Sveriges lantbruksuniversitet
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Does vaccination against Feline Parvovirus protect hospitalized raccoon kits from clinical outbreaks of parvoviral disease?

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Får tvättbjörnsungar på djursjukhus ett skydd mot kliniska utbrott av kattpest om de vaccineras mot felint parvovirus?

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ABSTRACT

The Northern Raccoon (Procyon lotor) belongs to the Carnivore-family and is a species endemic to North America. Every year hundreds of orphaned raccoon cubs are admitted into the Wildlife Rehabilitation Center of Minnesota (WRCM), a non-profit organization where all injured or orphaned wild animals are admitted and receive quality health care with the goal of being released out into the wild.

The WRCM routinely vaccinate all of the admitted raccoon cubs with a killed feline panleucopenia vaccine, but despite this there are outbreaks of parvovirus infection every year where up to 50% of the admitted cubs have been euthanized or died. The objective of this study was to determine whether vaccinating the admitted raccoon kits has any significant protective effect to developing clinical parvoviral disease.

A single-blinded cohort study was designed with two parallel, independent groups. One group was given a dose of killed feline parvovirus vaccine at admission, and the other group was not given any vaccination at all. Assignment to the vaccinated or unvaccinated group was on a per-litter basis and done randomly by drawing lots out of a box. A second dose of vaccine was administered to the vaccinated group after two weeks if the litter was still in-housed. In all other respects, the groups received the same treatment, handling and medical care. The two groups were housed in two different rooms of the same building. To accommodate room size, the two study groups were proportioned so that 1/3 of the study population was not vaccinated and 2/3 were vaccinated. The raccoon kits were continually assessed clinically and these observations served as the base for incidence calculations according to pre-defined criteria. The difference between the groups was calculated concerning overall disease, sickness and death/euthanasia. Two blood samples were collected from every admitted raccoon for serological testing.

A total of 201 individual raccoon cubs were admitted into the WRCM mammal nursery in 2012, and served as the study population for this study. The non-vaccinated study group held 46 individuals, and the vaccinated study group consisted of 116 individuals. The results indicate that orphaned raccoon kits taken into the WRCM benefit from vaccination against FPV. Vaccinated individuals had a 0.54 relative risk of acquiring clinical parvoviral disease compared to unvaccinated individuals ($p = 0.07$). Serological testing for antibody titers was done for a few individuals in each study group. No significant difference was found between the vaccinated and unvaccinated individuals the regarding antibody titers.

SAMMANFATTNING

Den nordamerikanska tvättbjörnen (*Procyon lotor*) tillhör däggdjursordningen rovdjur (*Carnivora*) och är endemiskt förekommande på hela den nordamerikanska kontinenten. Hundratals föräldralösa tvättbjörnsungar tas varje år emot på Wildlife Rehabilitation Center of Minnesota (WRCM), en icke-vinstdrivande organisation vars uppgift är att ta emot och vårda sjuka, skadade eller föräldralösa djur och därefter släppa ut dem i det vilda igen.

Genom åren har samtliga inskrivna tvättbjörnsungar rutinmässigt vaccinerats mot felint parvovirus (kattpest) med ett avdödat vaccin, men trots detta förekommer varje år kliniska utbrott av sjukdomen där upp till 50% av tvättbjörnsungarna har varit tvungna att avlivas som följd. Syftet med denna studie var att säkerställa huruvida vaccination mot felint parvovirus har någon signifikant skyddande effekt mot risken att utveckla klinisk kattpest.

En enkel-blindad kohort-studie designades med två parallella, oberoende grupper. Grupperna lottades fram per kull vid inskrivning. Ena gruppen vaccinerades vid inskrivning med ett avdödat vaccin mot felint parvovirus, samt ytterligare en gång efter två veckor om individen var kvar på centret, och den andra gruppen vaccinerades inte överhuvudtaget. De två grupperna behandlades lika i alla andra avseenden. De två grupperna hölls åtskilda i två olika rum i samma sjukhusbyggnad. På grund av rummens olika storlek delades även försöksgrupperna upp i proportionerna 1/3 ovaccinerade och 2/3 vaccinerade individer. Tvättbjörnarna övervakades och kliniska parametrar journalfördes kontinuerligt under hela sjukhusvistelsen. Dessa kliniska observationer stod sedan till grund för incidensberäkningarna där grupperna jämfördes gällande sjuklighet och dödlighet (självdöda respektive avlivade). En mindre serologisk studie gjordes också, för vilken två blodprov togs från samtliga inskrivna tvättbjörnar.

Totalt sett skrevs 201 tvättbjörnsungar in på WRCM under 2012, och samtliga ingick i studien. I den ovaccinerade gruppen ingick 46 individer, och den vaccinerade gruppen innehöll 116 individer. Resultaten pekar mot att vaccinering av tvättbjörnsungar som skrivs in på WRCM har en skyddande effekt. Vaccinerade individer hade en relativ risk på 0,54 att drabbas av klinisk kattpest jämfört med ovaccinerade individer (p= 0,07). Den serologiska studien innehöll endast ett par individer från varje grupp och visade inga signifikanta skillnader i antikropptitrar eller -stegringar.

INTRODUCTION

Background

The Wildlife Rehabilitation Center of Minnesota (WRCM) is a registered non-profit organization which relies entirely on donations and volunteers to operate. It is one of the biggest and busiest wildlife rehabilitation centers in the USA, every year admitting up to 8800 hurt, sick or orphaned patients. These wild animals are provided quality health care and rehabilitation with the goal to be released again into the wild.

A large part of the spring activity has to do with the nursing of orphaned young animals of varying kinds. A frequently admitted orphan is the raccoon (*Protyon locor*), a species native to North America in which the parents will leave their young to search for food. This search often brings them into more or less densely populated human areas as well as in close proximity to well-trafficked roads where accidents are common. Litters or single-found raccoon kits are brought into the WRCM by finders, where they are kept and nursed until they can fend for themselves and are released into the wild.

Hospital environments are very susceptible to disease-spread with limited space and personnel to cope with a large number of sick, weak and/or young potentially infectious animals/patients of unknown immune status. To prevent hospitals from becoming a hub of infection, it is very important to take preemptive measures of biosecurity, such as strict cleaning and handling routines and hygiene barriers. However, some infectious disease agents are extremely persistent in the environment as well as highly infectious with high mortality, and extra measures such as vaccination might need to be taken to prevent outbreaks.

Parvoviruses are well-known, highly environmentally persistent as well as highly infectious agents. Certain strains of parvovirus (feline parvoviruses, see further below) infect what is thought to be most carnivore species, both domesticated and wild, among which the raccoon is one. Feline parvovirus is endemic in the wild populations of carnivores in North America (Barker et al., 1983). Every year, there will be outbreaks of parvoviral disease at WRCM. This occurs even though strict preventive measures are being taken, such as screening at admission where only ”healthy” individuals – in a parvoviral sense – are admitted, and immediate euthanization of suspected cases followed by cleaning. To minimize the effects and spread of parvoviral disease, all admitted raccoon kits have in recent years been vaccinated with a killed feline panleukopeniavirus vaccine according to an arbitrary vaccination protocol extrapolated from other kinds of carnivore/feline housing facilities in the world. Despite this vaccination protocol, there are outbreaks of parvoviral disease every year among the in-housed raccoons, leading to (sometimes) extensive euthanization of the nursery inmates.

Considering this reoccurring scenario, debate arises whether vaccination helps at all to minimize parvoviral disease outbreak. Although parvoviral research has been going on for some time, the complete understanding of phylogeny, evolution, host ranges and factors contributing to disease still remains unknown (Battilani et al., 2011). As far as parvovirus infections in raccoons are concerned, hardly any new literature has been published for the past two decades (Kapil et al., 2010). A scientifically based vaccination protocol has therefore not been drawn up concerning raccoons as individuals or populations.

Wholly or partially unanswered questions in the literature regard which strain/s of parvovirus infect raccoons, to what degree infection results in clinical disease, quantification of disease-spread as opposed to infectious doses of viral agents, occurrence of parvovirus disease in the wild raccoon populations, as well as well-defined clinical signs, pathogenesis, mortality and lethality of diseased raccoons. Questions also as to how the immune system reacts to infection, how long-lasting the immune response might be, prevalence of circulating antibodies that could be excreted into colostrum, whether or not cross-immunity occurs for the different strains of virus, and to what degree, remain to be answered.

In addition, not a lot is known about early immune responses of raccoons, presence of maternal antibodies, possible interference of maternal antibodies in young and juvenile animals to the mounted immune response, at what age the maternal antibodies would stop interfering and juvenile raccoons start being able to mount their own immune response to infection or vaccination. The point on cross-immunity is particularly interesting since developed vaccines so far exist only for felines, canines and mink, which makes the choosing of a vaccine strain somewhat arbitrary.

