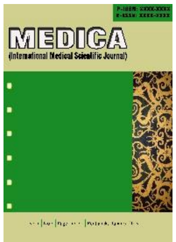
MEDICA

(International Medical Scientific Journal)
Vol.6, No.2, May 2024, pp. 76 – 87
ISSN 2622-660X (Online), ISSN 2622-6596 (Print)
https://journal.ahmareduc.or.id/index.php/medica



Analysis of LT and ST Genes of Enterotoxigenic Escherichia coli Using Polymerase Chain Reaction Method in Lawar Plek from Ketewel Village, Gianyar

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Info Article

Article History:
Received:
15 March 2024
Accepted:
16 April 2024
Published:
31 May 2024

Keywords:
Enterotoxigenic Escherichia coli
Lawar Plek
Polymerase Chain Reaction

Abstrak

Lawar plek is a traditional Balinese dish made from raw meat, which poses a potential risk of contamination by pathogenic bacteria, including Enterotoxigenic Escherichia coli (ETEC). This study aimed to identify the presence of ETEC in lawar plek samples from Ketewel Village, Gianyar. This research uses molecular methods. The identification was conducted in two stages: initial isolation of suspected bacteria on Eosin Methylene Blue Agar (EMBA), followed by molecular confirmation using Polymerase Chain Reaction (PCR). The study results show that in EMBA culture, 4 out of 12 samples (33.33%) showed colonies presumptively identified as E. coli. However, PCR analysis revealed that none of these isolates carried the LT (heat-labile toxin) or ST (heat-stable toxin) genes characteristic of ETEC. The conclusion shows that neither the LT (heat-labile toxin) nor the ST (heat-stable toxin) virulence genes of Enterotoxigenic Escherichia coli were found in any of the samples tested as suspected Escherichia coli colonies on EMBA.

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1. INTRODUCTION

Diarrhea remains one of the leading causes of mortality worldwide, especially among children under five years old, where it ranks as the second most common cause of death (World Health Organization, 2024). In Indonesia, the prevalence of diarrhea based on health professional diagnoses and symptoms across all age groups is 8% (Badan Penelitian dan Pengembangan Kesehatan, 2019). In Bali, diarrhea and gastroenteritis rank second among the top ten causes of hospitalization, with 3,061 cases reported (Dinas Kesehatan Provinsi Bali, 2017). Gianyar Regency has also reported a high number of diarrhea cases, reaching 13,829 across all age groups (Dinas Kesehatan Kabupaten Gianyar, 2019). In 2015, there were 18 recorded diarrhea outbreaks (KLB) involving 1,213 patients, resulting in 30 deaths (Kementerian Kesehatan Republik Indonesia, 2015). Diarrhea is defined as the condition of passing watery stools more than three times a day, commonly caused by Escherichia coli (World Health Organization, 2024).

Escherichia coli is a bacterium normally found in the human digestive tract and is generally harmless in normal quantities. However, pathogenic strains categorized as Diarrheagenic E. coli (DEC), such as Enterotoxigenic Escherichia coli (ETEC), are capable of causing disease. ETEC is responsible for over 200 million cases of diarrheal episodes annually, leading to an estimated 75,000 deaths, predominantly among infants and children in tropical areas with poor sanitation (Gomes et al., 2016).

Foodborne diseases can occur at any stage of food processing, distribution, or consumption (World Health Organization, 2019). Bali, as one of Indonesia’s premier tourist destinations, received 2,155,747 of the 5,471,277 international tourists visiting Indonesia in 2022 (Badan Pusat Statistik Provinsi Bali, 2023). As a culinary tourism hotspot, Bali’s traditional dishes—including lawar—are enjoyed by both domestic and foreign visitors (Yuda et al., 2019). Lawar is a traditional Balinese dish typically prepared by mixing raw ingredients, such as minced meat, blood, vegetables, and spices. The dish is usually hand-mixed by food handlers, and some types, such as lawar plek, are made from raw meat and are commonly found in Gianyar Regency (Soeprapto, & Yohana, 2021). Despite being raw, lawar plek remains popular and widely accepted by the community (Purwanata, & Ekayani, 2023; Purnama, Purnama, & Subrata, 2017).

