

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Sampling

Collections of water samples for the study were done from 15th May 2008 until 20th May 2008 in areas around Bachok, Kelantan. Water samples were collected from fifty sites around Bachok, Kelantan. Thirty-nine samples were river waters, nine were coastal waters, and two were water samples from waterfalls.

3.1.2 *E. coli* isolates

Three representative bacterial strains used as positive control strains in Polymerase Chain Reaction (PCR) assay were provided by Associate Prof. Dr. Richard C.Y. Kong (City University of Hong Kong). The control strains employed in the study were known LT2-positive and VT-positive isolate *E. coli* SA 53, known LT1-positive and ST-positive isolate *E. coli* ATCC 35401 and known VT-positive and *eaeA*-positive isolate *E. coli* 0157 (Kong *et al.*, 1999) (Table 3.1a).

The clinical strains for comparison studies were provided by Dr. Rohani Md. Yasin from Institute of Medical Research Malaysia (IMR). While food strains were previously isolated and identified by biochemical test by other researchers in Prof. Dr. Thong Kwai Lin's laboratory according to standard microbiological test.

Table 3.1a The bacterial control strains and their respective genes (Kong *et al.*, 1999)

Target Gene	SA 53	ATCC 35401	0157
Alkaline phosphatase gene (<i>phoA</i>)	+	+	+
Heat stable toxin 1 gene (ST1)	-	+	-
Heat labile toxin 1 gene (LT1)	-	+	-
Heat labile toxin 2 gene (LT2)	+	-	-
Verotoxin 1 & verotoxin 2 genes (VT1 & VT2)	+	-	+
Attachment and Effacement gene (<i>eaeA</i>)	-	-	+

3.1.3 Media and reagents

All the media used for bacterial growth were obtained from Oxoid Ltd, England and Merck Ltd, Germany.

3.1.3.1 Media for bacterial growth

Chromocult Coliform Agar for 100 mL (pH 6.8)

Chromocult Coliform Agar powder	2.65 g
Distilled water	100 mL

Nutrient Agar for 100 mL (pH7)

Nutrient Agar powder	2.8 g
Distilled water	100 mL

Luria-Bertani Broth for 100 mL (pH 7.5)

Tryptone	1 g
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Yeast extract	0.5 g
Sodium Chloride	0.5 g
Distilled water	100 mL

Luria-Bertani Agar for 100 mL (pH 7.5)

Bacteriological Agar	1.5 g
Tryptone	1 g
Yeast extract	0.5 g
Sodium Chloride	0.5 g
Distilled water	100mL

Eosin Methylene Blue (EMB) Agar for 100 mL (pH 7.1)

EMB Agar powder	3.75 g
Distilled water	100 mL

Sorbitol MacConkey Agar for 100 mL (pH 7.1)

Sorbitol MacConkey Agar powder	5.15 g
Distilled water	100 mL

Mueller-Hinton Agar for 100 mL (pH 7.3)

Mueller-Hinton Agar powder	3.8 g
Distilled water	100 mL

3.1.3.2 Materials for Biochemical Tests

Triple Sugar Iron (TSI) Agar for 1000mL (pH 7.4)

Triple Sugar Iron (TSI) Agar powder	65 g
Distilled water	1000 mL

Simmon Citrate agar for 1000mL (pH 6.9)

Simmon Citrate agar powder	23 g
Distilled water	1000 mL

SIM agar for 1000mL (pH 7.3)

SIM agar powder	36.3 g
Distilled water	1000 mL

Kovacs reagent

Isoamyl alcohol	150 mL
Concerntated Hydrochloric Acid	50 mL
<i>p</i> -dimethylaminobenzaldehyde	10 g

Methyl Red reagent

Methyl Red	0.1 g
95% ethyl alcohol	300 mL
Distilled water to	500 mL

Alpha Naphthol solution

Purified α - naphthol	5 g
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Ethyl alcohol	100 mL
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40% KOH

Potassium hydroxide	40 g
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Distilled water	100 mL
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3.1.3.3 Other Solutions

Buffered Peptone Water (BPW)

BPW powder	20 g
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Distilled water	1000 mL
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Saline Buffered water (0.85%)

NaCl	0.85 g
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Distilled water	100 mL
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Alcohol 70% for 500 mL (from 95%)

95% alcohol	395 mL
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Distilled water	132 mL
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3.1.4 PCR Materials and Equipments

