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Genetic Analysis of Feline Calicivirus (FCV) Isolates Associated with a Hemorrhagic-like Disease

Mohamed Mostafa Abd-Eldaim
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Mohamed Mostafa Abd-Eldaim entitled "Genetic Analysis of Feline Calicivirus (FCV) Isolates Associated with a Hemorrhagic-like Disease." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

L.N.D. Potgieter, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Major Professor

We have read this dissertation
and recommend its acceptance:

Stephen Kania

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Melissa Kennedy

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Michael Karlstad

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Accepted for the council:
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Vice Chancellor and
Dean of Graduate Studies

(Original signatures are on file with official student records)

Genetic Analysis of Feline Calicivirus (FCV) Isolates Associated with a Hemorrhagic-like Disease

A Dissertation Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Mohamed Mostafa Abd-Eldaim

May 2005

DEDICATION

To mother, Awatef Hassanin, my father Moustafa Abd –Eldaim, who have given their love and continuous courage.

To my lovely wife, Omaima Maamoun Ahmed, who has given, never ending support, courage and love.

To my wonderful daughters: Nada, Safa, and Hana, who make my life enjoyable and has a meaning.

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ABSTRACT

Feline Calicivirus (FCV) is one of the most common causes of upper respiratory tract disease in cats. Other disease syndromes associated with FCV infection have been reported. Recently, calicivirus infections associated with a hemorrhagic-like disease leading to significant mortality in cats has been reported. The clinical signs are similar to those observed with the Calicivirus of Rabbit Hemorrhagic Disease. This investigation characterized two FCV isolates associated with hemorrhagic-like disease. Nucleotide sequencing of the complete genome was done on these two isolates and four isolates representing other disease syndromes. Previously reported sequence data for the entire genome of classical FCV (six isolates) and a portion of the capsid gene for hemorrhagic-like FCV (three isolates) were used in the genetic analysis. Sequence data were used to determine relationships among the isolates and any correlates with phenotype. Nucleotide sequence comparisons of the entire genome and individual ORF's revealed high homology among all isolates. However, data from this study suggest that the virulence may have genetic determinants as revealed by phylogenetic clustering based on a specific 75 nucleotides stretch from

the capsid protein gene of the isolates associated with hemorrhagic-like disease.

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Sources and manufacturers

a- Liberty Research, Inc., Waverly, NY 14892 USA

b- Cambrex Bioscience Walkersville, Inc., Walkersville, MD, USA

c- Invitrogen Corporation, Carlsbad, California USA

d- TAKARA BIO INC, Seta 3-4-1, Otsu, Shiga 520-2193, Japan

e- Perkin Elmer Gene Amp PCR system 9600, Perkin Elmer, Inc., Norwalk, CA, USA

f- Sigma Chemical Co., St. Louis, Mo, USA

g- QIAGEN Inc., Valencia, CA, USA

h- Using an ABI prism dye terminator cycle sequencing reaction kit and an ABI 373 DNA sequencer, Perkin Elmer, Inc., Foster city, CA, USA.

j- Fort Dodge Animal Health, Wyeth, 5 Giralda Farm, Madison, NJ 07940, USA.

k- KPL, Gaithersburg, MD 20879 USA.

l- Zeiss, One Zeiss Drive, Thornwood, NY 10594, USA.

I-Introduction

Feline calicivirus (FCV) is an important pathogen of cats, producing a wide range of clinical diseases, the most important of which is acute upper respiratory tract disease [32]. Clinical signs associated with infection include ocular discharge, nasal discharge, lingual ulcers,[109] chronic gingivitis, pharyngitis,[149] chronic stomatitis,[146] and pneumonia [109]. Other disease manifestations have been reported and include jaundice, death from *in utero*[39], and acute arthritis [32,101]. Existence of a carrier state is not uncommon [157,159]. Recently, a virulent calicivirus- associated, systemic hemorrhagic disease with significant mortality has occurred in cat populations in the United States [61,100,130]. This latter disease is similar to rabbit hemorrhagic disease caused by a calicivirus (genus *Lagovirus*, family Caliciviridae)[93].

Virus neutralization tests indicated that all FCV associated with "classical" disease isolates are closely related to one another and comprise one serotype. Moreover, data from several studies suggested that viruses recovered from cats with different clinical signs or from disparate geographic regions are similar genetically [67,44,108]. The conclusion from several genetic studies on the FCV capsid protein also argued for only one

FCV genotype [6,44,48,59]. In contrast, some data suggest antigenic clustering of the isolates associated with similar clinical signs [34].

The nucleotide homology of the complete genome of different isolates ranges between 79.4-80 % [150]. Sequence analysis of ORF1 suggests that some variable areas exist within this region.[48] The nucleotide homology of the capsid gene among FCV isolates ranges from 77.1- 81% while the amino acid similarities are 88-91%. However, the nucleotide homology is only 55-75% when the hypervariable areas (E) were compared [23,48,50,132,134,150]. The nucleotide homology of the ORF3 is 84-86%, while the amino acid similarity is 90-95.3% [134,150].

The genetic relationship of a FCV isolate associated with hemorrhagic-like disease with other FCV viruses has been investigated by selectively comparing 180 to 210 of approximately 300 nucleotides of the hypervariable E region encoding the capsid protein. Researchers concluded that the new viruses were closely related to known FCV isolates [130]. On the basis of this fragmentary sequence data, it is difficult to explain any genetic basis for the pathogenesis variability of FCV. Unfortunately a systemic comparison of various regions of the genome has never been done to conclusively exclude the existence of genetic variants that may be

responsible for the newly identified FCV virulent isolates. Changes in pathogenicity of the viral diseases have been documented. The pathogenicity of some virus isolates has been associated with genetic changes in different parts of the genome and different types of mutations. Examples for these genomic alterations include insertions of cellular sequences, duplications of viral sequences, and genomic rearrangements in non-structural protein (bovine virus diarrhea virus) [141]. A large deletion in the 5'-region of the transmissible gastro-enteritis virus (TGEV) spike glycoprotein gene may have been responsible for a change in tissue tropism from gastrointestinal tract to respiratory epithelium [15,121], while point mutation of the capsid protein (VP1 and VP2 proteins) of the parvovirus is attributed to the genetic differences between canine parvovirus and feline panleukopenia [60]. More genetic analysis is needed to determine if the enhanced pathogenicity of FCV is due to genetic variation [61,100].

Researchers working with FCV isolates have focused their work on the genetic analysis of the capsid protein gene and its hypervariable region (E) while entire genome sequences have been determined only for six FCV isolates associated with classical disease. Pathogenicity markers have been

mapped to these regions in some other viruses such as bovine viral diarrhea (BVDV) [105].

The purpose of this research was to determine systematically the antigenic and the genetic basis which may be responsible for the FCV pathogenicity differences. Genetic data from other virus families, such as parvovirus, flavivirus and coronavirus in which mutations were considered the main reasons for their pathogenicity changes [60,121,141]. Genetic differences of the FCV associated with hemorrhagic-like disease may be the reason of the phenotypic change for FCV pathogenicity. This conclusion leads to my hypothesis that differences in the pathogenicity of FCV virus strains are genetically and consequently, antigenically determined.

In this investigation, the complete genome sequence of six FCV isolates was determined: two hemorrhagic disease-associated FCV isolates, three isolates representing other clinical "classical" diseases (isolated at the University of Tennessee, College of Veterinary Medicine, Clinical Virology laboratory over the last ten years), and one isolate obtained from National Veterinary Service Laboratories, USDA. The sequence data were used to determine the genetic relationship of the hemorrhagic disease-associated isolates to one another and to the other FCV isolates (including sequence

data deposited in GenBank). These analyses could reveal the genetic basis of FCV pathogenicity and identify variable and conserved regions of the FCV genome that could be exploited for FCV molecular diagnosis and molecular epidemiology. Serology on samples obtained from the animals infected with the hemorrhagic disease-associated FCV was done to compare antigenic relationships to genetic data.

II- Literature review

History and initial isolation

Feline herpes virus and feline calicivirus (FCV) are the most important pathogens involved with feline respiratory disease. Feline herpes virus produced inclusion bodies in cell culture while FCV did not [98].

Fastier from New Zealand isolated a cytopathogenic agent in 1957, from primary kitten kidney epithelial cells [40]. The isolated virus failed to produce obvious clinical signs in experimentally infected kittens; only transient anorexia and diarrhea were observed [40]. In the same year, in the United States, Bolin isolated another virus from a diseased cat [13]. It was identified as panleucopenia virus. By 1966, these two viruses were regrouped with other viruses that produce respiratory diseases in cats [30]. The first isolation of a virus that did not produce inclusion bodies in cell culture from cats with respiratory illness was reported in 1960 [28,29]. The latter virus was isolated from the blood and throat of a sick cat and the disease was reproduced when the isolate was administered to a group of cats [29]. In the same year, investigators reported a group of cytopathogenic viruses which had been isolated from cats with respiratory disease [12]. Viruses isolated by Fastier [40], Bolin [13], Crandell and Madin [28], Crandell et al. [29], and Bittle et al

[12], had common biochemical and biophysical properties and were grouped as feline picornaviruses [5,27,65].

Characterization

Burki was a pioneer in the effort to identify viruses that cause respiratory diseases in cats that did not produce inclusion bodies in cell culture [16]. They were classified as picornaviruses because they contained RNA, were resistant to ether and chloroform, and passed through filters of 50 nm average pore diameter. Burki and Zwillenberg continued the effort to identify these viruses using electron microscopy [162]. The morphology and structure of the capsid differed from other picornaviruses. Calicivirus virion had a size of about 40 nm in diameter and had a slightly radiate and reticulate appearance. Despite the shape and size differences of these viruses from picornaviruses, they were still classified as picornaviruses due to the similarities of the model structure. The difference between picornaviruses and the viruses causing feline disease were supported by other electron microscopy studies [2,14] and physico-chemical characters [103,142]. A later study presented data indicated that the virus replicates in cytoplasm and is released by cell lysis [103]. An electron microscopy study showed that the virus lacks an outer envelope [140]. The classification of these viruses was changed from

picornavirus to calicivirus, based on several criteria, summarized in report 1974 [18].

Clinical diseases

Feline calicivirus (FCV) is a significant pathogen of cats, producing a wide range of clinical diseases, the most important of which is acute upper respiratory tract disease [32]. Clinical signs associated with infection include anorexia, lethargy [28], ocular discharge, nasal discharge [5,28,70,109], lingual ulcers [58,70,109], soft palate epithelial necrosis and deep ulceration [158], chronic gingivitis, pharyngitis [149], chronic stomatitis [70,146], and pneumonia [58,109]. Other disease manifestations have been reported and include lameness and acute arthritis (limping syndrome), following vaccination and natural infections [24,32,101]. Virus antigens were demonstrated in various joints of the infected cats [32]. Jaundice and death in fetuses from *in utero* FCV infection have been reported [9,39,154]. Other reports have implicated calicivirus infection in urolithiasis and lower urinary tract disease of cats [73,123,124]. The presence of feline calicivirus particles in the lymphoid tissue (including the tonsils, pharyngyeal lymph node and spleen) of infected cats is a constant finding [57,66,109].

Experimental infection studies have been used to confirm the nature of clinical disease produced and to determine the pathogenesis of FCV infection. The route of infection played a role on the outcome of infection. The intra-nasal route was more efficient in eliciting respiratory tract disease than the aerosol route [95]. The pathogenicity was relatively constant when specific pathogen free cats were infected with a variety of antigenically and genetically different FCV strains [95,106,147]. Experimental infections have revealed the role of FCV in lower respiratory tract infection. Feline calicivirus often causes interstitial pneumonia which is characterized by diffuse molting due to pulmonary congestion and edema [57,95,109]. Ultrastructural studies revealed that the FCV has marked tropism for alveolar pneumocytes, and induced necrosis of pneumocytes within 12 hours of exposure [76]. FCV is associated also with acute serofibrinous and neutrophilic inflammation in the distal part of the respiratory tract [77].

A calicivirus-associated with systemic, hemorrhagic-like disease with significant mortality has been reported recently [81]. The clinical signs of the "new" syndrome included severe upper respiratory infection, oral vesicles on the margins of the tongue and soft palate and crusty, ulcerative lesions on face and pinnae [81]. The disease was characterized by diffuse cutaneous

edema of the face and submandibular area, over the muzzle, pinnae, and lower limbs. The latter signs gave the disease its name "hemorrhagic-like". Experimental inoculation resulted in edema of the face and limbs and hyperbilirubinemia of cats [81]. Histopathological lesions, of the naturally infected cats was subcutaneous edema on the lateral body wall, pinkish milky fluid in the pleural cavity and serosanguinous fluid in the abdominal cavity with fibrin clots [100]. The histopathological lesions associated with the same FCV virus in experimentally infected cats, included large areas of subcutaneous edema, hepatic necrosis, pancreatitis, intestinal crypt necrosis, and acute multifocal interstitial pneumonia [100]. Antibodies against the vaccine virus did not react significantly with the new isolate [61,100]. Other outbreaks have been reported throughout the United States [61,115,130]. Immuno-histochemistry detected viral antigen in endothelial and epithelial cells of the affected tissues [102]. This disease is similar to rabbit hemorrhagic disease caused by another calicivirus (genus *Lagovirus*, family Caliciviridae) [93].

Existence of a FCV carrier state in cats is not uncommon [157,159]. Cats, after recovery from the acute phase of primary infection with FCV, become persistently infected and shed the virus from the oro-pharynx for a prolonged period (more than 2.5 years) despite having a high titer of neutralizing

antibodies [112,156]. The tonsillar region (including the surface epithelium of the tonsil, and adjacent fossa mucosa) is considered the primary organ of persistence in asymptomatic carriers [36,112,158,158]. Kittens from queens persistently infected with FCV usually get mild or sub-clinical infection [64]. Isolation rates of FCV from cats with chronic stomatitis (up to 92%) are higher than the isolation rates of FCV from clinically healthy cats (from 7.3-43%) [26,69,149,157,159].

A number of viral agents, including FCV, feline-immunodeficiency virus, feline herpes virus type 1 and feline leukemia virus, are involved in causing chronic oral infection in cats [146]. Feline calicivirus, solely or in association with other viral agents, is considered one of the most frequent pathogens involved in chronic oral infection of cats [69,146]. Experimental infection, with virus isolated from cats with chronic stomatitis, failed to induce chronic infection [149].

Epidemiology

Feline calicivirus infection is widespread and has worldwide distribution. FCV was first described in New Zealand, and subsequently in many other countries [7,16,29,40,144,161]. The isolation rate of FCV from domestic cats was around 20% and suggested that younger cats are more susceptible

to infection than older cats (more than one year of age) [19,51,110]. The isolation rate was higher (26-31%) from cats in a rescue shelter [118].

Feline calicivirus has been isolated from other felidae including captive cheetahs [127]. Experimental infection of domestic cats with the virus, isolated from cheetah, produced classical clinical disease. The clinical signs included pneumonia, ulcerative glossitis, conjunctivitis and anorexia [80]. The antibody prevalence of FCV was 65% in free-ranging Namibian cheetahs [89], 26% in wild cats from Scotland [31], 33.3% in wild cats from Taiwan and Vietnam [62] and 40% in wild cats from Saudi Arabia [97].

Prevalence of antibodies against FCV was reported for many other free-ranging animal species around the world. Antibodies against FCV were detected in 70% of sera from free-ranging lions in East Africa (Tanzania's national park) (with different rates of samples from different locations) [55], 17% sera from free-ranging mountain lion in the United States [99] and 56% in the sera of free-ranging Florida panthers [126]. The antibody prevalence was 72% in spotted hyenas in the Serengeti ecosystem in Tanzania, Africa [52].

Taxonomy and viral properties

Caliciviridae has four genera; Vesivirus (swine vesicular exanthema virus, feline calicivirus, and others), Lagovirus (Rabbit hemorrhagic disease

virus), Norovirus (Norwalk virus) and Sappovirus (Sapporo virus). Vesivirus obtained its name from the prominent vesicles that characterize the clinical diseases [148]. Calicivirus particles have cup-shaped depressions on the virion (figure 2.1) visible by negative stain electron microscopy (Calyx; Latin for cup or chalice) [25]. The virus particles have a diameter of about 40 nm with 32 cup-shaped surface structures, each comprising 90 arch-like capsomeres, arranged in icosahedral symmetry [25]. The acid sensitivity of feline caliciviruses varies from one isolate to another [79].

The organization of the caliciviruses genome is different according to the genus; figure 2.2 shows the different genera and their genome organization. The FCV genome consists of a single-stranded RNA [1,38,81]. It has a poly-adenyl tail at the 3` end [38], and has positive polarity. The genome contains 7678 to 7693 nucleotides and encodes three open reading frames. A characteristic feature of FCV genome organization is a 5` non-structural polyprotein preceding the structural capsid protein. The 3` end of the genome has a small ORF which encodes a structural protein with unknown function (figure 2.2) [22,48,96]. In calicivirus-infected cells, two kinds of viral specific RNA are transcribed; genomic RNA and 2.4 kb subgenomic RNA. The subgenomic RNA encodes ORF-2 and ORF-3 (figure 2.3) [21,38,91].

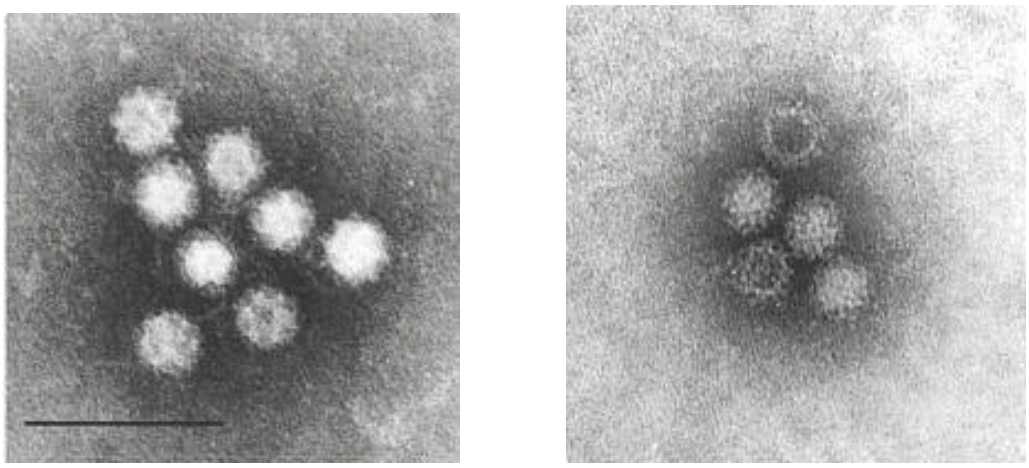


Figure 2.1: electron microscopy for the infectious calicivirus particles. Bar represents 100nm.