Previous studies using Eosin Methylene Blue Agar (EMBA) found positive results for E. coli contamination in minced fish samples (Fatiqin, Novita, & Apriani, 2019). Other studies using Polymerase Chain Reaction (PCR) have detected ETEC virulence genes in vegetable accompaniments and street-sold fruit juices (Jyoti, Agarwal, & Tomar, 2015), as well as in raw meat samples one of which was positive for the ST gene and another for both ST and LT genes (Abri, et al., 2015). These findings indicate the risks associated with consuming raw meat products, including the potential for foodborne illnesses like diarrhea.

Raw meat is known to be a potential carrier of diarrheagenic pathogenic bacteria. A microbiological quality assessment of lawar sold in tourist areas of Gianyar showed that 72.7% of samples were contaminated with E. coli, likely due to the use of raw ingredients and poor food handling practices (Purnama, Purnama, & Subrata, 2017). Contaminated food in tourist areas increases the risk of traveller’s diarrhea. According to previous research, gastrointestinal illnesses such as diarrhea are the most common health problems affecting tourists in Bali (Apsari, et al., 2023). ETEC is suspected to be the main cause of this illness, as the symptoms observed in tourists—such as rapid-onset, watery diarrhea—match those of ETEC-related infections (Masyeni, et al., 2017).

Health providers typically treat patients with antibiotics and supportive care (Sukrama et al., 2020), but antimicrobial resistance is increasingly observed, as noted in a study on travelers' diarrhea cases in Denpasar (Sukrama et al., 2020). Standard laboratory testing methods like culture are often insufficient for detecting ETEC, whereas molecular techniques such as PCR offer a faster and more accurate diagnostic approach. Rapid detection is essential to guide proper clinical management for travelers' diarrhea caused by ETEC (Mohammadzadeh, et al., 2015). ETEC produces two types of enterotoxins—heat-labile (LT) and heat-stable (ST)—which are critical for identifying this strain. Therefore, molecular detection methods such as PCR targeting the elt and est genes are needed (Gomes et al., 2016). For example, a study by Sukrama (2020) used the following primers: forward ACGGCGTTACTATCCTCTC and reverse TGGTCTCGGTCAGATATGTG for the elt gene (273 bp), and forward TTCACCTTTCCCTCAGGATG and reverse CTATTCATGCTTTCAGGACCA for the estA2-4 gene (120 bp), detecting the elt gene in two fecal samples. The objective of this study was to molecularly identify Enterotoxigenic Escherichia coli in lawar plek samples collected from Ketewel Village, Gianyar.

2. METHOD

This research is a descriptive study aimed at providing an overview and analysis of a phenomenon without the intention of generating broader generalizations (Adiputra et al., 2021). The study was carried out in April 2024 at the Integrated Laboratory of Poltekkes Kemenkes Denpasar, located on Jalan Pulau Moyo No. 33A, Pedungan, Denpasar. Samples were collected from vendors selling *lawar plek* in Ketewel Village, Gianyar Regency. The study population consisted of 14 vendors, with the actual samples being *lawar plek* dishes sold by them. A total of 12 samples were selected using Slovin’s formula, with inclusion criteria specifying dishes made from raw meat without any cooking process, while samples deemed no longer fresh were excluded (Nalendra et al., 2021). The sampling technique employed was non-probability purposive sampling, and the *lawar plek* samples were obtained through direct purchase from local vendors. Each sample was labeled and stored in an ice box for transport to the laboratory.

In the laboratory, Eosin Methylene Blue Agar (EMBA) media was prepared by weighing the powdered medium using an analytical balance and dissolving it in distilled water within an Erlenmeyer flask. The solution was homogenized and heated on a magnetic stirrer until fully dissolved, then covered with cotton and sterilized in an autoclave. Once cooled to a suitable temperature, the medium was poured into sterile Petri dishes inside a biosafety cabinet, allowed to solidify, and then stored in a refrigerator.

Agarose gel was prepared by weighing the agarose powder and mixing it with TAE buffer and distilled water. The mixture was heated until fully dissolved and transparent, then poured into a gel mold, with a comb inserted to form wells before being left to solidify.

