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Entry and release of canine coronavirus from polarized epithelial cells

Annamaria Pratelli

Department of Public Health and Animal Sciences, Faculty of Veterinary Medicine, Valenzano, Bari, Italy

SUMMARY

Virus entry into and release from epithelial cells are polarized as a result of the distribution of the viral receptors. In order to establish the polarity of entry and release of CCoV from epithelial cells, the interactions of the virus with A72 and CrFK cells grown on permeable supports was evaluated, and the amount of infective virus in the apical and in the basolateral media was determined and compared. Infection of A72 cells after different times post seeding demonstrated that CCoV grow after infection from both apical and basolateral sides. In CrFK cells, CCoV was observed in both compartments only in the later phase of the infection. To establish the reciprocal binding of CCoV on plasma membrane, A72 cells on a permeable support were preincubated with a mAb specific for CCoV. Infection from the apical side was blocked by mAb applied to that side; in contrast, such treatment on the basolateral side had no effect on the infectious process. Similarly, the low levels of CCoV observed after basolateral exposure to virus was abolished following mAb treatment of that side. The identification of CCoV into the basolateral medium could play an important role in the viral pathogenesis.

KEY WORDS: Coronavirus, Dog, Epithelial cells, A72, CrFK

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INTRODUCTION

Coronaviruses have been described for more than 50 years, but they remained relatively obscure for a long period, probably because there were no severe human diseases that could definitely be attributed to coronaviruses. However, in the spring of 2003 with the occurrence of the *Severe Acute Respiratory Syndrome* (*SARS*) epidemic, coronaviruses become much more recognized and are now considered emerging pathogens.

Coronaviruses are positive-stranded RNA viruses, 27 to 32kb in size, packaged in enveloped virions with *corona-like* morphology (Gonzalez *et al.,* 2003). The viral genome contains five major open reading frames (ORFs) that encode for the replicase polyprotein, for the structural proteins S, E, M and N, and for non-structural proteins (Huang *et al.,* 2004).

Coronaviruses infect many species of animals and, on the basis of phylogenetic analysis and antigenic cross reactivity, three distinct groups can be distinguished. Group I includes the human coronaviruses HCoV-229E and NL63, which cause respiratory infections, and the animal pathogens of swine, cat and dog (Pratelli, 2006). Primary replication of coronaviruses is often limited to epithelial cells of respiratory or gastrointestinal tracts and, in systemic infection, epithelial cells are the first host cells to be infected by incoming coronaviruses. Epithelia form highly organized sheets that line the body cavities and represent the first barrier to infections. In epithelial plasma membranes two domains can be distinguished: the apical face, exposed towards the external milieu, and the basolateral face, directed towards the internal environment. The tight junctions with neighbouring cells separate the two faces both defining the confine and restricting intercellular diffusion (Rossen *et al.,* 1995a). Indeed, each domain has a distinct lipid

Corresponding author
Annamaria Pratelli
Department of Public Health and Animal Sciences
Faculty of Veterinary Medicine
Strada per Casamassima km 3
70010 Valenzano, Bari, Italy
E-mail: a.pratelli@veterinaria.uniba.it

and protein composition that results from a sorting process and from the function of the tight junctions that prevent mixing of membrane components (Rossen *et al.,* 1995b).
Virus entry into and release from epithelial cells are polarized, i.e. viruses can enter epithelial cells or be released through either domain as a result of the distribution of viral receptors. Obviously, the presence of receptors only on the basolateral surface significantly hinders infection. Although not the only determinant, polarized virus release can influence viral spread: basolateral release from epithelial cells, allows the infection of underlying tissues and the spread of virus in the blood leading to systemic infection.
On the other hand, apical release can limit viral spread by preventing the infection of cells other than epithelial cells (Rossen *et al.,* 1995a). Entry and release from epithelial cells have been shown for different viruses and, in some of them, a plasma domain is involved.
This study investigated the polarity of entry and release of canine coronavirus (CCoV) in two polarized epithelial cell lines.

