Study on the Usefulness of Hypertonic Culture Media

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Specimens from 300 patients were studied using five to nine aerobic and anaerobic culture media, including five that were hypertonic. Groups studied included fever of unknown origin, suspected endocarditis, endocarditis during therapy, bacteremia during therapy, abscess and cellulitis, presumed infectious arthritis, renal transplantation during rejection, collagen disease, sarcoidosis, lymphoma, and colitis. Isolates in hypertonic media were reverted to parent form by agar passage. In only 5% of these selected cases were organisms found in hypertonic, but not conventional, media that appeared on the basis of repeated isolation and/or serological studies to come from the patient. Nine of the 16 appeared to be of major significance. The two groups in which use of highly enriched, hypertonic media seemed most helpful were suspected endocarditis and undefined meningitis with negative cultures using standard media. The most effective of the hypertonic media used was 0.3 M sucrose in brain heart infusion with 20% horse serum. In most instances, the organism grew only in the hypertonic sucrose, and in most cases it appeared in conventional rather than aberrant form. Hypertonic media, especially 0.3 M sucrose, are of substantial help in a small number of carefully selected cases.

The role of aberrant microbial forms in human disease remains unsettled. A number of reports show that aberrant or cell wall-defective variants, or both, of many microorganisms can be isolated on occasion from human body fluids or from human tissues (3-5). For the most part, the isolates have been found on reversion to the parent form to be gram-positive bacteria or, less frequently, yeasts.

It is still unclear whether these forms are merely markers indicating the presence of the pathogenic parent organisms, whether they are responsible for persistence of infection, or whether they are pathogenic in their own right (9). Aside from streptobacillary infections in man (6) and porcine *Haemophilus influenzae* experimental infections (10), pathogenicity has never been demonstrated convincingly.

In attempts to recover these fastidious and environmentally susceptible microorganisms, a variety of highly enriched culture media have been used. In the present study, we have tried to assess the usefulness of attempting to isolate these forms from human specimens. To do so, we have utilized first five, and then a total of nine, different aerobic and anaerobic liquid media for each specimen. Three hundred patients in 13 different categories have been studied; in most cases, at least two blood specimens were examined.

MATERIALS AND METHODS

Specimens of blood, spinal fluid, abscess material, or joint fluid were placed in tubes containing sterile heparin without preservative. A 0.5-ml portion of each specimen was placed in each of the liquid media designated for use. In some cases, the amount of fluid (particularly cerebrospinal fluid) was small, requiring that the amount in each tube be limited to 0.2 or 0.3 ml.

In the initial studies, three media were used: Trypticase soy broth, brain heart infusion broth containing 10% sucrose, and 77 medium (3% NaCl) (8). In the current study, the first 100 patient specimens were cultivated as follows.

Specimens were inoculated into five different liquid media and to the surface of two solid media. One-half milliliter was put in each tube or on a plate containing approximately 10 ml of medium.

The broth media used were: (i) 0.3 M sucrose in brain heart infusion with 20% horse serum added; (ii) 77 medium and Trypticase soy broth with 3% NaCl and 10 to 20% horse serum added; (iii) Trypticase soy broth; (iv) thioglycolate broth with 20% horse serum; and (v) thioglycolate with 0.3 M sucrose and 20% horse serum added. The plates used contained 5% sheep blood in a Trypticase soy agar base and 77 agar.

For the next 200 cases, the following liquid media were added: (i) 0.3 M sucrose broth with 20% horse serum and 1% Grobax (sodium polyanethol sulfonate); (ii) dithiothreitol broth, consisting of both 10^{-3} M dithiothreitol and 10^{-3} M L-cysteine in brain heart infusion broth (this was added to the broths

because of encouraging results with this medium [2]); and (iii) chopped meat glucose broth, a prereduced anaerobic medium. Additional agars used were chocolate (brucella agar base with hemoglobin plus X and V factors) and 5% horse blood in a Trypticase soy agar base, with a drop of *Staphylococcus aureus* culture in the center for satellite growth.

Additionally, 100 patient blood specimens were placed in 30% sucrose in brain heart infusion with 20% horse serum (14). No positive results were found with this medium, and it was discontinued after 100 patient specimens were studied. Similarly, chocolate and horse blood agars were discontinued after 100 patient specimens were studied with no positive results.

