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Development and validation of an insulated isothermal PCR assay for the rapid detection of *Pasteurella multocida*

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ABSTRACT

Pasteurella multocida, a ubiquitous opportunistic pathogen, is renowned for causing diverse respiratory tract diseases in animals. In the quest for on-site detection solutions, we developed a rapid insulated isothermal PCR (iiPCR) assay, targeting the *sodA* gene specific to *Pasteurella multocida*. This assay demonstrated remarkable specificity by successfully detecting clinical isolates of *Pasteurella multocida* types A, B, and D, while maintaining negative results for various other pathogens. Its robust sensitivity was evident in detecting positive strain DNA (2.03 copies/μL) and colonies (1.36×10^3 CFU/mL) as templates, with positive results for undiluted positive samples. The iiPCR method showed exceptional repeatability in triplicate detections of various diluted positive strains. In practical testing, encompassing 188 clinical nasal swab samples from goats, the iiPCR assay performed comparably to a reference PCR, underscoring its accuracy and reliability for on-site *Pasteurella multocida* detection in veterinary diagnostics.

Key words: insulated isothermal PCR (iiPCR); *Pasteurella multocida*; *sodA*; on-site detection; rapid detection

Introduction

Pasteurella multocida, recognized as a zoonotic pathogen with potential cross-species transmission, poses a substantial risk to poultry, livestock, wild animals, and even humans (STORZ et al., 2000; SMITH et al., 2021). Commonly residing in the oral, digestive, and respiratory tracts of animals as part of their normal microbiota, this pathogen, though generally non-pathogenic, can cause diseases under specific conditions (PENG et al., 2019; KUBATZKY, 2022). The clinical symptoms of goat pasteurellosis include high fever, pneumonia, and extensive internal organ bleeding, presenting significant challenges in both global livestock production and human health (MOGILNER and KATZ, 2019).

Despite advancements in molecular assays, that outperform traditional bacterial cultures in speed, sensitivity, and specificity, current *Pasteurella multocida* detection methods, such as bacterial isolation, agarose gel precipitation tests, and PCR, still rely heavily on laboratory testing. Traditional PCR, however, is time-consuming, limiting its application in rapid epidemic inspections and on-site detection, necessitating the development of on-site detection-compatible PCR methods (UJVÁRI et al., 2022; ABATE and FENTIE KASSA, 2023).

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An innovative solution has been found in Insulated Isothermal PCR (iiPCR), a thermal convection-driven PCR detection technology conducted within a specially designed portable device and capillary tubes (TSAI et al., 2012). The Pockit nucleic acid analyzer (GeneReach), a commercial instrument tailored for on-site iiPCR detection, exhibits promising capabilities, enabling the completion of iiPCR or reverse transcription-iiPCR reactions within just one hour, along with fluorescence signal detection and data interpretation. This technology has demonstrated success in the on-site detection of various veterinary pathogens (TSEN et al., 2013; CHUA et al., 2016; LUNG et al., 2016; AMBAGALA et al., 2017). The *sodA* gene, universally found in *Pasteurella* and related species, emerges as a superior target due to its discriminatory capability over the 16S rRNA gene (GAUTIER et al., 2005), so when employing iiPCR for *Pasteurella multocida* detection, the conservative *sodA* gene was selected as the target. Primers were designed to amplify the target fragment, and iiPCR tests were conducted to evaluate its efficacy. The results highlight the newly developed iiPCR's strong specificity, high sensitivity, stability, and the absence of cross-reactivity with other bacteria, making it a novel and effective technical approach for *Pasteurella multocida* detection.

Materials and methods

The nucleic acids of bacterial strains, including *Pasteurella multocida* types A, B, and D, *Mannheimia haemolytica*, *Mannheimia glucosida*, *Mannheimia ruminanlis*, *Mycoplasma ovipneumoniae*, *Mycoplasma mycoides, Staphylococcus*, *Salmonella*, *Escherichia coli*, and *Corynebacterium pseudotuberculosis*, are preserved at the Key Laboratory of Veterinary Medicine of Southwest Minzu University.

