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Research Article

Efficacy Test of Prototype Kit for Detection *Bacillus cereus* and *Listeria monocytogenes* in Processed Meat using Real-time PCR Method

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Abstract

According to the World Health Organization (WHO), harmful agents such as Bacillus cereus and Listeria monocytogenes are responsible for 600 million cases of disease and 420,000 deaths annually. This research aims to test the effectiveness of the real-time PCR method for developing a prototype kit to detect pathogenic bacteria in processed meat. As a comparison, the and conventional PCR methods were used to obtain the accuracy, specificity, sensitivity, and effectiveness of the real-time PCR method. All the samples were cultured in solid media agar, performed amplification using specific primers cyt-K 2 and hly using PCR and real-time PCR. Meatballs, nuggets, and sausages, five samples each, were found to be positive positively contaminated with all targeted bacteria. However, they did not provide specific results using solid media culture and the PCR method. In addition, the real-time PCR method using prototype kit formulas accomplished that all contaminated samples had a Ct value smaller than the negative control, NTC (No Template Control), and had a similar melting curve to the positive control. This establishes that the real-time PCR method clarifies that all samples were contaminated with target bacteria. A formula was developed in the prototype kit with real-time PCR methods that can be used and applied on a commercial scale efficiently and precisely.

Keywords: Bacillus cereus, efficacy test, listeria monocytogenes, processed meat, real-time PCR

1. INTRODUCTION

Food safety is directly and indirectly related to many of the Sustainable Development Goals (SDGs), especially on the issues of alleviating hunger, poverty, and improving health and well-being. Anything that can be harmful to consumers' health in food is a food safety concern, one of the causes is foodborne disease[1]. Contamination of food systems by foodborne pathogenic bacteria is a severe problem that can result in various illnesses and even death. According to WHO estimates, every year, at least 33 million years of healthy life are lost worldwide due to eating contaminated food. About 31 different pathogens are recorded as organisms that cause foodborne diseases, including *Bacillus cereus* and *Listeria monocytogenes*[2].

Meat products are increasingly popular among the public because they provide an easy-to-prepare meat presentation quickly, as well as a form of problem-solving regarding the high price of fresh meat. Although the price of meat products is low, it does not exclude the possibility that the source of fresh meat raw materials used can be contaminated in the manufacturing process, transportation, or from the equipment used, causing cross-contamination [3,4]. Apart from the processing process, unhygienic conditions when meat products pass through the packaging, storage, and distribution process can also cause the growth and proliferation of various bacteria. Thus, meat products are not suitable for consumption and may be harmful to health [5].

Considering the enormous number of people who consume ready-to-eat products, it is crucial to be aware of BPOM Regulation No. 13 of 2019 [6] regarding the Maximum Limit of Microbiological Contaminants in processed foods such as sausages, meatballs, nuggets, and other processed products. Therefore, the maximum number of pathogens in a processed meat product that is safe to consume must be determined using a feasible detection method.

Many methods have been developed to detect and identify pathogenic bacteria in food, including the agar media culture method, which is recognized as the gold standard. However, this method is time-consuming and unable to analyze specific organisms. It takes at least 2-3 days to determine the results and it needs to be followed by other biochemical tests [7,8]. Other identification methods have been used as an approach for rapid detection of bacteria in food samples using nucleic acid-based techniques, such as PCR [9,10]. Real-time PCR, as one of the development of the PCR method, is considered an effective method for detecting pathogenic [9,10].

Nurjayadi et al. has successfully developed a prototype kit formula using the real-time PCR to detect foodborne pathogenic bacteria [11]. This method was carried out using primer specificity testing to detect and validate the presence of pathogenic bacteria in intentionally contaminated food. The study successfully proved the specificity of primers on food intentionally contaminated by specific bacteria, namely Bacillus cereus and Listeria monocytogenes. This study was conducted to test the ability of the prototype formula using the real-time PCR method on direct food samples in the market by comparing the use of traditional culture methods and conventional PCR methods to assess the efficacy of the prototype detection kit on food samples and prove the accuracy and potential of the real-time PCR method to identify suspected contaminated food samples in the market.

2. RESEARCH METHODS

Instruments and Materials

The materials used in this study were meat processing products such as, nuggets; sausage; and beef meatball, bacteria *Bacillus cereus* ATCC 10876 and *Listeria monocytogenes* ATCC 13932 (KwikStick, Microbiologics, USA); Primers *Bacillus cereus* cyt-K 2 and *Listeria monocytogenes* hly gene (Macrogen); Mannitol Yolk Polymyxin Agar (MYPA) (Merck, US) for *Bacillus cereus* and Listeria Selective Agar (Oxford Agar) (Oxoid, UK) for *Listeria monocytogenes*; Brain Heart Infusion Broth (BHIB) (Merck, US), 0.85% glycerol, dry ice, GRS Genomic DNA Kit – Bacteria (Grisp, Portugal), GRS Genomic DNA Kit – Food (Grisp, Portugal); ExcelTaq 2X Q-PCR Master Mix (Smobio, Taiwan), Nuclease Free Water (Qiagen, Germany), NZYTaq II 2× Green Master Mix (NZYTech, Portugal), AgarOse (GRS, Portugal), 6x Loading Dye (Smobio, Taiwan), 50x TAE Buffer (Promega, US), greensafe (NZYTech, Portugal), 1 kb and 100 bp DNA ladder (Smobio, Taiwan), 96% ethanol, disinfectant, and distilled water.

The tools used in this study are 25 mL and 100 mL measuring cups; 250 mL and 500 mL beakers, 250 mL erlenmeyer, spatula, stirring rod, test tube, test tube rack, micropipette, micropipette tip, 0.2 mL and 1.5 mL microtube; mictube; Petri dish, analytical balance, bunsen, lighter, Ose wire, spreader, label, mask, latex gloves, tissue, parafilm paper, hot plate (Stuart SD 300, UK), 22 vortex (Type 37600 DAAD, Germany), centrifuge (Sorvall Legend Micro 17 R, Thermo Fisher Scientific, US), incubator (Memmert Type INB 400, Taiwan), shaking incubator (Yihder LM-400 D, China), autoclave (Hirayama HVE-50, Japan), Mic qPCR Cycler (Bio Molecular System, Australia), PCR Thermal Cycler (X960 Heal Force, Taiwan), freezer, showcase, shaking water bath (Stuart SBS-40, UK), Bio Safety Cabinet (Bio Base, China), UV Transilluminator (DAAD, Germany), nanodrop spectrophotometer (Nanovue Plus, Thermo Fisher Scientific, US), parafilm paper, and DNA

electrophoresis/Mini sub cell GT Power (BioRad, US).

