EVALUATION OF IMMUNOASSAYS FOR ELECTRON MICROSCOPY

Frances W. Doane, Nan Anderson, Francis Lee, Kathryn Pegg-Feige and John Hopley

Department of Microbiology
University of Toronto
Toronto, Ontario, Canada

INTRODUCTION

In recent years immunoelectron microscopy (IEM) has become one of the several immunoassays available to virologists for detection and identification of viruses. Like other immunological assays, the reliability depends on careful attention to test conditions (e.g. antigen/antibody concentration, incubation time, temperature, pH) and to a regard for incorporation of a range of appropriate controls. This chapter will discuss several IEM techniques that can be applied to fluid specimens commonly encountered in diagnostic virology. Consideration will be given to some of the variables that affect the efficiency of these tests, and to the advantages and disadvantages of IEM relative to other immunoassays. Additional practical details are available from several published sources (Doane, 1974, 1986, 1987, 1988a, 1988b; Doane and Anderson, 1977, 1987; Katz and Kohn, 1984; Kjeldsberg, 1986).

HISTORY

The prospect of using electron microscopy (EM) to examine antigen-antibody complexes was first reported in 1941 by Anderson and Stanley in the U.S.A. and by von Ardenne et al. in Germany. Both groups observed that visible aggregates were formed when tobacco mosaic virus (TMV) was incubated with homologous antibody. Virologists were slow to adopt this technique which has been termed "immune electron microscopy" (Almeida and Waterson, 1969) or "immunoelectron microscopy" (Kelen et al. 1971). With the introduction of the negative staining technique, however, (Brenner and Horne, 1959) the potential usefulness of IEM in the study of virus-antibody interaction gradually became apparent (Almeida et al. 1963; Bayer and Mannweiler, 1963; Kleczkowski, 1961; Lafferty and Oertelis, 1961, 1963). It also became apparent that IEM could be used to identify different viral antigens (Hummeler et al. 1961; Watson and Wildy, 1963), to identify elusive or fastidious viruses (Best et al. 1967; Kapikian et al. 1972a, 1972b, 1973; Paver et al. 1973), and to increase by several hundred-fold the sensitivity of detection of viruses by EM (Doane, 1974; Anderson and Doane, 1973). Recent improvements of IEM methodology have included the introduction of colloidal gold as a specific marker; this has re-

Medical Virology 9
Edited by L.M. de la Maza and E.M. Peterson
Plenum Press, New York, 1990

sulted in enhanced EM visualization of immune complexes and permits the detection of soluble viral antigens (Doane, 1987, 1988b; Kjeldsberg, 1986), and enables quantitation of low levels of viral antibody (Hopley and Doane, 1985; Vreeswijk et al. 1988).

MATERIALS

Viral Antigens

IEM can be used on crude clinical specimens such as fecal samples, on virus-infected cell culture lysates, or on purified virus preparations. When the interpretation of the test depends on the presence or absence of viral antibody aggregates, it is preferable to work with at least a partially purified specimen.

Antibodies

Either monoclonal or polyclonal antibodies can be used, although the majority of reports to date have employed polyclonal antibodies. Unfractionated serum is suitable in most instances, but where fine resolution (immunological or ultrastructural) is required, sera should be clarified by centrifugation for 1 hr at 100,000 x g to remove protein aggregates, or should be purified to obtain the globulin fraction. Antisera should be inactivated at 56^{o}C for 30 min to avoid complications arising from the presence of complement (Almeida and Waterson, 1969).

The concentration of antibody used, in relation to the amount of antigen, affects not only the sensitivity of the assay but also the appearance of the resultant immune complexes (Figure 1). Whereas higher concentrations may increase the sensitivity, and enlarge the immune complexes thereby making them easier to find by EM, too high a concentration may lead to antibody coating of individual virus particles and a reduction of antibody-antigen aggregates (Lee, 1977). In addition, higher antibody concentrations may produce non-specific cross reactions and increased background deposits of serum protein that tend to interfere with visualization of immune complexes. Ideally, a box titration on all antibody preparations to be used as IEM probes should be performed against positive and negative virus controls to obtain an "IEM endpoint" - viz. the highest dilution of antibody that produces a positive reading. Between 5-10 times that concentration should be used in subsequent tests.

Negative Stains

A variety of negative stains are suitable for IEM, but phosphotungstic acid (PTA) remains the universal favorite. We use 2% PTA in Millipore filtered distilled water, adjusted to pH 6.5 with 1N KOH.

Specimen Grids

Copper grids of 300-mesh size coated with Formvar or parlodion offer a stable support for negatively stained specimens. Maximum stability of the plastic support film is achieved by coating it with a thin layer of evaporated carbon prior to use.

