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# PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*

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**Objectives:** Firstly, to evaluate the current PBP3-S primers of Hasegawa *et al.* (*Microb Drug Resist* 2003; **9**: 39–46) and develop new primers for the amplification of N526 in isolates of *Haemophilus haemolyticus*. Secondly, to develop a new PCR assay for the detection (by amplification) of the N526K substitution, encoded by either the AAA or AAG single nucleotide polymorphism (SNP) at position 1576–1578 of the *ftsI* gene, in isolates of both *Haemophilus influenzae* and *H. haemolyticus*.

**Methods:** A total of 50 *H. influenzae* and 50 *H. haemolyticus* isolates, comprising N526 and N526K genotypes, were used to evaluate the performance of SNP-based PCR primers for the detection of the β-lactamase-negative ampicillin resistance (BLNAR)-defining N526K substitution in *H. influenzae* and *H. haemolyticus*, using a real-time PCR platform.

**Results:** The PBP3-S primers of Hasegawa *et al.* failed to amplify *H. haemolyticus* isolates, irrespective of their N526/N526K status, owing to an inability of the forward primer to bind the *H. haemolyticus ftsI* sequence, giving an overall sensitivity of 100% and a specificity of 40% when using all of the isolates. However, the PBP3-N526 and PBP3-N526K PCR primers designed in this study were 100% sensitive and specific, and 84% sensitive and 100% specific, respectively, for the detection of N526K-positive isolates.

**Conclusions:** Although antibiotic resistance surveillance studies on *H. influenzae* should include a definitive test for *H. influenzae*/*H. haemolyticus* identification, the new primers from this study will not only allow for PCR characterization of both *H. influenzae* and *H. haemolyticus* with respect to the N526K BLNAR substitutions, they will also stop incorrect characterization of susceptible *H. haemolyticus* isolates as low-BLNAR *H. influenzae*.

**Keywords:** *ftsI*, β-lactamase-negative ampicillin resistance, PBP3

## Introduction

Non-β-lactamase-mediated β-lactam resistance is emerging as an important resistance mechanism in clinical isolates of *Haemophilus influenzae* and usually infers low-level resistance to ampicillin and reduced susceptibility to some cephalosporins.[1] In these β-lactamase-negative ampicillin-resistant (BLNAR) isolates, resistance is attributed to two key amino acid substitutions in penicillin-binding protein 3 (PBP3) that line the active site pocket of the mature protein: N526K and, far less commonly, R517H.[1]

By definition, the BLNAR phenotype is characterized by the absence of a β-lactamase and an ampicillin MIC of ≥4 mg/L – according to CLSI breakpoints.[2] However, it is now routinely recognized that BLNAR isolates can have ampicillin MICs as low as 0.5 mg/L, which overlap the β-lactamase-negative ampicillin-susceptible (BLNAS) population, making MIC-based detection methods insensitive.[3] As a result, there has been a shift toward genotypic methods for BLNAR detection, to which there are two major approaches: *ftsI* gene sequencing or single nucleotide polymorphism (SNP)-based PCR. Sequencing of the *ftsI* gene allows the identification of both the BLNAR-defining substitutions (R517H and N526K), and also allows the detection of any additional substitutions surrounding adjacent conserved motifs of PBP3, which are being increasingly reported.[1,3] Admittedly, sequencing is the only method by which the R517H substitution can be detected, owing to an inability to design adequate SNP PCR primers for this substitution.[4–6] By contrast, current SNP-based PCR approaches interrogate the 526 position of PBP3 for detection of the N526K BLNAR genotype, and such isolates identified by genotype are referred to as genetically BLNAR (gBLNAR).[4,7]

The PBP3-S assay of Hasegawa *et al.*[4] is the most widely used of these SNP-based approaches, and is designed to amplify N526 of susceptible isolates but not N526K of gBLNAR isolates.[4] Interestingly, however, with this PCR method, the detection of the key

gBLNAR-defining N526K substitution is achieved through non-amplification, and may pose some interpretational and methodological issues.[8,9] Nakamura *et al*.[8] attempted to rectify this by designing a PCR primer set to separate N526 and N526K by amplification in the presence of the N526K substitution.[8]

A subsequent evaluation[9] of these primer sets highlighted a shortcoming in the N526K-specific primer design of both Hasegawa *et al*.[4] and Nakamura *et al*.[8] Witherden *et al*.[9] observed a previously unrecognized AAT to AAA nucleotide change at position 1576–1578 of the *ftsI* gene in isolates with N526K, in addition to the previously described AAT to AAG change, on which all previous 526 specific primer design was based.[4,8] In particular, the authors noted that although N526K-positive isolates were encoded by the AAA and AAG codons on a ∼50:50 basis, all N526K-positive isolates encoded by the AAA codon would be incorrectly categorized.[9]

