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Detection of the *chuA* gene encoding the invasive enterohemorrhagic species *Escherichia coli* 0157:H7 using qPCR in horse feces samples on Sumbawa Island, Indonesia

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Abstract

Background: Bacterial identification can be done using various testing techniques. Molecular techniques are often used to research dangerous diseases, an approach using genetic information on the pathogenic agent. The enterohemorrhagic invasive species *Escherichia coli* 0157:H7 was identified from the feces of working horses on the island of Sumbawa. Another advance in molecular technology is genome amplification with qPCR which is the gold standard for detecting *E. coli*.

Aim: This study aims to detect and identify the invasive species *E. coli* 0157:H7 using the gene encoding *chuA* with the qPCR method sourced from horse feces.

Methods: Fresh fecal samples from horses on Sumbawa Island were isolated and identified, then continued with molecular examination using the gene encoding *chuA* using the qPCR method.

Results: qPCR testing in this study showed that six sample isolates that were positive for *E. coli* 0157:H7 were detected for the presence of the *chuA* gene, which is a gene coding for an invasive species of *E. coli* bacteria. The highest to lowest Cq values and Tm from the qPCR results of the sample isolates were 15.98 (4KJ), 14.90 (19KG), 14.6 (3KJ), 13.77 (20KG), 12.56 (5KGB), and 12.20 (6KJ). Tm values are 86.7 (4KJ), 86.69 (3KJ), 86.56 (5KGB), 85.88 (20KGB), 85.81 (19KG), and 85.74 (6KJ).

Conclusion: Validation, standardization of the development, and modification of qPCR technology must be carried out to harmonize testing throughout to avoid wrong interpretation of the test results so that the determination of actions to eradicate and control diseases originating from animals in the field does not occur.

Keywords: *Escherichia coli* 0157:H7, *chuA*, qPCR, Horse, Invasive.

Introduction

The majority of facultative organisms in the gastrointestinal system are *Escherichia coli*. Due to the presence of particular virulence factors and genes linked with pathogenicity that are typically absent in other *E. coli*, pathogenic strains of *E. coli* can cause a range of diarrheal illnesses in hosts. However, some strains of *E. coli* can also lead to further intestinal infections (Darvishi *et al.*, 2020). *E. coli* is a common cause of meningitis, septicemia, diarrhea, and outpatient urinary tract infections (UTIs) in women worldwide (Pokharel *et al.*, 2023).

Various diseases found in animals and humans are caused by many pathogenic strains invasive enterohemorrhagic of *E. coli*, although most of them coexist in safe symbiosis (Hu *et al.*, 2022). However, the majority of them coexist in safe symbioses. Community- and nosocomial-related UTIs are primarily caused by *E. coli*. The second most typical infectious presentation in general practice is UTI. There is growing concern over the emergence and dissemination of different antibiotic resistance mechanisms among prevalent human pathogenic *Enterobacteriaceae* members, reducing the number of treatment alternatives (Boucher *et al.*, 2009). A significant clinical issue is the ongoing

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rise of antibiotic resistance among clinical bacterial strains. The most significant danger to managing infectious diseases is the rapid growth of antibiotic resistance among common pathogens (Adamus-Bialek et al., 2013).

In addition to sepsis, newborn meningitis, gastroenteritis, and several infections, such as UTIs, pathogenic strains of *E. coli* can cause a wide range of intestinal and extra-intestinal disorders (Coura et al., 2015; Khan et al., 2017).

The respiratory system is the second most common reported illness, while UTI is one of the most prevalent bacterial consequences. *Escherichia coli* is the most prevalent bacteria that can infect the urinary system. 80%–90% of UTIs are caused by *E. coli*, which is still the most common bacteria in the urinary tract worldwide (Lee et al., 2016).