The methods used in this study are bacterial culture, preparation of artificially contaminated samples, DNA isolation of *Bacillus cereus* & *Listeria monocytogenes* and artificially contaminated processed meat products, confirmation for positive controls, and efficacy test for processed meat product samples.

Bacterial culture media

Culture of *Bacillus cereus* ATCC 10876 and *Listeria monocytogenes* ATCC 13932 bacteria was carried out by resuspending the culture on an inoculating swab (kwik Stick with hydrating liquid, then the bacterial culture was taken by the swab method using an Ose needle. Then, the inoculated bacterial suspensions were incubated on selective media for each bacterium. Selective media for *Bacillus cereus* is Mannitol Yolk Polymyxin Agar, and selective media for *Listeria monocytogenes* is Listeria Selective Agar (LSA). Then, a single colony that grew was taken using a sterile Ose needle and dipped the Ose needle into 10 mL of Brain Heart Infusion Agar liquid media. Furthermore, incubation was carried out at 37 ºC for 18-24 hours (overnight culture) with shaker aeration.

Preparation of Artificially Contaminated Sample

The processed meat samples used in this study were bought from traditional markets; the samples were processed meat products; nuggets, meatballs, and sausages. Measured and then roughly chopped each sample. In a 2:1 ratio, distilled water was used to dissolve the crushed sample. The sample was briefly heated to ensure sterilized results. In traditional sterilizing, the product is heated between 110 °C and 125 °C for a briefly, equilibration at a lower temperature comes after this stage [12]. Then, a 1:1 ratio of 5 mL of a previously cultured targeted bacteria (*Bacillus cereus* and *Listeria monocytogenes*) was inoculated into the samples. The artificial food sample suspension was then incubated using orbital shaking incubation (YIHDER LM400D) for an entire night at 37 °C with a speed of 150 rpm. The artificially cultured samples were then subjected to DNA isolation to target bacterial DNA from artificially contaminated food samples.

DNA Isolation of Targeted Bacteria and Artificially Contaminated Samples

DNA isolation for targeted bacteria samples from previous steps was performed using the GRS Genomic Bacteria Kit (GRiSP) commercial kit and for artificially contaminated nugget samples using the GRS Genomic Food (GRiSP) commercial kit. 1.5 mL of cultured bacterial suspension was transferred to an Eppendorf tube and centrifuged at 14,000-16,000 x g for 1 min until a pellet was formed. The pellet formed was suspended with G+ buffer (Tris HCl; EDTA; Triton X-100; Lysozyme). For artificially contaminated food samples, 200 mg of artificial processed meat product suspension was put into an Eppendorf tube, and a Food lysis buffer was added. Both DNA isolations followed the DNA isolation protocol for Gram-positive bacteria. The extracted DNA isolates were placed in a freezer at -20 ºC and used as DNA templates for PCR and real-time PCR.

Efficacy test for Processed Meat Products

The efficacy test conducted in this study aims to determine whether the food samples suspected of being contaminated are contaminated by specific genes of the target bacteria. The efficacy test was carried out by comparing three different pathogenic bacteria detection methods, were conventional culture methods, PCR, and real-time PCR.

The culture methods were performed with the solid media agar technique. The surface of the food sample was swabbed using a sterile swab. Then the sterile cotton bud was streaked on selective agar media, Mannitol Egg-Yolk Polymixin Agar for specific identification of *B. cereus* bacteria and Listeria Selective Agar (Oxford Agar) for specific identification of *L. monocytogenes* bacteria, and incubation was carried out at 37 ºC for 18-24 hours (overnight culture). PCR methods were performed using DNA from DNA isolation each sample (sausage, meatballs, and nuggets), DNA from each sample were amplified using specific primer *cyt-K 2* and *hly*. The PCR product result was characterized with agarose gel electrophoresis. For the efficacy test, the pure culture of *B. cereus* and *L. monocytogenes* bacteria and artificially contaminated samples were used as positive controls for PCR and real-time PCR methods. The thermal cycling procedure began with an initial denaturation phase, followed by 40 cycles of amplification that included denaturation, primer annealing, and extension steps. NTC (No Template Control), NFW (Nuclease Free Water)+ MM (Master Mix) were used as negative controls.

3. RESULTS AND DISCUSSION

Growth of *Bacillus cereus* and *Listeria monocytogenes*

Pure cultures of *Bacillus cereus* and *Listeria monocytogenes* bacteria were cultured using two types of growth media: solid and liquid. Both bacteria were grown in the same liquid media, namely BHI (Brain Heart Infusion) Broth. Meanwhile, the solid media used is selective media for each bacterium. The selective media used to grow *Bacillus cereus* bacteria is Mannitol Yolk Polymyxin Agar, while *Listeria monocytogenes* bacteria use Listeria Selective Agar

(Oxford Agar). The results of culture on agar media can be seen in **Figure 1**, showing that *Bacillus cereus* bacterial colonies with pink edge zones (a) and *Listeria monocytogenes* bacterial colonies with black edge zones (b).

