Vietnam Journal of Food Control
A Scientific Journal of National Institute for Food Control
Journal homepage: https://vjfc.nifc.gov.vn

Research Article

Detection of alpha-amanitin encoding gene utilizing real-time PCR technique

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(Received: 14 Jan 2024; Revised: 08 Apr 2024; Accepted: 09 Apr 2024)

Abstract

Given the limited taxonomy of toxic fungi as well as the large number of mushroom species, identification of mycotoxins in mushroom samples can accelerate information gathering and progress, while increasing Enhanced testing accuracy of testing. In that situation, it is necessary to research methods to identify toxin-carrying genes in mushrooms, to help monitor and classify poisonous mushrooms, disseminate and increase people's awareness of food safety. done faster and more accurately. The research team succeeded in developing a real-time PCR method to detect the toxin alpha-amanitin gene on mushrooms with a detection limit of 10^1 copies/µL. The study has fully verified the correct parameters, 100% accuracy, and specificity.

Keywords: *Alpha-amanitin, poisonous mushrooms, food poisoning, real-time PCR.*

1. INTRODUCTION

Recently, the study of poisonous mushrooms and their toxins that exist in nature has become increasingly popular. In China more than 180 species of poisonous mushrooms have been discovered, of which 30 are fatal [1]. In the United States there are more than 5000 species of mushrooms, including nearly 100 species of poisonous mushrooms [2].

Common toxins of poisonous mushrooms include amatoxin, muscarin toxin, coprin toxin and gastrointestinal disturbance toxin in green white palm mushrooms (Chlorophyllum molybdites). The majority of these toxins are detected in various mushroom genera, e.g. amatoxin in the mushroom genera Amanita, Galerina and Lepiota; while muscarin-containing fungi are commonly found in the genera Inocybe, Clitocybe and Omphalotus; and coprin toxin found in the mushroom genus Coprinus.

Poisonous mushroom containing amatoxin are often the main cause of death from mushroom poisoning in the world, which is the cause for 90 - 95% of deaths, for this reason,

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Doi https://doi.org/10.47866/2615-9252/vjfc.4259

mushrooms containing this toxin have been widely studied worldwide. Amatoxin consists of 8 types: α-amanitin, β-amanitin, γ-amanitin, ε-amanitin, proamanullin, amanin, amanullin, and amanullinic acid. Amatoxin is present in the entire fruit body of the mushroom (caps, blades, peduncles) and filamentous bodies (mushroom roots) [3].

Studies from abroad on symptoms of mushroom poisoning containing amatoxin show that common symptoms in poisoning patients include nausea, vomiting, abdominal cramps and repeated diarrhea. Symptoms appear 6 - 24 hours after ingestion of poisonous mushrooms. Then there is an interval of 1 - 3 days when the patient resolves abdominal pain, diarrhea, vomiting. However, at a period of 4 - 5 days after eating mushrooms, hepatic, renal failure may occur with typical symptoms such as jaundice, hemorrhage, decreased urination or anuria, coma and concomitant death may take place at this stage due to complications of liver failure, renal failure [4].

The presentation of amatoxin-containing mushroom poisoning in poisoning patients is usually not highly specific (common symptoms such as nausea, bowel movements, abdominal pain, vomiting, etc.), so in-depth studies of toxin detection are needed to differentiate amatoxin poisoning from cases of intestinal illness or food poisoning caused by other causes from The time of detecting a case of poisoning, in order to provide an effective treatment regimen quickly for patients, avoiding causing dangerous complications. In addition, in cases of amatoxin poisoning, studies have been shown that the amount of toxins in the patient's body does not correlate with the presentation of poisoning. More specifically, the concentration of amatoxin identified in blood or urine samples does not correlate with the stage of intoxication or the severity of the poisoning [5, 6]. For this reason, in clinical cases, qualitative amatoxin is the primary target of trials, primarily α-amanitin toxin [7]. With the main objective of confirming the presence of amatoxin, scientists have developed different methods for analyzing toxins on a variety of sample substrates, typical methods applied for amatoxin analysis can be mentioned as liquid chromatography [8, 9], Thin plate chromatography [10], ELISA [11] testing, electrophoresis [12, 13]. However, the above methods have their own strengths and weaknesses of each method, such as requiring investment in expensive equipment, long sample preparation time or limited on different sample platforms.

