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Development and validation of an insulated isothermal polymerase chain reaction assay for the rapid detection of *Mycoplasma synoviae*

Lucai Wang, Lijia Liu, Huanrong Zhang*

Department of Veterinary Medicine, College of Animal and Veterinary Sciences, Southwest Minzu University, Chengdu, China.

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Abstract

Mycoplasma synoviae, which causes the disease known as chicken synovitis, causes serious immunosuppression. We developed a rapid insulated isothermal polymerase chain reaction (iiPCR) assay for on-site detection of *M. synoviae* using a primer and probe set targeting the *variable lipoprotein and haemagglutinin* (*vlhA*) gene. In addition, the specificity, sensitivity, repeatability, and clinical detection of this method were evaluated. Our iiPCR assay detected *M. synoviae* clinical isolates and samples successfully and produced negative results on *Mycoplasma galliscepticum,* avian viral arthritis, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Corynebacterium*, indicating that the PCR reactions were specific. Additionally, our iiPCR assay detected the prepared positive standard plasmid diluted 10 times (1.00×10^{-1} - 1.00×10^{-10}) as a template. The undiluted positive plasmid was positive and double distilled water was negative indicating that the PCR reactions were sensitive, respectively. Finally, the *vlhA* positive standard plasmid with dilution multiple of 1.00×10^{-4} - 1.00×10^{-6} was repeatedly detected three times to evaluate the repeatability of the iiPCR method established in this experiment showing that the iiPCR of *M. synoviae* is repeatable. The established iiPCR was also used to detect 50 chicken joint enlargement samples. The thermostatic detection PCR established in this experiment was comparable to a reference real-time PCR (qPCR).

Introduction

Mycoplasma synoviae (MS) mainly infects chickens and turkeys, causing clinical symptoms such as synovitis, joint swelling and balloon inflammation. In addition, infection of the fallopian tubes of hens by MS strain can cause eggshell apex abnormality. The feed intake of sick chickens decreases and they grow slowly resulting in feed intake, weight gain and growth retardation, as well as economic losses in the breeding industry.[1,2] Transmission of MS is accomplished laterally via direct contact and respiratory aerosols, and vertically within eggs.[3] The MS can be mixed with a variety of viruses, leading to more severe conditions, such as avian influenza, Newcastle disease, and other viral diseases.[4-6] It is prevalent in China and abroad and its infection has been detected in many countries such as Great Britain, the Netherlands, Australia, Brazil and most provinces of China. The disease causes severe immunosuppression, making it difficult to be treated.[7-10]

Molecular assays can provide more rapid, sensitive, and specific detection of pathogens than conventional bacterial culture. Currently, the methods recommended by Office International Des Epizooties for MS detection are bacterial isolation, serological assays and polymerase chain reaction (PCR). The gold standard method for MS detection is a slow process and may take up to 28 days to be completed within the laboratory.[3] The PCR and real-time PCR have good sensitivity and specificity for detection. However, these traditional detection methods are insufficient to support the current epidemic inspection. Traditional PCR takes a long time from the beginning to the end of nucleic acid extraction. Compared to the traditional PCR, real-time PCR has higher detection sensitivity and specificity; but, the specific operation needs special experimental personnel and laboratories. Furthermore, these two PCR-based methods cannot achieve efficient on-site detection. Therefore, the application of PCR for the on-site detection of pathogens in the field is currently needed. Our study aims to establish a thermo-

***Correspondence:**

Huanrong Zhang. PhD
Department of Veterinary Medicine, College of Animal and Veterinary Sciences, Southwest Minzu University, Chengdu, China
E-mail: zhrong05@163.com

static detection PCR to improve the detection efficiency of the disease significantly. Fluorescent probe hydrolysis insulated isothermal PCR (iiPCR) methodology has been developed specifically for on-site applications. The iiPCR is a newly developed technology that applies thermal convection to drive PCR cycles in a specially designed portable device and capillary tubes. The POCKIT nucleic acid analyzer (GeneReach) is a commercial device designed to perform iiPCR assays in the field. The device detects the fluorescence signal generated through probe hydrolysis during PCR cycles, converts to signal-to-noise (S/N) ratios (signal after/signal before), and ultimately interprets as positive or negative results. The iiPCR assay has been used successfully to detect a variety of veterinary pathogens, including influenza A (H7N9),[11] classical swine fever,[12] foot-and-month disease,[13] duck hepatitis A,[14] and canine distemper viruses,[15] as well as *Salmonella*.[16] The variable lipoprotein and hemagglutinin (vlhA) as a major membrane protein helps MS adhere to host cells and evade immune responses. The vlhA protein is cleaved into two parts during translation. One of them is a conserved protein that helps to bind to the receptors on the host cell surface; thus, firmly attaching the MS to the cell to obtain the nutrients needed for autogenic growth. To apply iiPCR for MS detection, we first selected the more conservative *vlhA* gene as a target. Then, primers were designed to amplify the target fragment, iiPCR test was carried out, and its detection effect was evaluated.

