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Coronavirus infection in mink (*Mustela vison*). Serological evidence of infection with a coronavirus related to transmissible gastroenteritis virus and porcine epidemic diarrhea virus

P. Have^a, V. Moving^a, V. Svansson^b, Å. Uttenthal^c, B. Bloch^b

^aState Veterinary Institute for Virus Research, Lindholm, DK-4771 Kalvehave, Denmark

^bDepartment of Veterinary Microbiology, Royal Veterinary and Agricultural University, DK-1870
Frederiksberg C., Denmark

^cDanish Furbreeders Association, DK-2600 Glostrup, Denmark

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ABSTRACT

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Antibodies to a transmissible gastroenteritis virus (TGEV)-related coronavirus have been demonstrated in mink sera by indirect immunofluorescence, peroxidase-linked antibody assays and immunoblotting. This is the first serological evidence of a specific coronavirus infection in mink. The putative mink coronavirus (MCV) seems to be widespread in the Danish mink population with a prevalence approaching 100%. Analysis by immunoblotting has shown that MCV is closely related to TGEV by the spike (S), matrix (M) and nucleoprotein (N) polypeptides. Furthermore, antibodies to MCV also cross-reacted with N and M polypeptides of porcine epidemic diarrhea virus (PEDV). Thus MCV may occupy an intermediate position between the TGEV group of coronaviruses and PEDV. The possibility that MCV may be associated with syndromes of acute enteritis in preweaning mink is discussed.

INTRODUCTION

Coronaviruses infect a number of mammalian and avian species and the mammalian viruses are usually placed in two major serological groups represented by mouse hepatitis virus (MHV) and transmissible gastroenteritis virus (TGEV) (Sturman and Holmes, 1983, Siddell et al., 1983). The MHV group includes bovine coronavirus (BCV), porcine hemagglutinating encephalomyelitis virus (HEV) and human coronavirus (HCV) OC-43. The TGEV group includes porcine respiratory coronavirus (PRCV) (Pensaert et

al., 1986, Sánchez et al., 1990), canine coronavirus (CCV), feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV). Mature virions of coronaviridae contain three major structural proteins (Cavanagh et al., 1990) of which the spike (S) glycoprotein and transmembrane matrix (M) glycoprotein are glycosylated. The nucleoprotein (N) is associated with the positive-stranded RNA genome. Coronaviruses are responsible for various disease manifestations in their host species, depending on virus tropism (e.g. pneumotropic, enterotropic or neurotropic viruses).

Porcine epidemic diarrhea coronavirus (PEDV) (Pensaert et al., 1981), causing a gastroenteritis similar to TGE in pigs, is as yet unclassified, although a slight serological relationship with FIPV has been demonstrated (Yaling et al., 1988). Until recently, no known cell culture system was available for propagation of PEDV *in vitro* but this has now been accomplished using VERO cells and medium including proteases such as trypsin (Hofmann and Wyler, 1988). The apparent M_r values of PEDV structural proteins have been found to be 85 000–135 000K for the S protein, 58 000 for the N protein and 20 000–32000 for the M protein (Egberink et al., 1988).

This serological study on mink coronavirus was initiated by our observations in electron microscopy of coronavirus-like particles in stool samples from neonatal mink kits suffering from diarrhea. Specific coronavirus infections have so far not been described in mink but coronavirus-like particles in feces have recently been associated with epizootic catarrhal gastroenteritis in mink (Gorham et al., 1990). The present paper provides the first serological evidence for infection in mink with a coronavirus (here designated MCV) serologically related to TGEV and PEDV.

MATERIAL AND METHODS

Viruses

TGEV (Purdue strain) and PRCV (Danish isolate DK1/86, Have, 1991) were grown in a swine testicle (ST) cell line (McClurkin and Norman, 1966). CCV (strain 1-71) was grown in A-72 cells (ATCC CRL 1542). FIPV (strain DF2, ATCC VR-2004) and FECV (strain WSU 79-1683, ATCC VR 989) were grown in Crandell feline kidney (CRFK) cells. PEDV (strain 1/87) was kindly provided by Dr. S. Cartwright, Weybridge, as an intestinal passage of PEDV-infected colostrum-deprived newborn piglets. After a further two passages in piglets the virus was adapted to grow in VERO cells by the method of Hofmann and Wyler (Hofmann and Wyler, 1988).