Because of the lack of information, WRCM has not been able to perform a scientifically-based cost-benefit analysis of vaccinating juvenile raccoons against parvovirus. The continuation of today's protocol is based on the assumption that it is beneficial to the raccoon nursery patients as a whole, and the fear of what might happen if the protocol was wrongly set aside. Being an organization wholly founded by donations, though, the WRCM risks putting substantial means into routines that might lack the needed effect.

The main objective of this study was to find out whether vaccinating juvenile raccoons taken into the nursery at WRCM had a protective effect on the risk to develop clinical parvoviral disease. Further objectives were to analyze the immune response after vaccination at a young age, and, if possible, to investigate the effects of maternal antibodies on the immune response.

The northern raccoon (*Procyon lotor*)

The northern raccoon (*Procyon lotor)* is of the family Procyonidae, belonging to the order *Carnivora*. The Procyonidae family is restricted to the North and South Americas (with the exception of the lesser panda of southeast Asia). Northern raccoons are furthermore of the genus Procyon, a genus of nocturnal animals all named raccoons, but in which only the Northern raccoon is native to North America. The other two species in this genus (the Crab-eating raccoon, *Procyon cancrivorus*, and the Pygmy raccoon, *Procyon pygmaeus*) are considerably lesser known and live only in the tropics (IUCN, 2012). From here on, *Procyon lotor* will be referred to as ”raccoon.”

The raccoon is of a medium size, adults measuring 46-71 cm with a 20-30 cm long tail and weighing approximately 5.4-15.8 kg depending on the season. It is easily recognized by its black face mask and rings of varying yellowish to white and black over the bushy tail. The body is stout and plantigrade, moving in a waddling gait. Each foot has five toes with non-retractable claws, and the forepaws have adapted to be unusually dextrous (Hazard, 1982).

The breeding season stretches from February to June, peaking in March. Raccoons have a polygynadrous reproduction, meaning that males and females both are polygamous and can have

several mates. After a gestation period of 63 days, a litter of 3-7 young are born blind and helpless in a tree den. Their eyes typically open at about 18-24 days of age, and the kits are fully weaned by 70 days. By the time the kits are 5 months old, they regularly forage with their mothers at night, but it takes them an average of 10 months to become fully independent, meaning that they spend their first winter with their mothers (Fox, 2001).

Raccoons are nocturnal opportunistic omnivores, meaning that they eat everything and anything from invertebrates, vertebrates, plants, fruits and vegetables to carrion and garbage in urban areas. As their hunting and search for food mainly takes place at night while they den up in trees during daytime, they naturally evade most of their potential predators. These predators include gray wolves, coyotes, snakes, large hawks and owls. Raccoons are excellent climbers and swimmers but somewhat reluctant to swim as their heavy fur carries a lot of extra weight when water-logged. Adults are more or less solitary, though young stay with their mothers, and males and females might den together for a month or so in breeding season (Fox, 2001).

Although raccoons are seen as mainly solitary animals, they do interact directly or indirectly on a regular basis. Although raccoons are territorial, their home ranges generally overlap with those of several other individuals/groups. This is called a fission-fusion social structure, with a large number of short-term acquaintances but also some long-term positive interactions (Prange et al., 2011).

In urban areas, raccoons may interact with domestic carnivores. This is of particular importance concerning the spread of some infectious diseases, as the raccoon is a carnivore and therefore susceptible. The diseases of utmost importance include rabies, canine distemper, leptospirosis, canine adenovirus, and parvovirus (Kapil et al., 2010).

Carnivore parvoviruses and their role in raccoons

Phylogeny and evolution

Feline parvoviruses are a subgroup of the genus Parvovirus within the family *Parvoviridae*. Clinical diseases caused by feline parvoviruses have been known since the early 1900s, but were at that time described as a number of different syndromes, such as feline infectious enteritis, malignant panleukopenia, feline distemper or spontaneous agranulocytosis. In 1928 Verge et al. were able to show that the cause of these syndromes was a filterable virus, what we today know as feline panleukopeniavirus (as reviewed by Steinel et al., 2001).

Similar viruses have since been isolated from many species of carnivores, and they have by tradition been named after either a virus of a related host or of the host animal from which the virus was isolated. In 1940, a parvovirus-like disease in a raccoon was first recognized by Waller. The causative agent was isolated in the 1980s and subsequently named raccoon parvovirus or RPV (as reviewed by Parrish et al., 1987). The tradition of naming parvoviruses for the species in which they were isolated has led to nomenclature that can be misleading, and which in describing the viruses can lead to misunderstandings about what strain is actually being described (Barker and Parrish, 2001).

However, the parvoviruses that affect carnivores are all grouped informally within the feline parvovirus subgroup. Of these, feline panleukopeniavirus (FPV), mink enteritis virus (MEV) and

canine parvovirus (CPV) are the three main subgroups which other subgroups are put in relationship to (Steinel et al., 2001).

Research regarding the evolution and phylogeny of carnivore parvoviruses has, on a genomic level, centered on defining VP1/VP2 as well as NS1 gene sequences. As found by Truyen et al. (1995), parvovirus isolates of cat, mink, raccoon and fox could not be readily differentiated. Isolates from canines were found to differ at most 1.3% from the feline isolates. Later sequencing has found raccoon isolates to be phylogenically in-between parvovirus strains with canine and/or feline host cell tropism. Virus isolation from parvovirus-infected raccoons has showed that raccoons are susceptible to modern canine as well as feline isolates (Allison et al., 2012).

Parvovirus infections of raccoons

Raccoon parvovirus (RPV) is described as a feline panleukopenia-like virus (Steinel et al., 2001). Appel and Parrish (1982) concluded that while RPV is not identical to FPV, the two strains share two common antigens, and RPV is therefore antigenically closer related to FPV than to CPV. Raccoons inoculated with CPV failed to become clinically ill and did not shed virus in their feces. Barker et al. (1983) showed that raccoons turned out to be highly susceptible to MEV and FPV, developing clinical disease, shedding virus in feces and seroconverting. Again, raccoons were not very susceptible to CPV, although a few individuals did shed virus and/or seroconvert after inoculation.

It has previously been reported that raccoons are not susceptible to CPV (Appel and Parrish, 1982), and studies on genetic sequencing have determined that isolates of RPV are very like, if not identical, to FPV (Steinel et al., 2001). However, in a case study from 2010, a canine parvovirus type 2-related virus (CPV-2) was isolated from a deceased raccoon at a wildlife rehabilitation center in Arkansas, USA (Kapil et al., 2010). Several other studies from around the world have reported CPV-2-related viruses being isolated from feline populations. More research is needed to determine whether this reflects on an evolvement of feline parvoviruses (CPV-2) leading to a broadened host range, or if these were sporadic cases. The focus of this review will mostly be on FPV, as RPV in its original sense is described as an FPV-like virus, included in the feline host range as opposed to the canine.

Characteristics

The parvovirus capsid is small, non-enveloped, and has a T=1 icosahedral symmetry (Hueffer and Parrish, 2003). It consists of single-stranded DNA of about 5100 bases in length and can remain infectious in nature for days or weeks (Parrish, 1995). Disinfection requires the use of formaldehyde, glutaraldehyde, or chlorine solutions, as the virus particles are resistant to many common disinfectants including alcohol and quarternary ammonium compounds (Barker and Parrish, 2001).

As its genome does not include a code for DNA polymerase (an enzyme that is necessary for the first step of DNA replication), parvoviruses can only replicate in the nucleus of dividing cells. In this way, the virus makes use of the cell's own enzymes for replication, and virus spread is therefore the quickest in the most rapidly dividing cells of the body (Steinel et al., 2001). Partly because of the need for mitosis for virus replication, the clinical signs developed are dependent on the age of the infected animal. As fetal and newborn tissues are still undergoing rapid growth, young animals

(generally classified as under 4-6 weeks of age) have a different presentation of disease than older individuals infected (Barker and Parrish, 2001).

Pathogenesis

As noted above, the pathogenesis of parvovirus infections is dependent on the replication of DNA in cells, meaning that rapidly dividing tissues such as epithelial cells in the intestines, are most affected. In animals older than 4-6 weeks the pathogenesis and clinical presentation of disease is similar in all hosts (Steinel et al., 2001).

The primary route of infection is oral, with the cells of the nasopharynx and/or the tonsils being the first site of virus replication. This initial replication goes on for 1-3 days, whereafter virus can be isolated from most lymphoid tissues in the body, including intestinal mesenteric lymph nodes and Peyer's patches (Parrish, 1995). Virus then spreads through a plasma viremia and migrating lymphoid cells, resulting in a systemically spread infection (Barker and Parrish, 2001). Virus-shedding in feces is prominent 5 days post-inoculation (Parrish, 1990) and typically lasts 1-2 days in felines, although there are recorded cases of cats shedding virus in their feces up to 6 weeks after recovery from clinical disease (Greene, 2006).