3.1.4.1 Materials and Equipments for PCR assays

Oligonucleotide primers

All oligonucleotide primers were synthesized by Operon Biotechnologies GmbH, Germany. The oligonucleotide primers used in PCR assay and the size of expected PCR product are listed in Table 3.1b and 3.1c. For *E. coli* confirmation test, *phoA* primer was used for detecting *E. coli* house keeping gene in monoplex PCR assay. While five pairs of primers which include ST1, LT1, LT2, VT and AE were used in multiplex PCR assay for the detection of virulence genes. These primers were used for detecting heat-stable toxin 1 gene (ST1), heat-labile toxin 1 gene (LT1), heat-labile toxin 2 gene (LT2), Verotoxin 1 gene (VT1), Verotoxin 2 gene (VT2) and Attachment and Effacement gene (*eaeA*).

Chemicals and enzymes

All the PCR chemicals and enzymes were purchased from Promega Corporation, USA. These include PCR buffer (5x Colourless and Green GoTaq® Flexi Buffer), deoxynucleotide triphosphates (dNTPs), Magnesium Chloride (MgCl₂) and *Taq* polymerase (GoTaq® DNA polymerase).

Equipments for PCR assay

Micropipettes, PCR cabinet (Cleansphere CA100 by Safetech Limited), microcentrifuge (MiniSpin® by Eppendorf), Robocycles (Stratagene) and PCR thermal cycler (Mastercycler personal by Eppendorf) were utilized for PCR assay.

Table 3.1b Primers used for detection of housekeeping gene (monoplex) and virulence genes (multiplex) of *E. coli*.

(Kong *et al.*, 1999)

Target gene	Primers	Sequences	Expected band
Alkaline phosphatase (<i>phoA</i>)	Pho-F Pho-R	GTC ACA AAA GCC CGG ACA CCA TAA ATG CCT TAC ACT GTC ATT ACG TTG CGG ATT TGG CGT	903bp
Heat stable toxin 1 (ST1)	ST1-F ST1-R	CTT TCC CCT CTT TTA GTC AG TAA CAT GGA GCA CAG GCA GG	175bp
Heat labile toxin 1 (LT1)	LT1-F LT1-R	TTA CGG CGT TAC TAT CCT CTC TA GGT CTC GGT CAG ATA TGT GAT TC	275bp
Heat labile toxin 2 (LT2)	LT2-F LT2-R	ATA TCA TTT TCT GTT TCA GCA AA CAA TAA AAT CAT CTT CGC TCA TG	720bp
Verotoxin 1 (VT1)	VT-F	GAA CGA AAT AAT TTA TAT GTG	523bp
Verotoxin 2 (VT2)	VT-R	CCT GAT GAT GGC AAT TCA GTA	520bp
Attachment and Effacement (<i>eaeA</i>)	AE22 AE20-2	ATT ACC ATC CAC ACA GAC GGT ACA GCG TGG TTG GAT CAA CCT	397bp

Table 3.1c Primers used for REP-PCR assays.

Primers	Sequences
REP	GCG CCG ICA TGC GGC ATT
REP1R(a)	III CGI CGI CAT CAT CTG GG
REP2I	ICG ICT TAT CIG GCC TAC
REP1R(b)	III ICG ICG ICA TCA TCT GG

3.1.4.2 Materials and Equipments for Agarose Gel Electrophoresis

Agarose Gel (1.2 %)

Agarose gel	1.2 g
0.5x TBE buffer	100 mL

Gel Loading Dye, 100bp DNA ladder and 1kb DNA ladder

6x Loading Dye, 100 bp DNA ladder and 1kb DNA ladder were purchased from Promega Corporation, USA.

10x Tris Borate EDTA Buffer (TBE) for 1000 mL

Tris	121.2 g
Boric acid	61.8 g
Na ₂ EDTA.2H ₂ O	0.745 g
Distilled water	1000 mL

The pH of the solution was adjusted to 8.3. The solution was diluted to 0.5X with distilled water before use.

Ethidium Bromide (10mg/10mL)

Ethidium Bromide	100 mg
Deionised water	10 mL

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

Phosphate buffered saline (PBS)

PBS tablets	10
Distilled water	1000 mL

The pH of the solution was adjusted to 8.3.