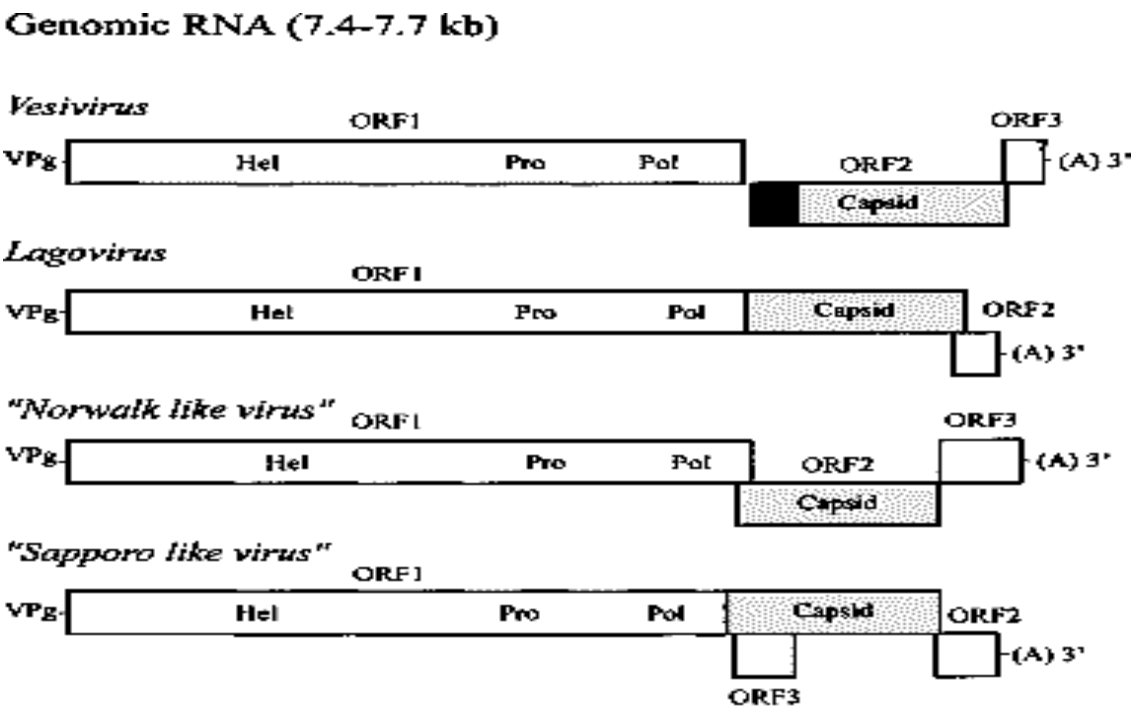


Figure 2.2: the genome RNA and its organization among different genera of Caliciviridae [148]

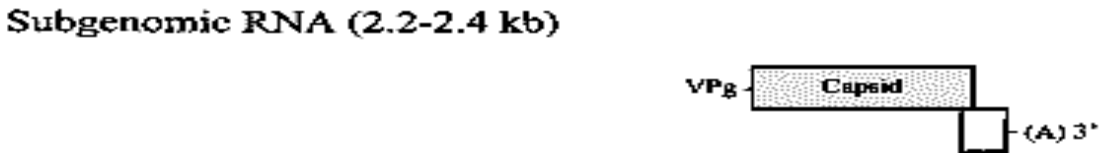


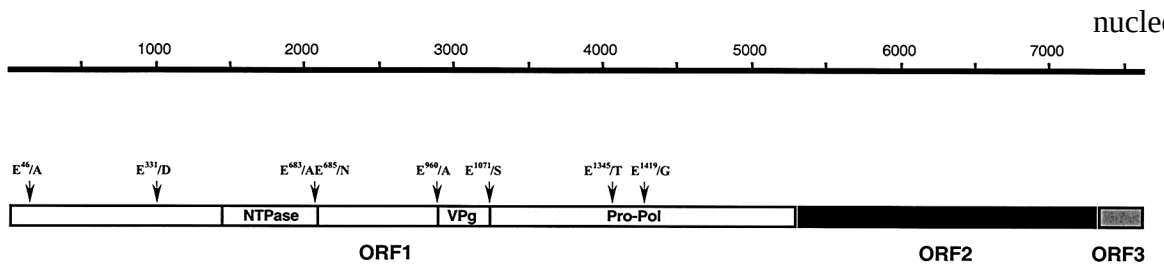
Figure 2.3: the position of sub-genomic RNA of the FCV [148]

ORF1 is 5308 nucleotides long (nucleotides 20 to 5305) encoding 1763 amino acids. It encodes a large polyprotein, which undergoes co-translational cleavage yielding six nonstructural proteins ranging in size from 5.6 kd to 75.6 kd. They are p5.6, p32, p39 (NTPase), p30, p13 (VPg), and p76 (proteinase-polymerase) (figure 2.4) [20,20,71,136,136,139,160]. The p76 (proteinase-polymerase) is similar to picornavirus 3C proteinase and 3D polymerase domain [139]. A 3C-like cysteine proteinase is responsible for co-translational proteolytic processing of ORF-1 and the capsid protein precursor [138,139]. The full-length proteinase-polymerase protein is the active form of RNA-dependent RNA polymerase [160].

The cleavage site between p5.6 and (p32) is E^{46}/A^{47}. The other cleavage sites are: E^{331}/D^{332} between p32 and p39 (NTPase), E^{685}/N^{686} between p39 and p30, E^{960}/A^{961} between p30 and p13 (VPg) and E^{1071}/S^{1072} between p13 VPg and pro-pol [136,139].

The ORF2 has approximately 2004 nucleotides (nucleotide 5314 to 7317-7326). It encodes the capsid protein and is predicted to have about 668 amino acids, the putative 76 kd capsid precursor protein [20,23,92,152]. The latter is subsequently cleaved by a viral-encoded proteinase, resulting in a 65-66 kd mature protein (major capsid protein) [20,71,138].

A)



B)

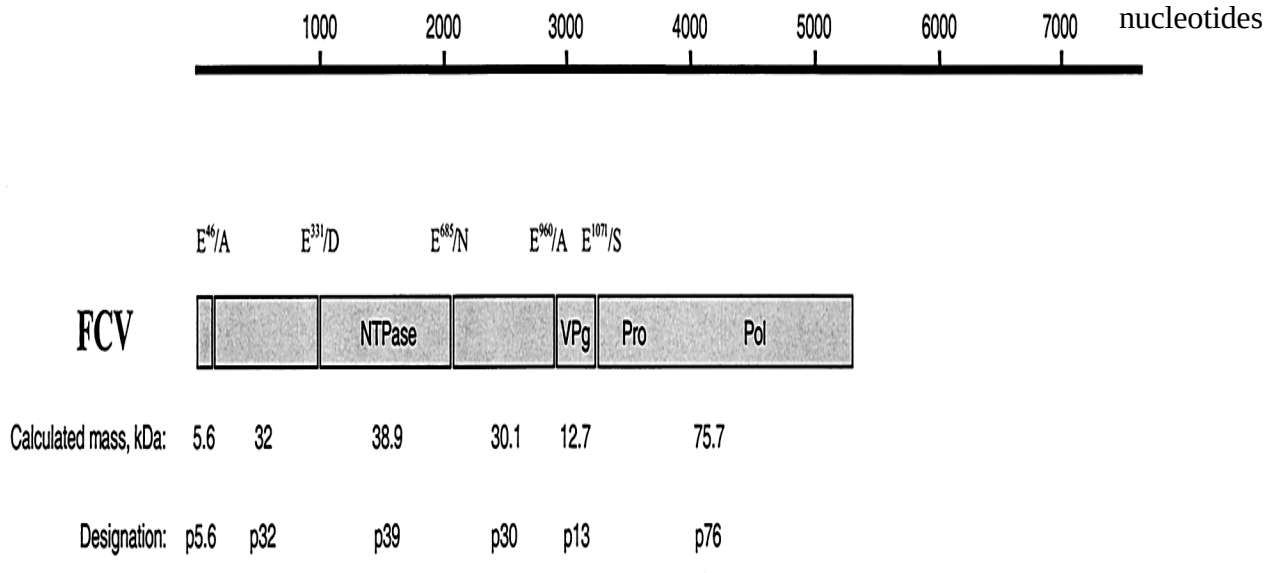


Figure 2.4: A) the organization of the three ORF of FCV, B) different proteins encoded by ORF-1 [136]

When the genetic bases encoding the mature form were cloned and then expressed in cell culture, the product was a virus-like particle resembling the FCV mature virion [42,43].

The capsid protein gene is divided into six areas based on genetic variability, and designated A, B, C, D, E, and F representing amino acids residues 1-120, 121-396, 397-401, 402-425, 426-520 and 521-668 respectively of the ORF2 [90,133]. Area E, identified as a hypervariable region of the capsid protein, is probably responsible for the feline calicivirus antigenic variations [6,44,48,90,133,134].

ORF3 contains 318 nucleotides and encodes a 106 amino acid protein. The start codon for ORF3 overlaps the stop codon of ORF2. It is a minor structural protein, VP2, with an unknown function and is found in small amounts relative to the major capsid protein [54,92,137,152]. VP2 has a calculated molecular weight of 12.5kd but it behaves like an 8.5 kd in polyacrylamide gel electrophoresis (PAGE) that may be attributed to an unusual mobility in SDS-PAGE [137].

The nucleotide homology of the complete genome sequences available from Genbank for six isolates associated with classical form of the disease ranges between 79.4-80 % [49,150]. Sequence analysis of ORF1 of these isolates suggested that some variable areas exist within this region.[48] The nucleotide homology of ORF1, the non-structural polyprotein, for these isolates, ranged from 77.8-80.7%, while deduced amino acid similarities ranged from 90-95.5% [48,96,150]. The nucleotide homology of the capsid gene among FCV isolates ranged from 77.1- 81% while the amino acid similarities were 88-91%. However, the nucleotide homology was only 55-75% when the hypervariable areas (E) were compared [6,23,48,50,132,134,150 49,85]. The nucleotide homology of the ORF3 among FCV isolates was 84-86%, while the amino acid similarity was 90-95.3% [85,134,150]. Genetic studies on the FCV capsid protein also suggested that only one genotype of FCV exists [6,44,48,59]. However, other genetic data suggest antigenic clustering of the isolates producing similar clinical signs [34,83].

The genetic and antigenic relationship among FCV isolates

The relationship among FCV isolates, associated with different clinical diseases and different geographical location, has been studied extensively. Analyses have included serological assay, plaque morphology

and some genetic analyses [67].[94] Virus neutralization patterns [17,34,67,68,78], immuno-diffusion [67], and an ELISA assay [83] have been used to study the relationship among different FCV isolates. These studies suggested that FCV isolates were closely related to one another and comprised one serotype. In addition, genetic diversity was not noted among viruses recovered from cats with different clinical signs or from disparate geographic regions. The phylogenetic analysis has been used to study the genetic relationship among different genera of caliciviruses [10]. It has been used also to analyze the relationship of FCV field stains among one another and their relationship to vaccine strains [10,114,128]. The capacity of the genetic studies to segregate virus strains has been compared to the antigenic analysis (virus neutralization) [117]. Virus neutralization data suggested that wide spectrum of antigenic variations existed among FCV isolates and in contrast the genetic analysis suggested that FCV isolates have a close relationship to one another [117].

Genetic studies on the genomic domain encoding the FCV capsid protein also suggest that only one genotype of FCV exists [6,44,48,59]. However, some data suggest antigenic clustering together of isolates producing similar clinical signs [34,83]. By using the neutralization cross reactivity, FCV isolated

from the same geographical area did not segregate together [68]. In contrast, phylogenetic analysis using the capsid gene sequence data, segregated 67% of the Japanese FCV isolates together (geographical segregation) [128]. Most of the genetic analyses of FCV have targeted the hypervariable area (E) [114,116,128]. It is considered the immuno-dominant region of the capsid gene [114,116,128]. The nucleotide distance, by sequencing the E area of the capsid protein gene, ranged between 0-16% for isolates from the same cat colony or for epidemiologically-related viruses, while it ranged between 20-40% for unrelated isolates [114,116].

The genetic relationship of a FCV isolate associated with hemorrhagic-like disease with other FCV viruses has been investigated by comparing nucleotide sequences of the hypervariable E region of the capsid protein gene. Researchers concluded that the new viruses are closely related to known FCV isolates [61,100,130]. The capsid protein gene was sequenced to determine the genetic relationship between the FCVs causing lower urinary tract disease to vaccine and other field FCV isolates [123]. Two FCV isolates associated with lower urinary tract disease had no relationship to one another [123].

Cross-neutralization tests revealed that FCV undergoes antigenic changes over the course of persistent infection in cats [63,72,119]. On the other hand, the frequent passages of FCV in cell culture did not result in antigenic changes measured by neutralization activities when the early passages of the viruses were compared to late passages of the virus [119]. Western blot analysis using polyclonal antibodies against the parent virus with putative progeny viruses from the persistently infected cats indicated antigenic homogeneity among all FCV isolates. In contrast, analysis of the hypervariable region of these isolates revealed changes affecting encoded amino acids [72].

Vaccination and immune response

Licensed modified-life and inactivated vaccine for FCV are available [41,111,131]. Furthermore, an experimental recombinant capsid protein vaccine elicited high antibody yields when administrated to cats [84]. A DNA vaccine using a plasmid encoding the mature capsid protein did not protect cats against clinical disease [,135]. Despite widespread vaccination, FCV-associated disease is still a significant clinical problem. Vaccination only protects against clinical disease and sub-clinical and persistent infections still occur [32,5133,107]. Neutralizing antibodies, from cat sera, produced against various vaccine strains only cross-reacted with 22 to 70% of the FCV field isolates

[56]. FCV strain F9 was the most used in vaccine preparations because it elicited antibodies with a higher degree of cross-neutralization against other FCV isolates [11,67].

The neutralizing antibody epitopes of FCV were mapped to the 5' end of the hypervariable E area of the capsid protein gene [45,88,120,151,153]. Milton *et al* identified that this area contains amino acid residues 422 to 458 of the capsid protein and was capable of binding to two kinds of neutralizing antibodies [88]. Tohya located two neutralizing epitopes in the region at amino acid residues 426 to 460 and 490 to 520 [153]. Non-neutralizing antibody epitopes were mapped to areas B, C, D and F of the capsid protein [45,88]. The antibodies induced in experimentally immunized mice with recombinant capsid protein, cross-reacted with homologous and heterologous FCV isolates by Western blot which suggested that these epitopes are localized within the conserved areas of the capsid protein [44,45,88].

Diagnosis of infection

Diagnosis of FCV is based mainly on virus isolation in cell culture followed by serological identification by immunofluorescence [46]. Immunohistochemistry in tissue from cats in the acute phase of disease efficiently detects FCV in formalin-fixed tissue [35]. Development of

diagnostic nucleic acid amplification techniques has been investigated. Reverse-transcription PCR (RT-PCR) has been developed using a primer set targeting a 670-680 base pair segment of the capsid protein gene [68,143]. A nested RT-PCR was more sensitive than a one step procedure [82]. A real-time PCR for FCV detection has been developed using SYBR Green instead the specific molecular probe [53,129].

III- Material and methods

Experimental design: Two FCV isolates causing the hemorrhagic-like disease were compared by genetic and antigenic analysis to four isolates associated with classical diseases and to one another. The entire genomes of these six FCV isolates were sequenced, by direct amplification of targeted irrelevant areas of the genomes, using RT-PCR followed by determination of the base sequences. The genetic relationships among the different isolates were determined using phylogenetic analysis and degree of similarities, using nucleotide sequences and deduced amino acid sequences, among different FCV isolates. These relationships were studied for each part of the genome including structural, non-structural proteins genes and untranslated 5' and 3' areas. The antigenic differences among these isolates were determined by cross neutralization and immuno-fluorescence.

Viruses: Six FCV isolates were included in the study. Five had been isolated at the Clinical Virology Laboratory, College of Veterinary Medicine, University of Tennessee over the last 10 years. Two of these isolates had been associated with hemorrhagic disease, designated UTCVM-H1 and UTCVM-H2 (University of Tennessee, College of Veterinary Medicine-

Hemorrhagic), three had been associated with classical signs of FCV, designated UTCVM-NH1, UTCVM-NH2, and UTCVM-NH3 (University of Tennessee, College of Veterinary Medicine-Non-Hemorrhagic), and one isolate was obtained from National Veterinary Service Laboratories, USDA. UTCVM-H1 isolate was identified as one associated with hemorrhagic disease based on the clinical signs and diffuse vasculitis found on necropsy (table 3.1). UTCVM-H2 isolate was identified as one associated with hemorrhagic disease based primarily on clinical signs described for the syndrome.[130] Laboratory tests supported the diagnosis and ruled out other etiologies (including FIV, FeLV, *Ehrlichia canis, Ehrlichia chaffeensis, Rickettsia rickettsii*, aerobic and anaerobic bacteremia, and Blastomyces dermatitidis). FCV was isolated from lung (H1) and oral swab (H2). Table 3.1 shows the clinical signs, date of isolation, geographical location of the infected case(s) and the necropsy findings (if available) for each FCV disease occurrence. Genome sequence data of six other FCV isolates were obtained from the GenBank (names and accession numbers are given in the results section), and used for genetic analysis. Partial sequence data (180-

Table 3.1: feline calicivirus isolates used in this study

Isolate	Isolation date	Geographic origin	Specimen used	Case history and clinical signs	Necropsy finding (if available)
UTCVM -NH1	1993	Mississippi	Lingual ulcer, buccal ulcer and tonsil	Depression, high fever, oral and lingual ulceration. Two other cats in the house hold died with similar signs	Not Done
UTCVM -NH2	1998	Kentucky	Nasal swab	Eye lesion and upper respiratory signs	Cat survived
UTCVM -NH3	2000	Tennessee	Lung, nostril, tissue	six years old, female Ragdoll cat suffered from: moderate ventral subcutaneous edema, fever, vomiting, and nasal discharge. Died within 24 hours.	Nasal turbinate mildly edematous, patchy necrosis of alveolar epithelium, alveolar edema, moderate to severe, acute, locally extensive, suppurative bronchointerstitial pneumonia.
UTCVM -H1	1999	Tennessee	Lung	Outbreak affected eleven cats. Pneumonia and vasculitis, with three deaths. The virus was easily transmitted and rapidly spread to other cats in the facility. Clinical signs were high fever, depression, anorexia, jaundice, and necrotic lesions of the pinnae. Blood work evidenced thrombocytopenia, bilirubinemia, leukopenia to leukocytosis, and elevated liver enzymes	Necropsy was done on two cats. The lung was markedly hemorrhagic with serosanguinous fluid on section, micro-vascular thrombosis, marked endothelial hyperplasia, intra alveolar hemorrhage with fibrin, marked vascular congestion and mild to moderate, diffuse pulmonary edema was observed. Kidney had hemorrhage at corticomedullary junction. Pinnae had sub-epidermal vesicle formation mild multifocal microvascular thrombosis
UTCVM -H2	2002	Tennessee	Oro-pharyngeal swab	Five year old male; Lameness, swollen, edematous limbs and joints, ventral abdominal edema, anorexia, fever, oral ulcer. Ulceration on mandibular lip. Ehrlichia canis, FELV/FIV, Blastomysis testing were negatives.	cat survived

210 nucleotides) of three FCV isolates associated with hemorrhagic disease in cats were obtained from published articles (FCV-Ari,[100] FCV-cat 15,[130] FCV-kaos [61]).

Serum samples: Five convalescent serum samples from cats infected with the UTCVM-H1, a serum sample from the cat infected with UTCVM-H2 and a negative control serum (obtained from Liberty Research [a]) were used in this study to detect the neutralization titer and for the cross-neutralization test. Eleven convalescent sera (including the five sera for virus neutralization) from the outbreak caused by UTCVM-H1 were examined by immuno-fluorescence to detect the antibody response against the FCV infection.

Virus propagation: Crandell-Reese feline kidney (CRFK) cells were used for virus isolation and serum virus neutralization assays. The CRFK cells were grown with Dulbecco's Modified Eagle Medium (DMEM) [b] supplemented with 5% heat-inactivated fetal bovine serum with standard concentrations of penicillin, streptomycin and amphotericin. After samples preparation (given in table 3.1), confluent monolayer of CRFK cells, grown in 75 cm^2 flask, were inoculated and observed daily for cytopathic effect

(CPE). The viruses were grown until 50-70% CPE developed and the cells were collected for RNA extraction.

Negative staining electron microscopy: the primary diagnosis was done using direct immuno-fluorescence and electron microscopy. Two of 75 cm^2 flask CRFK cells were used to grow the virus. The infected cells were collected along with the medium and then centrifuged at 14,000xg at 4 °C for 50 minutes. The supernatant was discarded and the pellet re-suspended in double distilled water and then centrifuged again. 100 ul from 3% PTA (phosphotungstic acid) was mixed with 100 ul of the cell suspension and mixed. Electron microscopy grids were sprayed 5-10 times and then examined. The FCV particles were detected by electron microscopy.

RNA extraction: RNA was extracted with Trizol LS reagent [c] according to the manufacturer's protocol. In brief, the infected cells were collected from the 75 cm^2 flask, and then pelleted using low speed centrifugation (1000 x g) for 5 minutes. The cell pellet was resuspended in 750 ul Trizol reagent, and incubated for five minutes at room temperature. Two hundred µl chloroform was added to the suspension, incubated for 2-3 minutes at room temperature, and then centrifuged at 12,000 x *g* for 15 minutes at 4°C. The RNA, the aqueous upper phase, was precipitated by adding 500 µl isopropyl alcohol;

the mixture was incubated for 10 minutes at room temperature and then centrifuged at 12,000 x g for another 10 minutes at 4°C. The pellet obtained after centrifugation was washed with one ml 75% ethyl alcohol and then centrifuged at 7500 x g for five minutes at 4 °C to obtain the RNA pellet. The RNA pellet was air dried and dissolved in 100 µl Rnase-free water.

