BIODIVERSITAS
Volume 22, Number 10, October 2021
Pages: 4566-4573

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d221050

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

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

Abstract. *Indraswari A, Suardana IW, Haryanto A, Widiasih 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* (DEC) (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 biochemistry 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 (Patel 2001; 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 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 (BHIB) media and incubated for 24 h at 37°C. Bacterial growth was evident with increasing turbidity of BHIB media.

DNA extraction

The *E. coli* isolates grown in BHIB 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 IDT™ of 2 µL, PCR mix from MyTaq™ HS Red Mix (Cat. No.: BIO-25048) of 25 µL, and sterile ddH_2O from UltraPure™ ddH_2O (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'-GGTTACCTTGTTACGACTT-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 (AFOB02000112), *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.

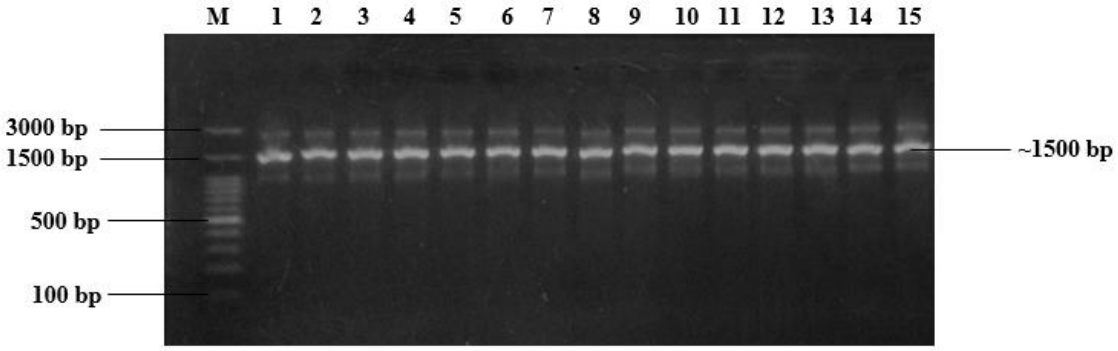

Figure 1. DNA band from 16S rRNA gene amplification in fifteen isolates of pathogenic *E. coli*. M: marker, 1: D1, 2: KR3-2, 3: KR1-3, 4: N2, 5: D1-2, 6: D2-2, 7: D4-2, 8: D1-3, 9: D4-3, 10: N1-2, 11: N2-2, 12: N2-3, 13: L2-2, 14: L1-2, and 15: N1-3

```
E.coli_Sakai      GTC GAA CGG TAA CAG GAA G-- --A AGC TT- --- -GC TTC  [  39]
E.coli_O104:H4    ... ... ... ... ... ... A-- --C ... ..- --- -.. .GT  [  39]
E.coli_EDL_933_W  ... ... ... ... ... ... .-- --. ... ..- --- -.. ...  [  39]
E._coli_SM25(1)   ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
E.coli_KL48(2)    ... ... ... ... ... ... .-- --. ... ..- --- -.. ...  [  39]
S._sonnei         ... ... ... ... ... ... A-- --C ... ..- --- -.. .GT  [  39]
E.coli_O111:H-    ... ... ... ... ... ... .-- --. ... ..- --- -.. ...  [  39]
E.coli_O121:H19   ... ... ... ... ... ... .-- --. ... ..- --- -.. ...  [  39]
D1                ... ... ... ... ... ... T-- --C ... ..- --- -.. .GA  [  39]
KR3-2             ... ... ... ... ... ... T-- --C ... ..- --- -.. .GA  [  39]
KR1-3             ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
N2                --- --- --- --- --- --- --- --- ... ..- --- -.. .GA  [  39]
D1-2              ... ... ... ... ... ... .-- --. ... ..- --- -.. .G.  [  39]
D2-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
D4-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
D1-3              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
D4-3              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
N1-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
N2-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
N2-3              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
L2-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
L1-2              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
N1-3              ... ... ... ... ... ... .-- --C ... ..- --- -.. .G.  [  39]
```

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

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-.

