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Establishment of a Fluorescence Quantitative PCR Assay in Detection of *Listeria monocytogenes* based on Internal Reference

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ABSTRACT

Background: *Listeria monocytogenes* is the only pathogenic bacterium of *Listeria* that causes disease to humans. The Dairy products, meat and other foods have been proved to be the transmission carriers of *Listeria monocytogenes*, endangering human health. Therefore, it is necessary to establish a fast, sensitive and specific fluorescent quantitative PCR method for detecting *Listeria monocytogenes* in milk based on internal reference.

Methods: According to the genome sequence of *Listeria monocytogenes* published in Genbank, we screened specific target gene Hly gene, designed specific primers and probes, optimized the reaction system and added internal reference (IAC) to the system. This IAC was detected by TaqMan probes labeled with different fluorophore. 25-100 CFU/25 g artificially contaminated sample was added to evaluate the performance of reaction system.

Result: The assay was could be used reliably for detection of *Listeria monocytogenes*with a sensitivity of 10^{-3}ng/uL. For the 10-fold dilutions bacteria DNA extracted by cooking water,the lowest detection limit was 4×10^2 cfu/mL. But for the plasmid with hly, the lowest detection limit can reach 10^4 cfu/mL. The standard curve of hly and hly-IAC was established and the quantification was linear between Ct and template copy number (r^2= 0.999). While the initial sample amount of artificially contaminatedbacteria was 25 CFU/25 g (Fresh milk, milk powder, fermented milk), *Listeria monocytogenes* could be detected in 14 h. The Hly IAC fluorescent quantitative PCR detection method established in this study,and added internalreference tothe system, can accurately detect *Listeria monocytogenes*in fresh milk.At the same time, real- time monitoring of the PCR reaction process ensures the reliability of the results, which is suitable for rapid detection of large batches of samples.

Key words: Fluorescence quantitative PCR, Fresh milk, Internal reference, *Listeria monocytogenes*, Test method.

INTRODUCTION

Listeria monocytogenes is the only pathogenic bacterium of *Listeria* that causes disease to human beings and it is one of the four foodborne pathogens that are mainly detected (Zhu *et al*., 2016). Vegetables, dairy products, meat and other foods can be infected (Liu *et al*., 2004). In recent years, it has been found that the contamination rate of this bacterium in raw milk and ready to eat food is relatively high (Hao *et al*., 2022). The bacteria has strong vitality and can still grow in cold storage. There is a risk of infection of the bacteria in ready to eat food in bulk (Zhao *et al*., 2012; Su *et al*., 2021; Wu *et al*., 2011). Infection with this bacterium generally leads to intestinal infection and can also cause human meningitis, sepsis and other symptoms. The incidence rate of this disease is not high, but the mortality can reach 30%~70% (Thønnings *et al*., 2016; Rietberg *et al*., 2016). In Michigan, the United States, 14 people died from eating “hot dogs” and cooked meat contaminated with the fungus (Wang *et al*., 2018). Therefore, *Listeria monocytogenes* has potential harm to human health, which should be paid attention to.

The poor hygiene level of dairy farms is the primary factor of *Listeria monocytogenes* contamination in fresh milk. Poor quality silage, poor sanitation of cow feeding environment, inadequate cleaning and disinfection of dairy farms and poor personal hygiene of breeders can all lead to the contamination of *Listeria monocytogenes* in fresh milk.

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In recent years, with the rapid development of entry-exit food trade, people have higher and higher requirements for products, not only for good quality, but also for fast operation time. This requires the intermediate link of inspection and quarantine to shorten the time and issue fast, accurate and reliable inspection reports as soon as possible. The traditional detection method is mainly bacterial culture method. Through isolation, culture, biochemical reaction and other tests, it takes 3-7 days, some even longer, to determine Listeria before serotyping, which requires a lot of manpower

and material resources (Liu *et al*., 2022; Wang *et al*., 2022; Hu *et al*., 2022). The detection method established in this study is a real-time fluorescent quantitative PCR method based on internal reference. It takes 55 minutes to use ABI7500 and the IAC added in the reaction system can successfully indicate the false negative of the sample, realizing the goal of fast, simple, sensitive, specific and reliable results.

