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APPLICATION MULTIPLEX PCR FOR EARLY DETECTION OF *ESCHERICHIA COLI* CONTAMINATION IN SOME DRINKING WATER RESOURCES IN ABEPURA, PAPUA INDONESIA

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Abstract

Microbial detection takes a long time to produce positive results, so a quicker detection method was chosen. Multiplex polymerase chain reaction (m-PCR) identified bacterial strains in less than 24 hours, detects E.coli specifically, and is faster than traditional methods. The goal of this study was to use m-PCR to detect early pathogenic E.coli bacteria in several drinking water sources in the Abepura district of Papua Indonesia as a parameter of pollution and water quality. The Chelex100 and microwave combination method were used to extract DNA. The first round of testing was done at four different concentrations: 0.125, 0.250, 0.375, and 0.500 M.. The optimum primer concentration for multiplexing applications is 0.25 uM for lt primer; 0.125 uM for stx2 and eae primer, with an annealing temperature of 55oC. m-PCR has been shown to quickly detect pathogenic E. coli in water samples. In the PCR process, the E.coli DNA template was obtained with high purity (1.80-1.94) and concentration (576.9-4301.6 ng/uL). Each multiplex set included three primer pairs for the target gene lt-eae-stx2 on ETEC-EPEC and EHEC respectively. The m-PCR process showed excellent results, and these findings can be considered as a reference for water analysis in several drinking sources in Papua Province.

Keywords: *Drinking Water Resources, Early Detection, M-PCR, Pathogenic E.Coli*

INTRODUCTION

According to World Health Organization data, bacterial contamination in drinking water exposes more than a third of the world's population to disease. Thousands of millions of people died as a result of microbial pathogens and gastrointestinal disorders. Drinking water is microbiologically feasible if it is free of pathogenic microbes and harmful substances, and aesthetically pleasing in terms of color, taste, odor, and turbidity (Soemirat, 2018).

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Several studies on the prevalence of pathogenic *Escherichia coli* contamination of frozen chicken meat, beef, and other foods have been conducted in Indonesia, such as the study of (Teertstra et al., 2017; Yunita, 2015) regarding the analysis of pathogenic *E.coli* contamination of frozen chicken meat, beef, and other foods. The findings revealed that these bacteria isolates were found in these samples. Meanwhile, the presence of *E.coli* in water samples has been extensively studied, with PCR identification proving to be sensitive and specific for assessing water quality and detecting pathogenic *E.coli* bacteria (Radji et al., 2010; Bintari et al., 2014; .

Water and soil are habitats for bacteria, including *Escherichia coli, Staphylococcus aureus, Bacillus cereus* and spore-forming bacteria (Rajkovic et al., 2013; Mailia et al., 2015). Microbes in drinking water sources require attention because poor water hygiene can result in poisoning and disease, the trigger being *E.coli*, especially in patients with compromised immune systems (Huang et al., 2006). Enteropathogenic *E.coli* (EPEC), Enteroaggregative *E.coli* (EAEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Diffusely Adherent *E.coli* (DAEC), and Enterohemorrhagic *E.coli* (EHEC) are all pathogenic *E.coli* strains (EHEC) (Food & Drug Administration, 2012). The presence of *E. coli* in drinking water products indicates contamination (Odonkor & Ampofo, 2013).

Microbiological tests that are commonly carried out are through culture and biochemical tests (Anggreini, 2015), but this method takes about 5-7 days to get positive results, even though the diversity of DNA of various bacterial species can be studied to identify their pathogenicity (Biswas et al., 2010). On the other hand, this methods is complicated, time-consuming, technically difficult, and low sensitivity (Hu et al., 2020) . Scientific studies on the application of multiplex PCR to detect pathogenic *E. coli* have been carried out by (Madic et al., 2011; Kagkli et al., 2012; Mohammed, 2012; Sjoling et al., 2015). The m-PCR method has an advantage over traditional PCR in that it simultaneously amplifies multiple target genes, making it very effective for use in drinking water sources with varying bacterial content. This method has been used on meat, water, milk, vegetables, and ready-to-eat food (Sen et al., 2011; Russo et al., 2014).