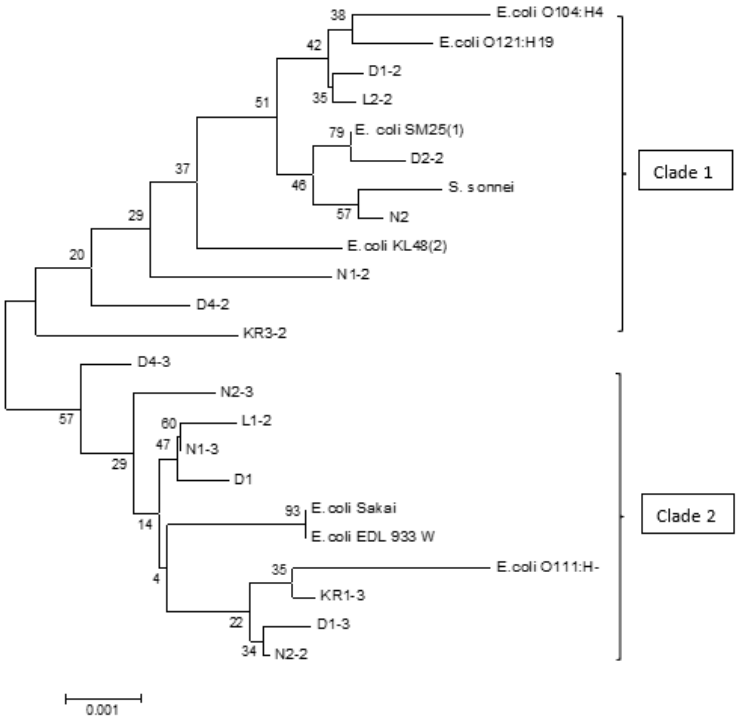

Figure 3. Phylogenetic tree among fifteen isolates and reference strains available on GenBank

Table 1. The genetic distance among fifteen isolates with data from GenBank of the 16S rRNA genes

	***E. coli* Sakai**	***E. coli* O104:H4**	***E. coli* EDL 933 W**	***E. coli* SM25(1)**	***E. coli* KL48(2)**	***S. sonnei***	***E. coli* O111:H-**	***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**
*E. coli*_Sakai																						
*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	0.004	0.010	0.002	0.005																	
*E. coli*_O111:H-	0.004	0.010	0.004	0.010	0.008	0.012																
*E. coli*_O121:H19	0.010	0.003	0.010	0.003	0.004	0.005	0.007															
D1	0.003	0.010	0.003	0.008	0.008	0.008	0.006	0.011														
KR3-2	0.007	0.007	0.007	0.004	0.004	0.004	0.010	0.007	0.004													
KR1-3	0.004	0.008	0.004	0.010	0.010	0.010	0.003	0.008	0.003	0.007												
N2	0.010	0.004	0.010	0.001	0.004	0.001	0.011	0.004	0.007	0.003	0.010											
D1-2	0.010	0.003	0.010	0.001	0.004	0.004	0.008	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	0.002									
D4-2	0.007	0.006	0.007	0.006	0.006	0.008	0.005	0.004	0.007	0.006	0.004	0.007	0.004	0.007								
D1-3	0.004	0.008	0.004	0.008	0.008	0.009	0.004	0.008	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	0.004	0.007	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	0.007	0.004	0.006	0.004	0.006	0.006					
N2-2	0.004	0.009	0.004	0.009	0.009	0.010	0.004	0.009	0.002	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	0.007	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	0.005	0.003	0.009	0.002	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	0.006	0.011	0.001	0.006	0.003	0.008	0.010	0.009	0.007	0.003	0.003	0.009	0.002	0.002	0.009	
N1-3	0.002	0.010	0.002	0.007	0.007	0.008	0.005	0.010	0.001	0.005	0.002	0.007	0.009	0.008	0.006	0.002	0.002	0.008	0.001	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:H-, 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 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 (Muniesa et al. 2012; Navarro-Garcia 2014). According to Bielaszewska 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 enteroaggregative *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:H-, *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 monophyly 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 Suardana (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 (Suardana et al. 2013a), genetic diversity (Suardana et al. 2013b), and the potential to induce lesion in Vero cells (Suardana 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 (Suardana 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

Abuelhassan NN, Mutalib SA, Gimba FI, Yusoff WM. 2016. Molecular characterization and phylogeny of Shiga toxin-producing *E. coli* (STEC) from imported beef meat in Malaysia. Environ Sci Pollut Res 23: 17553-17562. DOI: 10.1007/s11356-016-6954-0

ALatawi A, Sutarno, Susilowati A, Hailu HW. 2015. Biochemical and molecular characterization of food contaminating bacteria isolates from food stall vegetables. Br Microbiol Res J 5 (5): 405-411. DOI: 10.9734/bmrj/2015/13792

Amarantini C, Sembiring L, Kushadiwijaya H, Asmara W. 2011. Identification and characterization of *Salmonella typhi* isolates from Southwest Sumba District, East Nusa Tenggara based on 16S rRNA gene sequences. Biodiversitas 12 (1): 1-6. DOI: 10.13057/biodiv/d120101

