Detection of Enterohemorrhagic *Escherichia coli* (EHEC) in Consumption Water Source using Multiplex PCR Method

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Abstract : *Escherichia coli* is microorganism that often causes diarrhea. One of the most pathogenic *E. coli* bacteria for humans is *Escherichia coli* O157: H7. These bacteria are included in *E. coli* Enterohemorrhagic (EHEC) bacteria which produce shiga like toxin. Shiga like toxin causes Hemorrhagic Colitis (HC) and Hemolytic Ureamic Syndrome (HUS). Water is a basic need for living things. Water that is suitable for use as a source of consumption must fullfill certain chemical, physical and biological requirements. Biologically, the source of consumption water should not contain *E. coli*. The presence of these bacteria indicates fecal contamination. Water, especially consumption water, is a potential source for the spread of microorganisms that cause infection, including *E. coli* O157: H7 bacteria. The purpose of this research is to detect *Escherichia coli* O157: H7 bacteria in consumption water source sample using multiplex PCR method. The primers used in this study were *fliCh7* (625 bp), *rfbE* (296 bp), *stx1* (210 bp), *stx2* (484 bp), *eaeA* (397 bp), and *hly* (166 bp) genes. The process of bacterial isolation was carried out through several stages, namely presumptive test, confirmed test, and completed test. Isolation of bacterial DNA was carried out using boiling cell method. Amplification process was carried out in conditions of 94˚C-2 minutes, followed by 35 cycles of 94˚C-20 seconds, 63˚C-1 minute annealing, and 72˚C-1 extension minutes, and post-extension 72˚C-10 minutes. Next, 2% agarose gel electerophoresis was done at 50 volts for 65 minutes. The results showed that from the total 7 samples tested, all are positive samples of coliform bacteria, 6 are positive samples of *Escherichia coli bacteria*, and 3 are positive samples of *Escherichia coli* O157: H7 bacteria with virulent expression of different genes including *stx1* and *eaeA* genes. An analysis of all irrigation systems is needed to determine the entry of coliform contamination, *E. coli* and *E. coli* O157: H7.

1. INTRODUCTION

Escherichia coli is a normal flora microorganism in the human digestive tract and warm-blooded animals that are facultative anaerobic (Drasar and Hill, 1974). Based on its pathogenicity, *E. coli* is divided into 6 groups: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), and Diffusely Sdherent *E. coli* (DAEC) (Nataro and Kaper, 1998). *Escherichia coli* O157: H7 is part of the Enterohemorrhagic *E. coli* group (EHEC) which is the most pathogenic of other types due to its ability to produce shiga like toxin which can cause Thrombotic Thrombocytopenic Purpura (TTP), Hemorrhagic Colitis, and Hemolytic Ureamic Syndrome (HUS) (Law, 2000)

Water that is suitable for use as a source of consumption must meet the requirements chemically, physically, and biologically. Chemically, consumption water must not contain toxic chemicals. Physically, water should be odorless, tasteless and colorless. Meanwhile, biologically, consumption water should not contain *E. coli* bacteria. The presence of these bacteria indicates that the water has been contaminated by feces (Mubarak and Chayatin, 2009). Escherichia coli O157:H7 infections are mostly caused by food or milk originating from livestock, including fresh fruit, vegetables and water.

In America, the first incident reported Escherichia coli O157:H7 infection after drinking contaminated water occurred in 1989 in Missouri Village. Food and water contamination has been identified as a potential source of the spread of pathogenicity of EHEC O157 in humans (*Tutenel et al*., 2003). The Ministry of Health of the Republic of Indonesia has urged the public to be aware of diseases caused by *E. coli* bacteria. Because, according to the Ministry of Health data, the outbreak of this disease

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actually began to occur in Germany in mid-May 2011. Until June 2, 2011, Germany found 520 cases of haemolytic uraemic syndrome (HUS) with 11 deaths. There were 1,213 cases of enterohaemorrhagic *Escherichia coli* (EHEC); 6 of them died. In Germany, there are 1,733 cases and 17 deaths (Zakki, 2015). Cases of *E. coli* O157:H7 cases have been widely reported in developed countries such as the United States and Japan, but there are few data for cases that occur in Indonesia. This is due to the unavailability of selective media for the isolation of these pathogenic bacteria (Aziz *et al*., 2009). Hill and Jinneman (2000) explained that, for the purpose of epidemiological studies, a zoonotic agent should be used in the application of genetic techniques because the data produced have very high accuracy. One of the molecular methods that can be used is the multiplex PCR method. This method uses a variety of primers to produce amplicons of various sizes specific to different DNA sequences in one running-test.