One hundred and eighty-eight nasal swab samples were collected from a goat farm in Sichuan Province in September 2022.

*Main reagents and instruments***.** The Premix Ex Taq (Probe qPCR) and DNA Marker were procured from TaKaRa. The Pet NAD nucleic acid extraction kit, Uni iiPCR starter kit, the Insulated Isothermal PCR instrument, and a portable pocket item Intelligent nucleic acid analyzer were purchased from Jinruihongjie (Xiamen) Biotechnology Co., Ltd. The pMD19-T cloning vector was procured from TaKaRa. The DNA Gel Extraction Kit D2500, DH5α competent cells, and plasmid extraction kits were sourced from OMEGA, Bao Biological Engineering (Dalian) Co., Ltd., and TaKaRa, respectively. Additionally, equipment including a gel imaging system (Shanghai TianNeng Technology Co., Ltd., Shanghai, China) and a fluorescent quantitative PCR instrument (BIO-RAD, Hangzhou, China) were utilized.

*Nucleic acid extraction***.** DNA extraction from the laboratory-preserved strains was carried out using the phenol-chloroform method (BARNETT and LARSON, 2012). The extracted nucleic acid was stored at -20°C. For on-site applicability, nucleic acid extraction from clinical samples was performed using the commercial PetNAD nucleic acid extraction kit. The extraction procedure involved diluting clinical samples directly with PBS, followed by thorough mixing and centrifugation. Subsequently, 200 μL was transferred to a 1.5 mL centrifuge tube. The PetNAD nucleic acid extraction kit's protocol was then followed, extracting total nucleic acids, including DNA and RNA, from the centrifuge column. The extracted material was stored at -20°C for future use, as per the PetNAD nucleic acid extraction kit instructions.

Primer design. To develop an iiPCR assay for the detection of *Pasteurella multocida,* we initially selected the *sodA* gene as the target. The amplification primers and probes were designed through Beacon Designer. The amplified fragment was from the 16 bp site to the 134 bp site of the *sodA* gene, with a length of 119 bp. The forward primer was *sodA* F (5'-GGGCTTGTCGGTAGTCTTT-3'), reverse primer: sodA R (5'-CGGCAAATAACAATAAGCT GAGTA-3'), and probe: sodA P (5'-CGGCGCAAC TGATTGGACGTTATT-3').

Preparation of Pasteurella multocida positive standard. The *Pasteurella multocida* positive standard was prepared using the extracted nucleic acid samples as templates. A 119bp fragment of the *sodA* gene of *Pasteurella multocida* was

amplified with specific primers. The PCR product was identified by 2% agarose gel electrophoresis, and the target fragment was recovered using a gel recovery kit. Subsequently, the fragment was cloned into the pMD-19T vector and transformed into Escherichia coli DH5α competent cells. Positive clones were selected in LB liquid medium containing ampicillin, and cultured at 37°C for 8 hours. Recombinant plasmids were extracted using a plasmid extraction kit, and the correctly sequenced positive plasmid was used as the positive standard.

Optimization of reaction system. Optimization of the reaction system was conducted following the Uni-iiPCR Starter Kit instructions: A 50 μL system was employed to optimize the probe concentration (10 μ mol/μL, 0.05 μL to 0.5 μL), the concentration of upstream and downstream primers (10 μ mol/μL, 0.5 μL to 5 μL), Premix Ex Taq™ Enzyme (5 u/μL, 23 μL to 27 μL), and *Pasteurella multocida* template (0.5 μL to 5 μL). The ratio R1 of the fluorescence value read after the iiPCR reaction to the fluorescence value read before the reaction reached its maximum, indicating that this was the most suitable reaction system.

Analytical specificity of the Pasteurella multocida insulated isothermal PCR assay. The optimized iiPCR method was employed to detect *Mannheimia haemolytica, Mannheimia glucosida, Mannheimia ruminanlis, Mycoplasma* ovipneumoniae*, Mycoplasma mycoides, Staphylococcus, Salmonella, Escherichia coli,* and *Corynebacterium pseudotuberculosis*. The amplification products were correctly identified and sequenced to analyze the specificity of the method.