Especially where original mushroom specimens are collected, mushroom species identification may be a method that offers more promising results for clinical treatment [14]. Kotlowski and colleagues' research has successfully developed a traditional PCR method capable of identifying the *glyceraldehyde-3-phosphate dehydrogenase* (*gpd) gene fragment* from Amanita phalloides, but this test is not very effective when *gpd* is a low-copy- target, making the sensitivity of the experiment low-value and the authors do not offer an application for clinical cases. In 2008, Maeta et al. [15] developed a real-time PCR method to successfully detect four common poisonous mushroom species in Japan and also announced that their method could be applied on processed food samples, thereby expanding the sample platforms for PCR and real-time PCR tests to detect toxin-producing genes in fungi. Following that success, in 2010, Epis et al. [16] published their single-plex real-time

PCR experiment, which was capable of detecting the toxin-producing genes of poisonous fungi on different sample substrates (dried mushrooms, spaghetti with mushrooms, processed mushrooms, and gastric juice supplemented with dried mushrooms). Epis's study successfully identified *A. phalloides*, *Lepiota cristata*, *L. brunneoincarnata*, and *Inocybe asterospora*. The first three named species of fungi are responsible for the majority of mushroom poisonings in Italy. The primer sequence of the PCR reaction is based on the intervening transcribed sequence (ITS), which is widely used in mushroom classification research.

The process of amanitin biosynthesis in *Amanita* species is more complex and flexible than the amanitin biosynthesis pathway that occurs in *Galerina* mushrooms and *Lepiota* fungi. To date, 45 cyclic peptides have been recorded in lethal *Amanita* species, although some of these cyclic peptides, such as antamanide and CylA-D, are not known to be toxins [17]. Many studies have shown that the *Amanita* fungus produces more toxic peptides [17, 18]. Many genes encoding amatoxin are expressed at the transcriptional level, and the corresponding cyclic peptides, with or without post-translational modifications, have been detected by mass spectrometry [18]. However, three available sequenced *Galerina* genomes encode a single gene encoding amatoxin and *Galerina* mushrooms synthesize a single cyclic peptide, i.e., α-amanitin. γ-amanitin peptides are also synthesized by *Galerina*, and α-amanitin is derived from γ-amanitin by post-translational hydroxylation [18]. The presence of small amounts of β-amanitin in *G. marginata* mushrooms may be due to the chemical deamination of α-amanitin. Of the four *Lepiota* genomes sequenced, α-amanitin is the main metabolite of amanitin. Other small amanitins reported in *lepiota* (amanin, γ-amanitin, and amaninamide) are likely intermediates of α- or β-amanitin lacking post-translational hydroxylation(s) [19]. Current analysis of the representative genomes of *Amanita*, *Lepiota* and *Galerina* suggests that the gene encoding α-amanitin is shared by all amatoxin-producing species in these three families of fungi.

2. MATERIALS AND METHODS

2.1. Research subjects and materials

Mushroom samples collected from poisoning cases and from surveillance samples from 2019 to now in Son La, Ha Giang and Bac Kan provinces are sent to the National OSH Testing Institute.

2.2. Chemicals and standards

The chemicals used in this study include Luminaris HiGreen qPCR master mix, 2X (Thermo, USA), primer (IDT, Singapore), amanita exitialis alpha-amanitin (AMA) gene (code KC778585.1) (sequence published in Genbank) and proteinase K (Thermo).