Sera

Four groups of mink sera were selected for evaluation of MCV antibodies: A, 71 mink sera were obtained during 1990 from the Danish Fur Breeders Association as part of routine sampling for plasmacytosis testing (Aleutian

disease, ADV). B, 23 sera were obtained in 1989 from a mink farm showing a high level of neonatal mortality. C, 17 sera were obtained from mink kept for experimental purposes. D, 41 mink sera collected during 1981–1988 were examined in order to trace the history of coronavirus infection in mink. Hyperimmune or convalescent porcine, canine and feline sera against TGEV, PRCV, CCV, FIPV and PEDV were included as controls. Porcine sera were produced by experimental oronasal inoculation of SPF pigs with TGEV, PRCV or PEDV, respectively. Canine antiserum to CCV was obtained from a recovered dog in a kennel having an acute outbreak of CCV gastroenteritis. FIPV antiserum was from a cat (field case) with clinically diagnosed FIP.

Indirect fluorescent antibody test (IFAT)

Monolayers for IFAT were prepared by infecting secondary porcine kidney cells with TGEV and acetone fixation after 24 h. Sera were examined at a dilution of 1/2 in PBS using acetone-fixed monolayers of virus-infected cells on multitest slides. Following incubation in a moist chamber for 1 h at 37°C, the slides were rinsed three times in PBS and incubated with FITC-conjugated protein-A (Pharmacia) at a dilution of 1/50 in PBS. After incubation for 30 min at 37°C slides were rinsed and mounted for microscopy.

Peroxidase-linked antibody (PLA)-test

Virus-infected cells were prepared in 96-well tissue culture microplates by mixing cell suspensions with an appropriate dilution of virus. Following incubation for 24 h cells were fixed in 30% acetone in PBS for 10 min, air dried at 37°C for 1 h and stored at –20°C or used immediately (Jensen, 1981).

Monolayers were incubated with dilutions of test sera in PBS-0.1% Tween 20 (PBST) for 1 h at 37°C. Following washing in PBST, peroxidase-conjugated protein A (ZYMED) or peroxidase-conjugated anti-swine IgG (DAKO), both diluted 1/200 in PBST, were added and incubated for 30 min at 37°C. Finally, the substrate 3-amino-9-ethylcarbazole was added and left for 30 min. Serum titres were recorded as the highest dilution causing specific cytoplasmic staining.

Neutralization test

Neutralization tests were carried out in 96-well microplates. Sera were heat-inactivated and two-fold dilutions were mixed with 100 TCID₅₀ of virus and incubated for 1 h at 37°C. Following incubation, the appropriate cell suspension was added, plates were sealed and incubated at 37°C in a 7% CO₂-air mixture. Neutralizing titres were evaluated after 3–4 days using the method of Reed and Muench (1938).

Western blot

TGEV, CCV or PEDV culture supernatants were clarified at 3500 *g* in a JA 14 rotor for 20 min followed by pelleting at 20 000 *g* for 4 h in the same rotor. Pelleted virus was separated by SDS-PAGE on a 10% gel after solubilisation at 100°C for 2 min in sample buffer (0.06 M tris-HCl, 9% glycerol, 3% SDS, pH 6.8) under non-reducing conditions. Electrophoresis was performed using Laemmli buffers (Laemmli, 1970). Proteins were transferred electrophoretically (200 mA, 90 min) to nitrocellulose or polyvinylidene difluoride sheets by semi-dry blotting using Tris-glycine-methanol buffer (60 mM glycine, 50 mM Tris base, 0.03% (*w/v*) SDS, 20% methanol) pH 8.9. Strips were blocked by incubation for 1 h with 5% (*w/v*) skimmed milk powder in PBST. Sera were diluted 1/100 in the same buffer and applied for 1 h on a tilt shaker. Peroxidase-conjugated protein A (ZYMED) was diluted 1/500 in PBST and applied for 30 min. Specific bands were revealed using the substrate *o*-dianisidine dihydrochloride (Sigma, 0.3 mg/ml in PBS).

