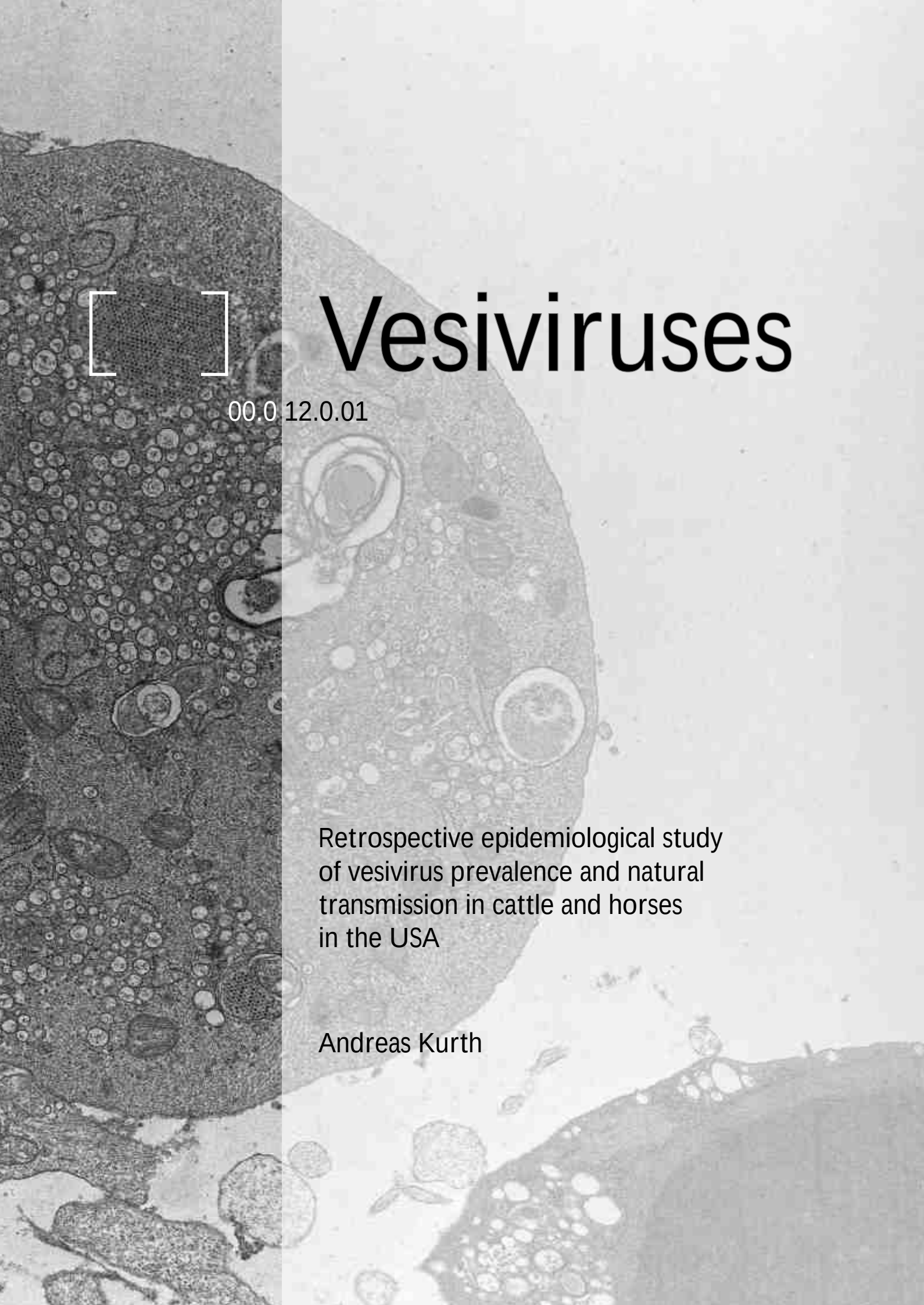

Vesiviruses

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Retrospective epidemiological study of vesivirus prevalence and natural transmission in cattle and horses in the USA

Andreas Kurth

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(Retrospektiv-epidemiologische Studie zur Prävalenz und natürlichen Übertragung von Vesiviren bei Rindern und Pferden in den USA)

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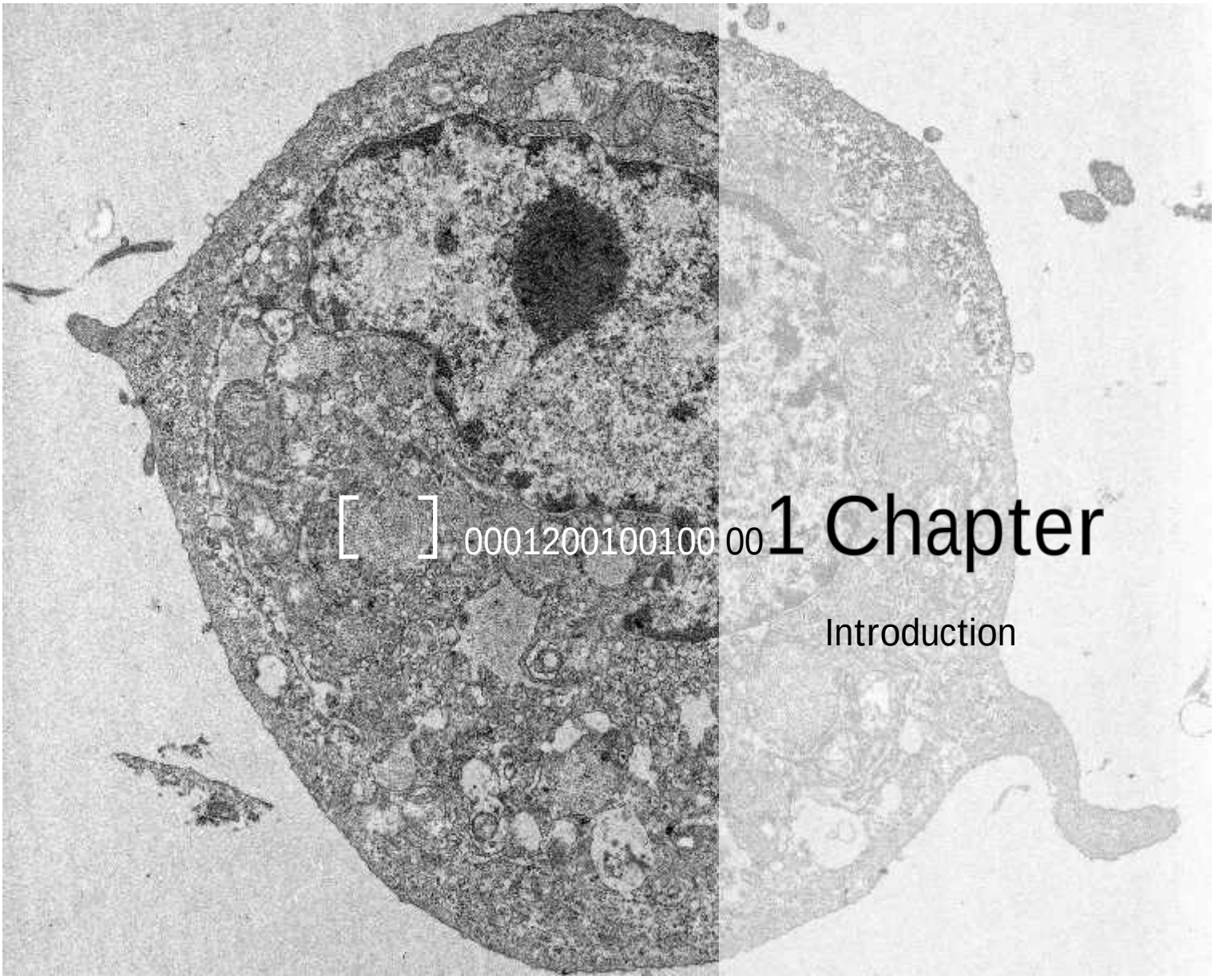
CONTENTS

Abbreviations

Ab	antibody
Aqua bidest.	bidestilled water (Aqua bidestillata)
BLV	Bovine leucosis virus
BoHV-1	Bovine herpesvirus 1
BRSV	Bovine respiratory syncytial virus
BSE	Bovine spongiform encephalopathy
BVDV	Bovine viral diarrhea virus
CCV	Cetacean calicivirus
CPE	cytopathic effect
CrFK	Crandell-Rees feline kidney
DNA	desoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
ETC	Eastern tent caterpillars
FA	fluorescent antibody test
FCV	Feline calicivirus
FMD	foot-and-mouth disease
HuCV	Human calicivirus
IBRV	Infectious bovine rhinotrachitis virus
kb	kilobase
kDa	kilo Dalton
LM	light microscopy
MRLS	mare reproductive loss syndrome
OD	optical density
OR	odds ratio
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RHDV	Rabbit haemorrhagic disease virus
RNA	ribonucleic acid
RT-PCR	reverse transcriptase - PCR
SMSV	San Miguel sea lion virus
SLV	Sapporo-like virus
VES	Vesicular exanthema of swine
VESV	Vesicular exanthema of swine virus



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1 Chapter

Introduction

A single bovine case: findings in tissue from an aborted fetus

In March of 1999, an aborted Holstein bovine fetus measuring 76 cm from crown to rump came to the Oregon State University, College of Veterinary Medicine Veterinary Diagnostic Laboratory for routine diagnostics. The mildly autolyzed female was necropsied and no important gross findings where noted. Samples of liver, spleen, thymus, uterus, large intestine, skeletal muscle, adrenal gland, tongue, eyelid, placenta and brain were taken for histological examination. Findings were a slightly autolytic kidney with no discernible lesions, a thymus showing moderate lymphoid depletion, and lungs with evidence of severe meconium aspiration, wispy protein and occasional macrophages in the alveoli. No other lesions were reported in the remaining tissues. Additional tests using a routine "abortion screen" panel for microbial agents were negative. No pathogenic microbe was isolated, all results of serologic tests for leptospires, bovine diarrhea virus (BVDV, member of the genus Pestivirus, family *Flaviviridae*) and infectious bovine rhinotrachitis virus (IBRV, family *Herpesviridae*) were negative, as were the results of fluorescent antibody tests (FA) run on kidney sections for IBRV and BVDV. Because the analysis for selenium of the fetal liver revealed a value of 389 ng/g, or 65% of the reference value, the final pathologic diagnosis was fetal stress syndrome associated with selenium deficiency (Smith et al., 2002b).

This represents just one case of approximately 75% of all bovine abortion cases processed by US Veterinary Diagnostic Laboratories that go without a definitive aetiologic diagnosis (personal communication with Dr. A.W. Smith). Since very specific tests are commonly used especially for viral diagnosis instead of working simultaneously with more general tests like electron microscopy with its "open view", only a limited number of mostly "reportable disease agents" are tested for in the United States and Germany. The undiagnosed 75% of all abortion cases include infectious agents, a small proportion of birth complications, chronic diseases, metabolic disorders and problems with the sample quality, handling and shipping. About the same ratio of diagnosed and undiagnosed causes for bovine abortions occurs in Germany (personal communication with Dr. F.K. Reckling, Landesveterinär- und Lebensmitteluntersuchungsamt Stendal).

However, because this diagnosis was felt unsatisfactory, further studies were initiated at a nearby university research laboratory to clarify the aetiology of this syndrome. Like many other cases with an uncertain aetiology the samples were tested for a number of emerging/reemerging microbes. Negative stain electron microscopy was performed on homogenized lung tissue from the aborted fetus. Individual virus particles with a distinctive "star of david" appearance and cup-like surface features were observed, i.e. a morphology typical for the family of *Caliciviridae* with its four genera *Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus* (Figure 1). This diagnostic finding was further explored using fluorescent antibody (*Vesivirus* genera specific monoclonal antibody) tests performed on impression smears taken at necropsy from cut lung and frozen sections of lung. Areas of green fluorescence consistent with a positive reaction in the cytoplasm were observed. Reverse transcription-polymerase chain reaction (RT-PCR) of a 500-base pair sequence from the vesivirus RNA polymerase region and sequencing of the resulting amplicon were performed on lung homogenate extract. The greatest identity of 99% was found for cetacean calicivirus (CCV), originally isolated in 1979 from an Atlantic bottlenose dolphin [*Tursiops truncatus*] (Smith et al., 1983d). But there were also identities of 88% - 98% with 17 other vesivirus sequences in GenBank. Thus, the cross-species infection of cattle by a marine vesivirus was suggested (Smith et al., 2002b). Despite this apparently clear-cut case of caliciviral abortion in cattle and less well documented further evidences derived by extrapolation from cases of calicivirus-induced abortion in several other species, the pathogenic potential of vesiviruses in cattle, in particular the viral role in abortion has not been carefully scrutinized yet.

History of Vesiviruses

Although vesiviruses are known to cause abortion and stillbirth in swine (Bankowski, 1981; Barlough et al., 1986; Dunne et al., 1965) they were first recognized as causing a more threatening disease. The history of vesiviruses started back in 1932, just three years after the eradication of an outbreak of foot-and-mouth disease (FMD) in the United States. The first outbreak occurred on 22 April 1932 in Orange County, California/USA, on a large farm where raw garbage was fed to swine. The animals developed vesicular lesions on the snout, tongue and feet and without further tests it

was assumed to be a new epizootic of FMD (Bankowski, 1981; Traum, 1934, 1936). More than 19,000 head of affected swine and adjacent exposed livestock of all species were slaughtered and disposed of by deep burial because of the very strict FMD eradication procedures. Each farm was thoroughly cleaned and disinfected with 2% lye solution. In retrospect, infectivity studies suggested the disease was atypical FMD because the agent in 1932 caused infection only in swine but not in other cloven-hoofed animals (Smith and Akers, 1976).

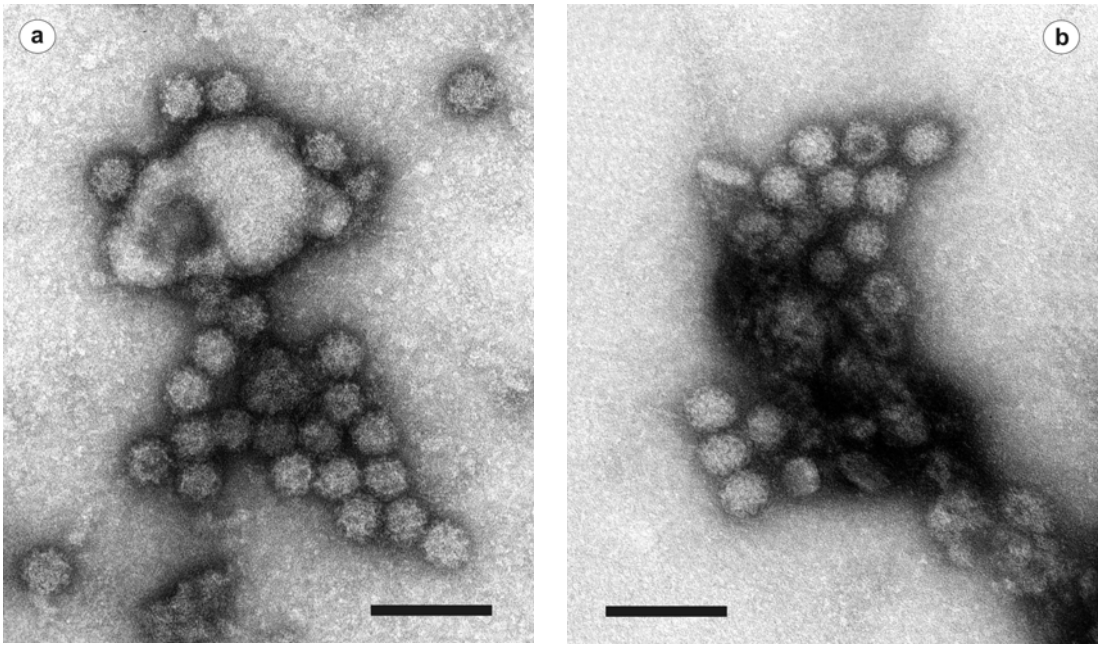


Figure 1. Electron micrographs of vesivirus partiles with their calivirus-typical cup-shape depressions (a) and *Noroviruses* with their more fuzzy-like appearance (b) after negative staining with 1% uranyl acetate. Size bar = 100nm

Foot-and-mouth disease has been around since the antique, first described as a vesicular disease in Europe in the 18th century. The United States has experienced nine outbreaks of FMD, the first in 1870 and the last in 1929 (Irwin, 2003). FMD outbreaks in Britain appeared between 1839 until recently (Woods, 2001). The standard procedure for FMD eradication in the United Kingdom demands that as soon as a case of FMD is confirmed, all animals on infected premises must be slaughtered and disposed of within 24 hours. That is because the FMD agent, an antigenetically heterogeneous RNA virus of the family *Picornaviridae*, is one of the most contagious agents on earth. The disease can be presumptively diagnosed on clinical signs by a veterinarian, or the diagnosis can be confirmed by a positive laboratory test result. During

the 2001 outbreak in the UK only 1.3 out of 6.5 million animals (5.3 mill sheep, 0.75 mill cattle, 0.45 mill pigs, 7,500 goats, 1,000 deer and 1,000 others) which were slaughtered, were FMD confirmed by a laboratory test (DAFRA, 2003). These numbers demonstrate the need for a more accurate and sensitive diagnose for FMD. In swine, besides FMD, three other known viruses can cause clinical diseases that mimic FMD. These are swine vesicular disease virus (also a picornavirus which has never been reported in the United States), vesicular stomatitis virus (a member of the family *Rhabdoviridae* and cyclic in the United States) and the vesicular exanthema of swine virus (endogenous to the United States) (Smith et al., 2002b; van Regenmortel et al., 2000).

During the second outbreak of *Vesiviral* disease, which occurred in 1933, lesion material from swine was examined and again, the virus would not experimentally infect all the usual hosts of FMD. Infected swine developed lesions indistinguishable from FMD while cattle and guinea pigs were unaffected. However, several inoculated horses developed fevers and lingual lesions, a clear indication that the vesicular disease could not be FMD. Therefore it was described as a new disease of swine and was called vesicular exanthema of swine (VES) (Crawford, 1937; Smith and Akers, 1976). A year later, the third outbreak occurred and again VES instead of FMD was diagnosed. Clinically VES was characterized as a contagious, febrile disease with vesicle formation on feet, tongue and snout of infected swine, was often implicated in reproductive failure with a variable morbidity of 30 - 90% and a low mortality, especially in older animals. All outbreaks, which occurred from 1932 to 1952 were in California swine herds and were shown by cross-immunity tests to be caused by many distinct but related VES virus serotypes and this could best be accounted for by introduction of the virus into swine from uncooked garbage (Bankowski, 1981; Smith and Akers, 1976). The original source of the aetiologic agent causing the disease were unknown, and swine were said to be the only naturally infected host (Smith and Akers, 1976). After the disease was determined to be a new disease of swine and not the feared FMD, clinically recovered pigs were allowed to be slaughtered and processed for food. However, because VESV was already widely disseminated into swine, the cycle was greatly amplified when in 1952 nationwide VES epidemics occurred by

feeding raw virus-containing pork scraps in garbage to susceptible pigs. From 1940 until 1956, the disease continued unabated. Quarantine did not provide effective control (Barlough et al., 1986) and within 14 month after the virus escaped California in 1952, all major swine-growing areas in 41 of the 48 contiguous States of the United States had reported VES.

Direct and indirect contact, especially by feeding raw garbage and offal, accounted for the vast majority of VES outbreaks among swine. Contaminated feed, water, urine and feces have been less frequently involved sources of viral transmission. It was shown that VESV shedding occurred shortly before and several days after vesiculation and could be found in feces, urine, nasal and oral secretions, and vesicular lesions (Bankowski, 1981; Smith et al., 1998b). The incubation period experimentally and under field conditions is usually 24 to 72 hours, although with milder strains it may be protracted to 10 days. The first signs of the vesicular disease in a herd is lameness and fever, followed by the occurrence of vesicles as blanched, flattened areas which enlarge and elevate to form blisters with clear fluid containing highly contagious virus. In natural uncomplicated infections without secondary infection, the lesions heal in usually 5 to 7 days. A typical complication involves sloughing of the hoof and requires more than 90 days to heal, by which time the animals lose most of their value. The proof of correlation between feeding raw garbage and VES infection was established through experimental transmission studies on feeding pork scraps and offal from infected swine to susceptible swine (Bankowski, 1981).

This historic event resembles in a very remarkable way the more recent bovine spongiform encephalopathy (BSE) epidemic in Europe. After enforcing federal laws, beginning in 1953, which required the cooking of all raw garbage used for feeding swine, the disease was contained and the last infected herd in the United States were identified at Secaucus, New Jersey, in late 1956 (Bankowski, 1981; Barlough et al., 1986). Strong eradication and quarantine measures against the disease VES, which was common in the US for 24 years appeared to have eliminated the disease by 1956. During this time 13 serotypes of VESV were described (Bankowski, 1981). Otherwise, the virus itself was not further characterized because neither EM nor serology data were available for comparison and clarification. The US Secretary of Agriculture

announced in 1959 that VES had been eradicated and designated it a Foreign Animal Disease (FAD) even though VESV had not been reported outside the United States with the exception of Iceland, where in 1955 the disease appeared among swine fed raw pork scraps from a US military base. Between 1932 and 1956, a total of 1,563 individual outbreaks of VESV were recorded in the US, mostly in California, according to the U.S. Livestock Sanitary Association (Bankowski, 1965). The direct cost of eradication was set at $ 39,000,000 (Barlough et al., 1986).

Roughly two decades later, while investigating the high incidence of abortion in California sea lions [*Zalophus californianus*] a virus was isolated in 1972 from a rectal swab of an aborting animal on San Miguel Island, California and was designated San Miguel sea lion virus (SMSV). Based on morphology after negative-stain and thin section electron microscopy, this newly emerged virus was assigned as a calicivirus like VESV (Smith et al., 1973). SMSV was classified by United States Government regulatory officials as "a virus indistinguishable from VESV". This was based on animal infectivity where infected swine developed a vesicular exanthema. The morphology and physiochemical characteristics, e.g. buoyant density, sedimentation coefficient, ether sensitivity and others were typical for caliciviruses. Neither VESV or SMSV cause disease in mice, rabbits, guinea pigs and hamsters, but both can produce lesions in swine exposed by contact or inoculation (Smith et al., 1973; Smith and Boyt, 1990). The origin and potential reservoir of VESV remained unknown and swine were thought to be the only natural infected host species until the 1972 discovery of the caliciviruses designated SMSV which proved to be additional serotypes of the 13 existent serotypes of vesiviruses isolated prior to 1956, which had been designated VESVs (Smith et al., 1973; Smith and Boyt, 1990; Smith et al., 1980). Subsequently, at least 16 additional SMSV serotypes have been isolated from a number of marine species including fish, shellfish, seals and whales. They differ by standard cross-neutralization testing, using 100$TCID_{50}$ (1:100 dilution of virus required to infect 50% of a given batch of inoculated cell cultures (Mahy and Kangro, 1995)) of test virus against 20 antibody units of typing sera; an antigenetic variant designated a serotype is based on not being neutralized by antiserum containing 20-times the antibody concentration needed to neutralize the homologous virus type

(Smith and Boyt, 1990). All 13 virus strains isolated before 1956 were called VESV, labeled alphabetically in order of isolation followed by the year of their collection, e.g. VESV-K_{56} was the last strain isolated in 1956; those isolated from marine mammals after 1972 were called SMSV, labeled with following numbers, e.g. SMSV-6 was the sixth serotype isolated (Bankowski, 1981) and still others were named after the species of first isolation such as bovine calicivirus, primate calicivirus and reptile calicivirus. However, there is evidence linking all *Vesiviruses* except canine calicivirus to an ocean presence and virtually all appear to be a continuum of the VESV/SMSV group within the genus *Vesivirus* (Smith, 2000). All VESV- and SMSV-strains are members of the two serotypes of VESV and SMSV, combined in a single species, named Vesicular Exanthema of Swine Virus within the genus *Vesivirus* (Tidona and Darai, 2002; van Regenmortel et al., 2000). Although, SMVS-4 and VESV A_{48} were shown to differ at the genetic level (Neill et al., 1998), when using neutralization tests no serotype of SMSV was neutralized by any other SMSV or VESV antiserum nor were any of the 13 existent VESV serotypes neutralized by antiserum to any other VESV or SMSV serotype (Barlough et al., 1986; Neill et al., 1995; Smith and Akers, 1976).

Principles of emerging viruses

Apparently SMSV and VESV induce productive infections in marine as well as terrestrial mammals. The viruses, causing the devastating epidemics of vesiviral disease in swine, were eventually shown to be present in the ocean. This was the first report of "viral trafficking" involving pathogenic viruses from marine mammals, which produced a disease that spread among terrestrial mammals (Smith et al., 1973).

But where did the vesivirus causing the FMD-like epidemic in 1932 come from? And if VESV/SMSV was really the same virus where was it hidden between 1955 and 1972?