All aerobic tubes and plates were incubated at 37°C under 5% CO₂. This temperature was chosen arbitrarily because of our feeling, based on our prior studies, that the aberrant organisms we had isolated grew better at this temperature than at 37°C. However, we have not done a systematic study of growth patterns at various temperatures. Anaerobic tubes and plates were incubated in an anaerobic Gas-Pak jar (BBL) at 37°C.

Broths were subcultured at intervals of 5 to 7 days. If turbidity or obvious growth occurred, subcultures were made sooner. Approximately 0.1 ml was streaked on each of the agars, and 0.2 ml was placed in 0.3 M sucrose broth. All other tubes were subcultured, and Gram stains were made several times before they were discarded after being observed for 3 to 4 months.

The groups studied are indicated in Table 1. In most instances at least two blood specimens were studied. However, in those with suspected or proved endocarditis and in the bacteremia cases, only one specimen was studied in 30% of the cases. In cases of meningitis under treatment and presumed infected (nontuberculous) arthritis, only one specimen of blood and either spinal or joint fluid were studied. In those with subcutaneous abscess, material from the abscess was studied in addition to blood. In 7 of 22

patients with lymphoma and in 10 of 23 with sarcoidosis, lymph nodes were studied in addition to blood specimens.

All bacterial isolates were reverted to a parent organism by serial agar passage. For serological tests, the organism was incubated for 24 h at 30°C in 0.3 M sucrose, washed three times with saline, and resuspended in saline. The suspension, containing 5×10^7 to 5×10^8 colony-forming units/ml according to pour plate enumeration techniques, was heat-killed (56°C, 1 h), and 1 ml was inoculated three times weekly intravenously into rabbits for 7 weeks. A fresh culture was used each week. Rabbits were bled by cardiac puncture 8 weeks after the initial injection of killed organisms. Rabbit antisera, serum from the patient, and at least three normal human controls were simultaneously tested for precipitins in Preer double-agar tube diffusion tests (11, 13). Each serum was tested undiluted and at a 1:10 dilution. In these studies, washed Pyrex tubes (75 by 10 mm) were coated with 0.5% Noble agar (Difco). Noble agar (2%) in 0.9% sodium citrate, 0.85% sodium chloride, and 1:100,000 merthiolate were mixed with equal parts of antigen (0.25 to 0.25 ml). The central layer (0.3 ml) consisted of equal parts of agar solution and saline. The patient's serum was used as the upper layer. Control tubes were prepared with equal parts of the agar solution and saline (instead of agar and antigen) in the lower layer. All tubes were incubated at 37°C for 2 days and, if results were negative, at room temperature for another 3 days. A positive serum gave a precipitin line tangential to the meniscus between the neutral and serum layers.

RESULTS

The overall yield in this series (Table 1) was small. In only 16 cases (5%) were organisms found that on the basis of repeated isolation or serological studies, or both, appeared to come from the patient. Of these, nine organisms appeared to be of major significance; the role of the other seven isolated organisms was difficult to assess. In each case, the organisms were recovered initially only from the 0.3 M sucrose medium, and either the original culture tube or the first subculture was positive. Organisms could always be seen in wet mounts and on Gram-stained smears of the positive broths.

In each case, cultures had been negative in the diagnostic bacteriology laboratory. Five different hospital laboratories were involved. In these laboratories, blood culture specimens were incubated for 7 to 10 days and cerebrospinal fluid for 2 to 7 days. Liquid media included Trypticase soy, chopped meat glucose, and thio-glycolate. Agars used were blood, chocolate, and eosin methylene blue. Thus, in no instance were cultures positive with conventional media.

Cases in which the organisms appeared

TABLE 1. Groups studied for the presence in blood, cerebrospinal fluid, or tissues of aberrant microbial forms

Group studied	No. of cases studied	No. positive ^a	Positive
Fever of unknown origin	32	1	3
Suspected endocarditis	32	6	18.8
Endocarditis during treatment ^b	41	2	4.8
Meningitis during treatment ^b	30	0	0
Undefined meningitis	7	2	28
Bacteremia during treatment ^b	30	0	0
Abscess, cellulitis, pneumonia	36	3	8.3
Acute arthritis	15	0	0
Renal transplant during rejection	5	0	0
Colitis	6	0	0
Sarcoidosis	23	1	4.3
Collagen disease	21	1	4.8
Lymphoma	22	0	0

^a Only 0.3 M sucrose broths showed growth initially.