Analytical sensitivity of the Pasteurella multocida insulated isothermal PCR assay. Genomic DNA was subjected to 10-fold serial dilutions and tested using the iiPCR assay. The positive strain was prepared in 10-fold dilutions (2.03×10^{5}~2.03×10^{-3} copies/μL) as a template, with positive results observed for the undiluted positive strain and negative results for ddH_2O. The established iiPCR method was employed for detection. In order to evaluate the lower limit of the CFU detection of suspension, the bacterial concentration of the isolated strain was determined by the plate colony counting method (HASNAN et al., 2022). A gradient dilution of the suspension was carried out, and DNA extracted from the diluted suspension. It was detected by two methods: ordinary PCR and iiPCR, respectively. The minimum concentration of bacteria that can be detected by both methods was calculated. At the same time, the detection was performed by the ordinary PCR method in order to calculate and compare the sensitivity of the two methods.

Analytical stability of Pasteurella multocida insulated isothermal PCR assay. Template DNA extracted from three dilution gradients (1×10^{-5} to 1×10^{-7}) of pure cultured *Pasteurella multocida* was tested in triplicate using the iiPCR method to assess intra-batch reproducibility. Additionally, at three different time points, three diluted gradients of template DNA were detected using the established iiPCR method to evaluate batch-to-batch repeatability.

Clinical application of Pasteurella multocida insulated isothermal PCR assay. To validate the clinical applicability of our iiPCR assay, DNA extracted from 188 goat samples was subjected to testing using both the iiPCR and the conventional PCR assays (HU et al., 2018). Concurrently, five iiPCR-positive products were randomly selected and sent to Shanghai Biotechnology Bioengineering Co., Ltd. for sequencing verification, ensuring accurate detection.

Results

Optimization of reaction system. In this experiment, the optimal iiPCR system was established, comprising 0.35 μL of the probe, 4.0 μL each of upstream and downstream primers, 25 μL of Premix EX Taq™ enzyme, and 2 μL of DNA template, with the remaining 14.65 μL filled with ddH2O. The fluorescence ratio (R1), indicating the system's performance after the reaction in relation to before, reached a maximum value of 2.8.

Analytical specificity of Pasteurella multocida insulated isothermal PCR assay. The iiPCR method developed in this study demonstrated specificity by producing negative results for *Mannheimia haemolytica, Mannheimia glucosid, Mannheimia ruminanlis, Mycoplasma ovipneumoniae, Mycoplasma mycoides, Staphylococcus, Salmonella, Escherichia coli*, and *Corynebacterium pseudotu-*

berculosis. In contrast, it exhibited positive results for *Pasteurella multocida* DNA, encompassing *Pasteurella multocida* type A, *Pasteurella multocida* type B, and *Pasteurella multocida* type D, as illustrated in Figure 1. These outcomes align with the results obtained from 2% gel electrophoresis, affirming the excellent specificity of the developed iiPCR method.

Analytical sensitivity of Pasteurella multocida insulated isothermal PCR Assay. The prepared positive strain underwent 10-fold dilution (2.03×10^{5}~2.03×10^{-3} copies/μL) as a template for testing. The undiluted positive strain yielded a positive result, while ddH_2O produced a negative result when using the established iiPCR method for detection. The detection limit of this iiPCR method was determined to be 2.03 copies/μL (Fig. 2). In contrast, the ordinary PCR method exhibited a lower detection limit for the bacterial genome at 2.03×10^{2} copies/μL (Fig. 3).

To determine the bacterial concentration of the isolated strains, the plate colony counting method was used, revealing a concentration of 1.36×10^{8} CFU/mL. After subjecting this concentration to a 10-fold gradient dilution (ranging from 1.36×10^{8} CFU/mL to 1.36×10^{-1} CFU/mL), the iiPCR method established in this study demonstrated a lower limit of detectability at 1.36×10^{3} CFU/mL (Fig. 4). Conversely, the ordinary PCR method's lower limit of CFU detection was 1.36×10^{5} CFU/mL (Fig. 5).