MATERIALS AND METHODS

Strain preservation

The experiment began in 2022 and the strains used were kept by the strain laboratory of Tangshan Normal University.

Design of primer and probe

Primer and probe were designed with Primer Express 3.0 software and blast tool was used for comparison. The amplified fragment size was 95 bp. The 5′ end of the probe was labeled FAM and the 3′ end was labeled BHQ1. Primer and probe sequences (Table 1).

Amplified internal reference

The amplified internal reference was constructed by using the complex primer method Siebert *et al*. (1993). The Nmi gene of BHK-21 was used to design and amplify the internal reference primer and probe. The internal reference probe was labeled with CY5 fluorescence signal. The internal reference fragment was connected to the vector pMD19-T, transformed into competent cells and sequenced for verification; The plasmid was extracted, the concentration was determined and stored at -20°C for standby and the bacterial solution was stored at -80°C.

Determination of nucleic acid concentration

Add 1 mL of bacterial culture solution cultured overnight into a 1.5 mL centrifuge tube, centrifuge it for 5 min with a high-speed centrifuge 8000-12000 rpm, discard the supernatant, collect the bacteria and then extract DNA according to the instructions of the kit mention the name of the kit and manufacturer of kit.measure the concentration and purity and store at -20°C for standby.

Optimization of the reaction system of hly fluorescence quantitative PCR

Using genomic DNA as template, the best combination is the combination of primers and probes with different concentrations, which can give the lowest Ct value and the highest fluorescence intensity. Real time PCR amplification system is 25 μL: 2×Taq PCR Master Mix 12.5 μL, Upstream and downstream primers (10 pmol·μL^{-1}) Set the final concentration as 0.2 pmol·μL^{-1}, 0.4 pmol·μL^{-1}, 0.6 pmol· μL^{-1}, 0.8 pmol·μL^{-1}, Probe (3 pmol·μL^{-1}) Set the final concentration as 0.12 pmol·μL^{-1}, 0.16 pmol·μL^{-1}, 0.18 pmol·μL^{-1}, 0.2 pmol·μL^{-1}, ROX II 0.5 uL,Template DNA 50~100 ng, The system was supplemented with ddH_2O. Amplification conditions: 95°C 30 s, 95°C 5 s, 60°C 35 s, 35 cycles. Collect fluorescence at 60°C.

Optimization of amplified internal parameters

The reaction system contains 10^6 copies of IAC. The PCR reaction conditions are the same as above. The optimal concentration is the minimum concentration of IAC probe that does not cause a significant increase in Ct value. On the basis of other conditions unchanged, Take the IAC of 10^2, 10^3, 10^4, 10^5 and 10^6 copies/uL respectively and select the smallest IAC that can give positive signals without affecting the optimal addition amount of target gene amplification.

Construction of standard curve

Determine the concentration of plasmid, make 10 times serial dilution and finally make 1 copy/uL to make standard template solution. Take 1 μL to make quantitative PCR, establish standard curve through different Ct values and concentrations and finally get the linear equation.

Specificity of Hly IAC fluorescence quantitative PCR

The specificity experiment was carried out for*Listeria monocytogenes* and other pathogenic bacteria. The selected strains were *Escherichia coli* (CICC21530O157:H7), *Staphylococcus aureus* (ATCC25923), *Salmonella* (CICC22956), *Vibrioparahae molyticus* (CICC21617), *Enterobacter sakazakii* (ATCC29544), *Shigella* (CICC21678), *Campylobacter jejuni* (ATCC33291), *Listeria monocytogenes* (ATCC19114). Under the conditions of no IAC and adding IAC, 1 ng of each genome DNA was taken for quantitative PCR reaction to verify the specificity of the reaction system.

