CHAPTER 2

MATERIALS AND METHODOLOGY

2.1 Materials

2.1.1 Bacterial strains

A total of 110 strains of *Shigella* spp. were obtained from the Institute for Medical Research (IMR), Malaysia (84 strains of *S. flexneri*, 15 strains of *S. sonnei*, 10 strains of *S. dysenteriae* and 1 strain of *S. boydii*). These strains were isolated during 1997 to 2000 from sporadic cases of shigellosis in different parts of Peninsular Malaysia and serotyped by the Bacteriological Department, IMR (Appendix 1). All the strains were restreaked onto *Salmonella-Shigella* (*SS*) agar plates for confirmation of shigellae identity, before being transferred to LB agar plates, incubated at 37^0C overnight and kept at 4^0C for screening of virulence-associated genes.

2.1.2 Clinical specimens

A total of ten clinical specimens from the University of Malaya Medical Centre (UMMC) were screened. Faecal suspensions from patients afflicted with diarrhoea were used as DNA templates for mPCR assays.

2.1.3 Chemicals

Whenever possible, all the chemicals, media and reagents were of Analar grade or of the highest grade available commercially. Details of chemicals and reagents are listed in Table 2.1.

Table 2.1
Chemicals and reagents used in this study

Chemical/Reagent	Manufacturer
Absolute alcohol	R & M Chemical, UK
Agarose powder (LE Analytical Grade)	Promega Corp., USA
Bacteriological agar (agar No. 1)	Oxoid Ltd., UK
Blue/Orange 6X loading dye (15% Ficoll 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orangeG, 10 mM Tris-HCl, 50 mM EDTA)	Promega Corp., USA
Brain Heart Infusion (BHI)	Oxoid Ltd., UK
Ethidium bromide	Sigma Chemical Co., USA
Ethylenediamine-tetraacetic acid (EDTA)	Sigma Chemical Co., USA
MacConkey agar	Difco Laboratories, USA
Mineral oil	Sigma Chemical Co., USA
Orthoboric acid	BDH Lab Supplies, UK
Phenol:chloroform:isoamyl alcohol (25:24:1) pH 8.0	United States Biochemical Corp., USA
RNase A	Sigma Chemical Co., USA
Salmonella-Shigella (*SS*) agar	Oxoid Ltd., UK
Sodium acetate	Sigma Chemical Co., USA
Sodium chloride (NaCl)	Sigma Chemical Co., USA
Sodium dodecyl sulphate (SDS)	Sigma Chemical Co., USA
Sodium perchlorate	Sigma Chemical Co., USA
Tris base	Genaxis Biotech.
Tris-HCl	Gibco BRL, USA
Tryptone	Oxoid Ltd., UK
Yeast extract	Becton Dickson & Co., USA

2.1.4 Media for bacteria growth

2.1.4.1 Luria-Bertani (LB) broth

(For every 100 ml)

Tryptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Dissolved in	100 ml dH_2O

The broth was autoclaved at 15 p.s.i (121^{0}C) for 15 min.

2.1.4.2 LB agar

(For every 100 ml)

Tryptone	1.0 g
Yeast extract	0.5 g
NaCl	0.5 g
Bacteriological agar (agar No. 1)	1.5 g
Dissolved in	100 ml dH_2O

The agar was autoclaved at 15 p.s.i for 15 min and poured into petri dishes in a biosafety cabinet Class II type A (Alnor Instrument Co., USA). They were dried in the presence of ultraviolet (UV) irradiation for approximately 30 min and cooled before use.

2.1.5 Medium for bacteria enrichment

2.1.5.1 Brain Heart Infusion (BHI)

(For every 100 ml)

BHI powder	3.7 g
Dissolved in	100 ml dH_2O

The broth was autoclaved at 15 p.s.i for 15 min.

2.1.6 Media for bacteria selection

2.1.6.1 MacConkey agar

(For every 100 ml)

MacConkey powder	5.0 g
Dissolved in	100 ml dH_2O

Boiling dissolved the powder and the solution was autoclaved at 15 p.s.i. for 15 min.

2.1.6.2 *Salmonella-Shigella* (*SS*) agar

(For every 100 ml)

SS agar powder	6.3 g
Dissolved in	100 ml dH_2O

The powder was dissolved on a heating plate, cooled to 50^0C, mixed and poured into petri dishes in a biosafety cabinet. The plates were then dried in the presence of UV irradiation for approximately 30 min before use.

