Raccoon-pox (RCN)
cross-reacting antibodies in prairie dogs

Do they interfere with RCN-vectored plague vaccination?

Research report

Student: drs. G.M. Bron
Supervisors: Dr. T. Rocke and Prof. A. Gröne

Dutch Wildlife Health Centre, Utrecht University, Faculty of Veterinary Medicine, Utrecht, NL
USGS, National Wildlife Health Center, Madison, Wisconsin, USA
December 2010

Preface

This research report describes the journey of a three month research project that is part of the Veterinary Medicine rotations from the Faculty of Veterinary Medicine of the Utrecht University, The Netherlands. The research was conducted at the USGS - National Wildlife Health Center, Madison, Wisconsin, United States.

The aim of this research report is to reach out to people who are curious about research and scientists. The first I would like to tell an exciting story on research. The latter I would like to challenge to think about the time when you got your feet wet in research and of course I am interested in your opinion on my methods, results and conclusion.

This research was conducted at the plague-research group, where an oral virus-vectored vaccine against plague in prairie dogs is being developed. The vector of this vaccine is a raccoon poxvirus (RCN). The first objective of this research was to develop an ELISA to detect (cross-reacting) antibodies against RCN in prairie dog serum samples. The second objective was to see if (cross-reacting) antibodies to RCN interfere with the vaccine.

It was a journey in which I got my feet wet in the research- and wildlife world. It is an exciting world that kept me up at night, more than once.

Enjoy!

Index

Contents

Summary 4
1. Introduction 5
  1.1 Prairie dogs 5
  1.2 Plague 5
  1.3 The National Wildlife Health Center plague research 5
  1.4 Orthopoxvirus 5
  1.5 Aim of study 5
2. Study design 5
3. Methods and Materials 5
  3.1 Population 5
  3.2 Samples 5
  3.3 Vaccine groups 5
  3.4 Optimizing ELISA 5
  3.5 Screening ELISA 5
  3.6 Side-issue: serum color 5
4 Results 5
  4.1 Optimizing ELISA 5
  4.2 Screening ELISA 5
  4.3 Side-issue: serum color 5
5. Discussion 5
  5.1 Optimizing ELISA 5
  5.2 Screening ELISA results 5
6 Conclusion 5
Acknowledgement 5
Reference list 5
Appendix 5

Summary

Black-footed ferret *(Mustulus nigripedes*) families and prairie dog (*Cynomys*) colonies can be decimated by 90% due to plague (*Yersinia pestis* infection). The prairie dog has a key role in the lifestyle of the black-footed ferret and various other endangered species. Besides that, one of the four prairie dog species of the United States is endangered itself, the Utah prairie dog. To protect the prairie dogs from great losses due to *Yersinia pestis* infection, the USGS – National Wildlife Health Center is developing a vaccine against *Yersinia pestis* in cooperation with other institutes. This vaccine is a raccoon poxvirus-vectored plague vaccine, which is administered orally. Raccoon pox (RCN) is an orthopoxvirus (large, DNA virus) and one of the three orthopoxviruses (OPX) endemic in North America. Skunkpox, volepox and RCN cause mostly asymptomatic infection in rodents and are known to give cross-immunity. The prevalence and host distribution of these viruses is not known. Prior exposure of the prairie dog to OPX could therefore interfere with the immune response to a RCN vectored vaccine.

In this research an indirect ELISA against prairie dog RCN antibodies was developed. Pre-vaccination and post-vaccination samples (from previous NWHC research) of 200 prairie dogs (3 different species) were screened by this ELISA. The aim was to answer the research questions: Are prairie dogs previously exposed to orthopoxviruses and does this interfere with the oral raccoon poxvirus plague vaccine? The results (optical density) of the ELISA were converted to ELISA values (test OD – negative control OD) / (positive control OD – negative control OD) and combined with the survival data. These data were analyzed. The screened animals were either vaccinated with a RCN-vectored plague vaccine, a RCN-vectored placebo or a plague vaccine injection. All animals were challenged with *Yersinia pestis.* If animals received the RCN-vectored plague vaccine a higher ELISA value ratio (EV post-/ EV pre-vaccination) gave significant better survival chances. In a plot of the RCN vectored plague vaccine animals (96) pre-vaccination ELISA values (pre-EV) were shown against post-vaccination ELISA value (post-EV) by survival. This plot showed 2 animals that had high pre-vaccination EV% (>90%) and a lower EV% post-vaccination, these animals died. This suggested that previous exposure to an OPX and enough cross-reacting antibodies to prevent the vaccine from replicating, F1 and V titers post-vacination were identical to pre-vaccination. In the plot 3 animals with an average EV% pre-vaccination and a high EV-ratio (1:2) died. The strong immune response to OPX, without enough antibody development against *Yersinia pestis*, can be explained by a booster reaction to OPX.

The method used in this research gave very low optical densities and no positive or negative control could be used as a known reference. Further development of the ELISA is recommended. Furthermore the prairie dogs used for this research were from different species (sensibility to *Yersinia pestis* varies between species) and different vaccine types were used. Also the samples used were stored for different periods and probably frozen and thawed multiple times.

This research has shown that the RCN-vectored plague vaccine gives better survival chances. These higher survival chances seem due to the response to the vaccine and not to individual pre-vaccination or post-vaccination statuses. However, the answer to the main question whether prairie dogs were previously exposed to orthopoxviruses and whether this interferes with the oral raccoon poxvirus-vectored plague vaccine remains uncertain. Further research can be done on various scales to answer these questions. First the post-booster vaccination samples could be screened. From the animals that are suspected of previous exposure, PCR for OPX virus can be performed on tissue samples. An animal trial can be done, by infecting prairie dogs with an OPX followed by vaccination. On a scientific level it would be interesting to look into the immune system of the prairie dog. The immune response needed to survive a plague infection in different species is known to be humoral and cellular.

1. Introduction

Nature is a complex system, a lot of (if not all) processes are connected. Human beings are in a continuously need of more land to produce food or create homes. Less space is left for wildlife and species are going extinct or adapt to their changing surroundings. In the United States, a large part of the country consisted of grassland, but with the arrival of the European settlers grassland was quickly converted to agriculture land. The prairieland flora and fauna were disrupted in their delicate balance. An intriguing example is the story of the black-footed ferret (*Mustulus nigripes*) and the prairie dog (*Cynomys sp.*). The black footed ferret is almost solely dependent on the prairie dog for food and uses their burrows for housing. The ferret was thought to be extinct in 1979, but in 1981 a family was discovered and a captive breeding program was started. This family later died of plague. The breeding program, luckily, has produced over 6500 young up to 2009 and over 2300 animals have been reintroduced into the wild. Since black-footed ferrets are dependent on prairie dogs for their existence, the latter species also became a subject of concern. One of the major reasons in the decline of the black-footed ferrets and the prairie dogs is the decline in habitat, furthermore prairie dogs are killed by humans since they are a curse to agriculture. Besides that, ferrets and prairie dogs are susceptible to plague (*Yersinia pestis* infection). A *Yersinia pestis* infection can kill 99% of a prairie dog colony. Recently a plague infection killed prairie dogs living over 10,700 acres. The ferrets living in this and surrounding areas were quarantined [Black-footed Ferret Recovery Implementation Team]. To decrease the impact of plague in these species an oral vaccine is being developed. The plague research group of the National Wildlife Health Center is an important partner in this research and it was the host for this research project on possible interference of natural OPX antibodies in prairie dogs on the oral OPX vectored plague vaccine.

1.1 Prairie dogs

Prairie dogs were first described by Lewis and Clark in 1804 [Lewis 1804]. They grow up to be 30-40 centimeters (including tail) and weigh approximately 0.5 – 1.5 kilogram. They are burrowing squirrels and most of them hibernate in their burrows during winter. There are 5 different species: Black-tailed (*Cynomys ludovicianus*), White-tailed (*Cynomys leucurus*), Gunnison (*Cynomys gunnisoni*), Utah (*Cynomys parvidens*) and Mexican (*Cynomys mexicanus*) prairie dogs. [Hoogland 1995, ITIS 2004] The genus Cynomys (prairie dog) is part of the family Sciuridae in the order of Rodentia [ITIS 2004]. Its closest relative in the Netherlands is most likely a member of the Sciurus genus, the Eurasian red squirrel (*Sciurus vulgaris*).

1.1.1 Population

The prairie dogs are inhabitants of the United States and Mexico. Because of its capability of destroying crops they are seen as a pest to agriculture and eradication programs try to eliminate prairie dogs from cropland. Up to 2003, prairie dogs could be caught in the wild and sold as a pet. Besides the wild prairie dog population in the US, there is a domestic prairie dog population throughout the world. It is difficult to estimate the number of prairie dogs from both populations. The US Fish and Wildlife service (FWS) estimates the wild population sizes based on “occupied land”. Because occupied habitat seems to be declining, it is presumed that the prairie dog populations are declining as well. While black-tailed and white-tailed prairie dogs were both investigated by the FWS in order to get them listed under the Endangered species act, they were however not considered threatened or endangered species. The Utah (49 FR 22330, May 29, 1984) prairie dog is considered a threatened species and Mexican prairie dog (35 FR 8491, June 2, 1970) is an endangered species. The Gunnison’s prairie dog is currently a candidate species (73 FR 6660, February 5, 2008). [www.fws.gov/endangered]

In the early 2000’s it the FWS estimated that, based on density figures, 18,420,000 wild black-tailed prairie dogs were living in the US. In 2004 the service estimated 8000 Utah prairie dogs in Utah. For

the Gunnison's the number will vary between 100.000 and 1.000.000 animals, (FWS 2006). Looking at the maps below, the white-tailed prairie dog population will be in the same range. [Natureserve.org]

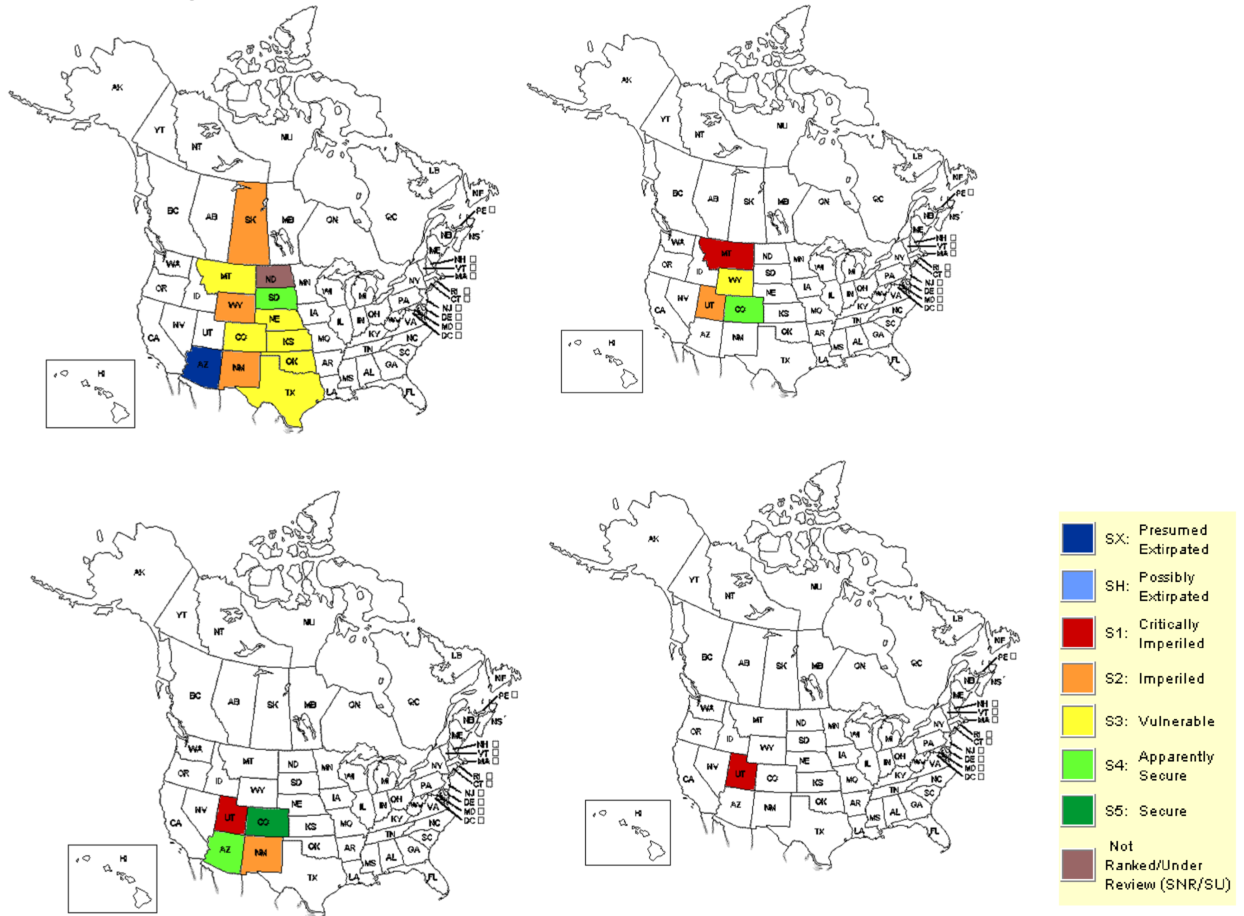

Figure 1: Map of US with prairie dog status per state. From left to right and head to toe: Black-tailed, white-tailed, Gunnison and Utah prairie dog. Blank, no wild prairie dogs found in these states. [Natureserve.org]

Prairie dogs are essential in the prairie grassland ecosystem, because they are a prey for many species and there burrows are used as nesting places by many other animals. The main threats these dogs are facing are declining grassland, chemicals and sylvatic plague. The latter is of concern because it can give local and even regional extinction [NWHC Factsheet, 2008].

1.2 Plague

The bacterium *Yersinia pestis* is the pathogen of plague. The bacterium is transmitted through fleas and it is foremost a rodent disease. But it also causes disease in humans: bubonic or pneumonic plague. Better known as the "Black Death" that killed 17-28 million Europeans from 1327-1351 a.d., (approximately 30-40% of the population) [Perry 1997]. No such outbreaks are recently known, but the gram negative bacterium is endemic in China, central and south Africa, parts of Asia and south-west United States [Plague Manual]. At the moment it is considered a re-emerging disease by the World Health Organization. Not without reason, the bacterium was considered to have disappeared from Madagascar since 1930. In 1990 it reappeared and in a 1996 the first multi-drug resistant strain was isolated. [Chanteau 1998, Galitant 1996]

In the United States its main host are the Gunnison prairie dog (*Cynomys Gunnisonni)* and the rock squirrel (*Spermophilus variegatus)*, but other prairie dog species are also hosts for the plague bacterium. The prairie dog is considered a minimal risk to public health concerning plague, since the flea is not likely to feed voluntary on humans [Plague manual, Ch 4 Gratz]. Plague however is a problem in wildlife. To protect prairie dogs from plague and to prevent the bacterium from spreading, burrows are sprayed with insecticide to eradicate fleas. Furthermore ferrets are trapped and administered parental vaccine. These solutions are expensive and labor intensive, therefore research is done on an oral plague vaccine for black-footed ferrets and prairie dogs. The National Wildlife Health Centre conducts research on the oral plague vaccine in cooperation with other organizations.

1.3 The National Wildlife Health Center plague research

The National Wildlife Health Center (NWHC) is located in Madison, Wisconsin and is part of the United States Geological Survey, which is a bureau of the US Department of Interior (DOI). The DOI has the following mission: "Protect America's natural resources and heritage, honor our cultures and tribal communities, and supply the energy to power our future." The DOI is focused mainly on US issues, whereas the USGS slogan states: "Science for a changing world." As part of the USGS the NWHC formulated the following mission in their 2006 strategic science plan:

> *" To serve the nation and its natural resources by providing the best available science and technical support, and to disseminate information to promote science-based decisions affecting wildlife and ecosystem health. The NWHC provides information, technical assistance, research, education, and leadership on national and international wildlife health issues." [NWHC Strategic Science Plan, April 18 2006].*

The mission of the NWHC has a worldwide focus and their vision is to become a world leader in wildlife health through preeminent science, innovative technology, and responsive service *[NWHC Strategic Science Plan, April 18 2006].* Because of these statements, their research is ongoing and handling as the problem is recognized. The NWHC is divided in a diagnostic (f.e. avian influenza surveillance) and a research part. Research is conducted on prion-disease, west nile virus, avian influenza (high and low pathogenic), *Yersinia pestis* and others. Every pathogen is investigated by a special research group. In the plague (*Yersinia pestis*) research group, this research was done.

1.3.1 The NWHC plague-group

The NWHC plague-group, in cooperation with other research groups, has been working on the development of a vaccine to protect prairie dogs and black-footed ferrets against *Yersinia pestis* infections since the early 2000's. In black-footed ferrets an oral vaccine has been found to give sufficient immunization [Rocke 2008] and in black-tailed prairie dogs an oral vaccine also gave immunity against the disease [Mencher 2004, Rocke 2008]. Before field trials can be conducted, further research on the oral vaccine was done at the NWHC in the summer of 2010.

1.3.2 The vaccine

The oral plague vaccine that is being developed, is based on a recombinant raccoon poxvirus (RCN). The virus expresses plague genes: Fraction 1 and Virulence protein genes. These proteins are essential in preventing phagocytosis of the bacterium and they regulate the type three secretion of the bacterium. Type three secretion is secretion of a complex needle-like protein, which is directly secreted from bacterium into the host cell. Inhibition of these mechanisms will prevent disease. Formulated in a vaccine these proteins gave sufficient immunization to protect several other species (mice, guinea pig, brown Norway rat and three non-human primates) against a challenge with *Yersinia pestis* [Williamson 2000]. Previous prairie dog plague research showed 56% survival rate

after an initial and booster RCN F1 vaccination compared to 12% survival rate for the control animals [Mencher 2004].
Before a vaccine can be commercially available, several characteristics of the vaccine have to be proven. Janeway gives 6 features of effective vaccines:

- safety
- protection
- sustained protection
- induction of neutralizing antibody
- induction of protective T-cells
- practical considerations

At the NWHC animals are vaccinated once or twice and challenged with *Yersinia pestis*, to test if the vaccine has these features. The vaccine is virus vectored, therefore replication of the virus is necessary before the plague antigens are exposed to the immune system. Interference in the replication of the virus will possibly prevent immunization against plague. Three weeks after vaccination blood is drawn and the F1 and V titers (antibody level against plague-antigen) are determined by serology as a measure for immunization.