The issues surrounding these PCR-based detection methods for the N526K substitution are further compounded by the recent identification of the N526K substitution (encoded by both AAA and AAG) in other *Haemophilus* species, including the phylogenetically closely related *Haemophilus haemolyticus*.[10] The report of N526K in isolates of *H. haemolyticus* is of particular concern, as non-haemolytic *H. haemolyticus* is often misidentified as *H. influenzae* in *H. influenzae* prevalence and surveillance studies using nasopharyngeal or other upper respiratory specimens.[11–13]

In this study, we set out to evaluate whether the commonly used PBP3-S primers of Hasegawa *et al*.[4] could be used to screen for the N526K substitution in isolates of *H. haemolyticus*, whilst also attempting to design new SNP PCR primers that could detect N526 of genetically BLNAS (gBLNAS) isolates and the N526K substitution of gBLNAR isolates by positive amplification, irrespective of how it may be encoded in both isolates of *H. influenzae* and *H. haemolyticus*.

## Materials and methods

### Bacterial isolates

A total of 100 upper respiratory tract (URT) normal flora isolates, comprising 50 *H. influenzae* and 50 *H. haemolyticus*, were included in this study. All the isolates had been partially characterized as part of another study with respect to species designation by PCR for *sodC*, *fucK* and *hpd* genes, and partial *ftsI* gene sequences were also available.[9,10] Isolates could be classified into one of four genotypes: *H. influenzae* gBLNAS isolates ($n=25$), *fucK/hpd* positive and N526; *H. influenzae* gBLNAR ($n=25$), *fucK/hpd* positive and N526K; *H. haemolyticus* gBLNAS ($n=30$), *fucK/hpd* negative, *sodC* positive and N526; and *H. haemolyticus* gBLNAR ($n=20$), *fucK/hpd* negative, *sodC* positive and N526K. Full characteristics of the study isolates and the GenBank accession numbers for the *ftsI* gene sequences are given in Table S1 (available as Supplementary data at *JAC* Online).

### Real-time PCR

Three sets of primers were used to interrogate position 526 of PBP3 (position 1576–1578 of the encoding *ftsI* gene) and are given in Table 1. In brief, the PBP3-S primers of Hasegawa *et al*.[4] and the modified PBP3-N526 primers designed in this study, target and amplify isolates with the normal amino acid at position 526 of PBP3 encoded by AAT. However, the PBP3-N526K primers designed in this study amplify N526K encoded by either the AAA or AAG codon SNP.

Real-time PCR reactions were performed on the IQ5 Cycler (Bio-Rad, Hercules, CA, USA) using 12.5 μL of SYBRGreen Supermix (Bio-Rad, Gladsville, Australia) and 1 μL of genomic DNA as template in a total reaction volume of 25 μL. In both the PBP3-S and PBP3-N526 assays 0.25 μL of 20 μM forward and reverse primers were used, whilst in the PBP3-N526K assay 0.25 μL of the forward primer and 0.5 μL of the degenerate reverse primers mixed in a 2:1 ratio (N526K-A:N526K-G) were used. PCR cycling conditions were as follows: 30 cycles at 95°C for 10 s, 53°C for 30 s and 72°C for 30 s.

**Table 1.** PCR primers used for the interrogation of position 526 of PBP3

| Primer set | Sequence (5′-3′)[a] | Position[b] | Primer specificity[c] | Reference |
|---|---|---|---|---|
| PBP3-S | | | | |
| PBP3S-F | GATACTACGTCCTTTAAATTAAG | 1048–1070 | Hi | Hasegawa *et al*.[4] |
| PBP3S-R | GCAGTAAATGCCACATACTT**A** | 1598–1578 | 3′ A amplifies N526 | Hasegawa *et al*.[4] |
| PBP3-N526 | | | | |
| *ftsI*-FL | ACGGTATTGAGGGCATTGAG | 620–639 | Hi/Hh | this study |
| PBP3S-R | GCAGTAAATGCCACATACTT**A** | 1598–1578 | 3′ A amplifies N526 | Hasegawa *et al*.[4] |
| PBP3-N526K | | | | |
| *ftsI*-FL | ACGGTATTGAGGGCATTGAG | 620–639 | Hi/Hh | this study |
| N526K-R-A | GCAGTAAATGCCACATATGT**T**[d] | 1598–1578 | 3′ T amplifies N526K (AAA) | this study |
| N526K-R-G | GCAGTAAATGCCACATATCT**C**[d] | 1598–1578 | 3′ C amplifies N526K (AAG) | this study |

