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Micron and Microscopica Acta, Vol. 22, No. 4, pp. 449–460, 1991.
Printed in Great Britain.

0739–6260/91 $3.00 + 0.00
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NEGATIVE STAINING IN THE DETECTION OF VIRUSES IN CLINICAL SPECIMENS

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(*Received* 28 *September* 1991)

Abstract—Viruses have unique morphology and are therefore good candidates for negative staining. Negative staining with phosphotungstic acid (PTA) or uranyl acetate has facilitated the detection of many viruses in clinical specimens. Enhancement procedures have included the use of centrifugation and agar diffusion for concentrating virus particles, the use of solid phase capture reagents to trap virus particles and the use of secondary antibodies and electron dense markers to help visualize them. Techniques currently in use and employing negative staining include direct EM, immune electron microscopy (IEM), solid phase immune electron microscopy (SPIEM), colloidal gold-labeled protein A (PAG), solid phase IEM employing a second decorator antibody (SPIEMDAT), and solid phase IEM using colloided gold-labeled secondary antibodies (SPEIMDAGT). IEM methods assist with the detection of small viruses or viruses present in low numbers while PAG offers increased sensitivity over direct EM and IEM. In our experience the serum-in-agar (SIA) method is the most sensitive of the PAG IEM techniques for detection of rotavirus particles in clinical specimens. SPIEMDAT enhances the detection of small viruses which are often missed by other techniques due to background staining in specimens. SPEIMDAGT employing colloidal gold-labeled secondary antibody has increased sensitivity and offers the advantage of detecting viral antigen when whole virus particles are not visible. IEM techniques have recently been used for typing viruses using either monospecific antisera or monoclonal antibodies and colloidal gold-labeled secondary antibody.

INTRODUCTION

Prior to the development of immunoassay techniques for detecting viral antigens, direct electron microscopy (DEM) with negative staining was the only method of providing a definitive diagnosis of several viral infections. Diseases for which electron microscopy (EM) has played a role in rapid diagnosis include gastroenteritis, herpes simplex infections, varicella zoster, variola, vaccinia, congenital cytomegalovirus infection, viral hepatitis, pustular contagious dermatitis, molluscum contagiosum, and warts (Chernesky *et al.*, 1979, 1982; Kapikian *et al.*, 1980; McIntosh *et al.*, 1980; Palmer and Martin, 1988). The major limitation of DEM is that when virus particles are present in low concentrations($< 10^6$ particles per ml) in clinical specimens, virus is difficult to detect. Several methods have been developed to concentrate viruses in clinical specimens, the simplest being ultracentrifugation. The techniques of pseudo-replication and agar diffusion allow diffusion of the liquid phase of the specimen into an agar substrate with subsequent concentration of virus particles on the surface. IEM employs specific antibodies to aggregate virus particles and facilitate their detection but requires a prior knowledge of virus serotype and the availability of specific antisera.

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MATERIALS AND METHODS

Negative stains

Negative staining is the most appropriate technique for the preparation of clinical specimens. Thin sectioning techniques are not useful for making a rapid diagnosis; however, a rapid embedding method that takes only 2 h has been described (Doane and Anderson, 1977) and thin sectioning can be useful where speed of diagnosis is not important. The basic requirements for negative staining have been described in detail by Almeida (1980). The most commonly used stain is phosphotungstic acid (PTA). Uranyl acetate has been used for some SPIEM techniques. Formvar- or carbon-coated 400-mesh copper grids provide the most versatile characteristics for specimen viewing.

Clinical specimens

Electron microscopy techniques are most applicable for the investigation of infections when virus may be present in specimens in concentrations of at least 10^6 particles per ml. Specimens such as feces, vesicle fluid, brain tissue, wart tissue, urine, or serum can be negatively stained with minimum preparation to yield positive results. Specimens should be collected and transported at ambient temperature in sealed containers to prevent drying. Depending upon the type of clinical specimen, centrifugation may help to prepare it better. Feces can be prepared by making a 10–20% suspension in distilled water and then clarifying with a bench centrifuge if necessary. Rotaviruses, adenoviruses, picornaviruses, astroviruses, caliciviruses, coronaviruses, and Norwalk agents have been viewed in feces from patients with gastroenteritis.

