ACETIC ACID INHIBITION OF GRAM-NEGATIVE BACILLI IN CULTURE MEDIA

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Many substances have been added to culture media in attempts to inhibit the growth of gram-negative bacilli, especially those of the coli-proteus-pyocyaneus group, thereby facilitating the isolation of gram-positive organisms (Floyd and Dack, 1939; Snyder and Lichstein, 1940; Lichstein and Snyder, 1941). However, with effective concentrations of these substances, many of the gram-positive bacteria are also inhibited, their colonial characteristics are altered, or typical hemolysis is suppressed or disguised by changes in the medium. Further, in liquid media, the gram-negative bacilli soon outnumber the gram-positive organisms and, when the culture is plated, the appearance of isolated gram-positive colonies is more a matter of luck than of good management. Sporulating forms may be isolated by the use of heat, but the cocci are often exceedingly difficult to obtain in pure culture.

For some years it has been the practice of clinicians to irrigate with dilute acetic acid such wounds as are contaminated or infected with *Pseudomonas aeruginosa* or related species in attempts to decrease the activity of these organisms. Levine and Fellers (1939, 1940), studying the effect of acetic acid on microorganisms involved in the spoilage of food, demonstrated that the toxic effect of this substance is due in part at least, to the undissociated molecule and not entirely to the changes in the hydrogen-ion concentration.

This laboratory has been engaged in the study of the bacterial flora of traumatic and surgical wounds and has found the isolation of gram-positive organisms from gram-negative overgrowths a frequent and discouraging problem. Therefore, an assay of the efficacy of acetic acid as an inhibitory agent for gram-negative organisms was made.

Technique. Meat tubes, consisting, on an average, of about 1 inch of ground beef heart in 15 ml of beef heart infusion broth, are boiled in a water bath and cooled rapidly in running water. Glacial acetic acid is diluted to 10 per cent and 1 per cent in sterile distilled water and added to the meat tubes as follows:

Tube 1—0.1 ml of 1 per cent
Tube 2—0.5 ml of 1 per cent
Tube 3—0.1 ml of 10 per cent
Tube 4—0.5 ml of 10 per cent
Tube 5—1.0 ml of 10 per cent

The tubes are shaken slightly to distribute the acid, which forms a heavy, white precipitate in the broth. Each tube is then inoculated with 0.1 ml of an 18- to

24-hour meat tube culture of the specimen under study and incubated at 37 C, aerobically or anaerobically according to the preference of the organisms sought. The cultures are examined microscopically at 24-hour intervals, and, when good growth of the gram-positive or moderate to poor growth of the gram-negative organisms is observed, the material in that tube is streaked on horse blood agar plates (plus 5 to 6 per cent of 95 per cent ethyl alcohol if the flora includes *Proteus*). After overnight incubation aerobically and anaerobically at 37 C, the plates are examined for colonies of the desired types.

Results. Table 1 gives the typical pH determinations in the series of meat tubes after the addition of acetic acid. The pH was determined by the use of BDH universal indicator.

The results obtained by the use of this method are typified by the following summarized protocols:

Case 1, chronic leg ulcer: Staphylococci and streptococci were seen microscopically in meat tube cultures but were overgrown by *P. aeruginosa* when the

TABLE 1

Changes in the pH of meat tubes upon addition of acetic acid

TUBE NO.	pH BEFORE ADDITION OF ACID	AMOUNT OF ACID ADDED	pH IMMEDIATE	pH AFTER 18-HOUR INCUBATION UNINOCULATED
		ml		
1	7.5	0.1 of 1 per cent	6.5	6.5–7.0
2	7.5	0.5 of 1 per cent	6.0	6.5
3	7.5	0.1 of 10 per cent	5.0–5.5	6.0
4	7.5	0.5 of 10 per cent	4.5	5.0–5.5
5	7.5	1.0 of 10 per cent	4.0	4.0–5.5

tubes were plated out. Beta hemolytic streptococci, group C Lancefield, and coagulase-positive hemolytic *Staphylococcus aureus* were isolated from acetic acid tubes 3 and 4, thus accounting for all morphologic types seen with the microscope.

Case 2, chronic draining sinus from perisplenic abscess: Staphylococci and streptococci were seen microscopically but were overgrown by *P. aeruginosa* and *Escherichia coli* on plates. Coagulase-positive *S. aureus*, coagulase-negative *Staphylococcus albus*, aerobic and anaerobic nonhemolytic streptococci, and *Fusobacterium* sp. were isolated from tubes 3, 4, and 5.

Case 3, chronic leg ulcer: Streptococci, seen in smears from meat tubes, were overgrown by *Proteus*, even on 5 per cent alcohol plates. Alpha hemolytic streptococci were isolated on alcohol blood agar plates streaked from tube 5.

