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# An Enteric Coronavirus of the Rabbit: Detection by Immunoelectron Microscopy and Identification of Structural Polypeptides*

By

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With 3 Figures

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## Summary

The immunoelectron microscopy (IEM) technique has been used for the detection of a rabbit enteric coronavirus (RECV). Immune serum was prepared in guinea pigs; the viral antigen used for the immunization procedure was obtained from the caecum of a sick rabbit, concentrated by centrifugation and purified on Percoll gradient. In order to identify the viral particles used in the immunization procedure, the protein pattern of the particles was determined by electrophoresis and compared with the pattern of other known coronaviruses. Analysis of structural polypeptides of the purified viral particles revealed a pattern similar to that reported for other coronaviruses. These polypeptides cross reacted with two other coronavirus specific immune sera (IBV and TGE). IEM assay of fecal samples collected from healthy and sick rabbits showed the presence of immune aggregates in specimens from both sick and healthy rabbits. Those aggregates contained viral particules sharing morphological characteristics with other coronaviruses. Furthermore, IEM assay was shown to be more sensitive than a direct EM procedure to detect coronavirus particles in rabbit feces. This assay also allowed the detection of a larger number of chronic carriers.

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## Introduction

The electron microscopy (E.M.) technique is commonly used to detect the presence of coronavirus particles in fecal samples collected from different animal species (3, 8, 11, 14, 18) including man (13). However, in suspensions of fecal materials, numerous particles do not always exhibit the typical coronavirus morphology and consequently are often refered to as virus-like or coronavirus-like particles (5, 7). This is particularly the case when rabbit fecal material is evaluated for the presence of coronaviruses. Particles have been observed in fecal material collected from young rabbits with clinical signs of enteritis (6, 11, 12). However, very little is known about these particles. They share some morphological characteristics with other members of the family *Coronaviridae* but most often do not show typical surface projections. Those structures are either shorter than the reported length or not clearly defined and appear as a fuzzy coat. Furthermore, according to our experience, coronavirus particles are often present in low titer in rabbit feces. Proper identification is then difficult to achieve and it has become necessary to develop a more sensitive assay. For that purpose, we characterized the structural polypeptides of the viral particles in rabbit feces and we adapted an immunoelectron microscopy (IEM) technique to evaluate the presence of rabbit enteric coronavirus (RECV) in rabbit feces.

## Materials and Methods

### *Specimens*

Healthy rabbits were obtained from a commercial colony with a low incidence of enteritis. Sick animals were randomly selected among the specimens submitted to The Laboratory Animal Diagnostic Service of the Armand-Frappier Institute.

A total of 30 fecal samples were examined. Seventeen were collected from healthy animals and 13 were obtained from young rabbits showing clinical signs of enteritis. The samples were collected from the caecum diluted in phosphate buffered saline as a 20 per cent suspension, centrifuged at $3000 \times g$ for 30 minutes in a refrigerated centrifuge and the supernatant frozen at —70° C until used.

### *Virus Purification and Characterization*

Fecal material collected from the caecum of a sick rabbit was prepared as above, purified and characterized.

For purification procedures, the material was first diluted 1:5 in NTE buffer (0.15 M NaCl, 0.05 M Tris HCl, 0.001 M EDTA, pH 7.4) and examined by E. M. for the presence of viral particles. Aliquots (100 ml) were clarified by low speed centrifugation and banded isopycnically on Percoll™ self generating isoosmotic gradients (Pharmacia, Uppsala, Sweden) adjusted at 1.1 g/cc. Centrifugation was carried out in a type 30 rotor (Beckman, Palo, Alto. Co.) at 30,000 rpm for 30 minutes at 4° C. Sucrose gradient centrifugation was carried out on 20—50 per cent gradient to determine particle density, using an SW 40 rotor. The HA activity was used to monitor the presence of viral particles in the fractions. The assay was done in U-type microplates using rabbit erythrocytes as previously described (11). Peak fractions were stored at —70° C until further use. An aliquot of peak fractions showing an elevated HA activity and con-

taining RECV as observed by EM was used to determine the protein pattern of the viral particles.

