Suitability of Peracetic Acid for Sterilization of Media for Mycoplasma Cultures

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Received for publication 15 January 1975

Mycoplasms require very complex media, a fact that complicates identification procedures in laboratory diagnosis. The sterilization of horse serum and yeast extract is of particular importance in preparation of medium. Attempts to replace the time-consuming filtration generally used for heat-labile culture media, by chemical sterilization with ethylene oxide (30), betapropiolactone (2, 10, 15, 30, 31), formaldehyde, or peracetic acid (21, 27, 33) have already been described by various authors. Cold sterilization with peracetic acid has proven to be a useful method for the preparation of bacterial and cell culture media (21, 23, 27, 33). Peracetic acid has broad antimicrobial action in low concentration, and within short times of action (5, 6, 13, 14, 23–26, 29; I. J. Hutchings and H. Xezzone, unpublished data), factors which are only approximately reached by betapropiolactone (10, 12, 15, 17–20). The present studies were carried out to determine if horse serum and yeast extract can be sterilized with peracetic acid and used for growth of mycoplasms.

MATERIAL AND METHODS

The mycoplasma medium used was that described by Hayflick et al. (3, 8, 9) which contained 70% of basic medium (PPL0 agar [Difco]), 20% inactivated horse serum, 10% yeast extract, 0.5 mg of thallium acetate per ml and 1,000 IU of penicillin per ml. For the preparation of the yeast extract, equal portions of yeast (baking yeasts of VEB Bramsch, Dresden) and distilled water (1 kilo of yeast in 1 liter of distilled water) were suspended and heated in an autoclave at 80°C for 30 min. Subsequently, the extracts were filtered twice by paper.

The serum and yeast extract were sterilized with peracetic acid concentrations (commercial 40% peracetic acid solution "Wofasteril") of 0.1, 0.02, 0.04, and 0.05% for a period of 30 min. These concentrations are known to effectively kill bacteria (21). For comparison, samples were Seitz-filtered. Adjustment of pH was carried out with 1 N NaOH. For the reduction of the peracetic acid residues in the serum and yeast extract, sodium bisulphite solution in amounts equivalent to the peracetic acid content was added after 30 min (Table 1).

Growth measurements. For an appraisal of the differently sterilized culture medium additives, the following prototype strains of human origin were used: Mycoplasma fermentans (PG 18), Mycoplasma arthritidis (PG 27), Mycoplasma orale type 1 (CH 19299), Mycoplasma pneumoniae (FH), and Mycoplasma salivarium (PG 20).

For evaluation of growth, agar media were prepared where either the yeast extract or the horse serum or both additives had been sterilized with various concentrations of peracetic acid. These plates were inoculated with 0.5-ml suspensions of the six Mycoplasma species. Media containing Seitz-filtered additions were used as controls.

Mycoplasma growth on the various media was evaluated as follows: as soon as colonies could be detected using low magnification (about 3 days at 37°C aerobically) colonies were counted at 4 to 6 days or until colonies stopped growing. Five microscope fields distributed at random on the agar surface of each plate were counted using a magnification of ×56. The average numbers obtained in the counts were represented graphically.

Cultivation of Mycoplasma. The strains M. orale types 1 (CH 19299) and type 2 (CH 20247), M. pneumoniae (FH) and M. salivarium (PG 20), were serially propagated through 16 alternating broth-agar passages with serum additions that had been sterilized with 0.05% peracetic acid, and for comparison, Seitz-filtered additions. After each agar passage, we counted the colonies semi-quantitatively as described. The cultures were regularly transferred after 5 days of incubation at 37°C by inoculating each of the plates with 0.1 ml of mycoplasma-containing broth and adding to the fresh broth a colonized piece of agar of about 8 × 15 mm.

RESULTS

The semi-quantitative growth measurements carried out repeatedly on the six Mycoplasma
species showed that the use of horse serum and yeast extract sterilized with 0.02 to 0.1% peracetic acid offered the microbes equally good growing conditions as compared to media with filtered additions (Fig. 1 and 2). On agar plates on which only one addition had been sterilized with peracetic acid, the results were usually better. In one case *M. fermentans* showed clearly inferior growth after sterilizing the two additions with 0.1% peracetic acid. This result could, however, not be corroborated in the repeated test. For *M. pneumoniae*, the results were more favorable with peracetic acid sterilization than with filtered supplements. However, when both additions were sterilized with 0.1% peracetic acid, the observed growth was approximately the same.