Patients presenting with vesicular lesions (ex- or enanthematous) may yield herpes simplex, varicella, or vaccinia viruses in vesicle fluid. Vesicle fluid should be collected from unbroken lesions using a tuberculin syringe containing a small amount of distilled water. The EM morphology of the herpes viruses allows identification to group, and other laboratory techniques may be used for typing. EM can be used to detect herpes virus particles in brain biopsy material from a patient with encephalitis, but may not be as sensitive as immunofluorescent staining. Human papillomavirus (common warts), Orf virus, and molluscum contagiosum pox virus can be viewed in homogenates of solid biopsy tissue.

Fluid specimens can be examined directly or centrifuged for 1 h at 15,000 ***g*** to concentrate virus with resuspension of the pellet in a small amount of distilled water before staining. Urine should be centrifuged at 2500 ***g*** for 30 min. The supernatant fluid will usually yield virus more readily if as large a volume as possible is used. Serum contains many low-molecular-weight proteins that must be washed out by diluting with an equal volume of distilled water. Centrifugation for 1 h at 1500 ***g*** is necessary, and the serum may even require recentrifugation to provide a cleaner preparation. CSF seldom yields a virus. Sputum specimens should be diluted in saline and then homogenized or treated with 20% *n*-acetyl cysteine.

Biopsy or autopsy tissue is cut into small (1 mm) cubes and placed on a metal planchet, which enables several cycles of freezing and thawing to help release viral particles. An alternate method involves mechanical homogenization using a mortar and pestle or homogenizer. Following this, a relatively smooth suspension can be achieved by mixing with small amounts of distilled water using a fine-bore Pasteur pipette. Best results are then achieved by differential centrifugation before staining with PTA. Conjunctival scrapings usually contain small amounts of tissue and should be examined directly without centrifugation.

Electron microscopy

Direct electron microscopy. The easiest laboratory technique for examination of clinical specimens is direct EM using negative staining. The most commonly used stain is phosphotungstic acid (PTA) as a 2–4% solution adjusted to a pH of 7; uranyl acetate at a concentration of 1% is an alternative. Formvar- and carbon-coated, 400-mesh copper grids provide the most versatile carriers for specimen viewing. Twenty microliters of virus suspension are placed onto Formvar/carbon-coated 300-mesh copper grids for 3 min, the drop is removed by blotting from the edge of the grid with filter paper and the grids are negatively stained for 30 s in a drop of PTA.

Immune electron microscopy.

(1) *Direct method.* Equal volumes (20 μl) of antigen and antiserum (optimally diluted, usually 1/800) are mixed in a microtiter well. Formvar/carbon-coated grids are placed into wells and incubated for 60 min at 37°C in a humidified environment. After incubation, the grids are washed and negatively stained.

(2) *Agarose method.* For this method, all reagents are mixed and incubated in microtiter plastic wells, as described by Hopley and Doane (1985). A 20 μl volume of viral suspension is mixed with 20 μl of specific antiserum (dilution 1/800) and incubated at 37°C for 45 min in a humidified environment. After incubation, a 20 μl volume of PAG is added into the well, and incubated for another 45 min at 37°C. PAG complexes (PAG) of 16 nm diameter are prepared according to Hopley and Doane (1985) and diluted in buffer consisting of 0.5 M Tris (hydroxymethyl) aminomethane hydrochloride, pH 7.0, 15% NaCl, 0.5 mg/ml polyethylene glycol 20,000, 0.1% NaN_3, standardized by absorption spectrophotometry at 580 nm to 0.2/ml and stored at 4°C until use. The total volume of the antigen–antisera–PAG mixture (60 μl) is then transferred to another well containing a Formvar/carbon-coated 300-mesh copper EM grid placed on top of 2% agarose, and the fluid phase of the mixture allowed to diffuse into the agarose for 30 min at room temperature. After washing with three drops of PBS and three drops of distilled water, the grid is negatively stained.