Amézquita-Montes Z, Tamborski M, Kopsombut UG, Zhang C, Arzuza OS, Gómez-Duarte OG. 2015. Genetic relatedness among *Escherichia coli* pathotypes isolated from food products for human consumption in Cartagena, Colombia. Foodborne Pathog Dis 12 (5): 454-461. DOI: 10.1089/fpd.2014.1881

Antaki-zukoski EM, Li X, Pesavento PA, Nguyen THB, Hoar BR, Atwill ER. 2018. Comparative pathogenicity of wildlife and bovine *Escherichia coli* O157 : H7 strains in experimentally inoculated neonatal jersey calves. Vet Sci 5 (88): 1-11. DOI: 10.3390/vetsci5040088

Ayoade F, Oguzie J, Eromon P, Omotosho OE, Ogunbiyi T, Olumade T, Akano K, Folarin O, Happi C. 2021. Molecular surveillance of shiga toxigenic *Escherichia coli* in selected beef abattoirs in Osun State Nigeria. Nature portofolio 11: 13966. DOI: 10.1038/s41598-021-93347-w

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

Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, Peters G, Karch H. 2011. Characterisation of the *Escherichia coli* strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: A microbiological study. Lancet Infect Dis 11 (9): 671-676. DOI: 10.1016/S1473-3099(11)70165-7

Blanco M, Blanco JE, Mora A, Dahbi G, Alonso MP, González EA, Bernárdez MI, Blanco, J. 2004. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain and identification of a new intimin variant gene (*eae-ζ*). J Clin Microbiol 42 (2): 645-651. DOI: 10.1128/JCM.42.2.645–651.2004

Botkin DJ, Galli L, Sankarapani V, Soler M, Rivas M, Torres AG. 2012. Development of a multiplex PCR assay for detection of Shiga toxin-producing *Escherichia coli*, enterohemorrhagic *E. coli*, and enteropathogenic *E. coli* strains. Front Cell Infect Microbiol 2 (8): 1-10. DOI: 10.3389/fcimb.2012.00008

CDC. 2014. Questions and answers *E. coli*. https://www.cdc.gov/ecoli/general/index.html

CFSPH. 2016. Enterohemorrhagic *Escherichia coli* and other *E. coli* causing hemolytic uremic syndrome Center for Food Security and Public Health Technical Factsheets 61. Iowa State University Center for Food Security and Public Health. https://lib.dr.iastate.edu/cfsph_factsheets/61

Croxen MA, Finlay BB. 2010. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol 8: 26-38. DOI: 10.1038/nrmicro2265

Dharmayanti NLPI. 2011. Molecular phylogenetic: organism taxonomy method based on evolution history. Wartazoa 21 (1): 1-10. [Indonesian]

Donnenberg MS, Tzipori S, McKee ML, O'Brien AD, Alroy J, Kaper JB. 1993. The role of the *eae* gene of enterohemorrhagic *Escherichia coli* in intimate attachment in vitro and in a porcine model. J Clin Invest 92 (3): 1418-1424. DOI: 10.1172/JCI116718

Fialho OB, de Souza EM, de Borba Dallagassa C, de Oliveira Pedrosa F, Klassen G, Irino K, Paludo KS, de Assis FEA, Surek M, Farah SMDS, Fadel-Picheth CMT. 2013. Detection of diarrheagenic *Escherichia coli* using a two-system multiplex-PCR protocol. J Clin Lab Anal 27: 155-161. DOI: 10.1002/jcla.21578

Fujioka M, Kasai K, Miura T, Sato T, Otomo Y. 2009. Rapid diagnostic method for the detection of diarrheagenic *Escherichia coli* by multiplex PCR. Jpn J Infect Dis 62: 476-480.

Ghazali NSH, Rashid NHA. 2019. Molecular identification of bacterial communities from vegetables samples as revealed by DNA sequencing of universal primer 16S rRNA gene. Int J Med Sci 4 (1): 19-26.

Gomes TAT, Elias WP, Scaletsky ICA, Guth BEC, Rodrigues JF, Piazza RMF, Ferreira LCS, Martinez MB. 2016. Diarrheagenic *Escherichia coli*. Braz J Microbiol 47: 3-30. DOI: 10.1016/j.bjm.2016.10.015

Habets A, Engelen F, Duprez J, Devleesschauwer B, Heyndrickx M, Zutter LD, Thiry D, Cox E, Mainil J. 2020. Identification of Shigatoxigenic and enteropathogenic *Escherichia coli* serotypes in healthy young dairy calves in Belgium by recto-anal mucosal swabbing. Vet Sci 7 (167): 1-10. DOI: 10.3390/vetsci7040167

Indraswari A, Haryanto A, Suardana IW, Widiasih DA. 2021. Isolation and detection of four major virulence genes in O157:H7 and non-O157 *E. coli* from beef at Yogyakarta Special Province, Indonesia. J Anim Heal Prod.