1.4 Orthopoxvirus

The vector used is RCN, an *orthopoxvirus*. *Orthopoxviruses* (OPX) are part of the *Poxviridae* family. Poxviridae have a large genome (130.000 – 375.000 nucleotides). This large genome is one of the reasons why orthopoxviruses are subject to research on vaccine development, its genome is relatively easy to manipulate [Jacobs 1995].

1.4.1 Phylogeny

The *Poxviridae* can be divided into a subfamily that infects vertebrates (*Chordopoxvirinae*) and a subfamily that infects non-vertebrates (*Entomopoxvirinae)*. The *Entomopoxviruses* are divided into three genera: *A,B* and *C*. The *Chordopoxviruses* can be divided in eight genera [International Committee on Taxonomy of Viruses, Gubser 2003]:

- *Avipox*
- *Orthopoxvirus*
- *Parapoxvirus*
- *Yatapoxvirus*
- *Capripoxvirus*
- *Suipoxvirus*
- *Leporipoxvirus*
- *Molluscipoxvirus*

Emerson et al 2009, adds *Deerpoxvirus* and *Crocodillepoxvirus.* Relatively well known orthopoxviruses are variola-, cowpox- and vaccinia virus [Quinn 2002]. The complete genus consists of ten species (*Vole-, Variola-, Vaccinia-, Raccoon-, Skunk-, Camel-, Monkey-, Tartera-, Ectromilia- and Cowpoxvirus*). Three of which are endemic in North America: *Raccoonpoxvirus, Skunkpoxvirus* and *Volepoxvirus*. These three species are phylogenic sisters of the European, Asian and African O*rthopoxviruses* [Emerson 2009]. In figure 2 the phylogenetic tree is shown.

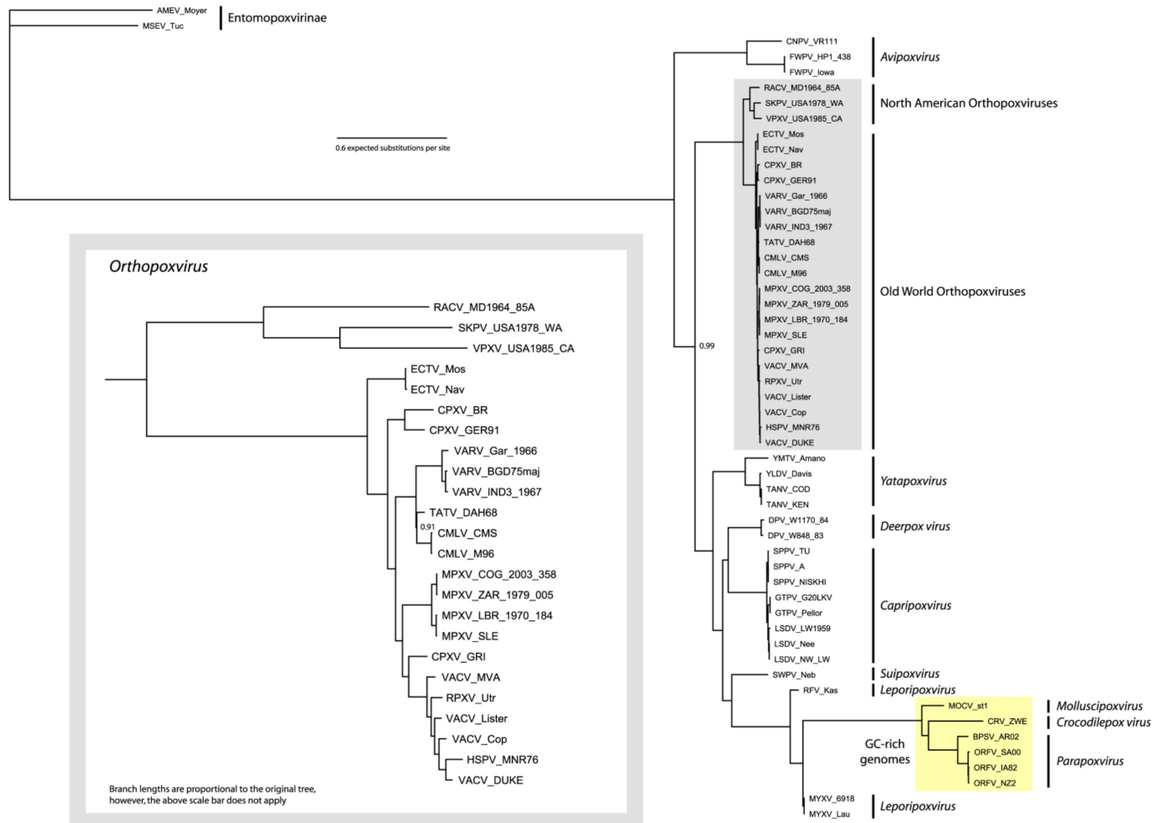

Figure 2: The phylogenetic tree of *Poxviruses* by Emerson**,** inferred from Bayesian analysis of the DNA sequences from 9 conserved genes.

Orthopoxviruses are the only poxvirus that produce haemaglutinin [Ropp 1995]. Gubser sequenced the central 100 kb of 11 othopoxviruses and compared 26 poxviruses (including the 11 sequenced OPX). They concluded that: "The central region of the genome of *Chordopoxvirunae* (ChPVs) is highly conserved in gene content and arrangement, except for some gene inversions in *Fowlpox virus* and species-specific gene insertions in *Fowlpox* and *Molluscum contagiosum virus*. In the central region 90 genes are conserved in all ChPV's, but no gene from near the termini is conserved throughout the subfamily." [Gubser 2002] For the OPX genus these results closely resemble Emerson's phylogenetic tree. Even though it might look like the OPX species are pretty distinct to the 'freshman reader', to the immune system they might look the same.

1.4.2 Cross-protection and reactivity

Theoretically genetic recombination within genera results in extensive serological cross-reactions and cross-protection [Quinn 2002]. For orthopoxviruses (OPX) immunological cross-protection is known [Fenner 1989]. The viruses have highly conserved structural proteins as written earlier, which is one of the reasons why they are used in vaccines because they give cross-protection against other orthopoxviruses [Jacobs 2009]. An example is Vaccinia virus, it has been used more extensively for human immunization than any other vaccine according to Jacobs. Vaccinia was first used to provide cross protection against variola virus (pathogen of small pox). After eradication of the pathogen of small pox, further development of vaccines using vaccinia as carrier were carried out. Besides attenuation it became possible to add genetic material to the virus. A result of this invention was the oral rabies vaccine for wild animals. Vaccinia virus infects mucosal tissue and therefore it is a suitable oral vaccine vector. A success story is the recombinant vaccinia virus encoding the rabies virus glucoprotein, which will provide immunity after oral ingestion [Jacobs 2009]. In several other studies it was noted that serological surveys based on detection of antibodies to an OPX gave cross reactivity

to another OPX [Alexander 1972, Boulanger 1996, Czerny 1996, Marennikova 1981]. Figure 3 shows cross-reaction. It is unknown whether previous exposure to orthopoxviruses, inhibits immunization by a RCN vectored vaccine in prairie dogs. In raccoons pre-immunization with raccoon poxvirus resulted in limited immunization or no immunization against rabies after administration of a recombinant vaccinia-rabies vaccine [Root 2008]. This suggests that the same could happen in prairie dogs. It is unknown if prairie dogs are carriers of orthopoxviruses. An acute response to monkey poxvirus suggest that there is no previous immunization. But monkey poxvirus appears to give minimal to no cross-reactivity with raccoon poxvirus [Alexander 1972].

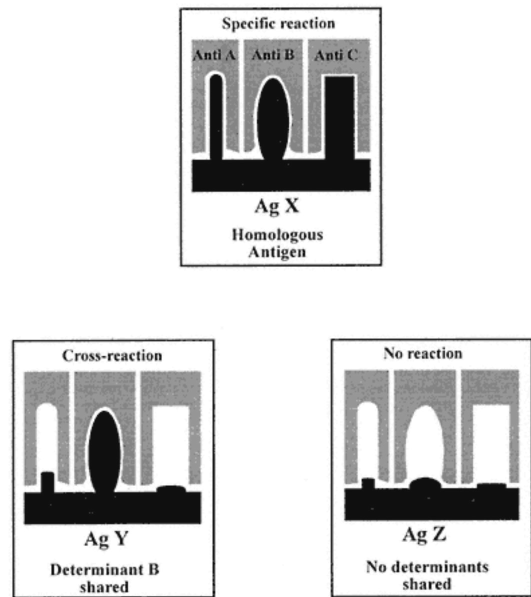

Figure 3: A homolog reaction is Ag X, also Ag Y is recognized this is a cross-reaction.

Further research is done on potential recombination between natural orthopoxviruses and OPX vaccines in rodents in Europe. In Norway cow poxvirus was found in 8 rodent species and the common shrew (*Sorex araneus*). There are concerns on distributing orthopoxvirus vaccine because: "Recombinant poxviruses may represent hazards to immune suppressed individuals, and the possibility of spontaneous recombination with naturally occurring orthopoxviruses resulting in progeny with altered characteristics can not be excluded at this stage." [Tryland 1998]. A survey in Belgium determined a low risk of recombination after testing 11 mammal species. All 125 foxes (*Vulpes vulpes*) tested negative for orthopoxviruses, two of the rodent species (bank vole and woodmice) tested positive [Boulanger 1996]. In Germany a survey of the Brandenburg area tested 46 of 703 red foxes positive to orthopoxvirus [Henning 1995]. All studies used a serological screening method and a confirmation test.

1.5 Aim of study

Introduction of the oral RCN vectored plague vaccine to the wild prairie dog population brings up the same questions on recombination and cross-reactivity as described in the previous section. Especially since an oral vaccine has to be distributed on the prairie grassland, therefore other rodents can ingest the vaccine too. This research will focus on prairie dogs and the potential cross-reactivity between natural orthopoxviruses and the vaccine. It is not known if prairie dogs are carriers or hosts for the endemic orthopoxviruses, but there is a chance they are involved. When prairie dogs develop antibodies against natural OPX, the antibodies could eliminate the virus vectored vaccine before sufficient replication of the virus is established. Without extensive replication of the virus, the plague antigens are not or merely temporarily presented to the immune system. The immune system might not respond and the animal is not adequately protected against an infection (challenge) with *Yersinia pestis.* The aim of this research was to determine if prairie dogs have RCN (cross)-reacting antibodies and if these antibodies interfere with the RCN vectored plague vaccine.

In the next chapter the study design will be explained. After this section the details can be found in the material & methods part, results shall be presented in the adjacent chapter. In the discussion the methods and results will be discussed. Suggestions for further research will be done and in the conclusion the aim of the study will be restated and the results summarized.

2. Study design

The aim of this research was to determine if prairie dogs have RCN (cross)-reacting antibodies and if these antibodies interfere with the RCN vectored plague vaccine. The set-up of this research is presented in this section

The aim was to measure RCN (cross-reacting) antibodies in prairie dog serum. Serum (drawn before and after vaccination) was available from this summer and previous prairie dog studies. An indirect Enzyme Linked Immuno Sorbent Assay (ELISA) was used to measure antibody levels. It is relatively cheap and sensitive. A lot of ELISA's have been performed with orthopoxviruses as antigen, also anti-RCN ELISA's were previously described [Alexander 1972]. Furthermore prairie dog serum has been used in ELISA's coated with plague protein F1 and V [Rocke 2008, Mencer 2004] and Vaccinia virus [Hutson 2009]. But an anti-RCN ELISA for prairie dog serum was only performed once. This was done by the NWHC plague group using a mouse anti-RCN ELISA protocol [Appendix D] and the plague ELISA reagents [appendix E].

- **The first objective of this study was to develop an ELISA that could be used to screen for RCN cross-reacting antibodies in prairie dog serum.**

The basic principle of the indirect ELISA: The ELISA is like a brick house, it is build in layers. If the bricks don't glue to the layer underneath they will be washed away. The first layer consist of antigen or antibody, the second layer is the test fluid. Present antibody or antigen can bind to the first layer and form an antigen-antibody complex. The third layer is a conjugated antibody against the test species antibodies (rabbit anti prairie dog) or against the antigen. If there are antigen-antibody complexes the conjugate will bind to the complex. The fourth layer is a substrate which activates the conjugate and color will develop and the plate can be read. More information on ELISA and optimizing an ELISA can be read in appendix C

The idea was to determine negative and positive animals, but without a known negative or positive control this was difficult. An ELISA to compare immune status between animals before and after vaccination remained the goal of the test. Therefore continuous data were used and the Elisa Value (EV%) [Samson 2000] was used to compare optical densities between plates. Hypothetically the post-RCN-vaccination group should have higher values than the pre-vaccination group. Since after vaccination with a RCN vectored vaccine, animals should develop antibodies against RCN before plague antigens are expressed and plague antibodies are formed.

- **The second objective of the study was to determine if pre-vaccination RCN cross-reacting antibodies interfere with the RCN-vectored plague vaccine.**

This had to be established by data analysis of the EV% of pre-vaccination and post-vaccination serum, survival data and F1/V titer. It will be shown that animals in the RCN F1 + V with a high EV% pre-vaccination status (natural RCN cross reacting antibodies) have significantly smaller survival chances than animals with a low EV% pre-vaccination (no exposure). The following hypotheses were tested to see if this relationship was present or if other relations between EV%, vaccine type and survival were present:

A: Is there a relation between EV% (pre, post, ratio (post/pre) or delta (post-pre)), vaccine type and survival chances.

H_0: There is no relation between any of these parameters

H_1: There is a relation between these parameters

If a relation is shown for set(s) of parameters, this relation will be further defined. Mencher showed better survival chances for RCN F1 vaccinated animals, therefore a relation between RCN F1 +V vaccinated animals and survival chances is expected. The relation that is expected in "naïve" animals is: higher EV% post, ratio and delta give better survival chances. The object of this research is to analyze the relation between EV% pre and survival chances in the RCN F1 + V vaccinated animals.

	Naïve	Previously exposed	Recently exposed
Pre EV%	Low	Low-moderate	High
Post EV%	Higher	High	Moderate - higher

Table 1: Hypothetical pre and post EV% for naïve, previously exposed and recently exposed animals

As shown in table 1, 3 different groups of animals with different immune status could be present in the population. Post EV% status are based on the following principals: In naïve animals the virus has to replicate and the immune system will respond to these unknown particles and produces antibodies (primary response). In previously exposed animals a secondary response is seen. The antigen is recognized and cells immediately release and produce a lot of antibody, like a booster reaction in vaccination schedules (see lower part figure 4). This could also happen in recently infected animals, but the high antibody titer present in serum might be sufficient to kill the virus before it starts full force replication. There is no need to produce more antibodies.

The ELISA measures RCN antibodies, RCN has to replicate to express F1 and V. Depending on the primary antigen doses (primary immunization) the amount of F1 and V antibodies formed will fluctuate as shown in the upper part of figure 4. These antibodies will protect the prairie dog against a *Yersinia pestis* infection in cooperation with the cellular immune response. Hypothetical more RCN replication gives more F1 and V expression and more antibodies of both antigens are expected. To see if RCN EV% post has a relation to post-vaccination F1 and V titer the following hypothesis is tested:

B: A high F1 or V titer (>1:160) has a significantly different post EV% than low F1 and V titers (0 and 1:160)

H_0: There is no difference

H_1: There is a difference

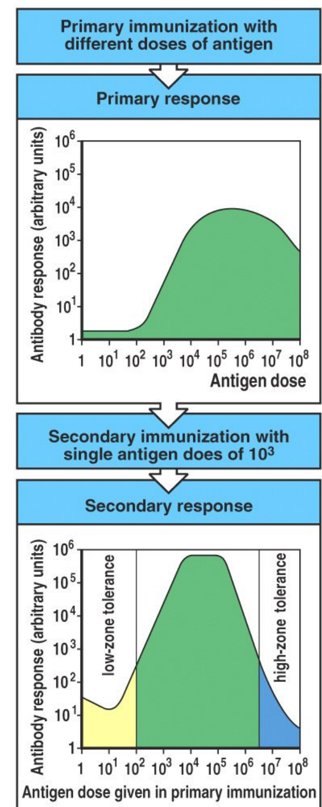

Figure 4: Primary and secondary immunization based on different primary doses of antigen

Side issue: serum color

During blood withdrawal it was noticed that prairie dog blood clotted differently than expected. Furthermore, collection of serum was difficult and approximately 2/3 of the serum samples were either hemolytic or contained erythrocytes. In a TNF-alfa ELISA, hemoglobine reacted with the monoclonal antibody, which is not likely to happen to RCN antigen. But hemoglobine also is capable of catalytic peroxidase-like activity, suggesting it might mimic the action of horseradish peroxidase (HRP) and produce false positives [Siminoni 1995]. HRP is the same conjugate that is used in the RCN prairie dog ELISA. Hemoglobine is one of the products of hemolysis. The absorption values of methemoglobin and oxyhemoglobin are 406 nm and 415 nm [Norvving 1979]. There is no definite answer in the literature to the question whether the hemolytic samples would interfere with the ELISA. A small sample (8) experiment was conducted. Optical color was scored, a full wavelength (200-800 nm) scan was performed on a 1:12 dilution of the sample and the end product of the ELISA in the well.

3. Methods and Materials

This section will describe all materials and methods used for this research. Furthermore it contains the facts concerning the populations tested, the optimizing process, the screening ELISA and the side experiment. The first ELISA's were performed in a different lab than the screening ELISA's, therefore the material section is repeated. In appendix C more information can be found on ELISA's and optimizing an indirect ELISA. In appendix D, E and G ELISA protocols can be found for respectively a anti-RCN ELISA using mouse serum samples, the first anti-RCN prairie dog sample trial and the anti-RCN screening ELISA using prairie dog serum samples.

3.1 Population

In this study the population of concern is wild prairie dogs in the United States. This population consists of four species (black-tailed-, Utah-, Gunnison- and white tailed prairie dogs). The wild populations of these species ranges from 8.000 to 33.000.000 (see introduction: prairie dog population). The prairie dogs that are part of this study, were used as research animals for plague research at the National Wildlife Health Center between 2006 and September 2010. The studies are summarized in table 4. Three of the four prairie dog species were used in the NWHC research and the animals from different studies were captured in different regions. Their age and medical history are unknown. The number of animals per study is an indication for the number of samples available and used during this research. Not all samples were available. Samples that were used are shown in table 6.

