COMPARISON OF HOYLE'S MEDIUM AND BILLINGS' MODIFICATION OF TINSDALE'S MEDIUM FOR THE BACTERIOLOGICAL DIAGNOSIS OF DIPHTHERIA

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THE laboratory diagnosis of diphtheria is still important, in spite of the declining incidence of the disease. One effect of this declining incidence is that few workers are experienced in the recognition of *Corynebacterium diphtheriae* on primary cultures from patients, as Mueller and Miller predicted in 1946. In 1947, Tinsdale introduced a medium on which colonies of *C. diphtheriae* were surrounded by a brownish-black halo and could be recognised by inexperienced workers. These haloes, due to the formation of tellurium sulphide from hydrogen sulphide produced by *C. diphtheriae*, develop around the colonies of these organisms and clearly distinguish them from diphtheroid bacilli and other organisms that grow on the medium. Billings (1956) modified the medium because the preparation of Tinsdale's original medium was complicated and required reagents that were not obtainable in North America. This modification was enthusiastically referred to by Moore and Parsons (1958), who recommended it for routine use.

This paper compares Billings' modification of Tinsdale's medium with Hoyle's (1941) lysed-blood tellurite medium which is commonly used in Britain. The latter is not as easy to use as Tinsdale's medium. Diagnosis depends on the recognition of colonial characteristics and this can be learned only by working with *C. diphtheriae* in the laboratory.

C. diphtheriae infection is more common among the Indian, Eskimo, and Metis peoples of Northern Alberta and the adjoining Northwest Territories than in many other populations (Dixon and Thorsteinson, 1969). Consequently there is a steady flow of material into this laboratory, which enabled a comparison of the two media to be made in the course of the routine work.

MATERIALS AND METHODS

Hoyle's medium (Hoyle, 1941)

Agar base. Lab-Lemco beef extract 10 g; peptone (Oxoid L37) 10 g; NaCl 5 g; agar 15 g; distilled water 1000 ml. Dissolve ingredients, adjust pH to 7·8; sterilise for 15 min. at 121°C.

Lysed sheep-blood. Sterilise 10 per cent. solution of saponin in water for 15 min. at 121°C. Add 0·5 ml saponin solution to 10 ml of blood, mix gently to avoid formation of air bubbles, incubate for 30 min. at 37°C, and store at 4°C.

Potassium tellurite solution. Potassium tellurite 0·7 g; sterile water 20 ml.

Preparation of medium for use. Melt agar base, cool to 50°C, add lysed blood and tellurite solution, and pour plates. Agar base 200 ml; potassium tellurite solution 2 ml; lysed blood 10 ml. The medium is very stable and may be kept at room temperature.

Billings' modification of Tinsdale's medium (1956)

Agar base. Proteose peptone (Difco) 20 g; NaCl 5 g; agar 19·2 g; distilled water 1000 ml. Dissolve ingredients, adjust to pH 7·4, sterilise for 15 min. at 121°C. To 200 ml melted base, cooled to 56°C, add aseptically in the following order: bovine serum 20 ml; N/10-NaOH 12 ml; 0·4 per cent. L-cystine in N/10-HCl 12 ml; 1 per cent. potassium

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tellurite in sterile water 6 ml; 2.5 per cent. sodium thiosulphate (anhydrous) in sterile water 3.4 ml. The HCl and NaOH solutions should neutralise each other. The sodium thiosulphate solution should be freshly made. As some batches of L-cystine are inhibitory to *C. diphtheriae*, each new batch should be tested. Because the complete medium is not stable, it should be kept at 4°C, and should not be used after 4 days; for this reason *C. diphtheriae* gravis, intermedius, and mitis types were subcultured and incubated daily on Tinsdale's medium as a quality control.

Swabs from nose, throat, ear, wound, and skin infections were inoculated on half segments of 9-cm petri dishes of Hoyle's (H), modified Tinsdale's medium (MT) and 10 per

TABLE

*Number of isolations of C. diphtheriae from swabs inoculated on Hoyle's medium and Billings' modification of Tinsdale's medium**

Culture result on indicated medium	Number of specimens positive for <i>C. diphtheriae</i>	Number of strains of indicated type			
		gravis	mitis	intermedius	atypical
H+ve MT+ve	381†	155	125	87	14
H+ve MT-ve	156	56	67	20	13
H-ve MT+ve	44‡	9	20	15	0
Totals	581	220	212	122	27

* Hoyle's medium was incubated for 18–21 hr. Modified Tinsdale's medium was incubated for 18–21 hr and, if negative, for a further 24 hr (45 hr in all).

† 309 of the 381 were MT-negative at 18–21 hr.

