Assessing the *ecpA* Gene as a Target for Detecting *Klebsiella pneumoniae* Using *real-time Polymerase Chain Reaction* Method

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Abstract. *Klebsiella pneumoniae* is the most nosocomial pathogenic bacteria found in the hospital that causes infections such as pneumonia, liver abscesses, and urinary tract infections. The *ecpA* gene is one of the target genes of *Klebsiella pneumoniae* that functions in biofilm development to increase the ability of bacteria to colonize and as the main subunit of fimbriae. This study aims to determine the confirmation, sensitivity, and specificity test of pathogenic bacteria by *real-time Polymerase Chain Reaction* method. The *ecpA* primer has an amplicons length of 170 bp and has an annealing temperature of 60°C. These primer pair produced Ct test at 16.78±0.04 and Tm value of 86.33°C±0.12. The specificity test of the *ecpA* gene successfully differentiated between target and non-target bacteria based on Ct and Tm value. Therefore, these primers can detect *Klebsiella pneumoniae* bacteria at the smallest concentration until 1.06 pg/μL equivalent to 1.47 x 10^4 CFU. Based on these results, the *ecpA* primer successfully detected *Klebsiella pneumoniae* bacteria quickly, specifically and sensitively using the rt-PCR method. In the next step, these primer pairs can detect the artificial contaminated samples using rt-PCR method.

Keywords: *Klebsiella pneumoniae*, Pathogenic Bacteria, *ecpA* Gene, *real-time Polymerase Chain Reaction*.

1 Introduction

Klebsiella pneumoniae is one of the pathogenic bacteria of the *Enterobacteriaceae* family which is gram-negative, facultative anaerobic, rod-shaped, and unable to move (non-flagellate). *K. pneumoniae* can generally be found in the environment including water, soil, food, human or animal digestive tract, and is most commonly found in hospital environments [1]. This bacteria can cause infection because it can spread rapidly in hospital environments with the main transmission from the digestive tract and hands of officers so that it tends to cause nosocomial infections such as pneumonia, liver abscesses, central nervous system infections to urinary tract infections [2].

The *ecpA* gene is one of the target genes of *Klebsiella pneumoniae* which functions as the main subunit of fimbriae which has the ability as an adhesion tool [3]. The transmission of the initial process of infection by attaching bacteria to the surface of host cells by encoding proteins (fimbriae) is called adhesion. After the adhesion process, bacteria begin to multiply to form biofilms which play a role in increasing the ability of bacteria to colonize in large numbers and virulence against antibiotics [4]. Therefore, a rapid detection method at the genomic level is needed to assess the increase in bacterial contamination in samples.

real-time Polymerase Chain Reaction (rt-PCR) is a rapid detection method in identifying bacteria because it amplifies and determines the number of target DNA molecules resulting from the amplification in one stage during the reaction. The advantage of using rt-PCR is that it can monitor quickly, accurately, sensitively and specifically to detect pathogenic bacteria in samples [5]. The purpose of this study is expected to be able to detect *Klebsiella pneumoniae* bacteria to obtain confirmation data, specificity, and sensitivity of the *ecpA* gene primer. These results

will then be developed for detection of artificial food contamination using the rt-PCR method.

2 Material and Methods

2.1 Target Gene Primer Pair Design

The primer design aims to obtain the right gene primer pair to be used in the DNA amplification process. The target gene primer pair was designed using an in-silico approach using software, namely the National Center for Biotechnology Information (NCBI) Primer-Blast website. The designed gene primer pair will be used on *K. pneumoniae* strain ATCC 35657 bacteria with accession number NZ_CP015134.1. Furthermore, the right gene primer pair was checked using the NetPrimer program according to parameters such as primer length, Tm,% GC, dimer, and hairpin. The gene primer pair was synthesized at the Macrogen, Inc. Laboratory in Korea.

