DISSERTATION

INVESTIGATION INTO DISEASE EVENTS AT THE WILDLIFE/LIVESTOCK INTERFACE: LESSONS LEARNED FROM BOVINE VIRAL DIARRHEA VIRUS IN COLORADO CERVIDS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY COLLEEN DUNCAN ENTITLED 'INVESTIGATION INTO DISEASE EVENTS AT THE WILDLIFE/LIVESTOCK INTERFACE: LESSONS LEARNED FROM BOVINE VIRAL DIARRHEA VIRUS IN COLORADO CERVIDS' BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

INVESTIGATION INTO DISEASE EVENTS AT THE WILDLIFE/LIVESTOCK INTERFACE: LESSONS LEARNED FROM BOVINE VIRAL DIARRHEA VIRUS IN COLORADO CERVIDS

The study of infectious disease in free-ranging, wild animals is important on a number of levels. Disease can serve as a regulating factor for population distribution and abundance; as such it is necessary to understand the regional epidemiology so that significant changes may be identified where necessary. Infectious agents may also be transmitted between wild and domestic animals, or to humans; these events can have significant economic or public health consequences. For these reasons, insight into tools and techniques with which to study distribution and determinants of disease in wild species is essential.

Identification of, and investigation into, important health related events requires appropriate preparedness. Principles of wildlife disease surveillance were reviewed and it was concluded that although investigation into disease events may require unique logistical adaptations, basic principles of surveillance remain the same. A review of sources of information that may contribute to an opportunistic surveillance in the Rocky Mountain Region of the United States of America revealed that information collected, and shared, is dependent on the group involved and that there are opportunities to improve the type and quality of data available for evaluation. When information was deemed significant, reports of health events tended to aggregate at the level of the state wildlife agency; as such these groups need the training and resources necessary to follow-up on the report.

But wild animals are not always the source of the problem; infectious agents may be transmitted from domestic animals into the free-ranging population. Bovine viral diarrhea virus (BVDV) is an important virus of domestic cattle that has recently been identified in wild ruminants worldwide. To investigate the presence, prevalence, distribution and significance of BVDV in wild cervids of Colorado a series of projects were conducted. Persistently infected deer were studied post mortem; immunohistochemical (IHC) and molecular laboratory techniques used to look for viral antigen in deer tissue were found to be effective supporting the use of these tests in further studies. The prevalence and distribution of the virus in the state was estimated using an opportunistic sampling technique and IHC; a single persistently infected animal was identified suggesting the prevalence is extremely low, but that naturally occurring infection is present.

The cost associated with testing animals for an uncommon disease may be very high; techniques like pooling samples can help to keep costs down during such investigations. To evaluate the sensitivity and specificity of RT-PCR on pooled earnotch supernatant an experimental study was conducted, results showed that a supernatant from a single PI deer skin sample may be diluted up to 10,000 times and still be detected, however follow up work is needed to narrow the confidence interval on the sensitivity of this assay. Another technique to focus research efforts on high risk areas is the use of simulation modeling. A stochastic risk assessment model was developed to identify regions in Colorado where PI cattle were likely to be born following exposure to a PI deer. Results of the model were consistent with both the cross-sectional survey for persistently infected cervids and other published reports on BVDV in wildlife of Colorado; these finding suggest that simulation modeling may be used as an effective technique for directing research or control programs when resources are limited.

Through the study of BVDV in free-ranging cervids, a number of knowledge gaps were identified that need to be addressed in order to most effectively investigate disease in new species. For new

diseases, often little is known about pathogenesis and disease manifestation in new species; this clinical picture is an important part of the identification of a health event by many agencies and can dictate the sharing of information. Diagnostic testing modalities designed for use in one species may not be effectively used in another; therefore, whenever possible diagnostic test validation should be conducted prior to the use of this test in a population level project. Modification of testing protocols, including pooling of samples, can aid in cost-effectiveness. Disease modeling may be an effective tool to direct research efforts, however in many cases insufficient data of appropriate quality is available for inclusion in simulation studies. There are many examples where the role of wild animals in the epidemiology of infectious disease is significant and this is unlikely to change as opportunities for disease transmission between wild and domestic animals increase. For this reason an effective framework and foundation for the investigation of health related events in wild animals is essential.

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Chapter 1: Introduction

Centuries of research by veterinarians and others have provided immense information on infectious animal disease. Our understanding of disease causation has evolved from wayward spirits to microorganisms as astute researchers learn from their own, and others', successes and failures. Although our depth of knowledge and ability to specialize is unquestionably commendable, it could be argued that it is equally important to maintain a broad perspective. The ability to consider variables beyond just the affected host and single agent remains critical in the study of infectious diseases today; unfortunately this type of research often presents new logistical challenges.

Epidemiology is most literally defined as 'the study of that which falls upon the population'. While scholars have chosen to modify this translation to varying degrees, the beauty of this simple definition is its breadth of application. 'That' could be a syndrome or condition of any definition and needn't be restricted to a single etiology. Population is loosely defined and may include individuals who share any common feature or risk factor irrespective of location, age, behavior or even species. This flexibility is extremely important as, with increasing frequency, we are identifying aspects of disease that transcend species or traditionally defined field of study boundaries such as veterinary vs. human medicine or small vs. large animal practice.