^b Duration of treatment ranged from 1 to 14 days.

clinically significant. The nine cases are summarized in Table 2. In cases 1 and 2, the alpha-hemolytic streptococcus was isolated only once, but in each case precipitin tests were positive, the patient was known to suffer from alpha streptococcal endocarditis, and the antibiotic sensitivity pattern of the pretherapy isolate was identical to the pattern observed in the isolate recovered in hypertonic media (after reversion). In cases 3 to 6, the clinical and microbiological evidence seemed to us to clearly implicate the isolated organisms as the cause of the endocarditis. Patient 7, a heroin user, could also have had endocarditis, since such persons often do not have demonstrable cardiac murmurs; her response to therapy was dramatic. Case 8 is similar to a case we previously reported (8) in which initially only hypertonic media were positive, but subsequently, during amphotericin B therapy, the yeast grew well in standard media. In case 9, only a single cerebrospinal fluid specimen was available to us. The confirmatory precipitin test was strongly positive, and the patient responded well to amphotericin B therapy.

Cases in which organisms were recovered but could not be shown to be clinically significant. In 7 cases (Table 3), aberrant organisms were isolated, but their role in the patients'

diseases was unclear for a variety of reasons. In cases 1 and 2, the single isolate could have been a contaminant. The clinical and pathological picture in case 3 showed convincingly that the patient had endocarditis and the failure to show organisms in the valve may have been due to the 12 days of antimicrobial therapy. This case may well belong in the clinically significant group, but it is included in the uncertain category because of the combination of only a single isolate pre-mortem and failure to demonstrate organisms at autopsy. Case 4 cannot be interpreted because of a positive serological reaction in one of the controls. In case 5, change in the precipitin test from negative to positive during the illness suggests a transient bacteremia. Additionally, sera from other patients with proven sarcoidosis did not show precipitins against the organism isolated twice from the blood of this patient. Case 6 is unique; unfortunately, there was only a single positive culture, this from a carefully obtained surgical specimen from a deep-seated site well ahead of the clinically obvious necrotic lesion. All blood cultures were negative. Eventually, despite multiple antimicrobials, the burrowing necrotic lesions eroded into the trachea. Case 7 is also interesting. The patient clearly had a propensity to develop massive inflammatory lesions, but their nature and

TABLE 2. Cases in which aberrant organisms isolated appeared to be significant in patient's illness

Case	Age	Sex	Antibiotic therapy	Disease	Organism isolated ^a (no. of cultures positive)	Precipitin test ^b	Comments
1	47	M	Penicillin, streptomycin	Endocarditis	Alpha-hemolytic streptococcus (1)	+	Isolated 2 days after treatment for proven alpha streptococcal endocarditis
2	59	M	Penicillin	Endocarditis	Alpha-hemolytic streptococcus (1)	+	On long-term therapy for recurrent alpha streptococcal endocarditis
3	42	M	Multiple	Endocarditis	<i>Pseudomonas aeruginosa</i> (2)		Same organism isolated at autopsy
4	51	F	None	Endocarditis	<i>Staphylococcus epidermidis</i> (2)	+	Died with aortic insufficiency; no autopsy
5	45	F	None	Endocarditis after cardiac surgery	Alpha-hemolytic streptococcus (2)	+	Died of cerebral emboli
6	50	M	None	Endocarditis with aortic insufficiency	Alpha-hemolytic streptococcus (2)	+	Recovered after penicillin-streptomycin treatment
7	20	F	None	Heroin abuse	<i>Corynebacterium</i> sp. (2)	+	Bacteremia without endocarditis; recovered after appropriate therapy
8	16	F	None	Meningitis	<i>Cryptococcus neoformans</i> (1)	Antigen negative, antibody 1:40 ^c	Later cultures positive on regular media
9	24	M	None	Meningitis	<i>Candida albicans</i> (1)	+	Meningitis indolent; responded to amphotericin B

^a After reversion; organism isolated from blood cases 1-7, spinal fluid from cases 8-9.

^b Positive precipitin test with controls negative.

^c Antigen determined by latex agglutination; antibody determined by cryptococcal cell agglutination.

