Comparative Properties of Feline Coronaviruses *in vitro*

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ABSTRACT

Two feline coronaviruses were characterized to determine their biological properties *in vitro* and their antigenic relatedness to a previously recognized feline infectious peritonitis virus and canine coronavirus. The viruses, designated WSU 79-1146 and WSU 79-1683, were shown to have comparable growth curves with the prototype feline infectious peritonitis virus. Treatment of the feline infectious peritonitis virus strains with 0.25% trypsin indicated that they were relatively resistant to proteolytic inactivation when compared with the feline enteric coronavirus strain. This observation may serve as a useful *in vitro* marker to distinguish closely related members of the feline coronavirus group. Plaque assay results indicated that the feline infectious peritonitis virus strains produced large homogeneous plaques in comparison to the feline enteric coronavirus strain and canine coronavirus, which showed a heterogenous plaque size distribution. No naturally temperature sensitive mutants were detected in either of the feline coronavirus populations. Both of the viruses were antigenically related to feline infectious peritonitis virus and to a lesser extent to canine coronavirus by virus neutralization.

Key words: Feline coronavirus, feline infectious peritonitis, feline enteric coronavirus, canine coronavirus.

RÉSUMÉ

Cette expérience consistait à caractériser deux coronavirus félins, afin de déterminer leurs propriétés biologiques *in vitro*, ainsi que leur relation antigénique avec un virus de la péritonite infectieuse féline, précédemment identifié, et un coronavirus canin. On démontra que ces deux coronavirus félins, à savoir : WSU 79-1146 et WSU 79-1683, affichaient une courbe de croissance comparable à celle du virus prototype de la péritonite infectieuse féline. Leur traitement avec une solution de trypsine 0,25% révéla leur résistance à l'inactivation protéolytique, à la faveur d'une comparaison avec la souche du coronavirus entérique félin. Cette propriété pourrait servir de marqueur *in vitro*, utile pour différencier les membres étroitement liés du groupe des coronavirus félins. Les résultats de la méthode des plages démontrèrent que les souches du virus de la péritonite infectieuse féline en produisaient des grandes et homogènes, tandis que la souche du coronavirus entérique félin et celle du coronavirus canin en produisaient, de dimensions variables. On ne détecta pas de mutants, naturellement sensibles à la température, chez l'un ou l'autre des coronavirus félins. L'épreuve de neutralisation virale permit de constater la parenté antigénique des deux coronavirus expérimentaux avec celui de la péritonite infectieuse féline et, à un degré moindre, avec le coronavirus canin.

Mots clés: coronavirus félin, péritonite infectieuse féline, coronavirus entérique félin, coronavirus canin.

INTRODUCTION

The feline coronaviruses are represented by a diverse number of isolates which have been shown to be indistinguishable by standard biological and biochemical criteria (1). Yet, these strains have a disease spectrum which ranges from mild enteric infections in domestic cats to a fatal immune-mediated vasculitis in both domestic and exotic cats, such as the cheetah (*Acinonyx jubatus*) (2). The classification of the feline coronaviruses currently relies upon this *in vivo* disease spectrum to identify the most widely divergent isolates (3). Those strains causing a mild to asymptomatic gastrointestinal infection are referred to as the feline enteric coronaviruses (FECV), and those strains which induce the immune-mediated vasculitis are referred to as feline infectious peritonitis viruses (FIPV) (4,5,6).

The recognition of different pathogenic variants of feline coronavirus has led to an increased emphasis on identifying *in vitro* properties of the strains which make them unique (7). Differentiation of FECV from FIPV may allow for an increased understanding of the mechanism of disease and the fatal immune-mediated vasculitis resulting from infection with FIPV (5,6).

The purpose of this report is to present some *in vitro* studies comparing two recently recognized feline coronaviruses, which have been classified as FECV and FIPV on the basis of their virulence in cats (4,5,8).

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MATERIALS AND METHODS

CELL CULTURE

Crandell feline kidney (CrFK) cells were obtained from the Naval Bioscience Laboratory (NBL-CrFK), University of California, Oakland, California, at the 175th passage. Cells were propagated in Eagle's minimum essential media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 50 μg/mL gentamicin. The cells were maintained in a humidified 5% CO_2 incubator at 37°C.

