COMPARISON OF A MODIFIED ENZYME-LINKED IMMUNOSORBENT ASSAY WITH IMMUNOSORBENT ELECTRON MICROSCOPY TO DETECT CORONAVIRUS IN HUMAN FAECAL SPECIMENS

Author(s): PRANEE LEECHANACHAI, CHALOBON YOOSOOK, SURANGA SAGUANWONGSE and PORNCHAI MATANGKASOMBUT

Source: *Journal of Diarrhoeal Diseases Research*, Vol. 5, No. 1 (March 1987), pp. 24-29
Published by: icddr,b
Stable URL: http://www.jstor.org/stable/23497982

J Diarrhoeal Dis Res 1987 Mar;5(1):24-29
0253-8768/87 $ 1.00+0.10

COMPARISON OF A MODIFIED ENZYME-LINKED IMMUNOSORBENT ASSAY WITH IMMUNOSORBENT ELECTRON MICROSCOPY TO DETECT CORONAVIRUS IN HUMAN FAECAL SPECIMENS

PRANEE LEECHANACHAI[1], CHALOBON YOOSOOK[1], SURANGA SAGUANWONGSE[2] AND PORNCHAI MATANGKASOMBUT[1]

[1]*Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400;*
[2]*Virus Research Institute, Department of Medical Sciences, Ministry of Public Health, Yod-se, Bangkok 10100, Thailand*

Received : 12 Jan 1987

Abstract

Two commonly used methods to detect human enteric coronavirus in faecal specimens were compared. All samples were screened by a modified enzyme-linked immunosorbent assay (ELISA). Positive samples were confirmed by an ELISA with a blocking test. Randomly selected negative and positive samples by the ELISA with a blocking test were then subjected to immunosorbent electron microscopy using a protein A coated grid technique (PA-CGT). Out of 39 negative specimens using an ELISA with a blocking test, 38 (97.4%) were still negative by PA-CGT whereas coronaviruses were seen in only 26 out of 32 (81.2%) samples positive by the ELISA with a blocking test. This indicated that the ELISA with a blocking test is superior to PA-CGT for detecting human enteric coronaviruses.

Key words: ELISA; Coronaviruses; Electron microscopy; Gastroenteritis.

Introduction

The role of coronaviruses in causing respiratory and intestinal diseases in animals and respiratory illness in man is well documented (1). These organisms have been detected by electron microscopy in stool specimens collected from patients with gastroenteritis during outbreaks in India (2), England (3), Australia (4, 5), Germany (6), and South America (7, 8), as well as in Thailand (9). Studies of the general population in several developing countries have shown that the virus can also be detected in large numbers in stool specimens from healthy individuals (2,5,8). Thus, attempts to associate human enteric coronavirus with gastroenteritis have yielded conflicting opinions. Many recent investigations have been directed towards resolving the causative role of enteric coronavirus in human gastroenteritis. The slow progress in this regard is largely due to the difficulty of growing the virus in tissue culture and the lack of simple serological tests. The identification of coronaviruses so far has been based mainly on the appearance of the virus in faecal extracts examined by electron microscopy – a procedure which is cumbersome for routine diagnosis or for large-scale studies. Unlike rotaviruses which are easily counted under the electron microscope because of their characteristic appearance, coronaviruses are rather fragile and those found in faeces have often collapsed (10, 11).

The aim of our study was to compare a modified ELISA and an immunosorbent electron microscopical technique for detecting coronavirus in human faeces.

Correspondence and reprint requests should be addressed to : Dr C Yoosook.

Materials and methods

Faecal specimens from 896 healthy individuals and 267 patients with diarrhoea were collected

and preserved at −70°C. Each sample was suspended (at 10% w/v) in phosphate-buffered saline (PBS), pH 7.4, and then centrifuged at 1000 x g in a refrigerated centrifuge for 10 minutes.