MATERIAL AND METHODS

Cell lines and Minicell Cell Culture Device
The origin of A-72 cells was reported by Binn *et al.* (1980). This canine fibroma cell line was established from a tumor surgically removed from a female 8-year-old Golden Retriever dog. They are susceptible to infection with infectious canine hepatitis virus (CAdV-1), canine parvovirus type 2 (CPV2), canine adenovirus type 2 (CAdV2), canine herpesvirus (CaHV), canine parainfluenza virus (CPiV), and CCoV.
CrFK cell line, derived from kidney tissue of a normal domestic kitten, was initiated in 1964. With intermittent periods of storage in the frozen state, it has been grown in vitro during more than 200 passages, without apparent loss of susceptibility to selected viruses. Various herpesviruses and feline and canine viruses grow readily and with distinct cytophatic features. They are susceptible to infection with FCoVs, feline parvovirus (FPV), feline herpesvirus (FHV), feline calicivirus (FCV), and with canine viruses CPV2 and CCoV. The cells grow as a smooth monolayer of epithelial-like cells; most have 37 chromosomes (2n-1) and are thus aneuploid for cat karyotype (Crandell *et al.,* 1973).
A72 and CrFK cells were maintained at 37°C and 5% CO_2 in plastic culture flasks (*Falcon, Becton Dickinson*) in culture medium, Eagle Minimal Essential Medium (EMEM) (*BioWhittaker, Cambrex BioScience*) supplemented with 10% foetal calf serum, 100IU/mL penicillin, 100 l/mL streptomycin and 10µg/mL amphotericin. Both cell lines were passaged as reported. A confluent cell cultures in a 75cm^2 culture flask was rinsed twice with phosphate-buffered saline containing 0.01% EDTA (PBS-EDTA) and incubated for 15s at 37°C in PBS-EDTA. The detached cells, collected after centrifugation at 1500xg for 10min, were thoroughly resuspended into 24 mL of culture medium and employed for the preparation of polarized cell monolayers. The Minicell 24-Well Cell Culture Device (*Millipore Corporation, Billerica, MA 01821*), designed to support suspension and adherent cell growth and differentiation, was employed in the present study. The cells grown on filter support allowed inoculation both from the apical and the basolateral side. Four hundred µl of cell suspension were added per filter plate well, while 800 µl/well of cell growth medium was added to the 24-well receiver plate. The tightness of the monolayers was checked by adding medium to the upper chambers up to a slightly higher level than in the lower chambers. Routinely every day growth medium was replaced to achieve optimal cell growth. No leakage of culture medium from the apical to the basolateral compartment occurred from 12h after cells were seeded on filters and before 12h post infection (p.i.).

Virus and antisera
The 257/98-3c strain of CCoV, originally isolated from a dog with enteritis on A72 cells, was used for all the infections. The monoclonal antibody (mAb) against CCoV and the mAb specific for FCoV type I were kindly provided by dr. Gilles Chappuis (Merial, France) and used to monitored virus growth.

Transepithelial resistance measurements
Transepithelial resistance (TER) was measured using a Minicell-ERS (Electrical Resistance System) apparatus (*Millipore Corporation, Billerica, MA 01821*) according to the manufac-

turer's instructions. Measurements were performed by using A72 and CrFK cells grown on the Minicell 24-Well Cell Culture Device at room temperature in culture medium at fixed time points: every 6h for 3 days. TERs were measured over monolayers of non infected cells.