Year	Study nr	Species	Region	# of animals
2010	10-08	Black tailed	South Dakota	90
2010	10-09	Utah	Utah	42
2009	09-10	Black tailed	Unknown	37
2009-10	09-15 & 10-02	Gunnison	Arizona: Espee & Aubrey Valley	66
2007	07-05	Black tailed	Unknown	58
2006	06-11	Black tailed	Unknown	51
2010*	Colorado	Black tailed	Colorado	10

Table 4: Overview of NWHC-plague prairie dog research
*Not a NWHC plague prairie dog serum sample. Dan Tripp research-group

Beside these research animals, 10 plasma samples from a population of black tailed prairie dogs in Texas were used. Age, sex, weight and medical history are unknown. In the habitat of these prairie dogs an oral recombinant Vaccinia rabies vaccine was distributed.

3.2 Samples

Three different lots of serum samples will be described, including collection and storage methods, when known. Exact numbers on samples that were screened can be found in table 5 in the next section.

3.2.1 Lots tested

Lot 1: Prairie dog samples collected at the NWHC in summer 2010

In the summer of 2010 90 black-tailed prairie dogs en 42 Utah prairie dogs were housed at the NWHC. Blood was drawn from all animals at arrival. 30 Black tailed prairie dogs and all (42) Utah prairie dogs were baited with RCN tK- or RCN F1/V (see vaccination groups 4.3). Three weeks after vaccination new blood samples were taken (post initial vaccination).

Lot 2: Samples stored at the NWHC from previous prairie dog-plague studies

Serum from all prairie dog studies conducted by the plague group are stored at -20ºC. For this study serum from studies from 2006 to January 2010 was used. Animals were bled three weeks after each vaccination and blood was collected at euthanasia. Pre-vaccination and post initial vaccination samples were used for the screening. The same blood withdrawal procedure was used as was described for Lot 1.

Lot 3: Colorado samples

Ten samples from a prairie dog population in Colorado were send to the NWHC this summer. It is unknown how this blood had been collected.

3.2.2 Bleeding procedure

The standard technique for blood withdrawal in prairie dogs at the NWHC (lot 1 and 2) is as follows: two persons, a handler and a bleeder, will be conducting the procedure. The animal will be caught using protective gloves (leather handling gloves) and allowed to tunnel into a narrow-necked appropriately sized plastic bottle (0.5 or 1 liter Nalgene) with the bottom cut off and the cap off to allow breathing. The animal can then be restrained in the bottle in dorsal recumbence and held against the handler's body with the medial rear limbs exposed. Thumb pressure will be placed on the medial aspect of either limb to hold off the vein. A rear limb will be grasped with one hand and extended by the blood collector. The femoral and medial saphenous veins can then be visualized. Blood collection via venipuncture is carried out using 27 Gauge needle and a 1ml syringe. A minimum of 0.5 ml blood was collected and put into a 1.5 ml plain tube (Fischerbrand). [Application for Standard technique ACUS Trackin No: #ST100405]

3.2.3 Processing of samples

Lot 1: Summer 2010 NWHC samples

A minimum of 0.5 ml blood was collected and kept at room temperature for approximately 15 minutes. Thereafter it was placed in a cooler +/- 10-15ºC for 30 minutes to 3.5 hours, while other prairie dogs were bleed. The blood was centrifuged (Eppendorf centrifuge), for 10 minutes at 5000 rpm. When possible serum was collected in 0.6 ml eppendorf tubes, labeled and stored at -20ºC. If serum was not available after the first spin cycle, samples were spun a second and eventually a third time (5000 rpm for 5-10 minutes).

Lot 2: previous NWHC samples

The same procedure was followed as for lot 1. Unknown are the times and storage temperatures between collection and centrifugation. The sera were stored at -20˚C. Samples were used for F1 and V ELISA's. How often a sample was frozen and thawed is unknown, it is assumed that all post vaccination samples were at least frozen and thawed twice.

Lot 3: Newly collected samples from Texas

Approximately 1 ml blood was drawn on the 13th of July 2010 and collected in an EDTA K_2 tube (BD purple top microtainer, lot KM 0131). The samples were shipped to the NWHC. At the NWHC they were stored at 4˚C. The 26th of July the samples were spun at 5000 rpm for 10 minutes and plasma was collected in 0.6 ml tubes [Fischerbrand]. The tubes were labeled and stored at -20˚C.

3.2.4 Control sera

3.2.4.1 Optimizing ELISA

Negative control: The negative control sera for the optimizing ELISA were chosen ad random from the pre-vaccination sera. After three plates were run samples that showed the lowest optical densities (OD) were used as negative control.

Positive control: The positive control for the first plates was chosen based on F1 and V titer. Samples (post initial vaccination and post booster vaccination) with a F1 or V titer > 1:640 were used. When samples showed a higher OD value than the positive control, these samples were used when necessary.

3.2.4.2 Screening ELISA

Negative control: The negative control was TBST 1% (dilution- and wash buffer). Since there was no known negative control and by picking the lowest OD value samples, our negative control would be biased.

Positive control: The positive control pool had to consist of at least 20 animals (10% of samples tested), with a minimal of 2 animals per study. From all different (6) studies 4-6 post initial vaccination or when available 4-6 post booster animals were randomly selected. 52 Animals were screened in duplicate, 26 per plate. These samples were screened at a 1:40 dilution, based on previous dilution curves. Per plate the average of the samples was calculated and the samples that had an OD value above average were tested in duplicate at 1:20 to 1:640 in twofold dilutions. The selected samples (20 or more) were tested and the raw optical densities were plotted in a graph without corrections between plates. The dilution series had to show a gradually decreasing curve to be included in the positive control sample pool.

3.3 Vaccine groups

As mentioned above the animals were part of different studies. In the different studies animals were assigned to different groups and were given different kinds of vaccine or no vaccine. Different principles were used and more types of vaccine were used. To combine animals from different studies, the different methods were reduced to 4 treatment groups:

- Containing RCN
  - With plague-antigen: RCN-F1, RCN-V or RCN F1/V — RCN F1 + V
  - Without plague-antigen: RCN tK- — RCN tK-
- Without RCN
  - With plague-antigen: F1- V injection — F1/V protein
  - Without plague-antigen: placebo bait, no bait — none

The animals were randomly assigned to the different vaccine groups in the different studies. A summary of the number of animals tested in the screening ELISA per vaccine group and study (including challenge doses) is shown in table 5. The Texas samples are not included since these animals were not challenged with plague. Therefore no survival data were generated. The 10 samples tested for this study are part of the none-group.

Research number Vaccine type	06-11	07-05	09-10	09-15	10-02	10-08	10-09	Total
RCN F1/V	-	-	*13/13*	-	*6/6*	*21/21*	*14/14*	*54/54*
RCN F1 + RCN V	*18/18*	*11/11*	*12/12*	-	-	-	-	*41/41*
Protein F1/V	6/6	18/18	-	-	-	-	-	24/24
RCN tK-	17/17	14/14	12/12	-	-	21/21	13/13	77/77
Placebo-none	8/8	-	-	12/0	-	-	2/2	22/10
Challenge dose [cfu]	5 10^7	5 10^7	5 10^7	50,5000,50000	5 10^7	5 10^7	5 10^7	
Number of vaccinations	2	2	2	2	1	1	1	

Table 5: Number of pre and post initial vaccination samples tested in different vaccine group. Vaccine type and survival data had to be known otherwise samples were not included.

3.4 Optimizing ELISA

3.4.1 Materials

Coating

- 96 well flat bottom immunoplates (Thermo Scientific - Nunc)
- Crude lysate RCN wt (March 4, 2009)
- Carbonate coating buffer (NWHC production)
- Formalin 10% (NWHC production)

Samples, Reagents and buffer

- Samples: study 09-10 pre vaccination, post initial and booster vaccination
- TBST 10X, 3 different lots, prepared using following ingredients:
    - 48 gram Trizma base (Sigma) add 490 ml H_2O pH to 7,4 with concentrated HCl
    - 160 gram NaCl (Sigma)
    - 4 gram KCl (Sigma)
    - 10 ml Tween 20 (Sigma)
- Fetal Bovine Serum. (Without GG fraction, delta inactivated, NWHC)
- Rabbit anti prairie dog IgG HRP conjugated 28.0 mg/22ml (Bethyl laboratories)
- ABTS
    - Solution A: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate) 0.3 g/l (Kirkegaard & Perry Laboratories)
    - Solution B: H_2O_2 0.02% (Kirkegaard & Perry Laboratories)
    - 10% SDS, 2 lots prepared:
        - 20 gram Sodium Dodecyl Sulfate
        - ddH_2O added to 200 ml

Equipment

- Platewasher: Bio-tek ELx405, wash program see Appendix F
- Shake-incubator: Brinkmann Orbimex 1010 & Brinkmann incubator 1000
- Shaker in frigde: Orbit shaker lab line
- Platereader: Bio-tek instruments universal microplate reader El 800
- Read software: Bio-tek instruments 1998-2003 KC Junior version 1.41.3
- Parafilm, pipets (Pipet lite, Gilson, Rainin), tips, glassware, vortex, picofuge, fridge

3.4.2 Method

A mouse RCN protocol established by Willy Berlier (appendix D) was used as basis for Judy Williamson's trial for a prairie dog RCN ELISA on January 29th, 2010. This trail used the reagents available at the NWHC-plague lab. The protocol is shown in appendix E and described below. During the optimizing process different parameters were adjusted. Therefore dilutions are not specified in the protocol but summarized below the protocol.

Antigen coating: The inner wells (60) of the plates were coated with a dilution of raccoon pox virus wild type crude lysate in a carbonate coating buffer (pH 9,6), 50ul/well. The outer wells (36) were coated (50ul/well) with carbonate coating buffer and the plate was sealed with parafilm. Plates were incubated overnight at 3.5˚C on a shaker. The next morning 50ul of 10 % Formaline was administered in the wells. The plates were incubated for 10 minutes at room temperature on the Brinkmann shaker. After incubation the plates were washed. Appendix F contains the wash protocol, this protocol was used in all following steps. Plates were immediately used or stacked and stored in a Ziploc bag at 3.5˚C.

Blocking: 200 ul of 5% Fetal Bovine Serum in TBST1% was administered per well. Plates were incubated for 1 hour at 37˚C on the shaker incubator and washed.

Antibody: Serum samples were administered to the inner wells and the outer wells were filled with 50ul TBST1% per well. The plates were incubated for 2 hours at 37˚C on the shaker incubator and washed as previously described.

Conjugate: The inner wells were filled with 50ul rabbit anti prairie dog IgG, the outer wells were filled with 50ul of TBST1%. The plates were incubated for 1 hour at 37˚C on the shaker incubator and washed.

Substrate: Aliquots from solution A and B used at room temperature. Solution A and B were combined while washing the last plate. 50 ul of the ABTS solution was administered in all wells and incubated for 10 or 20 minutes at room temperature. After incubation, stop solution (1% SDS) was administered 50 ul/well

Reading of plate: Plates were immediately read at 405 nm and transformed to data by the software, KC junior.

Data analysis: Plate readings were processed in Excel (Microsoft XP®). The duplicate wells were checked for consistency. Duplicate OD values more than 10-20% apart were highlighted as were there means. Means were calculated for duplicate wells.
The negative control (ran in quadruplicate) mean and standard deviation were calculated. The coefficient of variance (CV%) = standard deviation (SD) / mean * 100 was established to measure precision. The cut-off value was determined by: negative mean + 2*SD. A sample was highlighted as positive when it was larger than the cut-off value. Positive control dilutions had to be positive for at least the dilution at which the samples were tested and preferably a higher dilution. The OD values of the dilutions had to decrease. Further interpretation of the plate was dependent on the plate lay-out.

Summary of variables tested during optimizing process:
- Plates: maxisorp, polysorp coated the day before and polysorp coated 5 months ago
- Coating dilution: 1:800 to 1:2000
- Sample incubation time: 1 and 2 hour
- Conjugate dilution: 4 ug/ml to 40 ug/ml
- Conjugate type: protein A and rabbit anti prairie dog IgG
- Substrate incubation time
- Wash protocol: dispensing rate

3.5 Screening ELISA

3.5.1 Materials

Coating, reagents and buffer: Identical to 4.4.1 Optimizing ELISA materials

Samples
Pre-vaccination and post-vaccination samples from different studies and vaccine groups were tested., see table 5, section 4.3. A positive control pool was used and buffer as negative control.

Equipment
- Platewasher Bio-tek ELx405, washprogram see Appendix F
- Shaker: Daigger minishaker
- Incubator: Imperial III incubator lab-line
- Platereader: Bio-tek ELx808 iu, ultra micoplate reader
- Readsoftware: BioTek instruments 2006, Gen 5. Version 1.01.9,
- Parafilm, pipets, tips, glassware, fridge, lunchbox (Mepal rosti 163)

3.5.2 Method

Determination of screening dilution

A dilution series of the positive control samples was made and used as final criterium for the determination of the screening dilution. The screening dilution was determined by three criteria:

1. Minimal screening dilution: At which titer a plateau was formed in the dilution series (1:20, 1:40,1:80, 1:160, 1:320, 1:640)
2. Maximal screening dilution: All samples should be above 2*negative control OD
3. A convenient work dilution
4. (Ideally you would like to screen at the dilution at which the curves are parallel)

Screening ELISA

The screening ELISA was performed during 5 days, 4 days of first time screening and 1 day of repeating plates. All plates were coated on the same day. Four plates were ran on the first day and on the other 4 days 8 plates divided into 2 sets of 4 plates were processed. Every morning TBST 1% was prepared and four plates were removed from the fridge and put on the counter to adjust to room temperature for ±15 minutes. TBST 1% from the day before was used as wash buffer. All procedures were started with plate 1, thereafter plate 2, 3, 4. The positive controls were filled after a plate was loaded. The positive controls were aliquot and frozen at the same time, therefore all positive controls endured the same thawing and freeze process.

Antigen Coating: 46 Plates were coated. The inner wells (60) of the plates were coated with a 1:1000 dilution of RCN crude lysate in a carbonate coating buffer (pH 9,6), 50ul/well. The outer wells (36) were coated (50ul/well) with carbonate coating buffer. Plates were incubated overnight on the Orbit-shaker at 3,5˚C. The next day plates 50 ul of 10% formaline was administered in the wells and the plates were incubated for 10 minutes at room temperature on the shaker (110 rpm). After incubation the plates were washed and “banged/slammed” on the lab counter.

Storage of plates: As before, four plates were stacked and the upper one was sealed with parafilm. They were rapped with a plastic sheet and 8 plates were stored in a plastic bag upside down at 4˚C until they had to be used.

Blocking: 200 ul of 5% Fetal Bovine Serum in TBST1% was administered per well. Plates were stacked and covered with an empty plate. The stack was shaken for 2 minutes at 110 rpm at room temperature and incubated for 1 hour (often longer up to 2 hours) at 37˚C.

Sample preparation: While plates were incubating, serum samples were prepared. Serum samples were thawed per plate in the following order: Plate 1 samples were taken from the freezer, 8 minutes later plate 2 samples were taken from the freezer. While the samples for plate 2 were thawing, plate 1 samples were fortexed and aliquot into the tubes and these tubes were fortexed. When all samples (1:50) and positive control dilution (1:50, 1:100, 1:200) were prepared, plates were removed from incubator and washed according to previous described protocol.

Sample loading: Serum samples were administered in the inner wells (see figure 5) and the outer wells were filled with 50ul TBST1% per well. The plates were shaken for 2 minutes at room temperature at 110 rpm. After shaking, individual plates were covered with an empty plate and put into a lunchbox with a moist paper towel and the lit was placed on top of the combination. These boxes were incubated for 2 hours at 37˚C, after incubation they were washed as previously described.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1	2	3	4	5	6	7	8	9	10	
C		5	6	7	1	2	3	4	5	6	7	
D		8	9	10	8	9	10	1	2	3	4	
E		11	12	13	14	15	16	17	18	19	20	
F		18	19	20	11	12	13	14	15	16	17	
G		15	16	17	18	19	20	11	12	13	14	
H												

Figure 5: Plate layout for screening ELISA, blank is outer well, color is inner well 1-16 serum samples, 17-19 positive control dilutions, 20 negative control

Conjugate: The innerwells were filled with 50 ul of 8 ug/ml rabbit anti prairie dog HRP conjugate, the outerwells were filled with 50 ul of TBST1%. The plates were shaken and incubated for 1 hour at 37˚C as previously described. After incubation plates were washed.

Substrate: Aliquots from solution A and B were adjusted to room temperature. Solution A&B were combined while washing the last plate. 50ul of the ABTS solution was administered in all wells. Plates were stacked and covered with an empty plate and aluminum paper. They were incubated for 10 minutes at room temperature at 110 rpm. After incubation, stop solution (1% SDS) was administered 50 ul/well and plates were read at 405 nm.

Reading of plate: Plates were read by the plate reader. The mean, standard deviation and Coefficent of Variance (mean / standard deviation * 100) of the sample triplicates were calculated by software Gen 5 (version 1.01.9).

Data analysis: Gen 5 results were exported to Excel (Microsoft XP®). Study number, animal number and date of blood withdrawal were added. Mean and standard deviation of the positive control dilutions were calculated. When the 1:50, 1:100 and 1:200 OD values were not decreasing, a plate was marked as doubtful. The mean of the triplicate samples were marked doubtful in two ways:

1. By hand: CV% > 10
2. By Excel: standard deviation of 0.010 and higher

Individual OD values were highlighted when OD values were 0.080 or lower (approximately 2*negative control). If more than 25% of a plate was highlighted the 1:50 positive control was compared to the standard curve. When the positive control was within the limits of the mean +/ – 2*SD of the standard curve, the plate stayed in the analysis. Plates were removed from analysis when the positive control did not consist of a triplicate or a mistake was made during the process. Individual wells were removed from the mean calculation, if it was clear by eye that a well was "over responding" (approximately 5 times OD value of other two wells).

Data were transferred from Excel to JMP SAS (version 8.0.2.2). The distribution of the EV% pre and EV% post values were analyzed. Delta EV% (EV% post – EV% pre) and EV% ratio (EV% post / EV% pre) were calculated. A proportional hazard analysis (Cox Model) was done for EV% pre, post, ratio and delta on survival data (censored by survival) and vaccine type. If a significant combination was found, this vaccine group was plotted in a Kaplan Meier survival curve were the EV% was grouped by quartiles. An optical analysis of a pre EV% versus post EV% plot was done.