Of infected animals over 6 weeks of age, many will acquire subclinical and/or mild disease. The most prominent clinical sign is usually diarrhea/enteritis, seen together with pyrexia, vomiting, lethargy, and/or leukopenia and/or lymphopenia. Severe infection will cause death, usually by destroyed gut epithelium giving rise to dehydration and possibly endotoxic shock (Parrish, 1990).

The range of clinical signs is mostly dependent on which cells are infected, which, as noted above, depends on the activity, growth, and rate of cellular turnover in that tissue. As can be detected from the clinical signs that follow, parvovirus in carnivores has tropism for mainly lymphoid and hematopoetic cells, as well as intestinal epithelium (Parrish, 1995). Hueffer and Parrish (2003) also confirmed the importance of cell-receptor-binding to parvovirus infection, and these cell-surface receptors appear to be most frequently displayed in intestinal crypt epithelium and on hematopoetic cells.

Diarrhea is a result of the Liberkühn cells in the intestinal crypts (the cells that generate the gut epithelium) being one of the main targets for parvovirus infection. Because the infection is lytic it leads to a substantial loss of gut epithelium (Steinel et al., 2001). This can be seen as flattened epithelium and shortened intestinal villi, leading to loss of osmotic regulation which, in turn, gives rise to the clinical signs of diarrhea, dehydration, pyrexia and endotoxic impact. The severity of disease is partly due to the turnover rate of the intestinal cells of that individual at the time of infection. Earlier devitalization of the epithelium might cause the turnover rate to increase and could lead to a more severely affected individual (Parrish, 1990).

The occurence of leukopenia or lymphopenia varies with the different viruses. Panleukopenia is a classical, prominent clinical sign in FPV infections of felines, but very uncommon in CPV infections of canines. Canines do, however, often display a relative lymphopenia (Parrish, 1990). Most infected animals show decreased cellularity of the bone marrow, with lessened numbers of myeloid, erythroid and megakaryotic cells. This is thought to be one of the reasons for depleted neutrophil levels (Parrish, 1995). The infection of lymphoid tissue causes cellular depletion and lymphocytolysis. The effects on lymphoid cell lines and bone marrow are not chronic, though,

meaning that they will be completely reversed if the animal survives the infection (Barker and Parrish, 2001).

The total white cell count of cats with feline panleukopenia can fall below 1000-2000 cells/mm^3, where neutrophils can account for less than 200 cells/mm^3. Lymphocyte numbers decline to a lesser degree, and there is usually little effect on eosinophil, basophil, monocyte or red cell counts unless blood is lost through gut lesions (Barker and Parrish, 2001). The lack of effect on erythrocytes may also have to do with their relatively long life span compared to the course of the disease (Parrish, 1995). Adding to the changes of hemogram (including anemia), parvovirus disease will also lead to electrolyte- and blood-gas imbalances due to vomiting, diarrhea and dehydration (Barker and Parrish, 2001).

In pregnant animals, the fetus is readily infected, leading to abortion or resorption in early pregnancies and abnormalities in later ones. Kittens born to queens infected by FPV are known to develop ”feline ataxia syndrome,” in which an infection of the cerebellum causes hypoplasia, giving rise to the clinical signs. The same can be true for young individuals (under 4-6 weeks of age) who are infected. On the other hand, enteritis is rarely developed as a sign of parvovirus infection in very young animals (Parrish, 1995).

Clinical parvoviral disease in raccoons

Wild raccoons in captivity are known to hide any signs of disease. In addition to this, literature suggests that parvovirus often gives rise to less prominent clinical signs in raccoons than in other carnivores. As mentioned before, the clinical signs of parvovirus are mainly generalized and unspecific such as lethargy/depression, diarrhea, vomiting, loss of appetite, loss of body condition, etc. The combination of mild, unspecific and generalized signs of illness even when severely affected by parvovirus makes it hard to define a clinical case either specifically or sensitively (Barker and Parrish, 2001; Kapil et al., 2010; Parrish et al., 1987; Steinel et al., 2001).

Canine Distemper Virus and its role in raccoons

Canine distemper is caused by canine distempervirus (CDV) within the family *Paramyxoviridae*, subfamily *Paramyxovirinae* and genus *Morbillivirus*. CDV has a wide host range; all families within the order *Carnivora* (therein *Procyonidae*) are susceptible to infection and disease (Williams, 2001). The disease is endemic in raccoon populations across North America, and these have even been implied as a reservoir for infection, spreading disease in turn to captive carnivores in zoological collections and conservation parks (Paré et al., 1999).

CDV is an important differential diagnosis when considering parvovirus infections in raccoons, as the clinical presentation can be almost exactly the same. Canine distemper classically presents with depression and a mild serous oculonasal discharge that turns mucopurulent as the disease progresses. Other very often-occurring signs are vomiting, fever and diarrhea, and animals that recover may be in very poor body condition. In raccoons there are many reports of a marked hyperkeratosis of the foot pads seen as thickening and the development of deep cracks. Neurological signs are commonly seen during advanced stages, or even up to 1-5 weeks after recovery, and the presentation depends on what part of the brain is affected, which can differ

between species. Raccoons often exhibit ”abnormal” behavior, but rarely turn aggressive (as some canines might). They more often present signs of cerebellar or vestibular damage, paresis or paralysis, aimless wandering, incoordination, myoclonus or convulsions that take the form of ”chewing gum” seizures (Williams, 2001; Paré et al., 1999).

Immunology

Innate immunity

All animals must be able to defend themselves against invading organisms, and have therefore developed defense mechanisms of varying degree. All vertebrates have innate immunity, which can be described as a second line of defense against infection after the physical barriers of the body. The innate immune response is a quick but non-specific reaction, carried out in minutes to hours. It consists of both cellular and chemical elements, whose purpose is to recognize and eliminate any foreign material. Antigen is recognized by being chemically different from normal body components, and detection initiates several cascade reactions to lead the response to effectively eliminate that certain foreign agent (Tizard, 2004).

One of the key aspects of the innate immunity is the body's ability to focus the defense mechanisms to the affected area, creating a site of inflammation. The chemical components (cytokines and complement) act both as messengers, calling white blood cells to the area, as well as contributing to and potentiating the elimination and breakdown of the antigens themselves. The cellular components work mainly to recognize and eliminate antigens and include macrophages, dendritic cells, NK cells and neutrophils (Tizard, 2004).

Innate immunity lacks any kind of memory, meaning that the response will be the same in duration and intensity no matter how many times a specific invader is encountered. Although memory might seem beneficial, it might also increase the time needed to initiate a full response when coming across a ”new” antigen. As it is, the innate immune system is always ready to respond immediately once a pathogen is encountered (Tizard, 2004).

Acquired immunity

An evolutionarily younger immunity has developed in advanced vertebrates, called acquired immunity. It is a specific response in the sense that it recognizes invaders, destroys them, and learns from the process. This response is described as the ultimate defense system of the body, its importance illustrated in human AIDS patients where loss of acquired immunity leads to overwhelming, uncontrolled infections and death. However, although acquired immunity is very effective, it is also quite slow at onset, taking days to weeks until the full response is mounted and effective (Tizard, 2004).

The main difference between acquired and innate immunity lies in the way foreign material is recognized. Where innate immunity uses preexisting receptors that bind to common antigens or molecular patterns that many antigens have in common, acquired immunity develops new and unique receptors randomly. Once it has found one that recognizes and/or neutralizes an antigen, the system remembers that pattern and will be able to respond more quickly if the invader is encountered a second time. Innate and acquired immunity are not two separate entities, but rather

work together for a common cause. Acquired immunity responds to many of the same chemical substances that control innate immunity, and the innate immune reaction itself is in a way the initiation of an acquired immune reaction. Acquired immunity is operated by way of T-lymphocytes and B-lymphocytes as effector cells and antibody-producing cells respectively. Acquired immunity also uses cells for processing and recognizing antigen as well as cells responsible for regulating the immune system so that the response is at the appropriate level (Tizard, 2004).

Microbial invaders can generally be divided into two broad categories. The first consists of organisms that originate outside of the body's cells, such as most bacteria and fungi as well as many protozoa and helminths. The second category includes organisms that generate or live intracellularly such as viruses, some bacteria, and some protozoa. Accordingly, the acquired immune system consists of two major branches called ”the humoral immune response” and ”the cell-mediated immune response.” The humoral immune response focuses mainly on extracellular pathogens and includes B-cells, Th2-cells and antibodies, while the cell-mediated immune response focuses on intracellular organisms brought into effect by Th1-cells (Tizard, 2004).