According to an *in vitro* genome analysis of *E. coli* strains belonging to various phylogenetic groups, particular genes or bacterial DNA fragments can be utilized as specific markers for the phylogenetic classification of the *E. coli* strain (Coura et al., 2017). The *chuA* gene, which is required for the transmission of invasive enterohemorrhagic species *E. coli* O157: H7, is present in three proposed particular markers. qPCR is employed as a straightforward and quick method with higher sensitivity and specificity because other techniques need more involved and time-consuming procedures needing a collection of model strains (Gordon et al., 2008). According to host niche, pathogenicity, antibiotic resistance, and most significantly certain virulence factors, bacteria exhibit distinct characteristics (Shaheen et al., 2013).

Horses are non-ruminant herbivorous mammals with one-toed hooves that belong to the Equidae family. These animals are well-built and capable of covering lengths of up to 16 km per day, making them popular choices for usage as draft animals and riders (Wibisono et al., 2017). In Indonesia, riding horses and horse-drawn carriages are common forms of transportation for equines (*Equus caballus Linnaeus*, 1758). This animal serves as a vital mode of transportation and a source of money for the neighborhood. Observations show that Sumbawa Island has many working horses, many of which are employed for transport and serve as a tourist draw in and of themselves. The upkeep of working horses necessitates consideration for the implementation of animal welfare based on upholding the five principles of animal freedom (the “five freedoms”), free from harm, illness, and disease, one of which is infection by microorganisms that cause disease in horses and reduce the performance and productivity of working horses (Scala et al., 2020).

Materials and Methods

Sample collection

A total of 52 samples of working horse feces from Sumbawa Island, the province of west Nusa Tenggara,

Indonesia. Samples were collected and put into buffered peptone water media, and each sample was labeled and explained accordingly. In the sample transportation process, all sample specimens were put into a coolbox containing an ice pack and immediately transported to the laboratory.

The tools used in testing *E. coli* O157:H7 isolates and the *chuA* gene from workhorse feces samples, using the qPCR method, namely scales, rollers, scissors, cutting boards, water baths, incubators, micropipettes, tips, Schott Duran, pellets, centrifuges, vortex, PCR tube, stomacher bag, Laminar air flow, Spindown, real-time PCR machine, cutting board and ruler. Materials used include: pure culture *E. coli* isolate O157:H7, buffered peptone water, Go Taq qPCR Master Mix (GoTaq® 1-Step RT-qPCR System) (Promega, 2023), Primer *chuA* R (1,000 µM Solution), Primer *chuA* F (1,000 µM Solution), internal amplification control (IAC) DNA template (0.75 µg/µl), 20% sucrose (0.22 µM filter-sterilized) and PCR grade water, lysis buffered. The primer sequences used in this study can be seen in Table 1.

DNA isolation of *E. coli* O157:H7 *chuA* gene

The preparation stage consists of isolating the culture of *E. coli* on eosin methylene blue agar (EMBA) and isolation of DNA genomes of *E. coli* all samples were weighed in a microtube, and then tissue lysis buffer and proteinase K solution were added. The mixture was vortexed and incubated at 57°C for 20 hours. The binding buffer solution was added to all the samples that had been incubated and after homogenization, they were re-incubated at a temperature of ±70°C for 10 minutes. Then the sample was added with absolute isopropanol and homogenized. The mixture is put into a filter tube, and then centrifuged. The liquid that passes through the filter is discarded, then the inhibitor removal buffer is added and centrifuged again. Next, washing buffer is added, and centrifuged, and then the solution is discarded. Finally, DNA was eluted by adding elution buffer at 70°C, then centrifuged. The microtubes contain DNA isolates. The DNA isolate obtained was analyzed for its presence using agarose electrophoresis and DNA concentration and purity were analyzed using ultraviolet (UV) spectrophotometry.

In the screen system, the “nucleic acid” panel is selected for the sample to be cleaned with sterile tissue. An elution buffer was used as a blank. A total of 2 µl of sample DNA was placed on the sample holder. Then the “measure” button is clicked. DNA was analyzed at wavelengths of 260 and 280 nm. The results of the instrumentation will provide data on DNA concentration in ng/µl and DNA purity by comparing the ratio of A280 and A260 (BioDrop, 2015).