*Electrophoresis and Immunodetection*

Electrophoresis under dissocating conditions was carried out using the discontinuous buffer system described by LAEMMLI (1970). Stacking and resolving gels consisted respectively of 3 per cent acrylamide (0.08 per cent bis-acrylamide) and 10 per cent acrylamide (0.32 per cent bis-acrylamide). Electrophoresis was carried out at 65 mA. Separated proteins were then transfered electrophoretically on nitrocellulose sheets (20). Blotting was performed at 50 v for 2 hours in buffer. Nitrocellulose sheets were then prepared for immunodetection by washing with buffer and incubating with guinea pig immune serum specific for RECV prepared in our laboratory. Bound specific antibodies were detected using a fluorescent goat anti-guinea pig IgG serum (Miles Laboratory, CA, U.S.A.). Bands were visualised by direct illumination by short wave UV light (Chromato-vue cabinet; UVP Inc. CA) and photographed with a Polaroid type 107 film using a 480 filter.

Anti IBV (avian infectious bronchitis), and anti TGE (porcine transmissible gastroenteritis sera were obtained from the Centre de recherche en médecine vétérinaire, Institut Armand-Frappier. Fluorescent antisera to guinea pig, chicken and porcine IgG were obtained commercially (Miles Laboratories, CA, U.S.A.).

*Production of Guinea Pig Immunoserum to RECV*

Percoll gradient peak fractions showing an elevated HA activity and containing purified RECV as observed by EM were used to immunize two guinea pigs. The animals were first bled from the venous orbital sinus to obtain pre-immunization serum and then immunized with 0.5 ml of the HA fraction mixed with an equal amount of complete Freund adjuvant. The preparation was inoculated subcutaneously and the animals were bled six weeks after to determine the presence of specific hemagglutination inhibiting (HI) antibody titers (11). The sera obtained were frozen at —70° C. Whole unheated serum was used throughout the evaluation procedure.

*Electron Microscopy Examination*

Aliquots (25 µl) of fecal suspensions and of peak fractions used for immunization procedure were deposited onto the surface of an agar microwell to concentrate the samples and a carbon-Formvar coated copper grid was placed face downward on top of the drop. After complete extraction of the aqueous phase into the agar phase, the grid was picked up, stained with 3 per cent phosphotungstic acid (PTA) pH 6.8 and examined with a Philips EM 300 electron microscope. Each specimen was examined for 10 minutes or less if viral particles were observed.

*Immunoelectron Microscopy Examination*

HA fractions from the Percoll gradient used for immunization and fecal samples collected from healthy and sick rabbits were evaluated for the presence of RECV. The specimens were first filtered through a 0.22 nm filter to eliminate natural aggregates. Twenty five µl of the filtrate obtained were then mixed with an equal quantity of specific antiserum diluted 1/40 and deposited on an agar well as described above. A carbon formvar-coated grid was overlaid on top of the drop and after almost complete hydroextraction, the grid was stained with PTA and examined by EM. Control grids were prepared using pre-immunization serum sample. Each specimen were examined for 10 minutes or less if immune aggregates were observed.

*Statistical Analysis*

The exact Fisher's test (1) was used to analyse the results obtained.

## Results

RECV banded isopycnically at a density of 1.06 g/cc on Percoll gradients (Fig. 1). The presence of complete viral particles in the peak fractions (9—12) were identified by HA assay (Fig. 1) and confirmed by EM examination (Fig. 2a). On sucrose gradients, the viral particles banded at a density of 1.18.

