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Research article

Multiplex PCR for simultaneous identification and differentiation of methicillin-resistant *Staphylococcus aureus* and *Staphylococcus pseudintermedius* isolated from dogs

Nguyen Pham Thao Nhi[1], Bui Nguyen Thanh Vy[1], Nguyen Thuy Y Vi[1], Nguy Ho Hoang Tho[2], To My Quyen[1], Dao Huyen Tran[1], Nguyen Tran Phuoc Chien[1], Nguyen Khanh Thuan[1], Nguyen Thi Lan Anh[3] and Lam Thanh Nguyen[1,*]

[1] *Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Can Tho city 900000, Vietnam*
[2] *Faculty of Applied Biology, College of Agriculture, Can Tho University, Can Tho city 900000, Vietnam*
[3] *Ho Chi Minh City University of Technology (HUTECH), Binh Thanh district, Ho Chi Minh city 700000, Vietnam.*

Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) are opportunistic pathogens that cause significant diseases in both human and veterinary medicine, particularly in pet animals. In recent decades, the emergence of MRSA and MRSP in pet animals has raised substantial public health concerns due to the high multidrug resistance exhibited by these zoonotic bacteria. Therefore, a rapid and accurate diagnosis is crucial as one of the key strategies for controlling infections caused by MRSA and MRSP. In this study, we applied a simple and cost-effective DNA extraction method, establishing a multiplex PCR assay for the identification and differentiation of MRSA and MRSP isolated from dogs in Vietnam. The results of this study indicate that DNA extraction of MRSA and MRSP using the Tris-EDTA-NaCl-Triton X100 (TENT) method yielded comparable efficacy to using a commercial DNA extraction kit, and the TENT method proved to be more cost-effective. Additionally, a multiplex PCR assay with four primer pairs (27F/1492R, mecA_F/mecA_R, au-F3/au-nucR, and pse-F2/pse-R5) successfully identified and discriminated between MRSA and MRSP. The multiplex PCR demonstrated high specificity with the desired amplification of targeted amplicons and no cross-reactions. Furthermore, the applicability of the diagnostic procedure was demonstrated by identifying field MRSA and MRSP isolated from clinical samples of dogs in Vietnam. Our study, therefore, provides an effective identification procedure for MRSA and MRSP, serving as a prompt countermeasure against the rapid emergence of MRSA and MRSP in Vietnam.

Keywords: *DNA extraction,* Dogs, M*ethicillin resistance,* M*ultiplex PCR, S. aureus, S. pseudintermedius*

[*]**Corresponding author**: Lam Thanh Nguyen, Faculty of Veterinary Medicine, College of Agriculture, Can Tho University, Can Tho city, Vietnam. Tel: (+84) 939.468.525, Email: ntlam@ctu.edu.vn.

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INTRODUCTION

Staphylococci are Gram-positive cocci that are nonmotile, non-spore-forming, and facultative anaerobes, constituting part of the commensal microbiota of the skin and entire upper respiratory tract in both humans and animals (Foster, 1996; McClure and Zhang, 2022). The Staphylococci genus belongs to the *Micrococcaceae* family, comprising at least 70 species and subspecies known to be both pathogenic and nonpathogenic across a wide range of host species (Parte et al., 2020). Traditionally, Staphylococci are classified into two groups: coagulase-positive Staphylococci (CoPS) and coagulase-negative Staphylococci (CoNS), based on their ability to clot plasma (Becker et al., 2014). CoPS includes nine species, such as *Staphylococcus (S.) aureus* (including *subsp. anaerobius*), *S. intermedius, S. pseudintermedius, S. delphini, S. hyicus, S. schleiferi subsp. coagulans, S. lutrae, S. agnetis* and *S. cornubiensis*. Notably, *S. aureus* and *S. pseudintermedius* are the most prevalent and clinically relevant in both human and veterinary medicine. Conversely, CoNS have traditionally been considered commensal and nonpathogenic (González-Martín et al., 2020).