(3) *Well method.* This method is similar to the agrose method except that the agarose is omitted (Wu *et al.*, 1989). Grids are floated on top of the mixture of virus, antibody and PAG in wells for 45 min at 37°C. The grids are thoroughly washed and negatively stained as outlined above.

(4) *Serum in agar (SIA) method.* The SIA PAG procedure is performed according to Hopley and Doane (1985). Antiserum (0.1 ml undiluted) is incorporated into 5 ml of 2% Noble agarose (Difco) in a 55°C waterbath and 0.25 ml of the molten agarose is dispensed into wells of a polyvinyl carbonate microtiter plate and allowed to solidify. A mixed suspension containing 20 μl of virus preparation and 20 μl of PAG is added to a grid on the surface of the agar and allowed to dry down for about 20 min at room temperature. The grid is then washed and negatively stained with 1% PTA (Wu *et al.*, 1989).

(5) *SPIEMDAT.* Giraldo *et al.* (1982) have used this method to detect papovaviruses in urine. Formvar-coated grids (300 mesh) are floated for 15 min on 10 μl of protein A solution and drained. Grids are then floated on a drop of specific antisera (in this case anti-BK papovavirus antisera) for 15 min at room temperature to immobilize capture antibody. Grids are next sequentially floated for 15 min in a drop of urine followed by decorator antibody before being stained with 1% uranyl acetate for 2 min. The decorator antibody facilitates easier viewing of viruses while coating grids with protein A allows a lower amount of capture antibody to be used.

(6) *SPEIMDAGT.* This technique is a modification of the SPIEMDAT procedure and employs gold-labeled secondary antibody (Wu *et al.*, 1990). Formvar-coated grids (300 mesh) are floated for 15 min on 10 μl of protein A solution and drained. They are then floated sequentially on 10 μl of guinea pig antirotavirus antiserum at room temperature for 20 min, on 20 μl of virus suspension at 37°C for 45 min, on 10 μl of rabbit antirotavirus antiserum at room temperature for 20 min, and on 10 μl of

gold-labeled goat anti-rabbit IgG antibody at room temperature for 20 min, with five washes with PBS after each step. Grids are washed twice with distilled water before being negatively stained with 1% PTA. The specificity of SPIEMDAGT is assessed by using preimmune guinea pig serum and rabbit antiserum as capture antibody, guinea pig antiserum as detector antibody, and adenovirus-positive, enterovirus-positive or -negative stool specimens. Background gold labeling is determined from a control grid containing all reagents except virus then subtracted from particle counts.

Examination of grids. In our laboratory, clinical specimens are examined in a Philips 301 transmission electron microscope for 10 min at a direct magnification of 35,000 ×. If quantitative deteminations are required five grids are usually prepared of each sample. The numbers of virus particles visible within five squares of each of three grids (total 15 squares of each sample) are counted and the average number of virus particles per grid square (3600 μm^2) is calculated.

RESULTS

Direct electron microscopy

Many viruses are present in clinical specimens in sufficient amounts to be detected by direct EM. Diagnostic virology laboratories routinely detect herpes virus, poxvirus, and molluscum contagiosum virus in skin lesions and rotavirus, adenovirus, astrovirus, calicivirus, picornavirus, and Norwalk-like viruses in stool specimens (Fig. 1A–D).

Centrifugation

The simplest way to enhance visualization of viruses present in low concentrations in clinical specimens is to use ultracentrifugation to concentrate the virus before negative staining. Rice and Phillips (1980), using conventional ultracentrifugation, detected 6.5% more rotavirus positive specimens compared to direct EM without centrifugation. Hammond *et al.* (1981) used the Beckman Airfuge air turbine ultracentrifuge (with a specially adapted rotor holding an EM grid) and detected 14% more rotavirus positive specimens. They also detected increased numbers of specimens containing herpesviruses, adenoviruses and enteroviruses. Jansons *et al.* (1985) using the Airfuge reported a three-fold increase in the detection rate of skin lesion specimens containing herpesviruses and poxviruses. They estimated the concentration factor of centrifuged specimens to be in excess of 1000-fold using the Airfuge.

