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Evaluation of zoonotic potency of *Escherichia coli* O157:H7 through arbitrarily primed PCR methods

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ABSTRACT

Objective: To evaluate the zoonotic potency of *Escherichia coli* O157:H7 through arbitrarily primed-PCR (AP-PCR) methods as one of the DNA fingerprinting methods.
Methods: A total of 14 isolates consisted of 11 isolates originated from human feces with renal failure symptoms, 2 isolates originated from cattle feces, and 1 control isolate were used in this study. DNA of each isolate was extracted, and their profiles were studied by using AP-PCR method with M13 F and M13 R arbitrary primers.
Results: The results founded that all of 14 isolates had similarity range from 54.6% to 88.5%. Isolates KL-106(3) and KL-55(6) originated from humans showed the degree of similarity with isolates SM-25(1) and SM-7(1) originated from cattle as high as 85% and 77%, respectively.
Conclusions: The high degree of similarity between isolates originated from cattle and human indicated the high potency of zoonoses. The results also concluded AP-PCR method as a briefly fingerprinting method in order to trace the epidemiological of *E. coli* O157:H7.

1. Introduction

Escherichia coli (*E. coli*) O157:H7 is a zoonotic agent of the type of Shiga toxin-producing *E. coli* that can cause disease in humans at some parts of the world [1–4], and cattle are known as the main reservoir of these bacteria [5,6]. Researchers had succeeded in isolating these bacteria from pigs, beef, pork, water or human [7,8] and proved the food products obtained from supermarkets were contaminated with *E. coli* O157:H7 [9,10].

The infection by these bacteria in animals is usually asymptomatic, whereas these bacterial infections in humans usually show clinical symptoms of diarrhea and hemolytic uremic syndrome [1,11,12]. The human infection usually occurred when proper hygiene is not strictly implemented especially in many developing countries and human consumed undercooked food products [2,13].

In Indonesia, several studies related to *E. coli* O157:H7, have been carried out by researchers. The study of Drastini in 2007 had found that 59% of vero toxin *E. coli* consisting of vero toxin *E. coli* O157 and non-O157 can be isolated from all types of livestock namely, dairy cattle, beef, pigs and goats/sheep [14]. Other researchers reported their findings of *E. coli* O157:H7 in Badung, Bali in beef and in healthy human feces were 5.62% and 1.30%, respectively [15]. The slight titer toxins of Stx1 and Stx2 resulted from local isolates of *E. coli* O157:H7 were

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originated from cattle feces and beef had been reported [16]. Moreover, the pheno-genotypic adherence of *E. coli* O157:H7 isolated from beef, feces of cattle, chicken and human had also been studied [17,18].

According to theory, the detection of pathogenic microorganisms including *E. coli* O157:H7 was preferable to using molecular methods, considering the highly sensitivity and specificity of these methods. Molecular method in order to study the epidemiology of food-borne diseases is usually using DNA subtyping. Genomic DNA profiles in DNA subtyping are divided according to the size, and the resulting size will form the patterns which can be used as a characterization of each isolate [19]. Some DNA subtyping methods are commonly used for the characterization of bacterial genomes including pulsed-field gel electrophoresis, ribotyping, and random amplified polymorphic DNA (RAPD) [20,21].

Pulsed-field gel electrophoresis subtyping as one of the fingerprinting methods usually use restriction enzymes that cut genomic DNA randomly to generate approximately 20–30 fragments. The size of the fragments formed quite varied between 10 and 1 000 kbp and this method requires a separation method with special electrophoresis [19,20]. On the other hand, ribotyping method also uses restriction enzymes. Restriction enzyme will cut the DNA fragment in the gene encoding ribosomal RNA. The number of fragments produced will vary ranges from 500 to 1 000 fragments with measure between 1 and 20 kbp. This method has limitation because the results can only be detected using a specific probe for the gene encoding ribosomal RNA from bacteria [22].

RAPD method was developed by researcher using short oligonucleotides (10 bases) as random primers. This method was successfully performed by researchers to differentiate the bacterial genome with only a small amount of genomic DNA [23,24]. RAPD method had been successfully used in an effort to study the zoonotic potency of *E. coli* O157:H7 [25,26].

In the development of this method, the other researchers developed a similar method that used approximately 15 nucleotides as random primers with different amplification conditions with RAPD which is known as the method of arbitrarily primed PCR (AP-PCR). The use of AP-PCR method as a variant of RAPD method for epidemiological investigation has been widely used by researchers [27,28].