To test correlation between EV% post (RCN) and the F1 or V titer the RCN F1+V samples were tested in a one-way analysis (x, F1 or V titer; y, EV%) on these data an ANOVA and students T test were performed. The F1 and V data were grouped in 3 groups: titer = 0, titer 1:160, titer >1:160.

3.6 Side-issue: serum color

3.6.1 Material

- Beckman Coulter DU 800 Spectrophotometer (8003365)
- 100 ul microcell
- 3.0 version of the software
- Samples:
    - 1:12 dilution
    - Immuno-plate well content
    - TBST 1%
- Optical scoring chart (appendix I)

3.6.2 Method

The Wavelength II scan was performed, which is an absorbance scanning mode, from 200 to 800 nanometers, with a wavelength interval of 1.0nm and a scan speed of 1200 nm/min.

- The first trial: 8 Samples were randomly selected from an ELISA left-over dilution (1:12) of TR09-10 pre- and post-vaccination samples. The spectrophotometer was blanked with deionized water. TBST 1% (dilution buffer) and the selected samples were tested. 100 ul was pipette into a microcell and the scans were performed. In between scans, the microcell was rinsed with deionized water.
- The second trial: A 1:12 dilution and the final content of a immuno-plate well (after RCN ELISA), from the same sample were scanned. Samples were selected after the ELISA was performed, an optical color score of the serum sample had to be known and a 1:12 dilution of the sample had to be available before a “well” was used. 8 Samples were selected.

Data analysis

- First trial: An absorbance – wavelength graph was generated by the spectrophotometer software. Absorbance peaks were noted per sample. If peaks were close to 405 nanometer, the exact data (Excell) were analyzed to find the maximum absorbance wavelength.
- Second trial: The absorbance – wavelength graph was used as a first step to analyze if the content of the immuno-plate well showed a difference with the 1:12 dilutions. Furthermore a graph was made to visualize the optical serum color score to the absorbance data.

4 Results

Firstly the results of the optimizing process will be described, secondly the results of the screening ELISA. The results of the screening ELISA also contain data analysis of previous established data (f.e. F1 and V titer). Also the survival analysis of different species and all vaccine groups are shown. The third part consist of the Side-issue, spectrophotometer readings.

4.1 Optimizing ELISA

At the start of the optimizing process plates were interpreted by using a technique to establish negative and positive samples (OD value> negative control mean + 2*SD).

Plates: maxisorp, polysorp coated the day before and polysorp coated 5 months earlier
The results of these plates were compared to the first anti RCN prairie dog trail in January.

- The maxisorp plate: negative control 1:20 and 1:640 gave blanked optical densities of 0,641 CV 5% and 0,342 CV 25%. A TBST1% showed up at an OD of 0,040. No positive control showed up positive.
- The polysorp plates coated in January 2010 and July 2010 showed similar positive dilutions (minus a dilution, but plates were incubated for 1 hour instead of 2 hours) as in the January trial.

Coating dilution: An optimal dilution of 1:1024 was established by Berlier in mouse. Therefore the dilutions tested were 1:800, 1:1000, 1:1200, 1:1400, 1:500 and 1:2000. Positive and negative samples were determined, but not much difference was seen in 1:800-1000-1200. The 1:800 dilution gave the highest OD's.

Sample incubation time: Plates were run with either 1 or 2 hour incubation. Plates that were incubated for 1 hour gave lower OD values and fewer positive samples. During 2 hour incubation the negative control did not increase drastically.

Conjugate dilution: As shown in figure 6, a 5 time increase in conjugate concentration gives a 2 time increase in OD value for a 1:80 dilution, the increase is less in higher sample dilutions.

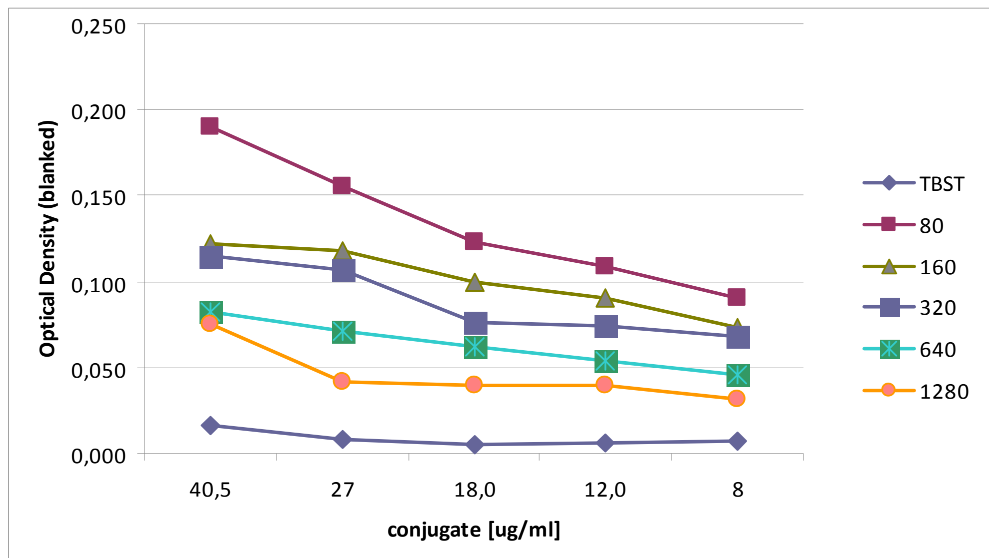

Figure 6: Graph of 5 dilutions (reciprocal is shown in index) and TBST 1% OD mean for 5 different conjugate dilutions.

The conjugate is expensive and the aim was to use as little as possible. 7, 6 and 4 ug/ml gave an average decrease of OD value of 50% compared to 8,35 ul/ml, 24%, 61% compared to 8 ug/ml. 8 ug/ml was used.

Conjugate type: protein A and rabbit anti prairie dog IgG. In the protein A immuno-plate no response was measured (OD <0,03). The protein was last used in 2009.

Substrate incubation time: 2 Plates were read at 10 minutes and 20 minutes. The evaluation of the plates was done in two ways:

- Cut-off method:
  - Plate 1: the same samples were positive after 10 (CV 19%) and 20 minutes (CV 6%)
  - Plate 2: after 10 minutes 6 out of 16 samples were positive (CV 15%), after 20 minutes (CV 18%) 4 samples remained positive.
- EV%: in both plates the EV > 60% was higher after 20 minutes, the EV <10% was mostly more negative after 20 minutes and the Elisa Values in between were not consistently going up or down.

Since EV% was going to be used in the screening ELISA, it was shown that the incubation time should be the same for every plate. All OD values increased, but a 10 minute incubation time was chosen.

Wash protocol: dispensing rate, the rate was decreased to 5 instead of 9. No stronger response was seen in the samples tested with a lower dispensing rate, therefore it is assumed that at a dispensing rate of 9 no significant amount of bound material is washed away.

4.2 Screening ELISA

4.2.1 Controls and screening dilution

The 52 post vaccination samples tested, resulted in 21 samples with an OD value above average. This group of samples included a minimum of 2 samples of each study. The dilution series of each of these samples is shown in figure 7.

The raw OD values are shown and they are not standardized to compare between plates. This graph shows that all samples had gradually decreasing OD values. Therefore all samples were included in the positive control pool.

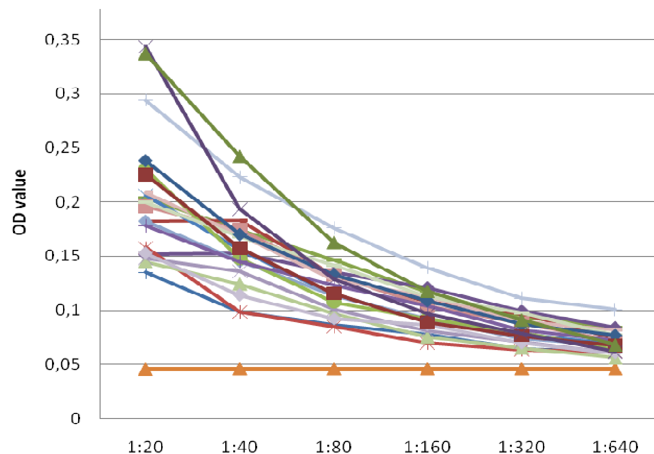

Figure 7: Dilution series of individual serum samples of positive control pool. (TBST1% ---)

Three samples (TR09-10 51, TR10-09 cp 12 and TR10-08 cl 181) show flattening of the curve from 1:40 to 1:20 dilution. This indicates that the sera are reacting maximally with the antigen at a 1:40 dilution, since there is no increase of OD value at a higher concentration. Therefore serum had to be screened at at least a 1:40 dilution, a 1:50 dilution was chosen. The curves are getting more parallel as dilutions are getting higher, put these dilutions are too high to screen with a reasonable chance of detecting antibodies in serum. All samples at 1:50 were above the 2*[-] control cut off.

Samples were screened at 1:50 dilution and positive controls were used at 1:50, 1:100 and 1:200 dilution. The positive control dilutions per plate are plotted in the column chart shown in figure 8. The mean OD value for 1:50 dilution is 0,177 with a standard deviation of 0,0164. The lowest value is 145 (8/9/2010 #4) and the highest 206 (8/11/2010 #1).

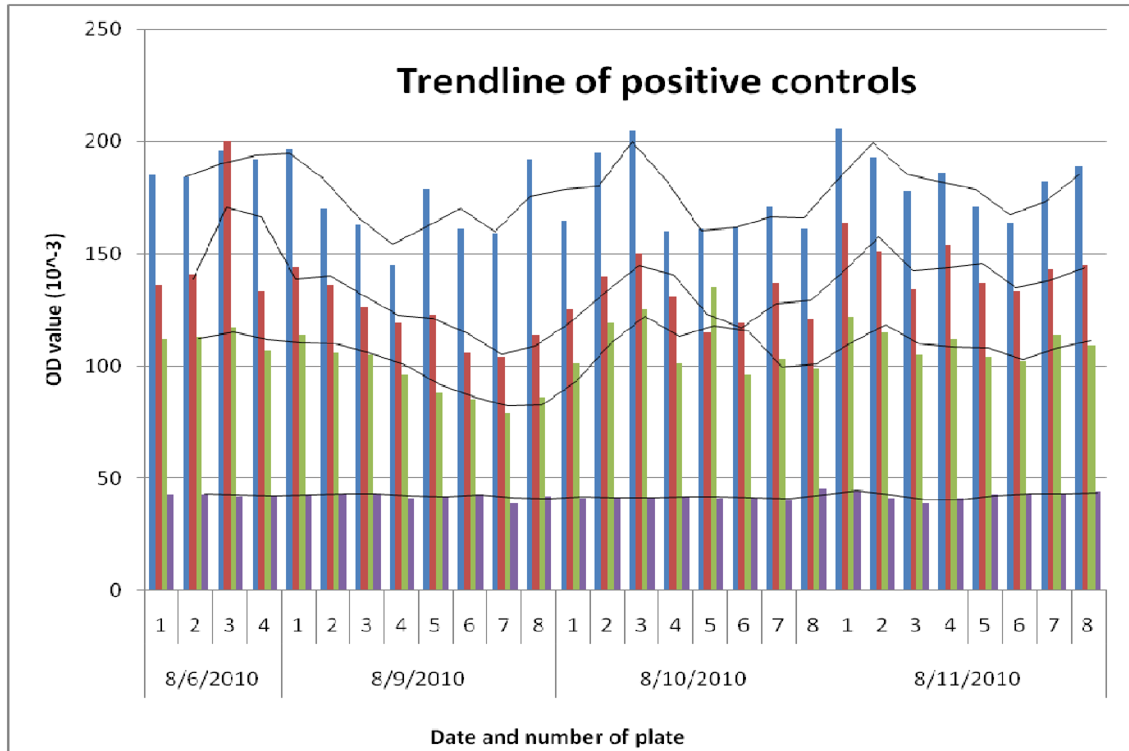

Figure 8: Trendline of postive controls in screening ELISA, in chronological order
Blue 1:50, red 1:100, green 1:200, purple negative control.

The plates positive control pool show fluctuations in OD values on all 3 three dilutions. Most plates show a simular decrease for all 3 dilutions, as shown by the trendline. Plate 8/6/2010 #3 and plate 8/10/2010 #5 show an inconsistent dilution series. In #3 the 1:100 dilution has a higher OD than the 1:50, in plate #4 the 1:200 dilution a has a higher OD value than 1:100. For the 8/10/2010, 6 samples were doubtful, but stayed in the analysis since the overall response of the plate was consistent. Eight plates were repeated, but only data from one plate (8/10/2010 #4)were replaced after consulting a statistical expert. The replaced plate had duplicates of the 1:50 positive control instead of triplicates.

4.2.2 Samples

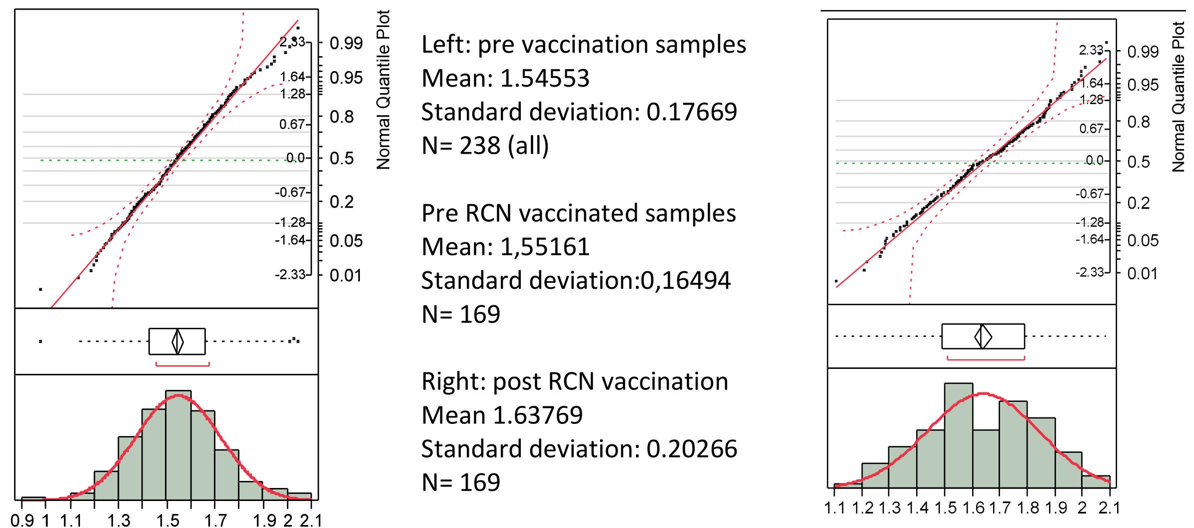

Figure 9: Log distribution EV% of pre and post vaccination samples

(——) resembles normal distribution

Both groups are shown to be lognormal distributed by the normal quantile plot with Lilliefors 95% confidence limits and the histogram.

The differences of the mean between the log pre EV% value and log post EV% value were calculated. The differences were analyzed in a one-way analysis of difference by vaccine type. The confidence intervals of the 4 vaccine groups are shown in table 6.

	N=	Lower 95%	Upper 95 %
RCN F1 + V	94	0.0568	0.13578
RCN tK-	75	0.0235	0.11245
F1/V Protein	22	-0.0996	0.10083
None	13	-0.0617	0.18032

Table 6: One way analysis of difference by vaccine type, confidence intervals

When the confidence interval does not include zero, there is a significant difference between the pre and post EV%. RCN vaccine groups do not include zero, thus the post vaccination EV% means are significantly higher than the pre vaccination EV%.

A potential hazard analysis per vaccine group was conducted for the EV% pre, post, delta (post - pre), ratio (post / pre). The only significant ($P < 0.05$) relation is shown in the RCN F1 + V group EV% ratio.

	EV% pre	EV% post	Delta EV%	EV% ratio	N=
RCN F1 + V	0.2233	0.3860	0.0638	0.0112*	94
RCN tK-	0.9346	0.4848	0.5194	0.6517	75
F1-V protein	0.3384	0.5971	0.7737	0.7384	13
None	0.2717	0.9528	0.5907	0.6893	22

Table 7: Potential Hazard Analysis of EV%, vaccine group and survival.

The RCN F1 + V group was split in quartiles, the Kaplan Meijer survival graphs are shown below for EV% ratio, pre and post EV%.

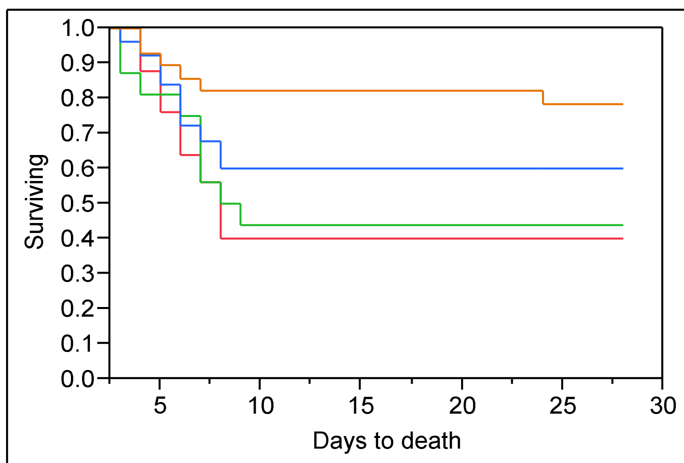

Figure 10 a: EV% ratio*

1 — 1st quartile (n= 25)
2 — 2nd quartile (n= 16)
3 — 3rd quartile (n= 25)
4 — 4th quartile (n= 28)

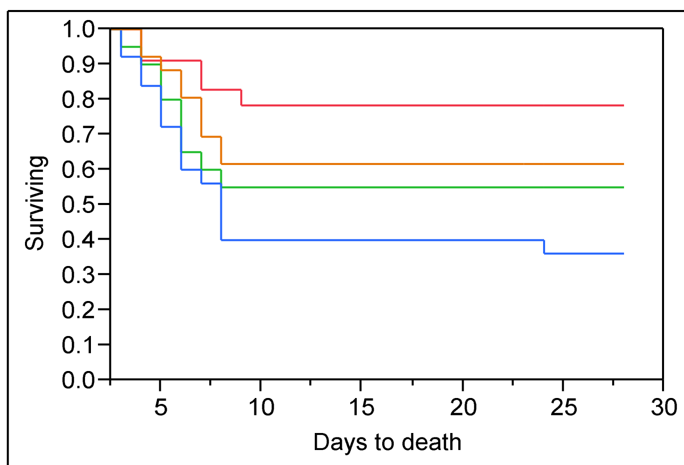

Figure 10 b: Pre-vaccination EV%*

1 — 1st quartile (n= 23)
2 — 2nd quartile (n= 20)
3 — 3rd quartile (n= 25)
4 — 4th quartile (n= 26)

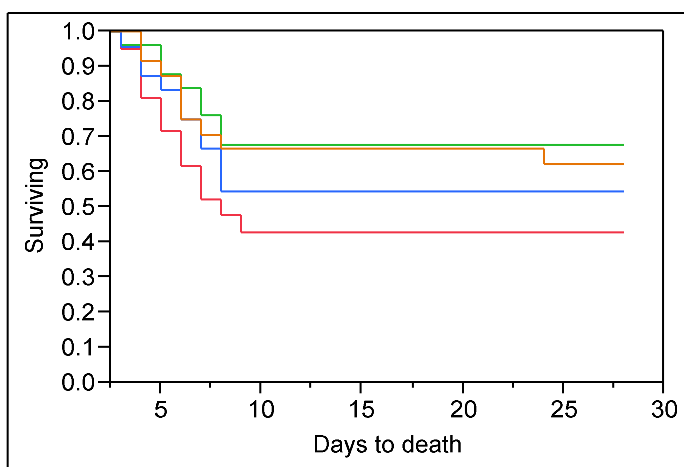

Figure 10 c: Post-vaccination EV%

1 — 1st quartile (n = 21)
2 — 2nd quartile (n = 25)
3 — 3rd quartile (n = 24)
4 — 4th quartile (n = 24)

Figure 10 a, b, c: Graphs of the survival analysis of the EV% ratio (a) pre- (b) and post-vaccination (c)
* Significant (p <0,05) difference between quartiles.

