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Original Article

Innovative exploration of Hep-2 cell culture in the isolation and culture of *Mycoplasma pneumoniae*

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ABSTRACT

Background: The isolation and culture of *Mycoplasma pneumoniae* (MP) is time-consuming and has a low success rate. On the basis of the fact that cell lines are susceptible to MP contamination, we explored the possibility of using Hep-2 cell culture for the isolation and culture of MP, to overcome this long-standing technical problem.
Methods: Quantitative Real-time PCR (qPCR) was used to detect MP in the nucleic acid samples of clinically suspected mycoplasma-infected patients. Positive samples were cultured in Hep-2 cells, with the classical commercial MP liquid culture medium serving as a control. For successful isolation of MP, the broth culture medium was used for subculture, then transferred to solid agar medium for isolation. The isolated strains were identified by nucleic acid and whole-genome sequencing.
Results: Among the 20 throat swab samples collected from individuals with influenza-like illness, 10 MP-positive samples were detected by qPCR. Five strains of *Mycoplasma* were successfully cultured in Hep-2 cells within 7–10 days, while one strain was cultured in commercial MP broth after 21 days, with isolation rates of 50% and 10%, respectively. After repeated subculturing in liquid medium and inoculation onto solid medium, “fried-egg”-like colonies emerged. The isolated strains were identified by nucleic acid and whole-genome sequencing.
Conclusions: The use of cell culture enables the rapid and effective isolation and culture of MP, addressing the long-standing challenge of MP cultivation. This advancement may contribute to improved antibiotic development, vaccine research, and the maintenance of global public health security.

1. Introduction

Mycoplasmas are a group of microorganisms that lack cell walls, and *Mycoplasma pneumoniae* (MP) is one of the major pathogens associated with human respiratory diseases.[1] As the smallest microorganism capable of growing on artificial media, mycoplasmas are challenging to culture.[2] Previous studies have shown that the primary difficulty in culturing mycoplasmas lies in their highly specific growth requirements, including strict nutritional needs, specialized culture media, and precise environmental conditions. The slow growth rate and long culture period add complexity to mycoplasma research,[3] and mycoplasma contamination is an issue in the cell lines used for *in vitro* virus culture.[4,5] Despite its slow growth rate, mycoplasma contamination in cell culture media can reach 10^6–10^8 cells/mL.[6] Previous research efforts focused on eliminating mycoplasma contamination from cell lines, rather than exploring the potential to leverage their tendency to contaminate cells for the isolation and culture of mycoplasmas.

Abbreviations: MP, *Mycoplasma pneumoniae*; qPCR, Quantitative Real-time PCR; CT value, cycle threshold value; NCBI, National Center for Biotechnology Information; SNP, single nucleotide polymorphism.

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2. Materials and methods

2.1. Sample collection and nucleic acid testing

Throat swabs were collected from 20 patients with clinically suspected mycoplasma infection using commercial MP sampling tubes containing yeast extract powder, beef extract powder, pig stomach digestive powder, glucose, phenol red, serum, and penicillin. Nucleic acid extraction was carried out using the QIAamp DNA Micro Kit (50) (Cat. No./ID: 56304) according to the manufacturer's protocol. Quantitative Real-time PCR (qPCR) detection was conducted using an MP-specific nucleic acid detection kit (X-ABT, Beijing, China) with a lower detection limit of 500 copies/mL, and human-derived gene detection as an internal standard. For qPCR, 5 μL of DNA extracted from the sample, 18 μL of MP reaction mix, and 2 μL of Taq DNA polymerase were mixed in a total volume of 25 μL. The reaction parameters were one cycle of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Fluorescence was measured after each of the repetitive cycles.

2.2. Isolation and culture

Positive qPCR samples were subjected to filtration using 0.45 μm syringe filters (Millipore, Boston, MA, USA) before inoculation into 1 mL of commercial MP broth culture medium (with a similar composition to transport medium, hereafter referred to as liquid culture medium). Parallel cultures were established using Hep-2 cells maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. All cultures were incubated at 37°C under 5% CO_2 with daily monitoring of both liquid culture medium colorimetric changes and cytopathic effects in cell cultures, and the nucleic acid was extracted to detect the growth of MP using the aforementioned qPCR kit. After 4 weeks of culture, isolation was considered unsuccessful if Hep-2 cells showed complete detachment or yielded two consecutive negative nucleic acid test results.

