VIRUS EVOLUTION IN A NOVEL HOST: STUDIES OF HOST ADAPTATION BY CANINE PARVOVIRUS

by Karla Marie Stucker

VIRUS EVOLUTION IN A NOVEL HOST:
STUDIES OF HOST ADAPTATION BY CANINE PARVOVIRUS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Karla Marie Stucker

August 2010

VIRUS EVOLUTION IN A NOVEL HOST:
STUDIES OF HOST ADAPTATION BY CANINE PARVOVIRUS

Karla Marie Stucker, DVM, PhD

Cornell University 2010

Virus emergence continues to pose a threat to the health of humans, companion animals, domestic agriculture and wildlife species. The processes of emergence by host switching and the subsequent adaptation of viruses to their new hosts remain poorly understood. Canine parvovirus (CPV) represents an excellent model for studying these processes. CPV emerged in the late 1970s as a host range variant of feline panleukopenia virus (FPV). The original variant, CPV-2, was rapidly replaced by a newer, presumably better-adapted variant, CPV-2a, that differs from CPV-2 in several genomic mutations, including four that alter capsid residue sequences.

These studies examine the evolution and adaptation of CPV in dogs by studying the differences among CPV variants. Viruses containing intermediate capsid sequences between the original and newer CPV variants were constructed and their relative fitness was assessed by various *in vitro* measures. These studies suggest that CPV adaptation in dogs required intermediate viruses that had lower fitness than either wildtype virus.

CPV capsid sequences have continued to mutate and multiple strains are in circulation today, including an antigenic variant, CPV-2c. These studies also examine this more recent CPV capsid variation and show that, although newer CPV variants are being selected in nature, there are currently no

significant differences in disease severity or clinical outcomes among these variants. While surveillance for novel CPV variants remains important, prevention, diagnosis and treatment of parvoviral enteritis has not changed.

Finally, a preliminary characterization of an *in vitro* model for CPV infection is presented and shows promise for use in defining additional cellular requirements for infection, in addition to the previously identified canine transferrin receptor. The model may be useful for identifying how the original CPV-2 and newer CPV variants differ in their requirements for productive infection, and may one day help us better understand the molecular mechanisms involved in host adaptation by viruses.

BIOGRAPHICAL SKETCH

Karla Marie Stucker was born in upstate New York and was fortunate to grow up in the foothills of the Adirondacks. Her interests in animals, nature, and the outdoors were sparked at an early age, while growing up in the rural communities of Hebron and Salem, NY. Toward the end of elementary school, Karla moved with her family to the suburban town of Queensbury, NY, and she finished grade school in the nearby Glens Falls School District. At age 13, Karla was introduced to Nordic skiing, a sport she pursued competitively in high school and college, and has enjoyed recreationally ever since. In high school, a student exchange program with Saga City, Japan opened her eyes to the wonders of international travel and the value of cultural exchange.

After high school classes solidified her interests in biological research and veterinary medicine, Karla attended Cornell University as a Cell and Molecular Biology major in the College of Agriculture and Life Sciences. During her senior year, she completed an undergraduate research project in the laboratory of Dr. Marci Scidmore, entitled “Identification of Possible Host-Protein Interactions with *C. trachomatis* Inclusion Membrane Protein, IncD.” Karla graduated in May 2002 with a Bachelor of Science degree, *Magna cum Laude* with Distinction in Research.

Following graduation, Karla continued at Cornell University as a member of the first student cohort admitted to the College of Veterinary Medicine’s Dual DVM/PhD Program. She began the program as a graduate student for two years, completing her laboratory rotations and graduate coursework. By the end of her first year, she had chosen to pursue her doctoral research in the laboratory of Dr. Colin Parrish. Her veterinary training

was completed in four consecutive years, and included a traditional mixed-animal medicine and surgery pathway during her clinical rotations.

As a component of her veterinary training, Karla participated in a year-long course in international agriculture and rural development, which included a two-week trip to southern India where she traveled with classmates from the U.S. and India and developed a group project entitled, “Strategies for Increasing Indian Dairy Production.” Karla has also been fortunate to travel to Kenya, Tanzania and Ecuador, countries rich in culture and natural beauty. These trips allowed her to realize two personal dreams: summiting Africa’s highest peak, Mount Kilimanjaro, and exploring the naturalist’s paradise, the Galapagos.

During graduate school, Karla raised two puppies for the Guiding Eyes for the Blind Finger Lakes Region (GEB-FLR). Natchez became an Alcohol, Tobacco and Firearms explosion detection dog, and Faye became a guide dog. Karla has also been a class instructor for the GEB-FLR and a member of the region’s leadership team. She now has two wonderful canine companions of her own, Lily and Clover, who are both American Kennel Club Canine Good Citizens.

After receiving her Doctor of Veterinary Medicine degree in May 2008, Karla turned her attention back to the laboratory to complete her dissertation. However, she also continued to develop her clinical training by working part time as a staff veterinarian for Shelter Outreach Services, a nonprofit organization providing shelter medicine consultation and spay/neuter services to local animal shelters. In August 2009, she was accepted into Cornell University’s inaugural group of Future Faculty Fellows who took part in a professional development and pedagogical training program developed by the

University's Center for Teaching Excellence. Her final project for this program was a preliminary synthesis of educational pedagogy and veterinary education to develop strategies for promoting research thinking skills among all veterinary students.

With the completion of her dissertation, Karla ventures into the next stage of her professional development. She will pursue postdoctoral training in the ecology and evolution of emerging viral diseases, with the objective of securing a faculty position at a veterinary college, establishing a research program in emerging infectious diseases, and continuing her studies of veterinary pedagogy. Her goals as an academician are 1) to contribute to improvements in human, animal and environmental health by performing rigorous basic and applied biomedical research, 2) to be a respected researcher who promotes and performs the collaborative, multidisciplinary science that is needed for advancing the limits of our knowledge, and 3) to be a competent, motivating teacher for undergraduate, graduate and veterinary students alike. She hopes to incorporate her interests in global issues and international development into both her research and teaching pursuits.

For my parents,

Jeffrey and Margaret Stucker,

and my sisters,

Rebekka Joslin and Lindsay Richardson,

with much love and many thanks!

ACKNOWLEDGMENTS

"I won't thank half of you half as well as I should like, and I can't thank less than half of you half as well as you deserve."
– modified quote from Bilbo Baggins

There are many people in need of thanking, all of whom deserve much more than a page of text for the support they have provided me over the years. First and foremost, I thank my advisor, Dr. Colin Parrish, for his patience, support and inspiration throughout the years of my dual degree program. He is an excellent role model for demonstrating how engaging and productive multidisciplinary collaborative science can be, and how important it is for the advancement of scientific knowledge. In addition, I thank my special committee members, Drs. Bill Brown, Robert Oswald and Volker Vogt, for their insightful feedback on my projects and their patience and flexibility when I was juggling my research and clinical commitments.

I also thank the many collaborators with whom I was very fortunate to work. I appreciated the enthusiasm and optimism of Drs. Jessie Markovich and Jan Scarlett, who helped facilitate the clinical study in Arizona, and I thoroughly enjoyed my engaging discussions with Drs. Eddie Holmes, Laura Shackelton, and Israel Pagán, who collaborated on the intermediate virus project and other phylogenetic studies.

During my tenure in the Parrish laboratory, I worked with many excellent trainees and staff, many of whom have become close personal friends. Wendy Weichert, Virginia Scarpino, Shelagh Johnston, and Gail Sullivan provided me with excellent technical and emotional support over the years. I greatly valued the scientific and personal discussions I've shared with

all of the Parrish lab trainees, past and present: Karsten Hueffer, Laura Palermo, Christian Nelson, Karin Hoelzer, Carole Harbison, Laura Goodman, Michael Lyi, and Jess Hayward. I also greatly appreciated the help that Tyler Lillie and Jason Kaelber – undergraduates in our laboratory – provided for several of my projects.

In addition, I was very fortunate to have the support of many friends and colleagues during my time at Cornell. These included the faculty, staff and trainees at Baker Institute, members of the Department of Microbiology and Immunology, and colleagues and friends in the College of Veterinary Medicine. Unfortunately, there are too many individuals to provide all their names here, but I sincerely thank each of them for their friendship and encouragement.

Finally, I give a huge thank you to my family, who has provided unwavering love and support throughout my years of training. I would not be where I am today if I had not had them to rely upon along the way. I am truly thankful for having such an encouraging and supportive family, which includes parents and sisters, as well as grandparents, aunts, uncles, and cousins.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

PREFACE

The year is 1979, and the manager of a prestigious colony of beagle hunting dogs in the southeastern United States is awakened by an early morning call from one of his kennel staff who is quite shaken by what she has just found in the nursery. Two newborn pups are dead and three more appear very sick with profuse bloody diarrhea. He tells her he'll be right there, and as he dresses, he wonders what could be happening. His kennels are known for their healthy dogs and pristine facilities. He recalls a recent report about a devastating new disease of dogs that has a high morbidity and mortality rate among puppies, and as he tries to remember the details, he hurries to his truck. Could this be what is killing his pups?

Meanwhile, similar emerging infectious disease events are occurring around the world. For example, just as the manager arrives at his nursery to witness the life-threatening illness caused by the newly identified canine parvovirus, a southern gastric-brooding frog over 9000 miles away in eastern Australia takes its last gulp of air and collapses, dead. While no human is present to witness her death, it is particularly tragic, as she was the last of her species. It will be some time before humans recognize that this unique species has been lost forever. The likely culprit is the highly invasive chytrid fungus, which will cause dramatic losses in amphibian numbers and biodiversity worldwide in years to come.

That same morning, eight hours earlier in a remote west African village, a woman attempts to give her two-year-old son some water. They have both been feeling poorly for several weeks, and her son has been slow to develop his motor skills. Now, they both have fevers and the son is having difficulty

breathing. A gastrointestinal illness has made the mother weak, and she struggles to care for her son. A more insidious disease has invaded their village and they have been unsuccessful in stopping its spread. Although this agent will soon be identified as human immunodeficiency virus, millions of people around the world will continue to suffer from this terrible disease for decades to come.

Meanwhile, as the sun begins to set in Northern India, a farmer stares helplessly at his rice paddy, from which he begrudgingly anticipates his lowest yield ever. Yellowish stripes with wavy margins have appeared on many of the leaves and opaque bacterial ooze hangs like evening dew off some of the blades. In this case, the causative agent – bacterial leaf blight – is known, although it is the first time this region has witnessed its deadly effects. Greater than 50% losses will be reported from this epidemic, the impact of which will weigh heavily on those who rely on rice for their livelihood.

Emerging infectious diseases represent real and constant threats to all taxa of life. They have helped shape the history of life on earth, and they will continue to influence life on our planet for the foreseeable future and beyond. Some suggest that given our present day circumstances, which include a burgeoning human population, global commerce, and international travel, we are accelerating the rate of infectious disease emergence. While this is an upsetting prospect, hope remains in our ever-improving ability to identify and combat these emerging pathogens. Through the union of scientific research, public policy, and international cooperation, we can improve the state of our planet, curtail the negative effects of global warming, reduce risks for disease emergence, and alleviate human and animal suffering. While some forecasts for our planet's future are grave, we should not accept these projections as

inevitable truths, and instead we can all work toward reshaping the Earth's future for the benefit of all living organisms.

This thesis represents almost eight years of research, my small sliver of a contribution to the complex challenges that face our planet. While I cannot offer any real answers to these looming global threats, I hope these studies will contribute to an ongoing scientific dialogue that may one day lead to solutions that benefit our world. These studies were designed to answer both basic scientific questions about how pathogens – specifically viruses – adapt to novel hosts, as well as to address the clinically relevant concern of how new virus variants may affect disease pathogenesis and the management of patients. These studies demonstrate one small way in which basic and clinical research can be unified for the benefit of both. Here's to making the world a better place, one small step at a time!

Karla M. Stucker

August 2010

CHAPTER 1

INTRODUCTION

1.1 EMERGING INFECTIOUS DISEASES

Emerging infectious diseases (EIDs) occur when the incidence of a known disease rapidly increases or expands its geographic range, when a more virulent or drug-resistant pathogen becomes established in a host population, when a pathogen is newly recognized as a disease-causing agent, or when a pathogen acquires a new host range (53). A wide range of pathogens can cause EIDs, from prions to helminths, although bacteria and viruses make up more than three-quarters of recognized EIDs (77). EIDs may occur as local epidemics or spread to global pandemics. Through direct and indirect effects, EIDs threaten the health of humans and companion animals, agricultural plants and animals, and diverse wildlife species. They also hinder international development efforts, threaten global biodiversity, and disrupt global commerce and travel (17, 53).

Many causes for EIDs have been described, and some argue that EIDs are increasing as human influences on our planet increase (53). Drivers for EIDs include agricultural intensification, urbanization, global travel, regional and international translocation of species, and human-induced land use changes (18, 53). These activities, among others, increase contact among humans, domestic animals and wildlife, thus increasing chances for pathogen sharing. These drivers may act at many levels, including pathogen exposure, the infection of individual hosts, the transmission potential among a new host population, or the likelihood of pathogen adaptation (51).

Of particular concern are EIDs resulting from host range switching, and global surveillance and research efforts are being promoted to attempt to determine when and how new pathogen host jumps might occur (30). For a host switch to occur, a pathogen must overcome an interspecies transmission

barrier between the donor and recipient hosts (33), and the pathogen must either be sufficiently competent for infection in the new (recipient) host or be able to adapt sufficiently to the recipient host to maintain transmission. It is generally assumed that more closely related hosts have an increased chance of sharing pathogens, although there are exceptions to this, as in the case of influenza A viruses which infect both avian and mammalian species.

The processes of host switching have been divided into three potential steps: infection of a recipient host with no onward transmission (spillover), spillover followed by local chains of transmission before fade-out (outbreak), and sustained recipient host-to-host transmission (epidemic) (51). These steps can also be defined in relation to R_0, the reproductive number of a transmissible pathogen that describes the number of new cases generated from each infected individual (4). When $R_0 = 0$, as in the first example of host switching, a spillover infection results in a dead-end recipient host with no further transmission. In the second type of transfer leading to a limited outbreak, R_0 is on average < 1 and transmission will end. True emergence only occurs where $R_0 \geq 1$ over a given period of time, resulting in sustained transmission.

1.2 CANINE PARVOVIRUS EMERGENCE

In the late 1970s, a new canine virus emerged and spread around the world in less than one year, often causing the loss of entire litters of puppies, as well as affecting some older dogs (reviewed in (44)). Infected dogs developed an acute disease with signs of general malaise, bloody diarrhea and vomiting. Blood cell counts showed a severe drop in circulating white blood cells, ranging from a relative lymphopenia to a generalized leukopenia

(11). Morbidity and mortality rates were high, especially among younger animals, and aggressive therapy was often needed. Treatment relied on supportive care to maintain proper hydration and antibiotics to treat the sepsis that can develop when bacteria translocated across the damaged gut epithelium into the bloodstream. These remain the mainstay of therapy for parvoviral enteritis today.

The causative agent was identified during late 1978 and named canine parvovirus type-2 (CPV-2) to distinguish it from a previously discovered but distinct parvovirus, the minute virus of canine (50). CPV-2 is believed to have arisen as a host-range variant of feline panleukopenia virus (FPV) (70), which causes a similar disease in cats. Within a year of its identification, a second antigenically variant strain, CPV type-2a (CPV-2a), emerged and achieved rapid global dissemination, displacing CPV-2 worldwide (46, 52). In 1984, an additional antigenic variant was identified (named CPV type-2b (CPV-2b)) that also achieved a widespread distribution (45, 52). Interestingly, CPV-2a and CPV-2b have both been abundant in canine populations until recently, indicating that the newer variants have a selective advantage over CPV-2 and are likely better adapted to their canine hosts.

1.3 PARVOVIRUSES AND THEIR PATHOGENESIS

The *Parvoviridae* family of viruses consists of two subfamilies: *Parvovirinae*, whose members infect mammalian and other vertebrate hosts, and *Densovirinae*, whose members infect insects and other invertebrates. *Parvovirinae* includes the autonomously replicating parvoviruses, as well as the dependoviruses, such as adeno-associated viruses (AAVs), which require coinfection by a helper virus to complete their replication cycle.

Parvoviruses have small, non-enveloped, 26-nm diameter capsids, containing an approximately 5-kb linear single-stranded DNA genome. These viruses generally resist desiccation, pHs ranging from 3 to 10, and temperatures up to 60ºC for 1 hour, making them highly stable in the environment and contributing to their transmission and widespread distribution. However, upon entry of these viruses into host cells, their DNA must be released from the highly stable capsid and delivered to the nucleus to allow replication and the production of new virions, suggesting that these viruses depend on specific cellular interactions to mediate capsid conformational changes and genome release.

The parvovirus genome encodes two major open reading frames (ORFs): one encoding the nonstructural proteins NS1 and NS2, and the other encoding the capsid proteins VP1 and VP2. Since these viruses do not carry their own polymerases and are unable to promote cell division in their host cells, parvovirus replication is restricted to host cells with active DNA replication machinery that are undergoing mitosis. This helps explain why parvoviruses tend to cause more severe disease in fetuses and newborns, where rapid cell division is occurring in most tissues, and why in adults, parvovirus replication is generally restricted to tissues containing actively dividing cells, such as hematopoietic cells and the crypt cells of the small intestine.

The autonomous parvoviruses are ubiquitous in nature, and most mammals appear to serve as hosts for a number of different parvoviruses that may cause subclinical to severe disease, depending on the virus and the age of the host. The feline subgroup of parvoviruses includes feline panleukopenia virus (FPV), mink enteritis virus (MEV), raccoon parvovirus (RPV), and canine

parvovirus (CPV) (59), and these can cause severe, often lethal, developmental abnormalities, such as cerebellar hypoplasia or myocarditis, in fetuses and newborns, as well as enteritis and leukopenia in adults (11). The human parvovirus, B19, replicates only in erythrocyte precursor cell populations and can cause severe anemia and hydrops fetalis in fetuses, erythema infectiosum (fifth disease) in children, and arthralgia and arthritis in adults (reviewed in (24)).

1.4 CPV EVOLUTION AND HOST ADAPTATION

CPV variants were initially identified by differences in binding properties against panels of monoclonal antibodies (mAbs) (48, 50, 52), although these antigenically distinguishable viruses differ in other biologically significant properties as well. For example, the *in vivo* and *in vitro* host ranges and tissue tropisms of these viruses differ in complex ways, and elucidating the molecular mechanisms behind these complexities will shed light on how the virus gained access and adapted to its canine hosts. FPV infects and can cause clinical disease in cats, but not dogs, although FPV replication in cells within the thymus of dogs has been detected (46, 69). CPV-2 infects dogs, but not cats, while the newer variants, CPV-2a and CPV-2b, regained the ability to infect cats in addition to dogs (69). Furthermore, FPV causes cerebellar hypoplasia in kittens, while CPV causes myocarditis in puppies (11). *In vitro*, FPV will infect only feline cells, while all CPV variants will infect and replicate in both feline and canine cells (71).

In addition, these viruses also differ in their hemagglutination (HA) properties. FPV requires a lower pH for HA than CPV (12, 60). CPV-2, but not CPV-2a, requires a lower temperature for optimal HA (57). This suggests

that CPV can bind sialic acid under the physiological conditions of the animal body, while FPV cannot (42). Other receptor binding characteristics also differ among these viruses, as discussed below. These differences in virus properties, taken together with the global replacement of the original CPV-2 with newer variants, indicate a biologically and clinically significant role for virus evolution and host adaptation, as initially defined by studies of viral antigenicity.

Sequencing and recombinant DNA technology have allowed the identification of specific nucleotide differences (and their predicted amino acid changes in coding regions) among the FPV and CPV genomes and the mapping of specific functions to those sequence differences. As summarized above and in subsequent sections, the various viral strains differ in their antigenic epitopes, *in vitro* and *in vivo* host ranges, HA and receptor binding properties, distribution in nature, and pathogenesis. As the virus capsid is the first site of contact between the virus and its host, the capsid structure plays a significant role in establishing these characteristics of each virus. The structural proteins VP1 and VP2 overlap so that all of the sequence of VP2 (which make up about 90% of the CPV capsid) is contained within that of VP1 (40). Studies with virus mutants mapped many of these properties to the VP2 gene (45, 46, 55). In addition, more recent phylogenetic analyses have shown that most of the genome sites that are under positive selection are found in the capsid ORF (25).

Important VP2 amino acid differences between FPV, CPV-2, CPV-2a, and CPV-2b are shown in Figure 1.1. *In vivo* and *in vitro* canine host range is controlled by amino acids difference at residues 93, 103, and 323 between FPV and CPV-2 (13, 45, 46, 68), while in experimental studies, residues 299,

300, 301, and 387 also influenced canine host range (42, 49). Important determinants of feline host range include residues 80, 564, and 568 (68). HA and sialic acid binding properties of the viruses are determined by VP2 amino acids 323 and 375, which control the pH dependence of HA, and by residues 377, 396 and 397 that can prevent HA (6, 45-47, 60, 67). Two major neutralizing antigenic epitopes, sites A and B, have been mapped to specific capsid sequences (62), and are discussed in more detail below.

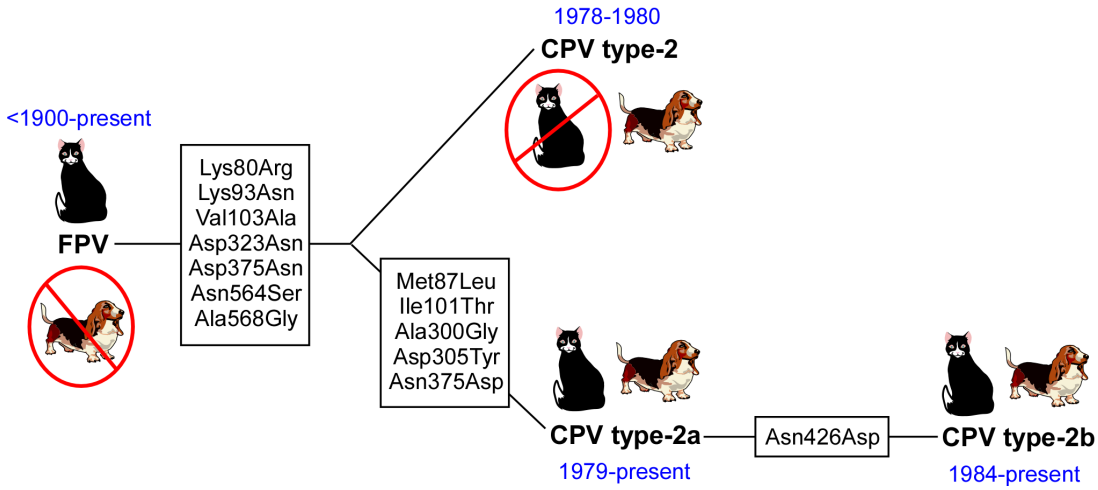


Figure 1.1. Model of CPV evolution showing VP2 amino acid differences between each virus and indicating the virus host ranges.