VIRUSES

A previously characterized feline coronavirus, NOR 15, was used as a FIPV prototype in the comparison studies (8). The NOR 15 strain was originally isolated using a spleen explant technique followed by three serial passages of infected spleen homogenates in specific-pathogen-free (SPF) cats. The two field isolates, designated WSU 79-1146 and WSU 79-1683, were originally derived from feline cases submitted to the diagnostic laboratory (4). The clinical data on these two cases and the preliminary characterization of these viruses has been reported previously (4). The infectivity titer of NOR 15 was $10^{5.5}$ $TCID_{50}$/0.025 mL. The infectivity titers of WSU 79-1146 and WSU 79-1683 were $10^{4.0}$ and $10^{4.7}$ $TCID_{50}$/0.025 mL, respectively. All three viruses were plaque purified prior to characterization studies (8).

A temperature sensitive (ts) mutant of NOR 15 (NOR ts), which was chemically derived (patent pending), was included in the study as a marker for identifying possible ts mutants of WSU 79-1146 and WSU 79-1683. Stock virus was prepared in a manner similar to NOR 15 and the field isolates, except that the cell incubation temperature was 31°C. When NOR ts was propagated and assayed at 31°C, the titer was $10^{4.8}$ $TCID_{50}$/0.025 mL.

Canine coronavirus (CCV), strain 1-71, was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland. Previous adaptation to CrFK cells and *in vitro* characteristics of CCV have been reported (9,10). The CCV was used to determine the antigenic relationship amongst the feline and canine coronaviruses and plaque variations (11).

GROWTH CURVES

Viral growth curves were conducted using a method modified from Otsuki *et al* (12). Briefly, NOR 15, WSU 79-1146 or WSU 79-1683 were inoculated onto 2 × 10^5 freshly trypsinized NBL-CrFK cells at a multiplicity of infection (MOI) of approximately 0.1 in 24-well plastic trays. Cell-associated virus (CAV) and extracellular virus (ECV) were collected for each of the viruses at predetermined times over 96 hours. Duplicate wells were sampled for each virus at designated hours post-inoculation (hpi), stored at -70°C, and assayed for infectivity at the completion of the growth curve.

EFFECT OF TEMPERATURE ON *in vitro* VIRAL INFECTIVITY

Duplicate cell culture flasks (25 cm^2) containing confluent monolayers of NBL-CrFK cells were used for infection with each virus (NOR 15, WSU 79-1146, WSU 79-1683 or NOR ts). After removal of the growth media, cells were infected with each virus at a MOI of approximately 0.01, and allowed to absorb for one hour at 37°C. At the end of one hour, MEM with 2% FBS and gentamicin was added to each flask. Infected cell cultures for each virus were incubated for 48 hours at either 31, 36 or 40°C. (The incubation period of 48 hours was chosen because maximum yields of virus were attained by 48 hours at 36°C in pilot growth curve studies.) The flasks with the virus-infected cells were treated by a single freeze-thaw cycle and the supernatant fluid clarified by centrifugation before being aliquoted and stored at -70°C. The supernatant fluids were assayed for viral infectivity at parallel incubation temperatures of 31, 36 and 40°C.

TRYPSIN SENSITIVITY

Sensitivity to 0.25% trypsin (DIFCO) was conducted as outlined by Otsuki *et al* (13). At the end of 6 h incubation at 37°C, a 1% trypsin inhibitor (MILES) solution was added to stop the reaction. The infectivity of each virus was assayed in duplicate by titrating the aliquots taken from the trypsin-treated and untreated tubes.

PLAQUE ASSAY

The viruses were plaque purified in NBL-CrFK cells three times prior to determining the plaquing profiles of CCV, NOR 15, WSU 79-1146, and WSU 79-1683 (8).

ANTIGENIC DETERMINATION

The microtiter method for virus neutralization (constant serum-dilute virus) was used to assess the antigenic relationship amongst NOR 15, WSU 79-1146, WSU 79-1683, and CCV. An equal volume of 1:20 dilution of serum and serial tenfold dilutions of virus were used. All sera were heat inactivated at 56°C for 30 minutes. Hyperimmune rabbit antiserum to NOR 15 virus was used as a feline coronavirus reference serum (8). Canine coronavirus antiserum was kindly supplied by Dr. L. Carmichael, Cornell University, Ithaca, New York.

RESULTS

GROWTH CURVES OF FELINE CORONAVIRUSES

The field isolates and NOR 15 appeared to have comparable growth curves at 36°C (Fig. 1). The ECV appeared in the cell culture supernate 8 hpi for NOR 15 and WSU 79-1146, and 16 hpi for WSU 79-1683. The ECV increased rapidly thereafter reaching maximum yields at 36 hpi for WSU 79-1146, and at 48 hpi for NOR 15 and WSU 79-1683. Virus yield declined between 72-96 hpi. The CAV titers consistently increased by 8 hpi for each virus and generally matched the optimum and decline of ECV titers with slightly lower virus yields. At times of maximum yields (36-48 hpi), the ratio of CAV to ECV was essentially equal.