To answer those questions we have to understand some principles of emerging viruses. Following the theory of Darwinian evolution, "newly evolving or emerging" viruses will descent from a parent that already exists in nature. There are three sources for "new" viruses: 1) evolution *de novo* and selection of a new virus strain; 2) intro

duction of an existing virus from another species; 3) dissemination of a virus from a smaller population in which the virus might have arisen or originally been introduced. The latter two are also referred as "viral traffic" (Morse, 1993).

Viruses, especially RNA viruses demonstrate high mutation rates, which provides them with a great potential for change and adaptation. RNA viruses show ten- to hundred-fold higher rates of mutations than DNA viruses and represent the majority of all plant, animal and human viruses (Domingo and Holland, 1994). Without the proof-reading and repair system of DNA organisms their replication is an extremely error-prone process, one necessity for evolutionary success of the phenotype. They are mostly small, simple, rapidly–replicating and efficient with a genetically plastic genome offering the advantage for an enormous adaptability to changing environments and possibly hosts since they consist of extremely heterogenous (quasispecies) populations. Quasispecies swarms evolve from a single virus particle (a clone) due to the enormous mutation rate. Some master sequences with the maximum fitness might become the product of selection and will represent an average (consensus) sequence for the entire genome, resulting in a serotype like cetacean calicivirus (CCV). Different serotypes within 85-100% homology for conserved regions at the genome representing the genus *Vesivirus*. Within the family *Caliciviridae*, the genome differences of the four genera, named *Vesivirus*, *Lagovirus*, *Norovirus* and *Sapovirus*, are greater than 25%.

DNA organisms developed a variety of mechanisms working against spontaneous mutations, maintaining or restoring their genetic code with the increasing complexity of the organism. In response, DNA viruses evolved secondary mechanisms to avoid the proof-reading of their genome and to induce a higher mutation rate as an advantage over their hosts. Despite the missing proof-reading and repair system in RNA viruses, the mutation rates can vary with different polymerases, pH, temperature, oxygen levels, pressure, base substitution pairs, genome regions and other factors. It is assumed that a reasonable approximation of the average RNA virus, replicase error frequency, including those of caliciviruses, is of the order of 10^{-4} to 10^{-5} base substitutions per site (0.1 to 1 transcriptional errors per replication) exceeding the average error frequencies by a million-fold or more of their animal, plant or human hosts

(Holland, 1993), meaning their evolution rate is proceeding a million-fold faster. But, obviously there are strict constraints such as virus genome size, replication strategy, genome organization, host range and so on, and therefore a limit of possible viable mutants. RNA virus genomes were shown to be able to accommodate half of it's genome as mutations (Holland, 1993). That means for the 8,000 (8 kb) base vesivirus RNA genome, about 4^{4000} sequence mutations might be possible. Sequence analysis of a hypervariable region within the capsid protein gene of a member of the vesiviruses, feline calicivirus (FCV), isolated from oropharyngeal swabs taken from cats, showed an evolution rate of about 1 base substitution per nucleotide per year (Radford et al., 1998), meaning an average error frequency of about 10^{-4} when calculating with a genome of about 8,000 nucleotides. Vesiviruses can replicate rapidly in 4 to 8 hours to very high copy numbers (1,000 to 10,000) per infected cell (Smith et al., 2002a), which results in virtually every progeny of a given viral particle being a genetic variant. Therefore, a single calicivirus virion does not reproduce exact copies of itself but instead a cluster of closely related but genetically slightly different replicates, which are referred to as quasispecies (Eigen and Biebricher, 1988). Similar mutation rates are described for HIV, influenza virus and foot-and-mouth disease virus in contrast to less than 10^{-9} base substitutions per nucleotide per year in higher eucaryotes (Holland, 1993). But although new sequences arising gradually and continuously with infinite possibilities, which produce a great diversity of viruses, VESV's and other marine caliciviruses appear to have been maintained in an antigenetically unchanged state for many years. Recent exposure to the same serotypes of VESV isolated over 40 years ago have been detected in both the marine and terrestrial environments (Smith and Boyt, 1990). In light of this, it seems unlikely that rapid mutation and genetic instability entirely explain the antigenetic variability of vesiviruses. Alternatively, some of the apparent stability could be attributed to viruses becoming locked in polar and glacial ice reservoirs and then, preserved there for decades or perhaps even thousands of years before being released back into the ocean by ice melt (Castello et al., 1999).

But although the significance of evolution (mutation) as a source of new viral diseases is hard to determine and may play a greater importance over long time periods, it is likely to be more important for increasing adaptedness in a new host immediately after

a virus is introduced into a new species or population, perfectly demonstrated by the co-evolution of virus and host. There appear to be relatively few documented examples in nature where a new viral variant shows a greatly increased virulence or causes a different type of disease. One of the most notable examples is demonstrated by influenza where different variants arise by mutation, but more importantly by recombination, becoming infectious for humans after evolving in alternative hosts, such as chicken (Morse, 1993).

In contrast, the majority of emerging viruses already existed in nature and gained access to new host populations via viral traffic. The increasing interactions between humans, wildlife and domestic animals and their changing environments provide a heightened potential for increased contact with previously inaccessible viruses. The translocation of wildlife for conservation and hunting occurs on a global scale, with an inherent risk of exposure of wildlife species to exotic infectious agents (Daszak et al., 2000). Other examples for the complex host-parasite ecological continuum are agricultural intensification, global travel, pathogen pollution, urbanization, human encroachment, ecological and biomedical manipulation. The mechanics of pathogen pollution involve global traffic in agricultural materials, domesticated animals, food crops, timber, contaminated wastes such as landfill and ballast water, human organ transplants and blood donations (Daszak et al., 2001). Most human and animal emerging infectious diseases (EIDs) are resulting from exposure to zoonotic pathogens, that is, those agents transmitted from animal species to humans, with or without establishment of a new life-cycle. Wildlife plays a key role in disease emergence by providing a large “zoonotic pool” from which previously unknown pathogens may emerge (Daszak et al., 2000). This seems logical, considering that the total number and variety of viruses in animal species is very large, demonstrated by arthropod-borne viruses as one example of the great variety of microorganisms in nature, which are transmitted from one host to another by hematophagous arthropods such as mosquitos, ticks, midges and sandflies. From more than 500 identified arthropod-borne viruses only about 120 are known to cause illness in humans or livestock (Calisher and Beaty, 1992). A similar situation would apply to viruses already present in a limited or isolated human or animal population. The examples of HIV, Marburg-

and Ebolavirus demonstrate that human activities can be especially important for dissemination of newly introduced viruses. Human infections by yellow fever, Dengue, Lassa, Hanta, Hendra and Nipah virus, and the recently emerged SARS-associated coronavirus represent successful introductions of viruses into mankind, whereas the emergence is forced by or depend on the right conditions (Morse, 1993). The zoonotic pool can also include organisms in environments that have been little studied like the ocean environment.

Vesiviruses as emerging agents

With the additional knowledge of the past 70 years we can now try to answer the open question about the emergence of VESV in 1932. There is still no certainty about the time of emergence of VESV in swine or other mammals. It could have been present for a long time in isolated places. But even if VESV was endemic, smoldering in swine populations, an important parameter changed around 1932. Feeding of raw garbage containing fish viscera and frames that had been collected from institutions in port cities of California to swine in adjacent herds and also trimmings from dead or slaughtered swine, was not uncommon prior to 1932. But, apparently the combination of agricultural intensification and pathogen pollution became an ideal condition for the spread of VESV or a new series of virus pathotypes suddenly emerged carrying a new vesiculogenic virulence factor. After strong regulations were introduced and enforced prohibiting the feeding of raw garbage and mandating “sterilizing” by cooking, the spreading of VESV was controlled and the disease vanished for 20 years until scrutiny in another environment got new attention. Viruses associated with ocean mammals were not studied before the end of 1960s. The first virus isolated from any marine mammal occurred in 1968. From then on marine caliciviruses were shown to be geographically widely distributed in a variety of mammals. The first vesivirus (SMSV-1) was discovered in California sea lion pups [*Zalophus californianus*] in 1972 then subsequently an additional 30 serotypes and over 160 strains have been isolated from a variety of sources at the Pacific Rim of North America (Table 1).

In 1988, after reexamination of the 1968 virus isolate from a California gray whale [*Eschrichtius robustus*] (the first virus ever isolated from any marine mammal) which was thought to be an enterovirus, was instead, shown by negative staining electron

microscopy to be a calicivirus. Furthermore, the serotype of this virus was found identical to VESV-A_{48} as revealed by cross-neutralization. VESV-A_{48}, which was originally isolated from diseased swine in 1948, is the prototype for the family *Caliciviridae*. In experimentally exposed swine the gray whale isolate caused clinical vesicular disease (Smith, 2000).

The 2001 finding of a vesivirus from a bovine fetus was not the first time vesiviruses were shown in terrestrial species after VESV was eradicated. While expanding the search for caliciviruses and their respective antibodies in terrestrial animals, a complex picture of antigenic diversity and host nonspecificity emerged for vesiviruses. In addition to the serotypes designated SMSV from 4 pinniped species, 13 distinctive additional serotypes were isolated from 18 species (mink, human, primates-5 species, dolphin-2 species, gray whale, reptiles-3 species, amphibians, cattle, rabbits, walrus, and domestic swine) (Table 2).

The ease with which vesiviruses of ocean origin could be replicated *in vitro* in primate and human cell lines and cause disease in primates following experimental infection gave early warnings of possible human health implications for these viruses (Smith et al., 1978). The first report of human disease caused by vesiviruses was published 20 years later, after the first human calicivirus isolate designated San Miguel sea lion virus serotype 5 Homosapiens-1 (SMSV-5 Hom-1) was recovered from a laboratory worker, who was processing caliciviruses for transmission experiments in calves. He developed systemic illness, including vesicular lesions on all four extremities. After identifying caliciviruses as the causal agent in the vesicular fluid by electron microscopy, a close relationship to SMSV-5 (97% nucleotide identity) was determined for the virus isolate using RT-PCR amplification and sequencing of a 500 base amplicon from the viral RNA polymerase region (Smith et al., 1998a).

Table 1. Vesivirus isolates from marine species

Serotype/strain	Species of origin	Source	Where isolated	Year	Reference
SMSV-1/1MR	California sea lion [*Zalophus californianus*] Northern fur seal [*Calorhinus ursinus*]	rectal swab nose swab	California, USA Alaska, USA	1972 1972	(Smith et al., 1973) (Smith et al., 1974)
SMSV-2/2MR	California sea lion	throat and rectal swab	California, USA	1972	(Smith et al., 1974)
SMSV-3/1FT	California sea lion	nose swab	California, USA	1972	(Smith et al., 1974)
SMSV-4/15FT	California sea lion Swine	throat swab throat and rectal swab	California, USA California, USA	1973 1976	(Smith et al., 1977b) (Smith et al., 1978)
SMSV-5/205	Northern fur seal Animal feed Human	vesicular lesion mink food blisters	Alaska, USA Alaska, USA Oregon, USA	1973 1974 1985	(Smith et al., 1977b) (Sawyer et al., 1978) (Smith et al., 1998a)
SMSV-6/436	California sea lion Northern fur seal Opaleye fish [*Girella nigricans*] Stellar sea lion [*Eumetopias jubatus*]	vesicular lesion throat swab spleen rectal swab	California, USA California, USA California, USA Oregon, USA	1975 1977 1976 1985	(Smith et al., 1979) (Smith et al., 1980) (Smith et al., 1980) (Skilling et al., 1987)
SMSV-7/Gn-21	Northern elephant seal [*Mirounga angustirostris*] Opaleye fish [*Girella nigricans*]	throat and rectal swab visceral tissue	California, USA California, USA	1976 1976	(Smith et al., 1980) (Smith et al., 1980)
SMSV-8/274	Northern fur seal	vesicular lesion	Alaska, USA	1976	(Smith et al., 1981a)
SMSV-9/427T	California sea lion Pacific bottlenose dolphin [*Tursiops gillii*]	throat swab	California, USA Hawaii, USA	1975 1987	(Smith et al., 1981a) (Smith, 2000)
SMSV-10/V53	Northern fur seal	vesicular lesion	Alaska, USA	1977	(Smith et al., 1981a)
SMSV-11/Zc-03-77	Northern fur seal	throat and rectal swab	California, USA	1977	(Smith et al., 1981a)
SMSV-12/913T	Northern fur seal California sea lion	throat and rectal swab throat and rectal swab	California, USA California, USA	1977 1977	(Smith et al., 1981a) (Smith et al., 1981a)
SMSV-13/CSL=461	California sea lion	vesicular lesion	California, USA	1984	(Smith, 2000)
SMSV-14/A1280	Stellar sea lion California sea lion	clinically normal	Oregon, USA California, USA	1987 1987	(Smith, 2000) (Smith, 2000)
SMSV-15/220R	California sea lion	throat and rectal swab	California, USA	1988	(Smith, 2000)

Table 1. Vesivirus isolates from marine species (continued)

SMSV-16/Zc-698	California sea lion	feces	California, USA	1988	(Smith, 2000)
SMSV-17/Zc-91PG	California sea lion Pacific mussels [*Mytilus californianus*]	nasal swab intestine	California, USA	1991 1992	(Smith, 2000) (Smith, 2000)
Walrus CV (WCV)/7420	Walrus [*Odobenus rosmarus*]	feces	Chukchi Sea	1977	(Smith et al., 1983b)
Cetacean CV (CCV Tur-1)/0041	Atlantic bottlenose dolphin [*Tursiops truncatus*] California sea lion	tattoo pox lesion	California, USA	1979 1979	(Smith et al., 1983d) (Smith et al., 1983d)

Table 2. Vesivirus isolates from terrestrial species

Serotype/strain	Species of origin	Source	Where isolated	Year	Reference
FCV/F-9	Cat	diagnostic cell culture	New Zealand	1957	(Fastier, 1957)
Mink CV (MCV)	Mink [*Mustela vision*]	pharyngeal and rectal swab	Idaho	1977	(Evermann et al., 1983)
Reptile CV (RCV)/0002	Reptiles and amphibians California sea lion Northern fur seal Stellar sea lion	various tissues and rectal swabs	San Diego Zoo, California	1978 1986 1986 1987	(Smith et al., 1986a) (Smith, 2000) (Smith, 2000) (Smith, 2000)
Skunk Calicivirus (SCV)	Skunk	feces	North-central USA	1994	(Seal et al., 1995)
Canine calicivirus (CaCV)	Dog	feces	Tennessee, USA	1982	(Schaffer et al., 1985)
Bovine calicivirus (BCV Bos-1)/217	Cattle	throat and rectal swab	Oregon, USA	1981	(Smith et al., 1983a)
Bovine calicivirus (BCV Bos-2)	Cattle amplicon only	lung	Oregon, USA	2001	(Smith et al., 2002b)
Non-cultivatable vesivirus	White tern hatchlings [*Gygis alba rothschildi*]	vesicular lesion	Hawaii, USA	1992	(Poet et al., 1996)
Primate CV (PCV Pan-1)/022	Pygmy chimpanzee [*Pan paniscus*] Lowland gorilla [*Gorilla gorilla*] Silverleaf langur [*Presbytis cristata*] Spider monkey [*Ateles fusciceps*] Douc langur [*Pygathrix nemaeus*]	herpes-like lip lesion spleen tissue throat swab throat swab brain tissue	San Diego Zoo, California, USA	1978 1978 1979 1978 1979	(Smith et al., 1983c) (Smith et al., 1985b) (Smith et al., 1985b) (Smith et al., 1985b) (Smith et al., 1985a)

In other mammalian species, the same and similar SMSV types have also been implicated in an array of diseases after natural or experimental infection, including abortion in swine, cattle and seals, pneumonia in cattle and swine, diarrhea in cattle and swine, myocarditis in seals and swine, and encephalitis in primates and swine (Smith, 2000). In 2003, the identification of a vesivirus isolate (isolate 2117) from Chinese hamster ovary cells in Germany was reported (Oehmig et al., 2003). Aside from the cultured cells, the fetal bovine serum (FBS) used as a culture supplement was described as the most likely source of viral contamination since the contamination of FBS with other viruses has already been published (Erickson et al., 1991). In addition, transplacental passage of caliciviruses has already been described for sea lions and cattle, where SMSV have been detected in aborted fetuses. Finally, there is recently documented evidence for an association between an emerging zoonotic vesivirus from the sea and hepatitis in humans. *Vesivirus* amplicons generated from sera of hepatitis patients matched closely to PCV Pan-1, SMSV-6 and –5 (Smith and Skilling, 2000).

Serological surveys in marine mammals adjacent to countries bordering the north Pacific Ocean such as the United States, Canada, Russia and Mexico have shown a much broader prevalence of vesivirus types than virus types actually isolated from animals in each of these locations (Smith, 2000). In addition to the animals already described, antibodies, and thus also evidence of contact with vesiviruses, were found in both ocean and terrestrial animals. Antibodies and sometimes isolates of additional SMSV serotypes were detected not only in the already mentioned marine mammals but also in bowhead whale, fin whale, sperm whale, sei whale, swine, donkey, gray fox, musk ox, bison, cattle, sheep and human. In addition to swine, the known historic host for all VESV serotypes, antibodies against VESV were detected in the donkey, California sea lion, bowhead whale, gray whale, fin whale, sei whale and Hawaiian monk seal (Smith, 2000). Often the same serotype has been isolated from different species on land and sea, over widely dispersed geographic regions, and in widely separated years. For example, when bowhead whales sera were examined in the 1980's, type-specific antibodies were found to three SMSV- serotypes in addition to VESV-J_{56} and –K_{56}, which had not been re-isolated since their discovery in New Jersey's domestic swine in 1956 (O'Hara et al., 1998; Smith et al., 1987). And a single

serotype, SMSV-5, was shown to infect naturally four species of pinnipeds, six species of cetaceans, rabbit, domestic swine, feral fox, musk ox, domestic cattle, and human with clinical disease observed in seals, humans, swine and monkeys (Smith and Boyt, 1990).

Thus, evidence from serological surveys and experimental transmission studies indicate that the land-ocean interface is not an effective barrier to these viruses. The repeated isolation of the same serotypes from so many different species provided evidence for transmission and potential reservoirs of vesiviruses. In comparison to other RNA viruses, mutation frequency and the introduction into other species are not the only reasons for *Vesiviruses* infecting such a broad range of different hosts. So why are *Vesiviruses* different? The vesivirus itself and its genetic characteristics are the keys to the unusual high host species versatility.

What exactly we are looking at?

Members of the family *Caliciviridae* [*Latin* = calix] are small, non-enveloped viruses with a single-stranded, linear, positive-sense polyadenylated RNA of 7.5 to 8.5 kb in length. The genome is protected by an icosahedral capsid composed of 180 molecules of a single structural protein linked as dimers to form 90 arch-like capsomeres. The three-dimensional structure of the capsid has a T=3 icosahedral symmetry, representing 2-, 3- and 5 fold axes (Prasad et al., 1994). When visualized by direct negative-stain electron microscopy (EM) or electron cryomicroscopy it appears with its typical 32 cup-shape depressions, except for *Noroviruses* with their more fuzzy-like appearance, 27 to 40 nm in diameter (Figure 1). Caliciviruses are easily recognized as clearly distinct from the members of all other virus families by shape and size. The calicivirus family comprises four genera based on tentative genomic organization and are designated *Norovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus* (Table 3) (Green et al., 2000a; International Committee on Taxonomy of Viruses, 2002; Tidona and Darai, 2002; van Regenmortel et al., 2000). The latter two have been further divided into the following species: Feline calicivirus (FCV) and Vesicular exanthema of swine virus (genus *Vesivirus*), and Rabbit hemorrhagic disease virus and European brown hare syndrome virus (genus *Lagovirus*), respectively. The International Committee on Taxonomy of Viruses (ICTV) gives the following definition for a species within the *Caliciviridae*:

"A calicivirus species will be defined as a cluster of viruses that constitutes a major phylogenetic branch within a genus and is also distinguishable from other branch(es) by one or more of the following biologic properties: natural host range, natural cell and tissue tropism, and antigenicity" (Green et al., 2000b; International Committee on Taxonomy of Viruses, 2002). FCV strains, associated with respiratory disease and conjunctivitis in cats, represent only one serotype according to the neutralization test while VESV/SMSV represent a very heterogeneous group of viruses. According to the Decimal Code for virus nomenclature (Tidona and Darai, 2002), the species *Vesivirus* contains a variety of serotypes, e.g. San Miguel sea lion virus, which contains a variety of isolates/strains, e.g. SMSV-1 or VESV-A_{48} (Table 3).

All genera of the *Caliciviridae* except vesiviruses were found resistant to *in vitro* propagation. While FCV is easy to grow in CrFK (Crandell-Rees feline kidney) cells (Fastier, 1957), vesiviruses often resist *in vitro* propagation on initial isolation. However, once adapted to cell culture after three to six blind passages they frequently replicate to 10^9 tissue culture infective particles per ml of stock culture. Infected cells undergoe a loss of microvilli and pseudopodia, become round and detach from the substrate as viral infection progresses. Marine mammal cell lines (sea lion skin cells, dolphin skin cells) have been uniformly disappointing for calicivirus isolation and propagation, but primate cell lines (Vero, African green monkey kidney), pig kidney (PK-15) cells and CrFK cells have been especially sensitive for this purpose. Members of the marine vesiviruses remain viable in buffered solutions at 4°C for indefinite periods and survive in seawater at 15°C for more than 14 days (Smith and Boyt, 1990).

Viruses of the Norwalk-like group are infectious mainly for humans. They cause gastroenteritis and were discovered prior to 1972 (Kapikian et al., 1972). Viruses of the Sapporo group, primarily isolated in 1983 in Japan, also cause gastroenteritis, particularly in children (Nakata et al., 1985). The third non-cultivable group is the calicivirus associated with rabbit hemorrhagic disease (RHD), first documented in rabbits in China in 1984 (Liu et al., 1984) and designated as the prototype strain (Green et al., 2000b). From time of exposure, hemorrhagic death can occur within 48 hours in up to 95% of exposed rabbits (Smith, 2000), making this one of the most

deadly viruses known. In 1980, the first lagovirus was found in Denmark in livers of infected hares with a mortality rate of ~40% and was designated European brown hare syndrome virus (EBHS) (Gavier-Widen and Morner, 1991).

Table 3. Taxonomy and Decimal Code of the family *Caliciviridae* (Tidona and Darai, 2002).

Taxonomic Level	Decimal Code	Nomenclature
Family	00.012	*Caliciviridae*
Genus	00.012.0.01	*Vesivirus*
Species	00.012.0.01.001	Vesicular exanthema of swine virus (VESV)
Serotype	00.012.0.01.001.00.001	VESV-A48
Serotype	00.012.0.01.001.00.002	Bovine calicivirus (BCV Bos-1)
Serotype	00.012.0.01.001.00.003	Cetacean calicivirus (CCV Tur-1)
Serotype	00.012.0.01.001.00.004	Primate calicivirus (PCV Pan-1)
Serotype	00.012.0.01.001.00.005	Reptile calicivirus (RCV Cro-1)
Serotype	00.012.0.01.001.00.006	San Miguel sea lion virus (SMSV)
Isolate/Strain	00.012.0.01.001.00.006.001	SMSV - serotype 1
Isolate/Strain	00.012.0.01.001.00.006.004	SMSV - serotype 4
Isolate/Strain	00.012.0.01.001.00.006.017	SMSV - serotype 17
Serotype	00.012.0.01.001.00.00	Skunk calicivirus (SCV)
Species	00.012.0.01.003	Feline calicivirus (FCV)
Genus	00.012.0.02	*Lagovirus*
Species	00.012.0.02.001	Rabbit hemorrhagic disease virus (RHDV)
Species	00.012.0.02.002	European brown hare syndrome virus (EBHSV)
Genus	00.012.0.03	*Norovirus* ("*Norwalk-like viruses*")
Species	00.012.0.03.001	Norwalk virus (NV)
Genus	00.012.0.04	*Sapovirus* ("*Sapporo-like viruses*")
Species	00.012.0.04.001	Sapporo virus (SV)
Unassigned	00.012.0.00.010	Walrus calicivirus (WCV)

The vesivirus genome is very similar to that of the other three genera of the *Caliciviridae* in that it encodes viral proteins in three separated open reading frames (ORFs) (Figure 2). ORF1 encodes a large polyprotein in the 5' region of the genome, i.e. the precursor to a number of non-structural proteins including a helicase, a protease, and a RNA-dependent RNA polymerase (Neill et al., 1995). The second ORF encodes the single major structural capsid protein in the 3' region with a molecular mass of ~58 kDa (Clarke and Lambden, 2000). Vesiviruses synthesize the capsid

protein initially as a larger precursor with ~78 kDa that undergoes a maturation cleavage by the viral 3C-like protease (Rinehart-Kim et al., 1999; van Regenmortel et al., 2000) giving rise to a mature capsid protein very similar in size to those of other members of the *Caliciviridae*.