2.2 Culture and Cultivation of *Klebsiella pneumoniae* Bacteria

Bacterial culture and cultivation aims to obtain pure cultures by growing bacteria into media and inoculating bacteria into other media to facilitate bacterial identification. Bacterial growth is carried out in liquid media.started by taking the bacteria *K. pneumoniae* strain ATCC 35657 from KWIK STICK™ with a sterile loop needle into 10 mL Nutrient Broth (NB). Bacterial cultures were incubated at 37°C for 18 hours in an orbital shaking incubator (YIHODER LM-400 D) at a speed of 150 rpm. Furthermore, the formation of turbidity is checked which indicates that the bacteria are growing well and the OD value is checked at a wavelength of 600 nm using a UV/Vis spectrophotometer.

Growth of *K. pneumoniae* bacteria on MCA agar media (*MacConkey* Agar) with the streak plate method using a sterile ose needle and incubated for 18 hours in an incubator (Memmert Type INB 400). Furthermore, the resulting colonies were inoculated into NB media using a sterile ose for use in the isolation process.

2.3 DNA Isolation

DNA isolation aims to obtain pure bacterial DNA from the separation of DNA from other particles to be tested both qualitatively and quantitatively. *K. pneumoniae* bacterial culture from NB media as much as 1.5 mL was transferred into a 1.5 mL tube and centrifuged (Sorvall Legend Micro 17 R) at a speed of 5000 × g for 10 minutes until a pellet was formed. DNA isolation was carried out from the pellet using the Viogene-Geno Plus Genomic DNA Extraction Miniprep System according to the instructions for isolating gram-negative bacterial DNA. The results of DNA isolation were analyzed quantitatively using a nanodrop spectrophotometer (Nanovue Plus Spectrophotometer) to determine the concentration and purity of

DNA with an A260/A280 ratio. Furthermore, qualitatively the results of DNA isolation were carried out by agarose gel electrophoresis with 0.7% agarose gel and visualized under a UV Trans-illuminator (Vilber Lourmat). Store the resulting DNA isolation at a temperature of -20°C in the freezer (Aqua Freezer Box).

2.4 Target Gene Primer Anneling Temperature Optimization

Annealing temperature optimization aims to determine the optimal temperature in the annealing process. This test uses Gradient PCR (X-960 Heal Force) with a temperature range of 54-62°C. A reaction mixture of 25 µL was used consisting of 5 µL of *K. pneumoniae* DNA isolate, 5 µL of forward and reverse primers for the *ecpA* gene, 12.5 µL of NZYTaq II 2x Colorless Master Mix (nyztech), and 2.5 µL of Qiagen Nuclease Free Water (NFW). The negative control used consisted ofNon-Template Control (NTC) and Nuclease Free Water (NFW) + Master Mix (MM) and positive control used *Bacillus subtilis* bacteria with a 175 bp *cod*Y gene primer pair.

Next, amplification was carried out, starting with the initial denaturation process at a temperature of 95°C for 100 seconds, denaturation at a temperature of 95°C for 30 seconds, annealing at a temperature of 54°C - 62°C for 30 seconds, extension at a temperature of 72°C for 1 minute, and final extension at a temperature of 72°C for 10 minutes. A total of 35 PCR cycles were repeated in the process. The optimized annealing temperature was determined by visualizing the results on a 2% agarose gel stained with Green Safe (NZYTech). The 100 bp Marker (SMOBIO) was used as a size reference. Agarose gel electrophoresis was carried out using 10 µL of Sterile Aquades, 5 µL of the amplified DNA Isolate and 3 µL of ExcelDyeTM 6X DNA Loading Dye (SMOBIO) as a loading buffer. The electrophoresis process was carried out for 70 minutes at 70 volts and 400 amperes. The results were visualized using a UV-Transilluminator (Vilber Lourmat).

2.5 Target Gene Confirmation Test

Confirmation test aimsto confirm the specific primers for detecting bacteria used with rt-PCR.Primary confirmation test of the *ecpA* gene to detect *Klebsiella pneumoniae* bacteria using the rt-PCR (Bio Molecular System Mic qPCR) method with 20 µL of reaction mixture. The sample (duplo) consists of 10 µL of ExcelTaq 2X qPCR Master Mix, 5µL of forward and reverse primer *ecpA*, and 5µL of pure *Klebsiella pneumoniae* DNA isolate template. Positive controls used include *Listeria monocytogenes* bacteria DNA template, *hly* primer pair and NFW. There are 2 negative controls used, namely, the first is Non-Template Control (NTC) which contains a primer pair, Nuclease Free Water (NFW), and Master Mix (MM). The second is a mixture of Nuclease Free Water (NFW) + Master Mix (MM). Negative and positive controls are made as much as 20 µL. The results of the confirmation test using *real-time* PCR are in the form of an amplification curve and a melting curve.