For bacterial inoculation, 25 grams of *lawar plek* was placed into an autoclave-safe plastic bag, followed by the addition of 225 mL sodium chloride solution. The mixture was homogenized, and an inoculating loop was used to streak the extract onto EMBA plates. These plates were then incubated, and any grown colonies were identified.

DNA extraction began by isolating a single bacterial colony into a microtube containing 500 µL of lysis buffer, to which 12.5 µL of Proteinase K was added. The mixture was vortexed and incubated at 56°C for 30 minutes. Subsequently, 500 µL of PCIA reagent was added, followed by mixing and a 10-minute incubation. The sample was then centrifuged at 15,000 g for 5 minutes, and the supernatant was transferred to a new microtube. Next, 700 µL of ethanol was added, the sample was mixed and incubated at 20°C for 24 hours, and centrifuged again at 15,000 g for 10 minutes. After discarding the supernatant, 500 µL of ethanol was added once more, followed by a 30-minute incubation and final centrifugation. The resulting pellet was air-dried for 30 minutes before being resuspended in 50 µL of TE buffer.

PCR identification was carried out by preparing the extracted DNA in microtubes and adding PCR reagents along with specific primers.

Table 1. LT and ST Primers

Target Gene	Primer Design	Primer	PCR Product (bp)	Variant
elt *(LT)*	Forward: ACGGCGTTACTATCCTCTC	LT	273	ETEC
estA2-4 *(ST)*	Reverse: TGGTCTCGGTCAGATATGTG / Forward: TTCACCTTTCCCTCAGGATG / Reverse: CTATTCATGCTTTCAGGACCA	STh	120	ETEC

Table 2. PCR Conditions

Step	Temperature (°C)	Time	Cycles
Pre-denaturation	95	3 minutes	1
Denaturation	95	10 seconds	35
Annealing	55	10 seconds	
Extension	72	20 seconds	
Final Extension	72	5 minutes	

PCR products were subjected to electrophoresis using agarose gel prepared in an electrophoresis tray and submerged in TAE buffer. The DNA samples were carefully loaded into the wells, and an electric current was applied to facilitate the migration of DNA fragments through the gel matrix. The resulting DNA bands were then visualized under ultraviolet (UV) light using a UV Doc imaging system.

Data collection encompassed both primary and secondary sources. Primary data consisted of laboratory results and field observations obtained during the sample collection process. Secondary data were derived from scientific journals, previous research findings, government publications, and applicable regulations. Data were collected through direct observation and laboratory analysis, utilizing tools such as writing instruments, documentation devices, and laboratory equipment. The collected data were analyzed using descriptive statistics in the form of percentage calculations.

3. RESULTS

Table 3. Observation Results of Lawar Plek Samples

No	Sample Code	Meat Type	Use of Spices	Cooking Process	Description
1	LP 1 A	Goat meat	Yes	Not cooked	Raw
2	LP 2 A	Fish	Yes	Not cooked	Raw
3	LP 3 A	Chicken	Yes	Not cooked	Raw
4	LP 4 A	Chicken	Yes	Not cooked	Raw
5	LP 5 A	Chicken	Yes	Not cooked	Raw
6	LP 6 A	Chicken	Yes	Not cooked	Raw
7	LP 7 A	Pork	Yes	Not cooked	Raw
8	LP 8 A	Pork	Yes	Not cooked	Raw
9	LP 9 A	Pork	Yes	Not cooked	Raw
10	LP 10 A	Pork	Yes	Not cooked	Raw
11	LP 11 A	Pork	Yes	Not cooked	Raw
12	LP 12 A	Pork	Yes	Not cooked	Raw

Table 3 shows that the *lawar plek* samples used in this study were characterized by the use of raw meat mixed with vegetables and various spices without any cooking process.

Table 4. EMBA Inoculation Results

Inoculation Result	Frequency	Percentage (%)
Presence of Bacterial Colonies	12	100
No Bacterial Growth	0	0
Total	12	100

Table 4 shows that the identification of *Escherichia coli* in 12 *lawar plek* samples showed that all samples (100%) exhibited bacterial colony growth suspected to be *Escherichia coli* on EMBA medium.