RESULTS

Initially, mink sera were examined in IFAT using acetone fixed monolayers of secondary swine kidney cells infected with TGEV as substrate and FITC-conjugated protein-A to detect specific binding of antibodies to TGEV. Of 71 sera collected during 1990 for routine testing of ADV antibodies, 68 were found to contain TGEV cross-reacting antibodies in IFAT at a dilution of 1/2. The pattern of fluorescence was most often diffusely cytoplasmic and very similar to that seen with sera from FIPV-infected cats. The serological data on mink sera have been summarized in Table 1.

All of 23 sera from a farm showing high neonatal mortality contained TGEV

TABLE 1

Serological results obtained with mink sera by IFAT, PLA and neutralization test using TGEV, PRCV, CCV, FIPV and FECV

Serum Group	Year	Method						
		IFAT	PLA	Neutralization test				
		Virus						
		TGEV	TGEV	TGEV	PRCV	CCV	FIPV	FECV
A	1990	68/71 ¹						
B	1989	23/23						
C	1988	17/17		0/17	0/17	0/17	0/17	0/17
D	1981-1988		31/41	0/20	0/20		0/16	0/16

¹No. positive/No. tested.

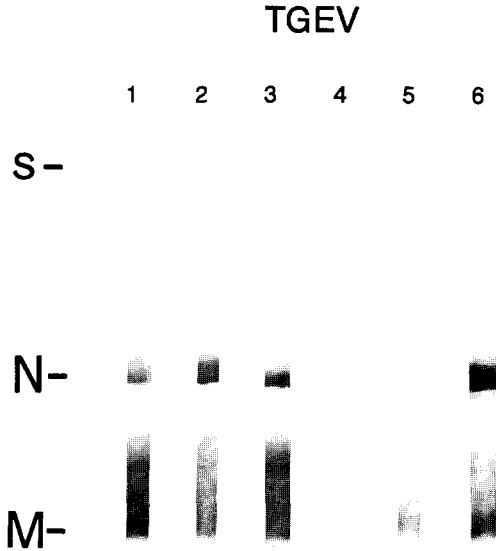


Fig. 1. Immunoreactivity of polyclonal coronavirus antisera against TGEV structural proteins. TGEV was separated by SDS-PAGE and blotted onto nitrocellulose sheets. Strips were reacted with antisera diluted 1/100 followed by peroxidase-labelled protein A. Antisera: lane 1: MCV, lane 2: FIPV, lane 3: CCV, lane 4: PEDV, lane 5: PRCV, lane 6: TGEV.

cross-reacting antibodies in IFAT at a dilution of 1/2. These sera represented adult females as well as 6 months old male and female kits. Also, all of 17 sera from experimental animals were positive in IFAT for TGEV cross-reacting antibodies.

Sera obtained during 1981–1988 were titrated in TGEV PLA and 31 of 41 samples were positive with titres ranging from 5 to 320. Positive samples were evenly distributed over the years with no obvious time-clustering.

Due to cytotoxicity some of the sera included in this study could not be examined in neutralization test. All of the 17 sera originating from experimental animals were tested in neutralization tests against TGEV and PRCV (initial dilution 1/10), CCV, FIPV and FECV (initial dilution 1/5) and none of the sera neutralized any of these viruses. Furthermore, 20 sera (13/20 positive by PLA) tested against TGEV and PRCV and 16 sera (16/16 positive by PLA or IFAT) tested against FIPV and FECV were also negative for neutralizing antibodies.

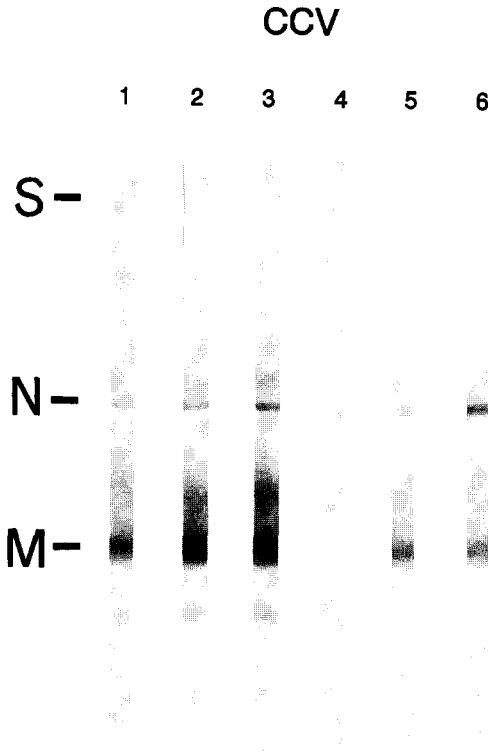


Fig. 2. Immunoreactivity of polyclonal coronavirus antisera against CCV structural proteins. See legend of Fig. 1 for a description of antisera.

