Molecular analysis of pathogenic Escherichia coli isolated from cow meat in Yogyakarta, Indonesia using 16S rRNA gene

ALVITA INDRASWARI1, I WAYAN SUARDANA2, ARIS HARYANTO3, DYAH AYU WIDIASIH4*
1Doctoral Program of Veterinary Science, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Jl. Fauna No 2 Karangmalang, Sleman 55281, Yogyakarta, Indonesia
2Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Udayana. Jl. P.B. Sudirman, Denpasar 80232, Bali, Indonesia
3Department of Biochemistry, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Jl. Fauna No 2, Karangmalang, Sleman 55281, Yogyakarta, Indonesia
4Department of Veterinary Public Health, Faculty of Veterinary Medicine, Universitas Gadjah Mada. Jl. Fauna No 2 Karangmalang, Sleman 55281, Yogyakarta, Indonesia. Tel.: +62-274-560866, Fax.: +62-274-560866, *email: dyahav@ugm.ac.id

Manuscript received: 3 August 2021. Revision accepted: 28 September 2021.

Abstract. Indraswari A, Suardana IW, Haryanto A, Widiash DA. 2021. Molecular analysis of pathogenic Escherichia coli isolated from cow meat in Yogyakarta, Indonesia using 16S rRNA gene. Biodiversitas 22: 4566-4573. Meat has been recognized as a major source of foodborne disease and a public health problem. The characteristics of meat become an ideal growth medium for various microorganisms if not handled properly. Pathogenic Escherichia coli is one of the foodborne disease agents that causes diarrhea. Identification of pathogenic E. coli isolated from cow meat needs to be done. This research aims to study nucleotide sequence of 16S rRNA gene of pathogenic E. coli isolated from cow meat in Yogyakarta, Indonesia using Polymerase Chain Reaction (PCR). These fifteen isolates have been detected for eae target gene, then amplification of the 16S rRNA gene was carried out using primers 27F and 1492R. Phylogenetic tree reconstruction was performed on the fifteen isolates of pathogenic E. coli to figure out the relationship to reference strains available at the GenBank. Results show that nucleotide sequence among the fifteen isolates from different traditional markets in Yogyakarta, Indonesia and reference strains are very similar. The fifteen isolates have small genetic distance to the reference strains, and these fifteen isolates are also in the same clade with reference strains. This research shows that the fifteen isolates under investigation are closely related to the reference strains, which is Shiga-toxin producing E. coli (STEC). People should pay more attention in processing food stock, especially cow meat. Further research may focus on determining the strain of those fifteen isolates.

Keywords: 16S rRNA, cow meat, DNA sequencing, pathogenic E. coli, phylogenetic

INTRODUCTION

Some strains of pathogenic Escherichia coli may cause diarrhea or other extraintestinal diseases among healthy individuals and those with immunocompromised (Gomes et al. 2016; Antaki-zukoski et al. 2018; Habets et al. 2020; Vidal et al. 2020). There are six pathotypes related to diarrhea and commonly referred to as diarrheagenic E. coli (DE) (Nataro and Kaper 1998; Jafari et al. 2012; CDC 2014; Gomes et al. 2016; CFSPH 2016). One of the pathotypes is Shiga-toxin producing E. coli (STEC), STEC is also known as Verocytotoxin-producing E. coli (VTEC) or enterohemorrhagic E. coli (EHEC). This pathotype is one of the most commonly recognized associated with foodborne disease (CDC 2014). Shiga-toxin producing E. coli has the virulence factors of Shiga-toxin (Stx) 1 and 2, encoded by the Stx1 and Stx2 genes, respectively. In addition, most STECs have an adhesin protein called intimin which is encoded by the eae gene and allows the attachment of bacteria to the intestinal epithelium. The attachment induces histopathological lesions defined as attaching and effacing (A/E). This lesion is regulated by a large pathogenicity island called the locus of enterocyte effacement (LEE) (Donnenberg et al. 1993; Nataro and Kaper 1998; Blanco et al. 2004; Croxen and Finlay 2010). It has been found the eae gene in both the feces of cows suffering from diarrhea or not (Yousif and Hussein 2015). There is a strong association with the discovery of the eae gene and the ability of STEC to cause severe disease in humans, particularly hemolytic uremic syndrome (HUS) (Oswald et al. 2000).