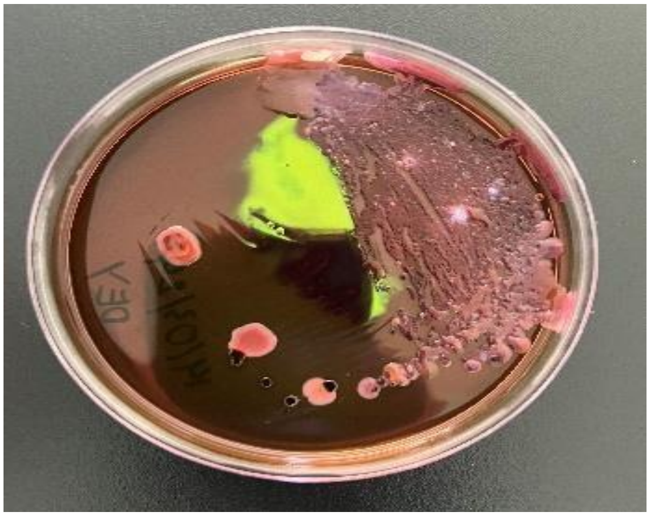

Figure 1. Suspected *Escherichia coli* Colonies on EMBA Medium

Table 5. Colony Identification Results

No	Sample Code	Colony Characteristics	Description
1	LP 1 A	Round colony, pink to colorless	Negative for *Escherichia coli*
2	LP 2 A	Round colony, pink to colorless	Negative for *Escherichia coli*
3	LP 3 A	Round colony, pink to colorless	Negative for *Escherichia coli*
4	LP 4 A	Round colony, metallic green with dark blackish center	Suspected *Escherichia coli* colony
5	LP 5 A	Round colony, metallic green with dark blackish center	Suspected *Escherichia coli* colony
6	LP 6 A	Round colony, pink to colorless	Negative for *Escherichia coli*
7	LP 7 A	Round colony, metallic green with dark blackish center	Suspected *Escherichia coli* colony
8	LP 8 A	Round colony, pink to colorless	Negative for *Escherichia coli*
9	LP 9 A	Round colony, pink to colorless	Negative for *Escherichia coli*
10	LP 10 A	Round colony, pink to colorless	Negative for *Escherichia coli*
11	LP 11 A	Round colony, pink to colorless	Negative for *Escherichia coli*
12	LP 12 A	Round colony, metallic green with dark blackish center	Suspected *Escherichia coli* colony

Table 5 shows that the results of colony identification grown on EMBA medium.

Table 6. Percentage Results of Colony Identification on EMBA

Colony Identification Results	Number	Percentage (%)
Suspected *Escherichia coli* Colony	4	33.33
Negative *Escherichia coli* Colony	8	66.66
Total	12	100

Table 6 shows that out of the 12 samples showing colony growth, 4 samples showed suspected *Escherichia coli* colonies, and 8 samples were negative for *Escherichia coli*. For the samples with suspected *Escherichia coli* colonies, the next step was DNA extraction from these colonies. To assess the quality of the extraction process, electrophoresis and spectrophotometry were performed.

Table 7. Extraction Results of Suspected *Escherichia coli* Colony

No	Sample Code	Electrophoresis	Nanodrop Concentration (ng/μl)	Purity A260/280	Purity A260/230
1	LP 4 A	Band present	107.9	1.87	2.14
2	LP 5 A	Band present	113.3	1.93	2.11
3	LP 7 A	Band present	110.4	1.81	2.17
4	LP 12 A	Band present	112.4	1.90	2.10

Table 7 shows that the extraction results were then followed by PCR identification. The PCR results were further processed by electrophoresis to detect the presence of the target gene, which was then visualized under ultraviolet light.

