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# Clathrin- and serine proteases-dependent uptake of porcine epidemic diarrhea virus into Vero cells



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## ABSTRACT

Porcine epidemic diarrhea virus (PEDV), a member of the genus *Alphacoronavirus*, is a causative agent of porcine enteric disease characterized by acute watery diarrhea and dehydration in suckling piglet. Similar to other coronaviruses, PEDV spike protein mediates its cell entry by binding to cellular receptors and inducing membrane fusion between viral envelopes and cellular membranes. However, the entry mechanism of PEDV is not studied. Here, we determined the entry mechanism of PEDV into Vero cells. Our data confirmed that PEDV entry followed clathrin-mediated endocytosis independence of caveolae-coated pit assembly. The internalized PEDV was co-localized with the clathrin-mediated endocytic marker, but not with the caveolae-mediated endocytic marker. In addition, cells treated with lysosomotropic agents and serine protease inhibitors were resistant to PEDV. Our data revealed that PEDV entry followed clathrin-mediated endocytosis and was dependent on a low pH and serine proteolysis for successful entry into cells.

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## 1. Introduction

Infection of enveloped viruses is initiated by binding of surface proteins with specific receptor(s) on the surface of the cell membrane, which leads to internalization of the virus into cells. The second step of infection following virus attachment is the uncoating of the viral genome into the cytoplasm after the viral envelope has fused with the host membrane. There are two major routes for enveloped viruses to enter host cells; the non-endosomal and the endosomal pathways (Pelkmans and Helenius, 2003; Smith and Helenius, 2004). Both pathways require the release of the viral genome by fusion of the viral envelope with the respective target membrane of the host cells such as the plasma or endosomal membrane, respectively (Matlin et al., 1981). In the non-endosomal pathway, the viral envelope directly fuses with the plasma membrane. Membrane fusion is mediated by a conformational change of the viral glycoprotein, which is induced by its interaction with the corresponding receptor on the host cell surface and/or proteolytic processing (Blumenthal et al., 2002). The endocytic pathway can

further differentiate into two well-characterized pathways; those acting via the clathrin-coated pit and the caveolae-mediated lipid raft (Brodsky et al., 2001; Pelkmans and Helenius, 2002). After internalization, viruses require a low-pH environment in the endosome to trigger conformational changes in the viral glycoproteins. The acidic pH environment is also important for proteolytic activation of viral glycoproteins by endosomal proteases (Qiu et al., 2006; Simmons et al., 2005).

The porcine epidemic diarrhea virus (PEDV) is classified as *Alphacoronavirus* together with transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and human coronavirus 229E (hCoV-229E). PEDV causes an acute watery diarrhea in suckling piglets, which results in approximately 50% mortality among suckling piglets and reduces the weight among fattening pigs (Debouck and Pensaert, 1980). Porcine epidemic diarrhea (PED) is first recognized in pigs in the United Kingdoms in 1971 (Wood, 1977). Although no evidence of PED is currently reported from Canada, similar coronavirus-like particles were reported from herds in Quebec in 1980 (Turgeon et al., 1980). Since then, outbreaks of PED have been documented in many European and Asian countries such as Czech Republic, Hungary, Korea, the Philippines, China, Italy, Thailand, Germany, Spain, and Japan (Song and Park, 2012). Recently, PEDV is spreading rapidly in swine farms in the United States, resulting in high mortality in piglets in more than 17 states (Mole, 2013).

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As typical for the *Alphacoronavirus*, the PEDV spike (S) protein encounters virus entry into host cells by interacting with its receptor, porcine aminopeptidase N (APN), in porcine enterocytes and by mediating membrane fusion with host cell membranes (Li et al., 2007; Oh et al., 2003). Upon receptor binding, several coronaviruses in *Alphacoronavirus* enter cells via endocytosis. For example, extensive studies on hCoV-229E have shown that upon binding with the human APN receptor, it is taken up in lipid rafts and enters via caveolae-dependent endocytosis (Nomura et al., 2004). Inside the endosome, cellular proteases that are active in a low-pH environment facilitate membrane fusion (Kawase et al., 2009). Similarly, TGEV binds to porcine APN (Weingartl and Derbyshire, 1994), and has been shown to enter MDCK cells over-expressing porcine APN via endocytosis and acidification of the intracellular compartment facilitated membrane fusion (Hansen et al., 1998). FIPV also requires acidification of endosomes for successful entry (Takano et al., 2008). Inhibition of FIPV infection with nystatin, a pharmacological reagent that causes caveolae to flatten and disrupt the coat structure, and dynamin 2 inhibitor suggests that FIPV entry might actually involve some types of caveolae-dependent endocytosis (Van Hamme et al., 2008).

Although several studies have examined the mechanism of entry of other coronaviruses, the mechanism of PEDV entry is still unknown. In this study, we studied the entry mechanism of PEDV by measuring virus infectivity in the presence of chemical inhibitors and co-localization of PEDV with endocytic pathway markers. We found that PEDV infection was diminished by treatment with chlorpromazine (CPZ) and lysosomotropic agents. In addition, we also investigated that PEDV required serine-like proteases for their entry through endocytosis and for cell–cell fusion. Taken together, our findings reveal that PEDV enters Vero cells via clathrin-mediated endocytosis and requires serine proteolysis during infection.

## 2. Materials and methods

### 2.1. Cells and viruses

Vero cells were maintained in Eagle's minimum essential medium (MEM, Gibco) containing with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin and 34 µg/ml amphotericin B. KPEDV-9, a Vero cell-adapted Korean strain, was propagated in Vero cells as described previously (Hofmann and Wyler, 1988). Briefly, Vero cells were inoculated with the KPEDV-9 at a multiplicity of infection (MOI) of 10 and cultured in serum-free MEM for 72 h at 37 °C with 5% CO<sub>2</sub>. The progeny viruses were titrated using the focus formation assay following a method described previously (Cruz and Shin, 2007).