2.3. Research methodology

2.3.1. Method of sample preparation and DNA extraction

Sample preparation: grind the mushroom flesh in liquid nitrogen using pestle & mortar for 3-5 times until very smooth.

DNA extraction uses the SEVAG method [20]. Prepare a 1% solution of β-mercaptor and a solution of SEVAG (Chloroform : Isoamylcohol 24:1, etc.) immediately before use. Add 660 - 700 µL Lysis buffer, 10 µL β-mercaptor. Vortex evenly, incubated at 65°C for 1h. Centrifuge the tube at 3400 rpm for 5 min at room temperature (RT) then transfer the top layer to a new tube (1). Add SEVAG mixture in a 1:1 ratio, vortex, and follow by centrifugation at 12,000 rpm for 10 min at RT. Transfer the upper phase to a new tube (2). Add 20 µL 3M NaOAC, fill the tube with isopropanol tightly close the lid then mix well by reversing the tube. Centrifuge the tube at 15,000 rpm for 2 min at RT. Remove supernatant. Add 300 µL EB and 1 µL to 100 mg/mL RNAse, and incubate at 65°C for 15 min. Add 250 µL 7.5 M ammonium acetate, centrifuge at 15,000 rpm for 5 min at RT, and transfer the upper phase to a new Eppendorf tube (3). After adding 750 µL of isopropanol, the Eppendorf tube was centrifuged at 15,000 rpm for 2 min, RT then removed the supernatant. Adding 1 mL EtOH 100%, vortex a few seconds the Eppendorf tube was centrifuged at 15,000 rpm for 2 min, RT, discard the supernatant. Add 1 mL of EtOH 70%, vortex, then centrifuge at 15,000 rpm for 2 min at RT, and discard the supernatant. Dry DNA. Re-dissolve DNA in 50 - 70 µL of TE buffer and store at 4°C. Check DNA quality with a Nanodrop meter.

2.3.2. Real-time PCR technique

**** Primer design***

The primers were designed based on the sequences of alpha-amanitin toxin gene fragment published on GenBank. After the research group find the Amanita exitialis alpha-amanitin gene, we utilize the primer 3 website for primer design (https://primer3.ut.ee/).

Primers are designed based on the following principles:

+ Primer has a length of 18 - 30 bases

+ GC content is about 40 - 60% and the best primer ending base is G or C

+ Avoid secondary structure (hairpin, self-dimer), repeating base C or G more than 3 times

+ Selecting the best template sequence region for primer design is about 300 - 1000 bases.

+ The bait should have a pairing temperature of 55 - 60°C

+ Check the sequence of the forward and reverse primers to ensure that there is no complementary pairing at the 3' end (avoid creating primer-dimer)

+ The primer pair is designed so that the melting temperature Tm of the forward and reverse primers is not too far apart.

**** Real-time PCR technique***

Ingredients for each reaction include Luminaris HiGreen qPCR master mix 2X, 10 µM downstream primer and 10 µM reverse primer, DNA template (concentration from 100 - 200 ng), total reaction volume is 20 µL. The thermal cycle is carried out in a sequence of 10 minutes at 95°C, 40 repeat cycles with 30 seconds at 95°C, 30 seconds at 59°C, 45 seconds at 72°C and lasting 10 minutes at 72°C, ending with a melting curve from 65 - 95°C.

The required validation parameters include detection limit (LOD), accuracy, ≥ requirement 90%, sensitivity, requirement ≥ 90%, specificity, requirement ≥ 90%.

**** Method limits of detection (LOD)***

Surveys at various DNA concentrations: 10^9,10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies. After finding the lowest density to be detected, we repeated PCR reaction 10 times. If more than 90% of the reactions are positive, it demonstrates a detection limit lower than or equal to the level of positive control material.

LOD = DNA concentration at which at least 90% of samples analyzed tested positive.

