Detection of attaching and effacing virulence gene of *E. coli*

Maratu Soleha

Center for Biomedical and Applied Health Technology, National Institute of Health Research and Development, Ministry of Health Republic Indonesia

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Abstrak

Latar belakang: *Bakteri Escherichia coli (E. coli) ada yang telah bermutasi menjadi patogen yang menimbulkan berbagai penyakit seperti hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), sepsis, pnemonia, neonatal meningitis, dan infeksi saluran kemih. Mutasi terjadi karena bakteri ini menerima transfer gen yang virulen dari bakteri lain yang hidup di sekitarnya. E. coli yang biasanya hidup normal di dalam usus manusia telah beradaptasi sehingga bisa hidup di tanah, makanan, dan saluran kemih. Penelitian ini mendeteksi gene yang virulen pada DNA isolat E. coli.*

Metode: *Untuk deteksi E. coli yang virulen pada penelitian ini digunakan metode Real-time PCR dengan mencocokkan hasil sekuensing dengan sekuens E. coli virulen yang telah di publikasikan sebagai rujukan.*

Hasil: *Sekuens RT PCR menggambarkan DNA gen eae pada BLAST mempunyai kesesuaian dengan rujukan segmen E. coli yang virulen. Dari sampel yang berasal dari E. coli di sekitar perairan lingkungan didapatkan gen Eae sebagai gen yang menyebabkan E. coli menjadi virulen sebesar 7,3%.*

Kesimpulan: *E. coli yang virulen ditemukan pada sampel E. coli yang berasal dari perairan lingkungan dengan metode realtime PCR.* ***(Health Science Indones 2013;1:41-6)***

Kata kunci: *gen virulen E. coli, real-time PCR, perairan lingkungan*

Abstract

Background: *Escherichia coli* (*E. coli*) bacteria have developed into pathogenic bacteria that caused diseases such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), sepsis, pneumonia, neonatal meningitis, and urinary tract infections. Pathogenic *E. coli* have acquired pathogenic/virulence genes from other bacteria in their environment. *E. coli* that normally lived in the human gut had adapted to other niches such as soil, food and the urinary tract. This study investigated the presence of pathogenic *E. coli* from water samples by examining *E. coli* virulence genes present in *E. coli* genomes of water sourced isolates.

Methods: This study used Real-time PCR to detect virulent *E. coli* and sequencing which was then matched to virulent *E. coli* listed in BLAST as a reference.

Results: *E. coli* sequence from RT PCR was matched with *eae* DNA segment pattern in BLAST. *E. coli* isolates tested with eae gene were detected in 7.3% of *E. coli* strains isolated from environmental water sources.

Conclusion: The presence of virulent *E. coli* strains was detected in environmental water sources using Real time PCR. ***(Health Science Indones 2013;1:41-6)***

Keywords: *E. coli* virulence genes, real-time PCR, environmental water

Corresponding author: Maratu Soleha

E-mail: maratu@litbang.depkes.go.id or maratu15@yahoo.com

Pathogenic Escherichia coli have been a threat to human health for many years due to this organism's virulence/pathogenic properties that provide these bacteria with the potential to cause disease in humans. The presence of virulent genes in *E. coli* has been linked to several disease manifestations such as hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), sepsis, pneumonia, neonatal meningitis and urinary tract infections.[1,2] *E. coli* had become pathogenic due to acquisition to pathogenic genes from other bacteria by many methods, such as conjugation, transduction, and as a mobile genetic element.[3] There are many modes of transmission of pathogenic *E. coli*, such as through food, water, and from the environment to humans. Studies have revealed that more than 100 genes from different ETEC, Enterophatogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DAEC), extra intestinal pathogenic *E. coli* (ExPEC), Enterohaemoragic *E. coli* (EHEC) and enteroinvasive *E. coli* (EIEC) strains.[6,7] Pathogenic *E. coli* could be present in the environment due to possible contamination by human or animal fecal material and also as a result of sewage spills into environmental water sources.

Humans frequently interact to environmental water, especially during either their daily activities or other recreational activities, which will potentially, exposed them to the existing biological health hazard including pathogenic E. *coli*. Therefore the identification of biological health hazards especially pathogenic *E. coli* in environmental water is necessary. Virulent *E. coli* have been reported in many countries, for example *E. coli* serotype O157:H7 was found to cause bloody diarrhea in a northern Dutchess County.[4] The bacteria were transmitted to the host by ingestion of *E. coli* bacteria while swimming in the lake.[4] *E. coli* virulent genes from enteroinvasive *E. coli* (EIEC), Enterohaemoragic *E. coli* (EHEC), UPEC and Enterotoxigenic *E. coli* (ETEC) strains have previously been detected in Australian water sources.[5] These studies indicated that there might be multiple virulence genes distributed in the environment. Furthermore every niche has specific organisms with different gene characteristic. Therefore the identification of potential bacteria pathogenic gene at the community contacted environmental water is needed.