Multiplex Polymerase Chain Reaction is a type of PCR technique that uses several primary pairs in one reaction to produce amplicons from various target genes with different DNA sizes. The use of molecular techniques is done because it is specific to the target genes to be detected. Besides that, the time used is shorter compared to conventional testing. Therefore, the purpose of this study is to detect the presence of *Escherichia coli* O157: H7 bacteria in consumption water sources samples using Multiplex PCR method.

2. RESEARCH METHODS

2.1. Tools and materials

The tools used in this study are Laminar Air Flow (LAF), glass beaker, hot plate, ose needle, measuring pipette, 10 µl micropipette, 20 µl, 200 µl, and 1000 µl, analytic balance sheet, centrifuge, autoclave, incubator, water heater, thermocycler (Labnet), spectrophotometer (Bio-Drop), electrophoresis devices (Mupid-Exu), and gel documentation tool (Enduro GDS-1302 Labnet).

The materials used in this study include 7 samples of consumption water, Lactose Broth (LB) (Merck) media, Eosin Methylen Blue (EMB) selective media (Merck), Nutrient Agar (NA) media (Merck), sterile aquadest, Go Taq® Green mastermix, six pairs of forward and reverse primary for multiplex PCR detection including *fliCh7* (flagellar antigen), *rbf E* (antigen O157), *stx 1* (shiga toxin 1), *stx 2* (shiga toxin 2), *eaeA* (intimin), and *hly* (haemolysin) (Macrogen) (Table 1), agarose (Promega), DNA scavengers Diamond Nucleic Acid Dye (Promega), TAE Buffer solutions (Promega), loading dye (Promega), DNA Ladder 100 bp (Promega), isolates of *Escherichia coli* O157: H7 bacteria as positive controls obtained from the Veterinary Public Health Laboratory of the Faculty of Veterinary Medicine, Universitas Gadjah Mada of Yogyakarta, and isolates *Candida albicans* as a negative control.

2.2. Sampling Method

The sampling process was carried out using purposive sampling method. Total samples used in this study are 7 samples including tap water and ice cubes.

2.3. Presumptive Test

1 ml of water sample was inoculated into 9 ml Lactose Broth (LB) media which was sterilized using autoclave at 121^{o} C for 15 minutes. Then, it was incubated at 37° C for 24-48 hours. This process was aimed at detecting the presence or absence of coliform bacteria in a sample. Positive results of coliform are indicated by changes in the color of the media from cloudy to clear and gas appears on the durham tube.

2.4. Confirmed Test

The testing process was carried out using Eosyn Methylen Blue Agar (EMBA) media which is a selective medium of *E. coli* bacteria. Positive results on LB media were then inoculated on EMBA media using needle ose, then incubated at 37° C for 24-48 hours. The positive results of *E. coli* bacteria are marked by the appearance of a metallic green colony.

2.5. Completed Test

Purposed to multiply and purify *E. coli* bacteria obtained from positive results in the confirmed test. Performed using Nutrient Agar (NA) media which is a universal medium for bacterial growth.

2.6. DNA Extraction

Isolation of bacterial DNA was carried out using boiling cell method (BPOM Work Instructions, 2008). DNA isolates from NA media were taken as 2-8 colonies using ose needles, then suspended with 500 µl sterile aquadest and distorted. The suspension results were then roasted at a temperature of ± 100^{o} C for 15 minutes, then put into the freezer at ± -4^{o} C for

3 minutes. After that, the suspension was centrifuged at 12.000 rpm for 5 minutes. supernatant was taken as much as 400 µl as a result of DNA isolates. The results of DNA isolates were then measured in terms of concentration and purity using a spectrophotometer (Bio-Drop).