Over the past few decades, there has been increasing concern regarding the emergence of antibiotic-resistant Staphylococci, which pose a potential threat of life-threatening infections in both humans and animals. Of particular note is the rise and spread of methicillin-resistant *S. aureus* (MRSA) and methicillin-resistant *S. pseudintermedius* (MRSP) (van Duijkeren et al., 2011; Feßler et al., 2018; Cuny et al., 2022). While MRSA is commonly found in humans and various animal species, MRSP is more prevalent and clinically significant among dogs and cats (Cohn and Middleton, 2010; Cuny et al., 2022). For instance, a recent study by the European Food Safety Authority revealed that 5.8% of clinical *S. pseudintermedius* strains in dogs and cats exhibited methicillin resistance. Similarly, a study conducted in Germany, involving 16,103 clinical samples from different animal species, found that 94.4% of the *S. pseudintermedius*-positive samples originated from dogs, with the remaining samples coming from cats (3.0%) and horses (2.6%) (Ruscher et al., 2009). Moreover, MRSA and MRSP are crucial zoonotic pathogens, with widespread reports of transmission between humans and animals (Feßler et al., 2018; Cuny et al., 2022).

The diagnosis of *Staphylococcus* species is routinely conducted through the morphological and biochemical identification of bacterial cultures (Hoffman et al., 2006). Nevertheless, these methods have inherent limitations, being both time-consuming and costly. Therefore, there is a critical need for rapid and accurate detection approaches to mitigate risks and enable effective treatment for infections caused by MRSA and MRSP. Over the past decades, various molecular detection methods, including conventional PCR, multiplex PCR, and real-time PCR, have been proposed for *Staphylococcus* identification. The application of these molecular approaches has been demonstrated in several previous studies (Costa et al., 2005; Rahman et al., 2018; Li et al., 2020). Nevertheless, there has been no study conducted on the molecular detection of MRSA and MRSP in Vietnam.

In Vietnam, research on MRSA and MRSP remains limited, with the majority of previous studies on MRSA within the context of human medicine (Van Nguyen et al., 2014; Quyet et al., 2019; Thai et al., 2019). No studies have been conducted on either MRSP or MRSA in pet animals in Vietnam. Therefore, the objective of the present study is to apply a simple DNA extraction in combination with establishing a multiplex PCR to identify and differentiate MRSA and MRSP isolated from dogs in Vietnam. This study enables the rapid and effective detection of MRSA and MRSP, making it a significant contribution to further investigations into MRSA and MRSP in veterinary medicine in Vietnam.

MATERIALS AND METHODS

Bacterial strains and sample collection

In this study, two reference strains, *S. aureus*, and *S. pseudintermedius*, provided by the Laboratory of Microbiology, Faculty of Veterinary Medicine and Animal Husbandry, HUTECH University, Ho Chi Minh, Vietnam, were utilized to optimize the DNA extraction procedure, as well as conventional and multiplex PCR. These strains had been previously isolated from dogs and identified through morphological and biochemical tests. Additionally, the reference strains were confirmed to exhibit oxacillin (methicillin) resistance using standard antimicrobial susceptibility tests. All procedures and interpretations of antibiotic susceptibility followed the guidelines established by the Clinical and Laboratory Standards Institute (CLSI).

Field *Staphylococcus* strains were isolated from nasal and skin swabs of dogs in various veterinary clinics in Ho Chi Minh from January 2022 to June 2022. A total of 36 specimens were randomly selected from dogs showing suspected respiratory infections. Inclusion criteria for dogs diagnosed with bacterial infections included a history of an acute onset of respiratory signs, such as nasal discharge, sneezing, cough, and dyspnea. The collected swabs were initially plated onto mannitol salt agar (Oxoid, UK) and incubated for 24 hours at 37 °C. One to three suspicious colonies from different samples were selected for subculture overnight at 37 °C in Brain Heart Infusion Broth (BHI) with 30% glycerol at the final concentration. A 500-µL aliquot of each culture suspension was then prepared for DNA extraction.

DNA extraction

The genomic DNA of *Staphylococcus* isolates was manually extracted using the Tris-EDTA-NaCl-Triton X100 (TENT) method, following the protocol previously described (Hassanzadeh et al., 2016). In summary, a 500-µL cell culture in LB broth was centrifuged at 10,000 rpm for 10 minutes, and the pellets were suspended in 50-µL of TENT buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 5% Triton X100, pH 8.0). The cell suspension was then boiled at 100 °C and centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into a new sterile tube. Subsequently, cold 95% ethanol was added to the supernatant and kept at -20 °C for 20 minutes. After centrifugation at 12,000 rpm for 5 minutes, the DNA template was dissolved in 50-µL sterile distilled water and stored at -20 °C until PCR amplification.

