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Genetic Characterization of Shiga Toxin-producing *Escherichia coli* Strains Isolated from Imported Beef Meat in Malaysia Using Polymerase Chain Reaction Analysis

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Abstract: This work was to genetically characterized Shiga toxin-producing *Escherichia coli* strains isolated from imported beef meat in Malaysia using polymerase chain reaction (PCR) base analysis, seventy four (74) frozen beef meats samples (imported) (n=74) tested originated forty two of the sample were from India and thirty two of the samples were from Australia. The samples were bought from the frozen meat units of five different supermarkets in diverse settings in Malaysia which start from 2012 April to 2013 October on a weekly basis. *E. coli* and shiga toxin producing *E. coli* isolation was done Isolation of plasmid was carried out using the PureYield™ Plasmid Miniprep System. Enterobacterial Repetitive Intergenic Consensus (ERIC) primer was use in genetically isolating the bacteria: We used seven types of primers for Random Amplified Polymorphic DNA-PCR (RAPD-PCR) namely Gen8, Gen9, A1, A7, A10, OPAR8 and OPAR20. Our result revealed that Plasmid profiling showed 16 patterns based on size and 4 patterns on gel. Combination of phenotypically and genotypically approaches revealed a varied heterogeneity among imported beef isolates of *E. coli*. Isolated plasmids from shiga toxin producing *E. coli* varied in sizes. The sizes are from 4.3 -23.1 kilo base (kb). Sixty two (62) isolates were found to harbor plasmid with size 23.2 kb while 43 isolates harbored more than a single plasmid. The analysis of data through the use of average linkage (UPGMA, unweight group pair technique with arithmetic averages) using Gel compare 11 software which was displayed in dendrograms, for the, ERIC-PCR and RAPD-PCR (RAPD Gen 8, Gen 9), (Opar 8 and Opar 20), (RAPD A1, RAPD A7and RAPD A10) analysis, produced various cluster. The PCR analysis using OPAR 8 and OPAR 20 also produces various clusters.

Keywords: Genetic Characterization, Shiga Toxin-producing *Escherichia coli,* Imported Beef Meat, Polymerase Chain Reaction (PCR) and Malaysia

1. Introduction

Pathogenic bacteria identification was formally through traditional isolation of the bacteria and studying it phenotypically via culture, biochemical analysis and Gram

staining which were the gold standards for identifying pathogenic bacteria [1]. With the discovery of polymerase chain reaction (PCR) and DNA sequencing, several bacteria genome have been completely sequenced. Comparing the genomic sequences of different bacterial species revealed that 16S ribosomal RNA (rRNA) gene is extremely a conserved gene amongst species of similar genus and within a species and can be employed as the novel benchmark for specifying bacteria [2]. The 16S rRNA gene sequences are very useful in studying bacterial phylogeny and nomenclature. With the existence of 16S gene in virtually all bacteria, which exist as a multigene family the 16S rRNA gene function for some time has never been altered, which suggest that indiscriminate sequence variations are more precise measures of time and 16S rRNA gene (1500 base pair) is big and good for informatics [3]. With the use of 16S rRNA sequence, several bacterial classes and even species have been renamed and reclassified; uncultivable bacteria grouping were possible through sequencing. Also phylogenetic relations have been determined, and the detection and classifications of new bacterial species by molecular characterization [4]. This technique has been fruitful in classifying Enterobacteriaceae species from a recipient of bone marrow transplant [4].

Several molecular subtyping techniques have been advanced to enhance the epidemiology of *E. coli* O157: H7 outbreaks understanding [5]. It is of importance to identify *E. coli,* and shiga toxin producing *E. coli* using molecular methods as culture based method may not properly identify this pathogen. Most media only identify bacteria to genus level and not species level. Proper identification is required so as to understand the type of measures to control this pathogen in food chain. However, the continuous advancement in molecular biology has resulted in advance of different molecular technique that can be used to identify and characterize bacteria including *E. coli* 0157: H to specie levels [6]. Molecular subtyping, also known as fingerprinting of bacteria makes it promising to generate molecular profile of any bacteria [7]. Among the commonly use molecular tools are pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), bacteriophage typing and Enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR). This is a molecular subtyping method that is based on the use of PCR for analysis of the repetitive chromosomal sequences, called the Enterobacterial repetitive intergenic consensus (ERIC) [10]. It is said to be mostly utilized for clonal classification of diverse species of Enterobacteriaceae [8, 9] and microbial diversity [10]. This molecular method generates genomic finger print that can different between bacterial strains [11]. According to [10], this molecular method or technique is fast, less laborious, simple and only require small amount of genomic DNA and set up equipment. ERIC-PCR is carried out using ERIC primers and amplified using PCR. Amplified PCR product will then be resolved using gel agarose electrophoresis. Obtained gel picture will then be analyzed using software before cluster analysis is performed and dendrogram constructed [10]. Random Amplified Polymorphic DNA-PCR (RAPD-PCR) is a molecular typing tool that requires minimal DNA template [12]. This technique involves the use of primers of arbitrary sequences to randomly amplify template DNA using PCR [12].

RAPD-DNA is been utilized for strain-typing of bacteria [13, 14]. In 2001, Radu et al [15] used this method for the DNA fingerprinting of 28 strains of *E. coli* O157: H7 isolated from haunch beef and three chicken meat burger samples correspondingly. Similarly, Sahilah [16] used this molecular method for genetic diversity and epidemiological study of the relationships of *E. coli* O157: H7 isolated from eggs and imported beef meat.

Shiga toxin-producing strains of *E. coli* O157: H7 are clonal in origin and they are phenotypically and genotypically closely alike [17]. In a recent review by, Etcheverria and Padola, [18] it was stated that the major virulence factor of *E. coli* O157: H7 is Shiga toxins encoded by *stx*1 and *stx*2 genes which can be produced singly or in combination. These toxins belongs to the AB5 protein toxins family containing enzymatically active A subunit, and 5 B subunits that are accountable for binding to the glycolipid globotriaosylceramide Gb3 which is the cellular receptor existing in organs of the body such as kidney, brain, liver, and pancreas [18]. The two main forms of Shiga toxins (*stx*1 and *stx*2) are further characterized as *stx1a*, *stx1c* and *stx1d*, and highly heterogeneous *stx2* with *stx2a, stx2b, stx2c*, *stx2dact, stx2e, stx2f* and *stx2g* subtypes [19, 20]. However, result from molecular epidemiological investigation shows that *stx2,* not *stx1* shiga toxins produced by this pathogen are responsible for the haemolytic uremic syndrome (HUS) [21].

Radu [22] revealed 12 strains of *E. coli* O157: H7 in 9 out of the 25 beef meat samples obtained from wholesale and retailing shops in Malaysia. However, it is necessary constantly monitor beef meat imported into Malaysia through the use of different molecular approach. Consequently, our study is to genetically characterized Shiga toxin-producing *Escherichia coli* strains isolated from imported beef meat in Malaysia using polymerase chain reaction (PCR) base analysis.

2. Material and Methods

2.1. Meat Sample Collection

Imported frozen beef meat samples totaling 74 were tested which came from two nations, including Australia (32 samples) and India (42 samples). The meat samples were bought from five different superstores in diverse places that include Tesco (Kajang), Carrefour (Mid-valley, Kuala Lumpur), Carrefour (Alamanda, Putrajaya), Jusco (Balakong) and Giant (Seri Kembangan) which are located in Selangor, Malaysia which begin from 2012 (April) to 2013 (October) weekly. The entire beef samples were raw, packed typically 4 to 6 samples were bought and kept at -20°C before usage and the testing was done within one week. Table 3 showed the distribution of sample sources and purchase location.

2.2. Isolation of E. coli and Shiga Toxin Producing E. coli from Frozen Beef Meat Imported

The isolation of *E. coli* and shiga toxin producing *E. coli* was done following the method described by Charimba et al. [23]. 25g of beef samples were improved into 225 ml of Maximum Recovery Dilution (MRD). Samples were positioned in sterilized stomacher bags (Nasco Whirl-Pak^{TM}) and regimented at 300 rpm for 90 s through the use of Lab Blender 400 (Interscience, France). Samples to be analyzed were diluted successively (10^{-1} to 10^{-3}). Flurocult medium specific for *E. coli* was prepared and autoclaved then poured into petri dishes and allow for solidification. One mile of the three dissimilar dilutions for every sample was spread through the use of sterile glass spreader which submerged into a bottle of alcohol and passed it through the Bunsen burner swiftly so that the residue of alcohol will be burn. Samples to be analyzed were makes in triple and kept for 18-24 hours at 37°C. Sample with having positive colonies on the discriminatory medium (selective medium) revealed purple shine coloration, shown as probable of *E. coli* and later confirmed additionally through most probable number (MPN), standard biochemical tests and API20E kit to ratify the strains and were later utilized for PCR subsequently.

2.3. Molecular Identification and Dna Extraction

Extraction of *E. coli* DNA was carried out using the method of Jothikumar and Griffiths [24] with minor adjustment. *E. coli* colony isolated through selective agar from isolation part was enriched by suspending a loopful of colony in 20 ml nutrient broth before *E. coli* DNA extraction was carry out,. The nutrient broth was later incubated over the night (±24 hours) at 37°C. After the over the night incubations, 1.5 ml of solution that consist of bacteria (*E. coli*) were transfer into 1.5 ml micro-centrifuge tubes and the tubes were centrifuged using Minispin (Eppendorf, German) at 10 000 r.p.m. for 10 mins. The supernatant was removed; this is done to get clear off the bacteria pellets which could remain in the micro-centrifuge tubes. 1.5 ml of the broth solution were transfer once more to 2 ml micro-centrifuge tubes through pipetting which consists of bacterial pellets and the tube was spin at 10000 rpm, for 10 mins through the use of Minispin (Eppendorf, German). The bacteria pellets were later utilized for DNA extraction.

The DNA extraction was achieved through addition of 1 ml of sterilized purified water into the micro-centrifuge tubes which consists of the pellets of the bacteria. The tube containing the bacteria pellets was later vortexed for about 10s for proper mixture of the bacterial pellets with purified water and it was subsequently wrapped using parafilm sheet. After that, the tubes containing the samples were floats using water bath for 10 mins at 98°C. After 10 mins the tube was immediately transferred into the freezer of -20°C for 10 mins. All of these steps were repeated again and the tubes finally were centrifuged at 10000 rpm, for 5 mins by the use of Minispin (Eppendorf, German) so that debris from the sample can be removed. The supernatant was move to a fresh sterilized 1.5 ml micro-centrifuge tubes and the DNA concentration was measured through Maestronano Spectrophotometer (Maestrogen, USA). The entire DNA extracted was kept in -20°C until use for subsequent test using PCR [25, 26].