2.6 Specificity and Sensitivity Test of *Klebsiella pneumoniae* Target Gene

The specificity test aims to ensure that the gene detected is a specific target gene.The *ecpA* primer specificity test was carried out using the *ecpA* primer pair that would be tested to determine the level of selectivity of the primer to the target bacterial DNA isolate. The *ecpA* primer that should only be used to detect *Klebsiella pneumoniae* ATCC 35657 (target) would be reacted with the target DNA template (positive control) and other non-target bacterial DNA, namely *S. flexneri, S. typhi, E. coli, P. mirabilis, C. jejuni, P. aureginosa, E. faecallis, L. Monocytogenes, C. Sakazaki, and Y. Entrocolitica*. Each reaction mixture was made as much as 20 µL. The *ecpA* primer was mixed into the reaction mixture with each different bacterial DNA template. The concentration of each bacterial isolate was equalized. The results obtained from the specificity test were in the form of an amplification curve and a melting curve.

The sensitivity test aims to determine the smallest concentration of DNA isolate used in sensitive rt-PCR detection.Sensitivity testing was carried out by diluting the pure *Klebsiella pneumoniae* DNA isolate 6 times, namely 50; 10; 2; 0.4; 0.08; and 0.016 ng/µL. Pure isolates of *Klebsiella pneumoniae* bacteria will be taken as much as 10 µL and added with 40 µL of Nuclease Free Water (NFW). Then each dilution result is entered into *real-time PCR*. The results obtained from the sensitivity test are in the form of an amplification curve and a standard curve.

3 Result and Discussion

3.1 Primer Pair Design of *Klebsiella pneumoniae ecpA* Gene

This study tested *Klebsiella pneumoniae* strain ATCC 35657 with accession number NZ_CP015134.1. Whole Genome Sequence shows the size of the bacterial genome is 5.229.229bp. The primer pair of *K. pneumoniae* bacteria was designed to target the *ecpA* gene sequence. The *ecpA* gene in *Klebsiella pneumoniae* strain ATCC 35657 has a size of 588 bp and is located in the sequence 2.335.746 to 2.336.333. Based on the NCBI page in the BLAST section, it can be obtained that the *ecpA* gene is specific to *Klebsiella pneumoniae* strain ATCC 35657. The *ecpA* gene was chosen because it has a role in the development of biofilms to increase the ability of bacteria to colonize and as the main subunit of fimbriae. The primers that have been designed obtain 20 pairs of nucleotide bases for both forward and reverse primers, the primers from the *ecpA* gene start from the sequence 2.335.910 – 2.335.929 for forward and 2.336.079 – 2.336.060 for reverse. The *ecpA* primer obtained an amplicon length of 170 bp, as in **Figure 1.**

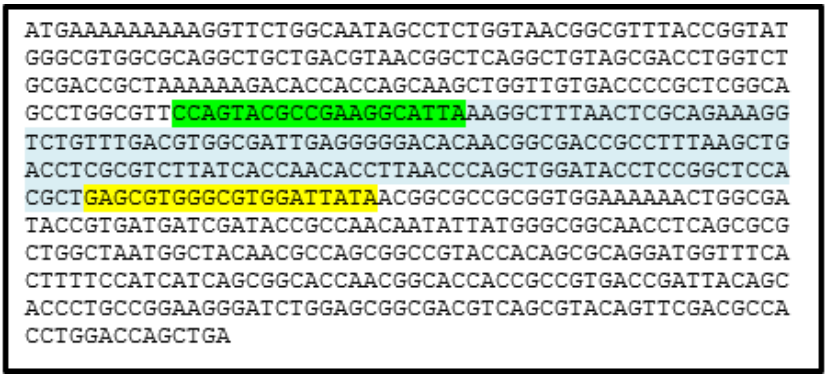


Fig. 1. In-Silico Genome Sequencing of the *ecpA* gene of *Klebsiella pneumoniae* strain ATCC 35657. (Yellow) Forward Primer; (Green) Reverse Primary; (Blue Highlight) Amplicon Length of *ecpA*-f and *ecpA*-r primer pairs.

