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Research on Rapid Detection of Salmonella and Listeria monocytogenes Using Colloidal Gold Immunoassay and PCR Methods

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ABSTRACT

This study used colloidal gold immunoassay strips and PCR (probe method) kit methods to detect Salmonella and Listeria monocytogenes. The accuracy and precision of the colloidal gold immunoassay strips and PCR (probe method) kit methods were validated using proficiency testing blind samples, and the consistency with the current national gold standard method was compared. The results of the two rapid detection methods were consistent with the results of the national standard method. This paper also analyzed the detection time and cost of the two rapid detection methods and the national standard method. The PCR (probe method) kit method takes less time for detection and can be used for high-throughput detection, with high sensitivity and low interference. It requires high equipment and personnel capabilities. The colloidal gold immunoassay strips method has the lowest detection cost, but its sensitivity is lower than that of the PCR method. It is easy to operate and requires low personnel and equipment requirements. Laboratories can choose the appropriate method based on their actual needs.

KEYWORDS

Salmonella; Listeria monocytogenes; Colloidal gold immunoassay strips; PCR (probe method) rapid detection kits; Detection methods; Accuracy

1. INTRODUCTION

Considering the current situation of food safety testing industry in China, food safety testing is mainly concentrated on illegal additives and antibiotic detection at present. With the strict supervision and monitoring of raw materials, the food safety problems caused by illegal additives are gradually decreasing. However, microorganisms have long existed in nature and cannot be avoided. China's food safety-related standards have clearly specified microorganisms and microbial pathogens as a single item for special detection. In the future, there will be higher requirements for detection speed and precision. Detection of microbial pathogens will be a more long-term and promising detection area.

Salmonellosis is one of the important zoonotic diseases in public health, which can cause serious systemic infectious diseases. The onset of the disease is usually slow, and the main symptoms are persistent fever, systemic toxic symptoms, and gastrointestinal symptoms. The most important complication is intestinal hemorrhage and perforation. Some symptoms are similar to sepsis, and complications such as pneumonia, toxic myocarditis, and toxic hepatitis may occur. Non-typhoidal Salmonella-induced food poisoning mainly manifests as gastrointestinal symptoms such as vomiting, diarrhea, and abdominal pain. Severe cases may cause dehydration and shock. According to the European Food Safety Agency (EFSA) statistics, more than 100,000 cases of Salmonellosis occur

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annually in Europe. According to the statistics of the US Food and Drug Administration (FDA), the number of Salmonellosis infections in the United States has increased to 12 million people per year, causing about 19,000 hospitalizations and 450 deaths. Food poisoning caused by Salmonella in the inland areas of China accounts for the largest proportion of bacterial food poisoning.

Listeria monocytogenes is a Gram-positive small bacillus. It is a highly pathogenic foodborne pathogen with a very high mortality rate. Patients infected with it will exhibit symptoms such as fever, muscle aches, nausea, and vomiting, leading to severe meningitis and sepsis. The mortality rate can reach 20% to 30%. Vulnerable populations include infants with immature or defective immune systems, pregnant women, patients with chronic diseases, and the elderly. Among them, pregnant women infected with Listeria monocytogenes can cause fetal infection, leading to miscarriage, abortion, and other serious consequences. Listeria monocytogenes can survive at temperatures ranging from 0°C to 45°C and can still grow and multiply in refrigerators, which is an important characteristic that distinguishes it from other foodborne pathogens. Global outbreaks of Listeria monocytogenes have occurred in the past, with the most serious being the contamination of meat products with ST6 of Listeria monocytogenes in South Africa from 2017 to 2018, causing 937 infections, half of which were pregnant women. In the report on "Attribution, Characteristics, and Surveillance of Listeriosis Caused by Listeria monocytogenes in Ready-to-Eat Foods" issued by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) in October 2022, meat products were ranked as the top cause of Listeria monocytogenes disease in ready-to-eat foods. In recent years, multiple outbreaks of Listeria monocytogenes infection caused by consuming ready-to-eat foods have occurred in China.

Currently, there are traditional culture methods, immunological detection techniques, molecular biology detection techniques, and recently developed techniques such as biosensor detection technology, nanotechnology, and aptamer-based detection technology for detecting Salmonella and Listeria monocytogenes. However, each method has its own advantages and disadvantages. In this study, the advantages and precision of the most commonly used immunological method and molecular biology method for rapid detection were studied and analyzed.