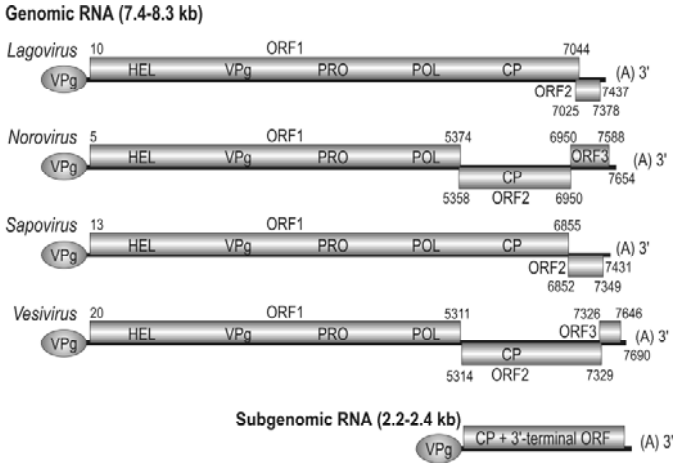


Figure 2. Calicivirus genomic organization and reading frame usage are shown for representative strains in *Lagovirus* (Ra/LV/RHDV/GH/1989/GE, GenBank accession no. M67473), *Norovirus* (Hu/NLV/NV/1968/US, GenBank accession no. M87661), *Sapovirus* (Hu/SLV/Man/1993/UK, GenBank accession no. X86560) and *Vesivirus* (Fe/VV/FCV/F9/1958/US, GenBank accession no. M86379). Predicted RNA helicase (HEL), protease (PRO) and polymerase (POL) regions present in all calicivirus genomes are indicated. The capsid protein (CP) coding sequence is located either on ORF1 (*Lagovirus* and *Sapovirus*) or ORF2 (*Norovirus* and *Vesivirus*). A subgenomic-sized (sg) RNA, being coterminal with the 3' end of the genome, is a template for translation of the major viral CP. The 3'-terminal ORF product has been identified as a minor structural protein in RHDV (Green et al., 2000b; van Regenmortel et al., 2000).

Sequence alignments of the capsid protein from different vesiviruses led to the identification of six distinct regions (A-F) based on amino acid homology, which differ in their degree of conservation (Neill, 1992). These regions denote the structural and antigenetic domains of the capsid protein. The third ORF was shown recently in *Noroviruses* to encode two basic proteins with molecular masses of 23 and 35 kDa that are able to interact with the ORF2 capsid protein (Glass et al., 2003). The virus replication occurs in the cytoplasm of the host cell via a negative strand intermediate. Central to the replication is the capability of the genomic RNA to serve as mRNA after infection. Enzymes, responsible for the replication of the genome are made after

infection and need therefore not to be brought into the cell by the virion. That is why RNA extracted from vesiviruses is still infectious (Roizman and Palese, 1996).

Genetic diversity within the *Caliciviridae*

Information concerning the genomic relatedness of caliciviruses has become available only recently after total RNA sequences were generated for several members of the family (Tidona and Darai, 2002). Homology comparison studies at the nucleotide and amino acid sequence levels demonstrated that caliciviruses have a number of highly conserved regions, e.g. motifs of the 2C RNA helicase or RNA-dependent RNA polymerase within ORF1, and of the capsid gene encoded within ORF2, although the overall sequence diversity within the family is high. Sequence comparisons of the highly conserved RNA polymerase genome region between vesiviruses and members of other genera of the *Caliciviridae* demonstrated high genetic variability with at least 25% amino acid sequence divergence between the different genera (Neill et al., 1995). The amino acid identity between different strains of the species VESV is 83 to 96%, but they have only 45 to 54% identity within the FCV species, 32 to 41% identity in *Sapovirus*, 27 to 32% identity within RHDV and only 16 to 21% identity in *Norovirus* (Matson et al., 1996; van Regenmortel et al., 2000).

Looking closer into the genus *Vesivirus*, strains of the species FCV have been shown to be very closely related, with differences in one or two regions of the capsid protein (Carter et al., 1992), representing only one serotype. In contrast to FCV, a broad cluster of serotypes within the species VESV exists. Nevertheless, the degree of genetic homology for different VESV and SMSV serotypes is high enough to demonstrate that they belong to the same species (van Regenmortel et al., 2000). For example, region D of the capsid sequence contains 15 amino acids, which are conserved at a level of ≥ 95% in all animal caliciviruses (Neill et al., 1998). A sequence comparison of the non-structural 2C helicase and RNA polymerase protein coding regions showed that the otherwise most divergent members of the species VESV differed by only 14% from each other (Neill et al., 1995). One group of viruses, skunk calicivirus (SCV) and SMSV-1, were identical in both proteins (Neill et al., 1995) although preliminary serum neutralization tests point to a significant difference between the two viruses. These data suggest the possibility that SCV may be SMSV-1 that was introduced into

the skunk population although SMSV-1 was first isolated 50 miles off the California coast and SCV was isolated 3000 miles to the East near the Atlantic Coast.

When the RNA polymerase genomic regions of the BCV Bos-2 virus, isolated from an aborted bovine fetus (Smith et al., 2002b) were compared with other vesiviruses, a 99% sequence identity was revealed with CCV, 98% identity with BCV Bos-1 and VESV-C_{52}. Greater than 88% identity in the RNA polymerase gene region to 15 other serotypes was shown including VESV-A_{48}, SMSV-4, PCV Pan-1, WCV, RCV and SCV (Smith et al., 2002b) confirming earlier sequence results from Neill et al. in 1995 about the close relationship within the genus *Vesivirus*. Altogether a picture emerges where more conserved regions of the genome can be separated even within heterogeneous regions. Two mechanisms driving viral divergence can be distinguished: point mutations of single bases and recombination events, which are leading to exchanges of whole genetic blocks (Morse, 1994).

Possibilities and limitations for the diagnosis of Vesiviruses

Vesiviruses can cause vesicular disease very different in clinical severity in a wide variety of host animals. Therefore it is important for veterinary authorities to have reliable diagnostic tools for the detection of as many serotypes and strains of vesiviruses as possible. The first twenty years after VESV was discovered in 1932, the only means available for identifying and differentiating VESV from other agents was by inoculation of a number of species of animals, e.g. horses, cattle, swine, goats and guinea pigs, with infectious material to determine in which species the typical disease would be produced since the susceptibility to several vesicular disease viruses was different. But even under the best conditions, often a definite and accurate diagnosis was not achieved. With the growing progress in biochemistry and biotechnology over the last decades other more precise, less expensive and time-consuming tests were developed. To date, to diagnose VESV and other vesiviruses a variety of more or less specific tests are used at the Laboratory of Calicivirus Studies, College of Veterinary Medicine at the Oregon State University, USA: virus neutralization with all 30 isolated VESV serotypes and strains (Smith and Skilling, 1979), RT-PCR with a variety of type- and group specific primer sets (CaliciAmp) (Reid et al., 1999; Smith et al., 2002b), the peptide-based antigen D3A (CaliciScreen) for a group-specific ELISA, a

fluorescence antibody test using a vesivirus-specific monoclonal antibody (Calici-Glow) which binds all known vesivirus serotypes, including FCV and a 300 base cDNA hybridization probe (CaliciComp), which identifies every known serotype of VESV/SMSV and FCV (Smith et al., 2002b) and electron microscopy. The first two procedures are type-specific while the remaining three tests can be used for the general laboratory detection of vesivirus infection.

Experimental infections

Experimental transmission studies with different vesiviruses have yielded the first insights into susceptible hosts, resultant diseases and possible transmission routes. Swine were inoculated experimentally with the gray whale isolate (Smith, 2000) and with several other vesiviruses, including CCV (Smith et al., 1983d), SMSV-2 and VESV-A_{48} (Gelberg and Lewis, 1982), SMSV-5 (Berry et al., 1990), SMSV-7 (Smith et al., 1980), SMSV-13, WCV (Berry et al., 1990), BCV Bos-1 (Smith et al., 1983a) and RCV (Smith et al., 1986a) using stock virus containing 10^6 $TCID_{50}$ of virus/ml given intradermally and orally. The outcomes were always vesicular lesions indistinguishable from those caused by the more virulent serotypes of VESV (some VESV types such as VESV-J_{56} and K_{56} were incidental findings and did not cause clinical disease in experimentally infected swine) except for RCD and WCV. In addition, horizontal spread of virus to other animals by pen contact occurred. Infectious virus was recovered from vesicle fluid, blood samples, rectal swabs, brain and other tissues of infected swine. Calves have been inoculated experimentally with SMSV-5, SMSV-13 and BCV. While SMSV-5 failed to produce visible lesions, SMSV-13 produced severe vesicle formation followed by erosions at the site of intradermal inoculation. By the fourth day post-inoculation, secondary vesicular lesions developed, and disease spread to the pen-contact calf, producing large vesicles on the feet. Cattle inoculated with BCV developed skin lesions at the injection site without secondary vesicle formation. In a further comparative study, BCV produced classic vesicular exanthema in swine (Smith and Boyt, 1990; Smith et al., 1983a). The only experimental inoculation of horses with SMSV types 1-5 in 1977 caused erosions at the sites of intradermal inoculation and mildly elevated body temperature. However, none of the lesions spread by extension (Wilder et al., 1977). African green monkeys developed vesicular

lesions after experimental inoculation of VESV-C_{52}, SMSV-4 and SMSV-5 using 10^8 $TCID_{50}$ of stock virus/ml given intradermally (Smith et al., 1978). Opaleye fish infected orally and intraperitonealy with SMSV-5, then held in a saltwater aquarium at 15°C did not develop observable clinical disease. Nevertheless, the virus replicated up to 10^7 infective particles per gram of spleen and the fish remained productively infected for at least 31 days. At that time they were killed and their frozen tissues fed to Northern fur seal pups, which subsequently developed vesicular and other lesions yielding isolates of SMSV-5 (Smith et al., 1981b). No diseases developed in mink and rattlesnakes using MCV and RCD respectively and common laboratory animals such as rabbits, hamsters, rats, mice and guinea pig after inoculation of VESV and SMSV types 1-5 respectively (Smith et al., 1986a; Smith and Boyt, 1990; Smith et al., 1977a). In cell culture, however, many of the SMSVs produce high titers and cytopathic effects (CPE) within 8-10 hrs p.i. while other vesivirus serotypes have replicated to a high titer without causing CPE (Smith et al., 1977a). Most SMSV/VESV serotypes can be propagated to high titers in multiple cell lines, including Vero (African green monkey kidney), PK-15 (porcine kidney) and CrFK (Crandell-Rees feline kidney) reaching up to 10^9 infective particles per ml of culture (Smith and Boyt, 1990).

As previously mentioned, vesiviruses have been isolated from apparently healthy animals, which may represent clinically recovered animals still shedding virus, asymptomatic persistently infected animals, or animals experiencing subclinical self-limiting infections. The causal relationships between vesivirus infections other than FCV and other diseases except vesicular exanthema and abortion in swine remain unproven. And, although the vesicular disease first seen in swine has not been officially reported since 1956, and the virus was officially declared eradicated in 1959 in the US, it is endemic in US swine and has been isolated repeatedly from domestic herds in California, Minnesota and Pennsylvania (Smith, 2000). One of these isolates was WCV, another SMSV-4 and a third, which was isolated from piglets dying with porcine reproductive respiratory syndrome (PRRS) caused abortion and neonatal death of piglets in three experimentally infected, 95 day pregnant sows. This pathotype was not typeable with 27 neutralizing typing sera available at that time

(Smith et al., 1992). This infrequent appearance of a new and pathogenic *Vesivirus* pathotype could be explained by assuming that marine caliciviruses continue to be introduced into swine populations, presumably through inadequately heat-treated fish lysate protein or fish meal. The implications of fish protein supplements added into animal foods may also be involved in transmitting caliciviruses to other terrestrial species (Munday and Ryan, 1982; Smith et al., 1980).

Studies of trans-species transmission cycles were completed where swine were infected and developed vesicular disease by feeding naturally vesivirus-infected opaleye fish (Smith et al., 1981b). In swine, vesiviruses were experimentally transmitted successfully by intradermal, intranasal and oral routes (Berry et al., 1990; Gelberg et al., 1982; Gelberg and Lewis, 1982; Wilder and Dardiri, 1978). Direct contact transmission has been reported repeatedly in pen-contact animals like swine, cattle and reptiles during experimental infectivity studies and could represent the most common transmission route in nature (Berry et al., 1990; Smith, 2000; Smith et al., 1986a; Smith et al., 1983a; Smith et al., 1980). And finally, SMSV-5 infectivity was shown to survive for 14 days in seawater held at 15°C, suggesting transmission of vesiviruses through contaminated water (Smith et al., 1981b).

Immunological response

Information on the immunity to calicivirus infections still remains incomplete. Several studies have tried to determine whether pre-existing levels of serum antibody correlate with protection to the same or different serotypes. Short-term (6-14 weeks) immunity has been demonstrated consistently by homologous rechallenge of human volunteers with different strains of *Norovirus*, whereas a rechallenge after 27-42 month did not induce immunity (Parrino et al., 1977). A similar short-term immunity was demonstrated in swine after rechallenge with different strains of VESV (Bankowski, 1965). And although long-term immunity has not been shown in humans, swine were solidly immune as long as 30 month against VESV-B_{51} (Bankowski, 1965). Results from cross-infectivity studies demonstrated the complex relationships among members of both the *Norovirus* and *Vesivirus* genera. Cross-protective immunity could not be demonstrated after inoculation with genetically closely related but distinct strains that caused the same clinical diseases (Bankowski, 1965; Hale et al., 1998; Matsui and

Greenberg, 2000; Parrino et al., 1977). Although a cross-reactive epitope has recently been described for vesiviruses on the basis of production of cross-reactive monoclonal antibodies against all isolated strains (Smith et al., 2002a), this is not a neutralizing epitope.

The immune response following an infection with vesiviruses is serotype specific and occurs soon after exposure. Neutralizing antibodies generally appear very early within 3 to 5 days postinfection (Gelberg et al., 1982) and are maintained at a high titer for more than 90 days (Smith et al., 1978). Shedding of BCV in experimentally infected calves was observed for 45-days in the presence of neutralizing antibody titers as high as 1:640 following a booster inoculation of BCV given 14 days post infection. Neutralizing antibody titers to other SMSVs in both naturally or experimentally infected species range from a screening dilution of 1:10 up to 1:1280 in cattle and some neutralization titers as high as 1:2560 in marine populations have been recorded (O'Hara et al., 1998; Smith and Boyt, 1990).

Serologic surveys in walrus showed only weak neutralizing antibody activity against WCV. The same walrus populations did show modest seroactivity against other vesivirus types like SMSV-5 and SMSV-8 (Smith and Boyt, 1990). When WCV was given experimentally to nine swine, all shed virus and developed hepatitis but did not develop any vesicular lesions or neutralizing antibody even at a 1:2 screening dilution (Smith and Boyt, 1990; Smith et al., 1988). In another experiment, swine fed seal meat infected with SMSV-2 failed to seroconvert, while another group fed tissues from seals infected with VESV-A_{48} developed high levels of neutralizing antibodies without developing clinical symptoms (Gelberg et al., 1982). This seroconversion is indicative of a self limiting infection in these animals and demonstrated clearly that wildlife, even when inapparently infected, may pose a threat to domestic livestock.

Pathogen pollution as a mechanism for spreading Vesiviruses

How serious are the threats of possible reintroduction of vesiviruses into swine, other livestock and humans? They have high mutability, the ability to cause different diseases in different hosts and the likelyhood for continued spreading between wildlife, domestic livestock and humans. In addition to this agent’s ability to infect

and then spread through new host species, the geographic distribution of virus carriers is an important and unusual part of viral traffic for the VESV/SMSV *Vesiviruses*. Given the distributions of species known to carry marine vesiviruses and their estimated range, it appears that southern California waters are a central focus of vesivirus activity. Most outbreaks with VESV in swine occurred in California and most of the different vesiviruses were shown to infect California sea lions (Table 4). Opaleye fish, which in nature serves as a prime food source for California sea lions, is a primary reservoir for marine caliciviruses. Their habitat extends throughout the California sea lion breeding range and they have direct access to the terrestrial environment through the distribution of fish scraps generated by the commercial and sports fishing industries. Many of the marine mammals shown to carry vesiviruses, such as Stellar sea lions, Northern fur seals and California gray whales pass through this area during their seasonal migrations. Among species that are more isolated such as walrus, Hawaiian monk seal or bowhead whale, the virus could be either maintained entirely within each species, where their food sources may serve as virus reservoirs or other intermediary hosts may exist. But, because most species carry multiple serotypes of vesiviruses, it is unlikely that their numerically small populations would serve as the primary reservoir and maintain numerous serotypes simultaneously within these small populations. Each serotype would be expected to induce type specific immunity in infected animals thereby suggesting that individual animals are being repeatedly exposed to sources of new virus introduced from outside their population.

Table 4. Vesivirus isolation and type-specific neutralizing antibodies in ocean mammals (Smith and Boyt, 1990; Smith et al., 1986b)

Species of origin	Serotype/strain being isolated	Serum antibodies
California sea lion [*Zalophus californianus*]	SMSV 1-4, 6, 9, 11-17 CCV, RCV	VESV A, C-G, I-K SMSV 1-6, 8, 10, 13, BCV, CCV
Stellar sea lion [*Eumetopias jubatus*]	SMSV 6, RCV	SMSV 1, 2, 5-8, 10, 13 BCV
Northern fur seal [*Calorhinus ursinus*]	SMSV 1, 5, 6, 8, 10-12 RCV	SMSV 2, 5, 6, 10
California gray whale [*Eschrichtius robustus*]	VESV A	VESV A, D-G, I, J SMSV 2, 5

There are almost certain to be other types of fish or poikilotherms in addition to the opal-eye perch that exist as reservoirs and zoonotic pools for vesiviruses. Compliant

cell lines (Vero) that are usually lysed by SMSV-5 within 24 hours postinfection at 37°C were permissive for viral replication for 14 days at 15°C, where lysis was not observed even though viral titers reached 10^6 tissue culture infective doses (Smith, 2000). These findings suggest that the primary lytic cycle of vesiviruses may occur during replication in warm temperatures. Thus, mammals would not be the natural reservoir hosts (the lytic cycle with rapid cell death could suggest poor adaptation to such host species), whereas poikilotherm hosts may better support virus survival through noncytolytic replication (Smith, 2000). Alternatively since vesiviruses seemed to adapt quite well over time to putative new host species such as marine mammals perhaps because of reversion to or predominance of non-lytic biotypes (small plaque variants) of the viruses, these host species might serve as a reservoir by themselves. Surveys have shown an incidence of visicular lesions with certain vesivirus strains between 0.1% and 2% annually in fur seals despite the high prevalence of the virus strains (Smith and Boyt, 1990). Besides the mechanics of pathogen pollution already mentioned we should not forget the ocean water itself as a pool for infectious virus. The general viral burden in oceans are typically 10^{10} per liter in surface waters (Fuhrman 1999) and 10^9 per ml in sediments (Danovaro and Serresi, 2000). Life forms such as zooplankton, filter feeders and whales can amplify viruses to very high titers. For example, one 35-ton gray whale is known to shed 10^6 vesiviruses per gram in a fecal sample, can eat 5% or more of its body weight per day and eliminate an equivalent quantity of feces containing an estimated 10^{13} vesiviruses daily (Smith et al., 1998b). Thus, gray whales migrating offshore for 10,000 km twice a year may discharge tremendous numbers of vesiviruses when they become persistently infected. This may indeed happen, because the animal which was shedding 10^6 caliciviruses per gram of feces also had a serum neutralizing antibody titer of 1:80 for the specific virus type that was continuously shed (Smith, 2000). A vesiviral disease for gray whales is unknown, but they apparently do become infected and can greatly impact the dynamic of viral transport. Introduction of whale or fish scraps into diets of terrestrial animals, seals breeding on lands adjacent to terrestrial animal populations, translocation of zoo animals and migrating birds are some potential mechanisms for extending the range of possible VESV infections.

Looking for answers

Considering the extend of migration of vesivirus hosts, it is understandable, that VESV could cross the land-ocean barrier and become manifested in terrestrial domestic livestock in 1932. Regardless of vesivirus reservoirs, the land-ocean transmission lines appear to be open and operating. To assume that these viruses are confined to the North Pacific Ocean is not realistic. Migrating marine mammals and fish, translocated domestic livestock and wildlife and humans transporting agricultural goods in a global way are not constrained by national and other geographical boundaries and are capable of spreading these viruses throughout the world. Such transmissions may have already occurred. Outbreaks of vesicular diseases with typical lesions were reported in swine in Australia, New Zealand, and Tasmania (Munday and Ryan, 1982) and in seals in Britain (Stack et al., 1993). After occational introducing vesiviruses into domestic animals and humans, the virus appears to be evolving and adapting to new ecologic niches.

Regarding the prior question of VESV/SMSV existence between 1955 and 1972, strains were surely present then as they are now in both ocean reservoirs and terrestrial animal populations, but were apparently not causing obvious vesicular diseases in livestock. Our knowledge of viral host range determinants and natural transmission routs for vesiviruses are too rudimentary to allow us to predict which agents are most likely to and how they emerge in the future from oceanic and terrestrial pools as new disease agents of animals or humans. Past evidence indicates that microbial pathogens of one species can be an important source of new emerging disease in another species after transspecies transmission and this is likely to continue. In addition, there might be other natural transmission routes of vesiviruses besides the already known mechanism of feeding infected meat. The study of hitherto unknown diseases might lead to insight into new transmission routes. Therefore, we focussed in the second half of this research project on the evaluation of a newly emerged disease in horses.

Something is causing an unprecedented number of broodmares to lose their foals in Kentucky, USA

In late April 2001, central Kentucky horse breeding farms began reporting an unusually high abortion rate, late fetal loss (LFL), in thoroughbred mares up to 10 times the

normal rate (United States Animal Health Association, 2001; USDA - National Agriculture Statistic Service, 2003). On some farms, the rate of spontaneous abortion was as high as 75%. Also noted was an unusually high rate of stillborn foals. Early fetal loss (EFL), as a result of "red bag" births, where the placenta separates prior to birthing resulted in suffocation of the foals. Those born alive exhibited breathing problem, severe dehydration with low white blood cells counts, and suffered a 50% mortality rate. The mares themselves in general were not showing any signs of illness. LFL and EFL together were called mare reproductive loss syndrome (MRLS) (United States Animal Health Association, 2001; USDA - National Agriculture Statistic Service, 2003). A report released by Kentucky Governor Paul Patton estimated the number of thoroughbred EFL caused by MRLS in 2001 to be 3,000 and an additional 1,000 in 2002 (Riddle, 2002). For the second clinical scenario, the LFL, 500 losses in 2001 and 150 losses in 2002 were counted (Brown, 2002). The only comparable experience worldwide was in 1980 and 1981 when smaller numbers of EFL were seen in Central Kentucky. Estimates were 256 in 1980 and 162 in 1981. No aetiology was ever determined for the losses in either year (Bryans, 1981).

Kentucky is currently the world leader in thoroughbred breeding, with more than 20.000 mares bred in Kentucky in 2000. MRLS in 2001 caused more than $300 million dollars in losses to the Kentucky's $2 billion-income and a $5 billion-impact on the horse industry (United States Animal Health Association, 2001; USDA - National Agriculture Statistic Service, 2003).

Clinical symptoms for EFL occurred in 30 to 100 day old fetuses. They palpitated normally, but ultrasound showed no heartbeat and cloudy echogenic amniotic fluid, which was often followed by exclusion of the fetus. LFL also showed characteristic pathology, including funisitis and pneumonic changes (Williams et al., 2002). Tissue samples from lung, kidney, spleen, thymus, liver, stomach and placenta (if submitted) of LFL cases were microbiological analyzed using isolation in culture media and fluorescent antibody tests (FAT) for bacteria and viruses. Two anaerobe bacteria, actinobacilli and non-betahemolytic streptococci, both common mouth commensals in horses, were recovered from 14.4% and 51.8%, respectively. Only 4 out of 682 fetuses (0.6%) were FAT-positive for equines herpesvirus 1 (EHV-1). No evidence for other

viral infections was observed using tests for specific agents. The common infectious causes of equine abortion, such as EHV-1, *S. zooepidemicus*, *E. coli* and *Leptospira spp.*, were recovered from less than 9% of the fetuses received (Donahue et al., 2002). Fetuses suffering from these infections probably represented the normal fetal loss that would occur without the presence of MRLS. During a six-year study of 3,500 fetuses from 1986 to 1991 submitted to the University of Kentucky Livestock Disease Diagnostic Center (UKLDDC), actinobacilli and non-betahemolytic streptococci were isolated from only 14 and 26 fetuses (0.4% and 0.7%), respectively (Giles et al., 1993).

A retrospective epidemiological study in 2001 established a strong positive correlations between early breeding, exposure to Black Cherry trees, Eastern Tent Caterpillars (ETC) and MRLS. Absence of caterpillars and supplementary hey were protective (Dwyer, 2002). Extensive tests and surveys revealed no individual causal correlation between MRLS and the following: existence of nitrite/nitrate, estrogenic activity, cyanide or cyanogenic compounds, unbalanced mineral level, toxins associated with pasture grass like mycotoxins, ergot alkaloids and hemlock alkaloids (Henning, 2003).