Materials and Methods

Clinical Samples. *M. synoviae*, *Mycoplasma galliscepticum,* avian viral arthritis virus, chicken *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Corynebacterium* were all preserved in the laboratory. Fifty chicken joint enlargement clinical samples were collected from 10 chicken farms in Sichuan province, China, and the extracted DNA was stored at –20.00 ˚C.

Main reagents and instruments. Premix Ex Taq ™ (probe qPCR) enzyme, Takara Ex Taq enzyme and cloning vector pMD19-T were purchased from Takara Bio Inc., Beijing, China. Gel Recovery Kit and Plasmid Extraction Kit were purchased from Omega Bio-Tek, Guangzhou, China. Competent cell DH5α and ampicillin were purchased from Guangzhou Saiguo Biotech Co., Ltd., Guangzhou, China. Luria-Bertani culture liquid and solid medium were purchased from Hangzhou Microbiological reagent Company, Hangzhou, China. Pet NAD Nucleic Acid Extraction Kit, Uni-iiPCR Starter Kit and the iiPCR instrument, a portable pocket item Intelligent Nucleic Acid Analyzer, were purchased from Jinruihongjie (Xiamen) Biotechnology Co., Ltd., Xiamen, China.

Nucleic acid extraction. The 200 μL of MS culture was inoculated into 1.80 mL of modified Frey's liquid medium (Merck, Darmstadt, Germany) for expanded culture. The culture solution was collected when the color of the medium turned yellow, centrifuged at 12,000 RPM for 10 min, and the bacteria were collected. The DNA was extracted by the phenol-chloroform method, and the extracted DNA was placed at –20.00 ˚C.

Primer design. To develop an iiPCR assay for the detection of MS*,* we initially selected the *vlhA* gene as a target. In the results of whole-genome sequencing analysis of seven MS strains, the sequenced *vlhA* gene was found and compared with 11 *vlhA* sequences being included in GenBank on MEGA Software (version 7.0; Biodesign Institute, Tempe, USA) to find the conserved region. The amplification primers and probes were designed through Primer Premier (version 5.0; Premier, Vancouver, Canada). The amplified fragment was from 16 bp site to 134 bp site of *vlhA* gene, with a length of 119 bp. The forward primer (*vlhA* F [5'-ATTAAATTACTATTAGCA GCTAGTGC-3']), reverse primer (*vlhA* R [5'-AGTGGCCATTGCTCCTGCTGTTATA-3']), and probe (*vlhA* P [5'-TTTGGGTTTCCAGGTGTTG-3']) were listed in Table 1.

Preparation of MS positive standard. The extracted MS DNA was thawed from the refrigerator at – 20.00 ˚C and used as a template. The target fragment of *vlhA* was amplified by PCR with the above primers and then, the product was recovered by gel. After purification, the target fragment was connected with the cloning vector pMD19-T and transformed to the competent cell DH5α. The transformed colonies were identified by PCR and sent to Shanghai Biological Company (Shanghai, China) for sequencing. After sequencing, the colonies with the same sequence were expanded for culture, the plasmid was extracted as a positive standard, and the concentration was measured by nucleic acid and protein detector.

Optimization of reaction system. According to the instructions of Uni-iiPCR Starter Kit, the 50.00 μL system was used to optimize the probe concentration of 10.00 μM (0.05 μL ~ 0.50 μL), the concentration of upstream and downstream primers 10.00 μM (0.50 μL ~ 5.00 μL), Premix Ex Taq ™ enzyme 5.00 U μL^{-1} (23.00 μL ~ 27.00 μL) and MS template (0.50 μL ~ 5.00 μL). The ratio R1 of the fluorescence value read after the iiPCR reaction to the fluorescence value read before the reaction is the largest, which is the most suitable reaction system.