Virus

Human rectal tumor adenocarcinoma cells (HRT 18) were used to cultivate human enteric coronavirus (Ric 13). Both the tumor cells and viruses were provided by Dr. Jacques Laporte of the Institute National De La Researches Agronomique, Thiverval-Grignon, France. Cells were grown in RPMI 1640 medium containing 10% foetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml fungizone, and subcultured at 3-day intervals with a splitting ratio of 1:3–1:4. Virus infection was carried out in serum-free medium for 4 days. The infected culture was freeze-thawed once and clarified by a step-wise centrifugation at 1100 x g for 20 minutes and at 12,000 x g for 30 minutes. The supernatant was concentrated to one-twentieth of the original volume using an ultrafiltration membrane (Diaflo, Amicon Corp., Mass., USA). The virus was further purified as described by Garwes *et al.* (12) except that a discontinuous gradient of 20, 30, 40, and 50% sucrose was used instead of a continuous one. The gradient was centrifuged in a SW40 rotor at 74,000 x g for 5 hours (Beckman LB-70 Ultracentrifuge, Beckman Instruments Inc., Calif., USA). The virus band at the 40%/50% sucrose interface was pooled, the residual sucrose was removed and the virus was resuspended in 10 mM Tris, pH 7.4. The total protein content in the purified virus suspension was measured using the method of Lowry *et al.* (13).

Antisera

Rabbit and guinea pig anti-human enteric coronavirus (Ric 13) were prepared using essentially the method described by Schmidt *et al.* (14). Animals were injected subcutaneously at several sites with the purified virus emulsified in complete Freund's adjuvant. The total protein content of the antigenic dose for the rabbits was 100 µg in 2ml and for the guinea pigs was 50 µg in 1ml. Subsequent injections were given intramuscularly at 3-week intervals for 9 weeks. The animals were bled 10 days after the last injection.

The IgG fraction of the rabbit and guinea pig antisera was purified by routine methods (15, 16), and the protein content of each was estimated by measuring its absorbance at 280nm. Anti-coronavirus activity was assayed by an ELISA using purified coronavirus-coated polystyrene microwell plates (Nunc-Immunoplate IIF, Denmark).

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA to detect coronavirus antigens in the faecal extracts was a modified double antibody sandwich method (17) using rabbit anti-coronavirus as the capture layer and guinea pig anti-coronavirus as the detecting antibody. The optimum concentrations of antisera and peroxidase-labelled rabbit anti-guinea pig globulin (DAKOPATT, Copenhagen, Denmark) were determined using a checkerboard titration as described by Voller *et al.* (18). Washing and diluting buffers were 0.1% Tween 20 in PBS, pH 7.4 (PBS-Tween) and 0.2% ovalbumin in PBS-Tween, unless indicated otherwise.

A 100 µl volume of pre-titrated rabbit anti-coronavirus IgG in carbonate buffer, pH 9.6, was used to coat the wells of 96-well polystyrene microwell plates overnight at 4°C. Wells were washed three times for 4 minutes each with PBS. Unbound sites were saturated by 150 µl of 5% ovalbumin in PBS, incubated at 4°C for 3 hours, after which plates were washed three times with PBS-Tween. Twenty-five µl of 10% faecal extract in PBS was added to two wells in duplicate and the final volume was adjusted to 100 µl with PBS-Tween containing 0, 01 M ethylene diamine tetraacetic acid (EDTA). Plates were incubated overnight at 4°C and washed three times with PBS-Tween to remove unreacted materials. Diluted guinea pig anti-coronavirus IgG (100 µl) was added, incubated at 37°C for 120 minutes and then washed 3 times, then 100 µl of peroxidase-labelled rabbit anti-guinea pig globulin, diluted 1:1000 (DAKOPATT) was added, incubated for an hour and a half at 37°C, and the plate was again washed three times. The colour reaction was then developed using a substrate solution containing 0.5mg/ml of O-phenylenediamine (OPD) and 0.02% H_2O_2 in 0.05M citrate phosphate buffer, pH 5.0. The reaction was

stopped after incubation for 30 minutes at room temperature by adding of 75 µl of 4N H_2SO_4 to each well. The colour that had developed was measured at 490nm in an ELISA reader (Dynatech Laboratory, Inc., Va., USA). The sample was considered to contain coronavirus when the mean optical density (OD) value was at least two times that of the PBS control.

