A Simple, Semiselective Medium for Anaerobic Isolation of Anginosus Group Streptococci from Patients with Chronic Lung Disease

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The anaerobic isolation of anginosus group streptococci (AGS) from respiratory specimens containing diverse microbiota using a semiselective blood agar medium incorporating nalidixic acid and sulfamethazine (NAS) is described. AGS were detected in 60% of tested sputa from patients with cystic fibrosis, chronic obstructive pulmonary disease, and bronchiectasis. This demonstrates NAS to be a diagnostic tool for detecting AGS within the complex microbial communities associated with chronic lung disorders.

Respiratory infections in patients with chronic lung disorders (CLDs) are a frequent and important cause of morbidity. In patients with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), and bronchiectasis, recurrent intermittent inflammatory events or pulmonary “exacerbations” (PE) contribute to the progressive decline in lung function that is characteristic of these diseases. Among the triggers of PE, changes in the dynamics of the commensal and colonizing microbial flora are increasingly recognized as important (1, 2, 4, 6, 9). In CF PE, a potential role of the oropharyngeal commensal anginosus group streptococci (AGS) (Streptococcus anginosus, Streptococcus intermedius, Streptococcus constellatus subsp. constellatus, and Streptococcus constellatus subsp. pharyngis) has been highlighted. AGS, which are often overlooked as oropharyngeal contaminants in respiratory samples, have been shown to proliferate within the lung prior to the onset of and during PE. Furthermore, a better response to antimicrobial therapy has been observed when agents targeting AGS rather than traditionally recognized pathogens, such as Pseudomonas aeruginosa, have been used to treat PE (5, 6). The increased recognition of AGS in the pathophysiology of PE and chronic lung disease highlights the need for effective methods for routine isolation, quantification, and identification. We have previously employed a simple, semiselective agar containing nalidixic acid and sulfamethazine (NAS agar) to recover and enumerate all three AGS species from other diverse microbial consortia, including oral specimens such as saliva and dental plaque (11). Here we report on the use of NAS agar under anaerobic conditions to selectively isolate AGS from the sputa of patients suffering from CLDs.

In initial studies, growths of reference and laboratory strains of AGS and other oropharyngeal streptococci were compared using NAS agar and the recently described semiselective McKay medium (7). NAS plates consisted of 40 g liter⁻¹ sensitivity test agar (STA) (STA Lab012; Lab M Ltd., Lancs, United Kingdom), 30 µg ml⁻¹ nalidixic acid (Sigma-Aldrich), 1,000 µg ml⁻¹ sulfamethazine (Sigma-Aldrich), and 6% defibrinated horse blood (TCS biosciences Ltd., Bucks, United Kingdom). Both media were tested for growth of the following strains: Streptococcus intermedius (NCDO 2227T and strain UNS38), S. anginosus (NCTC10713T and strain 3a), S. constellatus subsp. constellatus (NCDO 2226T and strain 13a), S. constellatus subsp. pharyngis (MM9889aT), Streptococcus sanguinis (NCTC 7863T), Streptococcus salivarius (NCTC 8168T), Streptococcus oralis (NCTC 11427T), Streptococcus mitis (NS51T), Streptococcus mutans (NCTC10449T), Streptococcus pneumoniae (strain 9V), and Streptococcus vestibularis (MM1T). Plates were incubated in an anaerobic atmosphere (80% N₂, 10% H₂, 10% CO₂) or in 5% CO₂ at 37°C for 72 h. All type strains of AGS species and subspecies grew on NAS and McKay agar in both anaerobic and 5% CO₂ atmospheres. Streptococcus mutans strain NCTC 10449T also grew on both agars in these atmospheres, although this species has a distinct colonial morphology (white, matte, and opaque), thereby precluding confusion with AGS on NAS agar (Fig. 1). Streptococcus salivarius strain NCTC 8618T grew only on McKay agar incubated in 5% CO₂. No other type or reference strain was able to grow on either NAS or McKay agar when incubated anaerobically or in 5% CO₂ in these studies. A quantitative comparison of AGS...
Colonies were subcultured for identification. Over the course of 2 weeks, the strains isolated were identified further by phenotypic and molecular techniques. Biochemical identification was undertaken using 4-methylumbelliferyl-\(N\)-acetylgalactosaminidase, \(N\)-acetylglucosaminidase, \(N\)-acetyl-

 recovery on NAS agar, McKay agar, and several nonselective blood agar media incubated anaerobically was performed. The strains listed above were diluted in a 10-fold series and plated onto the semiselective agars and the following nonselective agars containing 6% (vol/vol) defibrinated horse blood (TCS biosciences Ltd., Bucks, United Kingdom): brain heart infusion agar (Oxoid Ltd., Hampshire, United Kingdom), blood agar base no. 2 (Oxoid Ltd.), and Columbia agar (Oxoid Ltd.). NAS agar showed AGS recovery comparable to that of McKay agar and the nonselective agar media tested, with a \( \log_{10} \) difference between the numbers of CFU per ml obtained from all agars.