Many methods are useful to screen pathogenic *E. coli* such as PCR, multiplex PCR, Microarray, however Real-time PCR (RT-PCR) is a more efficient way of testing large numbers of samples and it is a single-step, closed-tube method that eliminates contamination. RT-PCR is very sensitive (<2% of standard deviation) and can detect small amounts of samples (< 5 copies).[8] Additionally, this method is highly sensitive making it more robust and reproducible than conventional PCR.

This study was aimed to find the presence of *eae* gene from water sources in Queensland Australia by using Real-time PCR methods followed by sequencing to describe the eae gene sequence. The role of the eae gene in *E. coli* is to promote attachment to epithelial cells in the microvilli of intestinal guts.

METHODS

Environmental water samples were collected during the period of 2008 - 2009 from Coomera River region in southeast Queensland. Samples were taken from Marina, Santa Barbara, Paradise point, and Coombabah River located near the Gold Coast where more people gather for recreation, swimming, and surfing.

The *E. coli* isolates recovered were previously identified and DNA was extracted by Maxim Sheludchenko method using the Corbett extractor protocol NO.141404 version 02 with DNA/RNAse-free water. In order to be efficient in time and cost, 96 DNA samples were screened by conventional PCR. Initially, all primers were subjected to gradient PCR to determine the optimal annealing temperature for each primer set. Subsequently, these conditions were applied to real-time PCR. Once optimized, virulence gene detection was done on 96 DNA samples using the Corbett 6000 real-time PCR instrument. Real-time PCR positive samples were selected for sequencing to validate the presence of each virulence gene found. Gene sequences were compared to previously characterized sequences using the BLAST online algorithm. The steps used in this study are described as below.

Culture method for *E. coli*

Isolation of *E. coli* from water sample was done by mTec (Difco, Detroit, USA) membrane filtration method, as recommended by the US Environmental Protection Agency. This method was used to screen for thermo tolerant type II *E. coli* from the environmental water samples. By using this media, only *E. coli* will grow and colonies will have a typical magenta color that possessed β-D-glucuronidase activity. This media is highly selective for thermotolerant type II E. coli. To keep the purity of *E. coli*, isolates were sub-cultured onto MacConkey agar. Nutrient broth

cultures (Oxoid, Cambridge, UK) were subsequently used to enrich and extract DNA from E. coli.

DNA extraction method

The overnight broth culture was centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the *E. coli* pellet was resuspended in 180 µL of DNAase/RNAase-free water. The Corbett protocol NO.141404 version 02 was used to extract the DNA from each sample using the high-throughput CAS-1200 Corbett DNA extractor instrument.

Primer design and optimation

The primer sequences used in this study were obtained from published papers. Forward primer 5'TGAGCGGCTGGCATGAGTCATAC3' reverse primer 5'TCGATCCCCATCGTCACCAGAGG3'. All 14 primer pairs were tested using gradient conventional PCR.

Virulence gene detection using real-time PCR

Following primer optimization, primers were selected for real-time PCR based on the presence of a single clear band and corresponding to the correct size of the gene fragment on the agarose gel. Real-time PCR was performed on the Corbett Rotorgene 6000 using the Type-it HRM PCR reagent (Qiagen, Hamburg, Germany). Each PCR reaction contained 10 µL of the Type-it (Qiagen, Hamburg, Germany), 1.25 µL forward primer with concentration of 10µM, 1.25 µL reverse primer with concentration of 10µM, 5.5 µL water and 2 µL DNA at a concentration of 10 ng/mL. The Corbett CAS-1200 liquid handler was used to prepare 96 reactions simultaneously. The cycling conditions for the three virulence genes *eae* detected by real-time PCR are shown in table 1.

Table.1 Realtime PCR Running Condition

Genes	Innitiation	Denature	Annealling	Extention	Final Extention
eae	94° C	95° C	57.1° C	72° C	72° C
	5 minutes	60 seconds	60 seconds	240 seconds	7 minutes

Sequence validation of virulence genes

The preparation of DNA sequencing reactions requires many steps. The first step is to clean up the PCR products for each virulence gene using the PCR purification kit (Bioline, London, UK). The second step is to prepare the sequencing reaction by combining 0.5 µL Big dye terminator mix (ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits, Original and Version 2.0, Applied Biosystems, CA, US) together with 1 µL forward primer at 3.2 µM in concentration, 3.75 µL Big dye buffer, 2 µL of DNA at a concentration of 0.2 ng/µL, and 12.75 µL Millipore water. The PCR cycling conditions were: 96°C for 1 minutes (initial denature) then repeat the cycle for 30 cycle 96°C for 20 second, 50°C for 5 second, 60°C followed by 4°C for 10 minutes.