ELISA-positive samples were confirmed by a blocking test modified from Yolken *et al.* (19). In brief, 25 µl of 10% faecal extract was mixed with 50 µl of rabbit anti-coronavirus antiserum containing 50 µg IgG/ml, or with pre-immune rabbit serum containing approximately the same amount of IgG. The volume was adjusted to 100 µl with PBS-Tween containing 0.01 M EDTA and this sample was then used as the antigen in the sandwich ELISA described above. The percentage blocking was calculated as

$$\frac{\text{O.D. (pre)} - \text{O.D. (post)}}{\text{O.D. (pre)}} \times 100.$$

O.D. (pre) and O.D. (post) represent the optical density values obtained from equivalent samples after incubation with pre-immune rabbit IgG and hyperimmune rabbit IgG, respectively. Antigen was considered to be present if blocking of 50% or greater was noted (19).

Electron Microscopical Technique

Immunosorbent electron microscopy was used to examine viral particles in 71 samples randomly selected from both ELISA blocking test-positive and test-negative results. Immunosorbent electron microscopy was performed using a protein A coated grid technique (PA-CGT) as described by Nicolaieff *et al.* (20). The faecal extract was clarified by centrifugation at 12,000 x g for 20 minutes before ultracentrifugation using a Beckman LB-70 Ultracentrifuge with a SW40 rotor at 70,000 x g for 90 minutes at 4°C. The pellet was resuspended in 1 drop of distilled water. A carbon-shadowed, Formvar-coated 250 mesh copper grid was floated for 10 minutes on a drop of PBS, pH 7.4, containing 0.1 mg/ml protein A (Sigma Chemical Company, St. Louis, Mo., USA). The excess fluid was blotted off. The grid was transferred onto a drop of rabbit anti-coronavirus IgG (diluted to a concentration of 0.05 mg protein/ml in PBS) for 10 minutes, then blotted, placed on a drop of suspended pellet for 10 minutes and washed twice with drops of distilled water for 30 seconds each time. The grid was then placed onto a drop of 1.5% PTA, pH 6.5 for 1 minute, blotted dry, then examined under an electron microscope.

Results

Of 1163 stool specimens collected from normal individuals and patients with diarrhoea, 380 were presumptively positive by the ELISA procedure. However, when the ELISA blocking test was used to confirm the presence of viral antigens, inhibition of 50% or greater was observed with only 91 samples (23.9%). Therefore, 76% of the 380 initially ELISA-positive samples were considered to be negative. This false positive ELISA reaction was rather common and can be partially explained by the presence of material in faecal specimens which can bind non-specifically to the antibody molecules. These results are consistent with previous reports of detecting other enteric viruses, such as rotaviruses (26) employing similar ELISAs. Thus, the blocking test eliminated false positives in the original ELISA to detect viral antigens in stool specimens. The characteristic appearance of the virion is illustrated in Fig. 1.

Of 32 ELISA blocking test positive samples, 26 (81.2%) were seen to contain coronaviruses by PA-CGT, while in all except one of 39 ELISA blocking test negative samples no coronaviruses were seen (Fig. 2).

Discussion

Immunosorbent electron microscopy can detect viruses at concentrations as low as 2-10 ng/ml (23, 24) and has been shown to be equally sensitive as an ELISA with a blocking test to detect rotavirus (23, 25). The ELISA with blocking test described here was more sensitive than electron microscopy: the PA-CGT detected only 26 (81.2%) of 32 specimens positive by the ELISA, though it confirmed all 39 negative samples except one. The lone exception (PA-CGT positive and ELISA blocking-negative) gave a borderline ELISA blocking value of 49%, just below the cut-off point of a 50% reduction in OD value.