2.1.7 Solutions for phenol-chloroform DNA extraction

2.1.7.1 0.5M EDTA, pH 8.0

(For every 100 ml)

Disodium EDTA	18.61 g
Deionized water	60 ml

(pH adjusted to 8.0 with the addition of 1M sodium hydroxide)

Addition of deionized water to make up a final volume of 100 ml

2.1.7.2 0.15M NaCl, 0.1M EDTA, pH 8.0

1M NaCl	15 ml
0.5M EDTA, pH 8.0	20 ml

Addition of deionized water to make up a final volume of 100 ml

2.1.7.3 1M Tris-HCl, pH 8.0

(For every 100 ml)	
Tris-HCl powder	15.76 g
Dissolved in	100 ml deionized water

2.1.7.4 Lysis buffer (1% SDS, 0.1M NaCl, 0.1M Tris-HCl, pH 8.0)

10% SDS	10 ml
1M NaCl	10 ml
1M Tris-HCl, pH 8.0	10 ml

Addition of deionized water to make up a final volume of 100 ml

2.1.7.5 5M Sodium perchlorate

(For every 100 ml)	
Sodium perchlorate powder	61.2 g
Dissolved in	100 ml deionized water

2.1.7.6 RNase A (10 mg/ml)

RNase powder	100 mg
10 mM Tris-HCl, pH 7.5, 10 mM NaCl	10 ml

The mixture was heated at 90^0C for 15 min to inactivate any DNase present and allowed to cool to room temperature. It was filter-sterilized, aliquoted and stored at – 20^0C.

2.1.8 Solutions for agarose gel electrophoresis and gel visualization

2.1.8.1 10X Tris-borate EDTA (TBE) buffer

(For every 100 ml)	
Tris base	10.8 g
Orthoboric acid	5.5 g
Disodium EDTA	0.93 g
Dissolved in	100 ml dH_2O

The pH of the buffer was adjusted to 8.3, and subsequently was diluted with distilled water to a 0.5X concentration for gel making and electrophoresis.

2.1.8.2 2% agarose gel

(For every 100 ml)	
Agarose powder	2.0 g
Dissolved in	100 ml 0.5X TBE buffer

The powder in 0.5X TBE buffer was dissolved by heating it in a microwave oven until a clear solution was obtained.

2.1.8.3 Ethidium bromide (10 mg/ml)

(For every 100 ml)	
Ethidium bromide powder	100 mg
Dissolved in	10 ml deionized water

The solution was stored at room temperature in a dark bottle and diluted to 0.5 µg/ml with distilled water before use.

2.1.9 Oligonucleotide primers

The oligonucleotide primers used in this study were synthesized by Integrated DNA Technologies, Inc., USA. Sterile deionized distilled water (ddH_2O) was used to dissolve lyophilized primers to give a concentration of 100 µM stock solutions. Smaller aliquots of 50 µM were then prepared as working solutions and stored at -20^0C.

Five sets of primers were used: four sets (i.e. ShET1A, ShET1B, ial, Shig1 and Shig2) were co-amplified in a mPCR, and the remaining set (ShET2) in a monoplex PCR. Details of the primers are shown in Table 2.2.

Table 2.2
Primer sets used for identification of virulence-associated genes in *Shigella* spp.

Gene	Primer	Primer sequence (5' → 3')	Location within gene	Size of amplicon (bp)	Reference
set1B	ShET1B	(F) GTG AAC CTG CTG CCG ATA TC	70-89	147	Fasano *et al.* (1995)
		(R) ATT TGT GGA TAA AAA TGA CG	197-216		Vargas *et al.* (1999)
set1A	ShET1A	(F) TCA CGC TAC CAT CAA AGA	461-478	309	Fasano *et al.* (1995)
		(R) TAT CCC CCT TTG GTG GTA	752-769		Vargas *et al.* (1999)
ial	ial	(F) CTG GAT GGT ATG GTG AGG	5340-5357	320	Frankel *et al.* (1989)
		(R) GGA GGC CAA CAA TTA TTT CC	5640-5659		
ipaH	Shig1	(F) TGG AAA AAC TCA GTG CCT CT	1063-1083	423	Lüscher & Altwegg (1994)
	Shig2	(R) CCA GTC CGT AAA TTC ATT CT	1466-1485		
sen	ShET2	(F) ATG TGC CTG CTA TTA TTT AT	809-828	798	Nataro *et al.* (1995)
		(R) CAT AAT AAT AAG CGG TCA GC	1587-1606		Vargas *et al.* (1999)

(F) denotes the forward/sense strand (R) denotes the reverse/antisense strand

2.2 Methodology

The general experimental steps are outlined in Fig. 2.1.

