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SHORT COMMUNICATION

Assessment of the Optochin Susceptibility Test to Differentiate *Streptococcus pneumoniae* from other Viridans Group Streptococci

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SUMMARY

Background: ***Streptococcus pneumoniae*** **identification has traditionally been based on two biochemical tests, susceptibility of pneumococci to optochin and solubility in bile-salt solution. Due to slowness and sometimes difficulty in interpretation, the bile solubility test has fallen into disuse. The main objective of this work was to assess the current effectiveness of the optochin susceptibility test in pneumococcal identification in clinical practice.**
Materials and Methods: **Overall 126 viridans group streptococci consecutively isolated from respiratory samples were analyzed using the optochin susceptibility test by picking one colony from the culture. Sixty-two were initially considered optochin susceptible, and 64 were considered optochin resistant and analyzed with the bile solubility test. If a discrepancy between the tests was observed (i.e., whether an isolate was optochin susceptible and bile insoluble or optochin resistant and bile soluble), then the optochin susceptibility test was repeated, adjusting the inoculum to a McFarland standard of 0.5. Species were identified by sequencing the *lytA* and *recA* genes.**
Results: **Twelve discrepancies were initially observed. The result of the repeated optochin test showed that the initial optochin test of 4 isolates had been wrongly interpreted. Of the remaining 8 discrepancies, 2 optochin-resistant bile-soluble isolates were identified by gene sequencing as *S. pneumoniae*, and of the 6 optochin-susceptible bile-nonsoluble isolates, 3 were identified as *Streptococcus mitis* and 3 as *Streptococcus pseudopneumoniae*.**
Conclusions: **The optochin test correctly identified 90.5% of all recent viridans group streptococci clinical isolates which include both optochin susceptible (62/126 = 49.2%) and optochin resistant (64/126 = 50.8%) strains. Of the group of optochin susceptible viridans, 87.5% were correctly identified, and 93.5% of the optochin resistant group were correctly identified. However, this technique does not correctly differentiate between *S. pneumoniae* from other viridans group streptococci in the clinical setting. Additional testing is needed for that identification.**
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KEY WORDS

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INTRODUCTION

Historically, *Streptococcus pneumoniae* identification has been based on its optochin susceptibility and bile solubility. However, largely due to laborious and time-consuming technical issues, the bile solubility test has fallen into disuse, leaving the optochin susceptibility test as the main method for pneumococcal identification [1]. Optochin (ethylhydrocuprein hydrochloride), a de-

rivative of quinine, was originally proposed as a possible treatment of pneumonia [2]. However, since 1955 its use has been reduced to laboratory diagnosis due to its high toxicity for humans [3,4]. Bowers and Jeffries reported that a filter paper moistened with optochin positioned on the surface of a horse-blood agar plate previously inoculated with *S. pneumoniae* produced an inhibition zone [4]. This simple test allowed the differentiation of *S. pneumoniae* from other viridans group streptococci (VGS), which were consistently optochin resistant. The first optochin-resistant *S. pneumoniae* strain was described in 1987 [5], and in the subsequent 3 decades an increasing number of optochin-resistant *S. pneumoniae* isolates has been reported [6,7]. However, in contrast to what occurs with optochin-resistant pneumococci, the description of optochin-susceptible VGS is extremely limited [8,9].

The aim of this work was to establish the reliability of the optochin susceptibility test as a unique tool to differentiate between *S. pneumoniae* and VGS in clinical practice.

MATERIALS AND METHODS

During the winter of 2017, the optochin susceptibility of 126 alpha-hemolytic streptococci isolates was tested. The isolates were collected from lower respiratory tract samples of patients with suspected lower respiratory tract infection. The optochin susceptibility test was directly performed on one or two isolated colonies (direct test) using 6 mm disks impregnated with 5 µg of ethylhydrocupreine hydrochloride per disk (Becton, Dickinson and Co, Franklin Lakes, NJ, USA) on Trypticase Soy Agar with 5% sheep blood (bioMérieux, Marcy l'Étoile, France) and incubation for 18 - 24 hours in a 5% CO_2 atmosphere. Isolates were considered optochin susceptible (Opt-S) if they showed an inhibition zone ≥ 14 mm and optochin resistant (Opt-R) if no inhibition zone (0 mm) was observed. Isolates with an inhibition zone < 14 mm (13/62) were grouped as *S. pneumoniae* and included in the Opt-S group [10]. The first 62 Opt-S and 64 Opt-R alpha-hemolytic streptococci isolates were arbitrarily selected for the study.

