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The use of Mueller-Hinton agar supplemented with citrated human or citrated sheep blood was compared with the use of routinely used Mueller-Hinton agar supplemented with defibrinated sheep blood for antimicrobial susceptibility testing of Streptococcus pneumoniae. The alternate supplements were found to be unsatisfactory, particularly for testing resistant isolates, and therefore are not recommended.

Streptococcus pneumoniae is a major cause of morbidity and mortality in children worldwide (11, 15, 18). It is a fastidious organism that requires a blood source for proper growth in vitro and antimicrobial susceptibility testing (AST). Defibrinated sheep or horse blood is a common supplement for blood agars, but pig or goat blood may also be used (2, 6, 14). Defibrinated sheep blood Mueller-Hinton agar (DSBMHA) is a standard medium used for AST of S. pneumoniae (1, 5).

Many resource-poor countries lack a regular supply of defibrinated animal blood and often use expired human blood samples obtained from blood donors for medium preparation (16; K. Edmond, personal communication) or blood samples obtained from volunteers, such as laboratory members (19). We previously reported that agar supplemented with citrated sheep blood (easily collected in citrated human blood donor packs) was suitable for the growth of S. pneumoniae but that agar supplemented with citrated human blood was not (16). We postulated that citrated sheep blood may be a suitable supplement for AST of S. pneumoniae (16), and a similar suggestion was also recently made by Yeh et al. (19). However, when these studies are combined, only four different strains, two of which being reference strains, were tested. Therefore, the use of citrated human or citrated sheep blood for AST of S. pneumoniae clinical isolates has not been appropriately validated.

The aim of this study was to determine if Mueller-Hinton agar containing citrated human or citrated sheep blood is a suitable medium for AST of S. pneumoniae. We compared the AST results obtained using the standardized disk diffusion and Etest methods on citrated sheep blood Mueller-Hinton agar (CSBMHA) and citrated human blood Mueller-Hinton agar (CHuBMHA) to those obtained with the recommended medium, DSBMHA. This study was approved by the Fiji National Research Ethics Review Committee and the University of Melbourne Human Research Ethics Committee.

Invasive S. pneumoniae isolates (in blood [n = 59], cerebrospinal fluid [CSF] [n = 7], pleural fluid [n = 1], and joint aspirate [n = 1]) collected from the Colonial War Memorial Hospital laboratory in Suva, Fiji, from July 2004 to October 2007 were tested for their susceptibility to antimicrobials using disk diffusion. In order to include a substantial number of nonsusceptible isolates, we also included 20 nasopharyngeal carriage isolates (obtained from 17 infants participating in a vaccine trial [17]) that were nonsusceptible (nonsusceptible [NS], intermediate [I], or resistant [R]), or borderline nonsusceptible, to oxacillin, erythromycin, or co-trimoxazole by disk diffusion on DSBMHA. These isolates were tested on the other agars as described below.

DSBMHA, CSBMHA, and CHuBMHA plates were prepared according to standard methods as previously described (16). Blood was obtained from sheep of the breed Fiji Fantasia, a hair sheep adapted for tropical conditions (3, 10), housed at the Koronivia Research Station. To conduct disk diffusion testing, isolates were made up to a 0.5 McFarland standard (up to 1.0 if mucoid for repeat testing) in brain heart infusion broth (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom). Lawn cultures were created, and disks (BD Difco, Franklin Lakes, NJ) containing 1 μg oxacillin, 15 μg erythromycin, 1.25 μg trimethoprim-23.75 μg sulfamethoxazole (co-trimoxazole), or 30 μg chloramphenicol were added onto the 3 different agar plates according to the Kirby-Bauer disk diffusion method (4), as per the CLSI guidelines (5). This included weekly testing of appropriate control strains (5). Plates were incubated in 5%
CO₂ at 35°C for 20 to 24 h. Isolates were categorized as sensitive (S), intermediate (I), or resistant (R) for erythromycin and co-trimoxazole and susceptible or nonsusceptible (NS) for oxacillin and chloramphenicol, according to standard guidelines (13). Of the 68 invasive isolates tested, 13%, 4%, 25%, and 0% were nonsusceptible (NS, I, or R) to oxacillin, erythromycin, co-trimoxazole, and chloramphenicol, respectively, by disk diffusion testing on DSBMHA. All included isolates were sensitive to chloramphenicol, so this antibiotic was not examined further.