Table 1. The primers in relation to the DNA sequence of *variable lipoprotein and haemagglutinin* of *Mycoplasma synoviae* (accession No. AE017245).

Primer name	Primer sequence	Amplified fragment (bp)
MS F16	ATTAAATTACTATTAGCAGCTAGTGC	
MS P42	AGTGGCCATTGCTCCTGCTGTTATA	119
MS R134	TTTGGGTTTCCAGGTGTTG	

Analytical sensitivity of MS iiPCR assay. Ten-fold serial dilutions of the genomic DNA were prepared and then tested using the iiPCR assay. The prepared positive standard plasmid was diluted 10 times (1.00×10^{-1} - 1.00×10^{-10}) as a template; the undiluted positive plasmid was positive and double distilled water (ddH_2O) was negative. The established iiPCR method was used for detection. The copy number of vlhA positive plasmid was 2.53×10^{10} copies μL^{-1}.

Analytical specificity of MS iiPCR assay. The optimized iiPCR was used to detect MS, *M. galliscepticum*, avian viral arthritis virus, chicken *E. coli*, *Salmonella*, *S. aureus* and *Corynebacterium*. The positive plasmid prepared above was positive and ddH_2O was negative. The reaction products were added with 6X loading buffer and detected by 2.00% agarose gel electrophoresis to confirm the specificity of the method.

Analytical stability of MS iiPCR assay. The iiPCR established in this experiment detected the vlhA positive standard plasmid with a dilution ratio of $1.00 \times 10^{-4} \sim 1.00 \times 10^{-6}$. The experiment was repeated three times to evaluate its stability.

Clinical application of MS iiPCR assay. To validate the clinical application of our iiPCR assay, the DNA extracted from 50 samples of chicken joint enlargement was tested using both the iiPCR and real-time PCR assays. The reference MS real-time PCR reaction system is as follows. The 25.00 µL reaction mixture contained 12.50 µL SYBR Premix^{EX} Taq, 9.50 µL ddH_2O, 0.50 µL Primer F2 (5'-GAGAAGCAAAATAGTGATATC-3'), 0.50 µL Primer R2 (5'-TCGTCTCCGAAGTTAACAA-3') and 0.50 µL templete. The cycling program included one cycle at 95.00 ˚C for 4 min, followed by 30 cycles of 95.00 ˚C for 40 sec, 56.00 ˚C for 30 sec, and 72.00 ˚C for 40 sec. The amplicon was 208 base pairs long.

Results

Optimization of reaction system. The optimal iiPCR system established in this experiment was 0.35 µL probe, and 2.50 µL upstream and downstream primers. The premix EX Taq™ enzyme was 25.00 µL, the DNA template was 2.00 µL, and the ddH_2O was 17.65 µL. The fluorescence ratio R1 of the system after reaction and before the reaction was 2.80, which is the maximum.

Analytical sensitivity of MS iiPCR assay. The prepared positive standard plasmid was diluted 10 times (1.00×10^{-1} - 1.00×10^{-10}) as a template, the undiluted positive plasmid was positive and ddH2O was negative. The established iiPCR method was used for detection. The copy number of vlhA positive plasmid was 2.53×10^{10} copies μL^{-1}. The results showed that the detection limit of this method is 25.30 copies μL^{-1} (Fig. 1).

Analytical specificity of MS iiPCR assay. The iiPCR established in this experiment was negative for *M. galliscepticum*, avian viral arthritis virus, chicken *E. coli*, *Salmonella*, *S. aureus* and *Corynebacterium*, and positive for MS DNA, as shown in Figure 2. The 2.00% gel electrophoresis results were consistent with the above results, indicating that the established iiPCR has a good specificity (Fig. 2).

Analytical stability of MS iiPCR assay. The results of three repeated tests within and between batches were consistent, indicating that the established iiPCR detection method has a good stability.

Clinical application of MS iiPCR assay. In this study, iiPCR assay was positive for 18 of 50 (36.00%) clinical samples. The real-time PCR assay was positive for 18 of 50 (36.00%) samples (Table 2). All real-time PCR positive samples were also detected successfully by our iiPCR assay. These results demonstrated that the iiPCR assay provides a better on-site detection capability than real-time PCR assay for clinical samples and it takes less time.