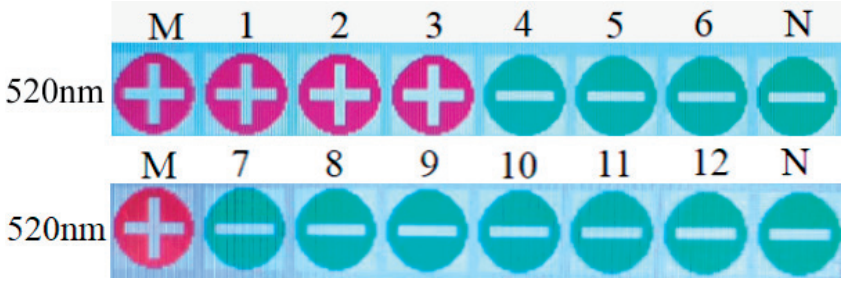

Fig. 1. *Pasteurella multocida* iiPCR specificity evaluation results

M: *Pasteurella multocida* DNA sample; N: negative control; 1~12: *Pasteurella multocida* type A, *Pasteurella multocida* type B, *Pasteurella multocida* type D, *Mannheimia haemolytica*, *Mannheimia glucosida*, *Mannheimia ruminanlis*，*Mycoplasma ovipneumoniae*, *Mycoplasma mycoides*, *Staphylococcus*, *Salmonella*, *Escherichia coli*, *Corynebacterium pseudotuberculosis*

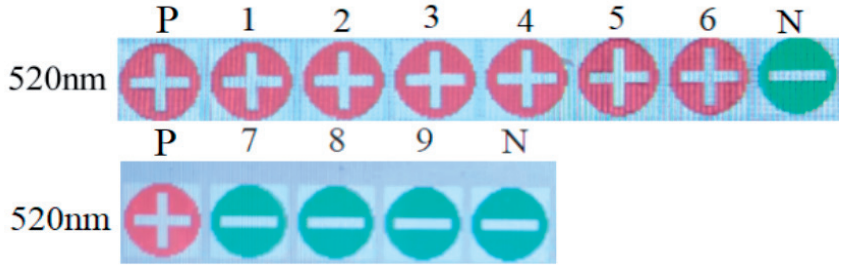

Fig. 2. Sensitivity results of *Pasteurella multocida* iiPCR DNA

P: *Pasteurella multocida* DNA sample; 1~9: 2.03×10^{5}~2.03×10^{-3} copies/μL； N: Negative control

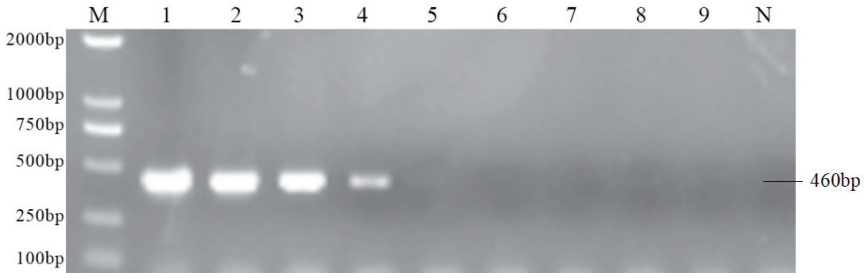

Fig. 3. Sensitivity results of common PCR method for DNA
M: DL2000 DNA Marker; 1-9: 2.03×10^{5} copies/μl~2.03×10^{-3} copies/μl; N: Negative control