Table 1. The specific qualities of *ecpA* primary pairs

Primary	Sequence (5'-3')	Primary Length	Tm (oC)	% GC	Self-Dimer (kcal/mol)	Hairpin (kcal/mol)	Cross Dimer (kcal/mol)	Amplicon Length
ecpA-f	CCAGTACGCCGAAGGCATTA	20 bp	60.18	55	-3.65	-1.01	-5.38	170 bp
ecpA-r	GAGCGTGGGCGTGGATTATA	20 bp	59.97	55	-3.4	-		

Based on **Table 1** parameter analysis of *ecpA* primer design, both primers have a primer length of 20 bp pairs. According to the theory, a good primer length ranges from 18-22 bases. Primers with higher lengths do not show higher specificity [6]. Furthermore, it is known that the Tm (melting temperature) of the *ecpA* primer pair is at a temperature of 60.18°C for forward and 59.97°C for reverse. A good primer has the same melting temperature between forward and reverse, but a difference of 5°C is still tolerable [7]. A high Tm value can cause a high denaturation temperature which will later cause an imperfect denaturation process. While a low Tm will reduce the annealing temperature which can cause non-specific amplification [8]. In addition, the *ecpA* gene primer pair has a GC percentage of 55% for both the forward and reverse primers. The GC percentage of 45-65% is a good GC percentage, where this parameter affects the stability of the primer and the dimer formed [9]. Parameters that also have an effect are the presence or absence of secondary

structures such as dimers or hairpins. The presence of secondary structures can reduce the specificity and success of PCR. Based on the analysis results in **Table 1**, there are still self-dimers, cross dimers, hairpins. However, it can still be tolerated because dimers can be tolerated with a maximum ΔG value of -6 kcal/mol for self-dimers, -5 kcal/mol for cross dimers, and hairpins of -3 kcal/mol [7].

3.2 Culture and Cultivation of *Klebsiella pneumoniae* Bacteria

K. pneumoniae bacteria grown on NB media were analyzed after incubation for 18 hours at 37°C. The results obtained showed turbidity in NB media which indicated that *K. pneumoniae* bacteria grew well. The turbidity formed was measured by the Optical Density value at a wavelength of 600nm (OD600) using a UV Vis spectrophotometer instrument, resulting in an OD600 value of *K. pneumoniae* of 1,635. Optical density measurements with a wavelength of 600 nm because this wavelength states the amount of light transmitted can be used to calculate the concentration of particles in the solution because the amount of light absorbed by a sample is related to its concentration [10].

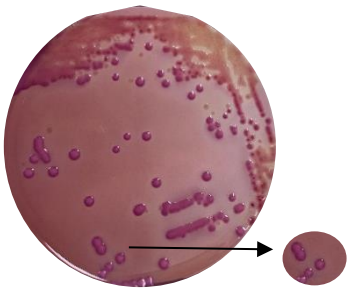


Fig. 2. Growth of *Klebsiella pneumoniae* bacteria on MCA media

The growth of *K. pneumoniae* on agar media based on **Figure 2** produces pink colonies because it has the ability to ferment lactose. MCA media as a selective differential media is able to inhibit the growth of most gram-positive bacteria and as one of the primary isolation media. In this media, bacteria that ferment lactose such as *K. pneumoniae* can form mucoid and pink colonies. MCA is a common media that has a composition of peptone, lactose, crystal violet, sodium chloride, Neutral red, Bile salts and agar [11]. Furthermore, the single colony found in the MCA media from *K. pneumoniae* is inoculated. The inoculation process occurs to increase/enrich the number of bacteria to obtain the maximum number during the next process, namely the bacterial DNA extraction process (DNA isolation).