To evaluate the efficacy of DNA extraction using the TENT method, a comparison was made with DNA extraction using a commercial DNA extraction kit, TopPURE® genomic DNA extraction kit (TBR, Vietnam). The procedure for DNA extraction using the commercial kit followed the manufacturer's instructions. The DNA templates were also stored at -20 °C until use.

Primer selection

Four primer pairs were employed for both conventional and multiplex PCR to distinguish MRSA and MRSP. The initial primer pair (27F/1492R) targets the highly conserved regions of the *16S rRNA* gene, common among Staphylococci and other eubacterial species (Weisburg et al., 1991). The second primer pair (mecA_F/mecA_R) targets the *mecA* gene, responsible for methicillin resistance in all Staphylococci (Murakami et al., 1991). The third primer set (au-F3/au-nucR) and the fourth primer set (pse-F2/pse-R5) target the *nuc* gene, facilitating the identification and differentiation of all *S. aureus* and *S. pseudintermedius*, respectively (Sasaki et al., 2010). The details of primers used in this study is described in Table 1.

Table 1 Nucleotide sequences of primers used for detection of MRSA and MRSP.

Target gene	Primer	Sequence (5' – 3')	Amplicon size (bp)	Annealing Tm (°C)	Reference
16S rRNA	27F 1492R	CAGAGTTTGATCCTGGCT AGGAGGTGATCCAGCCGCA	1,465	55	Weisburg et al., 1991
mecA	mecA_F mecA_R	AAAATCGATGGTAAAGGTTGGC AGTTCTGCAGTACCGGATTTGC	533	55	Murakami et al., 1991
nuc *(S. aureus)*	au-F3 au-nucR	TCGCTTGCTATGATTGTGG GCCAATGTTCTACCATAGC	359	57	Sasaki et al., 2010
nuc *(S. pseudintermedius)*	pse-F2 pse-R5	TRGGCAGTAGGATTCGTTAA CTTTTGTGCTYCMTTTTGG	926	57	Sasaki et al., 2010

Conventional and multiplex PCR

Conventional PCR reactions were conducted in a total volume of 20-µL, consisting of 2-µL of genomic DNA template, and 18-µL of 2X Colorless Go-Taq master mix containing $MgCl_2$, 10X PCR buffer, dNTPs, 10 units of Taq DNA polymerase (Cat #M7132, Promega, USA), and 0.5-µL of each 10-µM forward and reverse primers. PCR amplification followed this thermal profile: 94 °C for 4 min, followed by 35 cycles of 94 °C for 60 sec, 30 sec for annealing at temperatures specified in Table 1, and 72 °C for 1.5 min, with a final extension cycle at 72 °C for 10 min.

Multiplex PCR reactions followed a similar procedure to conventional PCR. They included 2-µL of DNA template in a total volume of 20-µL, composed of 2X Colorless Go-Taq master mix (Cat #M7132, Promega, USA), and 0.5-µL of each of the four primer pairs. PCR amplification was carried out under the same conditions as conventional PCR, except for a fixed annealing temperature at 56 °C for 35 sec.

Following amplification, 5-µL of the resulting PCR products were visualized on 1.3% tris-acetate-EDTA agarose gels stained with ethidium bromide alongside a 1,000 bp ladder (Bioline, UK) and photographed under UV transillumination.

RESULTS

Evaluation of DNA extraction using TENT method

Conventional PCR reactions were conducted using two primer pairs targeting the *16S rRNA* and *mecA* genes to assess the efficacy of DNA extraction with both the manual TENT method and a commercial DNA extraction kit. Figure 1 displays the amplicon products from conventional PCR reactions for the *16S rRNA* and *mecA* genes of reference strains MRSA and MRSP. The results indicate that DNA extracted using both the TENT method and the commercial kit produced positive amplification for both *16S rRNA* and *mecA* amplicons. The amplicons were observed at the expected sizes of 1,465 bp and 533 bp, respectively, with equivalent intensity of bands.

