A METHOD OF PERFORMING SURFACE VIABLE COUNTS WITH *CLOSTRIDIUM TETANI*

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PLATES LXII-LXIV

IN an earlier paper (Willis and Williams, 1970) we reported that the surface swarming of *Clostridium tetani* in plate cultures is prevented by incorporation into the medium of commercial tetanus antitoxic serum to give a concentration of 40–60 units of tetanus antitoxin per ml. The serum appeared to have no adverse effect on the growth of the organism, but allowed the development of discrete colonies.

In the present study, we report upon the use of antitoxin-blood agar as an alternative to firm agar for obtaining surface viable counts of *Cl. tetani*.

MATERIALS AND METHODS

Organisms. Fifteen strains of *Cl. tetani* were examined. These were kindly provided by Dr S. P. Lapage, and were National Collection of Type Cultures strains NCTC no. 2933, 2934, 2935, 2936, 2937, 2938, 5404, 9573 (Tulloch type II), 5409 (Tulloch type III), 9570 (Tulloch type IV), 9571 (Tulloch type V), 9569 (Tulloch type VI), 9568 (Tulloch type VII), 9574 (Tulloch type VIII) and 9575 (Tulloch type IX).

Media. Fresh horse blood agar was used as the plating medium, and was prepared in the usual way from an ox heart digest broth base (Cruickshank, 1965); layer plates containing 5 per cent. of defibrinated horse blood (Wellcome) were used. The ordinary agar medium contained 1·5 per cent. of New Zealand agar; firm agar plates contained 4 per cent. of New Zealand agar.

Tetanus antitoxin blood agar was prepared either by the addition of horse tetanus antitoxic serum (Wellcome or Lister Institute) to the ordinary blood agar medium (to give a final concentration of 60 units per ml) just before plates were poured, or by spreading ordinary blood agar plates with 4–6 drops of the undiluted antitoxin (4000 units per ml).

Selective blood agar plates contained a range of concentrations of one or another of the various special reagents listed below. The chemical reagents were added to the nutrient agar (1·5 per cent.) base prior to sterilisation; the antibiotics were added to the ordinary blood agar medium just before plates were poured.

Special reagents. These were chloral hydrate (May and Baker), boric acid (Hopkin and Williams), sodium azide (BDH), neomycin sulphate (Upjohn) and streptomycin sulphate (Glaxo).

Seeding of plates. In order to assess inhibition of swarming growth, organisms were inoculated on plates in a lateral segment only (Willis, 1964) or were streak-inoculated in the usual way. Surface viable counts were performed by the method of Miles, Misra and Irwin (1938) with 10-fold dilutions of the inoculum prepared in 0·9 per cent. NaCl in water containing 0·05 per cent. of cysteine hydrochloride (BDH). Cultures were incubated for 24 hr in an anaerobic atmosphere containing 10 per cent. CO₂. In all cases the inoculum was the supernatant from a fresh 18-hr cooked meat broth culture of the organisms under test.

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RESULTS

Early experiments with media separately supplemented with various chemicals and antibiotics indicated that these reagents are inappropriate for use in obtaining surface viable counts of *Cl. tetani*; chloral hydrate, boric acid, and sodium azide were commonly inhibitory at concentrations that did not abolish swarming growth. In the same way, non-inhibitory concentrations of neomycin sulphate and of streptomycin sulphate failed to restrict the colonies sufficiently to permit counting.

TABLE

Surface viable counts and type of growth of 15 strains of Clostridium tetani performed on antitoxin blood agar and firm blood agar

Strain of <i>Cl. tetani</i> (NCTC no.)	Surface viable count per ml on	
	antitoxin blood agar	firm blood agar
2933	85×10^8 D*	35×10^6 R†
2934	85×10^9 R	95×10^9 R
2935	28×10^{10} D	RNCP
2936	31×10^{10} D	RNCP
2937	60×10^{10} D	RNCP
2938	44×10^{11} D	RNCP
5404	10×10^{12} D	50×10^{11} R
5409	19×10^{11} D	19×10^{11} R
9568	RNCP‡	RNCP
9569	21×10^{10} D	18×10^{10} D
9570	14×10^9 D	65×10^6 R
9571	40×10^8 D	RNCP
9573	26×10^{11} D	22×10^{11} R
9574	55×10^{10} D	35×10^{10} R
9575	10×10^{10} D	65×10^9 R

* D = Discrete colonies.

† R = Rhizoidal colonies.

‡ RNCP = Rhizoidal, no count possible.