The onset of MRLS in 2001 coincided with an exceptional abundance of ETC, *Malacosoma americanum* (Fitzgerald, 2002), limited by its distribution to the eastern half of the United States and Canada and restricted to trees of the plant family *Roseaceae*, greatly preferring the Black Cherry tree. The overwintered eggs of ETC hatch in early spring and the larvae build a communal silk shelter (tent), which they expand daily to accommodate their growth. They are ravenous feeders of Black cherry leaves, completing their larval growth in as few as seven to eight weeks. When fully grown, the caterpillars drop from their host tree and start traveling, creeping over the ground in search of sheltered sites, spinning cocoons and metamorphose. The female moth, emerges from the pupae stage after two to three weeks, lives only for another two to three weeks without feeding and during this time mates with a male moth and produces a single egg mass that contains approximately 300 eggs. The eggs are wrapped around a cherry tree branch in June or July where the small larvae lie quiescent until the follow spring (Fitzgerald, 2002). Caterpillars in general have more than 200 known predators and are also susceptible to viruses, bacteria and microsporidians,

particularly when reaching high population densities. When a tentative association between ETC and MRLS was first made in 2001, the caterpillars were near the peak of their population cycle in many of the effected areas. Although the exact cause of MRLS remained unknown, a reasonable working hypothesis was that ETC serves as a vector for a microbe that is a causal agent of MRLS (Fitzgerald, 2002). To prove the correlation between ETC and MRLS, several experiments were set up in 2001 and 2002, where pregnant mares were grazed on pastures without hay supplement and fed starved ETC and insect's frass. Control animals were not fed ETCs or their frass. This provided the first experimental evidence of ETC to induce pregnancy loss in horses and was the first time to reproduce the syndrome under experimental conditions (Bernard et al., 2002).

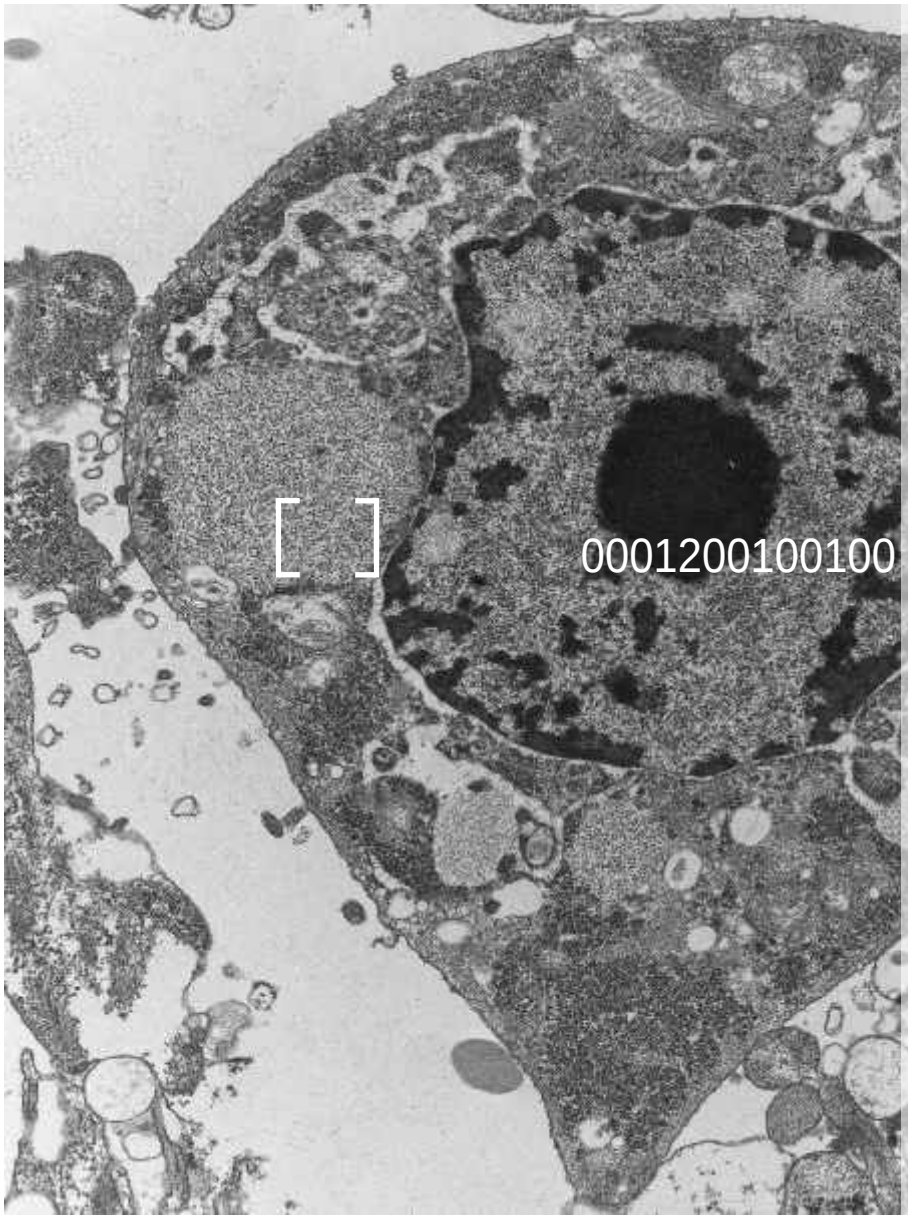
Aims of the study

Screening of cattle sera: distribution of vesiviruses in terrestrial domestic animals

To broaden the knowledge of vesivirus occurrence and distribution dynamics in domestic livestock, we attempted the first extensive study in cattle to assess the distribution of vesiviruses by examining the seroprevalence. Cattle were chosen to be studied here, because this livestock is well characterized, they are suffering diseases with high percentages of unknown aetiologies and extensive animal data are available. Furthermore, several case reports exist on the isolation of vesiviruses from cattle (Smith et al., 1983a; Smith et al., 2002b). The objective was to evaluate the distribution of vesiviruses in cattle, correlate vesivirus infection with disease manifestations and estimate the possible impact on other animals and humans. Roughly seven hundred bovine sera from nine States within the United States were acquired and were tested for antibodies against vesiviruses. The sera tested were drawn from a total population of about 9 million cattle in nine States (USDA - National Agriculture Statistic Service, 2003) with a geographical distribution ranging from northern Alaska to tropical Hawaii.

Screening of horse sera and tissue: correlation of vesivirus prevalence and mare reproductive loss syndrome (MRLS)?

A newly emerged disease in horses was analyzed in the second half of this research project. Since all tests for the common toxic abortogenic agents had failed, a further nationwide investigation was initiated for uncommon and unknown infectious agent(s). Caliciviruses, and vesiviruses in particular, are a well established cause of abortion in swine and seals. Nevertheless, these viruses are not routinely tested for or diagnosed. When the Laboratory for Calicivirus Studies at Oregon State University was asked for specific help in this investigation, pertinent seroepidemiological studies were entrusted to me under the guidance of Professor Dr. Alvin W. Smith. After the first serological examination of equine blood samples from mares that had aborted in 2001 and an equal number of sera from unaffected animals from 2000 were examined, tissue samples from aborted fetuses and two other sets of blood samples from ETC-exposed mares from two experiments were acquired. These have been tested for vesivirus-antibodies and antigen. Furthermore, additional samples of ETC were tested for vesiviruses by RT-PCR.



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Materials and Methods

Sample collection

Cattle sera sampling

To evaluate the occurrence and distribution of vesiviruses in cattle, 693 sera collected 1999 and 2000 from nine States within the USA (Figure 3) were acquired and have been tested for antibodies against vesiviruses. All sera samples were frozen and held on dry ice for transport to the laboratory and were kept frozen at –70°C until testing by indirect ELISA for the presence of vesivirus antibody without knowledge of the accession numbers (blinded study).

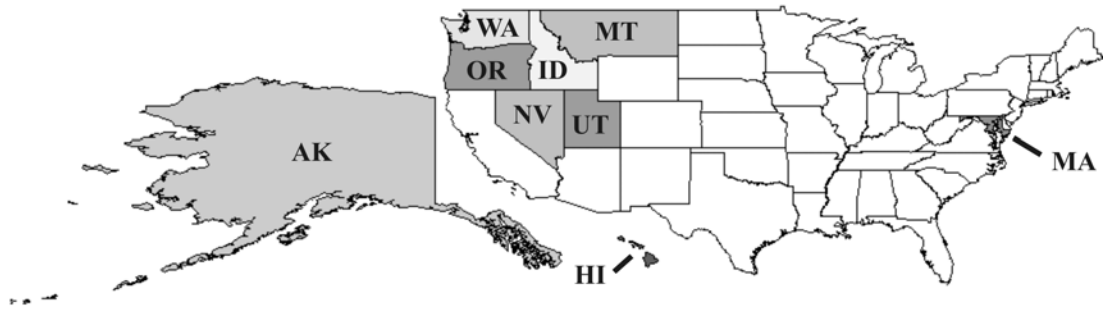


Figure 3. Bovine sera from nine States within the USA were tested for antibodies against vesiviruses: Alaska (AK), Hawaii (HI), Idaho (ID), Maryland (MA), Montana (MT), Nevada (NV), Oregon (OR), Utah (UT) and Washington (WA).

Since no disease correlation for vesiviruses in cattle are known and proven jet, and therefore no true negative control group could be chosen, 41 healthy male and female juvenile cattle up to 18 month of age were selected as a negative reference group. The remaining cattle samples were divided into distinct groups associated with their health status: 208 sera from cattle with abortions, 119 sera from cattle with respiratory diseases and 325 others who were enrolled in a survey to monitor Bovine Leucosis Virus (BLV), that were collected for pre-sale health testing or had other various problems (hereafter referred to as others-group). Those groups were divided further into various subgroups: beef and diary cattle, male and female, and their geographic location. The same sera have previously been screened for the presence of antibodies to a variety of

parasites, bacteria and viruses at the Washington Animal Disease Diagnostic Laboratory (WADDL), Washington State University, USA. Those results were used to assess the ELISA specificity by examining patterns of antibodies against other common veterinary relevant agents.

Horse sera and tissue sampling, ETC sampling

As part of the investigation of the unknown cause of MRLS in horses, sera and tissue samples were acquired in 2001 and 2002 from the University of Kentucky, USA (**Figure 4**). All sera samples were frozen and held on dry ice for transport to the laboratory and were kept frozen at –70°C until testing them by indirect ELISA for the presence of vesivirus antibody without knowledge of the accession numbers (blinded study). A variety of tissue samples from 20 aborted fetuses were sent improperly frozen, including liver, lung, intestine, spleen, kidney and thymus. Many samples were already thawed when they arrived at the laboratory. Freezing them again to –20°C for storage until further handling destroyed most of their tissue and cell structure. Anyway, all samples were tested by immune fluorescence and RT-PCR for the presence of vesiviruses. In addition, collected eastern tent caterpillars from areas of equine abortion in Kentucky were tested by RT-PCR for vesiviruses.

Figure 4. Equine sera, equine tissue and eastern tent caterpillars from Kentucky (KY) were tested for vesivirus antibodies and antigen.

Four groups of sera sample sets containing a total of 390 sera from horses of different locations were tested by indirect ELISA.

Within the initial 112 sera samples from 2001, 58 sera were from mares on farms having abortion problems, 25 sera were from randomly selected mares 3 to 13 years

old that were being sold at auctions (hereafter referred to as sale-barn mares) and 29 samples were from mixed age males and female juveniles also being pre-sale tested and sold at auctions.

The second sample set contained 28 paired (acute/convalescent) sera from mares diagnosed with MRLS in 2001 from locations different than the Group1 samples.

The third and fourth sample set contained samples from experimentally infected mares. The first experiment was designed in 2002 at the University of Kentucky to determine if there might be a causal association between MRLS and Eastern Tent Caterpillars (ETC). The intent was to mimic on-farm conditions. In addition to their normal diets pregnant mares were exposed for two 10-day periods to ETC and their frass (n = 10 mares) in the field on which the mares grazed. A second similarly managed group of mares (n = 9) was exposed to frass only and for a control group (n = 10 mares) the exposure of ETC and frass was minimized. The first serum sample was collected pre-experimental, followed by at least two other samplings during the experimental exposure. This study was compromised by some movement of caterpillars between the three groups and MRLS occurred in all three groups. Nevertheless, this experimental field study with 29 pregnant mares suggested that ETC and/or frass were causally related to MRLS. Seven of 10 mares exposed to ETC and frass, and 7 of 9 mares exposed to frass only had experienced abortion. Three of 10 mares in the control group also aborted, but were accidentally exposed to ETC larvae during the study (Webb et al., 2002).

The second ETC exposure study consisted of three groups of pregnant mares that received their respective 10-day treatments by gastric administration. One group (group A) of 5 mares received crushed fresh ETC, another 5 mares (group B) were inoculated with the same material, which was autoclaved before feeding and reference sera were obtained from 4 horses (group C) that were fed Gypsy moth larvae as a negative control. At least 6 pre- and post-infection sera were obtained for each mare during a 4-week period and were tested for antibody against vesiviruses. Three of five mares in group A aborted and all were MRLS type. In contrast, there were no abortions in group B and only one abortion in the control group and it was not of the MRLS type.

Chemicals and devices

Samples

Bovine sera	Dr. Jim Evermann Washington State University, USA
Equine sera, equine tissue, ETC tissue	Dr. Bruce Webb University of Kentucky, USA

TBS

1M Tris, pH7.4	50.00 ml
5M NaCl	30.00 ml
Aqua bidest.	ad 1000.00 ml

TBST (0.05% Tween 20)

Tween 20	0.10 ml
TBS	200.00 ml

Blocking buffer

Bring 1 packet of SuperBlock™ Dry Blend to 200ml H_2O, stir for 10 minutes. Unused can be stored in refrigerator for 6 month.

Antigen buffer (Coating Buffer): Sodium Carbonate/Sodium Bicarbonate, 0.1 M, pH 9.6

Stock "A": 0.2 M Na_2CO_3 (Anhydrous) 21.2 g/l H_2O	0.50 ml
Stock "B": 0.2 M $NaHCO_3$ 16.8 g/l H_2O	1.10 ml
Aqua bidest.	1.60 ml

Diluting buffer (for diluting sera and secondary antibody)

BSA (Bovine Serum Albumin)	0.10 g
TBST	ad 40.00 ml

TAE-buffer

Tris	400 mM
Na_2EDTA x 2 H_2O	20 mM
Sodium acetate	200 mM
Acetic acid	296 mM

Fluorescent antibody test (FA)

Premium Microscopic slides, frosted (Fisher Scientific, Pittsburgh, USA)

Peel-Away® base molds (Fisher Scientific, Pittsburgh, USA)

Frozen tissue matrix (Fisher Scientific, Pittsburgh, USA)

Cryostat Leica CM1900 (Leica Microsystems, USA)

FITC labelled vesivirus group-specific monoclonal antibody designated 4AD8D8 (OSU, Oregon, USA)

Reverse transcriptase polymerase chain reaction (RT-PCR)

TRIzol® (Gibco BRL, UK)

cDNA synthesis mix (Boehringer Mannheim, UK)

dNTP (Biogene)

RNAse inhibitor (Invitrogen Suppl., USA)

Moloney-murine leukaemia virus reverse-transcriptase (Gibco BRL, UK)

Primer set (Cruachem Ltd, UK)

Taq-Polymerase (Gibco BRL, UK)

Thermocycler (Hybaid, UK)

100 bp DNA ladder (Gibco BRL, UK)

Agarose, ultra pure (Bio-Rad, USA)

Ethanol (Gibco BRL, UK)

Ethidium bromide (Gibco BRL, UK)

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates (#76-371-04, ICN Biomedicals, USA)

Adhesive ELISA plate covers (ICN Biomedicals, USA)

ELISA plate reader (Titertek Multiskan, ICN Biomedicals, USA)

Tween 20 (ICN Biomedicals, USA)

SuperBlock™ Dry Blend (PIERCE, USA)

Rabbit-α-Bovine-IgG, Alkaline Phosphate Conjugate, 1:25,000 in diluting buffer (SIGMA, USA)

Rabbit-α-Horse-IgG, Alkaline Phosphate Conjugate, 1:35,000 in diluting buffer (SIGMA, USA)

Recombinant calicivirus antigen D3A (Abbott Diagnostic Core R&D, USA)

Blue Phos™ Microwell, Phosphatase Substrate System (KPL, USA)

Bovine Serum Albumin (SIGMA, USA)

Methods

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was used for the determination of vesiviral antibodies in equine and bovine sera. Vesiviral antigen was adsorbed to the solid-phase ELISA plate. Next, test serum was added, binding all vesivirus specific antibodies. Then enzyme-conjugated anti-bovine or –equine gamma globuline (IgG) was added, binding all specific antibodies of cattle or horses from which the serum was tested. After adding a substrate solution, the coulor-reaction was measured spectrophotometrically.

The recombinant test antigen named D3A (CaliciScreen) used for the ELISA in this study was developed by the Laboratory for Calicivirus Studies at Oregon State University (US patent number 6,593,080). The Calicivirus D3A recombinant with the size of 882 bp (Table 5) was inserted as a fragment into an *E. coli* vector and expressed by growing transformed bacteria (Bolling and Mandecki, 1990). D3A antigen with its 293 amino acids was extracted after several biochemical procedures to a final concentration of 1.2 mg/ml (Abbott Diagnostic Core R&D, USA).

Table 5. Linear sequence map of D3A, a cDNA that encodes an amino acid sequence useful for detecting vesiviral group-specific binding antibodies.

1	cttcatgcag aaaccaaagc caacaaccgt tggttccatg tgatcgacac agacaaagcc	60
61	ctggtgccag gcttgcctga tggttggcct gacactacaa tcccagaaag tgtgacagca	120
121	accaatggtg acttcgcgta cgcgaccgat ttctacaatc cggcaaccaa aactgttgac	180
181	cctaccaaga acaccacgcc cttcaaaggc acatacatct gtggcacttt atcaacggtc	240
241	accatacccg aggttgacaa tcagaactac gcaaagaagg aagcacaaaa gaaatcccaa	300
301	acaatgtaca taacaactgc tgacattggg gatggcaatg ccagtccaca acacaaaatt	360
361	tcacctcaga gattgattgt cttcttcgac ggtccggaga gcacgatgga catcaacgtc	420
421	acgttgagtc cgcttgggtt cacacttgtg gacggtcaac caattggctc cagttccagc	480
481	aaagttgtca ggattgctac actcccagaa gccattacac aaggagggaa ctacccaatc	540
541	ttctatgtga acaaagtcaa gattggatac tttgacaggc aaaccacaga gtgttacaac	600
601	agccaagttc tgatgacatc gcagaaactt gccgagggaa attacaacct ccccccctgac	660
661	tcccttgccg tgtacagaat cacagactct tcttctcaat ggttcgacat cgggatcaac	720
721	catgatggtt tctcgtttgt tgggctgtct gaccttccct ctgatctaga atttcccctc	780
781	acttcgacct tcatgggagt gcagctagca cgtgtcaagc tagcatcaaa ggtcaaaagc	840
841	acagccagaa caatagacta caaggacgac gatgacaagt aa	882

The test antigen consisted of 50µl of diluted recombinant calicivirus antigen in coating buffer (1µg/ml) and was used to sensitise 96-well ELISA plates for 2 hours at 37°C while rotating. Wells were washed twice with Tris-buffered saline (TBS). Free bindings at the plate were blocked with three changes of 200µl per well of SuperBlock™DryBlend, followed by adding of 100µl of each serum (diluted 1:100 in diluting buffer) for 2 hours at 37°C while rotating. Plates were washed six times with

TBST (TBS containing Tween 20). After serum incubation and washing, 100µl of the secondary antibody IgG-alkaline phosphatase, diluted appropriately in diluting buffer, was added and incubated for 2 hours at 37°C while rotating and then washed six times with TBST and twice with TBS without Tween 20. The pre-warmed chromogenic substrate Blue Phos™Microwell was added (100µl per well) and incubated for 3 hours at 37°C in the dark while rotating. Optical densities (ODs) were determined at 620 nm on a microplate reader. The net OD value used was the test OD value minus the OD of a chromogenic substrate control well and fusion peptide control. The optimal dilutions of the reagents were determined in previous experiments.

ELISA for bovine samples

Since no disease correlation for vesiviruses and cattle are known and proven jet, and therefore no true negative control group could be set up, 41 healthy male and female juvenile cattle up to 18 month of age were chosen as a negative reference population. The cut-off value of 0.29 for the used ELISA was established after calculating the mean (0.104) plus two-fold standard deviation (2x0.092) of the OD-result of those 41 samples. This approach leads out to a specificity of approx. 97.5% (Barajas-Rojas et al., 1993).

A randomly taken OD-negative bovine serum sample (#109480/01) was used as a negative control by running on each plate. The arithmetic mean of all 30 readings (min 0.052, max 0.095) were 0.074 with a standard deviation of 0.021. As a positive control a randomly taken OD-positive bovine serum sample (#105572/07) was used. The arithmetic mean of all 30 readings (min 0.906, max 1.359) were 1.159 with a standard deviation of 0.155.

ELISA for equine samples

Because no disease correlation for vesiviruses and horses are known and proven jet, and therefore no true negative control group could be set up, 29 samples from mixed age males and female juveniles from 2000 were used as a negative reference population. The cut-off value of 0.14 for the used ELISA was established after calculating the mean (0.065) plus two-fold standard deviation (2x0.034) of the OD-result of those

29 samples. This approach leads out to a specificity of approx. 97.5% (Barajas-Rojas et al., 1993).

A randomly taken OD-negative equine serum sample (#943/1) was used as a negative control by running on each plate. The arithmetic mean of all 14 readings (min 0.041, max 0.065) were 0.055 with a standard deviation of 0.007. As a positive control, a randomly taken OD-positive equine serum sample (#996/1) was used. The arithmetic mean of all 14 readings (min 0.274, max 0.425) were 0.35 with a standard deviation of 0.038.

Direct fluorescent antibody test (FA)

Tissue samples of aborted horse fetuses were examined by direct fluorescent antibody testing after frozen sectioning. The frozen tissues were trimmed and cut into pieces of no more than 5 mm thickness and placed in pre-labeled base molds filled with frozen tissue matrix. The base molds with tissue were placed into a stainless steel beaker in liquid nitrogen and allowed to cool adequately. The blocks were stored at -70°C until sectioning.

Before cutting sections, the temperature of the block was allowed to equilibrate to the temperature of the cryostat (-20°C). Sections of 5 µm were cut with a cryostat, placed on Fisher Superfrost slides and stored until the staining process at -70°C in a sealed slide box.

Slides were warmed in a freezer to -20°C and then fixed in -20°C acetone for 20 minutes. After the slides were air dried and transferred into TBS buffer for 10 minutes at room temperature, they were incubated 20 minutes at 37°C with 100 µl of a 1:100 dilution (in TBS Buffer) of fluorescein isothiocyanate (FITC) labelled with vesivirus group-specific monoclonal antibody designated 4AD8D8, which binds all known vesivirus serotypes, including FCV (Smith et al., 2002b). After washing with TBS buffer and air drying them, areas of green fluorescence consistent with a positive reaction were expected while examining the slides at 20x or 40x with a high dry objective under a fluorescent microscope.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA extraction and cDNA cloning

Reverse transcription-polymerase chain reaction (RT-PCR) was performed on horse tissue and ETC samples. Total cellular RNA was extracted in TRIzol® reagent (Simms et al., 1993) and stored at –70°C until tested by RT-PCR. Before using, the samples were thawed at room temperature and 200 µl chloroform added to each. The tubes were left at room temperature for 3 min before centrifugation for 15 min at 28,000 x g. The upper aqueous layer containing the RNA was removed to a fresh tube, recovered by precipitation with isopropyl alcohol and washed with 70% ethanol. The RNA was resuspended in RNase-free water and 5 µl used for RT-PCR.

First strand cDNA synthesis was performed using a commercial random hexanucleotide mix. The RNA was heated at 70°C for 5 min with 10 pmol of the random primer mix, cooled at room temperature for 10 min and subjected to reverse transcription for 45 min at 37°C in a 20 µl reaction mix. This mix contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3mM $MgCl_2$, 0.5 mM (each) dNTP, 10 mM dithiothreitol and 200 units of Moloney-murine leukaemia virus reverse-transcriptase (Huang et al., 2000).

Synthetic oligonucleotide primers

The primer set used for this study were designed to amplify a 768 base pair sequence from a vesivirus capsid-coding region (Table 6) (Reid et al., 1999).

Table 6. Used primer set for RT-PCR

1F (sense)	5‘ – GTG AGG TGT TTG AGA ATT AG
1R (antisense)	5‘ – ACA TCA ATT CCG CCA GAC CA

PCR amplification

A 1 µl RT reaction mix was amplified in a PCR reaction mixture (final volume 50 µl) which also contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 mM (each) dNTP, 10 µM of each primer and 0.5 units of Taq polymerase (Table 7). The virus DNA was amplified in a “Touchdown” thermocycler using the following programme: 94°C for 2.5 min, 1 cycle; 94°C for 45 sec, 55°C for 30 sec, 72°C for 1 min, 35 cycles; 72°C for 7 min, 1 cycle.

