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Research Article

Development of a multiplex PCR procedure for the simultaneous detection of *Clostridium botulinum* serotypes A, B, E, F

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Abstract

The anaerobic, spore-forming, Gram-positive bacterium *Clostridium botulinum* (*C. botulinum*) is responsible for botulism caused by the production of the botulinum neurotoxins (BoNTs), of which four serotypes, A, B, E, and F, are known to be lethal to humans. In 2020, nearly a hundred cases of botulinum poisoning were reported in Vietnam for the first time. Culture-based methods, including mouse bioassays routinely used for botulism diagnosis, may take weeks, resulting in the patients not receiving timely treatment. Thus, nucleic acid amplification tests (NAATs) using specific primers have become a promising alternative. In this study, we selected four primer pairs that would produce four distinct fragments of 240 bp, 205 bp, 140 bp, and 415 bp corresponding to 4 target genes that encode BoNT/A, /B, /E, and /F for convenient separation using regular agarose electrophoresis. The modified thermal cycle that combined annealing and extension steps allowed completion of the multiplex PCR reaction in one hour. The analytical specificity of the method was also evaluated based on two strains of the same genus Clostridium and six strains of different genera species. The detection limits of the method were determined to be 10^3 copies/µL for serotypes A, B, and E with both pure plasmids and DNA extracted from spiked samples. The total time of the established procedure was 1.5 hours, including electrophoresis

Keywords*: Multiplex PCR, Clostridium botulinum, serotype A, B, E, F, BoNT, agarose electrophoresis*

1. INTRODUCTION

Clostridium botulinum is a gram-positive, anaerobic, spore-forming bacterium that produces botulinum neurotoxin. Botulinum neurotoxin is divided into 7 toxin serotypes, of which 4 serotypes—A, B, E, and F—cause poisoning in humans in very small amounts of 25–50 ng. In Vietnam, from 2020 to the present, 12 cases of poisoning due to *C. botulinum* have been recorded, with about 100 patients and 20 deaths. The standard method for determining BoNT toxin is the mouse bioassay [6]. However, in the past 20 years, this method has been less used due to the issue of using live animals for experiments; the alternative tests are ELISA, anaerobic culture, and molecular biological tests. In 2001, Lindström et al. [1] developed a multiplex PCR method using 4 primer pairs to simultaneously detect the genes encoding toxins A, B, E, and F [2]. In the same year, 2001, the United States Food and Drug Administration (FDA) also issued a handbook on PCR standards for detecting *C. botulinum* bacteria [20]. These two studies are considered the premise for later works using multiplex PCR to detect toxin genes [2]. However, the target gene fragment amplified by the primer pairs used in these two

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studies was long and could not cover all subtypes of the recently described serotypes [9]. Due to the genetic diversity, specifically as reported by Pillai *et al.* in 2024, the A-G types differed by 24-42% at the nucleotide level, so the research team aimed to develop a multiplex PCR method for the simultaneous, specific, and high-coverage detection of 4 toxin genes of *C. botulinum* bacteria within 1.5 hours.

2. MATERIALS AND METHOD

2.1. Materials

The synthetic plasmids containing the genes encoding BoNT/A and BoNT/B were synthesized by the cloning method, and the synthetic plasmids containing the genes encoding BoNT/E and BoNT/F were synthesized by the artificial gene synthesis method, as shown in **Table 1**. The sequences of primer pairs used to amplify the specific target genes encoding BoNT/A, /B, /E, and /F are shown in **Table 2**.

***Table 1**. Structures and sizes of synthetic inserts with in the plasmids (designated PA, PB, PE, and PF)*

Sample name	Vector	Insert size (bp)	Synthetic method
PA	pLUG	1300	Clone
PB	pGEM-t	1300	Clone
PE	pUCIDT	300	Artificial synthesis (Integrated DNA Technologies, USA)
PF	pUCIDT	455	Artificial synthesis (Integrated DNA Technologies, USA)