More recently such conditions are often referred to as 'interface' diseases; in veterinary medicine this term is used to describe diseases that affect different types of animals that have some degree of interaction such as the wildlife/livestock interface. Diseases may

behave differently when multiple species are involved; thus investigators must keep a broad perspective and include in their analysis information that may affect all species involved.

The aim of this dissertation is to examination issues related to the investigation of disease issues involving the wildlife/livestock interface and the spread of infectious animal diseases using bovine viral diarrhea virus (BVDV) as a model. Specific aims include investigation into techniques and tools for disease surveillance in non-domestic species, prevalence estimates for BVDV in cervids, pathogenesis of infection in cervids and tools for disease diagnosis and finally, simulation modeling to estimate the significance of this virus in a free-ranging population and identify future work that needs to be done.

BVDV is a pestivirus of the family Flaviviridae. Two distinct genotypes of BVDV, type 1 and type 2, have been identified with further subclassifications based on genetic variation.[1,2] There are two biotypes, non-cytopathic (NCP) and cytopathic (CP), of BVDV based on the effect of the virus on cells in tissue culture. The virus is distributed worldwide[3,4] and results in significant economic losses to the both beef and dairy industries.[5,6] The virus has been identified in free ranging and captive non-bovid animals however it is unknown if this is the result of spillover from cattle, or if the virus can be maintained in wildlife; the latter scenario could complicate control programs that focus only on livestock.

The clinical spectrum of BVDV associated disease in cattle is broad and dependent on both host and viral characteristics. The virus is highly contagious and natural transmission occurs horizontally and vertically. Important variables influencing the outcome of exposure include host immunity, pregnancy status, transplacental infection and age of the fetus and environmental stressors. Immunocompetent, non-pregnant cattle develop a variety of disease conditions, from sub-clinical infection to diarrhea, thrombocytopenic and hemorrhagic syndrome, and immunosuppression.[2,7]

Pregnant animals exposed to the virus can experience reproductive failure or reduced performance before breeding up to approximately 45 days of gestation. Fetal infection occurs following this period. Infection of a pregnant animal between approximately 45-125 days of gestation may result in several different outcomes including fetal death and/or congenital abnormalities. One possible outcome is persistent infection (PI). PI occurs when a fetus is exposed to a non-cytopathic strain and develops immunotolerance specific to that BVDV strain. The result of this immunotolerance is that the fetus will be unable to clear the virus and therefore becomes PI.[8] Calves born PI may be weak or small but others are clinically normal.[8,9] Fetuses exposed later in gestation (approximately 125-170 days) can have congenital defects, are aborted or appear normal at birth. After approximately 150 days, the fetus has a fully competent immune system, eliminates the virus and is born with viral antibodies.

The role of PI cattle is well-documented in literature and PI animals are the primary source of new infections within a cattle population.[3] Persistently infected cattle shed

more virus into their environment than acutely-infected individuals and provide constant challenge doses to contact animals.[8,10] As such, PI individuals are central to the maintenance of BVDV in cattle populations.

Diagnostic tests used for BVDV include antigen detection, antibodies, virus isolation and PCR. The direct antigen detection methods include immunofluorsecence, ELISA and immunohistochemistry. These tests can be conducted on both tissues and peripheral blood and their application depends largely on the objective of the testing regime. The application of antigen detection tests are useful for the diagnosis of acute or persistent infection and differentiating between the two can be challenging. Although definitive proof of persistent infection requires isolation of virus over time, both IHC and AC-ELISA have been shown to be both sensitive and specific tests for the identification of PI animals.[11,12]

The most commonly employed antibody tests are virus neutralization and ELISAs and there are a number of commercially available tests. The problem with antibody detection is that routine vaccination of cattle has resulted in high titers, and a positive result must be interpreted in context of vaccination history or in conjunction with a convalescent serum sample. Persistently infected cattle do not develop antibodies against BVDV.

A number of PCR assays have been designed for the diagnosis and characterization of BVDV; given the variability in the nucleotide sequencing, selection of target sequences is important.[13] PCR can be used on multiple samples including tissue homogenates[14] and

can be used to diagnose both acute infections and persistent infections. Similarly, virus isolation can be used to diagnose acute and persistent infection although repeat testing is necessary to confirm the latter.

This dissertation is organized into eight chapters. The majority of the work is presented as independent projects that represent individual papers already submitted to peer reviewed journals; these manuscripts have been modified only slightly for inclusion here and changes incurred are summarized as follows. Chapters 2 and 3 are a review of general disease surveillance principles as they may apply to free ranging wildlife and an evaluation of how wildlife health information that may be used in surveillance is shared within the Rocky Mountain Region of the United States of America. These chapters will be submitted to or published in Animal Health Research Reviews[a] and the Journal of Transboundary and Emerging Disease[15] respectively. Modifications of the original works are only related to formatting.

