World Journal of Microbiology & Biotechnology (2006) 22:661–667
DOI 10.1007/s11274-005-9086-0
© Springer 2006

Clonal diversity of *Escherichia coli* isolates from marketed beef in East Malaysia

K. Apun*, P.P. Chang, E.U.H Sim and V. Micky
Resource Biotechnology Programme, Faculty of Resource Science and Technology, University Malaysia Sarawak, 94300, Kota Samarahan, Sarawak, Malaysia
**Author for correspondence: Tel.: +6082-671000 ext. 7203, Fax: +6082-672275, E-mail: kasing@frst.unimas.my*

Received 25 July 2005; accepted 31 October 2005

Keywords: beef, clonal diversity, *Escherichia coli*, East Malaysia, pulsed field gel electrophoresis

Summary

Escherichia coli, including Shiga-like toxin producing *E. coli* (STEC), serogroup O157:H7 and *E. coli* O157, were isolated from raw beef marketed in Sarawak and Sabah, East Malaysia. Molecular subtyping by pulsed-field gel electrophoresis (PFGE) was performed on 51 confirmed *E. coli* isolates. Of the 51 isolates, five were *E. coli* O157:H7, four *E. coli* O157, two non-O157 STEC and 40 other *E. coli* isolates (non-STEC). Digestion of chromosomal DNA from these *E. coli* isolates with restriction endonuclease *Xba*I (5′-TCTAGA-3′), followed by PFGE, produced 45 restriction endonuclease digestion profiles (REDPs) of 10–18 bands. *E. coli* O157:H7 isolates from one beef sample were found to have identical PFGE profiles. In contrast, *E. coli* serogroup O157 from different beef samples displayed considerable differences in their PFGE profiles. These suggested that *E. coli* isolates of both serogroups were not closely related. A large variety of PFGE patterns among non-STEC isolates were observed, demonstrating a high clonal diversity of *E. coli* in the beef marketed in East Malaysia. The distance matrix values (*D*), calculated showed that none of the pathogenic *E. coli* strains displayed close genetic relationship with the non-STEC strains. Based on the PFGE profiles, a dendrogram was generated and the isolates were grouped into five PFGE clusters (A–E). From the dendrogram, the most related isolates were *E. coli* O157:H7, grouped within cluster B. The STEC O157:H7 beef isolates were more closely related to the clinical *E. coli* O157:H7 isolate than the *E. coli* O157:H7 reference culture, EDL933. Cluster A, comprising many of other *E. coli* isolates was shown to be the most heterogeneous. PFGE was shown to possess high discriminatory power in typing pathogenic and non-pathogenic *E. coli* strains, and useful in studying possible clonal relationship among strains.

Introduction

Escherichia coli is a common commensal of the human gastrointestinal tract. However, under certain conditions strains of *E. coli* can cause disease. Shiga-like toxin-producing *E. coli* (STEC) has been implicated as the causative agent in several human diseases (Nataro & Kaper 1998; Paton & Paton 1998), with *E. coli* O157:H7 being the most well known among them. These diseases range from mild diarrhea, hemorrhagic colitis (HC), to complicated conditions, such as hemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Cattle are generally considered to be the major reservoir of both *E. coli* O157 and non-O157 STEC (Bettelheim 2000). Due to the threat pathogenic *E. coli* pose to public health, characterisation of the *E. coli* in food samples is essential. Several different methods for *E. coli* characterisation are being used to identify genetic differences among *E. coli* isolates. This included ribotyping (Dalla-Costa *et al.* 1998), phage typing (Khakhria *et al.* 1990), randomly amplified polymorphic DNA (RAPD) (Hopkins & Hilton 2000), and pulsed-field gel electrophoresis (Preston *et al.* 2000). Among these, pulsed-field gel electrophoresis (PFGE) was shown to produce highly discriminant genotyping and it was identified as the 'gold standard' for molecular subtyping of *E. coli* isolates (Preston *et al.* 2000; Rios *et al.* 1999). The purpose of this study was to evaluate by PFGE the genetic diversity as well as relatedness of *E. coli* O157:H7, *E. coli* O157, STEC and other *E. coli* strains (non-STEC) isolated from raw beef marketed in East Malaysia.

