BMC Microbiology

RESEARCH Open Access

# Comparative evaluation of proteinase K and dithiothreitol as pretreatments for extracting nucleic acids from respiratory samples for multiplex PCR

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## Abstract

**Background** Lower respiratory tract infections are frequently caused by bacteria, and the rapid identification of pathogens is crucial for guiding treatment. Multiplex PCR(M-PCR) can detect multiple pathogens simultaneously, but mucus and other cells in lower respiratory tract samples may interfere with nucleic acid detection. In this study, we compared the effectiveness of two pretreatment methods—proteinase K(PK) and dithiothreitol(DTT)—in detecting multiple pathogens using M-PCR in bronchoalveolar lavage fluid(BALF) and sputum samples.

**Methods** A total of 30 BALF samples and 20 sputum samples were collected. These samples were pretreated with PK and DTT, respectively. Bacterial structural changes and background material were examined using Gram staining. Nucleic acid purity and concentration were assessed following extraction. Finally, the detection rate of several common pathogens associated with lower respiratory tract infections was analyzed using M-PCR.

**Results** Gram staining indicated that both PK and DTT effectively destroyed the bacterial structure and reduced background material in BALF samples, while DTT was more effective samples compared to PK in sputum. The M-PCR results indicated no significant difference in Ct values for Streptococcus pneumoniae, Klebsiella pneumoniae, Haemophilus influenzae, and Pseudomonas aeruginosa between PK and DTT-treated BALF samples. The results of nucleic acid extraction showed no difference in purity and concentration of nucleic acids after treatment with PK and DTT in BALF and sputum samples. After PK treatment, the Ct values for the four bacteria in sputum samples were different from those in BALF samples treated with the three methods, while after DTT treatment, only *K. pneumoniae* and *H. influenzae* showed differences compared to BALF. There was no difference in bacterial detection rates between PK and DTT treatments of BALF, both of which were 100%. In sputum samples, the bacterial detection rate after DTT treatment was 100%, significantly higher than the 87.5% detection rate after PK treatment ($P < 0.05$).

**Conclusion** PK and DTT exhibited similar pretreatment effects on BALF samples, with neither having an impact on the results. However, DTT was superior to PK in reducing interference and enhancing the sensitivity of M-PCR for bacterial detection in sputum samples, making it the preferred pretreatment for sputum.

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**Keywords** Multiplex PCR(M-PCR), Proteinase K(PK), Dithiothreitol(DTT), Sputum, Bronchoalveolar lavage fluid(BALF), Homogenisation, Comparison

## Introduction

Respiratory tract infections are classified into upper and respiratory tract infections (URTIs and LRTIs), which differ markedly in etiology and clinical significance. URTIs, such as the common cold and pharyngitis, are predominantly caused by viruses and typically present with mild, self-limiting symptoms [1]. In contrast, LRTIs including pneumonia and bronchitis often involve bacterial pathogens and may lead to severe complications such as respiratory failure, particularly in immunocompromised populations [2]. The accurate identification of bacterial pathogens in LRTIs is critical, as delayed diagnosis can result in prolonged hospital stays and increased mortality rates [3].

Multiplex PCR (M-PCR) technology amplifies multiple targets in a single reaction, enabling simultaneous diagnosis of various pathogens. Compared to traditional culture methods, M-PCR reduces detection time from days to hours while maintaining high specificity [4, 5]. Other rapid molecular techniques such as real-time quantitative PCR (qPCR) offer superior sensitivity for single-pathogen detection, and next-generation sequencing (NGS) provides broader pathogen profiling but requires higher costs and longer turnaround times (24–72 h) [6]. M-PCR thus strikes a balance between efficiency, cost-effectiveness, and multiplexing capability for routine clinical use [7].

Bronchoalveolar lavage fluid (BALF) and sputum are common samples for pathogen testing in LRTIs [8]. However, mucus and cellular debris in these specimens can entrap pathogens and inhibit nucleic acid extraction, potentially causing false-negative results [9]. Sputum samples, in particular, require homogenization to disrupt viscous mucin networks prior to molecular analysis. Effective pre-processing of BALF and sputum is therefore crucial to improve detection sensitivity [10].