### 2.2. Effect of trypsin in PEDV infection

KPEDV-9 infection in Vero cells under trypsin and non-trypsin conditions was compared for 48 h. Vero cells in 4-well tissue culture (TC) plate (SPL Labware) were inoculated with KPEDV-9 and cultured in either serum-free MEM or MEM supplemented with trypsin (10 µg/ml). Infection was stopped by addition of 5% paraformaldehyde (PFA) at the indicated times for immunocytochemistry.

### 2.3. Treatment with endocytosis inhibitors

Vero cells were treated with various concentrations of either CPZ for 30 min or 0.45 M sucrose for 10 min to inhibit the formation of clathrin-coated pits. To block the caveolae-dependent pathway, cells were incubated with various concentrations of nystatin for

30 min. Control cells were incubated with or without dimethyl sulfoxide (DMSO). Cells were inoculated with KPEDV-9 at a MOI of 10 for 2 h, and then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

### 2.4. Co-localization of PEDV with endocytic markers

To prepare ultra-purified trypsin-free viruses, Vero cells were inoculated with the KPEDV-9 at a MOI of 10 and cultured in serum-free MEM for 72 h. Supernatant was clarified by centrifugation at 20,000 × g for 20 min at 4 °C, followed by ultra-centrifugation using a 20% sucrose cushion at 150,000 × g for 3.5 h. Following resuspension in buffer A (1 M Tris, pH 8, 5 M NaCl, 0.1 M CaCl<sub>2</sub>), protein concentration of purified virus stock was determined by the Bradford assay. Fluorochrome conjugation of KPEDV-9 with Alexa Fluor 594 (AF594) carboxylic acid-succinimidyl ester (Molecular probes) was performed according to manufacturer's instructions. Briefly, 5.0 mg of ultrapurified KPEDV-9 was dialyzed in labeling buffer (0.1 M NaHCO<sub>3</sub>, pH 8.3) at 4 °C overnight. Virus was then incubated for 1 h on a platform rocker at room temperature with 1 µg of AF594 succinimidyl ester in 100 µl of DMSO. The AF594-labeled KPEDV-9 was extensively dialyzed in buffer A.

Vero cells were prepared on cover glasses a day before assay. For AF594-KPEDV-9 co-localization with endocytic markers, the cells were incubated with AF594-KPEDV-9 combination with 10 µg/ml of Alexa Fluor 488-conjugated transferrin (AF488-TF) or 5.0 µg/ml of Alexa Fluor 488-cholera toxin subunit B (AF488-CT-B) for 30 min on ice to synchronize entry, and then shifted to 37 °C. Unbound viruses were removed, and the cells were fixed in 2% PFA at indicated times and analyzed at magnification of 63× on the laser scanning confocal microscope.

### 2.5. Neutralization of intracellular pH

Vero cells were treated with either 50 mM NH<sub>4</sub>Cl or 1 µg/ml Baf-A1 to neutralize the intracellular pH. The cells were then inoculated with KPEDV-9 at a MOI of 10 for 2 h in the presence of lysosomotropic agents. The virus inoculums were removed by washing with PBS. Cells were then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

The effect of low pH on the fusion activity of the S protein was investigated by subjecting PEDV-infected Vero cells to a low pH range. Vero cells were inoculated with KPEDV-9 and cultured in trypsin-free MEM for 20 h. Afterwards, the cell monolayer was washed thrice with PBS and replenished with serum-free MEM adjusted to pH 3, 4, 5, 6, 7. MEM containing trypsin (10 µg/ml) at pH 7 was used as positive control. The cultures were further incubated at 37 °C for 4 h, and then fixed with 5% PFA. PEDV-infected cells were detected by immunocytochemistry.

### 2.6. Protease inhibitor assay

Cells were pretreated with various protease inhibitors such as E-64 (10 µM), AEBSF-HCl (500 µM), pepstatin A (10 µg/ml), and phosphoramidon (10 µM) for 1 h. For examination the synergistic antiviral activity of AEBSF-HCl and lysosomotropic agent, cells were treated with AEBSF-HCl and/or NH<sub>4</sub>Cl for 1 h. Treated cells were then infected with KPEDV-9 at a MOI of 1 for 1 h in the presence of inhibitors. After 1 h adsorption, virus inoculums were removed by washing with PBS. Cells were then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

### 2.7. Trypsin bypass

Vero cells were pretreated with either 50 mM NH<sub>4</sub>Cl or 10 μg/ml CPZ and then inoculated with KPEDV-9 at a MOI of 10 for 2 h. After adsorption, virus inoculums were removed by washing with PBS. Cells were then overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. At 10 hpi, PEDV-infected cells were detected by immunocytochemistry.

### 2.8. Immunocytochemistry

To detect expression of viral proteins, KPEDV-9 infected Vero cells were fixed with 5% PFA for 5 min and permeabilized with 1% NP40. Following three washes with PBS, cells were incubated with 1:5000 dilution of mouse anti-PEDV polyclonal antibody for 1 h. Cells were washed three times with PBS and then incubated with 1:1000 dilution of goat anti-mouse IgG conjugated with horseradish peroxidase. KPEDV-9 infected Vero cells were stained using a 3, 3'-diaminobenzidine tetrahydrochloride solution containing NiCl<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> (Vector Laboratories). Clusters of immunostained cells were observed under the inverted microscope (Zeiss) and were presented as the ratio between mock-treated and DMSO treated cells.