Infection

Both A72 and CrFK cells grown on the Minicell 24-Well Cell Culture Device were infected with CCoV strain 257/98-3c. The infectivity titres of the stock viruses cultured on A72 and CrFK cells and employed to perform the experiences, were $10^{6.75}$ $TCID_{50}$/50 µl and 10^4 $TCID_{50}$/50 µl, respectively. The filter-grown A72 and CrFK cells were both rinsed twice with EMEM and were inoculated with 100$TCID_{50}$/50 µl of the respective viral suspension from either the apical or the basolateral side according to the manufacturer's instructions at different time post seeding (p.s.): 6h p.s., 12h p.s., 24 h p.s., and 48h p.s. Infection was allowed to take place at 37°C for 45 min, after which the inoculum was removed. The filters were washed twice with EMEM and further incubated in culture medium. Infected cells were employed for titration, immunofluorescence assays and for inhibition of infection trials. All the experiments were repeated twice under the same conditions.

Virus titration

The amount of infectious virus released into the cultured medium of both A72 and CrFK infected cells, was determined at regular time points p.i.: 24 h p.i., 48 h p.i., 72h p.i. Serial tenfold dilutions of CCoV-257/98-3c in EMEM were inoculated into monolayers of both A72 and CrFK cells grown in 96-well microtitration plates (*Falcon, Becton Dickinson Labware*). After 45 min the inoculum was removed and replaced by culture medium. Cells were incubated at 37°C for three days and observed daily for cytophatic effects. Viral titres were determined using the 50% tissue culture infectious dose assay ($TCID_{50}$)/50 µl, using the *Spearman/Kaerber* formula (Kaerber *et al.,* 1931).

Indirect immunofluorescence assay

A72 and CrFK cells grown on permeable supports were inoculated with CCoV-257/98-3c from both the apical and the basolateral side. At 24h p.i. cells were rinsed once with PBS and fixed with 3% paraformaldehyde overnight at 4°C. Subsequently the filters, after being washed three time for 10 min each with PBS containing 50nM glycine (PBS-G), were cut and removed from their holders. Next, cells were incubated for 30min at 37°C with the mAb for CCoV diluted 1/100 in PBS-G. Cells were washed three times for 10min each with PBS-G and then incubated with a 1/60 dilution of secondary goat anti-mouse IgG fluorescein isothiocyanate-labeled antibody (*Sigma Chemicals*). Finally, filters were washed three time for 10min in PBS-G, mounted with buffered glycerol (pH 7.2) on glass slides, and observed with a fluorescence microscope for the presence of CCoV infected cells.

Inhibition of infection and localization of CCoV receptor

A72 cells grown on glass slides were pre-incubated for 60 min at 37°C in a humidified chamber with 1/100 dilution of the mAb against CCoV. As control, glass slides were pre-incubated with serial dilution of the mAb specific for FCoV type I. Cell lines grown on permeable supports for 24h were pre-incubated with 1/100 dilution of mAb against CCoV, either from the apical or from the basolateral side. The cells were incubated for 60min at 4°C instead of 37°C to prevent possible transcytosis of antibodies (Rossen *et al.,* 1995b). Subsequently, cells grown on permeable supports were inoculated with CCoV (100 $TCID_{50}$/50 µl) from both the apical and the basolateral side and incubated at 4°C. Cells grown on glass slides also were inoculated and incubated at 37°C. Infection was monitored both by visual inspection of cytophatic changes under a light microscope (cells grown on glass slides) and by titration of released virus in the collected media. Culture media were collected at 24 h p.i. and employed in an endpoint dilution infectivity assay on A72 cells.

RESULTS

Transepithelial resistance

In order to establish the polarity of entry and release of CCoV in the epithelial cell lines A72 and CrFK, the cells must grow in order to form tight monolayers. To verify the tightness of the monolayers, the development of tight junctions was monitored by measuring transepithelial resistance at different p.i. time points. An evident TER on

A72 cells was observed at 6h p.s., reaching about 350 cm^2 between 24 h p.s. and 72 h p.s. (Figure 1a). In contrast, the TER of the CrFK cells was found to increase slowly reaching a steady-state value of about 280 cm^2 at 72 h p.s. (Figure 1b). During the experiments, there was no leaking of culture medium between the two compartments, confirming that both A72 and CrFK cells had formed a completely tight monolayers.