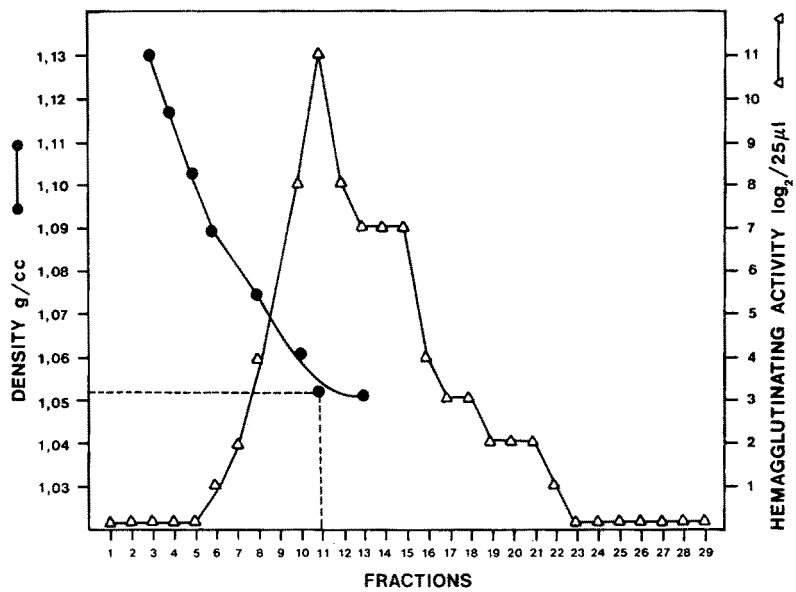


Fig. 1. Isopycnic purification of RECV in Percoll self generating gradient (p = 1.1 g/cc). Centrifugation was carried out in a type 30 rotor (25,000 rpm, 45 minutes 4° C). Fractions of 1 ml were collected and assayed for hemagglutinating activity. Density was determined using calibration beads (Pharmacia)

Coronavirus particles (Fig. 2a) appeared as spherical enveloped particles with a diameter ranging between 40 and 50 nm without peplomers. An inner tongue shape and a double shell envelope were also seen in a large number of particles. Peplomers between 10—12 nm in length were observed but were often broken. Fractions 13 to 22 contained mostly viral fragments or aggregated hemagglutinin.

HI antibody titers were found in guinea pig serum following immunization procedure. No detectable titers were observed in pre-immune serum. When an aliquot of diluted immune serum was reacted with the viral HA fraction used for immunization, immune aggregates were observed by IEM (Fig. 2b). The immune aggregates formed showed the presence of numerous coronavirus particles of variable sizes surrounded by antibodies.

Electrophoresis pattern reacted with guinea pig anti-RECV revealed 8 structural proteins ranging from 100,000 to 34,000 molecular weight: 7 were associated with the envelope (100,000, 82,000, 81,000, 76,000,