TABLE 3. Cases in which aberrant organisms were isolated but could not be shown to be clinically significant

Case	Age	Sex	Disease	Organism isolated ^a (no. of times recovered)	Precipitin test ^b	Comments
1	21	M	Pericarditis	<i>Corynebacterium</i> sp. (1)	+	One culture positive from pericardium on second pericardiocentesis; blood culture negative
2	27	F	Dermatomyositis	Alpha-hemolytic streptococcus (1)	+	Later cultures negative; possibly transient bacteremia in compromised host
3	60	F	Endocarditis	Alpha-hemolytic streptococcus (1)	+	Probably was significant, but only 1 positive culture and organism not seen in vegetations at autopsy
4	66	M	Rheumatic heart disease; fever	<i>Corynebacterium</i> sp. (2)	— ^c	Improved without therapy; serological tests uninterpretable
5	25	M	Sarcoidosis with fever	<i>Corynebacterium</i> sp.	Negative, then +	Probably represented transient bacteremia
6	24	F	Diabetes with burrowing subcutaneous necrosis	<i>Corynebacterium</i> sp. from deep surgical specimen (1)	+	Organism alternated between antibiotic-sensitive <i>Corynebacterium</i> sp. and antibiotic-resistant alpha streptococcus; single isolate makes significance uninterpretable
7	19	M	Subcutaneous necrosis of leg Multiple subcutaneous abscesses	<i>Actinomyces viscosus</i> ^d from subcutaneous lesion (1) <i>Staphylococcus epidermidis</i> (2); abscesses also positive	+	Isolated once from open lesion; significance uninterpretable Organisms seen in abscess Gram stain, but lesions did not behave like staphylococcal abscesses

^a Organism found on reversion; organism in blood unless otherwise indicated.^b Positive precipitin test with controls negative.^c —, Control also positive.^d Organism identified by Center for Disease Control.

underlying host defects have eluded definition. The staphylococcus isolated repeatedly during his second illness certainly was in the lesions, but they fulminated despite adequate drainage and the administration of appropriate antimicrobials; thus, there was a divergence between the microbiological findings and the clinical pattern, making the role of the isolated organisms unclear.

Cases in which infection was not detected in hypertonic studies. In three instances, we think that the use of hypertonic media should have produced positive results, but it did not. In one patient with documented staphylococcal endocarditis, fever recrudesced 1 day after methicillin treatment was completed. Although all cultures, including specimens placed in hypertonic media, were unproductive, the patient responded to antistaphylococcal therapy when it was reinstituted. In a second case, indolent lymphocytic meningitis supervened in an elderly woman being treated with steroids for myasthenia gravis. Three cerebrospinal fluid cultures showed no growth in routine and hypertonic media. Eventually, intracerebral candida abscesses ruptured into the meninges, all cultures in routine media were positive, and the patient died. In this case, specimens took 1

to 4 days to be delivered to our laboratory, and this could have accounted for our lack of success. In the third instance, a patient with candida endocarditis on a Starr-Edwards prosthesis had six positive blood cultures in the routine diagnostic laboratory. Six days later, before amphotericin B treatment was started, two blood specimens were studied in the routine diagnostic laboratory and two in our laboratory. All four cultures showed no growth.

DISCUSSION

In addition to the 16 cases summarized, we have previously reported 8 other cases in which microorganisms of apparent clinical importance were recovered from hypertonic but not isotonic media (7, 8). We have now studied over 600 individual patients, with a total yield of 17 organisms clearly appearing to be of clinical significance. This is a small percentage, considering the enormous amount of work involved. This small yield is similar to that found by Phair and colleagues in a study of 42 patients (22 with fever of unknown origin, 6 with abacteremic endocarditis, 14 with endocarditis during therapy) (12). Four points are worth stressing.