In Indonesia, diarrheagenic E. coli has been found in cow meat obtained from qurban animals (Ningrum et al. 2016) and slaughterhouses (Rananda et al. 2016; Suardana et al. 2007). In the meat of slaughtered cattle obtained from Jakarta, 5.3% of non-O157 Shiga-toxin (STEC) producing E. coli were found (Ningrum et al. 2016). It was found that 70% of cow meat samples obtained from the Lubuk Buaya abattoir, Padang, were found to be contaminated with E. coli O157:H7 with the number of colonies that had exceeded the maximum permissible contamination limit (Rananda et al. 2016). According to Suardana et al. (2007), 5.62% E. coli O157:H7 in cow meat obtained from slaughterhouses and traditional markets in Badung Regency, Bali. The discovery of diarrheagenic E. coli in cow meat indicates a critical role as a reservoir for strains that transmit disease to humans. Food produce from animals such as cow may be contaminated with DEC at the slaughterhouse, processing facility, or the consumer’s kitchen. DEC infection may also come from consumption...
of raw or rare meat, unpasteurized milk, and fruits and vegetables contaminated with feces. Bacterial transmission may also occur from one human to another (WHO 2018).

DEC strain is one of the first pathogens to be observed using today's advanced molecular diagnostic methods. These methods are the most popular and reliable in differentiating DEC strain from those of non-pathogenic bacteria. Moreover, the phylogeny and taxonomy of bacteria can also be studied using 16S rRNA gene sequence as genetic markers (Fujioka et al. 2009; Amarantini et al. 2011; Botkin et al. 2012; Fialho et al. 2013; Suardana 2014). The gene sequence of 16S rRNA is used because 16S rRNA gene is found in almost all bacteria, 16S rRNA gene does not change its function over time, and 16S rRNA gene (1500 bp) is large enough for information purposes. One of the most interesting potentials of 16S rRNA gene is its ability to provide genus and species identification for isolates that do not match standard biochemical profiles, for strains that only result in low likelihood or acceptable identification according to commercial systems, or for taxa that are rarely associated with communicable diseases in human (Pangastuti 2006; Janda and Abbott 2007).

Characteristics of molecular targets from these methods allow the study of bacterial phylogenetic, both for bacteria detection or identification in clinical laboratories (Rahmani et al. 2006; Amarantini et al. 2011; Rinanda 2011; Suardana 2014; Ghazali and Rashid 2019). This research aims to study nucleotide sequence of 16S rRNA gene of pathogenic E. coli isolated from cow meat in Yogyakarta using Polymerase Chain Reaction (PCR). Results from this research can be used to understand the diversity of pathogenic E. coli based on nucleotide sequence and relationships among isolates that are isolated from cow meat available in traditional markets around Yogyakarta Special Region. In turn, it may increase people’s awareness of E. coli.

MATERIALS AND METHODS

Bacteria isolates
This research employed 15 E. coli isolates coded as samples D1, KR3-2, KR1-3, N2, D1-2, D2-2, D4-2, D1-3, D4-3, N1-2, N2-2, N2-3, L2-2, L1-2, and N1-3. The bacterial isolates were obtained from the previous studies which were isolated from 48 samples of meat in several traditional markets in Yogyakarta, Indonesia. There were 12 meat samples from the Demangan market (D) and 6 isolates, 26 meat samples from the Kranggan market (KR) and 2 isolates, 6 meat samples from the Ngasem market (N) and 5 isolates, and 4 meat samples from the Lempuyangan market (L) and 2 isolates were found. These fifteen isolates have been detected for eae target gene and have been identified as pathogenic E. coli with such gene present (Indraswari et al. 2021).

Cultivation of bacteria isolates
These fifteen isolates were taken from isolate stock kept in a freezer at -20 °C. These were then grown in Brain Heart Infusion Broth (BHB) media and incubated for 24 h at 37°C. Bacterial growth was evident with increasing turbidity of BHB media.

DNA extraction
The E. coli isolates grown in BHB then underwent DNA extraction using Presto™ Mini gDNA Bacteria Kit (Geneaid GBB300) according to the procedure of choice with little modification as in Indraswari et al. (2021).