2.3. Subculture

Positive mycoplasma cultures from Hep-2 cells were filtered and transferred to liquid medium for multiple rounds of subculture using a 0.5 mL inoculum. Repeated subculture liquid medium was inoculated onto solid culture medium and colony morphology was observed.

2.4. Preservation and recovery

Isolated strains were cryopreserved at −80°C using a mycoplasma strain preservation tube (liquid culture medium containing 20% glycerol). Then, 0.2 mL of cryopreservation solution was inoculated into both liquid medium and Hep-2 cells. The cycle threshold value (Ct value) was detected by a qPCR assay to determine the recovery and growth of MP.

2.5. Whole-genome sequencing analysis

Subcultured MP broth (20 mL) was added to a 50 mL centrifuge tube and centrifuged at 12,000 r/min for 15 min. The supernatant was then removed, leaving the precipitate containing the nucleic acid. The DNA library was constructed using an Illumina Nextera XT DNA Library Preparation kit (San Diego, CA, USA) and sequenced on an Illumina Miseq sequencer. Sequence splicing was performed using CLC Genomics Workbench (Version 22.0.2, QIAGEN Digital Insights, Netherlands) for both non-parametric and parametric splicing with reference genome 50648-A01-3 (strain NCTC10119).

2.6. Virulence gene and 23S rRNA gene mutations

Virulence genes of the three strains of MP were compared using the Virulence Factors of Bacterial Pathogens Database. The *23S rRNA* gene sequence of the standard strain (NCTC10119) was downloaded from the National Center for Biotechnology Information (NCBI) database and compared with the genomic sequences of each strain using BLAST+ software. (https://blast.ncbi.nlm.nih.gov/Blast.cgi. NCBI, USA)

2.7. Construction of a whole-genome SNP phylogenetic tree

The whole-genome sequences of 34 MP isolates collected from diverse geographical regions and time periods were retrieved from the NCBI database. Single nucleotide polymorphisms (SNPs) were identified through reference-based alignment against the NCTC10119 genome. Phylogenetic reconstruction was performed using MGAP-Tree software (v2.0.0) with the approximately maximum-likelihood algorithm. For bacterial samples, the nucleotide alignment was analyzed under the general time reversible model with category discrete rate variation (GTR+CAT) from the MGAP-Tree, and then evolutionary trees were constructed.

2.8. Morphology identification

The morphological characteristics of *Mycoplasma* colonies on the agar medium were identified using an Olympus BX43 light microscope equipped with a smartV950D digital camera system under 10 × objective magnification (total magnification 100 ×).

2.9. Statistical analysis

Statistical analyses were conducted using R software (version 3.6.3). Student's *t*-test and Wilcoxon rank-sum

test were employed to assess the statistical significance of differences between the two culture methods. A two-tailed $p < 0.05$ was considered statistically significant.

3. Results

Among the 20 samples tested by qPCR, 10 were positive for MP. Five strains of MP were successfully isolated using Hep-2 cells, with the Ct values of the cell culture medium decreasing to 20 within 7 to 10 days (Fig. 1A). By contrast, only one strain (Sample No. 9) was isolated using liquid medium, showing a Ct value decrease from 27.35 to 23.33 over a 21-day period (Fig. 1B). For Sample No. 9, which was successfully isolated by both methods, an independent samples *t*-test indicated that the difference in Ct values between the two methods was statistically significant ($p < 0.05$). Contamination was observed in both Hep-2 cells and liquid medium (Sample No. 9) following the first inoculation using a 0.45 μm syringe filter. However, no contamination occurred during the second inoculation when a 0.22 μm filter was used. qPCR analysis was performed to determine the original Ct values of the 10 positive samples and to monitor Ct value changes in both Hep-2 cell and liquid culture media (Fig. 1).

Subsequent experiments showed that when MP was transferred from Hep-2 cell culture medium to liquid medium, the Ct values consistently decreased to below 20 by day 7. Human internal standard genes were initially detectable by qPCR in the liquid medium cultures, but their levels rapidly decreased below the detection limit as MP proliferation increased.

The typical "fried-egg"-like colonies on MP agar plates were observed after inoculation into liquid media for 2–3 generations or more. These characteristic colonies could be clearly identified under a microscope, with the earliest observation occurring approximately 10 days post-inoculation (Fig. 2A–C). However, when only transferring the first generation of mycoplasma liquid culture medium to agar plates, no typical "fried-egg"-like colonies were observed even after 4 weeks of culture. Instead, atypical colony morphology of mycoplasma was observed on the plates (Fig. 2D), which was subsequently confirmed by qPCR analysis with negative controls and the corresponding sites on the scraped plate.