For the EV% ratio, there is a significant difference between groups (log-rank 0.034* and Wilcoxon 0.050). A significant difference was also seen in the pre EV% (log-rank 0.028 and Wilcoxon 0.034). For the post EV% no significant difference between groups was calculated (log-rank 0.28 and Wilcoxon 0.26). The EV% ratio groups showed significant difference with quartile 4 (the highest response) giving the best survival chances and the 1st quartile (smallest chance) giving the lowest survival chances. This is expected in a population in which the major part was not previously exposed to OPX and in which humoral immunity plays an important role in the defense mechanism.

In a plot of EV% pre; EV% post by survival (•) or death (+), possible pre-exposed animals could be identified. The plot is shown in figure 11. The y=x line divides animals in 2 groups: above the line in which the RCN antibody status increased and below the line in which RCN antibody status decreased.

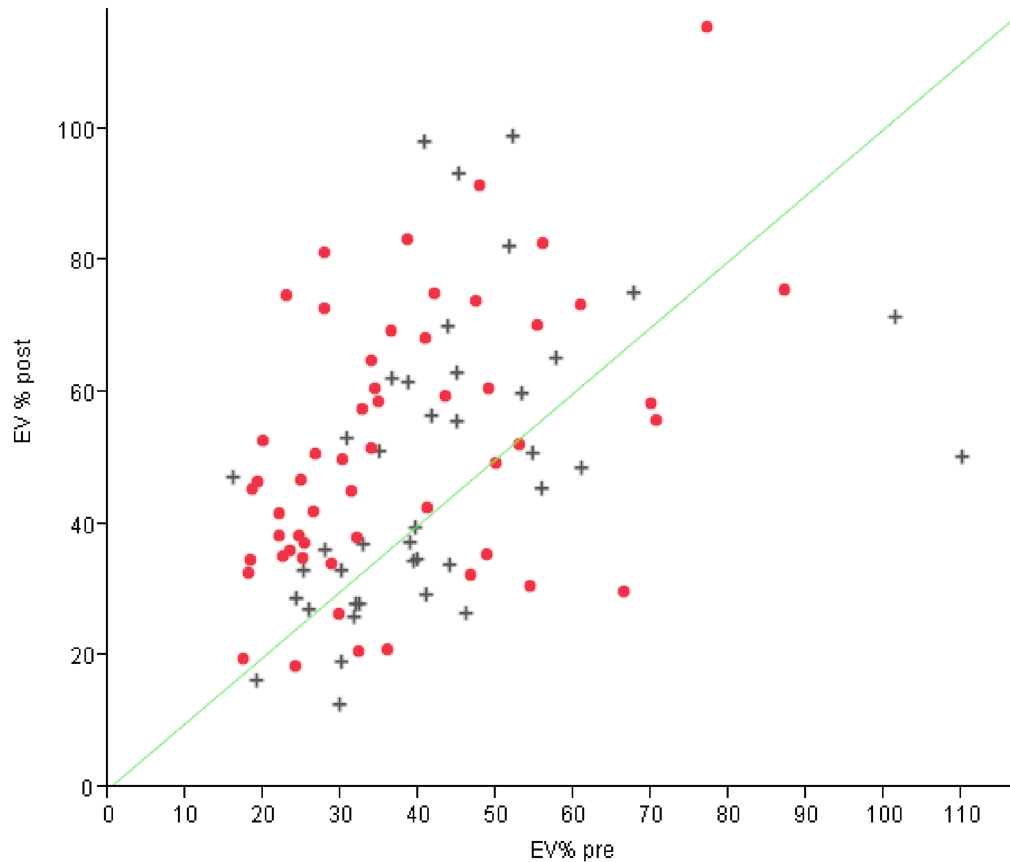

Figure 11: Plot of EV% pre; EV% post of RCN F1 + V group, marked by survival • and died **+**

Observations:

- Grouping: most marks are scattered throughout the graph (EV% post 15-75; EV% pre 10-100)
    - An unclear oval can be seen mostly above the line consisting of •,
    - A vague oval by a large part below the line of + can be seen
- 2 animals died, with high EV% pre (> 100%) and lower EV% post
- A trio of animals with pre EV +/- 45% increased to post EV +/- 100% and these animals died
- 14 animals survived and 18 died with a lower or the same EV% after vaccination

4.2.3 Relation between F1 and V titer – RCN EV%

In the ELISA (cross reacting) antibodies against RCN were measured on a continuous scale. Data of the F1 and V titers were measured in an ordinal way. Both were used to calculate correlation between EV% pre, post, ratio and F1/ V status. Test runs were done and are shown in table 8. First a oneway analysis was performed, the 95% confidence levels of the oneway ANOVA are shown. A student's T test was performed for mean comparison of each pair (each F1/V level in a EV% group), the letters in table 8 show which levels were significantly different.

	N	EV% pre		EV% post		EV% ratio	
F1 post = 0	30	33,6 - 46,3	A	44,8 – 60,6	A	1,17 – 1,60	A
F1 post = 1:160	10	30,6 - 53,4	A	37,1 – 64,9	A	0,86 – 1,62	A
F1 post > 1:160	20	29,3 - 45,5	A	46,8 – 66,5	A	**1,48 – 2,07**	B
V post = 0	27	*26,8 - 40,3*	B	40,6 – 55,5	B	1,29 – 1,77	A
V post = 1:160	15	33,9 - 52,8	AB	43,4 – 63,4	B	1,03 – 1,70	A
V post > 1:160	13	*39,2 – 58,7*	A	**62,7 – 84,2**	A	1,37 – 2,06	A

Table 8: 95% Confidence interval of the different levels of F1 and V titer for EV% pre, post and ratio. Levels not connected by the same letter are significantly different.

In the EV% pre analysis to V post, the V post group 0 and >1 :160 are significantly different. In the EV% post group, V level > 1:160 is significantly different from level 0 and 1:160. In the EV% ratio group F1 level >1:160 is significantly different from level 0 and 1:1:60. The graphs of EV% post versus F1 levels and V levels are shown in figure 12 a and b. The first one has no significant difference, figure 12 b shows a significant difference in level >1:160 to the other two levels. The black dots represent all samples. The green box shows the mean and the length shows the 95% confidence interval, the width shows the distribution of the number of samples per class.

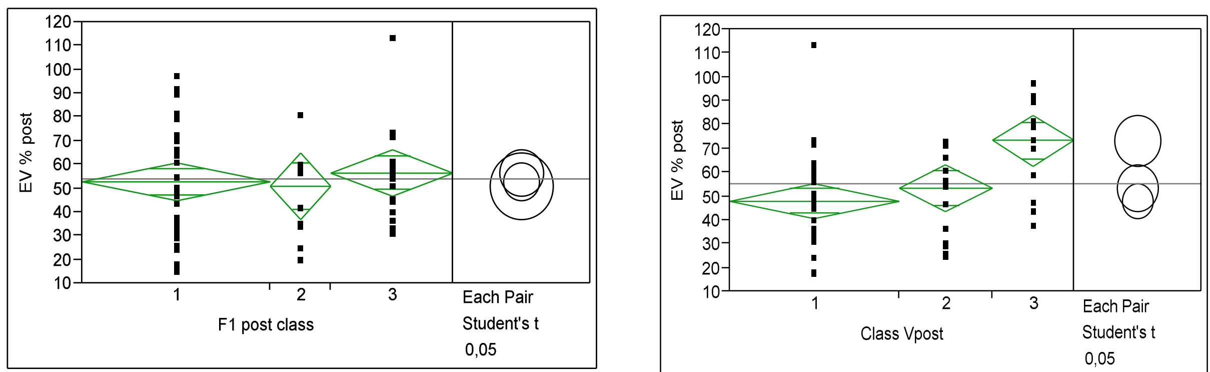

Figure 12 a, b: Plot of oneway analysis and Student's T test for EV% post and F1, V post.

4.2.4 Survival function of species and vaccine groups

A Kaplan Meier survival graph of control animals in shown in figure 13. The mean survival times of the different species were: Gunnisons 14.4 days (n=61), black-tailed 9.2 days (n=70) and Utah 5.6 days (n=21). Gunnisons were challenged with lower *Yersinia pestis* doses than the other species (see table 3) as they were part of a different research.

Difference between groups is significant (p<0,01)

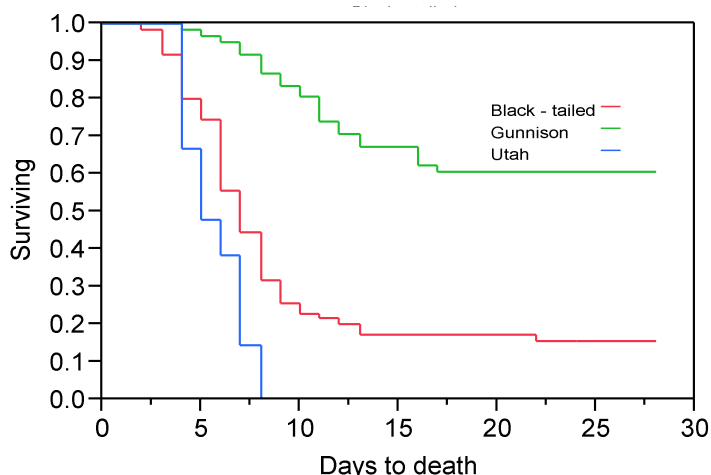

Figure 13: Kaplan Meier survival analysis of control animals, grouped by species

Survival probabilities for animals in different vaccine groups are shown in figure 14. The none groups includes the Gunnison prairie dogs that got a lower infection dose (see table 5) . RCN F1/V (n=40, mean 15.4 days) shows the highest mean survival days. In the former analysis this group is combined with RCN F1 + RCN V (n=62, mean 7.2 days) to RCN F1 + V. The mean survival of 66 animals that were not baited (none) is 13.7 days and animals vaccinated with F1/V protein (n=31) 7.3 days.

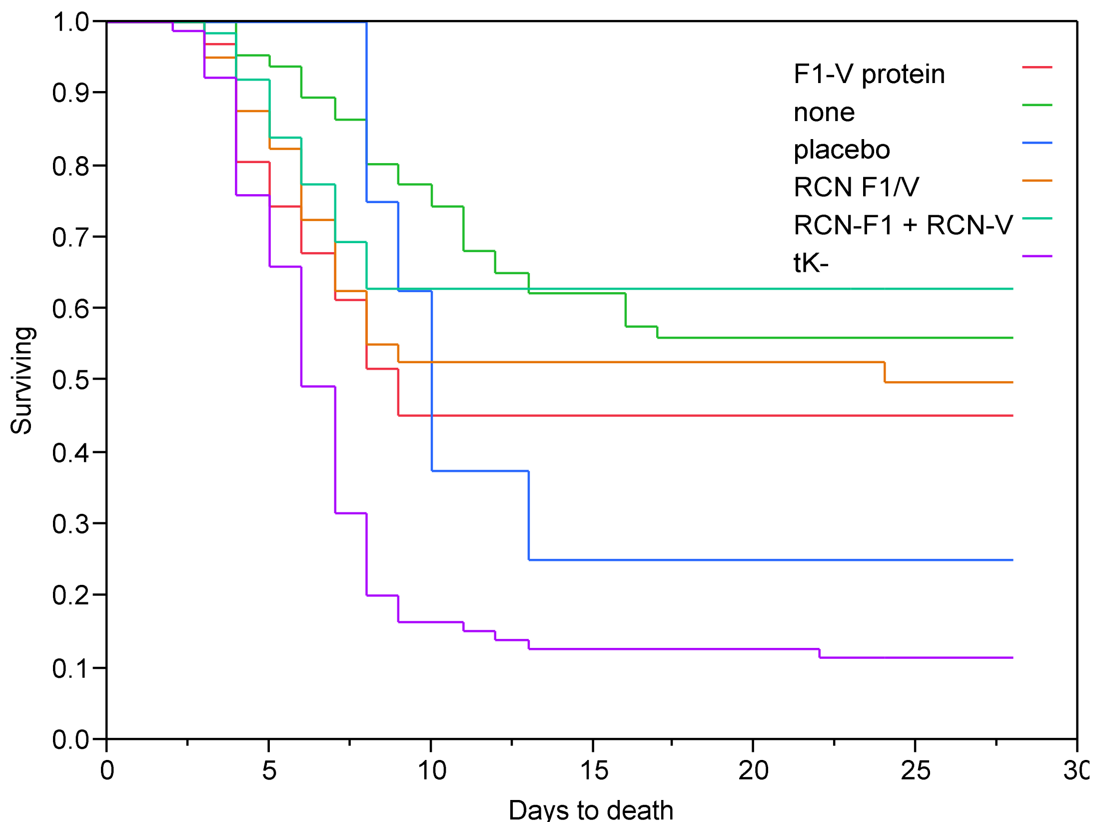

Figure 14: Kaplan Meier survival analysis of all vaccine groups

4.3 Side-issue: serum color

The graph of the first trial is shown in figure 15 , 3 peaks are visible (200-240 nm, 260-300 nm, 400-420 nm).

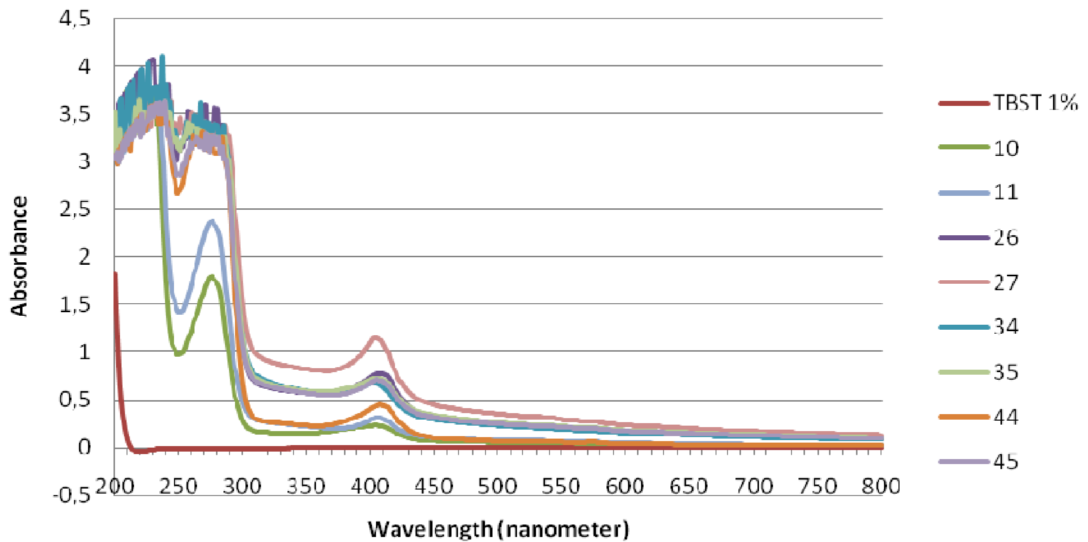

Figure 15: Spectrophotometer trial, 8 samples at 1:12 dilution, Wavelength scan

The peak at 400-420 nm, had a maximum absorbance wavelength of 405 nm in 3 animals. This is the same wavelength at which ELISA plates are being read.

The results of the second trial are shown in table 14. After the sample numbers, the absorbance of 1:12 dilution and absorbance of the immuno-plate well are shown. The OD value was measured by the plate-reader and the optical score was observed by the investigator (optical). The samples are arranged from lowest to highest OD value. The other numbers are not gradually increasing. The 3 samples that scored above 1 absorbance at 1:12 dilution, had an optical score of 5, 10 and 10,5. The OD value and well absorbance of the 10 and 10,5 samples were not increased as much.

	Absorbance at 405 nm			
Sample	1:12	Well	OD value	Optical
0910 #37	1.3160	0.1456	0.064	5
0910 #24	0.3881	0.1501	0.070	4
0910 #12	0.3231	0.1448	0.073	6
0910 #11	0.5386	0.1822	0.085	5
0910 #22	0.4555	0.2058	0.086	8.5
0910 #13	0.3990	0.2299	0.089	8.5
0910 #38	1.8114	0.2187	0.090	10.5
0910 #36	1.3856	0.2390	0.095	10

Table 9: 8 Samples scored at optical color by human, OD value by platereader, absorbance of 1:12 dilution and immuno-plate well content by spectrophotometer

5. Discussion

First the process of optimizing and the used ELISA will be discussed. The results of the screening ELISA and data analysis will be interpreted in context to all variables. The side issue will be addressed briefly at the end of the chapter.