PCR products were reprocessed to eliminate excessive reagents by adding 5µL of 125 mM EDTA, pH 8 and mixed by a quick spin or hand flicking. These were incubated with ethanol 100% at room temperature for 15 minutes. The tubes were covered with aluminum foil to protect the big dye reagent from decay. The DNA pellet was recovered by aspiration of the solution away after spinning 20 minutes. If necessary, another quick spin was applied again to remove any residual solution from pellet as unincorporated Big Dye terminator mix. The pellet was rinsed by adding 250 µL of 70% ethanol, vortexed briefly to mix the ethanol with the DNA. The DNA was separated from the ethanol by aspirating the solution after spinning down the DNA-ethanol mix at maximum speed for 5 minutes. Any residual solution was removed completely. The final step was to dry the DNA by air drying with an open lid at room temperature and again, the tubes were covered to protect from light. Sequencing was performed at the Genetic Research laboratory at QUT using the Applied Biosystems 3500 Genetic analyzer. The size of the DNA templates were below 400 bp in size, and therefore 3-10 ng of template DNA was used for each reaction.

RESULTS

Optimization of the primer

The primers were optimized to determine the optimal annealing temperatures using temperature gradient PCR. Among EPEC *E. coli* group only eae genes showed a single, clear PCR product of the correct size on the agarose gel. Annealing temperatures for eae primer sets was 57.1°C. Gel pictures of these results can be seen in Figure 1.

Environmental *E. coli* DNA samples were amplified using conventional PCR and the products were run on agarose gels (1.5%) for 45 minutes with a voltage of 90 V. A DNA molecular weight marker was used, namely, Hyper ladder IV (Bioline, London, UK). Lane 1 shows the *eae* genes which is 241 bp in length.

Realtime PCR

The RT-PCR results are presented in Figure 2. The eae genes were detected using Corbett realtime PCR instrument. There were 7 curves from 96 samples, which indicated the presence of eae genes. The curve that melted around 85°C indicated the presence of eae genes and was further confirmed by electrophoresis agar, then sequencing experiment.

Following the optimization of the *eae* primers, all 96 DNA extracts were screened for the presence of this gene using RT-PCR. There were seven positive *eae* samples out of the 96 samples tested (7.3%).

Sequencing validation of the virulence genes detected in the samples used in this study

EAE gene sequencing was performed to validate the RT-PCR results, and can be viewed in Figure 3. The *eae* genes had 98% query coverage, 98 % maximum identity with an E-value of 8e-88.

The sequence was the aligning comparation between sequence subject and query from BLAST. There were 98% of identities between subject and query of eae genes, 1% gaps with coding sequence length 426 bp.

The following letters were the *Eae* gene sequences from this study:

GAAKGARCSGCCCGGCAATGGTTTT-
GATATCCGCTTTAATGGCTATTTACCAT-
CATATCCGGCATTASGCYCCAAACTGAT-
GTACGAACAGTATTATGGTGATAATGTT-
GCTTTGTTTAATTCCGATAAGTTGCAGTC-
GAATCCTGGCGCGGCGACCGTTGGTG-
TAAACTACACTCCGATTCCTCTGGTGAC-
GATGGGGATCGAAAMGGCAACTTATCG-
GATTAAACAMMGCARGTTRTACMTAATACT-
GTTCTACTCAATTTGGCGCCKAATGCC-
GAWTGATGGTAAATAGCCTTAAGCSGATATC-
MAACATTTGCCGGGCGCTCATCAART-
CCTTCTTAYRTAYGACTTAGCCSCCCT-
CAAAAAAC

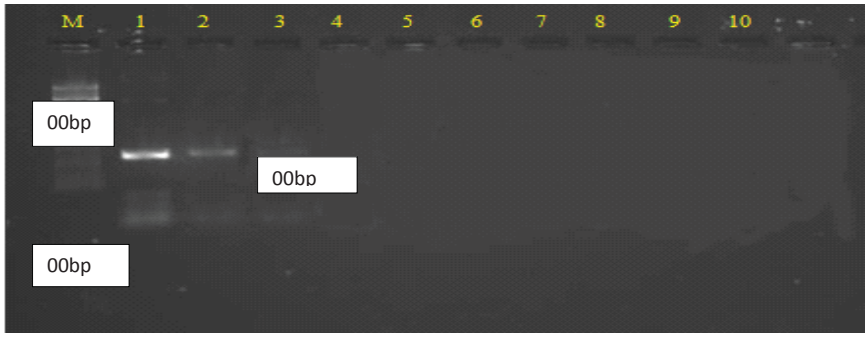

Figure 1. Gradient temperature test of eae genes

Lane 1=57.1°C, lane2= 57.4°C, lane3=58.0 °C, lane4=59.0°C, lane5=60.2°C, lane6= 61.6°C, lane7=63.0°C, lane8=64.3°C, lane9=65.5°C, lane10=66.4°C, lane11=67.0 °C, lane12= 67.1°C