Comparison of surface viable counts on antitoxin blood agar and firm blood agar

The surface viable counts for the 15 strains of *Cl. tetani*, performed on the two media in parallel, are summarised in the table; they represent the mean of quadruplicate counts in each case. None of the strains produced overt swarming growth on either of these media.

All but one of the 15 strains of *Cl. tetani* gave easily countable colonies on antitoxin blood agar (figs. 1 and 2), and in only one of these did rhizoidal growth develop. The exceptional strain (Tulloch type VII, NCTC no. 9568) produced growth that was too rhizoidal to allow an acceptable count to be obtained. In all other cases, colonies were small, circular and discrete, with a coarsely irregular edge and a total absence of rhizoidal outgrowths (fig. 3). On firm blood agar, on the other hand, six of the 15 strains produced growth that was so rhizoidal in structure that it was impossible to identify separate colonies

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FIG. 1.—Growth from inocula of serial dilutions of a culture of *Clostridium tetani* on tetanus anti-toxin horse blood agar. $\times c. 1$.

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FIG. 2.—Discrete colonies of *Cl. tetani* in a surface viable count inoculation zone on tetanus antitoxin horse blood agar. $\times 60$.

FIG. 3.—Higher-power view of some of the colonies shown in fig. 2. $\times 150$.

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FIG. 4.—Rhizoidal colonies of *Cl. tetani* in a surface viable count inoculation zone on firm (4 per cent.) agar. $\times 60$.

FIG. 5.—Higher-power view of some of the colonies shown in fig. 4. $\times 150$.

accurately and counts could not be obtained. Of the remaining nine strains, only the non-motile variant (Tulloch type VI, NCTC no. 9569) produced colonies that were virtually without rhizoidal outgrowths; the colonies of all other strains were markedly irregular (figs. 4 and 5). As might be expected, no strain produced haemolysis on antitoxin blood agar and haemolysis was usually difficult to detect on firm agar.

Among the nine strains for which surface viable counts were obtained on both media, five showed essentially comparable results, and the remaining four gave counts that were notably higher on the antitoxin medium. This difference is probably partly attributable to the subjective difficulty of counting closely adjacent rhizoidal colonies, but it is also a reflection of the fact that growth commonly occurred on antitoxin agar with inocula from dilutions that were 2–4 times greater than those yielding colonies on the corresponding firm agar plates.

DISCUSSION

The formation by *Cl. tetani* of discrete surface colonies in the presence of commercial horse tetanus antitoxic serum is doubtless due to the presence of agglutinating antibody to the vegetative cell. Although we have not followed the matter up experimentally, it seems probable that somatic antibody is the factor involved, since strains of *Cl. tetani* are known to possess a common O antigen, but may have type-specific H antigens. The possibility that preservative in the antitoxic serum was the factor responsible for inhibition of swarming was ruled out, for heterologous antisera containing similar preservative were without effect. Somewhat similar observations to ours were made by Beattie (1945) who added a polyvalent *Proteus* rabbit antiserum to fresh blood agar plates to inhibit the swarming of *Proteus* species.

Although firm agar is almost as effective as tetanus antitoxic serum in preventing surface swarming growth of *Cl. tetani*, it is much less satisfactory for the performance of surface viable counts. Colonies of *Cl. tetani* on firm agar are characteristically irregular and rhizoidal, so that visual separation of closely adjacent colonies is often impossible. Moreover, antitoxin agar allows excellent growth of *Cl. tetani* from inocula at higher dilutions (as much as 10^{-3} in the present study), whereas when corresponding serial dilutions are cultured on firm agar one is forced to count the colonies that develop within relatively crowded zones of growth. A combination of the inhibitory effect of firm agar on growth from inocula of highly diluted cultures, and of the markedly rhizoidal structure of colonies on firm agar probably accounts for its complete failure to provide a surface viable count for five of the strains tested. In the presence of antitoxic serum and at normal agar concentrations, the majority of our test strains of *Cl. tetani* produced discrete, almost entire colonies that were easily distinguishable by the naked eye.

SUMMARY

Accurate surface viable counts of *Cl. tetani* may be obtained by the method of Miles and Misra when horse blood agar containing commercial tetanus

antitoxic serum to yield a final concentration of 40–60 units tetanus antitoxin per ml is used as the plating medium. The inhibition of swarming is probably due to agglutinating antibodies, which are known to be present in commercial tetanus antitoxic serum. Firm horse blood agar is much less satisfactory for this purpose.

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