In many cases, virus strains differ in multiple sequences, including groups of residues that are exposed on the capsid surface. It is therefore likely that during CPV evolution, some of the critical residue changes were selected simultaneously or acted together to introduce altered phenotypes. These include differences between CPV and FPV, and the changes around amino acid 300 that differ between CPV-2 and CPV-2a, since virus recombinants in this area are non-viable and intermediate viruses with fewer than the full complement of changes have not been isolated from nature (13,

47, 62). This may reflect the complex selection pressures imposed on the virus and is an important consideration when studying CPV evolution.

CPV continues to evolve today and acquire changes in its capsid sequence, although we do not yet fully understand the significance of these changes for the pathogenesis and management of clinical cases. Some changes in the viruses are appearing more commonly and becoming widespread, suggesting they may confer an advantage to the virus, and indicating that CPV is continuing to evolve and adapt ((29, 37), and Parrish, unpublished data). In all cases, the currently circulating variants derive from a single common CPV-2a ancestor (58), so that all circulating strains of CPV are related to each other but distinct from the original CPV-2 variant. In some cases, a given capsid codon has changed more than once to express different residues. For example, FPV, CPV-2 and CPV-2a contain an Asn at VP2 position 426, while CPV-2b contains an Asp at the same position. Around 2000, a new variant containing a Glu at VP2 residue 426 was identified and named CPV-2c (10). VP2 residues 297, 300 and 305 also encode varying residues depending on the virus isolate.

As newer variants are capable of replicating in feline and canine hosts (69), the viruses may be influenced by both canine and feline selection pressures, as well as antigenic selection by antibodies. For example, changing VP2 residue 300 to an Asp results in antigenic changes in the virus, as well as the loss of ability to infect canine cells (49). It also appears to be associated with the ability to infect cats and raccoons (29). For example, multiple parvovirus isolates from sick raccoons have been shown to be CPV-2a-like with the VP2 300 Asp mutation ((32) and Allison and Parrish,

unpublished data), providing further evidence for the crucial role VP2 residue 300 plays in the host range properties of CPV.

1.5 CPV CAPSID STRUCTURE AND FUNCTIONS

Solution of the atomic structure of CPV-2 (72, 78) and FPV (2) has allowed differences in amino acid sequences to be understood in terms of the three-dimensional virus structure. The capsid protein folds into an eight-strand β-barrel typical of many other icosahedral viruses, with loops between β-strands largely determining capsid surface topology. The capsid has icosahedral symmetry with five-fold cylinders surrounded by a canyon, three-fold spikes, and two-fold depressions, or dimples (Figure 1.2). It remains largely intact even under physiological changes such as receptor binding, lower pH or removal of Ca^{2+}, with only subtle structural changes occurring that presumably alter capsid permeability sufficiently to release the viral DNA (38).

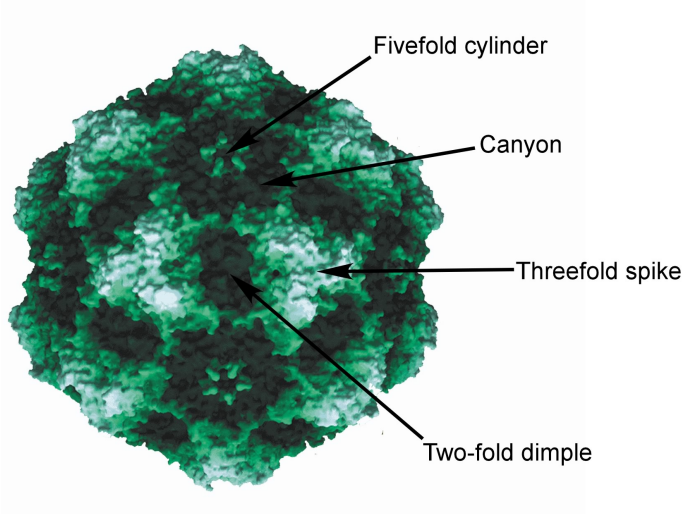


Figure 1.2. Surface rendered model of the CPV capsid, indicating the five-fold cylinder and surrounding canyon, three-fold spike, and two-fold depression. Modified from Hueffer and Parrish, 2003.

The N-termini of a proportion of VP2 molecules are found on the capsid exterior of full, DNA-containing capsids and those appear to pass through the five-fold cylinder, possibly aided by the flexible, relatively narrow polyglycine-rich sequence shared by VP1 and VP2 (72). Since the NS1-bound, 5'-end of the viral genome is also detected outside the capsid (15), the five-fold cylinder may also serve as a passageway for single-stranded DNA.

Locations of the key surface-exposed VP2 residues are mapped onto the capsid asymmetric unit in Figure 1.3. Residues 93, 300, and 323 mark the capsid regions on the three-fold spike and its shoulder that help determine canine host range and virus neutralization, indicating that the raised, exposed regions of the capsid are at least partially responsible for antibody binding and host cell interaction. The CPV-2b-specific epitope defined by VP2 residue 426 is also exposed on the three-fold spike. Residues 80, 564 and 568, which influence feline host range, are not exposed on the capsid surface, but lie just beneath the edge of the two-fold dimple, while resdues 101 and 103 are also found beneath the capsid surface but also influence important virus functions. Amino acids determining HA and sialic acid binding are found adjacent to the two-fold dimple, and are either on or just below the capsid surface (6, 67, 72).

Additional atomic structures of host range mutations in the CPV-2 capsid have also been solved. For example, changing the host-range-determining VP2 residue 300 from Ala to Asp introduced a salt bridge between 300 Asp and 81 Arg (36). In addition, structures of wildtype and mutant CPV-2, as well as FPV, have been solved at varying pH values and in the presence or absence of calcium ions (60). These structures indicate that a flexible loop between VP2 residues 359 and 375 differs in structure between CPV and FPV and changes conformation upon acidification via a mechanism regulated by

calcium binding. This flexible loop is adjacent to or contains important HA residues and its conformation is believed to influence the sialic acid binding properties of the viruses, as well as the involvement of residue 375 (Asn to Asp) in binding a calcium ion in the structure of FPV (2).

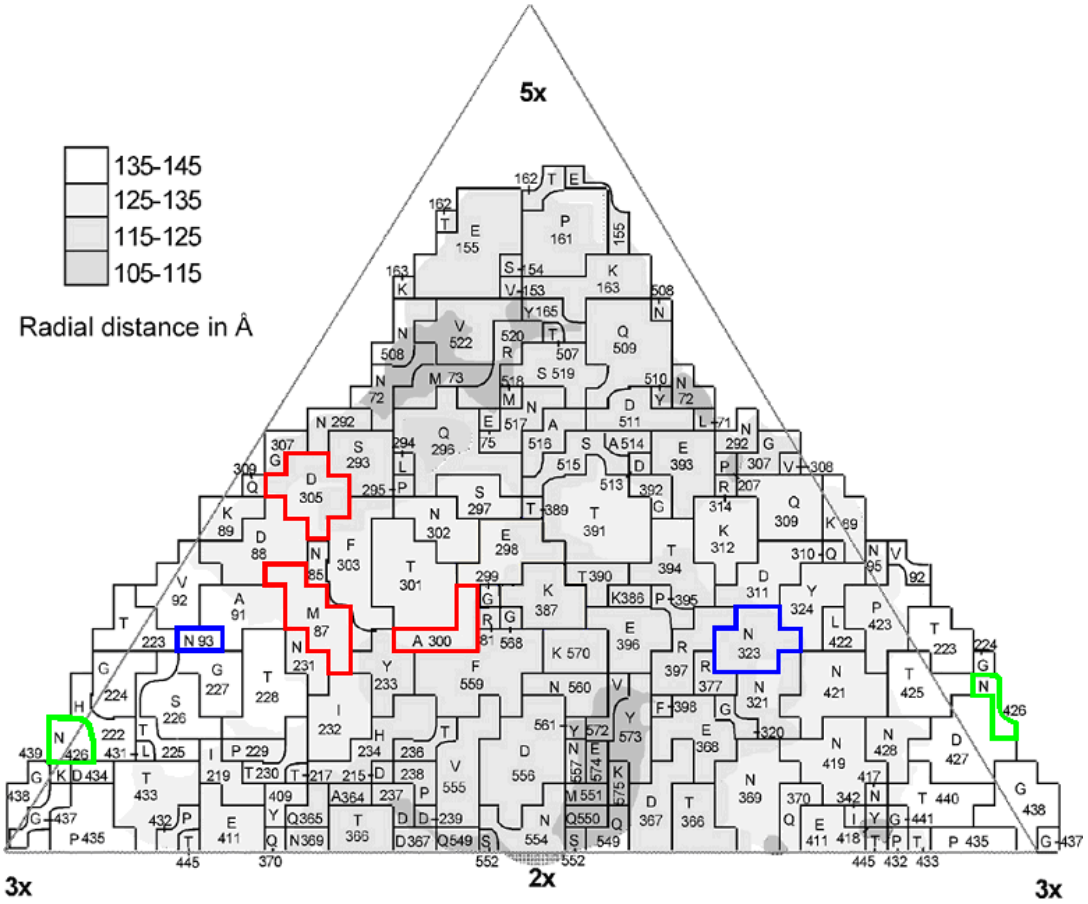


Figure 1.3. An asymmetric unit of the CPV-2 capsid showing surface-exposed VP2 residues. Shading indicates distance from the capsid center, with white showing raised regions on the capsid surface and dark grey showing recessed areas. This roadmap was made using a previously published method (56). Residues that differ between FPV and CPV are outlined in blue, while those that differ between CPV-2 and CPV-2a are outlined in red, and the residue that differs between CPV-2a and CPV-2b is outlined in green.

1.6 ANTIBODY INTERACTIONS WITH CPV

Two major neutralizing antigenic epitopes have been mapped to the capsid surface, and show that antibody binding is affected by amino acids 93, 426, 222, 224 (antigenic site A) and 299, 300 and 302 (antigenic site B) (13, 48, 49, 62). A Lys at amino acid 80 likely helps define an FPV-specific epitope, while an Asn residue at amino acid 93 results in a CPV-specific epitope, and an Asp or Glu at amino acid 426 gives the CPV-2b- and CPV-2c-specific epitopes (10, 13, 45, 68).

More recently, cryoelectron microscopy studies of capsids complexed with the antibody binding fragments (Fab) of CPV-specific mAbs have allowed mapping of specific Fab footprints to the surface of the capsid (21). These studies have confirmed and more precisely defined the site A and B epitopes, with site A lying over the three-fold spike and site B lying along the spike's shoulder. The two sites each have an overlapping region shared by several different mAbs (21).

1.7 CELLULAR RECEPTOR FOR CPV

FPV and CPV both use the cellular transferrin receptor (TfR) to bind and enter cells (41). The TfR is a membrane glycoprotein responsible for cellular uptake of iron-loaded transferrin and is expressed at highest levels in rapidly dividing cells (reviewed in (54)), which presumably helps the virus target the S-phase cells it requires for replication. The structure of the human TfR ectodomain has been solved (1, 8, 34), and canine and feline TfRs likely have similar structures since those three TfRs share ~80% sequence identity (28). The TfR functions as a dimer, with monomers associating primarily through their helical domains (Figure 1.4) (8, 14, 34).

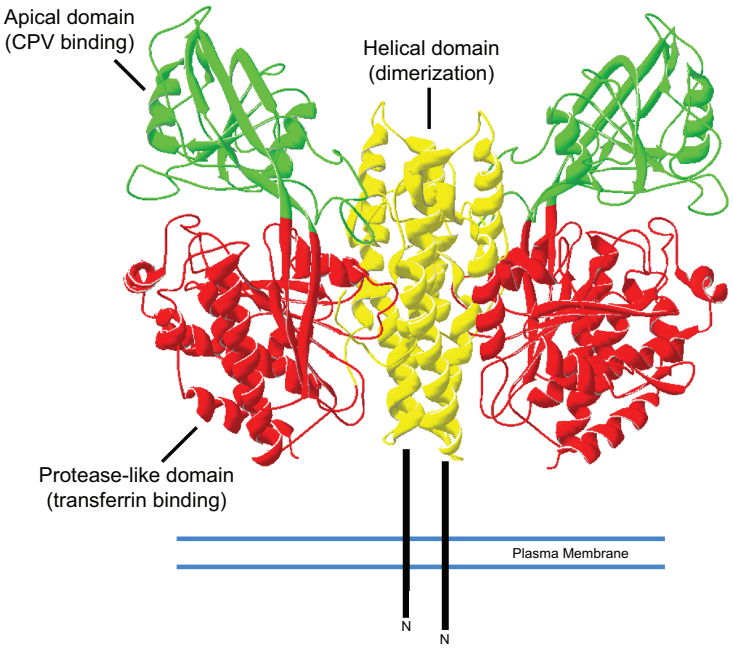


Figure 1.4. Ribbon diagram of the homodimeric human transferrin receptor ectodomain showing the helical domains in yellow, the protease-like domains in red, and the apical domains in green. To provide orientation in relation to the plasma membrane, the cytosolic N-termini of the TfR are depicted schematically.

Mutational analysis of the TfR shows that virus binding to the TfR involves the TfR apical domain, and recombinants and single amino acid substitutions between feline and canine TfRs in this region have identified a unique single-residue insertion and a potential glycosylation site in the canine TfR apical domain which allow CPV-specific binding (19, 39). Regions of the virus capsid have similarly been identified that allow binding of CPV, but not FPV, to the canine TfR. These include VP2 residues Asn93 and Asn323 (20, 26), as well as Gly299 (28), suggesting there is a broad contact interface between the virus and TfR (39). More recent cryoelectron microscopy studies have defined a binding footprint on the virus capsid that includes these residues and extends from the three-fold shoulder to the five-fold canyon,

primarily overlapping with epitope site B (22). These studies have also shown that receptor binds asymmetrically to the capsid, with only one to several receptors binding each capsid (22).

In vitro and *in vivo* properties of the virus indicate that the TfR may not be the only factor important in the cell infection and evolution of CPV. As mentioned previously, CPV-2 only infects dogs, while CPV-2b regained the ability to infect cats, in addition to dogs (69). Similar to what has been observed in the distribution of these viruses in nature, CPV-2b outcompeted CPV-2 in a limited tissue culture model where the canine TfR was expressed as the sole cellular receptor (28). In addition, CPV-2 showed 5-20 times more binding to canine and feline cells when compared to CPV-2b in a flow cytometry assay of cell binding and uptake (28).

Other parvoviruses have been shown to use a variety of glycans or oligosaccharides as receptors. For example, B19 uses the glycolipid globoside, as well as a not completely identified co-receptor, to enter and infect cells (9, 76). AAV2 binds proteoglycans (65), while AAV4 and AAV5 bind different sialic acids (31), and the minute virus of mice (MVM) binds sialic acid as well (16). Therefore, in addition to erythrocyte binding, sialic acid binding contributes to virus attachment to host cells for those cells. However, a CPV-2 mutant defective for sialic acid binding still bound host cells, and treatment of cells with neuraminidase to remove sialic acid did not reduce virus binding (28). Thus, the role of sialic acid binding during CPV infection of host cells remains uncertain, but does not appear to control primary infection of cells.

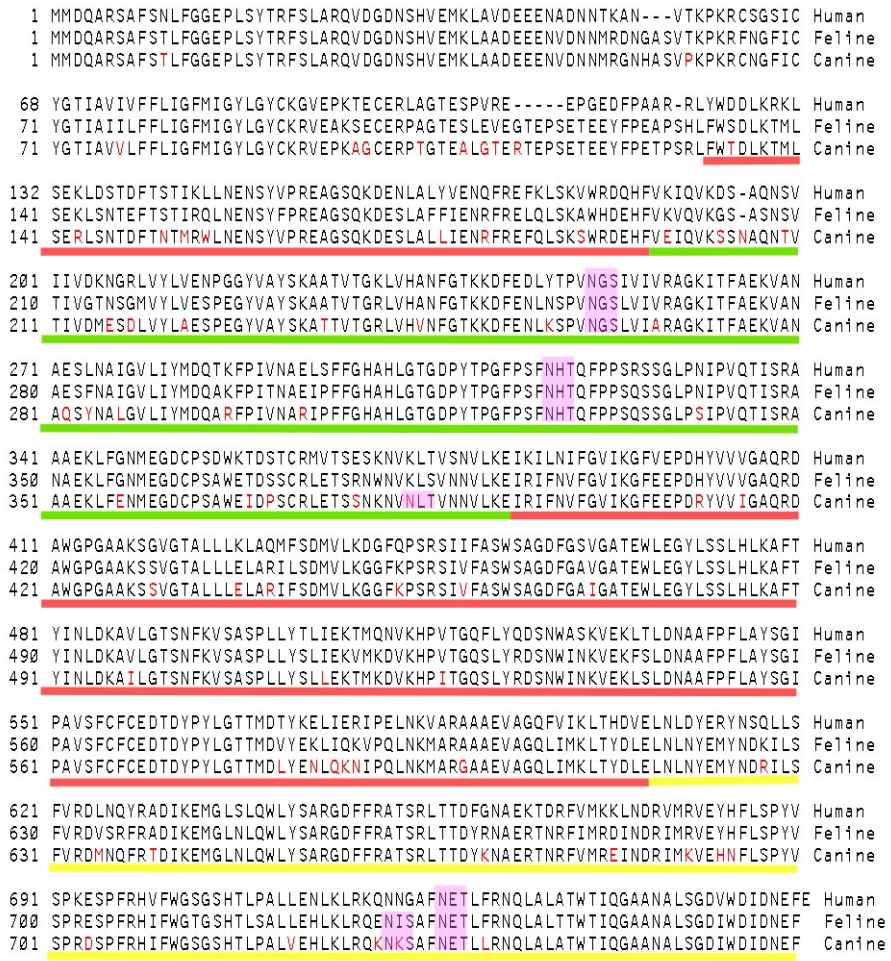


Figure 1.5. Alignment of the human, canine and feline TfR sequences. The helical, protease-like and apical domains are underlined in yellow, red and green, respectively. Known or potential glycosylation sites are shaded purple. Amino acids unique for the canine TfR are shown in red. Taken from (28).

Other potential interactions between cellular molecules and the virus have been identified. Virus overlay blots of membrane fractions from erythrocytes or feline or canine host cells, showed a number of erythrocyte proteins bands that bound virus in a sialic-acid dependent manner, as well as potential non-sialic-acid-specific, virus-binding proteins in host cells (6). Virus

overlay blots and immunoprecipitation of virus from infected A72 canine fibroma cells identified a 40- to 42-kD CPV attachment polypeptide (7).

1.8 CPV REPLICATION CYCLE

Although many events in the CPV replication cycle, as well as for nonenveloped viruses in general, are not fully understood at the molecular level, the basic steps involved in productive cellular infection by CPV have been described. Understanding the virus replication cycle is needed for a complete appreciation of how a virus interacts with its host, as this relationship determines the tissue tropism and host range of the virus. Blocks to infection in non-permissive cells and virus restriction by the host may occur at any point in the virus replication cycle. Restriction at the cell surface, due to viral requirements for specific attachment proteins and virus receptors, plays a particularly important role in determining tissue tropism and host range. Often, studies that propose intracellular blocks to infection in non-permissive cells do not rule out the possibility of an additional receptor-binding role. For example, two strains of MVM differing in their tissue tropism appear restricted at transcriptional initiation (61, 66). However, a key amino acid difference between the MVM strains aligns with amino acid 323 in CPV (3, 5), suggesting receptor binding may be playing a role in MVM strain differences as well, and effects on sialic acid binding have also been seen for this residue (60). The block to replication in cells non-permissive for B19 infection appears to involve aberrant transcription that produces an excess of NS1, which is toxic to cells and may induce apoptosis (35), although involvement of receptor binding has not been excluded.

Cell infection by CPV begins when the virus binds to the TfR on the host cell surface and is predominantly taken into the cell by relatively rapid, dynamin-regulated, clathrin-mediated endocytosis (43). Immunofluorescence colocalization studies with markers specific for a variety of vesicular compartments have suggested that CPV travels from early endosomes at the cell's periphery to perinuclear recycling endosomes and finally to late endosomes and lysosomes, the likely site of capsid release into the cytoplasm (23, 43, 64). Virus trafficking through these later endocytic compartments occurs relatively slowly, as the virus remains in vesicles at 90 minutes post infection and infection can be inhibited by cytoplasmically microinjected antibodies against the virus for 2-4 hours post infection (43, 74). However, recent live cell microscopy studies in cells expressing fluorescently tagged Rab proteins show that capsids reach late endosomes and lysosomes within 15 minutes of binding, suggesting a more rapid and potentially complex trafficking pattern (23). These studies also demonstrated different binding and uptake patterns for CPV-2 on feline and canine cells, with virus binding relatively uniformly over the feline cell surface, while it preferentially binds canine cell filopodia (23).

Staining with antibodies that recognize only intact virus capsids suggest that some CPV can be delivered relatively intact into the nucleus, possibly by transport through the nuclear pore complex (74, 75). In this case, extensive conformational changes and capsid disassembly are not required for nuclear entry, as supported by only subtle changes in capsid conformation being observed by cleavage studies under lower pH and receptor binding conditions (38). Presumably, however, some capsid modifications are occurring before nuclear entry, since the virus must escape into the cytoplasm. Capsid

changes may include the exposure of the N-terminal VP1 nuclear localization signal (NLS) and phospholipase A2 (PLA2)-like domain, cleavage of VP2 to VP3 and exposure of its glycine-rich sequence, and removal of capsid-bound calcium ions (40, 60, 73, 74). The identification of PLA2 sequence homology in the VP1 N-terminus of parvoviruses (80) and *in vitro* demonstration of CPV PLA2 activity after exposure of virions to acidic buffers or heating (63) has lead to the hypothesis that viral PLA2 activity aids in membrane penetration and escape of CPV from the endocytic pathway. Indeed, PLA2 appears necessary, but not sufficient, for membrane penetration and productive infection, while other factors required for successful membrane penetration may include specific receptor interactions or the exposed glycine-rich domain of VP3 (63). TfR binding also appears to play a role in capsid structural changes, as some mutant and chimeric TfRs allowed weak virus binding, but very little infection (27, 39).

Once in the nucleus, the virus uses the DNA replication and transcription machinery of its host to produce its copies of its genome and viral mRNA. Regulation of DNA replication and transcription is coordinated by viral NS1 and its interactions with host cell machinery. Capsid proteins are synthesized in the cytoplasm and imported into the nucleus, which is the primary site of virus assembly and DNA packaging (79). The virus replication cycle is complete following the death its host cell and release of progeny virions into the environment.

1.9 DISSERTATION OUTLINE

The research for this dissertation is divided into three sections, with the first two describing unique, but related, aspects of CPV evolution, and the third

discussing the preliminary development of an *in vitro* model for future studies of CPV host adaptation.

