A Growth Medium without Blood Cells for

*Pasteurella tularensis*

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SUMMARY

A modification of glucose cysteine blood agar (GCBA) is described for the cultivation of *Pasteurella tularensis* in which the blood is replaced by plasma and catalase. It has the advantage for colony counting of being clear, and is at least as good as GCBA for enumerating fastidious aged *P. tularensis* suspensions and aerosols. The liquid medium supports the growth of very small inocula.

INTRODUCTION

Glucose cysteine blood agar (GCBA) was described by Downs, Coriell, Chapman & Klauber (1947) as a suitable medium for the enumeration of *Pasteurella tularensis* by a plate colony counting method. The medium gives satisfactory counts in experienced hands but the need to use blood presents two problems: control of its quality is difficult, and it renders the medium opaque and therefore less satisfactory for colony counting. Won (1958) described a blood-free medium, but we have found it unable to promote full growth of aged organisms from stored suspensions or aerosols. This paper describes a clear medium which promotes the growth of *P. tularensis* as well as GCBA and gives consistent results.

METHODS

Glucose cysteine blood agar

GCBA was used throughout the work as the control medium against which variants of it were tested. The formulation was changed somewhat from that of Downs *et al.* (1947). The basal medium contained (g./100 ml.): Lab-Lemco (Oxoid), 0.3; peptone (Evans), 0.5–3.0; sodium chloride (Analar), 0.5; agar (Davis), 1.25; distilled water to 100 ml. The medium was completed by the addition of separately autoclaved solutions of L-cysteine HCl (10%) 1 ml.; L-histidine HCl (10%) 1 ml.; glucose (50%) 5 ml.; and 4 ml. of whole human blood (citrated).

The optimum amount of peptone was found to vary from batch to batch. The histidine decreased the required incubation time by several hours.

The basal medium was prepared by dissolving the Lab-Lemco, peptone and salt and adjusting to pH 7.6–8.4 (according to peptone batch and concentration) with sodium hydroxide so that the medium when completed was at pH 6.8–7.0. The agar was then added and, after steaming to melt it, the basal medium was autoclaved in convenient amounts for 15 min. at 120°. No deterioration of the basal medium was found to occur for at least 1 year when stored in screw-capped bottles in the dark at room temperature.
The medium was completed by melting the basal portion, cooling to 50–55°, and adding the remaining ingredients glucose, cysteine, histidine and finally blood. Penicillin to 10 units/ml. was frequently added; this was desirable to inhibit contaminants in certain uses of the medium, and had no detectable effect on the growth of Pasteurella tularensis. Petri plates were poured and ‘dried’ in the usual way for about 2 hr. at 37°.

Organism. Small-scale preparations were made on a laboratory shaker, using a modified casein partial hydrolysate medium. Strain Schu D was used for most of the work, and four other strains in confirmatory tests. The suspensions were stored at 0 to +4°.

Viable counts. Viable counts were made by the drop technique of Miles & Misra (1938). Dilutions were chosen to give about 150 colonies/plate on the control medium; six plates of each medium under test were counted. Colonies were counted when they reached 1–2 mm. diam. which was usually after 2 days at 37°; with some media longer incubation was necessary.

Use of ‘aged’ organisms. In the early stages of the work it was found that suspensions which had been stored longer than 3 or 4 weeks, and also samples from some ‘aged’ aerosols, were much more exacting in their requirements for growth than were ‘young’ suspensions or aerosols of high viability. Media were therefore tested with the more fastidious aged suspensions and sometimes also with aerosol samples. This was important, since media which were satisfactory in all other respects sometimes failed in this test.

RESULTS

Plate counts on the control GCBA medium were scaled to 100, and counts on test media in the range 85–115 were not considered significantly different from the control. A few typical results are shown in Table 1.

During preliminary work it was found that growth was completely inhibited when the glucose was autoclaved in the basal medium; this inhibition was partly annulled when the cysteine also was autoclaved in the basal medium. No inhibition was found when cysteine alone was autoclaved in the basal medium. Nevertheless, because of its instability it was decided to add it separately to the medium when required. A 10% solution of L-cysteine autoclaved 15 min. at 120° in a screw-capped bottle lost 8% of its cysteine. This loss could be safely ignored since it was found that the cysteine at 1000 μg./ml. in the medium was about 100 times minimal requirement: suspensions of Pasteurella tularensis up to 33 weeks old and aerosol samples of less than 0.5% viability gave plate counts equal to the control with cysteine down to 10 μg./ml. Cysteine was found to promote better growth than cystine in plain (i.e. no blood) agar, confirming the work of Downs et al. (1947). No difference could be demonstrated between them, however, when used at concentrations 1–1000 μg./ml. in the medium with blood. It was apparent that not only did blood eliminate the difference between growth promotion of the amino acids but it also stimulated the growth of aged P. tularensis which failed to grow on plain cysteine or cystine agar.

Attention was then turned to the blood. We usually used citrated whole human blood. Neither the cells nor the plasma would alone support full growth of aged P. tularensis.