Polarity of virus entry and release. Synthesis of virus particles

Supernatant fluids from the apical and basolateral infected cell compartments were tested for viral progeny by using the $TCID_{50}$/50 µl at different times p.i. Results of the evaluation are reported in Table 1. In A72 cells infected at 6h p.s., the release of infective particles were evident, as confirmed by indirect immunofluorescence assay (data not shown), but the amount of virus during the observation period was low, both in the apical and in the basolateral compartments. The infection of A72 cells after 12 h, 24 h and 48 h p.s. demonstrates that CCoV is able to grow, but with different titres after infection from the apical and basolateral sides. Infected cells also were also evident in the immunofluorescence assay (data not shown). In particular, when infections were performed 12 h p.s. the infectious titres in both A72 and CrFK cells, evaluated after 24 h p.i., were low for the A72 cells and negative for the CrFK cells. It seems likely, during this phase, even if the tightness of monolayers was complete, that there was a decrease in complete viral expression (latent phase of virus replication). CrFK cells appeared less accessible to CCoV infection as shown in Table 1b. In particular, the infectious titre evaluated after 24h p.i was negative (6h p.s. and 12h p.s.), i.e. less than 10^2 $TCID_{50}$/50 µl (24h p.s. and 48h p.s.). Significant virus titres were observed in the later phase of the infection (72h p.i.) both in the apical and in the basolateral compartments. Moreover, infectious virus was better expressed in the apical

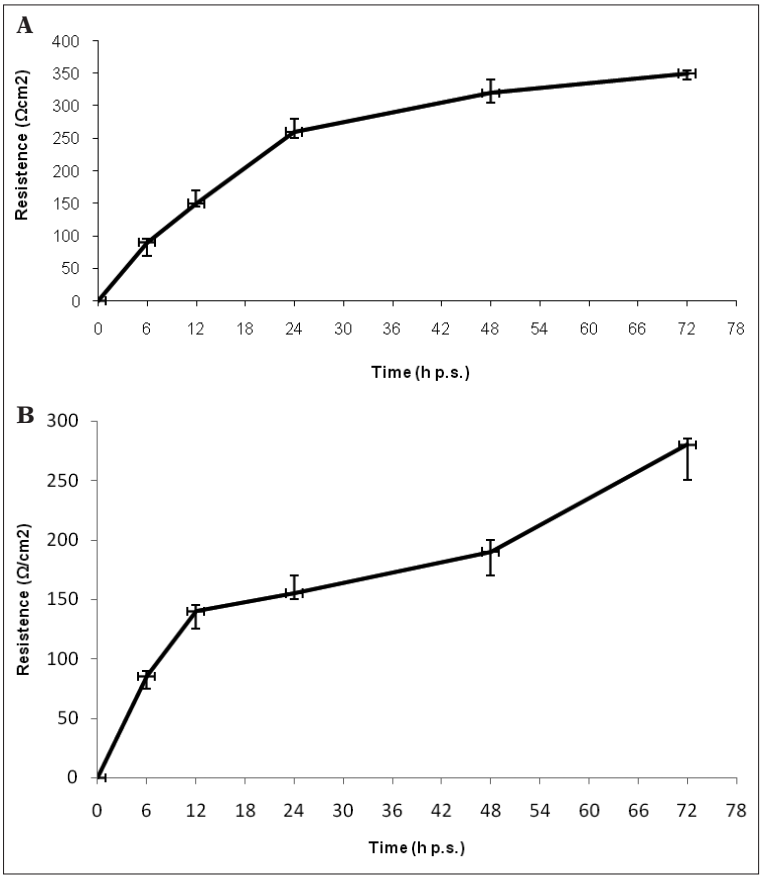

FIGURE 1 - *Transepithelial resistance (TER) measurements of A72 (a) and CrFK (b) cells. The development of TER was evaluated over time after seeding cells on filter support.*

medium as confirmed with the immunofluorescence test (data not shown). This was particularly evident in A72 cells, confirming the greater susceptibility of these cells to CCoV infection (data not shown).