Materials and methods

Bacterial strains

Fifty-one *E. coli* isolates previously isolated from raw beef marketed in East Malaysia were used in this study. Confirmation of the isolates were conducted using the latex agglutination test with *E. coli* 0157 test kit (Oxoid),

and using a multiplex PCR assay with four primers targeting the *stx1*, *stx2* (shiga-like toxin I and II), *RfbE* (O antigen) and *fliC*$_{h7}$ (H7 antigen) genes as described by Apun *et al.* (2003). Selection of isolates was based on their pathogenicity, phenotypic characteristics, and the source of isolation. These isolates included five *E. coli* O157:H7 isolates, four *E. coli* O157 isolates, two non-O157 STEC and 40 other *E. coli* isolates. The *E. coli* were isolated from raw beef purchased from six main towns in Sarawak, East Malaysia namely, Kuching (KC) Samarahan (SM), Sibu (SB), Sarikei (SA), Bintulu (BT), Miri (MI), and two main towns in Sabah, East Malaysia, namely Kota Kinabalu (KK) and Sandakan (SD). *E. coli* O157:H7 strain EDL933, was included as reference strain whereas *E. coli* O157:H7 strain SGH1, isolated from clinical sample, was included as comparison.

Bacterial culture conditions

All *E. coli* strains were cultured in LB (Luria Bertani) broth (Fluka) at 37 °C with agitation overnight before being subjected to DNA preparation.

DNA preparation

DNA for PFGE analysis was prepared by the method of Thong & Pang (1996), which required 3 days to complete, in contrast to the traditional protocol that requires 5 days to complete. Genomic DNA was prepared by embedding *E. coli* cells in agarose plugs (Ultrapure) and lysing the cells using 1 mg lysozyme/ml (2 ml per plug, 1 ml per subsequent plug) for 6 h at 37 °C, with gentle agitation. Then, the lysis solution was replaced with ES (0.5 M NA-EDTA, 1% sarcosyl) solution containing 0.5 mg/ml proteinase K and incubated overnight at 50 °C with gentle shaking. The plugs were dialysed with sterile TE for five times at room temperature with agitation (EYEL4, Multi Shaker MMS). The first two washes were performed at 1 h interval. Subsequent three washes were done at 2 h intervals.

Restriction endonuclease digestion

The chromosomal DNA digestion was performed while still embedded in agarose. The restriction enzyme *Xba*I (Promega), a six base cutter (5′-TCTAGA-3′), was used to digest the DNA for each isolate. The enzyme was selected because it yielded the restriction patterns that have been most reproducible. Following the restriction enzyme digestion, the DNA plug was equilibrated with 1.0 ml of 0.5× TBE buffer on ice for 45 min.

PFGE

The slice was then loaded into the well of 1.2% Pulsed-Field Certified agarose (Bio-Rad) prepared in 0.5× TBE buffer. PFGE of the digested DNA was carried out with a contour-clamped homogenous electric field apparatus (CHEF DRIII, Bio-Rad). For gel electrophoresis, two program blocks were used: block 1, ramped pulse times of 15–30 s (7 h of electrophoretic run), followed by block 2, ramped pulse times of 2.2–56.0 s (15 h of electrophoretic run). Other parameters included: voltage of 200 V (6 V/cm), an electric field angle of 120° and a temperature of 14 °C. Then, the gel was stained with 0.5 μg/ml ethidium bromide (Sigma) before viewing under an u.v. transluminator (Vilber Lourmat). The PFG lambda ladder (New England Biolabs) consisting of concatemers starting at 48.5 kb and increasing to approximately 1000 kb were used as molecular markers to estimate the size of the DNA fragments. Digested DNA of *E. coli* O157:H7 strain EDL933 was included in every gel for standardisation and as a reference.