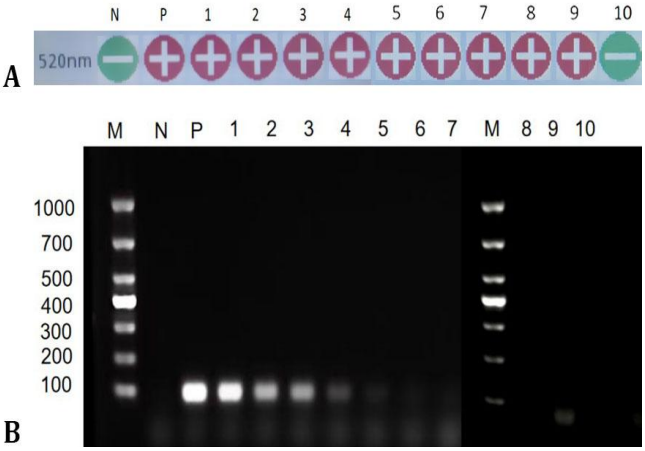

Fig. 1. A) Sensitivity results of insulated isothermal polymerase chain reaction (iiPCR). N means negative control, P means *Mycoplasma synoviae* (MS) variable lipoprotein and haemagglutinin (vlhA) positive plasmid as a positive control, and the instrument screen in iiPCR displayed No. 1 to 10 as 2.32×10^9 to 2.32×10^0 copies μL^{-1} in 520 nm; No. 1 to 9 show positive and No. 10 shows negative; **B)** Agarose gel electrophoresis of products from *vlhA* PCRs amplified from MS. Lane M: Molecular weight marker DL1000, Lane N: Negative control, Lane P: MS positive control, Lane 1: 2.32×10^9, Lane 2: 2.32×10^8, Lane 3: 2.32×10^7, Lane 4: 2.32×10^6, Lane 5: 2.32×10^5, Lane 6: 2.32×10^4, Lane 7: 2.32×10^3, Lane 8: 2.32×10^2, Lane 9: 2.32×10^1 and Lane 10: 2.32×10^0.

Table 2. Detection of *Mycoplasma synoviae* by insulated isothermal polymerase chain reaction (iiPCR) and real-time PCR testing clinical samples from chickens suspected of *M. synoviae* infection.

No.	Target gene	Test results of samples	Methods
1	*vlhA*	36.00% (18/50)	iiPCR
2	*vlhA*	36.00% (18/50)	real-time PCR

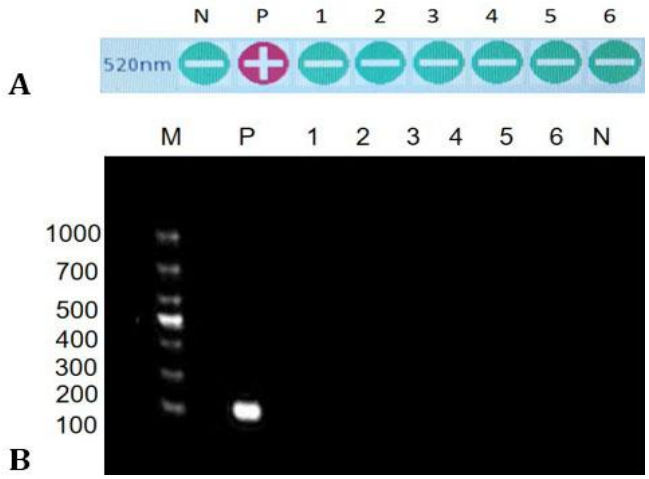

Fig. 2. A) Specificity results of insulated isothermal polymerase chain reaction (iiPCR). N means negative control, P means positive control, and No. 1 to 6 in the screen are *Mycoplasma galliscepticum*, avian reovirus, *Escherichia coli*, *Salmonella*, *Staphylococcus aureus* and *Corynebacterium*, respectively; The screen showed a positive control that was positive, and showed others negative; **B)** Agarose gel electrophoresis of products from *variable lipoprotein and haemagglutinin* PCR amplified from the control of *Mycoplasma synoviae* positive and the control of other strains. Lane 1: *M. galliscepticum*, Lane 2: Avian reovirus, Lane 3: *E. coli*, Lane 4: *Salmonella*, Lane 5: *S. aureus* and Lane 6: *Corynebacterium*. The results being shown through electrophoresis are consistent with iiPCR, indicating that iiPCR has a good specificity.