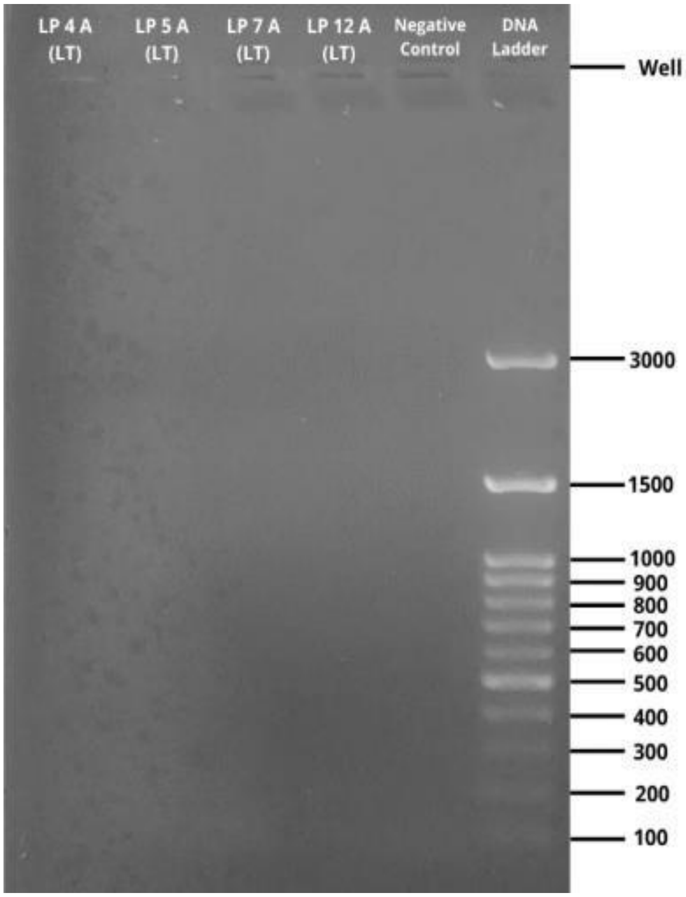

Figure 2. Ultraviolet Visualization of LT Genes Electrophoresis

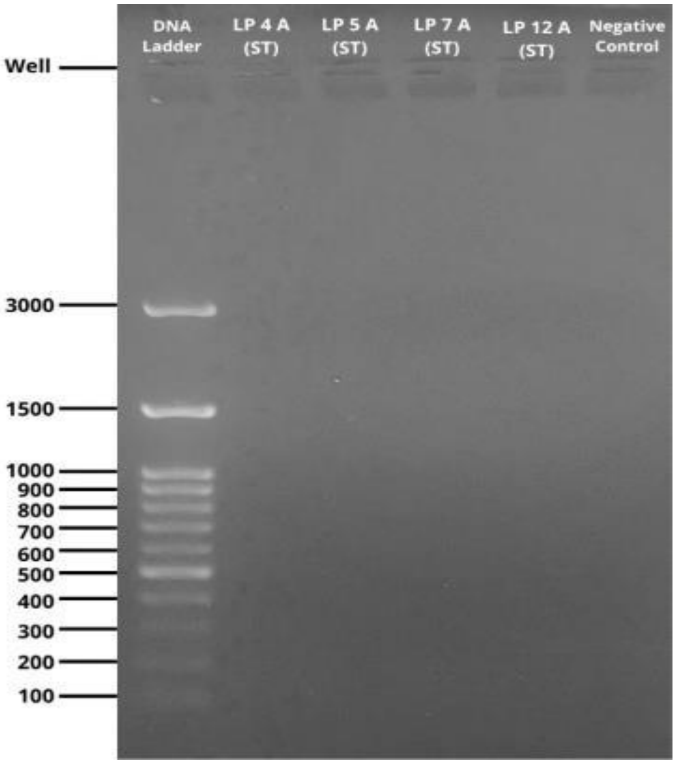

Figure 3. Ultraviolet Visualization of ST Genes Electrophoresis

Table 8. Electrophoresis Analysis of LT and ST Genes

No	Sample Code	Electrophoresis Results	Description
1	LP 4 A	No band appears	Negative for Enterotoxigenic *scherichia coli* (—)
2	LP 5 A	No band appears	Negative for Enterotoxigenic *scherichia coli* (—)
3	LP 7 A	No band appears	Negative for Enterotoxigenic *scherichia coli* (—)
4	LP 12 A	No band appears	Negative for Enterotoxigenic *scherichia coli* (—)

Table 9. Percentage of Electrophoresis Analysis of LT and ST Genes

Electrophoresis Result	Number	Percentage (%)
Positive for LT Virulence Gene of Enterotoxigenic *Escherichia coli*	0	0
Positive for ST Virulence Gene of Enterotoxigenic *Escherichia coli*	0	0
Positive for Both LT and ST Virulence Genes of Enterotoxigenic *Escherichia coli*	0	0
Negative for Virulence Genes of Enterotoxigenic *Escherichia coli*	8	100
Total	8	100

Table 9 shows that all samples did not contain the LT, ST, or both ETEC virulence genes.

