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A Microtechnique for the Isolation of Cell Wall Deficient Organisms from Very Small Samples

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A miniaturized procedure using a modified microtiter plate is described for the isolation of cell wall deficient organisms. It is particularly useful when the volume of a liquid specimen is very small.

THIS procedural paper describes a microtechnique designed to enhance the isolation of cell wall deficient forms (CWDF, also called bacterial L-phase variants). Our primary laboratory study is concerned with the microbiology of the aqueous humor of the eye in patients with uveitis.[1] Minute samples of aqueous humor are obtained by paracentesis from the anterior chamber. Because of the small specimen obtained, (0.10-0.15 ml aqueous), we can allow only one (0.02 ml) or two drops from a 27-gauge needle per tube of isolation media. In conventional isolation procedures the volume of liquid specimens is usually much larger.

L-phase isolates do not necessarily grow as do their classical counterparts. In 1970 Godzeski[2] suggested that we might obtain more consistent results by using microtechniques and that several advantages might be so obtained.

1. By reducing the volume of the media from 40 drops to 4 drops we can reduce the inoculum size from 2 drops to one drop and effect an increase in the relative amount of aqueous in each tube from 5% to 20%. It may be that the aqueous contains nutritional elements essential to a particularly fastidious micro-organism.

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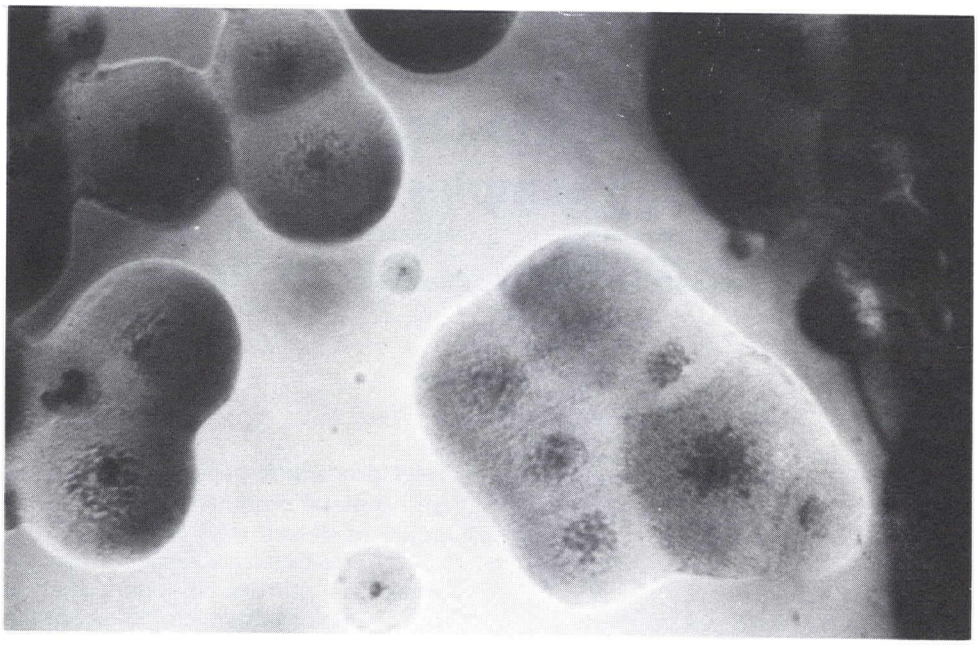

Figure 1
S. aureus (L-phase). Unstained X 620. Stock culture on L-agar. (5 day incubation).

2. Reducing the inoculum size to one drop per microwell allows a wider variety of media. Since work in bacterial L-forms is still hampered by great imprecision in media selection, this is thought to be of great importance.

3. Since the numbers of organisms present in the aqueous are thought to be small, it might be that their inoculation into a large volume of media simply allows them to be "lost in a sea of media".

Since it appeared potentially advantageous to employ the microtechniques in attempting to isolate fastidious L-phase organisms from very small samples, a comparison study was done between the standard macrotechnique and the microtechnique. Neither procedure demonstrated a significantly higher percentage of recovery over the other. The present study further investigates these microtechniques, using multiple media with stock cultures of L-phase bacteria.