- Determination of accuracy: AC, specificity (SP), sensitivity (SE), positive deviation (PD) and negative deviation (ND):

To calculate the sensitivity, we do 10 times at different sample backgrounds or spike the positive sample into the sample background.

- Calculating results:

Calculate the result according to the following formulas in Table 1.

Table 1. *Calculate the result*

Result	**Sample testimony (+)**	**Sample testimony (-)**
The sample tested positive (+)	HCMC	FP
Sample with negative result (-)	FN	TN

Where: TP: True positive (Positive sample, positive result, analysis result positive)

FP: False positive (Negative sample, positive analysis result)

FN: False negative (Positive control sample, negative analysis result)

TN: True negative (Negative sample, negative analysis result)

n = TP + FP + FN + TN: Total results

Accuracy, specificity, sensitivity are calculated according to the following formulas [21]:

Accuracy $$AC = \frac{TP + TN}{N} \times 100$$

Specificity $$SP = \frac{TN}{TN + FP} \times 100$$

Sensitivity $$SE = \frac{TP}{TP + FN} \times 100$$

- Evaluation of results:

Parameters should be evaluated according to the following standards: Accuracy (AC) ≥ 90%; Specificity (SP) ≥ 90%; Sensitivity (SE) ≥ 90% .

3. RESULTS AND DISCUSSION

3.1. Results of method development

Primer design

The primer was based on the sequence of the alpha-amanitin toxin gene fragment published on GenBank: Amanita exitialis alpha-amanitin (AMA) gene (code

KC778585.1). Primer design for the detection of α-amanitin toxin-producing genes in sequence in Table 2.

Table 2. *Specific primer pair sequences of AMA genes*

Primer sequence	***Product Dimensions***	***Target genes***
AMA 8585-10F: GCGAGGCGTAAGGACAATTT AMA 8585-10R: GTCATGGCAGCGGACAAAC	99 bp	*BUT*

According to the designed primer pair (AMA 8585-10), the pairing temperature is determined at 59°C.

The primer pair was used for screening tests to detect the gene encoding alpha-amanitin toxin in 20 mushroom samples stored at the Laboratory of Food Microbiology and Genetically Modified Food, NIFC, and 10 mushroom samples collected from Son La and Ha Giang provinces in April and May 2023. The results of the real-time PCR reaction showed no detection of the gene encoding alpha-amanitin toxin in 30 mushroom samples. The results of this real-time PCR reaction can be explained by the fact that of all 30 mushroom samples tested, none of the mushroom samples belonging to the *Amanita*, *Lepiota*, or *Galerina* species were based on ITS sequence (Sanger identification conducted at the Laboratory of Food Microbiology and Genetically modified food). Further experiments were conducted using positive DNA sequences based on the specific sequence of the positive sequence used in the study (253 bp):

3’ATCTGGGGCATCGGTTGCAACCCGTGCGTCGGTGACGACGTCACTTCAG
TCCTCACTCGTGGCGAGGCGTAAGGACAATTTTTCTCCAATAATAATATGCACT
CATGCGCTGCGTATTAGCCTTTGCTAAATACCCCATCCGTTTGTCCGCTGCCAT
GACACGAAGGTATTGCCATCTCACTTCATATAAGGCAGTTGTCCTGACTCAGA
CGTAGGAGTGGGCGATACAAGTTGTGGACCATATCAGGCTTGG 5’

3.2. Results of method validation

In order to determine the validation parameters according to CAC/GL 74-2010, guidelines on performance criteria and validation of methods for detection, identification and quantification for specific DNA sequences and specific proteins in foods [21], the parameters to be determined include: Detection limit (LOD), accuracy (AC ≥ 90%), sensitivity (SP ≥ 90%), specificity (SE ≥ 90%).

3.2.1 Alpha-amanitin gene detection primer pair amplification test

Primer pairs designed according to the AMA gene segment were tested for DNA detection on *the AMA* positive control (KC778585.1). The results are shown in Figure 1.