2.7. DNA Amplification

Amplification was done using the following 6 primary pairs as seen in Table 1. PCR reaction was carried out on a total volume of 40 µl in each tube consisting of 15 µl PCR Mastermix Solution, 9 µl DNA Template, 12 primary pairs of 0.5 Primary Primer and reverse µl, and 10 µl Free Water Nuclease. Amplification was carried out using Thermocycler with initial denaturation at 94˚C for 2 minutes, followed by 35 cycles of denaturation at 94˚C for 20 seconds, annealing at 63˚C for 1 minute, and extension for 1 minute at 72˚ C, with the final extension for 10 minutes at 72˚C ending with maintenance at 4˚C.

2.8. Electrophoresis

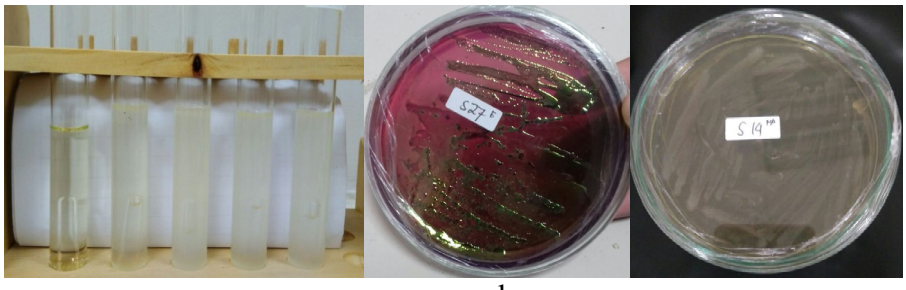
Electrophoresis was carried out using 2% agarose gel at 50 volts for 55 minutes

3. RESULTS AND DISCUSSION

The presumtive test results showed that of the total 7 samples tested; all of them were positive for coliform bacteria, characterized by acid formation, indicated by the color change of the media from clear to cloudy, and gas appearing on the durham tube. This is due to the ability of coliform group bacteria to ferment lactose found in the growth media. Coliform bacteria is one indicator of water quality. Coliform is found in nature like soil, but consumption water is not a natural environment for coliform bacteria (Sengupta and Saha, 2013). Meanwhile, the results of the connfirmed test showed that of the 7 samples tested, 6 samples were positive for *E. coli* bacteria and 1 was negative. Positive results are indicated by the appearance of a metallic green colony on EMBA media while on the NA media, the colonies appeared white as milk. The results of DNA isolates in this study have a good value of purity and concentration, it can be seen in the following table 2.

Table 1: Multiplex PCR Primer used in Amplification

Primers	Sequences (5'-3')	Target gene	Amplicon size (bp)	Reference
FLICH7-F	GCGCTGTCGAGTTCTATCGAGC	*fliCh7*	625	Sarimehmetoglu *et al.,* 2009
FLICH7-R	CAACGGTGACTTTATCGCCATTCC			
rfb E-F	CAGGTGAAGGTGGAATGGTTGTC	*rfb E*	296	Bertrand *et al.,* 2007
rfb E-R	TTAGAATTGAGACCATCCAATAAG			
SLT1-F	TGTAACTGGAAAGGTGGAGTATACA	*stx1*	210	Sarimehmetoglu *et al.,* 2009
SLT1-R	GCTATTCTGAGTCAACGAAAAATAAC			
SLT1 1-F	GTTTTTCTTCGGTATCCTATTCC	*stx2*	484	Sarimehmetoglu *et al.,* 2009
SLT1 1-R	GATGCATCTCTGGTCATTGTATTAC			
AE22	ATTACCATCCACACAGACGGT	*eaeA*	397	Sarimehmetoglu *et al.,* 2009
AE20-2	ACAGCGTGGTTGGATCAACCT			
MFS1-F	ACGATGTGGTTTATTCTGGA	*hly*	166	Sarimehmetoglu *et al.,* 2009
MFS1-R	CTTCACGTCACCATACATAT			



a b c

Figure 1. a. *Presumptive Test result*; b. *Confirmed Test result*; c. *Completed Test result.*

Table 2. DNA Concentration and Purity

Sample		Concentration (µg/ml)	Å260 /280
Ice cube 1	S1	6.720	1.994
Ice cube 2	S2	3.474	1.716
Tap water 1	S3	2.033	1.795
Ice cube 3	S4	3.853	1.973
Tap water 2	S5	0.755	1.361
Tap water 3	S7	0.949	1.901
PositiveControl	C+	0.768	1.838
NegativeControl	C -	0.536	2.269

High purity DNA and free contaminants are needed in molecular technology. The presence of contaminants can inhibit the molecular testing process. Generally contaminants found in DNA isolates are in the form of enzymes, proteins, and lipids (Padhye et al., 1997). DNA isolates are said to be pure if the ratio value in Å260/280 is between 1.8 and 2.0. If the ratio value at Å260/280 is less than 1.8, then DNA isolates are contaminated with phenol or too much solvent is used, and the DNA taken is too little. On the other hand, if the ratio value at Å260/280 is more than 2.0, then DNA isolates contain protein membrane contaminants or other compounds (Sambrook and Ruslle, 2001).