Lysis of erythrocytes takes place in a relatively short time even in cold storage,
A clear medium for *P. tularensis* and it was found that lysed blood was inferior to non-lysed blood when tested with aged *Pasteurella tularensis*. Erythrocytes were lysed in distilled water and washed by centrifugation until there were no intact cells in the deposit and the supernatant fluid was colourless. The deposit was used in the same proportion as the stroma content of whole blood in the control medium, and with 4% (v/v) human plasma it gave growth equal to the control; with 2% (v/v), the count was about 75% of control. The evidence suggests the presence of inhibitors in the erythrocytes rather than loss of growth factors when the erythrocytes were lysed.

Table 1. *Growth of Pasteurella tularensis on glucose cysteine agar with various additions*  

<table>
<thead>
<tr>
<th>Addition</th>
<th>Age of suspension (weeks)</th>
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<tbody>
<tr>
<td></td>
<td>6</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>4% human blood (control)</td>
<td>(100)</td>
</tr>
<tr>
<td>Human blood cell stroma (from 4% human blood)</td>
<td>101</td>
</tr>
<tr>
<td>Human blood cell stroma and 2% human plasma</td>
<td>92</td>
</tr>
<tr>
<td>Human blood cell stroma and 4% human plasma</td>
<td>102</td>
</tr>
<tr>
<td>4% human plasma and 10μg./ml. catalase (crude)*</td>
<td>101</td>
</tr>
<tr>
<td>4% human plasma and 100μg./ml. catalase</td>
<td>107</td>
</tr>
<tr>
<td>4% human plasma and 300μg./ml. catalase</td>
<td>90</td>
</tr>
<tr>
<td>4% horse plasma and 300 μg./ml. catalase</td>
<td>105</td>
</tr>
<tr>
<td>300μg./ml. catalase + 4% reconstituted freeze-dried human plasma</td>
<td>98</td>
</tr>
<tr>
<td>300μg./ml. catalase + 4% dialysed human plasma</td>
<td>93</td>
</tr>
</tbody>
</table>

* About 1 Kat.f./mg.

The growth-promoting action of the stroma seemed likely to be enzymic, and as it was found to have retained some catalase action preparations of this enzyme were tested. An impure beef-liver catalase extract of activity about 1 Kat.f./mg. was Seitz-filtered in 1% (w/v) solution in phosphate buffer (pH 7.4) and added to the medium, together with human plasma, in place of whole blood. With this catalase preparation at 300μg./ml. + 4% (v/v) plasma, the *Pasteurella tularensis* counts were equal to that of the control. The activity of the catalase required was about equal to that of 4% (v/v) of whole blood and much more than that of the separated stroma. The function of the stroma therefore cannot be attributed solely to the catalase content. Catalase was, however, capable of completely replacing the stroma. It was shown by tests with pure catalase that it was the enzymic activity that was responsible. Pure crystalline catalase (about 100 Kat.f./mg.) was equal in effect, at 3μg./ml., to the crude catalase (about 1 Kat.f./mg.) at 300μg./ml.

In the first tests with the 'blood-free' medium, citrated human plasma (about 4%, v/v) was used + 300μg./ml. crude catalase preparation. This plasma could be replaced by reconstituted freeze-dried human plasma and by human plasma dialysed against physiological saline. Ox plasma or horse plasma were also suitable but the pH value was rather critical at about pH 6.7, in contrast to human plasma which was satisfactory within the range pH 6.5–7.0.
Recommended medium

The composition of the medium finally adopted was as follows (g./100 ml.): Lab-Lemco (Oxoid), 0·3; peptone (Evans), 0·5–3; sodium chloride, 0·5; agar (Davis), 1·25; distilled water to 100 ml. The following ingredients were added separately as sterile solutions: glucose, 5 ml. (50 % solution); L-cysteine HCl, 1 ml. (10 % solution); L-histidine HCl, 1 ml. (10 % solution); catalase (pure), 0·1 ml. (1 % solution); plasma (human, ox, horse), 5 ml., pH 6·6–6·8. The method of preparation for this medium was as for GCBA. It should be noted that the catalase content given is about three times the adequate concentration of 3μg./ml.; this margin of safety may not be necessary. Poured plates of this medium keep about as well as GCBA when stored at 4°. For routine use plates kept for 2 weeks were satisfactory, but for extremely sensitive aged organisms 1 week was the limit.

The medium was tested with Pasteurella tularensis aerosols which had been held until the viability was considerably decreased (as determined on GCBA by the method of Harper, Hood & Morton, 1958). The bacteria remaining viable in such aerosols are in a sensitive state, and make demands on the culture medium that are similarly exacting to those of aged suspensions. In a series of five experiments, 12,376 colonies were counted on the new medium and 11,215 on the control, the ratio of 1·1 showing a small (possibly not significant) advantage in favour of the medium described here. The new medium was tested with aged suspensions of four other strains of P. tularensis (Fam, Jap, 403, and LV), and the results confirmed the performance with Schu D.

The success of the solid medium in growing Pasteurella tularensis suggested trial of the liquid medium, without agar, in shaken cultures. The yields of organisms obtained were similar to those from the casein hydrolysate medium (about 4×10^10 organisms/ml.). Unlike the casein hydrolysate medium the new medium supported growth from small inocula of less than 10 organisms/ml.

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REFERENCES


