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## **The Influence of the Host Cell and Trypsin Treatment on Bovine Coronavirus Infectivity**

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*With 5 figures*

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### **Summary**

We examined the effect of trypsin on the infectivity of bovine coronavirus strain L9 (BCV-L9). Sets of cultures of human rectal tumor (HRT-18) cells, four clones of HRT-18 cells, and bovine fetal spleen (D2BFS) cells were infected in duplicate with serial dilutions of BCV-L9. Infected cultures were propagated in trypsin-containing or trypsin-free medium, and virus yields were measured as plaque forming units (PFU) and hemagglutination (HA) titers. The effect of trypsin was assessed by comparing PFU:HA ratios obtained in the presence and absence of trypsin. Trypsin-enhanced infectivity was evident in cultures of HRT-18 parent cells, D2BFS cells, and 3 of the 4 HRT-18 clones when the cultures were inoculated with high dilutions of virus. The degree of enhancement varied between the different cell cultures. The data indicate that trypsin modification influences BCV infectivity, but this effect depends on the host cell. The effect of different trypsin-treatment conditions on the production of infectious virus was explored with D2BFS cells. Treatment of D2BFS cells with trypsin prior to infection had no effect on virus yield. The presence of trypsin in culture medium of infected cells resulted in significantly enhanced virus yields.

Key words: Bovine coronavirus infectivity, trypsin modification

### **Introduction**

Proteolytic cleavage of surface proteins is a prerequisite for infectivity in a number of virus systems. Specifically, orthomyxovirus requires cleavage of the 75–80 Kd HA protein into the 36 Kd HA1 and the 27 Kd HA2 species for the virus to be infectious. Activation of the cell-fusing potential and infectivity of paramyxovirus is a result of cleavage of the F<sub>0</sub> precursor protein into F1 and F2 (52 and 13 Kd, respectively) which are disulfide-linked subunits of the active F protein. Activation of the infectious potential of these two viruses is accomplished either by cellular proteases or by added trypsin (CHOPPIN and SCHEID, 1980; GARTEN et al., 1981; HOMMA, 1971; HOMMA, 1972; HOMMA and OHUCHI, 1973; HOMMA and TAMAGAWA, 1973; HUANG et al., 1981; PEEPLES and BRATT, 1984; ROTT, 1979; SCHEID and CHOPPIN, 1974). The infectivity of rotaviruses can be increased if the outer capsid polypeptide VP3 is cleaved into VP5 and VP8 (60 Kd and 28 Kd, respectively).

Cleavage by trypsin, elastase, or pancreatin causes an increase in infectivity of rotaviruses (ESTES et al., 1981; LOPEZ et al., 1986).

Proteolytic cleavage of the 180 Kd envelope protein of mouse hepatitis virus (MHV) into its 90 Kd subunits activates the cell-fusing activity of the virus, but this cleavage pattern has not been correlated to an increase in the infectivity of the virus. Cell-dependent differences in processing of MHV proteins were demonstrated (FRANA et al., 1985; STURMAN et al., 1985). Trypsin enhances the plaque development and the cell-fusing capacity of avian, bovine, and murine coronaviruses (OTSUKI and TSUBOKURA, 1981; STORZ et al., 1981; YOSHIKURA and TEJIMA, 1981), and the polypeptide profile of BCV is altered by trypsin treatment (WILLIAMS, 1983). TOTH (1982) found that the rate of replication and the infectivity of BCV was enhanced when the virus was propagated in bovine embryonic lung cells in the presence of trypsin.

We reported that the cytopathic expression and plaque development of bovine coronaviruses (BCV) are dependent upon the cell type used for virus propagation, the strain of virus assayed, and the presence of trypsin in the culture system (ST. CYR-COATS and STORZ, 1988). The purpose of the present investigation was to determine the influence of the host cell and trypsin treatment on the infectivity of BCV.