Based on the fluorescence amplification curve results on the positive control gene segment, in the real-time PCR reaction there is a positive concentration of 10^7 copies/µL for a typical amplification curve with a Ct value of about 16 (Figure 1).

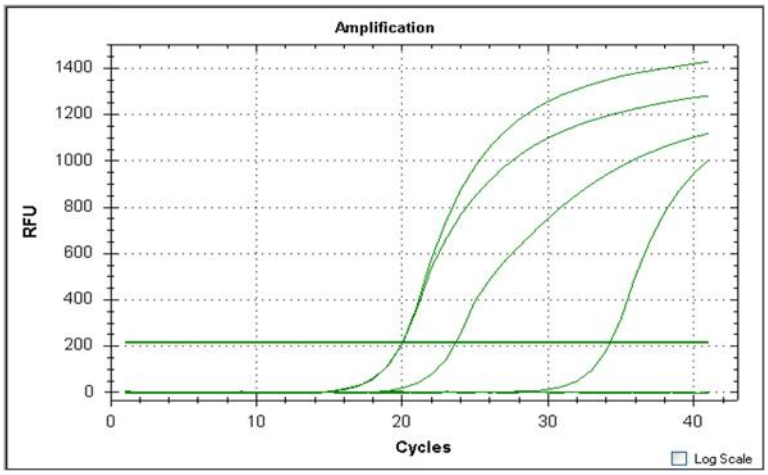

Figure 1. *The amplification path of the design primer pair on the AMA positive control gene segment (KC778585.1).*
(Ct primer pairs: 8585-1:16; 8585-10:16; 8585-7:19.5; 8585-8:30)

The results of the sequence of the product gene fragment of the PCR reaction showed that the designer primer multiplied the exact gene sequence for the α-amanitin toxin shown in Figure 2.

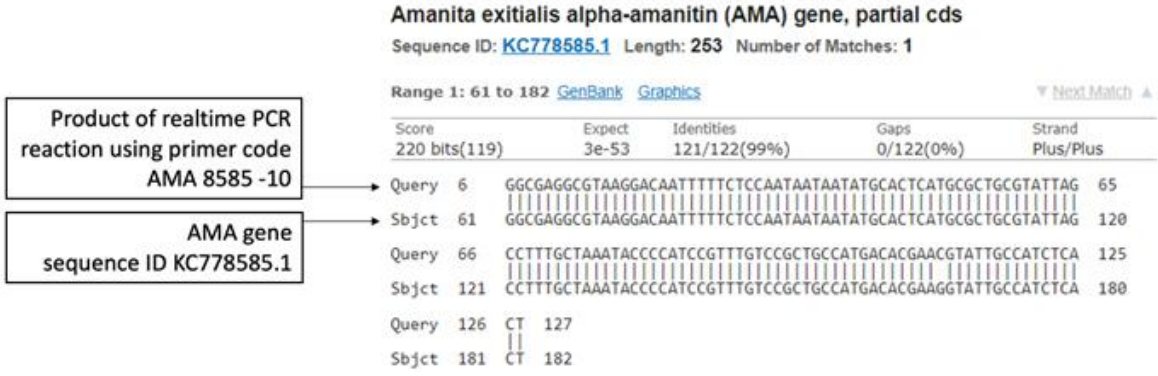

Figure 2. *Blast nucleotide sequencing results gene fragment product of Real-time PCR reaction*

The sequence of the DNA fragment multiplied in the real-time PCR reaction matches 100% with the sequence of the gene fragment encoding alpha amanitin toxin published in Genbank (ID: KC778585.1).

3.2.2. Validation of positive gene fragment

*** Limit of detection (LOD)**

The LOD of the method is calculated according to the lowest concentration of DNA in the sample, at which at least 90% of the sample is positive. To determine LOD, DNA from 1 µL DNA has a different number of copies: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies/µL used as molds in real-time PCR reaction using AMA gene-specific primer pairs. The obtained results are shown in Figure 3 and Table 3.