5.1 Optimizing ELISA

According to the ELISA guidebook the needs to be taken care of before optimizing an indirect ELISA are [Crowther 2001]:

- √ A sufficient amount of antigen coating to the wells to capture antibodies.
- ± At least one serum positive for the antigen.
- × At least one negative serum from the same species as the test samples.
- ± A anti-species conjugate.

In the setting where this research (anti-RCN indirect ELISA for prairie dog serum) was conducted, not all needs could be meet. The first need was met; 400 ul of RCN wt was readily available and new RCN could be prepared. The second need, a positive control, was theoretically available. Serum samples from vaccinated animals were assumed to have RCN antibody. This was not confirmed by plaque neutralization testing. The test was done once and gave a 1:16 titer [Rocke personal communication], therefore it might be difficult to detect antibodies with an ELISA and to pick a positive control. A positive sample from another rodent species with known RCN antibody titers was not available either. The third need, a negative sample, could be any of the pre-vaccinated animals, but none of them were confirmed negative. To get as close to a negative control as possible samples from companion prairie dogs could have been used. But samples from domestic prairie dogs (excluding zoo prairie dogs) were not available and these animals could still have been exposed to an orthopoxvirus.

The fourth need, the anti-species conjugate, was available in the laboratory, but it was also used for the plague research. Therefore as little as possible had to be used in this study. Protein A (cell wall component of *Staphylococcus aureus*, adhering to the heavy chain Fc region of most mammalian antibodies) could also serve as anti-species conjugate. It was successfully used in previous plague prairie dog ELISA's (personal communication). However the protein A trial showed no detection, this was probably due to the fact that an old lot was used. Reordering would have solved this problem. Due to time limitations no new protein A lot was ordered Therefore rabbit anti-prairie dog IgG (Bethyl laboratories) was used.

In an experiment with wild animals it is not always possible to meet al the needs as described above. A compromise must be found between certainty and being practical. During the optimizing process, the first anti-RCN indirect ELISA for prairie dog serum trial (appendix E) showed that the mouse protocol worked. Therefore the first focus was to look at the possibility to reduce reagents. To determine whether an ELISA was working, a cut off was established (negative mean + 2* standard deviation) and the positive controls were checked. When the positive controls tested positive, the plate was considered working. A working plate was the criterion for a possible reduction in reagents, instead of using wanted OD values as a criterion. In the end phase of the optimizing process a plastic coating was tested against a RCN coating. This showed that cut-off method would also give working plates in plates without antigen. Therefore this method it is doubtful to use as a criterion in the optimizing process. A more reliable approach is described in appendix C of this report: theory on ELISA and optimizing. This method, described by the ELISA guidebook, is a start from scratch and it based on the ideal process (assuming that OD values get over 1). In prairie dog ELISA's this was only described by Hutson, using a vaccinia virus coating, serum from animals infected with $10^{4,5}$ pfu of monkey poxvirus and 1:30.000 protein A/G conjugate [Hutson 2009].

The ELISA

The optimizing process was also a learning process for the student. Therefore there were inconsistencies in duplicates in the first couple of plates due to pipetting errors.

Plate: In the maxisorp plate OD values were high and inconsistent, because of high background this plate was not used. But, looking back, it is the only plate with "normal" OD values and it was the first plate coated and loaded by the student. For future research it is recommended to redo the ELISA on the maxisorp plate and when same results show, optimize the process. Maxisorp gives 4-5 times higher OD values than the polysorp plates.

Coating: Crude RCN was used in a 1:1000 dilution as coating. After an overnight incubation on shaker in the fridge, the virus was killed with 10% formaline. Crude virus at a 1:1024 dilution is the preferable antigen according to CDC and other poxvirus-ELISA experts in these ELISA's. The formaline step however could alter the configuration of the RCN antigens. It would be interesting to compare the process with and without this step.

Blocking: The blocking step was assumed to be necessary and efficient. The incubation time varied due to sample preparation time. This should not make any difference in results. Because the plastic plate did get signal, the blocking step might not be adequate and serum proteins might adhere to the matrix. In theory antibodies could bind to blocking buffer, but this is unlikely.

Sample- and conjugate incubation: The incubation times remained the same. The temperature during incubation was close to the average body temperature of prairie dogs. It was not changed, but varied from 35,4 to 38,8C during the screening ELISA's due to the equipment used. During the optimizing process the plates were shaking while incubating, this did not happen during the screening ELISA's. Either the mechanical movement or the heat induced movement has to generate movement of the molecules to get them in contact with the walls of the well. Incubation time could be reduced by shaking and incubating instead of incubation without shaking. However, incubation periods stayed the same, since longer incubation periods would be really inconvenient and no decrease in OD values was noticed.

Sample preparation and loading: Samples were thawed, but not stored on ice during this period. Before pipeting the sample were vortexed, not all samples were homogenous after vortexing. This makes the repeatability questionable. Loading of samples was done with an automatic pipette, this makes the loading really precise.

Conjugate: The way the optimal dilution was determined for the rabbit anti-prairie dog was based on the cut-off method instead of the OD value wanted. Next time a checkerboard titration should be done (see appendix C)

Substrate incubation: Based on the two plates that were run, a 20 minute incubation time could have been used as well. Furthermore is the producers advice to use 200 ul per well instead of 50ul. It is unclear what the influence of oxygen and light is on the chemical reaction, in this research all plates were treated the same (stacked and topped with aluminiumfoil). Open plates seem to give higher OD values in general. In next ELISA's this delicate balance could be explored further.

Recommendations:

Optimizing an ELISA should be done based on wanted OD values instead of the cut-off method used. Since the OD values in the anti-RCN ELISA for prairie dog serum are really low, it would be recommended to try and optimize a maxisorp plate protocol. When using the polysorp plate these questions should be answered: what does 10% formaline do to the crude virus coating, is the blocking buffer sufficient, does the hemolytic serum interfere with the plate readings? I would recommend to use more than 8 ug/ml conjugate (or try protein A) increase substrate incubation time and either homogenize samples completly or quickspin samples after fortexing.

5.2 Screening ELISA results

The samples used were from different species and regions. Animals were grouped in vaccine groups to have sufficient numbers for analysis. Animals in the RCN F1 + V group, that were vaccinated with RCN F1 + RCN V, got the double amount of RCN. This could have given higher EV% post. The combined groups gave approximately the same survival chances.

As shown in the positive control trendline (figure 8), two plates had one postive control dilution that was inconsistent. The positive control dilution series were prepared for 4 plates at the same time. One dilution being incompatible in one plate can therefore not be a mistake in dilution preparation as it not seen all 4 plates. For both plates that had a dilution off, the sample triplicates were looked at, the 8/6/2010 plate had perfect sample triplicates, therefore the coating was fine and a generalized problem of the plate is not likely. Both plates stayed in the analysis since no general plate issue was found.

On a plaque reduction test RCN titers came up to 1:16 after first vaccination [personal communication], which suggests that the difference might be difficult to detect in an ELISA . The anti-RCN indirect ELISA for prairie dog serum at 1:50 dilution did detect a significant difference in pre-vaccination and post-vaccination means. Therefore the ELISA is detecting RCN (cross-reacting) antibodies, but no cut-off could be established since the distribution where close together. A larger difference might have been established by testing post-booster samples, but the 70 animals from this summers study could not have been included since they were vaccinated once. This would have decreased the number of samples tested severely.

It seems that survival chance is not significantly influenced by EV% pre or EV% post, but it is significantly influenced by the EV% ratio. As expected this was only shown in the RCN F1 + V group, were animal received RCN and plague antigens. The non-RCN groups should not develop RCN antibodies and therefore show an EV% ratio close to 0. For the RCN –tK group the animals were not expected to survive as they did not receive plague antigens. Hence it was not expected that an EV% was related to survival in these animals. A relation between survival and EV% was expected in the RCN F1 + V group, but it was expected to be found for EV% pre, post and ratio. The fact that only EV% ratio gave a significant relation could be due to the method used. Since pre and post EV% are compared between plates and the EV% ratio is established within a plate. The ELISA value might not be accurate enough to compare between plates. This could be caused by the fact that our negative control is not in- and decreasing with the same percentage as the positive control. Variation in positive control and negative control is shown in figure 8 and the negative control does not seem to be responding corresponding to the positive control. Therefore different methods of comparing between plates could be tried to process the data in following experiments. Since there are no hyperimmune or negative samples, it is difficult to find a suitable method. A ‘negative control pool’ would give negative EV%, which are difficult to use in statistical analysis. In a ‘Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody’ Alcorn (2000) used a standard curve constituted of units/ml. This method can be used when the antibody dilution of the positive control is not known.

The relation between survival and EV% in RCN F1+V was as expected, the highest ratio was associated with the best survival chance and the lowest ratio gave the smallest chance of survival. High EV% ratio where related to post-vaccination F1 titers > 1:160 and EV% post was significantly correlated to post-vaccination V titers > 1:160. Even though high F1/V titers are not proven to be significantly related to survival [unpublished data 2009]. This is a confirmation that RCN post EV% is associated with F1 and V titers. It is shown that cellular immunity is necessary to survive a *Yersinia pestis* infection and this is one of the reasons why a dog with a lower titer may still survive. The fact that a lower EV% ratio (small or no increase in antibodies) in the RCN F1+V vaccine group gave low

survival chances is expected. This group could contain recently exposed animals that killed the virus after ingestion without replication of RCN. In the high EV% ratio naïve animals would survive and the previously exposed where expected too die, but they might have survived. From this result is concluded that the response to the vaccine is what matters, not the amount of RCN (cross reacting) antibodies after vaccination. Also cellular immunity should be kept in mind.

Hypothesis	Naïve	Previously exposed	Recently exposed
Pre EV%	Low	Low-moderate	High
Post EV%	Higher	High	Moderate - higher
Survival	Yes	?	No

Table 9: Hypothetical EV% and survival for 3 groups of animals

The aim of this research was to determine if animals with a high pre-vaccination status of RCN antibodies had a smaller survival chance. In the proportional hazard analysis no significant relation was found between pre EV% and survival. In the Kaplan Meyer graph the quartiles and survival chances were not ordered 4,3,2,1. They were aligned in the following order, highest to lowest survival chance quartile 1,4,2,3. The groups are significantly different, but no suitable explanation is found. Possible explanations for survivors with a low EV% ratio are: naïve animals with a strong cellular response or with no response to RCN but with a F1 and V titer because of previous exposure to plague or because the immune system does not respond to RCN. Quartile four is the group of animals with the strongest immune response, this group could represent naïve animals and some previously exposed animals. Group 2 and 3 can contain naïve, previously and recently exposed animals, therefore no conclusion can be drawn from these analysis.

The RCN F1 + V pre versus post EV% graph (figure 11), gives a clear visual on the “random” distribution of the samples. Two vague ovals can be seen, confirming that a higher ratio is related to a better survival chance, but imagination has to be used. Also between plate variability does matter since grouping of animals might be a little off. In the area’s where previously exposed animals were expected, two groups were found. On group of 4 samples was identified, 1 of these animals survived. It needs further research to determine if these animals where previously exposed to OPX and encountered a booster reaction and killed the virus before sufficient replication could take place or that their main immune response is humoral and the necessary cellular immune response is insufficient. In the other area two animals sampled with a high pre EV% died. These animals (TR06-11 31 and TR10-09 158) could be recently exposed animals since it seems they already had a RCN titer. Another explanation for high OD values could be hemoglobin interacting with the well, since the TR06 sample is very red (optical score 12, the TR10 sample scored a 7,5). The post-vaccination F1 and V titers were for the 10-08 black-tailed prairie dog 0 and for the black tailed 06-11, F1 – 0 and V – 1:160. This shows no antibody response to the vaccine (pre-vaccination titers were 0 and 1:160 too). These animals need further research, tissue samples of TR 10-08 158 will be sent to the pox-group of the Center of Disease Control to confirm the presence or absence of orthopoxvirus besides recombinant RCN in this animal. Another remarkable result is that 15 animals survived even though their RCN status decreased, which indicates that no sufficient F1 and V titers were established (is not confirmed). Because this happened in 15 out of 96 animals it is unlikely that all decreased statuses are due to method and material problems. Hence, cellular immunity could be the most important line of defense in these animals. Furthermore IgG was detected in the ELISA and IgM antibodies are not detected nor shown in the graph. IgM antibodies are the first antibodies to respond to a new agent, it is unknown how long they will last in prairie dog serum and when IgG is formed.

Based on the data that were collected over the past years a lot of analysis can be done. In this research only analysis has been done on survival of challenged control animals. Which showed that Gunnison prairie dogs are more resistant to plague, but these animals got a lower infection doses.

Utah prairie dogs seem more susceptible than both Gunnison and black tailed. More analyses can be done to look closer at this interesting finding, also other relations between prairie dog characteristics (gender, weight, region) and survival chances could be looked at. Difference in susceptibility per region is expected based on the introduction of Yersinia pestis in those regions. In Connata basin plague was seen for the first time 2 years ago whereas plague has been seen for over 50 years in other regions [Personal communication Rocke, unpublished data 2009].

Side-issue: serum color

The results of the trials are very inconsistent. Since only 8 samples are analyzed, no conclusion can be drawn. It was shown that a 405 nm peak of absorbance is a natural peak in prairie dog serum samples, probably because of hemoglobine and its derivates.

6 Conclusion

A three month project was conducted firstly to develop a method to detect RCN (cross-reacting) antibodies. A functional method to detect these antibodies in prairie dogs was developed. Further work has to been done on the anti-RCN prairie dog indirect ELISA to enhance the signal and establish cut-off values for screening.

The second aim of the project was to investigate wheter prairie dogs have RCN (cross-reacting) antibodies and if these antibodies interfered with the RCN vectored plague vaccine. 200 Animals from different vaccination groups, regions and species were tested using the developed method. As expected a significant relation between EV% ratio (post/pre) and survival in the RCN F1 + V vaccine group was found. This was confirmed by the Kaplan Meijer survival analysis, which indicated that a higher ratio is associated with better survival chances. Independently pre and post status did not matter, but this could be due to between-plate variability. A more consistent ELISA comparison method should be developed to be able to confirm these findings. The response to the vaccine, humoral and cellular, seems to be what matters. Higher EV% ratio's were also significant related to > 1:160 F1 post-vaccination titers. The V post vaccination titer >1:160 was significantly related to higher post vaccination EV%. This indicates that RCN-antibody measurement is a predictor for F1 and V titers. The answer to the main question remains uncertain; does previous exposure of prairie dogs to an orthopoxvirus interfere with the RCN vectored plague vaccine? To answer this question more research needs to be done, but 5 animals (out of 96) show evidence of interference with the vaccine. Two animals, with a high pre vaccination RCN status and no significant plague titers after vaccination, died after challenge. These animals could be infected with a wild orthopoxvirus and represent a group of concern. Three animals died with a strong RCN antibody response, it is unknown if these animals were previously exposed or did not have a sufficient cellular response.

Further research can be done on various scales. First the post-booster vaccination samples could be screened and processed by this or an improved research method. From the animals that are suspected of previous exposure, PCR on orthopoxviruses can be performed on tissue samples. A screening ELISA for RCN with a cut-off could be established to determine if animals are negative or positive. An animal trial can be done, by infecting the prairie dogs with an OPX (North American endemic or Vaccinia), vaccinate them afterwards and challenge them. On a scientific level it would be interesting to investigate the immune system of the prairie dog, since little is known about the prairie dog immune system. Furthermore the immune response needed to survive plague infections is unclear, both humoral and cellular responses seem needed.

The results of the research project can not exclude previous exposure to orthopoxviruses in prairie dogs nor confirm the interference of these OPX antibodies with the vaccine. Further research needs to be done.

Acknowledgement

In this part of the report I would like to express my gratitude to the National Wildlife Health Center, Utrecht University and everyone who assisted me in any way during this research. Thank you so much for your patience and expertise. A few people I would like to mention in person. This thesis would not have been possible without Andrea Gröne, professor at Utrecht University and director of the Dutch Wildlife Health Centre. She was my supervisor in the Netherlands and provided me with the inside contact at the NWHC. I owe my deepest gratitude to this contact at the NWHC, Carol Meteyer, without her enthusiastic response to my first email, her effort to find a project at the NWHC and her being my second mom made this project possible. I am grateful to Tonie Rocke as my supervisor and as primary investigator of the NWCH plague-group, she provided many projects to start on and helped me keep on track during the project with her practical approach and insights. It is my pleasure and honour to thank Judy Williamson for sharing all her knowledge on ELISA's with me, teaching me everything in the lab and for being a friend. I would like to thank Erik Hofmeister for adopting me in his lab and for his unlimited scientific mindset. Also I would like to express my gratitude to Susan Smith for assisting me when needed (how inconveniently my questions were), Dan Finley for sharing his spectrophotometer and Rozan van Rossum for supporting me in the writing process. It is my honour to thank Dennis Heisey for teaching me about and guiding me through the statistics. I also would like to thank Julia Rodriquez- Ramos Fernandez for teaching me prairie dog skills (general examination, handling, bleeding, microchipping etc) and being a good friend.

Utrecht University provided the opportunity to get your feet wet in research and the NWHC provided a research project and a great work environment. It was an extraordinary experience.

Reference list

Published papers and books:

Alcorn S.W., Pascho R.J., (2000) Single-dilution enzyme-linked immunosorbent assay for quantification of antigen-specific salmonid antibody, *Journal of Veterinary Diagnostic Investigation,* 12, 245-252

Boulanger, D., Brochier, B., Pastoret, P.P., Crouch, A., Bennett, M., Gaskell, R.M., Baxby, D., Clement, J., (1996), Serological survey for orthopoxvirus infection of wild mammals in areas where a recombinant rabies virus is used to vaccinate foxes, *The Veterinary record,* 138, 11-247

Chanteau, S., Ratsifasoamanana, L., Rasoamanana, B., Rahalison, L., Randriambelosoa, J., Roux, J., Rabeson, D. (1998), Plague, a reemerging disease in Madagascar, *Emerging infectious disease*, 4, 101-104

Czerny, C. P., Wagner, K., Gessler, K., Mayr, A., Kaaden, O.R., (1996) A monoclonal blocking-ELISA for detection of orthopoxvirus antibodies in feline sera, *Veterinary microbiology,* 52, 185-200

deSavigny, D., Voller A., (1980), The communication of ELISA data from laboratory to clinician. *Journal of immunoassay,* 1, 105-128

Fenner, F., Wittek, R., Dumbell, K.R., (1989) *The orthopoxviruses,* San Diego: Academic Press San

Gubser, C., Hue, S., Kellam, P., Smith, G.L. (2004) Poxvirus genomes: a phylogenetic analysis, *Journal of General Virology* , 85, 105–117

Henning, K., Czerny, C.P., Meyer, H., Müller, T., Kramer, M., (1995) A seroepidemiological survey for orthopox virus in the red fox (Vulpes vulpes), *Veterinary microbiology*, 43, 251-259

Hoogland, J.L., (1995), The *black-tailed prairie dog: social life of a burrowing mammal*, p 7-25, Chicago: University of Chigago press

Hughes, A.L., Irausquin, S., Friedman, R., (2010) The evolutionary biology of poxviruses, *Infection, Genetics and Evolution,* 10, 50-59

Gubser, C., Smith, G.L., (2002) The sequence of camelpox virus shows it is most closely related to variola virus, the cause of small pox, *Journal of General Virology*, 83, 855-872.