Chapter 4 is describes the pathology and distribution of bovine viral diarrhea (BVDV) antigen in tissues of experimentally induced, PI white tailed deer fawns. Information gleaned from this experiment provided information on diagnostic testing modalities that serve as the basis of subsequent chapters. Chapter 4 was published in the Journal of Veterinary Diagnostic Investigation[16] and has been slightly elaborated upon, and reformatted, for inclusion in this dissertation. Chapter 5 is a cross-sectional survey for BVDV in wild cervids of Colorado in attempt to estimate the prevalence of persistent infection of cervids in the state; this chapter has been published in the Journal of

[a] http://journals.cambridge.org/action/displayJournal?jid=AHR

Veterinary Diagnostic Investigation.[17] For this dissertation the manuscript has been reformatted and only slightly modified. Chapter 6 provides information on the use of pooled samples for the identification of BVDV infection in a population of cervids; at the time of writing this manuscript has not been submitted for publication

Chapter 7 uses information gleaned from previous chapters, along with published information to estimate the risk of BVDV transmission from wild cervids to domestic livestock in the state of Colorado and identify locations where surveillance efforts are best focused should further disease transmission be deemed a concern of producers or regulatory agencies. This research is written for submission to the Journal of Transboundary and Emerging Disease. Finally, chapters 1 and 8 are introductory and concluding chapters respectively; these sections will serve to synthesize the information presented in the remainder of the dissertation.

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Chapter 2: Wildlife Disease Surveillance: Review of principles and suggested implementation steps

Introduction

Wild animals are an important part of ecosystems worldwide; they represent most of the biological diversity within a system and are therefore a key stabilizing feature.[1] Animals provide nutrition and enrich the economy for humans through many means and they have abundant cultural, social and even religious connotations in all societies.[1] Despite these important roles, however, much remains unknown about wild animal populations including information on conditions that may impact the individual or population health of the animals themselves or represent a risk to humans or domestic animals. In recent years, the rapidly increasing human population has resulted in new demands on animal health programs and re-emphasized the importance of veterinarians in non-traditional roles including wildlife, ecosystem health and population level medicine.[2] Individuals and programs working in these fields need information on the epidemiology of disease such that appropriate actions can be taken where necessary; such information is often obtained through disease surveillance programs. The following is a review of key features of disease surveillance programs as they pertain to wild animal populations.

Disease surveillance

A large body of human and veterinary literature pertains to surveillance for disease. Although there exists no single, uniform definition of disease surveillance it does, in general, include the systematic collection of health related data for analysis, interpretation

and action to improve overall health; lists of published definitions are reported elsewhere.[3,4] Disease surveillance is distinguished from disease monitoring by the initiation of directed action based on results of the surveillance system. Due to the obvious overlap between surveillance and monitoring programs and the fact that monitoring is an inherent part of surveillance, the term 'monitoring and surveillance system' (MOSS) has been proposed,[4-6] widely adopted and will be used in this paper.

The objectives of surveillance programs are variable and influenced predominantly by the individuals conducting and using the system; however core principles of disease surveillance systems are similar irrespective of the agent or host of interest. In domestic animals MOS systems are most often implemented to facilitate trade of animals or animal products,[7] and to design and evaluate disease control programs.[6] Disease control programs traditionally focus on specific production limiting and economically significant diseases or those with potential for a substantial public health impact; mandates of programs range from prevention to control to eradication or demonstration of freedom from disease. In recent years there has been a recognized need for surveillance programs to identify emerging diseases defined as the expansion of a known pathogen to new host species and/or geographic range, or recent identification of a new infectious agent. New or emerging diseases are most commonly infectious, and introductions may be accidental or deliberate including biological or agroterrorism.

Surveillance systems are often sub classified based on the objectives of the system which dictate the method of collection and nature of data. A commonly used differentiation is

the intensity of effort of MOSS data acquisition, delineating 'active' from 'passive' surveillance. Passive surveillance implies that health related data collected for other, routine usage, such as diagnostic laboratory results or production data, is then used within a MOSS. Passive surveillance systems are inexpensive but often biased by sources of data and tend to underestimate the prevalence of disease.[4] Identification of cases within a passive system is influenced by the willingness of individuals to participate, awareness or detection pressure of for the disease, clinical manifestation and fatality rates, knowledge and education, and the availability of a diagnostic laboratory to support or confirm cases. Overall, such systems are inconsistent and it is difficult to compare the results of two passive systems. Passive surveillance systems do not improve the time to detection of a disease or important health event.

In contrast, active surveillance implies that a data collection scheme is predesigned specifically to meet the objectives of the MOSS. Appropriately designed active surveillance programs can provide unbiased prevalence estimates and can reduce the time to detection of disease. Active data collection systems are significantly more expensive to implement and maintain than passive systems.[4]

Many MOS systems involve both active and passive components. Given that the key feature differentiating surveillance from monitoring is the action taken following analysis of data collected within the surveillance activity, use of the term 'passive surveillance' system is an inherent contradiction.[4] For this reason, surveillance systems included in this paper will all be considered active even if they use 'passive' forms of data collection.

'Sentinel surveillance' refers to the testing of a select group of animals within a specific species, geographic location, production system or as representative of other animals that share similar risk factors (target population). Sentinel surveillance systems may be advantageous when costs or logistics of testing the target population are high. The value of using of sentinel animals is dependent on the knowledge and understanding of the relationship between the sentinel and target population as this knowledge dictates the interpretation and extrapolation of surveillance data.[4] When West Nile Virus emerged in North America, numerous avian species served as sentinels for the presence of virus and subsequent human risk within an area.[8,9]

The term 'targeted surveillance' most commonly refers to programs in which data collection efforts are focused on a specific group of individuals that possess attributes that increase their risk of infection and therefore disease detection efficiency is increased. The most significant limitation of targeted surveillance is that results obtained cannot be extrapolated beyond the target population without extensive knowledge of the differences between targeted individuals and the non-targeted individuals. For example, within some North American national parks, wild animals are reservoirs for diseases of cattle that have been eradicated from domestic animals. Surveillance efforts for diseases like brucellosis and tuberculosis can therefore be targeted toward animals in contact with these wild reservoirs.[10-13]

Based on the focused nature of the data collection, the term targeted surveillance has been suggested as a replacement for the commonly used and previously mentioned 'active surveillance.[3] Given the aforementioned limitations of defining surveillance systems based on intensity of effort and that all MOSS are targeted to some degree given the objectives of the system, the term 'targeted surveillance' offers little methodological information and is therefore of minimal value. Likewise, the converse term 'general disease surveillance', used to imply the collection of data from a broad range of individuals without focus on a specific demographic or agent, offers limited information to the user of the system; if the program has clearly defined objectives and data directed actions then it remains, at minimum, a MOSS regardless of its breadth.