Primer design: For RT/PCR, the FCV genome was divided arbitrarily into three major and three minor segments (Figure 3.1). Two primers (5 and 6) have been used before [1]. Various primer pairs used for RT-PCR amplification (Table 3.2) were based on published sequences; the most conserved areas of the genome were chosen. Additional sequencing primers were derived from the generated sequence data.

cDNA synthesis: Synthesis of cDNA was done using M-MLV Reverse Transcriptase [c] according to the manufacturer's protocol. In brief, 1 µl of the antisense primer (50µm), 4 µl from 10mM dNTP mix, and 1-4 ng of total RNA were mixed and made up to 12 µl with Rnase-free water. The mixture then was heated to 65°C for 5 minutes and quick-chilled on ice. Following this, 4 µl 5x first-strand buffers, 2 µl 0.1 M DTT and 1 µl RnaseOUT recombinant ribonuclease inhibitor (40units) [c], were added and the mixture incubated at 37°C for two minutes. After 1 µl (200units) of M-MLV \RTase

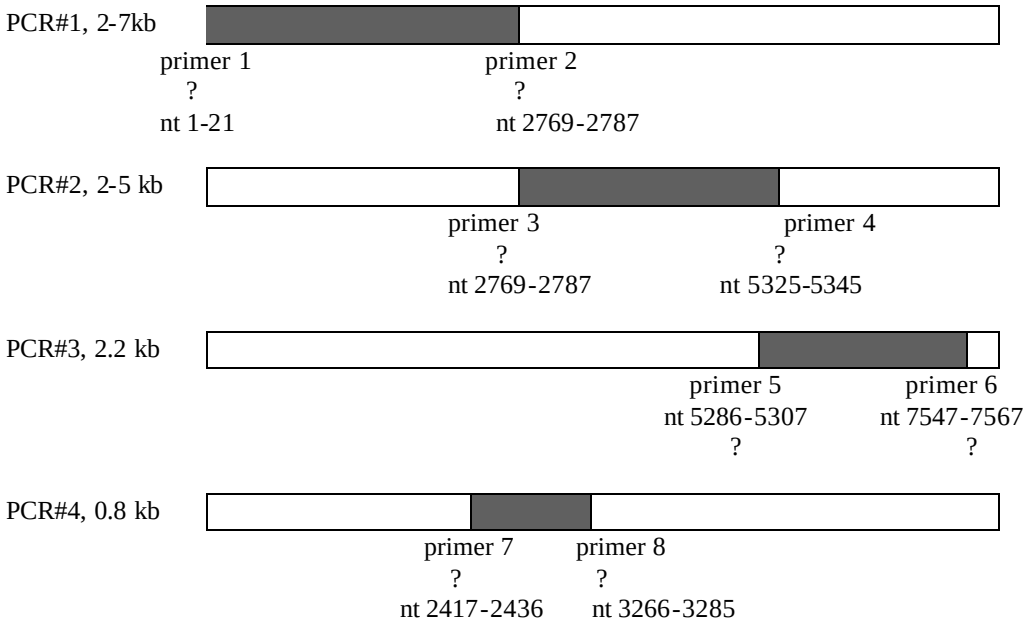


Figure 3.1: the genomic segments amplified by RT/PCR and the corresponding primers for each: nucleotides numbers indicate position in the genome.

Table 3.2: primer sequences used for RT, PCR reaction and sequencing

Primer	Primer sequence
Primer 1	5`-GTAAAAGAAATTTGAGACAAT-3`
Primer 2	5`-ATTTCAGACCATGGCATTG-3`
Primer 3	5`-CAATGCCATGGTCTGAAAT-3`
Primer 4	5`-TATTTAAGCACGTTAGCGCAG-3`
Primer 5	5`-TACACTGTGATGTGTTCGAAGT-3`
Primer 6	5`-GTGTATGAGTAAGGGTCAACC-3`
Primer 7	5`-GATGAACTACCCGCCAATCA-3`
Primer 8	5`-ACATCAGTGACTGATCCAAT-3`
Primer 9	5`-TAACTRGCACAAGAAGGR(A+G)CA-3`
Primer 10	5`-TGAGTCGATGACTCTAGCTTTTTTTTTTTTTTTTT3`
Primer11	5`-TGAGTCGATGACTCTAGC-3`
Primer 12	5`-CTGCCTCCTACATGGGAAT-3`

was added, the mixture was incubated for 50 minutes at 37℃, and then heated at 70℃ for 15 minutes to inactivate the enzymes.

Polymerase chain reaction (PCR); According to the manufacturer's protocol [d], 10 µl of 10X Ex Taq buffer, 8 µl dNTP mixture (2.5 mM each), 1 µl of the sense primer (50um), 1 µl of the antisense primer (50µm), 0.5 µl TaKaRa Ex Taq (5 units/µl), 4 µl of the cDNA were combined, and made up to 100 µl with Rnase-free water. The mixture was reacted in a thermal cycler [e] initiated by denaturation at 95℃ for 1.5 minutes, followed by 30, three-step cycles consisting of annealing at 58℃ for 30 seconds, extension at 72℃ for one minute and denaturation at 95℃ for one minute. At the end of the last cycle, the reaction was held for 2 minutes at 50℃ followed by 5 minutes at 72℃.

Genetic sequencing: The PCR products were purified by gel extraction. The products were electrophoresed in 1% agarose [f] gel in 1X TBE buffer. The band representing the expected size was purified from the gel using MinElute Gel Extraction kit and done according to the manufacturer's protocol [g] in a microcentrifuge. The gels, with the DNA fragment was excised with a clean, sharp scalpel and placed in a colorless tube, and weighed. Three volumes of buffer QG to one volume of gel weight were

added. The mixture was incubated at 50°C with frequent vortexing, for 10 minutes (or until the gel slices has completely dissolved). After complete dissolving of the gel, the color was checked to see if it was still yellow. One gel volume of isopropanol was added to the mixture and the tube was mixed by inverting it several times. The mixture was placed to a MinElute column in a 2 ml collection tube and centrifuged for 1 minute. The flow-through was discarded and 500 µl QG buffer was added to the column, centrifuged for 1 minute, and the flow-through was discarded. The MinElute column was washed with 750 µl of PE buffer and incubated for 5 minutes, centrifuged for 1 minute and the flow-through was discarded. Residual PE buffer was removed from the MinElute column by centrifugation for an additional 1 minute at maximum speed of the micro-centrifuge (~16,000g). The MinElute column was transferred into a clean 1.5 ml microcentrifuge tube, 25 µl of DNase and RNase free water was added and incubated for 1 minute. DNA was eluted from the column by centrifugation for 1 minute; DNA was kept at -20°C. Sequencing of the purified PCR products was done at the Molecular Biology Resources Service (University of Tennessee, Knoxville, TN) [h].

RACE (Rapid Amplification of cDNA Ends): The 3' and 5' ends of the FCV genome were determined using the RACE system. The 20 nucleotides at the 5' end were detected using the RACE system kit [d] and was done according the manufacturer's protocol using primer nine as anti-sense primer for cDNA synthesis as well as for PCR (table 3.2). The RACE system used for the 3' end, exploited primer ten, for cDNA synthesis (table 3.2). Polymerase chain reaction was done using primer 11 and primer 12, targeting about 437-446 bases of the genome representing nucleotides 7244-7681 (table 3.2)

Sequence analysis: Determination of ORFs, amino acid sequence, alignment, phylogenetic tree construction and nucleotide sequences analysis was done using the Wisconsin GCG package. Phylogenetic tree construction was performed using distances (neighbor-joining) and growtree programs available in the Wisconsin GCG package.

Virus titration: 200 $CCID_{50}$ was used in virus neutralization tests. The virus stock of each FCV isolate was aliquoted and kept at -70°C. The procedure for virus titration was done according to the following protocol: the viruses were titrated in CRFK cells were in 96 wells plates. Seeded with 100 µl of $1.0x10^{-5}$ cells/ml suspension, and then incubated at 37°C in the CO2 incubator. Ten-fold serial dilutions through 10^{-10} of the virus stock in

DMEM with 2% fetal bovine serum was prepared. After the cells became confluent, 100 µl of each virus dilution was placed in each of five wells. Starting with the 10^{-10} dilution, the plates were incubated at 37℃ in 5% CO2 four days. The plates were monitored for CPE. The $CCID_{50}$ was calculated using Reed and Munch formula [122].

Virus neutralizing antibody assay: Convalescent serum samples were tested for neutralizing antibodies against four of the six FCV strains used in this study (UTCVM-H1, UTCVM-H2, UTCVM-NH1 and USDA). The serum samples were heated at 56℃ for 30 minutes. Two-fold serial dilutions of a 1:5 through 1:2560 dilutions were done in disposable sterile test tubes in DMEM supplemented with 5% fetal bovine serum, and starting with 1: 5 dilution. 25 µl of each dilution was transferred into 96-well microtiter plates. Then 200 $CCID_{50}$ of a FCV isolate in 25ul medium was added to each well. The reaction was incubated at 37℃ for 45 minutes, and then 50 µl of 2 x 10^5 cells/ ml cell suspension was added to each well. These procedures were done in duplicate. The back titration of each FCV was done to verify the virus titer. The plates were incubated for three days at 37℃ and examined under an inverted microscope for typical FCV CPE. The reciprocal of the highest serum dilution without detectable CPE was read as the endpoint.

Serum titration by indirect fluorescent antibody test: Four different types of FCV antigen placed on slides representing different types of FCV isolates were used. They were: slide coated with UTCVM-H1, USDA and two types of slide obtained from Fort Dodge vaccine company [j] (type C is a field unknown strain and type D is the vaccine strain F9). Two-fold serial dilutions of the convalescent sera in PBS were made (1:40 through 1:10560). The dilutions were added to the wells, including monoclonal antibodies positive control and a negative control, and then incubated for 30 minute at 37°C. Three washes in PBS for 5 minutes each were made. FITC-labeled goat anti-cat IgG [k] was added to each well; the slide was incubated for 30 minutes, and then washed three times in PBS. The slides then were examined using a fluorescence microscope [l] and the titer was determined as the highest dilution with fluorescent reaction.

IX- Results

Sequence analysis

The nucleotide sequences and deduced amino acid sequences of isolates UTCVM-NH1, UTCVM-NH2, UTCVM-NH3, UTCVM-H1, UTCVM-H2, and USDA, were determined and analyzed. All of the sequence data has been sent to GenBank (GenBank information in table 4.1).

The 5' untranslated region (UTR) consisted of 19 nucleotides and was completely conserved among the sequence of both FCV isolates used in this study and the FCV isolates sequence data obtained from GenBank (figure 4.1).

The first 56 nucleotides, including the 19 nucleotides of UTR, of the genome were completely conserved in all FCV isolates except UTCVM-H1, which differed in nucleotides 51 and 52 (figure 4.1) resulting in a hydrophilic amino acid {asparagines (N)} replacing a neutral amino acid {threonine (T)} (figure 4.2). The change was in residue number 11 of the ORF1.

All of the FCV isolate genomes contained three potential ORFs: ORF1 encodes large polyprotein (non-structural proteins), ORF2 encodes

Table 4.1: FCV isolates with GenBank accession number and size of each area of the genome

	GenBank accession number	3'UTR	ORF1	ORF2	ORF3	Total genome length
UTCVM-NH1	AY560113	1-19	20-5308	5314-7317	7317-7634	7683
UTCVM-NH2	AY560114	1-19	20-5308	5314-7326	7326-7643	7690
UTCVM-NH3	AY560115	1-19	20-5308	5314-7317	7317-7634	7693
UTCVM-H1	AY560116	1-19	20-5308	5314-7317	7317-7634	7685
UTCVM-H2	AY560117	1-19	20-5305	5311-7314	7314-7631	7678
USDA	AY560118	1-19	20-5308	5314-7317	7317-7634	7681
F9	M86379	1-19	20-5308	5314-7326	7326-7643	7690
F4	D31836	1-19	20-5308	5314-7317	7317-7634	7681
F65	AF109465	1-19	20-5308	5314-7317	7317-7634	7681
FCV2024	AF479590	1-19	20-5308	5314-7317	7317-7634	7681
CFI/68	U13992	1-19	20-5305	5311-7314	7314-7631	7678
Urbana	L40021	1-19	20-5308	5314-7317	7317-7634	7683

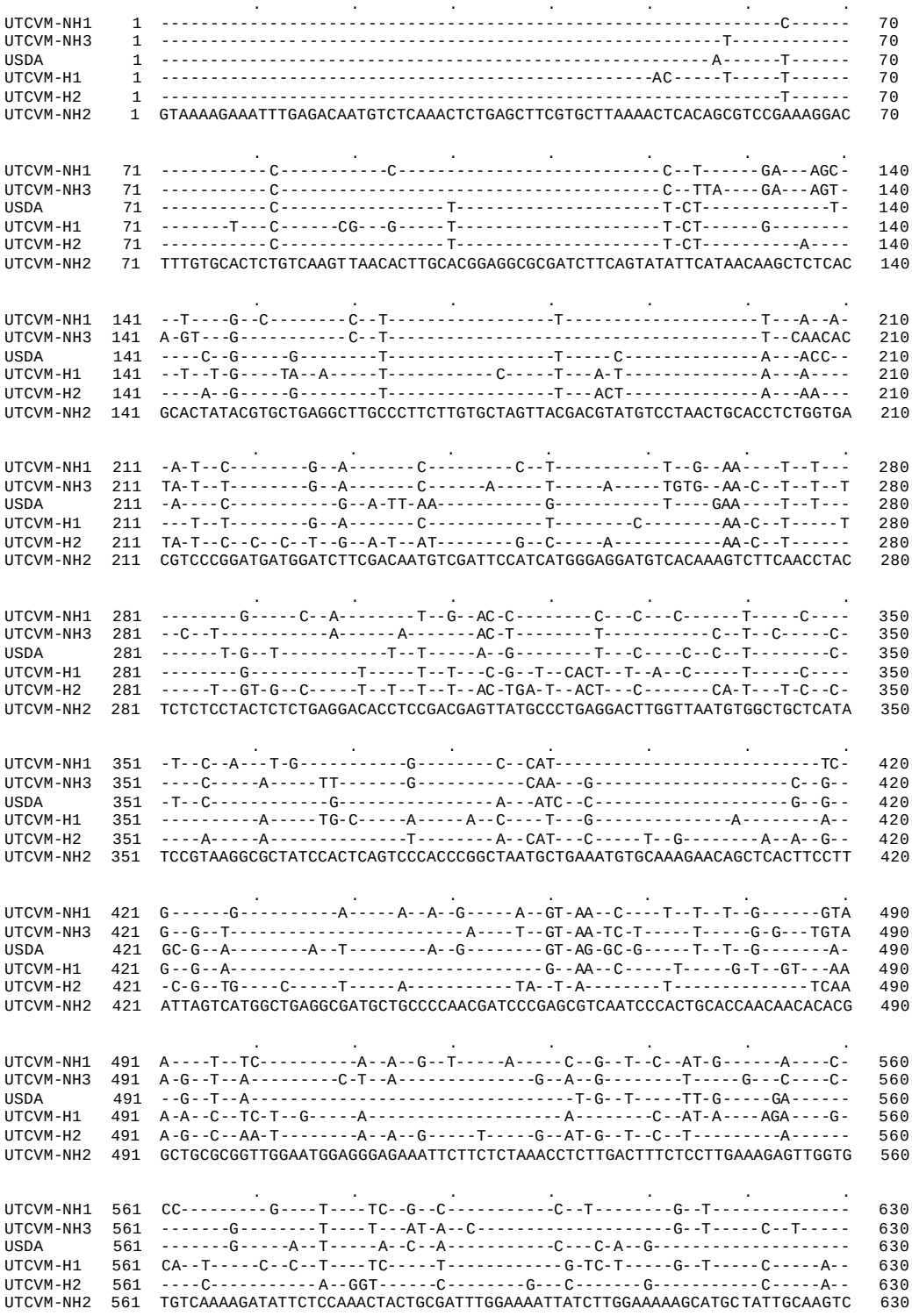


Figure 4.1: the nucleotide sequences of the complete genome for the six isolates of FCV used in this study. The sequence for UTCVM-NH2 isolate is shown; for the other isolates, only nucleotides that differ from UTCVM-NH2 are shown. The dots above the sequences are spaced every 10 residues

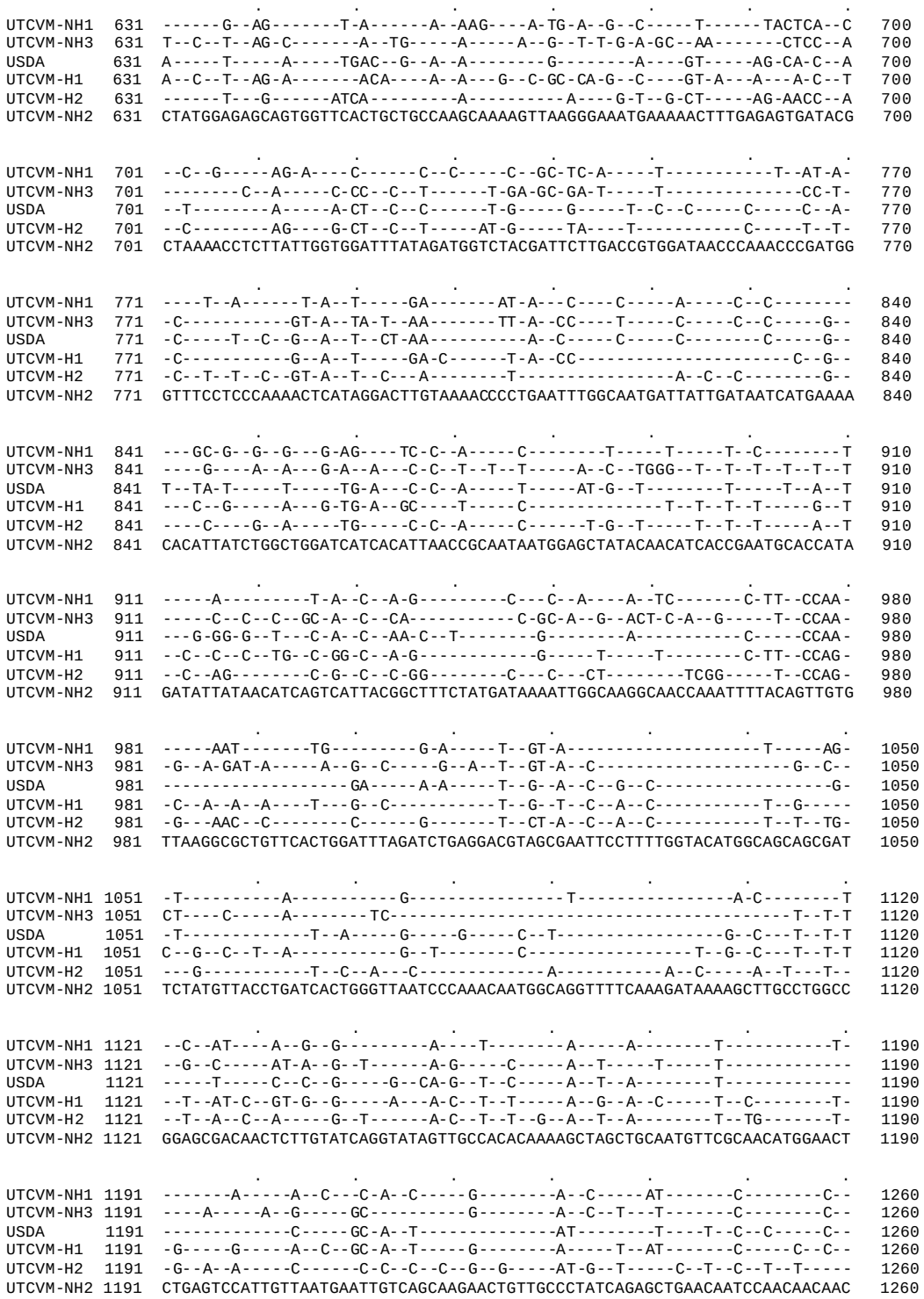
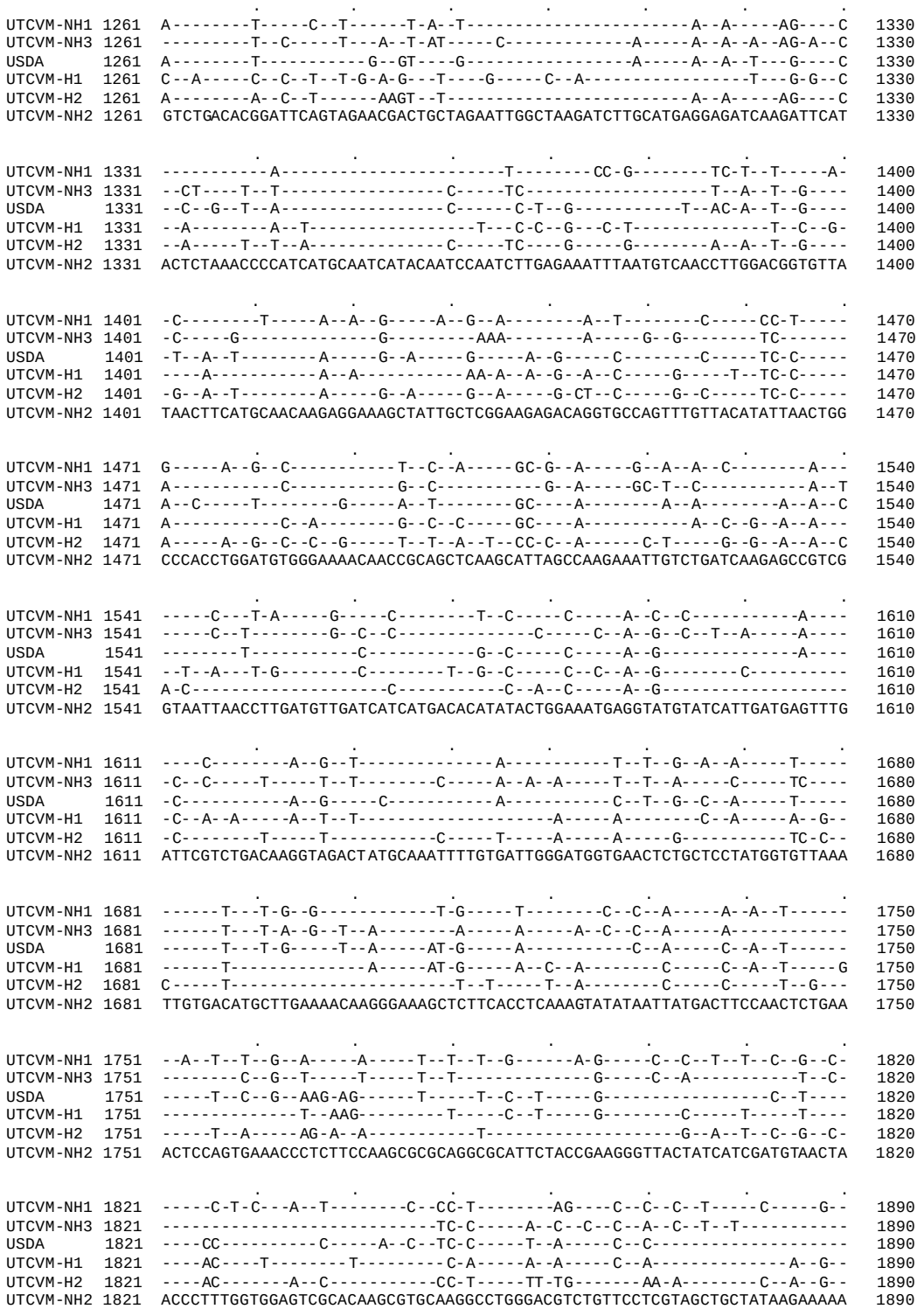