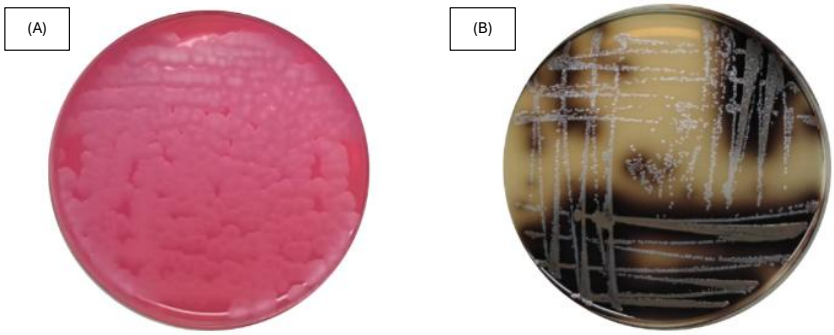


Figure 1. Bacterial culture results on selective media agar **(a)** *Bacillus cereus* colonies on MYPA are pink; **(b)** *Listeria monocytogenes* colonies on LSA are black

B. cereus could not ferment mannitol and thus produced pink colonies, colonies of Bacillus spp. Other than *B. cereus* caused the phenol red indicator to turn the colonies yellow. The lecithinase produced by *B. cereus* metabolizes lecithin and produces an opaque white precipitation zone. Meanwhile, polymyxin B is a selective agent that inhibits gram-negative bacilli and gram-positive cocci. Colonies that grow large, dry, pink in color, with a zone of egg yolk precipitate [13,14].

Differentiation of *Listeria spp*. The colony is based on the hydrolysis of agglucon and produces a black zone around the colony due to the formation of iron(II) ammonium citrate compounds derived from agglucon. Gram-negative bacteria are completely inhibited, and some other gram-positive bacteria, such as *Staphylococcus* produce a negative aesculin reaction [15].

After culturing bacteria on agar media, inoculation is carried out on liquid media or broth. Making inoculum is done by inoculating using a sterile wire Ose as many as 2 times bacterial colonies into Brain Heart Infusion Broth (BHIB) media for *Bacillus cereus* and *Listeria monocytogenes* bacteria and then incubating for 18 hours with 150 rpm shaker aeration at 37 ºC. Bacterial culture on BHI broth media produces a cloudy color, indicating that bacterial growth is good [16,17].

Qualitative and Quantitative Characterization DNA Sample

Isolation of pure culture DNA of *B. cereus* and *L. monocytogenes* using the GRS Genomic DNA Kit - Bacteria with additional lysis buffer, while isolation of DNA of processed meat samples, which are nuggets, meatballs, and sausages artificially contaminated by test bacteria, was isolated using the GRS Genomic DNA Kit - Food protocol. DNA isolation aims to obtain pure DNA without protein, RNA, or other matrix contamination[11]. The results of each isolation, including pure cultures of test bacteria (1), processed meat samples artificially contaminated with test bacteria (2), and meat product samples (3) were then analyzed qualitatively and quantitatively.

Quantitative analysis was conducted to determine the purity and concentration of DNA produced from the isolation process. An absorbance ratio of 1.8-2.0 measured at 260 and 280 nm wavelengths was used to assess DNA purity [18]. Values <1.8 indicate the presence of RNA and DNA contaminants that cannot be distinguished, while the opposite value indicates the presence of protein contaminants that absorb strongly in the 280 nm region [19]. The purity value of culture and sample DNA is shown in **Table 1**.

Qualitative analysis of pure cultures of *B. cereus and L. monocytogenes* was performed by characterization with 0.7% agarose gel electrophoresis in 1X TAE buffer solution and agarose gel added with fluorescent dyes, aiming to read the size of DNA bands resulting from the isolation process. Based on **Figure 2**, it shows that *B. cereus* bacteria have a genome size of 5.4 Mbp while *L. monocytogenes* is 2.9 Mbp.

Confirmation Test of *cyt-K 2* and *hly* primer by PCR

In previous studies, primer pairs *cyt-K 2* of *B. cereus* bacteria and *hly* of *L. monocytogenes* bacteria have been confirmed and validated [19]. The bacterial confirmation test on food samples aims to detect the presence of *Bacillus cereus* and *Listeria monocytogenes* bacteria in the test food samples, as well as to determine the ability of the *cyt-K 2* and *hly* gene primers as a component of the detection kit in recognizing target bacteria in food samples as evidenced by the production of appropriate amplicons. The food samples used in this study were processed meat: nuggets, meatballs, and sausages.

Table 1. Table of DNA Concentration and Purity of Pure culture and Artificially Contaminated Samples

Samples	Concentration (ng/µL)	Purity (A260/280)
Bacillus cereus pure culture	24,4	1,82
Listeria monocytogenes pure culture	70,4	1,83
Artificial contaminated meatball sample by *Bacillus cereus*	32,5	2,09
Artificial contaminated meatball sample by *Listeria monocytogenes*	152,1	1,85
Artificial contaminated sausage sample by *Bacillus cereus*	27,2	1,87
Artificial contaminated sausage sample by *Listeria monocytogenes*	12,4	1,80
Artificial contaminated nugget sample by *Bacillus cereus*	42,7	1,92
Artificial contaminated nugget sample by *Listeria monocytogenes*	23,4	1,80

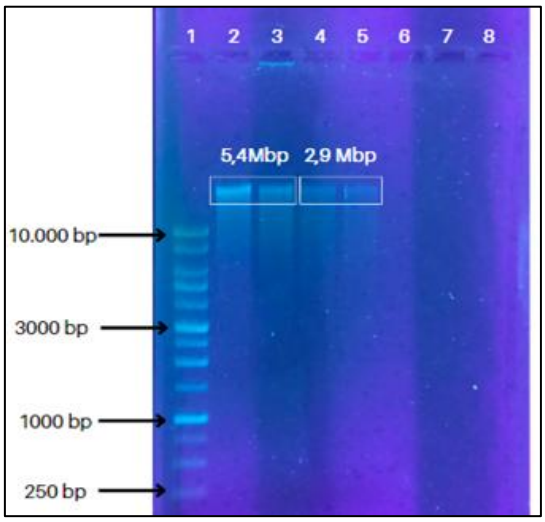


Figure 2. DNA electrophoresis of *Bacillus cereus* and *Listeria monocytogenes*. Notes: (1) 1 kB DNA Ladder (Smobio); (2) and (3) *Bacillus cereus* pure culture DNA isolate; (4) and (5) *Listeria monocytogenes* pure culture DNA isolate.