Proteinase K (PK) is commonly used in nucleic acid isolation kits for tissue samples. It is well-studied for its ability to degrade mucus and cellular debris, improving the extraction efficiency of pathogen DNA or RNA [11, 12]. Dithiothreitol (DTT) is a highly effective reagent that specifically breaks mucin disulfide bonds and is widely used to homogenize sputum samples [13, 14].

This study aimed to compare PK and DTT pretreatment for M-PCR detection of *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* in BALF and sputum samples. It also evaluated their effects on the sensitivity of pathogen detection in simulated clinical samples.

## Materials and methods

### Specimen source

BALF and sputum samples for this study were collected from the Department of Laboratory at Yuncheng Central Hospital affiliated to Shanxi Medical University, between July and August 2024. A total of 30 BALF samples (greater than 3 ml) and 20 sputum samples (greater than 1 ml) were collected. These samples were cultured and found negative for *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *P. aeruginosa*. No antibiotic treatment was administered before specimen collection. BALF and Sputum samples were collected from patients with suspected LRTIs at Yuncheng Central Hospital affiliated to Shanxi Medical University. Samples were subjected to Gram staining and microscopic examination to assess quality. BALF was obtained through bronchoscopy, following standard clinical protocols. Microscopic examination was performed to confirm the presence of alveolar macrophages (≥ 5% of total cells) and the absence of significant squamous epithelial cell contamination (< 1%), ensuring that the samples originated from the lower respiratory tract. Sputum samples were considered acceptable if they contained fewer than 10 squamous epithelial cells per low-power field (LPF, 100× magnification) and more than 25 polymorphonuclear leukocytes (PMNs) per LPF. All samples were transported to the laboratory within 2 h of collection and stored at 4 °C if immediate processing was not possible. Samples were processed within 24 h to minimize nucleic acid degradation.

The study was conducted in strict accordance with the regulations of the Ethics Committee of Yuncheng Central Hospital affiliated to Shanxi Medical University, and appropriate ethical approvals were obtained. All participating patients or their families signed an informed consent form, ensuring the legality and ethical integrity of the study.

### Sample Preparation and processing

The clinical isolates of *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *P. aeruginosa*. used in this study were obtained from the Microbiology Laboratory of Yuncheng Central Hospital affiliated to Shanxi Medical University. All strains were originally isolated from respiratory specimens (sputum or BALF) of patients diagnosed with lower respiratory tract infections (LRTIs) between July and August 2024. Prior to experimentation, the strains were subcultured on appropriate agar media (e.g., blood agar for *S. pneumoniae*, chocolate agar for *H. influenzae*) and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF

MS); The bacterial solution was prepared to a turbidity of 0.5 McFarland standard, which corresponds to approximately 1.5 × 10^8 CFU/ml. This stock was then diluted 100-fold to yield a concentration of approximately 1.5 × 10^6 CFU/ml. Prior to homogenization, 1 µL/mL of the diluted suspension of each bacterial pathogen was spiked into BALF or sputum samples, resulting in a final bacterial load of 1,500 CFU/mL in 200 µL of processed clinical samples. The mixture was then shaken for 20 s. The chosen bacterial concentration was based on two factors. First, it is approximately 1.5 times the lower detection limit of the reagent used in this study. Second, this concentration aligns with the bacterial load commonly found in BALF, where bacterial counts of ≥ 10^4 CFU/ml or ≥ 10^3 CFU/ml(for pollution prevention) are clinically significant for infection diagnosis.

BALF samples (3 ml) were centrifuged at 1,600 g for 10 min. The supernatant was discarded, and the remaining pellet was diluted with normal saline (NS) to a final volume of 1 ml. After bacterial contamination, the samples were pre-treated using one of three methods. The first method involved using treated BALF (NS-treated). The second method involved adding 20 µl of PK (20 mg/ml) to each milliliter of BALF, vortexing for 20 s, and incubating at 37 °C for 30 min (PK-treated BALF). The third method involved mixing equal volumes of BALF and DTT buffer (13.4 g of DTT per 1,000 ml of purified water), vortexing for 20 s, and incubating for 30 min (DTT-treated BALF).