Adsorption and elution to protein

Other methods of concentration have included the adsorption of viruses to meat protein followed by elution at elevated pH and salt concentration. Pontefract and Bergeron (1985) used this method to detect small picornalike viruses in stool specimens.

Immune elution microscopy

A number of IEM methods have been developed to detect viruses present in low concentrations. IEM is based on the formation of aggregates that occurs when viral particles are mixed with specific antibody. These complexes are then visualized by negative staining. Stanley and Anderson (1941) were the first to use IEM for the detection and identification of tobacco mosaic viruses. IEM has provided increased sensitivity over direct EM permitting better morphological and immunological identification of viruses. IEM has been employed for the detection of several viruses in cell culture as well as clinical specimens including rotavirus, enterovirus, adenovirus,

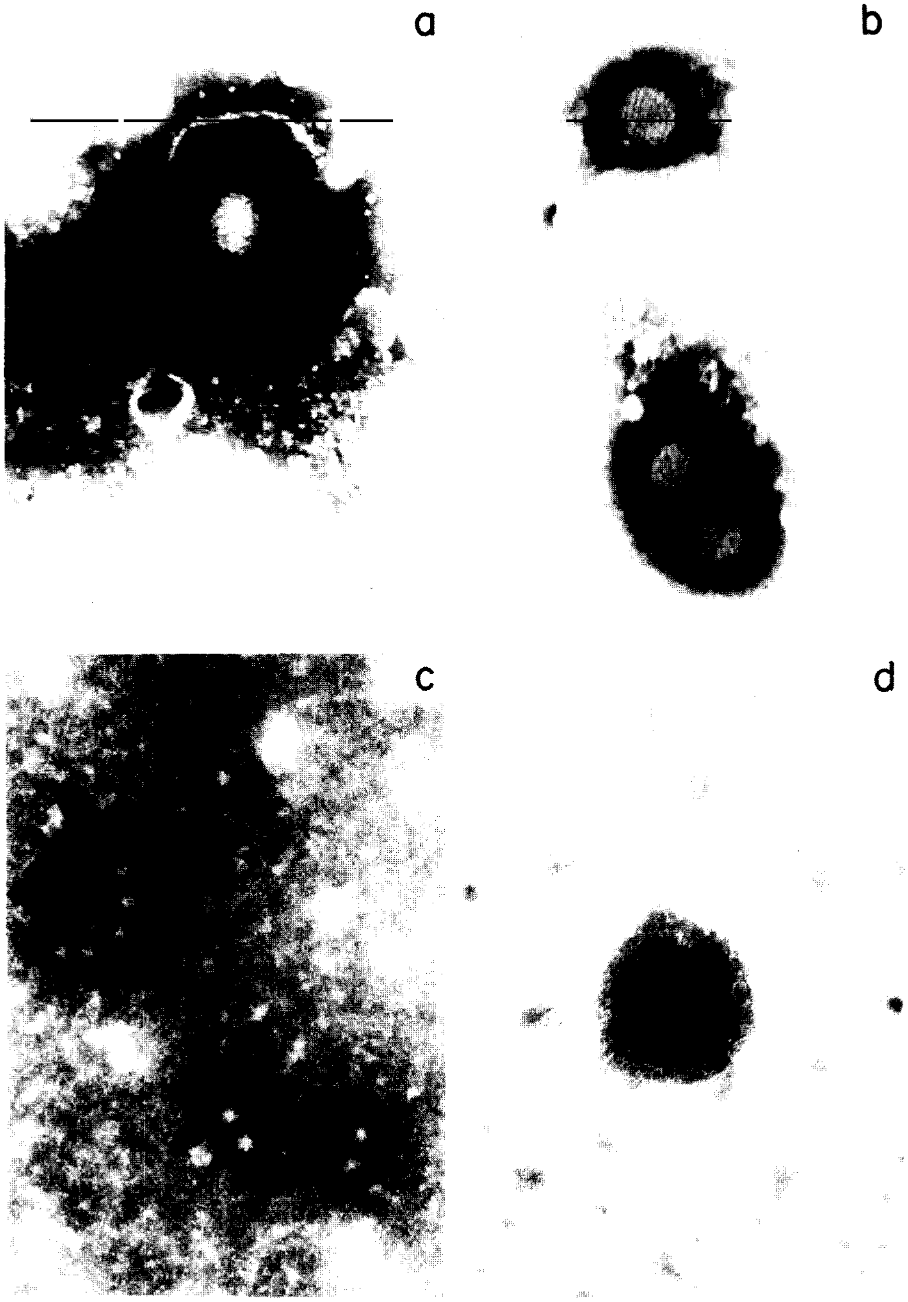

Fig. 1. Negatively stained viruses detected in clinical specimens by direct EM. (A) Herpes simplex virus from vesicular skin lesion. Magnification × 148,750. (B) Adenovirus from fecal specimen. Magnification × 123,250. (C) Astrovirus from fecal specimen. Magnification × 131,750. (D) Rotavirus from fecal specimen. Magnification × 106,250.

herpesvirus, rhinovirus, coronavirus, Norwalk and Norwalk-like viruses, hepatitis B, non A non B hepatitis and papovavirus (Almeida *et al.*, 1971; Chaudhary *et al.*, 1971; Kelen *et al.*, 1971; Kapikian *et al.*, 1972a, 1973; Anderson and Doane, 1973; Penney and Norayan, 1973; Flewett, *et al.*, 1974; Thornhill *et al.*, 1975, 1977; Valters *et al.*, 1975; Fauvel *et al.*, 1977; Muller and Baigent, 1980; Trepanier *et al.*, 1981; Brandt *et al.*, 1981; Dolin *et al.*, 1982; Stannard *et al.*, 1982; Petrie *et al.*, 1984; Louro and Lesemann, 1984; Lin, 1984; Sreenivasan *et al.*, 1984; Beasley and Betts, 1985; John *et al.*, 1974; Kjeldsberg, 1985; Wood and Bailey, 1987; Vreeswijk *et al.