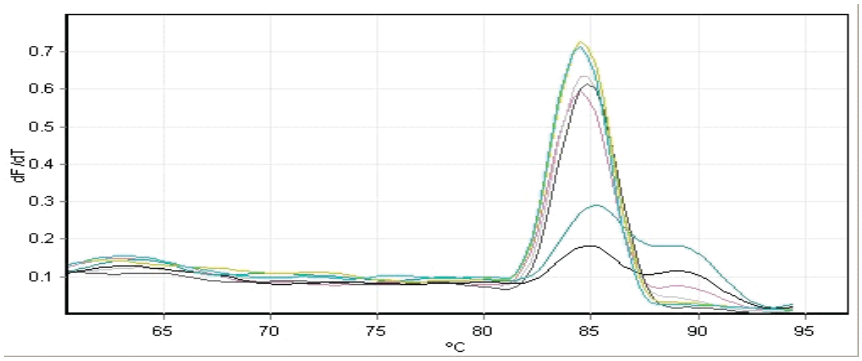

Figure 2. Melt report of eae genes

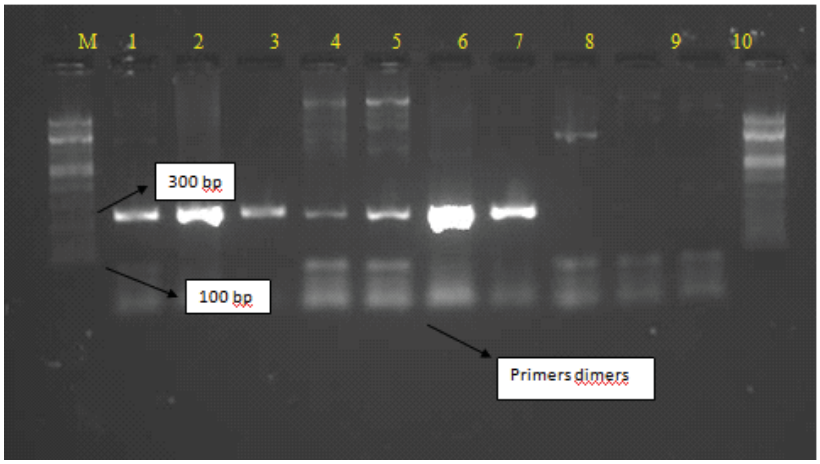

Figure 3. Eae genes RT-PCR confirmation test

Test of eae genes. 20 samples were amplified using conventional PCR and the products were run on agarose gels (1.5%) for 45 minutes with a voltage of 90 V. A DNA molecular weight marker was used, namely, Hyper ladder IV (Bioline, London, UK). Lanes 1,2,3,4,5,6,7,8 show the eae genes that are 241 bp in length.

DISCUSSION

The main objective of this study was to investigate *eae E. coli* virulence traits particularly in *E. coli* strains isolated from environmental water sources. *E. coli* strains harboring particular virulence genes have been found in food, animals, and humans. Previous reports have also found the presence of virulence genes in *E. coli* isolated from water sources. The results of this study showed that eae virulence genes were detected in environmental strains of E. *coli*. Every environment has specific characteristics, and each bacterial organism will adapt to that environment. In addition, the current study showed that virulence traits associated with the virulent *E. coli* O157:H7, known to contaminate food, was found in water. *E. coli* containg virulence genes might shed from humans or animals and then into the environment. Disposal of sewage into water sources can also result in the distribution of virulent *E. coli* into the environment.

This study result is an important finding as it highlights the risk to human and animal health associated with exposure to these virulent *E. coli* in environmental water sources.

Further studies are warranted to investigate the distribution of additional virulence traits in environmental E. *coli*. In particular, it is important to constantly monitor the presence and type of virulent *E. coli* in environmental water sources in order to determine the risk to human and animal health. This study focused only on environmental water from the Coomera River region in South East Queensland, and it is recommended that a larger geographical region is investigated in order to assess the presence and distribution of other virulence traits in *E. coli* isolated from water sources. Indonesia as a tropical country where bacteria in humid air easily grow, may have potential threat in virulent *E. coli*.

Conclusion

E. coli sequence from RT PCR was matched with *eae* DNA segment pattern in BLAST. *E. coli* isolates tested with eae gene were detected in 7.3% of *E. coli* strains isolated from environmental water sources.

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