Sputum samples, after bacterial contamination, were pre-treated using one of two methods. The first method involved adding 20 µl of PK (20 mg/ml) per milliliter of sputum and incubating at 37 °C for 30 min (PK-treated sputum). The second method involved mixing equal volumes of sputum and DTT buffer (13.4 g DTT per 1,000 ml of purified water) and incubating at room temperature for 30 min (DTT-treated sputum) [15, 16].

For both BALF and sputum samples, 500 µl of sample from each method was centrifuged at 12,000 rpm for 5 min, and the supernatant discarded. Finally, 10 µl of 0.9% NaCl was added to the pellet for Gram staining microscopy.

### DNA extraction and M-PCR

Nucleic acids were extracted using the magnetic bead method with an automatic nucleic acid extraction system (Hunan Shengxiang Biotechnology Co., China) and stored at -70 °C for future use. The nucleic acids were analyzed with a Nano-400 Ultra Micro Nucleic Acid Analyser (Hangzhou Aosheng Instrument Co., China) to determine the concentration and purity (A260/A280 ratio) of each DNA sample. Extracted DNA was detected by M-PCR using a nucleic acid detection kit(Hunan Shengxiang Biotechnology Co., China), and the threshold cycle (Ct value), inversely correlated with nucleic acid concentration, was recorded. Results were handled and analyzed according to the manufacturer's instructions, with internal quality control implemented for each batch.

### Statistical methods

Descriptive statistics were calculated using SPSS version 23.0 (Armonk, NY, USA). For hypothesis testing, a two-sample t-test and chi-square test were used, with a significance level set at $P < 0.05$.

## Results

### Microscopic observations

Gram staining results showed that in the NS-treated BALF, bacteria were structurally intact and morphologically consistent. In the PK-treated BALF, the number of bacteria was significantly reduced, and the amount of background material was notably decreased compared to the untreated BALF. In the DTT-treated BALF, intact bacteria were not observed, and the background material was almost completely eliminated. A large number of intact Gram-negative and Gram-positive bacteria were still present in the PK-treated sputum. In contrast, DTT-treated sputum showed a significant reduction in both the number of bacteria and the amount of background material compared to the PK-treated sputum (Fig. 1).

### Comparison of nucleic acid purity and concentration in BALF and sputum samples processed by different methods

The nucleic acid concentrations (ng/µl) of NS-treated, PK-treated, and DTT-treated BALF were 20.71 ± 2.38, 21.02 ± 1.96, and 21.55 ± 2.18, respectively. The A260/A280 ratios were 1.67 ± 0.06, 1.67 ± 0.05, and 1.84 ± 0.08, showing no significant difference between the methods (Fig. 2a and b). The nucleic acid concentrations (ng/µl) of PK-treated and DTT-treated sputum were 166.90 ± 32.99 and 174.40 ± 36.30, respectively, which were not significantly different from each other, but were significantly higher than the NS-treated BALF group ($P < 0.001$). The A260/A280 ratios of PK-treated and DTT-treated sputum were 1.83 ± 0.08 and 1.83 ± 0.09, respectively, and showed no statistically significant difference compared to the NS-treated BALF group or the two other treated groups, as shown in (Fig. 2c and d).

### Comparison of the results of M-PCR of BALF and sputum samples processed by different methods

The Ct values obtained from M-PCR for *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *P. aeruginosa* in NS-treated, PK-treated, and DTT-treated BALF were compared. The results showed no significant differences ($P > 0.05$) in Ct values for any of the four bacteria across the three BALF treatment groups. Similarly, after PK and DTT preprocessing of sputum samples, the CT values