*, 1988; Wu *et al.*, 1989, 1990). The availability of type specific antisera and monoclonal antibodies has facilitated the typing and detection of adenoviruses including non-cultivable types 40 and 41 (Luton, 1973; Wood *et al.*, 1989). The SIA IEM procedure has been used to detect rotavirus, adenovirus, and herpes virus (Anderson and Doane, 1973; Lamontagne *et al.*, 1980). The SIA IEM procedure can be modified by using PAG to enhance visualization (Hopley and Doane, 1985). We have used this method to detect rotavirus in fecal specimens (Wu *et al.*, 1989) (Fig. 2)

Solid phase IEM

Although IEM is considerably more sensitive than DEM, it is dependent upon the optimal concentrations of antibody and antigen and is susceptible to a prozone phenomenon. Derrick (1973) described an improved IEM method employing a solid phase (SPIEM) in which grids were coated with antibody and used to capture virus from the specimen. This method minimized or eliminated the prozone phenomenon and was later modified by precoating grids with staphylococcal protein A to anchor a larger concentration of antiviral antibodies for the detection of virus (Shuala *et al.*, 1979). A variation on this technique, introduced by Katz *et al.* (1980), is the use of whole *Staphylococcus aureus* organisms mixed with capture antibody as the solid phase. This technique similar to the other SPIEM methods avoids a prozone effect. Milne and Luisoni (1975, 1977), attempting to further improve virus detection, added a second layer of decorator antibody to captured virus. This solid phase double antibody technique (SPIEMDAT) produced a halo or decoration around virus particles. SPIEM has been used to detect rotavirus and Norwalk virus in stool specimens (Lewis *et al.*, 1988; Nicolaieff *et al.*, 1980, 1982; Kjeldsberg and Mortensson-Egnund, 1982;

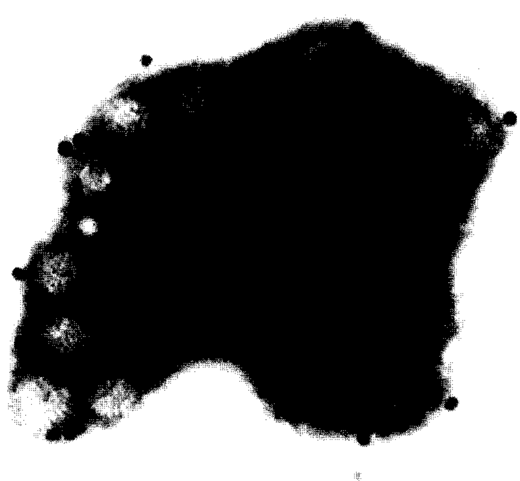

Fig. 2. SIA PAG IEM detection of rotavirus from fecal specimen, negatively stained with 1% PTA. Magnification × 130,000.