2.4. Plasmid Profiling

2.4.1. Plasmid Extraction

Plasmid isolation was done through the use of the PureYield™ Plasmid Miniprep System kit (Promega, USA) to get the high-quality plasmid DNA following manufacturer's instruction while 600 µl of culture bacteria was full-grown in Luria-Bertani (LB) medium was put into a 1.5 ml micro-centrifuge tubes. Addition and mixing through inverting the tubes about six times in one hundred microliter of cell lysis buffer was done. After the solution change to clear blue from cloudy, it indicates whole lysis. The next step was preceded in 2 min time. Excess lysis could leads in denaturing the plasmid DNA. The subsequent sets of ten samples were continued after the first set is completely neutralized and exhaustively mixed [27, 28]. Next, is the addition of 350 µl of cold (4-8°C) neutralization solution and mixed exhaustively through inverting tubes. The samples change to yellow after neutralization was completed which result to formation of yellow precipitate. The samples were inverted three more time to guarantee whole neutralization. Then samples were centrifuged at high speed for 3 min. After that, the supernatant (~900 µl) was put into a PureYield™ Minicolumn and the cell debris pellets were not upset. For maximum yield, the supernatant was transferred with a pipette. The mini column is later place to a PureYield™ collection tubes, and centrifuged at high revolution for 15 sec. The flow through was thrown away, while the mini column was replace back into the same PureYield™ collection tubes. Addition of 200 µl of Endotoxin Removal Wash to the mini column was done and centrifuged at high revolution for 15 sec. It's not essential to empty the PureYield™ collection tubes. Then, 400 µl of column wash solution was added to the mini column and centrifuged at high revolution for 30 sec. Next, the mini column was relocated to a clean 1.5 ml micro-centrifuge tubes, addition of 30 µl of Elution Buffer right to the mini column matrix was done. The micro centrifuge tube was incubated at room temperature for 1 min and centrifuged at high revolution for 15 sec for elution of the plasmid DNA. The micro centrifuge tube cap was closed and eluted plasmid DNA was kept at –20°C until use for analysis.

2.4.2. Plasmid DNA Analysis

After the plasmid DNA isolated, gel electrophoresis was done in accordance to the technique defined previously from section 3.3.3 (b) to analyse the DNA pattern on 1% (w/v) agarose gel. The gel electrophoresis was run at 85 V for 1 hour and Lambda DNA-HindIII Digest DNA ladder (New England BioLabs, USA) was utilized as DNA marker. Then the gels were view using UV light and documented by gel documentation of GeneSys G: BOX EF2 (Syngene, USA) and gel image was recorded and the plasmid DNA bands produced were analysed [28, 29].

2.5. Sub-typing of Shiga Toxin Producing E. coli

2.5.1. Enterobacterial Repetitive Intergenic Consensus-Polymerase Chain Reaction (ERIC-PCR)

In this section, we used one pair of Enterobacterial Repetitive Intergenic Consensus (ERIC) primer, as reported by Sahilah et al [16]: ERIC-1 (5'-CAC TTA GGG GTC CTC GAA TGT A -3') and ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G -3'). The PCR assay using ERIC primer was done in a concluding volume of 50 µl containing 25 µl of 2X DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 1 µl of 100 µM of each of the forward and reverse primers, 1 µl of about 100 ng DNA templates and nuclease free water marking-up to the volume. Negative controls and positive DNA sample were included in every reaction through addition of 1 µl of roughly 100 ng *E. coli* O157: H7 DNA templates and 10 µl of water (nuclease free) in PCR assay.

Amplification of PCR was done in the MyCycler™ Thermal Cycler (Bio-Rad, USA) with a temperature programme containing of primary denaturation for 1 min at 95°C which was follow by 35 cycles of denaturation's for 45 s at 92°C, annealing for at 52°C for a min and polymerization for 20 mins at 70°C. The concluding elongation was at 70°C for 20 mins. After complete running of the PCR, in the master cycler, the PCR products obtained was electrophoresed through 1.5% agarose gel (w/v) to observe the pattern of DNA band produced on gel surface. The amplicons were analyzed by gel electrophoresis using 1% (w/v) agarose gel in 1X TAE buffer (40 mM Tris-OH, 20 mM acetic acid and 1 mM of EDTA, pH 7.6) at 90 V for 45 mins and pre stained by MaestroSafe™ Nucleic Acid Prestained (Maestrogen, USA). A 1kb DNA ladder (Fermentas, Lithuania) was used as DNA size reference. The gels were view under UV light through gel documentation of GeneSys G: BOX EF^2 (Syngene, USA) and gel image was taken and documented concurrently. The DNA band pattern produced on gel image were captured and recorded then analyzed through dendogram software to get the final results of cluster and single band for this test.

2.5.2. Random Amplified Polymorphic DNA-PCR (RAPD-PCR)

There are seven types of primers used in this section of Random Amplified Polymorphic DNA-PCR (RAPD-PCR) namely Gen8, Gen9, A1, A7, A10, OPAR8 and OPAR20. All of these primers were reported by several different authors and all the primer sequences are in Table 1. The PCR assay for this section was done in 50 µl of total volume consisting of 25 µl of 2X DreamTaq Green PCR Master Mix (Thermo Scientific, USA), 1 µl of 100 µM each of the forward and reverse primers, 1 µl of approximately 100 ng DNA template and nuclease free water markin-up to the volume. A positive and negative control was included together each time when the PCR was carried out for different types of primers.

Temperature cycle of PCR amplification was set by the use MyCycler™ Thermal Cycler (Bio-Rad, USA) that carried out separately for each different type of primers. Table 2 showed temperature cycle of different primers used. Usually the PCR temperature cycle involved temperature of denaturation, annealing and extension or elongation. After finished PCR amplification, the amplified DNA was observed through 1% (primers of A1, A7 and A10) and 1.5% (primers of Gen8, Gen9, OPAR8 and OPAR20) of agarose gel at 100 V for 45 mins. The DNA band pattern produced on gel image were then analyzed through dendogram unweight average linkage analysis (UPGMA) software to get the final results.

Table 1. *List of primer sequences used in RAPD-PCR analysis.*

Primer Name	Sequences (5'-3')	Reference
Gen8	5'-GGA AGA CAA C -3'	Sahilah et al. (2010)
Gen9	5'-AGA AGC GAT G -3'	
A1	5'-TGC GGC TTA C -3'	Hurtado and Rodríguez-Valera (1999)
A7	5'-TCA CGG TGC A -3'	
A10	5'-GTA GAC GAG C -3'	
OPAR8	5'-TGG GGC TGT C -3'	Nor'aishah (2008)
OPAR20	5'-ACG GCA AGG A -3'	

Table 2. *Temperature cycle for RAPD-PCR amplification.*

Primer Name	PCR cycle
Gen8 Gen9	Initial denaturation at 94°C for 5 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and polymerization at 72°C for 2 min. Final elongation was at 72°C for 7 min.
A1 A7 A10	35 cycles of: denaturation at 94°C for 30 s, annealing at 36°C for 1 min, and extension at 72°C for 2 min. A final extension was performed at 72°C for 10 min.
OPAR8 OPAR20	Initial denaturation at 94°C for 5 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and polymerization at 72°C for 2 min. Final elongation was at 72°C for 7 min.

3. Result

3.1. Plasmid Profiling

Plasmid profiling showed 16 patterns based on size and 4 patterns on gel (Table 3 Figures 1 and 2). Using both the phenotypical and genotypical approaches revealed a wide heterogeneity among imported beef isolates of *E. coli*. The plasmid profiles of the isolates are listed in Table 3.

In this study, isolated plasmids from shiga toxin producing *E. coli* varied in sizes. The sizes are from 4.3 -23.1 kilo base

(kb) Table 3. All the isolates harbored plasmid except only one strain. Sixty two (62) isolates were found to harbor plasmid with size 23.2 kb while 43 isolates harbored more than a single plasmid.

Table 3. *Plasmid profiles analysis of shiga toxin producing E. coli.*

Strain No.	Locations	Plasmid profiles		
		Plasmid sizes (Kb) (patterns)		
1	Carrefour Alamanda-Indian Buffalo Block frozen	(A)	23.1	(P1)
2	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
3	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
4	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1, 23.2	(P2)
5	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
6	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
7	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1, 23.2	(P2)
8	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
9	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
10	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1, 20	(P3)
11	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
12	Tesco Kajang -Indian Buffalo Tlon FZ	(A)	23.1	(P1)
13	Tesco Kajang -Indian Buffalo Tlon FZ	A2	23.1	P1
14	Tesco Kajang -Indian Buffalo Tlon FZ	A2	23.1	P1
15	Tesco Kajang -Indian Buffalo Tlon FZ	A2	23.1	P1
16	Tesco Kajang -Indian Buffalo Tlon FZ	A2	23.1	P1
17	Tesco Kajang - India Buffalo CTEN FZ	A4	23.1	P1
18	Tesco Kajang - India Buffalo CTEN FZ	A3	23.1, 9.4	P4
19	India Buffalo CTEN FZ	A2	23.1, 9.4	P4
20	Carrefour Mid Valley-Indian topside	A5	23.1	P1
21	Carrefour Mid Valley-Indian topside	A2	23.1	P1
22	Tesco Kajang -Indian Cube FRZ	A2	23.1	P1
23	Carrefour Alamanda-Indian topside	A2	23.1	P1
24	Jusco Balakong-India buffalo FQ site L	A6	23.1	P1
25	Giant Seri Kembangan-Indian buffalo block	A6	23.1, 4.3	P5
26	Giant Seri Kembangan-Indian buffalo	A7	23.1, 23.2	P2
27	Giant Seri Kembangan-Indian buffalo block	A2	23.1, 23.2	P2
28	Giant Seri Kembangan-Indian buffalo block	A6	23.1, 23.2	P2
29	Giant Seri Kembangan-Indian buffalo block	A8	23.1, 23.2, 6.5	P7
30	Giant Seri Kembangan-Indian buffalo	A7	23.1, 23.2	P2
31	Giant Seri Kembangan-Indian buffalo block	A3	23.1, 23.2	P2
32	Giant Seri Kembangan-Indian buffalo block	A8	23.1	P1
33	Giant Seri Kembangan-Indian buffalo block	A8	23.1	P1
34	Giant Seri Kembangan-Indian buffalo	A8	23.1, 23.2,20	P6
35	Giant Seri Kembangan-Indian buffalo block	A2	23.1, 23.2	P2
36	Giant Seri Kembangan-Indian buffalo block	A2	23.1	P1
37	Giant Seri Kembangan-Indian buffalo block	A2	23.1	P1
38	Giant Seri Kembangan-Indian buffalo block	A8	23.1	P1
39	Giant Seri Kembangan-Indian buffalo block	A8	23.1	P1
40	Giant Seri Kembangan-Indian buffalo block	A9	23.1	P1
41	Giant Seri Kembangan-Indian buffalo block	A2	23.1,4.3	P5
42	Giant Seri Kembangan-Indian buffalo block	A8	23.1	P1
43	Carrefour Alamanda-AUST. TOPSIDE ST	A10	23.1,20, 4.3	P8
44	Carrefour Alamanda-AUST. TOPSIDE ST	A8	23.1, 23.2,20, 6.5	P9
45	Carrefour Mid Valley-AUST. Aris Tips	A10	23.1, 23.2,6.5, 4.3	P10
46	Carrefour Mid Valley-AUST. Rumpsteak	A8	23.1	P1
47	Carrefour Mid Valley-AUST. KNUCKEL BL	A8	23.1, 23.2,20	P6
48	Carrefour Mid Valley- AUST. outside BL	A8	23.1, 23.2	P2
49	Carrefour Mid Valley-AUST. TOPSIDE BL	A8	23.1	P1
50	Tesco Kajang-AUST. Daging pejal	A8	23.1, 23.2, 4.3	P11
51	Carrefour Mid Valley-AUST. Tender steak	A8	23.1, 9.4	P4
52	Carrefour Mid Valley-AUST. Round steak	A8	23.1, 23.2, 6.5	P7
53	Carrefour Mid Valley-AUST. For BBQ	A8	23.1, 20,4.3	P8
54	Carrefour Alamanda-AUST. Topside ST	A2	23.1,23.2,20	P6
55	Carrefour Mid Valley-AUST. SOUPM	A11	23.1,23.2,20,9.4	P12
56	Carrefour Mid Valley- AUST. Iris tipis	A8	23.1	P1
57	Carrefour Alamanda-AUST. Topside ST	A8	23.1	P1