2.2.1 Sterilization techniques

2.2.1.1 Moist heat

Culture media, chemical solutions, distilled and deionized water, mineral oil, Schott bottles, bijou bottles, toothpicks, micropipette tips, 0.6-ml microcentrifuge tubes, 1.5-ml microcentrifuge tubes, Millipore membrane filters (type GSWP, 0.2 µM, 25 mm) and Swinnex-25 filter holders were routinely steam-sterilized at 15 p.s.i for 15 min in an autoclave machine.

2.2.1.2 Membrane sterilization

RNase solutions were filtered through sterile membrane filters positioned in Swinnex-25 filter holders.

2.2.1.3 UV irradiation

Freshly poured agar plates were sterilized by UV light for 30 min during setting in a biosafety cabinet.

Prior to amplification processes, steam-sterilized microcentrifuge tubes, micropipette tips, ddH_2O, mineral oil and dedicated micropipettes were exposed to UV light in the Cleansphere CA100 (Safetech Ltd., Ireland) for 30 min.

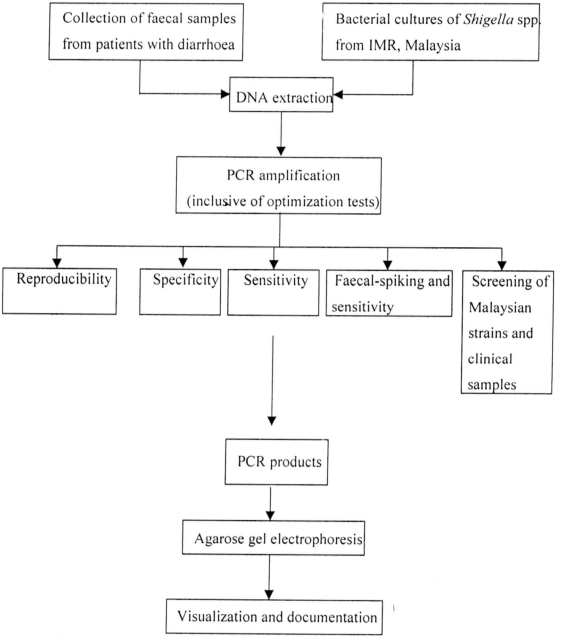

Fig. 2.1

General experimental steps

2.2.2 DNA extraction methods

2.2.2.1 Boiling

A sterile toothpick was used to pick up one or two colonies of shigellae from LB agar plates that had been incubated overnight at 37^{0}C. The colonies were suspended in 50 µl ddH_2O and vortexed to ensure homogeneity, before being boiled at 100^{0}C for 5 min in a Perkin-Elmer thermal cycler. The microcentrifuge tube was immediately snap-cooled in ice and centrifuged at 13 000 rpm for one min to obtain a clear lysate, which was used as DNA template for amplification purposes (2 µl per PCR reaction). The lysate could be used for several PCR reactions when stored at –20^{0}C.

2.2.2.2 Phenol-chloroform extraction (modified from Saito & Miura, 1963)

A single colony of shigellae was grown overnight in 5 ml LB broth. The bacteria in the broth were obtained by low-speed centrifugation at 5 000 rpm for 20 min. The resulting pellet was resuspended in 2 ml of ice-cold 0.15M NaCl and 0.1M EDTA (pH 8.0) (solution I). After a quick vortexing, the mixture was subjected to another round of centrifugation at 8 000 rpm for 10 min. The cell pellet was resuspended in 100 µl of solution I. The suspension was transferred to a fresh 1.5-ml microcentrifuge tube, and 100 µl of lysis buffer (1% SDS, 0.1M NaCl, 0.1M Tris-HCl, pH8.0) was added. Next, 50 µl of sodium perchlorate was added (to dissociate protein from nucleic acid), followed by 250 µl of buffered phenol-chloroform. The suspension was incubated at room temperature for 30 min with gentle mixing on a belly dancer. After a high-speed centrifugation at 10 000 rpm for 25 min, the aqueous phase of the solution was transferred to a fresh 1.5-ml microcentrifuge tube. Two microlitres of RNase (10 mg/ml) was added and the extracted DNA was incubated at 37^{0}C for 30 min. Approximately one-tenth volume of 3M sodium acetate (20 µl) was added to the tube before the DNA was precipitated by the addition of 400 µl of absolute alcohol,