AP-PCR method is known to have some advantages *i.e.* it can be applied to a wide range of organisms, fast and simple to generate fingerprint products. This method also used the selected primers without needing the initial information about the organism to be tested. Besides those, the data generated would be able to provide information about the difference or similarity of strains originated from the same species [29,30]. This method has been success to identify the number and the distribution of genotypes of *Streptococcus mutans* and *Streptococcus sobrinus* [31], to predict the degree of malignant cholangiocarcinoma as familiarly as the malignant neoplasm of biliary epithelium [32], and to describe an outbreak caused by extended-spectrum beta-lactamase producing *Klebsiella pneumoniae* [33]. Furthermore, this method also proved in good agreement with the results obtained for the 16S rRNA [34].

Based on these facts, in this study we reported the application of AP-PCR method in order to study the zoonotic potency of *E. coli* O157:H7 from animals to human and all at once as a clarification of previous study, which analyzed the zoonotic aspect of *E. coli* O157:H7 both animals and human origin by analysis of protein profile and 16S rRNA gene [35,36].

2. Materials and methods

2.1. Bacterial strains

Bacterial strains of *E. coli* O157:H7 that used in this study were the same as those used previously [25,36]. Fourteen isolates consisted of 11 isolates originated from human feces with renal failure symptoms *i.e.* KL-52(7), KL-87(7), KL-30(4), KL-45(1), KL-48(2), KL-85(1), KL-83(5), KL-24(5), KL-68(1), KL-106(3) and KL-55(6), 2 isolates originated from cattle feces *i.e.* SM-25(1) and SM-7(1), and 1 control isolate *i.e.* ATCC 43894 were used in this study.

2.2. Cultivation of bacterial strains

Cultivation of the bacterial isolates was done identically with previous study [35]. Fourteen isolates of *E. coli* O157:H7 were taken from stock (stored in 30% glycerol with a storage temperature of −20 °C) to subsequent cultivate on lactose broth medium at 37 °C for overnight. Isolates were re-confirmed by culturing on selective medium sorbitol MacConkey agar, followed by testing on latex agglutination test O157 and testing on H7 antiserum as a final confirmation.

2.3. Extraction of DNA and PCR

Bacterial DNA was extracted using QIAamp DNA mini kits (Qiagen) according to supplier's instruction as described previously [25,36]. The concentration of DNA was determined by spectrophotometer. Amplification of genomic DNA by using AP-PCR method was carried out in 40 μL reaction volumes. These reactions contained 2 μL DNA template (200 ng/μL), 36 μL PCR Supermix 2× (Invitrogen), and 2 μL (20 pmol/μL) of each primer. The primers used were M13F: 5′-TGTAAAACGACGGCCAG-3′ and M13R: 5′-CAGGAAACAGCTATGAC-3′ [37]. PCR program was performed on Eppendorf mastercycler personal. The PCR amplification had initial DNA denaturation at 94 °C for 5 min, followed by 39 cycles of denaturation at 94 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 1 min; finished by a final extension at 72 °C for 5 min at the end of the amplification. About 5 μL of PCR product was analyzed by electrophoresis (Bio-Rad) in 1.5% agarose (Gibco BRL) gel, at 90 v for 45 min. The gel then stained by 1% solution of ethidium bromide (50 μL/L) and destained with tetrabromoethane 1× for 20 min. Gel was visualized by UV transillumination and it was recorded by digital camera FE-270 7.1 megapixels.

2.4. Data analysis

The amplified fragments were scored in a descending manner from higher to lower molecular weight in a binary code. The appearance of product was designed as 1 and its absence as 0 [38]. Evolutionary distance was measured with algorithm of unweighted pair group method using arithmetic averages (UPGMA) and similarity coefficients of each cluster were showed near the branch of phenogram. The data were processed using multivariate statistical package 3.1 program [35,39].

3. Results

3.1. AP-PCR profile

The purified DNA of 14 isolates of *E. coli* O157:H7 including control isolate was subjected AP-PCR and different patterns of genomic DNA were obtained. The DNA profiles of 14 isolates generated using primer M13F were shown in Figure 1.