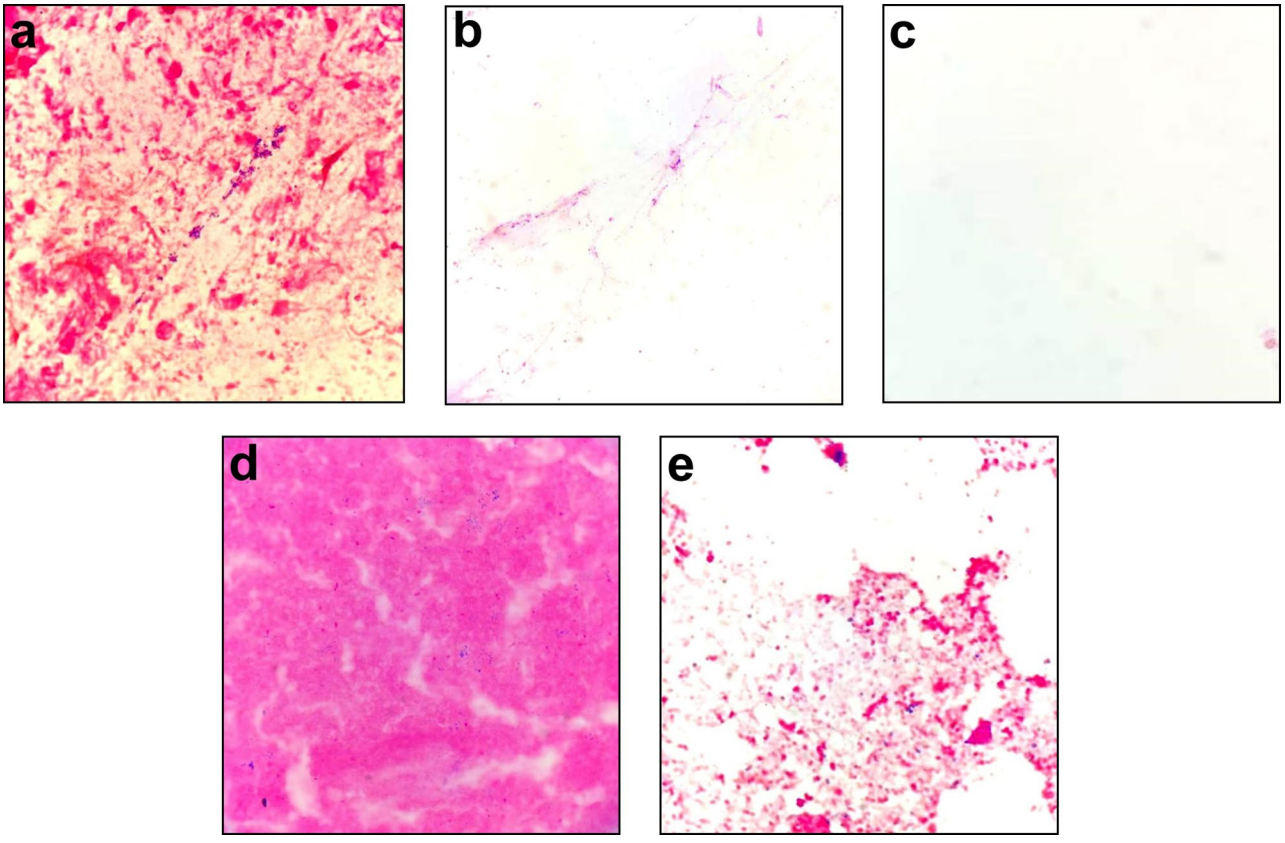


**Fig. 1** The effect of PK and DTT homogenization was observed using Gram staining (×1000). (**a**) NS-treated BALF; (**b**) PK-treated BALF; (**c**) DTT-treated BALF; (**d**) PK-treated sputum; (**e**) DTT-treated sputum