Data analysis

PFGE bands were scored for presence (1) or absence (0) of a distinct band. PFGE profiles obtained were analysed with RAPDistance Programs Package Version 1.04 (Armstrong *et al.* 1994). These programs were designed to help record and analyse the RAPD (Randomly Amplified Polymorphic DNA) or RFLP (Restriction Fragment Length Polymorphism) fragment of DNA. Distances calculation of similarity band profiles was based on Dice formulation (Nei & Li 1979). By this analysis, the degree of similarity among the strains was studied. A distance matrix value, *D* value, of 0 indicated identical patterns and a *D* value of 1.0 suggested complete dissimilarity. From the calculated Dice formulation, a dendrogram of neighbour-joining tree (NJTREE) description file was achieved by means of the Tdraw clustering algorithm program.

Results

Chromosomal DNA was digested with *Xba*I and yielded between 10 and 18 bands, with sizes of approximately 48.5–679.0 kb. A total of 45 distinct restriction endonuclease digestion profiles (REDPs) were generated from the 51 isolates of *E. coli* (Table 1). Two isolates repeatedly appeared as a smear of degraded DNA (Figure 1, lane 43 and 61). These may be due to endogeneous nuclease activity. These isolates were represented as untypeable (UT). The five STEC O157:H7 isolates from a beef sample from Bintulu shared identical PFGE pattern (Figure 1, lane 2–6). Two different PFGE patterns (differing by 14 bands) were observed among four *E. coli* O157 isolates from beef samples purchased in Kuching and Kota Kinabalu, one (PFGE pattern) of which was shared by three isolates. The PFGE patterns of STEC O157:H7 and *E. coli* O157 isolates differed by 16–18 bands. Two non-O157 STEC isolates, with different Shiga-like toxin profiles (i.e. Figure 1, lane 13 represented STEC harbouring *stx1* and *stx2* genes, lane 41 represented STEC harbouring *stx2* gene only) were found to be very different (21 band

Table 1. The source and PFGE patterns of *E. coli* isolates.

Isolate no.	Location	Serotype	PFGE pattern
BTC11	Bintulu	*E. coli* O157:H7	1
BTC14	Bintulu	*E. coli* O157:H7	1
BTC15	Bintulu	*E. coli* O157:H7	1
BTC19	Bintulu	*E. coli* O157:H7	1
BTC20	Bintulu	*E. coli* O157:H7	1
KCD8	Kuching	*E. coli* O157	2
KKW1	Kota Kinabalu	*E. coli* O157	3
KKW3	Kota Kinabalu	*E. coli* O157	3
KKW5	Kota Kinabalu	*E. coli* O157	3
BTE3	Bintulu	Non-O157 STEC	4
KCD32	Kuching	Non-O157 STEC	5
KCD23	Kuching	*E. coli* (NA)	6
KCB5	Kuching	*E. coli* (NA)	7
KCN1	Kuching	*E. coli* (NA)	8
KCO5	Kuching	*E. coli* (NA)	9
KCA25	Kuching	*E. coli* (NA)	10
KCA2	Kuching	*E. coli* (NA)	11
KCH5	Kuching	*E. coli* (NA)	12
KCM11	Kuching	*E. coli* (NA)	13
SMA2	Samarahan	*E. coli* (NA)	14
SMB2	Samarahan	*E. coli* (NA)	15
SMB3	Samarahan	*E. coli* (NA)	16
SAA1	Sarikei	*E. coli* (NA)	17
SAB1	Sarikei	*E. coli* (NA)	18
KKU16	Kota Kinabalu	*E. coli* (NA)	19
KKB8	Kota Kinabalu	*E. coli* (NA)	20
KKF5	Kota Kinabalu	*E. coli* (NA)	21
KKM6	Kota Kinabalu	*E. coli* (NA)	22
KKA7	Kota Kinabalu	*E. coli* (NA)	23
SDB1	Sandakan	*E. coli* (NA)	24
SDC5	Sandakan	*E. coli* (NA)	25
SDC10	Sandakan	*E. coli* (NA)	26
SDB18	Sandakan	*E. coli* (NA)	27
BTC4	Bintulu	*E. coli* (NA)	28
BTA1	Bintulu	*E. coli* (NA)	29
BTA14	Bintulu	*E. coli* (NA)	30
BTB8	Bintulu	*E. coli* (NA)	31
BTC9	Bintulu	*E. coli* (NA)	UT
BTF12	Bintulu	*E. coli* (NA)	32
SBD17	Sibu	*E. coli* (NA)	33
SBD11	Sibu	*E. coli* (NA)	34
SBF8	Sibu	*E. coli* (NA)	35
SBA9	Sibu	*E. coli* (NA)	36
MIO5	Miri	*E. coli* (NA)	UT
MIS4	Miri	*E. coli* (NA)	37
MIG1	Miri	*E. coli* (NA)	38
MIN2	Miri	*E. coli* (NA)	39
MIA9	Miri	*E. coli* (NA)	40
MIC1	Miri	*E. coli* (NA)	41
MIL10	Miri	*E. coli* (NA)	42
MIY2	Miri	*E. coli* (NA)	43
SGH1	Kuching	*E. coli* O157:H7	44
EDL933	–	*E. coli* O157:H7	45

