Agarose Soy Casein Digest Medium for Replacement of Blood Agar for Potency Determinations of Live Pasteurella Vaccines

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Received 22 July 1988/Accepted 21 October 1988

Blood agar, prepared with Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar and 5% defibrinated bovine blood, is used for testing the potency of live Pasteurella multocida and Pasteurella haemolytica vaccines, but its potential for variation makes it undesirable to use in a standard assay method. Tests done with RPMI 1640 and Trypticase soy agar medium indicated that the benefits obtained by adding defibrinated blood to the Trypticase soy agar medium were more likely due to neutralization of toxic components than to the presence of transferrin or iron as growth factors. Reduction of toxic components in the Trypticase soy agar medium was accomplished by replacing agar with agarose and by autoclaving glucose as a separate solution to produce the replacement medium. The replacement medium was prepared by autoclaving three separate solutions—Trypticase soy broth without glucose; glucose; and agarose—cooling to 55°C, and mixing and then pouring the mixtures into petri dishes. The growth obtained with this medium as judged by determination of the number of CFU and the colony sizes of P. multocida or P. haemolytica was equal to or better than those obtained with blood agar.

Commercial vaccines are required by law to be safe and effective. Producers of live Pasteurella multocida and Pasteurella haemolytica vaccines are required to monitor them for the number of CFU, which is a measure of their potency. Blood agar (BA) was chosen for the determination of the number of CFU by the National Veterinary Services Laboratories because the results are less variable and higher than with the dextrose starch agar medium which was previously used (Miles Bairey, National Veterinary Services Laboratories, personal communication).

The objective of the present work was to develop a new medium free of blood or serum which can be prepared with standardized commercially available components. To replace blood, experiments were carried out by supplying growth factors or by reducing the toxicity of the medium. Iron-transferrin complexes are a satisfactory replacement for fetal bovine serum in a liquid medium designed for P. haemolytica (6). Iron-transferrin complexes were added to two base media, RPMI 1640 and Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy (TSB) medium, as an alternative growth factor to blood.

Since serum acts as a detoxifying agent (3, 4), modifications were made that were expected to reduce the amount of toxins or growth inhibitors in the medium. Agarocetin, a component of agar, can be toxic for the growth of Mycoplasma and other bacteria (1, 2, 5, 7, 10). Since agarose, the gelling component of agar, is free of agarocetin, agarose was added to RPMI 1640 or TSB with optional additions of apotransferrin, ferrous sulfate, or both. Toxic components for some organisms can be produced (8) by autoclaving glucose in the presence of phosphate or protein components. The glucose was autoclaved separately from the base media to reduce the formation of toxic components (8). The replacement media were compared with BA by determining the number of CFU and measuring the size of the colonies of two Pasteurella strains grown on the various media. A medium free of blood or serum was developed in which the CFU and colony size of two Pasteurella vaccines were equal to or better than those obtained with BA.

MATERIALS AND METHODS

Bacteria. Two strains were used, the Wilkie strain of P. haemolytica serotype A1 (Internal Reference Product 280; original culture obtained from B. N. Wilkie, University of Guelph, Guelph, Ontario, Canada) and the bovine strain P-1062 of P. multocida serotype 3 (ATCC 15743; original culture obtained from Keith Rhoades, National Animal Disease Center, Ames, Iowa). Both were obtained from stock cultures at the National Veterinary Services Laboratories.

CFU. At 24 h before the experiment was to be conducted, a frozen liquid culture of the organism was thawed and plated on tryptose agar and incubated overnight at 35 to 37°C. At the time of the experiment, colonies were picked from the plates, suspended in tryptose broth, and adjusted to 68 to 70% transmittance at 630 nm. Serial 10-fold dilutions up to 10⁻⁷ were made with this suspension; 0.1 ml of each of the 10⁻⁵, 10⁻⁶, and 10⁻⁷ dilutions were plated in triplicate on each medium. All plates were incubated for 24 h at 35 to 37°C. The colonies were counted, and the average count per dilution was determined.

When counts for 2 dilutions were available in a usable range, such as 10⁻⁵ and 10⁻⁶ dilutions, the counts obtained with the 10⁻⁵ dilution were divided by 10 and analyzed together with counts obtained with the 10⁻⁶ dilution.

Statistical methods for CFU. Differences between CFU means from experiment 3 were evaluated by standard t tests as described by Snedecor and Cochran (9).

Colony size. Colony sizes were first estimated in experiment 1 and then measured by photographing the plates and making prints representing a 2× magnification of the original plate. Measurements of the colony sizes were made from plates containing approximately the same number of colonies with a measuring magnifier (Bausch & Lomb, Inc., Rochester, N.Y.) calibrated to read in tenths of a millimeter.

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Up to 20 colonies from each medium were measured, and the arithmetical average diameter was calculated.