The terms targeted and sentinel surveillance do overlap. Both systems rely on a risk based selection of the test population in attempt to optimize detection of an event of interest within the population of interest. Likewise the term 'risk-based surveillance' has been used and may impart more information to the user of the information than either 'targeted' or 'sentinel'.

'Syndromic surveillance' refers to the analysis of data regarding the occurrence of a pre-defined syndrome characterized by specific clinical symptoms or observations. The use of such systems is advocated for early detection of an event, focusing on the period of time between onset of illness and laboratory confirmation. Syndromic surveillance programs rely heavily on spatio-temporal patterns to detect clusters in attempt to differentiate syndromes from baseline. Syndromic surveillance has been used in

companion animal practice[14] as well as integrated human and veterinary data.[15] Recent advances in syndromic surveillance stem from the public health sector and are motivated by bioterrorism concerns.[16] Improvements in the integration and analysis of large human health datasets, including automation,[17] have made syndromic systems more practical for MOSS[18] and reviews of various syndromic surveillance programs have shown them to be very effective at detecting public health events if well designed.[19] Limitations to syndromic surveillance include difficulties in assessing how well the captured syndromes correlate with the target diseases, low positive predictive values and difficulties implementing these systems when few people are familiar with the concept.[20] The system will not detect new diseases that don't have a defined syndrome or rare events; in mathematical models simulating an outbreak of inhalational anthrax in people the timeliness and sensitivity of the system declined as the number of affected individuals decreased below 10,000.[21] Given the inherent lack of specificity of such systems they should not serve as a replacement of other more directed surveillance programs.[20]

Other modifiers are used for various MOSS programs; for example 'serosurveillance' refers to the use of serological techniques for the identification of exposure in the tested animal or population, 'slaughter surveillance' refers to systems utilizing animals at an abattoir and 'practice surveillance' refers to the use of animals presenting to clinical veterinarians. Other proposed classification schemes include the type of disease (exotic or endemic), the number of diseases, spatial scale of surveillance, characteristics of the monitored population or sampling strategy (sample based vs. exhaustive).[22] The use of such modifiers can be useful to remind users of the information about the source of the

data; however the principles behind the design and execution of the program will be consistent with other MOSS.

Surveillance for disease in wild animal populations

As with domestic animals, motivation for disease surveillance in wild animals is variable. Objectives are influenced by the rationale for the project, diseases under investigation, available resources, system users and scope of the MOSS. The value of wildlife comes from economic, nutritional, ecological and socio-culturally significant roles[1]; disease surveillance is therefore often motivated by species conservation to maintain healthy animal populations.[23]

Human or public health initiatives also provide incentive for ongoing MOSS in wild animals. Wild animals are frequently identified as reservoirs for infectious diseases of substantial public health significance.[23-25] In an international review of human pathogens, 62% reportedly had a primary animal reservoir and 75% of emerging human diseases were classified as zoonotic.[26] Wild animals are commonly involved in the epidemiology of these diseases[27] and therefore, in recent years, the role of wildlife in the epidemiology of zoonotic pathogens has received increased attention. Ecologic changes have altered both the natural environment of wildlife hosts and vectors involved in the lifecycle of some diseases. The expansion of people into previously undeveloped areas has increased the probability of contact between humans, wild species and some vectors.[28,29] Unfortunately there remain large gaps in our understanding of the epidemiology of these interspecies diseases within wild animal populations.

Wild animals can be reservoirs for diseases with significant trade impacts; an important issue of national and international interest.[30] Foreign animal disease preparedness should take into account wild animals as they can perpetuate and spread disease, prolong trade restrictions, and complicate eradication and control, all resulting in magnified impact.[31]

It is inherently more difficult to conduct disease surveillance in wild animals.[30] Basic population parameters used in epidemiology such as population size, population structure, density and general health status is often unknown for wildlife. Likewise, wild animals are not geographically restricted like domestic animals and can move around or migrate making conventionally used analysis techniques more challenging. Collecting data on wild animals can be logistically difficult, very expensive and often negatively impact the health of the animals through trap related stress, morbidity or mortality. Finally diagnostic modalities used in animals are rarely tested or validated in wild animals and interpretation of findings can be challenging.

