Incidence of Thymidine-Dependent Enterococci Detected on Mueller-Hinton Agar with Low Thymidine Content

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It was observed that 10% of urine culture isolates of enterococci tested for antimicrobial susceptibility failed to grow on commercially prepared Mueller-Hinton agar with low levels of thymidine and thymine. All strains could utilize exogenous thymidine and thymine and required only low levels (0.4-µg disk) to support growth. All thymidine-thymine-requiring strains were resistant to trimethoprim-sulfamethoxazole.

Trimethoprim-sulfamethoxazole (TMP-SMZ) is an effective antimicrobial combination which acts by sequential blockade in the metabolic pathways for synthesis of folates (8). Organisms which can use exogenous end products of the folate pathway will be resistant to TMP-SMZ when such end products are present (11). Therefore, in vitro susceptibility testing of TMP-SMZ is dependent upon utilization of a medium free of such exogenous metabolites since they will allow bypass of the blockade in a noncompetitive manner. Strains lacking thymidylate synthetase require folates (usually thymidine or thymine) and may not grow on media low in folate pathway end products. Clinical isolates of such strains are usually reported to the physician without antimicrobial susceptibility determinations, and the patient is at risk of being treated with an ineffective drug.

In 1972, Barker and co-workers (2) reported the isolation of six strains of *Enterobacteriaceae*, including two *Proteus mirabilis* and four *Escherichia coli* strains which were resistant to TMP-SMZ due to a requirement for exogenous thymidine. Five of the six isolates were from patients treated with TMP-SMZ. Subsequently, there have been a number of reports of clinical isolates resistant to TMP-SMZ due to a requirement for either exogenous thymidine or thymine. In 1973, Lacey and Lewis (12) reported the isolation of a thymidine-requiring *Staphylococcus aureus* from the sputum of a fibrocystic child previously treated with TMP-SMZ. Also in 1973, Okubadejo and Maskell (16) reported the isolation of three strains of thymine-requiring *P. mirabilis* from the urine of three patients on long-term TMP-SMZ therapy. In 1974, Tapsall et al. (18) reported the isolation of six strains of thymine-dependent *E. coli* from the urine of six patients receiving TMP-SMZ therapy. Also in 1974, Tanner and Bullin (17) reported on one blood and two urine culture-derived strains of thymidine-dependent *E. coli* from patients receiving TMP-SMZ. In 1976, Hayek and Netherway (10) reported the isolation of a thymine-requiring *Salmonella typhimurium* from the stool of a patient being treated with TMP-SMZ for infection with a wild-type *S. typhimurium*. Also in 1976, Maskell and co-workers (13) updated their work on thymine-requiring bacteria associated with TMP-SMZ therapy and expanded the list of species to include a coagulase-negative staphylococcus. They also reported the presence of a thymine-like compound in the urine of five patients infected with thymine-requiring strains. The work of Maskell et al. (14) was expanded again in 1977 and included four isolates of thymine-requiring *Streptococcus faecalis* from the urine of patients on TMP-SMZ therapy.

This paper records an unusually high incidence of thymidine-thymine-requiring (thy*) enterococci among urine cultures processed in our laboratory. The high rate of thy* enterococci became apparent when the manufacturer of the Mueller-Hinton agar (MHA) used in our microbiology (BBL Microbiology Systems, Cockeysville, Md.) modified the MHA (hereafter called MHII) to contain low levels of thymine and thymidine (amounts not specified) and controlled levels of calcium and magnesium (amounts not specified).

**MATERIALS AND METHODS**

All strains were clinical isolates from the Bacteriology Laboratory, Strong Memorial Hospital of the University of Rochester. Enterococci were identified by Gram stain, hydrolysis of esculin on a bile-esculin agar plate, and growth on a 6.5% NaCl agar plate (7). Identifications as to species and subspecies were accomplished by the methods of Facklam (5-7) and co-workers, and the isolates were serotyped by the Rantz-Randall modification of the Lancefield precipitin pro-

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P. aeruginosa was identified by the methods recommended by Gilardi (9).