The bile solubility test was performed using the tube test method and read by the naked eye. Streptococcal isolates showing a decrease in the turbidity of the tube containing the bacterial suspension (to which deoxycholate had been added) were considered bile soluble (Bil-S), whereas isolates without a change of turbidity after deoxycholate addition were considered bile nonsoluble (Bil-R). Using the direct plate method on blood agar plates, an isolate was considered Bil-S if the colony to which the deoxycholate had been added had disintegrated after incubation for 30 min at 35°C. If no disintegration was seen, then the isolate was considered Bil-R. When the results of the optochin and the bile susceptibility tests matched, a definitive identification of *S. pneumoniae* (Opt-S and Bil-S) or VGS (Opt-R and Bil-R) was made.

When the optochin and bile solubility tests did not agree, the optochin test was repeated, adjusting the inoculum to a turbidity of 0.5 McFarland standard (1.5 x 10^8 colony-forming units/mL).

If the data remained discordant, then a PCR of a 313 bp fragment of the *rec*A gene with DNA extracts obtained by the NUCLISENS easyMag® system (bioMérieux, France) was performed as previously described [12]. Subsequent sequencing analysis was performed using the leBIBI database (https://umr5558-bibiserv.univ-lyon1.fr/lebibi/lebibi.cgi).

In addition to *recA* gene sequencing, the 954 bp of the *lytA* gene was also sequenced as previously described [9]. Non-S. *pneumoniae* isolates characteristically show a deletion of 6 nucleotides (ACAGGC) at positions 868 to 873 corresponding to amino acids Thr290-Gly291, or a Val-317-Thr (V317T) mutation.

Serotyping was performed with the Quellung reaction using polyclonal antisera (Statem Serum Institut, Copenhagen, Denmark) and a single-tube multiplex PCR with fluorescently labelled primers followed by amplicon analysis using automated fluorescent capillary electrophoresis [13].

To confirm that nontypeable *S. pneumoniae* isolates were unencapsulated, a PCR for the amplification of the capsular *cpsA* gene, common to all types of capsular loci, was performed [14]. Isolates not amplifying the *cpsA* capsular gene were considered unencapsulated pneumococci.

RESULTS

Overall, 126 alpha-hemolytic streptococci isolates were included in the study, 62 Opt-R and 64 Opt-S as measured in the "direct test". There was an agreement between the screening with Optochin and LytA and RecA sequencing in 58/64 Opt-S, with 6 discrepancies identified as *S. mitis* (n = 3) and *S. pseudopneumoniae* (n = 3), and 60/62 Opt-R where 2 isolates were identified as pneumococcus.

Optochin-resistant clinical isolates

The bile solubility test showed that 6.4% (4/62) of Opt-R isolates were Bil-S. The repeated optochin susceptibility test adjusting the inoculum to a McFarland 0.5 suspension confirmed the 4 isolates as Opt-R, but the analysis of the *rec*A and *lyt*A genes identified these 4 Opt-R Bil-S isolates as *S. pneumoniae*.

To confirm the results of gene sequencing, 3 randomly selected Opt-R isolates were subjected to *recA* and *lyt*A gene sequencing, and the 3 were identified as *S. mitis* by sequencing both genes. The 3 isolates showed the characteristic 6-nucleotide deletion (ACAGGC) at positions 868 to 873.

Table 1. Optochin susceptibility results according to two different tests compared to LYT A, RecA sequencing, and bile solubility test.

Optochin susceptibility (BD Opt disc)	Optochin susceptibility (BD Opt disc) 0.5 McFarland	Bile Solubility test	LytA	RecA amplification and sequencing
Optochin susceptible (n = 54)	Optochin susceptible (n = 58)	64	negative (n = 6)	*S. mitis* (n = 3) *S. pseudopneumoniae* (n = 3)
Optochin resistant (n = 58)	Optochin resistant (n = 60)	62	positive (n = 2)	*S. pneumoniae* (n = 2)

Optochin-susceptible clinical isolates

Overall, 15.6% (10/64) of Opt-S isolates identified by the direct optochin test were Bil-R. The repeated optochin susceptibility test with an adjusted inoculum showed that 4 of these 10 isolates were indeed Opt-R. The 6 Opt-S Bil-R isolates were identified by *recA* gene sequencing as *mitis* (n = 3) and *S. pseudopneumoniae* (n = 3).