**Media.** Trypticase soy agar (TS agar; cat. no. 11043; BBL Microbiology Systems) was used to prepare BA plates by the addition of 5% sterile defibrinated bovine blood as specified by the manufacturer. TS agar was also prepared without blood. The following medium ingredients were used alone or as components of the media described for experiments 1, 2, and 3: RPMI 1640 (GIBCO Div., BBL Microbiology Systems), TSB containing 0.25% glucose (cat. no. 11768; BBL Microbiology Systems), TSB without glucose (TSB w/o G; cat. no. 11774; BBL Microbiology Systems); SeaKem LE Agarose (FMC BioProducts, Marine Colloids Div., Rockland, Maine).

**Media for experiment 1.** GIBCO’s RPMI 1640 tissue culture medium was made to a 2X concentration with glass-distilled water. Filter-sterilized fetuin bovine serum, ferrous sulfate, and apotransferrin were added. An equal volume of 2% agarose was autoclaved, cooled to 50 to 55°C, and mixed with the RPMI 1640 solutions. A 25-ml sample of the mixture was poured into a plastic petri dish (15 by 100 mm); the plates were checked for sterility by incubating them overnight at 37°C. The TSB media were made to 2X the listed concentration, autoclaved, and cooled to 50 to 55°C. Ferrous sulfate, transferrin, or both were filter sterilized and added to the TSB. The TSB solutions were mixed with an equal volume of 2% agarose at 50 to 55°C, and the plates were prepared as described above.

**Media for experiment 2.** TSB media were modified as indicated below and as shown in Table 1. All of the solutions were autoclaved except those that were filter sterilized. All contained 1% agarose except BA and TS agar; these contained 1.5% agar. A direct comparison was made between agar and agarose as the gelling agent by comparing TS agar with TSB agarose. The media can be divided into three groups: group A, reference media; group B, TSB media without additives; and group C, TSB media with additives. In group B, three types of sterilization were used. In the first, the TSB and agarose were autoclaved as a single solution. In the second, the main components were autoclaved as separate solutions. In the third, TSB was filter sterilized.

**Media for experiment 3.** To test the reproducibility of the results obtained with the TSB w/o G + G + agarose medium, two different lot numbers of the TSB w/o G were used in the preparation of the medium and the medium was prepared in two different laboratories. Blood agarose was prepared by adding 5% defibrinated blood to TSB w/o G + G + agarose medium. On the same day, six plates each of TSB w/o G + G + agarose medium with 0.6 and 0.8% agarose, nine plates with 1% agarose, six plates of blood agarose, and six plates of BA were inoculated with the same Pasteurella culture. The inoculation procedure was repeated with the other strain in the same week.

**RESULTS**

In experiment 1, comparisons were made of the growth of *P. multocida* and *P. haemolytica* on RPMI 1640 and TSB media and the modified media with growth on BA. The colony size and number of CFU of *P. multocida* and *P. haemolytica* were much reduced on RPMI 1640 as compared with BA unless fetuin bovine serum was added. The number of CFU obtained with the same two *Pasteurella* cultures with the TSB-1% agarose media was comparable to the number of CFU obtained with BA, but the colonies were smaller with TSB-1% agarose prepartions than with BA.

In experiment 2, the number of CFU and colony sizes of *P. multocida* and *P. haemolytica* grown on several TSB-agarose media were compared with those of the same strains grown on BA. The CFU obtained with TSB-agarose media were comparable to those with BA (Table 1), but the colony sizes obtained with TSB-agarose media were smaller. However, the number of CFU and colony sizes obtained with filter-sterilized TSB or with TSB w/o G + G + agarose medium were equal to or larger than those obtained with BA. Additions of ferrous sulfate or transferrin had little or no effect.

In experiment 3, CFU and colony sizes obtained for *P. multocida* and *P. haemolytica* with TSB w/o G + G + agarose medium containing 0.6% agarose, 0.8% agarose, 1.0% agarose, and blood agarose were compared with those obtained with BA (Table 2). The means of the CFU and colony sizes of *P. multocida* obtained with TSB w/o G + G + agarose medium were higher than those obtained with BA, and, in the case of *P. haemolytica*, the CFU and colony sizes were comparable to those obtained with BA at the 1% agarose level.

The concentration of agarose in the medium affected the size of the colonies. At lower agarose concentrations, larger colonies were obtained with both *Pasteurella* strains.