DNA amplification using qPCR

DNA amplification using the SYBR Green method is carried out in the following way. SYBR green mastermix was prepared with a total volume of 20 µl consisting of 5 µl of tested DNA; 3 µl aquabidest; 1 µl forward primer 10 µM; 1 µl reverse primer 10 µM; and 10 µl

Table 1. Target genes and DNA primers for bacterial identification of enterohemorrhagic invasive species *E. coli* 0157:H7.

Gene target	Primer	Amplicon	Reference
<i>chuA</i>	F: GAC GAA CCA ACG GTC AGG AT R: TCG CCA GTA CCA AAG ACA	279 bp	Machado <i>et al.</i> (2005)

Table 2. Cycle stages in qPCR.

Step	Time	Temperature	Number of cycles
Activation step	120s	95°	1
3-Step qPCR	-	-	40
Denaturation	10s	95°	-
Annealing	30s	52°	-
Extention	30s	72°	-

LightCycler® 480 SYBR Green. A total of 5 µl of DNA was to be tested and 15 µl of SYBR green Mastermix was put into the multiwell. The next mixture, real-time PCR is turned on, program settings are carried out, and the tool is run (Table 2) (Roche, 2012).

DNA amplification using a hydrolysis probe is carried out with the following procedure. Hydrolysis probe mastermix was prepared with a total volume of 20 µl consisting of 5 µl of tested DNA; 1.4 µl aquabidest; 1.6 µl forward primer 10 µM; 1.6 µl reverse primer 10 µM; 0.4 µl 10 µM probe; and 10 µl LightCycler® 480 Probe master (Taq DNA Polymerase enzyme, dNTP mix, and 6.4 mM MgCl₂). A total of 5 µl of DNA was to be tested and 15 µl of mastermix was put into a multiwell plate. The mixture is homogenized. Next, real-time PCR is turned on, program settings are carried out, and the tool is run (Roche, 2008).

Ethical approval

Not needed for this study.

Results

The qPCR method is an accurate and efficient method used to detect the presence of DNA more specifically for the detection of the *chuA* gene as the gene encoding enterohaemorrhagic invasive species *E. coli* 0157:H7 in feces samples from working horses on the island of Sumbawa. The results of this test explain the general conditions of the research, DNA amplification of working horse feces samples using qPCR.

Sample analysis is important to obtain data quality. This is related to the implementation of quality assurance (QA) and quality control (QC) systems to ensure that the working system can run correctly and appropriately so that the data obtained is of good quality. Apart from QA and QC, an important factor in obtaining data quality is to carry out validation analysis to determine the accuracy of the tests carried out, namely standardized and optimized correctly and appropriately (Hewajuli and Dharmayanti, 2014). The following are the results

of the C_q (quantification cycle) values in horse feces samples on Sumbawa Island which experienced an increase in the amplification curve.

Discussion

The method for detecting the *chuA* gene of enterohaemorrhagic invasive species *E. coli* 0157:H7 using the qPCR method can be carried out using the pre-enrichment method and without the pre-enrichment method. In this research, pre-enrichment was used. The pre-enrichment stage aims to increase the number of bacteria because PCR protocols generally require very high concentrations of target microorganisms which are usually obtained only after sample enrichment (Garrido-Maestu *et al.*, 2020). The pre-enrichment stage is important as an effort to reduce negative errors in testing (Nurjanah *et al.*, 2021).

qPCR is a reliable, effective, and reliable DNA-based analysis method (Zilhada *et al.*, 2017). This method has the advantages of being able to identify small samples, not having to visualize, the risk of contamination is small and can examine a large number of samples at once (Zhang *et al.*, 2021). qPCR with SYBR Green fluorescent staining is a detection method and is also able to differentiate between fresh and processed products (Law *et al.*, 2015). Analysis of DNA amplification from qPCR results based on the cycle threshold (C_t) value on the amplification curve. C_t is the number of cycles the sample begins to be read which indicates the beginning of the exponential growth phase. The lower the C_t value, the higher the amount of target DNA. Conversely, the higher the C_t value, the lower the amount of target DNA. The results of DNA amplification using qPCR on the *E. coli* 0157:H7 isolate sample carried out with SYBR green staining were seen through the increase in the curve and

Table 3. Results of C_q values in fecal samples from working horses on the island of Sumbawa.