Case 4, perineal abscess: The very heterogeneous flora was overgrown by the abundant *E. coli* present in the culture. Alpha hemolytic and nonhemolytic streptococci, *Clostridium welchii*, and *Clostridium bifermentans* were isolated from tubes 3, 4, and 5, the clostridia without the necessity of resorting to the application of heat. These organisms accounted for all the morphologic types seen in microscopic preparations of the culture.

Case 5, cystitis in a debilitated patient: Streptococci and spore-bearing bacilli, seen microscopically, were overgrown by *E. coli* and *P. aeruginosa* in the cultures. Nonhemolytic streptococci, *Fusobacterium* sp., and *C. welchii* were isolated from tubes 1, 2, and 3, again without the necessity of heating to obtain the clostridium.

Case 6, persistent sinus from osteomyelitis of ribs: Streptococci and staphylococci were overgrown by *P. aeruginosa.* Coagulase-positive hemolytic *S. aureus* and beta hemolytic streptococci, group A Lancefield, were isolated from tube 3.

Case 7, abscess of the upper arm: Staphylococci and streptococci were overgrown by *Proteus* even on 5 per cent alcohol blood agar plates. Anaerobic nonhemolytic streptococci, coagulase-positive hemolytic *S. aureus,* and coagulase-negative *S. albus* were isolated when material from tube 3 was streaked on alcohol plates.

As can be seen from the foregoing cases, a number of strains, interesting from a clinical point of view, can be recovered with the aid of this medium. Various species of clostridia have been isolated from anaerobic blood agar plates streaked directly from acetic acid medium. Some species of clostridia also sporulate well in this medium and can be recovered with greater ease by heating these cultures to 80 C for 20 minutes than by similarly heating meat tube cultures to which acid had not been added.

In approximately one-third of the specimens inoculated into this medium, staphylococci, seen microscopically in the original tubes, were inhibited at the concentrations necessary to suppress the gram-negative bacilli and could not be recovered by this method. Other staphylococci and the great majority of the streptococci encountered were easily isolated.

Several specimens were inoculated directly from the lesions into acetic acid meat tubes to ascertain whether primary culture in the acid-containing medium would further facilitate the isolation of the gram-positive organisms. No growth occurred in any of the tubes containing the acid, however, and direct inoculation was abandoned in favor of 18-hour incubation in routine media before transfer to the acetic acid tubes.

In order to be certain that growth in the acetic acid medium combination did not change any characteristic for which the organism might be tested in this laboratory, strains isolated without resort to this technique were grown in the medium, recovered by plating, and tested against the original strains as controls. In no case was any change observed in (1) color, hemolysis, or coagulating power of the staphylococci, (2) type of hemolysis of the streptococci, or (3) Lancefield grouping or *in vitro* virulence test (Ward and Lyons, 1935) of beta hemolytic streptococci.

It was found that tubes 1 and 2 (0.1 and 0.5 ml of 1 per cent acetic acid) contained concentrations usually too low to inhibit the gram-negative bacilli, and that tubes 4 and 5 (0.5 and 1.0 ml of 10 per cent) had concentrations usually too high to allow any bacterial growth. These tubes, therefore, are omitted unless demanded by the reactions of a particular culture.

The technique (using 0.1 and 0.2 ml of 10 per cent acetic acid) is now routinely employed in this laboratory whenever this type of mixed culture is found and has

proved to be a simple, inexpensive, and very valuable aid, especially in the isolation of the gram-positive cocci.

A few experiments were done using HCl and H_2SO_4 in parallel with acetic acid, but these were entirely unsuccessful.

SUMMARY AND CONCLUSIONS

The addition of acetic acid to meat tubes has been found to be a valuable aid in the isolation of gram-positive bacteria from cultures overgrown by gram-negative bacilli of the coli-proteus-pyocyaneous group. The method, which is practical for routine laboratories, is described, and a number of typical cases are cited.

REFERENCES

FLOYD, T. M., AND DACK, G. M. 1939 The isolation of *Bacterium necrophorum* in the presence of *Proteus*. J. Infectious Diseases, **64**, 269–272.

LEVINE, A. S., AND FELLERS, C. R. 1939 The action of acetic acid on food spoilage microorganisms. J. Bact., **39**, 499–516.

LEVINE, A. S., AND FELLERS, C. R. 1940 Inhibiting effect of acetic acid upon microorganisms in the presence of sodium chloride and sucrose. J. Bact., **40**, 255–269.

LICHSTEIN, H. C., AND SNYDER, M. L. 1941 The inhibition of the spreading growth of *Proteus* and other bacteria to permit the isolation of associated streptococci. J. Bact., **42**, 653–664.

SNYDER, M. L., AND LICHSTEIN, H. S. 1940 Sodium azide as an inhibiting substance for gram-negative bacteria. J. Infectious Diseases, **67**, 113–115.

WARD, H. K., AND LYONS, C. 1935 Studies on the hemolytic streptococcus of human origin. I. Observations on the virulent, attenuated, and avirulent variants. J. Exptl. Med., **61**, 515–531.