

Rapid Detection of Small Numbers of Airborne Bacteria by a Membrane Filter Fluorescent-Antibody Technique

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Received for publication 21 August 1970

Bacteria from stirred settling aerosols were recovered and identified by a rapid fluorescent-antibody technique previously described by Danielsson. Calibrated air samples from *Serratia marcescens* aerosols were drawn through a Millipore aerosol filter holder fitted out with a nonfluorescent membrane filter (type HAB 047). With the aid of a high-power incident-light ultraviolet microscope, the individual bacterial cells, trapped on the filter, could be inspected after staining with appropriate conjugate on the filter surface.

A sensitive and reliable microscopic technique for the examination of low-concentration bacterial aerosols in open or closed spaces, as well as in experimental aerosol systems, appears highly desirable. Membrane filters have been used as surfaces on which bacteria can be concentrated from water or air, and viability can be demonstrated in the form of small colonies after a short incubation of the filter on a solid nutrient medium.

Vlodavec (10) collected airborne *Serratia marcescens* on membrane filters which he colored with 3% carbol-erythrosin and rendered transparent for direct microscopic examination with immersion oil. A fluorescence microscope technique based on acridine orange staining was described by Brock (1), who collected bacteria from thermal springs on membrane filters which he inspected, after staining, with an incident-light ultraviolet microscope. Carter and Leise (2) were the first to use a nonfluorescent membrane filter and succeeded with the aid of a low-power dissecting microscope in demonstrating microcolonies of *Bacillus anthracis*, stained by fluorescent antibodies after incubation for 12 hr. Danielsson (3) and Danielsson and Laurell (4) used a nonfluorescent membrane filter [HABG (P) 047, Millipore Corp., Bedford, Mass.] to collect bacteria from water samples. The trapped cells were combined on the filter membrane with fluorescein isothiocyanate (FITC)-labeled antiserum, so that specifically fluorescent bacteria could be seen on the filter surface with the aid of the incident-light

fluorescence microscope. Recently, Guthrie and Reeder (5) used the same membrane filter (Millipore Corp.) for the detection of microcolonies of *Escherichia coli* by the fluorescent-antibody method.

By using the incident-light illuminator developed by Ploem (8), we succeeded in our laboratory in straining large and small numbers of *S. marcescens*, *E. coli*, and *B. cereus* cells by appropriate conjugates on HABP-type filters. Therefore, we initiated a study to recover bacteria on membrane filters from experimentally created aerosols and to identify the trapped cells by means of immunofluorescence (IF).

MATERIALS AND METHODS

Microorganisms and antisera. *E. coli* 78:80 B and *B. cereus* were grown in Trypticase soy broth enriched with 5% inactivated horse serum and 0.5% glucose. Stirred Erlenmeyer flask cultures of *E. coli* were incubated for 3 hr and cultures of *B. cereus* were incubated for 6 hr at 37 C.

S. marcescens was cultivated in a chemically defined medium (CDM) of the following composition (R. L. Dimmick, *personal communication*), per liter of distilled water: 2.5 g of ammonium citrate (dibasic), 5 ml of glycerol (USP), 3.9 g of K₂HPO₄ (anhydrous), 0.5 g of MgSO₄·7H₂O, 0.25 g of NaCl. For aerosol studies, *S. marcescens* was grown with continuous stirring in CDM for 36 hr in a water bath at 30 C. Cells were harvested by centrifugation at 4 C, washed twice with CDM, and resuspended in distilled water for atomizing.

Specific antisera were prepared in rabbits by five intravenous injections of formalinized cells, washed several times and suspended in phosphate buffer (pH 7.5), at an interval of 2 to 4 days each.

Preparation of conjugates. The antisera were frac-

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tionated with ammonium sulfate by the method of Stelos (9), and in the conjugation procedure we followed the method described by Holborow (6). However, we used a smaller fluorochrome to protein ratio: 2 mg of crystallized FITC was conjugated to 100 mg of protein as determined by the biuret method (7). Therefore, the ratio of FITC to protein for conjugation was $\frac{1}{50}$ instead of $\frac{1}{20}$ as suggested by Holborow (6). After conjugation, we purified our conjugates from unreacted dye by passing the whole conjugate through a column packed with Sephadex G50 (coarse) equilibrated in 0.01 M phosphate-buffered saline (PBS), pH 7.6. Crystalline FITC was obtained from BBL, and Sephadex G50 was obtained from Pharmacia, Uppsala.