- NA: not available (Serotype other than O157 and H7 was not performed).
- UT: untypeable; SF: sorbitol fermenting; GUD: β-glucuronidase activity.
- *E. coli* 0157 isolates were not tested for H serotype.

differences). The remaining *E. coli* isolates were assigned to 40 very different PFGE patterns.

The PFGE profiles were further analysed using the RAPDistance program and a dendrogram (Figure 2) was generated from the *E. coli* isolates. The dendrogram was constructed based on the differences of the genetic distance and distant matrix information gained from scoring the presence and absence of a band in the PFGE profile. *E. coli* isolates studied were grouped into five PFGE clusters, designated as cluster A–E. The lowest and highest *D* Values were detected at 0.2000 and 0.9130 for *E. coli* isolates in cluster B and D, respectively. The *E. coli* O157:H7, including STEC O157:H7 isolates from beef samples, the reference strain *E. coli* O157:H7 strain

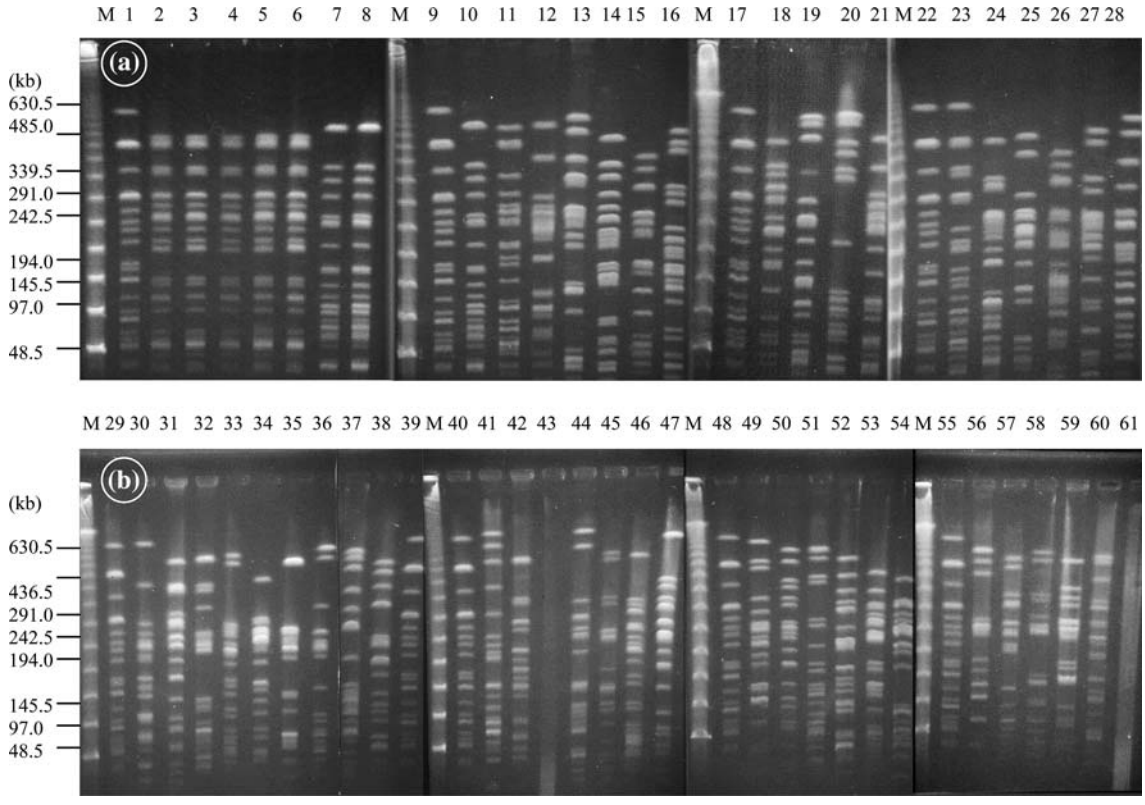

Figure 1. PFGE of chromosomal DNA digested with *Xba*I of 51 *E. coli* isolates with two program blocks. Lanes: M, PFG lambda ladder; 1, 9, 17, 22, 29, 39, 40, 48, 55, reference strain *E. coli* O157:H7 strain EDL 933; 2–6, STEC O157:H7 from Bintulu; 7, 8, 10, *E. coli* O157 from Kota Kinabalu; 11, *E. coli* O157 from Kuching; 12, *E. coli* from Kuching; 13, non-O157 STEC from Kuching; 14–16, 18–21, *E. coli* from Kuching; 23, *E. coli* O157:H7 strain SGH1 from clinical sample; 24–26, *E. coli* from Samarahan; 27–28, *E. coli* from Sarikei; 30–34: *E. coli* from Kota Kinabalu; 35–38: *E. coli* from Sandakan; 41, non-O157 STEC from Bintulu; 42–47, *E. coli* from Bintulu; 49–52, *E. coli* from Sibu; 53–54, 56–61: *E. coli* from Miri.