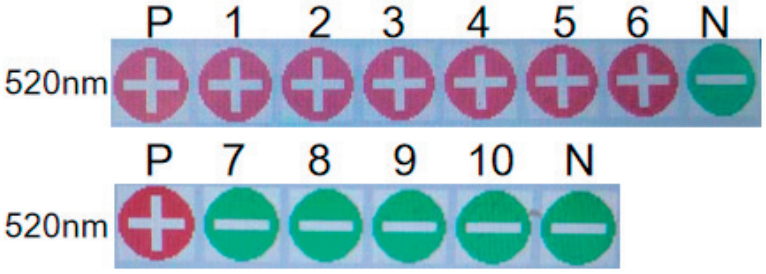

Fig. 4. Sensitivity results of iiPCR detecting *Pasteurella multocida* colony
P: *Pasteurella multocida* colony DNA sample; 1-10:1.36×10^{8} CFU/mL~1.36×10^{-1} CFU/mL； N: Negative control

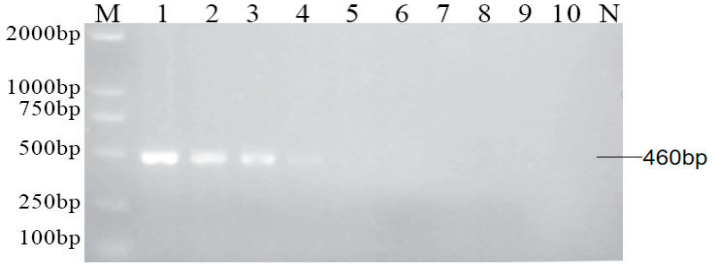

Fig. 5. Susceptibility of suspension by common PCR method
M: DL2000 DNA Marker; 1-10:1.36×10^{8} CFU/mL~1.36×10^{-1} CFU/mL; N: Negative control

Analytical stability of Pasteurella multocida insulated isothermal PCR assay. The results of three repeated tests within and between batches were consistent, indicating that the established iiPCR detection method has good stability.

Clinical application of Pasteurella multocida insulated isothermal PCR Assay. In this study, DNA was extracted from 188 goat nasal swabs collected during clinical practice. Both the iiPCR method and the ordinary PCR method were

employed for detecting the clinical sample DNA. Out of the 188 clinical samples tested, our iiPCR assay yielded positive results for 28 samples, accounting for 14.89% of the total. In contrast, the ordinary PCR assay detected positive results in 23 samples, making up 12.23% of the total (as shown in Table 1). Notably, all the samples that tested positive by the ordinary PCR method were successfully detected by our iiPCR assay as well. Moreover, sequencing of five randomly selected positive samples confirmed the test's accuracy.

These findings underscore the superior on-site detection capability of the iiPCR assay compared to the ordinary PCR assay for clinical samples. Moreover, the iiPCR method exhibited a quicker turnaround time. Overall, the results highlight the efficiency and effectiveness of the iiPCR method in clinical settings, positioning it as a promising choice for rapid and accurate detection.

Table 1. Comparative analysis of clinical sample detection using Conventional PCR and iiPCR

iiPCR	Conventional PCR		
	Positive	Negative	Total
Positive	23	5	28
Negative	0	160	160
Total	23	165	188

Discussion

Pasteurella multocida poses a significant threat to animal health, causing severe pneumonia and co-infections that adversely impact production efficiency and mortality rates (HURTADO et al., 2020). Traditional methods for detection suffer from time-consuming processes and potential omissions, emphasizing the need for efficient molecular techniques such as PCR. Various PCR-based approaches have been explored for *Pasteurella multocida* detection, each with limitations. However, their reliance on sophisticated setups and time-consuming procedures has limited their practicality (TOWNSEND et al., 1998; RAJKHOWA, 2015). Few studies have focused on rapid detection in goats, leaving a critical gap in on-site diagnostic tools (HU et al., 2018; LIU et al., 2021).

In response to the pressing demand for timely and on-site detection of *Pasteurella multocida* in goats, this study successfully introduced a novel and rapid diagnostic technology, namely thermostatic thermal isolation PCR (iiPCR). By leveraging the principles of thermal convection, iiPCR, implemented through the portable and user-friendly POCKIT™ instrument, demonstrated a remarkable capacity to perform automatic reactions within a mere 58 minutes, substantially enhancing on-site inspection efficiency.

