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Use of a Semiselective Medium to Culture *Legionella pneumophila* from Contaminated Lung Specimens

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Legionella pneumophila was successfully isolated, using a semiselective medium, from two of three lung specimens heavily contaminated with other organisms. This medium is composed of charcoal yeast extract agar, supplemented with vancomycin and polymyxin B. *L. pneumophila* was observed at 8 days on plates containing ≤40 units of polymyxin B and ≤1 μg of vancomycin per ml.

Bacteriological diagnosis of Legionnaires disease is difficult because of the lack of a good selective medium. Thus, in vitro isolation of *Legionella pneumophila* has usually been achieved only from normally sterile fluids and tissues (1, 2, 5). The organism is currently being isolated from contaminated specimens after intraperitoneal inoculation of guinea pigs (6). We report the use of a semiselective medium which may be useful in the rapid bacteriological isolation of the organism from contaminated specimens.

MATERIALS AND METHODS

Postmortem lung specimens were obtained (one by percutaneous aspiration) in an aseptic fashion from three patients with fatal Legionnaires disease. Examination of all three specimens by a direct immunofluorescent technique (DFA) for *L. pneumophila* was positive (3). Initial culture of each specimen onto charcoal yeast extract (CYE) agar yielded heavy growth of other organisms, making the isolation of *L. pneumophila* impossible (4).

CYE agar plates containing vancomycin and polymyxin B were made using vancomycin hydrochloride standard powder (Eli Lilly & Co., Indianapolis, Ind.) and polymyxin B sulfate standard powder (Pfizer Inc., New York, N.Y.). A "checkerboard" distribution of the antibiotics was used. The first set of plates, used to culture specimen 3, contained polymyxin B in concentrations of 40, 80, 160, and 320 units/ml and vancomycin in concentrations of 0.5, 1.0, 2.0, and 4.0 μg/ml, making 16 plates in all. The second set of plates, made after the successful isolation of *L. pneumophila* from specimen 3, was used to culture specimens 1 and 2. This set contained polymyxin B in concentrations of 10, 20, 40, and 80 units/ml and vancomycin in concentrations of 0.125, 0.250, 0.50, and 1.0 μg/ml, again making a total of 16 plates. Antibiotic-free plates were made from the same batches of media as the two sets of plates; an uninoculated plate and a plate inoculated with the specimen were used as controls. The antibiotics, at 10 times their desired final concentrations, were added to the molten, cooled CYE agar in a volume of 2.5 ml per 22.5 ml of medium. The CYE agar was made with 10% less water than usual. The vancomycin and polymyxin B solutions had previously been mixed together in equal volumes at 20 times their desired final concentrations. The pH of the CYE agar, before addition of the antibiotic solutions, was 6.90 ± 0.02 at 35 to 40°C. After the agar had solidified and cooled, the plates were dried and then inoculated.

All three specimens had been frozen at −70°C for a period of 1 to 4 months. The lungs were ground with Trypticase soy broth (TSB) in a Ten Broeck tissue grinder. Dilutions (1:10) of these were made with additional TSB, and 0.1-ml aliquots were plated onto the 16 antibiotic-containing plates as well as onto the antibiotic-free plates. The inoculum was distributed primarily in one quadrant of the plate. The other three quadrants were streaked for isolation. The lung aspirate, which was already diluted in TSB, was diluted to approximately 1:10 with TSB to obtain a sufficient volume to inoculate all plates with 0.1 ml. Smears of all the diluted specimens were examined by DFA.

The plates were incubated at 35 to 36°C in 3.0% CO_2 at 65% humidity. They were inspected daily for 2 weeks. Representative colonies were picked for Gram stain and DFA examination. DFA was performed as described by Cherry et al. using antibody conjugates directed against serogroups 1 to 4 (3). Serogroup 1 antibody conjugate was provided by the Biological Products Division, Center for Disease Control. Serogroups 2, 3, and 4 antibody conjugates were kindly provided by Roger McKinney of the Bureau of Laboratories, Center for Disease Control (7).

Two of these DFA-positive colonies from each specimen were subjected to further tests to confirm the identity of the organisms. Oxidase reaction was tested using 1% Kovacs reagent. Gelatinase was tested using undeveloped Plus-X Pan film strips (Eastman Kodak, Rochester, N.Y) incubated at 37°C with a heavy suspension of organisms in TSB.

L. pneumophila was identified on the basis of morphological, serological, and biochemical characteristics. A gram-negative filamentous bacillus that was

catalase and oxidase positive, failed to grow on both blood agar and Mueller-Hinton agar with X and V factors in CO_2, was gelatinase positive, and demonstrated strong fluorescence by DFA was identified as *L. pneumophila* (8). Gas-liquid chromatographic analysis of cellular fatty acids was not performed.

