NOTES

Agar Diffusion Method for Negative Staining of Microbial Suspensions in Salt Solutions

NAN ANDERSON and FRANCES W. DOANE

Department of Microbiology, School of Hygiene, University of Toronto, Toronto 5, Ontario, Canada

Received for publication 20 March 1972

Microbial suspensions in a saline medium can be prepared for negative staining by a simple agar diffusion method that achieves a high degree of particle concentration.

The negative staining of biological suspensions is frequently hindered by the presence in the suspending medium of salts which may crystallize in the electron microscope and obliterate specimen detail. To circumvent this problem, it is usually necessary to resuspend the specimen in water or in a volatile salt solution such as ammonium acetate. This is accomplished by techniques such as ultracentrifugation or dialysis, which are time consuming and may introduce structural artifacts. The problem of salt in the suspending medium is a common one in microbiology. Specimens are usually in the form of suspensions from broth or tissue cultures, egg fluids, or density gradient fractions, or they may be clinical specimens such as cerebrospinal fluid, vesicle fluid, or clarified stool suspensions.

We have previously reported a simple method of dialyzing salty suspensions on the surface of a water drop (1). Although this method is recommended for specimens containing greater than 10^6 particles per ml, for more dilute specimens, and especially for large numbers of specimens, we have found the following agar diffusion method to be more practicable. It is based on an agar diffusion principle originally employed by Kellenberger and Arber for the quantitative analysis of particle suspensions (3) and more recently employed by Kelen et al. in the detection of Australia antigen and antibody (2).

The present method uses micropip, disposable, microtiter plates (Cooke Engineering Co., Alexandria, Va.) Each cup is approximately three-fourths filled with 1% aqueous Noble agar (Difco Laboratories, Detroit, Mich.), and a 300-mesh, Formvar-carbon-coated copper specimen grid is placed on the solid agar surface. Plates so prepared can be used immediately, or they can be covered tightly with strips of adhesive sealing tape (Cooke Engineering Co.) and stored at 4°C for 2 to 3 weeks. To prepare a specimen for examination, one to two drops are added to a grid and allowed to air-dry for 15 to 30 min (under ultraviolet light if the sample is considered to be highly pathogenic). A drop of negative stain is then added (2% phosphotungstic acid, pH 6.5 or 4% ammonium molybdate, pH 6.5); after 1 min the grid is removed with forceps, air-dried briefly, and examined in the electron microscope.

Microbial suspensions prepared in this way show little evidence of salt when examined by electron microscopy. Figures 1 and 2 show the excellent distribution and resolution obtained when viruses and bacteria in clinical specimens or cultures are added directly to the agar-supported grid. Furthermore, a much higher concentration of the sample can be achieved by the agar diffusion method than by standard negative-staining techniques. Counts performed on a suspension of latex particles (88 nm) indicated that at a concentration of 10^7 particles per ml an average of five particles could be observed per grid square. This represents at least a 100-fold improvement over the previously described water drop dialysis method (1).

By using the microtiter plates it is possible to prepare a large number of specimens at one time, and without the usual requirement for separate forceps to hold each grid. If required, the plates can easily be cut into smaller sec-
Fig. 1. Electron micrographs of viruses observed in stool suspensions prepared by the agar diffusion method and negatively stained. A, Low magnification of adenoviruses in phosphotungstic acid showing even distribution and absence of salt; $\times$15,000. B, Reoviruses in ammonium molybdate; $\times$82,000.

Fig. 2. Microbial suspensions prepared by agar diffusion method and negatively stained with phosphotungstic acid. A, Parainfluenza viruses from tissue culture fluid; $\times$34,000. B, Escherichia coli from a broth culture; $\times$5,000.

We thank L. Pinteric, Department of Biochemistry, University of Toronto, for supplying the latex suspension and H. J. Weidinger for excellent technical assistance. This project was supported by Province of Ontario health research grant PR12.

LITERATURE CITED
1. Doane, F. W., N. Anderson, A. Zbitnew, and A. J. Rhodes. 1969. Application of electron microscopy to the diagnosis of virus infections. Can. Med. Ass. J. 100:1043-1049.
2. Kelen, A. E., A. E. Hathaway, and D. A. McLeod. 1971. Rapid detection of Australia/SH antigen and antibody by a simple and sensitive technique of immunoelectron microscopy. Can. J. Microbiol. 17:993-1000.
3. Kellenberger, E., and W. Arber. 1967. Electron microscopic studies of phage multiplication. 1. A method for quantitative analysis of particle suspensions. Virology 3:245-255.