incubated overnight at 4^0C and harvested by centrifugation at 10 000 rpm for 30 min. The supernatant was discarded and 100 µl of 70% alcohol was pipetted to dissolve any remaining salts in the DNA pellet. The tube was centrifuged at 10 000 rpm for 10 min and the alcohol was removed. The pellet was dried in a vacuum dryer for 10 min, dissolved in 100 µl sterile ddH_2O and stored at -20^0C. The quantity of DNA was determined spectrophotometrically at $OD_{260\ nm}$. For each amplification process, 100 ng of DNA template was used.

2.2.3 Polymerase chain reaction (PCR)

Positive and negative controls were included in each PCR: the positive control contained DNA extracted from a bacterial culture of strain TH13/00 (*S. flexneri* 2a), whilst the negative control had all the components of the reaction mixture excluding the DNA template.

2.2.3.1 Optimization of monoplex PCR

Prior to combining all the five primer sets (Table 2.2) in a multiplex PCR (mPCR), each pair of primers was optimized singly in separate PCR assays. Table 2.3 shows the composition of a typical 25-µl PCR reaction mixture for every primer set.

Table 2.3
Reaction mixture for optimization of monoplex PCR

Stock solution	Volume (µl)	Final concentration (µM)
10X PCR buffer B*	2.50	1X
25 mM $MgCl_2$*	4.00	4.00 mM
5 mM dNTP*	0.65	130.00
50 µM primer (F)	0.25	0.50
(R)	0.25	0.50
sterile ddH_2O	15.15	-
Taq DNA polymerase (5U/µl)*	0.20	1U
DNA template	2.00	-
Total	25.00	

* all the PCR solutions in this study were manufactured by Promega Corp., USA

A master mixture of all the PCR solutions (excluding DNA template) was always made in the following pipetting order: sterile ddH_2O→ PCR buffer → $MgCl_2$ → dNTP → primers → enzyme. Following a quick mixing of all the components, the master mixture was aliquotted into each microcentrifuge tube and the DNA template was added. Lastly, the reactants were overlaid with 20 µl of sterile mineral oil and subjected to the following amplification conditions in the Robocycler Gradient 40 Temperature Cycler (Strategene Cloning Systems, USA):

Initial denaturation at 95°C for 5 min → 1 cycle

Denaturation at 95°C for 50 sec
Annealing at 55°C for 1.5 min → 30 cycles
Extension at 72°C for 2 min

Final extension at 72°C for 7 min → 1 cycle

2.2.3.2 Optimization of multiplex PCR (mPCR)

Initial attempts to co-amplify all the five primer sets in a single mPCR assay following the protocol in Table 2.4 was carried out on three occasions.

Table 2.4
Reaction mixture for the initial attempts of mPCR optimization (incorporating 10 primers)

Stock solution	Volume (µl)	Final concentration (µM)
10X PCR buffer B	2.50	1X
25 mM $MgCl_2$	4.00	4.00 mM
5 mM dNTP	0.65	130.00
50 µM ShET1B (F + R)	0.25 + 0.25	0.50 each
ShET1A (F + R)	0.25 + 0.25	0.50 each
ial (F + R)	0.25 + 0.25	0.50 each
Shig1 + Shig 2	0.25 + 0.25	0.50 each
ShET2 (F + R)	0.25 + 0.25	0.50 each
Sterile ddH_2O	13.15	-
Taq DNA polymerase (5U/µl)	0.20	1U
DNA template	2.00	-
Total	25.00	

In spite of the attempts and troubleshooting steps to co-amplify all the five genes (Section 3.3), it was difficult to amplify the *sen* gene. Therefore, the multiplex PCR (mPCR) was developed to amplify *set1A*, *set1B*, *ial* and *ipaH* in a single tube (Table 2.5), and the *sen* gene was amplified in a separate monoplex PCR (Table 2.3). The PCR conditions for both the mPCR and monoplex PCR were as described in Section 2.2.3.1.