Sample origin	Sample code	C _q value <i>chuA</i> gene
Sumbawa Island	3KJ	14.61423596489081
	4KJ	15.983407355834174
	6KJ	12.20990450320965
	19KG	14.901781613978454
	20KG	13.776555518398498
	5KGB	12.468378742593433

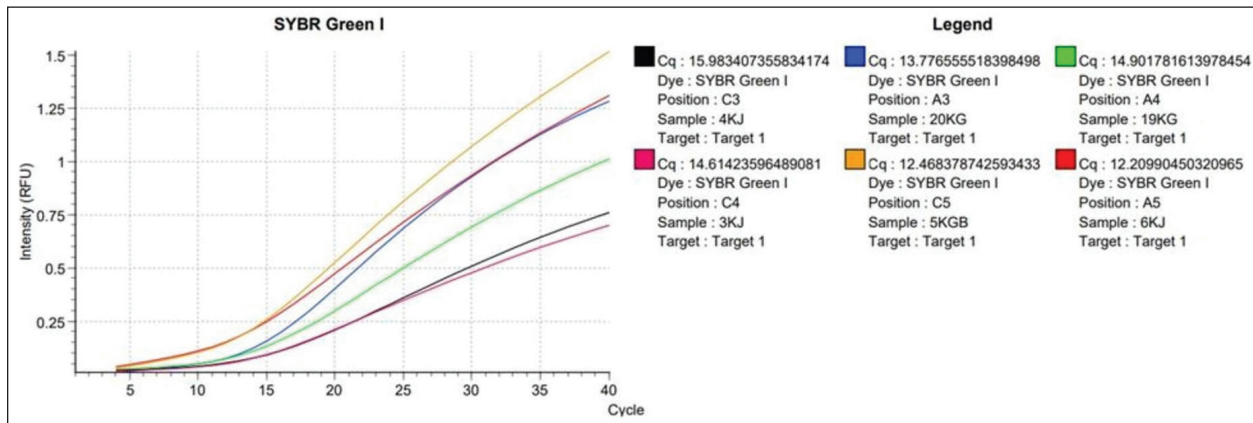


Fig. 1. Amplification curve using the SYBR Green method using the *chuA* primer. Color Description: Red: 3KJ, Black: 4KJ, Orange: 6KG, Green: 19KG, Blue: 20KG, Yellow: 5KGB.

the Ct value on the amplification curve. The specificity of the amplification results can be seen through the Tm value on the melt curve (Zilhadia *et al.*, 2017). Amplification stages of the qPCR process using Go Tag qPCR Master Mix (Promega), PCR amplification usually uses 35–40 cycles (Maulani *et al.*, 2020), but in this study, 40 cycles were used. This is because in cycle 40 there is amplification of the DNA sequence. Go Tag qPCR Master Mix (Promega), forward primer, reverse primer, nuclease-free water, and DNA (Promega, 2023). Next, the mixture is vortexed, which aims to help mix the solution perfectly (Nurhayati and Darmawati, 2017). The melting curve occurs at a temperature of 95°C for 5 seconds, at a temperature of 60°C, and at a temperature of 97°C, the melting curve aims to determine whether there are mutations or single changes in the DNA nucleotide bases (Tong and Giffard, 2012). In this study, the total number of qPCR cycles was 40 times. Based on data in Table 3, the qPCR results of the enterohaemorrhagic invasive species *E. coli* 0157:H7 *chuA* gene identified sample 3KJ with a Cq of 14.61, sample 4KJ with a value of Cq 15.98, sample 6KJ with a value of Cq 12.20, sample 19KG with a value of Cq 14.90, sample 20 KG with a value of Cq 13.77, and sample 5KGB with a Cq value of 12.46.