This study was intended to detect *E.coli* in several drinking water sources with the molecular m-PCR approach because this approach has more efficiency and sensitivity in identifying the presence of pathogenic bacteria. The selection of the Abepura district as the sampling location was based on data from the Jayapura City BPPS in 2019 that the amount of water distributed and the highest water customer in the PDAM was the Abepura district compared to other sub-districts (BPPS, 2020). Likewise, the information that has been gathered shows that drinking water sources such as bore-wells and bucket wells are increasing in number in Abepura, which is close to contaminant centers such as markets and waste disposal sites. So there is a high potential for gastrointestinal infections due to *E.coli* contamination.

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Based on this background, research on the early detection of *E.coli* in various drinking water sources in Abepura is very important to provide information about the quality of drinking water sources in the Abepura district, so that people can be more selective, prevent poisoning, diarrhea, and other gastrointestinal diseases. The purpose of this study was to detect the presence of pathogenic *Escherichia coli* bacteria early as parameters of microbiological pollution and water quality in several drinking water sources in the Abepura district using the multiplex Polymerase Chain Reaction (m-PCR) method.

The anticipated benefit of this research activity is the development of a pathogenic *E. coli* detection method that has specificity, sensitivity, and efficiency in detecting the presence of pathogenic *E. coli* in drinking water source samples by maximizing the multiplex PCR method.

METHODS

Water sampling took place in and around residents' homes in the Abepura district of Jayapura city. Multiplex PCR was performed in the laboratory of the Papua Agricultural Quarantine Center, while genomic DNA isolation was performed in the microbiology laboratory, Department of Biology, FMIPA Cenderawasih University.

The materials used were 10 mM TRIS-base, 1 mM EDTA, 0.5% SDS, proteinase-K, Rnase A, chelex100, 10% sodium acetate 3M, ethanol 70 and 95%, dreamTaq green, SYBR green, DNA template, primer, nuclease-free water, TSB media, TSA and BHIB. The tools used: thermal cycler, nanodrop, centrifuge, microwave, analytical balance, PCR tube, microcentrifuge tube, micropipette, freezer, petri dish, and Erlenmeyer.

This study started from the preparation stage (water sampling), DNA extraction, primer testing, multiplex PCR application, and analysis of multiplex PCR results.

Samples were collected from water sources in the Abepura district. Derived from drilled wells, bucket wells, and PDAM water. The negative control was *S.typhi* from the Microbiology Laboratory collection, FMIPA-UNCEN. The faucet water was cleaned and disinfected with 70% alcohol. The water was allowed to run for 3 seconds, then closed. The faucet was opened then the bottle was filled with water, sample was delivery from the field to the laboratory for a maximum of 24 hours at a temperature of 4^{o}C or a used a cool box.

Samples of drinking water sources were centrifuged at 3000 rpm for 30 minutes, then the sediment was collected for DNA extraction. A total of 100 L of sediment was put into Eppendorf and added 900 L of lysis buffer solution was then incubated for 24 hours after that DNA extraction was carried out.

Genomic DNA was extracted by a combination of chelex100 and microwave (Reyes-Escogido et al., 2010). The bacterial culture was put into a 15mL centrifuge tube and then centrifuged (25 min, 1500µg). The pellets were transferred to a 2mL tube for centrifugation for 5 minutes (8000µg at 4^{o}C). The pellet was added to 1000 L of TE buffer and then vortexed. The solution was then centrifuged again for 5 minutes (8000µg at 4^{o}C). The pellet was resuspended with 100µL TES lysis

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buffer then put in the microwave for 3 minutes. Added 150µg proteinase K+ 20µg RnaseA. The suspension was put back in the microwave for 3 minutes. Then it was incubated of 2 minutes at room temperature, added 150µL of TE buffer mixture and 25mg of Chelex100. The suspension was put in the microwave under the same conditions as before. Centrifuged at 12000 µg for 5 minutes at 4ºC. The supernatant containing DNA was precipitated with 10% 3M sodium acetate and 2.5µ volume of 95% ethanol. The supernatant mixture was incubated at -20ºC for 20 hours. DNA was washed twice with 1mL of 70% ethanol, dried, and dissolved in 100 L ddH2O. Then stored at -20^{0}C.

The multiplex PCR method was used to amplify the *E. coli* target genes ETEC, EPEC, and EHEC strains. Table 1 summarizes the primer sequences.