Sensitivity experiment and quantitative range of Hly IAC fluorescent quantitative PCR

The genomic DNA of the standard strain with known concentration for 10 times gradient dilution and each dilution is taken as 1 μL. According to the above reaction conditions, the determined amount of IAC is added at the same time for PCR amplification. The lowest concentration gradient that generates the amplification curve is the sensitivity of the PCR detection system. Use sterilized PBS for 10 times

Table 1: Information about the primers and probe.

Assay	Primers and probes sequence 5′-3′	Amplicon size (bp)
Hly	F: 5′-CGCAAAAGATGAAGTTCAAATCA-3′	95
	R: 5′-CTCCTGGTGTTTCTCGATTAAAAGT-3′	
IAC	5′-FAM-CGACGGCAACCTCGGAGACTTACG-Eclipse-3′	123
	5′-CY5-CACTGCGCATCTAGTCCCGCTGAAC-BHQ3-3′	

gradient dilution, count the three dilution degrees 10^5, 10^6 and 10^7 on the plate and calculate the original colony count. At the same time, take 1 mL of bacterial solution for each dilution, extract DNA with water boiling method, dissolve DNA with 50 μL sterile water, take 1 μL as template and conduct PCR amplification.

Clinical sample test

After the *Listeria monocytogenes* is cultured at night, it is diluted 10 times series with normal saline, 10^{-6} and 10^{-7} dilutions are selected for plate counting to calculate the initial concentration of *Listeria monocytogenes* pure culture. After dilution, select 25, 50 and 100 CFU of bacteria to add to 25 g of fresh milk, milk powder and fermented milk samples respectively and then add 225 mL of Half Fraser broth. At the same time, make a blank control and carry out 37°C shaking culture. Select 1 mL of culture solution with 8 h, 14 h and 20 h of pre enrichment time for nucleic acid extraction, add 50 μL for dissolution and take 1 μL for real-time fluorescent PCR detection.

RESULTS AND DISCUSSION

Optimization of fluorescence quantitative PCR for hlyIAC

IAC probe concentration is 0.16 pmol·μL^{-1} is the minimum probe concentration that will not cause a significant increase in its Ct value and is determined as the optimal concentration. When the addition of IAC is 10^5 copies, both the CY_5 signal can be detected without affecting the detection of hly. Finally, the optimal amount of IAC10^5 was selected for every 25 μL PCR reaction system.

Establishment of plasmid DNA standard curve

As shown in Fig 1 (A, B), the slope of the standard curve established under the optimized conditions of hly fluorescent quantitative PCR was -3.229, the reaction efficiency was 1.040 and r^2 was 0.999, which close to 1. The standard curve established under the optimized conditions of the hly-IAC fluorescent quantitative PCR has a slope was -3.126, a reaction efficiency was 1.089 and r^2 was 0.999, which is close to 1. This indicates that the logarithm of template copy number at different concentrations has a good linear relationship with the Ct value and the addition concentration of IAC does not affect the accuracy of the PCR reaction. The detection ability of fluorescence quantification determined by r^2 of the hly-IAC fluorescence quantitative PCR and amplification efficiency E (Cheng *et al*., 2019). The Ct value of the hly-IAC standard curve established in this experiment has a good linear relationship with the logarithm of the copy number. When the number of cycles is 35, the lowest 10^3 copies/uL reaction system can be detected.

The specificity of hly-IA Cfluorescence quantitative PCR

The genome of *Listeria monocytogenes* standard strain was subjected to fluorescent quantitative PCR reaction with the genome DNA of other 6 bacteria. The results showed that *Listeria monocytogenes*was positive amplification, with obvious amplification curve and none of the others had amplification curve and all IAC had obvious amplification curve (Fig 2 and Fig 3), indicating that the established method not only had good specificity, but also could effectively indicate the role of false negative.