## Material and Methods

### *Cells and Virus*

The human adenocarcinoma cell line HRT-18 (TOMPKINS et al., 1974) and HRT-18 cell clones 3F3, D2, 3E3, and 4B3, established by limiting dilution, were maintained in Dulbecco's modified minimum essential medium (DMEM) containing penicillin (100 units/ml) and streptomycin (100 µg/ml). The medium was supplemented with 5% fetal calf serum. D2BFS cells were derived from bovine fetal spleen cells. They are a population of cells which survived precrisis and continue to multiply well past the 30th passage. D2BFS cells were maintained in Eagle's minimum essential medium (MEM) containing 25 mM Hepes and penicillin (100 units/ml)-streptomycin (100 µg/ml). MEM was supplemented with 10% fetal calf serum. Serum was not used in the medium for virus propagation.

Bovine coronavirus strain L9 (BCV-L9), adapted to cell culture by MEBUS et al. (1973), was used in this study.

### *Comparative Infectivity of Virus Propagated in the Presence or Absence of Trypsin*

HRT-18 and D2BFS cells to be infected were cultured in 25 cm<sup>2</sup> tissue culture flasks until confluency was reached. Monolayers were washed with Dulbecco's phosphate buffered saline (PBS) and infected in duplicate with dilutions of BCV-L9 ranging from  $1 \times 10^{-2}$  to  $1 \times 10^{-6}$ . Virus was allowed to adsorb for 1 h at 37°C. For each cell type, paired flasks received either trypsin-free medium or medium containing trypsin. HRT-18 cell types received 5 µg/ml trypsin; D2BFS cells received 7.5 µg/ml trypsin. The flasks were incubated at 37°C for 48 h and then frozen at -70°C. Thawed cell lysates were sonicated for 15 sec, and aliquots were frozen. Samples were titrated for virus yields by hemagglutination and plaque assays.

### *Hemagglutination Assay*

Twenty-five microliter samples of infected cell lysates were diluted in a two-fold series in 96-well microtiter plates using PBS as diluent. Mouse erythrocytes at a 1% concentration were added to each well. Plates were agitated to assure mixing in wells, incubated at room temperature for 2 h or overnight at 4°C, and then examined for hemagglutination.

### *Plaque Assay*

Confluent monolayers of HRT-18 cells in 6-well polystyrene plates were used to quantitate the infectious virus yields. The washed monolayers were inoculated with 0.5 ml of 10-fold serially diluted samples of infected cell lysates. Plates were incubated for 1 h at 37°C in a 5% CO<sub>2</sub> atmosphere, and excess inoculum was removed. Overlay medium consisting of DMEM, 0.6% agarose (Gibco, electrophoresis grade), and 2.0 µg/ml trypsin (Difco, 1:250) was added to each well. The cells were incubated for 3-4 days, stained for 2 h with neutral red, and fixed for 10 min in formalin saline. The overlays were removed, and the plaques were counted.

*Quantitation of Viral Infectivity*

Virus yields titrated by plaque assay and hemagglutination assay were compared to give PFU:HA ratios. These values represent the number of plaque-forming virus particles per hemagglutinating unit. Graphs were plotted as  $\log_{10}$  PFU:HA versus inoculum virus dilution.

*Comparison of Virus Yields Using Different Trypsin-Treatment Conditions*

Duplicate 25 cm<sup>2</sup> flasks of confluent D2BFS cells were treated as follows:

Set 1 — Cells were washed three times with Dulbecco's PBS to remove residual fetal calf serum, then treated with 2 ml of MEM containing 1  $\mu$ g/ml trypsin (Sigma, TPCK treated). Cells were incubated for 10 min at 37°C. Cells were washed to remove trypsin, then infected with BCV-L9 from a stock which was propagated in D2BFS cells without trypsin (MOI =  $6 \times 10^{-4}$  PFU/cell). After adsorption for 1 h at 37°C, excess inoculum was removed, and cells were washed. MEM without trypsin was added to the cultures.

Set 2 — Untreated cells were infected with virus as described in set 1. After adsorption, MEM containing 1  $\mu$ g/ml trypsin was added to the cultures.

Set 3 — Virus was treated with 1  $\mu$ g/ml trypsin for 30 min at 37°C. Trypsin activity was stopped by adding 1  $\mu$ g/ml soybean trypsin inhibitor (STI, Sigma) and by incubating for 30 min at 4°C. Treated virus, diluted to an MOI of  $6 \times 10^{-4}$  PFU/cell, served as inoculum. After adsorption, medium lacking trypsin was added to the cultures.