Quantitative urine cultures were done by means of the calibrated loop method (0.001 ml) on both Trypticase soy agar (BBL Microbiology Systems) with 5% sheep blood and MacConkey agar. Organisms from cultures with growth of a predominant species at 10,000 colony-forming units per ml or greater were identified and tested for antibiotic susceptibility by the disk diffusion method set forth by the National Committee for Clinical Laboratory Standards (15). Enterococci which failed to grow, or to grow adequately, for susceptibility testing on MHII, or MHII with 5% sheep blood (MHBII), were retested on MHA, or MHA with 5% sheep blood (MHBAB), from another manufacturer (Scott Laboratories, Inc., Fiskeville, R.I.) with an apparently more variable thymidine content. Except for TMP-SMZ, results of susceptibility testing of enterococci on the media from the two manufacturers were quite similar.

To determine whether the lack of growth on MHII and MHBII was due to inhibition of growth or lack of adequate nutrients, we ascertained the abilities of a "staphylococcus streak" (S. aureus ATCC 25923), thymidine and thymine disks (0.4 μg/disk) (Aldrich Chemical Co., Milwaukee, Wis.), and X and V factor strips (Pfizer Diagnostics Division, New York, N.Y.) to support growth. A rough approximation of the thymidine and thymine requirement of each strain was made by diluting thymidine or thymine serially in Mueller-Hinton (MH) broth with low levels (amount not stated) of thymine and thymidine (Difco Laboratories, Inc., Detroit, Mich.) in microtiter plates.

RESULTS

During the month of December 1979, 96 enterococci (62 urine isolates and 34 from other body sources) were processed for susceptibility testing on MHII. A total of eight strains, all urine isolates, failed to grow for susceptibility testing on MHII or MHBII. A ninth strain, a clinical isolate of unknown origin used in our laboratory for several years for quality control of new lots of MHA, was also observed not to grow adequately on MHII or MHBII.

All nine isolates were serologically confirmed as being group D streptococci. Biochemically, one isolate was found to be S. faecalis subsp. faecalis, and eight were found to be S. faecalis subsp. liquefaciens. Ten randomly selected enterococcal isolates from urine cultures that grew on MHII were found to be comparably distributed (i.e., three subsp. faecalis and seven subsp. liquefaciens).

On repeat susceptibility testing on MHA and MHBAB, all strains grew sufficiently well on MHBAB to allow accurate zone measurement, as did three that grew on MHA. All nine strains were resistant to TMP-SMZ, susceptible to ampicillin and nitrofurantoin, but varied in susceptibility to cephalothin, tetracycline, and erythromycin.

All nine strains grew on MHII and MHBII adjacent to the staphylococcus streak and around the thymidine and thymine disks. No strains grew around the X or V factor strips.

MH broth supplemented with ≤0.63 μg of thymidine or thymine per ml supported growth of all nine strains, whereas all nine strains failed to grow in unsupplemented MH broth. The range of the thymine-thymidine supplementation required to support growth was from 0.075 to 0.63 μg/ml.

We also found six clinical isolates of organisms other than enterococci which were unable to grow adequately for susceptibility testing on MHII or MHBII. They included two strains of P. aeruginosa, three strains of E. coli, and one strain of P. mirabilis. These strains were screened to determine whether they required higher levels of thymidine or thymine for growth than that supplied by MHII agar. The strains of P. aeruginosa and E. coli did not grow adjacent to the staphylococcus streak on MHII or around thymine or thymine disks with concentrations up to 100 μg, suggesting a need for nutrients other than, or in addition to, thymine or thymidine and other supplements provided by the staphylococcus streak (e.g., nicotinamide adenine dinucleotide and vitamin B6). The strain of P. mirabilis, similar to the thy- enterococci reported here, grew on MHII agar with the addition of a 0.4-μg disk of either thymine or thymidine.

The eight thy- enterococci were isolated from six patients (Table 1). Charts of five of the patients were available for review. Two patients had been receiving TMP-SMZ therapy for a short period (1 week and 5 weeks) before isolation of the thy- organisms. Two patients were on long-term TMP-SMZ treatment for recurrent urinary tract infections. The fifth patient had no recorded history of treatment with TMP-SMZ and was reported to be allergic to sulfonamides.

