

Effect of Different Media on Determination of Novobiocin Resistance for Differentiation of Coagulase-Negative Staphylococci

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Species identification of coagulase-negative staphylococci often requires the determination of novobiocin susceptibility. Although previous investigators have recommended the use of P agar for this purpose, most clinical laboratories do not routinely utilize this medium. For this reason, studies were performed to compare novobiocin susceptibility results obtained with 11 different species of staphylococci (10 isolates of each species), using P agar, Trypticase soy agar with 5% sheep blood, and Mueller-Hinton agar. Tests performed on 70 susceptible isolates (minimal inhibitory concentration <1.6 µg/ml) resulted in zones of inhibition around 5-µg novobiocin disks ranging from 19.6 to 33.9, 16.2 to 26.6, and 21.3 to 36.4 mm on P agar, Trypticase soy agar with 5% sheep blood, and Mueller-Hinton agar, respectively. Forty resistant isolates (minimal inhibitory concentration ≥1.6 µg/ml) exhibited zones of inhibition ranging from 6.0 to 11.3 mm on P agar, 6.0 to 11.6 mm on Trypticase soy agar with 5% sheep blood, and 6.0 to 13.5 mm on Mueller-Hinton agar. Using the established cut off of 16 mm to define novobiocin resistance for the identification of coagulase-negative staphylococci, we correctly identified 100% of the strains tested, regardless of the media utilized.

Coagulase-negative staphylococci, once all identified as *Staphylococcus epidermidis* by clinical microbiologists, have recently been classified into 12 distinct species (5). Representatives of many of these species have been documented as etiological agents in urinary tract infections (4, 9, 12, 13), infections of indwelling intravenous catheters (2) and prosthetic devices (10), as well as in bacteremia in cancer patients (3). Because of this, clinical microbiologists are now faced with the integration into their daily laboratory practice of methods to identify these organisms.

Most accepted methods to identify coagulase-negative staphylococci, such as the simplified Kloos and Schleifer scheme (6) and the Staph-Ident System (Analytab Products, Plainview, N. Y.) (8), require the use of novobiocin susceptibility for a complete species level identification for a percentage of organisms tested. Previous investigators (6) have suggested two methods for testing novobiocin susceptibility for staphylococcal identifications. One method is to streak organisms onto P agar (PA) plates containing 1.6 µg of novobiocin per ml incorporated into the agar (6). Organisms capable of growth on this medium are considered novobiocin resistant. The second technique entails estimating novobi-

ocin susceptibility by using a 5-µg novobiocin disk on an inoculated PA plate (6). Strains that exhibit zones of inhibition of 5 mm or less from the edge of the disk (zone of inhibition ≥16 mm) are considered to be resistant to 1.6 µg of novobiocin per ml. Unfortunately, neither dehydrated PA nor prepared PA plates are commercially available. Also, clinical laboratories do not routinely utilize this medium for any other purpose. Therefore, clinical microbiologists wishing to identify coagulase-negative staphylococci must either prepare PA plates in their laboratories or test for novobiocin on a substitute agar contrary to the recommended technique (7).

The present investigation was undertaken to assess the utility of two media routinely used in clinical microbiology laboratories, Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 5% sheep blood (BA) and Mueller-Hinton agar (MH; BBL), for testing novobiocin susceptibility for the identification of coagulase-negative staphylococci. For this purpose, 10 representatives of each of seven susceptible coagulase-negative species and four resistant species were tested simultaneously on PA, BA, and MH and the results were compared.

MATERIALS AND METHODS

Staphylococcal isolates. Organisms consisted of 10 isolates each of 11 different species of staphylococci listed in Tables 1 and 2. These bacteria were selected from clinical isolates submitted to API Diagnostic Laboratories from various laboratories throughout the United States and Canada for confirmation of identification. In addition, several strains were obtained courtesy of W. E. Kloos, North Carolina State University, Raleigh, N.C. *Staphylococcus hyicus* and *Staphylococcus intermedius* were not included since to date these species have not been associated with human disease. All organisms were identified by using the Staph-Ident System as well as the Kloos and Schleifer (6) simplified scheme for the identification of staphylococci. Isolates were stored at -70°C in 15% glycerol broth.

Novobiocin testing. Initially, isolates were classified as susceptible or resistant to novobiocin by determining the ability of the isolate to grow on PA containing 1.6 μg of novobiocin per ml. (Sigma Chemical Co., St. Louis, Mo.) (6). PA was prepared as previously described (14). For the purposes of this investigation, isolates frozen at -70°C in 15% glycerol broth were subcultured onto BA and incubated at 35 to 37°C for 18 h. Organisms, after two additional subcultures on BA, were inoculated into 5 ml of Trypticase soy broth, and the turbidity was adjusted to equal a McFarland 0.5 standard. The resultant suspension was then used to simultaneously inoculate PA, BA, and MH in accordance with the National Committee for Clinical Laboratory Performance Standard for Antimicrobial Disk Susceptibility Tests (11) in sterile 100-mm petri dishes (20 ml per dish) as well as MH in 150 mm plates (60 ml per dish). A 5 μg novobiocin disk (BBL) was applied to each plate 10 min after inoculation. Plates were then incubated at 35 to 37°C for 18 h and removed from the incubator, and zones of inhibition around disks were measured to the nearest tenth of a millimeter with the aid of a sliding caliper.

Data analysis. Zones of inhibition for each isolate obtained on PA were compared with those obtained on BA and MH. In addition, results obtained on BA were compared to those obtained on MH. MH results obtained in 100- and 150-mm agar plates were also compared. Significant differences in zones of inhibition among different media were calculated by using the paired *t* test. Moreover, use of results for identification of the isolate in question was assessed on the

basis of the accepted cutoff of a 16-mm zone of inhibition to define resistance (6).

RESULTS

Results obtained for each staphylococcal species on each medium are summarized in Tables 1 and 2. The mean zone diameters (\pm standard deviation) obtained for susceptible species were 24.8 ± 2.3 , 20.9 ± 1.3 , and 27.2 ± 2.0 mm on PA, BA, and MH, respectively. Since no significant differences in zone sizes were found by using MH in 100- and 150-mm dishes, only data obtained in 100-mm plates are included. Zones of inhibition for each susceptible isolate (Table 2) were significantly larger on PA and MH than on BA ($P > 0.05$). No significant difference in zones was found between results obtained on PA and MH. *S. epidermidis* isolates tested on BA gave the smallest zones of inhibition. Regardless of the differences noted, when 16 mm was utilized as the cutoff to define novobiocin resistance for the identification of these species, all isolates were correctly interpreted as susceptible.

Resistant strains tested (Table 2) were also identified as such by utilizing a 5- μg novobiocin disk and the 16-mm cutoff to define resistance. Isolates gave the most consistent results when tested on PA. Three of the four novobiocin-resistant species (*Staphylococcus cohnii*, *Staphylococcus saprophyticus*, and *Staphylococcus xylosum*) showed no zone size variation when tested on PA. Testing of all isolates of these species resulted in no zone of inhibition around novobiocin disks. To the contrary, these same isolates produced zones of inhibition ranging from 6.0 to 11.5 mm and 6.0 to 13.5 mm when tested on BA and MH, respectively. Unlike the results obtained for susceptible strains, results obtained on PA were significantly different ($P > 0.05$) from those obtained with the same isolates on BA and MH. Furthermore, results obtained with resistant strains on BA and MH were not significantly different.

TABLE 1. Zone diameters of novobiocin-susceptible coagulase-negative staphylococci on different media^a

Organism	Zones of inhibition (mm)					
	PA		BA		MH	
	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
<i>S. aureus</i>	27.0 \pm 1.2	25.3–28.5	20.3 \pm 0.8	19.0–21.8	28.2 \pm 1.1	25.7–29.5
<i>S. capitis</i>	22.7 \pm 1.7	19.8–25.1	21.1 \pm 1.3	19.4–23.0	26.6 \pm 2.6	23.6–31.6
<i>S. epidermidis</i>	27.9 \pm 4.0	21.8–33.9	23.5 \pm 3.4	16.2–26.6	31.0 \pm 3.8	23.0–36.3
<i>S. haemolyticus</i>	24.7 \pm 2.5	19.8–28.2	19.4 \pm 1.8	17.7–23.7	26.7 \pm 3.8	24.3–36.4
<i>S. hominis</i>	22.0 \pm 1.8	19.6–25.0	20.5 \pm 1.6	18.8–23.5	24.6 \pm 2.5	21.3–29.7
<i>S. simulans</i>	26.1 \pm 2.6	21.7–29.2	20.2 \pm 1.8	17.5–22.3	27.5 \pm 2.7	23.3–32.3
<i>S. warneri</i>	23.3 \pm 1.7	21.3–26.8	21.3 \pm 1.5	19.3–24.2	26.0 \pm 1.6	23.0–28.7

^a Minimal inhibitory concentration <1.6 $\mu\text{g}/\text{ml}$.

TABLE 2. Zone diameters of novobiocin-resistant coagulase-negative staphylococci on different media^a

Organism	Zones of inhibition (mm) ^b					
	PA		BA		MH	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
<i>S. cohnii</i>	6.0 ± 0.0	6.0	6.2 ± 0.6	6.0–7.8	6.2 ± 0.7	6.0–8.3
<i>S. saprophyticus</i>	6.0 ± 0.0	6.0	6.0 ± 0.0	6.0	7.6 ± 0.9	6.0–8.6
<i>S. sciuri</i>	6.9 ± 1.7	6.0–11.3	9.6 ± 1.9	6.0–11.6	8.4 ± 2.3	6.0–11.5
<i>S. xylosus</i>	6.0 ± 0.0	6.0	6.6 ± 1.7	6.0–11.5	7.0 ± 1.6	6.0–13.5

^a Minimal inhibitory concentration <1.6 µg/ml.

^b The average mean ± standard deviation for each medium is: PA, 6.2 ± 0.5; BA, 7.1 ± 1.7; MH, 7.3 ± 0.9.

Several *Staphylococcus sciuri* isolates exhibited some degree of susceptibility to novobiocin on all three media. In addition, one *S. xylosus* isolate resulted in zones of inhibition of 6.0 mm when tested on PA but 11.5 and 13.5 mm on BA and MH, respectively. Regardless of these variations on different media, all resistant strains tested resulted in zones of inhibition of less than 16 mm and therefore were assessed as resistant by the novobiocin disk method.

DISCUSSION

Although previous investigators (4, 5, 6, 7) have recommended the use of PA for the determination of novobiocin resistance in coagulase-negative staphylococci, this medium is not commercially available nor used routinely by most clinical microbiology laboratories. Laboratories identifying coagulase-negative staphylococci are either preparing PA plates in-house or testing for novobiocin resistance by methods other than those recommended in the *Manual of Clinical Microbiology* (7). For this reason, it is imperative that data be generated on media routinely utilized in clinical laboratories, such as BA and MH, to determine the validity of test determinations of novobiocin resistance on these media.

Most investigators working with coagulase-negative staphylococci have utilized PA for the determination of novobiocin susceptibility according to the methods proposed by Kloos and Schleifer (6). One British laboratory early in 1979 (12) reported on the use of Mueller-Hinton agar, Wellcotest sensitivity agar (Wellcome Reagents; Ltd., London, England), and Iso-Sensitest agar (Oxoid, Basingstoke, England) for testing *S. saprophyticus* strains isolated from patients with urinary tract infections. These investigators demonstrated that testing novobiocin-susceptible coagulase-negative staphylococci yielded zones of inhibition measuring 8 mm or more from the edge of a 6-mm 5-µg novobiocin disk (zone of inhibition ≥22 mm). Using this criterion, 53 of 70 susceptible strains tested in the present investigation would have been misidentified. More recently, Almeida and Jorgen-

sen (1) reported on the use of MH as a substitute for PA in the determination of novobiocin susceptibilities for the species identification of coagulase-negative staphylococci. Using the 16-mm zone size criterion of Kloos and Schleifer to define novobiocin susceptibility in these organisms, these investigators demonstrated a 99% agreement between tests performed on PA and MH. Unfortunately, although several species of coagulase-negative novobiocin-susceptible species were represented in this investigation, data on resistant strains were limited to only one species, *S. saprophyticus*.