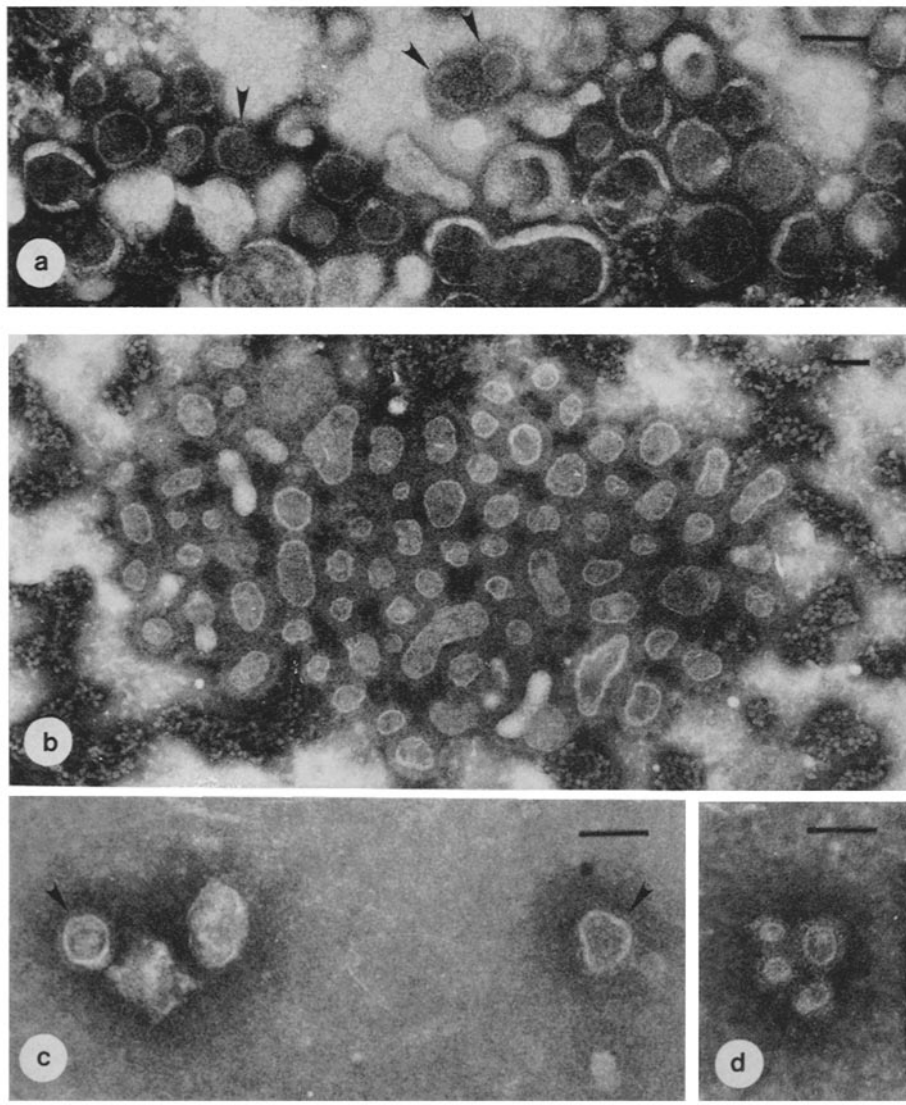


Fig. 2. A Coronavirus particles observed in fractions 9—12 of a Percoll gradient. Note the short surface projections seen in few particles (arrow). B Immune aggregates observed by IEM following reaction of an aliquot of diluted serum with the viral HA fraction used for immunization. C Coronavirus particles as observed in rabbit fecal samples. Surface projections (arrow) are generally poorly defined. D Immune aggregate observed by IEM in rabbit fecal sample. Immunoglobulins appear as a fuzzy coat surrounding the viral particles. Bar = 100 nm

71,000, 65,000 and 34,000) and one with the nucleocapsid (54,000) (Fig. 3A and Table 1). Immunoblotting of the same protein profile with a chicken anti-IBV serum, permit the identification, by crossed reaction, of only 6 of the polypeptides: 5 associated with the envelope (100,000, 82,000, 76,000, 71,000 and 34,000) and the 54,000 nucleoprotein (Fig. 3B and Table 1).

