Rapid Method for Detection of Adherent Bacteria on Foley Urinary Catheters

T. I. LADD,1 D. SCHMIEL,2 J. C. NICKEL,2 AND J. W. COSTERTON1*

Department of Biology, University of Calgary, Calgary, Alberta, T2N 1N4,1 and Department of Urology, Queens University, Kingston, Ontario K7L 2V7,2 Canada

Received 26 November 1984/Accepted 8 March 1985

An accurate, rapid, and inexpensive method was developed for detecting and enumerating bacteria adherent to Foley urinary catheters based on malachite green staining of acridine orange-prestained specimens. This method has proven to be quick and reliable and will find application in quantitative studies of biomaterial-related sepsis.

Bacteriuria and related sepsis associated with indwelling urinary catheterization is the most common nosocomial infection in medical practice (9). The etiology and persistence of this bacteriuria are related to bacterial adhesion and the formation of bacterial biofilms on the catheter surface (7; J. C. Nickel, A. G. Grinsta, and J. W. Costerton, Can. J. Surg., in press; J. C. Nickel, I. Ruseska, C. W. Whitefield, T. J. Marrie, and J. W. Costerton, Eur. J. Clin. Microbiol., in press). Previously, we have used scanning electron microscopy and direct recovery methods in which we scrape the biofilm from the surface, disperse the cells by sonication, and count the viable cells by plating, but neither method provides a rapid means of demonstrating the presence of bacteria on catheter surfaces. In this study we have developed a rapid and direct staining technique for epifluorescence light microscopy which, with the use of a simple counterstain, gives a quick and accurate means of detecting and quantitating biofilm bacteria on catheter surfaces. We expect that this very rapid and quantitative technique for the direct detection of adherent bacteria on the surfaces of biomaterials will find general application in quantitative studies of prosthesis-related sepsis.

A strain of Pseudomonas aeruginosa isolated from a patient with a urinary catheter-associated infection was grown at 37°C in a 1.5-liter continuous culture fermentor containing 1.0 liter of synthetic urine (composition of artificial urine) adapted from formula of Minuth et al. (8). Continuous culture was initiated while the batch culture was in the exponential phase of growth. The final flow rate was adjusted to 60 ml h⁻¹ to approximate the urinary output rates of catheterized patients. Foley catheters (Silkolatex; Rusch) encased in a sterile plastic envelope to prevent contamination of the outer surfaces were connected to the effluent port of the fermentor. The inner surfaces of these catheters were exposed to the effluent flow for 8 to 24 h. After the appropriate colonization period, the catheter was removed, drained, and rinsed three times with 10 ml of sterile phosphate-buffered saline (PBS). Under strict sterile conditions, the catheters were cut into premeasured 1.0-cm sections, transferred to separate sterile vials, and gently rinsed three times with PBS.

Sections to be used for viable plate counts were aseptically sliced longitudinally into equal halves and placed in individual vials containing 10 ml of sterile PBS. Cells were then removed from the surface of the catheter sections by scraping the inner surface with a sterile scalpel blade, by two 30-s low-level sonication cycles on a Virsonic cell disruptor, and by vortexing for 60 s. This method was selected because it was more effective in dislodging attached bacteria than were other techniques (Table 1). These dispersed samples were serially diluted between 10⁻² and 10⁻⁷ in PBS, and 0.1-ml aliquots were spread-plate onto nutrient agar (Difco). Viable counts were recorded as CFU per square centimeter. All counts were based on four replicate specimen sections counted in duplicate at each dilution.

Specimen sections for scanning electron microscopy were fixed in 5% glutaraldehyde in cacodylate buffer (pH 7.0, 0.1 M) for 1 h and then dehydrated in a series of ethanol solutions (20 to 100%). The specimens were passed through an ethanol-Freon 113 series and critical point dried (2) in Freon 13. Specimens were coated with gold and examined with a Hitachi S450 scanning electron microscope at 20 kV accelerating voltage.

Sections to be examined by epifluorescence were fixed in 0.5% glutaraldehyde in cacodylate buffer (pH 7.0, 0.1 M) for 1 h. The samples were sliced longitudinally in half and stained with acridine orange as described by Zimmerman and Meyer-Reil (12). The catheter surface fluoresced too brightly to see cells clearly; therefore, we attempted to stain the surface, and thereby reduce its fluorescence, with a number of dyes including malachite green, methylene blue, crystal violet, and basic fuchsins. Superior results were achieved by simply staining the specimen with a sterile 1% (wt/vol) aqueous solution of malachite green for 10 min. The