### 2.9. Western-blotting

Vero cells prepared in 6-well TC plates were treated with various chemicals to inhibit each endocytic pathway as described above. Cells were inoculated with KPEDV-9 at a MOI of 10 in the presence of drugs. At 2 hpi, unbound viruses were washed out and then cells were incubated in serum-free MEM. Infected cells were harvested and lysed in PRO-PREP protein extraction solution (iNtRON) at 36 hpi. The extracted proteins were diluted in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and electrophoresed in 10% SDS-PAGE system. Separated proteins were electrically transferred onto a polyvinylidene difluoride membrane and PEDV N protein was traced using anti-PEDV polyclonal antibodies. Bands were visualized using Supersignal West Dura with LAS-1000PLUS.

## 3. Results

### 3.1. The requirement of extracellular proteases in PEDV entry

It has been reported that PEDV requires an extracellular trypsin for its successful infection in vitro (Kusanagi et al., 1992; Lai and Cavanagh, 1997; Park et al., 2011). Early data on the infection of PEDV were first provided by Hofmann and Wyler when they demonstrated the formation of multinucleated cells in PEDV-infected Vero cells by supplementing the culture media with trypsin (Hofmann and Wyler, 1988). The trypsin-induced syncytium formation is corroborated by Li et al. when pAPN-expressing MDCK cells were infected with PEDV in the presence of trypsin (Li et al., 2007). Previous findings suggest that proteolytic processing of the S protein is required to facilitate viral membrane fusion with cellular membranes. And, it also raises the possibility that PEDV entry by direct fusion with the plasma membrane could take place in the presence of trypsin.

To confirm the role of exogenous proteases in PEDV infection, PEDV infected cells were treated with trypsin and evaluated viral infectivity by immunocytochemistry. As shown in Fig. 1, we found that PEDV could infect Vero cells even without trypsin treatment. Initial infection was confirmed as early as 8 hpi on both trypsin and non-trypsin conditions. The number of infected cells in both conditions was similar but the spreading into adjacent cells was faster in the presence of trypsin. At 24 hpi, more than 95% of the cell monolayer had formed large multi-nucleated cells. In sharp contrast,

PEDV growth without exogenous trypsin did not involve syncytial spread. At 48 hpi, more than 99% of the cells showed signs of infection, but still no cytopathic effect (CPE) was found. These results confirm that trypsin catalyzes PEDV S protein-mediated cell–cell fusion as demonstrated previously (Hofmann and Wyler, 1988; Park et al., 2011). Based on these observations, we concluded that an exogenous protease, like trypsin, was necessary to induce cell–cell fusion in PEDV-infected Vero cells but not essentially required for virus–cell entry. So, we hypothesized that PEDV entry into Vero cells under the trypsin-free condition most likely occurred inside endosomal compartments where cellular proteases might operate similar to trypsin, facilitating S-mediated fusion of PEDV with the endosomal membrane.

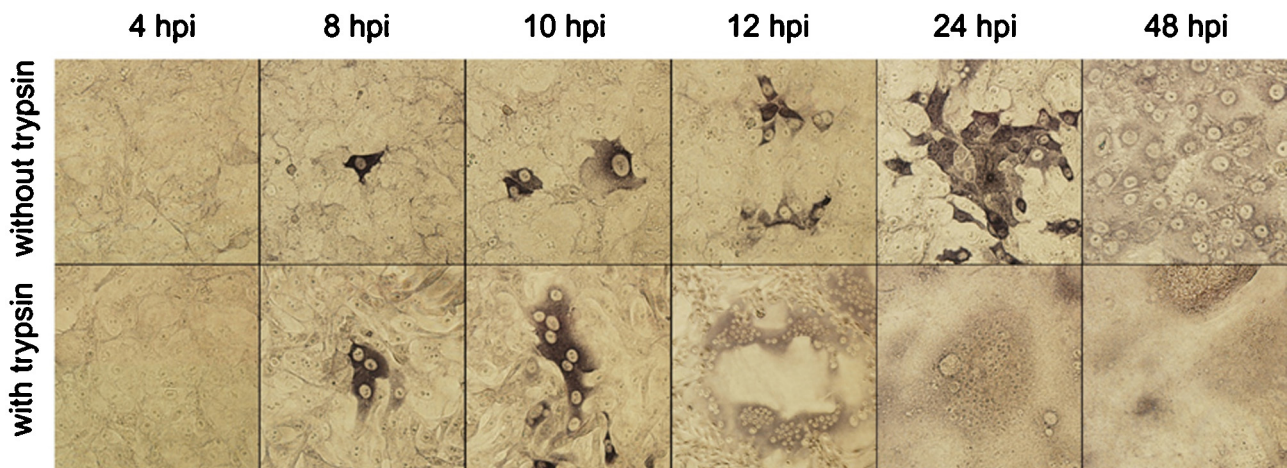
### 3.2. PEDV entry via clathrin-mediated endocytosis in Vero cells

The enveloped virus entry through endocytosis can differentiate into two well-characterized pathways; those acting via the clathrin-coated pit and the caveolae-mediated lipid raft (Brodsky et al., 2001; Pelkmans and Helenius, 2002). To explore whether the endocytic pathway supports PEDV entry into Vero cells and which endocytic pathway alters for PEDV infection, we inhibited pathway by using substances interfering either clathrin-mediated endocytosis or caveolae-mediated endocytosis. For the inhibition of clathrin-mediated endocytosis, we used either (i) CPZ, which is known to abolish the formation of clathrin-coated vesicles by interfering with the interaction of the adapter protein AP-2 with the clathrin-coated pit lattice and thus inhibiting clathrin-dependent endocytosis or (ii) hypertonic 0.45% sucrose, which inhibit clathrin-mediated endocytosis by inducing dispersion of clathrin lattices on the plasma membrane. For the inhibition of caveolae-mediated endocytosis, we used nystatin, a polyene antifungal agent that interacts with cholesterol and inhibits the lipid raft/caveolin pathway. Concentrations of substances were chosen according to previous studies showing the inhibition of other enveloped viruses entering the cells via these endocytic pathways.