Discussion

Based on iiPCR technology, a rapid, sensitive and on-site method for detecting MS was established, which not only shortened the detection time, but also improved the detection efficiency. Due to the slow *in vitro* growth of *Mycoplasm*a and relatively difficult culture, the traditional detection methods for MS, such as isolation, culture and identification, cannot be used as routine clinical detection methods because of their high cost. The real-time PCR requires professional technicians and takes a long time. Therefore, it is necessary to detect the results in time. The iiPCR method established in this study can meet this requirement.

The iiPCR is a fluorescent PCR amplification technology based on the principle of thermal convection. The hand held POCKIT™ small fluorescent PCR instrument was developed based on this principle. The PCR amplification and detection technology of POCKIT™ system based on TaqMan probe promotes the specificity and sensitivity of MS iiPCR detection in this test. The design of this system can be used for on-site detection. The POCKIT™ instrument is small and can be equipped with a battery for use at any time. The system only needs to set up a few assembly steps before starting the reaction. After the system preparation, fluorescence value selection, and pressing one key to start, the automatic reaction takes only 58 min. The results are directly displayed on the LCD screen, and the results can be read directly, showing "+" and "–"; "+" is positive and "-" is negative. Eight reaction systems can be carried out at the same time, and it is suitable for the on-site rapid detection. The Pocket™ device collects fluorescence signals before and after reaction to calculate the S/N ratio.[17] The detection technology has been widely used in rapid pathogen detection.[13,18,19]

Specificity is the key of molecular detection technology and is very important for the accurate diagnosis of diseases. The selection of target genes and design of primers and probes are the premises to ensure the specificity of iiPCR method. The *vlhA* gene encodes a membrane protein of MS,[20] in which hemagglutinin coding sequence has strong specificity and is one of the hot genes in molecular diagnosis.[21] In this study, highly conserved *vlhA* gene was selected to design primers and probes, and its specificity was preliminarily analyzed by BLAST tool. The specificity test showed that this method only produces positive results for MS, but does not amplify other six pathogens, showing negative results, indicating that the established iiPCR has a strong specificity. In addition, no sequence with significant homology with MS *vlhA* gene was found in the nucleotide BLAST analysis of GenBank database. It showed that the established MS iiPCR detection is unlikely to have cross reactivity with other species, which can ensure the reliability of detecting the pathogen. The sensitivity evaluation was carried out by diluting the positive plasmid with a 10 fold gradient. The results showed that the lower limit of iiPCR detection established was 25.30 copies per µL templates, and the higher limit of the iiPCR detection was 29.00 copies per µL templates established by Hung *et al*., indicating that our iiPCR was also sensitive.[3] In order to cooperate with the field application, the reaction system was also optimized.

In the process of pathogen detection, nucleic acid extraction is a necessary step of PCR detection, and the rapid nucleic acid extraction technology is a vital link to shorten the PCR detection time.[22] The traditional phenol chloroform method and DNA Extraction Kit need many links such as high-speed freezing centrifugation, water bath and ice bath. They can only be carried out in the laboratory. The extraction step takes much time in the detection process. In order to meet the needs of on-site PCR detection, Pet NAD Nucleic Acid Extraction Kit was used in this study. The extraction method can be completed within 15 min, shortening extraction time of the detection process. Moreover, the iiPCR method established in this study, combined with the nucleic acid extraction kit, can be completed within 2 hr from nucleic acid extraction to report the detection results, significantly saving the detection time, having simple operation and reliable operation results. It can meet the needs of MS *in situ* detection.

In conclusion, a method based on thermostatic PCR for the detection of MS was established in this study. The preliminary application of the method proved that the method has the characteristics of good specificity, high sensitivity and good stability. Furthermore, the method was combined with Pet NAD to quickly extract template DNA and Pocket™ by designing specific primers and probes; the hand held nucleic acid analyzer can realize the on-site rapid detection of MS.

The prevention and control of MS is a long-term and systematic work, in which the purification of breeding chickens is fundamental, and the premise of prevention and control is quick and accurate detection. The method established in this paper provides a good detection method for preventing the MS infection.

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Conflict of interest

The authors declare that there is no conflict of interest.

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