EDL933 and the clinical isolate *E. coli* O157:H7 strain SGH1, were grouped into cluster B. The *E. coli* O157:H7 beef isolates were more related to clinical isolate, SGH1 with *D* value of 0.2353 as compared to reference strain *E. coli* O157:H7 strain EDL933 (*D* value = 0.3939). There were five non-pathogenic *E. coli* isolates grouped into cluster B, indicating some genetic relatedness to the *E. coli* O157:H7. The most closely related isolates were the seven strains of *E. coli* O157:H7 (*D* value of 0.2–0.3939) within cluster B. In contrast, *E. coli* isolates in cluster A were shown to be the most heterogeneous. Three *E. coli* isolates grouped in cluster A were shown to have the most differences with the highest *D* value of 0.8261.

Discussion

PFGE has often been considered the 'gold standard' for the molecular typing of *E. coli* strains by many researchers (Izumiya *et al.* 1997 Preston *et al.* 2000; Welinder-Olsson *et al.* 2002), due to its high discriminatory power. The high discriminatory ability of PFGE analysis was clearly confirmed by the present study, generating 45 different REDPs on PFGE patterns of 49 *E. coli* strains. PFGE showed a good ability to discriminate between *E. coli* isolates of serogroup O157:H7 and O157, STEC and other *E. coli* isolated from beef marketed in East Malaysia. From the PFGE profiles and the distance matrix calculated, *E. coli* O157:H7 and *E. coli* O157 strains were not closely related. In addition, none of the pathogenic *E. coli* strains displayed close genetic relationship with the non-STEC strains. For further application, the different PFGE patterns would give invaluable information especially in the epidemiological area, whereby it is possible to assess the presence of an outbreak and to differentiate between outbreaks.