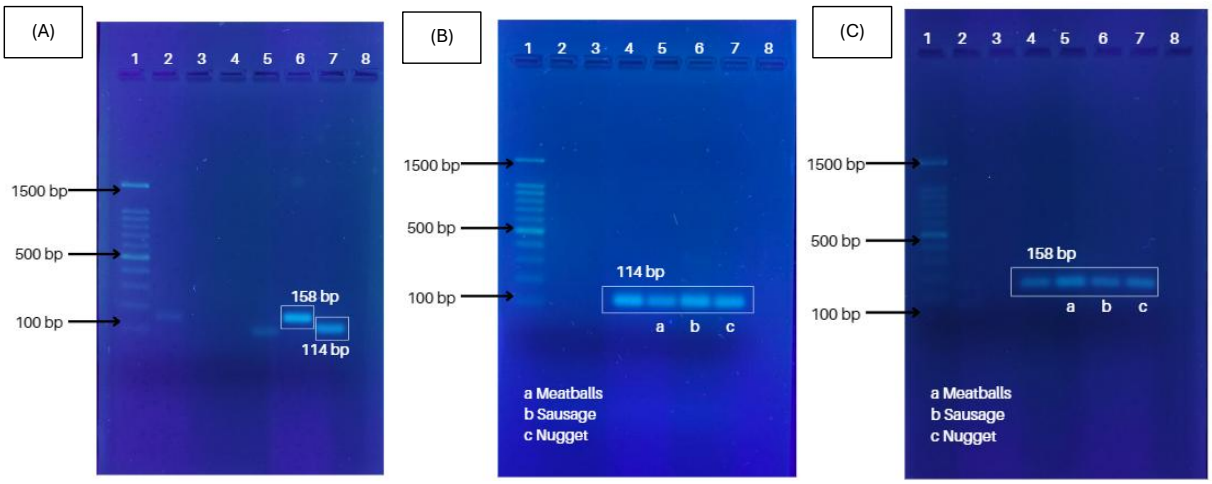


Figure 3. Characterization of *Bacillus cereus* and *Listeria monocytogenes* DNA fragments by agarose gel electrophoresis. [A] PCR electrophoresis results of pure culture [B] PCR electrophoresis results of artificially contaminated samples of *B. cereus* [C] PCR electrophoresis results of artificially contaminated samples of *L. monocytogenes* Lane: [A] (1) DNA Ladder 100 bp (2) cytk-2 gene NTC; (3) *hly* gene NTC; (4) NFW+MM (Negative control); (5) Positive control *fim-C Salmonella typhi* 95 bp; (6) DNA Fragment cytk-2 *B. cereus* 114 bp at 60ºC; (7) *L. monocytogenes hly* Fragment DNA 158 bp at 60ºC; [B] (1) DNA Ladder 100 bp (2) cytk-2 gene NTC; (3) NFW+MM (Negative control); (4-5) *B. cereus* cytk-2 Fragment DNA 114 bp at 60ºC and artificially contaminated samples 114 bp at 60ºC. [C] (1) DNA Ladder 100 bp (2) *hly* NTC gene; (3) NFW+MM (Negative control); (4-5) 158 bp *L. monocytogenes hly* Fragment DNA and 158 bp artificially contaminated samples at 60 ºC

Data from the confirmation of annealing temperature by the PCR method was further characterized by 2% agarose gel electrophoresis. Based on the results of data obtained from pure culture DNA isolates and samples artificially contaminated by the target bacteria, it shows that the *cyt-K 2* genes of *B. cereus* and *hly L. monocytogenes* can be well amplified during the PCR process with the optimum annealing temperature, which is at 60°C. The results of the bands formed on characterization with agarose

gel electrophoresis showed that the DNA fragments produced specific *cyt-K 2* gene amplicons with a product size of 114 bp and *hly* gene with a product size of 158 bp. The data shown at **Figure 3**.

Effication test for processed meat products

The efficacy test in this study aims to evaluate the accuracy and effectiveness of the prototype kit in directly detecting food samples suspected of being contaminated by *B. cereus* and *L. monocytogenes* bacteria. This study identified the differences between conventional culture, PCR, and rt-PCR methods in detecting test bacteria in food samples directly. The conventional culture method is carried out by swabbing food samples using sterile cotton buds and the results obtained are shown in **Figure 4**.

The conventional culture method efficacy test on 15 processed meat samples (5 nuggets, 5 meatballs, dan 5 sausages), gave positive results for contamination with the test bacteria. The left side shows MYPA media for identification of *B. cereus* bacteria. Samples a, b, c, d, e, h, k, and n showed the growth of pink bacterial colonies, while samples f, g, I, j, l, m, and o grew yellow colonies of other bacteria. The corresponding positive results are evidenced by the formation of pink colonies with large and dry characteristics. This is because *B. cereus* cannot ferment mannitol (mannitol negative) and can metabolize lecithin (lecithinase positive)[13]. Based on conventional culture methods, 15 processed meat samples were positive for bacterial contamination, but not specifically only *B. cereus*.

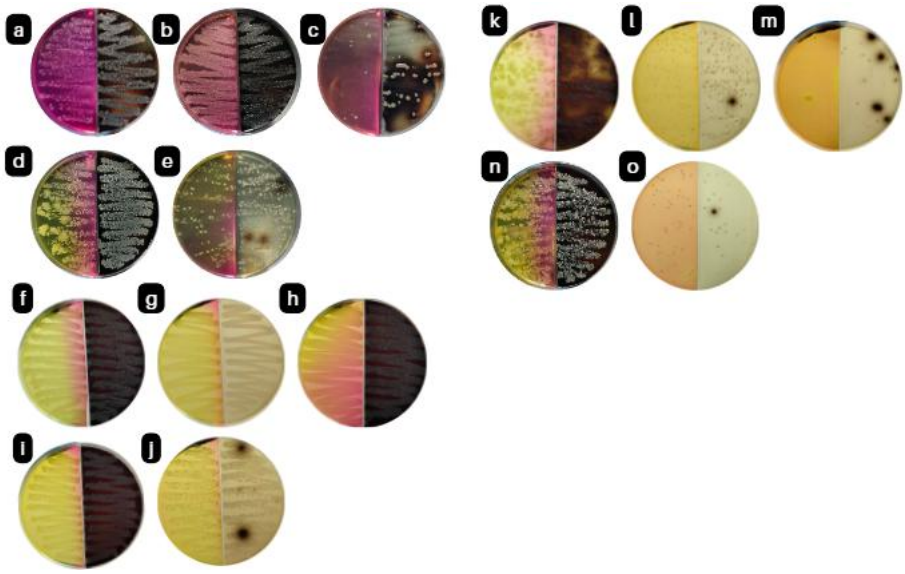


Figure 4. Agar culture efficacy tests on 15 food samples, left side MYPA for detect *B. cereus*, right side LSA for detect *L. monocytogenes*