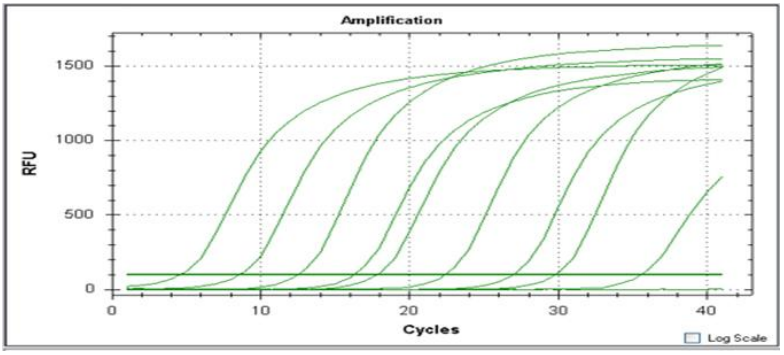

Figure 3. *Survey results limit detection of the method to samples with different DNA concentrations (10^1-10^9 copies/µL)*

Table 3. *Threshold cycle value (Ct) in real-time PCR reaction of samples with different DNA concentrations (10^1-10^9 copies/µL AMA gene)*

Samples	***Threshold cycle value (Ct)***		
	1st time	***2nd time***	***Average***
AMA (+) 101 copies/µL	4.62	4.78	4.70
AMA (+) 10^2 copies/µL	8.59	8.73	8.66
AMA (+) 10^3 copies/µL	12.48	12.60	12.54
AMA (+) 10^4 copies/µL	16.29	16.39	16.34
AMA (+) 10^5 copies/µL	17.86	18.01	17.94
AMA (+) 10^6 copies/µL	22.40	22.50	22.45
AMA (+) 10^7 copies/µL	27.04	27.12	27.08
AMA (+) 10^8 copies/µL	29.82	29.95	29.89
AMA (+) 10^9 copies/µL	35.55	35.67	35.61

The results of the detection limit survey showed that at DNA concentrations of 10^1 copies/µL, the Ct cycle threshold was approximately 35. These results indicated that DNA concentrations of 10^1 copies/µL were predicted to be the lowest concentrations at which this method could detect the presence of the toxin-producing gene alpha-amanitin. To confirm the detection limit, the reaction was repeated 10 times at a DNA concentration of 10^1 copies/µL, The results obtained are shown in Figure 4 and Table 4.

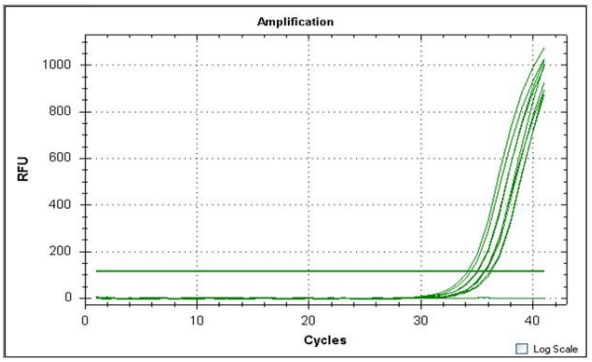

Figure 4. *The amplification path in 10 real-time PCR reaction repeats, each reaction contains 10^1 copies/µL of the AMA gene*

Table 4. *Threshold cycle values (Ct) in 10 real-time PCR reaction iterations, each containing 10^1 copies/µL of AMA gene*

Sample	***Threshold cycle value (Ct)***
AMA (+) 10^1 copies/µL – 1	35.79
AMA (+) 10^1 copies/µL – 2	35.63
AMA (+) 10^1 copies/µL - 3	35.32
AMA (+) 10^1 copies/µL - 4	35.49
AMA (+) 10^1 copies/µL - 5	35.03
AMA (+) 10^1 copies/µL - 6	34.14
AMA (+) 10^1 copies/µL - 7	35.96
AMA (+) 10^1 copies/µL - 8	35.67
AMA (+) 10^1 copies/µL - 9	35.91
AMA (+) 10^1 copies/µL - 10	35.54

A repeat of 10 tests at a concentration of 10^1 copies/µL of the AMA gene fragment showed a 100% positivity percentage. Therefore, the detection limit (LOD) of the alpha-amanitin toxin-producing gene detection method is 101 copies/µL.