[a]Bold text: SNP-specific nucleotides; 3′ A is specific for the AAT codon and 3′ T and C, respectively, are specific for the AAA and AAG codons.
[b]Numbering is based on the nucleotide sequence of *H. influenzae* Rd Kw20 (GenBank accession no. L42023).
[c]Hi primer specificity refers to the primer's ability to bind the *ftsI* gene sequence of *H. influenzae*; Hi/Hh primer specificity refers to the primer's ability to bind the *ftsI* gene sequence of both *H. influenzae* and *H. haemolyticus*; and 3′ primer specificity refers to the primer's ability to bind and amplify AAT or AAA/AAG, respectively, in isolates of both *H. influenzae* and *H. haemolyticus*.
[d]The N526K degenerate reverse primers were designed to have a 3′ mismatch specific for either the AAA or AAG codon, whilst an additional mismatch at the 3′-2 position was incorporated to prolong any amplification of isolates with the AAT codon. Furthermore, we incorporated the nucleotide T at the 3′-3 position as analysis of *ftsI* genes in GenBank revealed that nearly all isolates have an A at this position (1581 bp) instead of G at the corresponding position in the Rd KW20 sequence.

**Table 2.** Sensitivity and specificity of the SNP PCR primers evaluated for the interrogation of the 526 region of PBP3

| | Primer sets[a] | | |
|---|---|---|---|
| | PBP3-S | PBP3-N526 | PBP3-N526K |
| True positive, *n* | 45 | 45 | 38 |
| True negative, *n* | 22 | 55 | 55 |
| False positive, *n* | 33[b] | 0 | 0 |
| False negative, *n* | 0 | 0 | 7 |
| Sensitivity, %[c] | 100 | 100 | 84 |
| Specificity, %[c] | 40 | 100 | 100 |
| Very major errors, % | 0 | 0 | 7 |
| Major errors, % | 33 | 0 | 0 |
| Categorical agreement, % | 67 | 100 | 93 |

[a]Primer sets: PBP3-S, primers of Hasegawa *et al.*,[4]; PBP3-N526 and PBP3-N526K, primers designed in this study.
[b]None of the *H. haemolyticus* gBLNAS isolates ($n=30$) amplified and neither did three *H. influenzae* gBLNAS isolates.
[c]Sensitivity and specificity of primer sets was determined using the designation of an isolate as N526K positive by PCR (either by amplification or non-amplification, depending on the primer set) as a positive result, with the translated amino acids from the *ftsI* gene sequences used as the gold standard.

### *Data analysis*

The sensitivity and specificity of the primer sets were determined using the designation of an isolate as N526K positive by PCR (either by amplification or non-amplification, depending on the primer set) as a positive result, with translated amino acids from the *ftsI* gene sequences used as the gold standard.

## Results and discussion

The sensitivities and specificities of all PCR primers evaluated in this study are given in Table 2. In summary, the PBP3-S primers of Hasegawa *et al.*,[4] designed to amplify N526 in gBLNAS isolates, failed to amplify N526 in all *H. haemolyticus* gBLNAS isolates and three gBLNAS *H. influenzae* isolates. In the *H. haemolyticus* gBLNAS isolates this was attributed to a 12 bp mismatch between the *ftsI* sequence of *H. haemolyticus* ATCC 33390 (GenBank accession number AB267855) and the PBP3-S forward primer over its 22 bp length (data not shown). As a result, all *H. haemolyticus* isolates tested with these primers would be incorrectly categorized as N526K positive (designated low-BLNAR) according to the PCR algorithm of Hasegawa *et al.*,[4] irrespective of their *ftsI* gene status.