Generation of aerosols. Bacterial aerosols were generated with a Wells-type all-glass atomizer operated at 10 psi. The aerosol was equilibrated with partially saturated air to achieve a relative humidity of 70 to 80%. Relative humidity was determined several times in the course of an experiment by psychrometry. The aerosol was stored in a 420-liter stirred settling chamber with a fan operated at about 400 rev/min.

Air sampling. For the determination of the number of viable cells per liter of air, we used all-glass impingers (type AGI-30) with a constant flow rate of 12.5 liters/min. The collection fluid was 0.2% gelatine, a medium which requires the addition of an antifoam (Silicone).

For viable counting, small samples of the collection fluid were diluted and plated on nutrient broth agar (Difco) enriched with 0.5% glucose. Colonies were counted after 20 hr at 37 C.

Viable counting in aerosols containing less than 100 bacteria per liter was done by passing the collection fluid of an impinger (monitored for 2 to 4 min) through a membrane filter (Millipore AAWP 047) which had been previously washed and moistened with buffer and was, after the filtration, incubated on a nutrient agar plate. Microcolonies were counted at fivefold magnification after 12 hr at 37 C.

An Anderson sampler was equipped with glass petri dishes containing nutrient broth agar to obtain information about droplet size in the aerosol. It was found that, in aerosols of 70 to 80% relative humidity, 98% of the particles were $\geq 2 \mu\text{m}$ in diameter.

For IF studies, air samples were drawn through dry membrane filters (type HABP 047, Millipore Corp.) placed in a aerosol filter holder (Millipore Corp.). This instrument was operated with a membrane pump usually at a flow rate of 10 liters/min.

Immunofluorescence technique. Before use, the non-fluorescent filters were washed with about 50 ml of PBS. After air sampling, several pieces of a 30-mm² area were stamped out and placed on a nondefatted slide. A large droplet of diluted conjugate was put on each filter piece, and the slide was kept in a humid chamber for 50 min. The filter pieces were then washed by pouring through slowly about 100 ml of PBS and were finally mounted in carbonate-bicarbonate (pH 9.0)-buffered glycerol under a cover slip.

Fluorescence microscopy. We used a Leitz Ortholux microscope equipped with a mercury lamp (HBO 200). UG1 (2 mm) and BG38 (3 mm) filters, or BG12

instead of BG38, served as primary filters, and a yellow filter (510 nm) served as the secondary filter. The incident-light illuminator was used in combination with the following objectives: Leitz Apo-10, Apo-25, and Apo-40, all objectives being used without oil.

Standard IF preparations can be inspected as well by incident illumination as by transmitted light combined with a dark-field condenser.

RESULTS

The quality of the conjugates is of great importance. Undiluted conjugates produced a very bright fluorescent background and were therefore unsuitable for the detection of bacterial cells on HA filters.

In accordance with Danielsson (3), we found that only conjugates having a relatively high staining titer could be used successfully. Such conjugates should be diluted with PBS as much as possible. The dilutions for our conjugates were the following: anti-*S. marcescens*, up to $\frac{1}{20}$; anti-*E. coli*, $\frac{1}{10}$; anti-*B. cereus*, $\frac{1}{5}$ to $\frac{1}{10}$.

Even with excellent conjugates in an appropriate dilution, background fluorescence was still rather considerable. It did not, however, interfere with the identification of the bacteria because the background faded much faster under the exciting radiations than the cells. The cells were regularly distributed over the whole active filter surface, sticking more or less deeply in the membrane. Many of the cells were on the surface, others penetrated more deeply, and an unknown percentage of bacteria were hidden in the holes of the filter. In the case of *B. cereus*, most of the rods penetrated into the filter along their longer axis, so that the cells appeared shortened to the observer (Fig. 1). Fading of the preparation occurred very rapidly, a definite disadvantage of the incident-light technique.

The quality of the IF carried out with diluted, highly specific, and brilliant conjugates depends still largely on the structural integrity of the trapped cells. Flagella are usually lost during atomizing. It is unknown if changes in the external shape of single cells occur during atomizing and in the aerosol state. We believe that the described air sampling technique produces little structural damage to trapped cells, when the flow rate of aspirated air does not exceed 10 liters/min and when the membrane filters are rehydrated quickly after air sampling.