Strain No.	Locations	Plasmid profiles		
		Plasmid sizes (Kb) (patterns)		
58	Carrefour Mid Valley-AUST. Round steak	A2	23.1	P1
59	Carrefour Mid Valley-AUST. Rumpsteak	A8	23.1,23.2,20, 9.4, 6.5	P13
60	Giant Seri Kembangan-AUST. Beef BL OCK	A8	23.1, 23.2,20	P6
61	Giant Seri Kembangan-AUST. Beef CU BES	A8	23.1,20,4.3	P8
62	Jusco Mid Valley-AUST. CH Rump yak In IKU	A8	23.1	P1
63	Jusco Balakong-AUST. Chilled topside steak	A3	23.1, 20	P3
64	Jusco Balakong-AUST. Chilled topside steak	A8	23.1	P1
65	Carrefour Alamanda-AUST. Prime eye round	A8	23.1, 6.5,4.3	P14
66	Carrefour Alamanda-AUST. Topside ST	A3	23.1, 6.5,4.3	P14
67	Carrefour Alamanda-AUST. Topside ST	A3	-	
68	Carrefour Alamanda-AUST. Toside S	A12	4.3	P15
69	Carrefour Mid Valley-AUST. Chuck Tend	A8	6.5, 4.3	P16
70	Carrefour Alamanda-AUST. KNUCKEL BL	A8	23.1, 23.2,20	P6

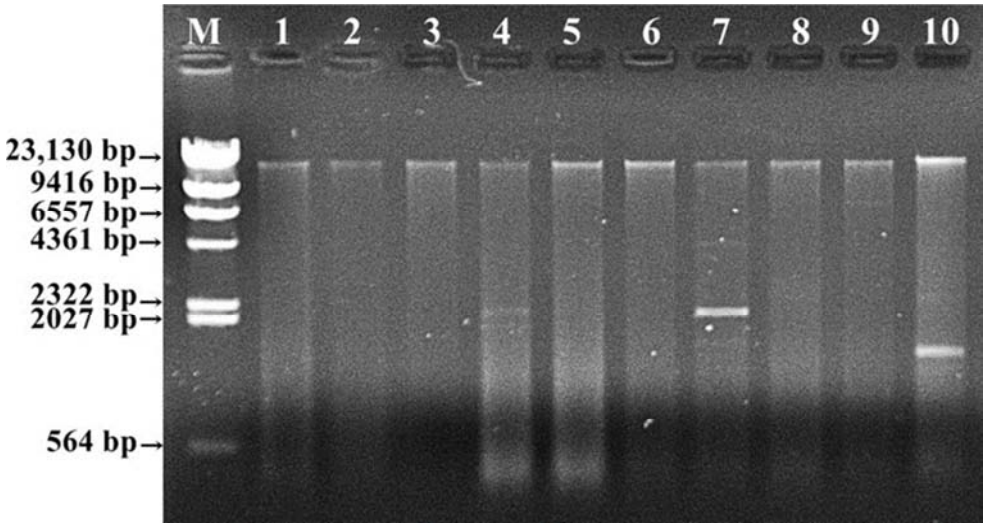

Figure 1. *Plasmid identification using PureYield™ Plasmid Miniprep System kit (Promega, USA) on 1% (w/v) agarose gel. Lane M: Lambda. DNA-HindIII Digest DNA ladder. Lane 1-10: EC.*

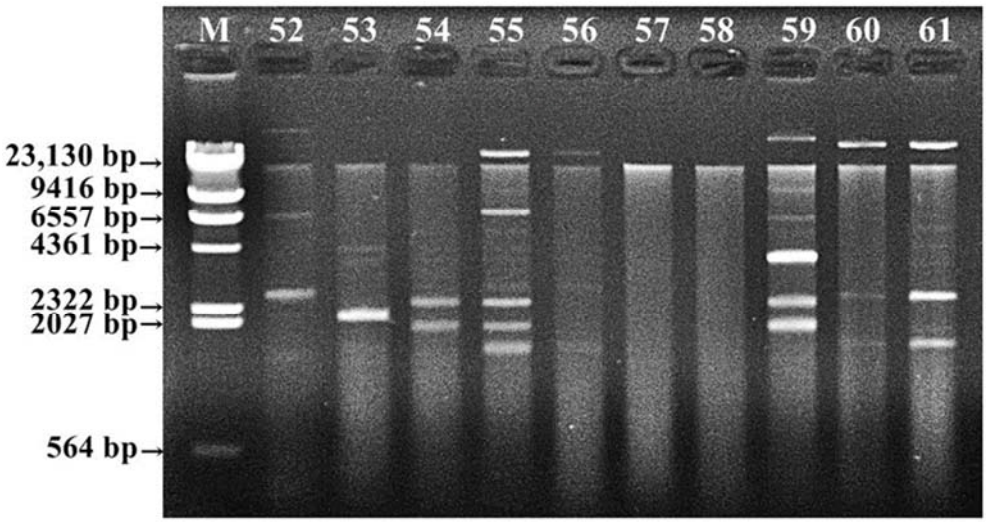

Figure 2. *Plasmid identification using PureYield™ Plasmid Miniprep System kit (Promega, USA) on 1% (w/v) agarose gel. Lane M: Lambda DNA-HindIII Digest DNA ladder. Lane 52-60: EC.*

3.2. Eric-pcr and Rapd-pcr Analysis

The outcomes of the ERIC (Figures 3 and 4), RAPD-PCR (Figures 5 and 6) were analyzed using Gel Compare 11 software, the rest of the results shown in figure 7 to 13. ERIC-PCR with primers ERIC1R and ERIC2 discriminated the *E. coli* strains into 4 clusters and 2 single isolates at a similarity level of 80% figure 14. RAPD-PCR with primer Gene 8 distinguished the *E.*

coli strains into 15 clusters and 8 single strains figure 15, RAPD-PCR using primer Gene 9 distinguished the *E.coli* into 16 clusters and 8 single strains figure 16, while RAPD-PCR using primer Opar 8 differentiated the *E. coli* into 8 clusters and 3 single strains figure 17, RAPD-PCR using primer Opar 20 differentiated the *E. coli* into 7 clusters and 4 single strains figure 18, RAPD–PCR using primer A1 differentiated the *E. coli* into 3 clusters and 6 single strains figure 19, RAPD-PCR using primer A7 differentiated the *E. coli* into 12clusters and 6 single strains figure 20 and RAPD using primer A10 differentiated the *E. coli* into 8 clusters and 4 single strains figure 21.

In this work, the use of molecular typing technique, ERIC-PCR, and RAPD-PCR methods were utilized to subtype *E. coli* isolated from imported frozen meat sold in Malaysia hyper and super markets. The analysis of data through the use of average linkage (UPGMA, unweight group pair technique with arithmetic averages) using Gel compare 11 software which was displayed in dendrograms, for the, ERIC-PCR and RAPD-PCR (RAPD Gen 8, Gen 9), (Opar 8 and Opar 20), (RAPD A1, RAPD A7and RAPD A10) analysis, produced various cluster as follows: ERIC-PCR produces 4 clusters and 2 single isolates and RAPD-PCR (RAPD Gen 8, Gen 9), produces 15 clusters and 8 single isolates, 16 clusters and 8 single isolates respectively figures 14 and 15. The PCR analysis using OPAR 8 and OPAR 20 produces 8 clusters and 3 single isolates and 7 clusters with 4 single isolates at a similar levels observed respectively while, RAPD A1, RAPD A7and RAPD A10 produces 3 clusters and 6 single isolates, 12 clusters and 6 single isolates and 8 clusters and 4 single isolates respectively figures 15

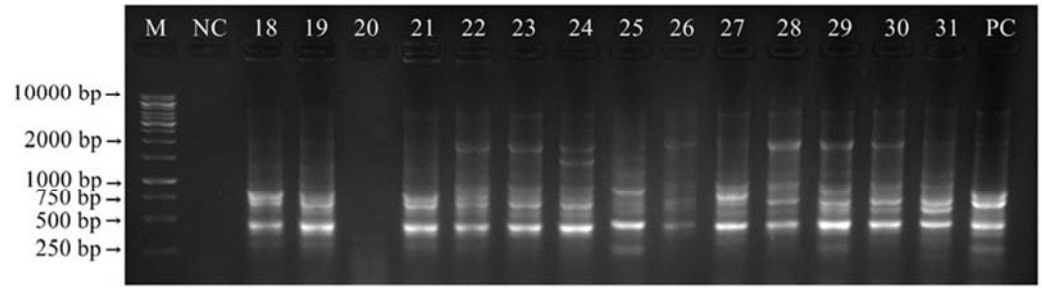

Figure 3. *ERIC-PCR using ERIC 1and ERIC 2 primers on 1.5% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 18-31: EC. Lane PC: positive control.*

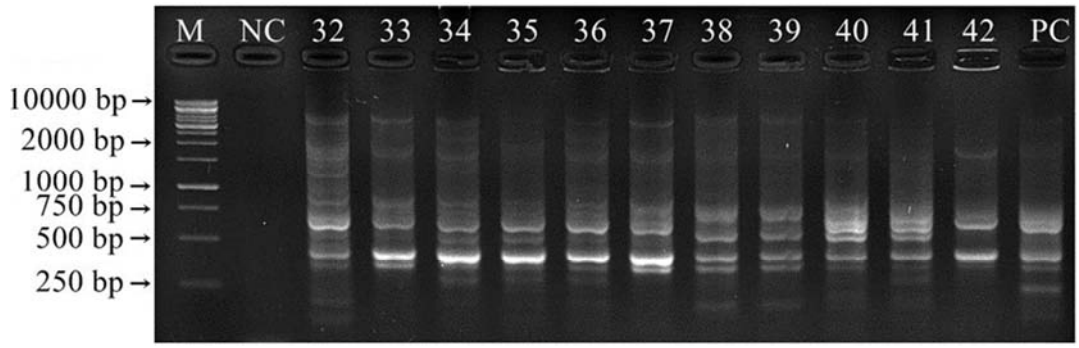

Figure 4. *ERIC-PCR using ERIC 1and ERIC 2 primers on 1.5% (w/v) agarose gel. Lane M: ladder. Lane NC: negative control. Lane 32-42: EC. Lane PC: positive control.*