Materials and Methods

A. Organisms used:

Three species of bacteria were used as test organisms: the L-phase of Staphylococcus aureus, the L-phase of Streptococcus pyogenes and the L-phase of Listeria monocytogenes*. These stock cultures were grown in L-broth, incubated in CO_2 (candle jar) at 37°C and stained with acridine orange and gram stain. The cultures were then subcultured to solid L-agar and characteristic colonies photographed.

*Cultures provided by York E. Crawford, Mycoplasma Division, Naval Medical Research Unit No. 4, U.S. Naval Hospital, Great Lakes, Illinois 60088. (Deceased)

Figure 2

S. aureus (L-phase). Acridine orange stain X 625. Smear of LB macrotube from LB micro-well. (5 day incubation).

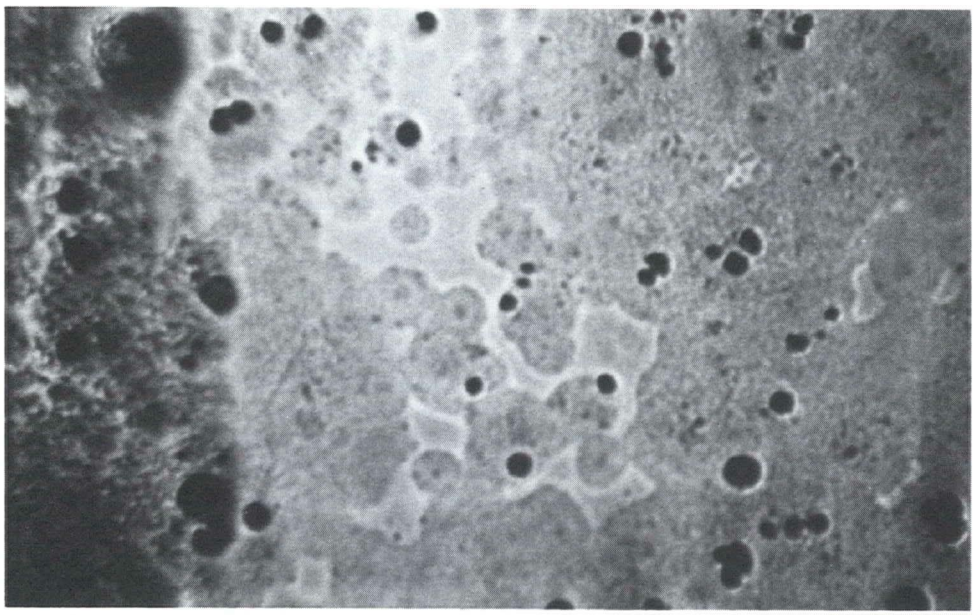

Figure 3

S. aureus (L-phase). Unstained X 620. "Fried-egg" colonies on L-agar of LB macrotube from LB micro-well. (5 day incubation).