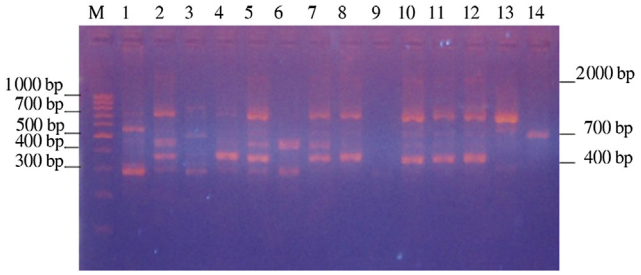

Figure 1. AP-PCR profile of genomic DNA of *E. coli* O157:H7 by using primer M13F on 1.5% agarose gel.
Line 1: ATCC 43894 (positive control); Line 2: KL52(7); Line 3: KL87(7); Line 4: KL30(4); Line 5: KL45(1); Line 6: KL(48(2); Line 7: KL85(1); Line 8: KL83(5); Line 9: KL24(5); Line 10: KL68(1); Line 11: KL-106(3); Line 12: KL-55(6); Line 13: SM-25(1); Line 14: SM-7(1); M: Marker 100 bp DNA ladder.

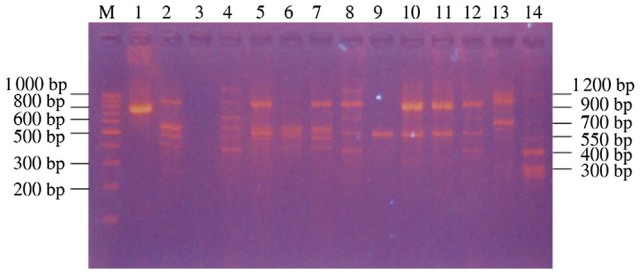

Figure 2. AP-PCR profile of genomic DNA of *E. coli* O157:H7 by using primer M13R on 1.5% agarose gel.
Line 1: ATCC 43894 (positive control); Line 2: KL52(7); Line 3: KL87(7); Line 4: KL30(4); Line 5: KL45(1); Line 6: KL(48(2); Line 7: KL85(1); Line 8: KL83(5); Line 9: KL24(5); Line 10: KL68(1); Line 11: KL-106(3); Line 12: KL-55(6); Line 13: SM-25(1); Line 14: SM-7(1); M: Marker 100 bp DNA ladder.

The use of primer M13R showed different patterns. The complete AP-PCR profiles of 14 isolates of *E. coli* O157:H7 including control isolate ATCC 43894 using primer M13R were shown in Figure 2.

According to Figures 1 and 2, the bands that were obtained from 14 isolates spread out the size range from 300 to 2 000 bp. Total bands and fragment variations of the 14 isolates of *E. coli* O157:H7 generated by AP-PCR using both primers M13F and M13R were summarized in Table 1.

3.2. Phenogram analysis

Total bands and fragments of all isolates obtained using AP-PCR method in Table 1 showed variation among isolates. These variations were specific for each isolate so that they can be used to differentiate between one isolate to others. Further analysis of the data in Table 1 showed the genetic relatedness among isolates. Genetic relatedness described as a tree-like structure was known as phenogram presented in Figure 3.

Figure 3 shows that *E. coli* O157:H7 strains were divided into some clades with each coefficient similarities. Isolate KL-87(7) showed closely related to ATCC 43894 with a coefficient similarity of 69.2 or having similarity of 69.2%. Moreover, both KL-45(1) and KL-30(4) isolates shared clade with coefficient similarity of 80.8 or having similarity of 80.8%, as well as the others. The complete matrix of similarity coefficient among isolates of *E. coli* O157:H7 was presented in Table 2.

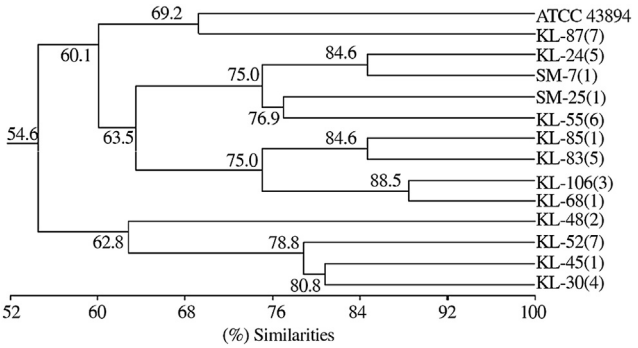

Figure 3. Phenogram of *E. coli* O157:H7.
Phenogram was constructed using simple matching coefficient (*Ssm*) and algorithm UPGMA based on 116 AP-PCR fragments generated by arbitrary primer M13F and M13R.