Amplification of 16S rRNA gene using PCR and DNA sequencing
DNA from extraction was used as DNA template for amplification using PCR master cycler personal. Component mixture for each reaction consisted of 4 µL DNA template, each with forward and reverse primer taken from IDTM of 2 µL, PCR mix from MyTaq™ HS Red Mix (Cat. No.: BIO-25048) of 25 µL, and sterile ddH₂O from UltraPure™ ddH₂O (Cat. No.: 10977015) was also added that the total volume required for each reaction was 50 µL. The primers used were 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R: 5'-GGTTACCTTGTACGACT-3' (Suardana 2014).

Amplification was performed at 95°C for 2 min (initial denaturation), and further 30 cycles at 95°C for 45 s (denaturation), at 50°C for 45 s (annealing), and at 72°C for 1 min (extension). Amplification program was ended at 72°C for 5 min (final extension). Around 5 µL of amplification result was then analyzed using electrophoresis in 1.5% agarose gel colored with SYBR® Safe DNA gel stain in TBE 1× solution. This gel was visualized using a UV transilluminator and documented using a digital camera. Obtained PCR products were then sent to PT. Genetika Science, Indonesia for DNA sequencing. Sequencing was performed using two primers of 27F and 1492R.

Analysis of 16S rRNA gene sequencing
The length of 16S rRNA gene amplification product was ~1500 bp and data from this sequencing were further analyzed using Molecular Evolutionary Genetics Analysis (MEGA)-X software. Nucleotide sequences of 16S rRNA gene from pathogenic E. coli available at the GenBank include E. coli Sakai (BA000007), E. coli O104:H4 (AF0B02000112), E. coli EDL 933 W (AE005174), E. coli SM-25(1) (KF768068), E. coli KL-48(2) (KF768069), E. coli O111:H:- (GU237022), and E. coli O121:H19 (JASV01000004) which were used as reference and Shigella sonnei (FR870445) which was used as an outgroup. Sequence of 16S rRNA gene was aligned using Clustal W program and was automatically compared using BLAST against bacteria sequence available at the GenBank (http://www.ncbi.nlm.nih.gov/). Analysis of genetic profile was determined by differences in nucleotide sequence of 16S rRNA gene. Genetic distance was measured using the Kimura 2-parameter method. Afterward, a phylogenetic tree was constructed using neighbor joining algorithm method with 1000× replication bootstrap test and the Kimura 2-parameter method using MEGA program (Saitou and Nei 1987; Suardana et al. 2017; Kumar et al. 2018).
Confirmation for species identification was also made based on the guidelines recommended by Janda and Abbott (2007).

RESULTS AND DISCUSSION

The fifteen *E. coli* isolates used in this research were isolated from cow meat sold in different traditional markets in Yogyakarta, Indonesia. They underwent detection for *eae* gene target and were identified as pathogenic *E. coli* as such gene was present (Indraswari et al. 2021). After bacteria isolate cultivation, DNA extraction was performed on them in order to obtain DNA template for amplification with PCR.

Amplification of 16S rRNA gene

Results of 16S rRNA gene amplification using primers 27F and 1492R are depicted in Figure 1. A positive result was indicated by the appearance of a DNA band measuring ~1500 bp. In all the studied isolates, DNA bands appeared at the appropriate size. Positive PCR results were sequenced to determine the base sequence of the 16S rRNA gene from each isolate.

Analysis of 16S rRNA gene sequencing

Alignment of 16S rRNA gene

The alignment of the 16S rRNA gene of the fifteen isolates against several reference strains available in Genbank is shown in Figure 2. Data from the alignment of the 16S rRNA gene showing similarities or differences between the aligned nucleotide sequences. The nitrogen base number 39 of isolates D1, KR3-2, and N2 is different from *E. coli* Sakai, *E. coli* O104:H4, and *Shigella sonnei* with base A replaced by bases C and T. Other than that, the nitrogen base number 24 from isolates D1, KR3-2, KR1-3, D2-2, D4-2, D1-3, D4-3, N1-2, N2-2, N2-3, L2-2, L1-2, and N1-3 only differs from *E. coli* Sakai with base C replaced by base A. The fifteen isolates studied tended to show a close nucleotide sequence with isolates from the same species. The nucleotide sequences among the fifteen isolates under investigation are also closely related.