Table 7. RT-PCR amounts for 1 sample

H_2O	26.5 µl
10xBuffer w/o Mg	5.0 µl
PCR Enhancer	5.0 µl
50 mM MgSO4	1.0 µl
dNTP (2.5 mM each)	6.0 µl
D3A reverse primer (10µM)	1.25 µl
D3A forward primer (10µM)	1.25 µl
Turbo Polymerase	1.0 µl
Reverse Transcriptase (RT)	1.0 µl
RNAse out (RNAse Inhibitor)	1.0 µl
RNA (1 µg total RNA)	1.0 µl

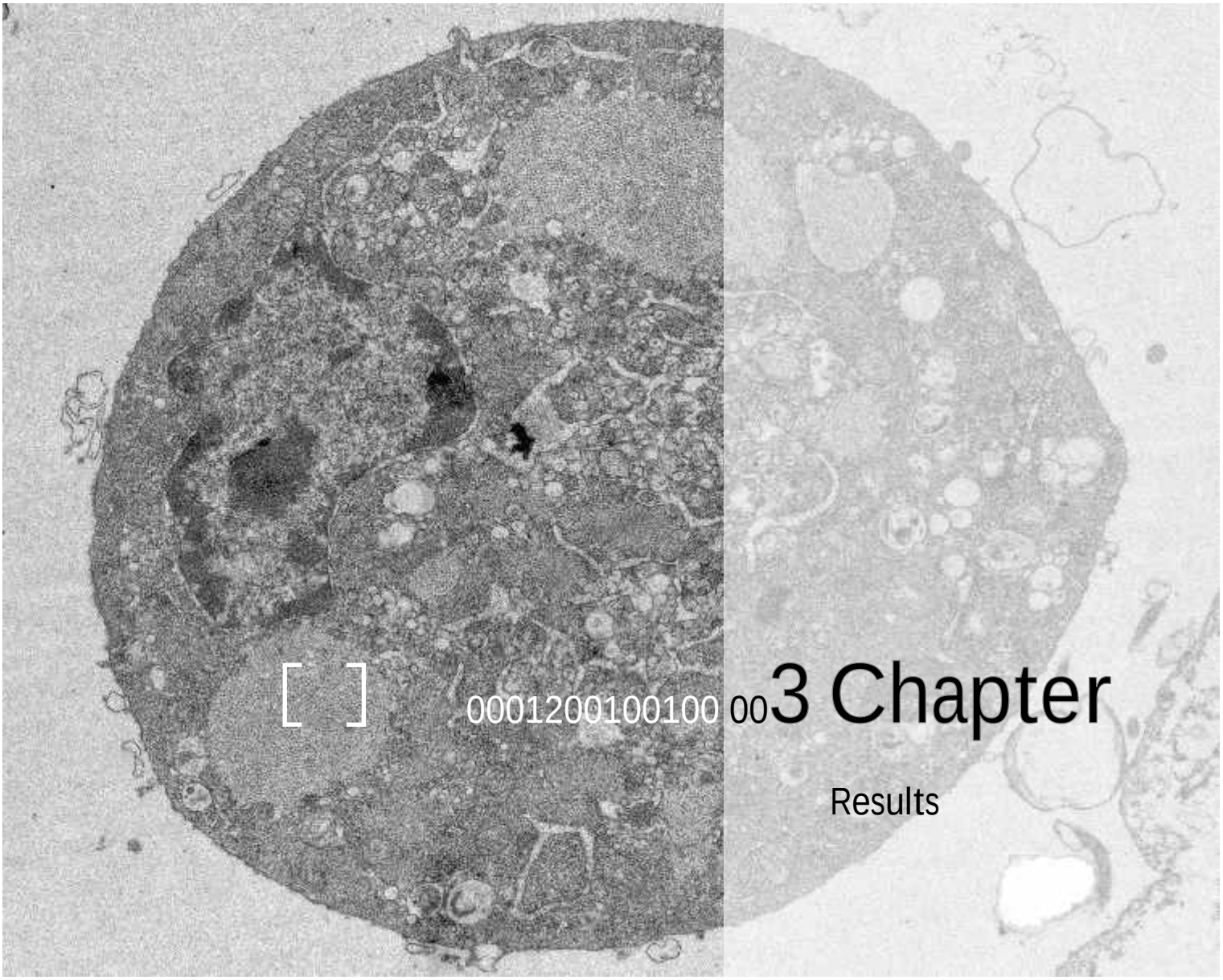
Electrophoresis

Following the amplification, 10 µl of each RT-PCR product was analysed by electrophoresis on a 1.5% agarose gel (in TAE-buffer) stained with ethidium bromide (0.5 µg/ml). A 100 bp DNA ladder was run on the gel as a molecular weight marker to measure the size of the product. After the gel was run for 75 min at 90 Volts at room temperature a digital picture was taken while UV illuminated.

Statistical analysis

To determine a cut-off for the indirect vesivirus ELISA used in this study, the mean plus two-fold standard deviation of the OD-result of the appropriate negative reference population was calculated. This approach was leading out to a specificity of approx. 97.5% (Barajas-Rojas et al., 1993).

The distribution of seropositivity among all examined cattle and horses was evaluated using the χ^2 (chi-square) or Fisher exact test (EpiInfo 2002; Centers for Disease Control and Prevention, USA). Chi-square calculations and two-tailed P-values were built into the computer model. P-values measure the degree of consistency between the data in an epidemiologic study and the hypothesis that there is no association between the exposure and the outcome. The strength for statistically significant associations was estimated by calculating odds ratio (OR). A P-value ≤ 0.05 was considered to indicate significant association, and OR with a lower limit of the 95% confidence interval (CI) >1 was interpreted as an indication of significant strength of the association (EpiInfo 2002).



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3 Chapter

Results

ELISA evaluation

To assess the sensitivity of the recombinant test antigen (D3A) for the indirect ELISA used in this study, which denotes the ability to detect the presence of vesivirus group-specific antibodies, 14 antisera of different vesiviruses were used as primary antibodies running the same ELISA protocoll. While all pre-immunized sera used as a negative control reached optical densities (OD) not higher than 0.008, all vesivirus antisera revealed high OD's between 0.43 and 3.61. Therefore, the D3A antigen reacted with all vesivirus serotypes tested.

To assess the specificity of the indirect ELISA used in this study, which denotes the ability to detect a single specific agent exclusively, the pattern of antibodies against other common veterinary relevant agents were cross-examined. The same 693 bovine sera examined in this study had previously been screened for the presence of antibodies to a variety of parasites, bacteria and viruses at the Washington Animal Disease Diagnostic Laboratory (WADDL), Washington State University, USA. For every of the examined agents (Table 8), all four possibilities were found to exclude cross-reactivity: agent seropositive/vesivirus seropositive, agent negative/vesivirus negative, agent positive/vesivirus negative and agent negative/vesivirus positive. No cross-reactivity of the vesivirus specific ELISA was detected.

Table 8. Common infectious agents in cattle being examined for cross-reactivity of the indirect vesivirus specific ELISA (International Committee on Taxonomy of Viruses, 2002)

Agent	Abbr.	Classification
Virus		
Bovine leucemia virus	BLV	*Retroviridae*
Bluetongue virus	BTV	*Reoviridae*
Bovine viral diarrhea virus	BVDV	*Flaviviridae*
Bovine respiratory syncytial virus	BRSV	*Paramyxoviridae*
Bovine herpesvirus 1	BoHV-1	*Herpesviridae*
Bovine parainfluenza virus 3	BPIV-3	*Paramyxoviridae*
Bacterial		
Mycobacterium paratuberculosis		Actinomycetales
Anaplasma marginale		Rickettsiales
Leptospira spec.		Spirochaetales
Brucella abortus		Rhizobiales
Parasite		
Neospora caninum		Protozoan

Epidemiological study of cattle sera

To evaluate the prevalence of vesiviruse infections in cattle, to correlate vesivirus infection with disease manifestations and to estimate the possible impact for other animals and humans, 693 bovine sera from nine States within the United States were acquired and have been tested for antibodies against vesiviruses (Figure 3).

Within the 693 bovine sera, received in 1999 and 2000 from the Washington State University, USA, 208 sera were from cattle with an abortion history and 119 sera were from cattle with respiratory diseases. The remaining 366 sera were enrolled in a survey to monitor Bovine Leucemia Virus (BLV), that were collected for pre-sale health testing or had various other problems (hereafter referred to as others-group), including 41 sera from healthy male and female juvenile cattle used as the negative reference group (Table 9).

Table 9. Vesivirus antibodies in 693 bovine sera collected in 1999 and 2000

Animal characteristics	Number of animals	Antibody positive (%)	Antibody negative (%)
Abortion associated	208	26 (12.5)	182 (87.5)
Diary	101	13 (12.9)	88 (87.1)
Beef	107	13 (12.1)	94 (87.9)
Respiratory disease associated	119	10 (8.4)	109 (91.6)
Diary	62	7 (11.3)	55 (88.7)
Beef	57	3 (5.3)	54 (94.7)
Others	366	69 (18.9)	297 (81.1)
Adult	325	67 (20.6)	258 (79.4)
Juveniles (control)	41	2 (4.9)	39 (95.1)
Diary	270	59 (21.9)	211 (88.1)
Beef	55	8 (14.5)	47 (85.5)
Diary	433	79 (18.2)	354 (81.8)
Beef	219	24 (11.0)	195 (89.0)
Female	579	94 (16.2)	485 (83.8)
Male	15	4 (26.7)	11 (73.3)
Mixed/NG	57	5 (8.8)	52 (91.2)
Total sample	693	110 (15.9)	583 (84.1)

Using the calculated index of 0.29 as the cut-off for the vesivirus antibody specific ELISA, the negative reference group revealed 4.9% seropositivity (2/41) (Figure 5). The abortion associated group of cattle were 12.5% seropositive (26/208). Ten positive sera (8.4%) out of 119 were revealed from the group of animals with respiratory diseases. Finally, the fourth group of other animals were 20.6% positive (67/325), excluding the 41 juveniles used as a control group.

Comparing those groups for statistical significance, an association was found between all four groups with a Chi-square value of 16.2 using three degrees of freedom (Table 10). The result is highly significant at a P-value of 0.001. Combining the groups of cattle with abortion and with respiratory problems into one group (36/327), the significant difference persists (P = 0.005). No statistically significance (P = 0.061) remains when comparing juveniles (control group) to all adults (other three groups combined) (103/652).

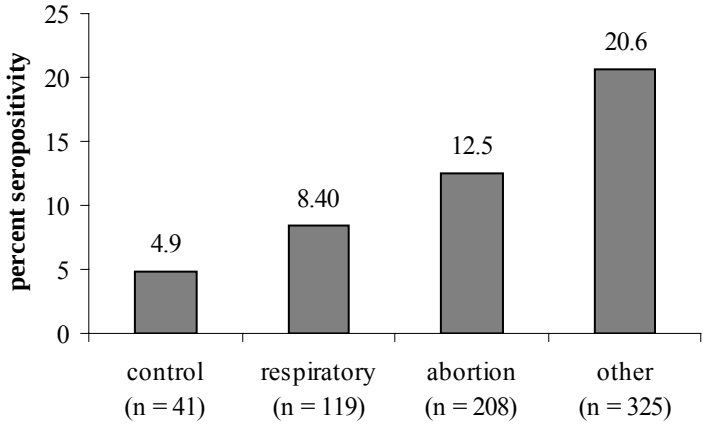


Figure 5. Distribution of vesivirus antibodies in different groups of cattle.

Comparing the control group with each of the three other groups, neither sera from cattle with abortion or respiratory problems were significant different with P-values of 0.12 and 0.36, respectively. In contrast, sera from the others-group revealed a significant higher seropositivity than the control sera (P = 0.015).

Table 10. Statistical association between the vesivirus seroprevalence and various animal characteristics[a]

Animal characteristics	χ^2 (Chi-square)	OR (95% CI)	P-value	Significant
Control vs. Abortion vs. Respiratory vs. Others	16.2	na[b]	0.001	Yes
Control vs. Abortion/Respiratory vs. Others	15.2	na[b]	0.0005	Yes
Control vs. Abortion/Respiratory/Others	3.5	na[b]	0.061	No
Control vs. Abortion	2.2	2.8 (0.6-12.2)	0.12	No
Control vs. Others	5.8	5.1 (1.2-21.5)	0.015	Yes
Control vs. Respiratory	1.5	1.8 (0.4-8.5)	0.36	No
Control vs. Diary	4.3	4.4 (1.03-18.4)	0.03	Yes
Control vs. Diary (Others)	6.5	5.5 (1.3-23.2)	0.011	Yes
Control vs. Males	4.1	7.1 (1.14-44)	0.038	Yes

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval.
[b]not applicable

Every group was divided into several subgroups of adult cattle, diary or beef cattle and male or female cattle. All subgroups were statistically compared against each other and the control group. In general, no significant difference was found between the seropositivity of sera from females (16.2%) and males (26.7%), between diary (18.2%) and beef cattle (11.0%), between abort/diary (12.9%) and abort/beef (12.1%), between respiratory/diary (11.3%) and respiratory/beef (5.3%), and between others/diary (21.9%) and others/beef (14.5%).

Nevertheless, compared to the control group, three subgroups revealed a significant higher seropositivity; all dairy cattle (79/433, 18.2%) with a P-value of 0.03, diary cattle from the others-group (59/270, 21.9%) with a P-value of 0.011 and all adult male cattle (4/15, 26.7%) with a P-value of 0.038 (Table 10). The confidence interval showed always a limit higher than 1, indicating significant strengths of the associations.

Samples within each of these groups were from animals in separate herds from distinct geographic locations. Forty-nine herds (71/428, 16.6%) with three or more samples showed the same trend for seroprevalence than all animals; 16 herds (15/112, 13.4%) from animals with abortion problems, 12 herds (7/89, 7.9%) from animals with respiratory problems, 17 herds (48/205, 23.4%) of animals from the others-group and 4 herds (1/22, 4.5%) of control animals (Figure 6).

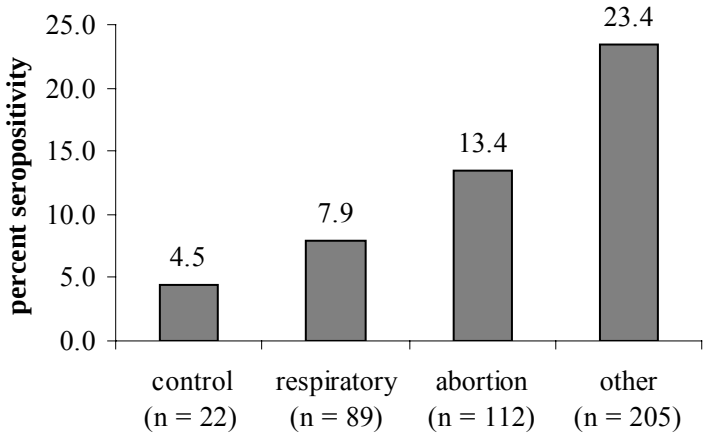


Figure 6. Distribution of vesivirus antibodies in forty-nine herds each with three or more samples in different groups of cattle.

In all but the control group, herds with high and low seroprevalence can be found (Figure 7). Only one herd within the control group revealed a seropositive result. Although in both groups of cattle with abortion and respiratory problems herds with a high vesivirus prevalence up to 33% (# 113020) were found, no significant difference between separate herds was revealed. In contrast, herds with low and high prevalence within the others-group showed significant differences. Examples of statistically significant associations are presented in Table 11.

One sample set of a herd (# 108278) containing 28 sera from adult dairy cattle from Washington, USA, screened for BLV antibodies only (11/28, 39.3%), revealed no vesivirus seropositive result. Another sample set of a different herd (# 105572) contained 42 sera from adult dairy cattle from the same location, also tested for BLV antibodies (12/42, 28.6%) and revealed a seropositivity of 35.7% (15/42) against vesiviruses. Both herds were statistically significant different at a P-value of 0.002 with an OR of 15, indicating significant strengths of the association. Herd #109649 contained 7 sera from male beef cattle from Alaska who were screened for export to Canada against bovine viral diarrhea virus (BVDV) and bluetongue virus (BTV) (0/7

for both agents), revealing a seropositivity of 42.9% (3/7) against vesiviruses. And a fourth example, herd #108506 contained 5 sera from adult dairy cattle from Washington (another location than the two herds # 108278 and 105572 mentioned above) were screened for BVDV, BLV and Johne's disease (*Mycobacterium paratuberculosis*) (0/5 for all three agents), revealed a seropositivity of 80% (4/5) against vesiviruses. The results from both herds (#109649 and 108506) are statistically significant different to herd #108278 (0/28) at P-values of 0.02 and 0.0006 respectively.

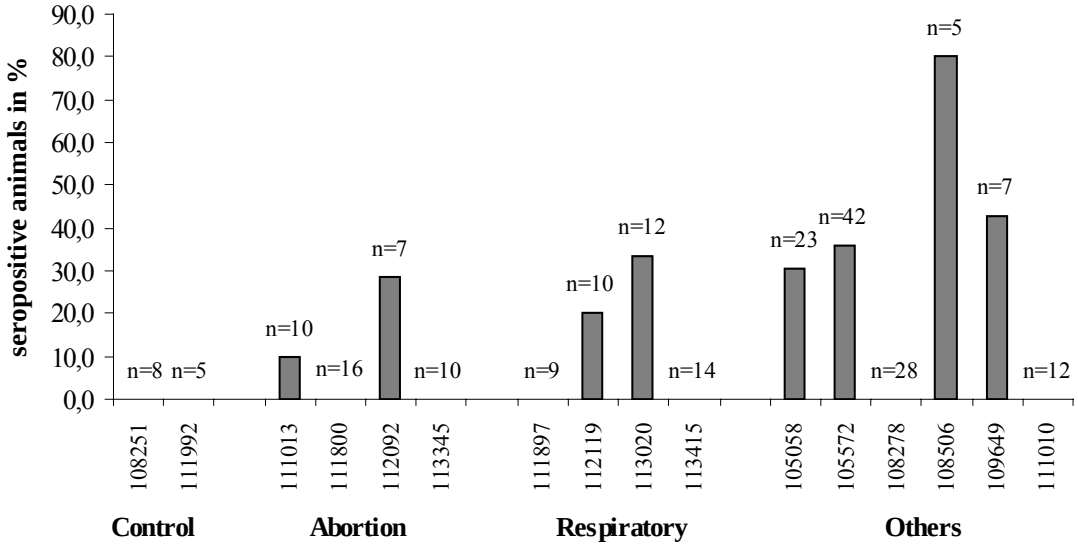


Figure 7. Percentage of vesivirus antibody positive sera of cattle from selected distinct herds within groups of cattle with abortion and respiratory problems, BLV-surveyed and control animals

Table 11. Statistical significant associations of vesivirus seroprevalence between selected distinct herds of the others-group[a]

Animal characteristics	OR (95% CI)	P-value	Significant
# 108278 (0/28) vs. # 105572 (15/42, 35.7%)	15 (1.8-121)[b]	0.002	Yes
# 108278 (0/28) vs. # 109649 (3/7, 42.9%)	20.3 (1.7-245)[b]	0.02	Yes
# 108278 (0/28) vs. # 105058 (7/23, 30.4%)	11.8 (1.3-105)[b]	0.011	Yes
# 108278 (0/28) vs. # 108506 (4/5, 80%)	108 (5.6-2092)[b]	0.0006	Yes

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval

[b]OR is underestimated due to the replacement of 0 by 1

When examining all available parameters of the animal case histories no general association to any specific disease manifestation could be found using serological screening for vesiviral antibodies. Nevertheless, within samples of herds with a general diagnosis for the cause of a manifested disease, differences and correlations to vesivirus infection were found and are demonstrated with the following examples.

Nine convalescent sera from adult females of a herd from Washington (#111177) that had aborted previously were sent to the Animal Disease Diagnostic Laboratory as a follow-up study, revealing only one increasing antibody titer change for *Neospora caninum* which was the official diagnosis for this abortion problem. In reality, only 8 out of 9 animals showed a seropositivity against neospora. The one animal who was negative against *Neospora caninum* was the only one being highly seropositive against vesiviruses (OD = 1.032). Since no acute sera from the same animals were available to test against vesiviruses, the hypothesis of vesiviruses being a cause for abortion in cattle could not be confirmed.

Another example for probable vesivirus induced abortion was revealed for 3 adult cattle from Washington, who had aborted 4, 1 and 14 days before acute sera (#112098) were submitted. The first and third animal were seropositive against BVDV, Bovine herpesvirus 1 (BoHV-1) and *Neospora caninum* while the second serum was seropositive for only BVDV and BoHV-1. As a follow-up, convalescent sera (#112538) were submitted 18 days after the acute sera, revealing no significant change of antibody titer. The final diagnosis for causing abortion was due to a *Neospora caninum* infection. Those sera except the acute serum of the second animal were tested for vesivirus antibodies. While no antibody titer change could be detected for the first animal (OD = 0.039 and 0.033) and the convalescent serum of the second animal was negative as well (OD = 0.041), the third showed a more than three-fold OD-increase from 0.543 to 1.895, indicating a recent exposure to vesiviruses.

One adult cattle from Idaho (#1014/03) with an idiopathic abortion, was negative for *Brucella abortus*, *Leptospira* and BoHV-1, and low positive for BVDV revealed a high seropositivity (OD = 1.656) against vesivirus antibodies. And finally, a 6 year old cattle from Washington (#111378) with a idiopathic diagnosed late term abortion for twins tourned out seronegative for *Neospora caninum*, *Brucella abortus* and

Leptospira while positive for BVDV and BoHV-1 (most likely due to previous vaccination), revealed a high seropositivity (OD = 1.92) against vesivirus antibodies.

The seropositive results from the previous screening against various infectious agents (Table 8) were compared with vesivirus seropositive results to examine probabilities of double infections. No association was found when statistically examining the ratio of vesivirus positive results to the total number of examined samples for a specific other agent and the ratio of the double-positive results for vesivirus and the specific agent to the total number of positive results for the other agent. It is therefore not likely for a cattle to be seropositive against both vesiviruses and one of the other agents examined. Interestingly, without the knowledge of the exact disease for every cattle being tested for antibodies against common agents within the others-group, a trend revealed when only 9.4% (5/53) of sera were positive for *Mycobacterium paratuberculosis* while 24.5% (13/53) of the same samples were positive for vesivirus antibodies including two sera which were positive for both agents. Another example was found for animals with abortion problems that were mainly idiopathic and were also tested for antibodies against *Brucella abortus*. While only 0.9% (2/215) were seropositive against *Brucella abortus* as a common cause for abortion, 12.1% (26/215) of the same samples were positive for vesivirus antibodies. No sera were positive for both agents.

Geographical distribution

Cattle sera were received from 9 States of the USA (Figure 3). The ELISA results revealed a seroprevalence against vesiviruses of an average 21% (Figure 8). The results from Utah (n = 1), Maryland (n =4), Montana (n = 10) and Nevada (n = 5) were not used for statistical calculation due to insufficient sample size. Alaska revealed the highest seropositivity of 28.6% (4/14), followed by Hawaii with 16% (4/25), Washington with 15.5% (73/467), Oregon with 13.6% (9/66) and Idaho with 12.9% (13/101). When compared the seropositive results of the remaining States, no statistically significant difference between seropositivity against vesiviruses in different States was detected. Nevertheless, a similar low seroprevalence can be seen over long geographic distances from northern Alaska along the north-western contiguous States to tropical Hawaii.

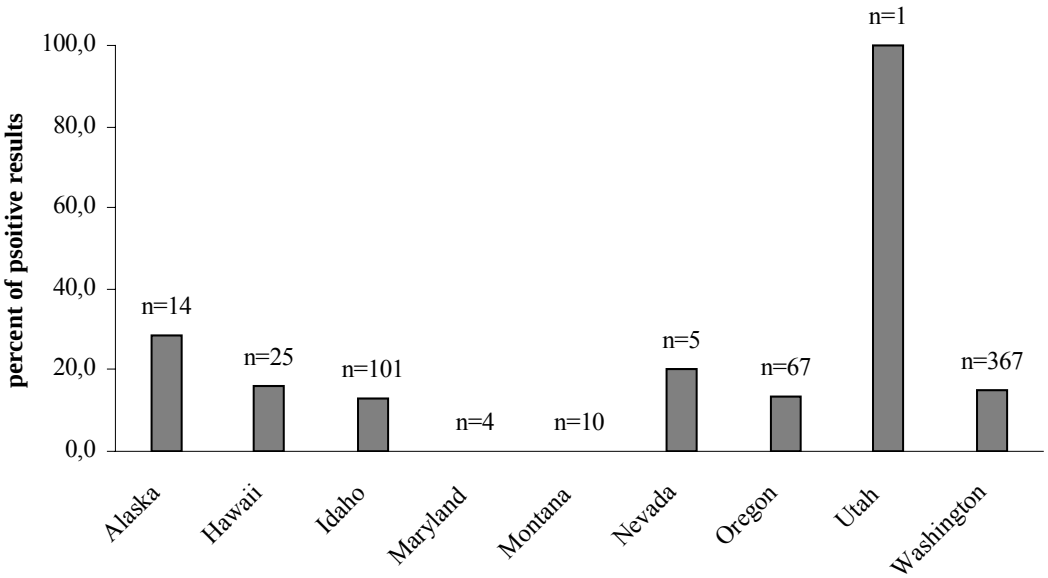


Figure 8. Percentage of vesivirus antibody positive sera from different States of the USA

Epidemiological studies of equine sera and tissue, and ETC tissue

To evaluate the possible correlation between vesivirus prevalence and Mare Reproductive Loss Syndrome (MRLS), sera and tissue samples were screened over a two-year period for the presence of vesivirus antibody and antigen. After the initial sample set of 112 randomly collected sera from mares with and without abortion problems were submitted and analysed by ELISA, three other sera sample sets, one from mares diagnosed with MRLS and two from mares with experimentally induced abortions were screened for vesivirus antibody. Additional tissue samples of fetuses and Eastern Tent Caterpillars (ETC) have been tested for vesivirus infection by FA and RT-PCR.