2. EXPERIMENT

2.1. Experimental Principle

2.1.1. The principle of rapid detection of pathogenic bacteria by colloidal gold immunoassay

The product uses nanogold particles as the marking indicator and applies the principle of "double antibody sandwich method". The specific pathogenic bacteria in the sample and the specific monoclonal antibody labeled with gold bind together, and the specific polyclonal antibody pre-coated on the T line position of the NC membrane also binds together. The aggregation of gold particles results in a clear red line. The presence of the T line can be determined by direct observation with the naked eye, and the sample is judged to contain the pathogenic bacteria if it appears.

2.1.2. Real-time Fluorescence Quantitative PCR Rapid Detection of Pathogenic Bacteria Principle

Based on Real Time PCR technology, by adding specific primers, fluorescent probes, and other reagents targeting the Salmonella-specific gene to the sample to be tested, an amplification reaction can be initiated. During the amplification process, the fluorescent probe binds to the target gene fragment, and the probe can be hydrolyzed by Taq enzyme to produce a fluorescent signal. At this time, the real-time fluorescence PCR instrument can recognize the fluorescent signal, and a real-time amplification curve can be drawn based on the change in its intensity, thereby determining whether the pathogenic bacteria are present.

2.2. Experimental Equipment

Electronic balance, ultra-clean workbench, biological safety cabinet, real-time fluorescence quantitative PCR instrument, biochemical incubator, autoclave, metal bath, water bath

2.3. Experimental Reagents and Culture Media

BPW Broth, SC, TTB, HE, BS, Sheffield Agar, LB1, LB2, PALCAM, Listeria Colour Plate, Blood Agar, Triple Sugar Iron Agar, Salmonella Biochemical Identification Kit, Monocytogenes Biochemical Identification Kit, RVS Broth, Physiological Saline, Monocytogenes Standard Strain, Salmonella Rapid Test Strips (Colloidal Gold), Monocytogenes Rapid Test Strips (Colloidal Gold), Salmonella Nucleic Acid Test Kit (PCR-Probe Method), Monocytogenes Nucleic Acid Test Kit (PCR-Probe Method)

2.4. Experimental Procedures

2.4.1. Rapid detection of Salmonella and Listeria monocytogenes by colloidal gold immunoassay:

Sample enrichment: For Salmonella detection, 25 g of sample was weighed into 225 mL PBW, mixed well, and incubated at 36°C for 18 hours. After gentle shaking, 0.1 mL of the culture liquid was transferred to 10 mL RVS culture medium and incubated at 42°C for 18 hours. For Listeria monocytogenes detection, 25 g of sample was weighed into 25 mL LB1, mixed well, and incubated at 30°C for 24 hours. After gentle shaking, 0.1 mL of the culture liquid was transferred to 10 mL LB2 culture medium and incubated at 30°C for 24 hours.

Detection: After gentle shaking, a certain amount of the enriched culture liquid was transferred to the sample well of the horizontal test strip using a disposable pipette. Boil water bath heating for 10 minutes. Return to normal temperature.Three drops of the sample culture liquid were then added to the sample well of the test strip. The strip was left at room temperature for 20 minutes, and the results were observed within 30 minutes.

2.4.2. Real-time Fluorescence Quantitative PCR Rapid Detection Method for Pathogenic Bacteria

Weigh 25 g of sample and add it to 225 mL of PBW. Mix well and incubate at 36 °C for 18 h for enrichment. After shaking the enrichment liquid, remove 40 μL of it and add it to the pre-packed lysis solution tube. Heat the mixture in a metal bath at 98 °C for 10 min to lyse the bacteria. Remove 5 μL of the sample to be tested and add it to the pre-packed PCR reaction tube (which contains 20 μL of reaction solution). The PCR reaction system is 25 μL. The fluorescence signal collection channel and quenching group selection, as well as the heating program, are carried out according to the instructions in the kit. The reaction is repeated 45 times.

2.4.3. Verification of the Accuracy of Lateral Flow Immunoassay and PCR (Probe Method) for the Detection of Salmonella and Listeria monocytogenes:

In this study, the blind samples were tested using two rapid detection methods, namely lateral flow immunoassay (immunological method) and PCR probe method (molecular biology method), with the help of proficiency testing blind samples. At the same time, the samples were tested according to the methods specified in GB 4789.4 and GB 4789.30, and the gold standard was set according to the national standard method[11.12]. The consistency of the results obtained by the two rapid detection methods was compared. The precision of the PCR (probe method) method was also verified at the same time, and three sets of parallel processing were selected for repeated testing, with each set of processing sample repeated twice. Single factor analysis of variance was used, and $p^* < 0.05$, indicating significant differences with statistical significance. Similarly, the three sets of parallel

processing samples were tested using the lateral flow immunoassay method, and the degree of color change of the colloidal gold should be consistent.