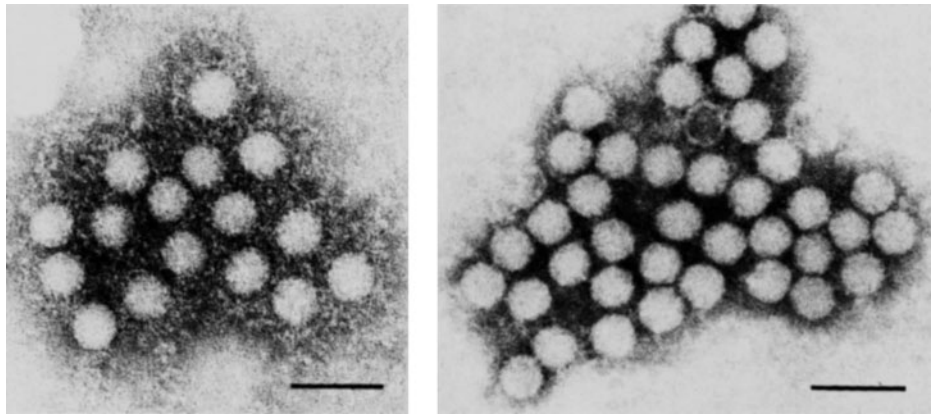

Figure 1. The effect of antibody concentration on the morphology of the immune complex. Left: high antibody concentration. Right: low antibody concentration. Bars equal 50 nm.

IMMUNOELECTRON MICROSCOPY METHODS

When the first two methods described below are used for serotyping, a virus-antibody complex is the indicator of a positive reaction, whereas the presence of predominantly single particles indicates a negative result. Unfortunately, many viruses tend to clump naturally, especially in fecal specimens (Narang and Codd, 1981); thus, one must always be cautious in interpreting the significance of aggregates in IEM assays, paying close attention to the accompanying controls (e.g. test sample exposed to antibodies of different specificities). Differentiation is facilitated by using an antibody concentration that produces a visible halo around the immune complexes. It is also possible to enhance or "decorate" the complex by the addition of anti-species antibody or protein A (Milne and Luisoni, 1977).

Although IEM is generally used to detect a specific viral antigen, it can also be adapted as an assay for viral antibody. Kapikian et al. (1976) measured the diameter of the antibody halo to determine the relative concentration of antibody in acute and convalescent phase sera. A major limitation is that the immune complexes produced at high dilutions of serum may be devoid of a visible halo. A more sensitive and readily detectable method is provided by decorating with an anti-species antibody or protein A labelled with a gold marker (Hopley and Doane, 1985).

Direct Immunoelectron Microscopy (DIEM) Method

In the original method described by Almeida and Waterson (1969), the virus preparation is mixed with an equal volume of antiserum, incubated at 37°C for 1 hr., then placed at 4°C overnight. Immune complexes are sedimented by centrifugation at 40,000 x g for 1 hr. The pellet is resuspended in distilled water, added to a grid, and negatively stained. We have found it possible to shorten the method, without an appreciable loss in sensitivity, by eliminating the overnight and centrifugation steps (Lee, 1977). After the initial incubation at 37°C, the mixture is processed by the agar diffusion method (Anderson

and Doane, 1972), in which a drop of mixture is allowed to air-dry on a coated EM specimen grid placed on agar.

Serum in Agar (SIA) Method

This is a modification of the agar diffusion method, incorporating the viral antiserum in the agar itself (Figure 2) (Anderson and Doane, 1973). As the specimen on the grid dries, homologous antibody diffuses through the agar to form immune complexes on the EM grid support film. The method has been used to increase the sensitivity of virus detection by EM (Anderson and Doane, 1973), and for serotyping enteroviruses (Anderson and Doane, 1973; Lamontagne et al. 1980; Petrovicova and Juck, 1977).

When screening a specimen for a variety of different viruses, human gamma globulin can be used in the agar (Berthiaume et al. 1981) This permits an initial family identification of a virus, or mixture of viruses, on the basis of morphology. Further identification can be performed by SIA using individual or pooled antisera.

The concentration of antiserum used for screening should be considerably higher than that used for serotyping. We routinely use immune serum globulin at a final dilution (in molten agar) of 1/50; pools of enterovirus antisera are also prepared to give a final dilution of 1/50 per serum (Doane 1986; Doane and Anderson, 1987).

A modification of this technique has been described by Furui (1986). The sample is placed directly on the agar surface, and a specimen grid coated with protein A is floated on the sample, coated side down. After 60 min, the grid is removed, washed and negatively stained.

Solid Phase Immunoelectron Microscopy (SPIEM) Method

This method is also known as immunosorbent electron microscopy (ISEM) (Katz and Kohn, 1984). Introduced by Derrick in 1973 for "trapping" plant viruses (Figure 3), it involves the coating of the specimen support film with viral antibodies. An even greater trapping effect can be achieved by the application of *Staphylococcus aureus* protein A to the film (SPIEM-SPA), prior to the antibody (Table 1) (Pegg-Feige, 1983; Pegg-Feige and Doane, 1983).

SPIEM provides a relatively simple and sensitive IEM method for detecting viruses. It has been successfully applied to rotaviruses (Katz et al. 1980; Kjeldsberg and Mortensson-Egnund, 1982; Nicolaieff et al. 1980; Obert et al. 1981; Rubenstein and Miller, 1982), adenoviruses (van Rij et al. 1982), enteroviruses (Pegg-Feige, 1983; Pegg-Feige and Doane, 1983, 1984), and hepatitis A virus (Kjeldsberg and Siebke, 1985), and Sindbis virus (Katz and Straussman, 1984).