During the recovery process of the five isolated MP strains, it was observed that the Ct values of the Hep-2 cell culture solution gradually decreased within the first 4 days, followed by an accelerated rate of decline, indicating significant MP growth (Fig. 3A). By contrast, the MP liquid medium did not show a significant decrease in Ct value until day 17 after inoculation (Fig. 3B). In the comparative analysis of both recovery methods for strains No. 1, 5, 8, and 9, independent sample *t*-tests revealed statistically significant differences in Ct values between resuscitation methods for strains No. 1, 8, and 9 ($p < 0.05$), whereas no significant difference was observed for strain No. 5 ($p > 0.05$). Strain No. 10 exhibited non-normally distributed Ct values, and non-parametric analysis using the Wilcoxon rank-sum test confirmed a statistically significant difference in Ct values ($p < 0.05$).

The nucleic acids were extracted from the MP liquid culture medium by centrifugation. Subsequently, three MP genomic libraries were constructed, sequenced, and assembled using the CLC Genomics Workbench platform, yielding 41, 45, and 47 contigs, respectively. Sequence analysis revealed no contamination with MP outer gene sequences, with the longest contig spanning approximately 200 kb, representing approximately one-quarter of the complete genome. All three isolates exhibited the *23S rRNA* A2063G mutation and contained multiple virulence-associated genes, including *P1, CRMA, P65, HMW1, HMW2, HMW3, MPN372*, and *P200*. The whole-genome SNP phylogenetic tree demonstrated that the three MP strains were mainly clustered on one branch with those from Beijing and Seoul (Fig. 4).

4. Discussion

MP demonstrates the ability to penetrate through 0.22 μm filters typically used for sterilization.[7] In our study,

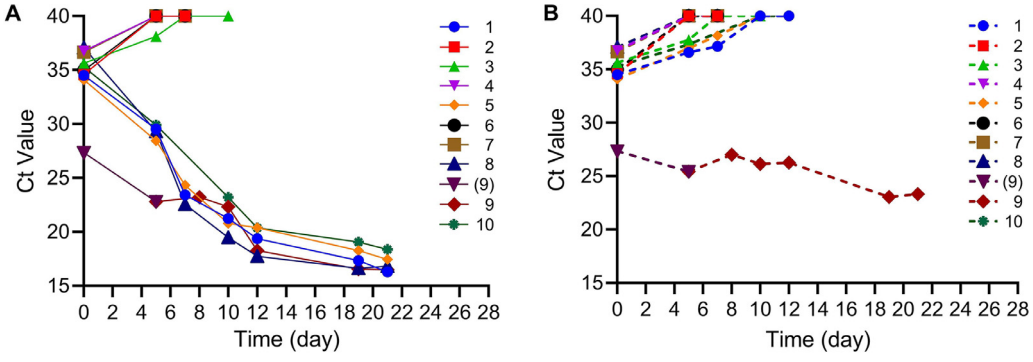

Fig. 1. Changes in Ct values in the qPCR assay for the separation and culture of 10 positive samples of *Mycoplasma pneumoniae* nucleic acid. Legend serial numbers indicate 10 sample numbers, (A) indicate culture using Hep-2 cells, and (B) indicate culture using liquid medium. Sample No. (9) was found to be contaminated on day 5 of culture, indicating contamination after re-filtration. When the nucleic acid test result was negative, the marker was 40. Day 0 is the Ct value of the sample, not the Ct value of the culture medium at the start of culture.