The culture media tested by serial transfer were sterilized with 0.05% peracetic acid and, for obtaining a comparison, given an addition of filtered horse serum. The concentration of 0.05% was chosen in view of the favorable results obtained in the preceding growth measurements. It moreover offers a certain reliability of sterility which does not exist with 0.02% because of the protein error (26). In the course of 16 alternating broth-agar passages of the test strains *M. orale* and *M. arthritidis*, *M. pneumoniae*, and *M. salivarium*, no differences in growth were found, either in semiquantitative (Fig. 3) or in qualitative respects—even in comparative microscopic studies of shape and size of the individual colonies.

**DISCUSSION**

All tested mycoplasms grew as well on solid and liquid media containing serum and yeast extract additions which had been sterilized with peracetic acid as they did on media containing filtered additions. According to our experience, concentrations of 0.05 to 0.1% with a time of action of 30 min are quite sufficient for sterilization, assuming that the horse serum is collected properly so that no pronounced bacterial contamination need be expected (15). Opinions vary as to whether the yeast extract can also be

<table>
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<tr>
<th>Peracetic acid concn (%)</th>
<th>Peracetic acid content (mg/100 ml)</th>
<th>Sodium bisulphite (mg/100 ml)</th>
<th>Sodium sulphite (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>100</td>
<td>137.0</td>
<td>335.0</td>
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<tr>
<td>0.05</td>
<td>50</td>
<td>68.5</td>
<td>167.5</td>
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<td>0.04</td>
<td>40</td>
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<td>134.0</td>
</tr>
<tr>
<td>0.02</td>
<td>20</td>
<td>27.4</td>
<td>67.0</td>
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*The reducing agents were dissolved in distilled water in high concentrations, autoclaved, and added to the medium in a sterile form.*

![Fig. 1. Growth measurements of Mycoplasma pneumoniae strain FH on culture media with filtered and peracetic acid (PE) sterilized horse serum additions.](http://jcm.asm.org/)
Fig. 2. Growth measurements of Mycoplasma arthritidis type 2 strain PG 27 on culture media with filtered and peracetic acid (PE) sterilized horse serum and yeast extract additions.

Fig. 3. Growth measurements of Mycoplasma salicarium strain PG 20 and Mycoplasma orale type 2 strain CH 20247 through 16 alternating bouillon-agar passages on culture media with filtered and peracetic acid (PE) sterilized additions of horse serum.

autoclaved. Hayflick holds that the active substances in the yeast are heat stable (8) so that cold sterilization of only the serum is necessary. Witzleb, however, finds a certain impairment of the active components of the yeast extract by heat (32). For the preparation of antigens, Seitz-filtration is certainly justified in spite of the well-known disadvantages because this process removes disturbing yeast residues at the same time (32).

The following possibilities may explain the better growth of some of the mycoplasma strains on media containing additions that were sterilized with peracetic acid: (i) peracetic acid denaturates antibodies (unpublished data), and hydrolyzes higher-molecular proteins into decomposition products which might be better utilized by microbes; (ii) filtration, which retains microbial growth-promoting substances (22); (iii) some filters, in particular Seitz filters, release toxic substances into the medium which inhibit microbial growth (4, 7, 16, 22, 28); (iv) possibly, the alpha-globulin fraction in certain horse sera which inhibit the growth of M. pneumoniae is removed (1, 32).

Most important it is difficult to remove L
forms of bacteria, mycoplasmas, and viruses by filtration (11, 16, 20, 22). The cold sterilization method with peracetic acid described by us, therefore, offers a sometimes considerable increase in growth of some Mycoplasma species. Laboratory work for the sterilization of horse serum and yeast extract additions is simplified since the technical shortcomings and the higher costs involved in filtration are excluded. Although peracetic acid would appear to have general utility for sterilization of serum with a spectrum including sporeforming bacteria, it is not presently known whether peracetic acid treatment would provide serum suitable for cultivation of the T strains.

ACKNOWLEDGMENTS

We should like to express our special gratitude to certificated chemist Mücke for his expert advice.

LITERATURE CITED