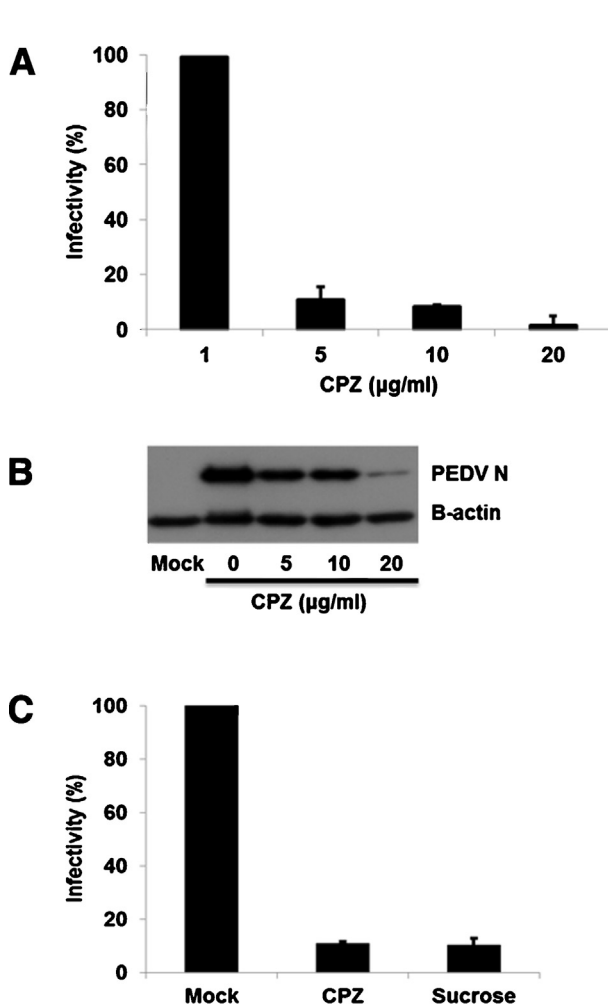
To access the inhibitory effect of CPZ on PEDV infection, Vero cells were treated with CPZ and infected with KPEDV-9. To measure the inhibitory effect of virus entry, cells were overlaid with 0.5% methyl cellulose in MEM containing trypsin for 10 h. Media containing methyl cellulose and trypsin blocks second-cycle infection, but allows the formation of syncytium to visualize infected cells. Infectivity was determined by measuring infected cells by immunocytochemistry staining at 10 hpi and normalized with “untreated cells” control. As shown in Fig. 2A, PEDV infection remarkably diminished (>90%) by CPZ treatment, and the inhibition rate was positively related with concentration of CPZ. To confirm decreased replication of PEDV, we determined the expression of PEDV nucleocapsid (N) proteins by western blotting. The PEDV N proteins were far less expressed in CPZ-treated cells compared to untreated cells (Fig. 2B), whereas β-actin expression was same. Consistent with previous results, PEDV infection with hypertonic sucrose treated was also significantly inhibited (Fig. 2C). Our result strongly suggested that PEDV uses clathrin-mediated endocytosis pathway for their entry into Vero cells.

Similar experiments were performed with nystatin treatment to determine whether PEDV also uses caveolae-mediated endocytosis. Unlike CPZ treatment, PEDV infection was only slightly decreased in highest concentrations (Fig. 3A). Likewise, the levels of PEDV N protein synthesized virtually identical in both presence and absence of nystatin (Fig. 3B).

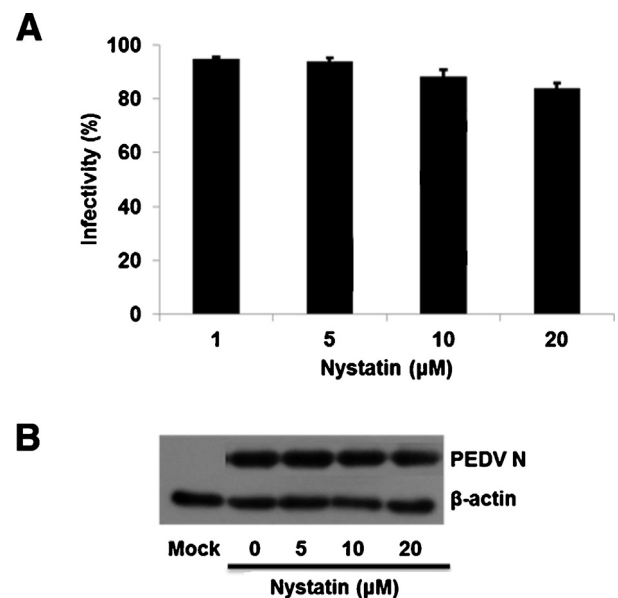
To verify and confirm our results, several other markers specific to the clathrin-mediated pathway or caveolae-mediated endocytosis were used to provide direct evidence that PEDV uses this pathway for entry. We traced and visualized PEDV location in Vero



**Fig. 1.** PEDV infection with or without trypsin. Vero cells were infected with KPEDV-9 at 37 °C for 2 h and then incubated in trypsin-free MEM (upper panel) or MEM containing 10  $\mu$ g/ml trypsin (lower panel). At the indicated time points, infected cells were fixed for immunocytochemistry (magnification: 20 $\times$ ).