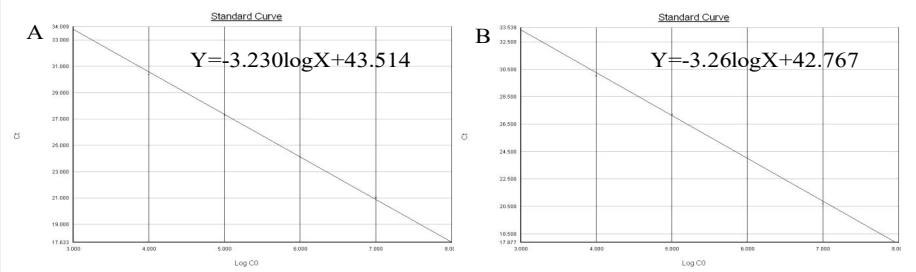
The sensitivity and quantitative experiment of hly-IAC fluorescence quantitative PCR

The *Listeria monocytogenes* genome was diluted with 10 times series gradient and different concentrations of DNA were taken as templates for real-time fluorescent quantitative PCR

Table 2: Real time fluorescence quantitative PCR detection results of nucleic acid extracted from pure culture by water boiling.

Bacterial liquid concentration (cfu/mL)	GE/uL	Ct
2×10^8	4×10^6	16.847/16.832
2×10^7	4×10^5	21.047/20.995
2×10^6	4×10^4	24.519/24.306
2×10^5	4×10^3	27.784/27.692
2×10^4	4×10^2	30.376/30.112
2×10^3	4×10^1	undet/undet
2×10^2	4×10^0	undet/undet

*DNA was extracted by 1 mL bacterial liquid boiling method and dissolved in 50 μL pure water. Assuming the extraction efficiency is 100%, the genome equivalent (GE)/μL was obtained.



A: Standard curve of the hly real-time fluorescence quantitative PCR method, the concentrations were10^8, 010^7, 010^6, 010^5, 010^4 10^3copies/uL; B: Standard curve of thehly-IAC real-time fluorescence quantitative PCR method, the concentrations were10^8, 010^7, 010^6, 010^5, 010^4, 010^3 copies/uL.

Fig 1: Standard curve of real-time fluorescent quantitative PCR for hly gene.

detection. As shown in Fig 4, no matter whether the corresponding IAC is added to the reaction system or not, there is no impact on the amplification of the target gene and the detection sensitivity has not changed. In 35 cycles, the sensitivity of target gene was 10^{-3} ng/uL. The DNA of each dilution of bacterial solution was extracted by boiling method (Table 2). Whether or not 10^5 copies of IAC was added to the reaction system, the hly IAC fluorescence quantitative PCR reaction was not affected and the lowest level was 10^4 cfu/mL.

Clinical sample test results

The test results show that (Table 3), For the hly-IAC real-time fluorescence quantitative PCR method established in this study, for the sample with the initial bacterial amount of 25 CFU/25 g, when the DNA template is extracted using the water washing and kit method, the sample can be enriched for 14 h and *Listeria monocytogenes* can be detected from the sample; When DNA was extracted by boiling method as a template for detection, it would take 20 h for enrichment to detect *Listeria monocytogenes* from the sample; IAC in the blank control and all samples were amplified, but the target gene in the blank control group was not amplified. The results were consistent with the parallel validation of traditional microbial culture methods, indicating that the Hly-IAC fluorescent quantitative PCR method established in this study has the advantages of short detection time, simple operation, high accuracy and good specificity.

Compared with the traditional bacterial culture method, the fluorescent quantitative PCR detection technology has the advantages of simple operation, high sensitivity, accurate results and good specificity and has been highly recognized and widely used in various scientific research fields (Wu *et al*., 2011; Guo *et al*., 2020). In this experiment, Hly target gene was selected to design primers and probes, IAC was added to the reaction system and the false negative was monitored during the reaction process through simultaneous amplification of target gene and IAC. In the actual detection

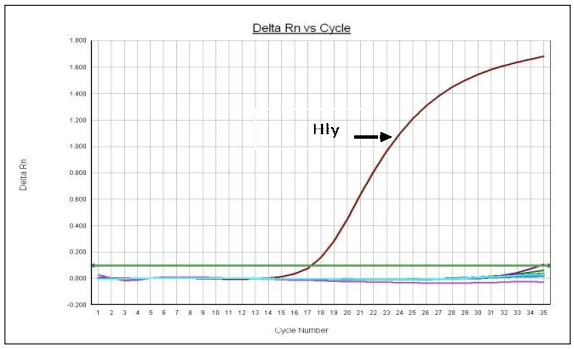


Fig 2: Specificity of the Real Time PCR assay for detection thehly nucleic acid.