TEMPERATURE SENSITIVITY STUDIES

The effect of incubation temperature upon the *in vitro* growth of the field isolates, as well as NOR 15, and NOR ts are presented in Figs. 2 and 3.

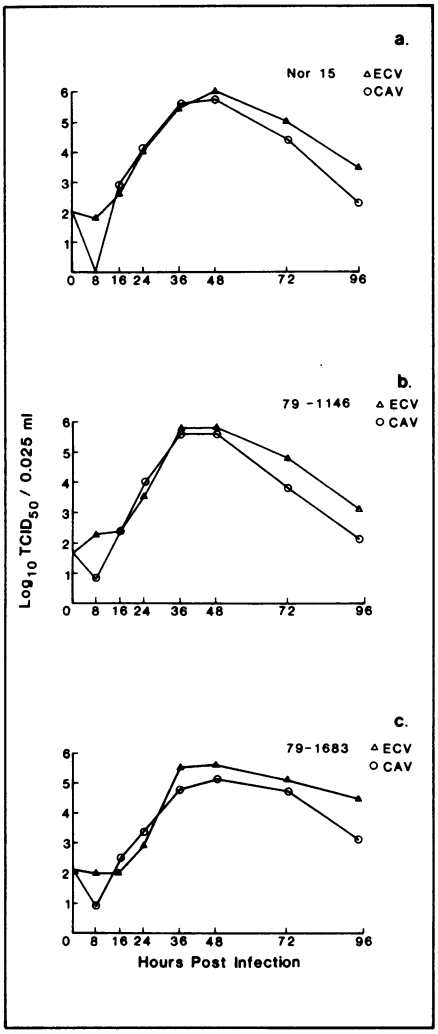

Fig. 1. Growth curves of feline coronavirus in NBL-CrFK cells at 36°C: 1) NOR 15; b) WSU 79-1146; and, c) WSU 79-1683. Extracellular virus (ECV), cell associated virus (CAV).

Both WSU 79-1146 and WSU 79-1683 had optimal infectivity when propagated at 36°C and subsequently assayed at 36°C or 40°C. When propagated at 31°C, WSU 79-1146 had no detectable infectivity when assayed at 31°C, but showed minimal infectivity (10^4 $TCID_{50}$ reduction of titer) when assayed at 36°C or 40°C. WSU 79-1683 failed to replicate when propagated at 31°C. When WSU 79-1683 was propagated at 36°C or 40°C, however, moderate to low yields of virus were obtained when assayed at 31°C. The prototype FIPV, NOR 15, had a temperature growth profile similar to that observed for WSU 79-1146. The chemically-induced temperature sensitive mutant, NOR ts, had maximum yields of virus when assayed at 31°C and to a lesser extent at 36°C subsequent to propagation at 31°C. NOR ts failed to replicate when either propagated or assayed at 40°C, which confirmed its restrictive growth at elevated temperatures (E.V. Davis, personal observation).

SUSCEPTIBILITY TO TRYPSIN

Treatment with 0.25% trypsin indicated that the FIPV strains, NOR 15 and WSU 79-1146, were relatively resistant ($\leq 10^1$ $TCID_{50}$ reduction) to proteolytic action when compared with the FECV strain, WSU 79-1683 which had its infectivity decreased by $10^{2.3}$ $TCID_{50}$ (Table I).

PLAQUING PROFILES

Plaque assay results showed three distinct plaque types (Fig. 4). Canine coronavirus plaques appeared as fuzzy diffuse areas; NOR 15 and WSU 79-1149 showed large (4 mm) distinct plaques; and WSU 79-1683 plaques were heterogeneous in size despite three cycles of plaque purification. Plaques for WSU 79-1683 showed a range in size from 1 mm to 5 mm.

ANTIGENIC RELATIONSHIPS

Serum neutralization with rabbit anti-NOR 15 serum resulted in a complete reduction in the infectivity of NOR 15, WSU 79-1146, WSU 79-1683, and CCV (Table II). Although there was significant neutralization ($> 10^2$ $TCID_{50}$/0.025 mL) of the feline coronaviruses by canine anti-CCV serum, the reduction in virus infectivity was not as great as that observed with the homologous system where CCV neutralization was complete.