The first section focuses on the early evolution of CPV, the stage where the original CPV-2 virus was replaced globally by the newer CPV-2a-derived viruses, such as CPV-2b. It uses molecular biological techniques to create a panel of potential capsid intermediate viruses with different combinations of four VP2 differences between those viruses. These intermediates are tested using various *in vitro* measures of fitness to elucidate the evolutionary constraints and pressures on CPV-2 during its initial adaptation to dogs. These intermediate viruses will also help evaluate the requirements for co-selection of specific capsid residues in CPV.

Section two approaches the question of CPV evolution from a clinical perspective and analyzes whether or not current strains circulating in the southwestern United States differ in their clinical presentations and outcomes. Strain variation has the potential to alter CPV detection by antibody-based diagnostic tests, reduce the effectiveness of vaccination at preventing disease, or change the severity of disease and prognosis for patients.

The final section describes studies that characterize cell line susceptibilities to CPV infection, with the aim of finding ways to dissect differences in biological functions between original and newer CPV variants. These preliminary data suggest potential uses of various cell lines for future *in vitro* studies of CPV replication and evolution.

REFERENCES

1. Abraham, J., K. D. Corbett, M. Farzan, H. Choe, and S. C. Harrison. Structural basis for receptor recognition by New World hemorrhagic fever arenaviruses. Nat Struct Mol Biol 17:438-44.

2. Agbandje, M., R. McKenna, M. G. Rossmann, M. L. Strassheim, and C. R. Parrish. 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16:155-71.

3. Agbandje-McKenna, M., A. L. Llamas-Saiz, F. Wang, P. Tattersall, and M. G. Rossmann. 1998. Functional implications of the structure of the murine parvovirus, minute virus of mice. Structure 6:1369-81.

4. Antia, R., R. R. Regoes, J. C. Koella, and C. T. Bergstrom. 2003. The role of evolution in the emergence of infectious diseases. Nature 426:658-61.

5. Ball-Goodrich, L. J., and P. Tattersall. 1992. Two amino acid substitutions within the capsid are coordinately required for acquisition of fibrotropism by the lymphotropic strain of minute virus of mice. J Virol 66:3415-23.

6. Barbis, D. P., S. F. Chang, and C. R. Parrish. 1992. Mutations adjacent to the dimple of the canine parvovirus capsid structure affect sialic acid binding. Virology 191:301-8.

7. Basak, S., H. Turner, and S. Parr. 1994. Identification of a 40- to 42-kDa attachment polypeptide for canine parvovirus in A72 cells. Virology 205:7-16.

8. Bennett, M. J., J. A. Lebron, and P. J. Bjorkman. 2000. Crystal structure of the hereditary haemochromatosis protein HFE complexed with transferrin receptor. Nature 403:46-53.

9. Brown, K. E., S. M. Anderson, and N. S. Young. 1993. Erythrocyte P antigen: cellular receptor for B19 parvovirus. Science 262:114-7.

10. Buonavoglia, C., V. Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, and L. Carmichael. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J Gen Virol 82:3021-5.

11. Carmichael, L. E., and L. N. Binn. 1981. New enteric diseases in the dog. Adv.Vet.Sci.Comp.Med. 25:1-37.

12. Carmichael, L. E., J. C. Joubert, and R. V. Pollock. 1980. Hemagglutination by canine parvovirus: serologic studies and diagnostic applications. Am J Vet Res 41:784-91.

13. Chang, S. F., J. Y. Sgro, and C. R. Parrish. 1992. Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties. J Virol 66:6858-67.

14. Cheng, Y., O. Zak, P. Aisen, S. C. Harrison, and T. Walz. 2004. Structure of the human transferrin receptor-transferrin complex. Cell 116:565-76.

15. Cotmore, S. F., and P. Tattersall. 1989. A genome-linked copy of the NS-1 polypeptide is located on the outside of infectious parvovirus particles. J Virol 63:3902-11.

16. Cotmore, S. F., and P. Tattersall. 1987. The autonomously replicating parvoviruses of vertebrates. Adv Virus Res 33:91-174.

17. Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Trop 78:103-16.

18. Daszak, P., A. A. Cunningham, and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife--threats to biodiversity and human health. Science 287:443-9.

19. Goodman, L. B., S. M. Lyi, N. C. Johnson, J. O. Cifuente, S. L. Hafenstein, and C. R. Parrish. Binding site on the transferrin receptor for the parvovirus capsid and effects of altered affinity on cell uptake and infection. J Virol 84:4969-78.

20. Govindasamy, L., K. Hueffer, C. R. Parrish, and M. Agbandje-McKenna. 2003. Structures of host range-controlling regions of the capsids of canine and feline parvoviruses and mutants. J Virol 77:12211-21.

21. Hafenstein, S., V. D. Bowman, T. Sun, C. D. Nelson, L. M. Palermo, P. R. Chipman, A. J. Battisti, C. R. Parrish, and M. G. Rossmann. 2009. Structural comparison of different antibodies interacting with parvovirus capsids. J Virol 83:5556-66.

22. Hafenstein, S., L. M. Palermo, V. A. Kostyuchenko, C. Xiao, M. C. Morais, C. D. Nelson, V. D. Bowman, A. J. Battisti, P. R. Chipman, C. R. Parrish, and M. G. Rossmann. 2007. Asymmetric binding of transferrin receptor to parvovirus capsids. Proc Natl Acad Sci U S A 104:6585-9.

23. Harbison, C. E., S. M. Lyi, W. S. Weichert, and C. R. Parrish. 2009. Early steps in cell infection by parvoviruses: host-specific differences in cell receptor binding but similar endosomal trafficking. J Virol 83:10504-14.

24. Heegaard, E. D., and K. E. Brown. 2002. Human parvovirus B19. Clin Microbiol Rev 15:485-505.

25. Hoelzer, K., L. A. Shackelton, C. R. Parrish, and E. C. Holmes. 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. J Gen Virol 89:2280-9.

26. Hueffer, K., L. Govindasamy, M. Agbandje-McKenna, and C. R. Parrish. 2003. Combinations of two capsid regions controlling canine host range determine canine transferrin receptor binding by canine and feline parvoviruses. J Virol 77:10099-105.

27. Hueffer, K., L. M. Palermo, and C. R. Parrish. 2004. Parvovirus infection of cells by using variants of the feline transferrin receptor altering clathrin-mediated endocytosis, membrane domain localization, and capsid-binding domains. J Virol 78:5601-11.

28. Hueffer, K., J. S. Parker, W. S. Weichert, R. E. Geisel, J. Y. Sgro, and C. R. Parrish. 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. 77:1718-1726.

29. Ikeda, Y., M. Mochizuki, R. Naito, K. Nakamura, T. Miyazawa, T. Mikami, and E. Takahashi. 2000. Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virology 278:13-9.

30. Jones, K. E., N. G. Patel, M. A. Levy, A. Storeygard, D. Balk, J. L. Gittleman, and P. Daszak. 2008. Global trends in emerging infectious diseases. Nature 451:990-3.

31. Kaludov, N., K. E. Brown, R. W. Walters, J. Zabner, and J. A. Chiorini. 2001. Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient

transduction but differ in sialic acid linkage specificity. J Virol 75:6884-93.

32. Kapil, S., G. Rezabek, B. Germany, and L. Johnston. Isolation of a virus related to canine parvovirus type 2 from a raccoon (Procyon lotor). Vet Rec 166:24-5.

33. Kuiken, T., E. C. Holmes, J. McCauley, G. F. Rimmelzwaan, C. S. Williams, and B. T. Grenfell. 2006. Host species barriers to influenza virus infections. Science 312:394-7.

34. Lawrence, C. M., S. Ray, M. Babyonyshev, R. Galluser, D. W. Borhani, and S. C. Harrison. 1999. Crystal structure of the ectodomain of human transferrin receptor. Science 286:779-82.

35. Liu, J. M., S. W. Green, T. Shimada, and N. S. Young. 1992. A block in full-length transcript maturation in cells nonpermissive for B19 parvovirus. J Virol 66:4686-92.

36. Llamas-Saiz, A. L., M. Agbandje-McKenna, J. S. Parker, A. T. Wahid, C. R. Parrish, and M. G. Rossmann. 1996. Structural analysis of a mutation in canine parvovirus which controls antigenicity and host range. Virology 225:65-71.

37. Martella, V., A. Cavalli, A. Pratelli, G. Bozzo, M. Camero, D. Buonavoglia, D. Narcisi, M. Tempesta, and C. Buonavoglia. 2004. A canine parvovirus mutant is spreading in Italy. J Clin Microbiol 42:1333-6.

38. Nelson, C. D., E. Minkkinen, M. Bergkvist, K. Hoelzer, M. Fisher, B. Bothner, and C. R. Parrish. 2008. Detecting small changes and additional peptides in the canine parvovirus capsid structure. J Virol 82:10397-407.

39. Palermo, L. M., K. Hueffer, and C. R. Parrish. 2003. Residues in the apical domain of the feline and canine transferrin receptors control host-specific binding and cell infection of canine and feline parvoviruses. J Virol 77:8915-8923.

40. Paradiso, P. R., S. L. Rhode, 3rd, and Singer, II. 1982. Canine parvovirus: a biochemical and ultrastructural characterization. J Gen Virol 62 (Pt 1):113-25.

41. Parker, J. S., W. J. Murphy, D. Wang, S. J. O'Brien, and C. R. Parrish. 2001. Canine and feline parvoviruses can use human or feline transferrin receptors to bind, enter, and infect cells. J Virol 75:3896-902.

42. Parker, J. S., and C. R. Parrish. 1997. Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. J Virol 71:9214-22.

43. Parker, J. S., and C. R. Parrish. 2000. Cellular uptake and infection by canine parvovirus involves rapid dynamin-regulated clathrin-mediated endocytosis, followed by slower intracellular trafficking. J Virol 74:1919-30.

44. Parrish, C. R. 1990. Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Adv Virus Res 38:403-450.

45. Parrish, C. R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology 183:195-205.

46. Parrish, C. R., C. F. Aquadro, and L. E. Carmichael. 1988. Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink and raccoon parvoviruses. Virology 166:293-307.

47. Parrish, C. R., G. Burtonboy, and L. E. Carmichael. 1988. Characterization of a nonhemagglutinating mutant of canine parvovirus. Virology 163:230-2.

48. Parrish, C. R., and L. E. Carmichael. 1983. Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. Virology 129:401-14.

49. Parrish, C. R., and L. E. Carmichael. 1986. Characterization and recombination mapping of an antigenic and host range mutation of canine parvovirus. Virology 148:121-32.

50. Parrish, C. R., Carmichael, L.E., Antczak, D.F. 1982. Antigenic relationships between canine parvovirus type-2, feline panleukopenia virus and mink enteritis virus using conventional antisera and monoclonal antibodies. Arch.Virol. 72: 267-278.

51. Parrish, C. R., E. C. Holmes, D. M. Morens, E. C. Park, D. S. Burke, C. H. Calisher, C. A. Laughlin, L. J. Saif, and P. Daszak. 2008. Cross-

species virus transmission and the emergence of new epidemic diseases. Microbiol Mol Biol Rev 72:457-70.

52. Parrish, C. R., P. H. O'Connell, J. F. Evermann, and L. E. Carmichael. 1985. Natural variation of canine parvovirus. Science 230:1046-1048.

53. Patz, J. A., P. Daszak, G. M. Tabor, A. A. Aguirre, M. Pearl, J. Epstein, N. D. Wolfe, A. M. Kilpatrick, J. Foufopoulos, D. Molyneux, and D. J. Bradley. 2004. Unhealthy landscapes: Policy recommendations on land use change and infectious disease emergence. Environ Health Perspect 112:1092-8.

54. Ponka, P., and C. N. Lok. 1999. The transferrin receptor: role in health and disease. Int J Biochem Cell Biol 31:1111-37.

55. Rhode, S. L., 3rd. 1985. Nucleotide sequence of the coat protein gene of canine parvovirus. J Virol 54:630-3.

56. Rossmann, M. G., and A. C. Palmenberg. 1988. Conservation of the putative receptor attachment site in picornaviruses. Virology 164:373-82.

57. Senda, M., N. Hirayama, O. Itoh, and H. Yamamoto. 1988. Canine parvovirus: strain difference in haemagglutination activity and antigenicity. J Gen Virol 69 (Pt 2):349-54.

58. Shackelton, L. A., C. R. Parrish, U. Truyen, and E. C. Holmes. 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci U S A 102:379-84.

59. Siegl, G., R. C. Bates, K. I. Berns, B. J. Carter, D. C. Kelly, E. Kurstak, and P. Tattersall. 1985. Characteristics and taxonomy of Parvoviridae. Intervirology 23:61-73.

60. Simpson, A. A., V. Chandrasekar, B. Hebert, G. M. Sullivan, M. G. Rossmann, and C. R. Parrish. 2000. Host range and variability of calcium binding by surface loops in the capsids of canine and feline parvoviruses. J Mol Biol 300:597-610.

61. Spalholz, B. A., and P. Tattersall. 1983. Interaction of minute virus of mice with differentiated cells: strain-dependent target cell specificity is mediated by intracellular factors. J Virol 46:937-43.

62. Strassheim, M. L., A. Gruenberg, P. Veijalainen, J. Y. Sgro, and C. R. Parrish. 1994. Two dominant neutralizing antigenic determinants of

canine parvovirus are found on the threefold spike of the virus capsid. Virology 198:175-84.

63. Suikkanen, S., M. Antila, A. Jaatinen, M. Vihinen-Ranta, and M. Vuento. 2003. Release of canine parvovirus from endocytic vesicles. Virology 316:267-80.

64. Suikkanen, S., K. Saajarvi, J. Hirsimaki, O. Valilehto, H. Reunanen, M. Vihinen-Ranta, and M. Vuento. 2002. Role of recycling endosomes and lysosomes in dynein-dependent entry of canine parvovirus. J Virol 76:4401-11.

65. Summerford, C., and R. J. Samulski. 1998. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. J Virol 72:1438-45.

66. Tattersall, P., and J. Bratton. 1983. Reciprocal productive and restrictive virus-cell interactions of immunosuppressive and prototype strains of minute virus of mice. J Virol 46:944-55.

67. Tresnan, D. B., L. Southard, W. Weichert, J. Y. Sgro, and C. R. Parrish. 1995. Analysis of the cell and erythrocyte binding activities of the dimple and canyon regions of the canine parvovirus capsid. Virology 211:123-32.

68. Truyen, U., M. Agbandje, and C. R. Parrish. 1994. Characterization of the feline host range and a specific epitope of feline panleukopenia virus. Virology 200:494-503.

69. Truyen, U., J. F. Evermann, E. Vieler, and C. R. Parrish. 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology 215:186-189.

70. Truyen, U., A. Gruenberg, S. F. Chang, B. Obermaier, P. Veijalainen, and C. R. Parrish. 1995. Evolution of the feline-subgroup parvoviruses and the control of canine host range in vivo. J. Virol. 69:4702-4710.

71. Truyen, U., and C. R. Parrish. 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: distinct host cell tropisms of each virus in vitro and in vivo. J Virol 66:5399-5408.

72. Tsao, J., M. S. Chapman, M. Agbandje, W. Keller, K. Smith, H. Wu, M. Luo, T. J. Smith, M. G. Rossmann, R. W. Compans, and et al. 1991. The three-dimensional structure of canine parvovirus and its functional implications. Science 251:1456-64.

73. Vihinen-Ranta, M., L. Kakkola, A. Kalela, P. Vilja, and M. Vuento. 1997. Characterization of a nuclear localization signal of canine parvovirus capsid proteins. Eur J Biochem 250:389-94.

74. Vihinen-Ranta, M., W. Yuan, and C. R. Parrish. 2000. Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. J Virol 74:4853-9.

75. Weichert, W. S., J. S. Parker, A. T. Wahid, S. F. Chang, E. Meier, and C. R. Parrish. 1998. Assaying for structural variation in the parvovirus capsid and its role in infection. Virology 250:106-17.

76. Weigel-Kelley, K. A., M. C. Yoder, and A. Srivastava. 2001. Recombinant human parvovirus B19 vectors: erythrocyte P antigen is necessary but not sufficient for successful transduction of human hematopoietic cells. J Virol 75:4110-6.

77. Woolhouse, M. E. 2008. Epidemiology: emerging diseases go global. Nature 451:898-9.

78. Xie, Q., and M. S. Chapman. 1996. Canine parvovirus capsid structure, analyzed at 2.9 A resolution. J Mol Biol 264:497-520.

79. Yuan, W., and C. R. Parrish. 2001. Canine parvovirus capsid assembly and differences in mammalian and insect cells. Virology 279:546-57.

80. Zadori, Z., J. Szelei, M. C. Lacoste, Y. Li, S. Gariepy, P. Raymond, M. Allaire, I. R. Nabi, and P. Tijssen. 2001. A viral phospholipase A2 is required for parvovirus infectivity. Dev Cell 1:291-302.

CHAPTER 2

THE ROLE OF INTERMEDIATE SEQUENCES IN THE EVOLUTION OF CANINE PARVOVIRUS*

* Modified from a manuscript being prepared with co-authors Tyler D. Lillie, Jason T. Kaelber, Edward C. Holmes, and Colin R. Parrish.

2.1 ABSTRACT

The emergence of viral host range variants frequently requires a number of mutations that often alter multiple virus properties simultaneously. The process by which these mutations arise and are selected remains poorly understood. Canine parvovirus (CPV) serves as an excellent model for studying viral emergence and adaptation to a novel host. Here, we characterize the individual and combinatorial effects of four clade-defining capsid (VP2) residues that differ between the original variant, CPV-2, and a newer strain, CPV-2b. We show that all four residues, including the buried VP2 residue 101, alter binding to several anti-capsid monoclonal antibodies, as well as binding of virus to feline and canine cells, likely by influencing transferrin receptor binding. We also demonstrate that adaptation to dogs likely required transient evolutionary intermediates that had reduced viral fitness, at least for cells in tissue culture.

2.2 INTRODUCTION

As viruses evolve, they may acquire new properties that alter their host range, transmission, tissue tropism, antigenicity, and/or virulence. Acquisition of novel biological functions often requires multiple mutations working together in complex ways to give the final viral phenotype. How these mutations arise and are selected is not well understood. This is particularly true for mutations that alter the ability of a virus to replicate in different hosts (i.e., those that change host range), where mutations that give higher fitness in the new host may reduce fitness in the original host, resulting in different selection pressures in each host. Mutations may also be under complex selection in the same host, for example, when receptor binding and antibody recognition sites

on the viral capsid overlap. In such cases, selection pressures will presumably differ between immunologically naïve and immune individuals and populations. Understanding the processes by which viruses acquire new phenotypes in the face of such complex selection environments is critical for improving the prediction, prevention and control strategies for emerging viral diseases.

One way to conceptualize these processes is to represent viral sequence space on a fitness landscape, an extension of Wright's adaptive landscape that describes the relative fitness of all possible genotypes of a given replicon in a given environment (28, 29). For example, Figure 2.1 shows a simplified, two-dimensional fitness landscape for a hypothetical virus in a novel host environment. A recently emerged virus would likely have a relatively low fitness in its new host and would subsequently gain fitness through the acquisition of adaptive mutations. This process may occur at the intra- or interhost level, and may or may not involve the generation of intermediate viral variants with lower fitness. Viral fitness refers to the contribution of a given variant's genotype to the next generation of viruses (4), and various experimental measures can be used to estimate the relative fitness of virus variants under different conditions, including *in vitro* measurements of antibody binding and neutralization, replication efficiency, receptor binding and uptake, and infectivity.

Canine parvovirus (CPV) provides an excellent system for studying these evolutionary processes because its evolution and biological properties have been characterized in detail since its emergence as a new pathogen of dogs in the late 1970s. The original virus was named CPV type-2 (CPV-2) and is a host-range variant of feline panleukopenia virus (FPV), or one of its

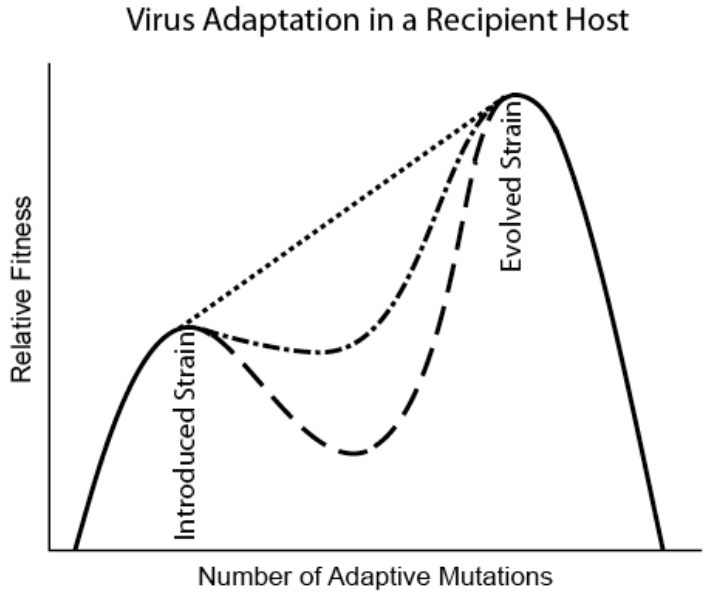


Figure 2.1. Two-dimensional representation of a fitness landscape for a virus in a novel recipient host species, where multiple mutations are required for adaptation. A virus transferred to a recipient host is relatively poorly adapted (peak on the left). Adaptation to the recipient host requires multiple mutations in the viral genome to increase its fitness (peak on the right). Multiple potential pathways exist for traversing the genotypic distance between these two peaks. There could be a steady increase in fitness (dotted line), or combinations of mutations may have varying degrees of reduced fitness (dashed lines). Knowing which of these evolutionary pathways is followed during an emergence event would aid in risk assessment and development of prevention strategies.

close relatives (26). CPV-2 gained the canine host range through the acquisition of several mutations found primarily in the capsid gene. Some of those changes caused it to simultaneously lose the ability to replicate in cats, although it is still able to infect feline cells *in vitro* (26). Within two to three years, the original CPV-2 was replaced globally with a genetically and antigenically distinct variant, CPV type-2a (CPV-2a), which had several additional nonsynonymous mutations in the capsid gene (16, 20). Phylogenetic analyses show that currently circulating strains of CPV form a

monophyletic clade that is derived from the original CPV-2 lineage (22). Interestingly, CPV-2a, and more recent variants derived from that virus, regained the feline host range, although with little reversion to the original FPV sequences (25). They also show reduced binding to their cellular receptor, the transferrin receptor (TfR) (9), compared with either FPV or CPV-2, and they have varying antigenicity as defined by monoclonal antibody (mAb) or sensitive polyclonal antibody analyses (20).