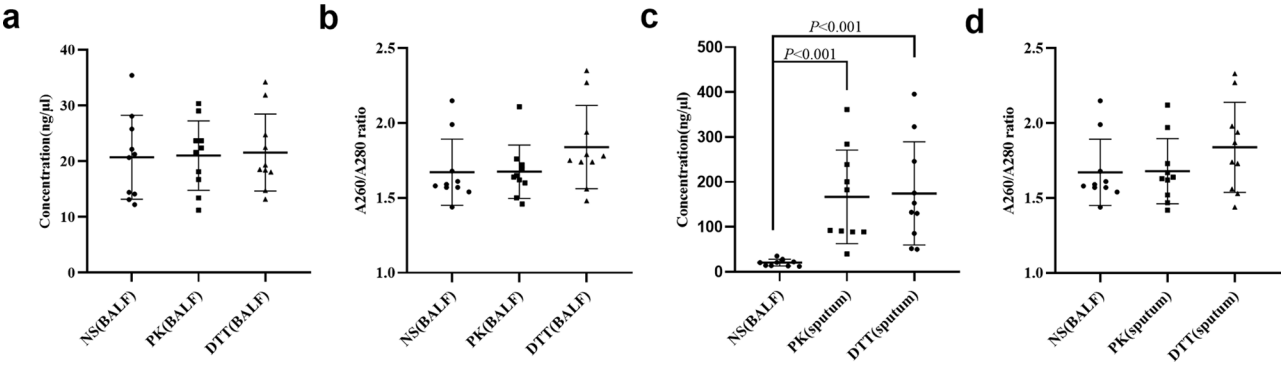


**Fig. 2** Comparison of nucleic acid concentration (ng/μl) and purity of BALF and sputum samples after homogenization with different treatments. (**a**) Nucleic acid concentration of BALF samples after processing; (**b**) A260/A280 ratio of BALF samples after processing; (**c**) Nucleic acid concentration of sputum samples after processing; (**d**) A260/A280 ratio of sputum samples after processing

of the four bacteria were measured. There were no significant differences in CT values between the two sputum treatment groups ($P > 0.05$). However, further analysis revealed significant differences ($P < 0.05$) in CT values for all four bacteria between sputum treated with PK and the three BALF treatment groups. Compared to the three BALF treatment groups, significant differences in CT values for sputum treated with DTT were observed only for *K. pneumoniae* and *H. influenzae* ($P < 0.05$) (Fig. 3).

### Comparison of positive rates of M-PCR results in BALF and sputum samples processed by different methods

In the BALF samples, the detection rates of *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *P. aeruginosa* were 100% ($n = 10/10$ for each species) in the NS-treated, PK-treated, and DTT-treated groups. However, in sputum samples, the detection rate was significantly lower in the PK-treated group. Specifically, the positive detection rates for *S. pneumoniae*, *H. influenzae*, and *P. aeruginosa*

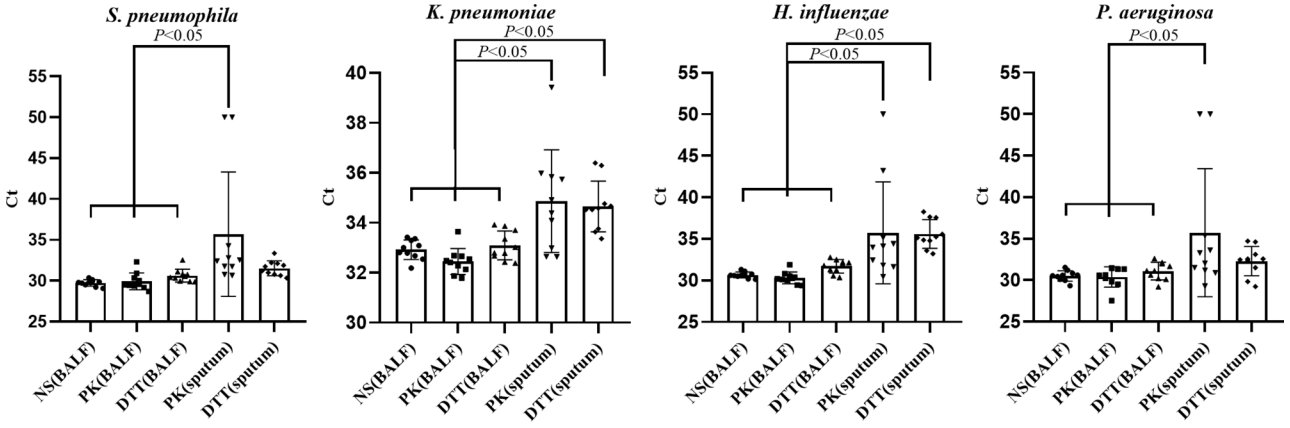


**Fig. 3** Comparison of Ct values for four bacterial species in BALF and sputum following pretreatment with different methods. For sputum samples that showed no amplification curves, Ct values were set to 50

were 80%, 90%, and 80%, respectively. The overall detection rate for the PK-treated group was 87.50% (35/40). In contrast, all target pathogens were successfully detected in the DTT-treated group (100%). Statistical analysis revealed that the detection rate in the DTT-treated group was significantly higher than that in the PK-treated group ($\chi^2$ = 5.33, $P < 0.05$). This indicates that DTT is more effective in releasing nucleic acids from sputum samples than PK.