Hutson, C.L., Olson V.A., Carroll D.S., Abel J.A., Hughes, C.M., Braden, Z.H., Weiss, S., Self, J., Osorio, J.E., Hudson, P.N., Dillon, M., Karem, K.L., Damon, I.K., Regnery, R.L., (2009), A prairie dog animal model of systemic orthopoxvirus disease using West African and Congo Basin strains of monkeypox virus, *Journal of General Virology*, 90, 323-333;

Jacobs,B. L., Langland,J. O., Kibler,K. V., Denzler,K. L., White,S. D., Holechek,S.A., Wong,S., Huynh,T., Baskin,C. R., (2009), Vaccinia virus vaccines: past, present and future, *Antiviral Research,* 84, 1-13

Lewis, M.; Clark, W., Journals of the Lewis and Clark expedition: August 25, 1804 – April 6, 1805. September 7th Friday 1804: A very cold morning. P52-53

Maltseva,N. N., Marennikova,S. S., Matsevich,G. R., Stepanova,L. G., Habahpasheva,N. A. (1998), Standardization of ELISA for detection of antibodies to orthopoxviruses, Acta virologica, 29, 294-298

Marennikova,S.S., Malceva,N.N., Habahpaševa,N.A., (1981), ELISA—a simple test for detecting and differentiating antibodies to closely related orthopoxviruses, Bulletin of the World Health Organization, 59, *3-365

Mencher, J. S., Smith,S.R., Powell,T.D., Stinchcomb,D.T., Osorio, J.E., Rocke,T.E., (2004) Protection of black-tailed prairie dogs (Cynomys ludovicianus) against plague after voluntary consumption of baits containing recombinant raccoon poxvirus vaccine, *Infection and immunity,* 72, 5502-5505

Meyer,H.;Damon,I. K.;Esposito,J. J., (2004) Orthopoxvirus diagnostics, Methods in Molecular biology 269,119-134

Perry, R.D., Fetherston J.D., (1997) *Yersinia pestis* – Etiologic Agent of Plague, *Clinical microbiology reviews,* 10, 45-66

Rocke,T. E., Mencher,J., Smith,S. R., Friedlander,A. M., Andrews,G. P., Baeten,L. A., (2004) Recombinant F1-V fusion protein protects black-footed ferrets (Mustela nigripes) against virulent Yersinia pestis infection, Journal of Zoo and Wildlife Medicine, 35, 142-146

Rocke,T. E., Smith,S., Marinari,P., Kreeger,J., Enama,J. T., Powell,B. S., (2008), Vaccination with f1-v fusion protein protects black-footed ferrets (Mustela nigripes) against plague upon oral challenge with Yersinia pestis, Journal of wildlife diseases, 44, 1-7

Rocke,T. E., Smith,S. R., Stinchcomb,D. T., Osorio,J. E., (2008), Immunization of black-tailed prairie dog against plague through consumption of vaccine-laden baits Journal of wildlife diseases, 44, 930-937

Root, J.J., McLean, R.G., Slate, D., MacCarthy, K.A., Osorio, J.E., (2008), Potential effect of prior raccoonpox virus infection in raccoons on vaccinia-based rabies immunization, *BMC immunology*, 3, 9 - 57

Ropp,S.L., Jin,Q., Knight,J.C., Massung,R.F., Esposito,J.J., (1995), PCR strategy for identification and differentiation of small pox and other orthopoxviruses, *Journal of clinical microbiology,* 33, 2069 - ….***

Samuel, M.D., Shadduck, D.J., Goldberg D.R., Baranyuk, V., Sileo, L., Price, J.I. (1999), Antibodies against Pasteurella Multocida in snow geese in the western arctic, Journal of Wildlife Diseases, 35, 440–449

Sittampalam, G.S., Smith, W.C., Miyakawa, T.W., Smith D.R., McMorris, D.J., (1996) Application of experimental design techniques to optimize a competitive ELISA, *Journal of Immunological Methods*, 190, 151-161

Simoni, J., Simoni, G., Lox, C.D., Prien, S.D., Shire, G.T., (1995), Hemoglobin interference with an enzyme-linked immunosorbent assay for the detection of tumor necrosis factor-alpha*, Analytica Chimica Acta*, 313, 11-14

Tryland, M., Sandvik, T., Mehl, R., Bennett, M., Traavik, T., Olsvik, O., (1998) Serosurvey for orthopoxviruses in rodents and shrews from Norway, *Journal of wildlife diseases*, 34, 2-240

Williamson, E.D., (2009), Plague, *Vaccine,* 27, 56-60

Unpublished papers:

NWHC Strategic Science Plan, April 18 2006
NWHC Factsheet 2008-3087: Protecting black-footed ferrets and prairie dogs against sylvatic plague

Websites:

Black-footed Ferret Recovery Implementation Team, Strategy and timelines.
http://www.blackfootedferret.org/strategy-timeline.htm.
Accessed August 19th, 2010.

Department of interior
http://www.doi.gov/
Accessed July 19th,2010.

US Fish and Wildlife Service, endangered species program. Search on Cynomys.
http://www.fws.gov/endangered/
Accessed August 18th, 2010

Interagency Taxonomic Information System, search on Cynomys.
http://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=180183
Accessed July 14th, 2010

Natureserve. 2009. NatureServe Explorer: An online encyclopedia of life [web application]. Version 7.1.
NatureServe, Arlington, VA. U.S.A.
http://www.natureserve.org/explorer.
Accessed: August 19th 2010.

Appendix

A. Abbreviations 5
B. Cynomys taxonomy 5
C. ELISA Theory: commonly used procedures & optimizing indirect ELISA 5
D. Anti-RCN ELISA with mouse serum samples protocol 5
E. Anti-RCN ELISA prairie dog serum samples trial 5
F. Wash protocol 5
G. Anti RCN screening ELISA with prairie dog serum samples 5
H. Summary of ELISA plates run 5
I. Optical scoring sheet 5

A. Abbreviations

ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid, substrate used in ELISA
ChPV: Chordopoxvirinae
DOI: Department of Interior
ELISA: enzyme linked immune sorbent assay
F1: Fraction 1 protein of *Yersinia pestis*
FBS: Fetal Bovine Serum, blocking protein used in ELISA
FWS: Fish and Wildlife Service
Ig: Immunoglobin
NWHC: National Wildlife Health Center
Nm: nanometer
OD: optical density
OPX: Orthopoxvirus
RCN: Raccoonpoxvirus
TBST: Triz Buffered Saline Tween 20, buffer used in ELISA
Ul: microliter (10^-6 liter)
USGS: United States Geological Survey
V: Virulence protein of *Yersinia pestis*

B. Cynomys taxonomy

Kingdom	*Animalia -- Animal, animals, animaux*	
	Phylum	Chordata -- chordates, cordado, cordés
	Subphylum	Vertebrata -- vertebrado, vertebrates, vertébrés
	Class	Mammalia Linnaeus, 1758 -- mamífero, mammals, mammifères
	Subclass	Theria Parker and Haswell, 1897
	Infraclass	Eutheria Gill, 1872
	Order	Rodentia Bowdich, 1821 -- esquilo, preá, rato, rodents, roedor, rongeurs
	Suborder	Sciuromorpha Brandt, 1855 -- squirrels
	Family	Sciuridae Hemprich, 1820 -- chipmunks, marmots, squirrels
	Subfamily	Sciurinae Hemprich, 1820
	Genus	Cynomys Rafinesque, 1817 -- prairie dogs
		Direct Children:
	Species	Cynomys gunnisoni (Baird, 1855) -- Gunnison's prairie dog
	Species	Cynomys leucurus Merriam, 1890 -- white-tailed prairie dog
	Species	Cynomys ludovicianus (Ord, 1815) -- Arizona black-tailed prairie dog, black-tailed prairie dog, Perro-llanero cola negra
	Species	Cynomys mexicanus Merriam, 1892 -- Mexican prairie dog, Mexican prairie marmot
	Species	Cynomys parvidens J. A. Allen, 1905 -- Utah prairie dog

Interagency Taxonomic Information System, last record review 2004.

C. ELISA Theory: commonly used procedures & optimizing indirect ELISA

This appendix gives a quick review of the steps and most commonly used procedures in the (indirect) ELISA. It is primarly based on The ELISA guidebook by Crowther 2001. An ELISA consists of various steps. Like in preparing a cake all the ingredients should be added in the right order and in the correct amount. A quick overview of the various stages and their relations is shown in figure 4*. Depending on the system used, stages may vary.

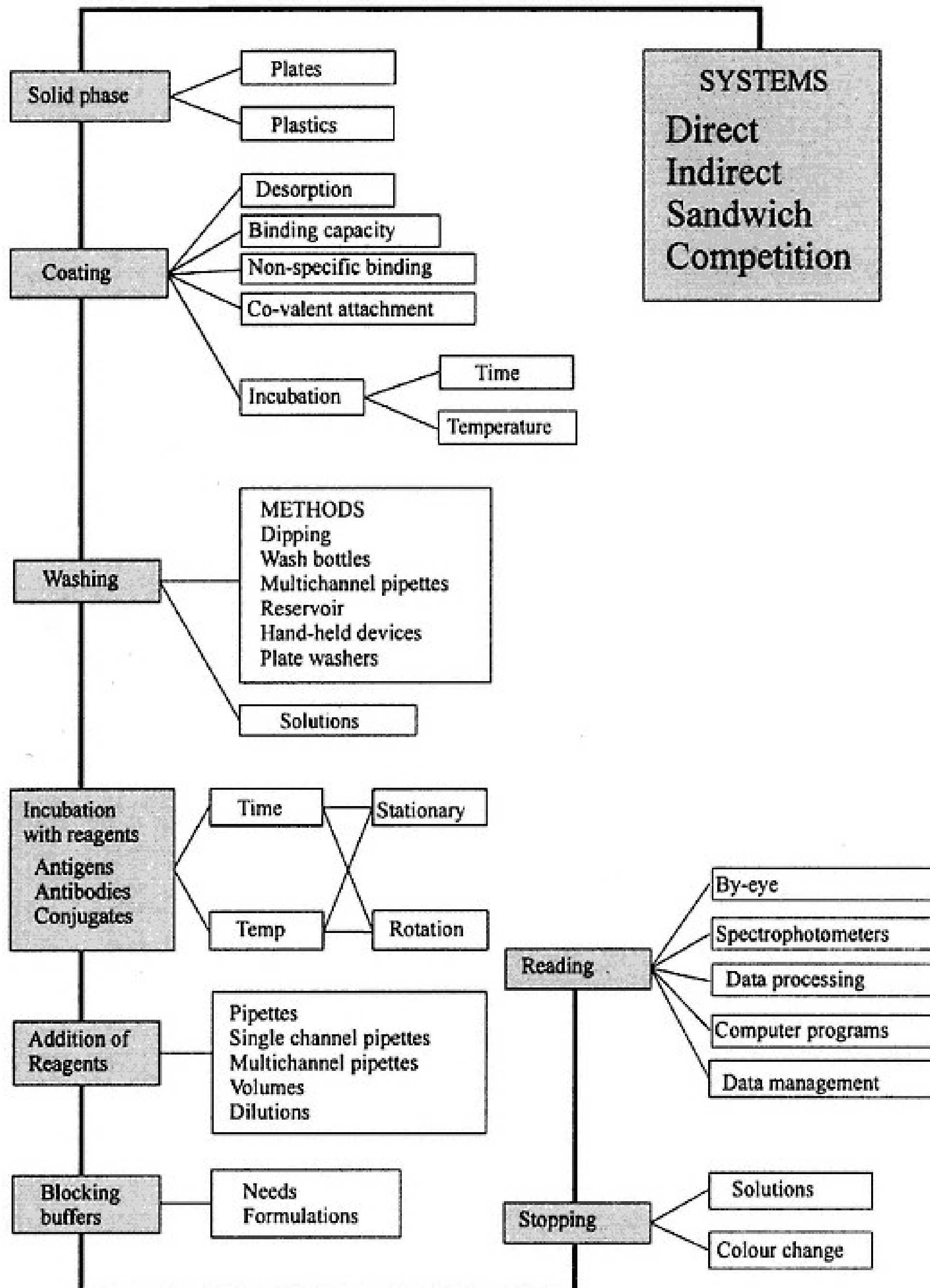

Figure 4: Scheme relating stages in ELISA. [Chrowter 2001]

Stages of the ELISA

1. Adsorption of antigen or antibody to the plastic solid phase

The most commonly solid phase used is the 96–well plate, with polystyrene or a polyvinyl chloride bottom. Also 384-well plates are in use. The bottom can have different shapes: flat bottom shaped wells are recommended for spectrophotometric reading and U or round bottomwell shaped wells are easier to read by eye. The protein will be adsorbed to the plastic matrix, probably as a result of the hydrophobic interaction. The amount of protein bound and speed is depending on:

- The diffusion coefficient of the attaching molecule.
- The ratio of the surface area to be coated to the volume of the coating solution.
- The concentration of the substance being adsorbed.
- The time and temperature in which the plate is incubated.
- There are many variations on incubation conditions (1-3 hours at 37*C to overnight at 4*C)

A covalent attachment of the antigen to the matrix is the most stable bond, but it isn't necessary. A way to configurate the protein is the pH of the buffer it is in. Theoretically the pH should be 1-2 units higher than the isoelectric point value of the protein. Since this is difficult to determine for each protein used, there are some frequently used buffers.

2. 50 mM carbonate pH 9,6
3. 20 mM TrisHCl pH 8,5
4. 10 mM phosphate-buffered saline pH 7,2

A risk during the further stages of the assay is desorption of the coating, however this isn't frequently reported. Another issue is non specific binding; substances may adsorb to the plastic during any stage of the assay. Therefore a blocking step is part of the protocol. A protein which doesn't react with the antigen or conjugate in diluted in buffer (5% commonly used)and is put in the wells and while incubating it adheres to the "empty" places in the plastic matrix.

2. Separation of bound and free reactants by washing

The wash procedure has to wash away unbound reagent, but it shouldn't be to intense and wash of the bound proteins. A isotone wash buffer is used, preferably. Therefore tapwater isn't recommended. PBS (0,1 M, pH 7,4) is commonly used. A three times repetition of emptying and filling the wells is the minimal advised. There're different methods to wash plates: manual methods as dipping, wash bottles, multichannel pipets and other special hand washing devices or automatic plate washers. During the wash process some procedure will soak and/or shake the plates. To add detergent (for example Tween-20) isn't recommended since it could foam and an air bubble on the bottom of the well might prevent the wash buffer from rinsing the complete well.

3. Addition of the test sample and subsequent reagents

Usual volumes to be add to a well are 50-100 ul. To make a dilution of a sample, 2-10 ul of sample might be used. An alert operator with an accurate pipetting technique is required. Different pipets and tips could give different result. A performance test of pipets can be performed. Furthermore there are tips available that prevent retention of fluid in the tip. When adjusting a tip to the pipet special care must be taken to make sure it securely on there, especially in multichannel pipets.

4. Incubation of reactants

During incubation of the reactants, the antibody and antigens need to bound as "closely" as possible. This is affected by concentrations of both proteins, the distribution, time and temperature of incubation and pH. The avidity of antibodies to the antigen is als inprmot*****

Incubation can be done while the plate is rotating or on a stationary bottom. A rotating incubation mixes the reactant completely during incubation. A stationary plate is dependent on diffusion and

therefore temperature is important. When stacking plates while incubating, it takes the wells on the inside more time to reach the temperature equilibrium. Therefore stacking of stationary plates isn't recommended, for rotating plates this isn't as important since the interactions are based on the rotation. Rotating while incubating can reduce incubation time. Especially in viscous samples (1:20 dilution for example). The temperature used for incubation is mostly 4*C, room temperature, 37*C. The interaction between antigen and antibody are most likely to happen at the body temperature of the species sample.

5. Addition of enzyme-labeled reagent

Usually antibodies are conjugated to enzymes. Antibodies against a certain species antibodies might not be available. A closely related species can be used, for example anti-dog antibody for a fox sample. Instead of antibodies Protein A and G can be used for mammalian species. Or indirect labeling techniques as biotin/avidin could be used. After adding and incubating the conjugate, the plates will be washed and substrate will be added. Color will develop, after which the plate can be read.

		Color		**Reading (nm)**		
Enzyme label	**System**	**Not stopped**	**Stopped**	**Not stopped**	**Stopped**	**Stop solution**
HRP	OPD	Green/orange	Orange/brown	450	492	1,25M sulphuric acid
	TMB	Blue	Yellow	650	450	SDS (1%)
	ABTS	Green	Green	414	414	No stop
	5-As	Brown	Brown	450	450	No stop
	Db	Brown	Brown	n/a	n/a	No stop
AP	Pnpp	Yellow/green	Yellow/green	405	405	2M sodium carbonate
B-galactosidase	ONPG	Yellow	Yellow	420	420	2M sodium carbonate
Urease	Urea b	Purple	Purple	588	588	Merthiolate (1%)

Table 3*: Enzyme label, color development and stop solution in ELISA. [Crowther 2001]

HRP: horseradisch peroxidase, AP: Alkaline Phosphatase, OPD: ortho-phenylenediamine, TMB: Tetramethy benzindine, ABTS: 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonate Db: Diaminiobenzidine, Pnpp: p-nitrophenylphosphate, ONPG: o-Nitrophenyl-B-d-Galactopyranoside, Urea b: urea bromoceral purple indicator.