Set 4 — The virus was pretreated with trypsin as described in Set 3, but STI was not added to this set. Instead, the action of trypsin was stopped by immediate dilution of the virus to  $6 \times 10^{-4}$  PFU/cell. Following infection, MEM containing 1  $\mu$ g/ml trypsin was added.

Set 5 — Untreated cells were infected with untreated virus. After adsorption MEM without trypsin was added.

All flasks were incubated for 24 h, then frozen at -70°C. Cells were thawed, sonicated, and aliquots were frozen. Yields were titrated from duplicate samples obtained from replicate flasks by plaque assay in HRT-18 cells. The four values were averaged to obtain the PFU titer.

*Statistical Methodology*

The Statistical Analysis System (SAS) was used to analyze the data with the general linear model procedure and Tukey's Studentized Range Test for analysis of individual mean differences. All individual mean separation tests were considered significant at a probability level of 0.05.

**Results***Effect of Trypsin on BCV Infectivity*

BCV-L9 propagated in D2BFS cells showed maximal increase in PFU:HA at dilutions  $1 \times 10^{-4}$  and  $1 \times 10^{-5}$ . Virus was not detectable at these dilutions in the absence of trypsin, while the PFU:HA ratio reached peak level in the presence of trypsin. Enhanced infectivity yields were not apparent at lower dilutions of the inoculum virus (Fig. 1). The infectivity ratios produced by BCV-L9 in HRT-18 parent cells remained nearly identical with and without trypsin through the  $1 \times 10^{-5}$  dilution of virus. At the  $1 \times 10^{-6}$  dilution of inoculum, virus was not detected in the absence of trypsin while infectivity was high in the presence of trypsin (Fig. 2).

PFU:HA values obtained with trypsin are compared to those obtained without trypsin are compared in Figure 3. Comparisons are made for each cell type at the highest limiting dilution producing detectable virus. In the presence of trypsin infectious virus yields were increased by 5:1, 2:1, and 3:1 margins in clones 3F3, D2, and 3E3, respectively. In contrast, the infectious virus titer was decreased in 4B3 cells in the presence of trypsin. It was numerically impossible to calculate with: without trypsin ratios for D2BFS and HRT-18 cells at the limiting virus dilutions because the PFU:HA value in the absence of trypsin was zero.

The PFU:HA ratios of with: without trypsin for each cell type at various inoculum dilutions are displayed graphically in Figure 4. This graph simplifies the comparison of the effects of trypsin on viral infectivity between the different cell types. Enhancement of infectivity was apparent in D2BFS cells at the  $10^{-4}$  and higher inoculum dilutions. This

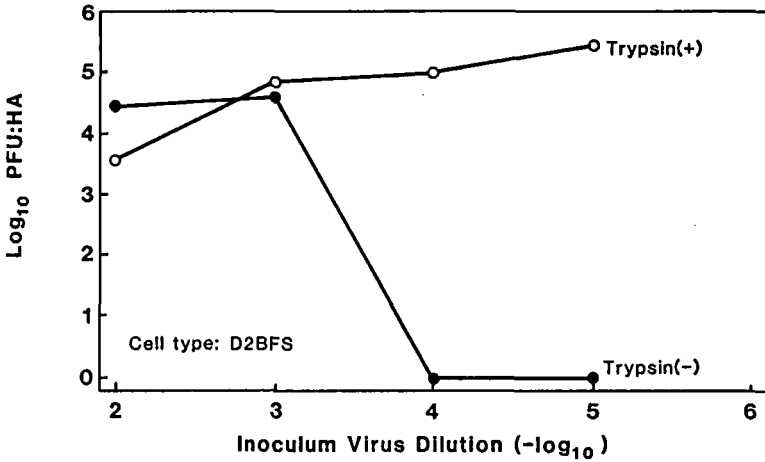


Fig. 1. Infectivity ratios of BCV-L9 propagated in D2BFS cells with or without trypsin

dilution represents an initial MOI of  $3 \times 10^{-4}$  PFU/cell. HRT-18 cells produced at comparable enhancement of viral infectivity in the presence of trypsin at the  $1 \times 10^{-6}$  dilution (initial MOI of  $3 \times 10^{-7}$  PFU/cell). When BCV was propagated in HRT-18 clones D2 and 3E3, trypsin enhancement of viral infectivity was evident at the  $10^{-4}$  dilution. A detectable increase in infectious virus yields was evident in clone 3F3 cells at  $10^{-6}$  dilution, but at the same dilution BCV infectivity yields were decreased by trypsin when the virus was propagated in clone 4B3.