In addition, **Figure 4** on the right side, which shows LSA media for identifying *L. monocytogenes* bacteria, shows 15 processed meat samples confirmed to be positively contaminated by bacteria. Samples a, b, c, d, f, h, I, k, j, l, m, n and o showed positive results as evidenced by the growth of black bacterial colonies, while sample g produced other bacterial colonies. The positive result is evidenced by the formation of black colonies caused by the formation of iron(ii) ammonium citrate compounds that produce a black zone around the colonies [16]. Based on the conventional culture method, the 15 processed meat samples were positive for amplicons, each sample was then compared with the results of the positive control amplicons. The positive control is a DNA template of a pure culture of test bacteria and processed meat artificially contaminated by test bacteria. The following presents the efficacy test results using the *cyt-K 2* and *hly* genes to determine bacterial contamination of *B. cereus* and *L. monocytogenes*.

Based on the data results shown in **Figure 5**, a single amplicon band was formed on the DNA of 15 samples with a 114 bp size, corresponding to the positive control amplicons. This shows that the *B. cereus cyt-K 2* gene primer can amplify DNA isolates in processed meat samples using the PCR method at the optimum annealing temperature of 60°C[23]. In addition, this shows that the primer can amplify specifically and produce amplicons that match the target size. Thus, based on the efficacy results of the PCR method, 15 samples were positively contaminated by *B. cereus* bacteria with the specific gene *cyt-K 2*[19].

The same thing was also done to determine the presence of *L. monocytogenes* in processed meat samples using the *hly* specific gene, which will produce an amplicons product size of 158 bp (**Figure 6**).

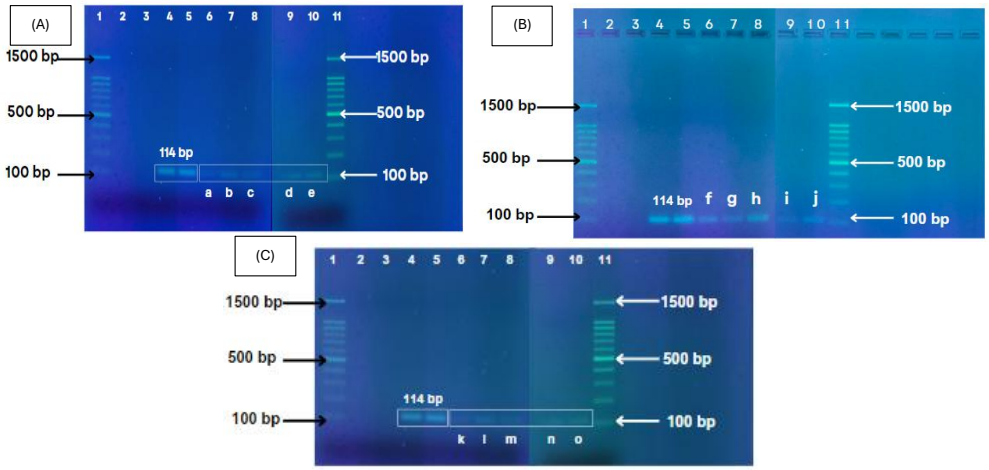


Figure 5. Characterization of *cyt-K 2* gene efficacy test of *B. cereus* bacteria in processed meat samples [A] a-e; nugget, [B] f-j; meatball, and [C] k-o; sausage