Figure (4.1) continued



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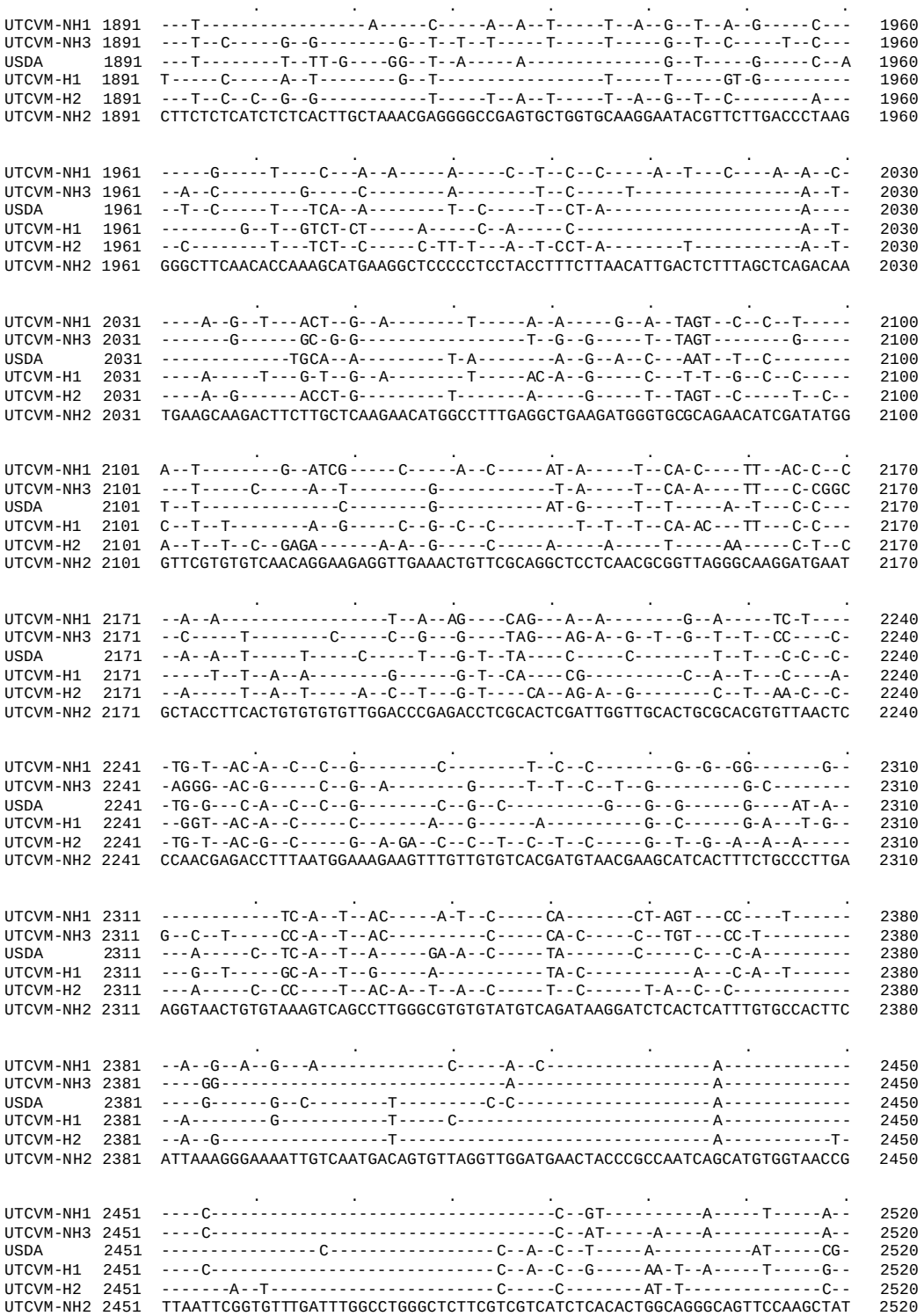
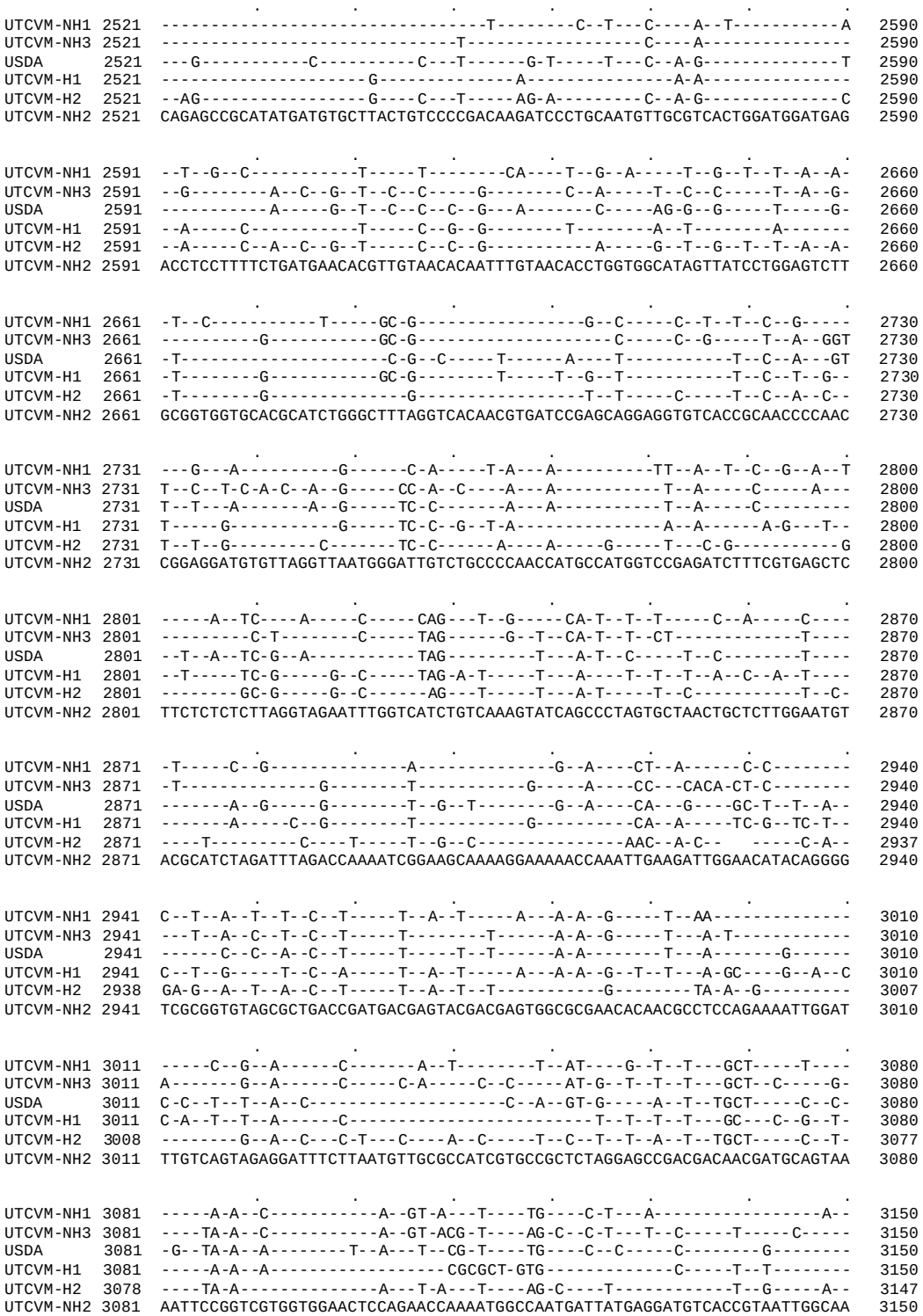


Figure (4.1) continued



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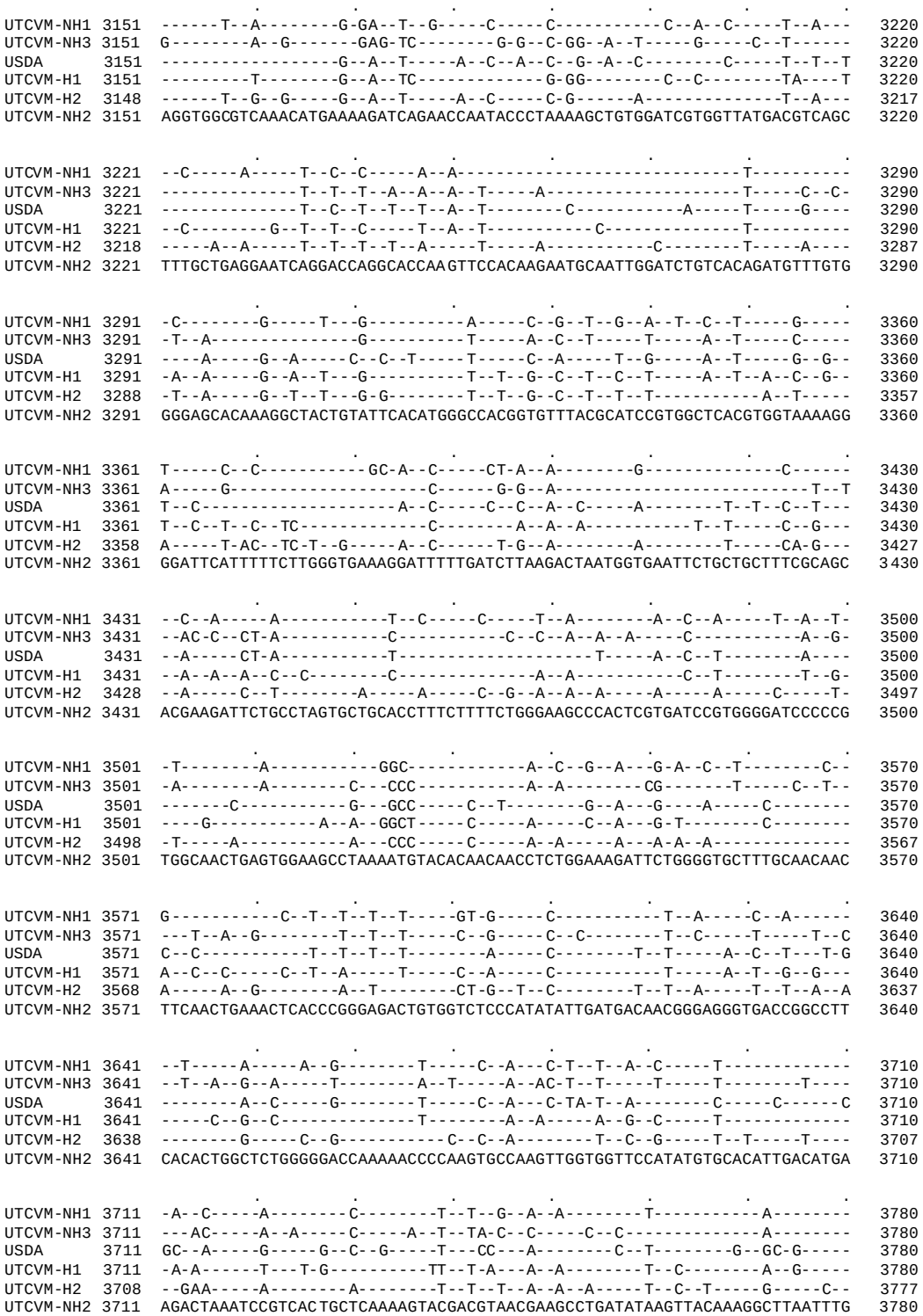

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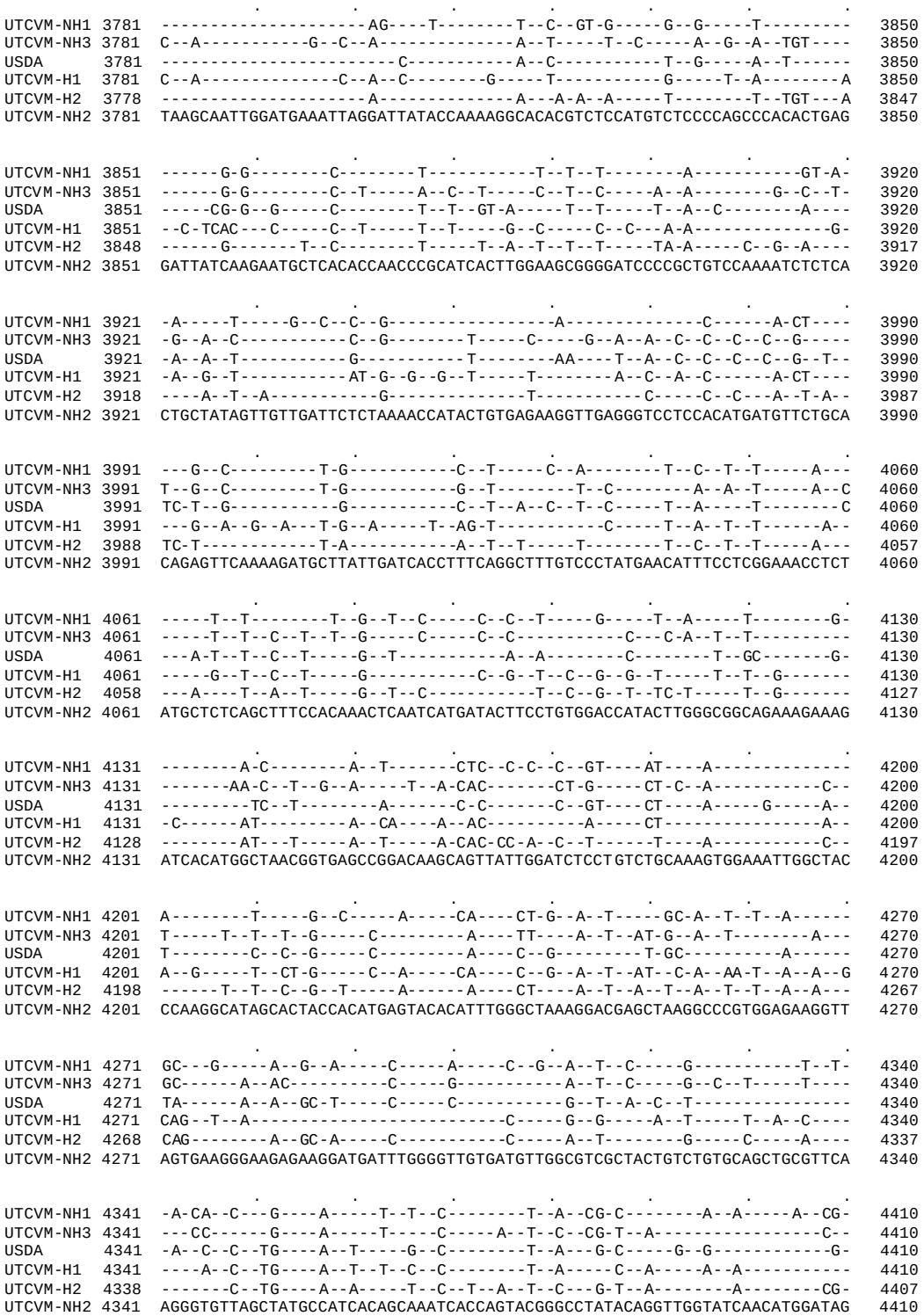

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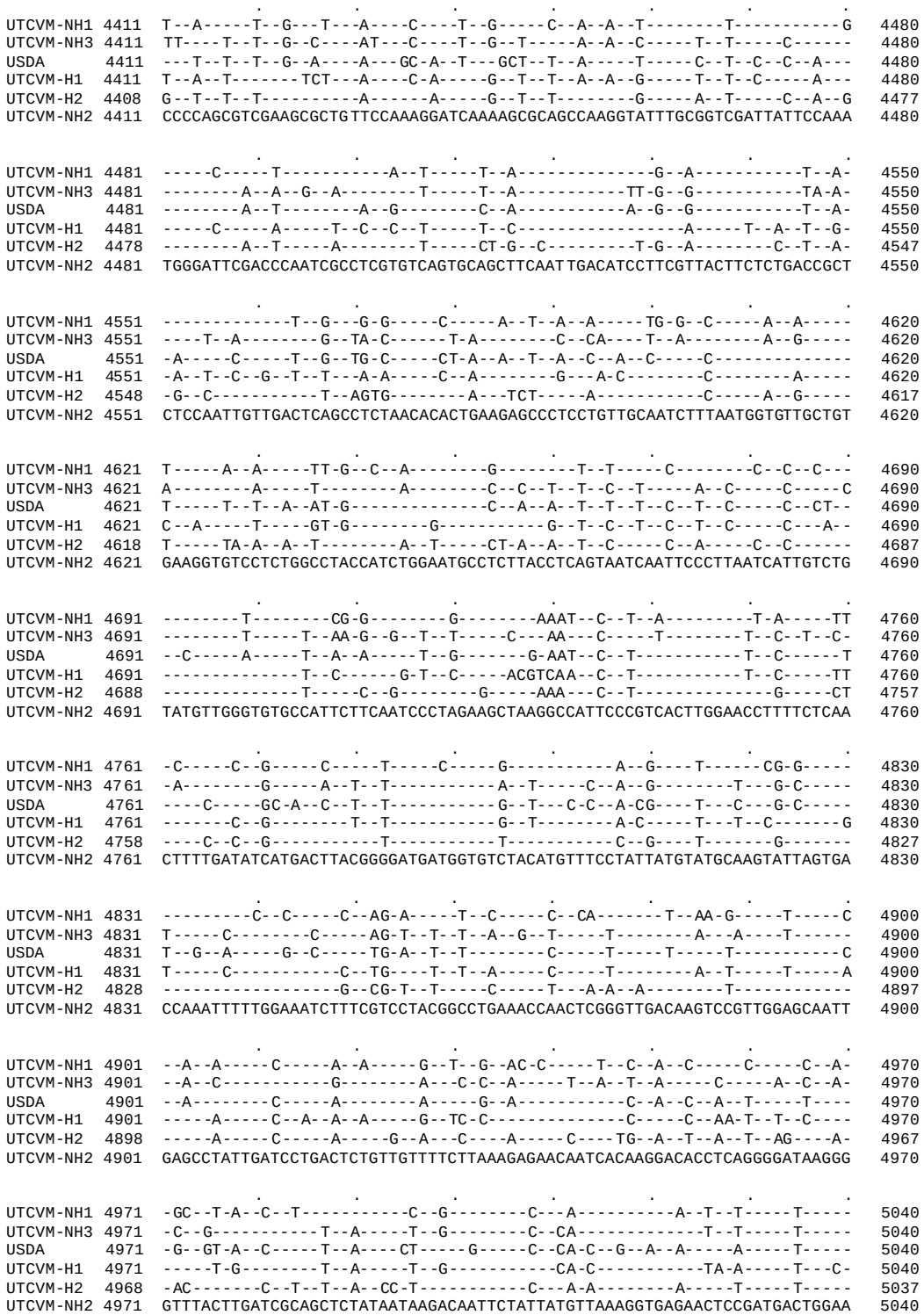