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU cm⁻²</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10⁵±10⁶</td>
<td>6</td>
</tr>
<tr>
<td>Scraping and vortexing</td>
<td>2.14 ± 1.39 × 10⁶</td>
<td>6</td>
</tr>
<tr>
<td>Scraping and sonication</td>
<td>1.52 ± 0.13 × 10⁶</td>
<td>6</td>
</tr>
<tr>
<td>Scraping, vortexing, and sonication</td>
<td>2.65 ± 0.93 × 10⁶</td>
<td>6</td>
</tr>
<tr>
<td>Vortexing</td>
<td>1.48 ± 0.66 × 10⁶</td>
<td>5</td>
</tr>
<tr>
<td>Sonication</td>
<td>2.41 ± 1.00 × 10⁶</td>
<td>5</td>
</tr>
<tr>
<td>MAOCC</td>
<td>4.10 ± 1.4 × 10⁶</td>
<td>4</td>
</tr>
</tbody>
</table>

* Corresponding author.

* Samples were vortexed for a total of 60 s, using three 20-s periods.
* Samples were sonicated with two 30-s bursts.
* Samples were prestained with acridine orange, stained with malachite green, and counted in situ by direct epifluorescence microscopy.
cells rather than the catheter surface took up the stain. Prestaining with acridine orange, 0.01% for 2 min, improved the visibility of the dark green cells against an orange fluorescent background. The prestained and stained specimens were rinsed with sterile PBS and air dried for 20 min. After staining, the catheter sections were folded back with the inner surface exposed, bonded with glue, and mounted on clean glass slides. Samples were examined under a Zeiss standard 16 microscope equipped for epifluorescence (5). Direct counts (acridine orange direct counts), based on the malachite green staining procedure, were designated MAODC (malachite green-acridine orange direct counts) and recorded as cells per square centimeter. Cell counts were made in duplicate, using at least four replicate sections.

Scanning electron microscopic examination (Fig. 1A) clearly demonstrated the presence of an attached bacterial biofilm on the catheter surface after 8 h. Bacteria could be dislodged from the surface by a number of techniques (Table 1), and viable counts for catheters exposed to infected artificial urine for 8 h ranged between $1.48 \times 10^6$ and $2.65 \times 10^6$ cells cm$^{-2}$, with the highest counts obtained after scraping, vortexing, and sonication. However, scanning electron micrographs of the inner surface of a Silkolatex Foley catheter clearly showed bacterial biofilm formation.
microscopy of the surfaces after the putative removal of the adherent bacteria showed clearly that none of these methods removed all of the attached cells. When bacterial recovery was assisted by scraping, vortexing, and sonication, the number of living cells recovered (2.65 \times 10^6) was usually lower than the total number seen by direct epifluorescence microscopy (4.10 \times 10^6). Similar discrepancies between these two counts have been reported and in part reflect an inability of the total direct count to distinguish between live and dead bacteria (3). Nonetheless, there was no significant difference between the MAODC and viable plate counts after adequate dispersal of the biofilm bacteria (Table 2). Thus, both direct and viable counts gave similar estimates of bacterial density on the surface.

Epifluorescence microscopy provides a rapid and very useful method for examining bacteria directly on opaque surfaces (1). In the present study, however, background fluorescence of the catheter itself was so intense that acridine orange-stained cells were poorly resolved. Moreover, none of the counterstains tested reduced this background fluorescence. On the other hand, malachite green, which is more commonly used as a counterstain, stained the adherent biofilm cells dark green and rendered them clearly visible against the orange autofluorescent background of the catheter surface (Fig. 1B and C). In contrast, free-living bacteria stained with acridine orange alone fluoresced orange-yellow (Fig. 1D) (3).

Malachite green in combination with glutaraldehyde has been shown to preserve certain lipid granules in mammalian spermatozoa (10) and in certain gram-positive and -negative bacteria (6) and to combine with fatty acids, phospholipids, glycolipids, and fatty aldehydes (11). The chemical nature of the components involved in the reaction between malachite green and the Pseudomonas species in this study is not known, but the staining procedure has since been used successfully in our laboratory to enumerate pure strains of staphylococci, streptococci, Escherichia coli, and Proteus mirabilis on catheter surfaces.

The MAODC estimates of the bacterial density adherent to catheter surfaces is accurate, rapid, and inexpensive compared with viable plate counts or electron microscopy examination. It cannot, however, replace standard microbiological plating procedures for identification and antibiotic sensitivity testing of pathogenic organisms or replace electron microscopy for the morphological examination of adherent biofilm organisms. This new MAODC technique may find practical application as a rapid, accurate method for enumerating the attached bacterial populations in studies of urinary catheter-associated infection. It may find wider applicability in the study of other biomaterial-related sepsis, and as the etiology and pathogenesis of such infections become clearer, it may prove useful as a valuable diagnostic test complementing routine microbiological procedures.

**LITERATURE CITED**