DISCUSSION

Table 3 shows that the 12 *lawar plek* samples were obtained. All samples were made using raw meat of various types such as goat, fish, chicken, and pork. Additionally, all samples were mixed with spice blends and not cooked, so the food was in a raw state. Despite being made from raw meat, *lawar plek* still has high commercial value because consumers are interested in buying it due to its uniqueness and distinct taste (Purwanata & Ekayani, 2023). Based on the results of the study conducted by inoculating on EMBA, as shown in Table 4, 12 samples yielded results from which 4 samples showed suspected *Escherichia coli* colonies and 8 samples were negative for *Escherichia coli*.

Nevertheless, all media showed bacterial growth. This is due to the use of raw materials and the lack of cooking. Foods made with raw meat that are not properly cooked can contain pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enteritidis* (Ballout et al., 2023). However, 8 samples did not show characteristics of *Escherichia coli* colonies. This is due to the use of spices known as "base genep" which contain ingredients such as galangal, ginger, kencur, turmeric, onion, chili, shrimp paste, bay leaves, and lemongrass. These ingredients are suspected to have antibacterial properties, which could inhibit the growth of pathogenic bacteria. The phytochemical content of these ingredients can damage bacterial cell walls and even reduce toxins or toxicity produced by the bacteria. However, no independent research was conducted on the antibacterial properties of these ingredients in this study. Previous research aligns with this study, showing that *lawar plek* with added "base genep" or spices affects the total plate count and most probable number (MPN) of *Escherichia coli*, showing a significant reduction trend in both measurements (Suastina et al., 2023).

The EMB medium is a selective differential medium containing eosin and methylene blue. This composition can inhibit the growth of gram-positive bacteria and is suitable for growing gram-negative bacteria. *Escherichia coli* on EMB medium will produce a metallic green color with a dark blackish center due to lactose fermentation present in EMB. The fermentation of lactose by *Escherichia coli* increases the acidity in the medium, causing the methylene blue to precipitate (Jamilatun & Aminah, 2016). In this study, 8 samples (66.66%) showed negative results, indicated by colonies that were pink to colorless. Pink to colorless colonies on EMB medium are suspected to belong to species such as *Enterobacter aerogenes* and *Klebsiella* (Khakim, & Rini, 2018; Trisno, Tono, & Suarjana, 2019).

Based on the results of this study, Table 6 shows that 4 samples (33.33%) were suspected to be *Escherichia coli* colonies in the EMBA identification test. After identifying suspected *Escherichia coli* colonies, the DNA was extracted using the Phenol-Chloroform-Isoamyl Alcohol (PCIA) method. The extraction results were then analyzed using electrophoresis and spectrophotometry. The next step was PCR testing. The PCR product size is shown in Table 3, and the PCR program is presented in Table 2. After PCR, electrophoresis was performed on the PCR product. The electrophoresis results showed that all samples (100%) were negative for ETEC virulence genes, as shown in Table 9. All samples did not contain the LT, ST, or both ETEC virulence genes.