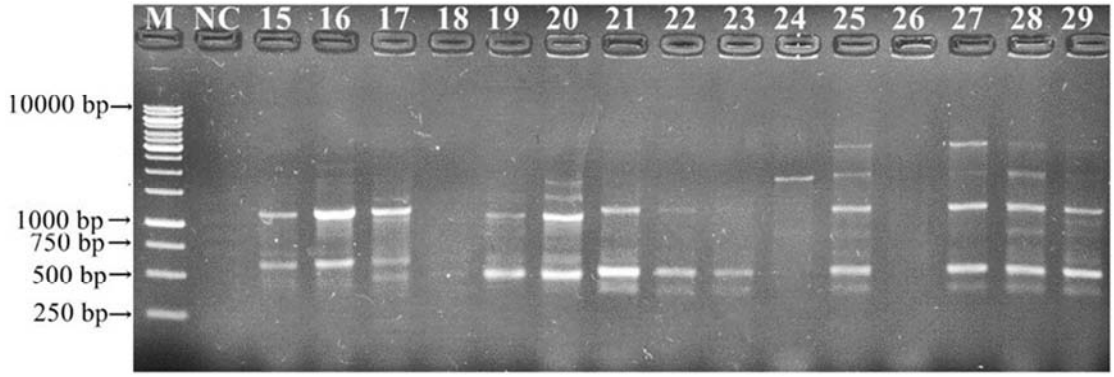

Figure 5. *RAPD-PCR using GENE 8 primer on 1.5% (w/v) agarose gel. M: 1 kb ladder. Lane NC: negative control. Lane 15-29: EC.*

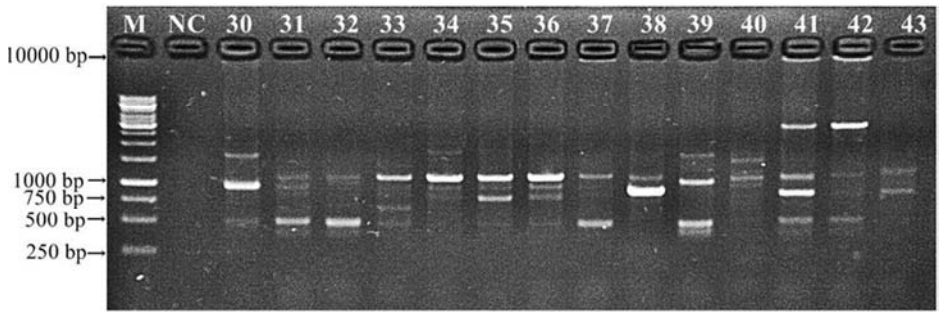

Figure 6. *RAPD-PCR using GENE 8 primer on 1.5% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 30-43: EC.*

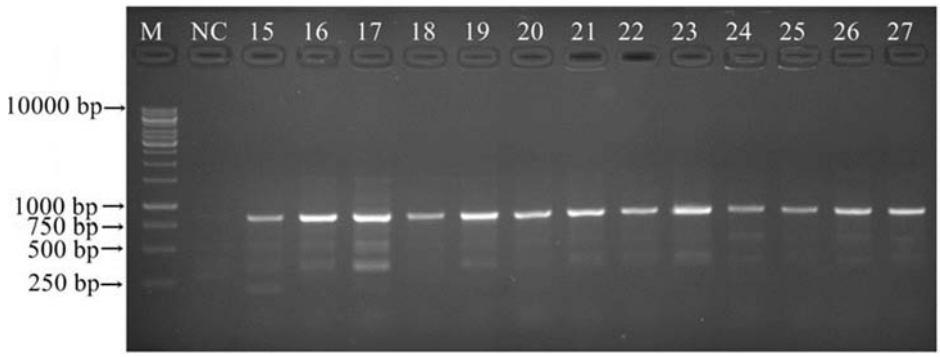

Figure 7. *RAPD-PCR using GENE 9 primer on 1.5% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 15-27: EC.*

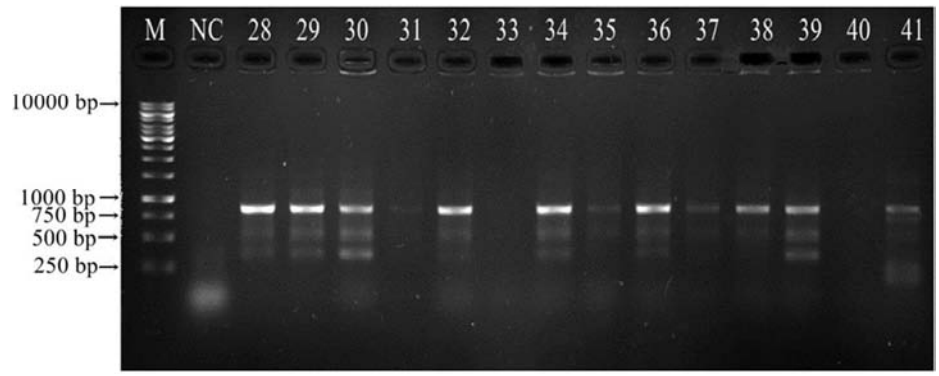

Figure 8. *RAPD-PCR using GENE 9 primer on 1.5% (w/v) agarose gel. Lane M: 1kb ladder. Lane NC: negative control. Lane 28-41: EC.*

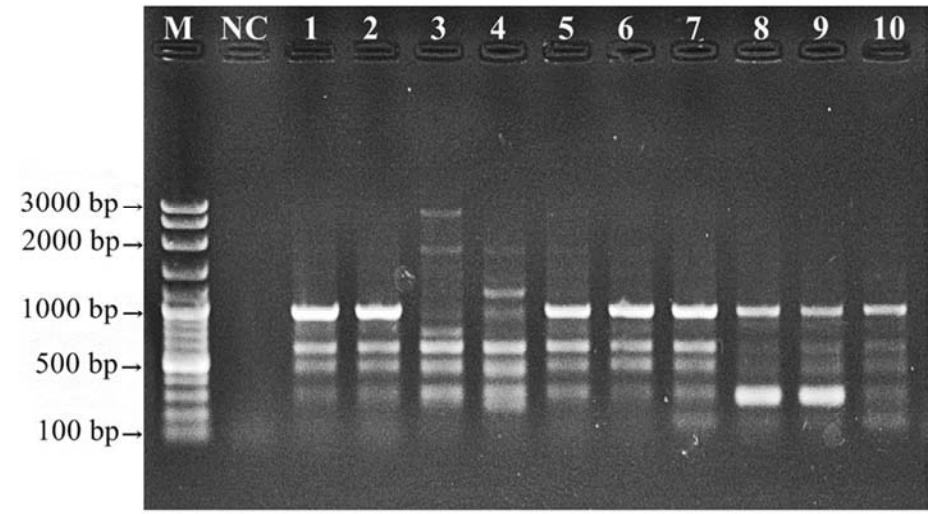

Figure 9. *RAPD-PCR using OPAR 8 primer on 1.5% (w/v) agarose gel. Lane M: 123 bp ladder. Lane NC: negative control. Lane 1-10: EC.*

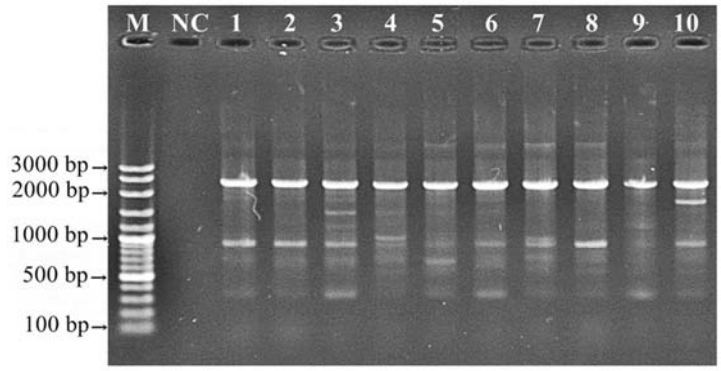

Figure 10. *RAPD-PCR using OPAR 20 primer on 1.5% (w/v) agarose gel. Lane M: 123 bp ladder. Lane NC: negative control. Lane 1-10: EC.*

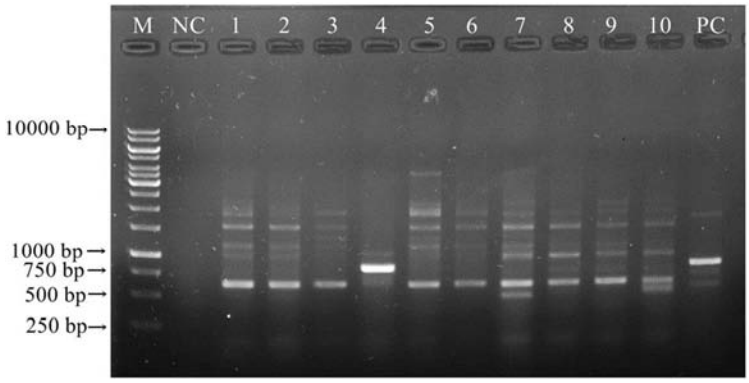

Figure 11. *RAPD-PCR using A1 primer on 1.0% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 1-10: EC. Lane PC: positive control.*

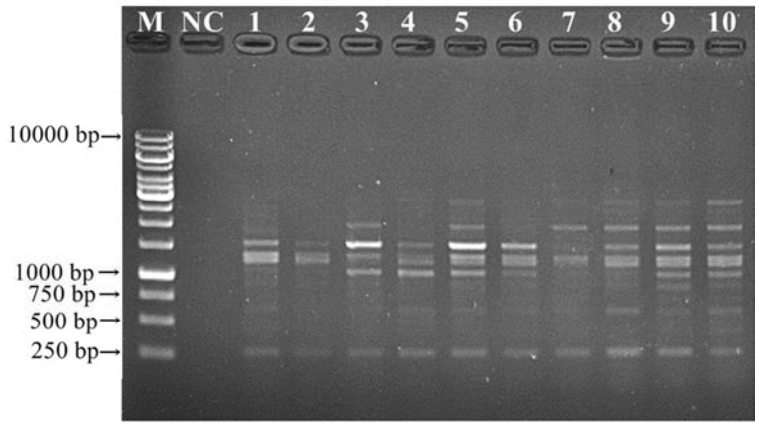

Figure 12. *RAPD-PCR using A7 primer on 1.0% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 1-10: EC.*

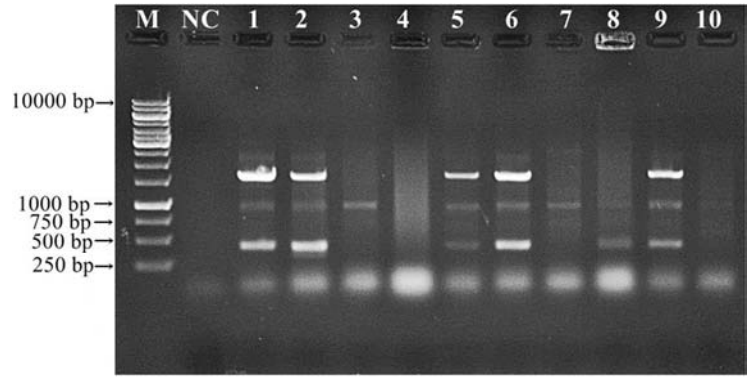

Figure 13. *RAPD-PCR using A10 primer on 1.0% (w/v) agarose gel. Lane M: 1 kb ladder. Lane NC: negative control. Lane 1-10.*