Table 2.5
Reaction mixture for optimization of mPCR (incorporating 8 primers)

Stock solution	Volume (µl)	Final concentration (µM)
10X PCR buffer B	4.50	1.8X
25 mM $MgCl_2$	4.00	4.00 mM
5 mM dNTP	0.65	130.00
50 µM ShET1B (F + R)	0.15 + 0.15	0.30 each
ShET1A (F + R)	0.25 + 0.25	0.50 each
ial (F + R)	0.25 + 0.25	0.50 each
Shig1 + Shig 2	0.15 + 0.15	0.30 each
Sterile ddH_2O	12.05	-
Taq DNA polymerase (5U/µl)	0.20	1U
DNA template	2.00	-
Total	25.00	

2.2.3.3 Purification of PCR products

A 6-volume reaction mixture of 25-µl each was prepared for each gene amplification. The PCR products were electrophoresed on a 1% agarose gel. The ethidium bromide-stained gel was visualized under a UV transilluminator, and the amplicon band was excised with a sterile scalpel.

Purification of the products was conducted using the QIAquick Gel Extraction Kit by QIAGEN (Germany). Three volumes of Buffer QG (a solubilization buffer) were added to a single volume of gel and incubated at 50°C for 10 min to dissolve the agarose gel; after which, one gel volume of isopropanol was pipetted in to increase the DNA yield for amplicons of less than 500 bp and more than 4 kb in size. The solution was pipetted into a spin column attached to a collection tube and centrifugation at 13 000 rpm for one min was performed. The flowthrough was discarded and 0.5 ml of Buffer QG was added into the column in order to remove residual agarose. After a quick spin at 13 000 rpm, 0.75 ml of Buffer PE (a wash buffer) was added into the column. Following an incubation period of 2 to 5 min and a quick spin, the flowthrough

was discarded and the column was centrifuged again to remove residual ethanol from the buffer. Purified DNA was eluted from the membrane of the column by the addition of 30 µl of sterile ddH_2O to the center of the membrane. The amount of purified DNA was determined spectrophotometrically at $OD_{260\ nm}$ and then stored at -20^0C until further use.

2.2.3.4 Sequencing

The five purified DNA templates were sent to Research Biolabs (Singapore) for sequencing. ABI PRISM BigDye Terminator chemistry was used for the sequencing reaction. The ABI PRISM 377-96 DNA sequencer analysed DNA molecules labeled with multiple fluorescent dyes by the BigDye Terminator sequencing reaction, on a vertical 5% Long Ranger DNA sequencing gel (Appendix 2).

2.2.3.5 Reproducibility

A total of 28 strains (from years 1997-2000) were randomly chosen and tested for their reproducibility of PCR results. Whenever a second amplification gave a different set of results from the first amplification, a third PCR assay was performed on that particular strain for confirmation.

2.2.3.6 Specificity

In addition to the 110 *Shigella* strains, 12 non-*Shigella* strains were screened for the presence of the virulence-associated genes. The strains were *Enterobacter cloacae*, *Salmonella* Paratyphi A (ATCC 9281), *Salmonella* Paratyphi C, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Typhi (ATCC 7251), *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *Escherichia coli* O157:H7 and *E. coli* O78:H11.

2.2.3.7 Sensitivity

An overnight culture of strain TH13/00 (*S. flexneri* 2a) was grown in 5 ml LB broth. Appropriate bacterial concentrations were obtained by preparing serial 10-fold dilutions in LB broth (10^{-1} dilution to 10^{-10} dilution). To enumerate the bacteria, 100 µl of each diluted culture were spread onto *SS* agar plates and incubated at 37 ^{0}C overnight. The bacteria concentration was estimated by calculating the average colony count on plates as given by (Penn, 1991):

$$\text{Viable count (cfu/ml)} = \frac{N}{V \times D}$$

whereby N = number of colonies
V = volume of culture plated (0.1 ml)
D = optimum counting dilution

DNA templates for mPCR assays were also prepared from each dilution by spinning down the remaining culture and washing the bacterial pellet twice with sterile dH_2O. The suspension was boiled at 100^{0}C for 5 min and the supernatant served as template for subsequent amplification processes.