**** Accuracy (AC), specificity (SP) and sensitivity (SE) of the method***

To determine the accuracy (AC), specificity (SP) and sensitivity (SE) parameters of the method, two groups of samples were included in real-time PCR testing: group 1 included samples of mushrooms supplemented with alpha-amanitin toxin-producing genes, group 2 included samples of mushrooms that did not belong to the Amanitin family and belonged to the Amanitin family but did not produce alpha-amanitin toxin. The real-time PCR test results of these two sample groups are shown in Figure 5 and Table 5.

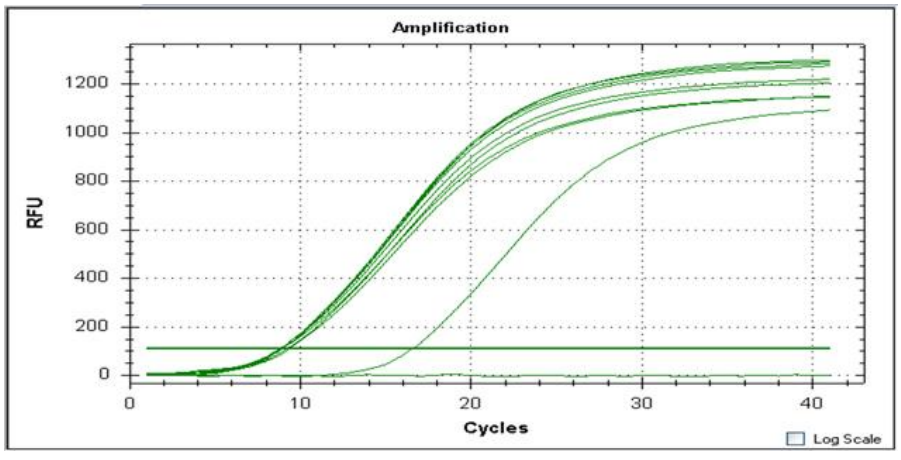

Figure 5. *Typical amplification lines of 2 sample groups (group 1: mushroom sample group with alpha-amanitin toxin-producing gene, group 2: mushroom sample group not in Amanitin family and no alpha-amanitin toxin-producing gene)*

Table 5. *Threshold cycle values (Ct) of the two sample groups*

No.	*Sample name*	*Ct*
Group 1: mushroom sample group spiked with α-amanitin toxin-producing gene		
1	AMA (070521-1)	8.79
2	AMA (070521-2)	8.97
3	AMA (070521-3)	8.84
4	AMA (070521-4)	9.21
5	AMA (220521-1)	8.90
6	AMA (220521-2)	8.94
7	AMA (220521-3)	8.93
8	AMA (190622-1)	9.38
9	AMA (190622-2)	16.55
10	AMA (190622-3)	9.25
Group 2: mushroom sample group that do not belong to the Amanita family and do not have the α-amanitin toxin-producing gene		
11	SL (070622-1)	ON
12	SL (070622-2)	ON
13	SL (070622-5)	ON
14	SL (250522-3)	ON
15	SL (250522-5)	ON
16	SL (250522-6)	ON
17	SL (250522-7)	ON
18	SL (220521-4)	ON
19	SL (220521-5)	ON
20	SL (220521-6)	ON

The results obtained in Figure 5 and Table 5 show that in group 1:10 different mushroom samples with alpha-amanitin toxin-producing gene at concentrations of 10^7 copies/µL all exhibited amplification curves at Ct threshold periods at about 8-9. In group 2, the control group of 10 mushroom samples that did not belong to the Amanitin family and did not have the alpha-amanitin toxin-producing gene all showed results No typical gain curve detected. From there, it shows that the method of detecting alpha-amanitin toxin-producing genes using real-time PCR method and AMA 8585-10 primer pairs specific to AMA toxin-producing *genes.* Accuracy (AC), specificity (SP) and sensitivity (SE) have also been calculated according to the formula given in the method section and have values of 100%.

