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Discovery of IS1515 transposition into the Spn9802 fragment of *Streptococcus pneumoniae* and its implications for PCR-based diagnostic assays

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Abstract

This study investigates a diagnostic challenge in detecting *Streptococcus pneumoniae* using a Spn9802-targeted PCR assay. Four *S. pneumoniae* isolates tested *lytA*-positive but Spn9802-negative due to an insertion sequence (IS1515) within Spn9802, which was confirmed by whole-genome sequencing. This insertion may lead to false-negative results, posing concerns for PCR-based diagnostics. The study emphasizes the need for dual-target PCR and regular updates to diagnostic primers, underscoring the importance of continuous assay evaluation and post-market surveillance to ensure reliable pathogen detection.

Main text

Streptococcus pneumoniae is a leading cause of pneumonia and bacterial meningitis [1]. Standard detection relies on culture, followed by optochin susceptibility and/or bile solubility testing [2]. These methods are time-consuming and may lack the sensitivity or specificity needed to distinguish *S. pneumoniae* from other viridans group streptococci [3]. Closely related *Streptococcus* species often have highly similar protein profiles, making identification by MALDI-TOF MS challenging.

PCR-based assays have improved *S. pneumoniae* detection. At our institution, a Spn9802-targeted PCR adapted from Suzuki et al. [4] is used for direct testing of clinical samples, and the same primers and probe are applied in a duplex PCR with *lytA*-specific oligonucleotides adapted from Carvalho et al. [5] to differentiate *S. pneumoniae* from *S. pseudopneumoniae* using cultured isolates [6]. Typically, *S. pneumoniae* isolates test positive for both targets, whereas Spn9802 positivity alone indicates *S. pseudopneumoniae*. Other streptococci, such as *S. mitis*,

generally test negative for Spn9802, and previously reported false-positives were most likely caused by wrong determination [6].

Between 2017 and 2024, duplex PCR analysis identified 107 *S. pneumoniae* isolates. Four isolates (two from our center and two referred from a nearby hospital) tested *lytA*-positive but Spn9802-negative, a profile also previously reported in Spain [7]. To confirm species identity and investigate Spn9802 negativity, whole-genome sequencing (WGS) was performed using Oxford Nanopore PromethION R10.4.1 chemistry, assembled with Flye v2.9.3 [8] and analysed using Geneious v10.2.6.

WGS revealed an 870-bp insertion within Spn9802 in all four isolates, explaining the false-negative PCR results (Fig. 1) and confirming *S. pneumoniae* identity via *tuf* [9] and *rpoB* [10] analysis. The insertion was highly conserved among the four isolates (≤ 1 nucleotide difference) and 99.8% similar to IS1515 (GenBank: Z86112.1). IS1515 has been reported within *cap1E* [11] and *ply* [12], affecting capsule production and pneumolysin expression, respectively. Our results demonstrate that IS1515 can also insert within Spn9802, although its phenotypic effect is unknown.

IS1515 is common, present in 5,788 (17%) of 33,303 *S. pneumoniae* genomes in the PubMLST Pneumococcal Genome Library [13], sometimes up to 13 copies per genome. In the four isolates, the flanking Spn9802 regions are highly AT-rich (77% over 35 bp), consistent with known