Inhibition of infection

To establish the receptorial binding of CCoV on A72 cell plasma membranes, an infection-inhibition experiment was performed. Prior to inoculation, cells on glass slides were preincubated

TABLE 1 - *Release of infectious virus from A72 (a) and CrFK (b) infected cells into apical and basolateral media at different time points.*

a)	*Titer (log_{10})**											
	6h p.s.°			*12h p.s.°*			*24h p.s.°*			*48h p.s.°*		
	24h p.i.	*48h p.i.*	*72h p.i.*	*24h p.i.*	*48h p.i.*	*72h p.i.*	*24h p.i.*	*48h p.i.*	*72h p.i.*	*24h p.i.*	*48h p.i.*	*72h p.i.*
A	$10^{2.5}$	$10^{3.5}$	$10^{3.5}$	$10^{2.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	$10^{6.5}$	10^{6}	$10^{6.5}$	10^{6}	10^{6}
B	10^{2}	10^{3}	10^{3}	10^{2}	$10^{6.5}$	$10^{5.5}$	10^{6}	$10^{5.5}$	$10^{7.5}$	$10^{5.5}$	10^{6}	$10^{5.5}$
C	$10^{2.5}$	$10^{2.5}$	$10^{3.5}$	10^{1}	$10^{7.5}$	10^{7}	10^{6}	$10^{6.5}$	10^{6}	10^{6}	10^{6}	10^{6}
D	10^{2}	10^{2}	10^{3}	10^{1}	10^{7}	10^{6}	$10^{5.5}$	$10^{6.5}$	10^{6}	$10^{5.5}$	10^{6}	$10^{5.5}$
b)	*Titer (log_{10}) **											
	6h p.s.°			*12h p.s.°*			*24h p.s.°*			*48h p.s.°*		
	24h p.i.	*48h p.i.*	*72h p.i.*	*24h p.i.*	*48h p.i.*	*72h .i.*	*24h p.i.*	*48h p.i.*	*72h p.i.*	*24h p.i.*	*48h p.i.*	*72h p.i.*
A	neg	10^{4}	$10^{4.5}$	neg	$10^{3.5}$	10^{4}	10^{2}	$10^{3.5}$	$10^{4.5}$	$10^{1.5}$	$10^{2.5}$	$10^{4.5}$
B	neg	10^{3}	10^{4}	neg	$10^{3.5}$	$10^{3.5}$	10^{1}	10^{3}	$10^{3.5}$	$<10^{1}$	$10^{2.5}$	$10^{3.5}$
C	neg	$10^{3.5}$	$10^{4.5}$	neg	$10^{1.5}$	$10^{3.5}$	$<10^{1}$	10^{3}	$10^{3.5}$	10^{2}	10^{3}	10^{5}
D	neg	$10^{3.5}$	$10^{4.5}$	neg	$10^{2.5}$	10^{3}	$<10^{1}$	10^{2}	$10^{2.5}$	10^{1}	10^{2}	$10^{3.5}$

*Titers were determined using the 50% tissue culture infectious dose assay/50µl. °Hours post seeding (h.p.s.). A: Infection by the apical route and harvesting by the apical route. B: Infection by the apical route and harvesting by the basolateral route. C: Infection by the basolateral route and harvesting by the apical route. D: Infection by the basolateral route and harvesting by the basolateral route.