Table 1. Multiplex primer sequences

Gene	Primer Sequence	Size (bp)
lt (ETEC)	AGCGGCGCAACATTTCAG TTGGTCTCGGTCAGATATGTGATTC	113
eae (EPEC)	TGATAAGCTGCAGTCGAATCC CTGAACCAGATCGTAACGGC	229
stx2 ((EHEC)	ATCCTATTCCCGGGAGTTTACG GCGTCATCGTATACACAGGAGC	587

A total of 5 µl of the PCR product was electrophoresed on a 2% agarose gel containing TAE buffer1x and EtBr. DNA bands were observed under a UV transilluminator. DNA purity and concentration were measured using a spectrophotometer and nano-dupe and then analyzed descriptively using tables and figures.

RESULTS AND DISCUSSION

The DNA extraction stage is extremely important in DNA analysis using the PCR method. DNA extraction is critical and has a significant impact on the success of the analysis process. The electropherogram displayed the results of DNA visualization with 1% gel electrophoresis from each extracted DNA. Furthermore, it is necessary to carry out quantitative testing of genomic DNA because the concentration of genomic DNA produced varies between 1.80 and 1.94. The DNA purity value (Table 2) clearly shows that the DNA produced by the chelex100-microwave method is pure because it falls within the specified range. If the purity ratio is less than 1.8 or more than 2.0, the DNA is still contaminated with phenol or other proteins.

DNA extraction quantity test showed that the ratio value of the DNA isolates produced from the combination of chelex and microwave methods (1.80-1.92) had a good level of purity. Chelex100 is a chelating resin that has a high affinity for polyvalent metal ions. Initially, chelex resin was used in the process of extracting DNA from blood for forensic purposes (Rathnayaka, 2011), but later, this resin has also been

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successful in extracting DNA from bacterial cells with good concentration and purity of DNA. The presence of chelex during the heating step prevents DNA degradation by chelating metal ions which can act as a catalyst in DNA cleavage/destruction at high temperatures.

Table 2. Concentration and purity of DNA extracted from water samples

Sample code	Concentration	Purity
	(ng/uL)	(A_{260}/A_{280})
Water 01	1659.9	1.80
Water 02	1738.4	1.85
Water 03	4301.6	1.91
Water 04	854.9	1.82
Water 05	3682.1	1.92

In addition, DNA that was treated with the addition of distilled water and heated at 100^{o}C when tested on PCR became undetectable. Unlike the case when the DNA was added with chelex and then heated, the test results using PCR were still detected. This proves that the presence or addition of chelex during the DNA heating process has a protective effect and prevents DNA degradation by chelating metal ions. Chelex 100 is known to be able to remove metals from culture media and reagents, purify dinucleotides, and remove metal ions from blood (Bio-Rad Laboratories, 2017). The amount of DNA extracted from food samples is critical because the PCR method's efficiency can be reduced by the presence of inhibitors derived from the food matrix or by nucleic acid degradation or damage.

Because primer annealing has a significant effect on temperature optimization, the consideration when selecting primer when using the multiplex PCR method is the same annealing value. The annealing temperature (Ta) is the temperature at which the primer begins to attach to the DNA fragment to start the elongation (polymerization) process. Ta value of length and GC content of primer. A non-specific product will result from a low Ta value, while a high Ta value will result in a low amplification efficiency. In an ideal situation, all mPCR primer pairs would support the same amplification efficiency for each target gene. At 55^{o}C, all primer pairs could attach to DNA, according to PCR results. In the multiplex PCR method, the temperature is determined as the annealing temperature.

The multiplex PCR method should allow detecting, differentiating, and quantification of multiple target genes without interfering with one another. Kagkli et al. (2012) stated that when multiplexing, it is very important to combine reactions with the same efficiency.

Confirmation results with 2% agarose revealed only three DNA bands, corresponding to the three products formed. The three DNA bands are lt (113 bp), stx2 (587 bp), and eae (229 bp). Differences in the products of the PCR process are caused

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by differences in GC content, size/length, and sequences, allowing multiple applications to be used. Based on melting curve analysis, not all PCR multiplex assays are suitable for identifying amplicons (Russo et al., 2014). Meanwhile, multiplex PCR testing with three primer pairs on three target genes revealed the presence of bands (Figure 1).

In general, multiplex reactions are limited to the amplification process. One reason is that with each additional primer set that enters the reaction, the degree of flexibility is reduced or even lost (Luyt et al., 2020). Increasing the number of primers also increases the possibility of primer-dimer formation and non-specific amplification. Multiplex test showed peak melting of the product with a low fluorescent value. The stx2 test on EHEC showed a higher fluorescent than others. These results are in accordance by the study of Fedio et al. (2011) which stated that there was a decreasing trend when several target genes were present in isolates for multiplex reactions.