Viruses in the CPV-2a clade contain four unique coding changes in the major capsid gene, VP2, compared with CPV-2 viruses, at residues 87, 101, 300, and 305 (Table 2.1). Since it emerged around 1979, CPV-2a clade viruses have acquired a number of additional mutations in the capsid protein, and in some cases, the same VP2 residue has changed multiple times. For example, VP2 residue 426 is Asn in CPV-2a, but an Asp in CPV-2b (15) and Glu in CPV-2c (2). Substitutions at residue 300 (from Ala to Gly, Val or Asp in different viruses) alter host range and antigenicity of CPV (12, 18), whereas substitutions at residue 426 alter the antigenicity of the capsid but do not appear to directly effect host range (15). Residues 87, 300 and 305 lie within the binding footprint of the TfR (7). Previous escape mutant analyses and cryoEM studies of mAb-derived antibody binding fragments (Fab) complexed with the capsids define two epitopes on the capsid surface: site A, which overlaps with much of the 3-fold spike, and site B, which lies further down on the shoulder of the 3-fold spike (6, 24). Residue 300 lies within site B, while residue 426 in within site A, and residues 87 and 305 lie within an overlapping region of the site A and B mAb binding footprints (6).

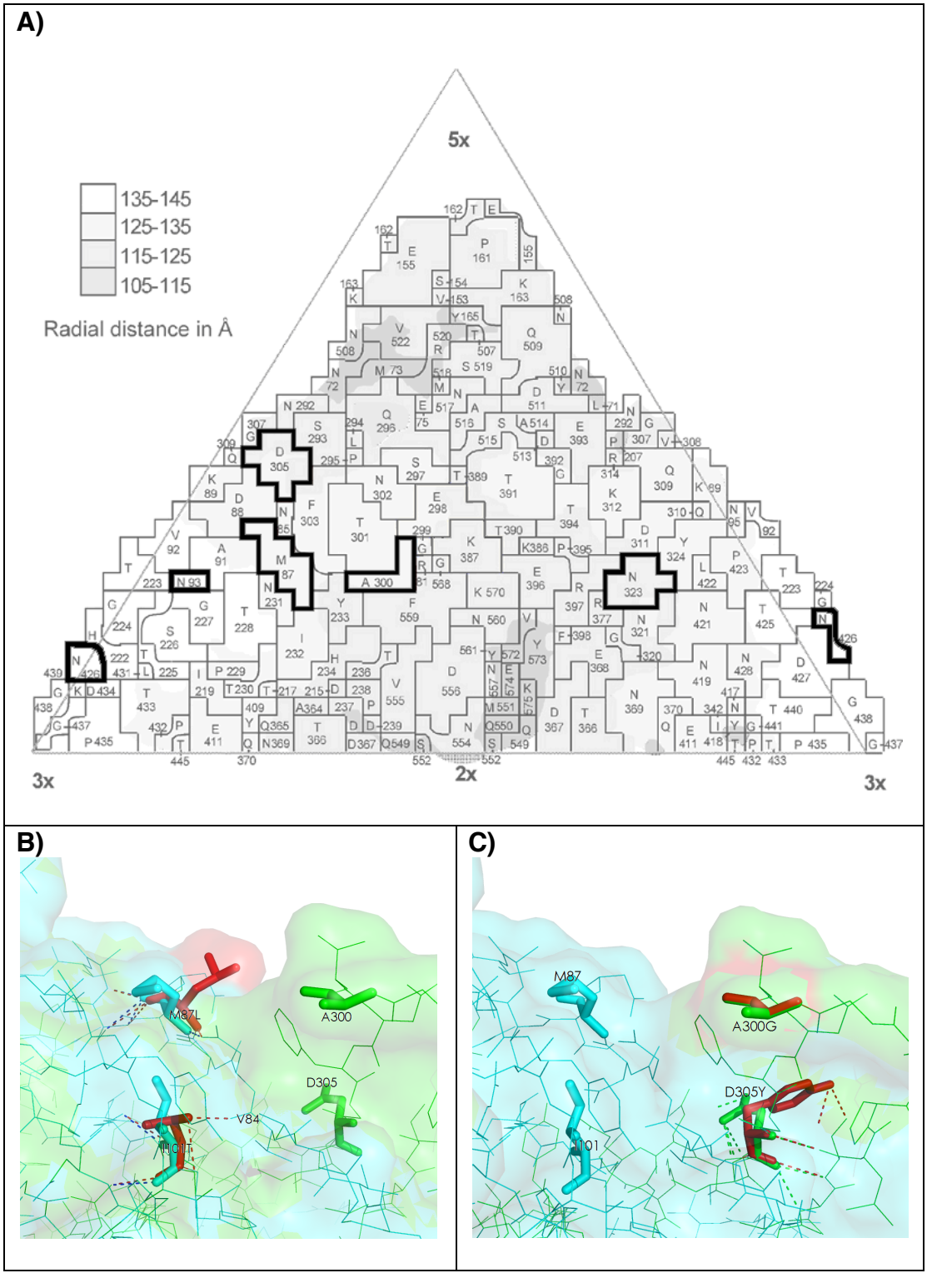
Table 2.1. Wildtype differences in the four VP2 residues examined in this study.

	VP2 Residue			
	87	101	300	305
CPV-2	Met	Ile	Ala	Asp
CPV-2b	Leu	Thr	Gly	Tyr

The atomic structures of CPV-2 and FPV, as well as some mutants (including VP2 300Asp), have been solved by X-ray crystallography (1, 12, 27, 30) and those show that VP2 residues 87, 101, 300, and 305 are found in a region of the shoulder of the three-fold spike (Figure 2.2 A). Residues 87, 300 and 305 are surface-exposed, while residue 101 is buried directly below residue 87. Within one asymmetric capsid unit, residues 87 and 101 are part of a flexible loop of one VP2 molecule, and that loop interacts closely with a flexible loop from a second VP2 molecule that contains residues 300 and 305 (Figure 2.2 B). Changes in the three surface exposed residues could therefore directly alter interactions with various ligands, including the TfR and a number of antibodies. Mutating residue 101 may also alter local structures enough to modify the capsid surface, and thus also contribute to changes in binding properties (Figure 2.2 C).

Here, we investigate the evolution of CPV-2 in dogs by examining the functions of viruses containing intermediate combinations of VP2 residues 87, 101, 300, and 305, the signature mutations of the CPV-2a variant that globally replaced the CPV-2 strain. Viruses containing these intermediate capsid sequence combinations have not been isolated from nature, suggesting they existed transiently and had lower fitness. By examining the properties of these

Figure 2.2. A) Asymmetric unit of the CPV-2 capsid showing surface-exposed VP2 residues and created using a previously published method (21). Surface-exposed residues that differ among wildtype FPV, CPV-2 and CPV-2b viruses are outlined in black. B, C) Crystal structure of the shoulder of the 3-fold spike of CPV-2 (Protein Data Bank accession no. 4DPV). Flexible surface loops from two distinct VP2 peptides are colored (cyan and green), and show that residues 87, 300 and 305 are surface-exposed, while residue 101 is buried directly below residue 87. CPV-2b VP2 residues 87 and 101 (B) or 300 and 305 (C) were introduced into the crystal structure using WinCOOT (5). The mutated structures were overlaid onto a capsid fragment generated with VIPERdb (3) and visualized using PyMOL. Hydrogen atoms are not displayed, but hydrogen bonds are indicated by dashed lines and show that the CPV-2b point mutations introduce new hydrogen bonds that may alter the surface structure of the capsid.


A)
5x
135-145
125-135
115-125
105-115
Radial distance in Å
3x
2x
3x
B)
M87L
A300
D305
V84
I101
C)
M87
A300G
D305Y
I101

22 intermediate viruses, we could assess the phenotypes and relative fitness of intermediates that may have occurred during the evolution of CPV.

2.3 MATERIALS AND METHODS

Cells and viruses. Norden Laboratory feline kidney (NLFK) cells and A72 canine fibroblasts were grown in a 1:1 mixture of McCoy's 5A and Liebovitz L15 media with 5% fetal calf serum (FCS) (growth medium).

Parvoviruses were derived from infectious plasmid clones of CPV-2 (CPV-d) and CPV-2b (CPV-39) strains, as previously described (15). Intermediate viruses were created from infectious plasmid clones using either the GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI) or the Phusion Site-Directed Mutagenesis Kit (Finnzymes, Woburn, MA). In all cases, the mutated region of the VP2 gene was sequenced to confirm that only the desired mutations were present, and in some cases, the mutated region was recloned into the appropriate infectious clone background to ensure no additional mutations were present in the genome. To prepare capsids for binding studies, viruses were concentrated by polyethylene glycol precipitation, followed by sucrose gradient centrifugation and dialysis against phosphate buffered saline (PBS) (1, 13).

Virus infectivity assays. For viability and infection assays, NLFK cells seeded at 2×10^4 cells/cm^2 in 25 or 75 cm^2 dishes were incubated overnight at 37°C. To assess viability, cells were incubated with Lipofectamine reagent (Invitrogen, Carlsbad, CA) and 5 μg plasmid DNA for 4 hours, and then with growth medium for 7 days, with one passage to maximize cell division and viral replication. To assess virus infectivity, cells were inoculated with first passage virus supernatant and then incubated for 7 days. Coverslips were

fixed with 4% paraformaldehyde two days post-transfection or post-infection, and analyzed for viral proteins by immunofluorescence microscopy (IFA). Transfected and infected cultures were frozen, thawed and spun at 10,000 x g for 10 minutes. Virus titers were tested in hemagglutination (HA) assays using feline erythrocytes in Bis-Tris buffered saline (pH6.2 at 4°C) (19, 23).

Virus detection by IFA. Fixed cells were stained for virus with Alexa488-labeled anti-nonstructural (NS) protein mAb (CE10) (31) and/or Alexa594-labeled anti-capsid mAb (A3B10) (19). Anti-viral antibodies were diluted in 1X PBS containing 1% bovine serum albumin, 0.1% Triton X100 and 0.05% sodium azide (permeabilization solution) and incubated with cells for 1 hour at room temperature. Stained coverslips were mounted on slides and analyzed with a Nikon Eclipse TE300 inverted epifluorescent microscope.

Antigenic analysis of viruses. Antigenic testing of wildtype and intermediate viruses was performed using a hemagglutination inhibition (HI) assay with a panel of mouse or rat mAbs prepared against FPV, CPV-2 or CPV-2b capsids (17, 19, 20) (Table 2.2). HI assays were performed with conditions described above.

Virus binding and uptake by feline and canine cells. Feline or canine cells were seeded at 2×10^4 cell/cm^2 in 12-well tissue culture plates. Following overnight incubation at 37°C, cells were washed twice with warm DMEM with 0.1% BSA (binding medium). Wildtype and intermediate virus binding and uptake was performed by incubating cells with 15 μg/ml appropriate purified virus for 1 hour at 37°C. Virus was then removed and cells were washed twice with binding medium before being dissociated with brief exposure to trypsin and transferred to a 96-well, V-bottom plate.

Table 2.2. Wildtype virus specificity of monoclonal antibodies used to test intermediate virus antigenicity in the hemagglutination inhibition assay. The capsid binding site it indicated, if known (24).

mAb	Species	Immunizing Antigen	Binding Site on Capsid	CPV Specificity
G	rat	FPV-c		FPV only
J	rat	FPV-c	B	CPV-2
I	rat	FPV-c	A	CPV-2
D	rat	FPV-c	B	CPV-2
E	rat	FPV-c	B	CPV-2
1D1	mouse	CPV-39 (2b)		CPV-2b
7D6	mouse	CPV-39 (2b)		CPV-2b
7E2	mouse	CPV-39 (2b)		CPV-2b
14	mouse	CPV-a (2)	A	All CPV
8	mouse	CPV-a (2)	B	All CPV

Cells were pelleted at 1000 x g for 5 minutes at 4°C and washed once with 1X PBS containing 1% ovalbumin, 1mM EDTA and 0.01% sodium azide (wash solution) before being fixed for 20 minutes in IC fixation buffer (eBioscience, San Diego, CA). Cells were then washed three times with 1X permeabilization buffer (eBioscience) and stained with Alexa488-labeled anti-capsid mAb 8 (19) for 30 minutes at room temperature. Following three final washes with permeabilization buffer, cells were resuspended in wash solution and analyzed using a GuavaCyte flow cytometer (Millipore, Billerica, MA). Flow cytometry data was analyzed using FlowJo v9 software (Treestar, Ashland, OR).

***In vitro* competition assays for relative viral fitness**. Pair-wise mixtures of wildtype and intermediate viral stocks were prepared at 10:1, 1:1 and 1:10 volume-to-volume ratios. For each replicate, feline or canine cells were seeded at 2×10^4 cell/cm^2 in two 96-well tissue culture plates. The next day, cells were washed twice with DMEM with 0.1% BSA (infection medium) at

37°C. Inoculations were performed in each plate for 1 hour at 37°C with 15-25 μl/well appropriate virus stock mixture plus 15-25 μl/well infection medium. Following inoculation, the virus was removed and the cells were washed twice with infection medium, then incubated at 37°C for either 2 days or 7.5 days in growth medium before being frozen at -80°C and thawed rapidly at 37°C to release cell-associated virus. Supernatants were transferred to a 96-well, V-bottom plate and centrifuged at 1000 × g for 5 minutes to pellet cellular debris. This clarified supernatant used for standard virus PCR and sequencing.

PCR, sequencing and analysis. Phusion hot start, high fidelity DNA polymerase (Finnzymes) and a standard set of CPV primers (forward 5'-GAAAACGGATGGGTGGAAATCACAGC-3' and reverse 5'-TATTTTGAATCCAATCTCCTTCTGG-3') and thermocycler settings (30 cycles of 0:10 at 98°C, 0:30 at 54°C, 2:15 at 72°C) were used to PCR amplify the mid-portion of the major capsid gene, VP2, that includes codons 87, 101, 300 and 305. DNA products were purified using QIAquick 96 PCR purification kits (Qiagen, Valencia, CA), and Sanger sequencing reactions were performed by Cornell University's Core Laboratories Center using the primers described above.

Peak heights were measured from sequence traces using 4Peaks software (Mekentosj, Amsterdam, The Netherlands), and peak height ratios (PHR) were calculated for nucleotides that differed in sequence between each set of input viruses. Changes in PHR over time were examined graphically, and fold increases or decreases in PHR after 7.5 days post infection were calculated. Where more than one nucleotide varied between the two input viruses, fold changes in PHR were averaged for all sites.

2.4 RESULTS

A series of 22 intermediate viruses was prepared between the wildtype viruses CPV-2 and CPV-2b, which represent prototype viruses for the original and newer CPV clades, respectively (Figure 2.3). CPV-2 codons for the VP2 residues 87, 101, 300, and 305 were mutated individually and in pairs to the CPV-2b sequences. An additional CPV-2-derived virus was also created with all 4 residues changed to the CPV-2b sequences. Equivalent intermediate viruses were made from the CPV-2b background by changing combinations of these same residues back to the CPV-2 sequence. Viruses were named by the virus from which they were derived, followed by a 4-digit subscript that indicates the amino acid present at VP2 positions 87, 101, 300, and 305, respectively. A subscript of 0 indicates the CPV-2 residue is present, while a subscript of 1 indicates the CPV-2b residue is present. Thus, wildtype CPV-2 is represented as CPV-2_{0000} and wildtype CPV-2b is CPV-$2b_{1111}$.

Viability and infectivity. All but 3 intermediate viruses (indicated by asterisks in Figure 2.3) were viable and infectious by IFA and HA testing after transfection and passaging in tissue culture (data not shown). The 3 intermediates that did not grow were each derived from CPV-2b and contained individual mutations at residues 101 or 300, or a double mutation at residues 101 and 305.

Antigenic variation. Each virus was tested in an HI assay for reactivity against a panel of mAbs that have various specificities for CPV or FPV strains (Table 2.2) (20). The FPV-specific mAb G did not bind any of the wildtype or intermediate viruses, while the broad-specificity mAbs 8 and 14 bound all the viruses (Figure 2.4 A). In CPV-2, changing VP2 residue 300 to the CPV-2b sequence alone, or in combination with residues 87, 101 and/or

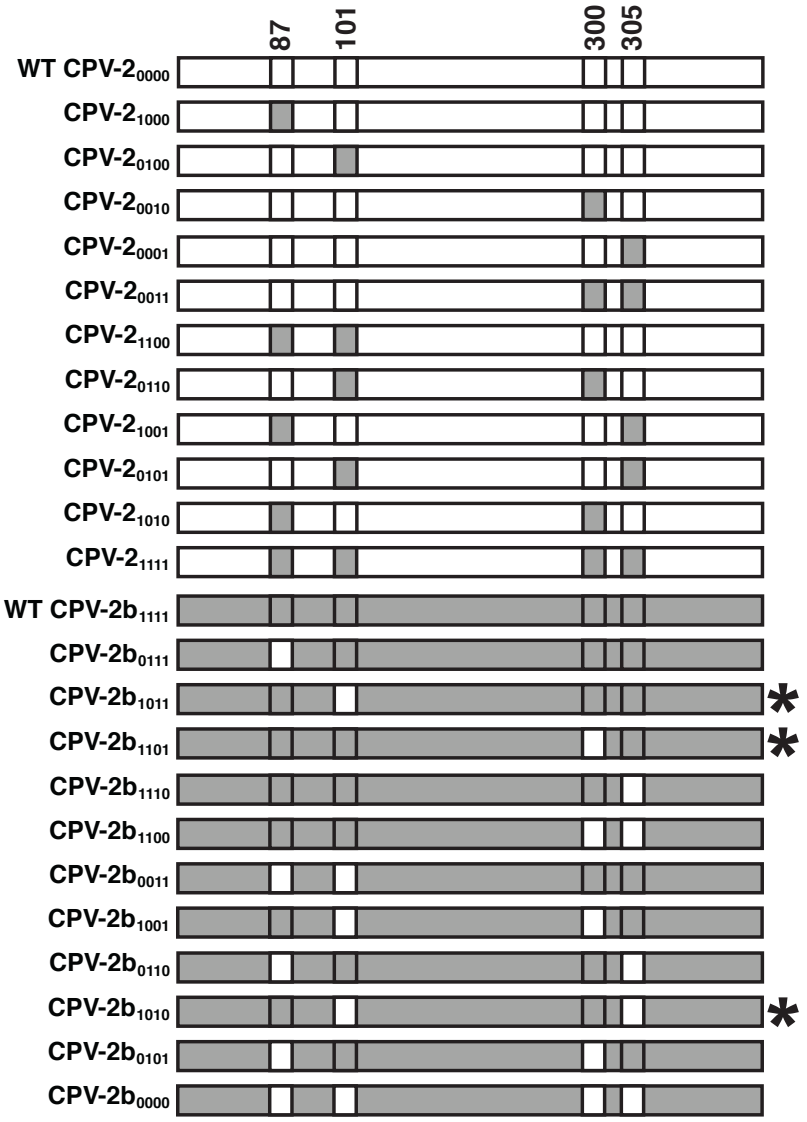
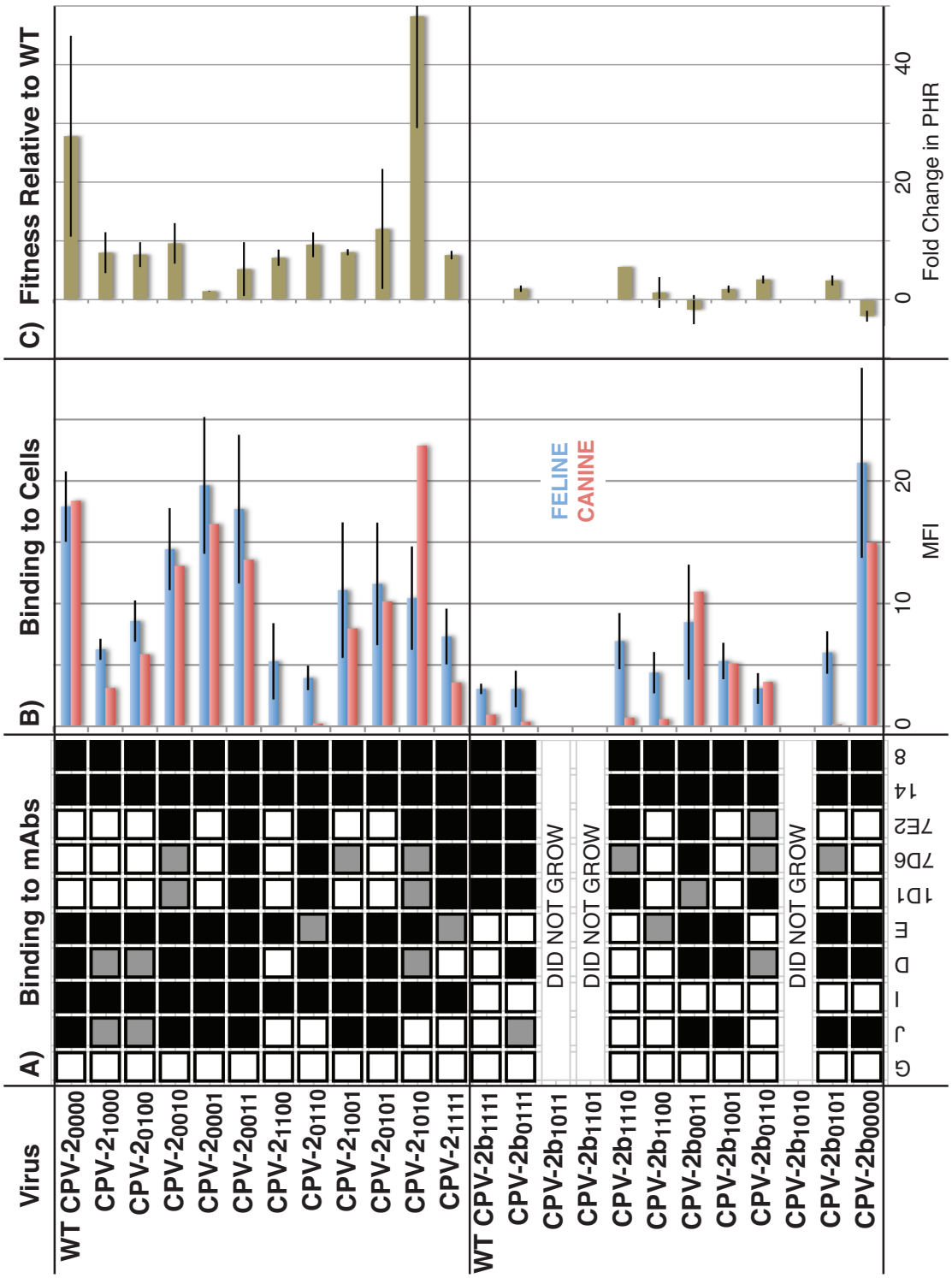


Figure 2.3. VP2 schematic for wild type viruses, CPV-2 (white) and CPV-2b (grey), as well as for the 22 intermediate viruses created for this study, indicating their changes at VP2 residues 87, 101, 300, and 305. Naming conventions are described in the main text. All viruses were prepared as infectious plasmid clones and tested in at least two independent experiments for viability and infectivity after transfection and passage in tissue culture, respectively. Asterisks indicate intermediate viruses that were noninfectious.