E. coli O157:H7 with single PFGE type was observed in this study. Five *E. coli* O157:H7 were isolated in one beef sample purchased from Bintulu. All of them were found to exhibit identical PFGE profile designated as pattern 1. This indicated that the *E. coli* O157:H7 positive beef sample marketed in East Malaysia, which may pose a threat to the public health, harboured only single *E. coli* O157:H7. In contrast to the result of this study, multiple PFGE types of *E. coli* isolates were indicated in some other studies. Kudva *et al.* (1997), in the characterisation of *E. coli* O157:H7 isolated from sheep had demonstrated that multiple *E. coli* O157:H7 strains could be simultaneously shed by a single animal. Faith *et al.* (1996) observed 20 distinct PFGE patterns

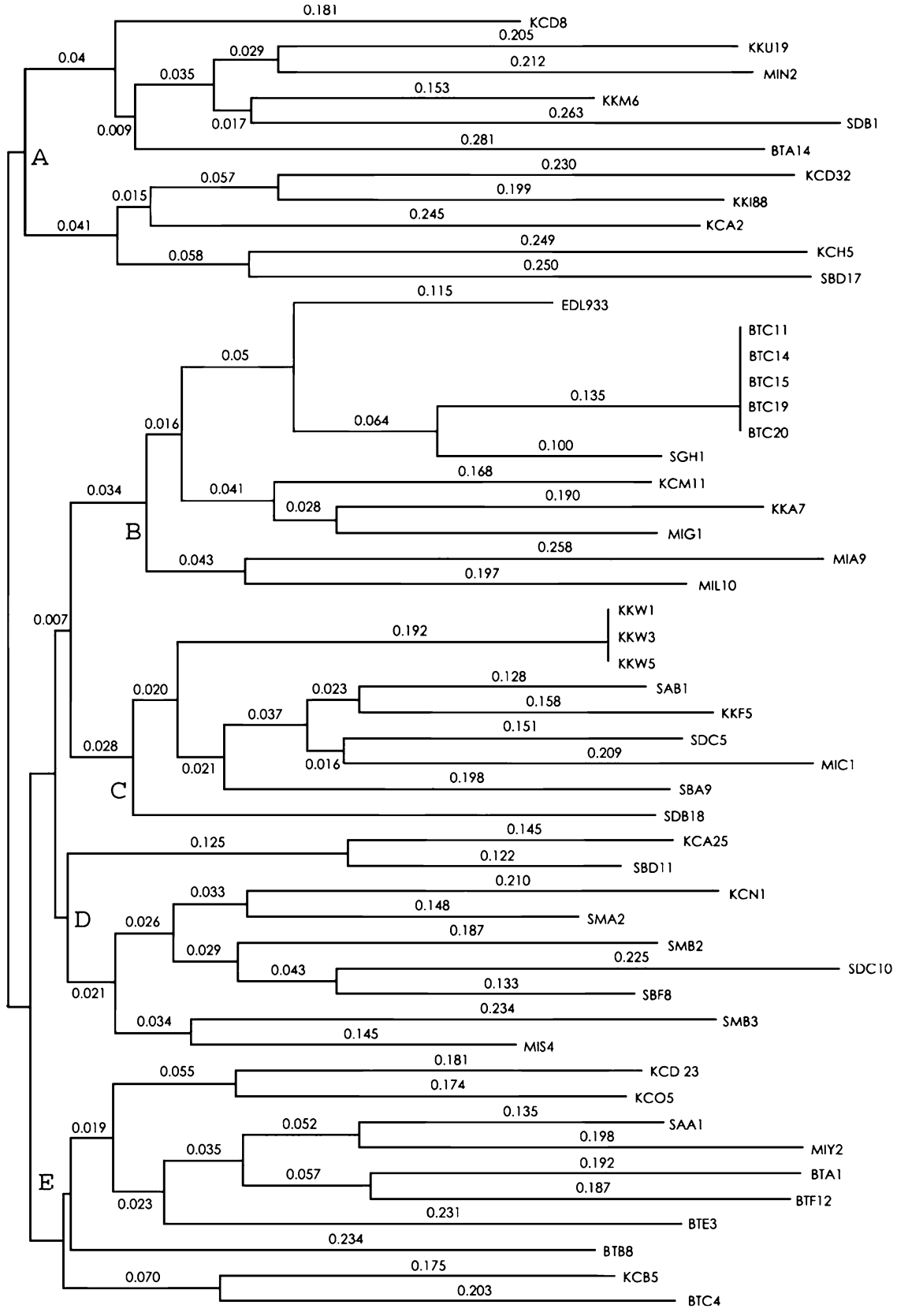

Figure 2. Dendrogram of *E. coli* O157:H7, *E. coli* O157, STEC, and *E. coli* strains isolated from beef marketed in East Malaysia. The dendrogram was constructed with the use of the NJTREE method by comparison of *Xba*I PFGE patterns. Numbers represent the distance values between the respective isolates.

among 160 *E. coli* O157:H7 isolates from 29 cattle over an 8-month period and they indicated that individual cattle could harbour more than one *E. coli* O157:H7 strain simultaneously. Both workers attributed the existence of multiple *E. coli* O157:H7 strains in the animals to a common environmental source (water, feed, pasture) that allowed the animals to become infected with these strains, or that they were passed from one infected animal to others. The presence of several strains of *E. coli* 0157:H7 would further complicate the safety of the beef consumed, and pose greater threat to consumers due to the fact that these strains are responsible for a range of disease ranging from mild-diarrhea to complicated conditions such as HUS. In addition, the existence of different strains will make it difficult to determine the type of strains responsible for any outbreaks. However, our present study indicated the presence of only one serotype of *E. coli* O157:H7 from one beef sample, thus this will make it easier to pinpoint the strains responsible for any possible outbreak and determine the source of infection.

From the PFGE analysis, a large variety of PFGE patterns among *E. coli* isolates were observed, demonstrating a high *E. coli* diversity in the beef marketed in East Malaysia. The great variety of *E. coli* isolates was also demonstrated through the cluster analysis in the dendrogram, with observation of great differences in their distant matrix values (i.e. the lowest *D* value of 0.2000 and the highest *D* value of 0.9130). This is in agreement with the findings reported by Rios *et al.* (1999), who also reported the high diversity of Chilean isolates of EHEC of human, animal, or food origin by using *Xba*I PFGE typing. In the Aslam *et al.* (2003) study, the RAPD technique was used to study the genetic diversity of *E. coli* in beef cattle, at various stage of beef production. In this study, high genetic diversity was reported with 121 *E. coli* genetic subtypes being recovered from beef cattle feces, hides, carcasses and ground beef. In our study, which targeted raw beef marketed in East Malaysia, only five *E. coli* O157:H7 isolates from one beef sample and three *E. coli* O157 isolates showed identical PFGE patterns respectively. The remaining 41 *E. coli* isolates were demonstrated to have different PFGE patterns, therefore exhibiting a great genetic diversity among the *E. coli* isolates from raw beef. No clear correlation could be established between the DNA profiles and locations (towns) where the beef samples were purchased.

Conclusion

In conclusion, although the *E. coli* strains were isolated from the same type of meat (beef), considerable genetic diversity was found among the strains originating from different locations in East Malaysia. The PFGE method has been successfully used in typing both pathogenic and non-pathogenic *E. coli* strains isolated from beef marketed in East Malaysia. Because of its usefulness in the analysis of level of diversity among strains originating from different location, it will be a useful typing method to study the possible clonal relationship among *E. coli* strains and to monitor the pathway of transmission of pathogenic *E. coli* that may pose threat to public health.

Acknowledgements

The authors acknowledged the financial support rendered by University Malaysia Sarawak through the Short-term Grant No. 243/2001 (2), and the Ministry of Science, Technology and Environment of Malaysia's National Science Fellowship (NSF) award.