Given the difficulties of procuring sufficient wild animal samples to meet the objectives of a MOSS when survey techniques are used, it is important to be aware of, and employ when necessary, other surveillance tools such as scenario trees and simulation modeling, expert opinions and Bayesian techniques.[4] Modeling techniques have been used to design and evaluate various disease detection techniques in wildlife such as FMD in feral pigs.[32] This study used simulation modeling to compare the number of FMD cases that would occur before the disease would be identified using opportunistic vs structured surveillance techniques. Simulation modeling is commonly employed in the case of

foreign animal diseases where there is a paucity of data in many disease free regions. As such, extrapolation of information gained in other areas to the regional animal population is necessary to make educated decisions regarding disease management policies. Likewise, in the case of wild animals, often insufficient information is available for the region in question and integration of information gained from other locations or species can be incorporated into a model to yield meaningful regional information.

Components of an effective MOSS

Designing a surveillance system requires abundant forethought pertaining to the objectives, data collection, data analysis and interpretation, reporting of results and directed action. Appropriate planning is essential to the implementation of an effective MOSS. The most important step is defining the objectives and the scope of the program. Proposed objective categories include surveillance for foreign animal diseases or emerging diseases and surveillance for endemic diseases[33] along with surveillance for specific risk-factors[6]; however, more important than categorization of the objectives are that the appropriate questions have been asked.[4] Important considerations are often peripheral to the disease itself and such as economics, politics, public health impact, cultural and social factors.

During design of the MOSS, objectives need to be agreed upon by all parties involved in the specific system. If agencies do not subscribe to the mandate of the program they will lack the intrinsic motivation to participate and, if they do participate, data may be of questionable quality.[31] Education can increase compliance; as individuals learn of the importance of the surveillance effort they will be more inclined to contribute.[34] Other

important considerations for enlisting participants into the program are the time commitment involved, confidentiality, or use of information. These factors must be thought of in advance. A MOSS will only be effective as long as participating individuals and organizations contribute to the system as outlined during system design and communication remains open among all parties involved. International solidarity and cooperation was one of the most important factors of a successful emerging bacterial zoonoses surveillance program.[35]

Some general guidelines can assist in the development of disease surveillance programs.[36] It is important to be practical and realistic with respect to human and financial resources. Where possible existing programs should not be re-duplicated; instead cooperative agreements should be sought.[34] In a review of outbreak detection networks it was concluded that outbreaks are identified by a number of different groups in a number of different ways and thus a surveillance system must incorporate various groups and levels.[37] For free-ranging species these contributing individuals and agencies may differ from those involved in domestic animal surveillance, extending well beyond the veterinary community to include other groups that may identify morbidity and mortality.

Data collection

Findings of a surveillance system are only as good as the data that they are based on; for this reason it is imperative that the data collected is appropriate for the desired analysis. Design of the MOSS must involve the development of an appropriate target population and case definition; only after these have been established one can identify data sources

and sampling strategies. Strong working knowledge about the agent, potential and known hosts and regional environment is necessary to establish criteria for inclusion. Accuracy and reliability for the entire data collection system involves understanding of limitations of all aspects of data collection such as diagnostic testing, animal sampling and reporting.

Defining the target population depends on the objectives of the system but must also take into account all susceptible animals including species that may serve as reservoirs for future re-introductions. Within the population of interest, clinical manifestation of disease or subclinical infection and any diagnostic tests must be well understood with respect to the pathogenesis of infection in that host. Ideally, diagnostic tests would be inexpensive, accurate and provide results quickly; in reality selection of a diagnostic test must optimize parameters pertinent to the objectives, for example a highly sensitive test would be chosen if identification of all positives was important and the cost of false positives is low while a highly specific test is warranted when it is important to minimize false positives. Other epidemiological characteristics such as modes of transmission, factors influencing population incidence, prevalence, morbidity and mortality and persistence of the agent within the environment must also be considered.

Once the target population has been defined a sampling strategy must be designed. Development of a sampling protocol is influenced by the event of interest, expected prevalence and available diagnostic tests. As previously, mentioned data collection methods may be active or passive and the decision to use each method depends on

objectives, resources and acceptable limitations. There are many animal health data sources available for use in MOSS that have been described elsewhere.[6] Compliance will be enhanced if those involved in data collection subscribe to the mandate of the surveillance system; thus, in the case of wildlife health a multidisciplinary team of biologists, ecologists, microbiologists and veterinarians should work together.

Information or materials collected may include information such as results of diagnostic testing, population structure data or tissues for testing in conjunction with information related to the samples in question. Ideally information pertaining to the host, agent and environment would be available including species, location, tests used, population at risk, type of production system and laboratory information.[38] The degree of resolution within the dataset is important as the data must be the right type to meet the objectives. In a retrospective review of skunk rabies in Texas, available information changed during the study period; early data often lacked details necessary to accurately evaluate spatial distribution, prevalence and risk.[39]

Sources of wildlife disease information

Opportunistic, post-mortem examination of dead animals identified by biologists, field personnel, outdoorsmen, naturalists and the public is a mainstay of wildlife disease surveillance.[30,34] This is an inexpensive means to gain insight into specific causes of mortality, however it is largely biased towards large, charismatic, easily identifiable species[40] and of limited epidemiologic value.[30] It is logistically difficult to count and recover sick and dead wild animals as has been exemplified in experimental studies

evaluating carcass recovery and mortality estimates.[41] Mass mortality events are likely to be detected and reported by biologists or the public; however these events represent only a fraction of mortality in wild animals.[30] Given the difficulty of identifying health related events in small and inconspicuous species, systematic trapping or other techniques would need to be employed.[30]