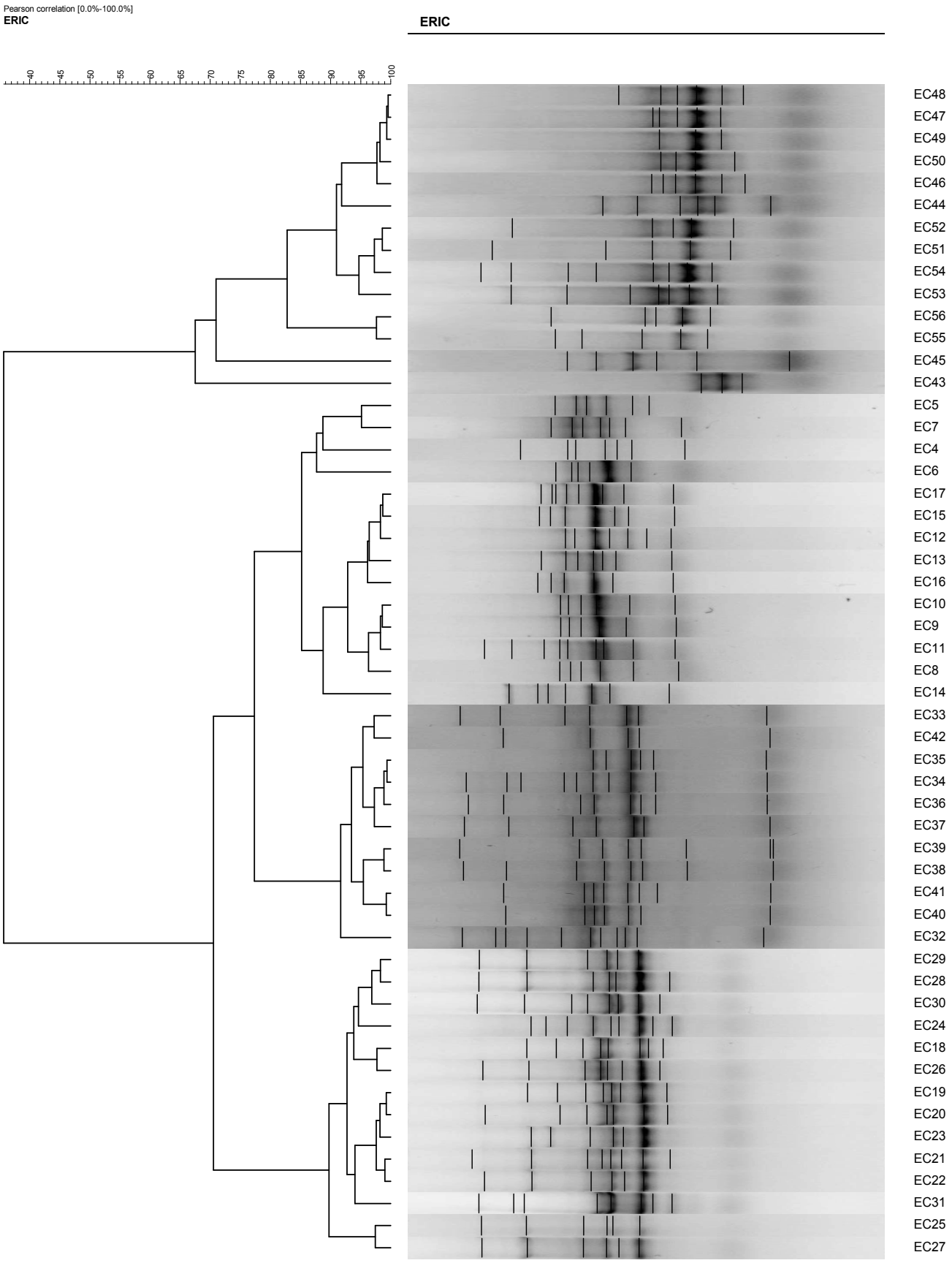

Figure 14. *Dendogram of typeable E. coli isolates produced from ERIC analysis using average linkage unweighted group pair method with arithmetic average (UPGMA).*

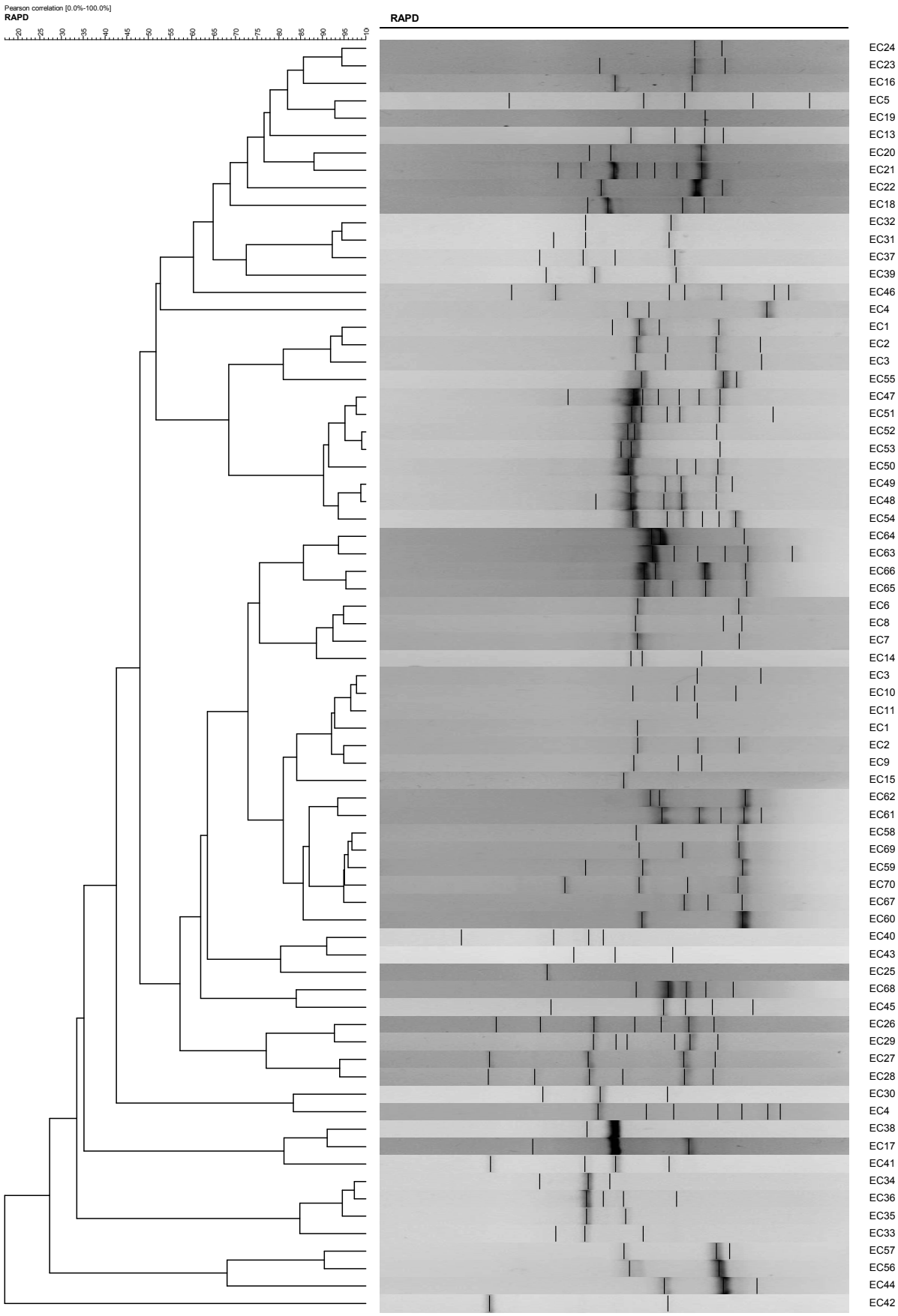

Figure 15. *Dendogram of typable E. coli isolates from RAPD analysis (Gen 8) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

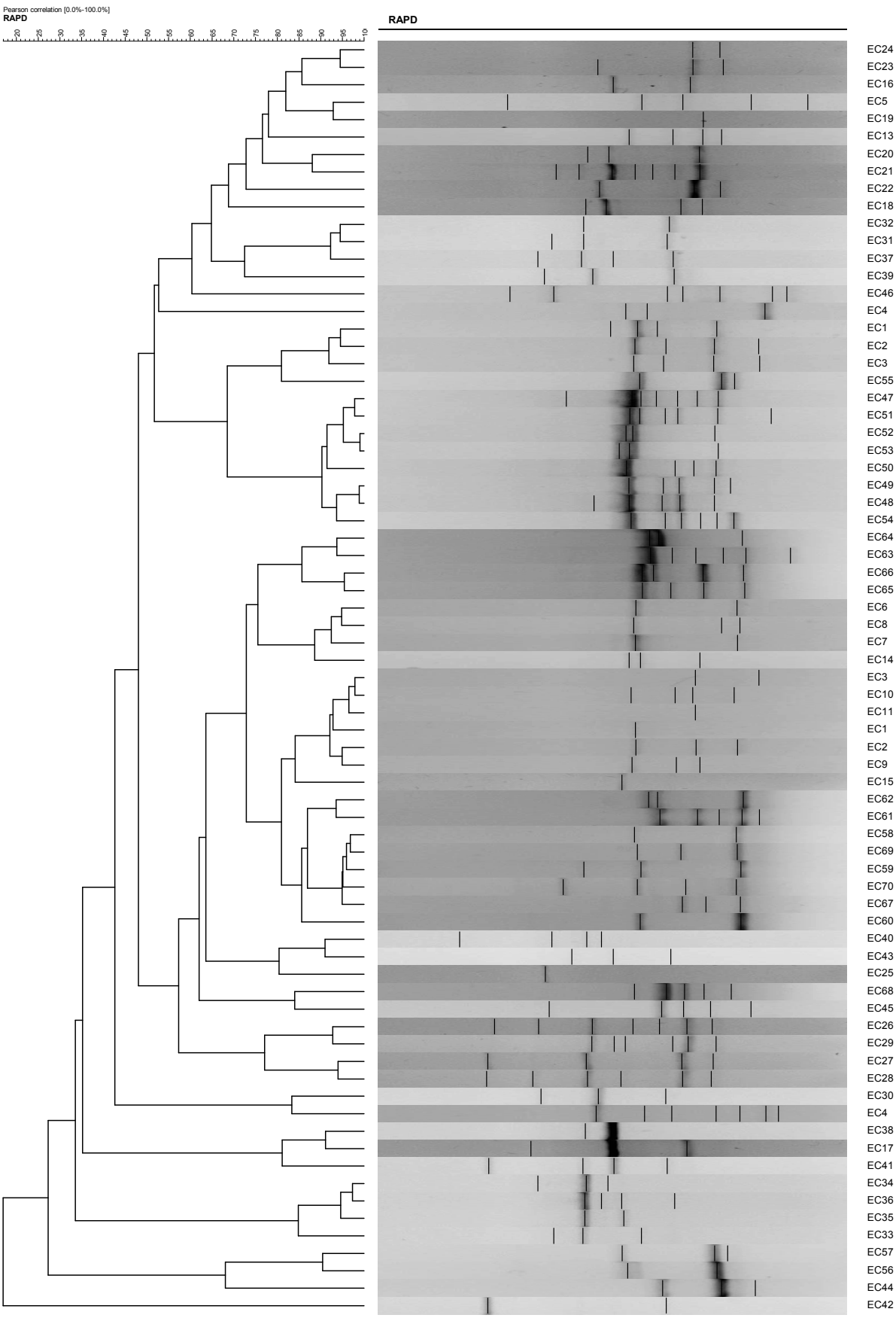

Figure 16. *Dendogram of typable E.coli isolates from RAPD analysis (Gen 9) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