Table 1
Fragment and total band of 14 isolates of *E. coli* O157:H7 generated by AP-PCR using primers M13F and M13R.

Isolates	Source	Fragments with primer M13F (bp)	Fragments with primer M13R (bp)	Total bands
ATCC 43894	Human feces control	300; 450; 550; 600; 700; 2000	750	7
KL-52(7)	Human feces	300; 400; 450; 550; 750; 1400	400; 450; 550; 800; 850	11
KL-87(7)	Human feces	300; 400; 500; 700; 800	–	5
KL-30(4)	Human feces	300; 400; 450; 750	400; 450; 500; 550; 700; 850; 1200	11
KL-45(1)	Human feces	300; 400; 450; 600; 750; 1400; 2000	400; 450; 500; 550; 850	12
KL-48(2)	Human feces	300; 500	400; 450; 500; 550; 700; 800; 1000	9
KL-85(1)	Human feces	400; 500; 600; 800; 1 400; 2000	400; 450; 500; 550; 900	11
KL-83(5)	Human feces	400; 500; 600; 800; 1400; 2000	400; 550; 700; 900; 1 200	11
KL-24(5)	Human feces	–	400; 550	2
KL-68(1)	Human feces	400; 500; 600; 800; 1400	350; 550; 900	8
KL-106(3)	Human feces	400; 700; 800; 1400	350; 550; 900	7
KL-55(6)	Human feces	400; 700; 900; 1400	400; 550; 700; 900	8
SM-25(1)	Cattle feces	400; 700; 900	550; 600; 700; 1000; 1200	8
SM-7(1)	Cattle feces	700	300; 400; 550; 700; 1200	6
Total general				116

Table 2
Similarity coefficient among isolates of *E. coli* O157:H7.

	ATCC 43894	KL-52(7)	KL-87(7)	KL-30(4)	KL-45(1)	KL-48(2)	KL-85(1)	KL-83(5)	KL-24(5)	KL-68(1)	KL-106(3)	KL-55(6)	SM-25(1)	SM-7(1)
ATCC 43894	1.00													
KL-52(7)	0.54	1.00												
KL-87(7)	0.69	0.54	1.00											
KL-30(4)	0.46	0.77	0.54	1.00										
KL-45(1)	0.58	0.81	0.50	0.81	1.00									
KL-48(2)	0.46	0.62	0.62	0.69	0.58	1.00								
KL-85(1)	0.46	0.54	0.62	0.54	0.73	0.62	1.00							
KL-83(5)	0.46	0.46	0.62	0.54	0.58	0.54	0.85	1.00						
KL-24(5)	0.65	0.65	0.73	0.65	0.62	0.73	0.65	0.65	1.00					
KL-68(1)	0.50	0.50	0.73	0.42	0.54	0.50	0.81	0.81	0.69	1.00				
KL-106(3)	0.54	0.54	0.77	0.46	0.50	0.46	0.69	0.69	0.73	0.89	1.00			
KL-55(6)	0.50	0.58	0.65	0.58	0.54	0.58	0.65	0.73	0.77	0.69	0.81	1.00		
SM-25(1)	0.50	0.42	0.65	0.58	0.39	0.58	0.42	0.58	0.69	0.54	0.65	0.77	1.00	
SM-7(1)	0.58	0.50	0.65	0.65	0.46	0.65	0.50	0.65	0.85	0.54	0.65	0.77	0.77	1.00

Similarity matrix was generated using value of simple matching coefficient (Ssm) and algorithm UPGMA.

4. Discussion

AP-PCR as one of the fingerprinting techniques was known to have many advantages such as its simplicity and shorter time consumption. This technique allows genetically different bacterial strains to be distinguished with great sensitivity and efficiency [28]. Sensitive and efficient technique for typing pathogenic microbes was important for tracing routes of infection and for understanding the spread and evolution of virulence agent [28,34]. In our study, AP-PCR was used as a technique to study zoonotic potency of *E. coli* O157:H7 in humans from animals' origin.

Our study showed AP-PCR technique as a powerful discrimination method to distinguish each isolate characterized by many bands or fragments that were amplified. Either primer M13F or M13R produced a number of lengthy fragments (Table 1). Total amplification produced by this method *i.e.* 116 bands consisting of 59 amplicons for primer M13F and 57 amplicons for primer M13R. The lengthy fragment resulted from both primers also showed variation. As many as 12 fragments were detected from primer M13F consist of 300, 400, 450, 500, 550, 600, 700, 750, 800, 900, 1 400 and 2 000 bp. Moreover, primer M13R detected 13 variations consisting of 300, 350, 400, 450, 500, 550, 700, 750, 800, 850, 900, 1 000 and 1 200 bp. The variation in the length and number of fragments was amplified from both primers for each isolate indicated the highly power discrimination of this method as one of the DNA fingerprinting methods to describe the genetic variation for each isolate.