***Table 2.** Sequences of primer pairs used to amplify the gene segments encoding BoNT/A, /B, /E, and /F*

Symbol	Sequence (5'- 3')	Length (bp)	Size (bp)
AF	TCAATACATTAGATTTAGCCCA	22	240
AR	CCCAAATGTTCTAAGTTCCT	20	240
BF	CAGGAGAAGTGGAGCGAAAA	20	205
BR	CTTGCGCCTTTGTTTTCTTG	20	205
EF	AAAAGCTAATCCATATTTAGGRAATGAT	28	140
ER	GGCTCTGCTCCCATTATAATAAC	23	140
FF	AACTAATGGATTCAGGTGGAG	21	415
FR	TCAGGAGGAGCACTATTAACT	21	415

The canned meat matrix was supplemented with *C. botulinum* serotype A(b) bacteria strain provided by the National Institute for Food Control. The bacterial strains used to evaluate the analytical specificity of the method were provided by the National Institute of Hygiene and Epidemiology and Phenikaa University and are listed in **Table 3.**

***Table 3**. Eight bacterial strains used to evaluate the analytical specificity of the multiplex PCR method*

No.	Strain name	No.	Strain name
1	*Clostridium perfingens* ATCC13124	5	*Escherichia coli* BL21
2	*Clostridium difficile* ATCC 43593	6	*Salmonella enterica* VTCC12270
3	*Escherichia coli* C118	7	*Salmonella enterica* VTCC12271
4	*Escherichia coli* VTCC12271	8	*Listeria monocytogenes* ATCC15313

2.2. Method

2.2.1. Select primer pair to multiply 4 target gene segments

The four primer pairs used to amplify the four gene segments encoding botulinum toxin serotypes A, B, E, and F were selected based on the criteria of being able to separate PCR products when running on an electrophoretic gel. In addition, the primer pairs were also tested for cross-pairing and self-pairing capabilities

using the OligoAnalyzer software of Integrated DNA Technologies (https://www.idtdna.com/pages/tools/oligoanalyzer) and the Multiple Primer Analyzer of ThermoFisher Scientific (https://www.thermofisher.com/vn/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html).

2.2.2. Single-target PCR assays

PCR reactions for each target gene were performed in a volume of 12 µL, including 6 µL 2X SP Master mix (Phu Sa genomics, Vietnam), 10 pmol of each forward and reverse primer, with the following thermal cycling protocol: (1) DNA denaturation at 95℃ for 4 minutes; (2) amplification for 35 cycles, each cycle consisting of 3 steps (step 1: 95℃ for 30 s, step 2: 57℃ for 45 s, step 3: 72℃ for 30 s); (3) 72℃ for 4 minutes and finally sample storage at 4℃ [21].

2.2.3. Electrophoresis of PCR products

After the PCR reaction was completed, 5 µL of the product was mixed well with 1 µL of 6X sample loading buffer, applied to a 2% agarose gel wells (supplemented with Red safe dye (Intron Biology, Korea)) and run in 1x TAE buffer (Biobasic, Canada). The 100 - 1500 bp DNA ladder (Cleaver Scientific, UK) was electrophoresed in parallel with the sample. The gel containing the bands was observed on a gel scanner. The electrophoresis results of the PCR products must show bright, clear bands, with the correct size compared to theoretical calculations for single-target PCR reactions and bands that can be separated from each other, with the correct size, bright and clear bands for multiplex PCR reactions.

2.2.4. Multiplex PCR method

The multiplex PCR reaction was performed in a volume of 20 µL, including 6ul 2X SP Master mix (Phu Sa genomics, Vietnam), 10 pmol each forward and reverse primer. With the thermal cycling: (1) DNA denaturation at 95℃ for 4 minutes; (2) amplification for 35 cycles, each cycle consisting of 3 steps (step 1: 95℃ for 30 s, step 2: 57℃ for 45 s, step 3: 72℃ for 30 s); (3) 72℃ for 4 minutes and finally storing the sample at 4℃. The multiplex PCR product was then electrophoresed on a 2% agarose gel prepared by mixing 2 g agarose with each 100 mL TAE buffer, using 0.5 µL RedSafe/ 12.5 mL gel. Each well on the gel was inoculated with 5 µL of PCR product, the gel was placed in an electrophoresis tank containing 1X TAE buffer, electrophoresis under 90 V, 30 minutes.