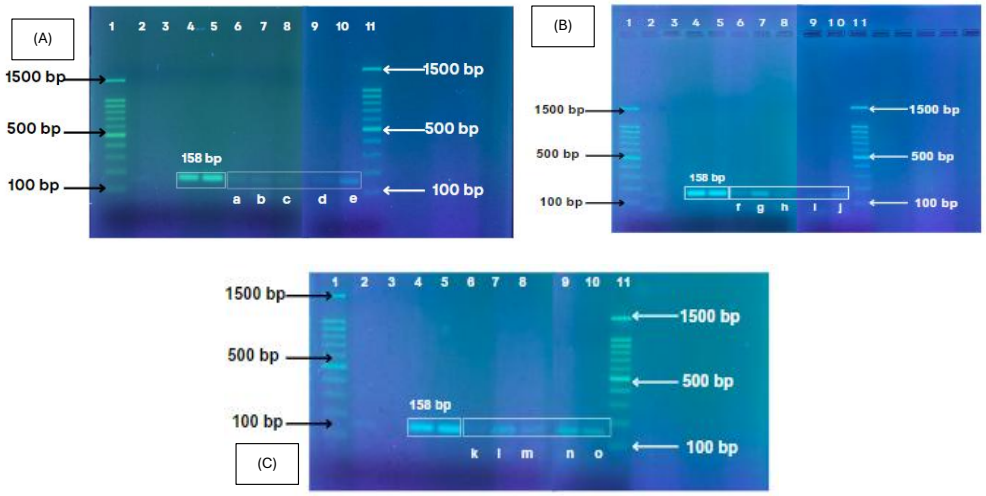


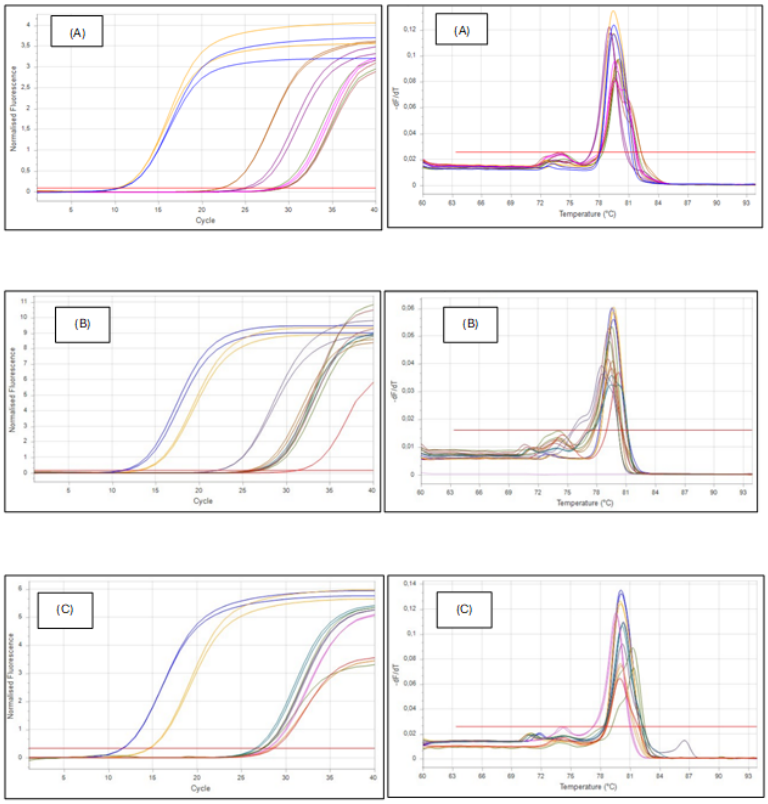
Figure 6. Characterization of *L. monocytogenes* bacterial *hly* gene efficacy test in processed meat samples [A] a-e; nugget, [B] f-j; meatball, and [C] k-o; sausage

Based on the data results shown in **Figure 6**, a single amplicon band was formed on the DNA of 15 samples with a size of 158 bp, corresponding to the positive control amplicons. This shows that the *L. monocytogenes hly* gene primer can amplify DNA isolates in processed meat samples using the PCR method at the optimum annealing temperature of 60 °C [20]. In addition, this shows that the primer can amplify specifically and produce amplicons that match the target size. Thus, based on the efficacy results of the PCR method, 15 samples were positively contaminated.

The efficacy test of the real-time PCR method on 15 processed meat samples was proven by looking at the Ct value formed and the melting temperature peak of the sample, and then comparing it with the positive control. The positive control used was a pure culture of *B. cereus* and a sample of processed meat artificially contaminated with *B. cereus* . cereus artificially. The reaction composition of the prototype detection kit used in the real-time PCR method with a total reaction volume of 20 µL includes MM (Master Mix), Forward and Reverse *cyt-K 2* primers, NFW (Nuclease Free Water), and Template DNA.

The following are the results of the amplification and melting temperature curves of 15 processed meat samples amplified by the *cyt-K 2* specific gene of *B. cereus* bacteria and *L. monocytogenes* bacteria with the specific gene *hly*.

The *cyt-K 2* primer pair could amplify the DNA template of the *B. cereus* pure culture positive control at Ct 10.31 and 10.35 (duplo) while the DNA template of the samples artificially contaminated by *B. cereus* was amplified at 10.45 and 10.58 (duplo); the meatball sample at 12.98 and 13.09; and the sausage sample at 14.63 and 14.64 The melting temperature curve of each positive control had a similar peak at 79 ± 1°C.



Line	Sample	*cyt-K 2*	
		Ct	Tm (°C)
	Sample Nugget A	22,24 & 22,24 (duplo)	79,84 & 79,90 (duplo)
	Sample Nugget B	25,39 & 24,87 (duplo)	79,12 & 79,03 (duplo)
	Sample Nugget C	28,20 & 29,20 (duplo)	80,06 & 79,86 (duplo)
	Sample Nugget D	29,37 & 29,32 (duplo)	79,72 & 79,53 (duplo)
	Sample Nugget E	28,84 & 28,49 (duplo)	79,60 & 79,60 (duplo)
	Sample Meatballs F	25,51 & 25,87 (duplo)	79,50 & 79,59 (duplo)
	Sample Meatballs G	26,52 7 26,21 (duplo)	79,55 & 79,57 (duplo)
	Sample Meatballs H	22,04 & 22,06 (duplo)	78,53 & 78,59 (duplo)
	Sample Meatballs I	26,67 & 27,06 (duplo)	79,55 & 79,28 (duplo)
	Sample Meatballs J	26,37 & 26,63 (duplo)	79,24 & 79,14 (duplo)
	Sample Sausage K	27,33 &27,46 (duplo)	80,33 & 80,22 (duplo)
	Sample Sausage L	26,51 & 26,76 (duplo)	80,30 & 80,18 (duplo)
	Sample Sausage M	27,92 & 28,07 (duplo)	79,66 & 79,58 (duplo)
	Sample Sausage N	27,30 & 27,40 (duplo)	81,25 & 81,34 (duplo)
	Sample Sausage O	28,37 & 28,32 (duplo)	80,09 & 80,02 (duplo)