305, allowed binding by CPV-2b-specific mAbs (1D1, 7D6 and 7E2). Similarly, changing VP2 residue 300 in CPV-2b to the CPV-2 sequence, in combination with residues 87, 101 and/or 305, prevented binding by these CPV-2b-specific mAbs. These results demonstrate that the epitope recognized by all three CPV-2b-specific mAbs is largely controlled by the presence of VP2 residue 300 Gly.

Of the CPV-2-specific mAbs, mAb I binds site A, while the others bind site B (24). mAb I recognized all intermediate viruses as their corresponding wildtype virus, and thus bound only to CPV-2-derived intermediates. mAbs D and J showed similar capsid binding properties, with reciprocal results for CPV-2 and CPV-2b intermediates that have the same residues changed. Binding of mAbs D and J to CPV-2 was reduced when residues 87 and 101 were individually changed to the CPV-2b sequence, and completely lost when residues 87 and 101 were changed together. Changing CPV-2 residues 300 and 87 together reduced or eliminated mAb D and J binding, respectively, while changing residue 300 alone did not alter CPV-2-specific mAb binding. Changing CPV-2 residues 101 and 300 simultaneously also eliminated mAb J binding. Similar reciprocal results were seen when residue 87 in CPV-2b was changed to the CPV-2 sequence alone, or in combination with changes at residues 101 or 300, as these changes partially or fully restored virus binding by mAbs D and J. Changing CPV-2b residues 101 and 300 in concert also restored mAb D and J binding. These results clearly show that the epitopes recognized by mAbs D and J are similar and primarily controlled by residue 87, with varying degrees of modulation by residues 101 and 300 that depended on the mAb.

Figure 2.4. Ligand binding properties and relative fitness of wildtype and intermediate viruses. A) Virus binding profiles for 10 mAbs as determined by HI assay. Strong (black), intermediate (grey) and weak (white) binding for each mAb was defined by its wildtype CPV-2 and CPV-2b binding titers, as wildtype virus specificity for these mAbs is well characterized (20). B) Virus binding and uptake in feline and canine cells as measured by flow cytometry. For feline cells, the mean fluorescent intensity (MFI) from three independent experiments was averaged and the standard error of the mean is shown. For canine cells, an average of two replicate wells from a single experiment is shown. C) Relative fitness of wildtype and intermediate viruses following one week of replication in feline cells. Average fold changes in wildtype:intermediate peak height ratios (PHR) from one to three independent competition assays is shown for 1:1 ratios of input virus. For WT CPV-2, the bar indicates WT CPV-2:CPV-2b PHR. Error bars represent standard deviation. Results for the 10:1 and 1:10 input virus ratios showed similar trends (data not shown).


A)
Binding to mAbs
Virus
G
J
I
D
E
1D1
7D6
7E2
14
8
WT CPV-2
CPV-2
CPV-2b
DID NOT GROW
B)
Binding to Cells
FELINE
CANINE
MFI
0
10
20
C)
Fitness Relative to WT
Fold Change in PHR
0
20
40

mAb E demonstrated a unique binding pattern, as results were not directly reciprocal between intermediate viruses where the same residues were changed in each wildtype virus, as generally observed with the other mAbs. mAb E showed reduced binding to CPV-2 intermediate viruses when residues 101 and 300 were simultaneously changed, but otherwise bound strongly to all CPV-2-derived intermediates. Conversely, more than one combination of changes in CPV-2b resulted in a partial to full return of mAb E binding. These included concerted changes of residue 300 together with residues 87, 101 and/or 305, as well as simultaneous changes in residues 87 and 101. These results suggest that there are additional structural differences between CPV-2 and CPV-2b that involve residues other than 87, 101, 300, and 305.

Receptor binding. The binding and uptake of each virus in feline NLFK and canine A72 cells were assessed using flow cytometry (Figure 2.4 B). Wildtype CPV-2 bound at 5- to 20-fold higher levels than wildtype CPV-2b in feline and canine cells, respectively, as reported previously (9). Changing VP2 residues 87 and 101 to the CPV-2b sequence, alone and in concert, reduced the binding of CPV-2 to feline and canine cells. Conversely, changing the CPV-2b residues 87 and 101 simultaneously to the CPV-2 sequence increased binding to feline and canine cells, although changing residue 87 alone did not.

CPV-2 binding to feline and canine cells was also reduced when VP2 residues 101 and 300 were changed together. However, changing those two residues in CPV-2b resulted in only a modest increase in cell binding, and CPV-2b cell binding only reached CPV-2 levels when all 4 VP2 residues were changed simultaneously.

Relative fitness. To measure the relative fitness of viruses in tissue culture, we developed a PCR-based assay that detects the proportions of wildtype and intermediate viruses produced during a 1-week incubation following inoculation with various ratios of input virus. In preliminary studies, we found that this method accurately detected the relative ratios of wildtype CPV-2 and CPV-2b viruses in mixtures containing known amounts of each virus (results not shown). For example, when similar quantities of each virus were amplified by PCR in the same reaction, two nucleotide peaks of comparable amplitude were detected by sequencing for each single nucleotide polymorphism (SNP). Figure 2.5 gives an example of the sequence trace data obtained from this assay and gives a graphical representation of changes in peak height ratios (PHR) over time. In feline cells, CPV-2 clearly replicated better than CPV-2b, resulting in an increase in CPV-2:CPV-2b PHR over time, regardless of whether cells were initially infected with more CPV-2 or more CPV-2b virus. In the case of competitions between wildtype viruses, all 4 codons (87, 101, 300, and 305) can be used for PHR calculations, and analysis of each of those sequence positions gave similar results (data not shown).

In general, most CPV-2-derived intermediate viruses had lower replication fitness than wildtype CPV-2 in feline cells, as demonstrated by a greater than 5-fold increase in CPV-2 compared to intermediate virus PHR after 7.5 days post infection. Changing VP2 residues 87 and 300 simultaneously in CPV-2 resulted in the most dramatic reduction in relative fitness of all CPV-2-derived intermediates, as that resulted in a 48-fold increase in CPV-2:intermediate PHR after 7.5 days incubation. Changing residue 305 alone resulted in minimal loss of fitness, and this intermediate

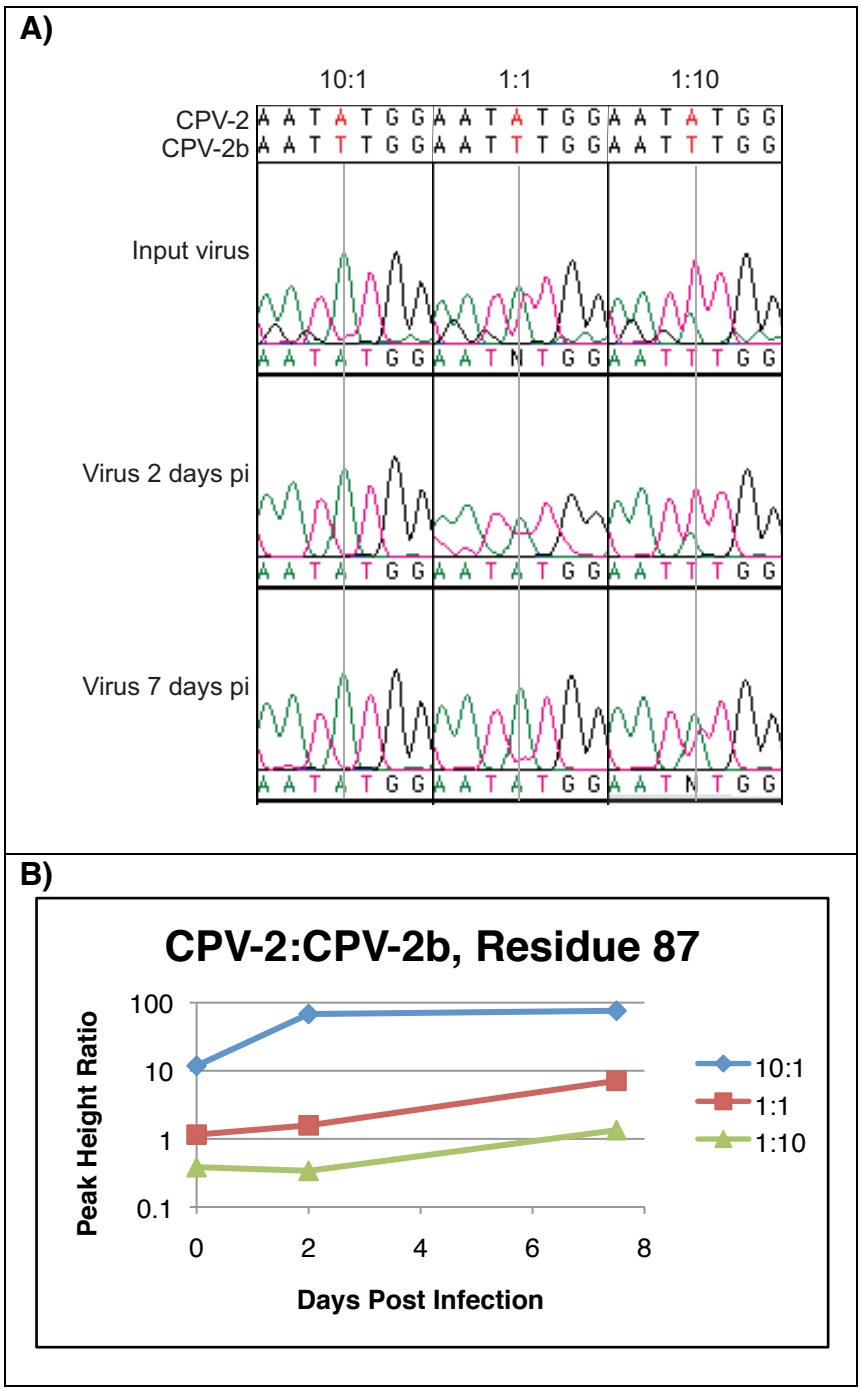


Figure 2.5. *In vitro* competition assay between wildtype CPV-2 and CPV-2b in NLFK cells. A) Sequence trace data for VP2 codon 87 (gray lines) showing an increase in CPV-2 (green peak for adenine) and a decrease in CPV-2b (red peak for thymine) over time at each of the three input ratios of virus. B) Graphical representation of the CPV-2:CPV-2b PHR over time at each of the input ratios. (pi – post-infection)

replicated to similar levels as wildtype CPV-2, as indicated by a fold increase in CPV-2:intermediate PHR of only 1.4 after 7.5 days incubation.

These results are in contrast to the replication of CPV-2b-derived intermediates, which generally showed similar replication fitness to wildtype CPV-2b viruses, as indicated by small fold increases or decreases in CPV-2b:intermediate PHR after 7.5 days post infection.

2.5 DISCUSSION

Emergence of the CPV strains currently circulating in dogs today involved two steps that each required the acquisition of multiple capsid mutations, one step being the change from FPV to CPV-2 and the other from CPV-2 to CPV-2a. In both cases, the group of mutations effected virus antigenicity, TfR binding and host range properties. We have previously shown that combinations of amino acid differences between the FPV and CPV-2 capsid proteins acted together to introduce the ability of CPV-2 to infect dogs and canine cells (25). However, we still know little about how groups of mutations that control host range or other complex biological properties arise and are selected, or how they interact to confer their novel phenotypes on the viruses. Here, we examined various properties of 4 conserved differences between the original and newer CPV strains and found that all four work in concert to convey various viral phenotypes.

Of the three surface-exposed VP2 residues (87, 300 and 305), residue 300 had previously been shown to influence TfR and antibody binding (12, 18), but here we have demonstrated that residues 87 and 305, as well as the buried residue 101, also play important roles in defining ligand-binding properties. Changing residue 101 in the CPV-2 structure from Ile to Thr

appears to introduce a new hydrogen bond with residue 84, which likely influences the structure of the flexible loop that contains residues 84, 87 and 101, including modification of its surface-exposed portion. More advanced modeling is needed to explore the structural alterations created by this residue change, as well as that of the surface residues 87, 300 and 305. Solving the atomic structure of CPV-2b would be particularly informative.

Our mAb-virus binding data confirmed previous findings of mAb specifities and mapped the contributing residues in more detail. The data shows that the CPV-2-specific epitopes, particularly within site B, interact with mAbs in a number of different conformations, since the same combinations of residue changes are recognized differently by mAb E compared with mAbs D and J. This is in line with variation in mAb binding footprints on the capsid surface that have been defined by cryoEM (6). Conversely, the CPV-2b-specific mAbs generally appear to bind in similar conformations, at least within the site B region of the capsid, since minimal variation was seen in the virus binding profiles between mAbs 1D1, 7D6 and 7E2.

CPV-2 is known to bind feline and canine cells to a higher level in flow cytometry assays than does CPV-2b (9), and our data confirmed these findings. The aspartic acid at residue 305 has previously been shown to contribute to the increased binding of CPV-2 to host cells (9), but our results showed that residues 87 and 101 were the primary contributors to increased CPV-2 binding, with minimal contribution from residue 305. There may be differences in binding to the feline and canine cells used in previous studies. We know that in canine Cf2Th cells, CPV-2 binds preferentially to filopodia, whereas in NLFKs, virus binds more uniformly over the cell surface (8). The binding data presented here suggest that there are subtle differences in how

viruses bind to feline and canine cells, with changes at residues 87 and 101 possibly having a greater effect on virus binding to canine cells, although additional studies with various canine cells are needed to confirm this.

Other mutations in the surface loop containing residues 300 and 305 have been shown to affect TfR and mAb binding. Specifically, a change in VP2 residue 300 from Ala to Asp was selected when CPV-2 was grown in feline cells, and this mutant had lost the ability to infect canine cells (12, 18). In addition, CPV-2a-dervied viruses carrying the 300Asp mutation have been isolated from naturally infected cats (10) and raccoons ((11), Allison and Parrish, unpublished data). An antibody-selected escape mutant carrying a Gly to Glu change at VP2 residue 299 also lost the ability to infect canine cells (14, 24). The crystallographic structure of CPV-2 with Asp at residue 300 has been determined, and shows that 300Asp forms a salt bridge with Arg81 in an adjacent VP2 subunit (12). The Gly found at VP2 position 300 in the newer CPV-2a variants likely introduces even more flexibility into its surface loop, which may aid virus binding to the canine TfR.

The four capsid residues examined in this study lie within the binding footprints of the TfR and several mAbs (6, 7), so it was not surprising that changes in these residues altered both cell binding and antigenicity in these studies. Since CPV-2 could not infect cats, selection for the newer CPV-2a viruses would have occurred in infected dogs and may have been influenced in part by vaccine-induced immunity. Since these newer CPV-2a viruses have completely replaced CPV-2 in nature, they presumably are better adapted for replication in dogs, although the specific advantage(s) these newer viruses have during natural infection remains unknown.

In addition to finely mapping specific cell and mAb interactions, we examined the relative fitness of each intermediate virus against its wildtype to begin addressing the question of how evolutionary intermediates arise and are selected. All 11 CPV-2-derived intermediate viruses were viable and infectious, suggesting that there were no strict structural or infectious barriers to the order in which this set of four amino acid mutations was acquired. Competition assays demonstrated that all but one of these 11 intermediates had lower replicative fitness in feline cells than wildtype CPV-2, suggesting that evolutionary intermediates may have been required to pass through a fitness trough during the process of CPV adaptation in dogs. Changing residue 305 alone to the CPV-2b sequence did not reduce virus fitness when compared with wildtype CPV-2, and in general, changing residue 305 alone had the smallest effects in these studies. Testing these 11 intermediates in competition assays against wildtype CPV-2b would offer additional insights into the evolution of CPV in dogs, and these studies are underway.

Three of the 11 CPV-2b-derived viruses were nonviable, suggesting that single mutations at residues 101 or 300 back to the CPV-2 sequence, or the double back mutation of residues 101 and 305, create viruses that fail to replicate. This suggests these residue changes interact epistatically with other regions of the virus genome to give the nonviable phenotype, since the equivalent forward mutations in CPV-2 are viable. Furthermore, relative fitness of the CPV-2b-derived intermediate virus that has all four residues changed back to the CPV-2 sequence remains similar to CPV-2b and fails to return to the higher CPV-2 levels, again suggesting the other difference between the wildtype CPV-2 and CPV-2b genomes play a role in determining replication phenotypes. In addition their differences at VP2 codons 87, 101,

300, and 305, the wildtype CPV-2 and CPV-2b viruses used in these studies also differ at 5 noncoding nucleotides, three nucleotides in the nonstructural ORF (including a nonsynonymous change at NS1 codon 544) and two nucleotides in the capsid ORF that result in nonsynonymous changes at VP2 codons 375 and 426. Additional mutagenesis mapping studies may allow us to determine which of these specific differences is responsible for the reduced replication of CPV-2b in feline cells.

Preliminary studies in various canine cells lines have shown that at 2 days post infection, virus titers are too low to be reliably detected by the PCR-based *in vitro* competition assay. This draws a parallel with previous findings from immunofluorescence microscopy that showed fewer CPV-infected cells in various canine cultures compared with feline cultures that were similarly inoculated (results not shown). Studies are on-going to determine if this assay can be used successfully at later time points in various canine cells lines. In addition, competition assays in primary feline and canine lymphoctyes would allow relative fitness of wildtype and intermediate viruses to be characterized in a more clinically relevant tissue type, as lymphocytes are the primary target of CPV in infected animals. Such cells may more closely mirror the CPV host range properties seen in nature.

2.6 ACKNOWLEDGEMENTS

We would like to thank Dr. Kai-Biu Shiu for his initial help with intermediate virus cloning. We also greatly appreciate the invaluable technical support provided by Ms. Virginia Scarpino and Ms. Wendy Weichert. These studies were supported by National Institutes of Health (NIH) grants AI28385

to CRP and GM080533 to ECH. KMS was supported by NIH training grant RR007059 to Dr. Douglas D. McGregor.

REFERENCES

1. Agbandje, M., R. McKenna, M. G. Rossmann, M. L. Strassheim, and C. R. Parrish. 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16:155-71.

2. Buonavoglia, C., V. Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, and L. Carmichael. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J Gen Virol 82:3021-5.

3. Carrillo-Tripp, M., C. M. Shepherd, I. A. Borelli, S. Venkataraman, G. Lander, P. Natarajan, J. E. Johnson, C. L. Brooks, 3rd, and V. S. Reddy. 2009. VIPERdb2: an enhanced and web API enabled relational database for structural virology. Nucleic Acids Res 37:D436-42.

4. Dykes, C., and L. M. Demeter. 2007. Clinical significance of human immunodeficiency virus type 1 replication fitness. Clin Microbiol Rev 20:550-78.

5. Emsley, P., B. Lohkamp, W. G. Scott, and K. Cowtan. Features and development of Coot. Acta Crystallogr D Biol Crystallogr 66:486-501.

6. Hafenstein, S., V. D. Bowman, T. Sun, C. D. Nelson, L. M. Palermo, P. R. Chipman, A. J. Battisti, C. R. Parrish, and M. G. Rossmann. 2009. Structural comparison of different antibodies interacting with parvovirus capsids. J Virol 83:5556-66.

7. Hafenstein, S., L. M. Palermo, V. A. Kostyuchenko, C. Xiao, M. C. Morais, C. D. Nelson, V. D. Bowman, A. J. Battisti, P. R. Chipman, C. R. Parrish, and M. G. Rossmann. 2007. Asymmetric binding of transferrin receptor to parvovirus capsids. Proc Natl Acad Sci U S A 104:6585-9.

8. Harbison, C. E., S. M. Lyi, W. S. Weichert, and C. R. Parrish. 2009. Early steps in cell infection by parvoviruses: host-specific differences in cell receptor binding but similar endosomal trafficking. J Virol 83:10504-14.

9. Hueffer, K., J. S. Parker, W. S. Weichert, R. E. Geisel, J. Y. Sgro, and C. R. Parrish. 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. 77:1718-1726.

10. Ikeda, Y., M. Mochizuki, R. Naito, K. Nakamura, T. Miyazawa, T. Mikami, and E. Takahashi. 2000. Predominance of canine parvovirus (CPV) in unvaccinated cat populations and emergence of new antigenic types of CPVs in cats. Virology 278:13-9.

11. Kapil, S., G. Rezabek, B. Germany, and L. Johnston. Isolation of a virus related to canine parvovirus type 2 from a raccoon (Procyon lotor). Vet Rec 166:24-5.

12. Llamas-Saiz, A. L., M. Agbandje-McKenna, J. S. Parker, A. T. Wahid, C. R. Parrish, and M. G. Rossmann. 1996. Structural analysis of a mutation in canine parvovirus which controls antigenicity and host range. Virology 225:65-71.

13. Nelson, C. D., E. Minkkinen, M. Bergkvist, K. Hoelzer, M. Fisher, B. Bothner, and C. R. Parrish. 2008. Detecting small changes and additional peptides in the canine parvovirus capsid structure. J Virol 82:10397-407.

14. Parker, J. S., and C. R. Parrish. 1997. Canine parvovirus host range is determined by the specific conformation of an additional region of the capsid. J Virol 71:9214-22.

15. Parrish, C. R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology 183:195-205.

16. Parrish, C. R., C. F. Aquadro, and L. E. Carmichael. 1988. Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink and raccoon parvoviruses. Virology 166:293-307.

17. Parrish, C. R., and L. E. Carmichael. 1983. Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. Virology 129:401-14.

18. Parrish, C. R., and L. E. Carmichael. 1986. Characterization and recombination mapping of an antigenic and host range mutation of canine parvovirus. Virology 148:121-32.

19. Parrish, C. R., Carmichael, L.E., Antczak, D.F. 1982. Antigenic relationships between canine parvovirus type-2, feline panleukopenia virus and mink enteritis virus using conventional antisera and monoclonal antibodies. Arch.Virol. 72: 267-278.

20. Parrish, C. R., P. H. O'Connell, J. F. Evermann, and L. E. Carmichael. 1985. Natural variation of canine parvovirus. Science 230:1046-1048.

21. Rossmann, M. G., and A. C. Palmenberg. 1988. Conservation of the putative receptor attachment site in picornaviruses. Virology 164:373-82.

22. Shackelton, L. A., C. R. Parrish, U. Truyen, and E. C. Holmes. 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci U S A 102:379-84.

23. Simpson, A. A., V. Chandrasekar, B. Hebert, G. M. Sullivan, M. G. Rossmann, and C. R. Parrish. 2000. Host range and variability of calcium binding by surface loops in the capsids of canine and feline parvoviruses. J Mol Biol 300:597-610.

24. Strassheim, M. L., A. Gruenberg, P. Veijalainen, J. Y. Sgro, and C. R. Parrish. 1994. Two dominant neutralizing antigenic determinants of canine parvovirus are found on the threefold spike of the virus capsid. Virology 198:175-84.

25. Truyen, U., J. F. Evermann, E. Vieler, and C. R. Parrish. 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology 215:186-189.