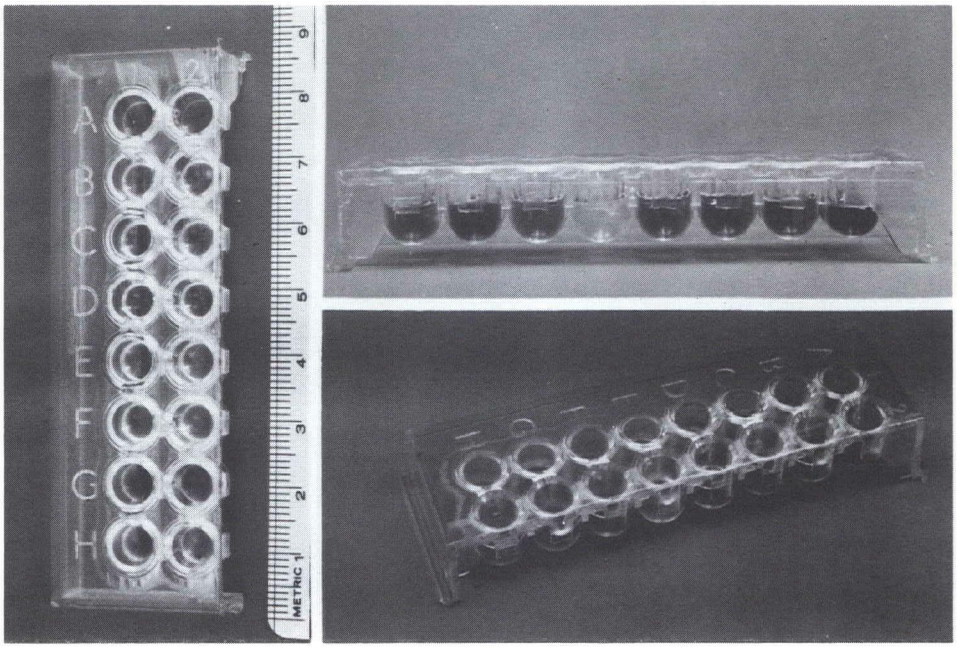

Figure 4
Micro-well plate (MWP) with 8 isolation media.

B. Micro-well construction:

The microtiter plate developed by Takatsy[3] has previously been modified for a variety of diagnostic procedures.[4,5] We further adapted it for these purposes of isolation. Commercially available* microtiter plates consist of a plexiglass plate molded to contain 96 U-wells. Each microtiter plate was cut into six small micro-well plates (MWP) having two parallel rows with eight wells in each row (Figure 4).

The volume of each well in the MWP is 0.2 ml. When compared to standard isolation techniques, this volume represents a 10-fold reduction in the amount of media used. The MWP were sterilized by washing in a series of three 95% ethanol baths. The first wash was for five seconds, the second for four hours, and the third for five seconds. The MWP were then aseptically placed in sterile bags and sealed until needed.

C. Media used:

Two side-by-side wells were assigned to each of eight different isolation media: Medill-O'Kane broth (MOK), L-Broth (LB), Thioglycollate broth (Thio), Heart Infusion broth (HIB), Kirschners broth (Kirsch.), Thioglycollate-X broth (Thio-X), KEI-4 broth with Cholesterol [KEI-4 (Chol.)] and KEI broth without Cholesterol [KEI-4 (w/o Chol.)]. For each medium one well was inoculated with two drops of the L-phase stock culture while the other was left uninoculated and served as a media control. After each of the different media had been inoculated, the plate was sealed by plac-

*Microtiter System Components, Microbiological Associates, Inc., 4733 Bethesda Ave., Bethesda, MD, 20014.

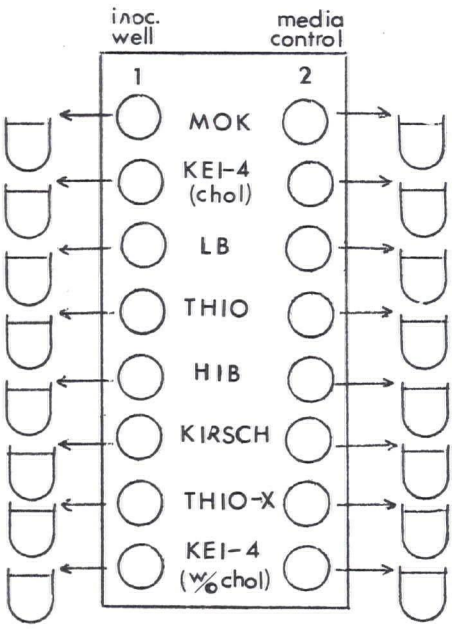

Diagram 1
Micro-well transfer to corresponding macrotube at 96 hours. Each microwell (circle) contains 0.2cc of media. Each macrotube (stirrup) contains 2.0cc of media.

ing a strip of steam-autoclaved transparent tape over the top. The tape allowed us to see into and through the wells. Inoculated micro-well plates were incubated in CO_2 at 37°C for 96 hours.

D. Gross examination of micro-wells:

The micro-wells were examined after 96 hours and their gross appearance described. Each inoculated micro-well was carefully compared to its media-control well by looking down through the taped top of the plate as well as examining it from the side. The micro-well plate was then sharply tapped to detect turbidity.

Inoculated micro-wells were considered probably positive when they did not appear identical to the media control. Colonial growth of CWDF often caused a turbidity which was easily discernible. More subtle changes in the color of the media also made us consider the inoculated well as probably positive.