Development of the immune system

Young mammals are capable of mounting both innate and acquired immune responses directly at birth. However, since the fetus has developed in a sterile environment, any acquired immune response must be primary, with a prolonged lag period and low concentrations of antibodies. Also, the newborn animals' acquired immune system is skewed towards a Th2 (humoral) response. This cytokine pattern prevails until about 2 months of age in general (Day, 2007).

Unless immunological help is provided, newborn animals may be killed by infections that would present little threat to an adult. Immunological help is usually presented in the form of maternal antibodies, i.e. antibodies produced by the mother transferred to the fetus/newborn (Tizard, 2004). In carnivores (canines and felines) 5-10 % of the mother's IgG-levels may transfer to the fetus before birth, but the bulk of passive immunity must be gained by way of colostrum. This is due to carnivores having an endotheliochorial placenta, where the chorionic epithelium is in contact with the endothelium of maternal capillaries, allowing some antibodies to pass through (Day, 2007).

Canines and felines are capable of absorbing the highest amount of maternal antibodies at 36-48 hours of age. The gained level of protection varies between litters as well as littermates. Therefore both the antibody level of the mother as well as individual differences in the young, such as appetite and absorption capability, influence to what degree each individual will be clinically protected against infectious disease in early life. Similarly, the level of residual antibodies is the main factor deciding clinical protection of the young, rather than the actual age of the young (Chappius, 1998).

Immunoprophylaxis in the neonate

Although vaccination in neonates is generally not only an accepted but also considered a vitally important event, not much is known about the immune development of carnivores up to 6 months of age. Most vaccination studies start at 12 months of age, when these species are considered to be fully immunocompetent (Day, 2007). Still, there is evidence that although the immune response of neonates is different, it is not altogether lacking. In the event of failure of passive transfer of maternal antibodies, puppies have been seen able to respond to antigen at 2 weeks of age (Toman et al., 2002). Newborn puppies lacking maternal antibodies developed a serological response to a

modified-live parvovirus vaccine 21-91 days post-vaccination (Chappuis, 1998). Antibodies developed in neonatal animals tend to have a shorter half-life than in adults, but there is evidence of memory B-cells being elicited as part of a vaccine-induced immunologic response (Siegrist, 2007).

Kittens in general have levels of maternal antibody high enough for protection until 9-12 weeks of age. This number can vary greatly and some kittens lose protection at 6 weeks or younger, risking exposure to infection before vaccination. Because of this risk, the American Association of Feline Practitioners (AAFP) emphasized the importance of early vaccination of kittens in their Feline Vaccine Advisory Panel Report (2006), this being especially important in environments of high disease pressure such as catteries or shelters. The guidelines developed by this group stated that the final vaccine of an initial vaccination-series should be given at no older than 16 weeks of age, with 3-4 weeks lapsing in-between each dose. In practice, one dose at 8 and 12 weeks are seen as sufficient. However, recent studies of FPV vaccination under field conditions have shown that over one-third of the kittens had not seroconverted at 20 weeks of age despite three vaccinations (an “extra” dose given at 16 weeks of age) done according to the standard recommendations. To be noted, most of the queens in this study were middle-aged breeding-cats and therefore subject to routine vaccination which gave them high levels of circulating antibodies (Jakel et al., 2012). In contrast, a large proportion of 6 week-old kittens in animal shelters were found seronegative regarding FPV, indicating the lack of maternally derived neutralizing antibodies as well as protection against disease (Dawson et al., 2001).

Vaccination

Vaccination against specific diseases has both direct and indirect effects on the individual and on the population. Firstly, vaccination protects the individual from a specific pathogen in the sense that the individual, when exposed, will not get ill or only mildly ill. However, vaccination also protects the rest of the population since the effectively vaccinated individual will not shed virus to the environment or other individuals. If enough individuals are vaccinated, the amount of pathogens will decrease to the point where even non-protected individuals will not be exposed to enough agents to fall ill. This is the concept of herd immunity, which is a very important indirect effect of vaccination to keep in mind when trying to measure the effect of a vaccine on individuals (Giesecke, 2001)

Giesecke (2001) states that performing a randomized, clinical trial with one vaccinated group and one unvaccinated group which is given a placebo is one of the best ways of measuring a vaccination's protective effect, or efficacy. Giving the unvaccinated group a placebo product reduces the risk of bias by ensuring that all parties working on the project are fully blinded. Vaccine efficacy is a percentile measurement of how much the incidence decreases in a vaccinated population, and can therefore be used as a measurement of how much protection the vaccination offers. If the incidence of disease is the same in both study groups, vaccine efficacy is zero and if there is no incidence of disease at all in the vaccinated group, vaccine efficacy is 100%.

Principles of infectious disease management in animal shelters

It has long been widely concluded that animals in shelters or other environments with a high animal density and rate of transition of individuals should be routinely vaccinated. However, it is important to note that only vaccines with a proven effect against common and serious shelter diseases are indicated for use. The reason for only using vaccines where there has been proven effect in a shelter environment lies in the reason behind vaccinating individuals in shelters. The protection is not meant for individuals so much as protecting the shelter itself from infectious agents and thereby diminishing disease spread through the population as a whole. Also, many shelters are non-profit organizations with limited resources that should be put to use based on sound reasoning and documented effect (Robertson, 2011).

Developing infectious disease protocols and policies is the first step to preventing disease spread in an animal shelter, as recent understanding of population management has shown proactive measures to be more efficient to the cause than reactive measures. This planning requires taking into account the aim of the shelter, the value of the individual animals as opposed to the population as a whole, which individual diseases the shelter stand risk of acquiring and their potential effects, costs, funding and other resources such as facilities and available work-force. Preventative measures such as biosecurity, planned use of facilities, cleaning and disinfection routines, intake procedures and environments and handling designed to decrease stress are equally if not more important than medically reacting to a disease outbreak. If, during this stage of planning, the facilities are recognized as poorly equipped to handle certain serious infectious diseases, routines concerning eliminating sick individuals should be decided upon, such as transfer to another center or humane euthanasia (Robertson, 2011; UC-Davis Koret Shelter Medicine Program, 2010).

If prevention of a certain disease is considered to be best handled by vaccination, the vaccination should be done as part of the intake routines, i.e. before the animal comes in contact with the actual living environment of the shelter. Newly admitted and vaccinated individuals should be kept separate (in quarantine) if there is a lag-time between the vaccination and the acquired protection (Robertson, 2011).

MATERIALS AND METHODS

Animals and admission criteria

Orphaned raccoon kits were found and submitted to the WRCM by the general public from April to August, 2012. All kits underwent a physical examination by a staff veterinarian who then, based on certain criteria (weight and health status – see below), decided whether the individual could be admitted into the mammal nursery or was to be euthanized. All submitted and admitted raccoon kits of 2012 were part of the clinical study.

Cut-off weight for admission was set to 400 g because past experience at WRCM has proved that kits under this limit generally are too young and do not survive the nursery. Raccoons weighing 400-700 grams were admitted or euthanized on a case-by-case basis where health, good attitude (bright, alert, responsive), good hydration and good body condition were mandatory for admission.

More mature raccoon cubs (over 700 grams) were admitted as long as any findings on the initial physical examination were deemed to be non-significant and/or easily restorable and/or not

contagious, such as external parasites, mild-moderate dehydration or mild-moderate undernourishment. In the case of uncertainty, extra diagnostics such as a CBC (complete blood count) and/or parvo SNAP-testing was applied.

If a cub was found to have evidence of parvovirus or distemper (diagnosed by way of positive SNAP testing and/or leukopenia found on CBC), the whole litter was euthanized, as all individuals in one litter would have encountered the same disease agents. Both parvovirus and distemper virus are said to often give mild to subclinical disease, and raccoons in general do not have strong reactions to the viruses (Williams, 2001). Euthanasia of a litter in which one individual was suspected of distemper or parvoviral disease was therefore a measure of biosecurity, so as to diminish disease spread as much as possible.

Six euthanized individuals of suspected parvoviral disease were sent to the University of Georgia on request as material for a study concerning parvovirus in wild carnivores. These individuals were necropsied as part of that study, and the results of the necropsies were sent back to the WRCM.

Every individual patient had a specific identification number and patient chart. All charts were computerized using FileMakerPro 5. Clinical data on the status of the patients were continually entered into the charts by the veterinarians. Animal caretakers did not have access to these data. At admission, circumstances surrounding the finding of the litter of orphans were entered, as well as individual clinical status/findings on physical examination, weight, body condition score, approximate age, gender, and any potential diagnostic procedures (including results) that were undertaken at this time.