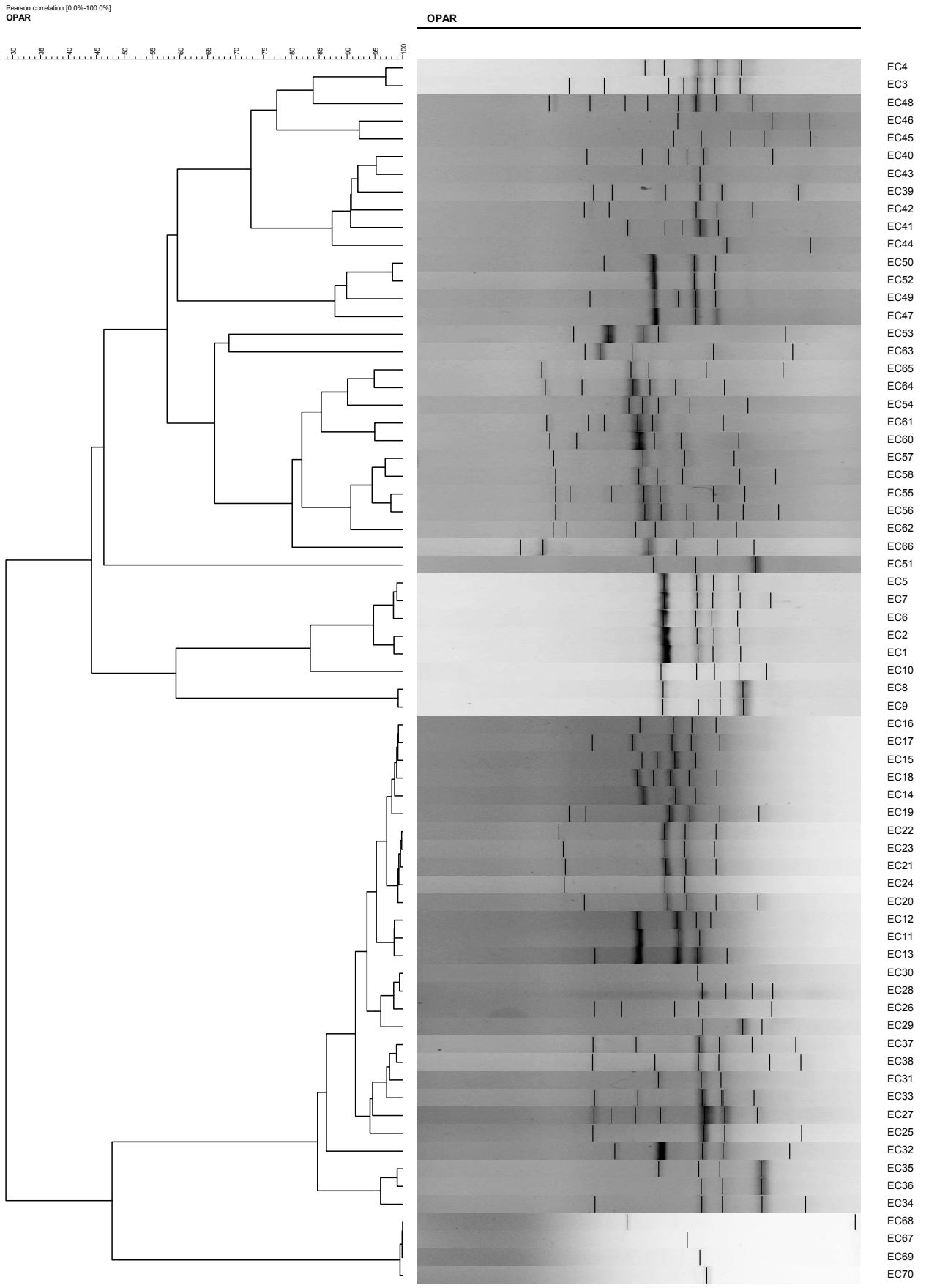

Figure 17. *Dendogram of typable E. coli isolates from RAPD analysis (primer Opar 8) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

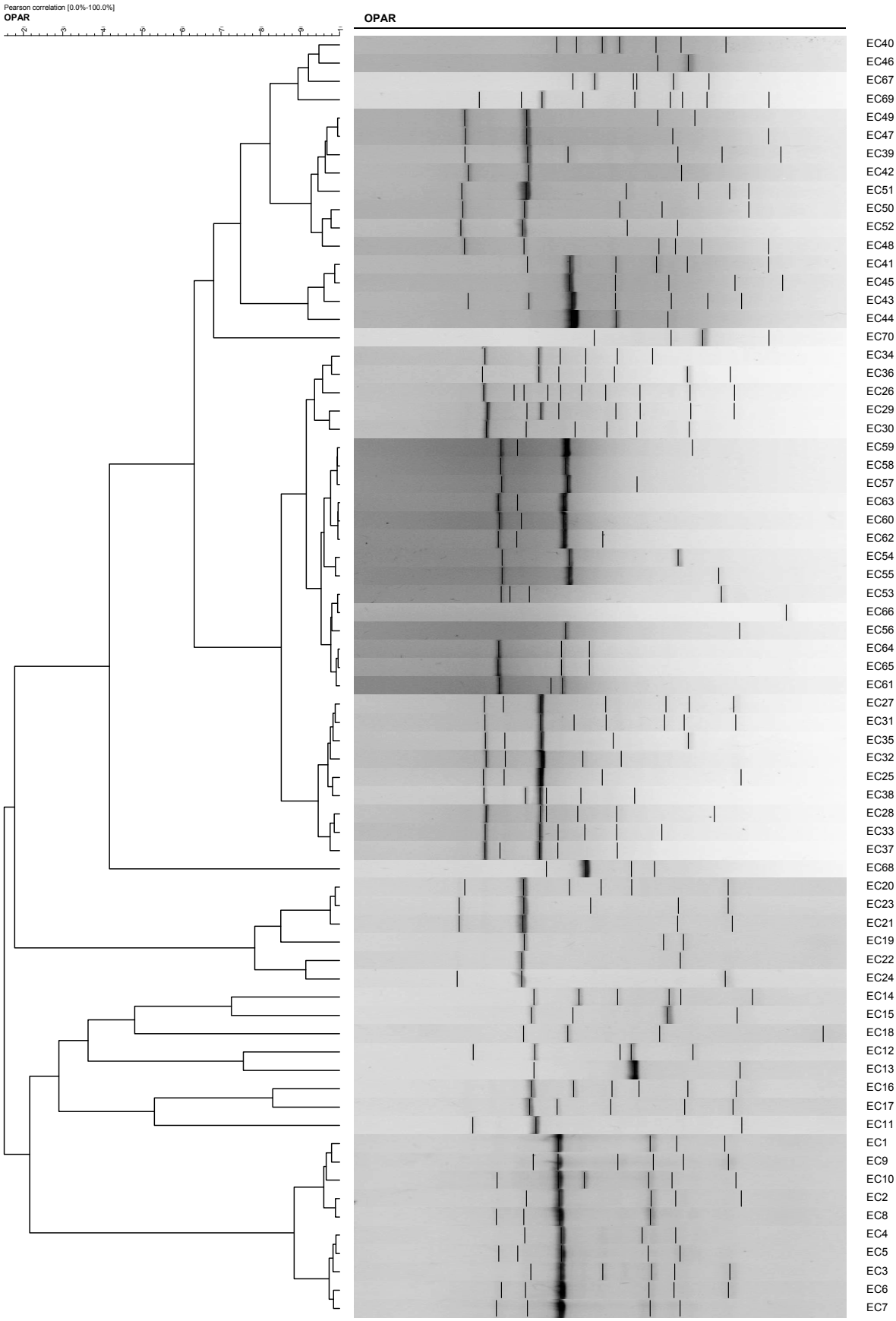

Figure 18. *Dendogram of typable E. coli isolates from RAPD analysis (primer Opar 20) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