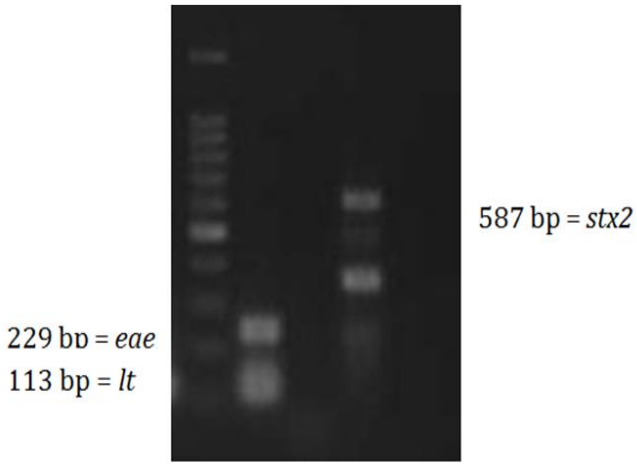

Figure 1. PCR multiplex electropherogram

Culture and biochemical tests are two types of microbiological tests that are commonly used (Anggreini, 2015). However, this method takes about 5-7 days to produce positive results, even though the diversity of DNA from various bacterial species can be studied to determine pathogenicity (Biswas et al., 2010). A scientific study on the use of multiplex PCR to detect pathogenic *E. coli* was conducted by (Madic et al., 2011; Kagkli et al., 2012; Mohammed, 2012). The m-PCR method that has been applied in this study was able to amplify different target genes simultaneously. The results of m-PCR have shown its effectiveness when applied to drinking water sources with various bacterial contents.

This finding has also been widely applied to detect *E.coli* microbes in meat, drinking water, milk, vegetables, and ready-to-eat food (Sen et al., 2011; Russo et al., 2014). Several studies have used the multiplex PCR method to detect and identify pathogenic strains of *E. coli* bacteria in food and non-food samples. The detection of E.coli type EHEC using multiplex PCR on various food

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samples including meat and its derivative products such as milk, apple cider, and lettuce (Fratamico & DebRoy, 2010; Fratamico et al., 2011).

Multiplex-PCR takes only 24-48 hours. This technique isolates bacterial cell DNA from water samples before performing DNA amplification (Yuwono, 2006). Moreover, It takes about 5-6 days to detect *E.coli* in drink and food samples, which is significantly longer than the traditional method, and it also requires more energy (Fedio et al., 2011; Shen et al., 2014). The detection of pathogenic bacteria in drinking water has been shown to have high sensitivity, specificity, and speed using mPCR-based molecular detection.

The mPCR application should have the lowest possible sensitivity in total DNA and be specific in the resulting target gene. The values generated from this study were more sensitive than V. parahaemolyticus mPCR with the toxR gene target (Malcolm et al., 2015; Kim et al., 2014).

The development of mPCR begins with the process of selecting the right primer (Sahu et al., 2019) and optimizing the optimal annealing temperature at 54 – 58 °C (Wang et al., 2019). The mPCR application should have the lowest possible sensitivity in total DNA and be specific in the resulting target gene. The values generated from this study were more sensitive than *V. parahaemolyticus* mPCR with the toxR gene target (Malcolm et al., 2015; Kim et al., 2014).

Previous research conducted by Nguyen et al. (2016) namely one-tube multiplex PCR on *E. coli, S. enterica,* and *L. monocytogenes bacteria* showed a qualitative decrease in the intensity of the PCR product. The results of another study using the one-tube multiplex PCR method on 6 pathogenic bacteria by Wang et al. (2019) showed the detection limits for the identification of *E. coli, P. multocida, P. mirabilis, P. aeruginosa, Salmonella spp.* and *S. aureus was* only able to detect up to low concentration about 500 pg/ml.