3. RESULTS

3.1. Accuracy Analysis

Analyzing the detection results, we found that the results of the colloidal gold test and PCR method for detection of Salmonella and Listeria monocytogenes were consistent with the results of the national standard method. The specific results are shown in Table 1 and Table 2.

Table 1. Consistency of Rapid Detection Method for Salmonella with National Standard Detection Method

Sample No.	Standard Method	Colloidal Gold Test Strip	PCR (Probe Method)
22-J278	Positive /25g	3+/3	Positive
22-S221	N.D/25 g	3-/3	N.D

Table 2. Consistency of Rapid Detection Method for Listeria monocytogenes with National Standard Detection Method

Sample No.	Standard Method	Colloidal Gold Test Strip	PCR (Probe Method)
22-N779	N.D /25 g	3-/3	N.D
22-P822	N.D /25 g	3-/3	N.D
Listeria monocytogenes	Positive /25mL	3+/3	Positive

3.2. Densitometry Analysis

From the detection results, we can see that in the positive reaction of the colloidal gold test strip, there are obvious red colloidal gold marks at both the t line and c line, and the depth of the parallel t line marks in the three groups of replicates is not significantly different. In the negative reaction, in addition to the c line, there are obvious red colloidal gold marks at all the t lines in the three groups of test strips. However, the detection cards may be affected by the background color of the sample solution. In addition, during the detection process of the positive group of Listeria monocytogenes, the t line is not as obvious as that of Salmonella. Please refer to Figure 1 and Figure 2.

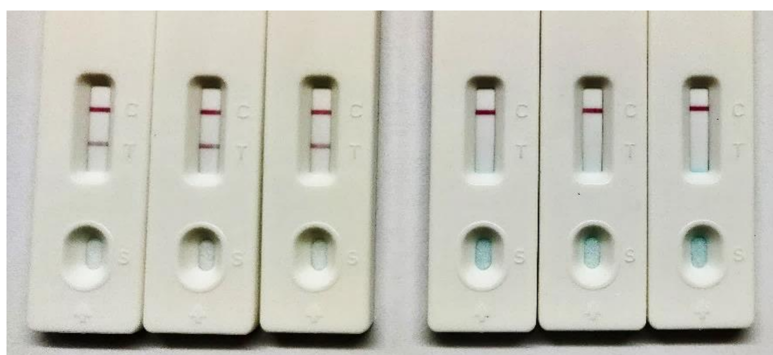

Figure 1. Results of Salmonella colloidal gold immunoassay test on samples

Note: The final report of proficiency testing indicates that Sample 22-J278 (the 3 on the left) is a lyophilized milk powder contaminated with Shigella flexneri and Escherichia coli interference, while Sample 22-S221 (the 3 on the right) is a lyophilized milk powder contaminated with Escherichia coli.

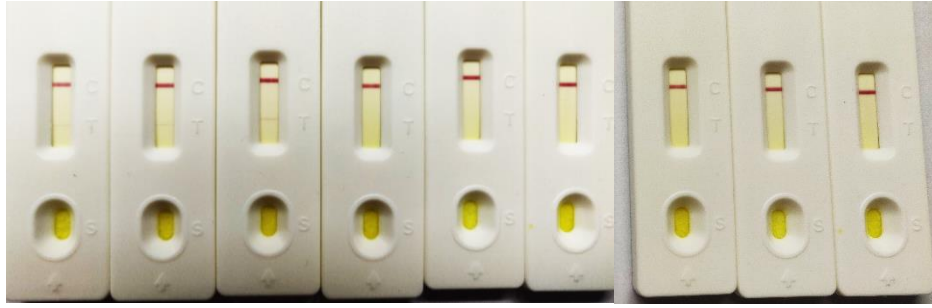

Figure 2. Results of Monocytophagus Listerii Lateral Flow Immunochromatographic Test on Samples

Note: The final report of proficiency testing indicates that Sample 22-N779 (middle 3) contains Listeria innocua spiked into dried milk powder, while Sample 22-P822 (right 3) contains Listeria iuanuii spiked into dried milk powder. The left 3 are diluted standard strains of Listeria monocytogenes.

For the PCR (probe-based) rapid detection method, we also designed 3 sets of parallel lysate extraction reagents and conducted PCR amplification reactions for each set of 2 replicates. After the specified 45-cycle reaction was completed, for the Salmonella samples, the ct values of the 3 sets of parallel samples were compared using a one-way ANOVA, with $p=0.93$, $p^*>0.05$, indicating no significant differences and no statistical significance. This indicates that there is no difference in ct values among the parallel groups and that the precision is good.