## Discussion

BALF and sputum are commonly used samples for detecting lower respiratory tract infections. However, these samples often contain high amounts of mucus, which complicates the nucleic acid extraction process. Mucus, mainly composed of glycoproteins like mucins, forms a dense, viscous network that can encapsulate bacterial cells and other pathogens [17], hindering extraction reagents from accessing the nucleic acids. Furthermore, cellular debris, including proteins and lipids, can interfere with enzymatic reactions during nucleic acid extraction [18]. These interactions may reduce nucleic acid yield and purity, leading to false-negative results in downstream applications such as PCR. Therefore, appropriate pre-treatment of these specimens is crucial to improve extraction efficiency [19]. PK and DTT are frequently used to homogenize sputum samples by digesting mucus proteins and disrupting epithelial cells, thereby facilitating automated extraction after treatment. PK, a serine protease, facilitates nucleic acid extraction and purification by degrading proteins in the sample [20, 21]. It is particularly effective at disrupting cellular and tissue structures, removing proteins that inhibit PCR reactions, and processing viscous samples. DTT, a reducing agent, is primarily used to break disulfide bonds in proteins, converting them into sulfhydryl groups. This property plays a crucial role in preserving protein activity, preventing RNA degradation by RNases, and enhancing the homogenization of viscous samples. These functions are essential for stable nucleic acid extraction and subsequent molecular biology studies [22–24].

In this study, BALF and sputum samples were treated with PK and DTT, respectively, followed by Gram staining. The results highlight the advantages of DTT in processing both BALF and sputum samples, particularly in disrupting bacterial structures and breaking down mucus [25]. The reducing properties of DTT enable it to efficiently cleave mucus and cellular components, thereby enhancing the efficiency of nucleic acid extraction [15]. In contrast, PK, while effective in BALF fluid, was less efficient in sputum samples. This difference may be attributed to the complex composition of sputum, which could inhibit the activity of PK.

In this study, we assessed the effects of PK and DTT treatments on the purity and concentration of nucleic acids extracted from BALF and sputum samples. The results showed that for BALF, None of the tested pretreatment methods caused significant differences in nucleic acid concentrations or A260/A280 ratios, suggesting that both methods are similarly effective in terms of purity and concentration of nucleic acid extraction. This finding aligns with previous studies that have demonstrated the efficacy of both PK and DTT in disrupting cellular structures and releasing nucleic acids for further processing [15, 16]. For sputum samples, although the A260/A280 ratios were not significantly different between the treatments, significant differences in nucleic acid concentrations were observed compared to NS-treated BALF. This variation may be attributed to the higher cellular and bacterial content in sputum, which can influence nucleic acid extraction efficiency [26–28].

We also performed M-PCR to evaluate the impact of these pretreatment methods on Ct values and positivity rates. For BALF samples, no significant effect on Ct values was observed with either PK or DTT, and the detection rate for all four bacteria was 100%. This suggests that

these treatments do not affect M-PCR sensitivity or efficiency for BALF, likely due to its relatively low bacterial and protein content, as well as its homogeneous matrix [29]. In contrast, for sputum samples, significant differences in Ct values were observed after PK treatment, compared to BALF treated with the same methods. This may be due to the complex mucus and cellular components in sputum, which inhibit PK activity and reduce nucleic acid extraction efficiency, thus affecting M-PCR sensitivity. This finding contrasts with some previous reports [30, 31]. After DTT treatment of sputum samples, significant differences in CT values were observed for *K. pneumoniae* and *H. influenzae* compared to all three BALF treatments. However, the detection rate of bacteria treated with DTT was 100%, which was significantly higher than that of sputum treated with PK. This suggests that DTT is more effective at disrupting sputum's mucus and cellular structures, improving nucleic acid extraction and M-PCR sensitivity [24]. However, the lower sensitivity of PK in sputum samples contrasts with findings from a previous study, which reported similar performance for PK and DTT in extracting and detecting influenza A virus nucleic acids from sputum [15]. This discrepancy may be due to differences in the target pathogens. Our study focused on bacterial detection, while the cited study investigated viral nucleic acids. Bacterial cells are often embedded in dense mucus and cellular debris, which may hinder PK's ability to fully penetrate and lyse these structures. In contrast, viral particles are smaller and less physically entangled in mucus, making them more accessible to PK-mediated digestion. These findings emphasize the need to tailor pretreatment methods to the specific characteristics of both the target pathogen and the sample matrix.