The use of opportunistic systems can be optimized by collection of appropriate information at the time of reporting including information related to the number of animals affected, location, environmental conditions, and, when possible, collection of carcasses and submission to the appropriate diagnostic facility.[34] Information reporting and submissions to diagnostic laboratories are increased when infrastructure facilitating delivery of the animal or tissue is optimized. The Canadian Cooperative Wildlife Health Center (CCWHC) maintains a toll free phone number for individuals to report mortality or possible disease events in wild animals; difficulty getting animals to the diagnostic laboratory reduces submissions.[34]

A review of potential sources of wildlife health data was conducted on Vancouver Island, British Columbia, Canada; in this area wildlife rehabilitators were considered to be an important resource as they encountered the largest number of animals and the greatest variety of taxa over the largest geographic area.[40] While rehabilitators in this study were willing to share information and participate in disease surveillance programs, observed limitations of their involvement included the lack of, incompleteness of or unsearchable nature of health records, and lack of data standardization of important information such

as etiological, pathologic or clinical diagnosis as samples were infrequently sent to diagnostic laboratories or done in house. Examinations were most commonly done by volunteers and rarely with veterinary involvement. Young animals and certain species are over represented at wildlife rehabilitation facilities and these biases in the data would need to be considered prior to the use of such agencies in disease surveillance. Similar findings were made in a review of opportunistic wildlife disease surveillance in the Rocky Mountain region.[42]

Other individuals and organizations identified as having a potential role in wildlife disease surveillance included veterinarians, government agencies, municipal public works, road maintenance crews, animal control agencies, public health agencies, universities and wildlife trappers.[40,42] In British Columbia, wild animals examined under the jurisdiction of government agencies were more likely to involve the provincial wildlife veterinarian, have a clinical or pathologic diagnosis and have samples collected for ancillary diagnostics; however the number of animals seen by these agencies were relatively low, turnaround time was slow with cases taking almost 50 days from the date of submission to necropsy and all necropsies were conducted on previously frozen carcasses which restricts the use of some diagnostic tests.[40,42] In the Rocky Mountain region of the United States, state wildlife agencies were identified as an important point for the aggregation of wildlife health information from various sources.[42]

Veterinarians in British Columbia, Canada and the Rocky mountain region of the United states were surveyed and the majority reported that they had limited involvement with

wild animals and most re-directed cases to wildlife rehabilitators.[40,42] The remaining agencies in both studies kept few to no records pertaining to wildlife health events or were unwilling to share information and were therefore considered of little value for early detection of emerging disease. Those that did keep records and were willing to share them already collaborated with provincial or national agencies involved in wildlife disease surveillance. When surveyed regarding willingness to participate in a wildlife disease surveillance program less that 15% of the agencies were willing to; the most commonly listed reasons for not participating included limited funding, staff time and manpower, lack of interest, insufficient numbers of animals observed, concern for animal welfare, concern for who data might be shared with, lack of training in wildlife disease, lack of equipment or facilities and legal issues.[40]

Active data collection for surveillance of disease in wild animals is predominantly employed in large initiatives for diseases of regulatory interest like brucellosis or tuberculosis. As previously mentioned these programs are expensive to initiate and maintain. Indirect testing such as collecting fecal or environmental samples may be a less expensive form of data collection that minimizes animal handling and inherent problems associated with wild animal capture.

Wildlife disease diagnosis

Laboratory based diagnostic tests central in wildlife disease surveillance include histopathology, microbiology, serology, histochemistry and immunohistochemistry. Such tests are usually extrapolated from veterinary or human medicine with little or no evaluation of the test in wild animals. Also, many species lack reference ranges and

established 'normal' values, so results of such tests are often subjective; where possible material should be saved for the establishment of test standards and validation of diagnostic tests.[43] It has been reported that surveillance for specific agents (focused) is more logistically simple than examining material (or data) for evidence of something new.[40] Where disease surveillance is not focused on a single agent, rather morbidity and mortality events in general, diagnosticians need access to a broad array of diagnostic tests[34]. The reliability of the diagnosis and ability to identify new diseases are influenced by the training and education of the diagnostic personnel and the facilities at the diagnostic laboratories.[44]

Post-mortem examination has been cited as 'the single most critical step in diagnosis for general wild animal disease surveillance.[44] The reasons for this are many; first of all wild animals are difficult to capture and examine in a safe, thorough manner. These capture events are expensive and can be associated with considerable post-trapping morbidity and mortality within the target species. Additionally, some significant pathogens that can be harbored in wild animals are difficult to detect in live animals. For instance diagnosis of tuberculosis in wild animals is rarely based on clinical disease[43] and some wild mammals can harbor foot and mouth disease in the pharynx without showing easily identifiable lesions.[45] Post-mortem examination is not only important for determining the cause of death in an individual or a group of animals, but it is also a valuable source of information on variations in 'normal' within a species or for the identification of other underlying disease or physiologic changes.[46]

Infrastructure to get tissues or carcasses to a diagnostic laboratory is critical as it dictates the condition of the samples and thus the test results. For post mortem examination a carcass should be in good condition.[44] It is particularly difficult for developing countries to get diagnostic help if samples have to be shipped internationally, and need permits such as health and CITES; the OIE can provide some assistance in this area.[43] Ideally people trained specifically in wildlife pathology should conduct necropsies; however this is often logistically difficult and therefore biologists and field personnel should know how to do a good field post-mortem examination using consistent methodology and take correct samples.[46] Thorough descriptions and photographs will aid in the interpretation of field necropsy findings. Field kits including personal protective equipment, necropsy tools, sample collection materials and instructions should be provided for all personnel. Regular training on technique is necessary to minimize spread of pathogens and zoonoses.[46] Quick turnaround on disease diagnostics to provide feedback to submitters is an important part of a disease surveillance program.[34] Timeliness of reporting has been cited as a factor inhibiting effective avian influenza management in some countries.[47]