Detecting coronavirus particles by electron

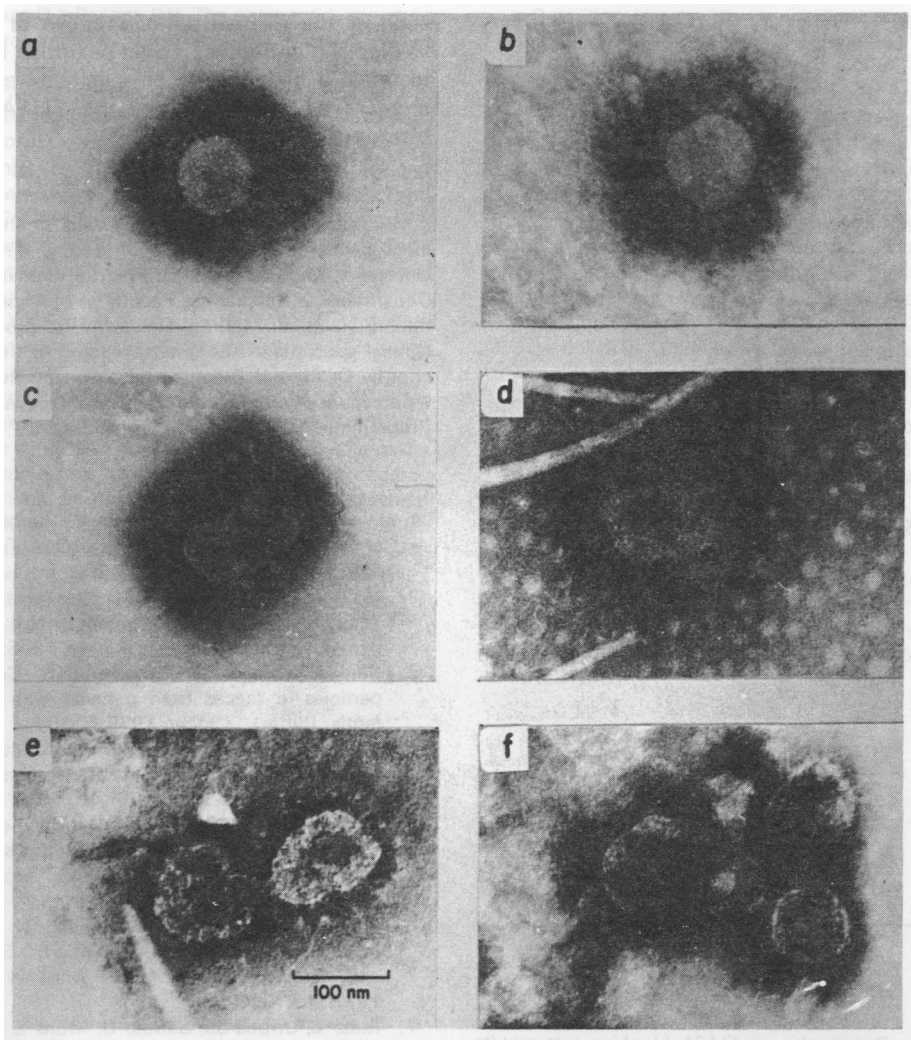

Fig. 1 – Appearance of enteric coronavirus seen by the immunosorbent electron microscopic technique. The virions are spherical (a and b) or pleomorphic (c and d) structures, approximately 100-300 nm in diameter, with characteristic club-shaped peplomers; virions with only a few or without projections on the surface (e and f) are also visualized. Bar = 100 nm.

microscopy depends entirely on the presence of the complete and whole virions in sufficient numbers. For this reason, the protein A-coated grid technique of immunoelectron microscopy appears to present some advantages over conventional techniques which do not use protein A. A preliminary comparison in our laboratories indicated that when protein A and antibodies are used, adherence of debris to the grids was essentially eliminated, thus allowing viral particles to be seen clearly. The presence of an additional layer of protein and the increased efficiency of coating antibodies using protein A apparently prevented much of the non-specific adherence of faecal debris to the grids, such as, membraneous fragments of intestinal epithelial cells, disintegrated bacteria and mycoplasma (21, 22).