TABLE I. Effects of Trypsin Upon Feline Coronavirus Infectivity

	Virus Infectivity[a]		
	FIPV		FECV
Treatment	NOR 15	79-1146	79-1683
Control, no treatment	5.6	4.8	4.6
Trypsin	5.2	4.4	2.3

[a] Titers expressed as $\log_{10}$ tissue culture infective dose 50% $TCID_{50}$/0.025 mL for duplicate cultures

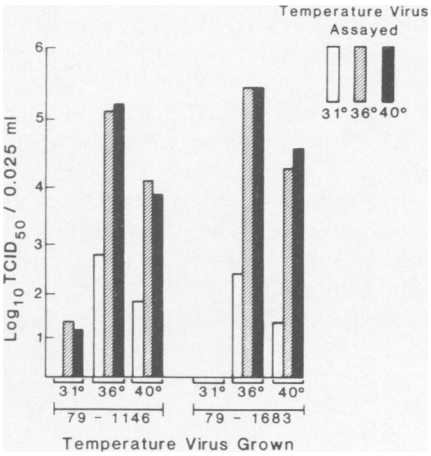

Fig. 2. The temperature assay of feline coronaviruses, WSU 79-1146 and WSU 79-1683, grown at 31, 36 and 40°C in NBL-CrFK cells. Bars indicate assay temperature for the viruses.

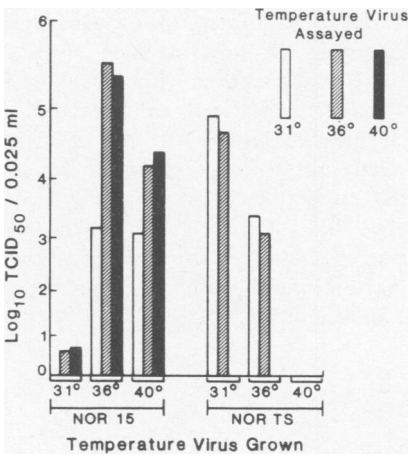

Fig. 3. The temperature assay of feline coronaviruses, NOR 15 and NOR ts, grown at 31, 36 and 40°C in NBL-CrFK cells. Bars indicate assay temperatures for the viruses.

DISCUSSION

The biological properties of the FIPV strains, NOR 15 and WSU 79-1146, were similar to the FECV, WSU 79-1683 in terms of growth curves in NBL-CrFK cells. Progeny virus was detected as early as 8 h post-infection, followed by a rapid increase

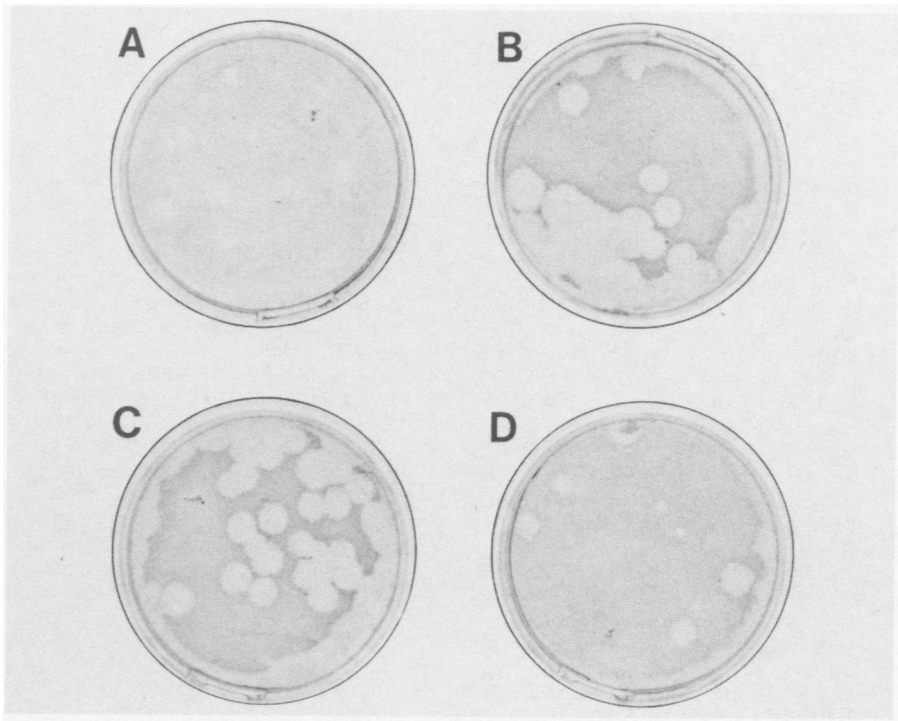

Fig. 4. Variation in plaque type among the canine and feline coronaviruses: A) canine coronavirus, 1-71; B) FIPV strain, NOR 15; C) FIPV strain, WSU 79-1146; and, D) FECV strain, WSU-1683. Note variation in plaque sizes for WSU 79-1683.