Following diagnosis or testing within a surveillance system the importance of maintaining tissue for reference collections have been reviewed.[43,46] These collections are important as reference ranges or normal controls as well as for use in retrospective investigations to look at similar species or at other individuals within the same geographic region when a new or interesting health related event is identified. Lack of tissue archives was cited as a problem when SARS emerged and there was no available material from Asian markets from which to look for the virus.[47] Reference collections

may be particularly important in threatened species where opportunities for future sampling are minimal. A complete archive should include fixed and frozen tissues, blood smears, sera, paraffin blocks, bacteriology and virology isolates.

Data management and analysis

The use of data differentiates surveillance from monitoring and therefore is it essential that information obtained within a MOSS be accessible and usable so that the objectives of the system are met. Data needs to be well indexed so that it can be searched and analyzed.[34] Computerization of records within widely available database management software provides the most flexible system. Ancillary case information such as photographs, slides, paraffin blocks, tissues can be filed in appropriate storage using identification numbers consistent with the computerized records. Data storage and processing can be challenging; use of companion animal health records from veterinary clinics resulted in greater than 4 terabytes of data that was difficult to manage.[14] Assistance of individuals trained in information technology and access to abundant storage and processing power can be crucial.

Unfortunately much of the current wildlife disease information is poorly centralized and relatively inaccessible. In Europe, computerization of disease records is variable and countries without search capacity may be unable to respond to requests for information.[44] Likewise wildlife rehabilitators, who were identified as a good source of data, most often lacked records of animals, had incomplete records or unsearchable records.[40,42]

Interpretation of surveillance data requires knowledge of the population at risk along with information that may impact the epidemiology of disease in an area. Information on species distribution and densities, movement patterns and behaviors, hunting and harvesting must be available in a central location. The data must remain up to date so that it can be utilized when needed for data analysis, designing management plans or control measures[31] Data on environmental conditions is critical when looking at wild animal diseases.[48] Concern is growing over the impact of anthropogenic changes and environmental disturbance on wild animal health. To elucidate these relationships, individuals involved in the collation and analysis of wildlife health information need to integrate information on environmental variables. Lack of access to quality data of sufficient resolution has been cited as a significant limitation to the investigation of wildlife population dynamics, disease and environmental factors.[49]

Risk analysis

Risk analysis techniques can be an effective tool for directing disease surveillance programs. In the World Trade Organization's (WTO) sanitary and phytosanitary measures agreement a scientific, evidence based risk assessment is central in the assessment trade regulation;[50] these same standards can be used to identify high risk areas so that limited resources can be allocated appropriately. Risk analysis techniques have been advocated as an important tool for focused disease control between deer and livestock in the UK.[51] Much of the literature on risk analysis in veterinary medicine pertains to the movement of animals,[52] however these guidelines can be useful to direct surveillance efforts as well. In order to evaluate risk of disease in wild animal

populations relevant information on the population at risk must be centralized as emphasized for foreign animal disease preparedness in Canada.[31]

Communication and use of surveillance information

The use of surveillance information is entirely dependent on the objectives of the MOSS and will not be discussed in detail here; however, regardless of the specific program objectives, communication of findings is an important part of all MOSS. Often the results of MOSS are used for directing policy, government regulation or other decisions. Decision makers are typically devoid of training in veterinary medicine, wildlife management or epidemiology. Findings must therefore be communicated in an effective manner without overwhelming technical language.[34]

Disconnect between individuals and organizations involved in wildlife disease surveillance was cited as a fundamental limitation to effective wildlife disease surveillance in the United Kingdom[33] and Canada.[40] In a review of wildlife disease surveillance in Europe, the degree of communication between field personnel and surveillance program coordinators was identified as a significant factor contributing to the frequency of case detection and submission; submitters needed to be well informed of the potential importance of wild animal disease, be involved in the process and receive reports or feedback in a timely fashion.[44] The CCWHC in Canada uses regular newsletters to communicate program information and current disease information.[34] Other effective media includes list servers, web pages and scientific literature.

Communication of surveillance findings to individuals and agencies peripheral to the immediate objectives is also important. Pathogen surveillance in humans, domestics and wildlife needs to be integrated.[47] Although the objectives of the wildlife disease surveillance program may focus on wildlife itself, communication with other agencies may result in collaboration and resources including funding, infrastructure, data access or expertise.

Evaluating MOSS

Following the design and implementation of a MOSS it is essential that the program be reviewed regularly to assess how well the objectives are being met. Standardized evaluation programs can assist the review process and facilitate the calculation of the system sensitivity, predictive values, flexibility, simplicity, representativeness, acceptability and timeliness of the system in general.[38,53-55]

Other considerations

Numerous logistical factors must be considered when implementing a MOSS. One of the most important aspects is a well defined, logical infrastructure so that information is not lost at various steps of the process. If existing infrastructure is in place it may be useful to use it as such relationships may be mutually beneficial. For some diseases, such as tuberculosis,[13] there may be a legal framework upon which MOSS can be implemented or regulated.