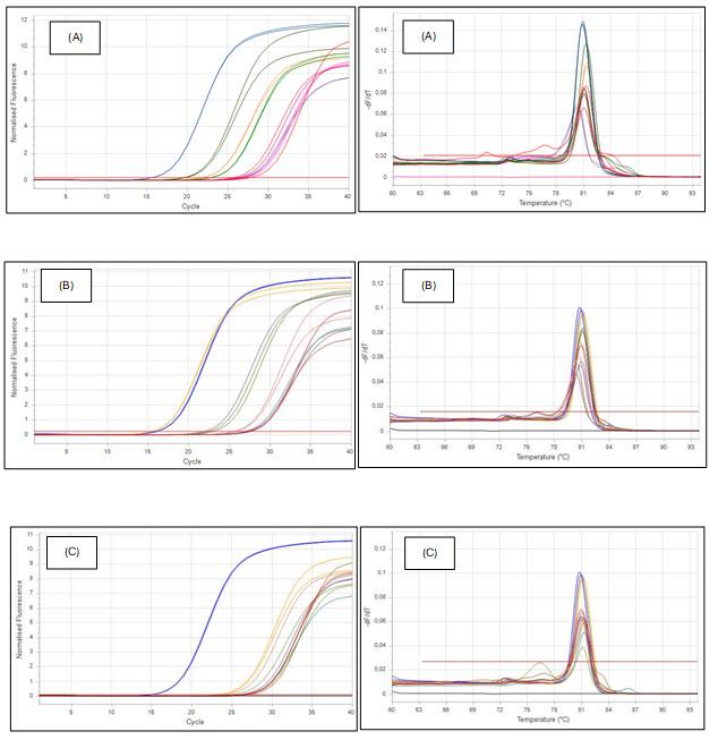
Figure 7. Efficacy Test of Samples A-E with *B. cereus* target gene *cyt-K 2* using real-time PCR Method. (Left) Amplification Curve and (Right) Melting Peak Curve of Efficacy Test Results of Primer Pair of *B. cereus cyt-K 2* gene on Samples by real-time-PCR. **(A)** Nugget **(B)** Meatballs **(C)** Sausage

The Ct and Tm values were used as positive controls for the efficacy test of 15 samples (A-E nugget samples, F-J meatball samples, and K-O sausage samples) with the *cyt-K 2* gene target of *B. cereus* bacteria. Based on the results obtained, all processed meat samples have a Ct value that is smaller than the Ct of the negative control NTC (No Template Control), which is 31.21. The smaller the Ct value reached the more DNA template of the nugget sample that was successfully amplified by the *cyt-K 2* gene primer pair.

In addition to the Ct value, a peak melting curve was also generated to see the peak point of the Tm value. The melting curve results are used to validate the results of the Ct. The Tm value shows the specific amplification of the bacterial DNA template; the melting temperature peak will have the same Tm value and peak point as the positive control. Based on the melting temperature curves of fifteen samples, all samples have the same Tm value as the positive control at 79 ± 1 °C, except sample N, which has a TM of 81 ± 1°C. Samples that produced one specific Tm peak with the Tm of the positive control indicated that the primer pair *cyt-K 2* successfully amplified the DNA template of samples suspected of being contaminated by *B. cereus* . Meanwhile, the Tm of the negative control NTC produced two peaks, indicating that the difference in temperature indicated the formation of non-target products.

Based on the results obtained, fifteen samples have a Ct value smaller than the Ct of the negative control NTC (No Template Control), which is at 27.59. The lower the Ct value reached the more *hly* gene DNA template that is successfully amplified, it can be said that the hly specific primer can amplify the DNA template from the suspected contaminated nugget sample. In addition to the Ct value, a melting peak curve was also generated to see the peak point and Tm value. The melting curve results are used to validate the results of the resulting Ct, this is because the specific amplification of bacterial DNA templates will have the same melting temperature peak as the positive control.

Based on the Tm results of fifteen samples, it can be seen that the Tm of all processed meat samples has the same Tm value as the positive control, which is at 80 ± 1°C. In addition, the results of the peak point of the sample have a point that is almost the same as the positive control peak point. So, based on these results, it can be proven that the processed meat samples are specifically amplified with hly primers. So, based on the results obtained, using the real-time PCR method, it can be proven that *L. monocytogenes* bacteria contaminate the positive sample in amplification with the hly specific gene.



Line	Sample	*hly*	
		Ct	Tm (°C)
	Sample Nugget A	22,17 &22,19 (duplo)	81,36 & 81,38 (duplo)
	Sample Nugget B	26,77 & 27,05 (duplo)	80,37 & 80,30 (duplo)
	Sample Nugget C	27,41 & 27,18 (duplo)	81,10 & 81,07 (duplo)
	Sample Nugget D	26,46 & 26,02 (duplo)	81,04 & 81,29 (duplo)
	Sample Nugget E	23,22 & 23,18 (duplo)	81,02 & 81,08 (duplo)
	Sample Meatballs F	22,00 & 22,01 (duplo)	81,06 & 81,08 (duplo)
	Sample Meatballs G	22,44 & 23,05 (duplo)	81,12 & 81,10 (duplo)
	Sample Meatballs H	27,03 & 27,24 (duplo)	80,42 & 80,36 (duplo)
	Sample Meatballs I	27,03 & 27,28 (duplo)	80,97 & 80,87 (duplo)
	Sample Meatballs J	25,82 & 25,63 (duplo)	80,68 & 80,81 (duplo)
	Sample Sausage K	24,02 & 24,08 (duplo)	80,97 & 80,87 (duplo)
	Sample Sausage L	26,47 & 25,05 (duplo)	81,12 & 81,28 (duplo)
	Sample Sausage M	26,30 & 26,35 (duplo)	81,28 & 81,38 (duplo)
	Sample Sausage N	26,28 & 25,90 (duplo)	81,07 & 81,06 (duplo)
	Sample Sausage O	26,38 & 26,97 (duplo)	81,32 & 80,97 (duplo)

Figure 8. Efficacy Test of Samples A-E with *L. monocytogenes* target gene *hly* using real-time PCR Method. (Left) Amplification Curve and (Right) Melting Peak Curve of Efficacy Test Results of Primer Pair of *L. monocytogenes hly* gene on Samples by real-time-PCR. **(a)** Nugget **(b)** Meatballs **(c)** Sausage

4. CONCLUSIONS

Based on the results of this study, *B. cereus* and *L. monocytogenes* bacteria have been successfully detected in fifteen samples of processed meat, namely nuggets, meatballs, and sausages directly, using three methods: conventional culture, PCR, and real-time PCR. The results of the efficacy test on the nugget samples showed that the five samples were confirmed positive for contamination with *Bacillus cereus* and *Listeria monocytogenes* bacteria as evidenced by the growth of specific colonies on selective media, with the PCR method forming amplicons that match the target *B. cereus* with primer *cyt-K 2* 114 bp and *L. monocytogenes* with primer *hly* 158 bp. The real-time PCR method can prove that the target gene primer pair is amplified and produces a Ct value lower than the cutoff value, the resulting Tm value and a specific peak. So based on the results obtained, it can be concluded that the use of detection methods using real-time PCR provides specific, sensitive, accurate, and fast results for detecting food samples suspected of being contaminated compared to the other two methods. Therefore, the efficacy test for the prototype kit was successful in detecting the presence of pathogenic bacteria in processed meat samples.