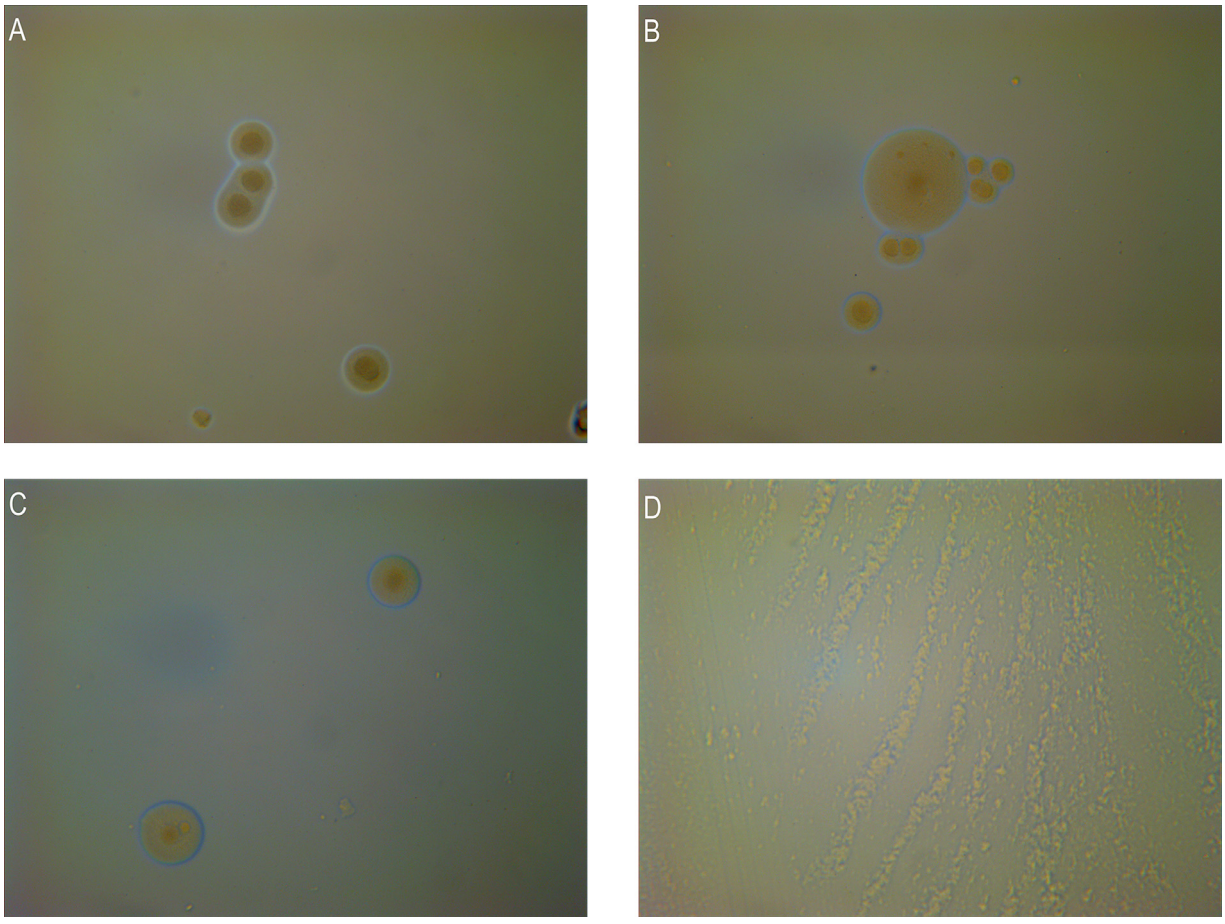

Fig. 2. Typical "fried-egg" like colonies (panels A–C) and scattered "granular" colonies (D) prior to the formation of "fried-eggs".

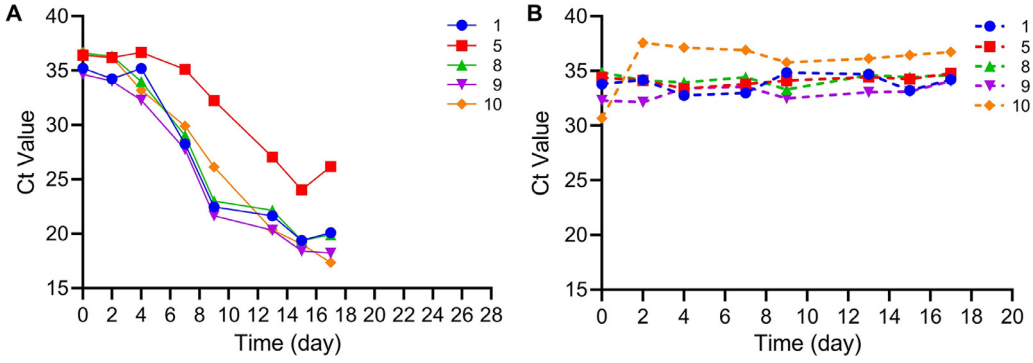

Fig. 3. Changes in Ct values in the qPCR assay for the resuscitation of five strains of *Mycoplasma pneumoniae.* (A) indicates the use of Hep-2 cells for *Mycoplasma pneumoniae* and (B) indicates the use of liquid medium for resuscitation. Day 0 indicates the Ct value of the culture medium at the start of culture.