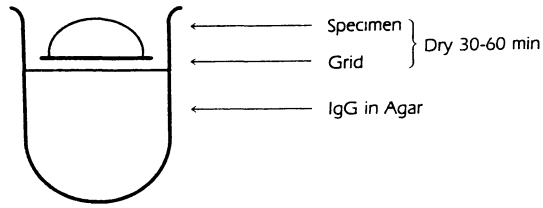

Figure 2. Serum-in-agar method.

TABLE 1. Sensitivity of Different EM Methods

	DEM	SPIEM	SPIEM-SPA	SIA	AIRFUGE
Poliovirus	6×10^5[a]	3×10^4	9×10^3	6×10^3	6×10^3

[a] Minimum detectable virus concentration ($TCID_{50}$/ml).

As discussed by Katz and Kohn (1984) in their comprehensive review of this technique, there are several variables that affect the efficiency of virus trapping; these include such factors as the nature of the specimen support film, the concentration and pH of the reactants, the duration and temperature of incubation steps. We have found that maximum trapping efficiency is obtained with parlodion-carbon films pretreated with ultraviolet light, or parlodion-carbon and Formvar-carbon films pretreated with glow discharge ionization (Table 2) (Pegg-Feige, 1983; Pegg-Feige and Doane, 1983, 1984). In our SPIEM protocol, pretreated grids are floated on drops of reactants at 25°C according to the following protocol: 25 µl of 0.1 mg/ml protein A, 10 min; 3 consecutive drops of Tris buffer, 1-2 min; 25 µl of diluted antiserum, 10 min; Tris buffer rinse, 1-2 min; 25 µl of test sample, 30 min; Tris buffer rinse, 1-2 min; 2% PTA pH 7.0. Each reference antiserum must be tested to determine the optimum working dilution necessary to achieve maximum virus trapping.

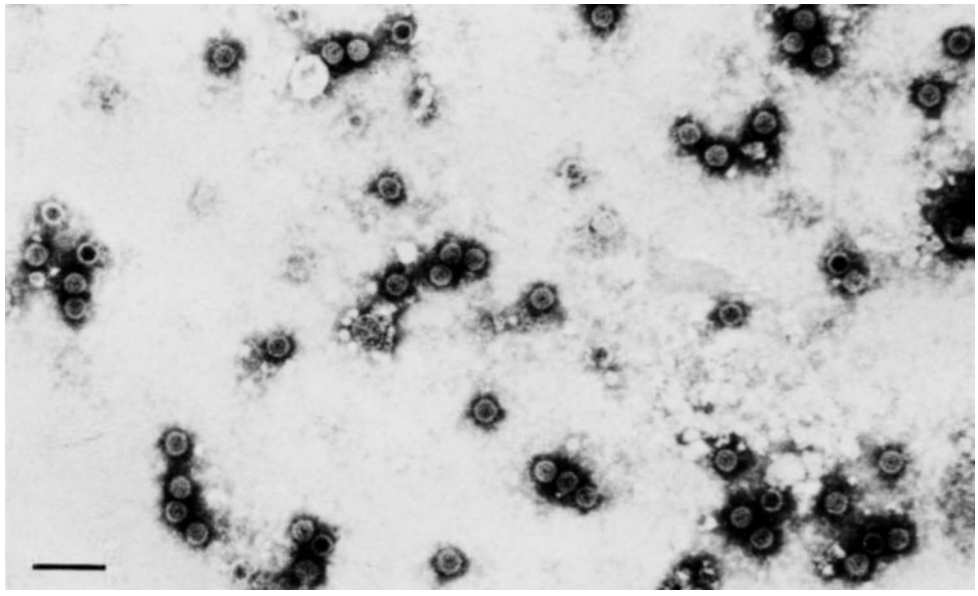

Figure 3. Rotavirus trapped on a specimen grid coated with protein A and rotavirus antiserum. Bar equals 200 nm.

TABLE 2. Effect on SPIEM Trapping Efficiency of Support Film Pretreatment

Film	Treatment		
	None	UV Light[b]	Glow Discharge[c]
Parlodion-carbon	71[a]	91	84
Formvar-carbon	9	8	74

[a] Average virus particles per grid square

[b] UV pretreatment: 1700 mw/cm^2 for 30 min.

[c] Glow discharge: 5-10 sec pink ionization of argon gas in a sputter coater.

SPIEM has also been used to serotype rotaviruses (Gerna et al. 1984, 1985, 1988) and enteric adenoviruses (Wood and Bailey, 1987) directly from fecal specimens. Individual grids were coated with a single type-specific rotavirus antiserum, and by a comparison of the number of virus particles trapped to each grid, rapid serotyping of human rotaviruses directly from stool specimens could be achieved.

Immunogold Method

The IEM methods described above rely on the presence of antibody-bound virus particles in the form of either virus-antibody aggregates or virus particles trapped to an antibody-coated grid. By using an electron-dense marker to tag the antibody probe, soluble viral antigen as well as virus particles can be detected by EM (Figure 4).