TABLE II. Comparative Serum Neutralization Among Feline and Canine Coronaviruses

	Virus Infectivity[a]			
	FIPV		FECV	CCV
Treatment	NOR 15	79-1146	79-1683	1-71
Control, no treatment	5.8	4.4	4.8	5.3
Virus treated with anti-NOR 15 serum	<1.0	<1.0	<1.0	<1.0
Virus treated with anti-CCV serum	1.8	1.5	2.8	<1.0

[a] Titers expressed as $\log_{10}$ tissue culture infective dose 50% $TCID_{50}$/0.025 mL for duplicate cultures

in infectivity with maximum viral titers attained at 36 to 48 h postinfection. The three viruses appeared to have primarily a cytolytic expression of cytopathogenic effect (CPE). The CAV closely followed the release of virus as detected from the ECV pattern. The feline coronavirus growth curves were consistent with growth curves observed with the avian coronavirus, infectious bronchitis virus (11), the prototype of the coronavirus group, and another cytolytic FIPV reported by Beesley *et al* (14).

Although temperature sensitivity has been proposed as one mechanism whereby coronaviruses can persist in nature and as a means to distinguish various strains *in vitro* (15), our studies indicated that the feline coronaviruses used in this study were able to replicate effectively at either 36°C or 40°C. There did not appear to be a substantial difference in the growth of the FIPV strains, NOR 15 and WSU 79-1146, when compared to the FECV strain, WSU 79-1683, at the various temperatures. The known temperature sensitive mutant, NOR ts, was shown to replicate effectively at 31°C and not at 40°C.

The observation that both the FIPV strains were relatively resistant to 0.25% trypsin, when compared to the FECV strain, indicated that this may serve as a useful *in vitro* marker to distinguish closely related members of the feline coronavirus group. The *in vitro* effects of digestive enzymes upon another coronavirus, transmissible gastroenteritis (TGE) of swine, indicated that virulent strains of TGE may have as much as 100 times the stability when compared to attenuated TGE virus strains (16). It was further proposed that the more virulent TGE strains correspondingly infect more sections of the gastrointestinal tract and induce higher levels of coproantibody (local antibody) than attenuated strains due to the apparent resistance to proteolytic enzymes (17).

The potential implications of naturally occurring trypsin-resistant strains of feline coronavirus are of interest and should be studied further in terms of explaining the pathogenesis of the various strains, as well as an approach toward effective prophylaxis (18). Trypsin has been used successfully to enhance the *in vitro* growth of certain feline coronaviruses (19). One mechanism whereby this may function could be the destruction of more attenuated portions of the virus population allowing for the most virulent to survive and, therefore, infect cells.

The occurrence of at least two populations of feline coronaviruses has been further documented by the plaquing profiles, which indicated that the FIPV strains had similar plaque types which differed from the FECV strain, WSU 79-1683, and CCV. The FIPV strains both produced clear, large, homogeneous plaques, while the FECV strain virus had a more heterogeneous population of plaque sizes. The CCV strain produced hazy plaques which were not uniform in size.

The antigenic similarity of WSU 79-1146 and WSU 79-1683 to NOR 15 substantiated the results of an earlier study which reported on the cross-reaction of these three viruses by indirect immunofluorescence (4). The partial virus neutralization of the three feline coronaviruses by CCV antisera indicated that there are some common type-specific antigenic sites between the feline and canine coronaviruses (11).

Despite their close antigenic relationship, Pedersen (1,5) and Davis (unpublished results) indicated that WSU 79-1146 and WSU 79-1683 exhibited different pathogenic poten-

tials in cats: WSU 79-1146 produced effusive FIP while WSU 79-1683 produced enteritis in specific-pathogen-free cats experimentally infected by the oronasal route. On the basis of disease production, WSU 79-1146 was classified as an FIPV strain and WSU 79-1683 as an FECV strain (1,3,4,5).

Similarly, there are strains of the murine coronavirus, mouse hepatitis virus (MHV) which are closely related antigenically, but differ greatly in their pathogenicity (20,21,22). In mice, MHV-A59 is weakly pathogenic while MHV-3 is virulent and primarily hepatotrophic. Nucleic acid sequence studies of the two MHV strains indicated a detectable difference between the strains in the mRNAs that produced peplomer protein and polymerase. It was concluded that subtle alterations in the peplomer structure may influence the host range and the tissue tropism of the respective viruses (20). In a recent report, monoclonal antibodies were used to identify antibody binding sites (epitopes) on MHV which detect differences among the viral strains (23). A similar approach may prove essential in distinguishing minor antigenic differences amongst the feline coronaviruses (24).

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