Jafari A, Aslani MM, Bouzari S. 2012. *Escherichia coli*: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran. Iran J Microbiol 4 (3): 102-117.

Janda JM, Abbott SL. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol 45 (9): 2761-2764. DOI: 10.1128/JCM.01228-07

Jenkins C, Ling CL, Ciesielczuk HL, Lockwood J, Hopkins S, McHugh TD, Gillespie SH, Kibbler CC. 2012. Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. J Med Microbiol 61: 483-488. DOI: 10.1099/jmm.0.030387-0

Kuczynski J, Lauber CL, Walters WA, Parfrey LW, Clemente JC, Gevers D, Knight R. 2012. Experimental and analytical tools for studying the human microbiome. Nat Rev Genet 13 (1): 47-58. DOI: 10.1038/nrg3129

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35 (6): 1547-1549. DOI: 10.1093/molbev/msy096

Magray MSUD, Kumar A, Rawat AK, Srivastava S. 2011. Identification of *Escherichia coli* through analysis of 16S rRNA and 16S-23S rRNA internal transcribed spacer region sequences. Bioinformation 6 (10): 370-371. DOI: 10.6026/97320630006370

McLennan DA. 2010. How to read a phylogenetic tree. Evol Educ Outreach 3: 506-519. DOI: 10.1007/s12052-010-0273-6

Miyazaki K, Sato M, Tsukuda M. 2017. PCR primer design for 16S rRNAs for experimental horizontal gene transfer test in *Escherichia coli*. Front Bioeng Biotechnol 5 (14): 1-7. DOI: 10.3389/fbioe.2017.00014

Muniesa M, Hammerl JA, Hertwig S, Appel B, Brüssow H. 2012. Shiga toxin-producing *Escherichia coli* O104:H4: a new challenge for microbiology. Appl Environ Microbiol 78 (12): 4065-4073. DOI: 10.1128/AEM.00217-12

Nataro JP, Kaper JB. 1998. Diarrheagenic *Escherichia coli*. Clin Microbiol Rev 11 (2): 403-403. DOI: 10.1128/CMR.11.2.403

Navarro-Garcia F. 2014. *Escherichia coli* O104:H4 pathogenesis: an enteroaggregative *E. coli* /Shiga toxin-producing *E. coli* explosive cocktail of high virulence. Microbiol Spectr 2 (6): 1-19. DOI: 0.1128/microbiolspec.ehec-0008-2013

Nei M. 1972. Genetic distance between populations. Am Nat 106 (949): 283-92.

Ningrum SG, Soejoedono RD, Latif H, Arnafia W, Wibawan IWT. 2016. Prevalence and characterization of shiga toxin-producing *Escherichia coli* isolated from slaughtered qurban animal in Jakarta Province. Media Peternakan 39 (2): 90-94. DOI: 10.5398/medpet.2016.39.2.90 [Indonesian]

Norman KN, Clawson ML, Strockbine NA, Mandrell RE, Johnson R, Ziebell K, Zhao S, Fratamico PM, Stones R, Allard MW, Bono JL. 2015. Comparison of whole genome sequences from human and non-human *Escherichia coli* O26 strains. Front Cell Infect Microbiol 5: 1-10. DOI: 10.3389/fcimb.2015.00021

Oswald E, Schmidt H, Morabito S, Karch H, Marchès O, Caprioli A. 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect Immun 68 (1): 64-71. DOI: 10.1128/IAI.68.1.64-71.2000

Pangastuti A. 2006. Species definition of procaryotes based on 16S rRNA and protein coding genes sequence. Biodiversitas 7 (3): 292-296. DOI: 10.13057/biodiv/d070319. [Indonesian]

Patel J. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. Mol Diagn 6 (4): 313-21. DOI: 10.1054/modi.2001.29158

Persad AK, Lejeune J. 2018. A review of current research and knowledge gaps in the epidemiology of Shiga toxin-producing *Escherichia coli* and *Salmonella* spp. in Trinidad and Tobago. Vet Sci 5 (42): 1-9. DOI: 10.3390/vetsci5020042

Rahmani S, Mosavp M, Rezaeej A. 2006. Detection of bacteria by amplifying the 16S rRNA gene with universal primers and RFLP. Med J Islam Repub Iran 19 (4): 333-338.