Colloidal gold has been shown to be an excellent marker for EM (Faulk and Taylor, 1971), and its application in diagnostic virology has been reported by several authors. (For review, see Kjeldsberg, 1986). The particles are relatively easy and inexpensive to prepare, and can be produced in a range of sizes. They can be conjugated via electrostatic attraction to a variety of probes including antibodies, protein A, protein G, and lectins (Horisberger, 1981). Because of their extreme electron-density, they greatly facilitate EM identification of antigen-antibody complexes. In IEM studies on partially purified rotavirus (Table 3) and rotavirus in fecal specimens (Table 4) we found the IEM method employing labelled protein A (PAG IEM) exhibited the greatest sensitivity of all EM methods tested (Hopley 1985; Hopley and Doane, 1985).

Despite the high sensitivity of immunogold labeling, it requires exceptional attention to controls. Under sub-optimal test conditions the non-specific background labeling can be high, and the various parameters involved in the assay should be thoroughly evaluated. Each antibody preparation to be used as a probe must be titrated before being assigned as a reference, to determine the dilution at which maximum specific labeling and minimum background labeling are obtained; the concentration of gold conjugate will also influence this ratio.

Several variations of the immunogold technique have been described. These include mixing the reactants directly in solution (Hopley and Doane, 1985; Stannard et al. 1982); adding each reactant by floating a specimen grid

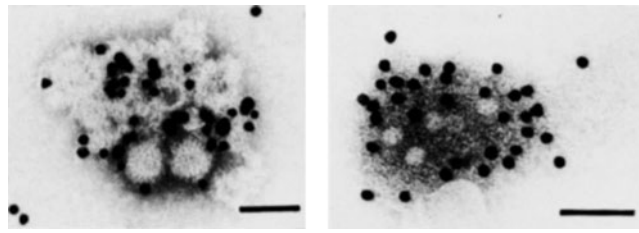

Figure 4. A colloidal gold marker greatly increases the visibility of virus-antibody aggregates that might otherwise be difficult to identify. Left: rotavirus. Right: enterovirus. Bars equal 100 nm.

TABLE 3. Sensitivity of Different EM Methods

	DEM	DIEM	PAG IEM (VP)[b]	PAG IEM (SA)[c]
Rotavirus	4×10^5[a]	2×10^4	1×10^4	2×10^3

[a] Minimum detectable virus concentration ($TCID_{50}$/ml)
[b] PAG IEM (VP): Endpoint read on basis of gold-labled virus particles.
[c] PAG IEM (SA): Endpoint read on basis of gold label.

TABLE 4. Comparison of Sensitivity of DIEM and PAG IEM for Detection of Rotavirus in Stool Suspensions

	Highest reciprocal titre	
Stool Specimen	DIEM[a]	PAG IEM[b]
HSC1	4,000	16,000
HSC2	2,000	16,000
HSC4	200	8,000
HSC5	1,000	8,000
HSC6	1,000	8,000
6749	200	4,000
5843	100	4,000
5706	2,000	4,000

[a] Based on presence of virus-antibody aggregates.
[b] Based on presence of gold label.

sequentially on the individual components (similar to the SPIEM) (Lin, 1984; Pares and Whitecross, 1982); combining with the SIA method (Doane, 1988b). A gold particle size of 5 nm constitutes a finer probe than larger particles, reducing steric hinderance. This may be appropriate in studies on the interaction of monoclonal antibodies with specific epitopes. For routine diagnostic IEM, however, a particle size of 15-20 nm has two advantages: (1) it is much easier to detect, therefore scanning a grid can be performed at a magnification of as low as 3,000x, whereas approximately 14,000x magnification is needed to detect 5 nm particles; (2) the signal-to-noise-ratio is much higher with 15-20 nm particles than with 5 nm particles (Hopley, 1985).

COMPARISON WITH OTHER IMMUNOASSAYS

IEM offers diagnostic virologists a multifunctional tool; because of its high sensitivity, it can be used to detect small amounts of viral antigen or antibody; as was first demonstrated by Best et al. (1967) with rubella virus and Bayer et al. (1968) with HBsAg, IEM can be used to reveal the identity of elusive viruses; in conjunction with antibody probes, it can be used to serotype viruses directly from clinical specimens or cell culture lysates, and to determine the location of specific epitopes on virus particles.

Various studies have shown that IEM is at least as sensitive as ELISA and RIA (Kjeldsberg and Mortensson-Egnund, 1982; Kjeldsberg and Siebke 1985; Morinet et al. 1984; Obert et al. 1981; Svensson et al. 1983). With weakly positive specimens it provides an advantage over ELISA in that the direct visualization of virus particles obviates the necessity for a confirmatory test (Obert et al. 1981).

The data in Table 1 indicate that a similar sensitivity can be achieved with the Airfuge ultracentrifuge, without the need for antibodies. With its impressive ability to concentrate viruses in small volumes, some diagnostic laboratories use this instrument routinely on many of their clinical specimens (Hammond et al. 1981).