26. Truyen, U., and C. R. Parrish. 1992. Canine and feline host ranges of canine parvovirus and feline panleukopenia virus: distinct host cell tropisms of each virus in vitro and in vivo. J Virol 66:5399-5408.

27. Tsao, J., M. S. Chapman, M. Agbandje, W. Keller, K. Smith, H. Wu, M. Luo, T. J. Smith, M. G. Rossmann, R. W. Compans, and et al. 1991. The three-dimensional structure of canine parvovirus and its functional implications. Science 251:1456-64.

28. Wright, S. 1931. Evolution in Mendelian Populations. Genetics 16:97-159.

29. Wright, S. 1932. General, Group and Special Size Factors. Genetics 17:603-19.

30. Xie, Q., and M. S. Chapman. 1996. Canine parvovirus capsid structure, analyzed at 2.9 A resolution. J Mol Biol 264:497-520.

31. Yeung, D. E., G. W. Brown, P. Tam, R. H. Russnak, G. Wilson, I. Clark-Lewis, and C. R. Astell. 1991. Monoclonal antibodies to the major nonstructural nuclear protein of minute virus of mice. Virology 181:35-45.

CHAPTER 3

PARVOVIRAL ENTERITIS: EFFECTS OF VIRUS STRAIN VARIATION ON DIAGNOSTIC TESTING AND CLINICAL MANAGEMENT*

* Modified from manuscripts being prepared with co-authors Jessica E. Markovich, Alaina Carr, Carole E. Harbison, Janet M. Scarlett, and Colin R. Parrish.

3.1 ABSTRACT

Objective – To estimate the prevalence of canine parvovirus (CPV) strains among dogs presenting to a referral hospital in the southwestern United States over a one-year period, and to determine if specific variation in capsid gene sequences is associated with different diagnostic test results, disease severity, or patient outcome.

Design – Prospective pilot study.

Sample Population – 72 dogs presenting to a single referral hospital in Mesa, Arizona, with clinical signs and history indicative of parvoviral enteritis, regardless of in-clinic parvovirus test results.

Procedures – Fecal samples or rectal swabs were tested for parvovirus with both commercial CPV in-clinic ELISA tests and CPV-specific PCR. Additionally, a subset of patients had their pharynx swabbed and similarly tested for parvovirus.

Results – Of the 42 fecal samples tested, 27 were positive for CPV by ELISA and PCR, while 6 were positive by PCR only. Of these 33 CPV-positive samples, 72.7% were CPV-type-2c-like and 27.2% were CPV-type-2b-like. Pharyngeal swabs were collected in 16 of the CPV-positive dogs, and 10 were CPV positive by PCR. No association was found between CPV strain and disease severity or clinical outcome, although the sample sizes were small.

Conclusions and Clinical Relevance – These findings suggest that CPV-2b and CPV-2c pose similar risks for dogs, making extensive diagnostic testing to identify CPV strain unnecessary at this time. Current diagnostic tests can detect the CPV-2c variant. Following national vaccination guidelines, proper disinfection protocols and standard clinical treatment procedures remain the mainstay for parvovirus prevention and disease management.

3.2 INTRODUCTION

Canine parvovirus (CPV) causes an acute disease in dogs, with signs ranging from subclinical infection to severe hemorrhagic enteritis, leukopenia, and possible death. The virus most commonly spreads via fecal-oral transmission. Following ingestion, CPV undergoes primary replication in oropharyngeal lymphoid tissues, such as the tonsils and retropharyngeal lymph nodes (15). From there, virus spreads hematogenously to the thymus and other lymphoid tissues, and eventually to the rapidly proliferating cells in the crypts of the gastrointestinal epithelium. This leads to sloughing of the intestinal epithelium and shedding of virus in the feces within 4 days of exposure (15). In most cases, virus shedding in the feces ceases within two weeks after the start of infection (15).

While severity of disease varies among individuals, the primary clinical sign is hemorrhagic diarrhea and complete blood cell counts reveal a severe leukopenia. Therefore, clinical management of sick individuals relies on supportive care to replenish fluid losses and antimicrobial therapy to prevent (or treat) septicemia. Vaccination with modified live vaccines is highly effective at preventing virus infection and disease in immunocompetent individuals and provides long-lasting immunity (reviewed in (23)). However, puppies may become ill if exposed to CPV during a window of susceptibility created as maternal antibodies wane to low levels, but before vaccine viruses can infect and generate protective immunity. This window of susceptibility varies among individuals, but in most cases occurs during the first 4 months of age, which reflects the current recommendations for the timing of puppy vaccine series (19, 20). Puppies housed with other dogs (e.g., in shelters, kennels) or exposed to areas with high canine traffic (e.g., community runs,

dog parks, shows, veterinary practices) during this window of susceptibility are particularly at risk for infection and development of parvoviral enteritis.

CPV first emerged in the canine population in the late 1970s, and was named CPV type-2 (CPV-2) to distinguish it from a different parvovirus of dogs, the minute virus of canines. CPV-2 quickly achieved a worldwide distribution, but was replaced globally in the early 1980s by an antigenically and genetically distinct strain, CPV type-2a (CPV-2a) (16). Since then, novel antigenic and genetic variants have continued to arise, often carrying mutations in the overlapping capsid genes, VP1 and VP2. Two such variants are defined by amino acid changes of VP2 residue 426, and those have been named CPV-2b and CPV-2c (1, 17). CPV-2 and CPV-2a, as well as feline panleukopenia virus (FPV), have an asparagine (Asn) at position 426, whereas CPV-2b has an aspartic acid (Asp) and CPV-2c has a glutamic acid (Glu) at this position. While these amino acid changes alter capsid antigenicity as defined by monoclonal antibody binding, they have only minor effects on recognition by polyclonal antibodies (15). Additional changes in other capsid amino acids have also arisen, creating multiple variants similar to CPV-2a, CPV-2b, and CPV-2c (6). Most of these remain unnamed and their functional consequences, if any, are not well characterized.

The significance of CPV genetic and antigenic variation for clinical disease and management remains uncertain. For example, there has been concern that alteration of capsid epitopes could reduce the effectiveness of vaccine-induced immunity or result in failures of rapid in-clinic CPV tests that rely on antibody binding. While CPV-2b (VP2 residue 426 Asp) has not been associated with vaccine failures or ineffective diagnostics since its recognition in 1984, some reports have suggested that CPV-2c (VP2 residue 426 Glu)

causes more severe disease, can infect properly vaccinated individuals, is not detected by in-clinic diagnostic tests, and results in worse patient outcomes (4, 9, 21). However, experimental studies have shown that dogs vaccinated with standard CPV-2-based vaccines are protected when challenged with CPV-2c (25), and earlier reports of natural infection suggested CPV-2c caused less severe signs (3).

Traditionally, diagnostic laboratory studies of CPV variants rely on sample submissions from a broad geographical area with limited clinical data for each patient. Conversely, hospital-based CPV studies often have extensive clinical data but rarely include sequence information. This work represents the first prospective study to combine CPV sequence analysis with an assessment of clinical presentation and outcome in a sample of dogs seen by an emergency and referral hospital. No CPV-2a variants were detected in this study, so we compared the effects on disease severity and outcome of CPV-2b-like and CPV-2c-like variants, as defined by the presence of Asp or Glu at VP2 amino acid 426, respectively. A separate phylogenetic analysis will be performed elsewhere to assess the clinical effects of all capsid protein variation observed among these samples (Stucker *et al.*, in preparation). This study also assessed the sensitivity of a commercial in-clinic CPV diagnostic test for detecting CPV-2b and CPV-2c, and compared outcomes based on at-home care versus hospitalization.

3.3 MATERIALS AND METHODS

Study design and data collection. The study population included patients presenting to VCA Animal Referral and Emergency Center of Arizona (ARECA) in Mesa, Arizona, with clinical signs (anorexia, vomiting, diarrhea)

and history (signalment, improper or unknown immunization schedule, known or high-risk exposure) indicative of CPV infection. Between August 2008 and June 2009, subjects were enrolled in this study as time and schedule constraints of the hospital allowed, creating a convenience sample representing a subset of the total potential parvovirus cases presented to ARECA during this time period. Complete histories were collected for all subjects and recorded in a standardized history form. Owners signed an informed consent form for inclusion of their pet in the study, and all subjects received a full physical examination. An in-clinic CPV antigen test kit (CPV SNAP, IDEXX Laboratories, Inc., Westbrook, ME) using ELISA technology was performed on a fecal swab for all suspect cases following standard hospital protocol. When possible, a fecal sample was collected within 24 hours of presentation and saved for subsequent CPV ELISA and PCR testing and sequencing. For a subset of subjects, a pharyngeal swab was also collected and stored in 1-2 ml sterile saline for CPV testing by ELISA and PCR. All fecal and pharyngeal samples were stored at 4°C prior to analysis.

Treatment options (at-home care versus hospitalization) were presented to owners and carried out according to client preference and financial situations. Depending on the care options chosen by the owner, various diagnostics and treatments were performed and recorded for this study, including blood glucose and albumin monitoring, complete blood cell counts, fecal parasite testing and administration of fresh frozen plasma. Patient outcomes were recorded when known, but some subjects receiving at-home care were lost to follow-up. This study complied with Cornell University's Institutional Animal Care and Use Committee and Institutional Review Board research guidelines.

Clinical severity scoring. Retrospective clinical severity scores were independently assigned by the study's two lead investigators based on history and clinical exam findings at initial presentation, as recorded by the admitting clinician in the medical record. A five-point scale was used, with one being least severe and five being most critical. Prior to CPV type determination, scores were assigned based on number of days sick, mentation, ambulatory ability, temperature, hydration status, and glycemic index.

PCR and sequencing. Fecal samples were diluted approximately 1:500 in sterile water before being added to a conventional PCR reaction using Phusion high fidelity, hot start DNA polymerase and standard Phusion polymerase HF buffer (Finnzymes, Woburn, MA). For pharyngeal swab samples, their saline diluent was added directly to conventional PCR reactions. To test for the presence or absence of CPV, forward primer 1F and reverse primer 1R were used (Table 3.1). To examine the 3'-proximal region of the VP2 gene that includes codon 426, CPV-positive samples were also amplified with forward primer 1F and reverse primer 2R (Table 3.1).

PCR reactions underwent 30 amplification cycles (10 seconds at 98°C, 30 seconds at 54°C, 2 minutes and 15 seconds at 72°C) with a final 10-minute extension (72°C), and products were analyzed by gel electrophoresis to

Table 3.1. CPV-specific PCR and sequencing primers.

Name	Sequence
Primer 1F	5'-GAAAACGGATGGGTGGAAATCACAGC-3'
Primer 1R	5'-TATTTTGAATCCAATCTCCTTCTGG-3'
Primer 2R	5'-CTAAGGGCAAACCAACCAACCAC-3'
Primer 2F	5'-AGATAGTAATAATACTATGCCATTT-3'
Primer 3F	5'-ACAGGAGAAACACCTGAGAGATTTA-3'

identify CPV-positive samples. Negative samples were retested by PCR, using a range of sample dilutions from 1:10 to 1:1600 before being deemed truly negative. For positive samples, the PCR product was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

Purified products were sequenced at Cornell University's Core Laboratories Center using big dye terminator chemistry and AmpliTaq-FS DNA polymerase on an automated 3730 Sanger sequence analyzer (Applied Biosystems, Foster City, CA). The above three primers were used in conjunction with forward primers 2F and 3F to obtain 2X sequence coverage (Table 3.1). Sequence contigs were aligned with prototype CPV-2 and CPV-2b genome sequences to generate a consensus sequence for each virus isolate. Identification of the sequence of VP2 codon 426 allowed typing of each isolate as CPV-2b-like or CPV-2c-like.

Since initial CPV SNAP tests may have been performed from a rectal swab or a separate fecal sample from that saved for PCR testing, all PCR-tested fecal samples were retested by CPV SNAP to ensure identical samples were tested for sensitivity and specificity calculations.

Statistical analysis. Most comparisons were made between the two CPV variant groups (CPV-2b and CPV-2c) for categorical variables (e.g., hydration status, prognosis, disease signs) using the Fisher's's exact test because of the small sample sizes. Otherwise, if the samples were large enough, the chi-square test of independence was used. For comparisons of continuous variables (e.g., clinical severity score, hospitalization time) between the two CPV variant groups, the Wilcoxon rank sum test was used. Nonparametric descriptive analyses and association testing were performed using Statistix v8.0 software (Analytical Software, Tallaassee, FL).

3.4 RESULTS

Case summaries. Seventy-two patients were enrolled in this study between August 2008 and June 2009 based on history and clinical signs, and fifty-six of these cases were positive for CPV by IDEXX SNAP and/or PCR testing. Of the 56 confirmed CPV cases, half were females (28, 50.0%) and half were males (28, 50.0%). Only 5 animals (3 females and 2 males, 10.0%) were neutered. Twenty-five patients (44.6%) were 4 months of age or younger on the day of presentation, and thus still within the window of susceptibility when they are potentially vulnerable to CPV even if they have been inoculated with a modified live vaccine (22). Twenty-three patients (41.1%) were at least 5 months old but less than 12 months, and eight (14.3%) were 12 months of age or older. The most commonly seen breeds were Chihuahuas (11, 19.6%) and pitbull-like breeds (8, 14.3%), with the remaining individuals representing various purebreds and mixed breeds.

Thirty-six owners (64.3%) of confirmed CPV-positive patients reported their pet had received at least one parvovirus vaccination, although vaccine histories from veterinary medical records were not available to confirm the number and date of vaccinations, or the vaccine manufacturer. In addition, owners in this area commonly administer their own vaccines, so it cannot be assumed that vaccines were stored and given appropriately. Of the 53 CPV-positive cases for which there were data, only 15 patients (28.3%) had previously been seen by a referring veterinarian for the presenting complaint in this study. Fifty-one owners of CPV-positive patients provided travel histories for their pets, and only one of these had been outside Arizona in the 2 months preceding their presentation for parvoviral enteritis.

Presenting complaints of CPV-positive cases included diarrhea (43, 76.8%), vomiting (51, 91.1%), lethargy (53, 94.6%), and inappetence (48, 88.9%). Physical exam findings also included ptyalism (22, 39.3%), abdominal pain (31, 55.4%), and dehydration (43, 78.2%). Blood glucose levels were tested for 22 patients, and 2 (9.1%) were severely hypoglycemic (< 50 mg/dL) on presentation. Albumin was tested for 21 patients, and 5 (23.8%) had marked hypoalbuminemia (<2.0 g/dL) on presentation. Fecal parasites were found in 2 of the 12 patients tested (16.7%), and included hookworms and Giardia. Complete blood cell counts were performed for 19 of the patients on presentation, and 10 (52.6%) had leukopenia.

Two owners each brought in two sick patients, and one brought in three; all 7 of these subjects were enrolled in this study and were CPV-positive. Twenty-three CPV-positive patients (41.1%) were hospitalized with a median hospital time of 4 days. Six hospitalized patients received fresh frozen plasma. Owners elected at-home care for 29 of the CPV-positive patients (51.8%), and four patients were either euthanized on presentation or were dead on arrival.

The outcome for 42 (75.0%) of the 56 CPV-positive cases was recorded, while the remaining 14 subjects were lost to follow-up. Thirty patients recovered (53.6%), eight were euthanized (14.3%), and four (7.1%) were reported by their owners to have died at home. Of those euthanized, half were due to deteriorating condition in the face of maximum supportive treatment, and half were due to financial constraints of the owner.

CPV variants detected. Forty-two fecal samples were collected for PCR testing and sequencing. Of these, 33 (78.6%) were positive for CPV by PCR. Sequencing showed that 9 samples (27.3%) encoded for Asp at VP2

codon 426, making them CPV-2b-like, while 24 samples (72.7%) had Glu at that codon, making them CPV-2c-like. Additional single nucleotide polymorphisms (SNPs) were found in the capsid genes of these samples and will be reported elsewhere (Stucker *et al.*, in preparation).

Sensitivity and specificity of diagnostic testing. To calculate the sensitivity and specificity of the IDEXX CPV SNAP test, SNAP results were compared with CPV-specific PCR amplification for all 42 fecal samples (Table 3.2). The overall sensitivity and specificity of the IDEXX CPV SNAP test in this study were 81.8% (95% CI: 36%-94%) and 100% (95% CI: 35%-100%), respectively. The sensitivity of the SNAP test was 100% (95% CI: 35%-100%) for the CPV-2b-like samples (n=9), and 75% (95% CI: 47%-90%) for the CPV-2c-like samples (n=24). A Fisher's's exact test showed no significant difference in the SNAP test's sensitivity for CPV-2b and CPV-2c ($p = 0.16$).

Table 3.2. CPV testing results on 42 fecal samples using IDEXX SNAP tests and PCR amplification.

	PCR Positive	PCR Negative	Total
SNAP Positive	27	0	27
SNAP Negative	6	9	15
Total	33	9	42

Pharyngeal swab testing for CPV. To determine whether CPV can be detected from the oropharynx of infected dogs at presentation, pharyngeal swabs were collected from 16 study subjects, each of which had a CPV-positive fecal sample. Of these 16 samples, 10 (62.5%) were positive for CPV by PCR and matched the CPV type obtained from the fecal sample by sequencing. None of the pharyngeal swabs were CPV-positive by IDEXX

SNAP. One patient had only a pharyngeal swab collected for CPV sequencing, with no fecal sample. This sample was identified as CPV-2c-like, bringing the total CPV-2c cases to 25, and the total typed cases to 34.

CPV variants and age. It has been suggested that CPV-2c causes disease in adult animals more frequently than do CPV-2a and CPV-2b (4). These data do not support this hypothesis, although the majority of our study population consisted of younger animals, as is conventional for CPV infections. The median patient age was 5 months (range 2 to 12 months) for CPV-2b and 4 months (range 2 to 36 months) for CPV-2c. A two-tailed Fisher's exact test showed there was no significant difference in CPV-2b and CPV-2c infections in patients older than 4 months compared with those 4 months or younger (p-value = 0.70).

CPV variants and vaccination history. Because patient vaccine histories varied and could not be confirmed, and since many subjects were unlikely to be vaccinated, this study did not address the question of whether CPV-2c infections are seen more frequently than CPV-2b infections in previously vaccinated individuals. However, it is worth noting that a two-tailed Fisher's exact test showed no significant difference in the proportion of CPV-2b cases (6/9, 66.7%) compared with the proportion of CPV-2c cases (13/24, 54.2%) that had received at least one parvovirus vaccination (p = 0.70).

CPV variants and disease severity. To address the concern that CPV-2c may cause more severe disease than CPV-2b, several parameters were examined: hydration status on presentation, clinical severity score, leukocyte count on presentation, and time in the hospital. Overall, our results suggest that CPV-2c does not cause more severe disease than CPV-2b.

All 9 CPV-2b cases were dehydrated, while 16 of 24 CPV-2c cases were dehydrated. Twenty-one patients had an estimated percentage dehydration recorded in their medical record on presentation. A two-tailed Fisher's exact test revealed no significant difference between CPV-2b (3/7, 42.9%) and CPV-2c (3/14, 21.4%) cases that were 8% or more dehydrated versus those that were less than 8% dehydrated ($p = 0.30$).

Clinical severity scores retrospectively assigned independently by both lead authors gave a high Spearman rank correlation coefficient of 0.78, suggesting that cases were scored similarly. One set of scores had a median severity score of 3 (range 2 to 5) for CPV-2b cases and a median score of 2 (range 2 to 5) for CPV-2c cases, while the second set of scores had medians of 3 (range 2 to 4) and 3 (range 1 to 5), respectively. Wilcoxon rank sum tests comparing the median clinical severity scores between CPV-2b and CPV-2c cases showed no significant difference with either of the two sets of scores ($p > 0.41$).

The median leukocyte count on admission was 5.0 K/μl and 5.2 K/μl for CPV-2b and CPV-2c patients, respectively, and a Wilcoxon rank sum test showed no significant difference between these medians ($p = 0.88$). For hospitalized patients, the median hospital time was 4 days (range 3 to 9 days) for CPV-2b and 5 days (range 3 to 8) for CPV-2c. An exact permutation test similarly showed no significant difference in medians ($p = 0.74$).

CPV variants and clinical outcome. A similar analysis was performed to determine if patients infected with CPV-2c had a different prognosis than those infected with CPV-2b. Prognosis was defined by clinical outcome (recovery versus death or euthanasia). Of the 30 PCR-typed cases with known outcomes, 4 of 8 CPV-2b patients (50.0%) recovered, and 17 of 22

CPV-2c patients (77.2%) recovered. A two-tailed Fisher's exact test indicated there was no significant difference in the number of recovered CPV-2b versus CPV-2c patients ($p = 0.20$).

Prognostic indicators for clinical outcome. Previous reports have suggested various prognostic indicators for parvoviral enteritis patients, including leukopenia, fecal parasites and early enteral nutrition (5, 13). We evaluated multiple parameters in our study for their use as prognostic indicators by comparing them to patient outcome (recovery versus death or euthanasia). Patients euthanized due to financial constraints of the owner were excluded from these analyses.

Wilcoxon rank sum tests showed no difference in outcome based on patient age in months ($p = 0.53$), leukocyte levels on presentation ($p = 0.43$), albumin on admission ($p = 0.53$), or glucose on admission ($p = 0.36$). A chi-square test of independence showed no difference in outcome based on breed ($p = 0.46$), while two-tailed Fisher's exact tests showed no difference in outcome based on patient sex ($p = 0.72$), owner-reported exposure to parvovirus ($p = 0.70$), administration of fresh frozen plasma ($p = 0.58$), or the presence on presentation of abdominal pain ($p = 0.47$), diarrhea ($p = 0.33$), vomiting ($p = 1.00$), lethargy ($p = 0.57$), inappetence ($p = 1.00$), or ptyalism ($p = 1.00$).

Patient dehydration on presentation was a negative prognostic indicator in this study. Of the 46 cases with known outcomes, all 16 patients that were fully hydrated on presentation survived, whereas only 21 of 30 of the dehydrated patients survived. A two-tailed Fisher's exact test demonstrated a statistically significant difference in outcome based on hydration status at presentation ($p = 0.02$).

Hospitalization versus at-home care. To determine if at-home management of CPV cases resulted in a poorer prognosis than did hospitalization, all 46 cases with known outcomes were compared. Nineteen of 24 hospitalized patients (79.2%) recovered, while 18 of 22 patients (81.1%) receiving at-home care recovered. A two-tailed Fisher's exact test showed no significant difference between the outcomes of the two treatment options ($p = 1.00$). This remained true when clinical severity scores were used to control for differences in disease severity at presentation between hospitalized and at-home care patients.