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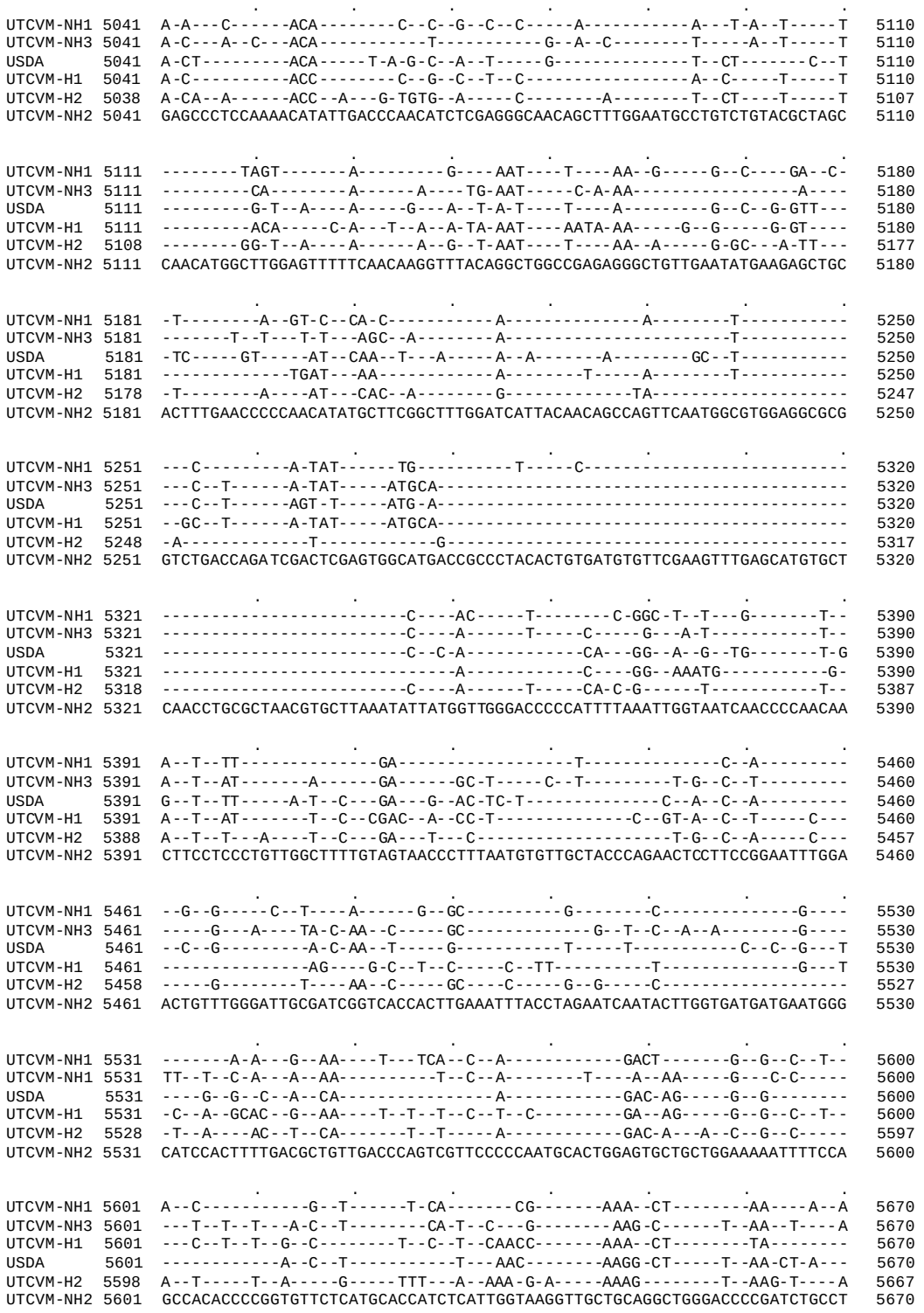

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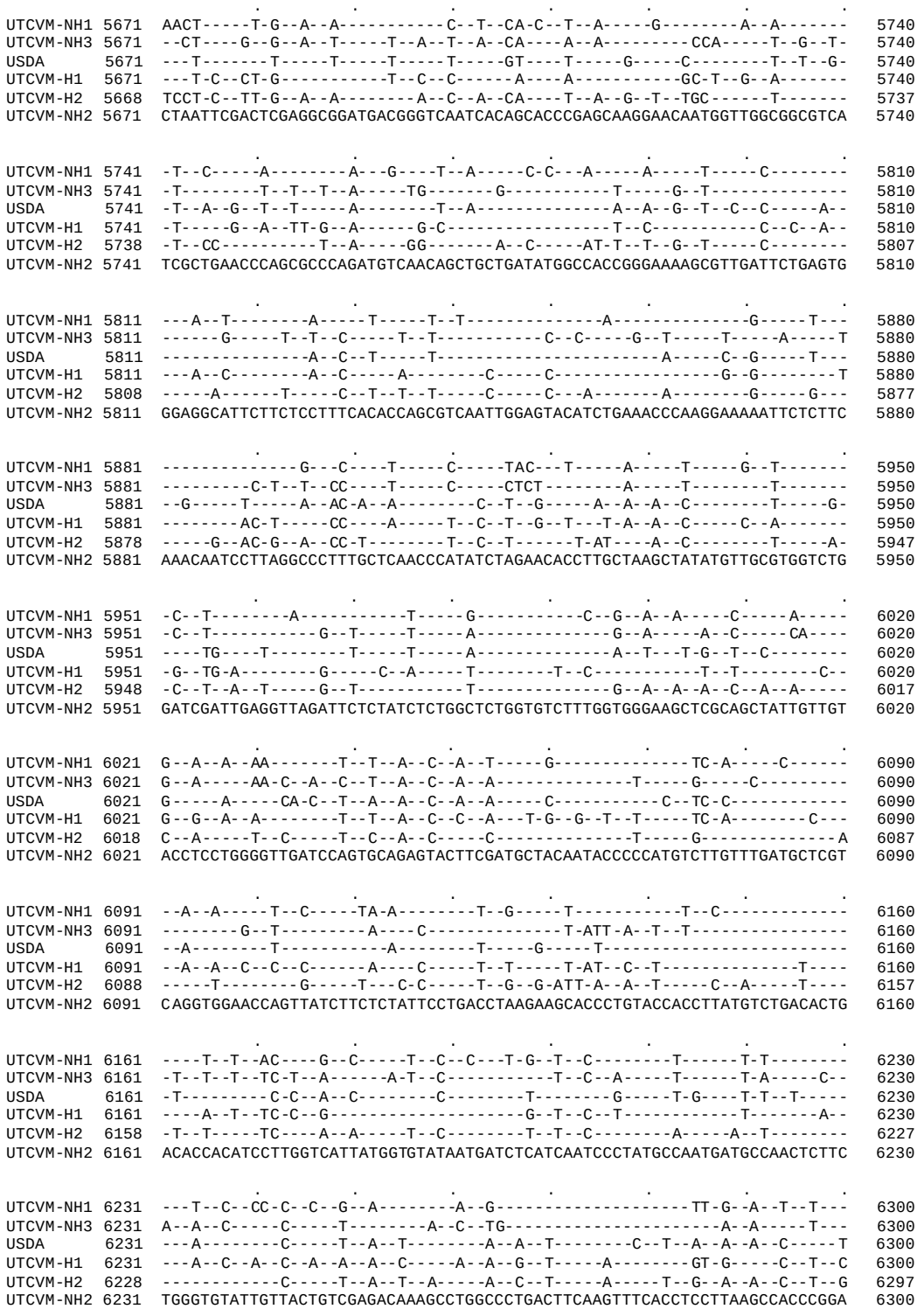

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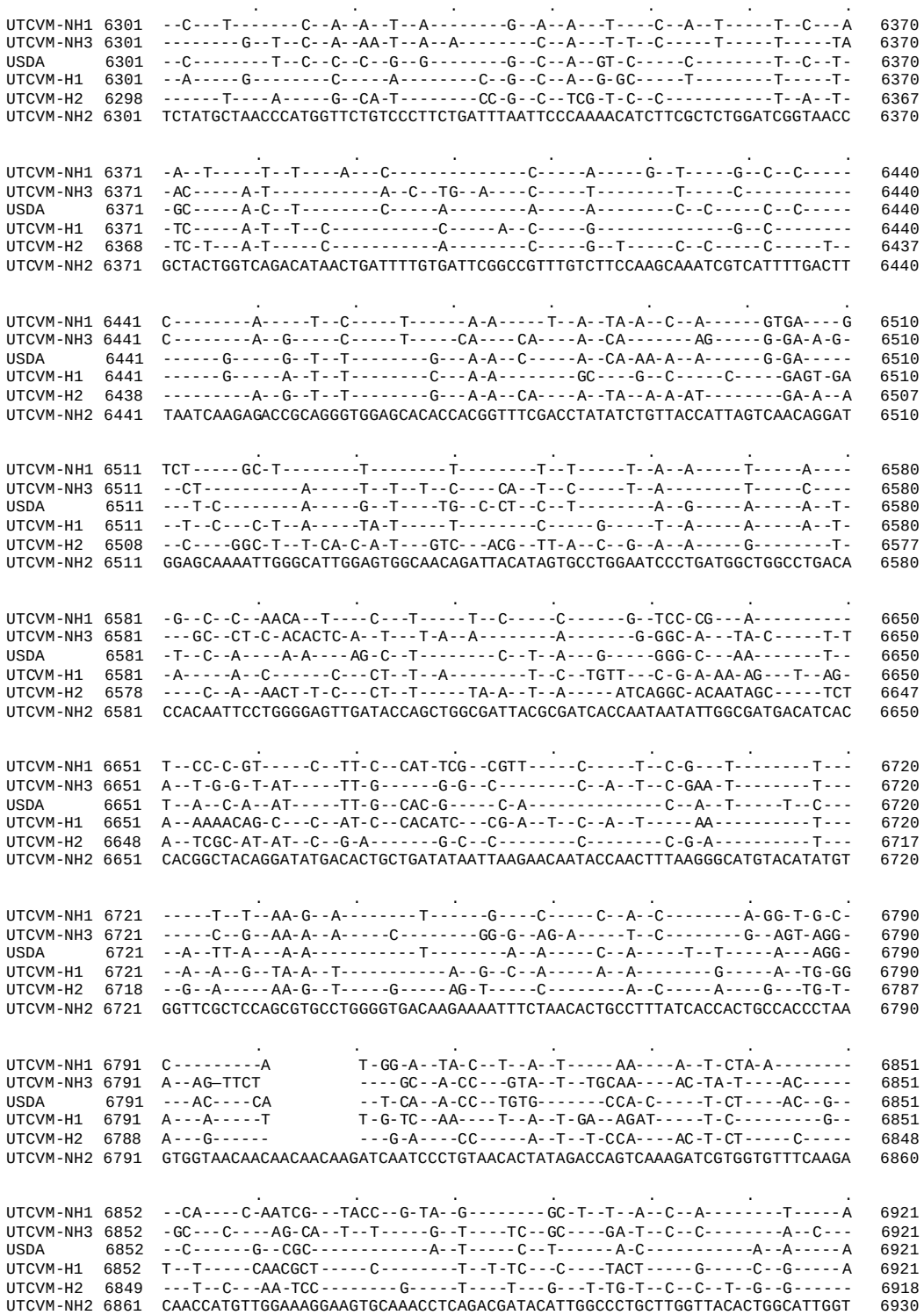

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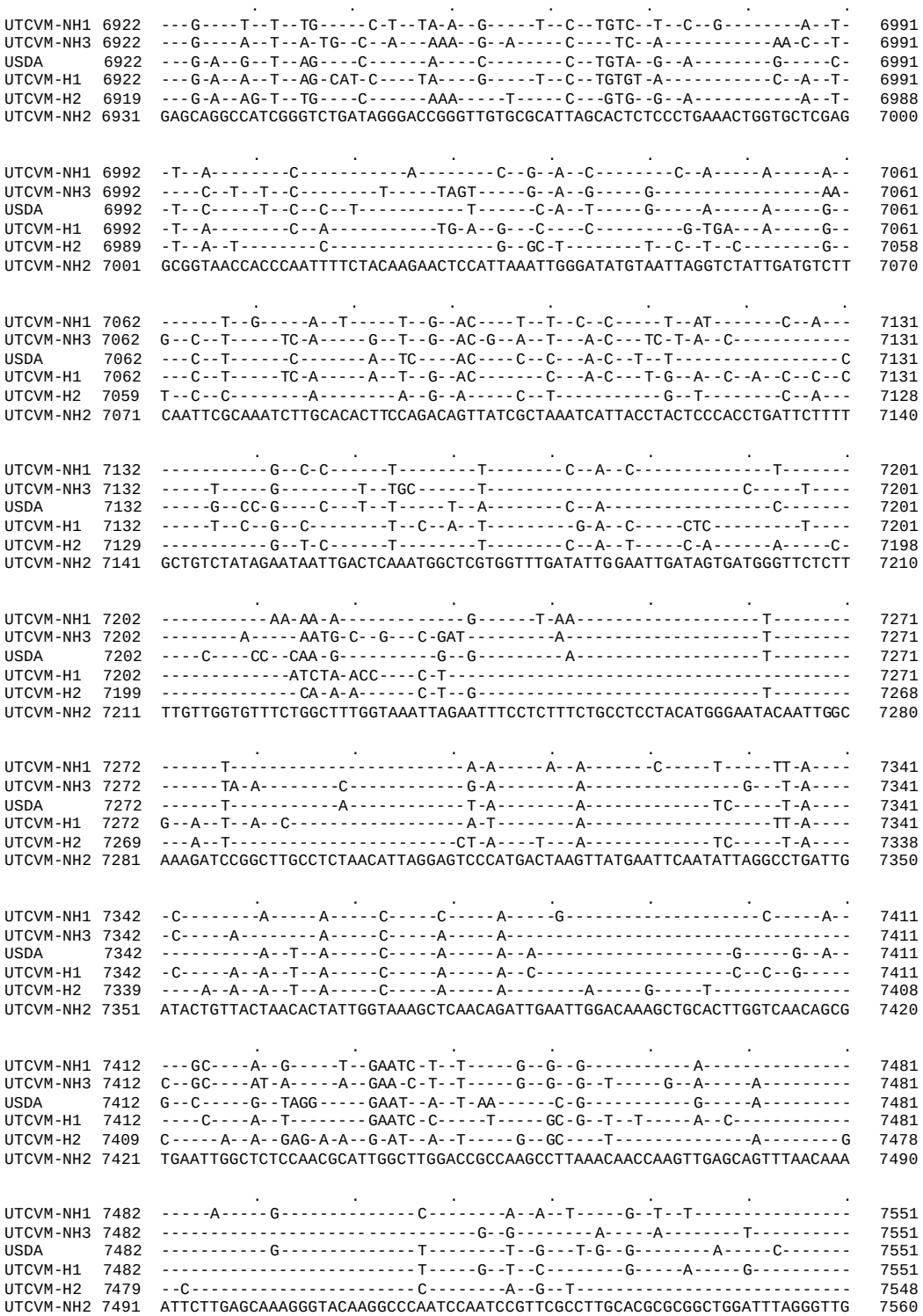

Figure (4.1) continued

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                          .         .         .         .         .         .         .
UTCVM-NH1 7552  ----------T--------T-----T--------T--------A---------C-G----------GG--   7621
UTCVM-NH3 7552  -------------------T--G--T--------T-----G--A---------C-G----------GG--   7621
USDA      7552  -------------------T-----T----------------------------------------G---   7621
UTCVM-H1  7552  -------------------T--G-----------T--------A---------C-G----------GG--   7621
UTCVM-H2  7549  -------------------T-----T-----------------A--------------T-------G---   7618
UTCVM-NH2 7561  ACCCTTACTCATACACAAACCAAAACTTTTATGACGATCAATTGAATGCAATTAGACTATCATATAAAAA   7630

