Increased Reliability in Detection of Enterotoxigenic Escherichia coli by DNA Colony Hybridization

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(Received for publication May 14, 1986)

ABSTRACT

Previously a DNA hybridization assay was designed to detect the presence of and to enumerate enterotoxigenic foodborne Escherichia coli. The determinative step in the method involves autoradiographic analysis of the DNA from foodborne isolates after hybridization with a $^{32}$P-labeled probe specific for an enterotoxin gene. Dark spots appearing on the X-ray film after exposure indicate which colonies carry genes encoding the pathogenic determinant. A problem with this assay is the tendency of some colonies to detach from the nitrocellulose filters during hybridization or washing to remove the unbound probe DNA; this results in a false-negative interpretation in up to 60% of the samples processed at 80°C. By lowering the temperature to 70°C and increasing the incubation time to 3 h during in vacuo baking of filters, detachment (floatation) of colonies is reduced to about 37%. At 65°C only 2% of the colonies came off the filter after in vacuo baking of filters for 24 h. Another problem has been the inadequacy of exposure of X-ray film at -20°C when a -70°C freezer is not available. This problem can be alleviated by exposing the X-ray film in cassette holders "sandwiched" between slabs of dry ice (CO$_2$ ice has a temperature of -78.5°C). These modifications improve the reliability and accuracy of this DNA colony hybridization method.

Isolates of Escherichia coli possessing the genetic material to produce toxins can be detected in a DNA colony hybridization assay described recently by Hill and Payne (4). This versatile assay allows for the simultaneous detection of a variety of E. coli strains producing the heat-labile enterotoxin (LT).

To perform this assay, a DNA fragment of the enterotoxin gene is labeled with $^{32}$P-deoxycytidine triphosphate using the in vitro nick translation technique (5). E. coli colonies having the potential for toxigenicity possess nucleotide sequences complementary to the radiolabeled probe DNA and therefore will bind the probe. The determinative step in the method involves autoradiographic analysis of the colonies to determine which have bound the toxin-specific probe DNA. The dark spots that appear on the X-ray film provide a direct measure of those bacterial isolates that encode a particular pathogenic determinant. Negative colonies appearing as (blanks) background or very faint spots do not have the genetic potential to produce toxins. One potentially serious problem encountered with this assay is that some colonies detach (float) from the nitrocellulose filters during hybridization and/or during the washing procedure employed to remove radiolabeled probe DNA not specifically bound to complementary nucleotide sequences.

Another problem involves the efficiency of exposure of the X-ray film when exposed to beta-radiation. This process is temperature and time-dependent; lower temperatures stabilize the light-sensitive crystals in the film emulsion providing more efficient conversion of the radiation energy to the chemical reaction leading to the deposition of elemental silver. It has been recommended (4) that X-ray film cassettes should be placed in a -70°C freezer to reduce the time for autoradiogram exposure. When a -70°C freezer is not available, a -20°C freezer may suffice. However, when a -70°C freezer is not available and -20°C is used, the quality of the autoradiograms exposed 18-24 h at -70°C was considerably better than that of film exposed for the same time at -20°C. Longer exposure times at -20°C were required to produce accurate autoradiograms to determine the presence of genes encoding labile toxin.

Consequently, the purpose of this study was to investigate the effects of time and temperature to optimize the adherence of the colonies to the filter and to minimize false-negative results as a consequence of having to use a -20°C freezer when a -70°C freezer is not available for accurate development of autoradiograms.

MATERIALS AND METHODS

The E. coli strains (Table 1) used in this study were kindly provided by Walter Hill, Division of Microbiology, Food and Drug Administration, Washington, D.C. and shipped on agar slants by overnight carrier to maximize stability of virulence. Upon receipt, they were immediately subcultured into brain heart infusion broth (BHI) and handled according to the procedure of Hill and Payne (4).

Overnight cultures of E. coli in BHI broth were spotted onto sterile nitrocellulose filters (BA85, Schleicher and Schuell 0.45-µm pore size, 82 mm diameter), placed on MacConkey agar and incubated at 37°C for 4-6 h following the procedure of Moseley et al. (8) as modified by Hill and Payne (4). Colonies were lysed for 20 min with the exception of filters incubated at 80°C, which were lysed for 10 min. Filters were air-dried on Whatman 3MM absorbent paper for approximately 30 min.
TABLE 1. Escherichia coli strains used in this study.  

<table>
<thead>
<tr>
<th>Strains</th>
<th>Heat-labile toxin production</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600 (pEWD 299)</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td>H10407</td>
<td>+</td>
<td>10</td>
</tr>
<tr>
<td>HB101 (pBR 313)</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>K334C2</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>TD427C2</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>TD225C4</td>
<td>+</td>
<td>7</td>
</tr>
</tbody>
</table>

*LT* was spotted four times on each filter.