A high-titered mink antiserum and representative high-titered antisera against TGEV, PRCV, CCV, FIPV and PEDV were selected for further studies of the serological relationship of the putative mink coronavirus to other coronaviruses. These sera were used to develop western blots of TGEV, CCV and PEDV. It should be noted, that antisera to CCV and FIPV are field sera and not homologous to the strains applied in this study.

It is apparent, that antisera against TGEV, PRCV, CCV and MCV all react strongly with all three major structural proteins S (200K), M (27K) and N (47K) of TGEV (Fig. 1). A fourth polypeptide (14–15K) is revealed by antiserum to TGEV only. In contrast, FIPV antiserum reacts primarily with N and M of TGEV. Antiserum against PEDV reacts weakly with S and N of TGEV. A similar pattern was found when using CCV instead of TGEV as antigen (Fig. 2) except that staining of the S protein was only evident with CCV antiserum. In parallel experiments weak staining of the S protein of CCV has been observed with TGEV, PRCV and MCV antisera (not shown). Antiserum against PEDV reacts with S, M (28K) and N (55K) of PEDV whereas

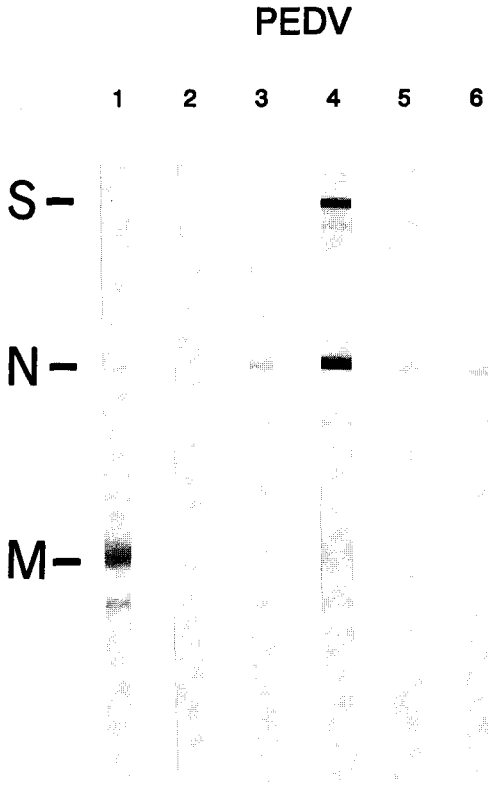


Fig. 3. Immunoreactivity of polyclonal coronavirus antisera against PEDV structural proteins. See legend of Fig. 1 for a description of antisera.

TABLE 2

Cross-neutralization of porcine, feline and canine coronaviruses by selected reference antisera

Serum	Virus neutralizing titre ($-\log_{10}$)				
	CCV	TGEV	PRCV	FIPV DF2	FECV 79-1683
TGEV 18/9/74	2.20	3.10	2.20	1.30	2.80
PRCV ref. 20/12/85	2.35	2.95	2.50	1.90	2.95
CCV 81.184.6	1.75	2.35	1.15	1.30	2.05
FIPV asc. 2 87.16	<1.00	<1.00	<1.00	<0.70	<0.70
PEDV gr. 3 4/11/87	<0.70	<0.70	<0.70	<0.70	<0.70
MCV 9f	<0.70	<1.00	<1.00	<0.70	<0.70

TABLE 3

Peroxidase-linked antibody titres of selected reference antisera against porcine and feline coronaviruses

Serum	PLA-titre (-log ₁₀)			
	TGEV	PEDV	FIPV DF2	FECV 79-1683
TGEV 18/9/74	2.50	0.60	2.50	2.50
PRCV ref. 20/12/85	2.50	<0.30	2.50	1.90
CCV 81.184.6	2.50	0.60	2.50	2.50
FIPV asc. 2 87.16	3.10	2.10	3.10	2.50
MCV 9f	2.50	2.10	2.50	2.50
PEDV gr. 3 4/11/87	<0.70	3.30	<0.70	0.70

antisera against TGEV, PRCV, CCV and FIPV barely reacts with N of PEDV (Fig. 3). Mink antiserum occupies an intermediate position in that there is a distinct reaction with both M and N of PEDV.