The test results are in the form of an amplification graph which can be seen in Figure 1. showing the results of the test amplification curve. The blue curve of the *E. coli* 0157:H7 *chuA* gene indicates the presence of the *chuA* gene or *E. coli* 0157:H7 DNA. The curve will be active if the enterohemorrhagic invasive species *E. coli* 0157:H7 *chuA* gene DNA is detected during the amplification process. *chuA* functions to detect the target DNA that you want to read. The intersection point between the sigmoid graph and the baseline threshold when reflected on the x-axis (cycle) is the threshold cycle (Ct) for the amplified sample (Pestana *et al.*, 2010). In the amplification curve for

testing the enterohaemorrhagic invasive species *E. coli* 0157:H7 *chuA* gene, the sigmoid graph intersects with the baseline threshold in the cycle (14.61, 15.98, 12.20, 14.90, 13.77, 12.46). The results of the increase in fluorescence are depicted through an amplification curve which shows three phases, namely the initial phase, exponential or peak phase, and plateau or stable phase (Vaerman *et al.*, 2004; Zilhadia *et al.*, 2017). In real-time PCR testing produces around one billion DNA molecules.

The data obtained is in the form of purity DNA and the graph shows the presence of DNA of the *chuA* gene as a gene encoding the enterohemorrhagic invasive species *E. coli* in fecal samples of working horses on Sumbawa Island using RT-PCR. All samples in this study were amplified with varying increases but were still in a small range. This increase shows and describes the number of products amplified in each sample. The 5KGB sample curve experienced the highest increase while the 3KJ sample curve experienced the lowest increase. This shows that the 5KGB sample has the highest number of amplified products, while the 3KJ has the lowest number of amplified products.

The results of testing samples on working horse feces showed that there were amplified samples, indicated by the quantification cycle (Cq) value). From the test results of 52 working horse feces samples, six sample isolates showed an increase in the amplification curve. The amplified samples have Cq values that are different from each other. The sample with the highest Cq value in detecting the *chuA* gene of the invasive enterohemorrhagic species *E. coli* 0157:H7 is shown in sample code 4KJ with a Cq value reaching 15.98. Meanwhile, the lowest Cq value was shown in the 6KG code sample with a Cq value reaching 12.20. qPCR uses fluorescence dyes and fluorescence probes. In this study, a fluorescence dye was used, namely a hydrolysis probe (TaqMan probe) using specific oligonucleotides that are complementary to the DNA to be amplified.

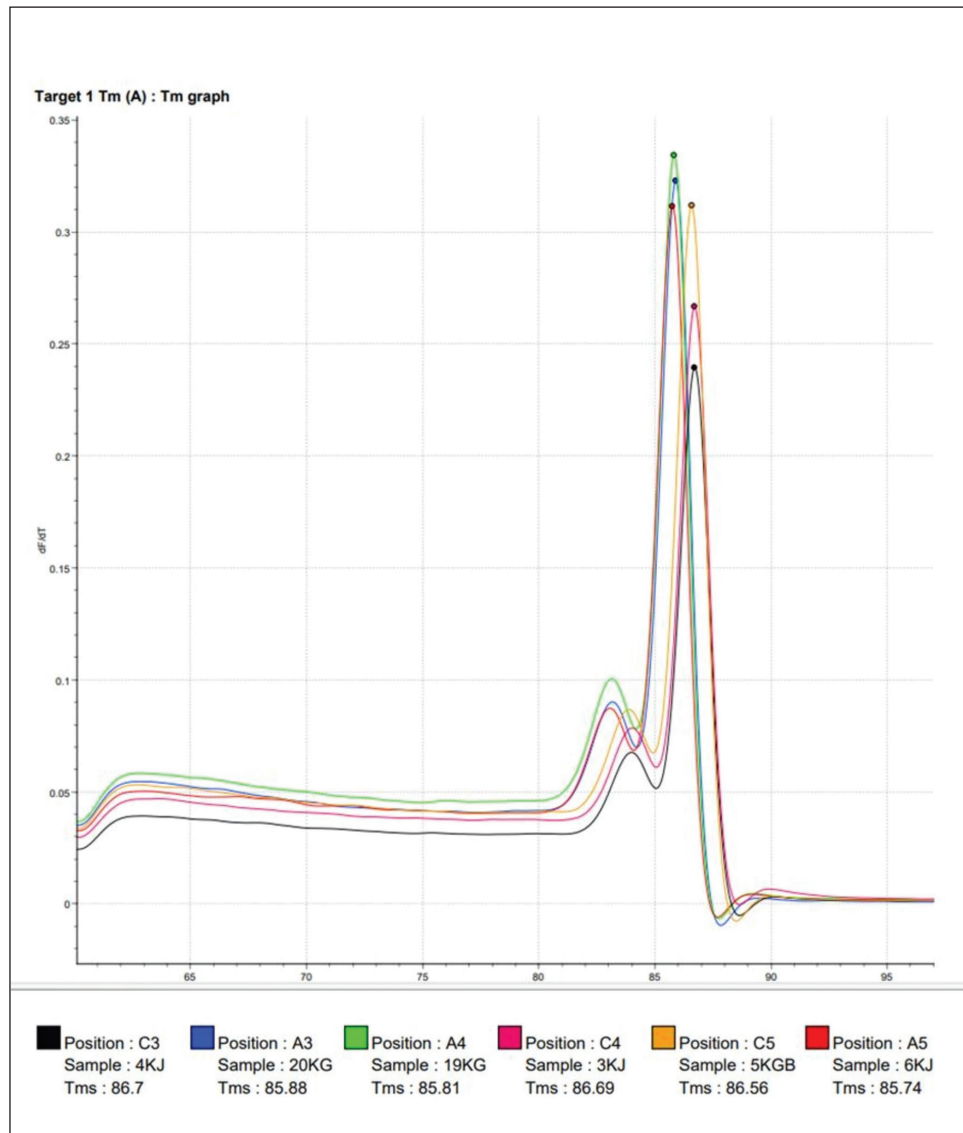


Fig. 2. Melting temperature (Tm) value from amplification using the SYBR Green method for working horse feces samples on Sumbawa Island using the *E. coli* 0157; H7 *chuA* gene primer. Color description: Red: 3KJ, Black: 4KJ, Orange: 6KG, Green: 19KG, Blue: 20KG, Yellow: 5KGB.

The fluorescent hydrolysis probe will glow when the reporter and quencher are separated through hydrolysis due to nuclease activity. The hydrolysis probe works specifically and can prevent the occurrence of primer-dimers and mispriming, thereby causing non-specific amplification products (Zilhadia *et al.*, 2017).

The use of specific probes will help increase the specificity of the qPCR test; however, qPCR requires relatively expensive equipment and reagents, and requires good understanding and correct techniques to get accurate results. qPCR has the working principle of detecting and quantifying fluorescent reporters. The signal from the fluorescent will increase as the DNA amplification product increases in the reaction.

Reactions during the exponential phase can be monitored by observing and recording the amount of fluorescent emission in each cycle. The increase in the amplification curve in the exponential phase is closely related to the number of target gene initiations. Therefore, the higher the expression level of the target gene, the faster the detection of fluorescent emissions will occur (Hewajuli and Dharmayanti, 2014).

An amplification curve will appear and form during the amplification process. The function of the amplification curve itself is to determine whether amplification occurs in the thermal cycler used. This amplification is determined based on the intensity of the fluorescent, that is, if more amplification products are produced,

the greater the accumulation of fluorescent that will be read (Pratama, 2015). qPCR has high sensitivity and has advantages compared to conventional PCR. qPCR is considered more dynamic, can be used for more testing, and has less risk of contamination. The results of amplification with the primer *chuA* gene of the invasive enterohemorrhagic species *E. coli* 0157:H7 using the Go Tag Master Mix protocol (GoTaq® 1-Step RT-qPCR System) and quantification kit will answer the core problem of this research, whether the *chuA* gene of invasive species is present and detected enterohaemorrhagic *E. coli* 0157:H7 in fecal samples of working horses on the island of Sumbawa.