After the first physical examination at intake, raccoon kits were assumed to be healthy unless suspected otherwise. Animal caretakers were in charge of overseeing the health-status of the animals on a day-to-day basis. If any signs of illness were observed, a veterinarian was notified and the individual animal received a new physical exam.

Study design

A cohort study was designed with two parallel, independent groups. The study was single-blinded in the sense that the daily animal caretakers did not receive any information regarding the two separate groups, but the veterinarians (who made diagnostic, treatment and/or euthanasia decisions) administered and kept track of vaccination and therefore were not blinded. One group was given a dose of killed feline parvovirus vaccination at admission, and the other group was not given any vaccination at all. Assignment to the vaccinated or unvaccinated group was on a per-litter basis and done by randomly by drawing lots out of a box. A second dose of vaccine was administered to the vaccinated group after two weeks if the litter was still in-housed. In all other respects, the groups received the same treatment and handling and, in those cases that called for it, medical care. The two groups were housed in two different rooms of the same building. To accommodate room size, the two study groups were not the same size, but proportioned so that 1/3 of the study population was not vaccinated and 2/3 were vaccinated.

When the raccoons were judged by the responsible veterinarians to be mature and healthy enough, they were moved to outside caging. All outside caging was situated in the same enclosure, meaning that raccoons from different litters and study groups from here on cannot be said to have been separated from each other. All tests for laboratory analysis were taken before the move to outdoor

caging, but the clinical data collection continued until the raccoon kits were released into the wild. The raccoons were released into the wild when they were old and mature enough (generally 10-12 weeks, weighing 2-3 kg), healthy, and uncomfortable with humans (i.e. wild).

Orphan outreach program

Raccoon cubs of less maturity (400-700 grams), which therefore were in need of more care and/or more frequent feedings than supplied at the WRCM, were submitted to the Orphan Outreach Program (OOP). Specially trained volunteers at WRCM would then take these individuals to their private homes for caretaking. All OOP-patients were vaccinated as a precaution against potential shedding of virus in the private homes of volunteers, as parvovirus particles are extremely resistant in the environment.

When OOP-patients were old enough for the care given at WRCM to be sufficient, they were transferred back to the hospital and housed in the same room as the vaccinated study population. From there on, they were treated in the same way as all other patients.

Sampling

Two blood samples were collected from every admitted raccoon, first at admit and second right before the transfer to outdoor caging. The blood samples were centrifuged and at least 1 ml of serum was collected and stored in a freezer (-18°C) for analysis of antibodies during the following autumn. After the clinical study period, the frozen serum samples were inventoried and cataloged. Criteria for samples to be chosen for analysis were:

1. Sample 1 (taken at admission) and Sample 2 (taken before moving into outside caging) both existed, were correctly marked and readable.
2. The individual raccoon kits from which the samples were taken had been released (not euthanized or found dead in cage).
3. The individual raccoons from which the samples were taken had not shown any signs of disease during their stay at WRCM.

Serum samples were sent to Antech Laboratories (Eden Prairie, MN) for antibody analysis with a hemagglutination-inhibition (HI) test. Interpretation of the serum titer results was done via the interpretation information available from Cornell University (Ithaca, NY) as they had designed the test used. The end-point dilution of the test was set to 1:160 which would give an antibody serum titer level of 160. The lowest possible dilution was 1:20, and undetectable serum titer levels were given the value of <1:20. According to Cornell University, titers for FPV usually fall between 20 and 10240.

Definition of a clinical case of parvoviral disease

As parvoviral disease is often mild or subclinical and gives rise to generalized, vague signs, there is no single way to accurately define a case of parvovirus that is both specific and sensitive. Therefore, several definitions of disease were used in this study.

All raccoon kits were given an ”intake status” depending on the results of their initial physical examination. The intake status ranged from 0-3, defined as follows.

- Intake status 0: No sign/s of disease.
- Intake status 1: Solitary and/or mild clinical sign of disease.
  Any sign of disease that is deemed to be mild (by being rated as an insignificant finding) and/or easily treatable (such as external parasites, or mild-moderate loss of body condition). Any individual displaying more than one mild or easily treatable disease symptom were excluded from this group and ranked as intake status 2.
- Intake status 2: Several and/or moderate sign/s of disease.
  More than one mild or easily treatable sign of disease (see intake status 1) or at least one moderate sign of disease, such as dehydration (not rated as insignificant/in need of treatment).
- Intake status 3: Severe sign/s of disease.
  Displayed signs of disease were rated as severe if the individual died or was euthanized within 4 days of the physical exam without the condition having deteriorated or not resolved. (The four-day period was based on the incubation time for parvovirus).

The same scale was used to identify clinical cases of clinical disease during the in-housed period.

Finally, an incidence of cases of parvoviral disease was calculated. Whether each individual raccoon had clinical parvoviral disease or not was decided on a case-by-case basis according to the comprehensive picture gathered from the available information. Any individual showing a positive result in a laboratory test (SNAP-test) was rated parvovirus positive. Any individual found to have parvovirus on necropsy was rated positive. In these cases the day of the first clinical sign/s was said to be the day of ”disease outbreak” in relation to days at risk.

Essentially, no attempt was made to differentiate clinical parvoviral disease from clinical canine distemper in cases presenting with gastrointestinal disease signs (vomit, diarrhea) or generalized disease signs (lethargy, loss of appetite). All of these cases were rated as parvoviral infections. Cases presenting with only classical signs of canine distemper (neurologic signs, ocular discharge) were, however, not rated as ”parvovirus,” even when combined with generalized signs of disease (lethargy, loss of appetite).

In a few cases, diarrhea would show up as a single, passing event not combined with any generalized signs of disease and with a quick recovery. These were not rated as cases of parvovirus infection and, accordingly, did not contribute to the calculated incidence rate.

Calculation and Analysis

After the last orphan had been released into the wild and the collection of clinical data was finished, the data was compiled using spreadsheets manufactured with Open Office Calc. Incidence rates (IR) were calculated based on days at risk (DAR). DAR was the total number of days for each individual raccoon to stay at WRCM, from the day of admittance to the day the raccoon started showing signs of disease, the day the raccoon was released into the wild or the end of the study period, whichever came first.

The relative difference in IR between vaccinated and non-vaccinated was expressed as the incidence rate ratio (IRR) and estimated as:

$$\widehat{IRR} = \frac{\frac{a}{DAR_a}}{\frac{b}{DAR_b}} = {IR_v}/{IR_{nv}}$$

Where $\widehat{IRR}$ is a point estimate of incidence rate ratio, *a* and *b* are the number of cases in the vaccinated and non-vaccinated groups, respectively and DAR_x is the sum of days at risk observed in the groups. Confidence intervals (lower (L), and upper (U)) of $\widehat{IRR}$ were calculated using the following formulas (Sahai et Kurshid, 1996):

$$\widehat{IRR_L} = \left(\frac{DAR_b}{DAR_a}\right)\left(\frac{a}{b+1}\right)\frac{1}{F_{\alpha/2;2(b+1),2a}}$$

$$\widehat{IRR_U} = \left(\frac{DAR_b}{DAR_a}\right)\left(\frac{a+1}{b}\right)F_{\alpha/2;2(a+1),2b}$$

Where *a*, *b*, $\widehat{IRR}$ and DAR_x is as defined before, and **F** is a quantile of the F distribution (denominator degrees of freedom are quoted last).

The calculations were made only with patients who met certain clinical requirements, namely having an intake status (see above) of 0 or 1 only. This was to achieve as standard and reliable results as possible while diminishing the risk of bias as much as possible. Signs that immediately excluded the patients from the calculations were any sign of infectious disease, lethargy, depression or severe loss of body condition at intake.

Vaccine efficacy (VE, %) was calculated according to the following formula:

$$VR = \frac{IR_{nv} - IR_v}{IR_{nv}} * 100$$

in which IR_{nv} and IR_v are the incidence rate of parvoviral disease in the unvaccinated and vaccinated group, respectively (Giesecke, 2001).

Differences in serum titer levels at Sample 1 according to age or group (vax/nonvax), as well as differences in change in serum titer levels between Sample 1 and 2 according to group, titer level at Sample 1 or number of days between samples was tested with linear regression methods, after the titer levels had been log-transformed to achieve reasonably normally distributed values. Undetectable levels (<1:20) were assigned 1:10 for these calculations.

RESULTS

A total of 201 individual raccoon cubs were admitted into the WRCM mammal nursery in 2012, and served as the study population for this study. Of these, 37 individuals were completely omitted from the calculations due to missing or confounding crucial data information such as whether the individuals were vaccinated or not. The non-vaccinated study group held 46 individuals, and the

vaccinated study group consisted of 116 individuals in which 22 were OOP-patients, meaning that the number of individuals in the vaccinated, completely in-housed study group was 94. Table 1 displays the average clinical parameters for admitted raccoonkits. Interestingly, there are a couple of parameters where the proportions differ between the groups. Some of them, such as sex, probably have no influence on responses to vaccination while others, such as initial health status, very well could.