3.3 Isolation of *Klebsiella pneumoniae* bacterial DNA

Table 2. Quantitative Test of *Klebsiella pneumoniae* DNA isolates

Bacteria	Concentration (ng/µL)	Purity (A260/A280)
Klebsiella pneumoniae strain ATCC 35657	83	1.80

The results of the isolation of *K. pneumoniae* bacterial DNA were subjected to quantitative tests using a nanodrop spectrophotometer to be analyzed with a small volume of 1-2 µL. This test was carried out measuring the A260/A280 wavelength ratio, because at a wavelength of 260 is the maximum wavelength absorbed by DNA, double-stranded DNA containing purine and pyrimidine bases can absorb UV light while at a wavelength of 280 is the maximum wavelength absorbed by protein. Based on **table 2**. The purity results of *K. pneumoniae* DNA isolates are in the range of 1.8-2.0. According to the literature, a good quality purity value at the A260/280 ratio is 1.8 to 2.0. If the purity value is below 1.8 indicates protein contamination in the isolate while the purity value above 2.0 indicates RNA contamination [12]. The results of this test meet the requirements for good DNA isolate purity.

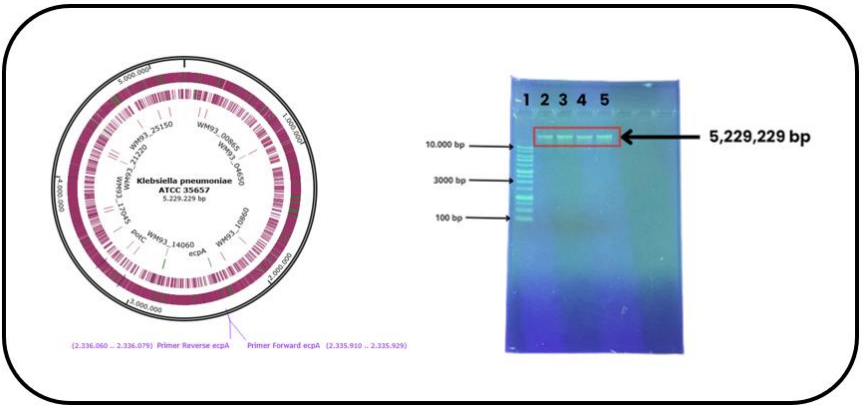


Fig. 3. Characterization of *K. pneumoniae* DNA isolates (Right) (1) DNA ladder 1kb; (2) *K. pneumoniae* DNA isolate; (3) *K. pneumoniae* DNA isolate; (4) *K. pneumoniae* DNA isolate; (5) *K. pneumoniae* DNA isolate. Whole genome of *K. pneumoniae* (Left)

K. pneumoniae DNA isolates were subjected to qualitative tests using agarose gel electrophoresis and visualized under UV to determine a single band that corresponds to the target size with a 10.000 bp DNA Ladder as a determinant of the DNA band size in DNA isolates. **Figure 3.** Shows the presence of a single bright DNA band above the 10.000 bp marker position, so it can be estimated that the results obtained correspond to the overall size of the *Klebsiella pneumoniae* strain ATCC 35657 genome sequence, which is 5.229.229 bp [13].

3.4 Annealing Temperature Optimization of *ecpA* Gene

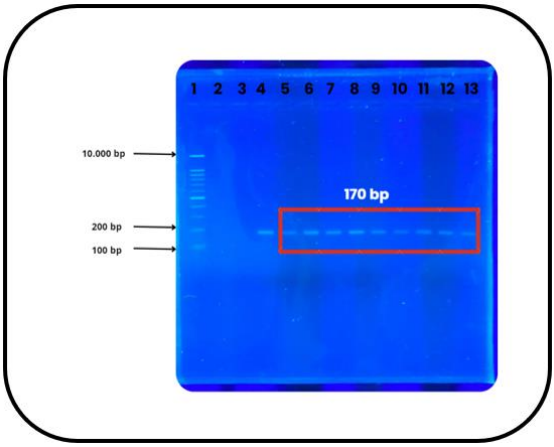


Fig. 4. Optimization of Annealing Temperature of *ecpA* Primer Pairs. (1) 100 bp DNA marker; (2) NTC (3) NFW+MM (Negative control); (4) Positive Control (*codY, B. Subtilis*); (5) – (13) DNA fragment of *ecpA* gene primers at a temperature of 54°C – 62°C.