This is of major concern, as there is a growing trend to use URT specimens in *H. influenzae* surveillance studies, many of which do not use adequate molecular tests for the differentiation of *H. influenzae* and *H. haemolyticus*.[14,15] In fact, recent retrospective analyses of phenotypically identified *H. influenzae* collections have shown that 17%–27% of nasopharyngeal isolates and 65% of oropharyngeal isolates were actually non-haemolytic *H. haemolyticus* on molecular characterization.[11,12] Antibiotic resistance surveillance studies of *H. influenzae* isolated from URT sources are not uncommon, and frequently use only PCR for confirmation of outer membrane protein P6 for *H. influenzae*,[4] a method that is now known to be inadequate for *H. influenzae* and *H. haemolyticus* separation.[11,16] In one such surveillance study on 264 presumptive *H. influenzae* isolates from URT specimens, the authors report a high incidence (37.1%) of the N526K genotype, based on non-amplification with the PBP3-S primers.[7] The authors of the study acknowledge that their methodology may not differentiate *H. influenzae* from *H. haemolyticus*; however, in light of the findings of our study, this high prevalence of the N526K genotype may actually represent a significant proportion of susceptible *H. haemolyticus* isolates that fail to amplify with the PBP3-S PCR. Unfortunately, as these studies did not perform *ftsI* gene sequencing for N526K confirmation, there are no data available in GenBank to confirm these findings. In addition, in another study using respiratory isolates, 175/229 (76%) presumptive *H. influenzae* isolates tested positive for N526K based on non-amplification using the PBP3-S PCR and were subjected to *ftsI* gene sequencing for confirmation. Of the 175 isolates, 86 were reported to have the N526K substitutions and were defined as gBLNAR.[6] However, the *ftsI* sequence data for the remaining 89 isolates were omitted without explanation in the paper.[6] Although purely speculative it is conceivable that, when sequenced, the *ftsI* genes of many of these isolates were not N526K (gBLNAR) and the non-amplification with the PBP3-S primers may have been because they were *H. haemolyticus*. Alternatively, if the PBP3-S PCR results were accurate, then the proportion of gBLNAR would be 76% – a prevalence much higher than ever reported elsewhere.

By contrast, the situation is different when examining clinical isolates of *H. influenzae*. Although Murphy *et al.*[11] showed that 40% of presumptive *H. influenzae* isolates from patients with chronic obstructive pulmonary disease (COPD) were actually non-haemolytic *H. haemolyticus*, more recent studies have been unable to replicate these findings.[13,17,18] In fact, Nørskov-Lauritsen[17] found only 0.4% (2/480) of presumptive *H. influenzae* isolated from a range of clinical samples actually to be *H. haemolyticus*, whilst Fenger *et al.*[13] found 0.5% (1/192) of *H. influenzae* isolates isolated from cystic fibrosis patients actually to be non-haemolytic *H. haemolyticus*. Furthermore, due to the limited association between *H. haemolyticus* and disease, the use of Hasegawa *et al.*[4] PBP3-S PCR primers on clinical samples is unlikely to be affected by the misidentification of non-haemolytic *H. haemolyticus*. Nevertheless, the findings of this study highlight the need for authors to use the Hasegawa *et al.*[4] PBP3-S PCR primers with caution and to optimize PCR protocols in-house against the gold standard of *ftsI* gene sequencing.[10]

In comparison, the PBP3-N526 primers of our study were 100% sensitive and specific for the separation of N526 isolates (which amplify) from N526K-positive BLNAR isolates (which fail to amplify) in isolates of both *H. influenzae* and *H. haemolyticus*, and thus can be used in surveillance studies when URT specimens are used.

Similarly, the degenerate reverse primers in the PBP3-N526K PCR of this study, designed to amplify N526K-positive isolates encoded by either the AAA or AAG codon, were sensitive (84%) and specific (100%). Although these primers consistently and correctly did not amplify N526 isolates within 30 cycles, they also failed to amplify seven N526K-positive *H. influenzae* isolates (giving a very major error rate of 7%). Analysis of the *ftsI* sequences of these isolates with respect to primer binding sites (no unexpected mismatches were identified within the forward or reverse primer

binding sites for these isolates) and repeating the PCRs did not elucidate the reasoning for the non-amplification noted.

As a result, we propose that the PBP3-N526 and PBP3-N526K primer sets be used together when screening for the N526K substitution, especially in surveillance studies when isolate speciation is not confirmed using adequate molecular tests. Furthermore, if any isolate either fails to amplify with both primer sets or amplifies with both primer sets, or if additional information about BLNAR-associated substitutions is preferred, we strongly recommend full *ftsI* gene sequencing be performed for confirmation.

In summary, we describe the use of a new highly sensitive and specific SNP-based PCR algorithm that positively detects the normal N526 and abnormal N526K genotypes in isolates of both *H. influenzae* and *H. haemolyticus*, which is an important tool for surveillance of the N526K BLNAR genotype in XV-dependent *Haemophilus* species.

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## Transparency declarations

None to declare.

## Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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