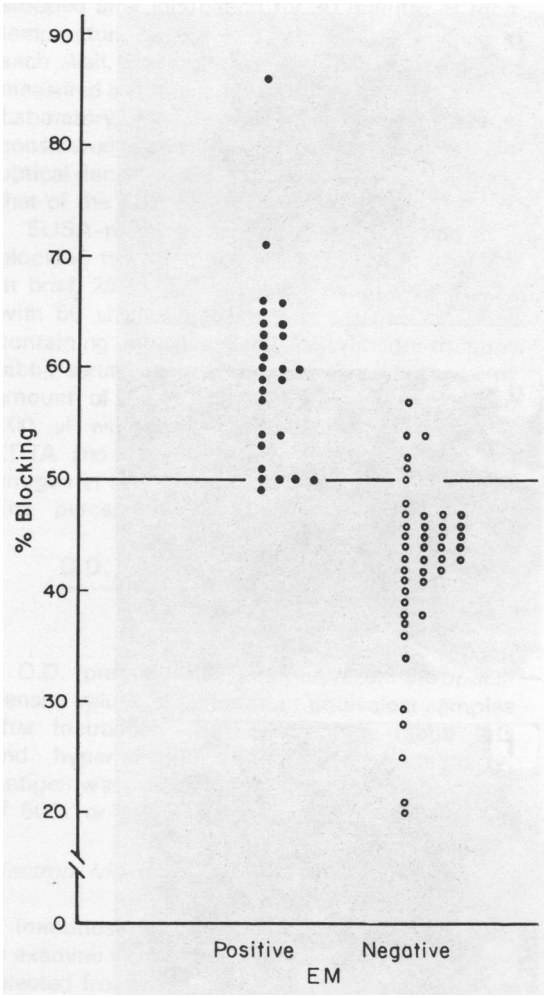

Fig. 2– Distribution of ELISA blocking test results expressed as% blocking among PA-CGT positive (●) and PA-CGT negative (o) stool specimens.

Only 91 of 380 specimens (24%) were confirmed to contain coronaviruses by an ELISA with a blocking test. This can be explained by the non-specific binding of faeces to the solid phase of the initial ELISA. Blocking by preincubation with excess anti-coronavirus antibodies discriminated against the non-specific binding, i.e., only binding by coronaviruses could be blocked. The present comparison of the ELISA blocking test and PA-CGT indicated that the blocking test is reasonably optimised. However, critical factors, such as the relative concentrations of trapping vs blocking antibodies, could be further improved to retain a high degree of specificity as well as increase the sensitivity of the ELISA with a blocking test.

Acknowledgements

We thank Dr Pramote Thongkrajai of the Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen and Dr Wandee Varavithya of the Department of Paediatrics, Faculty of Medicine, Ramathibodi Hospital, Mahidol University for providing the clinical specimens. The critical reading of this manuscript by Dr Peter B Billings is also acknowledged. This work was supported by the Diarrhoeal Diseases Control Programme, World Health Organization.