It appears that, in general, there is little difference in the sensitivity of virus detection that can be obtained with DIEM, SIA and SPIEM; depending on the virus and test conditions, the improvement over DEM can be as high as 1,000-fold (El-Ghorr et al. 1988). The decision as to which of the three IEM methods to choose is determined to a large extent by the nature of the specimen and the information being sought. We prefer to use DIEM in combination with the agar diffusion method when only small volumes of specimen or reference antibody are available. The SIA method, incorporating gamma globulin in the agar within the microtitre cups, is well suited to the broad screening of specimens for multiple viruses. Once a virus family has been identified on the basis of morphology, subsequent serotyping can be performed by the same system, using pooled or individual antisera. A major advantage of the SIA method is the ability to store prepared microtitre plates for long periods of time at 4°C. Because of the flexible nature of the plastic plate, pairs of cups can easily be cut away from the stored plate and set into a rubber holder in preparation for the test.

Both the DIEM and SIA methods produce virus-antibody aggregates in a positive reaction. At low virus concentrations the aggregates may be in isolated areas on the EM grid and many of the grid squares may be devoid of any virus, requiring a longer examination time (Morinet et al. 1984). This phenomenon is avoided by using SPIEM, where trapped virus tends to be more

uniformly distributed over the supporting film. Furthermore, virus morphology is usually more evident, as virus particles are not surrounded by a halo of antibody molecules.

The solid phase system has other advantages over DIEM and SIA methods. The antibody-coated grids can be stored for several weeks at 4°C without serious loss of trapping efficiency (drops below 50% after 4 weeks) (Pegg-Feige 1973). They can be dispatched to centers with no EM facilities, and returned later for examination. They are especially useful when dealing with crude samples such as fecal suspensions. Subsequent washing will remove background debris without removal of virus.

Even greater sensitivity can be achieved by the addition of a colloidal gold label. Provided optimum test parameters have been established and ample controls are included in every test, the immunogold method is a valuable diagnostic tool. Its full potential will undoubtedly come with the increasing availability of type specific monoclonal antibodies. Factors that remain to be established include the advantage of direct vs indirect labeling, and the relative sensitivities of antimurine antibody, protein A and protein G in the indirect immunogold assays employing monoclonal antibodies.

In assessing the value of techniques in diagnostic virology it is always necessary to consider not only the financial cost of supplies and equipment needed, but also the cost in terms of time required to perform these techniques. On this basis, ELISA is superior to IEM for screening large numbers of specimens. Using microtitre plates, automatic pipetters and readers, many dozens of specimens can be processed coincidentally by ELISA. Although multiple specimens can be prepared in parallel by IEM, each grid must be examined individually in the EM. We estimate that a single operator could process approximately 15-20 samples by IEM during an average working day (Doane, 1988b). But IEM can be applied more readily to selected specimens, is faster to process than ELISA, produces results more rapidly and provides comparably greater sensitivity. And, with the introduction of monoclonal antibodies to the diagnostic armamentarium, its full potential remains to be realized.

REFERENCES

Almeida J, Cinader B, Howatson A (1963) The structure of antigen-antibody complexes. A study by electron microscopy. J Exp Med 118:327-340.

Almeida JD, Waterson AP (1969) The morphology of virus-antibody interaction. Adv Virus Res 15:307-338.

Anderson N, Doane FW (1972) Agar diffusion method for negative staining of microbial suspensions in salt solutions. Appl Microbiol 24:495-496.

Anderson N, Doane FW (1973) Specific identification of enteroviruses by immuno-electron microscopy using a serum-in-agar method. Can J Microbiol 19:585-589.

Anderson TF, Stanley WM (1941) A study by means of the electron microscope of the reaction between tobacco mosaic virus and its antiserum. J Biol Chem 139:339-344.

Bayer ME, Mannweiler E (1963) Antigen-antibody reactions in influenza virus as seen in the electron microscope. Arch Gesamte Virusforsch 13:541-547.

Bayer ME, Blumberg BS, Werner B (1968) Particles associated with Australia antigen in the sera of patients with leukemia, Down's syndrome and hepatitis. Nature 218:1057-1059.

Berthiaume L, Alain R, McLaughlin B, Payment P (1981) Rapid detection of human viruses in faeces by a simple and routine immune electron microscopy technique. J Gen Virol 55:223-227.

Best JM, Bantvala JE, Almeida JD, Waterson AP (1967) Morphological characteristics of rubella virus. Lancet 2:237-239.

Brenner S ,Horne RW (1959) A negative staining method for high resolution microscopy of viruses. Biochem Biophys Acta 34:103-110.

Derrick KS (1973) Quantitative assay for plant viruses using serological specific electron microscopy. Virology 56:652-653.

Doane FW (1974) Identification of viruses by immunoelectron microscopy. In: Kurstak E, Morisset R (eds) Viral Immunodiagnosis. Academic Press, New York. pp 237-255.

Doane FW (1986) Electron microscopy and immunoelectron microscopy. In: Specter S, Lancz GJ (eds) Clinical Virology Manual. Elsevier, New York. pp 71-88.