The suspected *Escherichia coli* colonies identified by EMBA were further tested for *Enterotoxigenic Escherichia coli*. This began with the DNA extraction from the suspected *Escherichia coli* colonies. DNA extraction is a process that separates DNA from other unwanted components, such as fats, carbohydrates, and proteins (Hutami et al., 2018). The PCIA method was chosen because it provides good DNA concentration results. However, it has the drawback of using hazardous chemicals and a long processing time (Javadi et al., 2014). In this study, the extraction was identified by electrophoresis and spectrophotometry using a nanodrop. The electrophoresis results showed bands, indicating the presence of total DNA in the extraction. The extraction results, shown in Table 7, revealed high DNA concentrations, around 100 ng/µl, with purity levels between 1.8–2.0 for the A260/A280 ratio and 2.0–2.2 for the A260/A230 ratio. These values are within a good range (1.8–2.0 for A260/A280 and 2.0–2.2 for A260/A230). The A260/230 ratio measures contamination from unwanted components like trizol, phenol, guanidine HCl, and guanidine thiocyanate, while the A260/280 ratio measures contamination from proteins and phenol (Qamariya, Sulihingtyas, & Wirajana, 2016; Sophian, & Yustina, 2022). The results in this study align with previous research showing that pig meat extracted using PCIA had a purity of 2.0 for the A260/A280 ratio, which is within the good range (Hutami et al., 2018). Moreover, the findings were consistent with previous studies, where the purity values ranged between 2.0 and 2.2 for the A260/A230 ratio (Sophian, & Yustina, 2022).

After obtaining the DNA extraction results, PCR was performed to amplify the target DNA gene. The main steps in PCR include denaturation, annealing, and extension. During denaturation, double-stranded DNA is separated into two single strands. In the annealing step, primers bind to the target DNA strands formed in the previous step. The final step, extension, involves elongating the DNA strands. These reactions occur in a thermal cycler, using the PCR components. The molecular sequence formed from the target DNA and primers is the PCR product (Putra et al., 2020).

Electrophoresis was then performed on the PCR products. Electrophoresis is a technique used to separate cellular molecules based on size using an electric current through a medium. Negatively charged particles, like DNA, move towards the positive pole. In this study, 1.5% agarose gel was used as the medium, with 77 volts for 77 minutes. 8 µl of the PCR product and 2 µl of loading dye were loaded into the wells, and 6 µl of DNA ladder was used in a separate well. The loading dye helps visualize the DNA and ensures it stays at the bottom of the well, while the DNA ladder is used as a marker to estimate the size of the DNA fragments (Novitasari, Elvyra, & Roslim, 2014; Tilawah, Sari, & Apridamayanti, 2019). The negative control used was nuclease-free water. As presented in Table 9, the electrophoresis results showed that all samples were negative for ETEC virulence genes. This was evidenced by comparing the sample wells with the DNA ladder. In the LT gene test well (well 14), no band appeared when compared to the DNA ladder with a base pair size of 273. Similarly, in the ST gene test wells (wells 7-10), no band appeared when compared to the DNA ladder with base pairs of 120. The DNA ladder band appeared only in well 6, and negative control bands were found in wells 5 and 11. The negative results in PCR testing suggest that the suspected *Escherichia coli* colonies did not contain ETEC genes. These results are consistent with earlier research, where PCR tests for ETEC, EPEC, and EHEC genes on *Escherichia coli* samples from pork all showed negative results (Ekawati, Yusmiati, & Hamidi, 2017).

Enterotoxigenic Escherichia coli (ETEC) is a pathogenic strain of *Escherichia coli*. PCR is used to detect the enterotoxins produced by *Escherichia coli*. ETEC can have one or both of the enterotoxin genes, LT (heat-labile toxin) and ST (heat-stable toxin), which are used to detect its presence (Gomes et al., 2016). The study lacked positive controls for EMBA identification and PCR identification, meaning there were no comparative positive results. PCR programs were not optimized, leading to uniform PCR reactions on the thermal cycler. Additionally, the study only detected the *Enterotoxigenic Escherichia coli* (ETEC) genes and did not test for other strains such as *Enteropathogenic Escherichia coli* (EPEC), *Enteroaggregative Escherichia coli* (EAEC), *Enteroinvasive Escherichia coli* (EIEC), or *Diffusely-adherent Escherichia coli* (DAEC).

4. CONCLUSION

Based on the results of the study on LT and ST genes of Enterotoxigenic Escherichia coli using the polymerase chain reaction method on lawar plek in Ketewel Village, Gianyar, it can be concluded that neither the LT (heat-labile toxin) nor the ST (heat-stable toxin) virulence genes of Enterotoxigenic Escherichia coli were found in any of the samples tested as suspected Escherichia coli colonies on EMBA.

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