References

1. Wege H, Siddell S, ter Meulen V. The biology and pathogenesis of coronaviruses. *Curr Top Microbiol Immunol* 1982;99:165-200.
2. Mathan M, Mathan VI, Swaminathan SP, Yesudoss S, Baker SJ. Pleomorphic virus-like particles in human faeces. *Lancet* 1975;1:1068-9.
3. Caul EO, Paver WK, Clarke SKR. Coronavirus particles in faeces from patients with gastroenteritis (letter). *Lancet* 1975;1:1192.
4. Moore B, Lee P, Hewish M, Dixon B, Mukherjee T. Coronaviruses in training center for intellectually retarded. *Lancet* 1977;1:261.
5. Schnagl RD, Holmes IH, Mackay-Scollay EM. Coronavirus-like particles in aboriginals and non-aboriginals in Western Australia. *Med J Aust* 1978;1:307-9.
6. Maas G, Baumeister HH, Freitag N. Viren ais ursache der akuten gastroenteritis bei Sauglingen und Kleinkin dern. *Munch Med Wochenschr* 1977;119:1029-33.
7. Mata L, Urrutia JJ, Serrato G, Mohs E, Chin TDY. Viral infections during pregnancy and in early life. *Am J Clin Nutr* 1977;30:1834-42.
8. Sitbon M. Human-enteric-coronavirus-like particles (CVLP) with different epidemiological characteristics. *J Med Virol* 1985;16:67-76.
9. Wasi C, Louisirirotchanakul S, Thakerngpol K, Satrasook S, Surakhaka M, Varavithya W, Thongcharoen P. The epidemiological study on viral diarrhoea in Thailand. *J Med Assoc Thailand* 1984;67:369-76.
10. Apostolov, *et al. In:* Barry RD, Mahy BWJ, eds. The Biology of Large RNA Viruses. New York: Academic Press, 1970:3-26.
11. Caul EO, Egglestone SI. Coronaviruses in humans. *In:* Tyrrell DAJ, Kapikian AZ, eds. Virus Infections of the Gastrointestinal Tract. New York: Dekker, 1982:179-93.

12. Garwes DJ, Pocock DH. The polypeptide structure of transmissible gastroenteritis virus. *J Gen Virol* 1975;29:25-34.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
14. Schmidt OW, Kenny GE. Immunogenicity and antigenicity of human coronaviruses 229E and OC43. *Infect Immun* 1981;32:1000-6.
15. Williams CA, Chase MW. Method in immunology and immunochemistry Vol. 1. New York; Academic Press, 1967. 307 p.
16. Yagi Y, Maier P, Pressman D. Two different anti-insulin antibodies in guinea pig antisera. *J Immunol* 1962;89:442-51.
17. Yolken R, Wyatt RG, Kapikian AZ. ELISA for rotavirus (letter). *Lancet* 1977;2:819.
18. Voller A, Bidwell D, Bartlett A. Microplate enzyme immunoassays for the immunodiagnosis of virus infections. *In:* Rose NR, Friedman H, eds. Manual of Clinical Immunology. Washington, D.C.: American Society for Microbiology, 1976;506-12.
19. Yolken RH, Kim HW, Clem T, *et al.* Enzyme-linked immunosorbent assay (ELISA) for detection of human reovirus-like agent of infantile gastroenteritis. *Lancet* 1977;2:263-7.
20. Nicolaieff A, Obert G, van Regenmortel MHV. Detection of rotavirus by serological trapping on antibody-coated electron microscope grids. *J Clin Microbiol* 1980;12:101-4.
21. Caul EO, Ashley CR, Egglestone SI. Recognition of human enteric coronaviruses by electron microscopy. *Med Lab Sci* 1977;34:259-63.
22. Gourlay RN, Wyld SG. Isolation of mycoplasma from bovine faeces. *Lancet* 1975;2:231.
23. Obert G, Gloeçkler R, Burckard J, van Regenmortel MHV. Comparison of immunosorbent electron microscopy, enzyme immunoassay and counter-immunoelectrophoresis for detection of human rotavirus in stools. *J Virol Methods* 1981;3:99-107.
24. Derrick KS. Quantitative assay for plant viruses using serologically specific electron microscopy. *Virology* 1973;56:652-3.
25. Kjeldsberg E, Mortensson-Egnund K. Comparison of solid-phase immune electron microscopy, direct electron microscopy and enzyme-linked immunosorbent assay for detection of rotavirus in faecal samples. *J Virol Methods* 1982;4:45-53.
26. Brandt DC, Kim WH, Rodriguez WJ, *et al.* Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children. *J Clin Microbiol* 1981; 13:976-81.