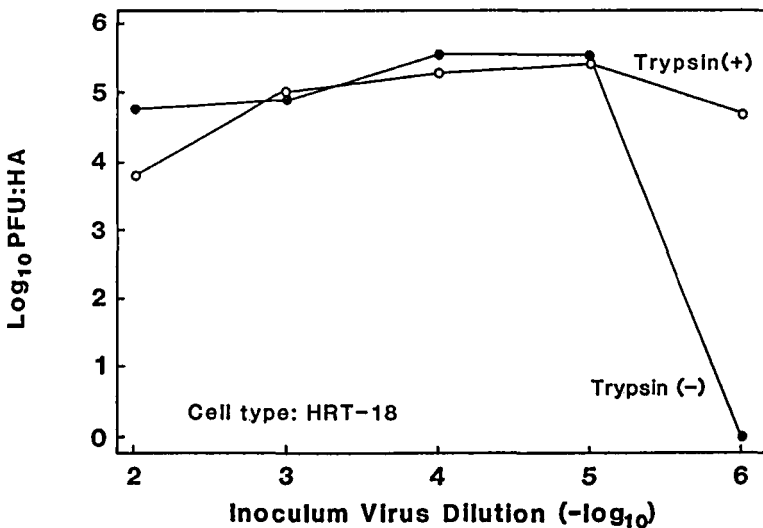


Fig. 2. Infectivity ratios of BCV-L9 propagated in HRT-18 cells with or without trypsin

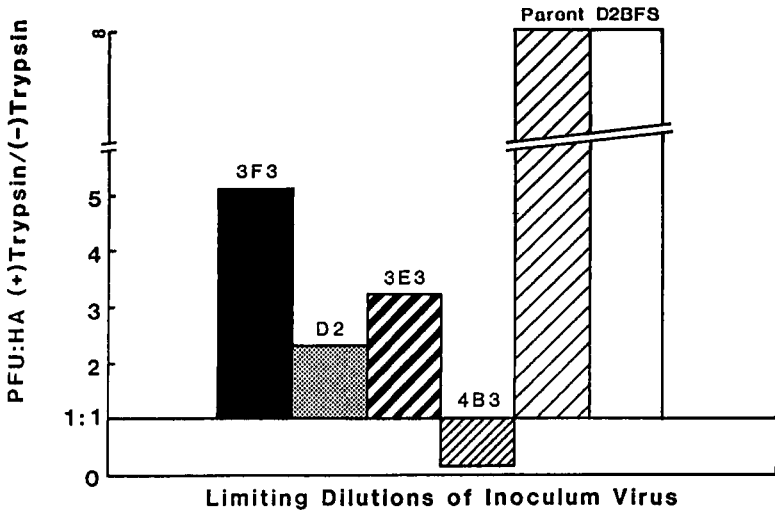


Fig. 3. Infectivity ratios with trypsin to without trypsin for BCV-L9 propagated in each cell type at the limiting dilution of virus

*Effect of Different Trypsin-Treatment Schemes on BCV Infectivity Yields*

The yields of BCV infectivity in D2BFS cells under different trypsin-treatment conditions are displayed as log<sub>10</sub> PFU in Figure 5. The PFU counts were essentially the same for trypsin-pretreated cells (set 1) and the non-trypsin-treated control system (set 5).

