposes for typhoid fever from blood specimens. However, by this method the separation on agarose gel gave distinct and sharp bands. The amount of bacteria was however very high that might be necessarily being present in blood obtained from a patient.

The third method tried was utilizing the commercial Qiagen kit method. Here sharp bands obtained after electrophoresis and bacteria up to 1.5x10 cfu/ml could be detected. Thus

this was the best procedure and was comparatively more sensitive than the above methods.

The commercial Qiagen kit was found to be the most sensitive method for DNA extraction from blood. The other two methods were not able to extract DNA for detect of *S.* Typhi as efficiently and was more sensitive. Based on this comparative study the Qiagen kit method was used for DNA extraction from blood of patients suspected of typhoid fever.

The DNA extraction from patient's blood using the Qiagen kit however did not look promising because only one patient was positive by PCR out of four blood culture positive specimens tested. Thus, another method which was described earlier by Boom's et al. [65] was used for detection of *S.* Typhi from blood of suspected typhoid fever patient. In this method it was observed that 5 patients were positive for PCR out of 14 blood culture confirmed patients. DNA extraction was also done in blood culture negative samples by this method (Boom's et al. [65]). Here, 12 (31%) positive for PCR out of 39 culture negative samples were found.

There are some drawbacks in DNA extraction procedures especially when specimens with low concentration of bacteria are used. In the method described by Haque et al [66] one unexpected band was found and same was observed by the direct boiling method. This could have been being due to contamination with *S.* Typhi.

In this exploratory study it was observed that, *S.* Typhi could be detected from the patient's blood specimen by PCR method for diagnostic purpose. More study is needed to evaluate the efficacy of PCR method for detecting *S.* Typhi at low concentration in blood.

Chapter Five

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Chapter Six

Appendices

Appendices

Appendix 1

Preparation of Phosphate buffered Saline (PBS) (PH-7.2) (1 ltr) :

NaCl	80.00 gm
Na2HPO4	11.50 gm
KH2PO4	2.00 gm
KCl	2.00 gm
Deionized water	1000.00 ml

Appendix 2

Reagent Preparation for DNA extraction (DNA extraction by R. Boom et al. [65]) :

Diatom Suspension

1. Deionized water (10 mL) was taken into a 50 mL Falcon tube.
2. Diatomaceous Earth (5 g) was added into it.
3. The tube was filled upto 50ml with deionized water (dH_2O).
4. The tube was shaken vigorously (to making MUD).
5. The diatoms allowed to settle down for 3 hours (30 minutes minimum).
6. The upper liquid layer was removed.
7. The tube was refilled upto 50 mL with dH_2O and mixed properly.
8. Then the tube was allowed to settle down for 2 hours (30 minutes minimum).
9. The upper liquid layer was removed.
10. A volume of dH_2O equal to the semi-solid MUD was added.

11. The diatom suspension after vortexed aliquoted into small volumes (0.5-1.0 ml) and autoclaved (15lbs for 15 minutes). Then stored at room temperature.

Buffers

Preparation of 50mM 100ml Tris-HCl (pH 6.4) :

1. Tris (0.606g) in a conical flask was taken and deionized water (90ml) was added into the flask.
2. The mixture was stired and the pH was adjusted to 6.4 by adding HCl.
3. The final volume was made 100ml by adding dH_2O.

Preparation of L2 extraction buffer (50ml):

1. GuSCN (32g) in a conical flask was taken and Tris-HCl was added upto 50ml.
2. The flask was heated at 65°C for 10-20 minutes to dissolve GuSCN.
3. The solution was filtered through 1.2µm filter and transferred to a 50 ml falcon tube. Then stored at RT.
4. The conical flask was covered by aluminium foil and falcon tube was covered by brown paper.

Preparation of L6 extraction buffer (50ml):

5. GuSCN (32g) in a conical flask was taken and Tris-HCl was added upto 50ml.
6. The flask was heated at 65°C for 10-20 minutes to dissolve GuSCN.
7. EDTA (0.292g) and Triton X-100 (100µl) were added. The mixture was stired for 10 min for mixing.
8. The solution was filtered through 1.2µm filter and transferred to a 50 ml falcon tube. Then stored at RT.

9. The conical flask was covered by aluminium foil and falcon tube was covered by brown paper.

Appendix 3

Reagents for DNA extraction using commercial Qiagen Kit :

The Reagents were provided by the Qiagen reagent company.

Appendix 4

Buffer preparation for DNA Extraction (DNA extraction by Haque et al. [66]) :

For 50 ml buffer

1. Tris HCl (10 ml of 50 mM) was taken in a bottle.
2. Deionized water (40 ml) was added into it.
3. The PH adjusted to 8.
4. EDTA (0.0146 g) was added into it and stired to dissolve.
5. Then triton X (200 µl) was added and mixed properly.

Appendix 5

Preparation of Tris-EDTA (TE) buffer (100ml) :

1. Deionized water (105 ml) with 0.2 µm was filtered and 95 ml was taken in a beaker.
2. Tris (0.1211g) was added into it.
3. The solution was stired and the pH was adjusted at 8.
4. EDTA (0.0292g) was added there and stired to dissolve.
5. The filtered deionized water was added upto 100ml.
6. The solution was filtered with 0.2 µm filter and autoclaved.
7. Then stored at +4°C.

Appendix 6

Preparation of TBE buffer (10x) :

Tris	12.1g
Boric Acid	6.0
Na-EDTA	0.74g

The deionized water was autoclaved upto 1L

Appendix 7

Loading dye composition (6X)

0.25% BPB (Bromo Phenol Blue)	: 0.025 g	(Stock 1% BPB	2.5 ml)
0.05% XC (Xylene Cyanol FF)	: 0.5 ml	(Stock 1% XC	0.5 ml)
100 mM EDTA	: 2 ml	(Stock 0.5 mM	2.0 ml)
50% Glycerol	: 5 ml	(Stock 100% Gly	5.0 ml)
Distilled water	: Rest of water		Nil
For	10 ml		10 ml