Rapid and Simple Method for Double Staining of Bacteria with 4′,6-Diamidino-2-Phenylindole and Fluorescein Isothiocyanate-Labeled Antibodies

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Bacteria were either heat fixed on microscope slides or filtered with 0.2 μm-pore-size Nuclepore filters. The samples were stained with 4′,6-diamidino-2-phenylindole (DAPI) for total staining and with polyvalent rabbit antibodies and fluorescein isothiocyanate-conjugated swine anti-rabbit antibodies for specific staining. By switching between two different optical filter packages in the microscope, only one sample was needed for determining both total and specific counts of bacteria. False-positive specific counts and other artifacts that occur with antibody staining were easily distinguished when individual fluorescent particles were checked for DAPI fluorescence. The method for applying the general stain to membrane filters was performed quickly and simply by using a DAPI-soaked polypropylene filter that lay beneath the Nuclepore filter which collected the sample.

Hobbie et al. incorporated filtration of water samples through polycarbonate filters to yield quantitative recovery of bacteria in the sample (5). They stained the filter with acridine orange to make the bacteria visible when using an epifluorescent microscope. Acridine orange has also been used for enumerating bacteria in inland waters and in soil. Use of acridine orange has some disadvantages, since this agent also stains particles other than bacteria and background fluorescence may be high. Other fluorochromes such as acriflavine, bisbenzimide (1, 10), 4′,6-diamidino-2-phenylindole (DAPI) (11), and rhodamine 123 (8) have since been used instead of acridine orange.

Specific recognition and counting of individual bacterial serotypes in mixed, natural populations may be done by using fluorochrome-labeled antibodies (2, 6, 9, 12). Both total and specific numbers of bacteria can be determined in one sample (9) by using both a specific stain and a general stain (e.g., DNA stain). It is possible to check for artifacts, such as false-positive specific counts, which occur with antibody staining by checking whether the antibody-stained particles have also been stained with a DNA-specific fluorochrome.

This report describes a double staining procedure which combines DAPI and fluorescein isothiocyanate (FITC)-labeled antibodies with bacteria that have been either fixed on glass slides or collected on membrane filters (Nuclepore Corp., Pleasanton, Calif.). Only one sample is necessary to obtain both the total and the specific population sizes for bacteria. This advantage will save both work and cost when the purpose is to enumerate bacteria or to examine the sample qualitatively. The samples used in this report were infected tissue and smears of gut content from moribund or newly dead salmon, which were fixed on glass slides. Feces from the same fish were collected on Nuclepore filters and analyzed.

MATERIALS AND METHODS

Staining of samples on glass slides. Atlantic salmon (Salmo salar) were infected with Vibrio salmonicida (4) by intraperitoneal injection. Moribund or newly dead fish suffering from cold-water vibriosis (3) were collected and examined. Bleeding areas on the skin or the trans-sectioned milt were laid gently on glass slides and then removed to produce imprints. Smears of gut content were also prepared on glass slides. Imprints and smears on glass slides were heat fixed before staining.

The rabbit antiserum specific for V. salmonicida used in this work is the same as that described by Egidius et al. (4). This antiserum shows low cross-reactivity against different strains of Vibrio anguillarum as measured by the enzyme-linked immunosorbent assay technique. There was very low cross-reactivity against Escherichia coli B and three different marine Pseudomonas spp. (data not shown).

Rabbit antiserum was diluted 1:100 in 0.01 M phosphate buffer, pH 7.2, supplemented with 20 g of NaCl, 4.3 g of MgCl·6H2O, and 1 g of NaN3 to give a final volume of 1,000 ml. Diluted antiserum was stored in sterile plastic syringes equipped with a 0.22-μm-pore-size filter unit (Millex-GV; Millipore Corp., Bedford, Mass.). The imprint was next covered with filtered antiserum for 20 min. The glass slide was gently washed with distilled water and allowed to drain. FITC-conjugated swine anti-rabbit immunoglobulins (DAKO, Copenhagen, Denmark) were diluted 1:100 in the buffer described above and stored in the same way. The imprint was covered with conjugate for 20 min, washed, and drained. The imprint was finally covered with freshly filtered (0.2-μm-pore-size filter) DAPI (1 μg/ml; Sigma Chemical Co., St. Louis, Mo.) for 5 min and then washed. The specimen was mounted in filtered (0.2-μm-pore-size filter) buffered glycerol, pH 7.5 (7).

Staining of samples on filters. Black polycarbonate membrane filters (5) with a diameter of 25 mm and a pore size of 0.2 μm (Nuclepore) were used. Fish feces were collected from the fish tanks with a dip net and mixed with 20 ml of incoming fresh seawater before fixation with formaldehyde (15% [vol/vol]). After thorough mixing, subsamples of 1 to 3 ml were filtered in a 12-funnel sampling manifold filter holder (Millipore). Polypropylene filters (Gelman Sciences, Inc., Ann Arbor, Mich.) supported the Nuclepore filters. Staining with DAPI was performed in two ways. (i) The polypropylene filters were soaked in DAPI solution (10 μg/ml) before use. In this way, the DAPI stain diffused upward through the
Nuclepore filter to the sample, which was simultaneously stained with fluorescent antibodies from above. This method also filters the DAPI stain before the stain reaches the sample. (ii) The filters were covered with freshly filtered (0.2-µm-pore-size filter) DAPI solution (1 µg/ml) for 5 min before the samples were stained with fluorescent antibodies. Ordinary polypropylene filters were used in this case.