2.2.5. Optimizing multiplex PCR reaction time

The reaction time was shortened by combining the annealing and extension steps in the synthetic thermal cycle as follows: (1) DNA denaturation at 95℃ for 4 minutes; (2) amplification of the BoNT/A, /B, /E, /F gene segments in 35 cycles, each cycle consisting of 2 steps (step 1: 95℃ for 15 s, step 2: 57℃ for 30 s); (3) 72℃ for 1 minute.

2.2.6. Determination of analytical specificity of multiplex PCR reaction

Two bacterial strains of the same genus *Clostridium* (Clostridium difficile 1, Clostridium difficile 3) and six strains of different genus *Clostridium* (*Escherichia coli* C118, *Escherichia coli* BL21, *Salmonella enterica* VTCC12270, *Escherichia coli* VTCC12271, *Salmonella enterica* VTCC12271, *Listeria monocytogenes* ATCC15313) were used to evaluate the analytical specificity of the method.

2.2.7. Limit of detection

Plasmid samples A, B, E, F were diluted at a stock concentration of 10^6 copies/µL by pipetting 2 µL of this stock plasmid into 18 µL of water to obtain a sample with a concentration of 10^5 copies/µL, similarly, 10-fold dilutions were performed for samples with concentrations of 10^4 10^3; 10^2; 10^1 copies/µL.

2.2.8. Tested on real samples supplemented with C. botulinum bacteria strain

To test the applicability of the multiplex PCR method in food samples, 1 g of canned meat was supplemented with 1 mL of a 10-fold dilution of a suspension containing 10^6 CFU/mL to 10^2 CFU/mL of spores or vegetative cells of *C. botulinum* strain A(b) (carrying both toxin-encoding genes A and B). The samples were then mixed with 9 mL of purified distilled water. In addition, because it was not possible to isolate *C. botulinum* serotypes E and F, the research team added a plasmid carrying the gene segment encoding

toxin types E and F to the canned fish sample. Finally, 1 mL of each concentration mixture was used to isolate DNA using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, USA).

3. RESULTS AND DISCUSSION

3.1. Selection of primer pairs for multiplex PCR reactions

The primer pairs used for the multiplex PCR reactions should have similar melting temperatures, and the amplified product sizes should have to be different for clear separation on the agarose gel [4]. Therefore, 4 primer pairs were selected with annealing temperatures ranging from 57 to 58°C, and the PCR product sizes were 415 bp, 240 bp, 205 bp, and 140 bp for BoNT/A, /B, /E, and /F, respectively. The similar annealing temperatures allowed the annealing step to take place at the same temperature, thus avoiding the amplification of non-specific products, and the sizes were suitable for separation.

Table 4. *Sequences of primer pairs used to amplify the gene segment encoding BoNT toxins/A, /B, /E, and /F*

Code	Order (5'- 3')	Length (bp)	Primer annealing temperature (℃)	Size (bp)	Energy to create hairpin structure (kcal/mol)	Energy creates the ability to self-pair (kcal/mol)	Energy that enables cross-pairing (kcal/mol)
AF	TCAATACATTAGATTTAGCCCA	22	57	240	1.96	-3.42	-6.71
AR	CCCAAATGTTCTAAGTTCCT	20	57	240	1.79	-1.95	-6.71
BF	CAGGAGAAGTGGAGCGAAAA	20	58	205	1.6	-3.61	-7.41
BR	CTTGCGCCTTTGTTTTCTTG	20	58	205	1.53	-9.89	-7.41
EF	AAAAGCTAATCCATATTTAGGRAATGAT	28	57	140	-0.22	-6.34	-5.37
ER	GGCTCTGCTCCCATTATAATAAC	23	57	140	0.1	-10.32	-5.37
FF	AACTAATGGATTCAGGTGGAG	21	58	415	0.27	-3.42	-4.38
FR	TCAGGAGGAGCACTATTAACT	21	58	415	0.19	-4.85	-4.38

Note: AF, BF, EF, FF: are forward primers that amplify the gene segment encoding the BoNT toxins/A, /B, /E, /F, respectively; AR, BR, ER, FR: reverse primers amplifying the gene segment encoding BoNT toxins/A, /B, /E, /F, respectively.

In addition, the parameters related to the ability to form hairpin structures, self-pairing ability and cross-pairing ability of the primers were all within the allowable limits (>-9 kcal/mol) (Integrated DNA Technologies), so they did not affect the amplification ability.