                          .         .         .         .         .         .
UTCVM-NH1 7622  ------------G-------G-G-AT-CC-TC-GGCTGC---A-C---GCCTAA--CCA-  7681
UTCVM-NH3 7622  ---------G-A--AG-TTG--T-GGTCA-AT-TAT--CTT-GG--CGTCGCATTTGC-C  7681
USDA      7622  -C---------A--------G-------------A-----T-TA----------------  7681
UTCVM-H1  7622  ------------G------C--G-AT-CC-TT-GGCTGA----CCTTGGCGC-TAACCCC  7681
UTCVM-H2  7619  -C-A----------------G--------C------A---AAT-----------------  7678
UTCVM-NH2 7631  TTTGTTTAAAATTTGATCATATATCCCTTTGGGCTGCCGCGCCTGCGCCTAACCCCAGGG  7690
```

Figure (4.1) continued

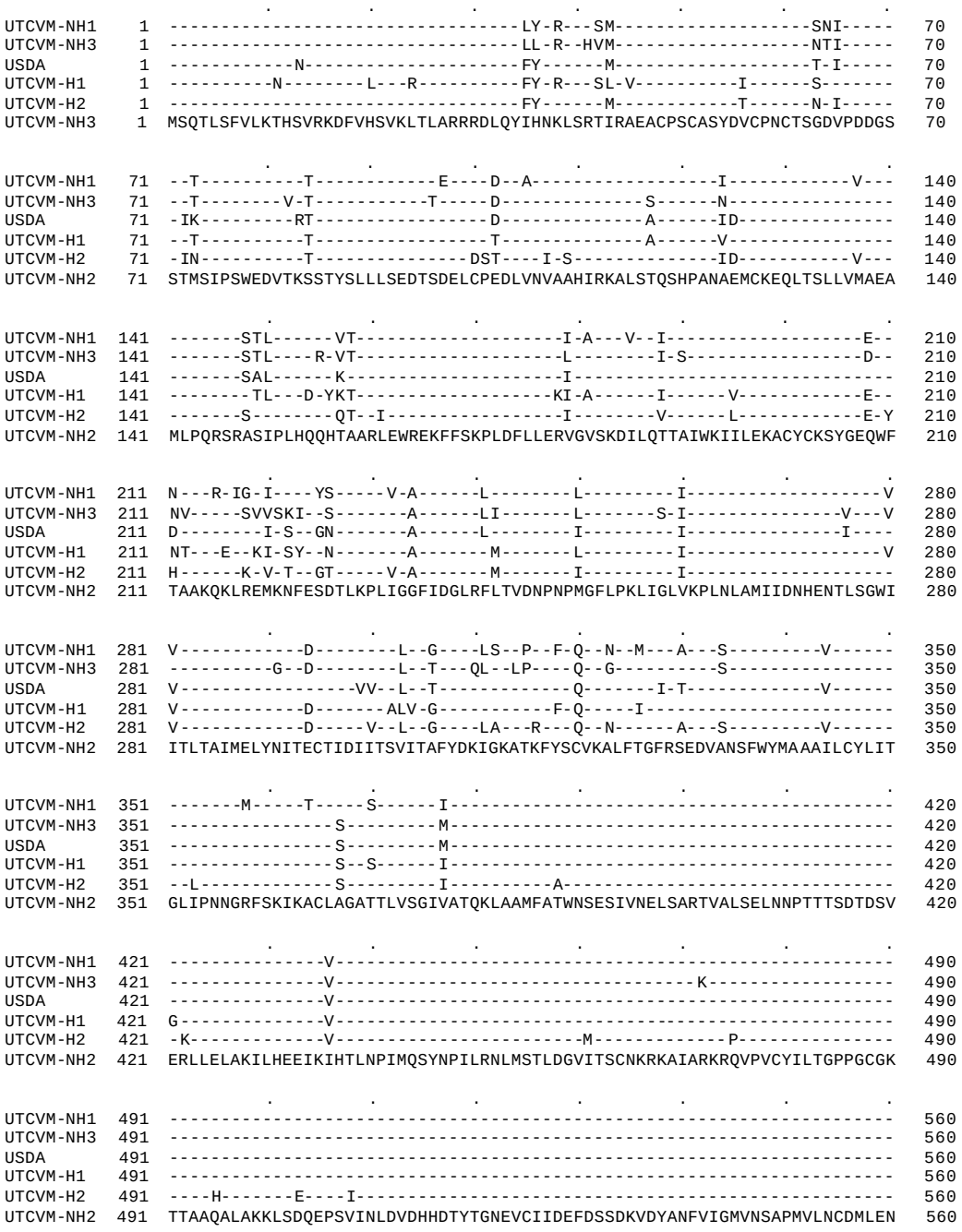
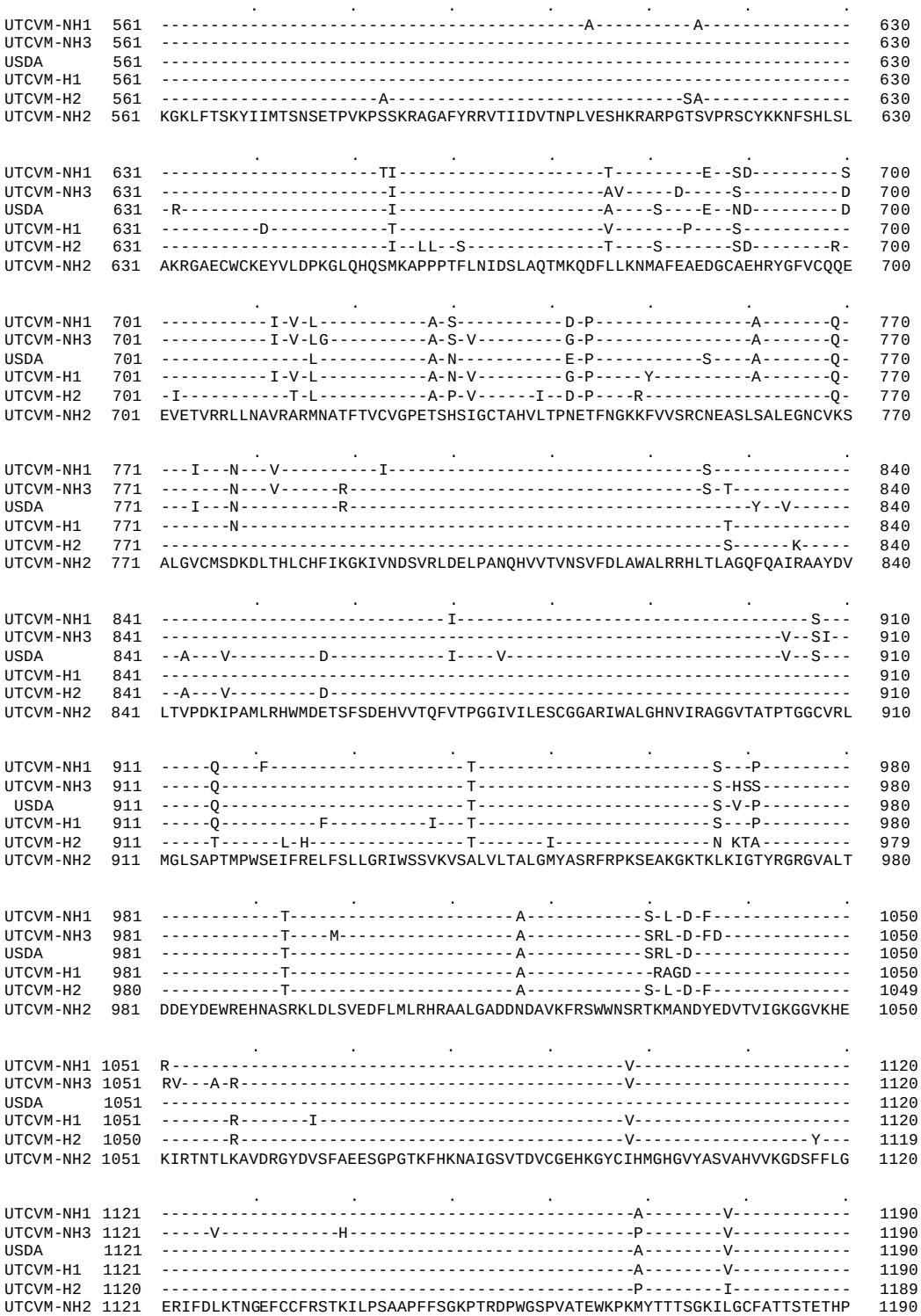


Figure 4.2: complete amino acid sequences of ORF-1 for the six isolates of Feline Calicivirus used in this study. The sequence for UTCVM-NH2 isolate is shown; for the other isolates, only residues that differ from UTCVM-NH2 are shown. The dots above the sequences are spaced every 10 residues



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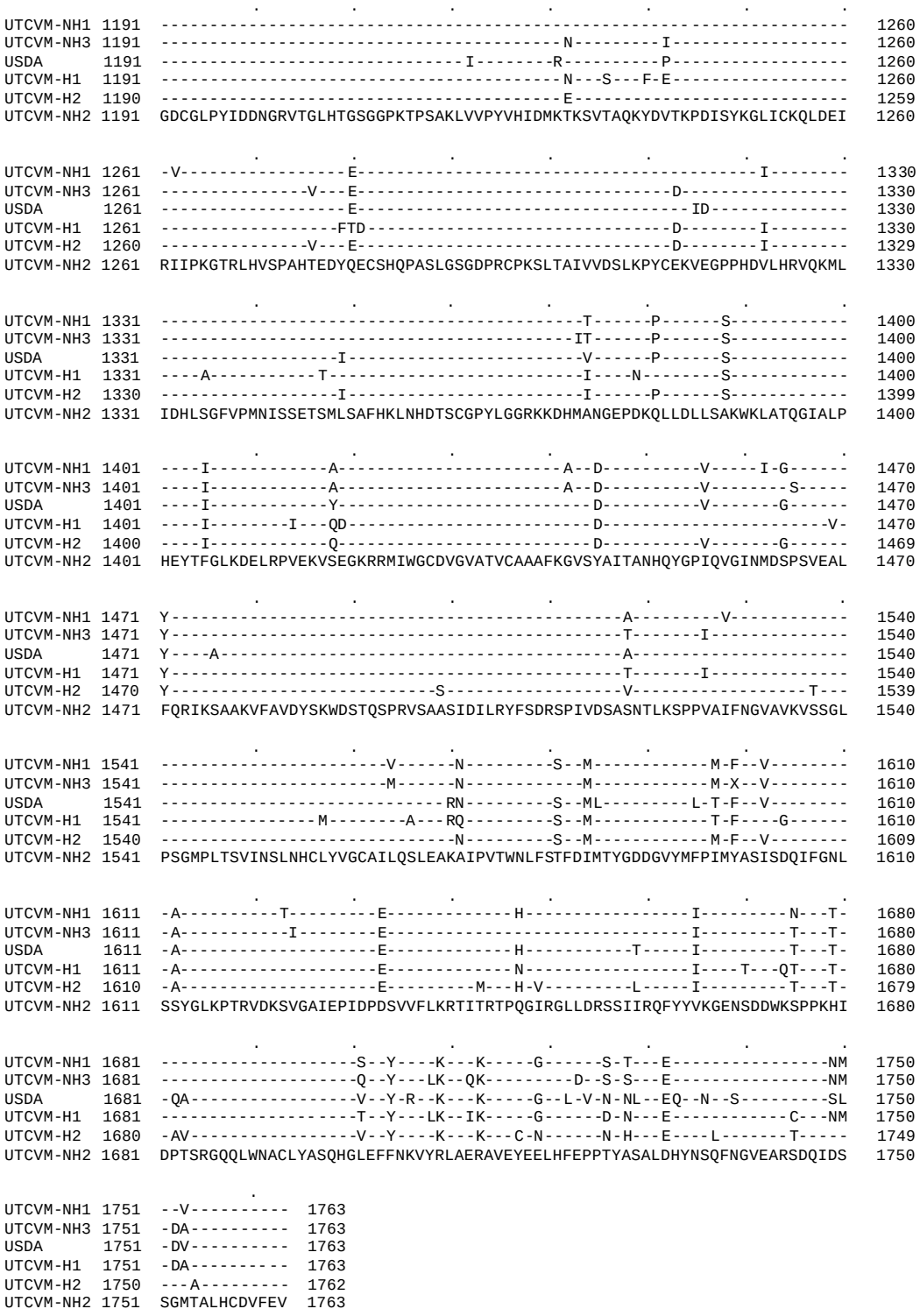

Figure (4.2) continued

the capsid protein, and the ORF3 encodes a minor protein with unknown function. The length of ORF1 was constant in most isolates (5308 nucleotides encoding 1763 amino acids). Two exceptions were encountered: FCV isolate UTCVM-H had a deletion mutation of three nucleotides (nucleotides 2928-2930 of the ORF1), and FCV isolate CFI/68 had a deletion mutation of a three nucleotides (nucleotides 1802-1804 of the ORF1) (figure 4.2).

The polyprotein encoded by ORF1 is cleaved into six proteins. These proteins are called p5.6, p32, p39 (NTPase), p30, p13 (VPg), and p 76 (proteinase-polymerase) respectively [136]. Except for an insignificant change of the cleavage site between P39 (NTPase) and P30 at amino acid residue 686, the cleavage sites of these proteins among isolates were conserved. The cleavage site between P39 (NTPase) and P30 was aspartic acid in isolates: UTCVM-NH2, UTCVM-NH3, UTCVM-H1 and UTCVM-H2, whereas it was glutamic acid in isolates, UTCVM-NH1 and USDA.

The capsid gene protein is located in the middle of the genome and is transcribed by both the genomic RNA and sub-genomic RNA 2.4 Kb. The capsid protein is encoded by the ORF2. In this study, it was predicted to have 668 amino acids in all isolates except UTCVM-NH2

which had three more amino acids in the hypervariable area E compared to the other isolates (figure 4.3).

ORF3 encodes 106 amino acids representing the VP2 structural protein. Its start codon overlaps the stop codon of ORF2 (figure 4.4).

The number of nucleotides in the 3' untranslated region was variable, ranging from 44 nucleotides (USDA, UTCVM-H2 and UTCVM-NH2), to 46 nucleotides in UTCVM-NH1, to 48 nucleotides in UTCVM-H1 and to 52 in UTCVM-NH3.

Comparison of the entire genome sequence among isolates indicated 78.3-80.6% homology (table 4.2 and figure 4.5). Comparison of ORF1 nucleotide sequence indicated 77.8-80.7% homology (table 4.3), whereas similarities among capsid gene nucleotide sequences were 74.8-81.1% among the FCV isolates used in this study (table 4.4). The ORF3 nucleotide homology among isolates was 82.4-90.3% (table 4.5). The similarities of the deduced amino acids among the ORF1 ranged from 90.3-95.9% (table 4.6). It was 88.3-94.3% for ORF2 (table 4.7) and 95.3-100% for the ORF3 (table 4.8) among the isolates examined. The genetic analysis of the isolates revealed a very high homology of UTCVM-NH2 to vaccine strain F9 (97.7-100.00%), and of FCV2024 to USDA FCV (99.9-100.00).

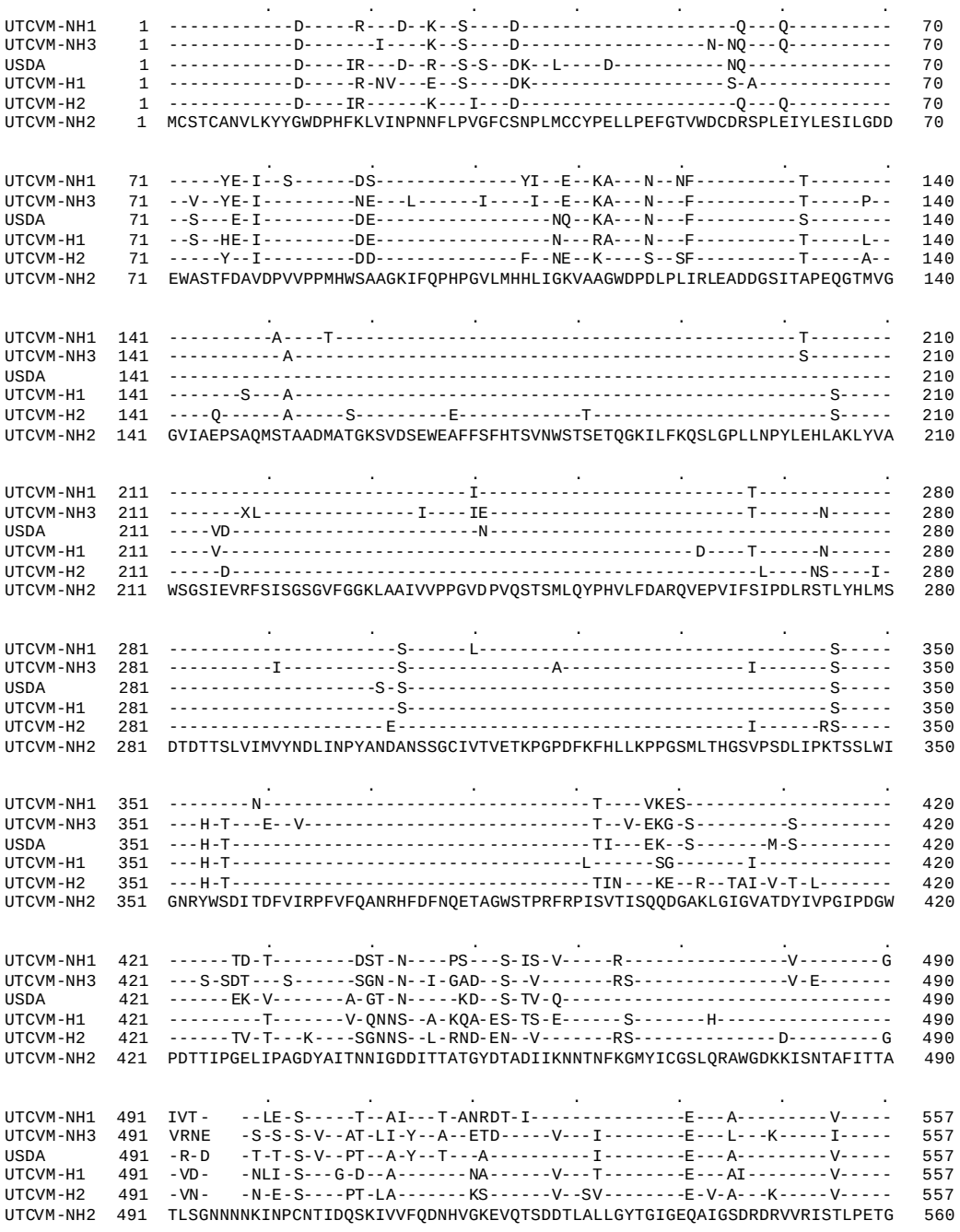

Figure 4.3: complete predicted amino acid sequences of ORF-2 for the six isolates of FCV used in this study. The sequence for UTCVM-NH2 isolate is shown; for the other isolates, only residues that differ from UTCVM-NH2 are shown. The dots above the sequences are spaced every 10 residues

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                      .         .         .         .         .         .         .
UTCVM-NH1  558  ------------------------------------------S---------T--------------   627
UTCVM-NH3  558  T----------------------M-------------N-S-------------A------------   627
UTCVM-H1   558  -------------------------------------N-------------T--------------   627
UTCVM-H2   558  ------------AM------GD-----------------N----------------------V------   627
USDA       558  -------------M------S-------------------------------T------------N--   627
UTCVM-NH2  561  ARGGNHPIFYKNSIKLGYVIRSIDVFNSQILHTSRQLSLNHYLLPPDSFAVYRIIDSNGSWFDIGIDSDG   630

                      .         .         .         .
UTCVM-NH1  628  ------NNI-------T------------------T----  668
UTCVM-NH3  628  -----I-NV---I----------------------A----  668
USDA       628  -----A-NV-------T------------------S----  668
UTCVM-H1   628  -------SIP-------------------------T----  668
UTCVM-H2   628  -------HI--------------------------S-I--  668
UTCVM-NH2  631  FSFVGVSGFGKLEFPLSASYMGIQLAKIRLASNIRSPMTKL  671
```

Figure (4.3) continued

```
                    .         .         .         .         .         .         .
UTCVM-NH1     1  -----------------------------------MN----------------------------   70
UTCVM-NH3     1  -----------------------------------MN----------------------------   70
USDA          1  --------------------------------D---R-MN--Q-------------------------   70
UTCVM-H1      1  -----------------------------------MN---------D--------------------   70
UTCVM-H2      1  ----------------------------------R-MD----------------------------   70
UTCVM-NH2     1  MNSILGLIDTVTNTIGKAQQIELDKAALGQQRELALQRIGLDRQALNNQVEQFNKILEQRVQGPIQSVRL   70

                         .         .         .
UTCVM-NH1    71  ----------------------------R----M  106
UTCVM-NH3    71  ----------------------------R----N  106
USDA         71  ----------------------------R----N  106
UTCVM-H1     71  ----------------------------R----M  106
UTCVM-H2     71  ----------------------------R-----  106
UTCVM-NH2    71  ARAAGFRVDPYSYTNQNFYDDQLNAIRLSYKNLFKI  106
```

Figure 4.4: complete predicted amino acid of ORF-3 for the six isolates of feline calicivirus used in this study. The sequence for UTCVM-NH2 isolate is shown; for the other isolates, only residues that differ from UTCVM-NH2 are shown. The dots above the sequences are spaced every 10 residues

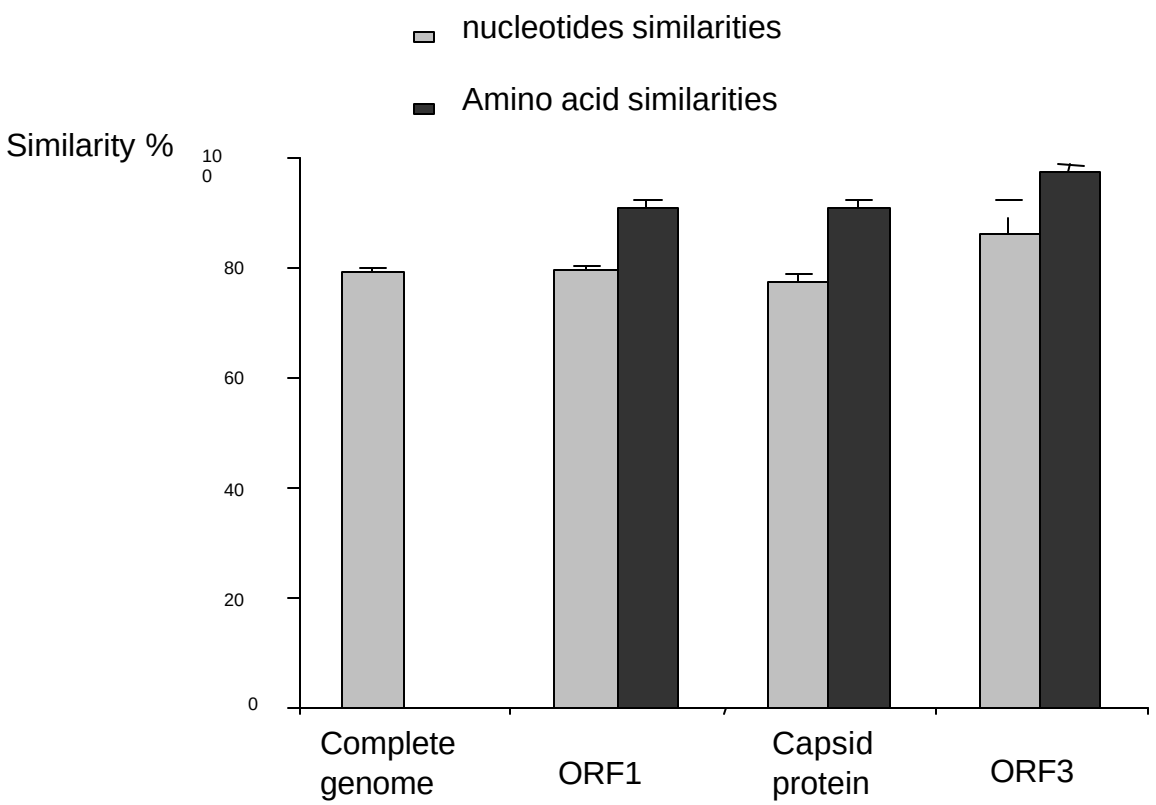

Figure 4.5: average nucleotide homology and % amino acids similarities % among the 12 FCV isolates used in this study

Table 4.2: nucleotide percentage similarities among the different FCV isolates; comparison of complete genome

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		79.7	79.2	79.9	79.6	79.2	79.7	98.2	78.9	78.7	78.4	79.9
F4	79.7		78.9	79.4	78.7	78.7	78.8	79.6	78.4	79.8	78.3	79.4
F65	79.2	78.9		79.4	79.4	79.7	80.0	79.3	78.7	79.5	79.4	79.4
FCV 2024	79.9	79.4	79.4		80.1	79.6	80.1	79.9	79.7	79.9	79.9	99.9
Urbana	79.6	78.7	79.4	80.1		79.7	79.5	79.7	79.9	78.9	78.9	80.1
CFI/68	79.2	78.7	79.7	79.6	79.7		80.6	79.2	79.0	79.0	79.4	79.7
UTCV M-NH1	79.7	78.8	80.0	80.1	79.5	80.6		79.6	80.6	79.5	79.8	80.2
UTCV M-NH2	98.2	79.6	79.3	79.9	79.7	79.2	79.6		79.0	78.6	78.4	79.9
UTCV M-NH3	78.9	78.4	78.7	79.7	79.9	79.0	80.6	79.0		78.6	78.4	79.7
UTCV M-H1	78.7	78.4	79.5	79.9	78.9	79.0	79.5	78.6	78.6		78.4	80.0
UTCV M-H2	78.4	78.3	79.4	79.9	78.9	79.4	79.8	78.4	78.4	78.4		79.8
USDA	79.9	79.4	79.4	99.9	80.1	79.7	80.2	79.9	79.7	80.0	79.8	