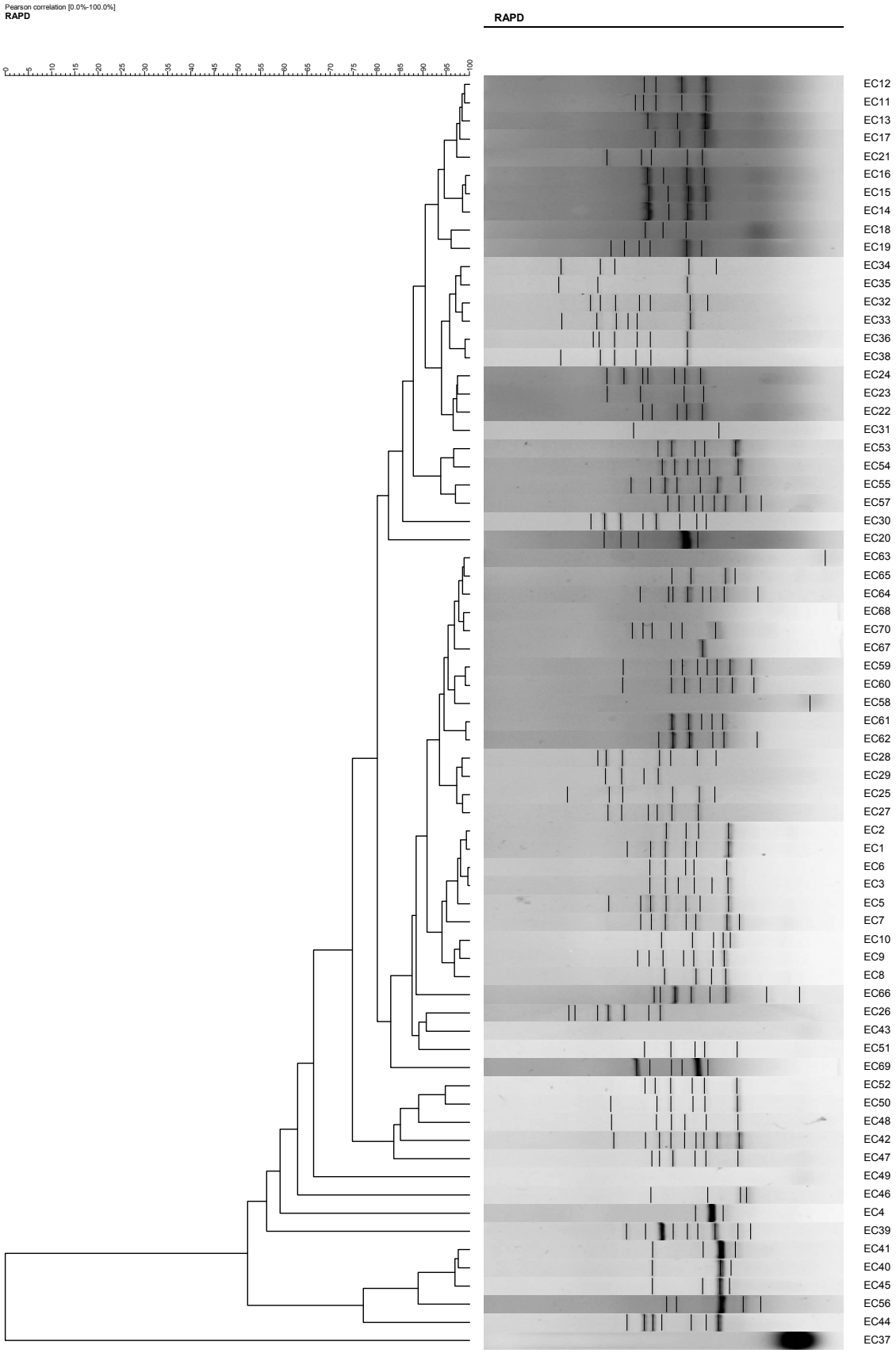

Figure 19. *Dendogram of typable E. coli isolates from RAPD analysis (primer A1) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

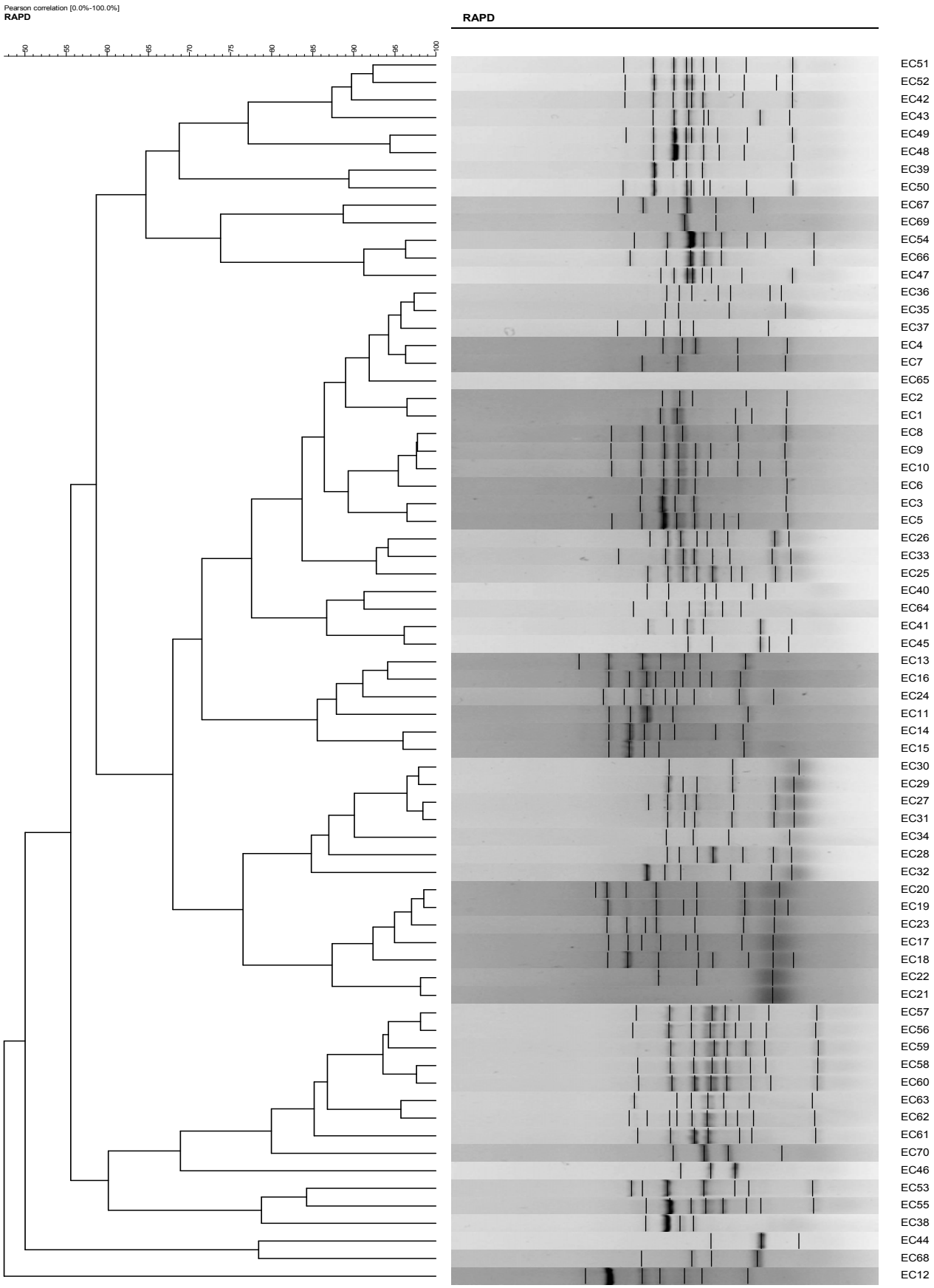

Figure 20. *Dendogram of typable E.coli isolates from RAPD analysis (primer A7) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

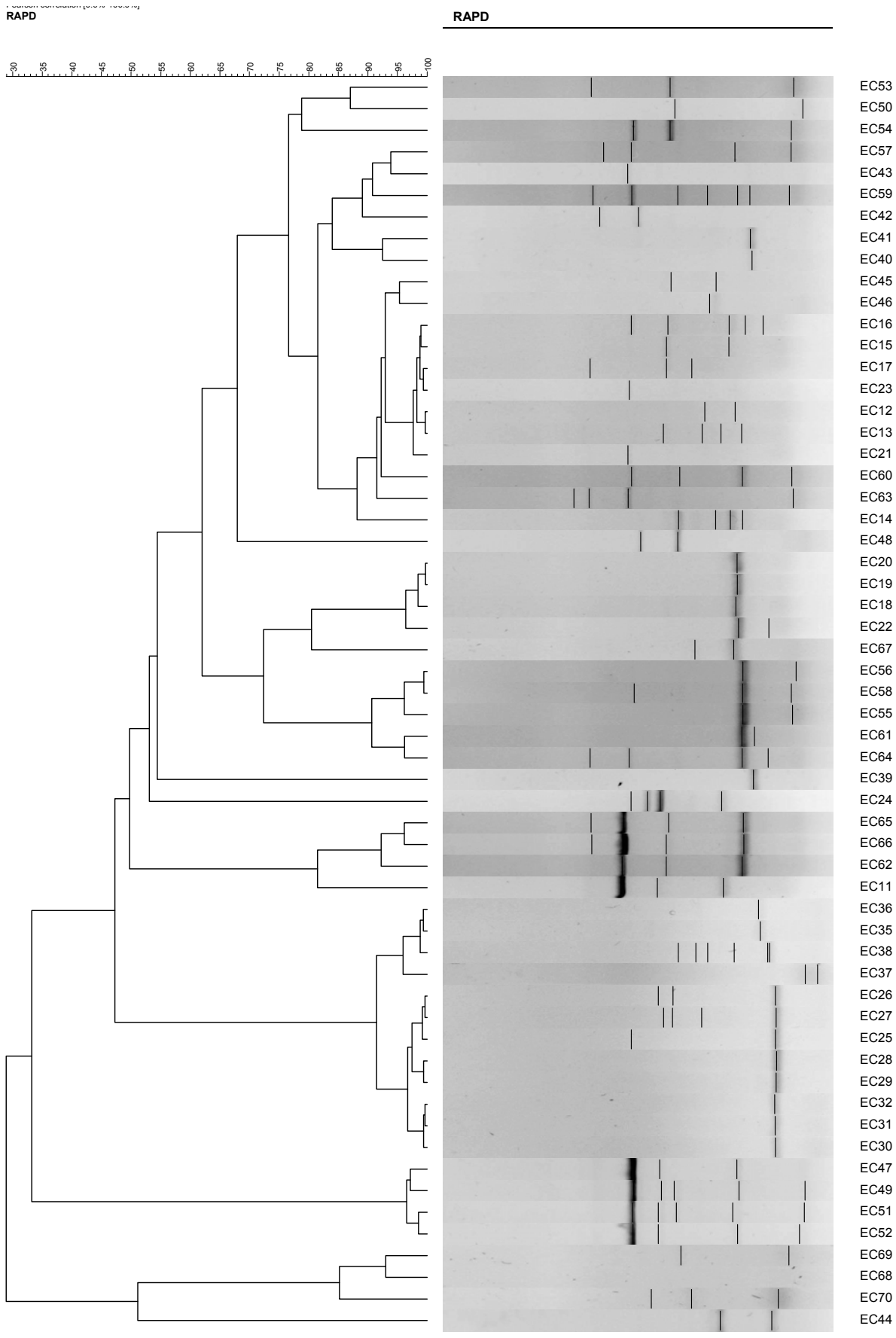

Figure 21. *Dendogram of typable E.coli isolates from RAPD analysis (primer A10) using average linkage unweighted group pair method with arithmetic average (UPGMA).*

4. Discussion

In this work, the applications of the molecular typing technique, ERIC-PCR, and RAPD-PCR methods were used to subtype *E. coli* isolated from imported frozen meat sold in Malaysia hyper and super markets. Sixty two (62) isolates were found to harbor plasmid with size 23.2 kb while 43 isolates harbored more than a single plasmid. According to Kesava et al [30], profiling of plasmid is among the many valuable techniques to determine the relationship or non-relationship of bacterial strains which consist of plasmid DNA, Isolates with single plasmids are mostly isolated from buffalo meat. Recently a work done by Kesava et al [30], comparable results were observed, in their study, it was observed that whereas the 21 kb plasmid was noticed in 23.3% (7) of shiga toxin producing *E. coli* and the smaller plasmids 3 kb and 2 kb plasmids were identified in 16.6% (5) of the isolates from beef.

Osaili et al [31], revealed that cows remain the major reservoirs of shiga toxin producing *E. coli* which may not show any clinical sign of the disease and the disease can cause ailment in human beings. Recently Osaili et al [31], in their work revealed that 7.8% of beef samples were found to be polluted with shiga toxin producing *E. coli*. In this work, occurrence of shiga toxin producing *E. coli* was considerably high comparing to the work done in South Africa that shows the rate of rate of shiga toxin producing *E. coli* to be74.5% [24, 32]. Both the genotypical and phenotypical methods revealed a varied heterogeneity amongst imported beef isolates of *E. coli*. Our result which shows the occurrence of shiga-toxin producing *E. coli* is rather disturbing and ought to draw a level of interest of the public health authorities of possible danger from imported beef meats.