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**Table 1.** Experiment 2: comparison of number of CFU and colony size of *P. multocida* and *P. haemolytica* obtained with modified TSB media with those obtained with BA

<table>
<thead>
<tr>
<th>Group</th>
<th>Growth medium</th>
<th><em>P. multocida</em></th>
<th><em>P. haemolytica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU*</td>
<td>Size*</td>
<td>CFU*</td>
</tr>
<tr>
<td>A</td>
<td>reference</td>
<td>39</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>TS agar</td>
<td>10</td>
<td>2.6</td>
</tr>
<tr>
<td>B</td>
<td>experimental TSB-1% agarose media without additives</td>
<td>39</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Filter-sterilized TSB</td>
<td>45</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>TSB w/o G + G + agarose</td>
<td>46</td>
<td>2.9</td>
</tr>
<tr>
<td>C</td>
<td>experimental TSB-1% agarose media with additives</td>
<td>45</td>
<td>4</td>
</tr>
</tbody>
</table>

* Each value is the average number of CFU from a 10^-6 dilution from six plates.
* For colony size the diameters were measured (in millimeters) from photographs for groups A and B; colony size was estimated for group C.
* Each value is the average number of CFU from a 10^-6 dilution from six plates plus the values from the 10^-5 dilution from six plates divided by 10.
* Autoclaved as a single solution.
* All media were autoclaved except filter-sterilized TSB, which was prepared by centrifuging TSB for 1 h at 15,000 x g, filtering the supernatant with a 0.45-μm-pore-size filter, mixing it with an equal volume of 2% agarose that had been autoclaved and cooled to 50 to 55°C, and then pouring.
* Separate solutions of TSB w/o glucose, glucose, and agarose, each autoclaved separately.

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TABLE 2. Experiment 3: comparison of colony sizes and of the means and standard errors of the number of CFU of *P. multocida* and *P. haemolytica* obtained with BA, blood agarose, and TSB w/o G + G + agarose medium

<table>
<thead>
<tr>
<th>Growth medium</th>
<th><em>P. multocida</em></th>
<th><em>P. haemolytica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean CFU ± SE</td>
<td>Size*</td>
</tr>
<tr>
<td>BA</td>
<td>44 ± 1.9</td>
<td>2.1</td>
</tr>
<tr>
<td>Blood agarose</td>
<td>55 ± 3.3</td>
<td>2.2</td>
</tr>
<tr>
<td>TSB w/o G + G + agarose, 0.6%</td>
<td>52 ± 4.9</td>
<td>3.6</td>
</tr>
<tr>
<td>TSB w/o G + G + agarose, 0.8%</td>
<td>54 ± 4.1</td>
<td>2.8</td>
</tr>
<tr>
<td>TSB w/o G + G + agarose, 1.0%</td>
<td>57 ± 2.2</td>
<td>2.3</td>
</tr>
</tbody>
</table>

* The colony size (in millimeters) was measured from photographs.

**DISCUSSION**

The objective of this study was to develop a new medium free of blood or serum that would give reproducible results and would be equivalent to BA for the growth of *P. multocida* and *P. haemolytica*. Each standard error shown in Table 2 represents a summary of the results from the replicate runs for each medium, and the small size of the standard error in relation to the mean is an indication of the excellent reproducibility of the results. An examination of the data in Table 2 gives a clear indication that the means of the number of CFU of *P. multocida* obtained with TSB w/o G + G agarose medium are equal to or greater than the means of the CFU obtained with BA. The results obtained for *P. haemolytica* with the two media are comparable; the differences are small and not statistically significant. Although the colony size does not enter into the determination of CFU, the size is important because if the colonies are too small they might be missed during counting. Furthermore, the size is an index of growth; the larger colonies presumably have more cells.

TSB was chosen for our initial experiments because casein and soy proteins are relatively well-defined and inexpensive proteins which are widely used in bacteriological media. Blood, serum, and other animal proteins are subject to greater variation. The hypothesis that agar might contain one or more toxic components, such as agarpectin, which are not present in agarose and that these toxic components would lower the number of CFU is supported by the results obtained with TS agar and TSB-agarose media and also by the results with BA and blood agarose.

The hypothesis that autoclaving TSB can result in the formation of inhibitors for *P. multocida* and *P. haemolytica* is supported by the effect of autoclaving on the colony size. The colony size of *P. multocida* was almost four times larger with filter-sterilized TSB than with autoclaved TSB. As predicted, the results obtained by autoclaving separate solutions of the ingredients, TSB w/o G + G agarose medium, were similar to those with filter-sterilized TSB. The procedure of autoclaving the separate solutions is more easily carried out than the sterile filtration procedure, and because of its simplicity, it was chosen for additional testing.

It is not clear why there appear to be two types of inhibitors. The first type affects the number of CFU and is due to the agarpectin component of agar. The second type, which affects colony size, is apparently generated by autoclaving glucose in the presence of protein and/or phosphate.

A replacement medium for BA for the determination of the number of CFU of *P. multocida* and *P. haemolytica* that gives equivalent numbers of CFU has been found. It is prepared by autoclaving three separate solutions—TSB without glucose; glucose; and agarose—which are cooled to 55°C, mixed, and poured.

**ACKNOWLEDGMENTS**

We thank Jerome Sacks of the National Animal Disease Center for editing suggestions. We also thank Randall Cutlip of the National Animal Disease Center for helpful advice in designing the experiments and Miles Bairey of the National Veterinary Services Laboratories for suggesting the need for the replacement medium.

**LITERATURE CITED**