The selection of the *sodA* gene of *Pasteurella multocida* as the target for detection stemmed from its well-established conservation within the pathogen, as documented in prior studies (GAUTIER et al., 2005). This gene's high conservation across all sequences in GenBank further validated its suitability for accurate and specific detection in our iiPCR method. Notably, the method exhibited exceptional specificity, as evidenced by the absence of signals for nine major pathogens of sheep origin, attesting to its precision and reliability. Comparative analysis of iiPCR and conventional PCR performance with clinical samples revealed not only a strong agreement but

also a significantly improved detection rate with iiPCR. The 14.89% detection rate for 188 clinical samples surpassed the 12.23% rate achieved by conventional PCR. Crucially, the positive results obtained with iiPCR completely mirrored those of conventional PCR, indicating the heightened sensitivity of the former. Consistent replication experiments served as a robust validation of the stability and reliability of the iiPCR method. The findings consistently demonstrated both sensitivity and dependability, positioning iiPCR as a valuable and promising tool for the rapid and precise detection of *Pasteurella multocida* in real-world clinical environments.

In conclusion, the results of this study underscore the potential of iiPCR as an efficient, sensitive, and reliable diagnostic approach for *Pasteurella multocida* in goats. The portability and rapid turnaround time of iiPCR make it particularly well-suited for on-site applications, offering a valuable contribution to the field of veterinary diagnostics. Further exploration of iiPCR's adaptability to diverse settings and its potential role in addressing emerging challenges in veterinary medicine is warranted.

Conclusions

In summary, the newly developed method not only enhances work efficiency but also significantly reduces detection time. Preliminary application of the method underscores its notable attributes, including high sensitivity, excellent specificity, and robust stability. The potential for on-site rapid detection of *Pasteurella multocida* is promising when combining this method with the portable POCKIT™ Intelligent Nucleic Acid Analyzer. By strategically designing specific primers and probes, the synergistic use of these technologies holds great promise for practical and swift *Pasteurella multocida* detection in various field settings. The positive outcomes of this study set the stage for further exploration and application of this method in broader veterinary contexts, highlighting its potential as a valuable tool in the field of rapid pathogen detection and diagnosis.

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Declaration of competing interest

All authors declare that they have no conflicts of interest.

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YANG, F., B. LI, L. ZHENG, Y. ZENG, H. ZHANG: Razvoj i validacija PCR testa za brzo otkrivanje i izolaciju bakterije *Pasteurella multocida.* Vet. arhiv 94, 365-374, 2024.

SAŽETAK

Pasteurella multocida, ubikvitaran je oportunistički patogen, poznat kao uzročnik različitih bolesti dišnog sustava u životinja. U potrazi za rješenjima za brzo otkrivanje i izolaciju ovog patogena razvijen je brzi izotermalni PCR test (iiPCR) usmjeren na gen *sod*A specifičan za bakteriju *Pasteurella multocida*. Ovaj je test pokazao znakovitu specifičnost u uspješnom otkrivanju kliničkih izolata *Pasteurella multocida* tipa A, B i D, dok su rezultati za druge patogene bili negativni. Njegova se iznimna osjetljivost očitovala detekcijom pozitivnog soja DNA (2,03 kopija/μL) i kolonija ($1{,}36\times10^3$ CFU/mL) kao predloška, s pozitivnim rezultatima za nerazrijeđene pozitivne uzorke. Metoda iiPCR pokazala je iznimnu ponovljivost u triplikatnom detektiranju različitih razrijeđenih pozitivnih sojeva. Praktično istraživanje je obuhvatilo 188 kliničkih uzoraka nazalnih brisova uzetih od koza. Test iiPCR proveden je usporedno s referentnim PCR-om. Rezultati upućuju na njegovu točnost i pouzdanost za otkrivanje bakterije *Pasteurella multocida* u praktičnim uvjetima veterinarske dijagnostike.

Ključne riječi: izotermalni PCR (iiPCR) za izolaciju; *Pasteurella multocida; sodA;* brza detekcija u praksi