Doane FW (1987) Immunoelectron microscopy in diagnostic virology. Ultrastruct Pathol 11:681-685.

Doane FW (1988a) Immunoelectron microscopy and its role in diagnostic virology. Clin Immunol News 9:159-162.

Doane FW (1988b) Electron Microscopy. In: Lennette EH, Halonen P, Murphy FA (eds) Laboratory Diagnosis of Infectious Diseases. Principles and Practice. Springer-Verlag, New York. pp 121-131.

Doane FW, Anderson N (1977) Electron and immunoelectron microscopic procedures for diagnosis of viral infections. In Kurstak E, Kurstak C (eds) Comparative Diagnosis of Viral Diseases, Vol II, part B. Academic Press, New York. pp 505-539.

Doane FW, Anderson N (1987) Electron Microscopy in Diagnostic Virology: A Practical Guide and Atlas. Cambridge University Press, New York.

El-Ghorr AA, Snodgrass DR, Scott FMM (1988) Evaluation of an immunogold electron microscopy technique for detecting bovine coronavirus. J Virol Methods 19:215-224.

Faulk WP, Taylor GM (1971) An immunocolloidal method for the electron microscope. Immunochemistry 8:1081-1083.

Furui S (1986) Use of protein A in the serum-in-agar diffusion method in immune electron microscopy for detection of virus particles in cell culture. Microbiol Immunol 30:1023-1035

Gerna G, Passarani N, Battaglia M, Percivalle E (1984) Rapid serotyping of human rotavirus strains by solid-phase immune electron microscopy. J Clin Microbiol 19:273-278.

Gerna G, Passarani N, Sarasini A, Battaglia M (1985) Characterization of serotypes of human rotavirus strains by solid-phase immune electron microscopy. J Infect Dis 152:1143-1151.

Gerna G, Sarasini A, Coulson BS, Parea M, Torsellini M, Arbustini E, Battaglia M (1988) Comparative sensitivities of solid-phase immune electron microscopy and enzyme-linked immunosorbent assay for serotyping of human rotavirus strains with neutralizing monoclonal antibodies. J Clin Microbiol 26:1383-1387.

Hammond GW, Hazelton PR, Chuang I, Klisko, B. (1981) Improved detection of viruses by electron microscopy after direct ultracentrifuge preparation of specimens. J Clin Microbiol 14:220-221.

Hopley, JFA (1985) Protein A-gold immunoelectron microscopy studies on the simian rotavirus SA11. M.Sc thesis, University of Toronto.

Hopley JFA, Doane FW (1985) Development of a sensitive protein A-gold immunoelectron microscopy method for detecting viral antigens in fluid specimens. J Virol Methods 12:135-147.

Horisberger M (1981) Colloidal gold. A cytochemical marker for light and fluorescent microscopy and for transmission and scanning electronmicroscopy. In: Johari O (ed) Scanning Electron Microscopy. AMF O'Hara, S.E.M., Inc., Chicago. vol II, pp 9-31.

Hummeler K, Anderson TF, Brown RA (1961) Identification of poliovirus particles of different antigenicity by specific agglutination as seen in the electron microscope. Virology 16:84-90.

Kapikian A, Almeida J, Stott E (1972a) Immune electron microscopy of rhinoviruses. J Virol 10:142-146.

Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM (1972b) Visualization by immune electron microscopy of a 27 nm particle associated with acute infectious non-bacterial gastroenteritis. J Virol 10:1075-1081.

Kapikian A, James H, Kelly S, Vaughn A (1973) Detection of coronavirus 692 by immune electron microscopy. Infect Immun 7:111-116.

Kapikian AZ, Dienstag JL, Purcell RH (1976) Immune electron microscopy as a method for the detection, identification, and characterization of agents not cultivable in an *in vitro* system. In: Rose NR, Friedman H (eds) Manual of Clinical Immunology. Amer Soc Microbiol, Washington D.C. pp 467-480.

Katz D, Straussman Y, Shahar A, Kohn (1980) Solid-phase immune electron microscopy (SPIEM) for rapid viral diagnosis. J Immunol Methods 38:171-174.

Katz D, Kohn A (1984) Immunosorbent electron microscopy for detection of viruses. Adv Virus Res 29:169-194

Katz D, Straussman Y (1984) Evaluation of immunoadsorbent electron microscopic techniques for detection of Sindbis virus. J Virol Methods 8:243-254.

Kelen AE, Hathaway AE, McLeod DA (1971) Rapid detection of Australia/SH antigen and antibody by a simple and sensitive technique of immunoelectron microscopy. Can J Microbiol 17:993-1000.

Kjeldsberg E (1985) Specific labelling of human rotaviruses and adenoviruses with gold-IgG complexes. J Virol Methods 12:47-57.

Kjeldsberg E (1986) Immunonegative stain techniques for electron microscopic detection of viruses in human faeces. Ultrastr Pathol 10:553-570.

Kjeldsberg E, Mortensson-Egnund K (1982) Comparison of solid-phase immune electron microscopy, direct electron microscopy and enzyme-linked immunosorbent assay for detection of rotaviruses in faecal samples. J Virol Methods 4:45-53.