In addition, many molecular-based analysis methods can be used because of the fast process and results in research, one of which is the DNA-based method which has been widely developed, such as fingerprinting using PCR, multiplex PCR assay, and real-time PCR; this method has been widely used for several last decades (Manalu and Rohman, 2019). The results of DNA amplification of the *chuA* gene of the invasive enterohemorrhagic species *E. coli* 0157:H7 using qPCR can be used as an analysis to detect the presence of DNA other than in horse feces. This is because the use of hydrolysis probes can be used for multiplex qPCR using target DNA and more than one partner in a reaction. After all, the probe will bind specifically to several different target DNAs (Harisah, 2017). The increasing amplification curve shows an increase in the concentration of DNA paired with the primer being tested. The increase in this curve occurs due to an increase in fluorescent light when it binds to double-stranded DNA. The resulting fluorescence is appropriate and proportional to the amount of amplified template DNA (Rasyid, 2015).

To test the specificity of the *chuA* primer using the SYBR green method, followed by analyzing the T_m value. The T_m value resulting from amplification using the SYBR Green method using the *chuA* primer can be seen in Figure 2. DNA isolates from workhorse feces samples with sample codes 3KJ and 4KJ have the same T_m value, namely at a temperature of 86.7°C. The T_m values of sample codes 6KJ (85.74), 19KG (85.81), and 20KG (85.88) have almost the same T_m values. Isolate with sample code 5KGB with a T_m value of 86.56. The highest T_m value was found in the isolate with sample code 4KJ (86.7) and the lowest T_m value was found in the isolate with sample code 6KJ (85.74). The T_m value is the T_m of the amplification target DNA. A good primer pair is that the two primers have a difference in T_m value of no more than 2°C. Thus, the bovine primers for analyzing bovine meat and gelatin DNA using the SYBR green method are specific primers (Smith and Osborn, 2008).

Melt curve analysis is carried out by increasing the temperature until it reaches the melting temperature, 50% of double-stranded DNA will separate into single strands at this melting temperature (Nur'utami, 2011).

Single-stranded DNA will bind to the specific primer *chuA* while non-specific double-stranded DNA will bind to SYBR green. Based on the melt curve in Figure 2, the six samples tested show that the melting temperature values are different and there are three melt peaks. Amplicons originating from the same target DNA will have almost the same T_m value. The qPCR method for this parameter was proven to identify the *chuA* gene as the coding gene for the invasive enterohemorrhagic species *E. coli* 0157:H7. This is following research that has been carried out previously using the same method (Septiani and Pandianto, 2018), which states that the qPCR method is fast and reliable so it is appropriate to use it as a method to detect the presence of the invasive enterohemorrhagic species *E. coli* 0157:H7 in the feces of working horses in Sumbawa Island.

Conclusion

In conclusion, the qPCR method can amplify the DNA of the *chuA* gene as a gene encoding the invasive enterohaemorrhagic species *E. coli* 0157:H7 using specific primers, with a denaturation temperature of 95°C for 10 seconds, annealing of 52°C for 30 seconds, and a temperature of 72°C for 30 seconds. An increase in the amplification curve occurred in all samples tested, based on the DNA amplification curve with qPCR of horse feces samples on the island of Sumbawa, the invasive enterohemorrhagic species *E. coli* 0157:H7 was detected. This research confirms that there is an invasive species of enterohemorrhagic *E. coli* 0157:H7 originating from the feces of working horses on the island of Sumbawa. Working horses are an important reservoir for the clinical isolation of invasive species, which pose potential public health risks. This must not be ignored and monitoring must be provided in the environment to prevent cases of infection from originating from animals to humans.

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Conflict of interest

There is no conflict of interest in this study.

Author's contribution

All authors conceptualized the idea and design of the study. KK and AS performed material preparation, data collection, and analysis. KHP, ARK, and SCK supervised the entire research process. All the authors read and approved the final manuscript.

Data availability

All data are available in the manuscript.

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