The overall incidence rate of any displayed sign of disease was 2.5 (81/3288) and the total mortality (death or euthanasia) was 33% (54/162) as seen in Table 2. These calculations are made including all of the raccoon nursery patients.

Table 1. Physical findings and clinical status in admitted raccoon-kits at admission

	OOP[a] (n=22)		VAX[b] (n=94)		NONVAX[c] (n=46)		ALL (n=162)	
Mean weight, *kg* (range)	0.47	(0.39-0.57)	0.81	(0.43-2.1)	0.76	(0.57-2.2)	0.68	(0.39-2.2)
Mean age, *weeks* (range)	4.4	(4-5)	5.8	(4-12)	5.6	(4-7)	5.3	(4-12)
Mean litter size *no. kits* (range)	2.8	(1-5)	2.7	(1-8)	2.6	(1-5)	2.7	(1-8)
Gender (M//F//Undetermined)	12 // 8 // 2		36 // 53 // 5		26 // 19 // 1		74 // 80 // 8	
Intake status 0	27.3%	(n=6)	71.3%	(n=67)	50.0%	(n=23)	59.3%	(n=96)
1	40.9%	(n=9)	13.8%	(n=13)	32.6%	(n=15)	22.8%	(n=37)
2	13.6%	(n=3)	9.6%	(n=9)	8.7%	(n=4)	9.9%	(n=16)
3	18.2%	(n=4)	5.5%	(n=5)	8.7%	(n=4)	8.0%	(n=13)

[a]OOP= Orphan outreach program, [b]VAX= vaccinated kits, [c]NONVAX= unvaccinated kits

Table 2. Clinical information for admitted raccoon-kits while in-housed

	OOP[a] (n=22)		VAX[b] (n=94)		NONVAX[c] (n=46)		ALL (n=162)	
Mean days in-house (range)	29	(2-56 d)	24	(1-44 d)	22	(1-48 d)	24	(1-56 d)
Additional diagnostic testing	9.0%	(n=2)	21.3%	(n=20)	32.6%	(n=15)	22.8%	(n=37)
Received medical treatment	50.0%	(n=11)	17.0%	(n=16)	21.7%	(n=10)	22.8%	(n=37)
Died in cage	9.0%	(n=2)	7.4%	(n=7)	13.0%	(n=6)	9.3%	(n=15)
Euthanized	22.7%	(n=5)	19.1%	(n=18)	34.8%	(n=16)	24.1%	(n=39)
Released	63.6%	(n=14)	74.5%	(n=70)	52.2%	(n=24)	66.7%	(n=108)

[a]OOP = Orphan outreach program (total time spent "in care"), [b]VAX = vaccinated kits, [c]NONVAX = unvaccinated kits

As seen in Table 3, 51.8% of the admitted raccoon cubs showed at least one sign of disease at least once during their stay. Cases when daily caretakers have alerted the veterinarians to clinical signs in a raccoon, but the physical exam reveals no findings (the individual is rated clinically healthy) are rated as ”no clinical signs”. If the veterinarians have been notified about ”diarrhea in cage” but the diarrhea cannot be connected to any individual, nor does any individual raccoon display any clinical signs at observation, the finding has not been included in the calculations.

Table 3. Clinical signs of disease presented while in-housed (n=162)

	Number of individuals	% of all individuals (n=162)	% of individuals with multiple clinical signs (n=84)
No clinical signs	78	48.1	-
Mulitple clinical signs	51	31.4	60.7
Only one clinical sign	33	20.4	39.3
Specific clinical signs			
Diarrhea	38	23.5	45.2
Eye discharge (bilateral)	22	13.6	26.1
Vomit	15	9.3	17.9
Leukopenia	13	8.0	15.5
Lethargy	12	7.4	14.2
Quiet	10	6.2	11.9
Dehydration	10	6.2	11.9
Loss of appetite	8	4.9	9.5
Ataxia	5	3.1	6.0
Raw feet	5	3.1	6.0
Bloated	5	3.1	6.0
Skin lesions	5	3.1	6.0
Anemia	4	2.5	4.8
Chewing-gum seizures	3	1.9	3.4
Blind	3	1.9	3.4
Raw nose	3	1.9	3.4
Partially blind	2	1.2	2.4
Hypothermia	2	1.2	2.4
Green feces	1	0.6	1.2
Crashing	1	0.6	1.2
Dyspnea	1	0.6	1.2
Pneumonia	1	0.6	1.2
Head tilt	1	0.6	1.2
Circling	1	0.6	1.2
Disoriented	1	0.6	1.2
Aggressive	1	0.6	1.2
Osteomyelitis	1	0.6	1.2

Only animals with intake status 0 or 1 were included in the calculation of effects of vaccinations. This left 95 individuals in the vaccinated group of which 15 were OOP-patients, and 38 individuals in the non-vaccinated group. Table 4 describes the calculated IR's of parvoviral disease in the study groups and the IRR, including a 95% confidence interval, comparing vaccinated with unvaccinated groups. Finally, the calculated vaccine efficacy is shown in the table.

Table 4. Incidence of parvoviral disease while in-housed

	VAX[b] (OOP[a] included)	NONVAX[c]	VAX[b] (OOP[a] excluded)
No. individuals	95	38	80
No. cases	28	18	24
Sum DAR[d]	2145	745	1667
Incidence rate of "parvovirus"	0.013	0.024	0.014
Incidence rate ratio	0.54		0.60
(95% confidence interval)	(0.29 – 1.04)		(0.31 – 1.17)
Vaccine efficacy	46%		40.4%

[a]OOP= Orphan outreach program, [b]VAX= vaccinated kits, [c]NONVAX= unvaccinated kits, [d]DAR=days at risk

Table 5 describes the overall disease outbreak concerning the in-housed raccoon cubs. Again, only individuals with intake status 0 and 1 have been included in the calculations. The scale of mild, moderate and severe clinical signs follows the same definitions as described for intake status (see Materials and Methods).

Table 5. Incidence of clinical signs of disease while in-housed

	Solitary/mild symptoms		Several/moderate symptoms		Severe symptoms	
	VAX[a]	NONVAX[b]	VAX[a]	NONVAX[b]	VAX[a]	NONVAX[b]
Number of cases	27	16	19	9	10	6
Sum DAR	1439	434	1634	558	1820	639
Incidence rate	0.019	0.037	0.012	0.016	0.0055	0.0094
Incidence rate ratio	0.51		0.72		0.58	
(95% confidence interval)	(0.26–1.01)		(0.31–1.81)		(0.33–3.30)	

[a]VAX= vaccinated kits, [b]NONVAX= unvaccinated kits

Table 6 shows the results from the serological analyses. There was no significant association between age at Sample 1 and the serum titer level (p=0.323) or group (p=0.303). This was the case

both in general (all individuals included) and when only comparing levels within a group (vax/nonvax). There was no significant difference in the change in serum titer levels between Sample 1 and Sample 2 between vaccinated and unvaccinated kits (p=0.434) or days between the samples (p=0.912).

Two raccoon cubs (12-4751 and 12-2823) had undetectable titer levels (<1:20) at admission and both experienced a rise in titers between sample 1 and sample 2. However, there was in general a significant negative association between titer level at Sample 1 and the change in levels (p=0.001).

Table 6. Results from the serological study

Raccoon ID	Age at admission (weeks)	Sample 1	Sample 2	Number of days between samples
VAX[a]				
12-4751	10	<1:20	1:40	7 days
12-4752	10	1:80	1:40	7 days
12-2652	7	1:640	1:20	11 days
12-3939	7	1:320	1:80	14 days
12-3427	7	1:2560	1:2560	15 days
12-3938	7	1:320	1:80	15 days
12-3941	6	1:160	1:40	16 days
12-3973	7	1:160	1:40	16 days
12-2389	5	1:1280	1:160	19 days
12-2391	6	1:640	1:160	19 days
12-3061	6	1:2560	1:320	19 days
12-3190	6	1:80	1:20	19 days
12-2650	7	1:40	<1:20	20 days
12-2860	7	1:320	1:80	20 days
12-3426	7	1:320	1:80	21 days
12-3187	6	1:640	1:160	24 days
12-2820	5	1:20	1:20	29 days
12-2823	5	<1:20	1:80	29 days
OOP[b]				
12-2196	4	1:40	1:20	31 days
NONVAX[c]				
12-3911	6	1:320	1:640	14 days
12-3336	7	1:640	1:80	22 days
12-3201	6	1:320	1:40	24 days
12-3202	6	1:160	1:40	24 days
12-3204	6	1:320	1:40	24 days
12-3203	6	1:320	1:40	24 days

[a]VAX= vaccinated kits, [b]NONVAX= unvaccinated kits

[c]OOP= orphan outreach program

DISCUSSION

Clinical results

As shown in Table 4, there was a higher incidence of parvoviral disease in the unvaccinated group than in the vaccinated group. According to these results, there is a reduced risk (relative risk = 0.54) to acquire parvoviral disease for an individual that is vaccinated compared to an individual that is not vaccinated against FPV. The effect of vaccination is nearly significant ($p = 0.07$) and indicates that vaccination against FPV may have a protective effect.