The direct optochin test was able to correctly identify 88.9% (112/126) of alpha-hemolytic strains, 93.5% (58/62) of Opt-R isolates, and 84.4% (54/64) of Opt-S isolates. The difference between the misidentifications of Opt-S and Opt-R strains with the direct optochin test was not statistically significant (p = 0.115).

DISCUSSION

In this work, we showed that the direct optochin test is able to differentiate most *S. pneumoniae* isolates from other related VGS. However, it was also proven that with independence of the optochin test used (direct test from one isolated colony or adjusted to a McFarland standard), there are *S. pneumoniae* isolates with intrinsic resistance to optochin and some VGS are intrinsically susceptible to optochin. On the other hand, the bile solubility test, in any of its variants (test tube as well as direct testing on blood agar plate), was able to discriminate *S. pneumoniae* from other very closely related species of the VGS with an accuracy of 100%. The bile solubility test has the advantage of being a reliable technique that can be done in all laboratories with no special equipment or skilled personnel required. The results of our study support Yahiaoui's recommendation for a new identification protocol that excludes the optochin susceptibility test for *S. pneumoniae* species identification and instead solely uses the bile solubility test [15]. Other authors have highlighted the inconveniences of the test tube bile solubility variant, such as the measure of subjectivity in the interpretation of results and difficulties in their reproduction from one laboratory to other [16].

It has been reported that the optochin inhibition zone can vary considerably depending on the serotype under analysis, with variations of up to 3 mm. Some serotypes (e.g., serotype 22F almost always) even have an inhibition zone that is usually < 14 mm [16]. Nevertheless, other authors have reported that optochin resistance is not related to the *S. pneumoniae* serotype, but rather to geographic distribution [17,18]. Of the two Opt-R *S. pneumoniae* isolates of our study, one was serotype 11A and the other was a non-encapsulated isolate. Serotype 11A has also been described as circulating among the most common Opt-R pneumococci serotypes in Portugal.

S. pneumoniae isolates presenting a variety of *atpC* gene mutations, including the characterization of 5 novel types of mutations, are associated with optochin resistance [19]. In previous studies on the utility of the optochin test to detect *S. pneumoniae* isolates, the focus had been placed on the isolation of Opt-R pneumococci, which seemed to have become increasingly common [7,20].

In our study, beyond the detection of Opt-R *S. pneumoniae* isolates, which only represented 3.2% (2/62) of pneumococci, what must be highlighted is the relatively high rate of Opt-S VGS isolates (6.3% [4/64]) that could lead to an over-diagnoses of *S. pneumoniae* infections and consequently to over-prescription of antibiotics. Although Opt-S VGS isolates have been previously described [8], their description is outdated [8,9,21] or limited to a case report, in which the severity of the infection caused by an Opt-S *S. mitis* is reported due to its singularity [22].

We shall briefly discuss using the matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) technique to support 100% differentiation of *S. pneumoniae* from other streptococci of the viridans group.

MALDI-TOF mass spectrometry (MS) is an automated method for identification of bacterial isolates. Although this technique has many advantages (e.g., it is fast, easy to use, and cost-effective), sometimes the Biotyper 3.1 (Bruker Daltonics, Bremen, Germany) database may provide untrustworthy results [23].

Recently, MALDI-TOF MS spectrum fingerprint analysis has been used not only as an automated identification tool in clinical microbiology laboratories, but also as a technology to differentiate organisms at the species

level and as a serotyping technique. It has shown very poor results and great measurement discrepancies within the reports published in the literature, as happens with VGS because the similarity in their mass spectra is too high. Therefore, it is sometimes difficult to differentiate the species of this group [23,24]. A new technology based on MALDI-TOF MS and the development of a new algorithm have recently been described as having great success at differentiating the species of the viridans group [25]. However, two things should be highlighted. The first differentiation is based on the bile solubility test and optochin test, so if we have followed this first differentiation for the differentiation among different species, then 87.5% of the optochin susceptible and 93.5% of the optochin resistant species had been erroneously identified. Second, although this technology seems promising, more strains of different geographic zones, clonal complexes, and serotypes should be tested to assess the viability for any type of strains among VGS.

To date, most laboratories base *S. pneumoniae* identification only on the direct optochin test. The infrequent but important misidentifications in the routine use of the optochin test for differentiation between *S. pneumoniae* and VGS makes it advisable to use the performance of the bile solubility test for *S. pneumoniae* identification. According to the results presented here, together with other previous reports, [15,21], the bile solubility test is currently the best technique for routine *S. pneumoniae* identification.

Declaration of Interest:

The author has declared that no competing interests exist.

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