Tris-EDTA (TE) buffer

1M Tris, pH 8.0	10 mL
0.5M EDTA, pH 8.0	2 mL
Distilled water	1000 mL

Equipments for Agarose Gel Electrophoresis

Micropipette, electrophoresis power supply and gel electrophoresis units, ethidium bromide staining equipments, Biorad Molecular Imager (Gel Doc™ XR) and computer for gel visualization and documentation.

3.2 Methods

3.2.1 Collection of water samples

Water samples were collected in sterile containers and immediately stored in a 4°C cooling box and analysed within 6h from the time samples were acquired at the sampling site.

3.2.2 Media preparation

All the media were autoclaved at 121°C, 15psi for 20 minutes with the cap bottle loosen before autoclaving, unless otherwise stated. Selective medium such as Chromocult Coliform Agar was not be autoclaved or overheated. Thus, media preparations were done according to the stated reference. Media were poured approximately into sterile petri plates in laminar flow. The agar plates were exposed to UV light in laminar flow for 10 minutes. Hardened and dried agar plates were refrigerated or kept in sterile environment.

3.2.3 Filtration of water samples

The membrane filtration method was carried out according to instruction by the United States Environmental Protection Agency (USEPA, 1986) to isolate *E. coli* and coliform from water samples. In this procedure, water samples were filtered through a sterile, 47-mm diameter membrane (pore size, 0.45±0.02 µm), which retained bacteria. Briefly, a sterile forceps used to place the membrane filter over porous plate of

receptacle. Funnel unit placed over the receptacle and locked in place. Samples were then filtered under partial vacuum which draw the water through and trapped all the bacteria present in the sample on the surface of the membrane filter. Filter equipments rinsed between samples to prevent carryover contamination.

3.2.4 Isolation of *E. coli* and other coliforms

After filtration, the membrane containing the bacteria was immediately placed on a selective differential medium, Chromocult Coliform Agar (CCA, Merck, Germany) (USEPA 1986) using a sterile forcep with a rolling motion to avoid entrapment of air. Plates were then incubated at 37°C for 24h to detect presumptive *E. coli* and total coliforms (Alonso *et al.*, 1998). After overnight incubation, coliform colonies turned salmon red and *E. coli* colonies turned dark blue or purple on this media. Presumptive colonies taken from the CCA were transferred first to an additional CCA plate and then to nutrient agar incubated at 37°C for 24 h to obtain pure single *E. coli* strains.

3.2.5 Biochemical tests

In order to distinguish *E. coli* from related species likely to be found naturally in the environment, a group of tests called the IMViC (Indole, Methyl red, Voges-Proskauer, and Citrate) reactions was used in order to differentiate fecal coliforms from nonfecal coliforms. The IMViC set of tests examines the ability of an organism to produce indole, produce sufficient acid to change the color of methyl red indicator, produce acetoin, an intermediate in the butanediol fermentation pathway (a positive result of

the voges-proskauer test) and the ability to grow on citrate as the sole source of carbon. Triple sugar iron (TSI) agar is a medium used in the identification of gram-negative enteric rods. The medium measures a bacterium's ability to utilize three sugars, glucose, sucrose and lactose, the concentrations of which are 0.1%, 1.0%, and 1.0%, respectively. A pH indicator included in the medium can detect acid production from fermentation of these carbohydrates.

3.2.5.1 Indole test

Tubes containing SIM medium were inoculated with a loop full of culture. Stab inoculation technique was used. Tubes were then incubated at 37°C for 24 hr. 1 mL of Kovacs reagents added to each tube and read immediately. A deep red colour in the top layer indicates the presence of indole. Negative reaction remains colourless or light yellow.

3.2.5.2 Methyl red test

Tubes containing MR-VP broth were inoculated with culture and incubated at 37°C for 72 hr. A few drops of methyl red solution were added to the culture and read immediately. It remains red at pH less than 4.4 (MR positive) and turns yellow if the pH goes above 6.0 (MR negative).

3.2.5.3 Voges Proskauer test

Tubes containing MR-VP broth were inoculated with culture and incubated at 37°C for 72 hr. Approximately 3 mL of alpha naphthol followed by 1 mL of 40% KOH were added to each tube. Positive reaction occurs at once or within 20 minutes. Positive reaction indicated by a change of colour to pink and negative reaction with no colour change.

3.2.5.4 Citrate utilization test

Simmon citrate agar slants were inoculated by streak inoculation technique and incubated at 37°C for 48 hr. Slant cultures were then examined for the presence of growth and change of colour from green to deep blue.