3.5 DISCUSSION

The acquisition of mutations by viruses can result in the emergence of viral variants with novel phenotypes, such as antigenic variation leading to immune escape, or alteration of host ranges, virulence, or efficiency of transmission. Since its emergence over 30 years ago, CPV has acquired a number of mutations throughout its genome and some of these changes alter specific viral properties. Phylogenetic and functional analyses have shown that the most dramatic change occurred in the late 1970s and early 1980s, when the original variant, CPV-2, was globally replaced by newer variants – the CPV-2a-like viruses – that differed from CPV-2 in 4 to 5 capsid residues (24). This change was associated with altered virus phenotypes for the CPV-2a viruses, including an expanded host range that allowed replication in cats, reduced affinity for host-cell receptor binding, and altered antigenicity (8, 18, 26). Although CPV-2a- and CPV-2b-based vaccines have been developed and are effective (10), CPV-2-derived vaccines have continued to provide adequate protection against these newer CPV variants (25).

Sequences of the newer (post-1980) CPV variants show that they are all derived from a single CPV-2a-like common ancestor, forming a monophyletic clade (24). Originally, new variants were identified by altered viral phenotypes, specifically changes in monoclonal antibody binding profiles, although today, variants are also readily defined at the genotypic level through DNA sequencing. Many of the CPV-2a-derived variants circulating today that have one to several additional changes in the capsid protein, including the named CPV-2b and CPV-2c variants which have unique substitutions at VP2 position 426. Some of these amino acid changes, including that of CPV-2c, have arisen independently in at least two different virus lineages (Pagan, unpublished data), so that not all 426-Glu-encoding CPV-2c viruses are identical and many isolates differ in other VP2 residues. Furthermore, while the capsid gene has been most closely studied due to its importance in receptor and antibody binding, mutations in the nonstructural genes also exist and could contribute to altered viral phenotypes. How various mutations within and between genes interact to alter virus properties, particularly in terms of clinical presentation and outcome, remains poorly understood.

Recognition of the CPV-2c variant in 2001 prompted concern that it may have had gained new properties that increased the severity of disease in patients, enabled it to infect properly vaccinated animals, and also allowed it to evade detection by standard in-clinic diagnostic tests that relied on antibody binding of viral antigens (4, 9, 21). CPV-2c-like viruses have achieved rapid global dissemination (2, 14, 21), similar to other variants in the past, such as CPV-2a, CPV-2b, and an unnamed variant with a serine to alanine change at VP2 amino acid 297 (11). This rapid dissemination suggests that genomes containing the 426 Glu mutation are under positive selection and that 426 Glu

provides an advantage for the virus. However, such selection does not necessarily equate with a more severe disease phenotype or the ability to infect properly vaccinated dogs, and other properties that could also result in selection include faster replication, greater shedding, more efficient transmission, or even less severe disease.

This pilot study is the first time that both clinical information and sequence data were collected from multiple parvoviral enteritis cases from an emergency and referral hospital over an extended period of time. The Arizona location was chosen, in part, because CPV-2c isolates had been previously identified in that region of the country (7). Comparing CPV-2b and CPV-2c cases from the same geographical region that were treated by the same hospital likely reduces bias from confounding factors such as population demographics and variation in hospital protocols and record keeping. However, biases may have been introduced because of several study design features including enrollment of only emergency and referral patients, enrollment of subjects by different clinicians, financial constraints of owners, and loss to follow-up for some at-home care patients. However, since the variant status of dogs was unknown during presentation and treatment, most of these potential sources of bias should affect CPV-2b and CPV-2c patients similarly. If parvovirus cases seen by general practitioners are generally less severe, it is possible that the proportion of CPV variants would differ from that seen by an emergency and referral hospital, such as the one in this study. Relatively small sample sizes limited our analyses and inferences, although none of these analyses suggested that CPV-2c cases were more severe or had worse outcomes than CPV-2b cases.

The overall sensitivity and specificity of a commonly used commercial CPV-detection kit (IDEXX CPV SNAP) was determined using CPV-specific PCR amplification as the gold standard. The SNAP test had an overall high specificity, as well as a high sensitivity for CPV-2b, as reported by the company (IDEXX Laboratories, Inc.). In addition, the SNAP test successfully detected CPV-2c variants, although possibly at a lower sensitivity. No significant difference in the sensitivity of the SNAP test by CPV variant was detected, but the samples sizes in each variant group were small and the confidence intervals were very broad. Clearly, any patient suspected as having parvoviral enteritis based on history and clinical signs should be treated as CPV-positive regardless of in-clinic test results. Because CPV is environmentally stable and highly contagious for naïve individuals, suspected CPV cases should be treated in isolation facilities, and any receiving areas or equipment having contact with these patients must be thoroughly disinfected.

The initial site for CPV replication is in the lymphoid tissue of the oropharynx, and virus has been detected in the tongues and tonsils of infected dogs (12). Detection of CPV infection in oropharyngeal tissue swabs may represent an alternative method for CPV diagnosis that can be used before overt clinical signs develop and virus is shed in the feces. This may be useful during intake exams for kennel populations by identifying CPV-positive animals before they show overt signs of illness, allowing for immediate isolation and reducing the risk of CPV spread. We assessed whether virus could be detected from the oropharynx of CPV-positive patients and found that CPV was detected by PCR in 10 of 16 pharyngeal swabs, but was not detected by the IDEXX SNAP test on the same samples. Storing the swabs in saline likely diluted the virus sample below the threshold of detection for the

SNAP test. Whether direct, undiluted pharyngeal swabs would reveal virus by the SNAP test at an earlier or more consistent time point than fecal swabs is unknown, but is worthy of further investigation.

In conclusion, the currently circulating CPV strains examined in this study do not appear to differ in their clinical presentation, disease severity, or outcome, suggesting that more extensive testing to identify CPV strain is unnecessary for the treatment and management of parvoviral enteritis cases. However, it is important for diagnostic laboratories and researchers to continue monitoring CPV variants and characterizing any that might cause alterations in clinical outcomes, diagnostic testing, or vaccine protection, using larger sample sizes. In this study, full compliance with recommended protocols was infrequently seen, emphasizing the importance of prevention through the timely administration of appropriate modified live parvovirus vaccines (reviewed in (20)). However, even when puppies are receiving the recommended vaccination schedule, there may still be a period when they are susceptible to CPV and this window of susceptibility varies among individuals. Therefore, it is important for puppies (generally 16 weeks of age or younger) and their owners to avoid high-risk areas where the virus is likely to be present. When animals do get parvoviral enteritis, prompt supportive care remains the mainstay for treatment, while thorough cleaning and disinfection of the patient's environment are crucial for preventing spread of the disease.

3.6 ACKNOWLEDGEMENTS

We thank Ms. Virginia Scarpino for her outstanding technical support. Funding was provided by VCA-Antech, Inc. KMS was supported by NIH training grant RR007059 to Dr. Douglas D. McGregor.

REFERENCES

1. Buonavoglia, C., V. Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, and L. Carmichael. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J Gen Virol 82:3021-5.

2. Decaro, N., C. Desario, D. D. Addie, V. Martella, M. J. Vieira, G. Elia, A. Zicola, C. Davis, G. Thompson, E. Thiry, U. Truyen, and C. Buonavoglia. 2007. Molecular epidemiology of canine parvovirus, Europe. Emerg Infect Dis 13:1222-4.

3. Decaro, N., C. Desario, M. Campolo, G. Elia, V. Martella, D. Ricci, E. Lorusso, and C. Buonavoglia. 2005. Clinical and virological findings in pups naturally infected by canine parvovirus type 2 Glu-426 mutant. J Vet Diagn Invest 17:133-8.

4. Decaro, N., C. Desario, G. Elia, V. Martella, V. Mari, A. Lavazza, M. Nardi, and C. Buonavoglia. 2008. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. New Microbiol 31:125-30.

5. Goddard, A., A. L. Leisewitz, M. M. Christopher, N. M. Duncan, and P. J. Becker. 2008. Prognostic usefulness of blood leukocyte changes in canine parvoviral enteritis. J Vet Intern Med 22:309-16.

6. Hoelzer, K., L. A. Shackelton, C. R. Parrish, and E. C. Holmes. 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. J Gen Virol 89:2280-9.

7. Hong, C., N. Decaro, C. Desario, P. Tanner, M. C. Pardo, S. Sanchez, C. Buonavoglia, and J. T. Saliki. 2007. Occurrence of canine parvovirus type 2c in the United States. J Vet Diagn Invest 19:535-9.

8. Hueffer, K., J. S. Parker, W. S. Weichert, R. E. Geisel, J. Y. Sgro, and C. R. Parrish. 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. 77:1718-1726.

9. Lamm, C. G., and G. B. Rezabek. 2008. Parvovirus infection in domestic companion animals. Vet Clin North Am Small Anim Pract 38:837-50, viii-ix.

10. Martella, V., A. Cavalli, N. Decaro, G. Elia, C. Desario, M. Campolo, G. Bozzo, E. Tarsitano, and C. Buonavoglia. 2005. Immunogenicity of an intranasally administered modified live canine parvovirus type 2b vaccine in pups with maternally derived antibodies. Clin Diagn Lab Immunol 12:1243-5.

11. Martella, V., N. Decaro, and C. Buonavoglia. 2006. Evolution of CPV-2 and implication for antigenic/genetic characterization. Virus Genes 33:11-3.

12. McKnight, C. A., R. K. Maes, A. G. Wise, and M. Kiupel. 2007. Evaluation of tongue as a complementary sample for the diagnosis of parvoviral infection in dogs and cats. J Vet Diagn Invest 19:409-13.

13. Mohr, A. J., A. L. Leisewitz, L. S. Jacobson, J. M. Steiner, C. G. Ruaux, and D. A. Williams. 2003. Effect of early enteral nutrition on intestinal permeability, intestinal protein loss, and outcome in dogs with severe parvoviral enteritis. J Vet Intern Med 17:791-8.

14. Nakamura, M., Y. Tohya, T. Miyazawa, M. Mochizuki, H. T. Phung, N. H. Nguyen, L. M. Huynh, L. T. Nguyen, P. N. Nguyen, P. V. Nguyen, N. P. Nguyen, and H. Akashi. 2004. A novel antigenic variant of Canine parvovirus from a Vietnamese dog. Arch Virol 149:2261-9.

15. Parrish, C. R. 1990. Emergence, natural history, and variation of canine, mink, and feline parvoviruses. Adv Virus Res 38:403-450.

16. Parrish, C. R., C. F. Aquadro, and L. E. Carmichael. 1988. Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink and raccoon parvoviruses. Virology 166:293-307.

17. Parrish, C. R., C. F. Aquadro, M. L. Strassheim, J. F. Evermann, J. Y. Sgro, and H. O. Mohammed. 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J Virol 65:6544-52.

18. Parrish, C. R., P. H. O'Connell, J. F. Evermann, and L. E. Carmichael. 1985. Natural variation of canine parvovirus. Science 230:1046-1048.

19. Paul, M. A., L. E. Carmichael, H. Childers, S. Cotter, A. Davidson, R. Ford, K. F. Hurley, J. A. Roth, R. D. Schultz, E. Thacker, and L. Welborn. 2006. 2006 AAHA canine vaccine guidelines. J Am Anim Hosp Assoc 42:80-9.

20. Paul, M. A., L. E. Carmichael, H. Childers, S. Cotter, A. Davidson, R. Ford, K. F. Hurley, J. A. Roth, R. D. Schultz, E. Thacker, and L. Welborn. 2007. 2006 AAHA Canine Vaccine Guidelines, Revised. Published on-line at www.aahanet.org.

21. Perez, R., L. Francia, V. Romero, L. Maya, I. Lopez, and M. Hernandez. 2007. First detection of canine parvovirus type 2c in South America. Vet Microbiol 124:147-52.

22. Pollock, R. V., and L. E. Carmichael. 1982. Maternally derived immunity to canine parvovirus infection: transfer, decline, and interference with vaccination. J Am Vet Med Assoc 180:37-42.

23. Schultz, R. D. 2006. Duration of immunity for canine and feline vaccines: a review. Vet Microbiol 117:75-9.

24. Shackelton, L. A., C. R. Parrish, U. Truyen, and E. C. Holmes. 2005. High rate of viral evolution associated with the emergence of carnivore parvovirus. Proc Natl Acad Sci U S A 102:379-84.

25. Spibey, N., N. M. Greenwood, D. Sutton, W. S. Chalmers, and I. Tarpey. 2008. Canine parvovirus type 2 vaccine protects against virulent challenge with type 2c virus. Vet Microbiol 128:48-55.

26. Truyen, U., J. F. Evermann, E. Vieler, and C. R. Parrish. 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology 215:186-189.

CHAPTER 4

IN VITRO MODEL FOR CANINE PARVOVIRUS INFECTION

4.1 ABSTRACT

Canine parvovirus (CPV) provides an excellent model for studying the process of viral emergence and adaptation to a novel host. The original virus, CPV-2, is a host-range variant of feline panleukopenia virus (FPV), and has been globally replaced by newer variants, including CPV-2a and CPV-2b. The binding of CPV, but not FPV, to the canine transferrin receptor (TfR) was required for CPV emergence, but canine TfR binding alone likely does not explain all of the subsequent adaptation that CPV underwent in dogs. The sequence differences between the original CPV-2 and newer, better-adapted variants, such as CPV-2a and CPV-2b, are known, but how these differences alter interactions in host cells and ultimately provide better fitness for the newer variants in dogs, remains poorly understood. Here, I characterize the differing susceptibilities of two lines of Madin-Darby canine kidney (MDCK) cells – Strain I and Strain II – to infection by CPV-2 and CPV-2b, establishing them as a model for future investigations of cellular requirements for CPV infection beyond receptor binding.

4.2 INTRODUCTION

The emergence of viral diseases in new host populations remains a threat to human and animal health, and a better understanding of the molecular mechanisms involved in virus host range switching is required. When a virus first jumps to a new host species, it presumably has a relatively low fitness for that new host, and over time the virus likely adapts to the new host by acquiring specific mutations that increase its fitness. Understanding how such mutations might result in improved fitness is necessary for improving our ability to predict, control and prevent viral emergence events.

CPV serves as an excellent model for such studies. The original virus, CPV type-2 (CPV-2), emerged in the late 1970s as a novel canine pathogen and host-range variant of FPV. Within a year, a new variant was identified and named CPV type-2a (CPV-2a) (22, 26). A fairly small set of mutations define the difference between CPV-2 and CPV-2a, including four amino acid changes in the capsid protein, VP2, at positions 87, 101, 300 and 305. Importantly, CPV-2a-derived viruses completely replaced the original variant (CPV-2) in nature within 1-2 years, suggesting they have an improved fitness for their canine hosts. CPV-2a-derived viruses in circulation today contain one to several additional capsid mutations, some of which contain changes at the same site. For example, CPV-2b and CPV-2c contain an Asp and Glu, respectively, at VP2 residue 426, whereas CPV-2a contains an Asn at that position (4, 23, 26).

The original variant, CPV-2, differs in several important biological properties from newer CPV variants. For example, CPV-2 is only able to infect dogs, but not cats, while CPV-2a and CPV-2b can infect both dogs and cats (30). CPV-2 binds more strongly to both canine and feline cells than does CPV-2b (14), and this seems to be primarily controlled by their differing affinities for the feline and canine TfRs (18). Older and newer CPV variants can also be distinguished by their monoclonal antibody binding profiles (24-26). CPV-2b has been shown to outcompete CPV-2 in a tissue culture model of canine TfR expression (14), and appears to have approximately 2-fold higher infection rates in cells expressing only the canine TfR (8). Not all of these differences between CPV-2 and CPV-2b are explained by differences in their TfR binding, suggesting there are additional host cell interactions that have played a role in CPV adaptation to dogs.

Capsid interactions with host cells and the immune system play a significant role in determining the host range and tissue tropism of CPV variants during infection. It is therefore not surprising that many of the sites under positive selection in the CPV genome fall within the VP2 coding region (12). However, additional sites for positive selection in the nonstructural (NS) ORF, particularly in the frame-shifted, overlapping regions of the NS1 and NS2 genes, have also been identified (12), suggesting that NS1, NS2 or both are also important in CPV host adaptation, although mutagenesis studies have shown the NS2 is not required for efficient *in vitro* and *in vivo* replication in canine cells (33). In addition, changes in the NS ORF have been shown to play a partial role in the restriction of FPV replication in canine cells (13), suggesting that NS1/NS2 may also be involved in determining host range.

Here, I develop an *in vitro* model of CPV infection that may allow further dissection of the CPV infectious pathway and help identify how it differs between CPV-2 and CPV-2b. This will help shed light on the selection pressures, in addition to receptor binding, that shaped CPV adaptation in dogs. To this end, I have characterized infection in two canine epithelial cells lines that are derived from the same genetic background: Strain I and Strain II MDCK cells. Previous work has shown increased susceptibility to CPV-2 infection in Strain I MDCK cells compared with Strain II cells (21, 33), suggesting that differences between these two cell lines could be used to identify additional cellular requirements for infection by CPV.

The MDCK cell line was established in the late 1950s from the kidney of a female cocker spaniel, and has since been used extensively as an *in vitro* epithelial cell model to study, among other topics, cell polarity, intracellular trafficking, and virus infection. Early work suggested that this cell line was

heterogeneous, and either underwent differentiation in culture or contained two clonal variants that consisted of epithelial cell types with distinct properties, likely originating from different segments of the kidney tubules (27). In the 1980s, two MDCK cell clones were derived which share properties similar to those earlier descriptions of differing MDCK cell types (16), and these are the Strain I and Strain II clones used in this study. The exact origins of Strain I and II cells from specific regions of the nephron remain unclear. Most evidence suggests that Strain I and Strain II cells are both derived from distal tubule or collecting duct epithelium (11, 31), although some properties of Strain II cells have lead to the alternative hypothesis that Strain II cells originate from proximal tubule epithelium (27).

Characterization of these MDCK clones showed that Strain I and Strain II cells differ in many properties, including morphology, rate of cell division, transepithelial resistance (27), glycosphingolipid composition and metabolism, hormonal responses (27), and glycoprotein (17) and prostaglandin distribution (9, 15-17, 27). At low densities, strain I cells are spindle-shaped, extending along their substrate, while Strain II cells are cuboidal and form cell clusters more readily (Figure 4.1). Similar to the varying apical morphologies observed for kidney tubule epithelium *in vivo*, the strains likely differ in the extent of their apical microvilli and the presence or absence of cilia, which may have important implications for virus entry. Indeed, scanning electron microscopy has shown that Strain II cells contain a single apical cilium, while Strain I cells lack cilia (16). Furthermore, strain II cells divide more slowly, having a doubling time during logarithmic growth of approximately 1.5 times that of Strain I cells (16), and Strain II confluent monolayers have a lower transepithelial resistance compared with Strain I monolayers.

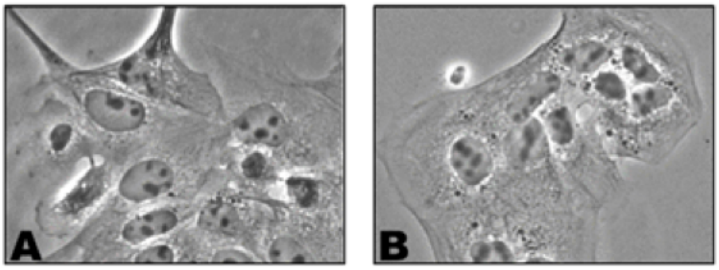


Figure 4.1. Comparison of Strain I (A) and Strain II (B) MDCK cell morphologies. Bright field images of cells fixed with 4% PFA were taken with a 40x objective.

Both MDCK strains can become polarized in tissue culture, forming confluent monolayers with apical and basolateral surfaces separated by tight junctions, and they contain unique protein and lipid compositions. The apical surface faces the lumen of the nephron *in vivo* and the growth media *in vitro*, while the basolateral surface faces the serosal side of the nephron *in vivo* and the substrate *in vitro*. MDCK cells are permissive for infection by many viruses, which may or may not cause natural infections in dogs, and in some cases virus entry and release is specific for either the apical or basolateral surface of polarized MDCK cells. For example, influenza can infect from either surface, but is preferentially released from the apical surface, while vesicular stomatitis virus primarily infects and is released from the basolateral surface (6, 28). In addition, TfR is predominantly expressed on the basolateral surface of polarized MDCK cell monolayers (7), and CPV binding and uptake occurs preferentially at the basolateral surface of polarized MDCK cells (2).

Here, I show that, in addition to showing strain differences in susceptibility to CPV-2 infection, CPV-2b infects MDCK cells to a higher level than does CPV-2. I show that both strains express functional TfR to similar

levels, suggesting differences in infection rates between the two strains might be caused by a cellular requirement for infection other than TfR.

4.3 MATERIALS AND METHODS

Cells, viruses and transferrin. Norden Laboratory feline kidney (NLFK) and Crandell-Reis feline kidney (CRFK) cells were grown in a 1:1 mixture of McCoy's 5A and Liebovitz L15 media with 5% fetal calf serum (FCS). MDCK Strain I and II cells were obtained from Dr. William Young (16) and cultured in Dulbecco's minimal essential media supplemented with 10% FCS and additional amino acids.

FPV, CPV-2 and CPV-2b viruses were derived from infectious plasmid clones as previously described (1, 20). For binding studies, viruses were concentrated by polyethylene glycol precipitation and purified on a sucrose gradient before being labeled with Alexa-488 fluorochrome (Molecular Probes, Eugene, OR) (10).

Canine transferrin (Tf) (Sigma, St. Louis, MO) was iron-loaded as previously described (3, 14) and labeled with Texas Red sulfonyl choloride (Molecular Probes) (14).

Virus infection assays. Cells were thinly seeded onto coverslips and inoculated the next day with equivalent amounts of either CPV-2 or CPV-2b. Two days post-inoculation, the cells were fixed and stained with a monoclonal antibody against the viral nonstructural protein, NS1, which is localized to the nucleus of infected cells (35). The percentage of infected cells was calculated by dividing the number of NS1-positive cells by the total number of cells present. Fields were counted until 100 positive cells were obtained, or until all cells on the coverslip were counted if there were fewer than 100 positive cells.

Antibody staining for TfR. Cells were thinly seeded onto coverslips and fixed the following day with 4% paraformaldehyde (PFA) and permeabilized with 0.5% Triton X-100. TfR was detected with a monoclonal primary antibody against the cytoplasmic tail of TfR (Zymed, San Francisco, CA), followed by an anti-mouse-IgG secondary antibody conjugated to the Alexa-488 fluorochrome (Molecular Probes). Cells were observed using a Nikon Eclipse TE300 inverted fluorescence microscope and images were taken using Simple PCI software and a Hammamatsu OrcaER digital camera using identical exposure and gain settings.

Western blotting for TfR. Protein content was normalized before loading using a BCA protein assay, and TfR was identified using the same anti-TfR primary antibody described above, followed by an anti-mouse-IgG secondary antibody conjugated to horse radish peroxidase and developed with SuperSignal chemiluminescent substrate solution (Pierce, Rockford, IL).

Tf binding and uptake. Cells were thinly seeded onto coverslips and incubated the next day in the presence of TexasRed-labeled transferrin for one hour before fixation in 4% PFA. Images were taken as described above.