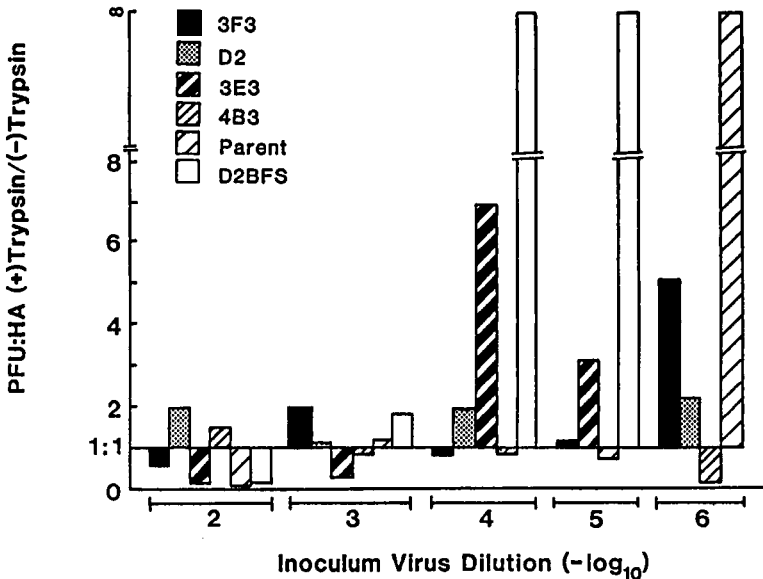


Fig. 4. Infectivity ratios with trypsin to without trypsin for BCV-L9 propagated in each cell type

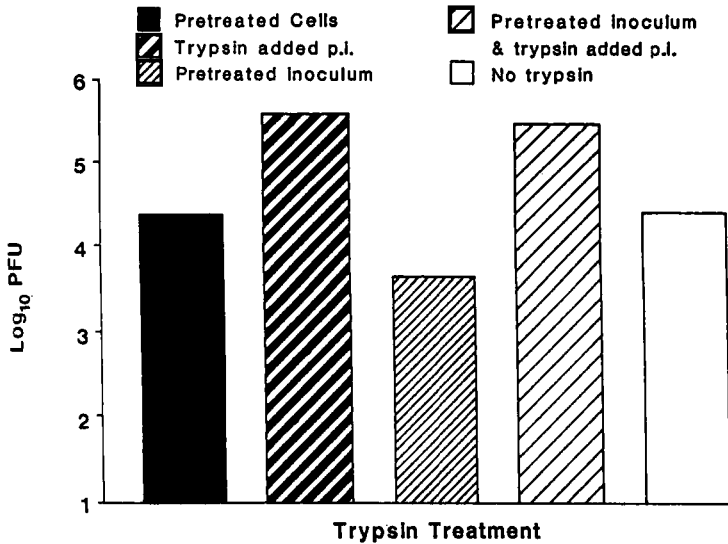


Fig. 5. Yields of infectious virus produced in D2BFS cells using different trypsin treatment schemes

Nearly identical infectivity yields were obtained when trypsin was added to infected cells after infection (set 2) and when the inoculum was pretreated, and trypsin remained present after infection (set 4). These values were significantly higher than those obtained from sets 1 and 5 ( $F = 134.56$ ,  $p < 0.0001$ ). The lowest yield was obtained from set 3 (inoculum pretreated with trypsin, then soybean trypsin inhibitor). This set was significantly lower than all other treatments.

### Discussion

The effect of trypsin on BCV infectivity was determined by comparing PFU:HA ratios of infected cultures propagated in the presence or absence of trypsin. This ratio represents the amount of infectious virus per hemagglutinating unit of given sample. This method of quantitation was chosen over a simple PFU titration because it demonstrated the effect of trypsin on a virus population consisting of infectious and non-infectious particles that both hemagglutinate.

Trypsin enhanced the infectivity of BCV-L9 propagated in HRT-18 parent and D2BFS cells to maximal levels. Where highest infectivity yields were obtained in the presence of trypsin, no virus was detectable without trypsin. This increase in infectivity was observed at a 100-fold lower inoculum dilution in D2BFS cells when compared with HRT cells. BCV propagated in the HRT-18 clones differed in trypsin enhancement and the dilution at which the increased yields appeared. A plausible explanation for these observations is that BCV-L9 requires proteolytic processing to activate their infectious potential. Progeny BCV consists of infectious and non-infectious virus in a given BCV-cell system, reflecting the cell's ability to process the virus to the infectious form by endogenous protease. Diluting the inoculum depletes infectious virus. Presumably, trypsin processes non-infectious progeny virus to the infectious form. If the amount of naturally-produced infectious virus exceeds non-infectious virus, then trypsin enhancement is not detectable. This interpretation would explain the marginal trypsin effect in the HRT-18 clones. Unlike D2BFS cells, which require trypsin for activation of virus infectivity, HRT-18 parent cells are innately capable of producing high yields of infectious BCV.