Cultures of *E. coli* 78:80 B showed a marked tendency of the cells to autolyze, even in very short incubation periods. Such samples produced a large amount of amorphous, fluorescent material after staining on the membrane filter. Our anti-*Serratia* conjugates appeared to contain a small proportion of antibodies, cross-reacting

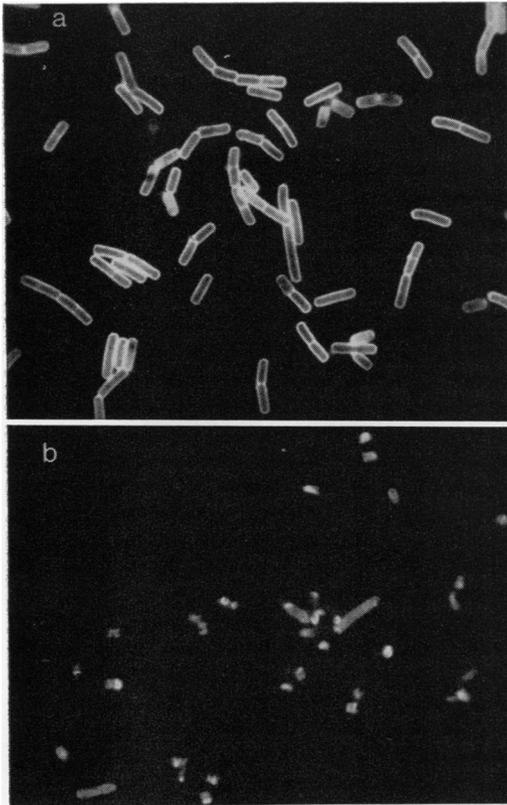


FIG. 1. (a) *Bacillus cereus* as seen in a Leitz Ortholux fluorescence microscope. Standard immunofluorescence preparation. $\times 900$. (b) *B. cereus* as seen on a nonfluorescent membrane filter. Leitz Ortholux microscope with incident-light illumination was used. $\times 600$.

with different strains of *E. coli*. We were, however, unable to demonstrate distinctly fluorescent cells of *E. coli* on the membrane filter when the membranes were stained with anti-*Serratia* conjugate and therefore did not absorb the conjugate.

A few quantitative measurements revealed the minimal number of bacteria which might theoretically be detected on a 30-mm² piece of the non-fluorescent membrane filter. In a Millipore aerosol filter holder, the radius of the active filter area was 17 mm. The active surface was therefore $3.14 \times 17^2 = 907.5$ mm². The small piece stamped out for IF was about 3.4% of this area.

The detection limits were experienced with a well-defined suspension of *S. marcescens*, obtained from a log-phase culture of this organism in CDM. Such a culture contained very few damaged or dead cells, and the results of a viable counting and a total count in the slide-counting chamber (Petroff and Hauser) differed only slightly. Small volumes of different suspen-

sions were filtered directly through HA-type filters. The number of cells on the filter was calculated on the basis of the known titer of the suspension. It was assumed that the cells were equally distributed over the filter area.

Calculated numbers of cells for the 3.4% pieces were compared with the results of the microscopic examination (Table 1). The tabulated data show that positive results can be expected when at least a few thousand bacteria are trapped on a filter. Considerably fewer cells were counted on the filter pieces than one might have expected from a calculation. Because we were unable to find the source of losses, we concluded that this effect was due to deep penetration of some of the bacteria with subsequent masking of their fluorescence.

The established sensitivity corresponded well with findings from stirred settling aerosols of *S. marcescens* (Fig. 2). Aerosols of high relative humidity (about 75%) were stored for 1 hr, after which time the number of viable cells in the aerosol dropped almost two logs. On the other hand, air samples with membrane filters, taken at 1, 30, and 60 min, revealed practically the same number of fluorescent cells in all cases. This indicates that the physical decay of the aerosol was much slower than the biological one.

For a number of aerosols with various initial viable cell concentrations, we determined the air volume required to obtain a clearly positive IF on filters (Table 2). We found it easy to demonstrate membrane-trapped bacteria by IF from aerosols containing only about 100 cells per liter. From this we deduce that in open-space studies even smaller concentrations of bacteria could be detected by the same technique.

After completion of this study, we were informed by L. J. Goldberg (Naval Biomedical Research Laboratory, Oakland, Calif.) that he uses a similar technique for the demonstration of airborne bacteria with IF. He is working with cellulose-TRI-acetate filters which he renders transparent by treatment with immersion oil. The incident-light energy is therefore absorbed less, the background appears black, and there is less of a bleaching effect (*personal communication*).

DISCUSSION

It seems that there is an urgent need for rapid and reliable detection of airborne bacteria. The value of direct microscopic examination of air samples could be enhanced greatly if the bacteria were stained with fluorescent antibodies of known specificity.

We found that the HA-filter method described by Danielsson (3) is suitable for the detection and identification of small numbers of different bacteria trapped from aerosols.