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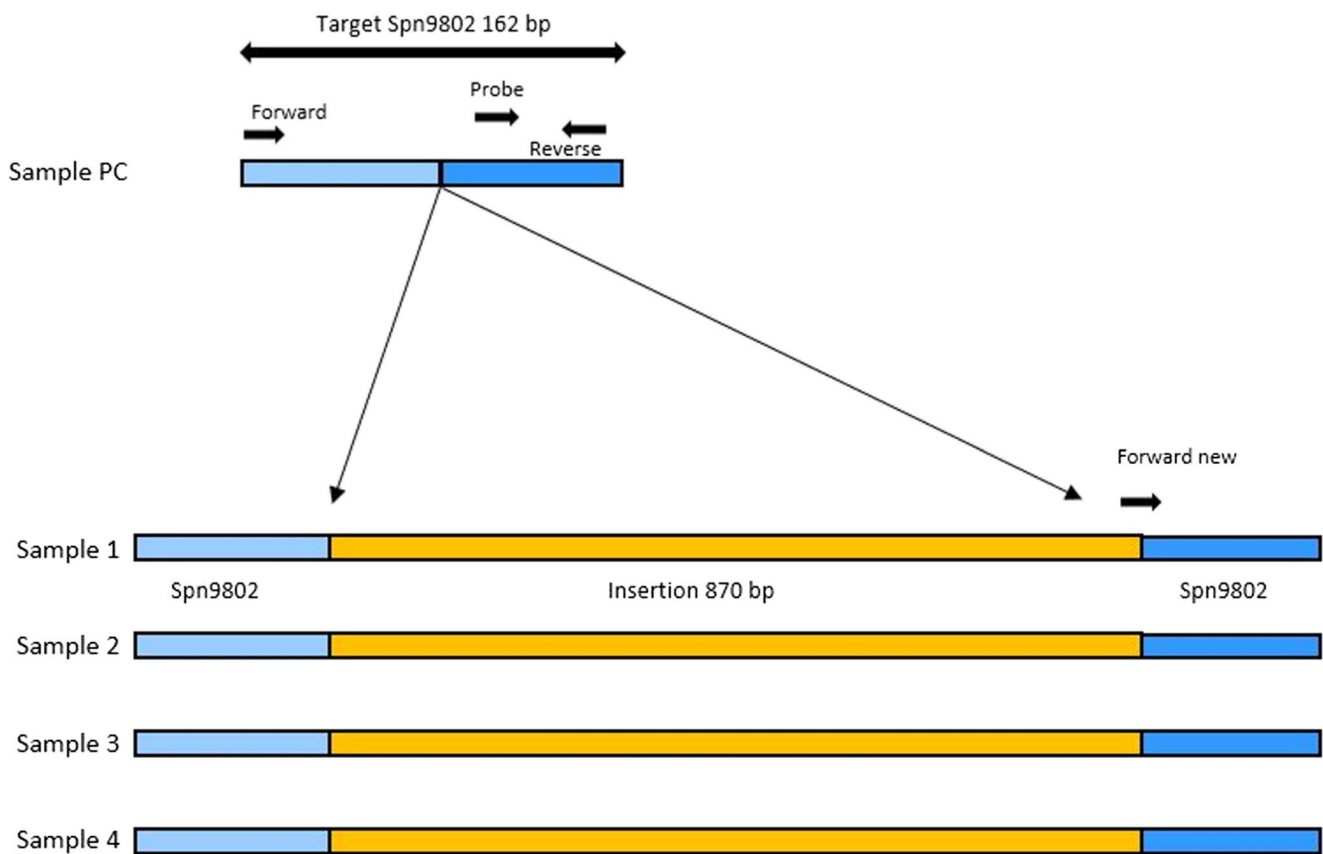


Fig. 1 Schematic overview of the Spn9802 region, showing the location of the original PCR oligonucleotides and the 870 bp insertion identified in four bacterial strains (Sample 1–4). In the updated PCR, a

new forward primer was added at the 3' end of the insertion, while the original probe and reverse primer remain unchanged

insertion hotspots [14]. Despite these favorable conditions, Spn9802 insertions appear rare, as none were observed in published genomes. This observation suggests that additional factors may influence transposition, or that insertions might occur more frequently but are subsequently lost, although this cannot be determined with the currently available data.

Nevertheless, the IS1515 insertion in Spn9802 may pose a diagnostic concern when Spn9802 PCR is used as a single-target assay to detect *S. pneumoniae* DNA directly from clinical samples [15–17], as this insertion could lead to missed infections. To mitigate this issue, a new PCR primer (5'CGAACAAGTCTATTGGATTTTATAA TGAG-3') was designed to anneal partly within IS1515 and partly within Spn9802 (Fig. 1), successfully detecting all four affected isolates. In addition to updating the PCR, targeting at least two independent genomic regions is recommended, as dual-target PCR can reveal future discrepancies and prompt additional analyses. Based on publicly available information, no commercial assays are known to rely solely on Spn9802, but the possibility cannot be excluded because the exact targets are generally

undisclosed. It also remains unclear whether this insertion affects *S. pseudopneumoniae* identification. Although the targeted Spn9802 region is nearly identical to *S. pneumoniae*, the limited GenBank genomes preclude confirmation of IS1515 presence in this species.

Insertion sequences disrupting PCR detection are widely reported, including *orfX*/SCCmec in methicillin-resistant *Staphylococcus aureus* (MRSA) [18] and *mcr-1* in *Enterobacteriaceae* [19], with Spn9802 providing an additional example in *S. pneumoniae*. These examples underscore the importance of dual-target testing and ongoing evaluation and vigilance regarding PCR primers and probes, including those used within CE marked assays. The In-Vitro Diagnostics Regulation (IVDR) requires clinical performance data and post-market surveillance for both CE-IVDs and in-house IVDs (IH-IVDs) [20], and the requirements for IH-IVD use may push laboratories towards commercial devices. Because the design and sequences of oligonucleotides are proprietary, laboratories should rely on the post-market surveillance of the manufacturers and assay performance reports of the users and the lacking information could compromise the reliability of pathogen detection.

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Author contributions Author contributions: E.C. and S.B. designed the research. E.C. developed and implemented the new PCR primer. M.K. and S.B. analyzed the WGS data. The duplex real-time PCR was developed by E.W. and she was responsible for the performance of the PCR in the routine diagnostics. M.T. and S.B. wrote the manuscript. S.B., M.K., E.C., and E.W. revised the paper for important intellectual content.

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Data availability The sequence data that are connected to this article are uploaded to the Sequence Read Archive database with accession number PRJNA1354391.

Declarations

Conflict of interest The authors declare no competing interests.

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