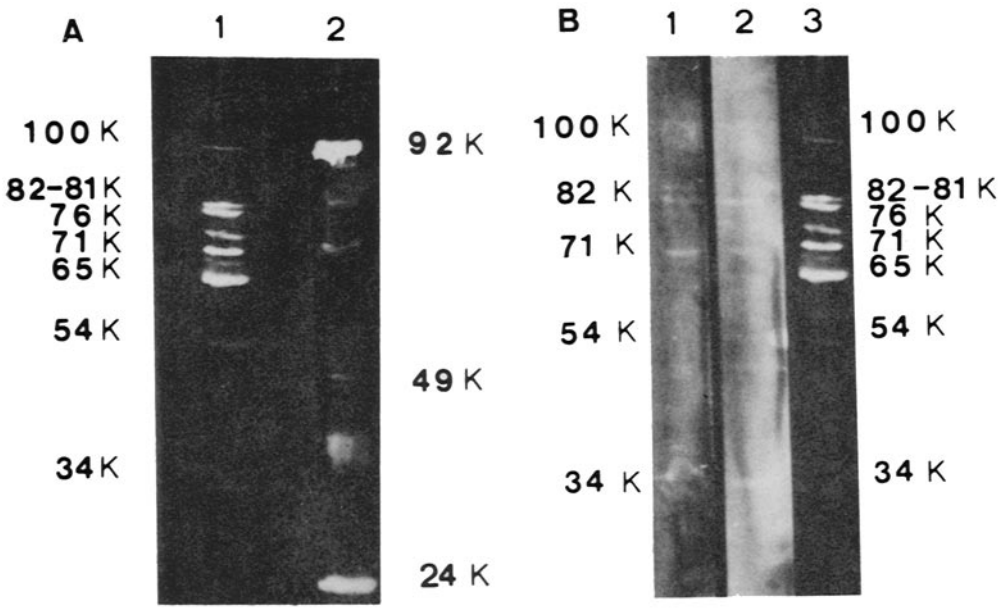


Fig. 3. SDS PAGE analysis of RECV. After electrophoresis proteins were transfered to nitrocellulose membrane and reacted with specific anti-coronavirus serum. Immunoblots were revealed with matching fluoresceine labelled goat anti IgG probes. *A* Track 1: RECV blot reacted with guinea pig immune sera revealed with goat anti guinea pig IgG labelled with fluoresceine. Track 2: Standard molecular weight proteins: phosphorylase B (92K), *Lens culinaris* lectin (49K) and trypsinogen (24K) revealed with rabbit immune sera and goat anti-rabbit IgG fluorescein probe. *B* Track 1: RECV blot revealed with a specific pig anti-TGE serum (Transmissible Gastroenteritis Coronavirus); Track 2: RECV blot revealed with a specific avian anti-IBV sera (infectious bronchitis coronavirus); Track 3: control RECV revealed with specific guinea pig anti-RECV

Immunoblotting of the RECV profile with the anti-TGE serum identified by cross reaction, 5 polypeptides: 4 associated with the envelope (100,000, 82,000, 76,000, 34,000) and the 54,000 nucleoprotein.

Table 2 summarizes the results obtained by EM and IEM techniques for detection of RECV in sick and healthy animals. By EM technique, 4 out of 17 healthy rabbits (23.5 per cent) and 3 out of 13 sick animals (23 per cent) were found positive for the presence of RECV. Using the IEM procedure, 14 out of 17 healthy rabbits (82.3 per cent) and 10 out of 13 sick animals (76.9 per cent) were found positive. In the positive specimens

Table 1. *RECV structural proteins as identified by a guinea pig anti RECV sera, and by cross reaction with anti IBV and TGE sera*[a]

| | Guinea pig anti-REC | Chicken anti-IBV | Porcine anti-TGE |
|---|---|---|---|
| | *100,000* | *100,000* | *100,000* |
| Envelope | 82,000/81,000 | 82,000 | 82,000 |
| | 76,000 | 76,000 | — |
| | 71,000 | 71,000 | 71,000 |
| | 65,000 | — | — |
| Nucleoprotein | 54,000 | 54,000 | 54,000 |
| Matrix | 34,000 | 34,000 | 34,000 |

[a] Data compiled from Figs. 3A and B

Table 2. *Detection of RECV in fecal material collected from healthy and sick rabbits using EM and IEM procedures*

| | N. positive/total N. (%) | |
|---|---|---|
| Procedures | Healthy animals[a] | Sick animals[b] |
| EM | 4/17 (23.5) | 3/13 (23) |
| IEM | 14/17 (82.3) | 10/13 (76.9) |

[a] Significantly different ($p=0.004$)
[b] Significantly different ($p=0.023$)

observed by EM, the viral particles were difficult to identify. They were in limited number not well defined and the surface projections were generally damaged (Fig. 2c). However when the IEM assay was used, immune aggregates were easily detected (Fig. 2d). These aggregates were formed by particles of various sizes surrounded by immunoglobulins. Although the surface projections were short and most often covered by antibodies, they were distinguishable in some particles. The results of the statistical analysis indicate a significant difference ($p=0.023$) between the number of positive specimens collected from sick animals. A significant difference was also obtained ($p=0.004$) between positive specimens collected from healthy animals.

## Discussion

For some time now, a coronavirus has been implicated in the etiology of rabbit enteritis (11). Morphological evidence based on EM studies of fecal samples had lead to this hypothesis. Data are now presented to support our preliminary report of a coronavirus enteric infection in rabbits.