3.3. Analysis on mushroom samples

Non-Amanita mushroom samples and alpha-amanitin non-toxin-producing mushroom samples were supplemented with 10^7 copies/µL. The results are shown in Figure 6 and Table 6.

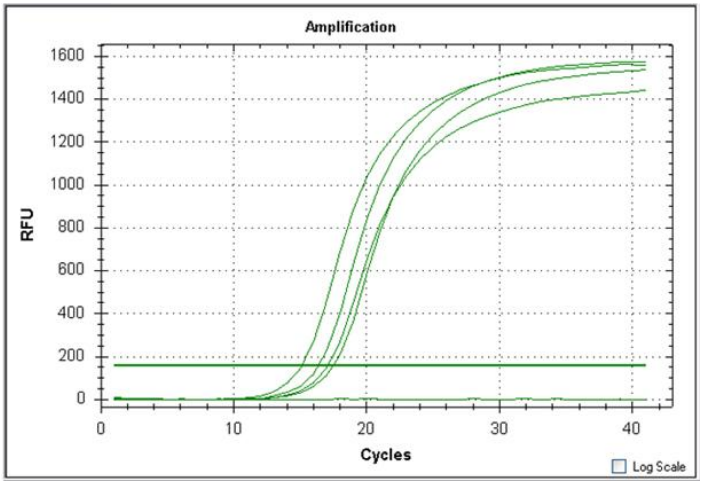

Figure 6. *The amplification curve of the alpha-amanintin toxin-producing gene on mushroom samples*

Table 6. *Threshold cycle (Ct) results of the test on mushroom samples at DNA concentrations of 10^7 copies/µL*

No.	*Sample name*	*Ct*
Group 1: mushroom sample group spiked with α-amanitin toxin-producing gene		
1	AMA (070521-1)	17.11
2	AMA (070521-2)	17.51
3	AMA (070521-3)	16.39
4	AMA (070521-4)	15.15
Group 2: mushroom sample group that do not belong to the Amanita family and do not have the α-amanitin toxin-producing gene		
1	SL (070622-1)	ON
2	SL (250522-3)	ON
3	SL (250522-7)	ON
4	SL (220521-4)	ON

The results above showed that the real-time PCR procedure is possible to specifically detect the alpha-amanitin toxin-producing gene, without crossing reaction with other mushroom species

3.4. Discussion

Our results indicate that the method of detecting the alpha-amanitin toxin-producing gene has yielded similar results with previous studies. Moreover, the assessment of specificity and sensitivity on 4 other mushroom samples showed that the designed primers and designed positive control were highly specific. However, to further confirmation, more research is needed to carry on with samples of alpha-amanitin toxin-producing mushroom.

4. CONCLUSION

A method for detecting alpha-amanitin toxin-producing genes on poisonous mushrooms has been developed completely, the method is validated according to CAC/GL 74-2010, validating methods in chemical & microbial analysis with accuracy, specificity, sensitivity parameters all achieved 100% and the limit of detection is 10^1 copies/µL. The results, evaluated on four mushroom samples, determined the presence of the toxin-producing gene alpha-amanitin in spiked samples and negative controls.

ACKNOWLEDGMENT

This research was funded by the National Institute for Food Control, project code NIFC.ĐTCS.2021.09. Thanks to all researchers at the Laboratory of Food Microbiology and Genetically Modified Food for participating and supporting the research project.

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