![Figure 1. DNA band from 16S rRNA gene amplification in fifteen isolates of pathogenic *E. coli*. M: marker, 1: D1, 2: KR3-2, 3: KR1-3, 4: N2, 5: D1-2, 6: D2-2, 7: D4-2, 8: D1-3, 9: D4-3, 10: N1-2, 11: N2-2, 12: N2-3, 13: L2-2, 14: L1-2, and 15: N1-3](image)

![Figure 2. The nucleotide sequences of the 16S rRNA gene from the fifteen isolates were compared with the nucleotide sequences available in GenBank. Data from the alignment results are not fully displayed](image)
**Genetic distance**

Results of genetic distance analysis from fifteen isolates under investigation against some strains of pathogenic *E. coli* and the outgroup of *S. sonnei* are given in Table 1. The fifteen isolates studied have different nucleotide sequence from 1 through 11 against reference strains available at the GenBank. Nucleotide analysis showed that the fifteen isolates studied were identical even though they had 1 to 11 nucleotide differences from the reference strain. For instance, between isolate D1 and *E. coli* Sakai only 3 nucleotides of 1000 being compared are found to be different. For isolate KR3-2, of 1000 nucleotide compared, only 4 are found to be different from those of *E. coli* SM-25(1). For isolate N2, only 1 nucleotide is different from those of *E. coli* SM-25(1) and *S. sonnei* out of 1000 being compared.

The closest genetic distance between the fifteen isolates under investigation and reference strains can be summarized as follows: isolate N2, D1-2, and D2-2 show the smallest genetic distance to *E. coli* SM-25(1) strain. Other than that, isolate N2 also shows the smallest genetic distance to *S. sonnei*, and isolate D1-2 shows the smallest genetic distance to *E. coli* O121:H19 strain. The biggest genetic distance is between isolate D1 and *E. coli* O121:H19, N2 and *E. coli* O111:H-, and L1-2 and *E. coli* O104:H4 and also *E. coli* O121:H19. Moreover, each of the fifteen isolates studied has a different nucleotide sequence from 1 through 10. The smallest genetic distance is between isolate D1 and L1-2 and also N1-3, KR1-3 and D1-3 and also N2-2, D1-2 and L2-2, D1-3 and N2-2, N2-2 and N1-3, N2-3 and N1-3, and L1-2 and N1-3. The biggest genetic distance is between isolate D1 and D1-2, KR1-3 and N2 and also D2-2, and D2-2 and N2-2.

**Phylogenetic tree**

Genetic distance measurements were then used to construct a phylogenetic tree of the fifteen isolates and reference strains available at the GenBank as depicted in Figure 3. The phylogenetic tree shows the existence of two clades, namely clade 1 and clade 2. Clade 1 consists of the isolate D1-2, L2-2, D2-2, N2, N1-2, D4-2, and KR3-2 which is in the same clade with the reference strains of *E. coli* O104:H4, *E. coli* O121:H19, *E. coli* SM-25(1) and *E. coli* KL-48(2). Clade 2 consists of the isolate D4-3, N2-3, L1-2, N1-3, D1, KR1-3, D1-3, and N2-2 which is in the same clade with the reference strains of *E. coli* Sakai, *E. coli* EDL 933 W, and *E. coli* O111:H-.

![Figure 3. Phylogenetic tree among fifteen isolates and reference strains available on GenBank](image-url)
Table 1. The genetic distance among fifteen isolates with data from GenBank of the 16S rRNA genes