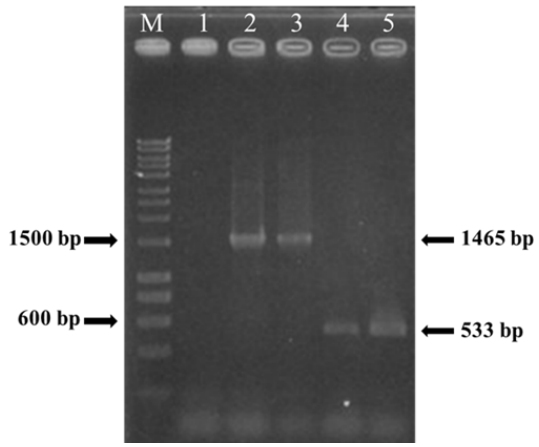

Figure 1 Conventional PCR reaction using DNA templates extracted by TENT and commercial DNA extraction kit for *16S rRNA* and *mecA* genes. Lane M: 1,000 bp ladder; lane 1: negative control; lane 2, 4: PCR amplification corresponding for *16S rRNA* and *mecA* genes using DNA template extracted from commercial kit; lane 3, 5: PCR amplification corresponding for *16S rRNA* and *mecA* genes using DNA template extracted from TENT.

Conventional PCR for evaluating specificity of individual primer pair

The specificity of each of the four primer pairs (27F/1492R, mecA_F/mecA_R, au-F3/au-nucR, and pse-F2/pse-R5) employed in this study was evaluated through conventional PCR. Figure 2 depicts the results of conventional PCR reactions, indicating successful identification of reference MRSA and MRSP strains. All targeted gene fragments exhibited single bands with expected sizes: 27F/1492R for the 16S rRNA gene at 1,465 bp, mecA_F/mecA_R for the *mecA* methicillin-resistant gene at 533 bp, au-F3/au-nucR for the *nuc* gene of MRSA at 359 bp, and pse-F2/pse-R5 for the *nuc* gene of MRSP at 926 bp.

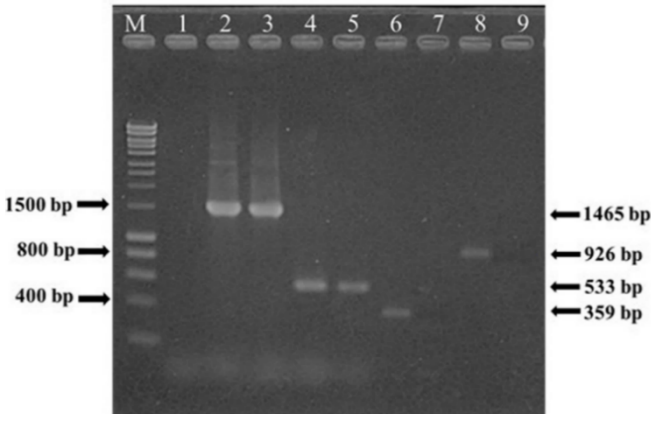

Figure 2 Conventional PCR reaction to detect and differentiate MRSA and MRSP using four primer pairs. Lane M: 1,000 bp ladder; lane 1: negative control; lanes 2, 4, 6, 8: PCR reaction using primer set targeting *16S rRNA*, *mecA*, *nuc* (*S. aureus*) and *nuc* (*S. pseudintermedius*) genes with genomic DNA template of reference MRSA; lanes 3, 5, 7, 9: PCR reaction using primer set targeting *16S_rRNA*, *mecA*, *nuc* (*S. aureus*) and *nuc* (*S. pseudintermedius*) genes with genomic DNA template of reference MRSP.

Multiplex PCR for identification of reference strains MRSA and MRSP

Multiplex PCR amplification, utilizing the four primer pair sets, was conducted to identify and differentiate MRSA and MRSP. The specific amplification produced three separate bands on the agarose gel for each DNA template of reference strains MRSA or MRSP (Figure 3). Specifically, amplicons at 1,645 bp, 533 bp, and 359 bp were observed to detect MRSA, characterized by a distinct pattern of 1,645 bp, 533 bp, and 926 bp for the identification of MRSP.