Kjeldsberg E, Siebke JC (1985) Use of immunosorbent electron microscopy for detection of rota- and hepatitis A virus in sucrose solutions. J Virol Methods 12:161-167.

Kleczkowski A (1961) Serological behaviour of tobacco mosaic virus and its protein fragments. Immunol. 4:130-141.

Lafferty KJ, Oertelis SJ (1961) Attachment of antibody to influenza virus. Nature 192:764-765.

Lafferty KJ, Oertelis S (1963) The interaction between virus and antibody. III. Examination of virus-antibody complexes with the electron microscope. Virology 21:91-99.

Lamontagne L, Marsolais G, Marois P, Assaf R (1980) Diagnosis of rotavirus, adenovirus, and herpesvirus infections by immune electron microscopy using a serum-in-agar diffusion method. Can J Microbiol 26:261-264.

Lee FK (1977) A study of enterovirus-antibody interaction in immunoelectron microscopy. M.Sc thesis, University of Toronto.
Lin N (1984) Gold IgG complexes improve the detection and identification of viruses in leaf dip preparations. J Virol Methods 8:181-190.
Milne RG, Luisoni E (1977) Rapid immune electron microscopy of virus preparations. In: Maramorosch K, Kaprowski H (eds) Methods in Virology. Academic Press, New York. vol 6, pp 265-281.
Morinet F, Ferchal F, Colimon R, Pérol Y (1984) Comparison of six methods for detecting human rotavirus in stools. Eur J Clin Microbiol 3:136-140.
Narang HK, Codd AA (1981) Frequency of pre-clumped virus in routine fecal specimens from patients with acute nonbacterial gastroenteritis. J Clin Microbiol 13:982-988.
Nicolaieff A, Obert G, Van Regenmortel MHV (1980) Detection of rotavirus by serological trapping on antibody-coated electron microscope grids. J Clin Microbiol 12:101-104.
Obert G, Gloekler R, Burckard J, Van Regenmortel MHV (1981) Comparison of immunosorbent electron microscopy, enzyme immunoassay and counter-immunoelectrophoresis for detection of human rotavirus in stools. J Virol Methods 3:99-107.
Pares RD, Whitecross MI (1982) Gold-labelled antibody decoration (GLAD) in the diagnosis of plant viruses by immune-electron microscopy. J Immunol Methods 51:23-28.
Paver W, Ashley C, Caul E, Clarke S (1973) A small virus in human faeces. Lancet 1:237-240.
Pegg-Feige (1983) Solid phase immunoelectron microscopy (SPIEM) in virus identification. M.Sc thesis. University of Toronto.
Pegg-Feige K, Doane FW (1983) Effect of specimen support film in solid phase immunoelectron microscopy. J Virol Methods 7:315-319.
Pegg-Feige K, Doane FW (1984) Solid-phase immunoelectron microscopy for rapid diagnosis of enteroviruses. Proc of the 42nd Annual Meeting of the Electron Microscopy Society of America. pp 226-227.
Petrovicova A, Juck AS (1977) Serotyping of coxsackieviruses by immune electron microscopy. Acta Virol 21:165-167.
Rubenstein AS, Miller MF (1982) Comparison of an enzyme immunoassay with electron microscopic procedures for detecting rotavirus. J Clin Microbiol 15:938-944.
Stannard LM, Lennon M, Hodgkiss M, Smuts H (1982) An electron microscopic demonstration of immune complexes of hepatitis B e-antigen using colloidal gold as a marker. J Med Virol 9:165-175.
Svensson L, Grandien M, Pettersson C-A (1983) Comparison of solid-phase immune electron microscopy by use of protein A with direct electron microscopy and enzyme-linked immunosorbent assay for detection of rotavirus in stool. J Clin Microbiol 18:1244-1249.
van Rij G, Klepper L, Peperkamp E, Schaap GJP (1982) Immune electron microscopy and a cultural test in the diagnosis of adenovirus ocular infection. Brit J Ophthamol 66:317-319.
von Ardenne M., Friedrich-Freska H, Schramm G (1941) Elektronmikroskopische untersuchung der pracipitinreaktion von tabakmosaikvirus mit kaninchenantiserum. Arch Gesamte Virusforsch 2:80-86.
Vreeswijk J, Folkers E, Wagenaar F, Kapsenberg JG (1988) The use of colloidal gold immunoelectron microscopy to diagnose varicella-zoster virus (VZV) infections by rapid discrimination between VZV, HSV-1 and HSV-2. J Virol Methods 22:255-271.

Watson DH, Wildy P (1963) Some serological properties of herpes virus particles studied with the electron microscope. Virology 21:100-111.
Wood DJ, Bailey AS (1987) Detection of adenovirus types 40 and 41 in stool specimens by immune electron microscopy. J Med Virol 21:191-199.

DISCUSSION

Riepenhoff-Talty M (Children's Hospital, Buffalo, NY):

How do you account for the two-log differences in detection between echo and coxsackie?