Gerna *et al.*, 1984; Doane, 1987). We have used SPIEMDAT to detect human papovaviruses in urine (Giraldo *et al.*, 1982). These highly specific immunosorbent grids have been shown to increase the sensitivity of electron microscopy for certain viruses 100- to 1000-fold (Hopley and Doane, 1985).

Electron dense markers

The most recent development in diagnostic virology has been the application of electron-dense markers for the detection, typing and quantitation of specific viruses. Ferritin-labeled anti-species antibodies were first used by Patterson (1975) to detect influenza virus. Immunoferritin labeling and negative staining have been used to demonstrate antibody attachement to hepatitis B core antigen (Huang and Neurath, 1979) and for the identification of rotavirus, adenovirus and Coxsackie virus B5 (Berthiaume *et al.*, 1981b). Gold-labeled IgG complexes have also been used as electron dense markers to enhance the visibility of antibody-coated viruses (Lin, 1984). Colloidal gold labeling has many advantages over ferritin labeling and has rapidly become the marker of choice (Faulk and Taylor, 1971). The first use of colloidal gold in clinical virology was the demonstration by Stannard *et al.* (1982) of the presence of hepatitis B virus e antigen immune complexes in the serum of patients with hepatitis B infection. The term gold-labeled antibody decorator or GLAD was coined by Pares and Whitecross in 1982 for the gold labeling of virus particles. Figure 3 shows Norwalk-like virus in a fecal specimen detected by colloidal gold-labeled secondary antibody. More recently, colloidal gold-labeled protein A (PAG) has been used to enhance the visibility of viruses. PAG IEM has been employed for the detection of rotavirus and enterovirus (Hopley and Doane, 1985; Wu *et al.*, 1989, 1990). Figure 4 compares rotavirus detection by direct EM, IEM and three PAG IEM techniques. Hopley and Doane (1985) reported that the sensitivity of the PAG IEM method was 40-fold greater than IEM and up to 1000-fold greater than DEM. Wu *et al.* (1989) reported that PAG IEM was 50 times more sensitive than DEM for the detection of rotavirus. These authors showed that the SIA method PAG IEM was the most sensitive of three PAG IEM techniques for the detection of rotavirus in fecal specimens. The use of collodial gold-labeled antibody together with solid-phase capture antibody has the additional advantage of being able to detect viral antigen in the absence of

Fig. 3. Detection of Norwalk-like virus in fecal specimen using colloidal gold-labeled secondary antibody. The bar represents 100 nm. (Photo courtesy of Dr F. Bishai, University of Toronto.)