3.2. Single-target PCR reaction, analytical specificity and detection limit of each toxin type

Single-target PCR reaction using primer pairs in **Table 2** with the aim of testing the amplification ability on templates which are plasmids carrying target gene segments encoding BoNT/A, /B, /E, /F. The results of electrophoresis on 2% agarose gel (**Figure 1**) showed that the primer pairs successfully amplified 4 target gene segments with product sizes as expected. However, bands smaller than 100 bp still appeared in the wells. These bands appeared because the amount of primer used in the reaction was not optimized, the remaining primers were not paired with the target template, so self-pairing occurred. This can be completely resolved by optimizing the concentration of template and primer in each reaction [19]. In addition, the bands on the gel

were blurred and the research team resolved this by reducing the electrophoresis time with the aim of making the electrophoretic bands clearer.

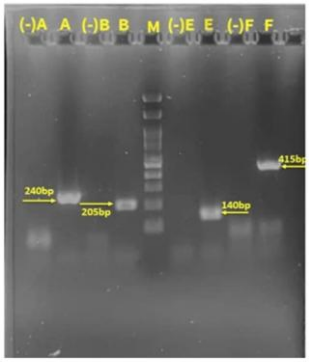

Figure 1. *Electrophoresis results of single-target PCR products of 4 serotypes A, B, E, and F on 2% agarose gel. (Notes: (-): negative controls; M: 100-1500 bp marker; A, B, E, F: 4 plasmids PA, PB, PE, and PF, respectively)*

Based on the electrophoresis results of the serial dilution with four serotypes in **Figure 2**, it can be seen that the 3 serotypes, including A, B, and E, had a single PCR detection limit of 10^2 copies/reaction, and serotype F has a single primer PCR limit of 10^3 copies/reaction. Serotype F has a lower detection limit than other serotypes, which can be explained by the fact that the amplified target gene segment of serotype F is long (415 bp), in which the A-T percentage accounts for 72%, which directly affects the amplification efficiency of the reaction [15].

In the electrophoresis results in **Figure 3**, it can be seen that the primer pairs used to detect *C. botulinum* serotypes A, B, E, and F were completely specific, not cross-reacting with other bacteria of the same genus and other food poisoning bacteria. The above results were consistent with the results of testing the primer pairs using *in silico* PCR. Thus, the selected primer pairs can be combined in the same multiplex PCR reaction to simultaneously detect 4 genes encoding the toxins of *C. botulinum* serotypes A, B, E, and F.

3.3. Multiplex PCR setup and optimization

The selected primer pairs were used to perform multiplex PCR reactions. The results showed that the 4 primer pairs successfully amplified the 4 corresponding target gene fragments and produced bands with clear separation on 2% agarose gel (**Figure 4**). The clear difference in the specific size of the target bands in the range of 0.2 – 1 kb allowed the products to have clear separation without the need to use high-resolution agarose [1, 4, 13].

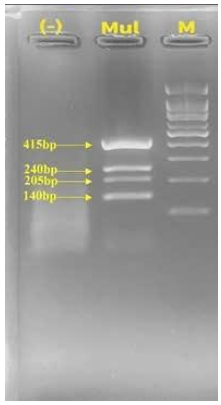

Figure 4. *Electrophoresis results of multiplex PCR products of 4 serotypes A, B, E, F on 2% agarose gel. (Note: (-): negative control; M: 100 – 1500 bp marker from Cleaver; Mul: multiplex PCR sample of 4 plasmids PA, PB, PE, PF)*