As the main goal of the study was to determine whether or not vaccination would give protection towards disease acquired while housed at WRCM, raccoon kits presenting with more than one trivial or at least one serious sign of disease (intake status 2-3, see Table 1) were omitted from the calculations. Signs of disease considered trivial and/or easily treatable included external parasites, mild-moderate decreased body condition and any other singularly occurring sign in a patient that was found ”clinically healthy” despite that sign. The proportion between the numbers of cubs admitted with a certain intake status varied between the groups (as is seen in Table 1) even though selection was made randomly. It is possible that these variations may have had effect on the outcome of the clinical study, as initial health is an important factor when disease is concerned. This study does not have the means for advanced calculations, but the question is intriguing and deserves to be looked at more thoroughly so as to come to a more definitive conclusion.

One reason to be careful while applying these results is seen in Table 5, where the incidence of mild or single clinical signs of disease is also less in the vaccinated than in the non-vaccinated group. There seems to be a relatively 0.49 lower risk of acquiring *any* sign of disease or illness (examples displayed in Table 3) if an individual raccoon kit is vaccinated against feline parvoviral disease. This finding seems to make no sense immunologically. However, parvovirus affects the cells of the immune system not only indirectly through the immune response but also directly, infecting the rapidly dividing hematopoietic cells and causing imbalances such as leukopenia. One could argue that since the majority of clinical cases of parvovirus are mild or subclinical, the raccoon kits could have gone through an unnoticed parvovirus infection which left them immunologically reduced. This would then make the kits more susceptible to other infectious agents which would cause signs of disease strong enough for the kits not to be able to hide them, and therefore be noticed by the animal care crew.

There are a few intriguing cases from the clinical study where the above hypothesis could be an explanation. Two litter mates in the unvaccinated group (12-2695 and 12-2696) were brought to the veterinarians because of being quiet and having bilateral ocular discharge. Clinically, these raccoon kits were suspected of CDV infection, but parvoviral SNAP testing was done “just in case” and turned out positive, meaning that these individuals were shedding parvovirus. The individuals were sent to Georgia for necropsy, which confirmed parvoviral infection. With these cases in mind, a vaccinated raccoon kit in the orphan outreach program (12-3142) was brought to the veterinarians severely lethargic and dehydrated with diarrhea but tested SNAP negative for parvovirus. The individual was euthanized with CDV as the primarily suspected diagnosis, but nevertheless sent to Georgia for necropsy and came back as positive with parvoviral infection. (The fact that this

individual was listed as vaccinated should not be taken too seriously as it had an intake status 3 and was euthanized within 4 days of admission which is within 4 days of receiving a first dose of vaccine).

In both these scenarios one could argue that a subclinical parvovirus infection might have weakened the immune system enough for the overall pressure of disease agents, which might otherwise have been contained and dealt with, to result in clinical disease. . Immunosuppression as a consequence of clinical parvovirus infections are well-known (Steinel et al., 2001) and the same is probably true for subclinical infections to a certain extent. Accordingly, this could help explain why the raccoon kits in the vaccinated group suffered less signs of disease and were euthanized or died to a lesser extent than the kits in the unvaccinated group.

It is important to keep in mind that the above argument is only valid as long as vaccination has any effect. As we know from past experience, vaccination of the raccoon cubs does not mean that they will not develop clinical parvoviral disease. An example of this can be seen in two litter mates in the vaccinated group (12-4006 and 12-4007) where one was found to have a slight loss in appetite and to be mildly quiet. The raccoon was tested SNAP positive. The cage mate had no clinical signs of disease and SNAP tested negative. They were euthanized and sent to Georgia where the diagnosis of parvovirus was confirmed for both. These individuals had an intake status of 0 and ten days had gone by since their admission and initial dose of vaccination.

Another aspect of the results of this study could be the low intake number of raccoon kits compared to other years. Lower numbers of animals mean less dense populations, giving a lower pressure of disease agents. The findings of this study may turn out to be valid only as long as the population density does not overcome a certain degree, where the pressure of disease agents would become too high for the difference in the kits' immunologic resilience (due to vaccination) to have significant clinical effect.

Finally, in any study exclusion and inclusion criteria are extremely important. However, in a clinical situation there will never be only easily distinguished cases. This is especially true in the case of parvovirus, as it is very difficult to define a parvovirus case either sensitively or specifically on a clinical basis (see the Introduction for further details). Displayed clinical signs are generalized, non-specific signs of illness, which could be due to a number of varying causes, infectious as well as non-infectious. Had this study been about a disease easily diagnosed both sensitively and specifically, the fact that any other signs of disease also diminished with vaccination might be taken as a warning of existing bias. However, as the study is on parvovirus, the finding of reduced illness in general is important and must be given serious consideration.

Serological results

In this study, only two out of twenty-five raccoon kits (12-4751 and 12-2823) had undetectable titer levels (<1:20) at admission. Both of them were in the vaccinated group and had, by the time of Sample 2, experienced a rise in titer levels. There is only one other raccoon kit sampled (12-3911) that had a rise in antibody levels during its stay and, interestingly, this raccoon was part of the unvaccinated group. In all other cases, the antibody levels dropped between sample 1 and sample 2 with the exception of case 12-2820 in which the levels stayed constant. To be noted, kits 12-2823

and 12-2820 were also of the same litter. As the sample size is quite small, caution must be taken when drawing conclusions. The fact that many of the raccoons come from the same litters further presses the need to be careful before applying the results.

There was no significant association between titer levels and age at admission, nor could any statistical significance be determined regarding the relative change in titer levels over time between the vaccinated and unvaccinated groups. Raccoon kits that came in with the highest titers had comparatively the lowest titer levels by the second sample. This finding is not surprising, as antibody levels decrease exponentially.

Paré et al. (1999) studied vaccine efficacy for Canine Distemper Virus in raccoon pups who were given three doses of modified-live CDV-vaccine at 8, 12, and 16 weeks of age. The study showed seronegative (no maternal antibodies) 8-week-old raccoon pups responding within 14 days after receiving the first dose, and reaching peak antibody levels after 4 weeks. However, in another study group, raccoon kits with maternal antibodies were vaccinated according to the same routine, and 7 out of 8 pups failed to develop a rise in their CDV antibody titers until they were at least 18 weeks old.

When comparing the study of Paré et al. (1999) to the results in Table 6, it is not far-fetched to presume that the antibody titers measured in Sample 1 are due to maternal immunity. The age of the raccoons suggests that maternal antibodies are still present, and the fact that most titer levels decreased in spite of vaccination seems to strengthen the theory. Had the antibodies been produced individually by the kits because of natural exposure to infection, the titers would most likely have risen. As there are at most 17 days between a raccoon's last dose of vaccine and Sample 2, it is highly unlikely that the antibody levels would have had time to first increase and then decrease several times below the level present at Sample 1.

Maternal antibodies interfering with vaccination has been seen in other studies as well. Jakel et al. (2012) performed a vaccination study of kittens under field conditions. Even kittens with maternal antibody levels as low as <1:10 were found not to seroconvert after several vaccinations. The authors concluded that even levels of maternal antibodies too low to give protection seem to interfere with vaccination.

The serological results displayed in Table 6 are at first a bit disappointing, as they do not explain how or why vaccination against FPV seems to have protective effect. However, when analyzing the results, one must not forget to take background information into account. As described in Materials and Methods, only serum samples from raccoons that were released and healthy throughout their stay at WRCM were chosen for analysis. Over half of the study population showed at least one clinical sign of illness sometime during their stay (see Table 3). The raccoon kits that did not fall ill during their inhoused period would logically be those with the most sufficient levels of maternal antibody at admission. It would have been interesting to also analyze samples (Sample 1) from raccoons that later started showing signs of disease and/or were euthanized. Perhaps these individuals generally had less maternal antibodies and therefore were more susceptible to disease? This would of course mean that they would also be the individuals who benefit from vaccination. The clinical part of the study has shown that vaccination does offer extra protection, and that finding cannot simply be cast aside because of the serological study on a limited number of samples not showing any significant differences.