As previously mentioned, appropriate training and education of individuals involved in the MOSS is important to ensure standardized data collection, analysis and interpretation

and appropriate actions. Training should occur at all levels of the MOSS including epidemiologists, decision makers, field personnel and laboratory staff.[4]

Funding

As previously mentioned, different data collection methods have different associated costs. Surveillance activities that rely on testing done under another mandate are inexpensive relative to a MOSS that requires collection of data to meet the specific objectives of the system. When opportunistic data collection schemes were employed, cost was reported to be a key limiting factor for the involvement of wildlife organizations of British Columbia, Canada to participate in disease surveillance.[40] Likewise when fees are charged for wildlife submitted for post-mortem examination the number of submissions were reduced both in Canada[34] and Europe.[44] When fees are charged for examination of a carcass the surveillance program becomes more biased toward specific species and associated disease conditions that are of immediate concern. Active data collection is usually more expensive but has more extensive applications that can be justified for some diseases or species.

Funding for the program needs to be sufficient to meet the objectives of the MOSS. The source of money will vary by program but will likely be the responsibility of the coordinating agency. Collaboration between public and private sectors may facilitate surveillance programs where a regulatory agency may be unable to bear all of the costs[4]; however the objectives of the MOSS needs to be in the interest of the private sector or producers in order for such collaborations to be effective. Motivation for collaboration of producers or private industry includes improved production, increased trade

opportunities, education, disease prevention and disease control products (vaccination, treatments etc). Funding also needs to be available to cover losses incurred as a result of findings of a surveillance system; failure to design appropriate compensation measures will result in noncompliance and under reporting of the event of interest.

Conclusions

Tools and techniques employed in wildlife disease surveillance can be improved. Investigation of disease in wild animals presents new challenges given the inherent difficulties of studying wildlife relative to domestic animals; therefore, alternative methodologies need to be explored.

Successful surveillance of disease in wild animal populations requires an interdisciplinary team of individuals with the capacity to share, store, search and utilize animal health and ecology data quickly and effectively. As diseases do not respect national boundaries, MOSS programs are best developed in collaboration with regions or countries that may be beyond the legislative area of interest.

The paucity of active sampling techniques is likely a function of available funding; this will only change if policymakers and funding agencies identify reasons to instigate such programs. As previously mentioned, capture of animals can be challenging, expensive and detrimental to the animal. When active animal capture events are conducted, collaboration amongst agencies for collection of sufficient samples to support multiple objectives, if possible, would increase available samples with minimal increase in cost and animal stress.

Opportunistic cases submitted to pathologists or wildlife disease centers for post-mortem examination are an important source of wildlife disease information; however this sample population is biased by identification of such cases in the field, proximity or access to a diagnostic facility with appropriate resources and sample quality. Tools to increase the probability of effective testing may include training of field personnel or rehabilitators to do field necropsies and collect correct samples. Digital photographs may provide an effective tool for sharing gross necropsy findings with individuals trained in pathology following a field post-mortem examination; further investigation into the use of such images is warranted.

Following the identification of animals for testing, diagnostic tests performed on wild animals need to have been evaluated in the species to which they are applied, so that interpretation of results is not ambiguous. Detailed knowledge of the test in question and appropriate controls is critical. Access to properly archived samples and appropriate control tissues is also important. Given the cost of diagnostic testing, development of methods to screen populations in a cost effective manner is desirable.

Principles of design and implementation of MOSS are the same irrespective of the species or disease in question; differences between systems are largely logistical. Challenges to the success of a MOSS in free-ranging animal populations are unique relative to most domestic animals and considerable forethought is required so that the system is appropriate for the objectives.

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Chapter 3: Passive, Opportunistic Wildlife Disease Surveillance in the Rocky Mountain Region, USA

Introduction

In recent years the role of wild animals in the epidemiology of emerging and zoonotic diseases has come under increased scrutiny. Many infectious diseases represent a threat to wild animal populations and biodiversity[1]: free-ranging animal populations can serve as important reservoirs for diseases with substantial public health and economic significance.[2-5] Despite the importance of this animal group, large gaps remain in our understanding of the behavior of these pathogens in wild animals and techniques with which to detect and study them.

Disease surveillance is the predefined, systematic collection of health related data for analysis, interpretation and action to improve health within populations. Such systems are often sub classified based on the method and intensity of data collection which delineates 'active' from 'passive' surveillance. Passive surveillance implies that health related data collected for other, routine usage is then used within the system whereas active surveillance entails a predefined data collection scheme specific to the objectives of the system. Although numerous resources exist to aid in the design and implementation of surveillance systems in domestic animals, conducting disease surveillance is inherently more difficult to in wild animals.[6] Collecting non-biased data on wild animals can be logistically difficult and expensive. Basic population parameters

are often unknown for wildlife, making the design and implementation of conventional surveillance techniques difficult. Thus, what is commonly referred to as passive surveillance can often be better described as 'opportunistic' in wild animals.

A key feature of opportunistic surveillance is the detection of a wildlife health event and communication of information to the appropriate individuals. The objective of this study was to identify key groups in positions to detect mammalian wildlife disease events within the Rocky Mountain Region of the United States and recognize pathways by which public health, domestic animal and wildlife surveillance information could be synergized.

Materials and Methods

The study was conducted in the Rocky Mountain Region (Colorado, Wyoming, North and South Dakota, