

## Growth of Coagulase-Negative Staphylococci on Colistin-Nalidixic Acid Agar and Susceptibility to Polymyxins

JOAN C. FUNG,<sup>1†</sup> GEOFFREY MCKINLEY,<sup>2\*</sup> MARY B. TYBURSKI,<sup>1</sup> MARJORIE BERMAN, AND JACK GOLDSTEIN<sup>2‡</sup>

North Shore University Hospital, Manhasset, New York 11030,<sup>1</sup> and Analytab Products, Plainview, New York 11803<sup>2</sup>

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Colistin-nalidixic acid agar, although recently recommended as a replacement for blood agar for primary plating of urine specimens (Fung et al., *J. Clin. Microbiol.* **16**:632-636, 1982), has also been reported to suppress the growth of some strains of staphylococci that are susceptible to colistin (polymyxin E). The susceptibility of 11 species of staphylococci to polymyxins was determined, and the ability of these species to grow on colistin-nalidixic acid agar was examined. Although the MICs for most of the strains tested were 8 µg/ml or less, only a few coagulase-negative staphylococci grew on or were inhibited by colistin-nalidixic acid agar. This discrepancy was explained by the antagonistic effects that medium components, such as physiological concentrations of magnesium and calcium and 5% sheep blood, had on the activity of polymyxin. Colistin-nalidixic acid agar is still recommended for routine urine processing; however, the poor growth of 13% of the *Staphylococcus saprophyticus* strains tested suggests that blood agar should be included in the primary plating battery of urine specimens obtained from female outpatients.

We recently reported that the best medium combination for routine urine processing is colistin-nalidixic acid agar (CNA) and MacConkey agar (MAC) (9). The CNA-MAC combination yielded an 11% higher isolation rate of suspected causative agent(s) of urinary tract infections than did the currently recommended combination of MAC and blood agar (BA). CNA, however, has been cited to be inhibitory for certain, unspecified strains of staphylococci (7), raising some concern as to the ability of CNA to recover *Staphylococcus saprophyticus* from urine specimens.

One explanation for the failure of some staphylococci to grow on CNA is their susceptibility to colistin (polymyxin E). Polymyxin antibiotics (polymyxins B and E) have commonly been used for selective media for gram-positive bacteria (5, 6, 8, 18) because of their activity against gram-negative bacilli (13). Although gram-positive bacteria have generally been considered resistant to polymyxins (13), this generalization has proven not to be true with strains of coagulase-negative staphylococci (7, 8, 17, 19).

This was of special interest to us since we had observed (9) that in the comparison between CNA and BA recovering gram-positive organisms from urine specimens, CNA yielded higher colony counts. In the study, discrepancies (a 10-fold difference between CFU per milliliter on CNA and that on BA) in colony counts were not most notable for coagulase-negative staphylococci; only three urine specimens involving coagulase-negative staphylococci demonstrated a quantitative difference between recovery on CNA and on BA. However, since only 18 coagulase-negative staphylococcal strains were isolated in >10<sup>5</sup> CFU/ml during the study and since the only *S. saprophyticus* isolated was recovered in fewer numbers on CNA than on BA, it became apparent that an examination of the ability of known species

of staphylococci to grow on CNA and their susceptibility to polymyxins was needed.

In the present study, we assessed the ability of staphylococci to grow on CNA, determined their susceptibility to polymyxins, and examined the possible antagonistic effects of medium components found in CNA on organism growth. Organisms were obtained from a variety of clinical sources and submitted to Analytab Products diagnostic laboratories from various laboratories throughout the United States for identification or confirmation. All isolates, which included 72 strains of 11 species of staphylococci (5 *S. aureus*, 4 *S. capitis*, 6 *S. cohnii*, 5 *S. epidermidis*, 5 *S. haemolyticus*, 5 *S. hominis*, 23 *S. saprophyticus*, 5 *S. sciuri*, 5 *S. simulans*, 5 *S. warneri*, and 4 *S. xylosus*), were identified by the API Staph-Ident system (Analytab Products, Plainview, N.Y.), as well as the Kloos and Schleifer scheme (12) for staphylococcal identification. Isolates were stored at -70°C in 15% glycerol broth.

The ability of the 72 isolates of staphylococci to grow on CNA and BA media was determined. Suspensions containing ca. 10<sup>4</sup> CFU of each staphylococcal strain per ml were made in Mueller-Hinton broth (MHB) (Difco Laboratories, Detroit, Mich.). Each suspension was quantitatively plated onto BA and CNA by using a calibrated 0.001-ml loop. Quantitation of growth was determined after 24 and 48 h of incubation at 35 to 37°C, and a 10-fold difference between the number of CFU per milliliter on BA and that on CNA was considered to be a discrepant result. The results showed that seven (10%) of the strains tested (which included three *S. saprophyticus*, one *S. capitis*, one *S. cohnii*, one *S. haemolyticus*, and one *S. sciuri*) showed a 10-fold increase in CFU on BA when compared to that on CNA.

The susceptibility of each isolate to polymyxins was determined by conventional and modified broth dilution and disk diffusion techniques to assess whether the inability of staphylococci to grow on CNA was a result of colistin or other components in CNA medium. MICs of polymyxins B and E were determined by the macrotube method (23), using either MHB alone or MHB supplemented to contain 50 mg of calcium (Ca<sup>2+</sup>) per liter and 25 mg of magnesium (Mg<sup>2+</sup>) per

\* Corresponding author.

† Present address: Department of Clinical Microbiology, University of Connecticut Health Center, Farmington, CT 06032.

‡ Present address: American MicroScan, 1715 Dell Avenue, Campbell, CA 95008.

liter (22). Serial twofold dilutions of colistin (1 to 32  $\mu\text{g/ml}$ ) and polymyxin B (3.12 to 100 U/ml) were prepared in MHB alone and MHB supplemented with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Each dilution was inoculated to achieve a final concentration of  $10^5$  to  $10^6$  CFU/ml. The MIC was determined as the concentration of polymyxin at which there was no visible growth of microorganisms after 18 to 24 h of incubation at 35°C. In addition, susceptibilities of each strain to polymyxins were determined by the disk diffusion method of Bauer et al. (1), in accordance with National Committee for Clinical Laboratory Standards (15), using colistin (10 mg) and polymyxin B (300 U) disks. Susceptibility to polymyxins was determined on Mueller-Hinton agar (MHA) plates with and without supplemented 5% sheep blood. Plates and antibiotic disks were obtained from BBL Microbiology Systems (Cockeysville, Md.).

The MICs for staphylococci indicated that when tested in MHB, 50 (69%) of the 72 strains were susceptible to  $\leq 8$   $\mu\text{g}$  of colistin per ml. The seven isolates which grew poorly on CNA had colistin MICs of  $\leq 8$   $\mu\text{g/ml}$ . Nevertheless, the ability of many staphylococci to grow at the inhibitory concentration (10  $\mu\text{g/ml}$ ) of colistin may be explained by the possible presence of medium components in CNA which have antagonistic effects on the activity of colistin. Since various lots of agar (22) have been found to contain physiological concentrations of magnesium (25  $\mu\text{g/ml}$ ) and calcium (50  $\mu\text{g/ml}$ ), the effect of divalent cations on the activity of colistin was examined. The MIC of colistin increased in the presence of physiological concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , in each case, from 2 to 4 doubling dilutions in the presence of divalent cations. A total of 33 strains of staphylococci required for inhibition MICs of  $\leq 8$   $\mu\text{g}$  of colistin per ml when tested in supplemented MHB, and 50 strains required MICs of  $\leq 8$   $\mu\text{g}$  of colistin per ml when tested in MHB alone.

The zones of inhibition to colistin were determined by disk diffusion on both MHA alone and MHA with 5% sheep blood, since CNA contains 5% sheep blood. A significant decrease in zone size was noted in the presence of 5% sheep blood. Zone diameters of  $\geq 11$  mm on MHA without blood were exhibited by 21 strains, whereas 1 strain exhibited this phenomenon on MHA containing 5% sheep blood. A zone diameter of  $\geq 11$  mm is interpreted as susceptible for a 10- $\mu\text{g}$  colistin disk by the National Committee for Clinical Laboratory Standards (15).

Cross susceptibility between colistin and polymyxin B is common, and since polymyxin B is used in selective media for gram-positive bacteria, the effects of divalent cations and of sheep blood on polymyxin B activity were also examined. As with colistin, physiological concentrations of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  had an antagonistic effect on polymyxin B activity against staphylococci, increasing the polymyxin B MIC from 2 to 4 doubling dilutions. A similar increase in resistance to a 300-U disk of polymyxin B was demonstrated by disk diffusion tests in which MHA alone and MHA with 5% sheep blood were used.

Our observations confirm previous findings that some strains of coagulase-negative staphylococci do not grow well on CNA (7), including strains of *S. saprophyticus*. Of the 23 *S. saprophyticus* isolates tested, 3 (13%) repeatedly grew more poorly on CNA than on BA. Whether this incidence, using stock strains, will reflect the primary isolation of *S. saprophyticus* from urine specimens on CNA remains to be examined. *S. saprophyticus* has been isolated in up to 30% of urine specimens from female outpatient populations with bacteriuria (10, 14, 21) and in less than 1% of urine specimens obtained from other patient populations with urinary

tract infections (20, 21). Laboratory personnel might consider that urine specimens obtained from female outpatients also be plated on BA. Nevertheless, the much higher isolation rate of etiological agent(s) from urine specimens on CNA than on BA warrants our continued recommendation of the CNA-MAC combination as the primary plating medium for routine urine processing (9).

Our results also confirm previous reports (7, 8, 17, 19) that some species of coagulase-negative staphylococci are susceptible to polymyxins. As with gram-negative organisms (11), cross susceptibility of staphylococci to polymyxins B and E also exists. Many staphylococcal strains with colistin MICs of  $\leq 8$   $\mu\text{g/ml}$  are able to grow at this 10- $\mu\text{g/ml}$  inhibitory concentration of colistin in CNA agar.

It appears that medium components present in CNA can influence the activity of colistin. Physiological concentrations of  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  have been found in various lots of agar (22), although wide variabilities in cation contents in agar do exist. Cations have been reported to affect the susceptibility of microorganisms to aminoglycosides, tetracycline, and polymyxins. Cations have an antagonistic effect on the activity of polymyxins to *Pseudomonas aeruginosa* (2-4, 16). This antagonism is believed to result from the prevention of polymyxin binding to bacterial cell wall phospholipids (3). Divalent cations influence the susceptibility of *P. aeruginosa* to aminoglycosides (22), as well as the activity of tetracycline on other microorganisms. The present study indicates that  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  also have an antagonistic effect on the activity of polymyxins against staphylococci.

Blood is often added to gram-positive selective media for nutritional enrichment. Our findings suggest that blood in agar media can also influence polymyxin activity. Physiological calcium concentrations present in 15% human serum have been shown to inhibit the effect of colistin on *P. aeruginosa* (4). This may not explain the antagonistic effect of 5% sheep blood on the influence of polymyxins against staphylococci. As determined by flame atomic absorption spectrometry, 5% sheep blood only increased the  $\text{Mg}^{2+}$  concentration in MHB by 1  $\mu\text{g/ml}$  and the  $\text{Ca}^{2+}$  concentration by 2  $\mu\text{g/ml}$ .

In summary, some strains of staphylococci have colistin MICs of  $\leq 8$   $\mu\text{g/ml}$ , although many can still grow at the 10- $\mu\text{g/ml}$  colistin concentration in CNA agar. Although there is no obvious correlation between growth ability on CNA and the degree of susceptibility to colistin, it appears that divalent cations and blood in CNA have an antagonistic effect on colistin activity. We continue to recommend the use of the CNA-MAC combination for routine urine processing. However, since 3 of 23 *S. saprophyticus* strains tested failed to grow well on CNA, laboratory personnel may consider including BA for culturing urine specimens obtained from female outpatients.

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