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REFERENCES

1. Grace D. Food safety Food safety is not salient in SDGs but may become more important. *J Agric Nutr Heal ILRI*. 2017;2015:1-11.
2. Shi C, Kang S. Foodborne Pathogenic Bacteria: Prevalence and Control—Volume I. *Foods*. 2024;13(10):1531. doi:10.3390/foods13101531
3. Alvarado PE, Barrios RMM, Xóchihua JAM, Hernández JFC. Fast and reliable DNA extraction protocol for products sold on the commercial market. *Open Agric*. 2017;2:469-472. https://www.degruyter.com/document/doi/10.1515/opag-2017-0051/html
4. Setiani BE, Pramono YB, Purwitasari L. Review on Pathogenic Bacteria Listeria monocytogenes, The Detection and The Sequencing Gene Methods Isolated from Meat Products. *J Appl Food Technol*. 2019;6(2):44-

51. doi:10.17728/jaft.6460
5. Younes O, Ibrahim H, Hassan M, Amin R, Maarouf A. Demonstration of some food borne pathogens in different meat products: a comparison between conventional and innovative methods. *Benha Vet Med J*. 2019;36(2):219-228. doi:10.21608/bvmj.2019.12557.1008
6. BPOM. Batas Maksimal Cemaran Mikroba Dalam Pangan Olahan Produk. *Badan Pengawas Obat dan Makanan*. Published online 2019:1-48.
7. Atmanto Y, Asri L, Kadir N. Media Pertumbuhan Kuman. *J Med Hutama*. 2022;4(1):3072-3073. http://jurnalmedikahutama.com
8. Stingl K, Heise J, Thieck M, Wulsten IF, Pacholewicz E, Iwobi AN, Govindaswamy J, Zeller-Peronnet V, Scheuring S, Luu HQ, Fridriksdottir V, Golz G, Priller F, Gruntar I, Jorgensen F, Koene M, Kovac J, Lick S, Reperant E, Rohlfing A, Huber I. Challenging the "gold standard" of colony-forming units - Validation of a multiplex real-time PCR for quantification of viable Campylobacter spp. in meat rinses. *Int J Food Microbiol*. 2021;359:109417. doi:10.1016/j.ijfoodmicro.2021.109417
9. Kim DH, Chon JW, Kim H, Kim HS, Choi D, Kim YJ, Yim, JH, Moon JS, Seo KH. Comparison of Culture, Conventional and Real-time PCR Methods for Listeria monocytogenes in Foods. *Korean J Food Sci Anim Resour*. 2014;34(5):665-673. doi:10.5851/kosfa.2014.34.5.665
10. Widyastuti DA. Deteksi Molekuler Mikroorganisme Patogen pada Bahan Pangan dengan Metode RT-PCR. *J Ilmu Pangan dan Has Pertan*. 2017;1(1):54-63. doi:10.26877/jiphp.v1i1.1356
11. Nurjayadi M, Maulana I, Pramudiyasih NA, Kusumawati RN, Azzahra M, Liman NK, Sangkara MA, Wibisono E, Kurniadewi F, Saamia V, Saputro DAO, Wiranatha M, El-Enshasy HA. Isolation and PCR Validation of Bacillus cereus and Listeria monocytogenes Bacteria for the Development of Prototype Kit Detection of Foodborne Pathogens Diseases. *AIP Conf. Proc.* 2023: 2614, 030015. https://doi.org/10.1063/5.0126315
12. Charis M. Galanakis, ed. *Sustainable Food Systems from Agriculture to Industry*. Elsevier; 2018. doi:10.1016/C2016-0-02157-5
13. Raplong HH, Odeleye PO, Hammuel C, Idoko MO, Asanato JI, Odeke EH. Production of Alpha Amylase by Bacillus cereus in Submerged Fermentation. *Aceh Int J Sci Technol*. 2014;3(3):124-130. doi:10.13170/aijst.3.3.1592
14. FDA. BAM Chapter 14: Bacillus cereus.
15. Law JWF, Ab Mutalib NS, Chan KG, Lee LH. An insight into the isolation, enumeration, and molecular detection of Listeria monocytogenes in food. *Front Microbiol*. 2015;6. doi:10.3389/fmicb.2015.01227
16. Mira P, Yeh P, Hall BG. Estimating microbial population data from optical density. Omri A, ed. *PLoS One*. 2022;17(10):e0276040. doi:10.1371/journal.pone.0276040
17. Brian C M. Differences in bacterial optical density measurements between UV-Visible spectrophotometers. *Thermo Sci*. 2019;(Mic).
18. Azzahra M, Nurjayadi M, Pramudiyasih NA, Kusumawati RN, Maulana I, Declan JL, Putri GI, Juliansyah DA, Krisdawati I, Kurniadewi F, Kartika IR, Sukmawati D, Nastassya L, Saamia V, Oktaviani DA, Wiranatha SM, El-Enshasy HA, Abomoelak B. Primer concentration and Pre-denaturation Time Effect on cyt-K Bacillus cereus Detection using Real-Time PCR Method. *E3S Web Conf*. 2023;400:0-4. doi:10.1051/e3sconf/202340004011
19. Utaminingsih S, Sophian A. Analysis of Purity and Concentration of DNA Isolation Results on Chondroitin Samples. *BiosciED J Biol Sci Educ*. 2022;3(2):56-61. doi:10.37304/bed.v3i2.5425
20. Azzahra M. *Validasi Formula Kit Deteksi Bakteri Patogen Bacillus Cereus & Listeria Monocytogenes Penyebab Keracunan Pangan dengan Real-Time Polymerase Chain Reaction*. Universitas Negeri Jakarta; 2022.