| Isolate           | E. coli Sakai | E. coli O104:H4 | E. coli EDL 933_W | E. coli SM25(1) | S. sonnei | E. coli O111:H2 | E. coli O121:H19 | D1 | KR3-2 | KR1-3 | N2 | D1-2 | D2-2 | D4-2 | D1-3 | D4-3 | N1-2 | N2-2 | N2-3 | L2-2 | L1-2 | N1-3 |
|-------------------|---------------|-----------------|-------------------|-----------------|-----------|----------------|-----------------|----|------|------|----|------|------|------|------|------|------|------|------|------|------|------|------|
| E. coli_O104:H4   | 0.013         |                 |                   |                 |           |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| E. coli_EDL_933_W | 0.000         | 0.013           |                   |                 |           |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| E. coli_SM25(1)   | 0.008         | 0.004           | 0.008             |                 |           |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| E. coli_KL48(2)   | 0.007         | 0.007           | 0.007             | 0.003          |           |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| S. sonnei        | 0.010         | 0.010           | 0.010             | 0.002          | 0.005    |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| E. coli_O111:H2   | 0.004         | 0.010           | 0.004             | 0.010          | 0.008    |                |                 |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| E. coli_O121:H19  | 0.010         | 0.003           | 0.010             | 0.003          | 0.005    | 0.007          | 0.007          |    |      |      |    |      |      |      |      |      |      |      |      |      |      |
| D1                | 0.003         | 0.010           | 0.003             | 0.008          | 0.008    | 0.008          | 0.006          | 0.11|      |      |    |      |      |      |      |      |      |      |      |      |      |
| KR3-2             | 0.007         | 0.007           | 0.007             | 0.004          | 0.004    | 0.010          | 0.007          | 0.004|      |      |    |      |      |      |      |      |      |      |      |      |      |
| KR1-3             | 0.004         | 0.008           | 0.004             | 0.010          | 0.010    | 0.003          | 0.008          | 0.003|      |      |    |      |      |      |      |      |      |      |      |      |      |
| N2                | 0.010         | 0.003           | 0.010             | 0.004          | 0.004    | 0.001          | 0.011          | 0.004| 0.007| 0.003| 0.010|      |      |      |      |      |      |      |      |      |      |
| D1-2              | 0.010         | 0.003           | 0.010             | 0.004          | 0.004    | 0.004          | 0.001          | 0.010| 0.006| 0.008| 0.003|      |      |      |      |      |      |      |      |      |
| D2-2              | 0.009         | 0.005           | 0.009             | 0.001          | 0.004    | 0.003          | 0.010          | 0.004| 0.009| 0.005| 0.010| 0.002|      |      |      |      |      |      |      |      |
| D4-2              | 0.007         | 0.006           | 0.007             | 0.006          | 0.008    | 0.005          | 0.007          | 0.006| 0.004| 0.007| 0.004| 0.007|      |      |      |      |      |      |      |
| D1-3              | 0.004         | 0.008           | 0.004             | 0.008          | 0.009    | 0.004          | 0.003          | 0.006| 0.001| 0.008| 0.007| 0.009| 0.004|      |      |      |      |      |
| D4-3              | 0.003         | 0.010           | 0.003             | 0.005          | 0.005    | 0.007          | 0.006          | 0.008| 0.003| 0.004| 0.007| 0.006| 0.005| 0.004|      |      |      |      |      |
| N1-2              | 0.009         | 0.007           | 0.009             | 0.005          | 0.005    | 0.007          | 0.007          | 0.005| 0.009| 0.007| 0.007| 0.004| 0.006| 0.004| 0.006| 0.006|      |      |      |
| N2-2              | 0.004         | 0.009           | 0.004             | 0.009          | 0.010    | 0.004          | 0.009          | 0.007| 0.001| 0.009| 0.007| 0.010| 0.004| 0.001| 0.004| 0.007|      |      |      |
| N2-3              | 0.004         | 0.010           | 0.004             | 0.007          | 0.007    | 0.007          | 0.010          | 0.002| 0.005| 0.004| 0.007| 0.008| 0.007| 0.007| 0.004| 0.002| 0.008| 0.003|
| L2-2              | 0.010         | 0.002           | 0.010             | 0.002          | 0.003    | 0.009          | 0.005          | 0.007| 0.002| 0.001| 0.003| 0.005| 0.006| 0.007| 0.004| 0.007| 0.007|      |      |
| L1-2              | 0.003         | 0.011           | 0.003             | 0.008          | 0.008    | 0.009          | 0.011          | 0.001| 0.006| 0.003| 0.008| 0.010| 0.009| 0.007| 0.003| 0.009| 0.002| 0.002| 0.009|
| N1-3              | 0.002         | 0.010           | 0.002             | 0.007          | 0.008    | 0.005          | 0.010          | 0.001| 0.005| 0.002| 0.007| 0.009| 0.008| 0.006| 0.002| 0.008| 0.001| 0.008| 0.001|
Discussion