After filtering of the samples and eventual staining with DAPI, fluorescent-antibody staining was performed by pre-washing the filters three times with 2 ml of distilled water, covering the filters with serum, again washing the filters three times, covering the filters with conjugate, and performing a third washing series. Staining time for serum and conjugate was 20 min. The samples were then placed on microscope slides for analysis as described above.

No improvement in fluorescence yield was noticed for either DAPI or FITC when the samples were washed with 0.01 M phosphate-buffered saline (8.75 g of NaCl per 1,000 ml), pH 7.2, instead of distilled water (7). This result was found both for the imprints on glass slides and for the filters. Therefore, distilled water was used as the wash solution.

Microscopy. The fluorescent-antibody-stained preparations were examined with a Leitz Orthoplan microscope (E. Leitz GmbH, Wetzlar, Federal Republic of Germany) with an O.SRAM HBO 200 W/4 mercury lamp as the light source. A Leitz Ploemopak A (BP 340-380 exciting filter, RKP 400 beam-splitting mirror, LP 430 suppression filter) was used for viewing DAPI staining, and a Leitz Ploemopak I 2 (BP 450-490 exciting filter, RKP 510 beam-splitting mirror, LP 515 suppression filter) was used for viewing FITC staining. A Plan-NEOFLUAR 63/1.25 oil immersion objective was used. Photomicrographs were taken with Kodacolor Gold 400 film (Eastman Kodak Co., Rochester, N.Y.), using a Wild MPS 45/51 Photoautomat.

RESULTS AND DISCUSSION

For the three methods described, some background fluorescence occurred. However, the contrast between stained bacteria and background was sufficient to allow the bacteria to be observed easily (Fig. 1 and 2). This was the case both for DAPI staining and staining with fluorescent antibodies. No interference between the two stainings was observed. Ordinary glass slides were used. They were not cleaned before use (13), nor were they of a special brand (14).

In some cases, staining fish feces with DAPI from above on Nuclepore filters resulted in high background fluorescence, which made counting impossible. This problem was overcome by staining from below as described. Bacteria in fish feces and from seawater were properly stained by using DAPI-soaked supporting filters (Fig. 1A).

No special washing step is required when staining with DAPI, and the process is easy and quickly performed. The work involved when double staining with fluorescent antibodies is negligible. When supporting filters soaked in DAPI solution are used, there is in fact no extra manipulation at all.

Since both total and specific counting may be performed on one sample (filter or imprint), work in preparing the specimens is reduced by half. Positive specific scores (FITC label) may be easily checked to verify that they are indeed due to bacteria by switching to the DAPI filter package of the microscope. A DAPI-stained bacterium should be visible at exactly the same spot. Artifacts with bacterium-shaped yellow-green rings are sometimes seen in antibody-stained specimens. Such fluorescent rings also appear as rings when viewed through the DAPI filter package, which indicates that they are artifacts associated with autofluorescence.

Differences between enumeration with DAPI staining and with antibody staining may be analyzed (Fig. 1). Newly divided cells appear as two distinct cells stained with DAPI.
FIG. 2. Fluorescence photomicrographs of an imprint on a glass slide of a bleeding area on fish skin. (A) DAPI stained; (B) the same field as shown in panel A viewed with filters for FITC. Note that the bleeding area is colonized almost entirely by V. salmonicida. Arrow in panel A indicates a bacterium that is not stained with antibodies. Bar = 5 μm.

(Fig. 1A). When stained with antibodies, however, the same cells appear as a single elongated cell without any visible constriction or cross wall (Fig. 1B). Thus, when pure cultures are enumerated, DAPI staining may result in higher counts than does staining with antibodies. During work with pure cultures of V. anguillarum and V. salmonicida starved in seawater for 41 weeks, the opposite observation was made. Bacterium-shaped fluorescent rings appeared in positions where no bacteria were stained with DAPI, which indicated the presence of intact cell envelopes (ghost cells) but leakage of DNA and other cytoplasm components (K. A. Hoff, manuscript in preparation).

A second application of this method of double staining is illustrated in Fig. 2. Imprints of infected tissue may be examined for a specific bacterium. It is possible to see whether this bacterium appears as a monoculture or whether there also are other bacteria in the infection. Analogously, bacterial colonization of wounds can be studied.

Muyzer et al. have used ethidium bromide in combination with FITC-conjugated antibodies for double staining of Thiobacillus ferrooxidans (9). By changing filter sets, it was possible to see either ethidium bromide-stained or FITC- and ethidium bromide-stained bacteria in the preparation simultaneously. Viewing both DNA-stained and immunologically stained bacteria with the same filter set is an advantage because there is no need for comparing two identical fields. However, in cases where there is a need to check for false-positive counts or when ghost cells are observed, both fluorochrome combinations, i.e., DAPI-FITC and ethidium bromide-FITC, require changing of the filter sets. Any overlapping or interference of the emission spectra of the fluorochromes, which may result in masking, is mutually excluded when only one stain is visible at a time. The advantages of using DAPI are the speed and simplicity of the staining procedure.

The methods described for double staining bacteria on filters with DAPI and fluorescent antibodies have been successfully applied to general staining of planktonic bacteria in seawater and specific staining of V. salmonicida in the same sample (unpublished data). In addition, these methods have been applied successfully to specific staining of populations with sulfate-reducing bacteria in samples collected from injection water and wastewater from oil wells in the North Sea (T. Torsvik, personal communication).

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LITERATURE CITED