3.2.5.5 Triple Sugar Iron (TSI) test

A single well isolated colony of bacteria was inoculated into TSI medium by means of a stab and streak inoculation. Tubes were incubated at 37°C for 24 hr. The ability to ferment glucose was observed by the colour change, while the gas production was determined by crack or air gap in the medium. H₂S production was indicated by the presence of a black precipitate.

3.2.6 API 20E

Presumptive *E. coli* strains were further identified by the API 20E test system (Biomérieux) according to the manufacturer's instructions. API 20E system is a ready to use, microtube system designed for the performance of 23 standard biochemical tests using well isolated colonies of bacteria on a plating medium. This system consists of a series of microtubes containing dehydrated substrates. The microtube is composed of a cupule (the upper portion of the microtube) and the tube (the lower portion of the microtube). The substrates are located in the tube portion of the microtube. These substrates are reconstituted by adding a bacterial suspension. The strips of reconstituted microtubes were incubated for the bacterial strains to react with the contents of the tubes. The strips were read after 18-24 hours when the various indicator systems in the microtubes are affected by the metabolites or added reagents.

3.2.7 Total DNA extraction from bacterial culture

Boiling method was used for the extraction of bacterial culture for PCR assay. *E. coli* isolates from the stock were inoculated on Luria-Bertani Agar incubated at 37°C for 24 h. Single colonies from fresh *E. coli* cultures were transferred to microfuge tube containing 50 µl PBS pH 7.3 and centrifuged at 13,400 rpm for 2 min. The supernatant was discarded. The cell pellet was resuspended in 50 µl sterile water, boiled in thermal cycler (Perkin Elmer) at 99°C for 5 min to release the contents of the cells and chilled on ice for 10 min to avoid the denatured DNA from forming back into double-stranded DNA. Then it was re-centrifuged at 13,400 rpm for 2 min to pellet the cell debris. An aliquot (5 µl) was used for subsequent PCR analysis.

3.2.8 Polymerase Chain Reaction (PCR) assay

3.2.8.1 PCR material and master mixture preparations

Before starting the PCR assays, proper preparation is necessary for a coordinated work flow. First, PCR materials such as 5 X PCR Buffer (GoTaq® Flexi Buffer), deoxynucleotide triphosphates (dNTPs) and Magnesium Chloride ($MgCl_2$) stock were thawed on ice. Then they were spun briefly before used. In order to prolong the shelf time of the PCR materials, they were prepared in batches and aliquot for storage to prevent contamination of stock solutions. In a multiplex PCR assay, five pairs of primers (LT1, LT2, VT, ST and AE primers) were mixed into a tube according to their volume and concentration before the PCR assay. Premixing of all the primers helps to shorten the time of master mixture preparation. Preparation of primer mix will also maintain the performance of the primers and furthermore it will ensure the outcome of the PCR assay.

For the preparation of master mixtures, the DNA template was thawed on ice and centrifuged at 13 400 rpm for 75 seconds. Primer or primer mix, 5x PCR Buffer, $MgCl_2$ and dNTPs were thawed on ice and spun briefly before being used. The amount of PCR tubes needed was estimated and they were labeled accordingly. The PCR cabinet was cleaned with ethanol before the preparation starts. The 0.2 mL PCR tubes, ddH₂O in 0.5 mL tubes, pipette tips, micropipettes and PCR tube rack were

exposed under UV in the PCR cabinet for 15 min before starting the work. Gloved hands were cleaned with ethanol as well.

After that, ddH₂O, 5x PCR Buffer, dNTPs, MgCl₂, primer or primer mix and *Taq* polymerase were added into a 0.5 mL PCR tube accordingly. The master mixture was then spun briefly before dispensing into 0.2 PCR tubes. Lastly, 5 µL of DNA template was added into the 0.2 mL PCR tube. Each reaction included a negative control, which was a reaction mixture that did not include a template DNA, and positive controls that included purified extracted DNA from a known strain of *E. coli*.

Finally, before loading the 0.2 mL PCR tubes in the Thermal Cycler, all the tubes were spun briefly. The PCR was run under the appropriate program. The PCR products were then stored at -20°C.

3.2.8.2 Confirmation of *E. coli* using monoplex PCR assay

Bacterial isolates that were screened from the conventional steps were further confirmed by PCR based assay targeting the *phoA* gene, which is the housekeeping gene for *E. coli* (Kong *et al.*, 1999). This monoplex PCR assay was carried out in total volume of 25 µL. The master mixtures were prepared as listed in Table 3.2a.