Viral particles purified from fecal material collected from the caecum of a rabbit showing clinical signs of enteritis were purified on Percoll gradient. Their morphology and antigenicity were well preserved. Particles banded

at the same density reported for other enveloped viruses (17). In sucrose gradients, virus banded at a density of 1.18, as reported for other coronaviruses (16). Furthermore, a specific antiserum was prepared in guinea pigs and was used to identify the structural proteins of the RECV by immunodetection of virus protein blots on nitrocellulose and to develop an immunoelectron microscopy assay for the rapid diagnosis of this specific virus in rabbit feces.

The electrophoretic profile of the structural proteins confirms that these virus particles had the same structural protein profile as member of the family *Coronaviridae*. The pattern of structural proteins should be exemplified by the group prototype, IBV which has been shown by Cavanagh (1981) to contain three membrane proteins of 94,000, 84,000 and 30,000 and a nucleoprotein of 54,000 molecular weight. The pattern of enveloped protein of RECV is more complex consisting of five proteins 100,000, 82,000, 81,000, 76,000, 71,000, 65,000 while good agreement is reached for the nucleoprotein at 54,000 and the matrix protein at 34,000. Since we already known, by EM studies, that the viral peplomers are damaged, it is not unexpected that we find multiple bands in the 82,000 to 65,000 molecular weight which could be broken peplomers.

Our results with the two other coronavirus specific sera (IBV and TGE) is even more interesting. Since both these sera recognized the 34,000 matrix protein, the 54,000 nucleoprotein and the 100,000, 82,000 and 71,000 molecular weight envelope protein. Both the 100,000 and 82,000 are recognised peplomer proteins (4, 16) and a protein between 60,000 to 70,000 has been described for mouse hepatitis, bovine coronavirus and porcine haemagglutinating encephalitis virus. Our results are in good agreement with the typical coronavirus profile. The 76,000 molecular weight protein recognised by the chicken anti-IBV is somewhat of a puzzle: it could be a higher molecular weight protein that has been partially degraded, since EM observation confirms that the spike protein is broken, but it is difficult to understand why the porcine anti-TGE does not recognize it. However, the data obtained support the hypothesis that this is indeed a coronavirus, and that there are cross reactions with two other coronaviruses. It is to be noted that by blotting we are identifying antibodies that recognise mostly primary and secondary structure epitopes which are likely to be more conserved that tertiary and quaternary epitopes. Those at the surface of the viral particle could be recognized by blocking biological activity such as infectivity or by IEM. This specific virus has not yet been cultured *in vitro*. However, its presence can still be easily detected by IEM assay. The results of this study indicate that this procedure is a useful method for the detection of RECV. The observation of immune aggregates not found in controls, the diameter of the particles observed and the presence of short peplomers indicate that these particles are coronaviruses. The electro-

phoresis study performed in the viral particles used for the immunization procedure confirm their identification. When these particles are observed by EM alone, they are found in limited number and are often difficult to identify. The use of specific antiserum contributes then to facilitate the diagnosis of RECV by eliminating the ambiguities on the identification of this specific virus and by reducing the observation time requested for its identification. The coronavirus particles are readily found in aggregates surrounded by antibodies and the observer does not have to rely only on a specific morphology for identification. Furthermore, the results of statistical analysis confirm, as expected, the superiority of IEM over EM. The IEM assay is used in the diagnostic field to evaluate the presence of virus in specimens from various sources (9, 15). In our laboratory it is used for the detection of human viruses in fecal material submitted for diagnosis purposes (2). It allows the identification of specific viruses and also the detection of viral particles present in limited numbers. The results of the present study indicate that this assay is also a valuable procedure for detection of RECV in rabbit feces.

Healthy carriers have also been detected in the rabbit population. Positive specimens in healthy animals were detected with both procedures used. However I.E.M. seems to detect more healthy carriers than E.M. It is not surprising to find healthy carriers since theyse have already been reported in different species (3, 8, 19) including man (13). However it is the first time that healthy carriers of coronavirus are reported in the rabbit species. These carriers are quite likely responsible for the persistence of the infection in certain colonies where mortalities due to enteritis are high. The IEM then becomes a useful tool to recognise the infection and to prevent the introduction of infected stock in a colony. The results of the screening of all new animals introduced could contribute to the control of enteritis due to coronavirus and thus considerably decrease the economical loss due to high mortality rate.

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