4.4 RESULTS

Cell susceptibility to infection. To determine MDCK susceptibility to infection, cells were inoculated with FPV, CPV-2 and CPV-2b and the percentage of infected cells was assessed using immunofluorescence microscopy. Feline cell lines showed the highest levels of infection for all three viruses, with FPV infecting the greatest percentage of cells (Figure 4.2). Strain I cells had an almost 10-fold higher level of infection by CPV-2b than did

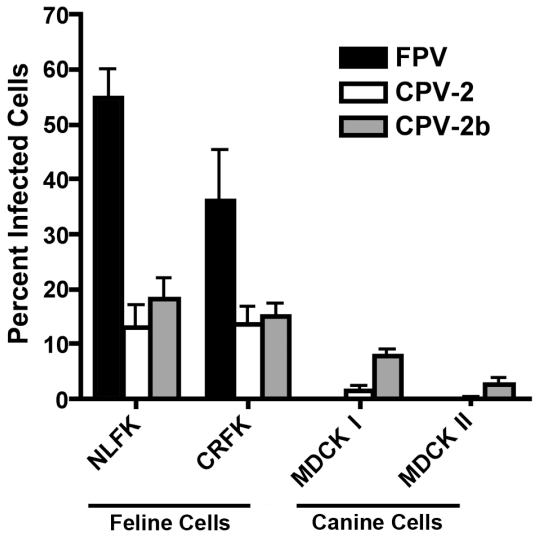


Figure 4.2. Infectivity of FPV, CPV-2 and CPV-2b in four cell lines showing that FPV results in higher infection rates in feline cells than CPV, but FPV cannot infect canine cells. MDCK cells are infected by CPV-2b better than CPV-2, particularly Strain I cells. Bars indicate the standard deviation for five to seven replicates.

Strain II cells. Also, Strain I cells were considerably more susceptible to infection by both CPV-2b than CPV-2 that were Strain II cells.

TfR expression. To determine if the differences seen in infection levels between Strain I and II MDCK cells is due to differential expression of TfR, I assessed total cellular TfR expression by Western blotting and immunofluroescence microscopy. Both strains expressed similar levels of TfR that were comparable to TfR expression in feline cells (Figure 4.3).

To ensure that the expressed TfR is functional, binding and uptake assays were also performed using fluorescently labeled transferrin (Figure 4.4). Both MDCK strains showed similar fluorescent intensities and localization patterns, indicating that Strain I and Strain II MDCK cells express similar levels of functional surface TfR, within the limits of detection afforded by fluorescence microscopy.

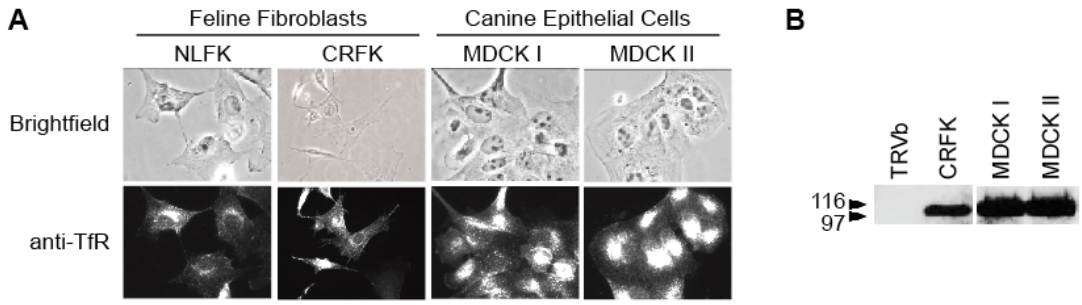


Figure 4.3. A) Antibody staining of fixed cells for total cellular TfR shows that all four cell lines express TfR with expected perinuclear localization patterns. B) Western blotting for TfR in cellular extracts confirms the findings in A. TRVb cells lack expression of endogenous TfR, and are used here as a negative control.

Taken together, these data suggest that functional surface TfR levels are similar between Strain I and Strain II MDCK cells and are therefore unlikely to be playing a role in the observed differences in CPV infection properties of MDCK cells.

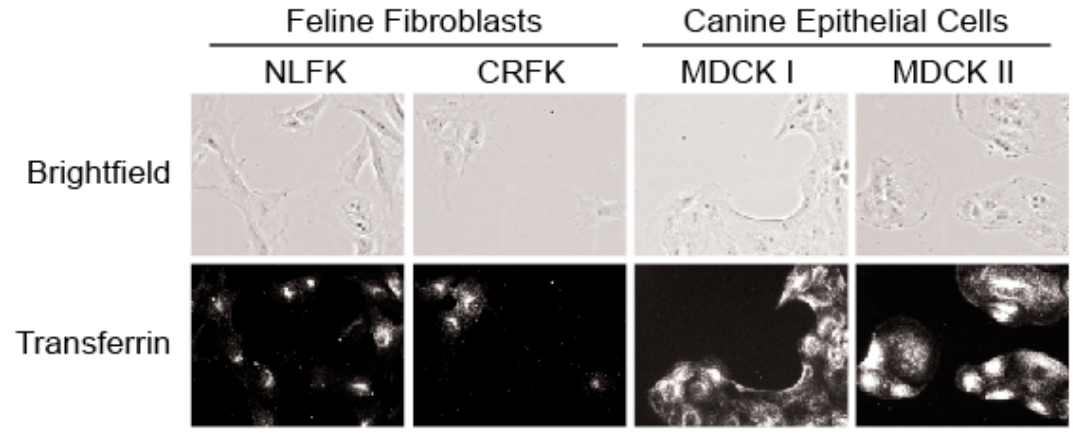


Figure 4.4. Comparison of fluorescently labeled transferrin binding and uptake in feline and canine cells, showing that both MDCK cell strains express functional TfR that is capable of binding and internalizing transferrin.

4.5 DISCUSSION

Despite strong selection for the newer CPV strains in nature, little is known about how differences between CPV-2 and CPV-2b affect virus-host interactions. Two clonal variants of MDCK cells – Strain I and Strain II – provide the first *in vitro* canine model that clearly displays differences in susceptibility to infection by CPV-2 and CPV-2b. There are also differences in susceptibility between strains, even though both are derived from the same genetic background. This establishes an *in vitro* system that can be used for testing differences in the infectious pathways of CPV-2 and CPV-2b.

Since binding to the TfR is a known requirement for CPV infection, I began characterization of the MDCK strains by examining their TfR expression. Strain I and II cells have previously been shown to express similar levels of TfR (7). Here, I have confirmed that expression of functional TfR is similar between Strain I and II cells. This suggests that the reduced CPV infection levels in Strain II cells are not caused by reduced expression of its cellular receptor. However, Strain II cells may contain a mutation in their TfR gene that alters important sequences in the TfR apical domain, necessary for virus binding, uptake and infection, but not required for Tf binding. For example, residue changes which alter the unique glycosylation site in the canine TfR would be expected to reduce CPV binding (19). Therefore, to determine if Strain I and Strain II cells are expressing wildtype TfR, TfR mRNA should be amplified, sequenced and compared with the known canine TfR sequence.

Since CPV does not encode or package its own polymerase, it is dependent on host cell machinery for its replication and requires actively dividing cells to complete its own replication. Therefore, since Strain I MDCK

cells divide almost two times more rapidly than Strain II MDCK cells (16), Strain I cells may support increased virus replication and production compared to Strain II cells at the same time point post infection. When Strain II cells are infected with either virus and assessed for viral antigen at various time points post infection up to 72 hours, the number of cells expressing viral antigen remains at a low, background level and does not increase (data not shown). By 72 hours post infection, the cells have reached confluency and are no longer rapidly dividing, so they are no longer expected to support additional virus replication. Since the percentage of infected cells in Strain II cells never reaches the level seen in Strain I cells at any time post infection, the slower rate of cell division in the MDCK Strain II cells likely does not account for their reduced susceptibility to CPV. In addition, early studies of parvoviruses showed that when they enter nondividing cells (e.g., cells arrested at the G1/S stage of the cell cycle by isoleucine deprivation and aphidicolin treatment), they could undergo replication if the cell subsequently begins to divide (e.g., when the cell cycle blockade is removed) (5). This suggests that if cell division is occurring, even if at a relatively slower rate, the virus has the ability to replicate in permissive cell lines, again making it unlikely that the slower rate of cell division in Strain II cells fully accounts for their relative resistance to CPV infection.

The infection assays described here suggest that restriction of infection in Strain II cells occurs before translation of viral proteins, since minimal to no anti-NS1 staining was observed in those cases. One possibility is that restriction occurs at the level of cell binding and entry, or during cytoplasmic trafficking to the nucleus. This hypothesis could be tested by transfection of MDCK cells with the infectious plasmid clones of CPV-2 and CPV-2b, which

would allow assessment of CPV infection in MDCK cells when normal viral entry and trafficking is bypassed. In addition, microinjection of capsids into the cytoplasm, with subsequent immunofluorescence staining for NS1, can be used to determine if the restriction of CPV infection occurs prior to or after capsid entry into the cytosol. It is known that microinjection will lead to infection (29, 32, 34), although the efficiency of infection may be low and dependent on the amount of virus microinjected.

Following the above characterizations of Strain I and Strain II MDCK cells, larger-scale analyses of differences between MDCK strains may help identify factors that restrict CPV infection in Strain II cells, as well as discover differences in cellular requirements for infection between CPV-2 and CPV-2b. For example, crude membrane preparations or whole-cell lysates from MDCK cells could be electrophoretically separated and analyzed using virus overlay blots to identify potential cellular binding partners for CPV-2 and CPV-2b. Differences in virus binding to proteins from Strain I and Strain II samples represent cellular molecules that may be influencing CPV susceptibility differences in MDCK strains. Similarly, differences between CPV-2 and CPV-2b binding to Strain I extracts represent potential cellular proteins that interact differently with each variant and may contribute the increased ability of CPV-2b to infect those cells. Proteins of interest can be cut out of the gel and analyzed by mass spectrometry to obtain amino acid sequence data, which can then be used to search for homologous proteins. Virus binding to any proteins identified by this method must be confirmed, and the functional significance, in the context of *in vitro* and *in vivo* virus infection, would need to be evaluated. In addition, co-immunoprecipitation experiments could be used

to identify cellular binding partners for NS1 and NS2 and differences between CPV-2 and CPV-2b variants could similarly be assessed.

Ultimately, this *in vitro* MDCK model of CPV infection may help us identify cellular factors, in addition to the TfR, that are important in the host range switch and subsequent evolution of CPV.

4.6 ACKNOWLEDGEMENTS

I would like to thank Ms. Tyler D. Lillie for her extensive help with the immunofluorescence microscopy analyses presented here, and Ms. Wendy Weichert for her excellent technical support. I would also like to thank my advisor, Dr. Colin R. Parrish, for his thoughts and discussions on this project. The research was supported by NIH grant AI28385 grant to CRP. I was supported by NIH training grant RR18269 to Dr. Mark S. Roberson during part of these studies.

REFERENCES

1. Agbandje, M., R. McKenna, M. G. Rossmann, M. L. Strassheim, and C. R. Parrish. 1993. Structure determination of feline panleukopenia virus empty particles. Proteins 16:155-71.

2. Basak, S., Compans, R.W. 1989. Polarized entry of canine parvovirus in an epithelial cell line. J.Virol. 63: 3164-3167.

3. Bates, G. W., and M. R. Schlabach. 1973. The reaction of ferric salts with transferrin. J Biol Chem 248:3228-32.

4. Buonavoglia, C., V. Martella, A. Pratelli, M. Tempesta, A. Cavalli, D. Buonavoglia, G. Bozzo, G. Elia, N. Decaro, and L. Carmichael. 2001. Evidence for evolution of canine parvovirus type 2 in Italy. J Gen Virol 82:3021-5.

5. Cotmore, S. F., and P. Tattersall. 1987. The autonomously replicating parvoviruses of vertebrates. Adv Virus Res 33:91-174.

6. Fuller, S., C.-H. von Bonsdorff, and K. Simons. 1984. Vesicular stomatitis virus infects and matures only through the basolateral surface of the polarized epithelial cell line, MDCK. Cell 36:65-77.

7. Fuller, S. D., and K. Simons. 1986. Transferrin receptor polarity and recycling accuracy in "tight" and "leaky" strains of Madin-Darby canine kidney cells. The Journal of Cell Biology 103:1767-1779.

8. Goodman, L. B., S. M. Lyi, N. C. Johnson, J. O. Cifuente, S. L. Hafenstein, and C. R. Parrish. 2010. Binding site on the transferrin receptor for the parvovirus capsid and effects of altered affinity on cell uptake and infection. J Virol 84:4969-78.

9. Hansson, G. C., K. Simons, and G. van Meer. 1986. Two strains of the Madin-Darby canine kidney (MDCK) cell line have distinct glycosphingolipid compositions. EMBO J 5:483-489.

10. Harbison, C. E., S. M. Lyi, W. S. Weichert, and C. R. Parrish. 2009. Early steps in cell infection by parvoviruses: host-specific differences in cell receptor binding but similar endosomal trafficking. J Virol 83:10504-14.

11. Herzlinger, D. A., T. G. Easton, and G. K. Ojakian. 1982. The MDCK epithelial cell line expresses a cell surface antigen of the kidney distal tubule. J Cell Biol 93:269-277.

12. Hoelzer, K., L. A. Shackelton, C. R. Parrish, and E. C. Holmes. 2008. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. J Gen Virol 89:2280-9.

13. Horiuchi, M., H. Goto, N. Ishiguro, and M. Shinagawa. 1994. Mapping of determinants of the host range for canine cells in the genome of canine parvovirus using canine parvovirus/mink enteritis virus chimeric viruses. J Gen Virol 75 (Pt 6):1319-28.

14. Hueffer, K., J. S. Parker, W. S. Weichert, R. E. Geisel, J. Y. Sgro, and C. R. Parrish. 2003. The natural host range shift and subsequent evolution of canine parvovirus resulted from virus-specific binding to the canine transferrin receptor. J. Virol. 77:1718-1726.

15. Lewis, M. G., and A. A. Spector. 1981. Differences in types of prostaglandins produced by two MDCK canine kidney cell sublines. Prostaglandins 21:1025-32.

16. Nichols, G. E., J. C. Lovejoy, C. A. Borgman, J. M. Sanders, and W. W. Young. 1986. Isolation and characterization of two types of MDCK epithelial cell clones based on glycosphingolipid pattern. Biochim Biophys Acta 887:1-12.

17. Ojakian, G. K., R. E. Romain, and R. E. Herz. 1987. A distal nephron glycoprotein that has different cell surface distributions on MDCK cell sublines. Am J Physiol 253:C433-43.

18. Palermo, L. M., S. L. Hafenstein, and C. R. Parrish. 2006. Purified feline and canine transferrin receptors reveal complex interactions with the capsids of canine and feline parvoviruses that correspond to their host ranges. J Virol 80:8482-92.

19. Palermo, L. M., K. Hueffer, and C. R. Parrish. 2003. Residues in the apical domain of the feline and canine transferrin receptors control host-specific binding and cell infection of canine and feline parvoviruses. J Virol 77:8915-8923.

20. Parrish, C. R. 1991. Mapping specific functions in the capsid structure of canine parvovirus and feline panleukopenia virus using infectious plasmid clones. Virology 183:195-205.

21. Parrish, C. R. unpublished data.

22. Parrish, C. R., C. F. Aquadro, and L. E. Carmichael. 1988. Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink and raccoon parvoviruses. Virology 166:293-307.

23. Parrish, C. R., C. F. Aquadro, M. L. Strassheim, J. F. Evermann, J. Y. Sgro, and H. O. Mohammed. 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. J Virol 65:6544-52.

24. Parrish, C. R., and L. E. Carmichael. 1983. Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. Virology 129:401-14.

25. Parrish, C. R., Carmichael, L.E., Antczak, D.F. 1982. Antigenic relationships between canine parvovirus type-2, feline panleukopenia virus and mink enteritis virus using conventional antisera and monoclonal antibodies. Arch.Virol. 72: 267-278.

26. Parrish, C. R., P. H. O'Connell, J. F. Evermann, and L. E. Carmichael. 1985. Natural variation of canine parvovirus. Science 230:1046-1048.

27. Richardson, J. C. W., V. Scalera, and N. L. Simmons. 1981. Identification of two strains of MDCK cells which resemble separate nephron tubule segments. Biochim Biophys Acta 673:26-36.

28. Rodriguez-Boulan, E., and D. D. Sabatini. 1978. Asymmetric budding of viruses in epithelial monolayers: a model system for study of epithelial polarity. Proc Natl Acad Sci U S A 75:5071-5075.

29. Suikkanen, S., T. Aaltonen, M. Nevalainen, O. Valilehto, L. Lindholm, M. Vuento, and M. Vihinen-Ranta. 2003. Exploitation of microtubule cytoskeleton and dynein during parvoviral traffic toward the nucleus. J Virol 77:10270-9.

30. Truyen, U., J. F. Evermann, E. Vieler, and C. R. Parrish. 1996. Evolution of canine parvovirus involved loss and gain of feline host range. Virology 215:186-189.

31. Valentich, J. D. 1981. Morphological similarities between the dog kidney cell line MDCK and the mammalian cortical collecting tubule, p. 384-405. *In* W. N. Scott and D. B. P. Goodman (ed.), Hormonal Regulation of Epithelial Transport of Ions and Water, vol. 372. Annals of the New York Academy of Sciences, New York.

32. Vihinen-Ranta, M., W. Yuan, and C. R. Parrish. 2000. Cytoplasmic trafficking of the canine parvovirus capsid and its role in infection and nuclear transport. J Virol 74:4853-4859.

33. Wang, D., W. Yuan, I. Davis, and C. R. Parrish. 1998. Nonstructural protein-2 and the replication of canine parvovirus. Virology 240:273-81.

34. Weichert, W. S., J. S. Parker, A. T. M. Wahid, S. F. Chang, E. Meier, and C. R. Parrish. 1998. Assaying for structural variation in the parvovirus capsid and its role in infection. Virology 250:106-117.

35. Yeung, D. E., G. W. Brown, P. Tam, R. H. Russnak, G. Wilson, I. Clark-Lewis, and C. R. Astell. 1991. Monoclonal antibodies to the major nonstructural nuclear protein of minute virus of mice. Virology 181:35-45.

CHAPTER 5

SUMMARY AND CONCLUSIONS

The emergence of new viruses by host switching remains a constant threat, possibly more-so today than in years past due to increased global movement of people and animals, and increased population overlap among humans, domestic animals and wildlife. The last couple decades have witnessed the emergence of many viruses, including SARS-CoV, novel Influenza A viruses, and the Henipah viruses. While emerging viruses are most widely publicized for their involvement in human illness, they also cause diseases in companion and domestic animals and wildlife, with direct effects on those populations and indirect effects on humans.

Global surveillance is increasing and international outbreak response is continually improving, thus aiding in the recognition and response to novel emerging pathogens. However, prediction algorithms and prevention strategies require more development to achieve widespread usefulness. In general, the prediction, prevention and control of emerging viruses will be aided by a better understanding of the factors contributing to emergence by host switching, from the ecological barriers to host switching to the molecular mechanisms involved in host adaptation.

Canine parvovirus (CPV) serves as an excellent model for studying viral emergence because its biological properties and evolution have been closely studied since it emerged worldwide as a novel canine pathogen in the late 1970s. The studies presented in this thesis were designed to specifically characterize the process of host adaptation that occurred in dogs after the primary host-switching event from cats took place.

For the first set of studies, I created a panel of intermediate viruses with various combinations of four capsid residues that help define the newer clade of currently circulating CPV strains (e.g., CPV-2b) from the original CPV-2

virus, which was replaced globally in a selective sweep during the early 1980s. These intermediate viruses represent potential evolutionary pathways for CPV adaptation, and I showed that all four residues work in concert to help define the receptor and antibody binding properties of the wildtype viruses. Furthermore, *in vitro* competition assays suggested that adaptation of CPV in dogs likely involved passage through a fitness valley, since the potential intermediates had lower fitness than CPV-2. Changing the four residues from the CPV-2b sequence back to the CPV-2 sequence did not restore its fitness compared to CPV-2, demonstrating that additional changes in the CPV-2b background also modulate CPV replication and viral fitness. These data represent one of the first fitness landscapes to be explored experimentally for a virus, and future testing of the same sequence space in additional environments (e.g., feline versus canine cells) will help reveal how selection pressures on the virus differ under varying conditions.

For this purpose, it would be helpful to have a canine cell line that differs in its susceptibility to CPV-2 and CPV-2b viruses. To this end, I developed an *in vitro* model using two strains of Madin-Darby canine kidney (MDCK) cells that display differential CPV infection between the two strains, as well as differential infection by CPV-2 and CPV-2b viruses. I showed that Strain II cells are largely resistant to infection by both viruses, whereas Strain I cells are infected to a higher level by CPV-2b than CPV-2. I also demonstrated that functional transferrin receptor expression levels do not control these differences in infection. These cell lines thus serve as a model for defining the cellular factors required during CPV infection, in addition to the TfR, and for exploring how these requirements for infection differ between

virus strains. Defining these differences would advance our understanding of how CPV adapted to dogs following its initial emergence.

This thesis also evaluates more recent changes among CPV variants from a clinical perspective. A year-long study of canine parvoviral enteritis cases in Arizona, where both clinical data and CPV capsid sequences were collected, showed that over two-thirds of the cases were caused by CPV-2c, while the remaining cases were caused by CPV-2b. These two viruses are defined by a difference in the VP2 capsid gene at codon 426, resulting in an Asp (CPV-2b) or Glu (CPV-2c) in the VP2 protein, although additional variation was also observed in the capsid sequences of the study isolates. Contrary to widely circulated suspicions among pet owners and clinicians, CPV-2c was not shown to cause more severe disease or result in a worse outcome than CPV-2b. Furthermore, a commonly used in-clinic diagnostic test for CPV was able to detect both variants, although false negative results were possible. The high number of CPV-2c cases found during this study, and reported elsewhere, indicate that CPV-2c is being positively selected and therefore likely confers some advantage to the virus, allowing it to contribute a proportionally higher number of progeny to the next generation of viruses compared with other variants. The specific advantage conveyed by the CPV-2c mutation remains unknown, but may be involved in maternal antibody escape, increased viral shedding or earlier shedding.

CPV is continuing to evolve today and multiple strains are co-circulating worldwide. While this study shows no indication that changes in case diagnostics or management are currently needed, CPV surveillance should continue to screen for new variants. This should include full VP2 sequencing, and full genome sequencing when possible, as these studies and others have

shown that multiple mutations, in various regions of the genome, are responsible for defining the biological phenotypes of the virus. How these sequence variants may be impacting *in vivo* replication and transmission remains largely undefined. Therefore, future studies should continue to combine phylogenetics, molecular biology and clinical studies to aid our understanding of how CPV variation influences CPV pathogenesis.