we selected a 0.45 μm filter to minimize excessive sample loss during filtration. Post-filtration analysis revealed no contamination, except in sample No. 9, demonstrating the effective filtration capability of the 0.45 μm filter for MP processing. We successfully isolated one MP strain using commercial liquid medium, although it exhibited relatively slow growth kinetics. The liquid culture medium could maintain the pathogen's viability but could not support recovery growth, which might also be related to the commercial mycoplasma cryopreservation tubes used. Overall, comparative analysis showed that Hep-2 cells enabled significantly higher MP isolation rates and recovery efficiency compared with liquid culture medium, establishing Hep-2 cells as a more effective culture system for MP isolation and recovery. From a research application perspective, Hep-2 cells are easily obtainable and have well-established culture conditions, making them feasible for mycoplasma isolation. We carried out repeated experiments under the same conditions across different laboratories and with different operators, and successively isolated 20 strains, confirming the stability of this methodology.

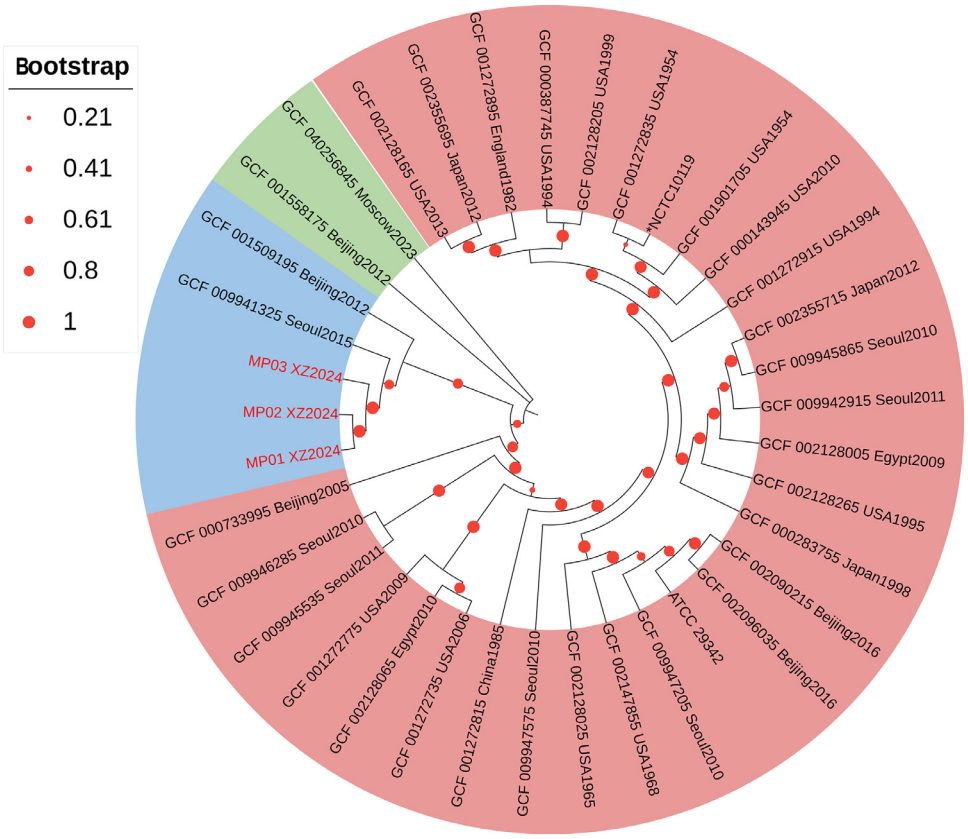

Fig. 4. The whole-genome SNP phylogenetic tree. The genome labels in the phylogenetic tree follow the format of NCBI RefSeq assembly, combined with the geographic location and collection date. Standard strains ATCC 29342 and NCTC 10119 are also included.
Abbreviation: SNP, single nucleotide polymorphisms.

Comparative genomic analysis of the three isolated MP strains revealed no detectable exogenous genetic material, demonstrating that the sequential isolation protocol (primary culture in Hep-2 cells followed by liquid medium transfer) effectively eliminates host cell interference in downstream analysis. The significantly higher isolation efficiency achieved through Hep-2 cell pre-enrichment compared with that with direct liquid medium inoculation suggests that this cellular system provides critical biological support for initial pathogen recovery. Subsequent subculture in liquid medium enables successful MP propagation, while maintaining genetic purity. Notably, first-generation liquid cultures showed limited capacity to form characteristic “fried-egg”-like colonies on solid media, potentially indicating strain adaptation requirements during culture transition. To address this, implementing strategic liquid subculturing with successive passages prior to solid medium plating may enhance colony formation efficiency and strain stability.