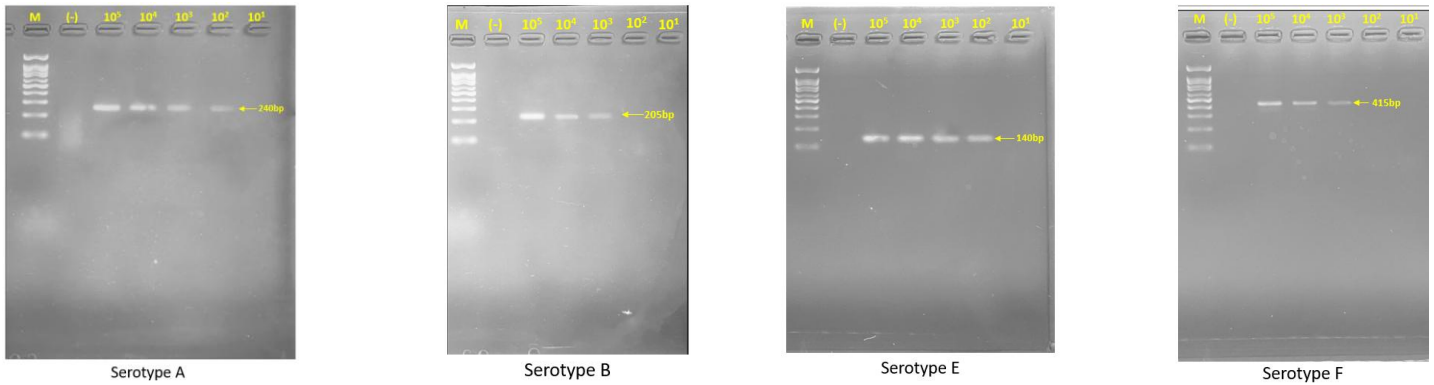

Figure 2. *Detection limit of single-target PCR method to detect the toxin-encoding gene of C. botulinum serotype A, B, E, F. (Note: (-): negative control; M: 100-1500bp marker from Cleaver, 10^6 – 10^1: wells with concentrations of 10^6 - 10^1 copies / reaction, respectively)*

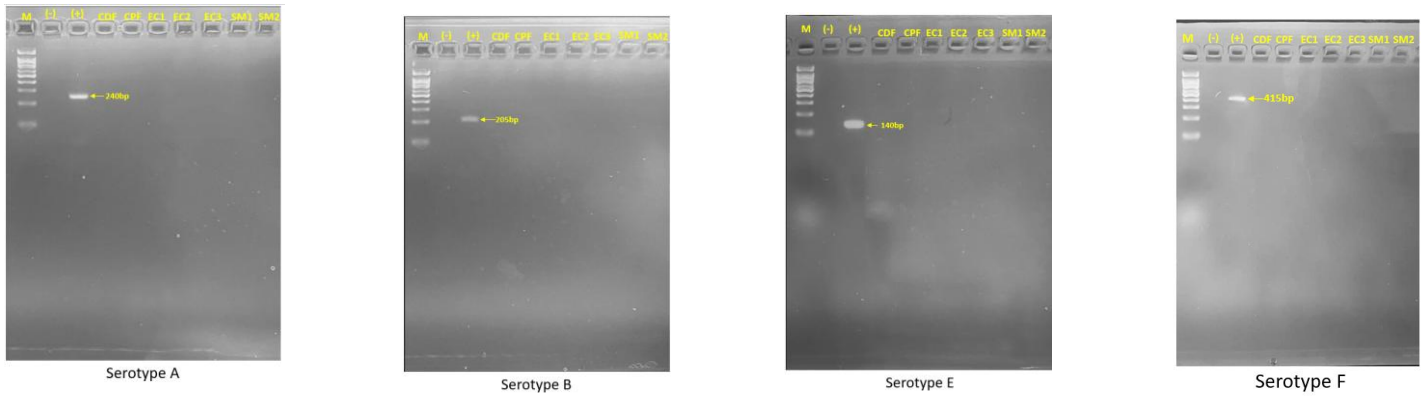

Figure 3. *Results of specificity testing of 4 serotypes A, B, E, F. (Notes: (-), (+): control samples; M: 100 – 1500 bp marker from Cleaver; CDF: Clostridium difficile strain ATCC 43593, CPF: Clostridium perfingens strain ATCC13124, EC1: Escherichia coli strain C118, EC2: Escherichia coli strain BL21, EC3: Escherichia coli strain VTCC12271, SM1: Salmonella enterica strain VTCC12270, SM2: Salmonella enterica strain VTCC12271, LM1: Listeria monocytogenes strain ATCC15313)*

By using a 2-step combination of annealing and extension, the multiplex PCR reaction time was shortened to approximately 1 hour compared to the reaction using the standard 3-step cycle (which takes up to approximately 2 hours).