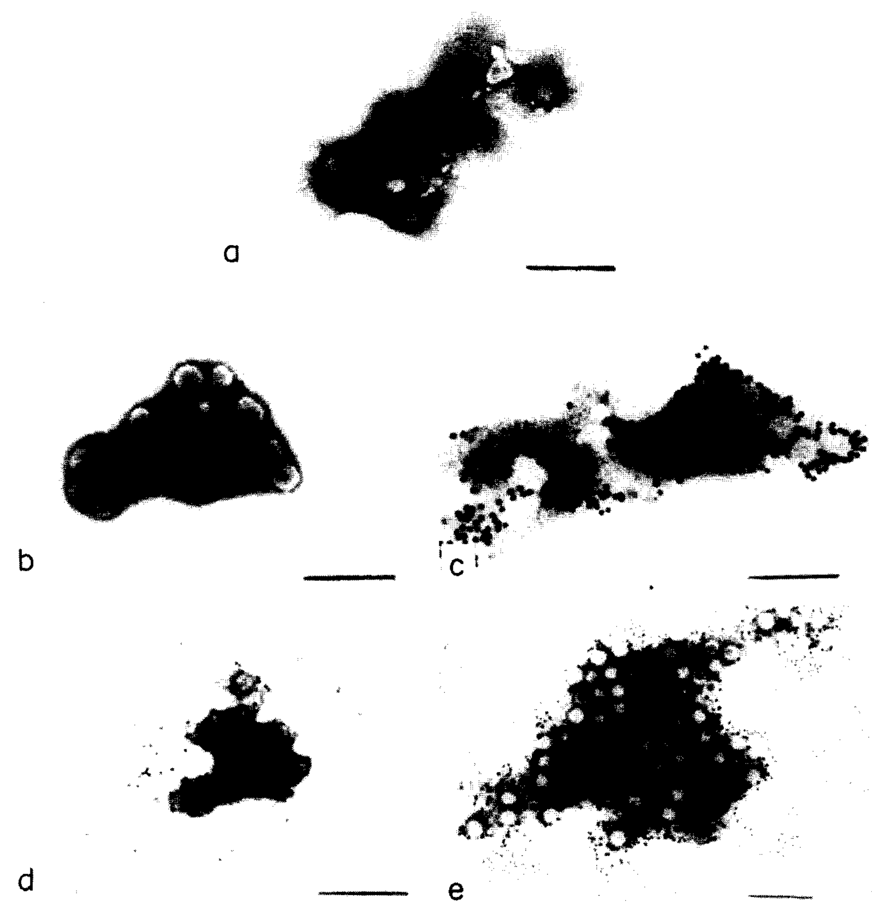

Fig. 4. Negatively stained rotavirus particles detected by: (a) direct EM, (b) direct IEM, (c) agarose PAG IEM, (d) well method PAG IEM and (e) SIA PAG IEM. Magnification × 59,500 (bar represents 200 nm).

morphologically recognizable virus particles. Wu *et al.* (1990) modified the SPIEM-DAT technique and employed a second antibody labeled with gold (SPIEMDAGT) (Fig. 5). These authors showed that SPIEMDAGT was 800 times more sensitive than DEM and detected 20% more rotavirus positive stools than commercially available enzyme immunoassays. Colloidal gold IEM has also been used to type viruses. Vreeswijk *et al.* (1988) have used specific gold-labeled antibodies to distinguish varicella zoster virus from herpes simplex virus in skin lesion specimens and to identify HSV type I and II viruses. We have used a modified SPIEMDAGT procedure employing type-specific monoclonal antibodies for the direct typing of rotavirus in stool specimens (Fig. 6).

CONCLUSIONS

Direct EM employing negative staining for the detection of virus particles in clinical specimens has contributed significantly to the diagnosis of viral infections. PTA and uranyl acetate have played important roles in newly developed IEM techniques.

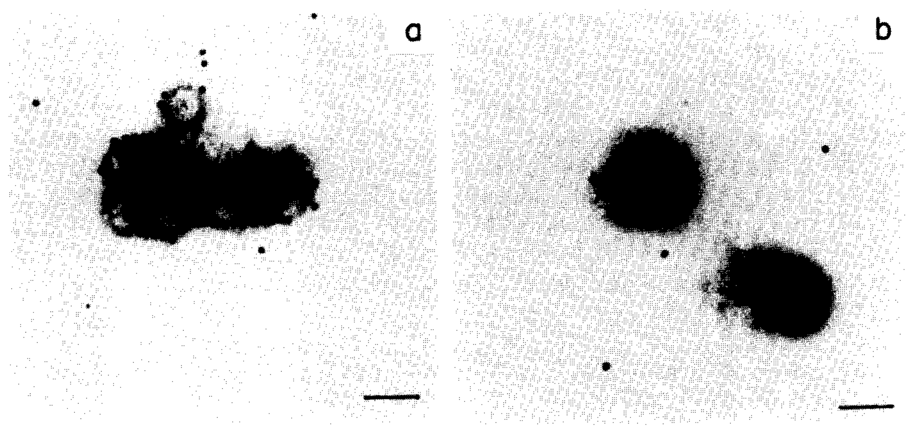

Fig. 5. SPIEMDAGT detection of rotavirus particles using solid phase capture antibody and gold-labeled secondary antibody. (A) Clumped rotavirus particles showing specific gold labeling. (B) Single virus particles showing detector antibody halo and gold-labeled secondary antibody, negatively stained with PTO. Bar represents 100 nm.