Equine Study 1

The initial 112 equine sera (Group 1) were received in 2000 and 2001 from the University of Kentucky, USA. Twenty five blood samples were collected for pre-sale health testing from randomly selected (breeding age) mares 3 to 13 years old that were being sold at auction (hereafter referred to as sale-barn mares) and 29 samples were from mixed age males and female juveniles also being pre-sale tested and sold at auction. The remaining 58 samples were from mares on farms having abortion problems (hereafter referred to as abortion associated mares).

Table 12. Serologic analysis of 112 equine sera in Group 1: numbers and percentages of seropositive results for vesivirus antibodies

Animal characteristics	Number of animals	Antibody positive (%)	Antibody negative (%)
Sale-barn horses			
Mares	25	10 (40)	15 (60)
Male and female juve-niles	29	0 (0)	29 (100)
Male	20	0 (0)	20 (100)
Female	34	10 (29.4)	24 (70.6)
Juvenile females	9	0 (0)	9 (100)
Abortion associated horses			
Mares	58	37 (63.8)	21 (36.2)
Total sample	112	47 (42)	65 (58)

Using an index of 0.14 as the optical density (OD) cut-off for the vesivirus antibody specific ELISA, the majority of the abortion associated mares were seropositive (63.8%, 37/58). While none of the males and female juvenile horses (0/29) showed a positive result, 40% of sale-barn mares sold at auction (10/25) were positive (Table 12). Within the group of horses auctioned, 29.4% of all females (10/34) were seropositive. In contrast, no sera of male horses (0/20) or of juvenile females (0/9) were positive (Figure 9).

Table 13. Statistical association between the vesivirus seroprevalence and various animal characteristics in Group 1[a]

Animal characteristics	OR (95% CI)	P-value	Significant
Sale-barn mares vs. Abortion associated mares	2.64 (1.01-6.92)	0.039	Yes
Male and female juveniles vs. Sale-barn mares	18.67 (2.2-160)[b]	<0.001	Yes
Male and female juveniles vs. Abortion associated mares	49.3 (6.3-389)[b]	<0.001	Yes
Sale-barn male vs. Sale-barn females	7.9 (1.14-181)[b]	0.005	Yes
Juvenile females vs. Sale-barn mares	5.3 (0.77-57.0)[b]	0.025	Yes

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval
[b]OR is underestimated due to the replacement of 0 by 1

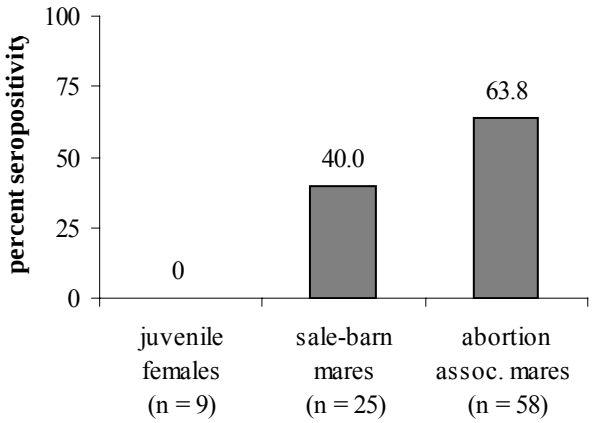


Figure 9. Vesivirus seroprevalence of all female horses within Group 1

A statistically significant difference was observed between the seropositive results of the samples from sale-barn mares and the abortion associated mares (P = 0.039) (Table 12). Interpreted with the calculated odds ratio (OR) as an indication of the

significant strength of the correlation, abortion associated mares had a 2.64 higher probability of being seropositive than the breeding-age sale-barn mares. Likewise, statistically significant differences were observed between the seronegative reference group (males and juvenile females sold at auction) and the sale-barn mares 3 to 13 years old sold at auction ($P < 0.001$) and also the abortion associated mares ($P < 0.001$) with ORs of 18.67 and 49.3 respectively. Female horses showed a statistically significant higher seroprevalence than males (0.005) with an OR of 7.9. Also, sale-barn mares (10/25) had a 5.3 times higher probability to be seropositive than female juveniles (0/9), showing a significant difference ($P = 0.025$).

Comparing the OD results from juveniles, sale-barn mares and abortion associated mares, different distributions of seropositivity are indicated (Figure 10). All samples of juvenile horses were below the cut-off of 0.14 showing almost a typical bell-shaped curve with a maximum at 0.06. While the peak of the curve of the OD results for the sale-barn mares is also at 0.14 and shows the same maximum with a broader distribution, a different shape of the curve with a maximum just below the cut-off at about 0.12 is seen for the OD results from abortion associated mares.

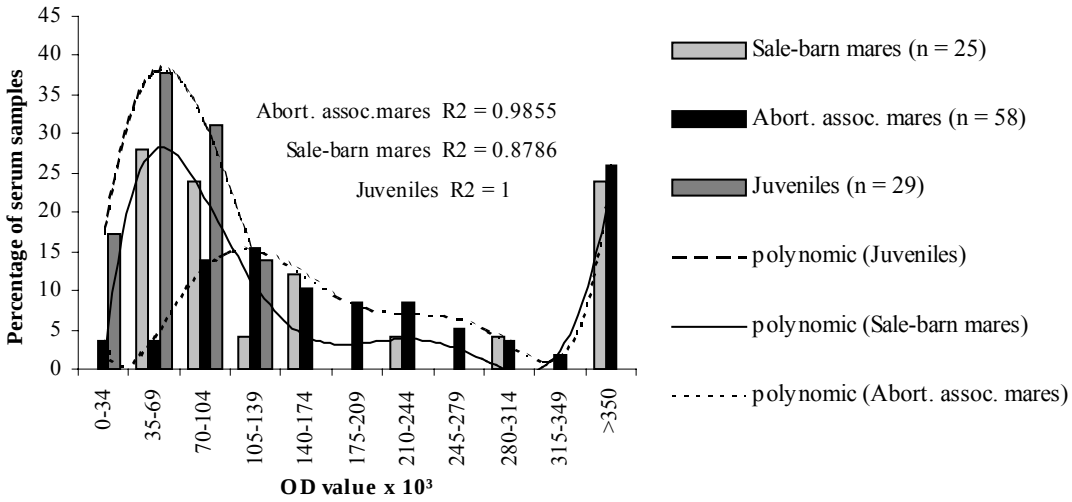


Figure 10. Results of the indirect ELISA in Group 1 shown as percentages of the frequency of seropositivity within selected OD ranges for sale-barn mares, Juveniles and mares associated with abortion problem farms. An index of 0.14 was calculated as the cut-off. Polynomic trend lines given for all animal characteristics indicating the distribution of the frequencies of OD results.

Equine Study 2

As a follow up study in 2001, 28 paired (acute/convalescent) sera (Group 2) from mares diagnosed with MRLS from the University of Kentucky were studied with regard to the correlation of vesivirus infetion and abortion in mares found in study 1. The sera were from locations different than the Group1 samples. The previously calculated cut-off of 0.14 was used for this comparative study.

The majorities of samples turned out to be seropositive, especially the acute sera (71.4%, 20/28) followed by convalescent sera (60.7%, 17/28) (Table 14).

Table 14. Serologic analysis of 28 paired (acute/convalescent) sera from mares diagnosed with MRLS from Group 2, seropositive for vesivirus antibodies

Animal characteristics	Number of animals	Antibody positive (%)	Antibody negative (%)
Acute sera	28	20 (71.4)	8 (28.6)
Convalescent sera	28	17 (60.7)	11 (39.3)

Despite the statistically significant difference between the acute sera (20/28) of Group 2 and sera from sale-barn mares (10/25) from Group 1 (P = 0.021) with an OR of 3.75, no association could be found between abortion and change in vesivirus antibody level (Table 15). When comparing acute and convalescent sera, not a single mare showed the typical threefold or higher change in vesivirus antibody level as could be anticipated following an immunological response after a suspected infection with vesiviruses (Figure 11).

Table 15. Statistical association between the vesivirus seroprevalence and various animal characteristics[a]

Animal characteristics	OR (95% CI)	P-value	Significant
Acute sera vs. Sale-barn mares (group 1, 10/25, 40%)	3.75 (1.19-11.8)	0.021	Yes
Acute sera vs. Convalescent sera	1.62 (0.53-4.94)	0.29	No

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval

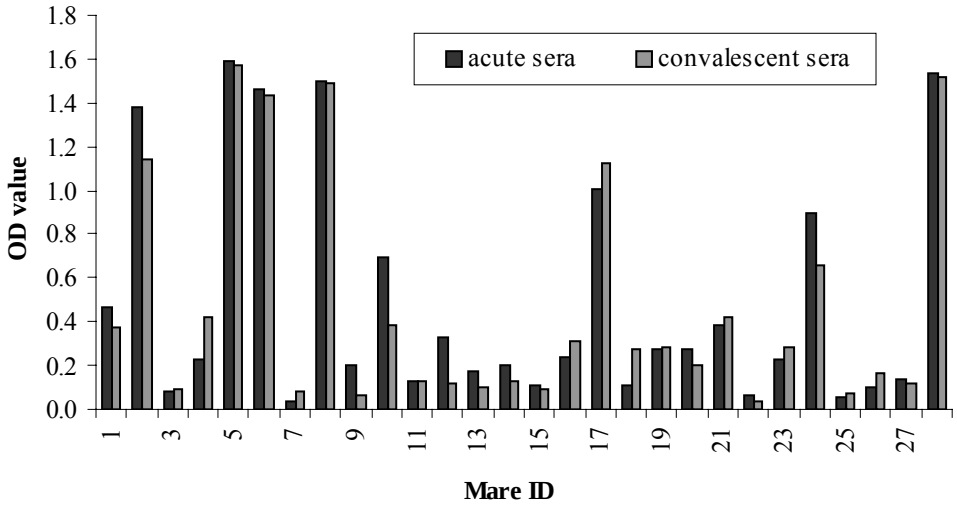


Figure 11. Results of the vesivirus specific indirect ELISA of 28 paired (acute/convalescent) sera from mares diagnosed with MRLS from group 2. Seropositivity is indicated with a cut-off of 0.14.

Equine Study 3

In order to determine if there might be a causal association between MRLS and Eastern Tent Caterpillars (ETC), the first experiment with the intent to mimic on-farm conditions was designed in 2002 at the University of Kentucky. In addition to their normal diets pregnant mares were exposed for two 10-day periods to ETC and their frass (n = 10 mares) in the field on which the mares grazed. A second similarly managed group of mares (n = 9) was exposed to frass only and for a control group (n = 10 mares) the exposure of ETC and frass was minimized. The first serum sample was collected pre-experimental, followed by at least two other samplings during the experimental exposure. Unfortunately, this study was compromised by some movement of caterpillars between the three groups with the result that MRLS occurred in all three experimental groups. Nevertheless, this experimental field study with 29 pregnant mares suggested that ETC and/or frass were causally related to MRLS. Seven of 10 mares exposed to ETC and frass, and 7 of 9 mares exposed to frass only had experienced abortion. Three of 10 mares in the control group also aborted, but were accidentally exposed to ETC larvae during the treatment (Webb et al., 2002).

The seroprevalence of vesiviral antibody in all pre-experimental sera was 62.1% (18/29) and increased during the experiment to 86.2% (25/29) for all sera 33 days post-exposure at the third bleed (Table 16). While the control group showed a constant seropositivity of 80% for all bleeds, the ETC-exposed group increased from 50% (5/10) seropositivity at the first bleed to 90% (9/10) at the third bleed (Figure 12). Likewise, the group being exposed to frass only, showed an increase from 55.6% (5/9) to 88.9% (8/9).

Table 16. Serologic analysis of serum samples from 29 mares in group 3, positive for vesivirus antibodies. Three pre- and post-experimental bleeds during treatment with ETC, frass only or none as a control. Seropositive mares and seropositivity in mares who aborted during the experiment are listed.

groups	1st bleed (pre-experimental)		2nd bleed		3rd bleed	
	(03/26/2002) pos/total (%)	(03/26/2002) pos/Abort (%)	(04/24/2002) pos/total (%)	(04/24/2002) pos/Abort (%)	(04/29/2002) pos/total (%)	(04/29/2002) pos/Abort (%)
control	8/10 (80)	1/3 (33.3)	8/10 (80)	2/3 (66.7)	8/10 (80)	2/3 (66.7)
frass	5/9 (55.6)	3/7 (42.9)	7/9 (77.8)	5/7 (71.4)	8/9 (88.9)	6/7 (85.7)
ETC	5/10 (50)	4/7 (57.1)	8/10 (80)	6/7 (85.7)	9/10 (90)	7/7 (100)
total	18/29 (62.1)	8/17 (47.1)	23/29 (79.3)	13/17 (76.5)	25/29 (86.2)	15/17 (88.2)

Eight out of 17 mares (47.1%) that aborted during the experiment were seropositive already pre-exposure (Table 16). Their number increased to 15 mares at the third sampling 33 days post exposure (88.2%). One out of three mares that aborted in the control group was positive already pre-exposure and two were positive at the end. Within the ETC-exposed group, 57.1% (4/7) of the mares that aborted were positive pre-exposure and this and increased to 100% (7/7) at the third bleeding (Figure 13). And finally, 3 out of 7 (42.9%) mares exposed to frass only who aborted were pre-exposure positive and this increased to 6 out of 7 (85.7%) at the end of the experiment.

Following statistical evaluation, a significant increase of the seroprevalence was seen between pre-experimental sera from all mares (18/29, 62.1%) and the third bleed (25/29, 86.2%) with an OR of 3.8 ($P = 0.03$) (Table 17). No statistically significant association was detected between seropositivity in mares for the same time of sera collection (1st, 2nd and 3rd bleed) within different groups (control, ETC and frass).

When evaluating statistical associations of seropositivity in mares that aborted during the experiment, a significant increase ($P = 0.013$) with an OR of 8.4 was detected

between the pre-experimental sera (8/17, 47.1%) and sera from the third bleeding (15/17, 88.2%) of all mares who aborted (Table 18).

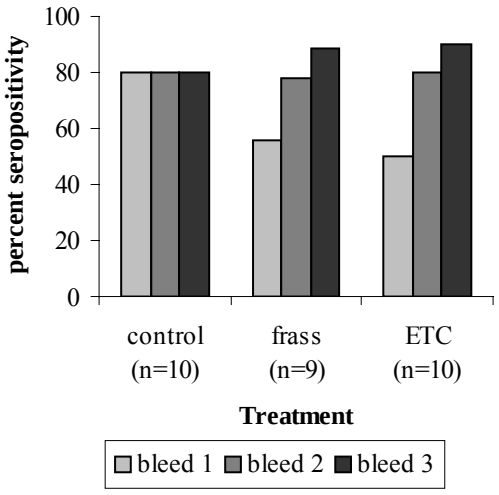


Figure 12. Results of the indirect ELISA in group 3 shown as percentages of seropositivity against vesiviruses in experimentally treated mares .

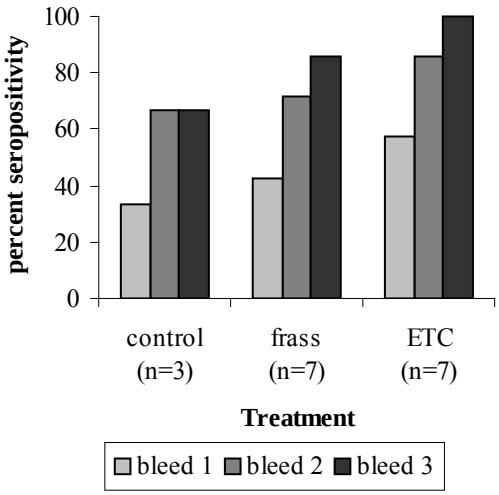


Figure 13. Results of the indirect ELISA in group 3 shown as percentages of seropositivity in mares who aborted during the experiment.

Table 17. Statistical association of seropositive mares in group 3 during the first experiment[a]

Animal characteristics	OR (95% CI)	P-value	Significant
1st bleed mares total (62.1%) vs. 2nd bleed mares total (79.3%)	2.3 (0.7-7.5)	0.12	No
1st bleed mares total (62.1%) vs. 3rd bleed mares total (86.2%)	3.8 (1.04-13.9)	0.03	Yes
1st bleed frass (55.6%) vs. 3rd bleed frass (88.9%)	6.4 (0.5-74)	0.15	No
1st bleed ETC (50%) vs. 3rd bleed ETC (90%)	9 (0.8-100)	0.07	No

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval

Table 18. Statistical association of seropositivity in mares of group 3 that aborted during the first experiment[a]

Animal characteristics	OR (95% CI)	P-value	Significant
1st bleed abort total (47.1%) vs. 2nd bleed abort total (76.5%)	3.6 (0.8-15.9)	0.08	No
1st bleed abort total (47.1%) vs. 3rd bleed abort total (88.2%)	8.4 (1.46-48.9)	0.013	Yes
1st bleed abort frass (42.9%) vs. 3rd bleed abort frass (85.7%)	8 (0.6-107)	0.13	No
1st bleed abort ETC (57.1%) vs. 3rd bleed abort ETC (100%)	n.a.[b]	0.1	No

[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval
[b]not applicable

Although no other statistical association between vesivirus infection and abortion for any of the three groups of the experiment and different blood samplings was detected, 5 mares showed a typical increase (three-fold and higher) of their immunological response against vesiviruses after a primary or secondary infection (Figure 14). Three of those mares (# 6, 8 and 12) aborted during the experiment. Their pre-experimental sera were all negative but converted during the experiment, thereby indicating a primary infection. The other two mares with raised titers (# 2 and 29), that did not abort during the experiment, were seropositive at the beginning, indicating a secondary infection during the experiment.

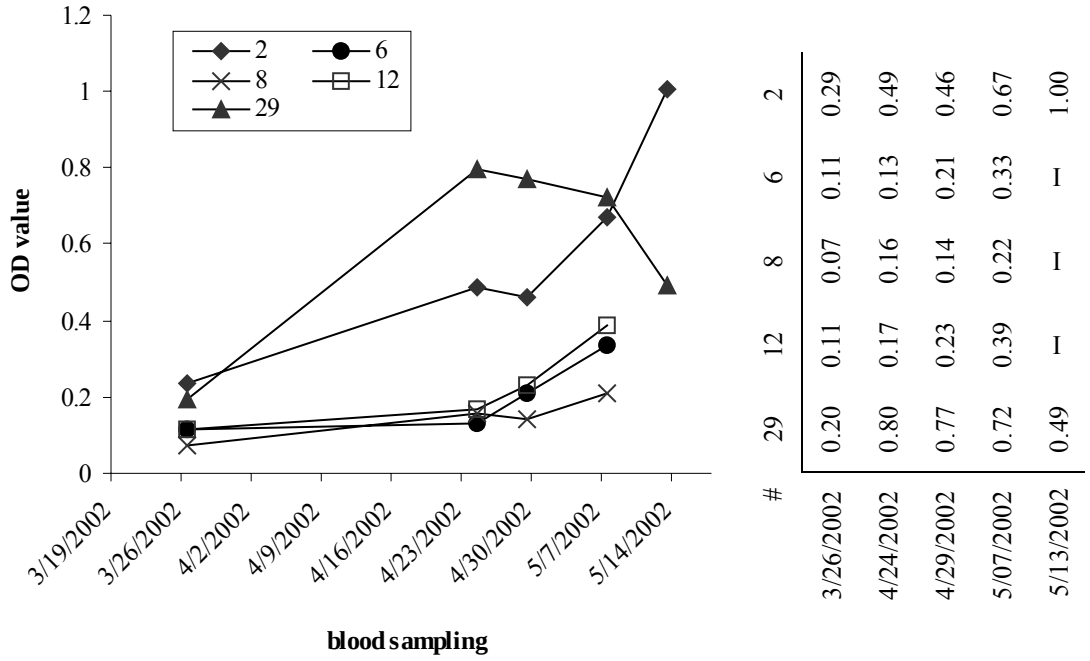


Figure 14. OD-values of serum samples from 5 mares taken over a period of 7 weeks and tested by indirect ELISA, who showed a threefold or higher increase of their immunological response during the experimental treatment. Mares #8 was fed with ETC; #2, 6 and 12 were fed with frass only, and #29 was from the control group. Mares #6, 8 and 12 aborted during the experiment.

Equine Study 4

To avoid the complications of the caterpillar movement during the first experimental study, another three groups of pregnant mares received their respective 10-day treatments by gastric administration at the second ETC exposure study. One group (group A) of 5 mares received crushed fresh ETC, another 5 mares (group B) were inoculated with the same material, which was autoclaved before feeding and reference sera were obtained from 4 horses (group C) that were fed Gypsy moth larvae as a negative
control. At least 6 pre- and post-infection sera were obtained for each mare during a 4-week period and were tested for antibody against vesiviruses. Three of five mares in group A aborted and all were MRLS type. In contrast, there were no abortions in group B and only one abortion in the control group and it was not of the MRLS type. The previously calculated ELISA cut-off of 0.14 was used for this comparative study.

The seroprevalence of all pre-experimental sera was 7.1% (1/14). During the experiment, no additional mares seroconverted. The only positive mare, which showed a constant low seropositivity for all sera had been inoculated with autoclaved ETC and did not abort during the experiment.

In this study no statistically significant association could be detected between vesivirus infection and ingesting ETC or abortion in any of the three groups of the experiment.

Table 19. Statistical correlation between seroprevalence of healthy and/or pre-experimental adult mares in equine studies[a]

Animal characteristics	OR (95% CI)	P-value	Significant
Equine study 1 (10/25, 40%) vs. Equine study 3 (18/29, 62.1%)	2.4 (0.8-7.3)	0.09	No
Equine study 1 (10/25, 40%) vs. Equine study 4 (1/14, 7.1%)	8.7 (0.97-77.1)	0.03	Yes
Equine study 3 (18/29, 62.1%) vs. Equine study 4 (1/14, 7.1%)	21.3 (2.4-185.9)	<0.001	Yes

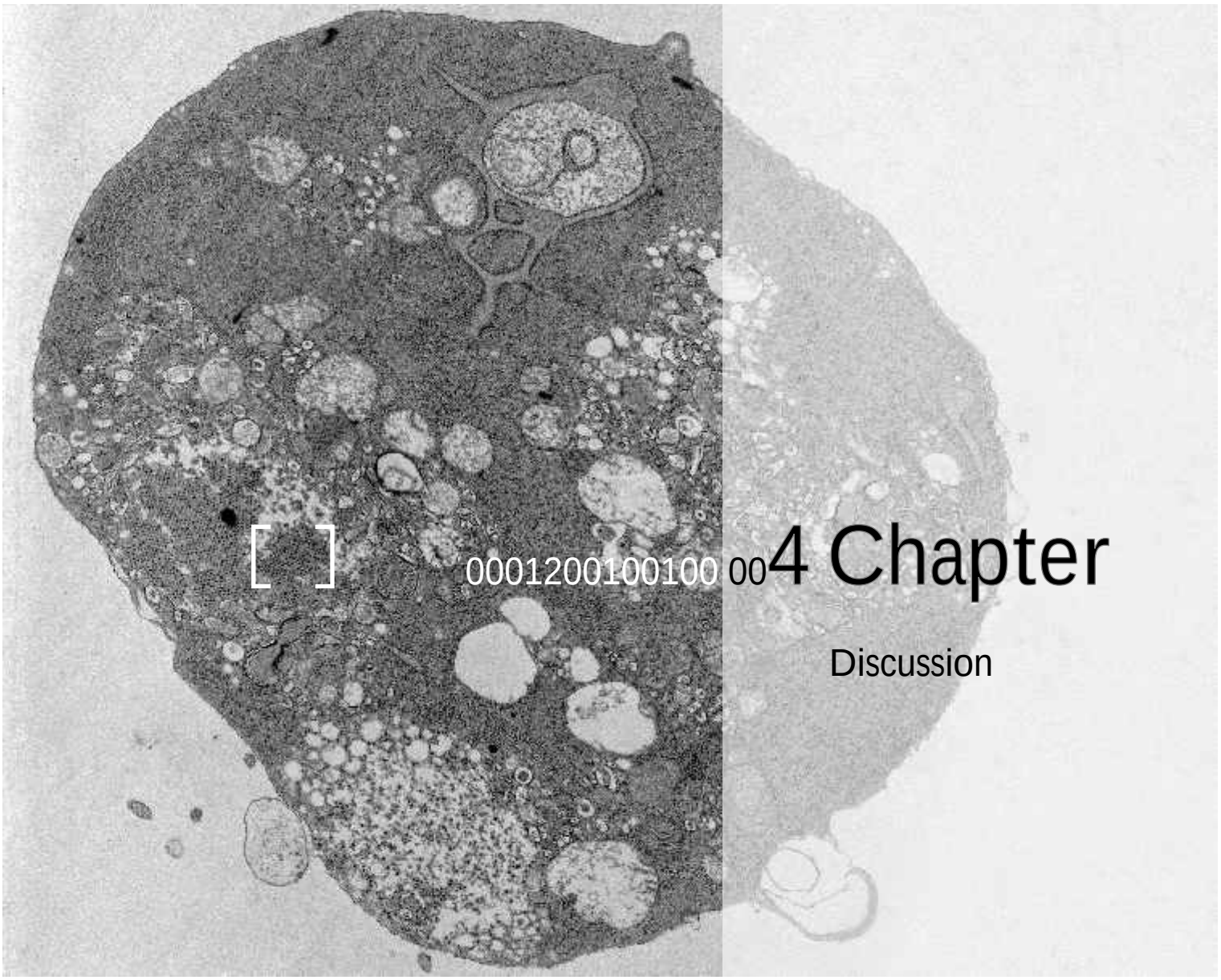
[a]Odds ratios (OR) and P values are for the comparison between antibody-positive animal characteristics. CI, confidence interval

Comparing seropositivity from sera of sale-barn mares from study 1 and from pre-experimental taken sera of mares from studies 3 and 4, the vesivirus prevalence was not significant different between studies 1 and 3 only ($P = 0.09$). Study 4 showed a significant correlation to study 1 ($P = 0.03$) and study 3 ($P < 0.001$) (Table 19).