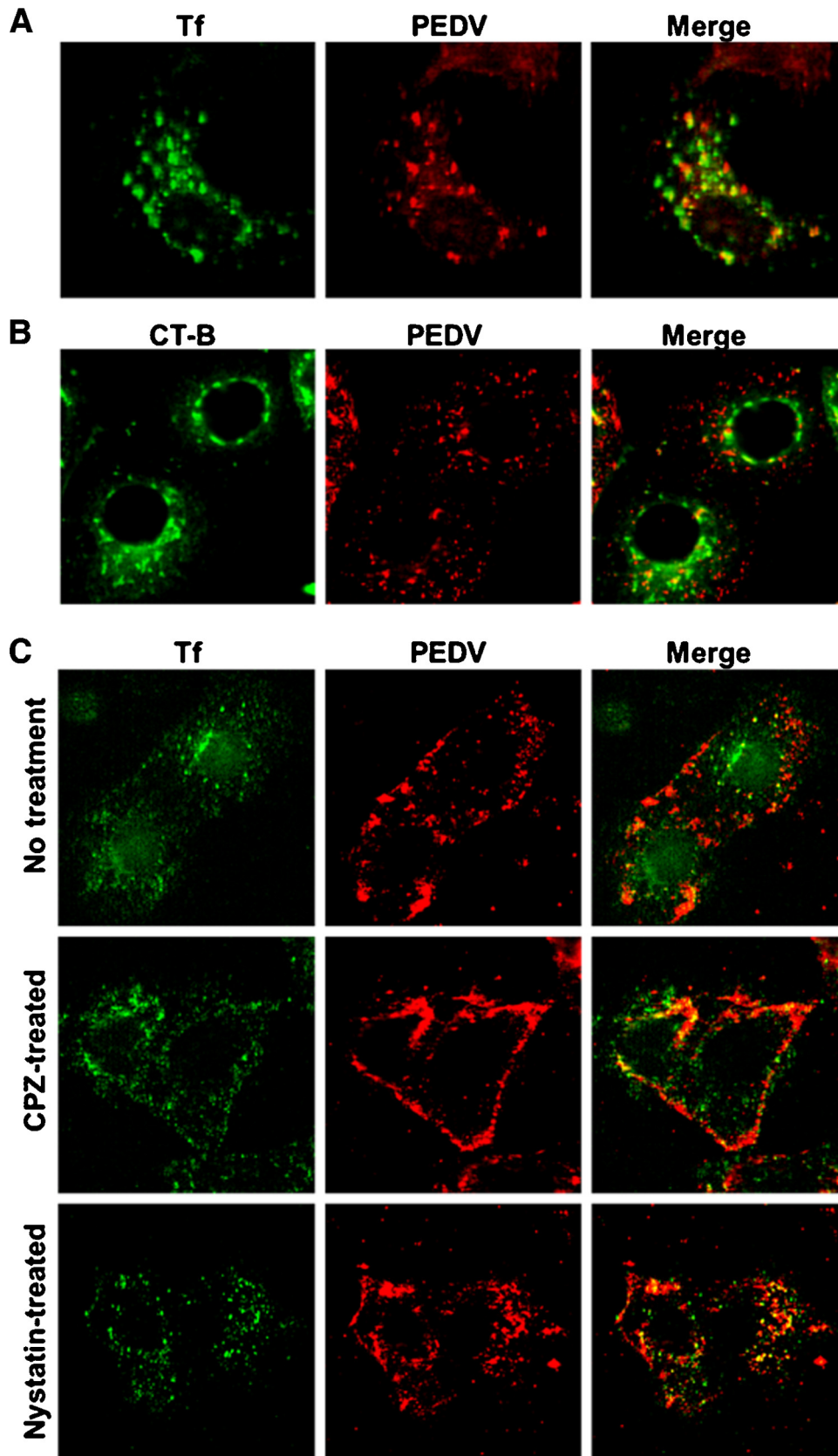


**Fig. 2.** PEDV entry via clathrin-mediated endocytosis. (A) Vero cells were treated with various concentrations of chlorpromazine (CPZ) prior to infection, and infected with KPEDV-9. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values. (B) PEDV N proteins in PEDV infected Vero cells were determined by western blotting at 24 hpi. The expression of endogenous  $\beta$ -actin was used as internal loading control. (C) Vero cells were treated with 0.45 M sucrose prior to infection, and infected with KPEDV-9. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values.



**Fig. 3.** PEDV entry via caveolae-mediated endocytosis. (A) Vero cells were treated with various concentrations of nystatin prior to infection, and infected with KPEDV-9. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values. (B) PEDV N proteins in PEDV infected Vero cells were determined by western blotting at 24 hpi. The expression of endogenous  $\beta$ -actin was used as internal loading control.

cells co-incubated with transferrin (Tf) and cholera toxin B (CT-B). Tf is transported into the cells in a vesicle by receptor-mediated clathrin-dependent endocytosis pathway. CT-B specifically binds to glycolipid monoganglioside at the plasma membrane and is internalized and delivered to the Golgi complex through caveolae-mediated endocytosis pathway (Nichols, 2002). Vero cells were incubated with fluorescence-labeled PEDV along with each marker, and then evaluated their subcellular localizations by confocal microscopy. At 4 h later, both endocytic markers, CT-B and Tf, were located in the cytoplasm. Co-localization was observed only between PEDV and Tf (Fig. 4A), but not with CT-B (Fig. 4B). To further confirm these findings, Vero cells were treated with CPZ and nystatin prior to PEDV inoculation. As shown in Fig. 4C, PEDV and Tf were co-localized in the cytoplasm in both nystatin and mock-treated, but not in CPZ-treated Vero cells. Both PEDV and Tf were found only on cell surface indicating that clathrin-mediated



**Fig. 4.** Colocalization of PEDV with transferrin (Tf) or cholera toxin B subunit (CT-B). (A, B) Colocalization (yellow) between KPEDV-9 (red) and Tf (green, A), but not between KPEDV-9 and CT-B (green, B), was observed in the merged images. (C) Colocalization of (yellow) KPEDV-9 and Tf was observed inside cells in the mock- and nystatin-treated Vero cells. By contrast, CPZ treatment inhibited internalization of both KPEDV-9 and Tf. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

endocytosis was successfully (or completely) inhibited by CPZ treatment. Taken together, we confirmed and concluded that the clathrin-mediated endocytosis pathway was important for PEDV entry.