Rananda RM, Djamal A, Julizar. 2016. Identification of *Escherichia coli* O157:H7 bacteria in beef from Lubuk Buaya slaughterhouse. J Kesehatan Andalas 5 (3): 614-618. DOI: 10.25077/jka.v5i3.586. [Indonesian]

Rinanda T. 2011. Analysis of 16S rRNA sequences in the field of microbiology. J Kedokt Syiah Kuala 3: 172-177. [Indonesian]

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4 (4): 406-425. DOI: 10.1093/oxfordjournals.molbev.a040454

Sambo F, Finotello F, Lavezzo E, Baruzzo G, Masi G, Peta E, Falda M, Toppo S, Barzon L, Di Camillo B. 2018. Optimizing PCR primers targeting the bacterial 16S ribosomal RNA gene. BMC Bioinformatics 19 (343): 1-10. DOI: 10.1186/s12859-018-2360-6

Srinivasan R, Karaoz U, Volegova M, MacKichan J, Kato-Maeda M, Miller S, Nadarajan R, Brodie EL, Lynch SV. 2015. Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. PLoS One 10 (2): e0117617. DOI: 10.1371/journal.pone.0117617

Suardana IW, Sumiarto B, Lukman DW. 2007. Isolation and identification *Escherichia coli* O157:H7 on beef at Badung Regency Province of Bali. J Vet 8 (1): 16-23. [Indonesian]

Suardana IW, Artama WT, Asmara W, Daryono BS. 2010. Identification of *Escherichia coli* O157:H7 and detection of Shiga like toxin 1 and 2 genes from animal feces beef, and human feces. J Vet 1 (4): 264-270. [Indonesian]

Suardana IW, Artama WT, Asmara W, Daryono BS. 2011. Adherence pheno-genotypic of *Escherichia coli* O157:H7 isolated from beef, feces of cattle, chicken and human. Indones J Biotechnol 16 (1): 46-52. DOI: 10.22146/ijbiotech.15990

Suardana IW, Pinatih KJP, Ratnawati NLKA, Widiasih DA. 2013a. Protein profile analysis of *Escherichia coli* O157:H7 from human and animals origin. Int J Curr Microbiol Appl Sci 2 (6): 204-214.

Suardana IW, Artama WT, Widiasih DA, Ngurah IG, Mahardika K. 2013b. Genetic diversity of *Escherichia coli* O157:H7 strains using random amplified polymorphic DNA (RAPD). Int Res J Microbiol 4 (2): 72-78.

Suardana IW, Widiasih DA, Nugroho WS, Wibowo MH, Suyasa IN. 2017. Frequency and risk-factors analysis of *Escherichia coli* O157:H7 in Bali-cattle. Acta Trop 172: 223-228. DOI: 10.1016/j.actatropica.2017.05.019

Suardana IW. 2014. Analysis of nucleotide sequences of the 16S rRNA gene of novel *Escherichia coli* strains isolated from feces of human and Bali cattle. J Nucleic Acids 2014: 1-7. DOI: 10.1155/2014/475754

Tan EY, Arifullah M, Soon JM. 2016. Identification of *Escherichia coli* strains from water vending machines of Kelantan, Malaysia using 16S rRNA gene sequence analysis. Expo Heal 8: 211-216. DOI: 10.1007/s12403-016-0194-x

Vidal A, Aguirre L, Seminati C, Tello M, Redondo N, Martin M, Darwich L. 2020. Antimicrobial resistance profiles and characterization of *Escherichia coli* strains from cases of neonatal diarrhea in Spanish Pig Farms. Vet Sci 7 (48): 1-12. DOI: 10.3390/vetsci7020048

Wardoyo EH, Suardana IW, Yasa IWPS, Sukrama IDM. 2021. Antibiotics susceptibility of *Escherichia coli* isolates from clinical specimens before and during COVID-19 pandemic. Iran J Microbiol 13 (2): 156-160. DOI: 10.18502/ijm.v13i2.5974

WHO. 2018. Key facts p. 1–7. https://www.who.int/news-room/fact-sheets/detail/e-coli

Yousif AA, Hussein MA. 2015. Prevalence and molecular detection of intimin (*eaeA*) virulence gene in *E. coli* O157:H7 in calves. Res J Vet Pract 3 (3): 47-52. DOI: 10.14737/journal.rjvp/2015/3.3.47.52