By cross-neutralization TGEV, PRCV, CCV, FIPV and FECV were found to be very similar, all being neutralized by antisera to TGEV, PRCV and CCV (Table 2). On the other hand antisera to FIPV, PEDV and MCV did not neutralize any of the viruses. In particular, antiserum to FIPV did not neutralize the strains of FIPV and FECV applied in this study. Neutralization of PEDV was not attempted, due to the extreme dependance of replication on trypsin.

The reactivity of these sera was also tested in PLA against TGEV, FECV/FIPV and PEDV (Table 3). The titres of antisera against TGEV, PRCV and CCV are higher against TGEV and FECV/FIPV than against PEDV. Antiserum against FIPV and MCV are broadly reacting with TGEV, FECV/FIPV and PEDV whereas antiserum against PEDV reacts strongly with the homologous virus and only weakly with FECV.

DISCUSSION

Examination of mink sera by IFAT has shown a high prevalence of antibodies reacting with TGEV. These results indicate that one or more coronaviruses related to TGEV are widespread in the Danish mink population and that such viruses have been present at least since 1981. The high prevalence of seropositive animals over several years points towards an enzootic coronavirus infection in balance with its host population. At present, it is unknown whether this coronavirus infection causes any disease but if so, it may be expected that the virus would manifest itself primarily in young animals either as a consequence of waning maternal antibodies or inadequate lactogenic immunity.

A number of clinical syndromes which might be associated with coronavirus infection have been described in mink. Epizootic catarrhal gastroenteritis (ECG) (Larsen and Gorham 1975) occurs primarily in mink four months and older exhibiting a mucoid diarrhea over 2–6 days. Coronavirus-like particles have recently been identified by negative contrast electron microscopy in feces of animals with ECG (Gorham et al., 1990). A similar observation has been made by one of us (Bloch, unpublished results), who found coronavirus-like particles in stool samples from neonatal mink kits. These mink kits originated from farms with a high neonatal mortality. The mink kits were suffering from diarrhea, referred to by farmers as “wet mink kits”. The epizootiology of this syndrom is still unclear (Henriksen 1989). FIP-like symptoms (pyogranulomas, vasculitis and immune-complex disease) have not been described in mink except for ADV-associated immune-complex disease (Porter et al., 1980) but such symptoms should be kept in mind when looking for possible effects of coronavirus infection.

From the data presented here it is clear that there is a close serological relationship between the putative MCV and both TGEV and CCV. The results of western blots show that antibodies to MCV react with all three major structural proteins of TGEV and CCV. However, none of the mink sera neutralized any of these viruses, indicating that the antigenic domains responsible for neutralization in the spike (S) protein of TGEV and CCV are probably absent from MCV. In this respect MCV may resemble field strains of FIPV/FECV as it has been found that feline field sera having IFAT and PLA titres of 20 480 or higher against TGEV generally do not neutralize TGEV or the laboratory strains of FIPV and FECV used in this study (Have, unpublished data). It would thus be interesting to have more recent isolates of feline coronavirus and analyze their relationship to MCV.

No clear relationship has been found between PEDV and any other known coronavirus (Pensaert et al., 1981) except for a weak cross-reaction with the N protein of FIPV (Yaling et al., 1988). The present results have confirmed this cross-reaction and further shown that it extends to TGEV, PRCV, CCV and MCV. In addition, the M proteins of PEDV and MCV are clearly related as seen by the reaction of mink antiserum with the M protein of PEDV.

The present study has shown that infection with a coronavirus serologically related to TGEV and PEDV is common in the Danish mink population. At present, nothing is known of the pathogenic potential of mink coronavirus, if any, nor the preferred site of replication in the host. Studies aimed at answering these questions as well as attempts to isolate the virus have been initiated.

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