2.2.3.8 Faecal-spiking and sensitivity (modified from Chiu & Ou, 1996)

Approximately 0.2 g of faeces from a healthy individual was suspended in 1 ml of BHI and diluted 10-fold. Next, 1 ml of the diluted faecal suspension was inoculated into 4 ml of BHI and vortexed to obtain a homogenous mixture of broth-faecal suspension. Meanwhile, an overnight culture of strain TH13/00 (grown in 5 ml of LB broth) was harvested and the bacterial pellet was serially diluted 10-fold with BHI. The next step involved combining 250 µl of each dilution of bacterial culture with 250 µl of the broth-faecal suspension and 500 µl of BHI into a new eppendorf tube. It was vortexed and preincubated at 37^{0}C for 4 h without shaking. Simultaneously, 100 µl of each diluted culture was plated onto *SS* agar to determine the number of viable bacteria in each dilution. Viable count was calculated as shown in Section 2.2.3.7.

After preincubation, mPCR assay was performed on the boiled lysates of the bacteria-broth-faecal suspensions, with strain TH13/00 as a positive control and an unspiked faecal sample as a negative control. The sensitivity of the mPCR was determined by the presence of all four genes in the highest dilution visible on an ethidium bromide-stained agarose gel. The test was carried out twice and the average detection limit was as reported in Section 3.8.

2.2.3.9 Screening of clinical specimens

Approximately 0.2 g of faecal material from each patient was suspended in 1 ml of BHI and diluted 10-fold. A volume of 250 µl of faecal suspension was inoculated into 5 ml of BHI and preincubated at 37^{0}C for 4 h without shaking. At the same time, 100 µl of the suspension was each plated onto MacConkey and *SS* agar plates and incubated overnight at 37^{0}C. mPCR assay was carried out on the boiled lysate of the suspension after preincubation. Both strain TH13/00 and a *Shigella*-spiked faecal sample served as positive controls, whilst a PCR reaction mixture without bacterial DNA and an unspiked faecal sample from a healthy donor functioned as negative controls.

2.2.3.10Agarose gel electrophoresis

PCR products were resolved by electrophoresis in a horizontal agarose gel (2% w/v) in 0.5X TBE buffer. Agarose powder was dissolved in 0.5X TBE buffer by heating the mixture in a microwave oven. The molten gel was cooled to 50^{0}C before being poured into a clean horizontal gel casting tray (either 12 X 9 cm or 210 X 100 cm), fitted with an 8-, 12-, 22- or 40-well comb. Prior to the pouring of gel, the teeth of the comb were cleaned with 70% ethanol. Caution was taken to ensure there was no

bubble formation in the gel on the tray; bubbles were removed by using a sterile micropipette tip. The gel was allowed to set for 30 min.

The tray with the set gel was then placed in an electrophoresis tank. A sufficient volume of 0.5X TBE buffer was poured and should cover the gel to a depth of ~ 3 mm. Each PCR product (=8 µl) was mixed well with 2 µl of 6X gel loading dye and loaded into the wells of the gel. A 100-bp DNA ladder (Promega Corp., USA) was also loaded into each gel in order to monitor the molecular weight of the amplicons. Electrophoresis was performed at room temperature with a constant voltage of 100 V, from cathode to anode. The progress of band separation was monitored by the migration of the loading dye. Electrophoresis was halted when the orange G dye came off the gel at the anode direction.

2.2.3.11Gel visualization and documentation

The gel was stained in ethidium bromide (0.5 µg/ml) for 5 to 10 min and destained with dH_2O for 15 min. The DNA-ethidium bromide complex was visualized on a 312 nm-UV transilluminator (Spectroline Model TVC-312A, USA). Gel documentation via photography was performed using a Polaroid MP-4 Land camera system fitted with a Kodak Wratten gelatin yellow filter and Polaroid Land black-and-white films (type 665).

Extreme care such as wearing gloves was taken with the ethidium bromide solution, stained gel and destaining water with ethidium bromide residue because the chemical is a mutagenic substance.

2.3 Data analyses

The efficiency of the different DNA extraction protocols, ie. boiling and phenol-chloroform extraction methods, and the differences in the prevalence of genes were

evaluated using paired samples t-test (SPSS Release 11 software). In addition, the association between the prevalence of each gene with *S. flexneri* 2a serotype was also determined using chi-square (χ^2) tests. All the tests were performed at a significance level (α) of 0.05. Null hypotheses (H_0) were rejected if the p-value was <0.05. To test for the strength of association, correlation coefficient Cramer's V was used. It is a measure from 0 (no association) to 1 (perfect association) (Dytham, 1999).