Agarose gel electrophoresis results and visualization using gel documentation showed the following results:

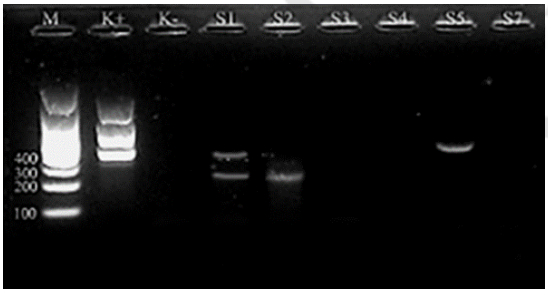


Figure 2. Multiplex PCR results in sample S1-S7
M = Marker (DNA Ladder 100 bp); C+ = Positive Control used E. coli O157:H7; C- = Negative Control

The picture above shows that from the 6 samples tested, 3 positive samples expressed the virulent gene of *E. coli* O157: H7 bacteria. Positive samples include the S1 sample (ice cube 1) DNA bands appear in the area of 210 bp and 397 bp which is an area of amplification products from the *stx1* and *eaeA* genes in sequence. Furthermore, in the S2 sample, bands appeared in the area of 210 bp (positive for *stx1* gene) and in the S5 sample, DNA bands appeared in the area of 397 bp (positive for *eaeA* gene).

E. coli O157: H7 is one of the most pathogenic bacteria in humans because it has a variety of different virulent genes, like a positive result in this study which found a positive sample of the *stx1* (shiga like toxin) gene resulting from the gene expression which can inhibit protein synthesis resulting in cell damage and death (Boerling et al., 1999; Lahtia et al., 2001; Rey et al., 2006; Bentancor et al., 2012). Meanwhile, the *eaeA* gene encodes intimate proteins can cause adherence, causing damage to the intestinal lining (Paton and Paton, 1998; Boerling et al., 1999; Fu et al., 2005; Rey et al., 2006). These bacteria should not be found in consumption water. Water pollution caused by microorganisms is an important problem that must be addressed immediately (Sengupta and Saha, 2013) because water is a medium that is very potential and fast in the spread of infectious diseases. Therefore, it is necessary to evaluate and monitor the consumption water (ice cubes) in circulation so that infection by bacteria *E. coli* O157: H7 can be prevented.

Multiplex PCR is one variation of the PCR technique. This method uses several primary sets in a single PCR mixture to produce amplicons of various sizes specific to different DNA sequences. With gene targeting at the same time, additional information can be obtained from a single running test that will not require multiple reagents and more time to do it. Annealing temperature for each primary set must be optimized to work correctly in a single reaction, and amplicon size. That is, the length of the base pair must be different enough to form a different band when visualized by agarose gel electrophoresis. Detection using multiplex PCR is faster than using conventional culture methods (microbiology) in sorbitol Mac Conkey agar (SMAC) media, other than that PCR also eliminates the possibility of errors in detecting the presence or absence of Escherichia coli O157: H7 bacteria in a sample because it uses a variety of primers specific to a particular gene.

4. CONCLUSION

Based on the research that has been done, 7 are positive samples of coliform bacteria, 6 are positive samples of *E. coli* bacteria, and 3 are positive samples of *E. coli* O157: H7 bacteria. Positive samples of *E. coli* O157: H7 have different virulent genes namely *stx1* and *eaeA*.

Various ways can be done to prevent the occurrence of infections due to these bacteria; one

of which is the correct and proper cooking process. It is known that the toxin produced by EHEC bacteria is damaged in the heating process with a temperature of 100^{o}C for 10 minutes, while the EHEC bacteria themselves will die during the heating process at 72^{o}C for 10 minutes.

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