THE USE OF THE SELECTIVE INHIBITORY ACTION OF TOLUENE ON COLIFORM BACILLI IN ROUTINE CULTURES

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Benians (1913) demonstrated that all Gram-negative organisms were destroyed by toluene, which had an inhibitory action on diphtheroid bacilli and streptococci but little effect on spores and staphylococci.

Webster (1942) suggested the use of toluene to suppress Gram-negative bacilli in mixed broth cultures; he added 10 per cent of toluene to broth cultures, shook them at intervals, and allowed them to stand for five minutes, by which time the toluene had risen to the surface. He removed a drop of the treated culture, taking precautions to avoid removing any toluene. Using this technique we have obtained results too variable to be of value, as sometimes coliform bacilli would survive or streptococci succumb. "Occasional" shaking is too haphazard, but vigorous shaking gives consistent results so long as the temperature of the broth is controlled.

Demonstration of the Effect of Temperature on the Action of Toluene

A set of bijou bottles, each containing 2 ml. measured from the same overnight broth culture of the organisms shown in the Table, were kept at each of the following temperatures: 4°, 23°, and 37° C. One of the bottles from each set was used as a control. A standard drop of the bacterial suspension was inoculated and spread evenly on to a MacConkey plate when coliform bacilli were being tested, or a blood plate for the other organisms; after overnight incubation a colony count was made. Similar counts were made after exposure of the organism to the action of toluene for times varying from 5 seconds to 3 minutes.

These results are summarized in the Table, which shows that *B. proteus*, *Ps. pyocyanea*, and the coliform bacilli generally were either suppressed by contact for 5 seconds at 37° C., or were reduced from hundreds of colonies per drop to about 7 or 10 in the same volume. At lower temperatures these Gram-negative organisms survived for much longer periods, so that at 4° C. they were present in large numbers after 2 minutes' exposure to toluene. The Gram-positive organisms withstood 2 minutes' exposure to toluene at 37° C., though there was a slight reduction in the number surviving after this time. Experiments with *Staph. aureus* and *Str. pyogenes* are duplicated to show the effect of toluene on small numbers of these organisms.

Organisms Tested and Shown to have Resistance to Toluene

Many strains of *Staph. albus*, *Staph. aureus*, α , β , and γ haemolytic streptococci, pneumococci, and diphtheroid bacilli encountered in routine work were tested and found to be resistant to toluene. As few strains of the following organisms were encountered, stock cultures were tested and were resistant: anaerobic streptococci 1 strain, *Cl. welchii* 6 strains, *Cl. tertius* 1 strain, *Cl. oedematiens* 1 strain.

The technique has now been employed for a period of over three years on routine cultures which included wound swabs, faeces, urines, and a few sputa, vaginal and cervical swabs, and throat swabs which have contained coliform bacilli: over five hundred strains of *B. proteus*

TABLE
REPRESENTATIVE RESULTS SHOWING THE EFFECT OF TEMPERATURE ON THE LETHAL ACTION OF TOLUENE

Organisms	Temp. °C.	Control	Time of exposure to toluene					
			5 secs.	15 secs.	30 secs.	1 min.	2 mins.	3 mins.
<i>Ps. pyocyanea</i>	4	+++	+++	+++	++	++	0	0
	23	+++	+++	++	+	12	0	0
	37	+++	2	—	—	—	0	0
<i>B. proteus</i>	4	+++	+++	++	+++	+++	+++	++
	23	+++	48	30	—	—	—	—
	37	+++	7	—	—	—	—	—
Diphtheroid bacilli	37	+++	+++	+++	+++	+++	++	0
<i>Staph. aureus</i>	37	+++	+++	+++	+++	+++	+++	+++
	37	7	0	5	6	9	0	0
Ana robic indifferent streptococci ..	37	+++	+++	+++	+	43	0	0
<i>Str. pyogenes</i>	37	+++	+++	+++	+++	+++	++	0
	37	25	0	37	42	40	0	0

+++ = About 500 to 800 colonies. ++ = 200 to 500 colonies. + = 50 to 200 colonies.
48, 42, 40, etc. = Number of colonies grown. — = Sterile. 0 = Not examined.

and three hundred strains of *Ps. pyocyaneus* have been encountered, and a few of these failed to be killed in our standard time of exposure to toluene, but when we obtained a fresh specimen the coliform bacilli were inhibited. Two insensitive strains of each organism were subcultured and each continued to be insensitive, but fresh specimens from the cases concerned contained *B. proteus* or *Ps. pyocyaneus* which were fully sensitive to toluene; if possible, therefore, when one of these organisms appears to be insensitive a fresh specimen is obtained as the insensitive variants may not be present.

Comparison of the Toluene Technique with the "Fry" (1932) Plate

Sixty-seven specimens containing a mixture of coliform bacilli and Gram-positive organisms were examined by both methods. The toluene technique was simpler and less time-consuming; the colonies were directly visible and colonial characteristics were unaltered, giving ease of identification and subculture. On the "Fry" plate, colonies—other than haemolytic streptococci—had to be subcultured for identification by pricking through the agar layer, a relatively difficult technique.