References

Apun, K., Chang, P.P., Sim, E.U.H. & Salleh, M.A. 2003 Survey of raw beef marketed in East Malaysia for *Escherichia coli* 0157 and 0157:H7. *Journal of Bioscience* **14**, 49–55.

Armstrong, J.S., Gibbs, A.J., Peakall, R. & Weiller, G. (1994) The RAPDistance package, version 1.04. In http://www.anu.edu.au/BoZo/software/.

Aslam, M., Nattress, F., Greer, G., Yost, C., Gill, C. & McMullen, L. 2003 Origin of contamination and genetic diversity of *Escherichia coli* in beef cattle. *Applied and Environmental Microbiology* **69**, 2794–2799.

Bettelheim, K.A 2000 Role of non-O157 VTEC. *Journal of Applied Microbiology* **88**, 38S–50S.

Dalla-Costa, L.M., Irino, K., Rodrigues, J., Rivera, I.N.G. & Trabulsi, L.R. 1998 Characterisation of diarrhoeagenic *Escherichia coli* clones by ribotyping and ERIC-PCR. *Journal of Medical Microbiology* **47**, 227–234.

Faith, N.G., Shere, J.A., Brosch, R., Arnold, K.W., Ansay, S.E., Lee, M.-S., Luchansky, J.B. & Kaspar, C.W. 1996 Prevalence and clonal nature of *Escherichia coli* O157:H7 on dairy farms in Wisconsin. *Applied and Environmental Microbiology* **62**, 1519–1525.

Hopkins, K.L. & Hilton, A.C. 2000 Simultaneous molecular subtyping and shiga toxin gene detection in *Escherichia coli* using multiplex polymerase chain reaction. *Letters in Applied Microbiology* **30**, 122–125.

Izumiya, H., Terajima, J., Wada, A., Inagaki, Y., Itoh, K.-I., Tamura, K. & Watanabe, H. 1997 Molecular typing of Enterohemorrhagic *Escherichia coli* O157:H7 isolates in Japan by using pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **35**, 1675–1680.

Khakhria, R., Duck, D. & Lior, H. 1990 Extended phagetyping scheme for *Escherichia coli* O157:H7. *Epidemiology and Infection* **105**, 511–520.

Kudva, I.T., Hatfield, P.G. & Hovde, C.J. 1997 Characterization of *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* serotypes isolated from sheep. *Journal of Clinical Microbiology* **35**, 892–899.

Nataro, J.P. & Kaper, J.B. 1998 Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews* **11**, 142–201.

Nei, M. & Li, W.H. 1979 Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences, USA* **76**, 5269–5273.

Paton, J.C. & Paton, A.W. 1998 Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clinical Microbiology Reviews* **11**, 450–479.

Preston, M.A., Johnson, W., Khakhria, R. & Borczyk, A. 2000 Epidemiologic subtyping of *Escherichia coli* serogroup O157 strains isolated in Ontario by phage typing and

pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* **38**, 2366–2368.

Rios, M., Prado, V., Trucksis, M., Arellano, C., Borie, C., Alexandre, M., Fica, A. & Levine, M.M. 1999 Clonal diversity of Chilean isolates of enterohemorrhagic *Escherichia coli* from patients with hemolytic-uremic syndrome, asymptomatic subjects, animal reservoirs, and food products. *Journal of Clinical Microbiology* **37**, 778–781.

Thong, K.L. & Pang, T. 1996 Method: a rapid, simplified method for preparation of chromosomal DNA from pathogenic bacteria for use in PFGE. *Asia Pacific Journal of Molecular Biology and Biotechnology* **4**, 59–62.

Welinder-Olsson, C., Badenfors, M., Cheasty, T., Kjellin, E. & Kaijser, B. 2002 Genetic profiling of enterohemorrhagic *Escherichia coli* strains in relation to clonality and clinical signs of infection. *Journal of Clinical Microbiology* **40**, 959–964.