6. Addition of enzyme detection system (color development)

The ability of the anti-antibody to bind to the antigen-antibody complex in combination with the specific enzyme activity is the basis for color development. These characteristics shouldn't be disrupted by chemical modification. For adding the substrate the same points of attention are noted as for adding the sample, since every variance in volume can result in more or less absorbance while reading the plate. Incubation time is dependent on substrate used, temperature of substrate and incubation chamber (room temperature most commonly used). A fixed time can be used or the reaction can be stopped when positive controls have developed enough coloring.

7. Visual or spectrophotometric reading of the assay

When the reaction is stopped by adding stop solution it is important to use a precise pipeting technique and prevent bubbles to develop. After a stop solution is administered plates should commonly be read within 30 minutes. It is recommended to read the plate directly after adding stop solutions. * The plate can be read by eye and scored by hand, when changes are officious and no precise values are needed. A plate reader can either read rows or whole plates. A default reading protocols can be used or a adjusted protocol can set by the matching software. Depending on plate reader and software program used, other variables can be set besides reading speed and wavelength.

8. Data analysis

Optical densities read by the plate reader are stored as a wordpad, excel or other file. Several plate reader software programs can perform basic calculations. Raw data or blanked data (OD value – mean of blank wells (most commonly the outer wells)) can be used for analysis. Depending on the aim of the plate a cut off value will be determined based on the negative control and the cut off value will be compared to the samples that were tested. Cut off values can be set according to different formulas:

- Negative mean + 1*Standard deviation of negative mean (seldom used)
- Negative mean + 2*Standard deviation of negative mean (commonly used)
- 2*Negative mean (standard at Center for Disease Control

A positive control is most commonly used to confirm if a plate worked or not. The positive control has to show up as high as you expect it to, based on previous experiments. At least the dilutions that samples are tested at have to show up positive (OD value higher than cut-off)

Based on the mean of multiple (triplicate of quadruplicates) the Coefficient of Variance (CV%) can be calculated to estimate the precision of a plate. CV% = standard deviation / mean * 100. A commonly used minimal precision limit is 10%.

Theory on optimizing an indirect ELISA

The ELISA Guidebook [Crowther 2001] gives a clear guide how to optimize reagents in an indirect ELISA. The needs that are specified are:

1. A sufficient amount of antigen coating to the wells to capture antibodies.
2. At least one serum positive for the antigen.
3. At least one negative serum from the same species as the test samples.
4. A antispecies conjugate.

3.1 Stages of optimizing reagents

The basis of optimizing reagents according to the ELISA guidebook are checkerboard titrations. Two reagents can be titrated, one following dilutions along the columns (1-12) and the other reagent titrated along the rows (A-H). See figure 5.

The start of optimizing is to relate the negative and positive controls to the coating antigen dilutions. A conjugate dilution that is recommended by the manufacturer or established by previous research can be you used. If this dilution isn't available a direct ELISA checkerboard titration can be used. In this checkerboard titration IgG or whole serum from the target species can be coated on the plate. For whole blood dilutions from 1:200 are diluted twofold from column 1 to 11, column 12 will be filled with buffer. The second layer will consist of a titration of the species conjugate from row A to H.

3.1.1 Stage I: optimizing antigen concentration

Establishing the optimal antigen concentration will be established by titration of the antigen against titrations of the positive control on one plate and on titration of the negative control on another plate. Row 12 will serve as a control, with no antigen. In the results of the antigen versus positive control, there is looked for an optimal dilution. The dilution yielding a plateau maximum or about

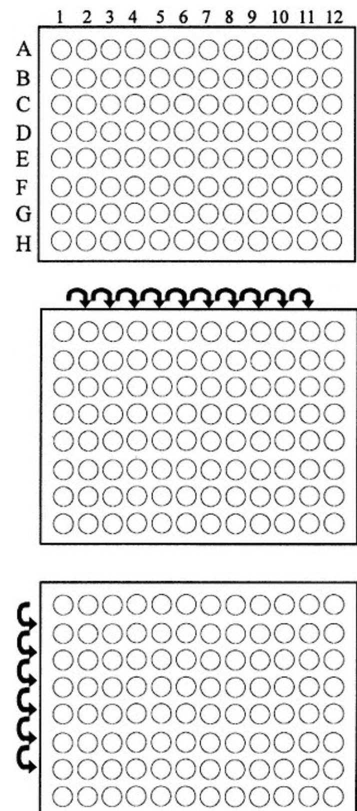

Figure 5: Principle of checkerboard

1.8 optical density with a good titration curve should be used in indirect ELISA assessment. The plate with antigen versus negative control is to examine background development. Especially in high concentrations the negative control will have unspecific binding and results in row 12 (without antigen) can be close to row 1 (highest concentration of antigen). The lowest dilution with good color development will be used, to prevent waste of antigen. [Crowther 2001]

3.1.2. Stage 2: optimizing conjugate concentration
The coating antigen concentration is established in stage 1. In stage 2, positive and negative sera (column 1-12) will be titrated against conjugate (A-H). For the positive control lower concentrations can be used if the endpoint wasn't reached in stage 1. The results of the plates will indicate which dilution will induce background and which dilutions doesn't detect antibody anymore. A dilution which produces minimal background and does detect antibodies should be used. Another approach to the data is to calculate binding ratio's (OD value positive serum at certain dilution / OD value negative serum same dilution). The highest numbers are indicating potential dilution, but care must be taken. Low OD values for positive sera may give a high result when the negative is low too. But the lower OD values being examined, the higher the potential variation in results. Therefore a compromise between high binding ratio and OD value must be found. [Crowther 2001]

3.2 Optimizing circumstances
According to * certain parts of the ELISA are more essential than others. A summary is shown in table 3. *We identified the specific antibody dilution (X5) and the enzyme label lots (X6) as critical factors determining the long-term performance of the assay. However, it appears that the assay temperature (X3), dilution of the enzyme label (X9) and the substrate incubation time (X10) also play an important role.*

Factors	**Optimum setting**
Number of plate wash steps	3
%-well plate lots	Lots with uniform blank absorbance < 0.05 absorbance/well
%-well plate edge effects	Edge wells not used
Reagent incubation time	60 min
Reagent volumes	200 ul/well total
Incubation temperature	Room temperature
Specific antibody concentration	1/3000 dilution of stock
Enzyme label lot	Calibration range: 0.1 -2.0 Absorbance
Enzyme label concentration	1/1000 dilution of stock
Stability of enzyme label *lot*	Single use of stock aliquots stored in 50% glycerol
Sample matrix (plasma/serum)	Serum (preferred)
Analyst-to-analyst variations	Trained analyst
Pipetting procedures	New tip for each well
Washers and plate readers	Fixed washer and reader

[Sittampalam 1996]

[Maltseva 1998]

D. Anti-RCN ELISA with mouse serum samples protocol

Willy Berlier July 2009

Materials:

Carbonate coating buffer

Na_2CO_3	1.59g
$NaHCO_3$	2.93g
H_2O	QS to 1000ml

Store at 4˚C.

Wash buffer

1X PBS

0.1% Tween

Blocking buffer

1X PBS

5% FBS

Secondary antibody: Rabbit anti-mouse IgG HRP-conjugated, diluted 1:4,000 in blocking buffer

Peroxidase substrate: TMB or OPD

Stop solution: 0.5 N (0.25 M) sulfuric acid.

Coating:

1. Dilute RCN wild-type to 5E+05 PFU/mL (1:1024). Coat plates with 100 µL/well overnight at 4° (seal with Parafilm).

2. Add 100 µL/well of 3.7% paraformaldehyde (or 10% formalin) in PBS for 10 minutes. Wash with 3 x 250µL/well wash buffer.

3. Block plates with 100µL/well blocking buffer for 1h at 37ºC.

4. While plates are blocking, make 1:200 dilutions of positive, negative, and control sera in Eppendorf tubes:
   Positive control, per plate: 1µL into 199uL buffer = 200µL total.
   Negative control, per plate: 2 µL into 398 µL buffer = 400µL total.
   Samples: 1.4µL into 278.6µL buffer = 280µL total.

5. Decant blocking buffer and wash plates.

1. Plate layout: pipet 100 uL blocking buffer into wells highlighted in blue below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	S2:1	S2:2	etc...			S3:1	S3:2			
B	-	-	S2:1	S2:2				S3:1	S3:2			
C	-	-	S4:1	S4:2				S5:1	S5:2			
D	S1:1	S1:1	S4:1	S4:2				S5:1	S5:2			
E	S1:2	S1:2	S6:1	S6:2				S7:1	S7:2			
F	S1:3	S1:3	S6:1	S6:2				S7:1	S7:2			
G	S1:4	S1:4	S8:1	S8:2				S9:1	S9:2			
H	S1:5	S1:5	S8:1	S8:2				S9:1	S9:2			

Pipet 100 uL into positive and negative control wells.
Pipet 133 uL of 1:200-diluted samples into first 2 wells (white wells) of each sample bloc.
Serially dilute the samples four-fold by transferring 33 uL from the 1:200 dilution into the next 2 wells containing 100 uL of buffer and so on. Discard the 33 uL from the last dilution so that all wells contain 100 uL.
Incubate plates at 37ºC for 2h.

2. Decant serum samples and wash plates 3 times.

3. Dilute anti-mouse HRP-conjugated secondary antibody 1:5000. Make 10mL per plate. Pipet 100µL per well.

4. Incubate plates at 37ºC for 1h.

5. Decant secondary antibody and wash plates 3 times.

6. Pipet 100µL/well of HRP substrate (TMB or OPD). Incubate covered at room temperature about 5min, checking color visually every min.

7. When positive control wells have a strong blue-green color, but before negative control wells change, stop the reaction with 50µL/well 0.5 N sulfuric acid.

8. Read plate. Determine cutoff as (mean of negative control wells) + 3*(standard deviation of negative control wells).

E. Anti-RCN ELISA prairie dog serum samples trial

Judy Williamson January 2010

Materials:

- <u>Carbonate coating buffer</u>
- <u>Raccoonpoxvirus wild-type</u>
- <u>10% Formalin</u>
- <u>TBST 10% working solution</u>
    - 200ml TBST 1% + 1800ml ddH_2O
- <u>5% FBS working solution</u>
    - 1.25ml 100% Foetal Bovine Serum + 23.75ml TBST 10%
- <u>Rabbit anti-prairie dog 8.35 ug/ml</u>
    - 39.4ul 1.273ug/ml stock + 5.96 ul TBST1%
- <u>ABTS:</u> solution A and solution B
- <u>Serum</u>: sample, positive and negative control

Methods:

<u>Coating:</u>

1. Dilute RCN wild-type to 1:1000. Coat (innerwell wtRCN, outerwell coating buffer) plates with 50 µL/well
2. Overnight at 4° (seal with Parafilm)
3. Add 50 µL/well of 10% formalin for 10 minutes
4. Wash with 4x 400µL/well TBST1% (platewasher and manual empitying)
5. Block plates with 200µL/well 5%FBS for 1h at 37ºC.
6. While plates are blocking, make dilutions of positive, negative, and control sera:
   Positive control:
    - Dilutions; 1:640, 1:2560, 1:10240, 1:40960.
    - Animal: # 48, control
    - Blood draw: post initial vaccination

   Negative control
    - Dilution; 1:640
    - Animal: #30
    - Blood draw: pre vax

   Samples:
    - Dilutions; 1:640, 1:2560. 1:10240
    - Animals:#29 & #31 vaccinated with RCN F1 and RCN Vt, #27 vaccinated with RCN F1/Vt
    - Blood draw: Post initial vax, Post boost vax, Post 2nd initial vax
7. Wash with 4x 400µL/well TBST1% (platewasher and manual empitying)
8. Plate lay-out

		Post Initial Vax (9/15-17/09)		Post Boost Vax (10/21/09)			Controls		Post 2nd Boost Vax (12/14/09)				
Dilutions		1:640	1:2,560	1:640	1:2,560	1:10240	(+) Control		1:640	1:2,560	1:10240		Dilutions
	x	x	x	x	x	x	x	x	x	x	X	x	
#29	x						1:640					x	RCN F1 +
	x						1:2,560					x	
#31	x						1:10,240					x	RCN Vt
	x						1:40,960					x	
#27	x						(-) Control					x	RCN

	x						1:160				x	F1/Vt
	x	x	x	x	x	x	x x	x	x	X	x	

Gray x = outerwell filled with TBST 1%

9. Wash with 4x 400µL/well TBST1% (platewasher and manual empitying)
10. Pipet 50 ug/ml of anti-praire dog-conjugated secondary antibody 8.35 ug/ml in innerwells and TBST 1% in outerwells.
11. Incubate plates at 37ºC for 1h.
12. Wash with 4x 400µL/well TBST1% (platewasher and manual empitying)
13. Pipet 50µL/well of ABTS. Incubate covered at room temperature about 10min.
14. Pipet 50 µL/well of 1% SDS and read plate

F. Wash protocol

The protocol consists of two parts: a automatic plate washer protocol and a manual part.

Plate washer

Reagent TBST 1%

- Method: wash for 4 times (no shaking or soaking)
- Dispense: 400 ul/well, with a flow rate of 9 (out of 9). Height (120). Horizontal -- . No bottomwash.
- Aspirate: 3.048 mm, rate 0.3, no delay or cross aspiration. Final aspiration without delay.

Manual

After the plate washer was finished, plates were banged upside down on the lab counter. 4 x 6 times, after each session of 6 bangs on the counter the plate was rotated 180˚

G. Anti RCN screening ELISA with prairie dog serum samples

Bieneke Bron August 2010

Materials:

- Immuno-plates
- Carbonate coating buffer
- Raccoonpoxvirus wild-type crude lysate
- 10% Formalin
- TBST 10% working solution
  - 200ml TBST 1% + 1800ml ddH_2O
- 5% FBS working solution
  - 100% Fetal Bovine Serum + TBST 10%
- Rabbit anti-prairie dog 8 ug/ml
  - 1.273ug/ml stock + TBST1%
- ABTS: solution A and solution B
- Serum: sample, positive

Methods:

1. Dilute RCN wild-type to 1:1000.
2. Coat (innerwell wtRCN, outerwell coating buffer) plates with 50 µL/well
3. Incubate overnight at 4° (seal with Parafilm)
4. Add 50 µL/well of 10% formalin for 10 minutes
5. Wash plates according to protocol
6. Store at 4°C or go to next step

7. Block plates with 200µL/well 5%FBS
8. Incubate for 1h at 37ºC
9. While plates are blocking, make dilutions of sample and positive control sera:
   Sample 1:50
   - 3,4 ul of sample + 166,6 ul of TBST 10%

   Positive control for 4 plates:
   - Dilutions: 1:50, 1:100, 1:200.
     - 1:50: 22 ul sample + 1078 ul TBST 1%
     - 1:100: 470 ul 1:50 dilution + 470 ul TBST 1%
     - 1:200: 310 ul 1:100 dilution + 310 ul TBST 1%
10. Wash plates according to protocol
11. Plate lay-out: pipet 50 ul of sample in the color wells and 50 ul TBST1% in the outerwells (white)

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		1	2	3	4	5	6	7	8	9	10	
C		5	6	7	1	2	3	4	5	6	7	
D		8	9	10	8	9	10	1	2	3	4	
E		11	12	13	14	15	16	17	18	19	20	
F		18	19	20	11	12	13	14	15	16	17	
G		15	16	17	18	19	20	11	12	13	14	
H												

12. Incubate for 2 hours at 37ºC

13. While incubating prepare conjugate dilution
    - For 4 plates 8 ug/ml rabbit anti-prairie dog: 78,6 ul stock + 12,471 ml TBST1%
14. Wash plates according to protocol
15. Pipet 50 ul of conjugate dilution in innerwells and 50 ul of TBST 1% in outerwells
16. Incubate for 1 hour at 37ºC
17. Aliquot A and B solution in aluminium foil wrapped tubes to adjust to roomtemperature
18. Wash plates according to protocol
19. Mix A and B solution and pipet 50 ul/well
20. Incubate for 10 minutes at room temperature on shaker
21. Prepare 5% stop solution
22. Pipet 50 ul/well of stop solution
23. Read plates at 405 nm

H. Summary of ELISA plates run

Elisa date	#	Goal
June 18, 2010	3	Test: Maxisorp plate. Fresh and old coating on polysorp plate
June 21, 2010	2	Compare within a plate: Antigen dilution (1:1000, 1500, 2000), conjugate (8,35; 6; 4 ug/ml)
June 25, 2010	2	Compare within a plate: Antigen dilution (1:1000, 1200, 1400), conjugate (8,35; 7; 6 ug/ml)
June 29, 2010	2	Compare within a plate: Antigen dilution (1:800, 1000, 1200, 1400), conjugate (9, 8,35; 8 ug/ml)
July 1, 2010	2	Compare between plates: Antigen dilution (1:1000, 1200) *Start pre vax baseline*
July 10, 2010	3	Test at lower dilutions, test protein A, different negative control dilutions *Start post vax baseline, prevax baseline*
July 14, 2010	2	Compare pre and post vax at one dilution, using cut off and EV% Test Utah prairie dog serum
July 16, 2010	3	Repeat Utah prairie dog plate Test screening dilution 1:20, 40, 80, 160 per sample, using cut off method
July 20, 2010	2	Test screening dilution 1:80, 1:160 in different plates Test 20 minute substrate incubation time
July 27, 2010	1	Test no coating versus RCN
July 29, 2010	2	Screen post initial and booster samples Test different conjugate dilutions (6,4 – 15,6 ug/ml)
July 30, 2010	2	Screen post initial and booster samples Test different conjugate dilutions (8 – 40,5 ug/ml)
August 3, 2010	5	Test positive control samples (1:20 – 1:640) Establish screening dilution
August 4, 2010	1	Test different wash protocol (3 cycles, dispensing rate 5) Positive control mix baseline (1:25 – 1:12800)
August 6, 2010	4	Screening of samples
August 9, 2010	8	Screening of samples
August 10, 2010	8	Screening of samples
August 11, 2010	8	Screening of samples
August 16, 2010	8	Repeat plates

I. Optical scoring sheet

Group A turned out to be the most efficient coloring scale. B and D were once in a while added.

	A	B	C	D
15	1	15	15	15
14	2	14	14	14
13	3	13	13	13
12	4	12	12	12
11	5	11	11	11
10	6	10	10	10
9	7	9	9	9
8	8	8	8	8
7	9	7	7	7
6	10	6	6	6
5	11	5	5	5
4	12	4	4	4
3	13	3	3	3
2	14	2	2	2
1		1	1	1