E. Transferring inoculation of micro-wells to macrotubes:

After 96 hours the contents of each well was aseptically pipetted into a regular (full size) tube containing 2.0 ml of fresh medium of the same type (Diagram 1). These regular tubes (macrotubes) were incubated in CO_2 at 37°C for five days at which time they were compared to uninoculated macrotubes containing the same medium (media controls). Changes in gross appearance such as turbidity or color changes were recorded.

F. Gross examination of macrotubes:

Discrepancies between inoculated and uninoculated macrotubes, usually either changes in turbidity or changes in color of the media, were tentatively attributed to microbial growth in the inoculated, macrotube pending microscopic confirmation.

G. Microscopic and macroscopic confirmation of microbial growth:

Acridine-orange stain (which demonstrates fluorescence of nucleic acids under UV microscopy)[6], gram stains, and subculture to corresponding agar plates, (ie, the respective broth media solidified with agar) were done on all macrotubes at five and thirty days. Acridine-orange stains (A.O.) and gram stains (G.S.) were considered positive (suggestive of growth) if the stain of the media control tube was negative and there were three or more colonies per smear of the macrotube inoculated with stock CWDF. Subcultures to solid agar plates were examined after 14 days' incubation in CO_2 at 37°C. They were considered positive if the plate inocu-

Results:

A. CHART NO. 1: APPEARANCE OF MEDIA MACROSCOPICALLY

Stock L-Form Culture:	Appearance of Media Macroscopically					
	Microwells at 96 hours		Macrotubes at 5 days		Macrotubes at 30 days	
	Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
	Neg.*	Pos.*	Neg.	Pos.	Neg.	Pos.
Staph. Aureus Stock L's	2	6	5	3	4	4
List. Mono-cytogenes Stock L's	2	6	5	3	6	2
Strep. Pyogenes Stock L's	2	6	3	5	6	2
TOTALS	6	18	13	11	16	8

*Negative = clear with no color change
*Positive = turbidity with a color change

1. Macroscopic appearance of the micro-wells at 96 hours (Columns 1 and 2):
   a. 6 of 24, (25%) were negative.
   b. 18 of 24, (75%), of the micro-wells showed evidence of CWDF or L-phase variant growth.

2. Macroscopic appearance of the mac-rotubes at 5 days incubation (Columns 3 and 4):
   a. 13 of 24 macrotubes, (55%, were negative after 5 days.
   b. 11 of 24 macrotubes, (45%), were positive after 5 days incubation.

3. Macroscopic appearance of macrotubes at 30 days of incubation (Columns 5 and 6):
   a. 16 of 24 macrotubes, (66%), were negative at 30 days.
   b. 8 of 24 macrotubes, (33%), were positive after 30 days incubation.

B. CHART NO. 2: FLUORESCENT STAINING (ACRIDINE ORANGE) OF MACROTUBES

Stock L-Form Culture:	Acridine-orange staining of Macrotubes			
	5 days		30 days	
	Col. 1	Col. 2	Col. 3	Col. 4
	Neg.*	Pos.*	Neg.	Pos.
Staph. Aureus Stock L's	3	4	3	4
List. Mono-cytogenes Stock L's	5	2	2	4
Strep. Pyogenes Stock L's	4	3	5	2
TOTALS*	12	9	10	10

*Negative stain results
*Positive stain results

1. Acridine-orange stains of macrotubes after 5 days incubation (Columns 1 and 2):
   a. 12 of 21 A.O. stains, (58%), from the macrotubes incubated for 5 days were negative.
   b. 9 of 21 A.O. stains, (42%), from the macrotubes incubated for 5 days were positive.

2. Acridine-orange stains of macrotubes after 30 days incubation (Columns 3 and 4):
   a. 10 of 20 A.O. stains, (50%), from the macrotubes incubated for 30 days were negative.
   b. 10 of 20 A.O. stains, (50%), from the macrotubes incubated for 30 days were positive.

*Totals of Columns 1+2 and 3+4 are less than 24 because of laboratory contamination with Paecilomyces sp.

lated with media-control remained unchanged, and characterisitc CWDF colonies appeared upon microscopic examination of the plates inoculated with media containing organisms. If the inoculated plate remained identical in appearance to its media-control, it was considered negative for support of CWDF.