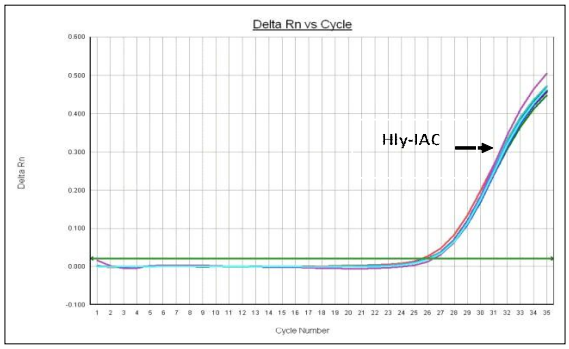


Fig 3: Specificity of the Real Time PCR assay for detection the Hly-IAC nucleic acid.

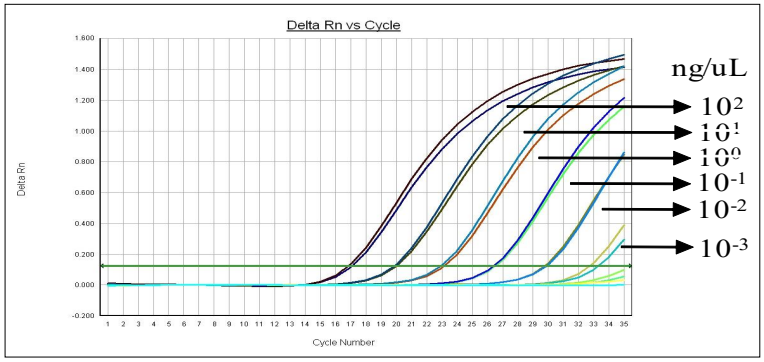


Fig 4: The sensitivity of hly-IAC fluorescence quantitative PCR.

Table 3: Detection of Listeria monocytogenes in dairy products by hly-IAC real-time fluorescent quantitative PCR.

Sample \ Time (h)	8			14			20		
CFU/25 g	25	50	100	25	25	100	25	50	100
Fresh milk	none	none	none	33.15	31.74	30.80	28.93	23.75	20.83
	none	none	none	none	none	32.24	31.05	26.26	24.25
Milk powder	none	none	none	32.97	31.85	29.36	25.35	23.02	18.13
	none	none	none	none	none	31.95	30.42	26.12	25.12
Fermented milk	none	none	none	31.54	30.12	29.08	28.37	25.80	24.67
	none	none	none	none	none	33.45	31.68	28.43	26.69

process, if the target gene detection is negative, but the IAC has no amplification signal, it is determined that the whole test is invalid and the PCR reaction is invalid, indicating that there may be reasons such as PCR inhibition reaction, which suggests that the reason needs to be found again and the detection must be repeated to ensure the accuracy and reliability of the detection results.

CONCLUSION

This study showed that the hly-IAC fluorescent quantitative PCR method established can detect the *Listeria monocytogenes* in dairy products rapidly within 55 min, which truly realizes the characteristics of fast, simple, sensitive and specific and does not need complex instruments and equipment. It can be applied to the on-site rapid detection of *Listeria monocytogenes* in food emergencies, which is of great significance to ensure food safety. It can not only rapidly detect pathogenic microorganisms, but also effectively prevent false negative results. It has high detection accuracy for *Listeria monocytogenes* in fresh milk and is suitable for rapid detection of *Listeria monocytogenes* in large quantities of fresh milk samples. In the follow-up work, we will continue to improve the detection and validation of the hly-IAC fluorescent quantitative PCR method for a large number of clinical samples, with a view to forming the corresponding national standard. At the same time, explore the multiple real-time RCR method of foodborne pathogens and the RCR method based on flow measurement chromatography strip detection.

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Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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