Based on **Figure 4**. Shows that all temperatures in the range of 54°C - 62°C the primers used are able to amplify the *ecpA* gene fragment, which is marked by the presence of a single band during electrophoresis indicating the absence of dimers, indicating the success of the primer in attaching and amplifying the targeted DNA sequence. The size of the *ecpA* gene amplicon produced during electrophoresis is 170 bp which is above the 100 bp DNA marker and below 200 bp. The results of the amplicon size are in accordance with those designed during the primer design and in-silico analysis. A good DNA band is the formation of a single band and is not smeared [14].

The optimal annealing temperature range in the PCR process is between 55°C - 61°C, the use of a temperature of 60°C is recommended because at that temperature no dimers are formed, either self-dimers or cross-dimers and no non-specific amplification is formed on the primers used. A temperature of 60°C will be used for the next test. In this test, both negative controls did not show bands indicating no contamination during the test. Therefore, at the annealing testing stage, the *ecpA* primer was able to amplify and attach to the designed DNA template and produce an amplicon of 170 bp at an optimum temperature of 60°C.

3.5 Confirmation Test of *K. pneumoniae* with *ecpA* Gene Using rt-PCR

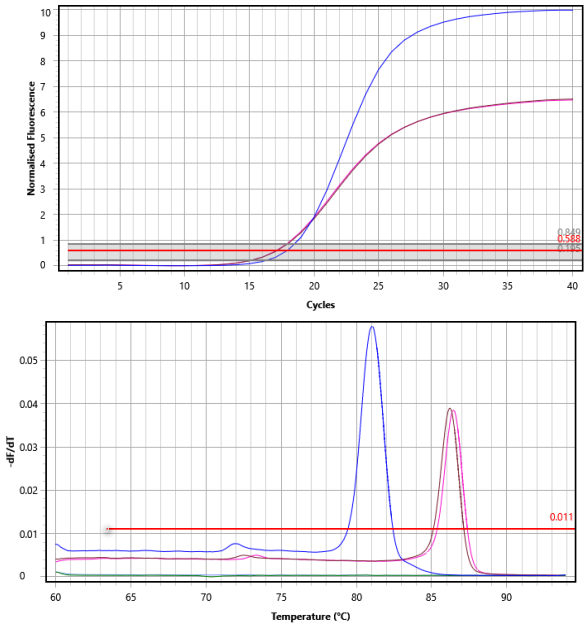


Fig. 5. Amplification curve of *ecpA* gene primer confirmation test (Left); Melting curve of *ecpA* gene primer (Right)

Confirmation testing using annealing temperature based on the results of the previous *ecpA* primer annealing temperature optimization, which was 60°C. This confirmation test was run for 40 cycles and produced a Ct (cycle threshold) value in the form of an amplification curve and a Tm value in the form of a melting curve. Ct is defined as the cycle number where the fluorescence signal from the reaction exceeds the baseline, where the Ct value correlates with the number of DNA copies and is inversely proportional to the sample concentration [7]. While the melting curve shows the change in fluorescence when DNA dissociates at a higher temperature, and shows the overall melting point through changes in the shape or slope of the curve as the temperature increases [15]. The target DNA template uses *K. pneumoniae* DNA with a concentration of 50 ng/µL. Based on **Figure 5**. shows that the primer can detect the presence of *K. pneumoniae* bacteria using a DNA concentration of 50 ng. The ability of the primer to detect the *ecpA* gene is indicated by the emergence of Ct and Tm values, as shown in **Table 3.** In addition, the Ct and Tm values for negative controls in the form of NTC and NFW+MM did not appear. If the Ct difference is more than ten cycles, the appearance of the sigmoid line in the negative control can be ignored [7].

Table 3. Primary Confirmation Test Results of *ecpA* Gene

Color	Sample	Ct	Tm (℃)
	Sample 1 *K. pneumoniae*	17.06	86.45
	Sample 2 *K. pneumoniae*	17.12	86.21
	Positive Control *L. Monocytogenes* (*hly* gene)	17.90	81.04
	Negative Control (NTC)	-	-
	Negative control (NFW + MM)	-	-

3.6 Specificity and Sensitivity Test of *ecpA* Gene Using rt-PCR

This test shows the results of the amplification curve found that the positive control of *K. pneumoniae ecpA* produced adjacent Ct values in two samples (duplo) namely 15.06 and 16.46 indicating the presence of the bacteria. Meanwhile, non-target bacteria appeared with Ct values from cycle 30 to none appeared and the NTC value did not appear. The primer can clearly separate target and non-target bacteria, as shown in **Figure 6** and **Table 4**. Each non-target bacteria appeared with a Ct value exceeding 10 cycles. The difference in the number of cycles of 10 is considered insignificant and is interpreted as a negative result [7].