Table 3.2a Monoplex PCR master mixture.

	[Working]	Volume
Sterile ddH ₂ O		13.05 µL
5x Buffer	1 X	5.00 µL
dNTPs	140 µM	0.35 µL
MgCl ₂	1.0 mM	1.00 µL
Primer Forward	0.1 µM	0.25 µL
Reverse	0.1 µM	0.25 µL
<i>Taq</i> DNA Pol	0.5 U	0.10 µL
DNA template		5.00 µL
Total Volume		25.00 µL

3.2.8.3 Toxin genes detection using multiplex PCR assay

Multiplex PCR was used to amplify six virulence gene (ST1, LT1, LT2, *eaeA* and VT genes) in pathogenic *E. coli* using primers as described previously (Kong *et al.*, 1999). The multiplex PCR assay was carried out only if the isolates were verified as *E. coli* by monoplex PCR assay. Multiplex PCR assay was carried out in total volume of 50 µL. The master mixtures were prepared as listed in Table 3.2b.

Table 3.2b Multiplex PCR master mixture.

	[Working]	Volume
Sterile ddH ₂ O		17.60 µL
5x Buffer	1 X	5.00 µL
dNTPs	200 µM	1.00 µL
MgCl ₂	3.0 mM	6.00 µL
Primer ST1-F	0.07 µM	0.35 µL
ST1-R	0.07 µM	0.35 µL
AE22	0.07 µM	0.35 µL
AE20-2	0.07 µM	0.35 µL
LT1-F	0.07 µM	0.35 µL
LT1-R	0.07 µM	0.35 µL
LT2-F	0.60 µM	3.00 µL

LT2-R	0.60 μ M	3.00 μ L
VT-F	0.70 μ M	3.50 μ L
VT-R	0.70 μ M	3.50 μ L
<i>Taq</i> DNA Pol	1.5 U	0.30 μ L
DNA template		5.00 μ L
Total Volume		50.00 μ L

3.2.8.4 PCR conditions

Monoplex and multiplex PCR assays employed same preparation procedures and amplification conditions though they were performed with different sets of master mixtures. Both PCR amplifications were performed in a thermal cycler

(Mastercycler personal by Eppendorf). The PCR conditions was set as listed in Table 3.2c.

Table 3.2c PCR amplification condition for monoplex and multiplex PCR assays.

Condition	Temperature ($^{\circ}$ C)	Duration (minutes)	Cycle
Initial denaturation	94	2	1
Denaturation	94	1	35
Annealing	56	1	
Extension	72	1	
Final elongation	72	10	1
Soaking	4		

3.2.8.5 Running Agarose Gel Electrophoresis

The procedures for running gel electrophoresis were stated briefly in the flow chart below.

The electrophoresis gel tank was set up



~~The gel forming block, gel tray and comb were cleaned with 70% alcohol and~~

assembled in balanced condition



The 1.2 % agarose gel was prepared in a clean bottle by mixing the powder with

0.5x TBE buffer.



The mixture was boiled in microwave oven using high temperature until the agarose

powder completely dissolved



The mixture was cooled to 50°C under the running tap water with gentle agitation



The mixture was then dispensed slowly into gel forming block without creating any

bubbles and left to be solidified for 10-15 min



0.5% TBE buffer was added to the gel forming block to avoid the agarose from

drying.



The comb was taken out without breaking the gel. Tray was then loaded into the gel

tank and filled up with 0.5x TBE buffer until it completely submerged the gel.



Loading dye and DNA ladder were thawed on the ice and spun briefly before being

used.



2 μL of loading dye was mixed with 5 μL of PCR products and DNA ladder.

Loading dye was not used for PCR products that used 5x colored buffer.



The mixtures were loaded into the well accordingly as fast as possible without damaging the wells as the mixture will diffuse out of well rapidly.



The gel was covered and connected to the power pack, with the current run from negative to positive. The voltage adjusted to 90V.