DISCUSSION

The laboratory had been alerted to the possibility of the failure of enterococci to grow on MHII and MHBII by the chance selection of the thy- quality control strain. We were, however, surprised at the incidence of thy- enterococci (10.0% of urine culture enterococcus isolates tested for antibiotic susceptibility if multiple thy- isolates from the same patient are eliminated). Because of the high rate of thy- strains,
we repeated the study over an additional 1-month period (1 to 31 March 1980) to verify our original observation and found the rate quite similar, but with several differences that should be noted. During March, 12 of 82 enterococci tested for susceptibility were found to be thy⁻. However, 6 of the 12 thy⁻ strains were repeat isolates detected throughout the month in the urine of one patient. Nine of the twelve strains were urine isolates, one was from dialysis fluid, one was from a wound exudate, and one was from a decubitus ulcer. Eliminating multiple thy⁻ strains from the same patient, the adjusted rate for March was 9.1% (7/77) from all body sites as compared with an adjusted rate of 6.4% (6/94) for December. Eight of the March isolates were identified as S. faecalis subsp. liquefaciens, and four were identified as S. faecalis subsp. faecalis (five S. faecalis subsp. liquefaciens strains were isolated from one patient throughout the month). Andrew (1) reported that laboratory-induced thy⁻ mutants of S. faecalis required low levels of thymine (2 μg/ml). Maskell et al. (14) reported that the four thy⁻ clinical isolates of S. faecalis studied grew around low-content (2 μg) thymine disks on Iso-Sensitest agar. Our observation of growth around a 0.4-μg thymidine or thymine disk on MHII and that 0.63 μg of thymidine or thymine per ml supported growth of all strains in MH broth suggests that the thy⁻ strains reported here also required only low levels of thymidine or thymine.

Maskell et al. (14) reported that only one thy⁻ strain (an E. coli) was isolated from a patient after a short course of TMP-SMZ therapy before July 1975, but after that date thy⁻ strains from patients on short-course therapy with TMP-SMZ were seen with increasing frequency. In this study, all but one of the patients (4 of 5) whose records were available for evaluation were receiving TMP-SMZ treatment before the isolation of the thy⁻ S. faecalis strains. Two of the four patients had received only a short course of TMP-SMZ therapy.

The high rate of the thy⁻ S. faecalis reported here may be due to an unusual patient population, over-utilization of TMP-SMZ, or other as yet unknown factors. On the other hand, utilization of MHA with low thymidine-thymine content may merely have made apparent a situation of long-standing duration.

It is our recommendation that enterococci which fail to grow on MHII, MHBII, or similar low thymidine-thymine agar be tested on MHA supplemented with 20 μg of thymidine per ml, if consultation with the physician suggests such testing is warranted.

Identification of an isolate as a thy⁻ strain can be accomplished by inoculating 20 μl of a 500-μg/ml solution of thymidine to a blank 1/4-in. (ca. 0.64-cm) paper disk (BBL Microbiology Systems) on MHII or MHBII and demonstrating growth around the disk but not around a blank control disk. Thymidine can be stored frozen at −20°C in small samples without significant loss of potency for at least 3 months.

**LITERATURE CITED**


**Table 1. Summary of data on patients with thy⁻ enterococci in urine**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Clinical diagnosis</th>
<th>Service location</th>
<th>Previous TMP-SMZ therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>86</td>
<td>F</td>
<td>Hip fracture, UTI*</td>
<td>Inpatient</td>
<td>5 wk</td>
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<tr>
<td>2</td>
<td>38</td>
<td>M</td>
<td>Colostomy, UTI</td>
<td>Emergency dept.</td>
<td>&gt;2 yr</td>
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<tr>
<td>3</td>
<td>8</td>
<td>F</td>
<td>Recurrent UTI</td>
<td>Outpatient</td>
<td>&gt;2 yr</td>
</tr>
<tr>
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<td>M</td>
<td>Quadruplegia, UTI</td>
<td>Inpatient</td>
<td>1 wk</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>M</td>
<td>Corneal transplant, UTI</td>
<td>Outpatient</td>
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</tr>
<tr>
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<td>73</td>
<td>M</td>
<td>Unknown</td>
<td>Other hospital</td>
<td>Unknown</td>
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</table>

* UTI, Urinary tract infection.