First, no medium was as effective as 0.3 M

sucrose. Others have found a variety of other media extremely valuable. Some of these have been included in this study. In no instance have other media shown positive results when the 0.3 M sucrose remained negative; indeed, in only 2 of the 24 cases were the other media positive. In the other 22 cases, only the 0.3 M sucrose was initially positive, and growth occurred in the other media only on subculture. The negative tubes were studied in original and subculture for many weeks, so the negative tubes probably would not have become positive with more prolonged observation. To some extent, this might represent a bias toward 0.3 M sucrose on the part of the single technician who performed these studies over 6 years. It could be argued that we should have compared the sucrose with brain heart infusion rather than Trypticase soy broth. We have used Trypticase soy broth for a long time and chose to continue using it as the "routine" medium. There is no evidence that brain heart infusion is better than Trypticase soy broth. Furthermore, the five hospital laboratories that received specimens from these patients for routine diagnostic purposes used at least one liquid medium with a Trypticase soy base. We considered it desirable to make observations with that standard medium for longer than the 7 to 10 days the hospitals used; such a study is necessary to obviate the argument that although hypertonic media permit more rapid isolation, they are not more frequently positive.

Second, the yield was greatest in putative cases of endocarditis in which routine cultures were negative. Indolent meningitis with negative cultures and a likely microbial etiology was another area in which hypertonic media proved beneficial. In each of three cases of meningitis (two reported here, and the other detailed elsewhere [8]) in which these studies were helpful, yeasts were the offending organisms. In our series, 8 of 39 cases of endocarditis or indolent meningitis were positive as contrasted to 8 of 261 cases in other categories, a highly significant statistical difference ($\chi^2 = 17.7$, $P < 0.001$).

Third, the yield in patients receiving antibiotics was disappointing, even though in most cases those antimicrobials were directed against the cell wall. In only two cases of bacteremia, meningitis, or endocarditis were the cultures positive. This is a smaller yield than reported by Phair et al., who found positive results in 3 of 14 such cases (12). In another of our cases, after 4 days of penicillin for pneumococcal meningitis, the 0.3 M sucrose broth became cloudy, and gram-positive cocci were seen

on Gram-stained smears, but no growth occurred on agar or in liquid media subcultures. Almost all the patients we studied were responding to antimicrobials; the yield may well have been higher if they had not been responding so well. Nevertheless, we certainly have adduced no evidence to suggest that wall-defective forms frequently continue to circulate after antibiotics that act at the cell wall level are administered.

Further, our yield might have been even higher if we had used a larger inoculum and more broth. The current recommendation is 5 ml of blood in 50 ml of medium. We used only 0.5 ml in 10 ml of liquid medium, because each specimen had to be inoculated into so many media.

Fourth, we never isolated aberrant organisms from the anaerobic media. Others have, especially in postoperative thrombophlebitis cases (1). Our failure may be due to deficiencies in our techniques, use of the wrong media, or selection of patients not harboring fastidious or aberrant anaerobic organisms.

We firmly believe that in cases 1 through 9 the organisms isolated were clinically significant, but the serological data must be interpreted cautiously. We used only a small number of controls, and we cannot rule out the possibility that in some cases positive reactions represented serological responses to their own skin flora, rather than serious microbial invasion. This possibility deserves particular consideration, since the organisms isolated can often be part of normal skin flora. Nevertheless, supporting serological data are highly desirable. Because the media are highly enriched, laboratory contamination is always a possibility; a confirmatory serological test eliminates that explanation for the positive cultures, although it does not remove the possibility that the isolate represents skin contamination of the specimen at the time the blood was drawn or the possibility that the bacteremia was only transient and clinically insignificant. Serological tests cannot establish the isolates as clinically significant, but they do add important additional information that must be interpreted in the light of the clinical picture and the number of positive cultures.

Clearly, the use of hypertonic media was extremely helpful in some cases. Without such media, the correct diagnosis would certainly have been delayed or perhaps not established at all. That, in turn, would have resulted, in at least some cases, in improper or inadequate therapy.

Since the amount of work per specimen is

enormous and the yield low, cases for study should be carefully selected. In addition to those with meningitis or suspected endocarditis with negative routine cultures, another promising group should be those with demonstrated bacteremia or meningitis who have clinical (or cerebrospinal fluid) evidence of persisting infection but whose routine cultures are nonrevealing.

Finally, it is well to reemphasize that these organisms may be wall defective, aberrant, or merely fastidious. In 12 of our 16 cases, the organism detected in 0.3 M sucrose was morphologically the same as that found with subcultures on agar. This is similar to the experience of Phair et al., who found classic microbial forms in all three of their positive cases (12). In the other four cases we observed, the organism initially appeared corynebacterium-like, but later reverted to an alpha-hemolytic streptococcus on solid-agar subcultures.

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