### 3.3. The effect of low pH on PEDV S-mediated entry and cell fusion

We next explored whether PEDV infection requires the acidic environment in endosomal compartments. For the inhibition of endosomal acidification, Vero cells were treated with either  $\text{NH}_4\text{Cl}$ , a relatively weak base accumulating inside endosomal vesicles, or Baf-A1, specific inhibitors of the vacuolar  $\text{H}^+$ -ATPase in animal and other eukaryotic cells. Neutralization of pH in acidic organelles was confirmed by a fluorescent pH indicator probe, LysoSensor (data not shown). We evaluated the inhibitory activities of different lysosomotropic reagents by measuring infected cells. Both lysosomotropic reagents showed strong inhibitory effects on production of progeny PEDV, especially with 60% reduction at 10 hpi output titer by  $\text{NH}_4\text{Cl}$  (Fig. 5A). PEDV replication was about 80% inhibited at concentrations as low as 100 nM of Baf-A1. These results indicated that PEDV entry was very sensitive to low pH and acidic condition in endosome and/or late endosome might be critical for its entry.

For further observation whether acidification solely induce PEDV S-mediated fusion, we evaluated PEDV S-mediated cell–cell fusion in acidic conditions. As shown in Fig. 5B, low pH did not induce cell–cell fusion. Syncytium formation was not observed in any cells under the condition between pH 3 and pH 6 (panels a–d). Similarly, no syncytium formation was found under neutral pH condition (panel e). This is in stark contrast to the cell–cell fusion observed after the addition of trypsin in the culture media at neutral pH (panel f). Collectively, we concluded that the acidic condition is important but still not sufficient for PEDV S-mediated membrane fusion. And also, the fusogenic property of S protein could be activated by proteolytic processing.

### 3.4. Importance of proteases in PEDV entry

To evaluate the role and effect of proteases other than trypsin in PEDV entry, we used protease inhibitors. First of all, we checked cytotoxicity of all those inhibitors to exclude false results with recommended concentrations, and confirmed no cell damaged by them (data not shown). As shown in Fig. 6A, we found AEBSF-HCl induced the strongest inhibitory activity with more than 90% inhibition. In contrast, E-64, pepstatin A, and phosphoramidon showed relatively lower inhibition with about 5–10% inhibition. These results suggested that serine proteases were importantly involved in PEDV entry into Vero cells.

For the cases of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and MHV-2, it has been reported that pH-dependent endosomal cellular factors were required for proteolytic activation of S proteins, rather than the virus requiring an acidic trigger itself (Qiu et al., 2006; Simmons et al., 2005). To evaluate this for PEDV S case, cells were treated with AEBSF-HCl in combination with  $\text{NH}_4\text{Cl}$  prior to infection. As shown in previous results, PEDV infection was significantly inhibited either by AEBSF-HCl or  $\text{NH}_4\text{Cl}$  treatment (Fig. 6B). The inhibitory effect on combination treatments by both was similar to that of AEBSF-HCl single treatment.

S protein activation by exogenous proteases renders coronavirus S mediated virus–cell fusion independent of cathepsin activity. Finally, we determine whether trypsin treatment bypass entry inhibition by endocytosis inhibitors. Vero cells were pretreated with either CPZ (10  $\mu\text{g}/\text{ml}$  for 30 min) or  $\text{NH}_4\text{Cl}$  (50 mM for 2 h) prior to virus infection and then infected with PEDV in the absence or presence of trypsin. PEDV infectivity was only slightly facilitated by trypsin treatment and trypsin treatment does not overcome the

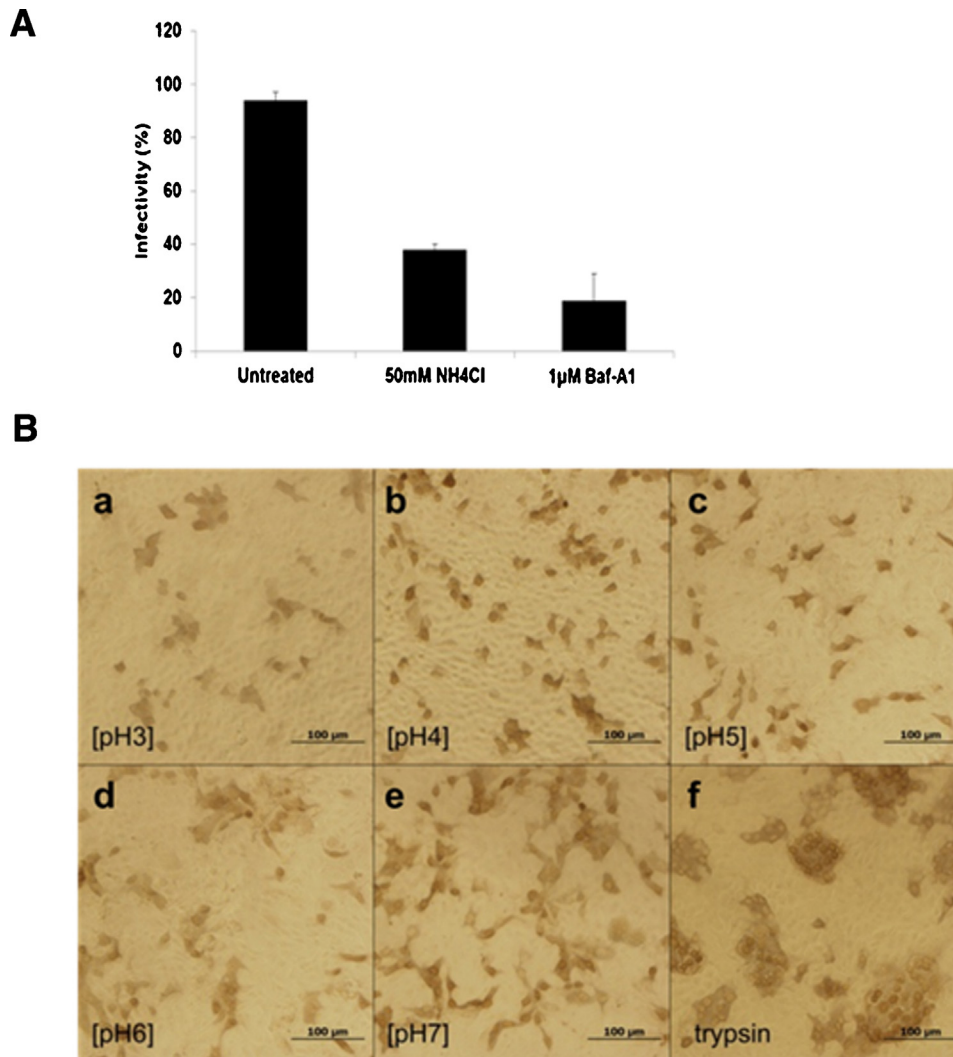
inhibitory effect of  $\text{NH}_4\text{Cl}$  and CPZ. The small increases of PEDV infectivity might be obtained by rapid spreading of virus infection via cell–cell fusion. These results suggested that trypsin did not support proteases-mediated virus–cell entry.