Glick and Pasternak revealed that the primer or short oligonucleotides used in genomic analysis would form pairs in many places with chromosomal DNA. The number of amplified DNA fragments would depend on the primers used, and the result could be used to determine the characteristic of the entire genome or the chromosome of an individual [19]. Accuracy of AP-PCR as one of the fingerprinting methods with a great sensitivity and efficiency had proved by researcher [40]. Their research reported this method was successfully used to distinguish *E. coli* O157:H7 strain using template from boiled stationary-phase cultures, without the need for time-consuming phenol extraction [41].

The use of AP-PCR method also successfully investigated clonal relatedness among the strains of epidemic isolates of *Vibrio cholerae* O1 recovered from an outbreak occurring in different parts of Iran [42] and to type *Clostridium difficile* isolated from different sources of Imam Reza hospital, Tabriz, Iran in order to control nosocomial infections produced by these bacteria [28].

Further analysis of the data in Table 1 showed the genetic relatedness among isolates of *E. coli* O157:H7. Genetic relatedness were described as a phenogram in Figure 3 showed 14 isolates tested exhibiting clustering into 13 clades. Clade 1 was formed by isolates KL-106(3) and KL-68(1) with 88.5% similarity; clade 2 was formed by isolates KL-85(1) and KL-83(5) with 84.6% similarity; as well as clade 3 was formed by isolates KL-24(5) and SM-7(1) with 84.6% similarity; clade 4 which was formed by isolates KL-45(1) and KL-30(4) was known to have similarity value of 80.8%; while clade 5 was formed as a joint of clade 4 with isolates KL-52(7) having 78.8% similarity and so on to clade 13 with a value of similarity 54.6%.

Phenogram on Figure 3, which was clarified by similarity values of each isolate that was showed in Table 2 exhibiting 2 out of 13 local isolates of *E. coli* O157:H7 *i.e.* isolates KL-24(5) and KL-87(7), respectively were known to have lower similarity *i.e.* 65% and 69%, respectively against control isolates ATCC 43894, which was known as the cause of humans' outbreaks in Japan. On the other hand, some isolates of human were also known to have a high similarity against isolates of cattle. Isolate SM-7(1) showed high similarity against isolates of human *i.e.* KL-24(5) and KL-55(6) as high as 85% and 77%, respectively. Isolate SM-25(1) also showed high similarity against isolate KL-55(6) originated from human with the degree of similarity 77%. The high degree of similarity between isolates of human and cattle origin, especially for isolates with degree of similarity 70% or more, it could be categorized as the same strains. This view was supported by Rosello-Mora and Amann who revealed in generally, organisms in prokaryotic species could be grouped into the same strain if they have similar genome as high as 70% or greater [43].

The high similarity of genomic DNA between isolates of *E. coli* O157:H7 in human and cattle origin indicated its potency as a zoonotic agent which transmitted from animals especially cattle as a main reservoir to humans. Researchers stated that cattle as a natural reservoir of *E. coli* O157:H7 could be

transferred to humans through beef or contaminated environment [3,5,6]. Other researches also reported *E. coli* O157:H7 as one strain of pathogenic *E. coli* known as a zoonotic agent could be detected in animals or humans [44,45] and the transmission usually associated with a complex factors involved environmental-host ecology that directly affects the likelihood of enterohemorrhagic *E. coli* O157 [8,46,47].

Zoonotic potency of *E. coli* O157:H7 local isolates was supported by closely contact between human and cattle. In the area of study, cattle were not always cared by farmers in their cage, but also the farmer brought them outside, and cattle got feed or drinking water from their environment. On the other hand, the beef sold in traditional market was generally with poor hygiene so that the bacteria could be transmitted from animals to humans, according to the statement of previously researchers [48,49]. In conclusion, the high degree of similarity of *E. coli* O157:H7 strains between isolates originated from cattle and human indicated the high potency of zoonoses, and AP-PCR method was proved to be a simple and rapid method to identify the zoonoses agent of *E. coli* O157:H7.

Conflict of interest statement

We declare that we have no conflict of interest.

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