For the Etest method, a subset of isolates was examined (32 invasive and 20 carriage isolates). The 15, 9, and 21 isolates that were nonsusceptible (that is, NS, I, or R) to oxacillin, erythromycin, and co-trimoxazole, respectively, by disk diffusion on DSBMHA had MICs determined for the corresponding antibiotics on all three agars by the Etest method. Etest strips (AB Biodisk, Solna, Sweden) containing benzylpenicillin, erythromycin, or co-trimoxazole were added to lawn culture agar plates, incubated under the same conditions as the disk diffusion plates, and interpreted using the manufacturer’s instructions (1). MIC results were generally consistent with disk diffusion testing. When tested on DSBMHA, 12/15 (80%), 9/9 (100%), and 15/21 (71%) isolates that were nonsusceptible (NS, I, or R) by disk diffusion were also nonsusceptible (NS, I, or R) by the Etest method for oxacillin-benzylpenicillin, erythromycin, and co-trimoxazole, respectively.

The results from the use of disk diffusion and Etest methods on CSBMHA and CHuBMHA were compared with the results from the use of these methods on the gold-standard DSBMHA for all isolates. Category agreement (disk diffusion), essential agreement (Etest method), and the number of interpretative category errors for each medium were determined for each antibiotic (7). Category agreement documents the proportion of isolates having the same category result (e.g., S/I/R) when tested on DSBMHA. Essential agreement documents results within one doubling dilution of the MIC obtained on DSBMHA. A category or essential agreement of ≥90% was used to initially assess for acceptability as a medium for AST. Interpretative category errors were defined as very major (classed as S when I, R, or NS on DSBMHA), major (classed as I, R, or NS when S on DSBMHA), or minor (all other errors). The percentages of very major, major, and total errors were determined by dividing by the number of R, S, and total isolates tested, respectively (7). A very major and major discrepancy rate of ≤3% each was also used as an acceptability criterion (7).

For disk diffusion testing (Table 1), the use of CHuBMHA and CSBMHA resulted in unacceptable (co-trimoxazole [59% and 68%, respectively]) and acceptable (oxacillin [both 93%] and erythromycin [92% and 90%, respectively]) category agreement in comparison with DSBMHA. There were also a large number of interpretative category errors, particularly for co-trimoxazole (Table 1).

For the Etest method (Table 2), the use of CHuBMHA and CSBMHA resulted in unacceptable essential agreement in comparison with DSBMHA for penicillin (67% and 60%, respectively), erythromycin (56% and 33%), and co-trimoxazole (52% and 57%), respectively. There were also a large number of interpretative category errors for all antibiotics (Table 2).

To our knowledge, this is the first examination of the validity of CHuBMHA for AST despite its common usage in resource-poor settings. Overall, the results for CHuBMHA and CSBMHA were too poor to recommend their use for AST, despite CSBMHA being a suitable medium for culture and identification of S. pneumoniae (16).

In terms of disk diffusion testing, CHuBMHA and CSBMHA were suitable for oxacillin and erythromycin, while both media were unsuitable for the testing of co-trimoxazole sensitivity when the category agreement alone was used as a measure. However, the characteristics of the isolates tested impacts on the calculation and assessment of errors. For example, very major errors will not be detected if the isolates are uniformly sensitive (8, 12). When we analyzed the resistant isolates separately by determining the percentage of very major discrepancies (7), we found that both media resulted in >3% very major discrepancies for all three antibiotics (Table 1), although for erythromycin, it must be noted that this was determined using only seven isolates. Overall, neither CHuBMHA nor CSBMHA were suitable for disk diffusion testing of oxacillin, erythromycin, or co-trimoxazole.

For the Etest method, both CHuBMHA and CSBMHA were unsatisfactory for all three antibiotics tested. This was also the case when the carriage isolates were analyzed separately (8), with all antibiotics on both media having an essential agreement of ≤73% (data not shown).

Although the blood agars were tested with only four antibi-
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