In samples of Listeria monocytogenes, no positive target bacteria were detected, and no typical amplification curve was observed in the expansion map within 45 cycles. To verify the precision, we used the positive MLP bacterial strain separately to lyse the 3 sets of parallel samples and compare the ct value differences between the groups using a one-way ANOVA. With $p=0.15$ and $p^*>0.05$, no significant differences were found, indicating no statistical significance. This indicates that there is no difference in ct values among the parallel groups and that the precision is good, as shown in Table 3, Table 4, and Figure 3 and Figure 4.

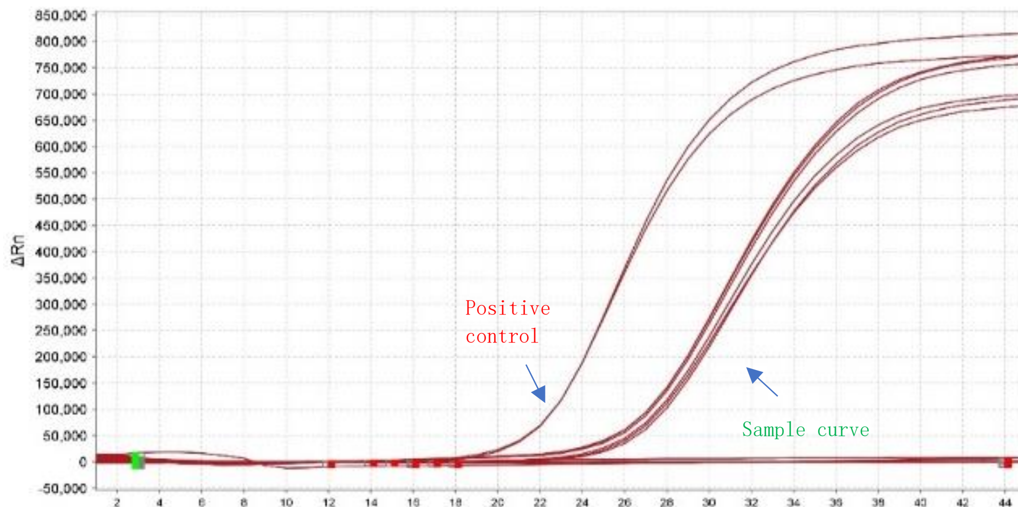

Figure 3. amplification curve of Salmonella by PCR (probe method)

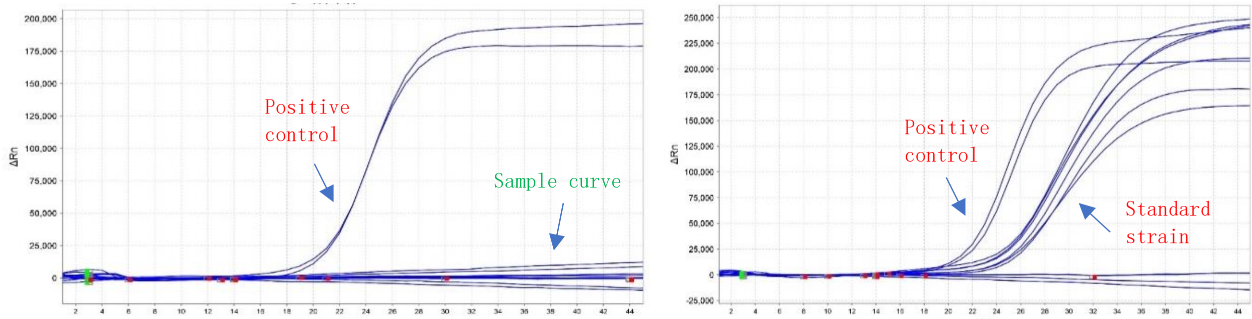

Figure 4. Amplification curve of Listeria monocytogenes by PCR (probe method)

Table 3. One-way ANOVA for PCR (probe method) detection results of Salmonella

Differences	SS	df	MS	F	P-value	F crit
between-group	0.028674	2	0.014337	0.07483	0.929579	9.552094
Within-group	0.574791	3	0.191597			
total	0.603465	5				

Table 4. One-way ANOVA for PCR (probe method) detection results of Listeria monocytogenes

Differences	SS	df	MS	F	P-value	F crit
between-group	1.637461	2	0.81873	3.828142	0.149373	9.552094
Within-group	0.641614	3	0.213872			
total	2.279076	5				

3.3. Time and Cost Analysis of Detection Methods

The time required for each detection method is different. The national standard method, which includes incubation, selection, isolation, microscopic examination, biochemical tests, and serological identification, usually takes 6-8 days. The colloidal gold immunochromatographic strip method is generally detected after the initial incubation and specific incubation, and directly on the plate, with a detection cycle of 36-48 hours. The PCR (probe method) generally involves initial incubation, lysis and extraction of nucleic acids, and then PCR amplification reaction, with a total detection cycle of 20-50 hours on average. The specific time required for each detection method is detailed in Table 5.