Doane F (University of Toronto, Toronto, Ontario, Canada):

Do you mean with respect to direct electron microscopy? I think it depends on the day; if you had taken another strain of echo and another strain of coxsackie, you may not have seen that difference. We weren't trying to compare one virus with the other, we were trying to compare those individual viruses under different conditions. It depends entirely on how many virus particles were being produced from the host cells by one virus as compared to the other.

Stewart J (Centers for Disease Control, Atlanta, GA):

When you were looking at trying to identify different viruses by the gold method, obviously if you had scattered particles with just one dot, you would not be really sure if that was specific. How many dots do you need; two, three? How do you evaluate that?

Doane F:

You have to compare the number of gold particles in the test grids versus those in the controls. On the basis of our experience with rotaviruses, we decided that it had to be more than a 14% difference in the total number of particles. If it's a very clear positive, you don't usually have to count the gold dots in your control. But if there is any question about it, then you really should count.

Stewart J:

That is in the positive specimen where you actually have a complex of particles and have several gold dots around it. As long as you don't see similar structures in your control, then you're in very good shape, I guess.

Doane F:

It's important that controls be run against negative or heterologous sera. You're treading on thin ice when you're identifying on the basis of labeled soluble antigen rather than labeled virus particles.

Merz P (Institute for Basic Research, New York, NY):

Do you have any idea what your particle to infectivity ratio is?

Doane F:

That varies with every single enterovirus preparation (especially with the enteroviruses). As you probably know, there is a great deal of variation. With enteroviruses, it can be 1,000:1 for one particular type and 100:1 for another. It depends on the kind of cells they have been grown in. For example, if a cell culture isolate is from a particularly susceptible cell line, you may have a very low particle to infectivity ratio.

Merz P:

I was actually more curious about clinical specimens.

Doane F:

We have not tried to analyze that. Very often these studies have been carried out on rotavirus, and because they are so difficult to culture, it's not that easy to determine the ratio. So, I can't answer that.

Stewart J:

In terms of the antibody in the agar, how important is that concentration? Do you have to do block titrations with this?

Doane F:

Most of our work with the serum-in-agar method has been with enterovirus and we always do block titrations to begin with. Once you've determined the optimum serum dilution for a particular reference serum, the titration doesn't have to be repeated.

Al-Nakib W (Kuwait University, Kuwait)

Have you tried the gold method with regard to respiratory viruses detection in nasal specimens, for example?

Doane F:

We have not tried it, but there should be no reason why it can't be used. However, over the years we have found that enveloped viruses are very very difficult to work with when it comes to immunoelectron microscopy. I would think that the gold technique for increasing your sensitivity of detection would work very well. But insofar as producing an immune complex and depending on that as your positive, you may run into difficulties because a lot of these enveloped viruses will aggregate even in the absence of antibody.

Al-Nakib W:

I feel this is an area that is very important. Say you take influenza, we do have antiviral chemotherapy available anyway, and this is a procedure that lends itself useful in terms of rapid diagnosis. You don't need to do it on many

specimens. Basically, you're going to have a few patients, say in hospitals, that you need to have a rapid diagnosis so you can make a decision as to whether to treat or not, and I think that this is an area that's very important.

Doane F:

I was thinking of RSV and parainfluenza, but certainly IEM has been used on influenza and it's been found to work very well. But I am not aware of the use of colloidal gold labeling with influenza assays. Something to try.

Bishop R (Royal Children's Hospital, Melbourne, Australia):

In regard to the serum-in-agar technique, you have emphasized how important it is to determine the concentration before adding it to the agar. You also said that when you don't know what you're looking for, you sometimes add gamma globulin to that agar. How do you determine in such a mixture what concentration to use?

Doane F:

We don't. Because when I mentioned that it's important to titrate, I really meant with respect to typing. With typing, of course, you want to be certain that your immune complex is a specific reaction. When you're using the serum-in-agar method for simply trapping a virus, it doesn't matter whether it's too concentrated or not. You just want to be able to find an aggregate and then look inside the aggregate and see what virus it is you've captured. So, pre-titration is essential if you're going to be doing typing tests, but if you're simply using a virus detection system, we just use immune serum globulin undiluted, or at a final concentration, in the agar, of 1/50.

Bishop R:

Do you mind telling me what glow discharge is?

Doane F:

It's a procedure that is performed in a sputter coating unit. Ionization of argon is carried out over the support film and that changes the charge on the formvar or the parlodian, making it more hydrophilic. The best thing is just to use parlodian, of course, and then you don't have to worry about pre-treating the film.

Oshiro L (California Department of Health Services, Berkeley, CA):

I have a couple of technical questions. First of all, how long are your grids treated with UV light?

Doane F:

They are pre-treated with 1,7000 mW/cm^2 of UV for 30 minutes, just prior to use.

Oshiro L:

The other question is, someone in England suggested that the commercial grade or the type of carbon might also change your sensitivity. Have you had any experience with that?

Doane F:

No, we haven't.

Oshiro L:

What type of carbon do you use?

Doane F:

We have always used Union Carbide spectroscopic electrodes.