RESULTS

All uninoculated plates had no growth after 2 weeks of incubation. Growth of non-*Legionella* organisms on the antibiotic-free plates was heavy after 24 h. Specimen 1 had a heavy growth of *Citrobacter* sp. and *Klebsiella* sp. Specimen 2 had heavy growth of *Enterobacter cloacae* and *Candida albicans*. Specimen 3 had a heavy growth of *Staphylococcus aureus* and light growth of *Enterobacter* sp.

Direct immunofluorescence examination of all specimens was positive with serogroup 1 antibody conjugate only. Specimen 1 had 2 to 3 fluorescent bacilli per ×650 field, specimen 2 had 100 fluorescent bacilli per entire smear, and specimen 3 had 20 fluorescent bacilli per entire smear.

All the antibiotic-containing plates of specimen 2 were overgrown with the same organisms present on the antibiotic-free plate, although in lesser numbers; *C. albicans* predominated. The antibiotics did effectively inhibit growth of non-*Legionella* organisms from the other two specimens. One to three colonies of the non-*Legionella* organisms from specimen 1 grew on the plates containing 10 units of polymyxin B per ml, and none grew on the plates containing higher concentrations of the drug. No non-*Legionella* organisms from specimen 3 grew on any of the antibiotic-containing plates. The results of growth of *L. pneumophila* on the antibiotic-containing plates are summarized in Table 1.

All isolated *L. pneumophila* were serogroup 1.

DISCUSSION

These results indicate that CYE agar supplemented with polymyxin B and vancomycin is effective as a semiselective medium because it enabled recovery of *L. pneumophila* from two of three contaminated specimens. It was not possible in two attempts each to recover this organism from the same specimens plated onto antibiotic-free CYE agar. The optimal concentrations of polymyxin B and vancomycin appear to be 40 units/ml and 0.5 μg/ml, respectively. The optimal concentration depends upon the susceptibility patterns of the contaminating organisms as well as upon the concentration of *L. pneumophila* in the specimen. It is possible that growth of small numbers of *L. pneumophila* may be inhibited by these drug concentrations, and that lower levels may need to be used. As demonstrated for specimen 2, the addition of an antifungal agent such as nystatin or cycloheximide would be wise. Whether or not this will affect the activity of the other antibiotics needs to be investigated. It is unlikely that VCN solution (BBL Microbiology Systems, Cockeysville, Md.) could be used to make this medium, since the 7.5:3 colistin-to-vancomycin ratio contained in this solution is not optimal.

We suggest that a single semiselective CYE plate, containing polymyxin B and vancomycin in concentrations of 40 units/ml and 0.5 μg/ml, respectively, be used as well as an antibiotic-free CYE plate when attempting to isolate *L. pneumophila* from contaminated specimens.

TABLE 1. *Growth of L. pneumophila on antibiotic-containing plates on selected days of incubation*

Specimen no.	Antibiotic concn[a]		Colonies per plate on day of incubation:				
	PB	V	1-4	5	8	10	14
1	10	1.0	0	15	50	50	50
	≤20	0.125-0.5	0	10-20	>300	>300	>300
	20	1.0	0	1	50	>300	>300
	40	0.125-0.5	0	0	100-200	100-200	100-200
	40	1.0	0	0	20	150	150
	80	0.125	0	0	5	25	50
	80	0.25	0	0	0	15	20
	80	0.5-1.0	0	0	0	1	15
3	40	0.5	0	1	18	18	18
	40	1	0	0	2	2	12
	40	2	0	0	0	0	1
	40	4	0	0	0	0	0
	>40	0.5-4	0	0	0	0	0

[a] Antibiotic concentrations in units per milliliter for polymyxin B (PB) and micrograms per milliliter for vancomycin (V).

Other modifications of the medium need to be made, so that swarming *Proteus* sp. will be inhibited. Possibilities include trimethoprim or nalidixic acid supplementation. A highly efficient selective medium might permit recovery of *L. pneumophila* from expectorated sputum and from soil and other environmental samples.

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ADDENDUM IN PROOF

Since submission of this manuscript, anisomycin (Pfizer Inc., New York, N.Y.) has appeared to provide suitable and stable antifungal activity in this medium without inhibiting *L. pneumophila*. The use of anisomycin was suggested by John Martin of the Center for Disease Control. A single lung specimen contaminated with *C. albicans*, *S. aureus*, and *Enterobacter* sp. yielded *L. pneumophila* after 3 days of incubation on this new formulation (vancomycin, 0.5 μg/ml; anisomycin, 80 μg/ml; polymyxin B, 40 units/ml). However, additional evaluation of the efficacy of anisomycin in this medium is needed.

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