## 4. Discussion

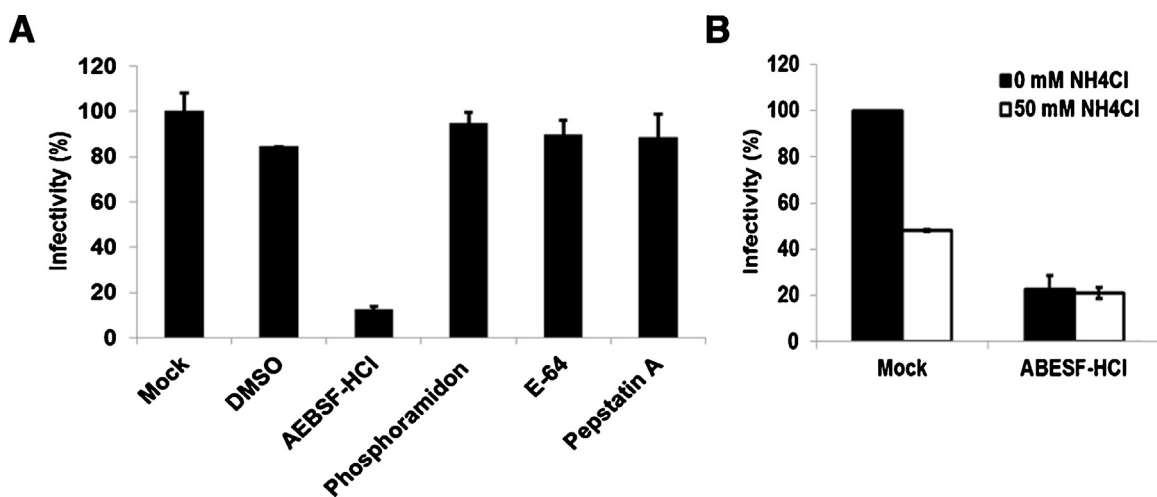
The entry mechanism of PEDV, a coronavirus, is largely unknown. Here, to examine the entry pathway of PEDV into Vero cells, we used essentially two independent and complementary approaches: (i) the focus formation assay to assess the level of infection by viruses in cells treated with various inhibitors, and (ii) fluorescence microscopy to monitor the entry of viruses into cells along with well-established markers. Both approaches provided similar conclusions on the mechanism of PEDV cell entry. The infection inhibition assay using various substrates that interfere with endocytosis or lysosomotropic agents revealed that PEDV enters Vero cells via clathrin-mediated endocytic uptake and delivery of virus to an acidic intracellular compartment. More interestingly, we found that PEDV requires serine proteolytic processes in early stages of infection. The serine proteolysis activates PEDV entry in independent manner with acidic pH environment, but did not bypass the infection reduction by lysosomotropic agents.

In the cell entry of many coronaviruses, the proteolytic activation of S proteins triggers viral membrane fusion and essentially required for virus entry (Huang et al., 2006; Qiu et al., 2006; Simmons et al., 2005). Cathepsins, which is a family of cysteine proteinases commonly found in acidified endosomes, have been associated with the proteolytic processing of S glycoproteins in SARS-CoV, MHV-2, and hCoV-229E and mediated viral membrane fusion with endosomal membranes within endosomes (Kawase et al., 2009; Qiu et al., 2006; Regan et al., 2008; Simmons et al., 2005; Turk et al., 1999). Similarly, various exogenous and cellular proteases such as trypsin, transmembrane proteases serine 2 enhance SARS-CoV entry by inducing virus–cell fusion at cell surface (Glowacka et al., 2011; Matsuyama et al., 2005, 2010; Shulla et al., 2011). The block to infection mediated by lysosomotropic agents could be bypassed by treating with exogenous or cellular proteases (Matsuyama et al., 2005; Simmons et al., 2005).

PEDV infection *in vitro* is also largely dependent on trypsin supplement (Hofmann and Wyler, 1988; Park et al., 2011). It raises possibility that PEDV S could fuse with plasma membrane by activation with proteases to deliver their genomes into cells. However, our study demonstrated that PEDV entry occurs without exogenous proteases. Although PEDV infection was likely facilitated by trypsin treatment as demonstrated earlier, PEDV also propagated even without trypsin (Fig. 1). Based on our results, we could confirm that exogenous protease, especially trypsin, might be critical factor for cell–cell fusion but not for viral envelope–cell membrane fusion. And also, we could conclude that trypsin is not essentially required for virus entry into Vero cells. Our results encouraged us to hypothesize that PEDV penetration must have been facilitated by fusion of its envelope with the host membrane in a fusion-permissive environment, which most likely occurs inside endosomal compartments. PEDV might take endosomal entry pathway rather than direct fusion. The experiment using various inhibitors supported our hypothesis that PEDV alters endosomal pathway for their entry. PEDV infection was greatly diminished by pre-treatment with CPZ and hypotonic sucrose (Fig. 2). It suggests that PEDV enters Vero cells via clathrin-mediated pathway. Furthermore, co-localization between endocytosed Tf and fluorochrome-labeled PEDV may support conclusion that clathrin mediated endocytic uptake is major pathway for entry (Fig. 4). Our data collectively propose that PEDV enters Vero cells via clathrin-mediated endocytosis similar to other coronaviruses.