Current research indicates that macrolide resistance in MP predominantly arises from mutations in the *23S rRNA* gene binding sites, with frequent reports of A2063 and A2064 position alterations.[8,9] Genomic sequencing revealed all three MP strains in this study carried the A2063G mutation in their *23S rRNA* genes. This molecular mechanism correlates with the observed high-level macrolide resistance and aligns with the nationwide MP infection outbreak documented in late 2023.[10]

This study has several limitations. First, bacterial and fungal contamination was not controlled during initial isolation, and non-filtration direct inoculation would lead to the loss of some samples, affecting the isolation rate. Hep-2 cells were selected for initial culture because of their laryngeal origin (a respiratory tract site colonized by mycoplasma) and their known susceptibility to mycoplasma contamination.[4] A comparative analysis was conducted using HeLa and Hep-2 cells for culturing an isolated MP strain. Preliminary results indicated the reduced MP proliferation efficiency in HeLa cells compared with that in Hep-2 cultures. However, this single comparison lacks statistical significance and requires validation through the use of additional cell lines. According to Uphoff *et al.*,[11] cell culture contaminants are predominantly derived from human, porcine, and bovine sources, with *Mycoplasma fermentans* (47%) as the most frequent contaminant, followed by *M. hyorhinis* (19%), *M. orale* (10%), *M. arginini* (9%), *Acholeplasma laidlawii* (6%), and *M. hominis* (3%). Future work should systematically evaluate additional cell lines (Vero, HEK-293, HeLa, RD, MDCK) to optimize isolation efficiency. It is crucial to note that our research merely offers a novel culture strategy for mycoplasma. Only one commercial culture medium was chosen for comparison, and this does not imply that our method outperforms all commercial culture media.

Acquiring higher-quality pathogen cultures and acquiring whole-genome sequences more rapidly is conducive to further exploring the growth and metabolism, gene regulation, and pathogenic mechanisms of MP. This not only promotes basic microbiological research but also enables a more comprehensive approach to addressing bacterial

resistance and screening of new antibiotics. Additionally, the availability of a large number of MP strains may optimize antigen screening, resulting in the production of purer and more representative antigens, thereby enhancing the immune efficacy of vaccines, which is of crucial significance for vaccine development.

5. Conclusions

The innovation of this study lies in the establishment of a highly efficient *Mycoplasma pneumoniae* isolation system utilizing Hep-2 cell culture. For the first time, we propose a two-stage isolation strategy comprising sequential enrichment and purification phases. In the initial phase, Hep-2 cells served as biological amplifiers for pathogen pre-enrichment, substantially enhancing the viability of MP in clinical specimens. During the subsequent purification phase, samples were subcultured in liquid medium for more than two passages, effectively eliminating residual host cell interference, and characteristic "fried-egg" like colonies were observed on solid medium. This optimized protocol demonstrated superior isolation efficiency compared with conventional methods.

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CRediT authorship contribution statement

Weiwei Wu: Conceptualization, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing. **Wenwen Zhu:** Conceptualization, Data curation, Methodology, Supervision, Writing – original draft. **Jing Tong:** Data curation, Funding acquisition, Project administration. **Qiang Zhou:** Data curation, Investigation, Project administration. **Yanping Xu:** Data curation, Methodology. **Xiuxiu Zhou:** Data curation, Methodology. **Yu Du:** Data curation, Methodology. **Jun Bi:** Data curation, Resources. **Liguo Zhu:** Writing – review & editing, Validation, Supervision, Software, Resources, Investigation, Formal analysis.

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Declaration of competing interest

The authors declare no conflicts of interest.

Data available statement

The strains and additional data are stored at the Xuzhou Center for Disease Control and Prevention, and can be provided on reasonable request.

Ethical statement

This study was approved by the Ethics Committee of Xuzhou Center for Disease Control and Prevention with approval number [XJK2025002].

Informed consent

Written informed consent was obtained from the subjects for publication of this manuscript and any accompanying images.

Organ donation

Not applicable.

Animal treatment

Not applicable.

Generative AI

None.

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