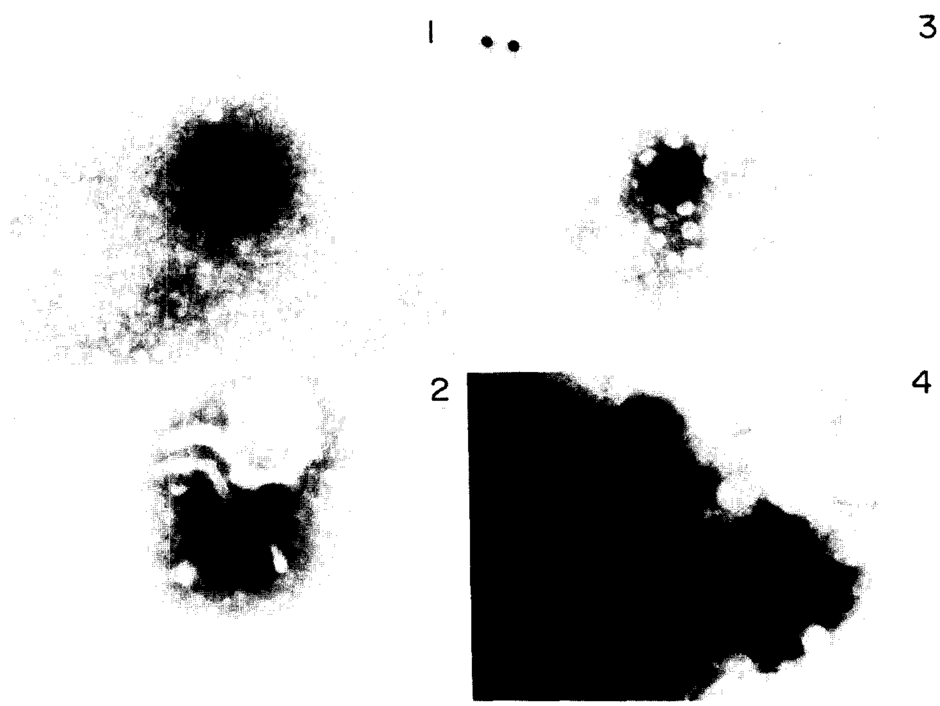

Fig. 6. Serotyping group A rotavirus by SPIEMDAGT using type-specific monoclonal antibodies. A rotavirus positive stool was tested by SPIEMDAGT with monoclonal antibodies to types 1, 2, 3 and 4 followed by gold-labeled anti-mouse IgG antibody. The figures shows gold staining only with monoclonal antibody to type 4 rotavirus (lower right panel). Magnification × 106,250.

Concentration techniques have increased our ability to detect viruses causing human disease. Application of indirect IEM techniques using a secondary antibody (unlabeled or labeled with colloidal gold) has had a major impact on facilitating the detection of small viruses or viruses present in low numbers. The development of solid phase techniques employing immobilized capture antibody together with gold-labeled decorator antibody (SPIEMDAGT) has only recently been employed in the diagnostic virology laboratory but will surely play a larger role in years to come. The role of direct EM however should not be overlooked since it remains the easiest, most rapid and versatile EM procedure for detecting viruses and for this reason should be used in conjunction with other procedures for diagnosing viral infections.

Acknowledgements—We would like to thank the technologists in our diagnostic laboratory for their dedicated efforts in employing these techniques for the detection of viruses in clinical specimens. We gratefully acknowledge the assistance of Ann Simpson Gomes, Rick McKenzie and Tom Daly with the colloidal-gold techniques, electron microscopy and photography and the typing of this manuscript by our secretaries, Lisa Rizzo and Ann Costie.

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