NAS medium was then used with a total of 25 sputa obtained from patients with CLDs attending Barts and The London NHS Trust. These consisted of 10 CF (5 outpatients and 5 exacerbations), 11 bronchiectasis (8 outpatients and 3 exacerbations), and 4 COPD (2 outpatients and 2 exacerbations) patients. Patients with exacerbations were undergoing treatment in hospital with intravenous antibiotics, whereas outpatients had stable respiratory function and were attending the clinic only for routine follow-up appointments. Sputa were liquefied in an equal volume of Sputasol (Oxoid Ltd.), and serial dilutions (20 \( \mu \)l in saline) were plated onto NAS and McKay agars as well as Columbia blood, chocolate, MacConkey, Pseudomonas isolation, and Pseudomonas cepacia agars according to the laboratory’s standard operating procedure. Plates were incubated in 5% CO\(_2\) at 37°C for 48 h (blood, chocolate, and MacConkey) or 72 h. NAS and McKay agar plates were incubated anaerobically for 72 h at 37°C.

Colonies recovered from the CLD samples on NAS plates were subcultured and identified further by phenotypic and molecular techniques. Biochemical identification was undertaken using 4-methylumbelliferyl-\(4\)-MU-linked fluorogenic substrates (Sigma) for the detection of \( \beta\)-N-acetylgalactosaminidase, \( \beta\)-N-acetylglucosaminidase, \( \alpha\)-arabinosidase, \( \alpha\)-fucosidase, \( \alpha\)-galactosidase, \( \beta\)-galactosidase, \( \alpha\)-glucosidase, \( \beta\)-glucuronidase, and sidasidase (neuraminidase) activity (10, 12). AGS-specific PCR was performed using primers specific for the penicillin-binding protein 2B gene (\(pbp2b\)), and isolates were identified to the species and subspecies levels by amplification of the hyaluronate lyase, 16S RNA, and intermedyis (\(iby\)) genes as described previously (8). Hemolysis after 24 h and 48 h of anaerobic incubation was determined on layered blood agar plates (blood agar no. 2; Oxoid Ltd., Hants, United Kingdom) containing 6% defibrinated horse blood (TCS biosciences Ltd., Bucks, United Kingdom). Lancefield serological grouping was carried out using a commercial kit (streptococcal grouping kit DR0585A; Oxoid Ltd., Hants, United Kingdom).

AGS were isolated from 15/25 sputa, including 6/10 CF cases, 3/4 COPD cases, and 6/11 bronchiectasis cases (overall prevalence rate = 60%), on NAS agar. Both NAS and McKay agars allowed AGS detection from sputa containing other microbial taxa (\(P. aeruginosa\)/nonfermentative Gram-negative rods [range, 0 to 10\(^7\) CFU ml\(^{-1}\)], Enterobacteriaceae [range, 0 to 10\(^7\) CFU ml\(^{-1}\)], yeasts [range, 0 to 10\(^7\) CFU ml\(^{-1}\)], Staphylococcus aureus [range, 0 to 10\(^2\) CFU ml\(^{-1}\)], and Aspergillus fumigatus [range, 0 to 10\(^5\) CFU ml\(^{-1}\)]) which were unable to grow on either of these media under anaerobic conditions. Using the protocol described above for processing sputa, NAS agar permitted routine isolation of AGS with a potential lower detection limit of 100 CFU ml\(^{-1}\). Anaerobic incubation of sputa on NAS agar typically yielded 3 to 4 distinct colony morphotypes per plate which were counted, and representative colonies were subcultured for identification. Over the course of the study, AGS constituted 46/107 (42.9%) of NAS-selected isolates that were screened by the phenotypic and molecular techniques described. Of the 61 non-AGS isolates, virtually all (60/61) were unidentified Gram-positive cocci except a single isolate of \(S. mutans\). As observed by Maeda et al. (3), \(Streptococcus anginosus\) was the AGS species most frequently isolated from CF sputa, while \(Streptococcus intermedius\) was not isolated from any of the CLD specimens. In the present study, \(S. anginosus\) was also isolated most frequently, with \(S. intermedius\) comprising 34% of the AGS isolated. In the present study, \(Streptococcus anginosus\) was detected in 13/25 (52%) sputa, \(S. constellatus\) subsp. \(constellatus\) was detected in 8/25 (32%) sputa, and \(S. anginosus\) and \(S. constellatus\) subsp. \(constellatus\) were detected together in 6/25 (24%) sputa; the species combination was also the most frequently occurring combination found by Sibley et al. (7). \(Streptococcus anginosus\) strains were more heterogeneous than \(S. constellatus\) strains with respect to Lancefield group reaction and hemolysis on blood agar (Table 1). All 8 \(S. constellatus\) strains isolated were nongroupable and alpha-hemolytic.

In summary, NAS agar incubated anaerobically appeared to be a relatively simple strategy for isolating and quantifying AGS from these challenging clinical specimens. We believe that this represents an important additional diagnostic tool which can be used by clinical microbiologists to monitor the involvement of AGS in respiratory disease and used in research to study the microbial dynamics of CLD infections.

**REFERENCES**


**TABLE 1** Characteristics of AGS from patients with CLDs

<table>
<thead>
<tr>
<th>CLD</th>
<th>No. of strains of each species in each Lancefield group*</th>
<th>S. anginosus (n = 15)</th>
<th>S. constellatus (n = 8)**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. constellatus</td>
<td>S. anginosus</td>
<td>S. constellatus</td>
</tr>
<tr>
<td>CF</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>COPD</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
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*NG, nongroupable.

**All strains of \(S. constellatus\) were alpha-hemolytic.

*One strain was beta-hemolytic.

*One strain was nonhemolytic.