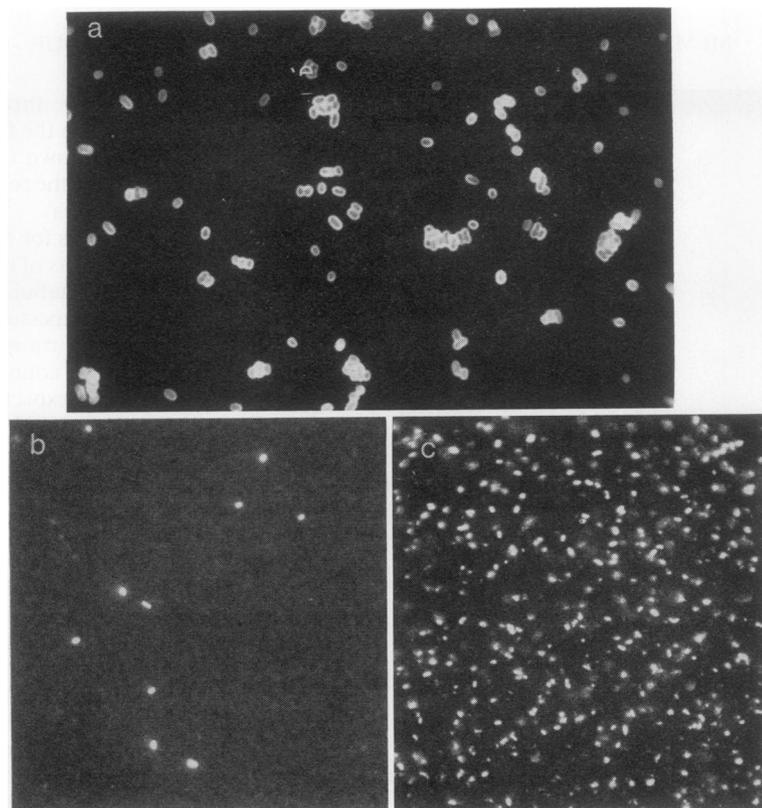


FIG. 2. (a) *Serratia marcescens* as seen in a Leitz Ortholux fluorescence microscope. Standard immunofluorescence preparation. $\times 900$. (b) *S. marcescens* as seen on a nonfluorescent membrane filter 5-min air sample from an aerosol containing 100 cells per liter. $\times 600$. (c) *S. marcescens* as seen on a nonfluorescent membrane filter 1-min air sample from an aerosol containing 10^5 cells per liter.

TABLE 1. Comparison of calculated numbers of cells for the 3.4% filter pieces with the results of microscopic examination

Total no. of cells filtered through HA filter	Expected no. of cells on 30-mm ² filter piece	Found by incident-light microscopy ^a		
		Filter 1	Filter 2	Filter 3
100	3	N, N, N, N, N	N, N, N, N, N	N, N, N, N, N
1,000	34	N, S, S, N, S	S, N, N, N, S	N, S, N, S, N
10,000	340	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P

^a Three filters were used for each concentration tested; five pieces were inspected from each filter. Abbreviations: N, negative result; S, single cells found; P, positive result, cells in each field of vision.

TABLE 2. Air volume required to obtain a clearly positive immunofluorescence on filters for aerosols with various initial viable cell concentrations

Initial concn	Results with various operation times at a flow rate of 10 liters/min ^a						Amt (liters) of air required for positive result	Approx. corresponding no. of bacteria
	10 sec	20 sec	40 sec	80 sec	160 sec	320 sec		
10^2	N, N, N, N, N	N, N, N, N, N	N, N, N, N, N	S, N, N, N, S	N, S, S, S, N	P, S, P, S, P	50	5,000
10^3	N, N, N, N, N	N, S, N, S, N	S, S, S, S, S	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P	10	10,000
10^4	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P	P, P, P, P, P	1.6	10,000

^a Abbreviations: N, negative result; S, single cells found; P, positive result, cells in each field of vision.

Many features of this method remain to be investigated. Its suitability for smaller bacterial cells like staphylococci and streptococci or for bacterial spores is still unknown. As the diagnostic value of the method depends on the structural integrity of the cell, we expect it to be especially valuable for microbial spores. A study on this subject is in progress at this institute. It is furthermore suggested that the method may be useful for the microscopic control of experimentally created microbial aerosols.

ACKNOWLEDGMENTS

This investigation was supported by a grant from the Fraunhofer Foundation (West Germany) and by grant In San 10/70 from the Fraunhofergesellschaft München.

We appreciate the kindness of S. H. Madin (School of Public Health, Berkeley) who introduced us to some techniques in aerobiology. We acknowledge gratefully the helpful advice of D. Danielsson. Due to the kindness of B. Sordat, Pathologisches Institut der Universität Bern, it was possible to use Leitz Orthomat equipment for microphotography.

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