Thus, the accuracy of selecting the target gene in determining the primer used is an important aspect in the development of the *E. coli* mPCR test to prevent misperceptions. Detection gene targets used to identify *E. coli* are stx1, stx2, bfpA, eae, lt, st, ial and ipaH specifically for detecting *E. coli* strains that have pathogenicity (Fialho et al., 2013). The primer will bind to the target gene for the targeted microorganism during this molecular process. The lt gene in the ETEC group, the eae gene in the EPEC group, and stx2 in the EHEC group are common gene targets amplified in pathogenic *E. coli.*

The sensitivity test in this study was performed to determine the multiplex PCR method's detection limit. According to Reyes-Escogido et al. (2010), One disadvantage of applying the multiplex method is that it has a lower sensitivity than simplex testing. The efficiency value generated in the 3-plex test is 84% to 180%. A good efficiency value is a value with a range between 90-110%. Efficiency above 110% is an indicator of the occurrence of pipeting errors, amplification of non-specific products, and the presence of dimer primers (Pestana et al., 2010).

Positive PCR results were obtained from the application of mPCR

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to water samples, where *E. coli* is a pathogenic bacterium that must be controlled in food safety standards. Based on the results shown in Figures 1 and 2, the presence of a target gene indicates that if *E. coli* is detected in high numbers in drinking water, consequently the water is unfit for consumption (Arlita, 2014). The results showed that pathogenic strains of *E. coli* may be derived from commensal strains through the acquisition of chromosomal or extrachromosomal malignancies, mutations that result in increased pathogenicity, and are more adaptive to extreme and pathogenic environments (Torres et al., 2010). The lt-eae-stx2 gene was found in ETEC-EPEC and EHEC, which both have the same malignant (virulence) factors, namely Enteropathogenic *E. coli*. It enables bacteria to adhere to intestinal cell mucosa. Pathogenicity and malignancy characteristics are influenced by different antigen content (Adamu et al., 2014; Brusa et al., 2015; Bintsis, 2017) have been associated with sporadic cases of human diseases (Friesema et al., 2015).

Measurement of water quality associated with feces is very important to do because it is a parameter of water pollution. Luyt et al. (2020) suggested that the current water quality monitoring and regulation approach uses fecal indicator bacteria (FIB) to indirectly assess health risks from fecal pathogens. Direct detection of waterborne pathogens is expected to provide a more accurate and comprehensive risk assessment, which has been hampered by a lack of methods for the simultaneous detection of many waterborne pathogens. The mPCR system was optimized for annealing temperature and primer concentration, and a bioinformatics procedure was developed to directly detect the target marker gene amplicons in NGS sequence readouts, indicating the simultaneous detection of 14 different target genes in a single reaction. The effectiveness of the mPCR-NGS approach was demonstrated by various target pathogen DNA, and all target genes affixed to environmental water samples were successfully detected.

The application of m-PCR to detect *E. coli* in drinking water in the Abepura District can provide an alternative procedure for microbial detection other than the microbiological test method, which often takes a long time to obtain positive results. This research will continue to be effective. Based on the results, m-PCR can quickly identify and differentiate several bacterial strains, whereas multiplex PCR can detect the presence of *E. coli* specifically and sensitively. Because it is performed directly on the substrate under study, the application of m-PCR on water samples in Papua Indonesia makes this method very simple and efficient.

In 2020, Luyt et al. developed multiplex PCR directly to test fresh samples to diagnose infections, including pneumonia. The findings cannot be ignored because the pathogenic *E. coli* has toxin and virulence properties that can cause pathogenicity. Pathogenic bacteria are a type of enteric bacteria that infect both human and animal cavities and intestinal tracts.

Clean water is a critical requirement for improving public health. Clean water plays an important

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role in reducing the number of people who suffer from water-related diseases, and it is also positively related to the community's quality of life. The availability of clean water sources must meet the needs of the community; if clean water is scarce, disease risk increases (Chandra & Widyastuti, 2007). Similarly, if the mechanism for preparing clean drinking water does not consider cleanliness, it has the potential to cause diseases.

CONCLUSIONS AND RECOMMENDATIONS

Pathogenic *E. coli* can be detected in water samples using multiplex PCR DNA was extracted using a combination method yielding good purity and high concentration Each multiplex set contains three primer pairs, one for each of the target genes lt-eae-stx2 in ETEC-EPEC and EHEC. The m-PCR process was carried out at a temperature of 55^{o}C with the optimum primer concentration for the target gene lt being 0.25 M, eae 0.125 M, and stx2 0.125 M. The multiplex PCR process will achieve stronger results if combined with quantitative PCR, making the detection of *E. coli* pathogens more complex. Further temperature optimization is required to make the PCR band appears brighter and more intact.

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