Another point of the discussion concerning vaccination against parvovirus in raccoons is the question of vaccine-type. Live strains of vaccine should be used only in the species they are manufactured for, as the risk of clinical illness is too great when inoculating a foreign species. Additionally, there is always a potential risk of reversion of the attenuated strain to virulence in an exotic host (Greene, 2006). Paré et al. (1999) used a modified live vaccine against CDV. As mentioned before, a killed vaccine is used at WRCM against FPV. There are no studies in which raccoons have been vaccinated using a modified-live FPV vaccine. Some modified-live vaccines have been shown to have a greater efficacy, giving rise to higher serum titer levels at younger ages and/or with fewer doses required, although studies showing that this would be true for all antigens are lacking (AAFP, 2006). As there is no evidence that modified-live vaccines against FPV would offer protection for raccoons, it is wise to keep using the killed alternative. However, if modified-live FPV vaccines were shown to protect raccoons and not lead to disease, they would be recommended because of their higher efficacy and less doses needed for protective antibody titers. The last statement is particularly important as the protection against disease is mainly of concern during the short period in which the kits are at the WRCM.

Virus strains

One of the drawbacks of this study is the lack of background information. Feline and canine parvovirus and their host-ranges in wild and domestic animals were extensively studied by Parrish et al. among others during the 1980's and 1990's. However, in the past two decades, hardly any new research has been done within this area regarding wild carnivores (Kapil et al., 2010). In recent years there have been increasing reports concerning suspected and/or confirmed CPV-related virus strains in cat populations worldwide (Ikedaa et al., 2000; Truyen et al., 1996; Decado and Buonavoglia, 2012). Indeed, in some studies over 80% of the virus isolates from feline populations in Taiwan and Vietnam were CPV-2-related rather than FPV-related (Ikeda et al., 2000). There is reason to believe that the new antigenic variants of CPV-2 have both increased pathogenicity for canines as well as an extended host range. The increased pathogenicity is among other things evident in the increasing incidence of severe disease in older dogs as well as dogs that have a sufficient vaccination record (Decado and Buonavoglia, 2012).

Appel and Parrish (1982) concluded that raccoons are not susceptible to CPV, but if that virus strain has evolved in 30 years (as we know it has), this conclusion might no longer be accurate. Indeed, Kapil et al. (2010) recently isolated a CPV-2-related virus from a raccoon in the United States, indicating that CPV-2 or CPV-2-related viruses are able to infect raccoons. Further confirmation regarding the significance of this finding is needed.

If future research should show that a large part (or even a majority) of northern raccoons are infected with CPV-related parvovirus and not FPV-related parvovirus, the vaccination routines at WRCM must certainly be revised. Perhaps vaccinating raccoons with canine parvovirus strains would give greater protection than feline. However, as of today, all CPV-vaccines are based on the original strains of CPV isolated in the 1970’s and 1980’s. There is an urgent need for controlled studies regarding different viral strains, cross-immunity and a development of new vaccines (Truyen, 2006). Before this has been done, there is no point in changing to CPV vaccination of the raccoons at WRCM, as Appel and Parrish (1982) have already concluded that raccoons are not susceptible to the original strain of CPV.

Prevalence

There are no studies concerning the prevalence of parvovirus in the northern raccoon population of Minnesota. The closest study is from Ontario, Canada in 1983 where sero prevalence was measured to 22.3% (Barker et al., 1983). This prevalence was, however, calculated from a very small study population of 112 individuals, which means it is not a strong study. Also, the number of survivors should logically be quite a bit lower than the morbidity, as individual cases often cannot survive without supportive therapy. In addition to this, there is no data concerning the half-life of antibodies developed during a parvoviral infection. If a female raccoon develops and survives parvoviral disease, the question is how long those titers will remain high enough for a significant amount to be able to pass over to the colostrum once that female starts reproducing.

However, as parvovirus particles are known for being very stable in the environment, surviving most common disinfectants as well as varying temperatures, there is in theory no need for clinical outbreaks in order to keep the infection endemic and active within the raccoon population. In fact, fomites are an important factor in spreading parvovirus, making the actual animal-to-animal contact time less important (Greene, 2006). The fission-fusion social structure of raccoons is dynamic with many short-term acquaintances and overlapping territories, meaning that new individuals and groups are continuously combining and separating (Prange et al, 2011). This system seems optimal for keeping the infection ubiquitous, which could in theory provide the ”booster” for keeping high neutralizing antibody titers.

Study design

The ultimate goal of this study was to provide the Wildlife Rehabilitation Center of Minnesota with a scientific background regarding the efficacy of vaccinating raccoon kits in the mammal nursery against feline parvovirus. For the results to be as accurate and practically applicable as possible, the study was performed under field conditions, knowing that these conditions lead to a greater risk of bias. Results must therefore be carefully analyzed in regard to surrounding factors and influences.

All in all, the study proceeded as planned. The subjective impressions of the WRC staff concluded that the summer of 2012 passed quite easily as far as the raccoon kits were considered, without any major disease-outbreaks or other mishaps. However, there are factors present which potentially contribute to bias in the data. Firstly, the number of admitted raccoon pups was substantially lower than expected, giving less power to the study and the results than if the number of admits had followed the same trend as in the last few years. As the total number of individuals was quite low (statistically speaking), a bigger difference between the groups would be needed to be able to say that the difference in incidence was indeed significant.

Secondly, the study was designed to be single-blinded, meaning that the crews of daily animal caretakers were not informed about which of the two groups was vaccinated. These crews handle the day-to-day caretaking of the animals, including feeding, cleaning cages and in other ways supervising. If a raccoon was showing signs of disease, the veterinarians were notified and the animal was taken in for a physical exam. Depending on the results of the physical exam, the individual raccoon was deemed healthy (no treatment), unhealthy but treatable (received treatment) or unhealthy and not treatable/poor prognosis (euthanasia).

All raccoons were given a physical examination at/before intake, and this is the only entirely comparable parameter for health. After the initial exam, raccoon kits were only examined if/when they were observed by the animal caretakers as displaying some sign of disease. Therefore, there is a certain bias in the animal caretakers' ability to notice signs of disease. This is particularly difficult in wild animals as they try to hide any signs of illness. More importantly, the caretakers were blinded, with respect to treatment, so any bias would most likely be non-differential, and thus err towards the null hypothesis of no difference between the groups. Again, the low intake number of 2012 would mean that each animal had more time and a bigger chance of being observed as showing signs of disease, even when mild, than in other years when the intake numbers have been twice as high.

As the veterinarians were in charge of performing vaccinations and keeping track of the two groups, they were not blinded, which makes up a risk for bias. One could argue that the veterinarians might have treated raccoons in the vaccinated group to a larger extent, rather than euthanizing them, thinking that they were protected or had less chance of spreading disease as room-mates would be protected. Also, the veterinarians could have been more prone to draw the conclusion that the clinical signs exhibited were not caused by an infective agent (parvovirus) in the vaccinated group. As the majority of clinical parvoviral disease is mild or subclinical, a slight imbalance in treatment versus euthanasia between the groups could have a large impact on the results, as symptomatic treatment is the described regime for parvovirus infections, and even severe cases can recover with adequate (albeit intensive) therapy (Greene, 2006).

Thirdly, there was also an aspect of housing. The WRCM is a wildlife hospital with a limited number of rooms, which are all in regular use. In past years, the raccoon nursery has been located in two rooms, where one room has been used in the manner of isolation or quarantine, providing housing for raccoon kits displaying clinical signs of what could be infectious disease. This year, it was decided to use one room for each of the two groups (vaccinated and unvaccinated), eliminating the opportunity of isolating potentially infectious individuals. This procedure can have led to an overall increase in the load of infectious agents compared to previous years. However, as there were no major disease outbreaks this year, the pressure of infection does not seem to have been overwhelming.

The use of these rooms for the study may also have skewed the results as the two rooms are of different sizes, with a ratio of about 2:3. To try to compensate for the difference in the load of infectious agents that this causes, the vaccinated and unvaccinated group have also been proportioned about 2:3. Still, the proportions are not exact, and this must be kept in mind while comparing and analyzing the results.

CONCLUSION

This study has shown that orphaned raccoon kits that are taken into the WRCM benefit from vaccination against FPV. Vaccinated individuals had a 0.54 relative risk of acquiring clinical parvoviral disease compared to unvaccinated individuals ($p = 0.07$). Serological testing for antibody titers was done for a few individuals in each study group. No significant difference was found between the vaccinated and unvaccinated individuals regarding antibody titers.

Vaccination against FPV seems to have protective clinical effect, and therefore the WRCM should continue vaccinating the orphaned raccoon kits at intake. However, as no significant findings could be seen using serology, the questions as to how and when vaccination should optimally be carried out regarding maternal antibodies as well as what kind of vaccine is the most effective (what strain of parvovirus raccoons are mainly infected by) still remain to be answered.

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