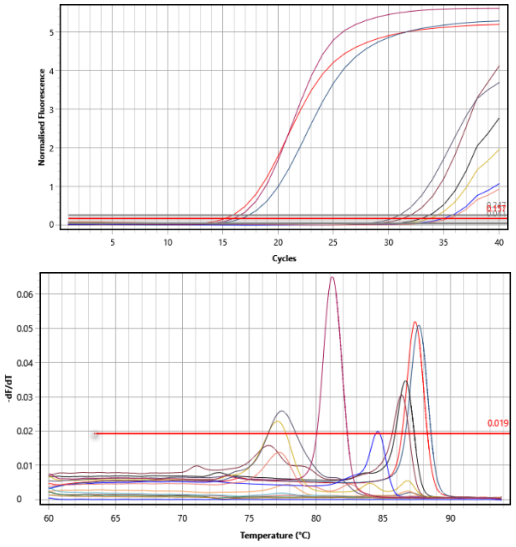


Fig. 6. Results of the amplification curve of the *ecpA* primer specificity test on *K. pneumoniae* against non-target bacteria.

Table 4. Results of primer specificity test of *ecpA* gene

Color	Sample	Ct	Tm (℃)
	Sample 1 *K. pneumoniae*	15.06	87.38
	Sample 2 *K. pneumoniae*	16.46	87.66
	Negative control NTC	32.79	86.65
	Positive control *L. Monocytogenes* (*hly*)	15.81	81.19
	C. jejuni	-	-
	C. sakazaki	-	-
	E. coli	34.74	84.58
	E. faecalis	-	-
	L. monocytogenes	-	-
	P. aeruginosa	-	-
	P. mirabilis	31.10	86.36
	S. flexneri	30.13	77.42
	S. typhi	33.50	77.10
	Y. enterocolitica	35.20	77.21
	NFW+MM	-	-

In addition to the Ct value, a melting curve analysis was carried out because each bacteria has a unique melting point (Tm) influenced by the sequence of the amplicon formed. **Figure 6** and **Table 4** show the results of the melting curve that the target bacteria *K. pneumoniae* with the *ecpA* primer have a Tm of around 87.38°C and 87.66°C. Meanwhile, in non-target bacteria, there is a shift in the Tm value towards lower and higher. In addition, the Tm value of non-target bacteria forms more than one peak, this indicates the occurrence of non-specific amplification in non-target bacteria. Thus, the *ecpA* primer pair is proven to be able to clearly separate target and non-target bacteria based on the difference in Tm values. Therefore, the primer is able to distinguish between target and non-target bacteria based on the Ct and Tm values of each non-target bacteria.

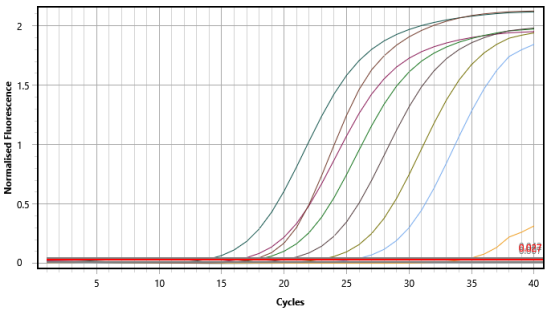
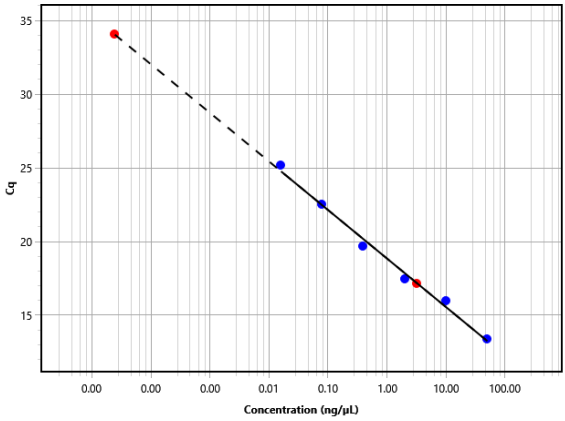