‡ All 44 MT-negative at 18–21 hr.

cent. sheep blood agar. The order of inoculation on H and MT was reversed with successive specimens. A wire inoculating loop was drawn through the heavy part of the inoculum on MT, penetrating the medium, because the dark halo first appears along this stab.

Both H and MT were incubated aerobically at 37°C. The H plates were examined only once, after overnight incubation (18–21 hr), with a ×8 magnifying hand lens and good illumination; suspected colonies were filmed and stained with Loeffler's methylene blue. The MT plates were similarly examined at 18–21 hr and then again after reincubation for 24 hr (42–45 hr).

A single colony from H or MT was subcultured on nutrient sheep-blood agar, incubated at 37°C for 18 hr and examined for haemolysis. For fermentation reactions, subculture was made to tubes of Hiss's sheep serum-water containing glucose, maltose, sucrose, and starch, and to a Christensen urea-agar slope to detect urease.

As an additional confirmatory test for specimens that were MT-negative on primary culture, a stab inoculum from colonies on H or blood agar was made on a segment of an MT plate, which was examined for halo formation after 18 hours' incubation at 37°C.

RESULTS

Stock strains of *Corynebacterium* species were cultured on MT and examined for halo formation after 48 hours' incubation at 37°C. *C. diphtheriae* and *C. ulcerans* produced haloes; *C. hofmannii*, *C. xerosis*, *C. pyogenes**, *C. equi**, *C. renale**, *C. murium**, and *C. ovis** did not.

Of 581 *C. diphtheriae* positive swabs (table), H was positive 537 times (92.4 per cent. of all positives), and was positive alone 156 times; MT was positive 425 times (73.1 per cent.), positive alone 44 times.

C. diphtheriae grew much more quickly and could be recognised earlier on H than on MT. The performance of the two media was comparable only when MT was incubated for 24 hr longer than H. Halo formation on MT was apparent at 18–21 hr only when the growth of *C. diphtheriae* was heavy, 72 of 425 MT-positive (17 per cent.) swabs being recognised at 18–21 hr. Gravis strains produced a darker and more conspicuous halo than intermedius and some mitis strains. It was easier to get pure single-colony subcultures from 18-hr H plates than from 42-hr MT plates, because the colonies on MT were often obscured by the overgrowth of other organisms. Colonies were picked from H and filmed less often when experience had been gained with this medium.

The different types of *C. diphtheriae* could not be distinguished on MT, the colonies of gravis, mitis, and intermedius types being similar in appearance and consistency; the organisms were rounded or diphtheroid in a stained film made from the colony. On H, however, type differentiation could usually be made from the colonial characteristics and from a stained film. During this work there were 16 patients with simultaneous infections with more than one type of *C. diphtheriae* (which are not included in the table); most of these multiple infections would not have been recognised if MT alone had been used.

The in-vitro toxigenicity test of Elek can conveniently be made by direct plating from H, but this test is less satisfactory when colonies are subcultured from MT, because the formation of the precipitin lines is slower.

The stab inoculum confirmatory test for the identification of *C. diphtheriae* proved to be very useful; under the conditions of this test, halo formation was present at 18 hr with all 581 subcultured strains of *C. diphtheriae*, confirming Tinsdale's claim for the specificity of halo formation. *Corynebacterium ulcerans* was the only other *Corynebacterium* sp. to give a halo.

DISCUSSION

It is most important that the laboratory diagnosis of diphtheria be made with as little delay as possible. The very rapid growth of *C. diphtheriae* is a valuable property of Hoyle's medium (H) and enables an early diagnosis to be made; cultures should be examined at only 18–21 hr after inoculation (Hoyle, 1941), in contrast with the longer incubation period required for other tellurite media. Unsuccessful attempts were made to combine the good qualities of both media—the rapid growth and good type differentiation of H, with the indicating halo of modified Tinsdale's (MT).

Both H and MT are used in this laboratory as primary selective media. In smaller laboratories the use of only one primary medium may be preferred, and H is recommended for routine use if experienced staff are available; if the limitations of MT are recognised, this medium is helpful when experienced staff are not available.

SUMMARY

A comparison of Hoyle's medium and Billings' modification of Tinsdale's medium was made. Hoyle's medium was significantly better for the primary isolation of *Corynebacterium diphtheriae* from swabs. The only advantage of Tinsdale's medium was that it could be used by untrained people, whereas the use of Hoyle's medium required experience. The

* Supplied by National Collection of Type Cultures, Colindale, London.

formation of a halo on Tinsdale's medium is recommended as a specific confirmatory test for the identification of *C. diphtheriae* and *C. ulcerans*.

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