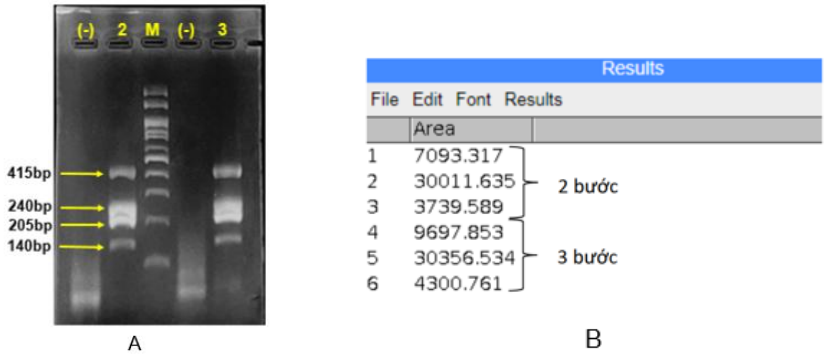

Figure 5. *A: Electrophoresis of optimized reaction time using 2-step cycle (Note: (-): negative control; M: 100–3000 bp marker; 2: multiplex PCR product using 2-step cycle; 3: Multiplex PCR product using 3-step cycle). B: Comparison results of band intensities when using 2-step and 3-step thermal cycles using ImageJ software*

The results displayed on the gel also showed that using the 2-step combination cycle had almost no effect on the amplification product (**Figure 5A**). Using ImageJ software showed that the brightness of the bands when using the 2-step or 3-step cycle was similar (**Figure 5B**). According to Lawyer et al., the optimal operating temperature of the Taq polymerase enzyme is from 75 to 80°C. However, at a temperature of 55-65°C, the enzyme still gave stable performance [14]. Therefore, if the annealing temperature is within 3°C of the extension temperature, both the annealing and extension temperatures can be combined into a single step called two-step PCR instead of the conventional three-step PCR. (https://www.thermofisher.com/vn/en/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/pcr-education/pcr-reagents-enzymes/pcr-cycling-considerations.html). Two-step PCR shortens the PCR procedure time because there is no need to switch and stabilize the temperature between annealing and extension.

3.4. Specificity of the analytical method

The electrophoresis results showed that in wells with specificity test samples, there was no band with the same size as the target band (**Figure 6**). Thus, it can be concluded that the primer pairs were selected specifically for the serotypes, and there was no cross-reaction with other species of the *Clostridium* genus that can cause food poisoning.

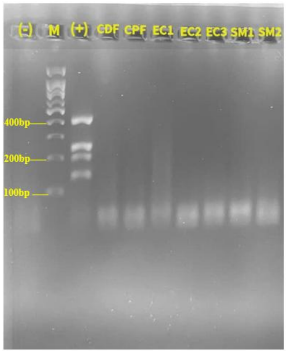

Figure 6. *Results of specificity testing of 4 primer pairs*

(Note: (-), (+): control samples; M: 100 – 1500 bp marker from Cleaver; CDF: Clostridium difficile strain ATCC 43593, CPF: Clostridium perfingens strain ATCC13124, EC1: Escherichia coli strain C118, EC2: Escherichia coli strain BL21, EC3: Escherichia coli strain VTCC12271, SM1: Salmonella enterica strain VTCC12270, SM2: Salmonella enterica strain VTCC12271, LM1: Listeria monocytogenes strain ATCC15313)

3.5. Limit of detection

Based on the electrophoresis results, the lowest detection limit of the multiplex PCR method for each serotype was determined to be: serotype A: 10^3 copies/reaction, serotype B: 10^3 copies/reaction, serotype E: 10^3 copies/reaction and serotype F was 10^6 copies/reaction (**Figure 7**). It was found that the detection limit of serotype F was lower than that of the other serotypes, possibly due to the largest target gene fragment size (415 bp). According to the study of Lindström *et al*., the detection limit of each serotype in the multiplex PCR reaction simultaneously detecting all 4 botulinum toxin-encoding genes was not uniform [1].