Table 4.3: nucleotide percentage similarities among the different FCV isolates; comparison of non structural poly-protein (ORF1)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		79.4	79.3	79.9	79.5	78.9	79.7	98.4	79.4	79.3	78.6	79.9
F4	79.4		78.5	79.0	77.8	78.4	78.7	79.2	79.4	80.2	78.0	79.1
F65	79.3	78.5		79.7	79.4	79.6	80.1	79.3	79.1	79.5	79.3	79.7
FCV 2024	79.9	79.0	79.7		80.3	79.8	80.6	79.9	80.3	80.5	80.2	99.9
Urbana	79.5	77.8	79.4	80.3		79.8	79.6	79.5	80.2	79.4	79.2	80.4
CFI/68	78.9	78.4	79.6	79.8	79.8		80.2	79.1	79.3	79.3	79.3	80.7
UTCV M-NH1	79.7	78.7	80.1	80.6	79.6	80.2		79.8	81.3	80.0	80.1	80.7
UTCV M-NH2	98.4	79.2	79.3	79.9	79.5	79.1	79.8		79.5	79.1	78.7	79.9
UTCV M-NH3	79.4	79.4	79.1	80.3	80.2	79.3	81.3	79.5		79.3	79.6	80.3
UTCV M-H1	79.3	79.4	79.5	80.5	79.4	79.3	80.0	79.1	79.3		78.7	80.5
UTCV M-H2	78.6	78.0	79.3	80.2	79.2	79.3	80.1	78.7	79.6	78.7		80.2
USDA	79.9	79.1	79.7	99.9	80.4	80.7	80.7	79.9	80.3	80.5	80.2	

Table 4.4: nucleotide percentage similarities among the different FCV isolates; comparison of capsid protein gene (ORF2)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		79.3	77.2	78.7	78.7	78.9	78.5	97.7	76.3	75.4	76.3	78.7
F4	79.3		78.1	78.9	79.0	77.9	77.7	79.0	78.1	77.4	77.8	78.9
F65	77.2	78.1		77.1	77.4	78.4	77.8	77.7	75.7	77.4	77.6	77.1
FCV 2024	78.7	78.9	77.1		78.1	77.8	77.8	78.7	77.3	77.0	77.1	99.9
Urbana	78.7	79.0	77.4	78.1		78.2	77.3	78.9	77.2	76.0	76.6	78.0
CFI/68	78.9	77.9	78.4	77.8	78.2		81.1	78.1	76.9	77.1	78.5	77.9
UTCV M-NH1	78.5	77.7	77.8	77.8	77.3	81.1		78.0	77.1	76.3	77.8	77.8
UTCV M-NH2	97.7	79.0	77.7	78.7	78.9	78.1	78.0		76.4	75.6	76.0	78.7
UTCV M-NH3	76.3	78.1	75.7	77.3	77.2	76.9	77.1	76.4		74.8	77.6	77.3
UTCV M-H1	75.4	78.1	77.4	77.0	76.0	77.1	76.3	75.6	74.8		76.0	77.1
UTCV M-H2	76.3	77.8	77.6	77.1	76.6	78.5	77.8	76.0	77.6	76.0		77.1
USDA	78.7	78.9	77.1	99.9	78.0	77.9	77.8	78.7	77.3	77.1	77.1	

Table 4.5: nucleotide percentage similarities among the different FCV isolates; comparison of VP2 (ORF3)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		85.2	87.1	85.2	83.3	84.3	84.6	98.7	84.9	84.9	84.9	85.2
F4	85.2		87.1	85.5	87.1	84.6	85.2	85.9	85.9	85.5	82.4	85.5
F65	87.1	87.1		86.5	89.9	86.8	88.4	86.5	89.3	88.4	88.1	86.5
FCV 2024	85.2	85.5	86.5		85.5	86.2	84.6	85.2	83.3	85.5	87.4	100.0
Urbana	83.3	87.1	89.9	85.5		86.8	89.3	84.0	90.3	86.8	86.2	85.5
CFI/68	84.3	84.6	86.8	86.2	86.8		84.6	84.9	87.4	84.9	84.9	86.2
UTCV M-NH1	84.6	85.2	88.4	84.6	89.3	84.6		84.6	88.7	89.3	85.2	84.6
UTCV M-NH2	98.7	85.9	86.5	85.2	84.0	84.9	84.6		85.5	84.9	85.5	85.2
UTCV M-NH3	84.9	85.9	89.3	83.3	90.3	87.4	88.7	85.5		88.7	85.2	83.3
UTCV M-H1	84.9	85.5	88.4	85.5	86.8	84.9	89.3	84.9	88.7		84.0	85.5
UTCV M-H2	84.9	82.4	88.1	87.4	86.2	84.9	85.2	85.5	85.2	84.0		87.4
USDA	85.2	85.5	86.5	100.0	85.5	86.2	84.6	85.2	85.5	85.5	87.4	

Table 4.6: amino acid percentage similarities among the different FCV isolates; comparison of non structural poly-protein (ORF1)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		91.7	93.0	94.8	95.3	94.1	94.7	99.6	93.9	94.4	94.3	94.8
F4	91.7		90.3	91.8	91.0	90.4	91.8	91.7	91.0	92.4	90.8	91.9
F65	93.0	90.3		93.7	93.0	92.5	93.4	93.2	92.3	93.4	92.7	93.8
FCV 2024	94.8	91.8	93.7		95.5	94.1	95.9	95.0	94.9	95.3	95.4	99.9
Urbana	95.3	91.0	93.0	95.5		94.0	95.7	95.5	94.6	94.4	94.8	95.5
CFI/68	94.1	90.4	92.5	94.1	94.0		94.0	94.2	93.0	93.9	93.6	94.2
UTCV M-NH1	94.7	91.8	93.4	95.9	95.7	94.0		94.8	95.8	95.2	95.1	95.8
UTCV M-NH2	99.6	91.7	93.2	95.0	95.5	94.2	94.8		94.0	94.6	94.4	95.0
UTCV M-NH3	93.9	91.0	92.3	94.9	94.6	93.0	95.8	94.0		94.6	93.9	95.0
UTCV M-H1	94.4	92.4	93.4	95.3	94.4	93.9	95.2	94.6	94.6		94.0	95.4
UTCV M-H2	94.3	90.8	92.7	95.4	94.8	93.6	95.1	94.4	93.9	94.0		95.3
USDA	94.8	91.9	93.8	99.9	95.5	94.2	95.8	95.0	95.0	95.4	95.3	

Table 4.7: amino acid percentage similarities among the different FCV isolates; comparison of capsid protein gene (ORF2)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		91.5	90.4	90.6	93.0	91.9	90.9	98.1	89.4	89.8	88.3	90.7
F4	91.5		91.3	91.9	94.3	91.5	91.8	91.3	91.5	90.1	90.0	92.9
F65	90.4	91.3		91.8	92.1	91.8	91.2	90.7	90.1	90.4	90.3	91.8
FCV 2024	90.6	91.9	91.8		92.5	91.8	91.6	90.7	91.6	90.4	90.0	99.9
Urbana	93.0	94.3	92.1	92.5		93.0	91.8	92.7	91.3	91.0	89.2	92.7
CFI/68	91.9	91.5	91.8	91.8	93.0		93.4	91.8	89.1	90.4	89.5	91.9
UTCV M-NH1	90.9	91.8	91.2	91.6	91.8	93.4		90.7	89.4	89.7	89.1	91.8
UTCV M-NH2	98.1	91.3	90.7	90.7	92.7	91.8	90.7		89.2	90.0	89.1	90.1
UTCV M-NH3	89.4	91.5	90.1	91.6	91.3	89.1	89.4	89.2		89.4	88.9	91.8
UTCV M-H1	89.8	90.1	90.4	90.4	91.0	90.4	89.7	90.0	89.4		89.7	90.1
UTCV M-H2	88.3	90.0	90.3	90.0	89.2	89.5	89.1	89.1	88.9	89.7		90.0
USDA	90.7	92.9	91.8	99.9	92.7	91.9	91.8	90.1	91.8	90.1	90.0	

Table 4.8: amino acid percentage similarities among the different FCV isolates; comparison of VP2 (ORF-3)

FCV isolate	F9	F4	F65	FCV 2024	Urb-ana	CFI/ 68	UTCV M-NH1	UTCV M-NH2	UTCV M-NH3	UTCV M-H1	UTCV M-H2	USDA
F9		95.3	96.2	95.3	97.2	97.2	97.2	100.0	97.2	97.2	97.2	95.3
F4	95.3		98.1	95.3	97.2	97.2	96.2	95.3	97.2	96.3	95.3	95.3
F65	96.2	98.1		97.2	99.1	99.1	98.1	96.2	99.1	98.1	96.2	97.2
FCV 2024	95.3	95.3	97.2		98.1	98.1	97.2	95.3	98.1	97.2	97.2	100.0
Urbana	97.2	97.2	99.1	98.1		100. 0	99.1	97.2	100.0	99.1	97.2	98.1
CFI/68	97.2	97.2	99.1	98.1	100. 0		99.1	97.2	100.0	99.1	97.2	98.1
UTCV M-NH1	97.2	96.2	98.1	97.2	99.1	99.1		97.2	99.1	100.0	97.2	97.2
UTCV M-NH2	100. 0	95.3	96.2	95.3	97.2	97.2	97.2		97.2	97.2	97.2	95.3
UTCV M-NH3	97.2	97.2	99.1	98.1	100. 0	100. 0	99.1	97.2		99.1	97.2	98.1
UTCV M-H1	97.2	96.3	98.1	97.2	99.1	99.1	100.0	97.2	99.1		97.2	97.2
UTCV M-H2	97.2	95.3	96.2	97.2	97.2	97.2	97.2	97.2	97.2	97.2		97.2
USDA	95.3	95.3	97.2	100. 0	98.1	98.1	97.2	95.3	98.1	97.2	97.2	

Phylogenetic analysis

The phylogenetic tree based on the complete genome nucleotide sequence revealed that all FCV isolates belong to the same genotype regardless of geographical origin or clinical signs (figure 4.6). The phylogenetic analysis, based on the complete genome nucleotide sequences, revealed that isolates F9 and UTCVM-NH2 segregate together as a result of a high degree of nucleotide sequence similarities. The only difference appears to be a few point mutations. Similarly, FCV2024 and USDA also segregate together. F9 and UTCVM-NH2 had a high degree of similarity of the base sequences (more than 99%) and they segregate together in phylogenetic analysis. The latter observations suggest that F9 and UTCVM-NH2 are virtually identical. The same conclusion was made with USDA and FCV2024 isolates. Both cases might represent the same virus with a few point mutations. UTCVM-H2 and CFI/68 isolates grouped together as a result of a three-nucleotide deletion in the ORF1, which is considered a conserved area.

Phylogenetic trees based on nucleotide sequences and deduced amino acid sequences of the six different proteins of ORF1 (figures 4.7, 4.8 and 4.9) showed also that the FCV isolates, included in this study belong to one

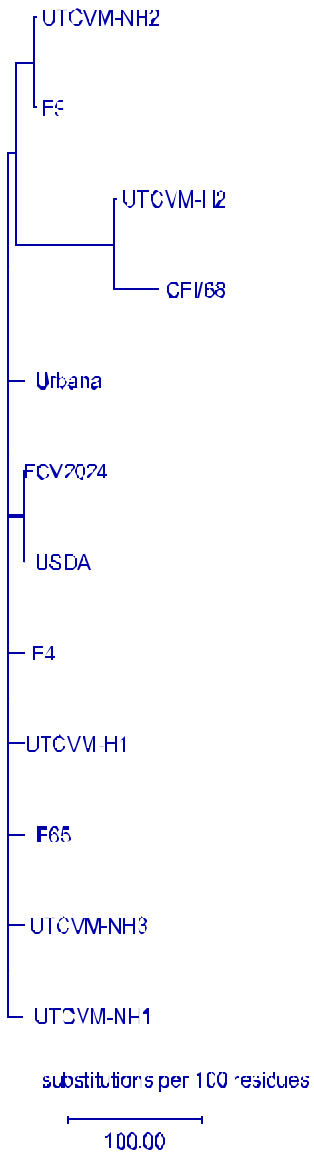

Figure 4.6: phylogenetic tree based on the nucleotide sequence of the complete genome among FCV isolates used in this study

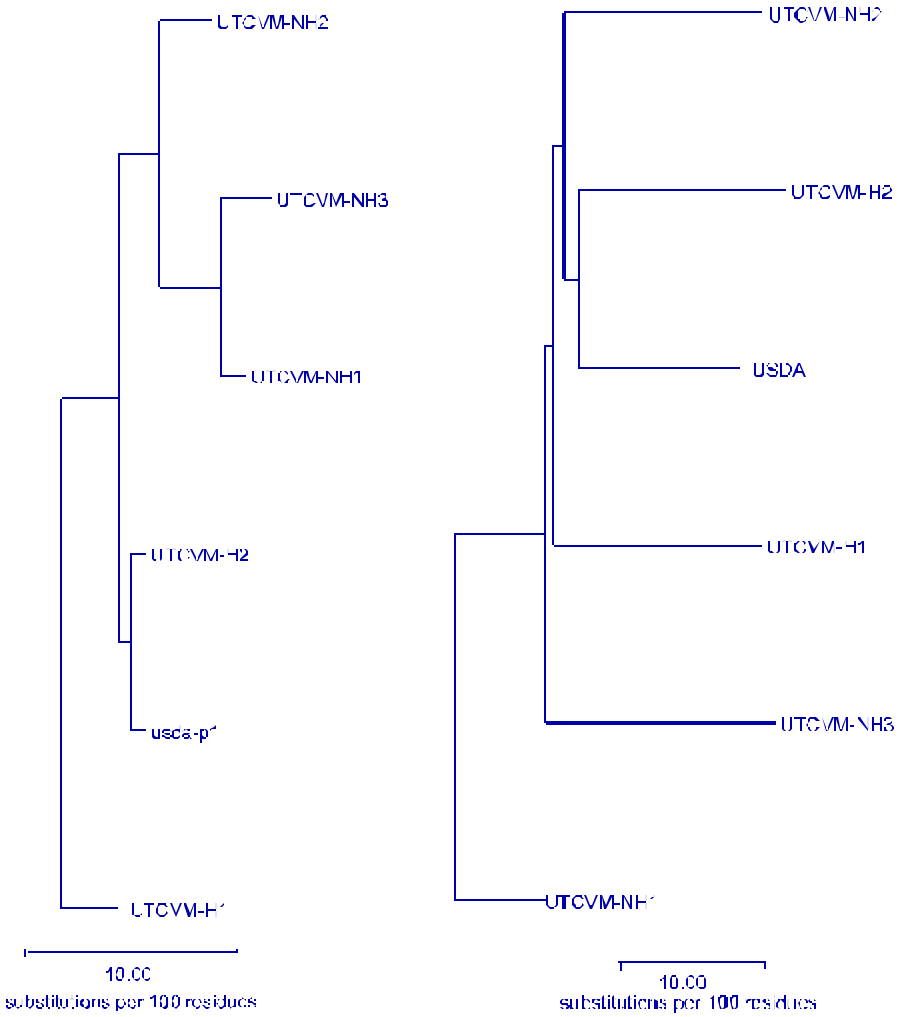

Figure 4.7: phylogenetic tree based on the amino acid sequences of; A) p6.5, and B) p32 of the ORF1

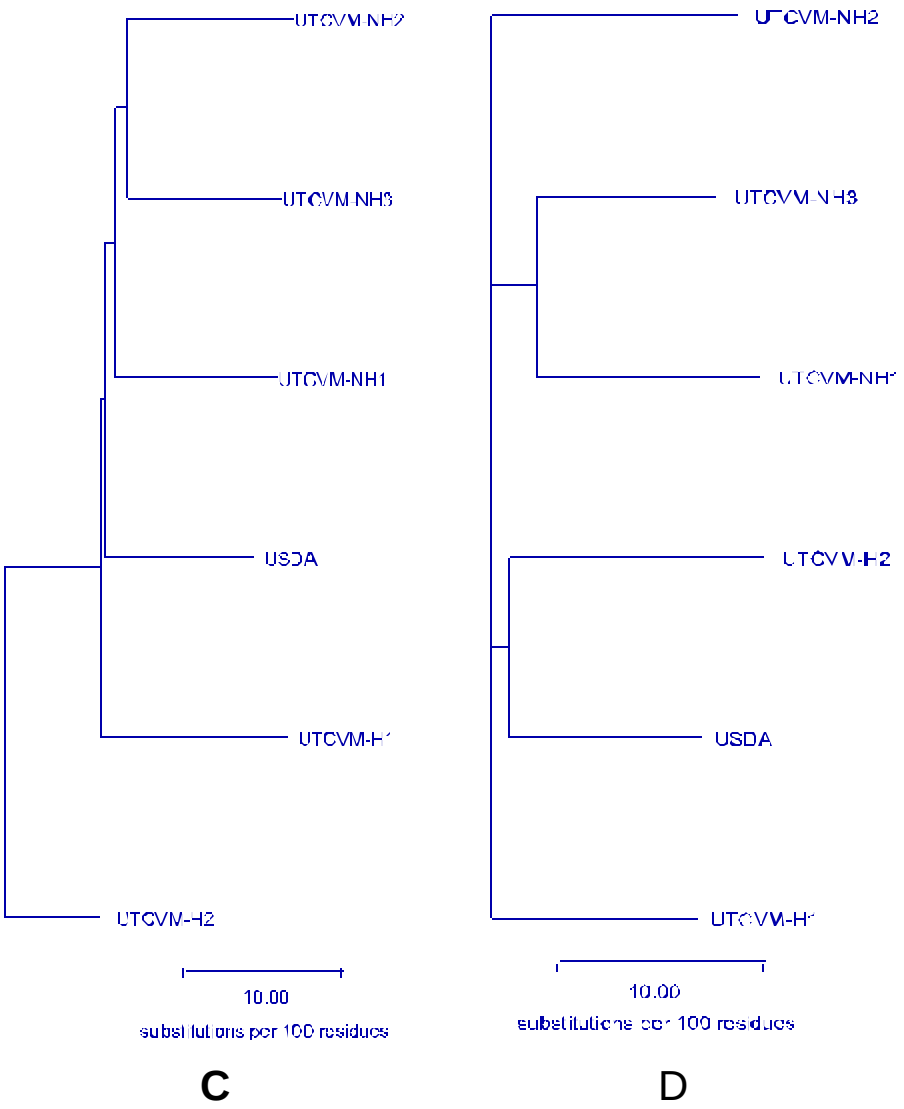

Figure 4.8: phylogenetic tree based on the amino acid sequences of; C) p39 (NTPase and D)) p30 of the ORF1

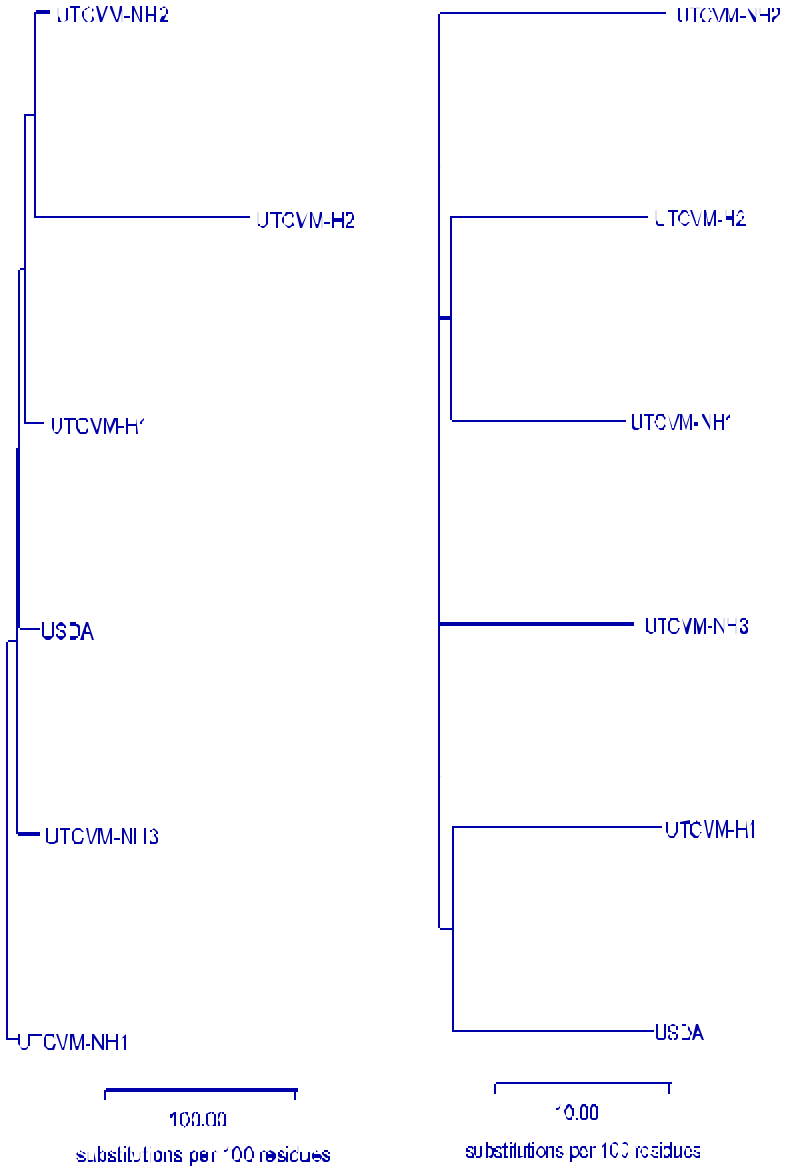

Figure 4.9: phylogenetic tree based on the amino acid sequences of; E) p13 (VPg) and F) p76 (proteinase-polymerase) of the ORF1

genotype. There was no closer relationship of any given FCV isolate to another FCV isolate. This applies also to the two FCV isolates associated with the hemorrhagic disease.