The Routine Plate for Wound Swabs

This is a blood agar plate that has a central ditch cut out and removed; the ditch prevents *B. proteus* swarming across to the toluene-treated side of the plate. Over the outer segment of each half-plate

gentian violet solution is spread (Fleming, 1942). As each batch of gentian violet varies in its inhibiting power, the optimum concentration must be established experimentally; our sample needed a dilution of 1/30,000. The gentian violet solution is made up in sterile industrial spirit (Robson, 1945), as it is found that the plates then dry much more quickly than with an aqueous solution of the dye. The plate is dried before planting as there is a tendency for the cultures to become confluent if the gentian violet is spread after inoculation.

1. The swab is first planted on one half of the plate described above and a smear made for a Gram stain.
2. The swab is emulsified in 2 to 3 ml. of broth, in a bijou screw-capped bottle; the bijou bottles are kept ready for use at 37° C.: if there is likely to be an interval before the toluene is added, the bijou bottle is replaced in the incubator to prevent cooling.
3. Toluene, approximately one-tenth of the volume of the broth, is added to the emulsion.
4. The bottle is recapped and immediately shaken vigorously for 15 to 20 seconds.
5. With a sterile Pasteur pipette one drop of the suspension is placed on the second half of the blood plate and is then spread in the routine manner; we do not wait for the toluene to rise and settle out before we remove the broth for planting.

This one plate allows the easy recognition of the relative numbers of the different organisms present. Any coliform bacilli present will grow on the side of the plate planted without treatment by toluene; if few coliform bacilli are present

they may be swamped by a heavy growth of staphylococci, whereas the use of gentian violet allows them to appear as discrete, recognizable colonies.

The toluene-treated culture will contain no Gram-negative organisms and the relative numbers of Gram-negative and positive organisms will be shown on the two halves of the plate. The part of the plate untreated by gentian violet will show the staphylococci and diphtheroid bacilli present, and alongside this area will be shown the

Discussion

Many techniques have been evolved to suppress coliform bacilli generally and *B. proteus* in particular, as their tendency to overgrow other organisms obscures the complete bacteriological picture in mixed infections. Inhibiting agents (phenol, alizarin red, etc.) can be incorporated in the medium, and Bray (1945) exposes the planted plate to ether vapour, but these methods necessitate the use of duplicate plates if the complete picture is to be given.

The 8 per cent agar plate, Hayward and Miles (1943) provides colonies unlike those on ordinary laboratory media, and therefore suffers from the same drawbacks as the "Fry" plate, although the latter was introduced primarily for the isolation of haemolytic streptococci in the presence of *B. proteus*. Beattie's (1945) method only inhibits the swarming of *B. proteus*, so if many coliform bacilli are present they may hide small numbers of Gram-positive organisms.

Toluene appears to inhibit the growth of all pathogenic organisms if it is left long enough in contact with them, the inhibiting action being accelerated by increased temperature. The Gram-negative organisms are very susceptible to its action, whereas the Gram-positive organisms are relatively resistant. We now employ toluene in the primary cultivation of any specimen that is likely to contain coliform bacilli; we use it on all wound swabs and faeces, and in some

cervical, urethral, and aural swabs, urines, post-mortem material, and sputa.

Summary

A method of inhibiting coliform bacilli in cultures is described; also a new method of applying gentian violet in a composite plate which gives a complete and unobscured bacteriological picture on the one culture.

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REFERENCES

- Beattie, M. K. (1945). *J. Path. Bact.*, **57**, 388.
 Benians, T. H. C. (1913). *Z. Chemoth.*, **2**, 28.
 Bray, J. (1945). *J. Path. Bact.*, **57**, 395.
 Fleming, A. (1942). *Brit. med. J.*, **1**, 547.
 Fry, R. M. (1932). *Brit. J. exp. Path.*, **13**, 456.
 Hayward, N. J., and Miles, A. A. (1943). *Lancet*, **2**, 116.
 Robson, R. G. (1945). *I.M.L.T. Bulletin*, **9**, 104.
 Webster, J. E. (1942). *I.M.L.T. Bulletin*, **8**, 7.

streptococci where the gentian violet has inhibited the staphylococci and diphtheroid bacilli (see Figure).

Faeces.—After the specimen has been planted on the usual special media for Salmonella-dysentery groups, an emulsion is made in broth and this is treated with toluene in the same fashion as a wound swab emulsion; occasionally the coliform bacilli fail to be killed by exposure to toluene for 15 seconds and this may be explained by the work of Benians (1913), who showed that fatty and albuminous substances inhibit the action of toluene.

Urines.—The centrifuged deposit is planted in the laboratory's usual manner, and then some of the deposit is added to the warm broth and treated with toluene as previously described.