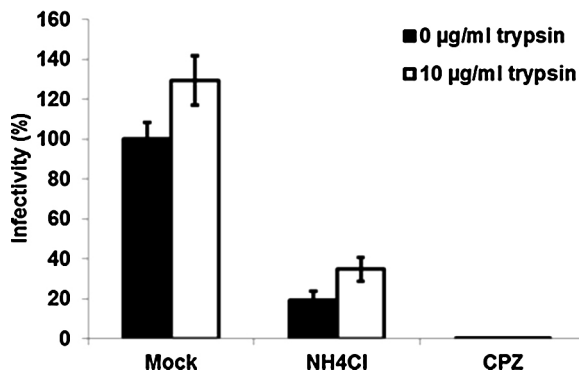


**Fig. 5.** PEDV infection in Vero cells treated with lysosomotropic agents. (A) Lysosomotropic agents inhibit PEDV entry. Vero cells were treated with lysosomotropic agents, either NH<sub>4</sub>Cl or Baf-A1, and then infected with KPEDV-9. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values. (B) Low pH does not convert the PEDV S protein to its fusogenic form. PEDV infected Vero cells were exposed to various pH conditions, and then incubated in serum-free media for 4 h. Low to neutral pH range (pH 3–7) did not induce cell–cell fusion of PEDV-infected Vero cells. In contrast, the addition of trypsin at neutral pH readily induced cell–cell fusion within 4 h after treatment (lower right).



**Fig. 6.** Serine proteases are involved PEDV entry in Vero cells. (A) Vero cells were treated with four different proteases inhibitors as described in Materials and Methods, and then infected with KPEDV-9. (B) Vero cells were treated with AEBSF-HCl and/or NH<sub>4</sub>Cl, and then infected KPEDV-9. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values.





**Fig. 7.** Trypsin did not bypass the infection inhibition of endocytosis. Vero cells were treated with NH<sub>4</sub>Cl or CPZ, and then infected KPEDV-9 in the presence or absence of trypsin. PEDV entry was scored by immunocytochemistry at 10 hpi. The relative infectivity was shown as percentages of infected cells to untreated cells. The error bars represent standard deviations of the mean values.

In order to mediate membrane fusion enveloped viruses, such as influenza and dengue, the low pH of acidified endosomal compartments is sufficient for their conformational changes (Plempner, 2011). Unlike these viruses, pH-dependent endosomal cellular factors were required for activation of low pH-dependent proteases, rather than the virus requiring an acidic trigger itself (Huang et al., 2006; Simmons et al., 2005). Similar to other coronaviruses, PEDV infection was significantly inhibited by both NH<sub>4</sub>Cl and Baf-A1 (Fig. 5A), but acidic pH did not induce PEDV S-mediated fusion (Fig. 5B), suggesting that low pH did not differently mediate virus fusion and another triggering factors such as proteolytic activation might be required for successful fusion.

While trypsin induce the fusion activity of PEDV S proteins on the plasma membrane as demonstrated by formation of syncytia (Fig. 1), it was not clear whether similar conditions are required for fusion between its envelope and endosomal membrane. PEDV entry into Vero cells was specifically inhibited by serine proteases inhibitor, but not by other proteases (Fig. 6A). It suggests that serine or serine-like proteases in the cytoplasm are involved in facilitating the fusion between PEDV envelope and host endosomal membrane. It is not clear which serine proteases are involved in PEDV entry, however, we are assuming that intracellular serine proteases may cleave the S protein so that induce membrane fusion during infection. As shown in Fig. 6B, inhibitory effect of serine proteases inhibitors was observed when the lysosomotropic reagents were treated, indicating that novel serine protease(s) is involved in PEDV entry with acidic pH independent manner unlike low pH-dependent endosomal cathepsins. In addition, our finding that trypsin treatment is not bypass the infection inhibition with lysosomotropic agents and CPZ as shown in Fig. 7, clearly indicates that PEDV entry requires pH-dependent step rather than the presence of pH-dependent proteolytic processing. Recently, similar possibilities were proposed by others in MHV-A59 S mediated infection study (Wicht et al., 2014).

Another question is how trypsin sufficiently mediates cell–cell fusion at neutral pH. It was reported that the furin cleavage of S on MHV and SARS-CoV is required for virus–cell fusion, but not for cell–cell fusion (de Haan et al., 2004; Follis et al., 2006). It seems like coronavirus S-mediated cell–cell fusion is regulated by different manner from virus–cell fusion. Acidic pH and serine proteolysis are sufficient for virus–cell fusion, in contrast, serine proteolysis and/or unknown other factor(s) which may provide similar condition to low pH, are required for cell–cell fusion at plasma membranes; thus S induces membrane fusion without low pH.

## 5. Conclusions

All our data confirmed that PEDV entry followed clathrin-mediated endocytosis independence of caveolae-coated pit assembly. The internalized PEDV was co-localized with the clathrin-mediated endocytic marker, but not with the caveolae-mediated endocytic marker. In addition, cells treated with lysosomotropic agents and serine protease inhibitors were resistant to PEDV. Our data revealed that PEDV entry followed clathrin-mediated endocytosis and was dependent on an acidic pH and serine proteolysis for successful entry into cells.

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