Table 5. Analysis of Testing Time

Testing Methods	Target Bacteria	Enrichment	Isolation	Biochemical Tests	Identification	Serological Tests	Inactivation and Extraction	Assembling and Amplification	Loading and Detection	Total Time
National Standard	Salmonella	36h	48h	48h	—	30min	—	—	—	6d
Colloidal gold		36h	—	—	—	—	10min	—	20min	37h
PCR		18h	—	—	—	—	10min	10min	90min	20h
National Standard	Listeria monocytogenes	48h	48h	96h	1h	—	—	—	—	8d
Colloidal gold		48h	—	—	—	—	10min	—	20min	49h
PCR		48h	—	—	—	—	10min	10min	90min	50h

Cost Analysis of Detection Methods: The cost of detection varies depending on the detection principle, and the consumables and reagents required for each method are also different. Generally, the national standard method uses culture medium, auxiliary reagents, culture plates, biochemical identification reagent kits, and serum diagnostic reagents, among others, which have a larger one-time investment but may be more suitable for continuous detection of large numbers of samples in a non-urgent situation. The cost of the colloidal gold immunochromatographic strip method is lower, with a usage cycle of one year. The cost of the PCR (probe method) reagent kit is higher than that of the colloidal gold method, with a usage cycle of six months, but because it has a small package size, purchasing a six-month supply at once results in a lower overall cost per sample, but a PCR instrument is required in the laboratory to perform the test.

4. DISCUSSION

Through this study, we found that colloidal gold immunoassay strips and PCR (probe method) kits can achieve consistent detection results with the national standard method for detecting Salmonella and Listeria monocytogenes. The colloidal gold immunoassay strips are susceptible to the influence of the background color of the sample being tested, as evidenced by the fact that the T line of the positive group of Listeria monocytogenes was less visibly colored in this experiment. The possible reasons for this are that the positive strain was diluted with its own bacterial culture supernatant, which may have a lower concentration, and the incubation time for enrichment was insufficient, the target bacterial concentration in the sample was not sufficient, and the color of the sample was not obvious. The PCR (probe method) is highly sensitive, so the amount of template in the reaction system is lower, and the influence of the background sample color is smaller.

In the PCR (probe method) kit method, good amplification curves can be obtained for both Salmonella and Listeria monocytogenes, indirectly verifying that the detection sensitivity of the colloidal gold immunoassay strips is lower than that of the PCR (probe method) kit method. When using colloidal gold immunoassay strips to detect Listeria monocytogenes, consideration should be given to extending the enrichment time appropriately.

From the analysis of detection time, both rapid detection methods take longer than the national standard method. The PCR (probe) kit method is slightly better than the colloidal gold immunochromatographic strip method, but considering the cost of the PCR (probe) kit itself and the situation of configuring real-time fluorescence quantitative PCR instruments in the laboratory, the overall cost of PCR (probe) kit method detection is higher. However, the PCR (probe) kit method can simultaneously handle a large number of sample throughput detection, and the detection sensitivity is higher. According to the instructions on the kit, the detection limit can reach 10 CFU, which is far superior to the identification limit of colloidal gold immunochromatographic strip (which is 10,000 CFU). From the operational aspect, the colloidal gold immunochromatographic strip method is simple to operate, and personnel do not need high technical ability, but the PCR method requires training in instrument operation, and certain requirements are needed for personnel's detection skills.

Both the lateral flow immunoassay test strips method and the PCR (probe) kit method can achieve consistency in precision and results as the gold standard national method for detection of Salmonella and Listeria monocytogenes. They are good methods to have on standby for rapid screening. Combining two or more detection techniques can appropriately compensate for the shortcomings of a single detection method, and combining traditional biochemical detection techniques, immunological techniques, and molecular biology techniques can improve the level of research and detection of pathogenic microorganisms. Whether a rapid detection method can be widely applied depends not only on its advantages of rapid detection, accurate results, and strong specificity, but also on its low cost and close proximity to actual application. With the close attention of people to food safety and the rapid detection requirements of food hygiene supervision, developing highly sensitive,

rapid and accurate, low-cost, and easy-to-use detection methods is an important research direction for detecting pathogenic microorganisms in food in the future.

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