*Toxin production determined by both biological (mouse Y-1 adrenal cell) and immunological (ELISA) methods.

TABLE 2. Percent colonies remaining on nitrocellulose filters.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Incubation time (h)</th>
<th>Colonies remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>2*</td>
<td>40</td>
</tr>
<tr>
<td>80</td>
<td>3.5</td>
<td>50</td>
</tr>
<tr>
<td>75</td>
<td>3.5</td>
<td>57</td>
</tr>
<tr>
<td>70</td>
<td>3</td>
<td>63</td>
</tr>
<tr>
<td>65</td>
<td>24</td>
<td>98</td>
</tr>
<tr>
<td>60</td>
<td>4.5</td>
<td>85</td>
</tr>
</tbody>
</table>

*Lysed for 10 min. Others lysed for 20 min.

and then baked in a vacuum oven at various times and temperatures (see Table 2). The filters were cooled and stored between paper filters in a vacuum desiccator at room temperature.

The hybridization mix and radioactive toxin gene DNA were prepared according to the procedure described and modified by Hill (3). Each filter was preincubated for 3 h at 37°C.

The methodology for autoradiography followed was that described by Hill and Payne (4) except autoradiograms were stored at -20°C. Generally X-ray films should be exposed at -70°C; the lower temperature will speed the exposure of autoradiograms. Radioactive colony hybridization filters contained in a Kodak film cassette holder with XAR-2 Kodak film and intensifying screens were sandwiched between two 10 lb. slabs of dry ice. The cassette, containing two filters and X-ray films, was taped closed in a plastic bag and placed between the dry ice as seen in Fig. 1. The resulting "sandwich" was placed in an outer plastic bag and stored in a -20°C freezer.

RESULTS AND DISCUSSION

This DNA colony hybridization method detects cells having the potential for toxigenicity because they possess nucleotide sequences complementary to the LT probe DNA. The LT-positive colonies appear as dark spots on the autoradiogram. It is important that DNA from colonies not be detached from filters during the hybridization and/or the washing procedures used to remove excess radiolabeled DNA that is not specifically bound. If the colonies came off the filter, autoradiograms could be misinterpreted because only very faint spots are observed where the colonies were lost. This may yield false-negative results.

Figure 1. *Sandwich technique.*

Figure 2. *Dry ice technique vs. simple storage at -20°C.* A) Exposed at -20°C for 24 h. B) Exposed in a dry ice sandwich for 24 h.
The results in Table 2 show the percentage of colonies remaining on the nitrocellulose filter after incubation at temperatures ranging from 60-80°C. The percent colonies remaining was determined by visually inspecting the filters. Dark spots for LT + colonies on the autoradiograms represent colonies remaining bound to the filter. Therefore, reliable results can be obtained if colonies are baked onto nitrocellulose filters at 65°C for 24 h which, unfortunately, adds an extra day to the test. Recently, paper filters (9) have been found to be more sensitive, less expensive and less troublesome for DNA colony hybridization than are nitrocellulose filters. Accurate results may be obtained without significantly increasing the length of the procedure.

To further shorten the method, the effect of exposing X-ray film at -20°C with and without the cassette being sandwiched between slabs of dry ice was examined. The cassette containing the filter and X-ray film was taped closed in a plastic bag (to minimize condensation and exposure to CO₂ vapors), and then placed between two 10 lb. pieces of dry ice. The resulting “sandwich” was enclosed in an outer plastic bag and stored at -20°C. Autoradiograms obtained with and without dry ice are compared in Fig. 2.

It is important to note that the same filter was used in A and B and that exposure of film A was done first. Faint spots 1-7 may be interpreted as negative; spot 8 would be read as positive. (Spots 1-4, which correspond with spots 9-12, are true negatives.) In autoradiogram B, spots 13-16 would be read as positives, even though 13-16 appear to be very faint when dry ice is not used as seen with spots 5-7. Dry ice may be used to reduce X-ray film exposure time and shorten the duration of the method when a -70°C freezer is not available.

ACKNOWLEDGMENTS

The author thanks Dr. Walter E. Hill, Division of Microbiology, FDA for providing the bacterial strains, probe DNA, and advice. Special thanks is also given to Dr. Neal Castagnoli, Science Advisor, San Francisco District, for his critical review of the manuscript and Dr. Ralph Kalinowski for assistance given in obtaining materials needed to expedite this study. I am grateful to Mrs. Shearl Mills for typing the manuscript.

REFERENCES