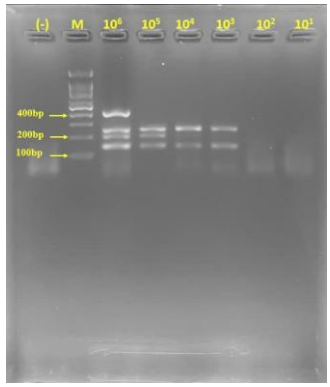

Figure 7. *Detection limit of the multiplex PCR method to detect the toxin-encoding gene of C. botulinum serotype A, B, E, F. (Note: (-): negative control; M: 100-1500 bp marker from Cleaver, 10^6 – 10^1: wells with concentrations of 10^6 - 10^1 copies / reaction, respectively)*

3.6. Detection of *C. botulinum* bacteria in food samples

Based on the electrophoresis results, it was found that the *C. botulinum* strain added to the canned meat sample carried both toxin-encoding genes A and B (**Figure 8A**). In fact, there have been many published reports of *C. botulinum* strains producing more than one type of toxin, for example Af, Bf, in which the main toxin is denoted by a capital letter and the secondary toxin is denoted by a lowercase letter [11]. According to the report of Cordoba *et al*. in 1995 [12], it was shown that the majority (43 out of 79 strains surveyed by PCR) of the BoNT/B toxin-encoding genes were found in the *C. botulinum* type A strain. In addition, the canned fish sample supplemented with PE and PF plasmids both detected the target gene fragment with the desired size (**Figure 8B**) by multiplex PCR method. It can be seen that the multiplex PCR method is useful in simultaneously detecting multiple genes encoding botulinum toxins of *C. botulinum* bacteria.

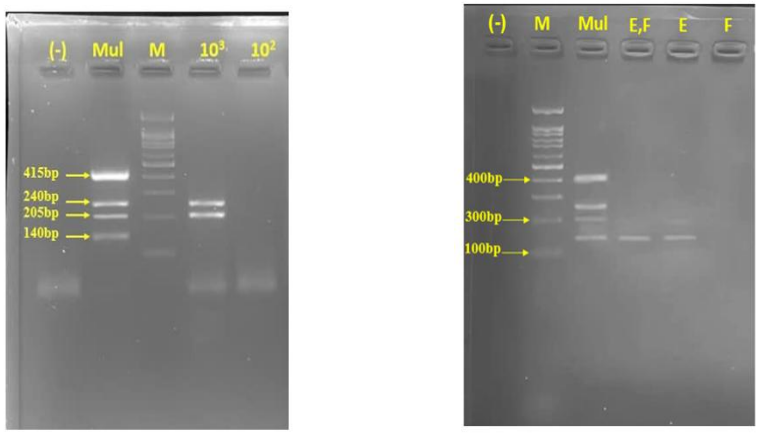

Figure 8. *Supplement A, B: Test on food sample (Note: (-): negative control; M: 100 – 1500 bp marker from Cleaver, 10^3 – 10^2: respectively are the concentrations of C. botulinum strain A(b) added, E/F: respectively are the samples supplemented with plasmid E/F with a concentration of 10^3 copies/ µL)*

4. CONCLUSION

Four specific primer pairs were selected and successfully used to set up a multiplex PCR reaction to detect four serotypes A, B, E, and F with the correct amplification product sizes as calculated. The established multiplex PCR reaction only took about 1 hour and 30 minutes compared to the previous 3-step multiplex PCR cycle of about 3 hours. In addition, the primer pairs used in this study had better coverage than the multiplex PCR method established by Lindström [1], and the target fragments were shorter, so it took less time than the FDA standard PCR procedure. The currently established procedure still needs further optimization because the detection limit for serotype F is quite high when only 106 copies/reaction can be detected. In fact, *C. botulinum* is one of the bacteria whose A-T ratio in the genome accounts for 70-72% [16-18], so primer design is difficult and the amplified target gene segment also has a high A-T ratio ranging from 66-72% for each serotype A, B, E, and F and has a direct impact on the results. To solve this problem, there are solutions such as using other Taq such as Phusion Plus DNA polymerase, continuing to optimize the annealing temperature, or other components in the PCR reaction [15]. In addition, the established multiplex PCR method can detect the presence of multiple genes encoding toxins of *C. botulinum* bacteria in food samples.

In the following experiments, the research team will compare the established multiplex PCR method for simultaneous detection of four genes encoding toxins A, B, E, and F of *Clostridium botulinum* bacteria with the PCR procedure of BAM Chapter 17: *Clostridium botulinum* on the detection limit of serotype F.

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