Equine Study 5: equine and ETC tissue

Tissue samples from the possible natural vector, eastern tent caterpillar (ETC), and from 20 aborted fetuses were acquired and have been tested for vesivirus antigen by FA and RT-PCR.

Testing tissue samples from aborted fetuses by FA using the well characterized vesivirus group-specific monoclonal antibody designated 4AD8D8, 12 out of 20 (60%) turned out positive. However, because of the low sample quality of the fetal tissues due to repeated freeze-thawing, no specific morphology could be identified. The positive results of the FA test could not be confirmed by a vesivirus-specific RT-PCR. Likewise, when tissue from ETC were tested by RT-PCR, there were no positive aetiologically links found.



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Discussion

This is the first extensive study to describe vesivirus prevalence in cattle and horses. The intent was to characterize distinct herds with the knowledge that in such studies, where individual animal data may not always be available, disease linkages may not always be obvious or even detectable. Single serum samples were drawn from most animals either randomly or to diagnose specific disease conditions and to test for the presence of vesivirus antibodies where sera that tested positive would be evidence of previous exposure to vesiviruses.

Epidemiological study of cattle sera

The overall vesiviral seroprevalence in cattle within different groupings was between 10 and 20%. This range is similar to results for other common veterinary relevant agents such as Bluetongue virus, Mycobacterium paratuberculosis, *Neospora caninum* and different species of *Leptospira*. The seroprevalence for *Brucella abortus* was even less. Other agents such as BVDV, BLV, BRSV, BoHV-1 generally exhibited a high prevalence for specific antibody tests in cattle due to routine vaccinations (personal communication with Dr. A. W. Smith). These serological data presented here did not statistically validate a previously reported association between abortion in cattle and vesivirus infection in cattle (Smith et al., 2002b). Likewise, when examining all available parameters of the animal case histories no general association to any specific disease manifestation could be found using serological screening for vesiviral antibodies.

Nevertheless, knowing that vesiviruses are pathogenic in other animals (Bankowski, 1965; Smith, 2000) and a high incidence of seropositive test results does occur in adult cattle (20/652 over OD=1.0) it is reasonable to suspect that vesiviruses also cause disease in cattle. Juvenile cattle had a seroprevalence of 4.9% (2/41) and the positive results on the two animals were low in range (OD<0.4). This emphasizes the strength of the significant difference when compared to adult cattle. Conclusive evidence of a general correlation was demonstrated but would be expected to show up more definitively if the bovine samples examined had been documented along with more specific and detailed health profiles, precise geographic locations, history of animal source, and the herd management details. The statistically significant differences shown between the four main groups of cattle suggests, that cattle with abortion

or respiratory problems, which were randomly sampled, and juvenile cattle represent different susceptibility groups for becoming vesivirus infected. Within those groups, different animal characteristics (beef or diary, male or female) represented no risk factors for vesivirus infections. After evaluating all subgroups for significant characteristics, only randomly sampled diary cattle (others-group) showed a statistically significant association for positive ELISA results.

The value of all ELISA test results depends upon both the quality and quantity of samples and the reliability of the ELISA test used for this study (Strongin, 1992). The recombinant test antigen used for this ELISA will detect most known vesivirus serotypes (Smith et al., 2002b). The calculated cut-off for the ELISA was set to give a specificity of approximately 97.5% (Barajas-Rojas et al., 1993). Since vesiviruses with a different virulence were detected simultaneously, no detailed statement could be made regarding the number of vesivirus strains and their pathogenic potential being found in individual samples. Nevertheless, cross-reactivities between the vesivirus group-specific ELISA and other common veterinary-relevant pathogenic agents (Table 8) could be excluded. Although all individual blood samples were of good quality when tested, sample collection was not exclusively random and therefore a disproportionate representation of samples from specific subpopulations could have skewed the results for the four main groups and their subgroups. Also, because of the lack of background information available on most animals, a complete evaluation for individual animals could not be achieved. For these reasons, the results comparing of distinct herds seem to provide the most indicative information. Concentrating on those results, associations were shown within the others-group and the group of cattle with abortion problems. Several herds with the same animal characteristics exhibited statistically significant differences for seroprevalence against vesiviruses. Two herds (#108278 and 105572) with randomly sampled healthy adult female dairy cattle revealed a vesivirus seropositivity of zero and 35.7% respectively, the second herd with high optical densities of over 1.5 for 4 samples. No clinical history of obvious differences could be found, because herd histories including data on abortions, pneumonias, diarrhea and other conditions sometimes occurring in other species as a result of vesivirus infections, were not available for these herds. Unknown risk factors will

exist and these might be the same or similar to those in other groups with high seroprevalence, e.g. herd #109649, which contained healthy male beef cattle being screened for export to Canada and herd #108506, consisting of adult dairy cows from Washington. Hypothetical risk factors might include contaminated food such as added fish protein supplements or fish meal (Munday and Ryan, 1982; Smith et al., 1980), introduction of infected animals into a herd and spread of infections by direct contact to other susceptible cattle, and the accessibility to waterways such as ocean, streams or lakes with infected fish or contaminated urine and feces from infected wildlife animals in areas where susceptible cattle are grazing. Apparently, a far more sensitive epidemiological approach would be necessary to confirm probable risk factors.

On the other hand, negative test results for other abortagenic agents in various herds of cattle with abortion problems, including several examples with an undiagnosed abortion cause, and high seropositive results for vesivirus antibodies indicated a possible vesivirus cause of abortion in cattle. One example (#112098-2 and # 112538-2) was shown with an increase of the vesivirus antibody response comparing acute and convalescent sera indicating a recent exposure to vesiviruses. As mentioned above, a more sensitive epidemiological approach would be necessary to more definitively examine probable risk factors. This could not be more precisely addressed due to limited animal information.

Another statistical approach revealed that it is unlikely for cattle to be seropositive against both vesiviruses and one of the other commonly tested agents. This suggests that vesiviruses are transmitted independent of other disease agents. Screenings for common veterinary relevant agents like *Mycobacterium paratuberculosis* and *Brucella abortus* in various cattle groups revealed a lower seropositivity than for vesiviruses, which may indicate or not the possible importance of vesiviruses as a cause of disease(s). Approximately 75% of all bovine abortion cases in the US and Germany remain without a definitive aetiologic diagnosis (personal communication with Dr. A.W. Smith and Dr. F.K. Reckling). This suggests to look for undiagnosed infections, such as those by vesiviruses. The finding of a high seroprevalence in distinct herds with or without manifested diseases, significant differences to the negative reference group and other herds with similar characteristics, the absence of other

common agents and the unlikelihood for vesiviruses being associated with other agents in mixed infections suggests, that vesivirus infections are manifested in certain groups of cattle. This concept receives strong extrapolatory support due to the pathogenic potential demonstrated by vesiviruses in a phylogenetically diverse array of other animal species (Smith, 2000).

Support for the hypothesis that vesiviruses are mainly transmitted by direct contact between animals (Bankowski, 1981; Smith et al., 1998b), is shown by a finding of significant differences for vesivirus seroprevalence in herds of healthy adult diary cattle within the same geographic location.

Because seroprevalences in cattle could not be linked to distinct geographical locations, this suggests that no geographic protective factor(s) exists. Consequently there is an equal probability of cattle getting infected with vesiviruses at all locations studied.

After chickens, cattle are the most common food animal in the United States numbering roughly 97 million head total in 2002, which includes 33 million beef cows and 9 million milk cows. Of about 36 million cattle slaughtered in 2002, about 200,000 (0.6%) were condemned at ante-mortem and post-mortem inspection by the Food Safety Inspection Service (USDA - National Agriculture Statistic Service, 2003). Using the prevalence of vesiviruses on the negative control group (4.9%) for the calculation of the total sum of vesivirus infected cattle, there are an estimated 5 million cattle in the USA that, at any one time, will have been exposed to vesiviruses and serve as an infected pool for cattle and other animals and a possible reservoir of zoonotic vesiviruses that could impact human health as a food safety issue (Smith and Boyt, 1990).

To estimate the possible impact of vesivirus infection for other animal species and humans using the results of cattle based on geographical distribution, we did not find any indication of decreased risk of vesivirus infection within the study areas ranging from Alaska over the western continent of America to tropical and isolated Hawaii. Further studies addressing the distribution of vesiviruses in adult sale-barn mares in the eastern USA, makes-up the second part of this study and reveals a high prevalence of vesivirus exposure without an obvious connection to sources of vesivirus infection

from the Pacific ocean, which suggests that vesivirus transmission can occur by-way-of terrestrial transmission routes independent from the ocean. Since vesiviruses are endemic in the United States of America and probably all countries surrounding the Pacific Ocean (Smith and Boyt, 1990), where both ocean and terrestrial animals including cattle and horses are infected at a high prevalence, cases of vesivirus induced vesicular diseases in cattle are likely to occur. The vesiculogenicity of these agents in cattle is already known and has been proven by experimental infectivity studies in cattle (Smith and Boyt, 1990; Smith et al., 1983a). Considering the broad host range of vesiviruses and the test results in cattle, we cannot exclude a similar vesivirus prevalence for other animals. Human infections with resultant disease has been observed and reported (Smith et al., 1998a; Smith et al., 1998b).

Epidemiological studies of horse sera and tissue

Four different study groups of horses were examined, and this represents a sufficient pool of animals to evaluate MRLS as a newly emerged disease in horses. All samples were chosen from one geographic location to exclude external influences like weather and pathogen pollution. Furthermore, all samples used as a negative reference group were randomly sampled. Although the twenty five blood samples from pre-sale health tested mares of breeding age (sale-barn mares) were randomly selected, they may not have been representative of the general population of healthy adult mares because the reasons for selling these animals were unknown and might have skewed the result. There was a surprisingly high seroprevalence for antibodies against vesiviruses among mares being sold at auction and also healthy mares that were used for experimental transmission studies (40% and 62% respectively). This demonstrates an extremely high seroprevalence compared to mixed-age male and juvenile female horses, which were used as a negative reference group and were all seronegative. All male horses were juveniles except 2, which were 1 and 3 years old. The absence of seropositive samples within this group suggests that horses are unlikely to become exposed to vesiviruses during their first year of life and indicates that infection with vesiviruses occurs in roughly half of all mares sometime after they become yearlings.

In study 1, a significant correlation between vesivirus infection and abortion could be shown. Not all the mares tested on farms with abortion problems actually experienced

abortion, therefore the result of 63.8% seropositivity might be an underestimation. On the other hand, it is not known whether mares being sold at auction had experienced a previous abortion and therefore the percent that were seropositive may be over-estimated. The different distribution of seropositive results (Figure 10) also suggests that mares associated with abortion are different when compared to the other two groups. Another difference was shown between sale-barn females and sale-barn males, indicating that female horses have a 7.9 times higher probability of being infected with vesiviruses than male horses, however, only two male horses were within this group. If the seroprevalence in male cattle (26.7%) is any indication of what might be seen in a larger sampling of male horses, we can assume that there is likely to also be an increase of seropositivity in adult male horses. As already mentioned, the disease history of each sale-barn horse is incomplete, therefore risk factors for exposure of females to vesiviruses could not be determined. In summary, adult female horses that were associated with a recent abortion were identified as the highest risk group for infection with vesiviruses and this leads to the question of whether or not vesiviruses are causing abortions in horses.

In study 2, acute and convalescent sera from mares having recently aborted revealed a seroprevalence slightly higher than the result in mares associated with abortion from study 1 and significantly different from the sale-barn mares from study 1. Although this result further validates the data from study 1, no additional evidence proving that vesiviruses causes abortion was found. Interestingly, any changes seen between the acute and convalescent sera reactivity were modest. Unfortunately, no additional data were submitted for the mares examined in study 2. Dates of the samplings as these relate to the time of abortion and whether any mare had experienced a previous abortion was not known. Usually, convalescent sera are taken two to four weeks after the first (acute) sampling. The exact time of the acute sampling could be estimated as a range from immediately after the abortion occurred up to a couple of days later when the veterinarian stopped by for a consultation. It seems unlikely that these very high levels of reactivity to a known pathogenic and sometimes abortogenic virus would be occurring and that this would not be relevant, especially when at least 6 aborted mares

(#2, 5, 6, 8, 17 and 28) developed antibody responses (Figure 11), which were among the highest values of the entire study.

In study 3, a high prevalence in both pre- and post-treatment samples was found. The 29 mares experimentally treated (Webb et al., 2002) had no previous history of abortions, but did have a prevalence of 62.1% seropositive, which was not a statistically significant difference from the sale-barn mares from group 1. An increase in prevalence occurred during the experiment, reaching 88.2% seropositivity for all mares that aborted after the feeding treatment. The statistically significant increase of over 20% from unaffected mares to mares associated with abortion is the same for both studies 1 and 3. Interestingly, the increase of seropositivity occurred only in mares who aborted (from 47.1% to 88.2%), mainly in mares of those groups which were fed with ETC or their frass only. In mares that were fed ETC and then aborted during the experiment the seropositivity reached the highest value of 100% (7/7). Mares without abortion showed no change of seropositivity against vesiviruses during the experiment (10/12, 83.3%). An increase of over 40% in mares that aborted demonstrates clearly an association between a positive test for vesivirus antibody and abortion in mares. Little is known about the immune response in animals after a primary or secondary vesivirus infection (Bankowski, 1965) and as already mentioned nothing is known for horses. A variety of responses has been demonstrated for different serotypes in other infected animals, ranging from an immediate appearance with very high titers to no production at all of neutralizing antibodies (Smith, 2000). Therefore, the greater than threefold increase in the serum antibody response during the treatment in five mares, three of whom aborted during the experiment, have to be interpreted carefully. The three mares that aborted (#6, 8 and 12) were pre-experimental negative for vesivirus antibodies but exhibited a similar pattern of antibody increase (Figure 14) which suggests, that they got infected with the same vesivirus serotype for the first time during the experiment. The other two mares which did not abort during the experiment were pre-experimental seropositive and showed a different pattern of antibody increase which suggests a secondary infection maybe with (a) different serotype(s). Despite the absence of a typical increase in their immune response subsequent to infection, the remaining 4 mares (#5, 10, 11 and 15)

that aborted experienced a primary infection with vesiviruses, therefore an association between abortion and vesivirus infection cannot be ruled out. Although only 3 sera were available from three of these mares (#10, 11 and 15), they all showed the same pattern of antibody increase (1.5- to 2.5-fold) and had a fourth serum sample been available, one would expect an even greater increase in antibody titer. Neither of two mares which were pre-experimental negative and did not abort during the experiment showed a similar pattern of antibody change. This leads to the assumption that only a slight seroconversion could take place in mares infected with a particular serotype of vesivirus, which might be the cause for the abortions that occurred in those mares.

In study 4, sufficient data about the origin and the health history of the experimentally treated mares were not available to explain the statistically significant low seropositivity (7.1% for all study 4 pre-experimental sera) compared to pre-study values for mares from study 1 and 3. Furthermore, since the seroprevalence for all post-experimental sera associated with abortion is also significantly different in this group from the appropriate samples from mares that experienced abortions in studies 1, 2 and 3, the result of this study differ from and lack correlation with studies 1-3 and between vesivirus infection and abortion in mares. As in study 3, other unknown factors must have contributed to the vesivirus prevalence, because no other mares seroconverted during the experiment, although 3 abortions occurred. Nevertheless, in study 4 the correlation between caterpillar extract as a source of abortion was confirmed (Bernard et al., 2002) but no correlation was shown between abortion and vesivirus infection. Even when the study 3 experiment was compromised by movement of the caterpillars between experimental and control groups, the differences between the outcome when compared to study 4, suggested a hypothesis of different causes being responsible for the abortions observed. The supporting data seen in the samples examined and the experimentally treated mares in studies 1 to 4 also support this observation. While something still unknown in ETCs is causing abortions in mares, vesiviruses appears to be another agent responsible for causing MRLS in mares. Since MRLS or a similar disease in horses was described to a lesser extent and only in 1980 and 1981 before the major outbreak in 2001 occurred and whereas the latter occurrence coincided with the exceptional abundance of ETC, natural changes

over years like certain weather pattern favourable for responsible vectors or circumstances seem to be responsible (Bryans, 1981; Hong et al., 1993). Because ETC, now shown to be associated with abortion in mares, have always been endemic in the eastern part of the US (Fitzgerald, 2002), it appears unlikely that this is the only factor resulting in MRLS. The exceptional damage in horses in 2001 (United States Animal Health Association, 2001) might be the result of overlapping cycles of different agents and if vesiviruses are among the abortogenic agents of horses, they may have routes of transmission that can operate independently of those explored in study 4.

In study 5, vesivirus antigen was found in tissue samples of aborted fetuses using fluorescent antibody testing, but these results could not be confirmed by RT-PCR. The differing result between the tests could be explained by the poor conditions of the tissue samples tested. During the transfer to the laboratory all samples thawed, arrived at ambient summer temperature and were likely somewhat degraded, thus not ideal for laboratory evaluation. It is likely that the viral RNA was more vulnerable to degeneration than the capsid protein. In general, viruses, especially if they are less pathogenic and cause less severe histological lesions, can show a persistent infection in organs of their hosts and low virus titers may result. This has already been described for vesiviruses (Smith, 2000; Smith et al., 1977b; Smith et al., 1981b). Viruses with low titers will be degraded more rapidly than highly cytolytic viruses because of intact cellular mechanisms. Disrupting virus structures make the virus genomic RNA susceptible for cellular RNA degenerating enzymes while capsid protein will be digested by cellular proteases. On the other hand, a successful RT-PCR depend on the preservation of several hundred specific base sequences of the agent's genome. With this scenario, it seems more likely that epitopes from capsid proteins would be detected with monoclonal antibodies using the fluorescent antibody test than intact segments of RNA using specific RT-PCR.

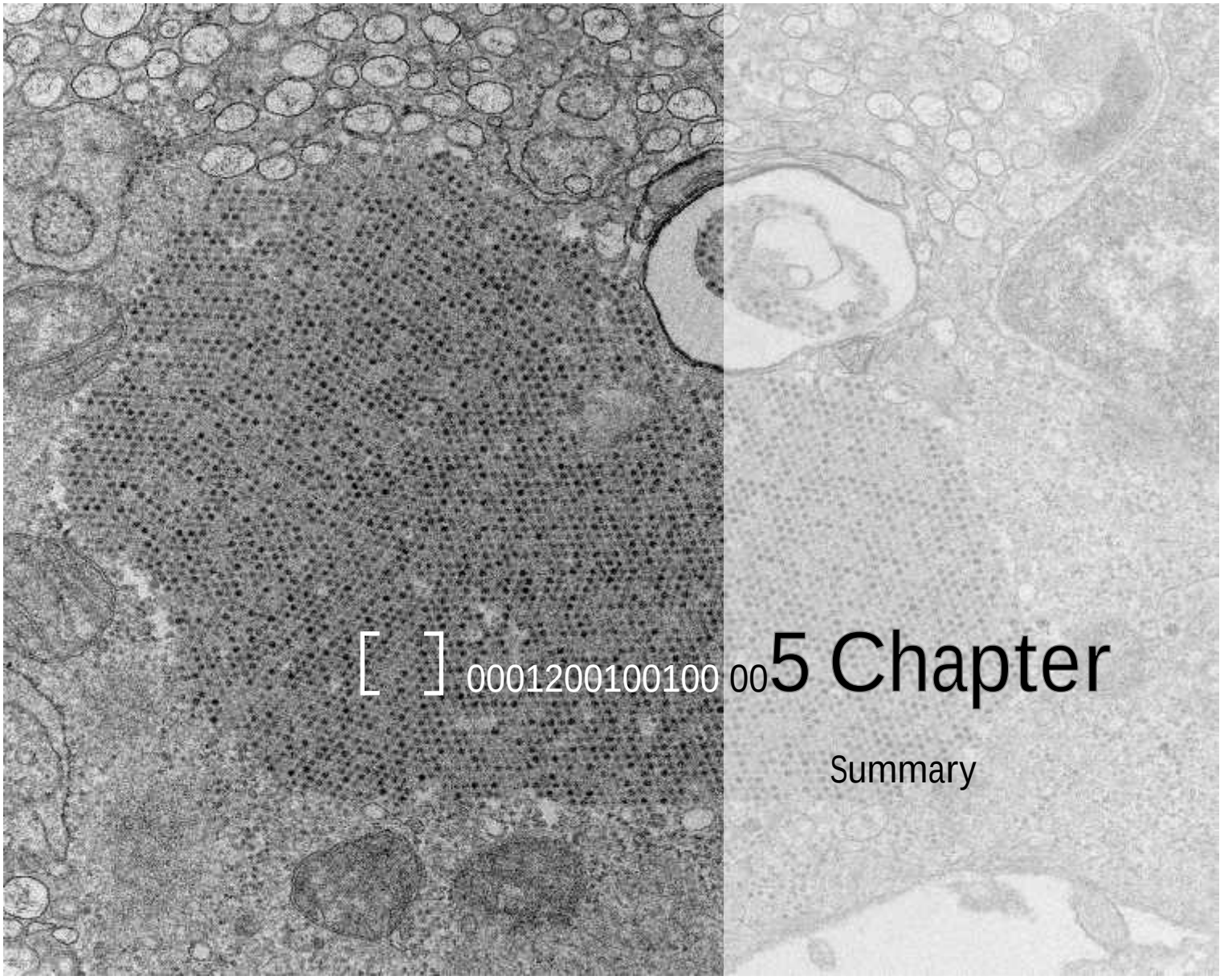
The successful visualization and identification of Cetacean vesivirus in the lungs of a stillborn bovine fetus using direct electron microscopy, immunohistochemistry and RT-PCR amplification (Smith et al., 2002b) demonstrated the propensity of vesiviruses for crossing the placental (mother-fetus) barrier. Still, since this finding has not been shown in horses, except for a positive immunohistochemisty result in

aborted fetal tissue the possibility of vesivirus infected fetal tissue and therefore a vesivirus infection passing across the placenta to the fetus as the cause of abortion remains an unproven but strong working hypothesis. That is supported primarily by sero-epidemiology and by extrapolations from other species serving as model systems (Bankowski, 1981; Barlough et al., 1986; Dunne et al., 1965; Smith, 2000; Smith et al., 2002b). Furthermore, the detection of no vesivirus seropositive blood samples from juvenile horses suggests, that either infection occurred early in gestation and the fetus did not seroconvert after a possible in-utero infection or that vesivirus infection of equine fetuses subsequent to their becoming immunocompetent is likely to terminate in an abortion which mimics MRLS.

In summary, because a very high vesivirus prevalence in mares with naturally occurring infections was proven and a correlation was shown between vesivirus seroconversion in experimentally induced abortions in mares, vesiviruses must now be considered a highly probable cause of equine abortion. And with such a high seroprevalence (up to 62% in unaffected mares) it seems unlikely that horses become infected exclusively by indirect contact through vectors or contaminated food such as raw garbage, offal and other animal products in their feed. A more likely source would be persistent infections with viral transmission from animal to animal by direct contact through vesivirus contaminated urine, feces, nasal or oral secretions (Bankowski, 1981; Smith, 2000). A persistent infection for vesiviruses has been previously described where they reach high titers in tissue culture both with and without CPE (Smith and Boyt, 1990). Also, experimentally inoculated animals may or may not show signs of disease even though a high antibody titer may occur in both symptomatic and asymptomatic animals with high prevalence in serologic surveys (Smith, 2000; Smith and Boyt, 1990; Smith et al., 1988). Furthermore, the natural seroprevalence of vesiviruses in horses rapidly increases with age, suggesting very effective unknown sources of infection and transmission routes for vesiviruses, and therefore a possible risk for other susceptible hosts such as swine, cattle and humans. To confirm the conclusion of vesiviruses as a cause of abortion in mares experimentally infection studies of pregnant mares with vesiviruses should be performed.