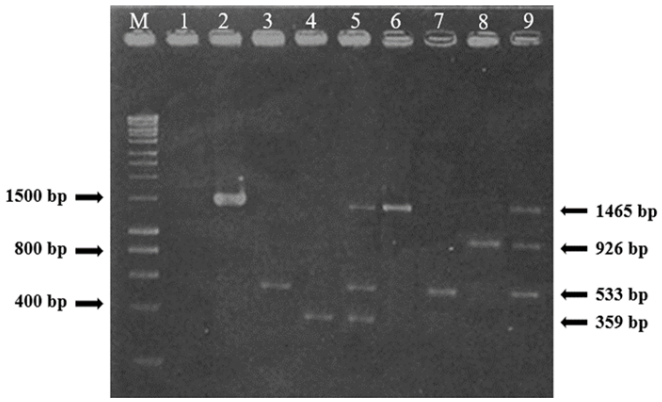

Figure 3 Multiplex PCR for identification and differentiation of reference strains MRSA and MRSP. Lane M: 1,000 bp marker; lane 1: negative control; lane 2, *16S rRNA* of *S. aureus*, 1,465 bp; lane 3: *mecA* of *S. aureus*, 533 bp; lane 4: *au-F3* of *S. aureus*, 359 bp; lane 5: multiplex amplification of *16S rRNA – mecA – au-F3* of *S. aureus*; lane 6: *16S rRNA* of *S. pseudintermedius*, 1,465 bp; lane 7: *mecA* of *S. pseudintermedius*, 533 bp; lane 8: *pse-F2* of S. *pseudintermedius* gene, 926 bp; lane 9: multiplex amplification of *16S rRNA – mecA – au-F3* of *S. pseudintermedius.*

Application of multiplex PCR for identification and differentiation of clinical isolates

To demonstrate the applicability of the multiplex PCR for detection and differentiation of clinical MRSA and MRSP isolates, a total of 11 colonies obtained from clinical samples in dogs, with unknown morphology and biological characterization, were subjected to the multiplex PCR assay. All isolates obtained were positive for the *16S rRNA* gene. Out of the 11 isolates, eight were positive for the *mecA* gene, which encodes methicillin resistance in *Staphylococcus* species; three were positive for the *nuc* gene, indicating the presence of *S. aureus*, and two were positive for the *nuc* gene, indicating the presence of *S. pseudintermedius*. No cross-reaction was observed in the multiplex PCR with the clinical isolates (Figure 4).

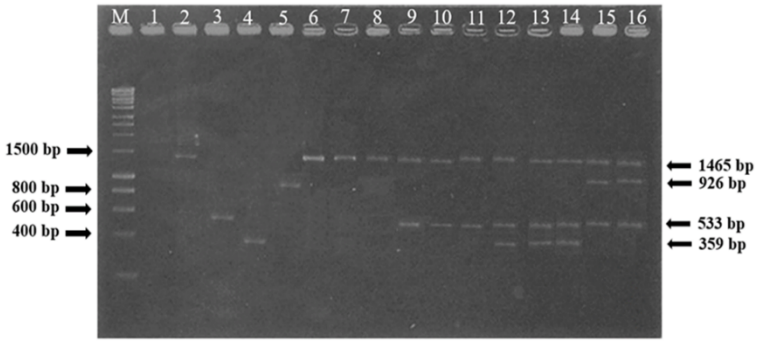

Figure 4 Multiplex PCR detection for isolates obtained from clinical samples in dogs. Lane M: 1,000 bp marker; lane 1: negative control; lane 2: positive control 16S rRNA gene; lane 3: positive control *mecA* gene; lane 4: positive control for *nuc* gene (*S. aureus*); lane 5: positive control for *nuc* gene (*S. pseudintermedius*); lanes 6–16: multiplex PCR detection for MRSA and MRSP isolated from clinical samples in dogs.