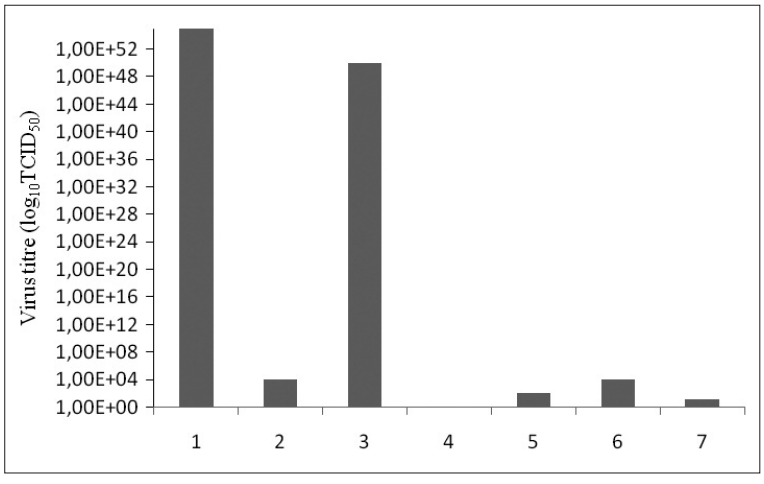

FIGURE 2 - *Filter-grown 24h-old A72 cells were preinoculated with mAb specific for CCoV from the apical (2, 6) and the basolateral (3, 7) sides or neither (1, 5) and then inoculated with CCoV from the apical (1, 2, 3) and the basolateral (5, 6, 7) sides.*

with 1:100 dilutions of a mAb specifically directed against CCoV. The production of progeny virus was then evaluated as a measure of infection. No release of infective virus was evident, confirming that the mAb was able to block viral infection. In contrast, inhibition was not found when cultured cells were preincubated with 1:100 dilution of a mAb specifically directed against FCoV type I.
In another experiment, A72 cells grown on permeable supports and preincubated 24h p.i. with 1:100 dilutions of mAb specific for CCoV from both the apical and the basolateral side, were employed to evaluate whether the polarity of CCoV entry was correlated with the receptor distribution. The infection from the apical side was specifically blocked by mAb employed to treated cells on apical side. In contrast, treatment with mAb from the basolateral side had no effect on infection through the apical membrane. Similarly, the low level of infection observed after basolateral inoculation was completely abolished after mAb treatment from this side (Figure 2). These observations suggest that the polarity of CCoV entry in epithelial A72 cells is correlated with receptor distribution.

DISCUSSION

The epithelial cell surface from which viruses are released is important in the development of pathogenesis (Tashiro *et al.,* 1990). To investigate whether the release of CCoV from both A72 and CrFK cells was polarized, the amounts of infective virus in the apical and in the basolateral media were determined and compared. Several studies have investigated the interaction of coronaviruses and other viruses with epithelial cells in an in vitro system. Vesicular stomatitis virus (VSV) uses the basolateral plasma membrane for both entry and release (Fuller *et al.,* 1984), while influenza virus can enter epithelial cells from both domains but is released from the apical membrane. Parainfluenza viruses, which cause a localized infection of the respiratory tract in humans, are released by budding through the apical membrane (Rodriguez-Boulan and Sabatini, 1978). Similarly, Sendai virus, which is exclusively pneumotropic in mice, also buds from the apical surface of epithelial cells, while a mutant Sendai virus that could infect multiple cell lines, was found to bud through both the apical and the basolateral domains (Tashiro *et al.,* 1990). More recent studies have focused on coronavirus infection in polarized epithelial cells and have demonstrated that both entry and release of TGEV from porcine epithelial kidney cells (LLC-PK1) are restricted to the apical membrane (Rossen *et al.,* 1994). In contrast, entry of murine hepatitis virus (MHV-A59) into murine epithelial kidney cells (mTAL) is restricted to the apical membrane and release occurs preferentially from the basolateral side (Rossen *et al.,* 1995b). However, release seems to depend on the type of cell infected, since MHV appeared to be released from the apical membrane when tested in Madin Darby Canine Kidney (MDCK) cells (Rossen *et al.,* 1997).
The same authors extended their studies on FCoV and CCoV focusing on the polarity of entry into and release from the murine epithelial kidney cells LLC-PK1. They observed that, as found for MHV and TGEV, FCoV and CCoV entered the cells preferentially through the apical side, but most infectious particles were found in the basolateral culture medium, similar to the release of MHV from LLC-PK1 (Rossen *et al.,* 2001).
To find out whether this was an exception, the study was designed to determine the polarity of entry and release of CCoV from epithelial cells of canine and feline origin, cells usually employed for virus cultivation and propagation for diagnostic and research. For this purpose monolayers of A72 and CrFK cells were grown on permeable supports which allowed inoculation from both the apical and the basolateral side. The filter-grown cells were infected from either side with CCoV at different time p.s. and apical and basolateral culture media were analysed for the appearance of infectious viral particles. In A72 cell it was observed that the virus enters the epithelial cells both through the apical and the basolateral side with the same efficiency. This observation means that the filter does not represent a physical barrier for CCoV. Apical exit of virus into intestinal lumen allows rapid infection of adjacent cells in the gut. The selective release of CCoV from the apical membrane domain of A72 cells appears early after infection. CrFK cell line is permeable to CCoV through both sides, therefore releases infectious particles later (not before 48h p.i.) and with less efficiency. Clearly, with the exception of CrFK cells infected 6 h p.s. and 12 h