This research has successfully amplified 16S rRNA gene from fifteen isolates of pathogenic E. coli isolated from cow meat. All isolates studied had DNA bands at the appropriate size indicated by the presence of bright thick bands. The DNA fragments of the E. coli genome were successfully amplified using primers 27F and 1492R adhered well to specific sites of the DNA template with the optimum temperature during primer annealing (ALatawi et al. 2015). The appearance of a single DNA band indicates that the primer pair used is specific and only attaches to the expected position (Kuczynski et al. 2012; Miyazaki et al. 2017; Sambo et al. 2018). The 16S rRNA gene of the fifteen isolates was successfully sequenced from isolates of pathogenic E. coli and aligned with the data available in GenBank. References are from the same and different species or genus. The reference strains are Shiga-toxin producing E. coli (STEC) of E. coli Sakai, E. coli O104:H4, E. coli EDL 933 W, E. coli SM-25(1), E. coli KL-48(2), E. coli O111:H1-, and E. coli O121:H19, while as an outgroup is S. sonnei. Alignment results from the fifteen isolates show that their nucleotide sequence is closely related to isolates from the same species. These results have been supported by 16S rRNA gene sequencing as an appropriate method for bacterial identification and agreed with previous studies.

Jenkins et al. (2012) have been successfully used 16S rRNA gene sequences to identify bacterial pathogens derived from clinical samples even though specific bacteria are difficult to recognize as they share more than 99% identity in their 16S rRNA gene sequences. However, sequencing the 16S rRNA gene has suggested making an essential contribution to patient management by detecting the pathogenic bacteria in culture-negative clinical samples. Srinivasan et al. (2015) have used the 16S rRNA gene to identify pathogenic bacteria from clinical samples and showed that between 16S rRNA gene-based and clinical identities, the concordance rate at the genus level was 96% and the species level was 87.5%. Meanwhile, Magray et al. (2011) and Tan et al. (2016) have succeeded in characterizing E. coli isolates and found two isolates of pathogenic E. coli isolated from drinking water through analysis of 16S rRNA gene sequences, respectively. In a previous study, the 16S rRNA gene sequence data have been compared in both conventional and commercial assay formats. Using the 16S rRNA gene sequence data has shown powerful results of bacterial species identification compared to conventional or commercial methods (Janda and Abbott 2007).

Nucleotide sequence of the fifteen isolates also has some similarities to that of S. sonnei. The reason for this is that S. sonnei has the same nucleotide sequence and virulence gene to pathogenic E. coli, especially the ones producing Shiga-toxin (Minuesa et al. 2012; Navarro-Garcia 2014). According to Bielaszewskas et al. (2011), the E. coli O104:H4 that caused the outbreak in Germany has a genome sequence and virulence gene profile of a combination of the STEC strain, namely Shiga-toxin and the enterohaemagglutinative E. coli (EAEC) strain, Shigella enterotoxin Set1. In addition, rRNA sequence-based analysis was used to understand not only microbial diversity within and across groups but also to identify new strains. The fifteen isolates studied had potential as new pathogenic E. coli strains and these strains were estimated to have the same characteristics as E. coli Sakai, E. coli O104:H4, E. coli EDL 933 W, E. coli SM-25(1), E. coli KL-48(2), E. coli O111:H1-, E. coli O121:H19, and S. sonnei.

Grouping and closeness of each isolate in this research and among reference strains are also supported with values of genetic distance and nucleotide differences. Values of genetic distance (Table 1) are in line with the concept of nucleotide similarity and differences put forward by earlier researchers. The genetic distance in this study showed low results, both between the fifteen isolates studied with the reference strain and among each of the fifteen isolates. According to Janda and Abbott (2007), 16S rRNA gene similarity of more than 95% or nucleotide differences of less than 1% (15 of 1500 bp), shows that a nucleotide must be categorized as of the same species. Genetic relationships among populations are depicted using values of genetic distance, with small genetic distance implying close genetic relation (Nei 1972). Similar to what was conveyed by Dharmayanti (2011), the lower the pairwise distance value (genetic distance) means the closer the relationships.