DISCUSSION

Staphylococci, especially CoPS, play a crucial role as commensal microorganisms and opportunistic pathogens in both human and veterinary medicine. The recent emergence of pathogenic CoPS carrying virulence and antibiotic resistance factors poses significant threats to human and animal health, primarily due to limited treatment options (Foster, 1996; Becker et al., 2015). Among these, MRSA and MRSP are recognized as the most important pathogenic species of CoPS. MRSA, in particular, is a major concern in human medicine, causing a variety of infections such as skin infections, pneumonia, endocarditis, osteomyelitis, and gastroenteritis (Harbarth, 2006; Feßler et al., 2018). In contrast, MRSP is frequently isolated from dogs and cats, primarily associated with skin diseases (van Duijkeren et al., 2011). Both MRSA and MRSP are significant zoonotic pathogens present in human and veterinary hospitals (Cuny et al., 2022). Despite the importance of MRSA and MRSP, their identification remains challenging. The diagnosis typically relies on the morphological and biological characterization of cultured bacteria, a time-consuming process. Moreover, distinguishing between MRSA and MRSP based on traditional phenotyping is difficult (Sasaki et al., 2007). Although various molecular methods have been reported (Costa et al., 2005; Rahman et al., 2018; Li et al., 2020), these techniques are often expensive, time-consuming, and the interpretation of results can be complicated. Therefore, there is a critical need for a simple and precise method for the identification and discrimination among MRSA, MRSP, and other CoPS species.

In our study, we devised a procedure for the effective identification of MRSA and MRSP isolated from dogs in Vietnam, employing rapid DNA extraction and multiplex PCR. This diagnostic approach combines the benefits of established strategies, featuring simplicity and cost-effectiveness in DNA extraction, while concurrently identifying molecular markers that discriminate between MRSA and MRSP, thereby enhancing control measures (Murakami et al., 1991; Weisburg et al., 1991; Sasaki et al., 2010; Hassanzadeh et al., 2016). For the multiplex PCR assay, we selected four primer pairs targeting crucial and conserved regions present in all *Staphylococcus* species, along with the methicillin-resistance gene (*mecA*) and the *16S rRNA* gene, which is ubiquitous in all bacteria. The inclusion of *nuc* genes specific to *S. aureus* and *S. pseudintermedius* further refines the discrimination process. Conducting the multiplex amplification reaction in a single PCR tube facilitates straightforward detection of *Staphylococcus* species and

differentiation of MRSA and MRSP. Additionally, this study implemented a simple and cost-effective method for genomic DNA extraction of *Staphylococcus* species using TENT, demonstrating comparable efficacy to a commercial DNA extraction kit. Described as simple, rapid, and cost-effective, this method imposes no quantitative limitations (Hassanzadeh et al., 2016).

The results of this study also demonstrate the utility of the developed multiplex PCR for detecting field MRSA and MRSP strains circulating in dogs in Vietnam. To demonstrate the applicability of its diagnostic procedure, 11 field bacterial strains, with unknown morphological and biological characteristics, were subjected to the multiplex PCR. These experiments involving field MRSA and MRSP strains were replicated to ensure the efficacy and reproducibility of our diagnostic procedure. Notably, similar success has been reported in previous studies that employed rapid detection of *Staphylococcus* species using clinical samples and animal products (Kim et al., 2001; Chiang et al., 2012; Kim et al., 2013).

It is important to acknowledge the limitations of our diagnostic procedure. Firstly, the multiplex PCR assay employed cannot identify virulence factors to distinguish pathogenic from nonpathogenic *Staphylococcus* strains. Additionally, the reliance on the culture and isolation process for DNA extraction means that the entire procedure remains lengthy and laborious. Therefore, further investigations are warranted to optimize the entire process for the identification of all *Staphylococcus* species, considering their antibiotic resistance capabilities and significance in both human and veterinary practices.

CONCLUSIONS

MRSA and MRSP have become widespread pathogens in both human and veterinary settings worldwide. The rapid detection and accurate identification of these species are crucial for the effective management of infections. This study marks the first application of a molecular diagnostic procedure using a multiplex PCR assay in Vietnam. Our method has demonstrated its utility as an effective and rapid means for the identification and differentiation of MRSA and MRSP isolated from dogs.

AUTHOR CONTRIBUTIONS

This work was conducted with contribution of all authors. **NPTN, BNTV, NTYV, NHHT, NTLA** and **LTN** designed the experimental procedures. **NPTN, BNTV, NTYV, NHHT** and **LTN** performed the experiments. **NPTN, BNTV, NTYV, NHHT, TMQ, DHT, NTPC, NKT**, **NTLA** and **LTN** interpreted the data and prepared the manuscript. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

We have no conflict of interest.

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