Incidence and incidences of shiga-toxin producing *E. coli* have been investigated in raw meats in diverse region of the globe. Recently a work done in Egypt by Ahmed and Shimamoto [33], revealed that 3.4% of the samples of raw meat were positive of shiga-toxin producing *E. coli* out of the total 800 meat sample tested. Likewise, in Saudi Arabia 11.33% were positive for shiga-toxin producing *E. coli* out of the total 150 raw meats samples examined [34]. Hessain et al [35] also revealed that 2% of the total 200 raw meat samples examined were positive of shiga-toxin producing *E. coli.* Though, biochemical examinations are not sufficient in confirming isolates as *E. coli*; hence genotypic approaches of Random amplified polymorphic DNA-polymerase PCR (RAPD-PCR), Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) and plasmid profiling are very important in confirming the biochemical test.

The outcome of the ERIC (Figures 3 and 4), RAPD-PCR (Figures 5 and 6) were analyzed using Gel Compare 11 software. RAPD-PCR analysis depends on the DNA polymorphisms within *E. coli* strains which are amplified during PCR procedure producing DNA fingerprints of different dimensions for differentiation purpose. According to Sahilah [16], application of DNA-based techniques in examining genetic relatedness of *E. coli* could aid in elucidating the epidemiology of STEC and EHEC isolates from different sources. The precise determinations of clones of *E. coli* are significant in the study of human epidemiologic outbreaks of STEC or EHEC. The method has been utilized successfully in determining the genetic similarity and epidemiological studies of *E. coli*. Recently, Sahilah et al [16] used the method to show the occurrence of varied heterogeneity within eggs and beef meat isolates of *E. coli*. Salehi et al. [36] used this technique for genetic differentiation of *E. coli* isolated from chicken. It has also been used to study epidemiology of *E. coli* isolated from human and animal feces [37]. The various genotypic patterns obtained in this studies based on the different genes used are as shown in Table 4. The patterns were obtained based on 7 genes analyzed. In this study, not all the isolates used were typeable using ERIC primers. Different detection pattern was observed among the RAPD primers used in this study. Five isolates (31, 37, 43, 58 and 63) were not detected by primer A1. A7 primer could not detect 2 isolates (21 and 65) (Table 4). However, primer A10 could not detect the highest number of the isolates (4, 10, 12, 33, 37, 45 and 68) compared to other primers. Gene 8 primer could not detect (ND) with 3 isolates (11, 12 and 26), Gene 9 could not detect 3 isolates (31, 33 and 40) likewise; Opar 8 could not detect 5 isolates (28, 30, 58, 67 and 70). Whereas Opar 20 (55 and 62) could not detect two isolates (Table 4), gel picture are not shown. Therefore, primer may not be good for genetic relatedness study of *E. coli* isolated from imported beef and also may not be useful for epidemiological study. The highest pattern was observed in A7 gene. Gene 9 has the lowest genotyping of the isolates while Gene 8 has 58 genotypes, A1 (56), Opar 20 (55) and Opar 8 (54). Based on the results in this study, ERIC PCR profiled the isolates into 64. Hence, ERIC PCR discriminated the isolates better than RAPD.

From our result, there was no specific order and clustering that will be associated to *E. coli* within the different source of the samples and also in the different shops or location of sample purchase using all the three technique (ERIC and RAPD -PCR), this shows a very wide heterogeneity of *E. coli* isolate within and without. It also shows that isolate of *E. coli* from different countries and location could be similar and isolate from the same country or location could be quietly different. The *E. coli* isolate in this study exhibit a level of genetic variation which was very clear using all the two technique (ERIC and RAPD-PCR), the RAPD-PCR revealed different amplification products leading to a configuration of bands usually from three to fifteen, with the size ranging from 200 bp and 3 k bp. The strength of the different band is not consistent; nevertheless both the size and strength of every band can be reproduced using similar situation. According to Yang et al [38], RAPD-PCR genotyping method has the leads because of the less labor-intensiveness involve in the procedure, is also fast to completing the process and the methods is based on nucleotide sequence differences in the whole genome.

Numerous molecular method of typing for example pulsed-field gel electrophoresis (PFGE), enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) ribosomal gene spacer sequence PCR (RS-PCR) and repetitive extragenic palindromic sequence PCR (REP-PCR) have been reported to subspecies differentiation of bacteria globally which could assist epidemiological investigations during disease outbreaks. RAPD molecular fingerprinting analysis was used in this study to principally understand the molecular relatedness of shiga toxin producing *E. coli* isolated from beef meats imported and sold in Malaysia from two major countries. In order to increase the reproducibility of RAPD analysis, 6 primers were used in this study. The discriminatory power (D-value) of this RAPD fingerprinting assay was 0.85. This was in support of the method of [39] who investigated molecular characterization of *E. coli* 0157: H7. According to Maurer et al [40], RAPD show higher genetic variances amongst avian *E. coli* strains than other molecular typing methods for example restriction fragment length polymorphism (RFLP) analysis. Similarly, Ji-Yeon Kim et al. [39] state that RAPD analysis used in this study has advantages of less time consuming or laborious, cost-effective, sensitive, and no distinctive talents needed to do it.

Table 4. *Molecular typing of E. coli (producing shiga toxin).*

Strain No.	Shiga toxin		ERIC profile	RAPD Profile using different primers							Genome type
	Stx_1	Stx_2		Gene 8	Gene 9	Opar8	Opar 20	A1	A7	A10	
1			E1	A1	B1	C1	D1	F1	G1	H1	1
2			E2	A1	B2	C1	D1	F1	G2	H1	2
3			E3	A1	B2	C2	D2	F2	G3	H2	3
4			E4	A2	B3	C3	D3	F3	G4	ND	4
5			E5	A3	B3	C4	D4	F4	G5	H3	5
6			E6	A4	B4	C4	D5	F5	G6	H3	6
7			E7	A4	B5	C5	D6	F6	G7	H4	7
8			E8	A5	B6	C6	D7	F7	G8	H5	8
9			E8	A5	B7	C6	D8	F7	G9	H5	9
10			E8	A6	B8	C7	D9	F8	G10	ND	10
11			E9	ND	B9	C8	D10	F9	G11	H6	11
12			E10	ND	B9	C8	D11	F10	G12	ND	12
13			E11	A7	B9	C9	D12	F11	G12	H7	13
14			E12	A8	B10	C10	D13	F12	G13	H8	14
15			E13	A9	B11	C11	D14	F12	G13	H9	15
16			E14	A10	B12	C12	D15	F12	G13	H10	16
17			E15	A11	B12	C13	D16	F13	G14	H11	17
18			E16	ND	B13	C14	D17	F14	G15	H12	18
19			E17	A12	B14	C15	D18	F15	G16	H13	19
20			ND	A13	B15	C15	D19	F16	G16	H14	20
21			E18	A14	B15	C16	D20	F17	ND	H15	21
22			E18	A15	B15	C16	D20	F17	G17	H16	22
23			E19	A16	B15	C16	D21	F19	G18	H17	23
24			E20	A17	B16	C16	D21	F20	G19	H18	24
25			E21	A18	B17	C17	D22	F21	G20	H19	25
26			E22	ND	B17	C18	D23	F22	G21	H19	26
27			E23	A18	B17	C19	D24	F23	G22	H19	27
28			E24	A19	B18	ND	D25	F24	G23	H19	28
29			E25	A20	B18	C20	D25	F25	G24	H19	29
30			E26	A21	B19	ND	D26	F26	G25	H19	30
31			E27	A22	ND	C21	D27	ND	G26	H19	31
32			E28	A23	B20	C22	D28	F27	G27	H19	32
33			E29	A24	ND	C23	D29	F28	G28	ND	33
34			E30	A25	B20	C24	D29	F29	G29	H20	34
35			E31	A26	B20	C25	D30	F30	G30	H21	35
36			E32	A27	B21	C26	D31	F31	G31	H22	36
37			E33	A28	B22	C27	D32	ND	G32	ND	37
38			E34	A29	B23	C27	D33	F32	G33	H23	38
39			E35	A30	B24	C28	D34	F33	G34	H24	39
40			E36	A31	ND	C29	D35	F34	G35	H24	40
41			E37	A32	B25	C30	D36	F34	G36	H25	41
42			E38	A32	B26	C31	D37	F35	G37	H26	42
43			E39	A33	B26	C32	D38	ND	G38	H27	43
45			E41	A35	B28	C34	D40	F37	G40	ND	45
46			E42	A36	B29	C35	D41	F38	G41	H29	46
47			E43	A37	B31	C36	D42	F39	G42	H30	47
48			E44	A38	B31	C36	D43	F40	G43	H31	48
49			E45	A38	B31	C37	D43	ND	G44	H32	49
50			E46	A39	B32	C38	D44	F41	G45	H32	50
51			E47	A40	B33	C39	D45	F42	G46	H33	51

Strain No.	Shiga toxin		ERIC profile	RAPD Profile using different primers							Genome type
	Stx_1	Stx_2		Gene 8	Gene 9	Opar8	Opar 20	A1	A7	A10	
52			E48	A41	B33	C40	D46	F43	G46	H34	52
53			E49	A42	B33	C41	D47	F44	G47	H35	53
54			E50	A43	B34	C42	D48	F45	G48	H36	54
55			E51	A44	B35	C43	ND	F46	G49	H37	55
56			E52	A45	B36	C44	D49	F47	G49	H38	56
57			E53	A46	B37	C45	D50	F48	G49	H39	57
58			E54	A47	B38	ND	D50	ND	G49	H39	58
59			E55	A48	B38	C46	D50	F48	G50	H40	59
60			E55	A49	B38	C47	D50	F48	G51	H40	60
61			E56	A50	B39	C48	D50	F48	G52	H41	61
62			E56	A51	B40	C49	ND	F49	G53	H42	62
63			E57	A52	B41	C50	D50	ND	G54	H43	63
64			E58	A53	B41	C51	D50	F50	G55	H44	64
65			E59	A54	B42	C51	D51	F51	ND	H45	65
66			E60	A54	B43	C52	D51	F52	G56	H45	66
67			E61	A55	B44	ND	D52	F53	G57	H46	67
68			E62	A56	B45	C53	D53	F54	G58	ND	68
69			E63	A57	B46	C54	D54	F55	G59	H47	69
70			E64	A58	B47	ND	D55	F56	G60	H48	70
Total			64	58	47	54	55	56	60	48	70

NOTE: Gene 8 = +A, Gene 9 = B, Opar8 = C, Opar20 = D, A1 = F, A7 = G, A10 = H, ERIC = E and ND = Not detected.

5. Conclusion

In conclusion cows remain the major reservoirs of shiga toxin producing *E. coli* which may not show any clinical sign of the disease and the disease can cause ailment in human beings. ERIC PCR profiled the isolates into 64 in this study. Hence, ERIC PCR discriminated the isolates better than RAPD. There was no specific order and clustering that will be associated to *E. coli* within the different source of the samples and also in the different shops or location of sample purchase using all the three technique (ERIC and RAPD -PCR) in this study, this shows a very wide heterogeneity of *E. coli* isolate within and without.

References

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