Vesicular exanthema of swine was declared eradicated and designated as a foreign animal disease (FAD) in 1959 (US Secretary of Agriculture). Since then there has been only one re-isolation of any of the original 13 original VESV serotypes and this was from a California gray whale in 1968 (Watkins et al., 1969). Although this first ever virus isolate from any marine mammal was originally identified as an entero-virus, it was subsequently proven in 1988 to be VESV-A_{48} (Smith, 2000). To date, type specific neutralizing antibodies against VESV A_{48} and at least two other VESV serotypes have been reported in domestic cattle and swine and a number of other wild and feral species (Smith, 2000) including various marine mammals, feral pigs and feral donkeys in the years between 1972 and 1976 (Smith and Latham, 1978). This demonstrates the presence of vesiviruses designated both SMSV and VESV in marine, terrestrial and domestic mammals including livestock.

The high prevalence of antibodies in cattle in Kansas and Minnesota (Smith and Boyt, 1990) and in Kentucky horses in 2001-2002 demonstrates that present-day transmission modes are not completely dependent upon the ocean as a sole source of infective vesivirus that can cause reportable vesicular lesions in domestic animals inside the US. Since many serotypes of VESV and SMSV are known to cause identical vesicular lesions in marine mammals, swine, sometimes horses and in one example cattle (Smith and Boyt, 1990), the results of this study supports the need for removing VESV from the FAD list. This study has correlated that vesiviruses of the VESV/SMSV classification are endemic in the USA. As a consequence, vesicular diseases of unknown aetiology should be routinely examined for vesiviruses. Such an approach would help evaluate the impact of vesiviruses on domestic animals including food animal populations. This is made all the more urgent because of the strong correlation between vesivirus infections and a Non A through E human hepatitis of unknown aetiology (Smith and Skilling, 2000).



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5 Chapter

Summary

Vesiviruses, a genus within the calicivirus family, are naturally transmitted common agents. This study is intended to evaluate the occurrence and distribution of vesiviruses in cattle and horses, two other livestock animals besides swine where vesiviruses were originally described in 1932. In swine they caused an epidemic vesicular disease (VES) indistinguishable from foot-and-mouth disease. Between 1932 and 1956, a total of 1,563 individual outbreaks of VES were recorded, caused by feeding infected pork scraps and offal to susceptible swine. After enforcing federal laws by 1953 requiring the cooking of all raw garbage used for feeding swine, the disease was contained and ultimately declared eradicated in 1959 in the USA. Since then, vesiviruses are not diagnosed routinely at US Animal Disease Diagnostic Laboratories, though they are known to infect an unusual broad range of different hosts like fish, reptiles, birds, primates and human, causing different and severe diseases like abortion, pneumonia, diarrhea, myocarditis and encephalitis.

To determine the distribution of vesiviruses in cattle for the first time in an extensive study, 693 sera from various areas of the USA were acquired and tested for vesivirus specific antibodies by ELISA in a blinded study. Sera were divided into distinct groups according to the animals health status: cattle with abortion problems (n = 208), cattle with respiratory diseases (n = 119), Bovine Leucosis Virus (BLV) -monitored cattle (n = 325) and a negative reference group (n = 41). These groups were divided further into subgroups according to various animal characteristics, e.g. beef and diary cattle, gender and geographic location. To assess the ELISA specificity, the pattern of antibodies against other common veterinary relevant agents, tested at another diagnostic lab with the same sera, were examined.

- No cross-reactivity of the ELISA to other common infectious agents of veterinary relevance was detected.
- When examining all available parameters of the animal case histories no general association to any specific disease manifestation could be found.
- BLV-surveyed cattle (20.6% positive) was the only group statistically associated to vesivirus infection compared to the reference group (4.9% positive). For all four groups, different animal characteristics (beef or diary, male or female, geographic location) represented no risk factors for vesivirus infections.
- In individual herds, several clusters of highly positive results up to 80% were detected within the abortion- and BLV-surveyed groups.

- One bovine abort case was shown with an increase of the vesivirus antibody response upon comparison of acute and convalescent sera, indicating an association between vesivirus infection and abortion.
- It appeared to be unlikely for cattle to be seropositive against vesiviruses and one of the other commonly tested agents, suggesting that vesiviruses are transmitted independently.

A similar vesivirus seroprevalence in various geographic locations ranging from Alaska over the western continent of America to tropical and isolated Hawaii was determined, indicating that vesiviruses are endemic in the United States of America and probably all countries surrounding the Pacific Ocean. Considering the broad host range of vesiviruses and the test results in cattle, we cannot exclude a similar vesivirus prevalence for other animals and humans.

The second aim of this study was to search for natural transmission routes of vesiviruses besides the already known mechanism of feeding infected meat. An opportunity opened in 2001, when horse breeding farms in the USA began reporting an unusually high abortion rate and all tests for common abortogenic agents had failed. This stimulated nationwide investigations for uncommon and unknown aetiologic agents. Caliciviruses, and vesiviruses in particular, are known to cause abortion in swine and seals but are not routinely tested for or diagnosed. When the Laboratory for Calicivirus Studies at Oregon State University was asked for specific help in this investigation, it was entrusted to me under the guidance of Dr. Alvin W. Smith.

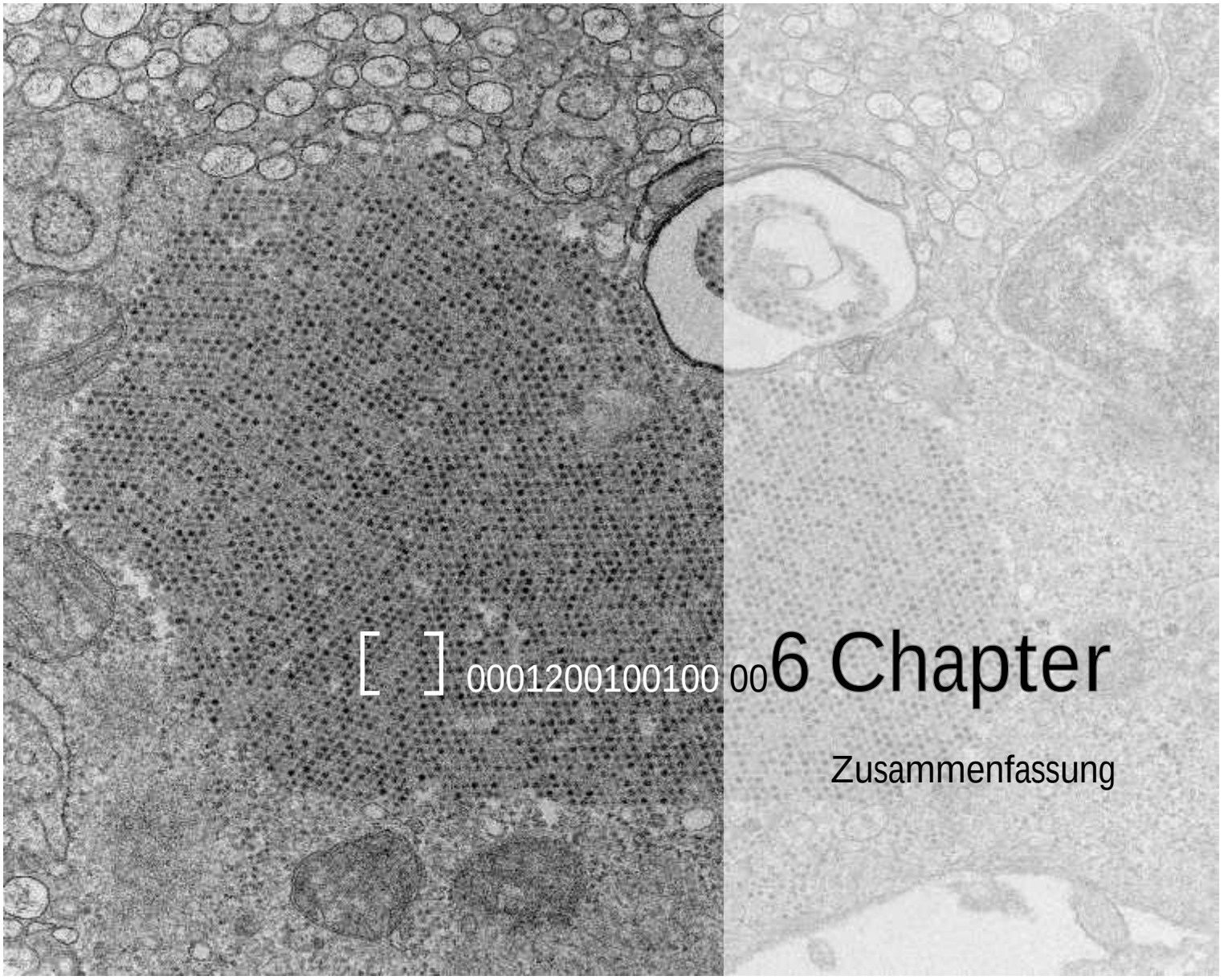
- In a first examination, 58 equine blood samples from mares that had aborted revealed 63.8% positive ELISA results while only 40% (10/25) of randomly sampled mares without health problems were positive indicating a statistically significant difference.
- Non of the male or juvenile females (n = 29) chosen as a negative reference group showed a seropositive result.

In light of this statistical result, further attempts were made to proof the hypothesis of vesiviruses being aetiologically associated to abortion in horses. In the meantime, a statistical link between a possible natural vector, the eastern tent caterpillar (ETC), and abortion had been determined. Three other sets of equine sera were tested by indirect ELISA for the presence of vesivirus antibodies. One set contained 28 paired (acute/convalescent) sera from mares with recent abortions. The other two panels

contained samples from mares being experimentally infected with the putative natural vector to proof the association between ingested ETC and abortion in mares. Furthermore, additional tissue samples from ETC and 20 aborted fetuses were acquired and have been tested for vesivirus antigen by fluorescent antibody testing (FA) and RT-PCR.

- Acute and convalescent sera from mares having recently aborted revealed a seroprevalence (71.4% and 60.7%, respectively) similar to the result in mares associated with abortion from study 1. This result further validates the data from the first study.
- Seropositivity of all collected pre- and post-experimental samples were between 40 and 90%. The increase of seropositivity occurred only in mares who aborted (from 47.1% to 88.2%), mainly in mares of those groups which were fed with ETC or their frass only. Mares without abortion showed no change of seropositivity against vesiviruses during the experiment (10/12, 83.3%).
- Three out of 5 mares, who showed a typical IgG-antibody increase (three-fold and higher) against vesiviruses, aborted during the experiment.
- No association could be detected between vesivirus infection and feeding ETC in any of the experiments. Therefore, ETC does not represent a vector for transmission of vesiviruses.
- When tissue samples from aborted fetuses were tested by FA using well characterized monoclonal antibodies, 12/20 turned out positive. However, because of the low sample quality of the fetus tissue due to repeated freeze-thawing, no specific morphological structures could be identified.
- The positive results of the FA test could not be confirmed by RT-PCR. Likewise, when tissue from ETC were tested by RT-PCR, no positive aetiologically linked results were achieved.

In conclusion, the natural seroprevalence of vesiviruses in horses rapidly increases with age. A very high vesivirus prevalence was proven in mares with abortions of unknown aetiology after naturally occurring infections, suggesting that vesiviruses should now be considered a highly probable cause of equine abortion. To confirm this conclusion experimentally infection studies of pregnant mares with vesiviruses should be performed. These serological data in horses are suggesting very effective unknown sources of infection and transmission routes for vesiviruses, and therefore a possible risk for other susceptible hosts such as swine, cattle and humans.



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6 Chapter

Zusammenfassung

Vesiviren, eine Genogruppe der *Caliciviridea*, sind bei Vertebraten häufig vorkommende, natürlich übertragene Erreger. In dieser Studie werden Vorkommen und Verbreitung von Vesiviren in Rindern und Pferden evaluiert, zwei weiteren Nutztieren neben Schweinen, wo Vesiviren bereits 1932 ursprünglich beschrieben wurden. Im Schwein verursachten Vesiviren ein epidemisches Vesikuläres Exanthem (VES), das klinisch nicht von der Maul- und Klauenseuche zu unterscheiden ist. Zwischen 1932 und 1956 wurden in den USA 1563 Ausbrüche von VESV dokumentiert, die durch das Verfüttern von infizierten Schweineabfällen auf andere Schweine übertragen wurden. Erst bei der Durchsetzung von Bundesgesetzen nach 1953, die das wirksame Desinfizieren von Tierabfällen für die Verwendung in Schweinefutter verlangten, konnte VES in den USA eingedämmt und 1959 als ausgerottet erklärt werden. Seitdem untersuchen die US Veterinäruntersuchungsämter Vesiviren nicht routinemäßig, obwohl sich diese Viren durch ein ungewöhnlich breites Wirtsspektrum in Fischen, Reptilien, Vögeln und Primaten inklusive dem Menschen auszeichnen. In diesen Spezies verursachen Vesiviren verschiedene zum Teil schwerwiegende Krankheiten wie Aborte, Pneumonie, Diarrhoe, Myokarditis und Enzephalitis.

In einer ersten ausgedehnten Studie wurde zunächst die Verbreitung von Vesiviren in Rindern ermittelt. Dazu wurden in einer Blindstudie mittels ELISA 693 Seren von unterschiedlichen Regionen der USA auf Vesivirus-spezifische Antikörper getestet. Die Seren wurden in Gruppen mit den folgenden Gesundheitsmerkmalen unterteilt: Rinder mit Aborten (n = 208), Rinder mit Respirationserkrankungen (n = 119), Bovine Leucosis Virus (BLV) –überwachte Rinder (n = 325) und die negative Referenzgruppe (n = 41). Diese Gruppen wurden weiter in Milch- und Fleischrinder, das jeweilige Geschlecht und die geographische Herkunft unterteilt. Um die Spezifität des ELISA zu beurteilen, wurden Muster von Antikörper-Reaktionen gegen andere weitverbreitete veterinärrelevante Erreger untersucht, die vorher in einem anderen diagnostischen Labor an den selben Seren ermittelt worden waren. Dabei ergab sich:

- Eine Kreuzreaktivität des ELISA mit anderen weitverbreiteten veterinärrelevanten Erregern wurde nicht festgestellt.

- Bei der Bewertung der verfügbaren Daten aller Anamnesen konnte allerdings noch keine Assoziation von ELISA-positiven Seren mit einer spezifischen Krankheit ermittelt werden.
- BLV-überwachte Rinder waren 20.6% seropositiv und damit die einzige Gruppe, für die eine Vesivirus-Infektion statistisch mit der Referenzgruppe (4.9% positiv) assoziiert werden konnte. In allen vier Gruppen representierten die unterschiedlichen Tiercharakteristika (Milch- oder Fleischrind, männlich oder weiblich, geographischen Herkunft) keine Risikofaktoren für eine Vesivirus-Infektion.
- Innerhalb einzelner Herden der Abort-Gruppe und der BLV-überwachten Gruppe wurden bis zu 80% Vesivirus-positive Tiere entdeckt.
- Am Beispiel eines Abort-Rindes wurde durch den beobachteten Anstieg Vesivirus-spezifischer IgG-Antikörper im Konvaleszenzserum ein Zusammenhang zwischen Vesivirus-Infektion und Abort aufgezeigt.
- Infektionen durch Vesiviren und anderer veterinärrelevanter Erreger sind statistisch nicht assoziiert, was auf die unabhängige Übertragung von Vesiviren hindeutet.

Für verschiedene geographische Regionen der USA einschließlich Alaska und Hawaii wurden sehr ähnliche Vesivirus-Seroprävalenzen ermittelt. Solche Werte lassen den Schluss zu, dass Vesiviren in den USA und wahrscheinlich auch in anderen Ländern, die den pazifischen Ozean umschließen, endemisch sind. Wenn man das breite Wirtsspektrum von Vesiviren bedenkt, ist eine ähnliche Vesivirus-Prävalenz in anderen Tierarten und in Menschen nicht auszuschließen.

Im zweiten Teil dieser Arbeit wurde untersucht, ob es neben der beim Schwein bekannten Übertragung durch Verfüttern von infektiösen Fleisch andere, natürliche Übertragungswege von Vesiviren gibt. Die Möglichkeit dazu ergab sich 2001, als in Pferdezuchtfarmen der USA eine ungewöhnliche Häufung von Aborten auftrat und alle Untersuchungen auf bekannte abortauslösende Erreger kein positives Ergebnis ergaben. Die Situation stimulierte die bundesweite Suche nach neuen, ungewöhnlichen und unbekannten ätiologischen Erregern. Da Caliciviren, und Vesiviren im besonderen, als Abort-Ursache bei Schwein und Robbe bekannt, aber bis dahin nicht

im Routine-Erregerspektrum getestet wurden, lag es nahe, das „Laboratory for Calicivirus Studies" an der Oregon State University um seine Expertise zu befragen. Unter der Leitung von Dr. Alvin W. Smith wurde mir diese Aufgabe anvertraut.

- In einer orientierenden Untersuchung von 58 Seren aus Stuten mit Abort-Anamnese erwiesen sich 63,8% Vesivirus-seropositiv. Dagegen waren Stuten ohne Gesundheitsproblemen aus einer Zufall-Stichprobe nur zu 40% (10/25) positiv. Dieser Unterschied war statistisch signifikant.
- Keine Probe der negativen Referenzgruppe von 29 männlichen oder weiblichen juvenilen Pferden enthielt Vesivirus-spezifische Antikörper.

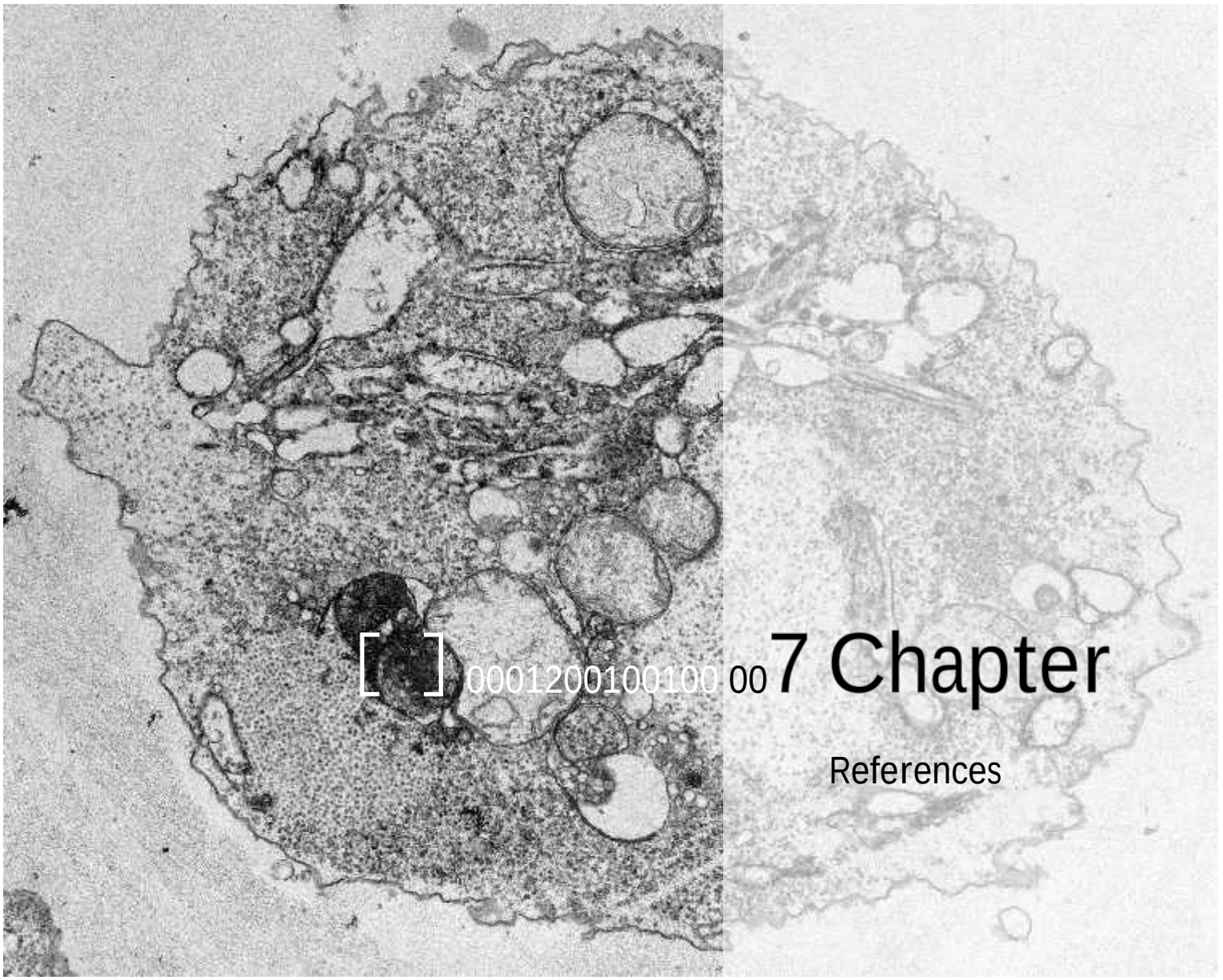
Nach diesem Ergebnis wurden weitere Versuche zur Sicherung der Hypothese von Vesiviren als ätiologischer Faktor für das Auftreten von Aborten in Pferden unternommen. In der Zwischenzeit war ein statistischer Zusammenhang zwischen der Raupe eines Falters (Eastern Tent Caterpillar (ETC)) als ein möglicher natürlicher Vektor und dem Abortgeschehen beschrieben worden. Drei weitere Gruppen von Pferdeseren wurden mittels des indirekten ELISA auf das Vorhandensein von Vesivirus-Antikörpern untersucht. Die erste Gruppe umfasste 28 gepaarte Seren (akut/konvaleszent) von Stuten mit kürzlichem Abort. Die zwei anderen Gruppen beinhalteten Seren von Stuten, die experimentell mit ETC, dem möglichen natürlichen Vektor infiziert worden waren, um der möglichen Assoziation zwischen aufgenommenen ETC und dem Stuten-Abort nachzugehen. Schließlich wurden auch Biopsieproben von ETC und von 20 Aborten im Fluoreszent-Antikörpertest (FA) und RT-PCR auf das Vorhandensein von Vesivirus-Antigen getestet.

- Die Seren abortierender Stuten und von Stuten nach Abort zeigten wieder eine hohe Seroprävalenz (71.4% und 60.7%), ähnlich wie schon die erste orientierende Studie 1. Dieses Resultat bestätigte also die Daten von Studie 1.
- Die Vesivirus-Seroprävalenz beider prä- und post-experimentell gesammelter Seren lag zwischen 40 und 90%. Ein Anstieg der Seroprävalenz zeigte sich allerdings nur in Stuten mit Aborten (von 47.1% auf 88.2%); überwiegend bei Stuten, die mit ETC oder deren Fraß gefüttert waren. Stuten ohne Aborte zeigten

während des Experimentes keine Änderung der Seroprävalenz gegen Vesiviren (10/12, 83.3%).

- Drei von 5 Stuten mit einem Vesivirus-spezifischen IgG-Anstieg (drei-fach und höher) abortierten während des Experimentes.
- Jedoch konnte in beiden Experimenten keine Assoziation zwischen einer Vesivirus-Infektion und der Fütterung von ETC festgestellt werden. Damit waren ETC als Vektor für eine Vesivirus-Übertragung ausgeschlossen.
- Bei der Untersuchung von Biopsieproben von Aborten im FA unter Verwendung gutcharakterisierter Vesivirus-spezifischer monoklonaler Antikörper waren zunächst 12 von 20 positiv. Wegen der schlechten Erhaltung der Gewebeproben, die mehrfach gefroren und getaut waren, ließ sich das Fluoreszenssignal keiner spezifischen zellulären Struktur zuordnen.
- Die möglicherweise weiterführenden Ergebnisse aus der FA konnten durch eine Vesivirus-spezifische RT-PCR nicht bestätigt werden. Ebenso ergaben die Gewebe von ETC in der RT-PCR kein Vesivirus-spezifisches Signal.

Zusammenfassend kann festgestellt werden: Die Häufigkeit Vesivirus-spezifischer Antikörper erhöht sich mit dem Alter der Pferde. Stuten mit natürlichen, in der Ursache ungeklärten Aborten, zeigen eine hohe Vesivirus-Seroprävalenz. Aufgrund dieser Assoziation sollten Vesiviren als eine wahrscheinliche Ursache des Aborts bei Stuten angesehen werden. Durch weiterführende experimentelle Infektionsversuche von trächtigen Stuten mit Vesiviren sollten die Ergebnisse dieser Studie abgeklärt werden. Die serologischen Befunde bei Pferden lassen erkennen, dass es noch unbekannte Übertagungswege für Vesiviren gibt und dass damit auch ein mögliches Risiko für andere empfängliche Wirte wie Schweine, Rinder und Menschen vorhanden ist.



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