Respiratory infections pose a significant challenge to global health [32]. Therefore, optimizing nucleic acid extraction and testing processes is essential for the rapid and accurate diagnosis of pathogens. Although numerous studies have investigated nucleic acid extraction methods for various respiratory samples, there has been no systematic comparison of PK and DTT in bacterial multiplex PCR pretreatment for respiratory samples. This study compares the PK and DTT methods and provides practical optimization recommendations to improve the sample preprocessing process in the laboratory. Furthermore, our study demonstrated the ability of DTT to detect pathogens at a load of 1500 CFU/mL, confirming the reliability and reproducibility of M-PCR results following respiratory sample pretreatment. This finding is especially significant for patients with early infections or those who have already received antibiotic treatment. It provides valuable support for clinicians in developing more accurate treatment plans.

Two major limitations of this study should be noted. First, In clinical settings, bacteria are often embedded in the mucus of sputum or interact with host cells in complex ways. However, this study artificially incorporated bacteria into BALF/sputum, which may not fully replicate the physiological conditions of natural infection. Although we carefully selected *S. pneumoniae*, *K. pneumoniae*, *H. influenzae*, and *P. aeruginosa*—important pathogens in both community-acquired and hospital-acquired pneumonia that are easy to cultivate and standardize in the laboratory—these bacteria are primarily extracellular. In contrast, intracellular or specialized pathogens require host cell lysis to release nucleic acids. Thus, the methods used in this study may not be suitable for evaluating nucleic acid release from such pathogens. To validate and extend these findings, future studies should consider using prospectively collected samples from actual infections. Second, this study did not investigate the specific impact of PK concentration on the experimental outcomes. Different concentrations of PK may influence bacterial degradation, sample component breakdown, extraction efficiency, and the accuracy of the final results. Therefore, future studies should assess the effect of PK concentration to determine the optimal amount for use in such experiments.

## Conclusions

In summary, for BALF samples, neither PK nor DTT pretreatment significantly affected the experimental results, and both methods can be used as pretreatment options when necessary. However, for sputum samples, DTT more effectively disrupted bacterial structures and reduced background interference compared to PK. This led to a significant improvement in PCR accuracy and pathogen detection rates. Therefore, DTT may be a more favorable option for enhancing nucleic acid extraction efficiency and the sensitivity of M-PCR in respiratory samples.

### Abbreviations

| | |
|---|---|
| PCR | Multiplex PCR |
| BALF | Bronchoalveolar lavage fluid |
| NS | Normal saline |
| PK | Proteinase K |
| DTT | Dithiothreitol |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03956-y.

Supplementary Material 1

### Acknowledgements

We would like to thank the Microbiology Department of Shanxi Medical University Affiliated Yuncheng Central Hospital for providing the strains used

in this study. We also express our gratitude to Junfang Pei and Shanshan Sun for their valuable assistance and support during the experiments.

**Author contributions**
All authors contributed to the conception and design of this study. YC, TL, YN, XG, and YX were involved in material preparation, data collection, and analysis. YC wrote the first draft of the manuscript, and all authors provided feedback on previous versions. Writing– review, and editing were performed by YC and YX. All authors read and approved the final manuscript.

**Data availability**
Data is provided within the manuscript or supplementary information files.

## Declarations

**Ethics approval and consent to participate**
The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was granted by the Ethics Committee of Yuncheng Central Hospital affiliated to Shanxi Medical University(YXLL2024212). Informed written consent was obtained from all participants.

**Consent for publication**
Not applicable.

**Competing interests**
The authors declare no competing interests.

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Received: 15 December 2024 / Accepted: 8 April 2025
Published online: 23 April 2025

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