After one of the loading dyes reaches the end of the gel, the power pack switched off and the gel transferred into 1 $\mu\text{g}/\text{mL}$ ethidium bromide (EtBr) solution and stained

for 3-5 min



Finally gel was destained in sterile water for 15-20 min. The bands in gel were visualized and photographed using Biorad Molecular Imager (Gel Doc™ XR)

3.2.9 Antimicrobial agent susceptibility testing (ARA)

Susceptibility testing was performed by an agar diffusion disk method as recommended by the National Committee for Clinical Laboratory Standards, 2004 (NCCLS, 2004). Antimicrobial agents used in the study were ampicillin (10 μg), chloramphenicol (30 μg), trimethoprim-sulfmethaxzole (25 μg), tetracycline (30 μg), gentamycin (10 μg), kanamycin (30 μg), ciprofloxacin (5 μg), nalidixic acid (30 μg), streptomycin (10 μg)

and ceftriazone (30 µg). *E. coli* ATCC 25922 was used as a reference strain for quality control.

3.2.9.1 Preparation of inoculum

A single bacteria colony was transferred from LB agar plate into 5 mL of LB broth and incubated at 37°C overnight. 2 mL of sterile saline (0.85% NaCl) was aliquot into 7 mL tubes and bacteria culture in LB broth was added. The value adjusted to 0.08-0.10 Abs that is equivalent to 0.5 McFarland turbidity standards.

3.2.9.2 Inoculation of Mueller-Hinton (MH) agar

Within 15 min after adjusting the turbidity of the culture mixture, culture was spread on MH agar by using sterile cotton swab. The sterile cotton swab was dipped into the culture and excess liquid was removed by pressing on the wall of the tube. The swab was streaked over the entire surface of the MH agar for 3 times by rotating the plate approximately 60° after each application to ensure an even distribution of the inoculum.

Then the plates were allowed to air dry in the laminar flow before application of antibiotic discs. The antibiotic discs were allowed to warm at room temperature before use. By using a sterile forceps, the antibiotic discs were gently placed and pressed onto the surface of the agar to ensure complete contact of discs with the agar. The plates were then inverted and incubated for 16-18 hr at 37°C. Finally the

plates were examined and the zone of inhibition and resistance were measured, recorded and interpreted. The breakpoints used to categorize isolates as resistant or not resistant to each antimicrobial agent were those recommended by NCCLS, 2004.

3.2.10 Repetitive extragenic palindromes (REP)-PCR amplification

REP-PCR was used to amplify repetitive elements from bacterial isolates to generate DNA fingerprint patterns. REP-PCR was carried out following the method previously described by Mclellan *et al.* (2003), with some modifications. The optimization of REP-PCR was carried out using four different primers to identify primer that gives discernible banding patterns for the *E. coli* strains. The primers used were stated in Table 3.1c. Briefly, the assay was done under the conditions stated in Table 3.2d. This PCR assay was carried out in total volume of 25 μ L. The master mixtures were prepared as listed in Table 3.2e.

Table 3.2d PCR amplification condition for REP- PCR assay.

Condition	Temperature (°C)	Duration (minutes)	Cycle
Initial denaturation	94	5	1
Annealing	33	5	
Extension	68	5	
Denaturation	94	1	35
Annealing	40	1	
Extension	68	2	
Final elongation	68	16	1
Soaking	4		

Table 3.2e REP-PCR Master mixture.

	[Working]	Volume
Sterile ddH ₂ O		12.35 μ L
5 X Buffer	1 X	5.00 μ L
dNTPs	240 μ M	0.60 μ L
MgCl ₂	1.5 mM	1.5 μ L
Primer (REP1R)	0.1 μ M	0.25 μ L
<i>Taq</i> DNA Pol	1.5 U	0.3 μ L
DNA template		5.00 μ L
Total Volume		25.00 μ L

The reaction was prepared with extracted DNA template. A control reaction mixture containing 5 μ l of water instead of *E. coli* was also included in each set of PCR. Separation of amplified genomic fragments was accomplished via gel electrophoresis by using 1.5% agarose gel and run at 60 V for 5 hr. A 1kb and 100bp size ladder (Promega Corporation USA) was loaded into the two terminal wells and in the middle of the gel. Gels were stained with ethidium bromide and visualized under UV. Banding patterns were digitally captured by using Biorad Molecular Imager (Gel Doc™ XR). REP-PCR assays were repeated at least twice for all the samples to demonstrate reproducibility of data.

3.2.11 Interpretation of fingerprints

Comparisons between fingerprint patterns generated from REP-PCR were performed using GelCompar software (Version 2.0, Applied Maths, Kortrijk, Belgium). Using this software, fingerprint images were added to a database and compared by performing a statistical analysis. Fingerprint similarities values were based on the presence or absence of bands between each profile. Blank lanes due to PCR failure and