The phylogenetic tree shows that the fifteen isolates may have genetic relationship with reference strains, but it will take more research to prove. Based on the phylogenetic tree, each species found in each traditional market forms a clade that spreads across all clades. A clade is a part of a phylogeny that includes an ancestral lineage and all descendants of that ancestor. This group of organisms has monophyletic characteristics, so it is called a monophyletic group. This causes members in monophyletic groups to be considered to have very close relationships and are assumed to carry the same genetic and biochemical traits or patterns (Baum 2008; McLennan 2010). This indicates that the bacteria isolated from each traditional market have close phylogenetic relationships. All fifteen isolates studied had the potential to be the same species although further research was needed. The 0.001 scale refers to the evolutionary distance in branch length. The high nucleotides similarity between 16S rRNA genes of isolates that originated from cow meat from the different markets with reference strain showed they have a close relationship with STEC. The same results have also been shown by previous studies from Abuelhassan et al. (2016) who explained in their phylogenetic tree that the studied E. coli isolates were in a clade close to and similar to those obtained in other parts of the world. The study showed that all E. coli isolates studied were similar to E. coli strains isolated in other countries which were pathogenic E. coli. Based on the phylogenetic tree, the closeness of the studied E. coli with E. coli O113:H21 which is a STEC has been reported by Ayoade et al. (2021). Phylogenetic analysis has shown to identify seven clusters among the O26 STEC strain (Norman et al. 2015) and prove the similarity of the pathogenic E. coli isolates studied with E. coli isolated from children with diarrhea (Ame´zquita-Montes et al. 2015). High nucleotide similarity between similar 16S
rRNA genes was also found in Saudarana (2014). The phylogenetic tree based on nucleotide sequence shows that D2-2 isolate is in one branch with _E. coli_ SM-25(1) with bootstrap value of 79%, both isolates show genetic similarities. Isolate D2-2 is potential of the same characteristics and cytopathic effect with the reference strains as proven by the presence of eae positive gene, and small genetic distance and insignificant nucleotide differences. The discovery of the eae gene in isolate D2-2 raises the potential that this isolate can colonize the host intestine and induce attaching-effacing lesions. _E. coli_ SM-25(1) and _E. coli_ KL–48(2) are the same species that have similarities in terms of profile protein (Saudarana et al. 2013a), genetic diversity (Saudarana et al. 2013b), and the potential to induce lesion in Vero cells (Saudarana et al. 2011). _E. coli_ SM-25(1) and _E. coli_ KL–48(2) were isolated from the feces of Balinese cow and a patient of kidney failure, respectively (Saudarana et al. 2010). Finding of pathogenic _E. coli_ in cow mean indicates the potential for zoonosis. Therefore, a proper treatment regime needs to be chosen in order to avoid antibiotic resistance of such bacteria, especially in this time of COVID-19 pandemic (Wardoyo et al. 2021). Moreover, similarities between isolate N2 and _S. sonnei_ show that isolate N2 could be a strain outside pathogenic _E. coli_ that may lead to _S. sonnei_. However, such an assumption requires further confirmation using other genetic markers to better understand the ecology and epidemiology of these two foodborne disease agents (Persad and Lejeune 2018).

In conclusion, the fifteen isolates have small genetic distance to the reference strains, and these fifteen isolates are also in the same clade with reference strains. This research shows that the fifteen isolates under investigation are closely related to the reference strains, which is Shiga-toxin producing _E. coli_ (STEC). People should pay more attention in processing food stock, especially cow meat. Further research may focus on determining the strain of those fifteen isolates.

**ACKNOWLEDGEMENTS**

The authors gratefully acknowledge the funding of this research provided by The Directorate of Research and Community Service, Directorate General of Research and Development Strengthening, The Ministry of Research, Technology and Higher Education, Republic of Indonesia in the scheme PMDSU research grant 2020 contract no. 3169/UN1.DITLIT/DIT-LIT/PT/2020.

**REFERENCES**


Baum D. 2008. Reading a phylogenetic tree: The meaning of monophyletic,