A miniaturized isolation procedure has demonstrated the successful

C. CHART NO. 3: GRAM STAINING OF MACROTUBES

Stock L-Form Culture:	Gram Staining of Macrotubes			
	5 days		30 Days	
	Col. 1	Col. 2	Col. 3	Col. 4
	Neg.*	Pos.*	Neg.	Pos.
Staph. aureus Stock L's	3	5	4	4
List. Mono-cytogenes Stock L's	5	3	5	3
Strep. Pyogenes Stock L's	5	3	6	2
TOTALS	13	11	15	9

*Negative stain results
*Positive stain results

1. Gram stains of macrotubes after 5 days incubation (Columns 1 and 2):
   a. 13 of 24 gram stains, (55%), of the macrotubes after 5 days incubation were negative.
   b. 11 of 24 gram stains, (45%), of the macrotubes after 5 days incubation were positive. (Of these 11 positive gram stains, 8 were identifies as "gram negative pleomorphic forms". The remaining 3 of 11 positive gram stains were identified as gram negative cocci).
2. Gram stains of the macrotubes after 30 days incubation (Columns 3 and 4):
   a. 15 of 24 gram stains, (62%), of the macrotubes after 30 days incubation were negative.
   b. 9 of 24 gram stains, (38%), of the macrotubes after 30 days incubation were positive. (Of these 9 positive gram stains, 6 were identified as "gram negative pleomorphic forms". The remaining 3 of 9 positive gram stains were identified as gram negative rods).

D. CHART NO. 4 SUBCULTURES TO AGAR MEDIAS

Stock L-Form Culture:	Subcultures to Agar Medias			
	5 days		30 Days	
	Col. 1	Col. 2	Col. 3	Col. 4
	Neg.*	Pos.*	Neg.	Pos.
Staph. Aureus Stock L's	5	1	4	1
List. Mono-cytogenes Stock L's	5	1	4	1
Strep. Pyogenes Stock L's	5	1	4	2
TOTALS*	15	3	12	4

*Negative for growth
*Positive for growth

1. Subcultures of macrotubes after 5 days incubation to corresponding agars. (Columns 1 and 2):
   a. 15 of 18 subcultures, (80%) from the macrotubes to their corresponding agars were negative after 5 days.
   b. 3 of 18 subcultures (20%), from the macrotubes to their corresponding agars were positive after 5 days.
2. Subcultures of macrotubes after 30 days incubation to corresponding agars. (Columns 3 and 4):
   a. 12 of 16 subcultures, (75%), from the macrotubes to their corresponding agars were negative after 30 days.
   b. 4 of 16 subcultures, (25%), from the macrotubes to their corresponding agars were positive after 30 days.

*Totals of Columns 1±2 and 3+4 are less than 24 because of laboratory contamination with Paecilomyces sp.

maintenance of stock L-phase cultures. A good example of the maintenance is evidenced by the L-broth sequence. When the micro-well containing L-broth medium is seeded with one drop of S. aureus (L-phase), a growth will be noted by turbidity of the medium when compared to its control at two days. After four days the contents of this well is aseptically pipetted into a macrotube

containing a ten-fold increase of the same medium and, again, good growth is noted within three days when compared to a control. A sample from the macrotube is acridine-orange positive and stains gram negative. When subcultured to a corresponding solid medium (L-agar) the classical "fried-egg" colonies are noted in 3-4 days (all incubation in CO_2 at 37°C).

Discussion

There are numerous reports of the isolation of L-forms and of transitional forms of bacteria from blood, urine or other clinical specimens in septicemia,[7] subacute bacterial endocarditis,[8] chronic pyelonephritis,[9] and following various surgical procedures.[10] Recently, cell wall deficient microbiological forms have been isolated from the aqueous of patients with active recurrent inflammatory intraocular disease (uveitis).[1]

To isolate L-variants from blood or urine, conventional macrotechniques are often adequate. When limited, however, to very small quantities of specimen, as with the aqueous humor of the eye, macrotechniques may be inadequate and the important isolate, (or some initial essential nutrient,) "lost in a sea of media". Thus, excessive dilution of the specimen by transfer to a large volume of liquid medium may be as inappropriate as not using the correct medium.

Conclusion

To allow the inoculation of very small amounts of specimen into much reduced volumes of media, we have devised a technique using a micro-well plate. We have shown this microtechnique to be capable of maintaining the growth of CWDF as long as the media itself are capable of this support.

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