We reported that the CPE produced in D2BFS and HRT-18 cells infected with BCV was enhanced by trypsin, and the four BCV-infected HRT-18 clones varied in response to trypsin treatment (ST. CYR-COATS and STORZ, 1988). Clone 4B3, which is minimally affected by BCV with or without trypsin, was the only infected cell line producing lower infectivity yields in the presence of trypsin.

Different trypsin-treatment conditions of BCV-L9-infected D2BFS cells were employed to discern the target of trypsin action. Pretreatment of cells with trypsin had no effect on the production of infectious virus. Any putative effect of trypsin on the host cell did not affect virus production. On the other hand, the presence of the enzyme in culture medium enhanced the BCV titer. Consequently, the trypsin effect appears to be exerted directly on the virus, activating non-infectious to infectious virions. Trypsin alters the polypeptide profile of BCV (WILLIAMS 1983; ST. CYR-COATS et al., submitted). The trypsin-pretreated inoculum produced the lowest yields. A potential deleterious effect of the added soybean trypsin inhibitor on host cells or virus were not examined but may have contributed to the low yield.

We conclude from this evidence that trypsin-dependent infectivity of BCV is determined by the host cell. Trypsin is a constituent enzyme of the intestinal tract, the site of BCV replication in the natural host. This enzyme may play a potential role in activation of infectious virus in natural infections.

Differences were observed in some structural proteins of BCV-L9 propagated in different cell types (ST. CYR-COATS et al., submitted). FRANA and coworkers (1985) found the murine coronavirus MHV-A59 to be dependent on the host cell or trypsin treatment for activation of the cell-fusing activity of the virus. Proteolytic activation of infectivity was documented in paramyxoviruses, orthomyxoviruses, and rotaviruses as well (CHOPPIN and SCHEID, 1980; ESTES et al., 1981; GARTEN et al., 1981; HOMMA, 1971, 1972; HOMMA and OHUCHI, 1973; HOMMA and TAMAGAWA, 1973; LOPEZ et al., 1986; HUANG et al., 1981; ROTT, 1978; SCHEID and CHOPPIN, 1974; PEEPLES and BRATT, 1984).

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### Zusammenfassung

#### Der Einfluß von Wirtszelle und Trypsinbehandlung auf die Infektiosität boviner Coronaviren

Der Einfluß von Trypsin auf die Infektiosität des bovinen Coronavirusstammes L9 (BCV-L9) wurde untersucht. Dazu wurden menschliche Rektaltumorzellen (HRT-18), vier Zellklone der HRT-18-Zellen und bovine fötale Milzzellen (D2BFS) im Doppelansatz mit Verdünnungsreihen von BCV-L9 infiziert. Infizierte Kulturen erhielten Medium mit oder ohne Trypsin, und die Virusernten wurden als Plaque-bildende Einheiten (PBE) bzw. Hämagglutinationstiter (HA) gemessen. Der Einfluß von Trypsin wurde durch das in An- oder Abwesenheit von Trypsin erhaltene Verhältnis von PBE zu HA abgeschätzt. Eine Erhöhung der Infektiosität durch Trypsin wurde in HRT-18- und D2BFS-Zellen sowie in drei der vier Subklone von HRT-18-Zellen beobachtet, wenn die Kulturen mit hohen Verdünnungen von BCV infiziert worden waren. Der Grad der Erhöhung war jedoch bei den einzelnen Zellkulturen unterschiedlich. Die Daten weisen darauf hin, daß Trypsin die Infektiosität von BCV beeinflusst, wobei dieser Effekt aber von der Wirtszelle abhängt. Der Effekt unterschiedlicher Trypsin-Behandlungsverfahren auf die Ausbeute an infektiösem BCV wurde in D2BFS-Zellen untersucht. Trypsinbehandlung dieser Zellen vor der Infektion hatte keinen Einfluß auf die Virusausbeute. Die Gegenwart von Trypsin im Medium infizierter Zellen ergab dagegen signifikant höhere Ausbeuten an infektiösem Virus.

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