In the present study, results obtained for novobiocin susceptibilities on PA were compared with those obtained on both MH and BA. Representatives of seven susceptible species and four resistant species were tested. Results obtained with sensitive strains indicated significant differences between zone sizes obtained on PA and BA as well as between MH and BA. Zones of inhibition on BA were consistently smaller than those obtained on either PA or MH. Regardless of these differences, all susceptible strains tested yielded zones of inhibition >16 mm on all media tested. Therefore, all were correctly identified as susceptible by this method. One *S. epidermidis* strain gave a zone of inhibition of 16.2 mm when tested on BA, which might have been misinterpreted as resistant as a result of subjective differences in the measurement of individual zone diameters in this disk diffusion test. Forty resistant strains representing four different species were also tested. Interestingly, with these isolates, results on PA were significantly different than those on BA or MH, which did not differ from one another. Again, regardless of these differences, since the smallest zone size exhibited with a susceptible strain was 16.2 mm and the largest zone obtained with a resistant strain was 13.5 mm (difference of 2.7 mm), strains were correctly identified when using the 16-mm cutoff.

In summary, clinical laboratories identifying coagulase-negative staphylococci must often perform a novobiocin susceptibility test for a

species level identification for a proportion of isolates tested. Results of the present investigation found both MH and BA to be satisfactory for this purpose as long as the accepted 16-mm cutoff is utilized as the interpretative standard to judge resistance.

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LITERATURE CITED

1. Almeida, R. J., and J. H. Jorgensen. 1982. Use of Mueller-Hinton agar to determine novobiocin susceptibility of coagulase-negative staphylococci. *J. Clin. Microbiol.* **16**:1155-1156.
2. Archer, G. L. 1978. Antimicrobial susceptibility and selection of resistance among *Staphylococcus epidermidis* isolates recovered from patients with infections of indwelling catheters. *Antimicrob. Agents Chemother.* **14**:353-359.
3. Eng, R. H. K., C. Wang, A. Person, T. E. Klehn, and D. Armstrong. 1982. Species identification of coagulase-negative staphylococcal isolates from blood cultures. *J. Clin. Microbiol.* **15**:439-442.
4. John, J. F., Jr., P. K. Gramling, and N. M. O'Dell. 1978. Species identification of coagulase-negative staphylococci from urinary tract isolates. *J. Clin. Microbiol.* **8**:435-437.
5. Kloos, W. E. 1982. Coagulase-negative staphylococci. *Clin. Microbiol. Newslett.* **4**:75-79.
6. Kloos, W. E., and K. H. Schleifer. 1975. Simplified scheme for routine identification of human *Staphylococcus* species. *J. Clin. Microbiol.* **1**:82-88.
7. Kloos, W. E., and P. B. Smith. 1980. Staphylococci, p. 83-87. In E. H. Lennette, A. Balows, W. J. Hausler, and J. P. Truant (ed.), *Manual of clinical microbiology*, 3rd ed. American Society for Microbiology, Washington, D.C.
8. Kloos, W. E., and J. F. Wolfshohl. 1982. Identification of *Staphylococcus* species by use of the API Staph-Ident System. *J. Clin. Microbiol.* **16**:509-516.
9. Lewis, J. F., S. R. Brake, D. J. Anderson, and G. N. Vredeveld. 1982. Urinary tract infection due to coagulase-negative *Staphylococcus*. *Am. J. Clin. Pathol.* **77**:736-739.
10. Liekweg, W. G., and L. J. Greenfield. 1977. Vascular prosthetic infections. *Surgery* **81**:335-342.
11. National Committee for Clinical Laboratory Standards. 1979. Performance standards for antimicrobial disk susceptibility tests, 2nd ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
12. Shrestha, T. L., and J. H. Darrell. 1979. Urinary infection with coagulase-negative staphylococci in a teaching hospital. *J. Clin. Pathol.* **32**:299-302.
13. Tselenis-Kotsowilis, A. D., M. P. Kollomichalis, and J. T. Papaouassilou. 1982. Acute pyelonephritis caused by *Staphylococcus xylosum*. *J. Clin. Microbiol.* **16**:593-594.
14. Vera, H. D., and D. A. Power. 1980. Culture media, p. 965-999. In E. H. Lennette, A. Balows, W. J. Hausler, and J. P. Truant (ed.), *Manual of clinical microbiology*, 3rd ed. American Society for Microbiology, Washington, D.C.