The phylogenetic tree based on the nucleotide sequence of the capsid protein gene (figure 4.10), strongly supported the finding that all the FCV isolates included in the study belong to the same genotype. The latter tree segregated F9 and UTCVM-NH2 isolates together because these two isolates represent the same virus (both have 9 nucleotide insertions in the hypervariable region E of the capsid protein gene).

The two isolates associated with hemorrhagic disease could not be distinguished from each other by phylogenetic analysis of the complete genome, ORF1 (using the 6 different cleaved proteins), ORF2 and ORF3 using both nucleotide and deduced amino acid sequences. The exception was using the area containing amino acids 426-445 of the capsid protein, (figure 4.11).

Sequence data was not available for the entire genome of the FCV hemorrhagic-like disease isolates other than UTCVM-H1 and UTCVM-H2, but nucleotide sequences of a portion of the capsid E area were used to create the phylogenetic tree of the hypervariable E area (data not shown).

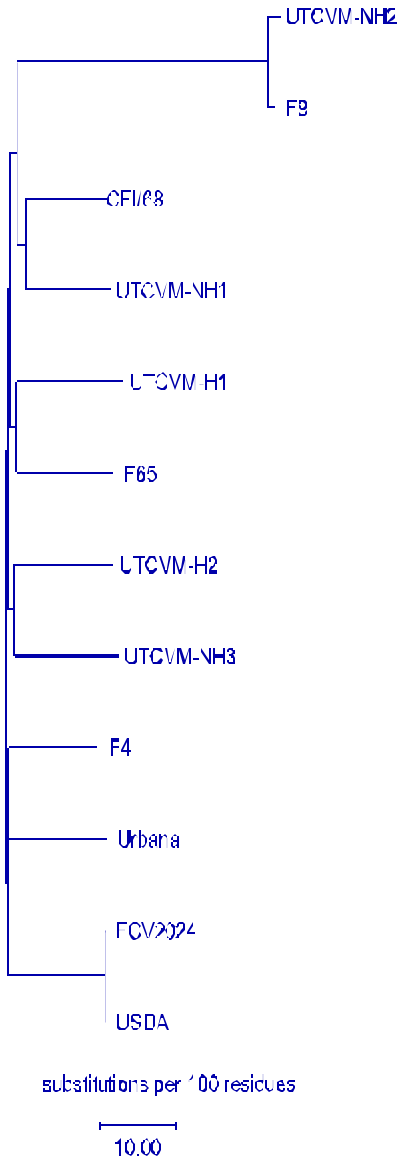

Figure 4.10: phylogenic tree based on the nucleotide sequence of the capsid protein gene among FCV isolates used in this study.

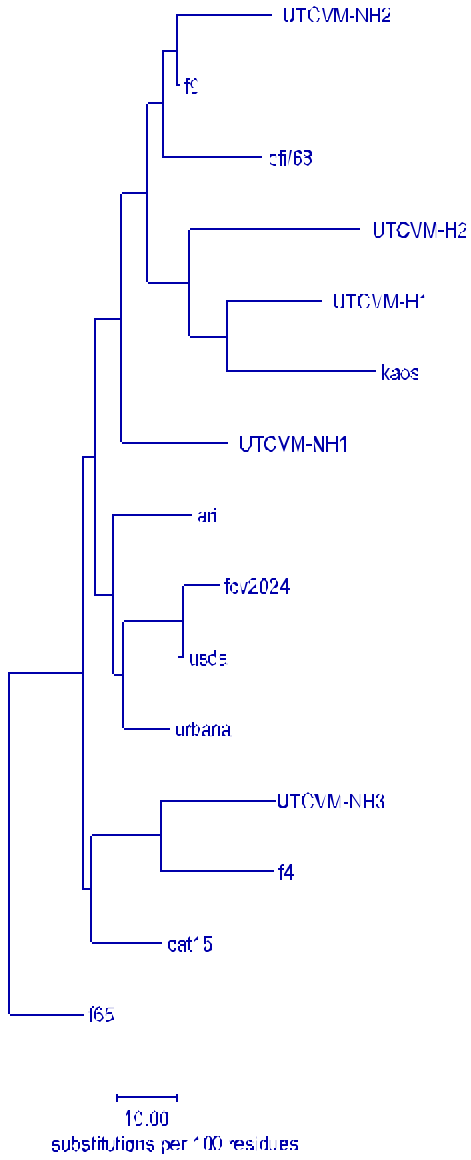

Figure 4.11: phylogenic tree based on the amino acids residues 426-445of the capsid protein among FCV isolates used in this study.

Using hypervariable E area to create a phylogenetic tree revealed one genotype of FCV. In contrast, a phylogenetic tree created by using deduced amino acid residues 426-445 of the capsid protein gene showed clustering of UTCVM-H1 and UTCVM-H2 along with the hemorrhagic-like isolate Kaos (figure 4.11).

Neutralization pattern

Seven serum samples were used to determine the neutralization pattern against four of the FCV isolates (UTCVM-H1, UTCVM-H2, UTCVM-NH1 and USDA). Five samples were from convalescent cats in the cattery from which UTCVM-H1 had been isolated. The five sera had very high antibody titers against the homologous isolate (UTCVM-H1), but not against other isolates. The titer against the UTCV-H1 was greater than 2560 in all of these sera while it was not greater than 320 against any of the other FCV isolates. A sample from a cat infected with UTCVM-H2 had the highest antibody titer (320) to homologous isolate (UTCVM-H2), and it did have cross reactivity with UTCVM-H1 at lower titer (80) while it had very low neutralization titer to the other two isolates associated with classical disease. The antibody titers for each sample against the four FCV isolates used are given in table 4.9.

Table 4.9: neutralization titer of the serum sample from cats infected with FCV associated with hemorrhagic-like disease against four FCV isolates

	Neutralization titer			
Serum samples	UTCVM-H1	UTCVM-H2	UTCVM-NH1	USDA
UTCVM-H1# 1	> 2560	40	10	40
UTCVM-H1 # 2	> 2560	80	20	40
UTCVM-H1 # 3	> 2560	40	< 10	40
UTCVM-H1 # 4	640	< 10	< 10	< 10
UTCVM-H1 #5	> 2560	80	20	320
UTCVM-H2 #1	80	320	10	10
Negative control	< 10	< 10	< 10	< 10

Immunofluorescence

The serum samples from the UTCVM-H1 outbreak were tested for antibodies by indirect immuno-fluorescence. All the samples reacted with high titer against all the FCV isolates (table 4.10). The results suggested that all cats infected with UTCVM-H1 had a high a cross-reactive antibody titer when the whole virus particles were used as antigen.

Table 4.10: immunofluorescence antibody titers of the serum sample from cats infected with FCV associated with hemorrhagic-like disease against four FCV isolates

FCV isolate	UTCVM-H1	Fort Dodge type C	Fort Dodge type D (F9)	USDA
serum samples				
UTCVM-H1 #1	2560	2560	1280	1280
UTCVM-H1 #2	2560	2560	5120	10240
UTCVM-H1 #3	5120	2560	5120	640
UTCVM-H1 #4	5120	5120	10240	10240
UTCVM-H1 #5	5120	10240	10240	10240
UTCVM-H1 #6	10240	2560	5120	2560
UTCVM-H1 #7	5120	5120	10240	10240
UTCVM-H1 #8	2560	1280	2560	2560
UTCVM-H1 #9	1280	1280	1280	1280
UTCVM-H1 #10	320	640	320	1280
UTCVM-H1 #11	640	640	640	640

X- Discussion

Changes of viral pathogenesis due to genomic mutation have been reported. Molecular analyses of virus isolates associated with altered pathogenesis defined various genomic alterations. These genomic alterations included insertions of cellular sequences, duplications of viral sequences, genomic rearrangements (bovine virus diarrhea virus), deletion mutation (swine coronavirus), and point mutation (parvovirus) [60,75, 121]. On the basis of fragmentary sequence data that exists on FCV associated with hemorrhagic-like disease, no conclusive hypothesis has been made to explain the genetic basis of FCV pathogenesis [61,100,130].

Bovine viral diarrhea virus (BVDV) is an RNA virus. Two biotypes of BVDV, distinguished by their effect on cell culture, are recognized; cytopathic (cp) and non-cytopathic (ncp) [47]. The difference between these two biotypes is attributed to genetic mutations. These mutations are due to insertion mutations in specific sites (p125/NS23 non-structural protein gene) of the viral RNA or point mutation within NS2 gene [75]. The insertion mutation is a consequence of genetic recombination of bovine cellular RNA [86,113,155] or rearrangement and duplication of viral RNA sequence [87,113,145] or, more interestingly, recombination of sequences from a vaccine strain [8].

While p125/NS23 non-structural protein gene has been expressed in both cytopathogenic and non-cytopathogenic BVDV, p80/NS3 (non-structure protein) has been expressed, exclusively by all cytopathogenic BVDV [37,104]. In addition to p80/NS3 expression, p54/NS2 has been expressed exclusively in some, not all cytopathogenic BVDV [4,74]. These mutations are the basis of a virulent disease (mucosal disease) developing in persistently infected animals and characterized by high case fatality with death occurring usually within two weeks [3]. The recombinant virus is antigenically identical to the parent virus but the biotype changes (non-cytopathic to cytopathic) [141]. The nucleotide sequence of the 5 untranslated region is the internal ribosome entry site and the translation initiation sequence, and is considered a virulent marker for different strains of BVDV [105,125].

Canine parvovirus and feline panleukopenia virus have over 98% DNA homology but their antigenic and hemagglutination properties differ and each has a distinct host range. The differences have been attributed to some amino acid variations of the capsid protein (VP1 and VP2 proteins) [60]. Another example of a pathogenicity changes due to genetic mutation is the porcine coronavirus associated with respiratory tract disease which likely originated from transmissible gastroenteritis virus (TGEV). A large deletion

in the 5'-region of the TGEV spike glycoprotein gene may have been responsible for a change in tissue tropism from gastrointestinal tract to respiratory epithelium.[15,121]

Calicivirus infections associated with a hemorrhagic-like disease with high mortality rates have occurred in cat populations.[61,100,130] FCV has been isolated from cats with fatal systemic infections and lesions attributable to vasculitis and severe necrotizing pneumonia. This novel presentation is similar to that observed with calicivirus infection of Rabbit Hemorrhagic Disease, and may represent an alteration in calicivirus tissue tropism in cats. Severe upper respiratory tract infection also was present in all reported outbreaks of the severe disease. Consequently, it is possible that FCV mutants with enhanced virulence may develop from a parent "classical" virus, in cats infected with the latter. Characteristic clinical signs were consistently reproducible when the FCV associated with hemorrhagic disease was experimentally administrated to research cats [100]. Because the disease is not always accompanied by hemorrhagic lesions, it has been suggested to rename the disease as a "virulent systemic FCV" disease instead of "hemorrhagic-like disease" [61]. The hemorrhagic-like disease was

an indication of the involvement of blood vessels resulting in vasculitis and edema.

Virus neutralization assays were done in this study to determine the antigenic relationship of FCV isolates associated with hemorrhagic-like disease to one another and to other FCV isolates associated with classical disease. Not surprisingly, the pattern of neutralization revealed that the homologous virus induced the highest neutralizing antibody titers. High titers were observed in convalescent serum samples from cats infected with UTCVM-H1. In these cats, the neutralizing antibodies titers, in most cases, were greater than 2560 (table 4.9). These results are in agreement with previous reports [100,130]. Neutralizing antibodies from cats infected with the two hemorrhagic isolates (UTCVM-H1 and UTCVM-H2) used in this study, had some cross-activities but titers against the other FCV isolates tested were relatively low. This suggests that these two virulent isolates share a degree of homology at the neutralizing epitope level but are distinct from the other isolates. As expected, the immuno-fluorescence assay that reacts with all structural and non-structural proteins, a group test, revealed that all the convalescent sera obtained from convalescent cats infected with UTCVM-

H1 had very high antibody titers against all FCV isolates. This confirms extensive antigenic cross-reactivity of several antigens in all FCV isolates.

In this study, the complete genome of two FCV isolates associated with hemorrhagic-like diseases and four FCV isolates associated with "classical" disease were sequenced. Data were analyzed to determine genetic diversity and to identify possible mutations in FCV isolates that may be correlated with disease manifestations. The sequences of the 5' and 3' untranslated regions were also determined because of the potential that these areas may affect viral pathogenesis and for differentiation of the isolates into different genetic subgroups. Sequence data for six additional isolates associated with "classical" disease from GenBank were included also in this study.

The nucleotide homology of the complete genome among various isolates (with two exceptions) was constant, at about 80% (table 4.2). This suggests the existence of a randomly heterogeneous population of viruses which is in agreement with data from previous studies [48,150]. The overall homogeneity existed in spite of isolate diversity (including clinical signs, geographical origin and date of isolation). Two FCV vaccine viruses (F9 and FCV2024) appeared to be identical to disease-associated field isolates

(UTCVM-NH2 and USDA respectively), suggesting that, in some circumstances, vaccine virus can produce disease. The overall length of the FCV viral genome was relatively constant and ranged from 7631 to 7643 nucleotides, which was in agreement with published data on FCV isolates. A lack of clustering among the all FCV isolates was identified in this study, when the complete genome sequence was used for phylogenetic analysis. The phylogenetic tree suggested that FCV exists as a single diverse genotype which is in agreement also with previous reports [44,48]. The phylogenetic analysis with nucleotide sequences of the complete genome nucleotide sequences failed to cluster the virulent isolates, UTCVM-NH1 and UTCVM-NH2, from the other FCV isolates. This contradicts the recent findings of Pesavento *et al* who speculated that the virulent systemic strains of FCV were genetically distinct from other FCV [102]. However the latter study did not present any genetic data for this conclusion.

The length of ORF1 was relatively constant and had 5305 to 5308 nucleotides, which is in agreement with the previous reports on FCV isolates associated with classical disease [48,96,150]. The genetic homogeneity of the ORF1 among the FCV isolates used in this study was approximately 80% which is similar to homogeneity analysis of FCV sequence data previously

reported[150] (table 4.3). The ORF1 encodes a polyprotein which is cleaved by viral-encoded proteinase into six non-structural proteins. The amino acid sequences at the cleavage sites of ORF1, proposed by recent studies, were conserved among FCV isolates included in this study [136,139]. These findings suggest that all non-structural proteins are constant products in FCV isolates including the FCV isolates associated with hemorrhagic-like disease. The phylogenetic tree using nucleotide and amino acid sequences of each of these six ORF1 proteins showed that FCV isolates (including FCV produce both hemorrhagic-like and classical disease) used in this study belong to the same genotype (Fig 4.7, 4.8 4.9). The phylogenetic trees of these proteins showed no clustering of any kind of the FCV isolates. It is clear that, these phylogenetic analyses did not segregate the virulent isolates. Collectively the data on ORF1 suggest that the non-structural polyprotein of FCV is a conserved product among all FCV isolates and does not have an obvious role in the change of viral pathogenesis.

Nucleotide sequences of the capsid protein gene of the various viruses had a homology of 74.8 to 81.1% (table 4.4) which reflects the variability of the capsid protein gene among the FCV isolates. The capsid protein gene is divided into six areas of variability, identified as A, B, C, D, E, and F [134].

The analysis of FCV isolates used in this study confirmed the existence of the same pattern. All FCV isolates, regardless the clinical disease produced, had a similar degree of sequence relatedness to one another over the majority of the capsid gene protein. In contrast, researchers, from Japan identified two genetic groups and an association of each to a geographical origin when a part of the capsid protein gene (area E through F) was used for phylogenetic analysis [128]. In the present study, there was little apparent clustering of isolates associated with the virulent disease when amino acid residues 426 to 445 of the E region was used in phylogenetic analysis (figure 4.11).

The ORF3 encodes a protein, VP2, of unknown function [54,137]. Its function, however, likely is not related to the viral pathogenicity as suggested by the high amino acid similarities of VP2 (95.3-100%) among different FCV isolates associated with a wide range of clinical diseases.

Vaccination against FCV is a common practice. The F9 strain has been widely used for preparation of killed and attenuated vaccines. The vaccine is effective at preventing disease, but occasionally FCV can be isolated from vaccinated cats that develop disease [24,33]. The nature and origin of FCV isolates causing disease in vaccinated cats have not been identified

[33]. Recent sequence studies of FCV isolates causing disease in vaccinated cats suggest these viruses are derived from a vaccine virus, based on very high nucleotide homology of the E hypervariable region (98-100% homology) with vaccine viruses [59,114]. The results in the present study support this conclusion. The UTCVM-NH2, E region, had 94.2% nucleotide homology (92.9% with the deduced amino acids) to that of the F9 isolate while it was 97.7% for the whole capsid protein gene. The E region may be responsible for inducing isolate-specific neutralizing antibodies and therefore may contain the immunologically-relevant part of the capsid protein [153]. Five out of seven amino acid differences in the capsid protein, between F9 and UTCVM-NH2, occurred in E region (amino acid residues 426 to 520) and two out of five differences in amino acid residues 440-443 of the predicted E region peptide. The latter may be responsible for change in viral pathogenicity because these differences occur in the putative neutralizing (and perhaps attachment) site. A recent study identified neutralizing antibodies that target amino acids residues 426 to 460 of the capsid protein [153]. Immunity induced by the F9 vaccine may not protect against UTCVM-NH2 because of the differences in the predicted immunologically relevant peptides (amino acid residue 441 to 443).

The amino acid residues 435 to 444 of various "classical" FCV capsid proteins were compared to the same residues of FCV associated with hemorrhagic-like disease because it is the most variable amino acid stretch between the two syndromes. The two hemorrhagic isolates (UTCVM-H1 and UTCVM-H2) and Kaos isolate (also a virulent isolate) have a similar point mutation replacing the amino acid asparagine with serine in position 443 of the capsid protein. More interestingly, UTCVM-H1 and UTCVM-H2 have the same sequence of amino acids at position 441 to 443 (NNS) which is a unique sequence found only in these two isolates. This mutation leads to an additional glycosylation site when compared to FCV isolates associated with "classical" disease (data not shown). The amino acid area unique to UTCVM-H1 and UTCVM-H2 was followed by two amino acid residues conserved among all FCV isolates investigated which suggests that this epitope has a role in virus neutralization and perhaps cell attachment and tropism changes. This hypothesis is in agreement with observations in a previous study which revealed that two closely-related FCV isolates, based on amino acid sequence of residues 439 to 441, had the highest cross neutralization titers [132]. In the present study, a phylogenetic tree based on twenty amino acids (426-445) segregated the three FCV isolates associated

with hemorrhagic like disease (UTCVM-H1 and UTCVM-H2 and Kaos) in a separate clade when all viruses were compared (Fig 4.11). The latter tree also segregated F9 and CFI/68 isolates in a separate clade. This is in agreement with a previous report in which MAbs produced against amino acid residues 422-448 of the F9 isolate capsid protein cross reacted with CFI/68 FCV isolate [88].

The extra glycosylation site for UTCVM-H1 and UTCVM-H2 found in the hypervariable area E, is present also (but in a different location), in other FCV isolates associated with hemorrhagic-like disease, including FCV-Ari and FCV cat 15. This is in disagreement with a previous report in which only one glycosylation site for FCV isolates was proposed; however, this work was done with "classical" FCV isolates only [132]. The potential role of glycosylation sites in the capsid protein of non-enveloped viruses with respect to virulence is unclear and merits further investigation.

It is likely that the viral changes producing an altered pathogenesis may be minor, even point mutations. After an infection, the mutation responsible for viral pathogenicity may be occurring in the E region and more specifically in residues 435-445. Further work is required to prove that

the twenty amino acid stretch within the E region of FCV may relate to relevant virus neutralization epitopes and virus virulence.

In conclusion, nucleotide homology and predicted amino acid sequence similarities between the two hemorrhagic isolates, either when the complete genome or individual ORFs were compared, were no greater than when either virus was compared to any other strain of FCV. This suggests that each FCV isolate has a distinct and unique origin. However data from the hypervariable E region suggests that disease phenotype may have a genetic basis. Further work is required to identify regions of FCV genome with emphasis on the E hypervariable region that may relate to virulence such as receptor binding sites.

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