p.s., both the apical and the basolateral transport pathways are rapidly established.

There are several possible mechanisms for the sorting of intracellular budding virus particles to the apical or basolateral plasma membrane domain. Coronaviruses are assembled at pre-Golgi compartment (Krijnse-Locker *et al.,* 1994) and are transported to the cell surface by vescicular transport. A possible mechanism involves the spike protein. In coronaviruses infected cells this protein is incorporated into virions, but a certain amount is independently transported to the plasma membrane. It may be hypothesized that CCoV virus particles are sorted into specific vesicles together with free spike proteins. Assuming this, we can suppose that the spike protein might confer specific targeting information to the vesicles transporting virus particles (Rossen *et al.,* 1994)

The distribution of viral receptors on epithelial membranes is essential in determining the susceptibility of cells to viral infection. The expression of receptors on apical surface of epithelial cells permits viral infection via the intestinal lumen. However, if the receptors are expressed exclusively on the basolateral surfaces, a virus must pass the epithelial barrier in order to interact with appropriate receptors. CCoV is considered an enteric pathogen, responsible essentially for enteric disease without systemic spread (Appel, 1987). By contrast, a recent study appears to call this hypothesis into question since certain CCoV variants are able to induce systemic infection (Buonavoglia *et al.,* 2006). The present study supports this novel pathogenetic characteristic of CCoV. The virus seems to be able to infect epithelial cells from both apical and basolateral compartments considering that, even with different titres, the virus was observed and released both in the apical and in the basolateral medium after infection.

Virus release by budding at basolateral membrane domains may play a role as a determinant of viral pathogenesis. Therefore, it will be necessary to modify our current view that CCoV is restricted to the intestine. The identification of CCoV in the basolateral medium indicates that the direction of release may be toward the blood stream, with subsequent spread to target tissues. It still needs to be examined whether the in vitro results are the same in vivo. Considering that CCoV, and other coronaviruses, are generally transmitted by the faecal-oral and/or the aerogenic routes, apical entry into epithelial cells seems plausible. By entering the organism's alimentary and respiratory tracts, CCoV first encounters the apical membranes of the epithelial cells that cover those tissues. Moreover, the ability to infect the apical surface of the epithelial cells, rather than being transported by M-cells to the basolateral surface of the epithelial cells before infection, represents an efficient way for the virus to initiate infection. The biological relevance of polar entry and the polar release of CCoV remains unclear since questions of how specific sorting within epithelial cells is accomplished, remain to be solved.

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