Fig. 7. Results of the amplification curve of the *ecpA* primer specificity test on *K. pneumoniae*

Sensitivity test produces a standard curve to determine the Limit of Detection (LoD) of the *ecpA* primer in detecting *K. pneumoniae* bacterial DNA. In this study, sensitivity testing indicates the ability of the primer to detect bacterial DNA in very small amounts. The standard curve is used to evaluate the qPCR test by calculating the efficiency and dynamic range of the test by diluting the sample series with a controlled amount of template [16]. This test was carried out with six dilutions on the *K. pneumoniae* DNA sample which initially had a concentration of around 83 ng/µL. The results of this test are in the form of an amplification curve in **Figure 7** and **Table 5**. It can be seen that there is a correlation between the Ct value and concentration, where at low DNA concentrations it has a large Ct value, and vice versa.

TABLE 5. Results of the *ecpA* gene primer sensitivity test

Color	Sample	Ct	Tm(℃)
	50 ng/µL	**13.36**	**86.33**
	10 ng/µL	**15.98**	**86.38**
	2 ng/µL	**17.43**	**86.46**
	0.4 ng/µL	**19.63**	**86.66**
	0.08 ng/µL	**22.48**	**86.48**
	0.016 ng/µL	**25.15**	**86.61**
	NTC	**34.04**	**83.79**
	Positive Control *L. Monocytogenes (hly)*	**17.15**	**80.68**
	NFW+MM	**-**	**-**



Equation : y = -3.29 x + 18.85

Efficiency : 1.01

R² : 0.9915

Fig. 8. Standard curve of primary sensitivity test *ecpA K. pneumoniae*

Based on the results of the sensitivity test, a standard curve was obtained as shown in **Figure 8.** The standard curve analysis produced the equation y = -3.29x + 18.85 and an R^2 value of 0.9915 was obtained for the *ecpA* primer. The coefficient of determination (R^2) value is used to measure the extent to which changes in results can be explained by the observed causes, with a maximum value generally of 0.999. The R^2 value obtained in this study has met the quality standards, with the requirement that a good R^2 value is equal to or greater than 0.98 [17]. The efficiency level of the real-time PCR reaction reached up to 100%, indicating that the reaction was efficient. Efficiency (E) reflects how well the target molecule is replicated each thermal cycle, and ideally reaches 100%. Several factors, such as fragment length, secondary structure, and GC composition in the amplicon, can affect efficiency. Other factors include reaction dynamics, reagent concentrations, and enzyme quality, which can result in efficiencies below 90% [18]. The slope of the log-linear phase is an indicator of reaction efficiency, a value close to -3.32 is desired for ideal results. This study obtained a slope value of -3.29 indicating adequate results.

The LoD value in this test is 1.06 pg/µL which is equivalent to 1.47 x 10^4 CFU of *K. pneumoniae* bacteria. The average Ct value is 16.78±0.04 and the Tm value is 86.33°C±0.12 real-time PCR results of *ecpA* primers. Based on the data obtained, the test using *real-time* PCR has the ability to detect more sensitively.

4 Conclusion

The *real-time* PCR method with the *ecpA* target gene showed that *K. pneumoniae* DNA was rapidly amplified at an optimal annealing temperature of 60°C with a 170 bp amplicon with a Ct value of 16.78±0.04 and a Tm of 86.33°C±0.12. In the specificity test, the primer was able to recognize the target bacteria *K. pneumoniae* and could not recognize non-target bacteria. In the sensitivity test results, the LoD value of *K. pneumoniae* bacteria was 1.47 x 10^4 CFU at the smallest concentration of 1.06 pg/µL. The *ecpA* primer produced a line equation of y = - 3.29 x + 18.85 with a coefficient of determination (R^2) of 0.9915 and an efficiency of 1.01.

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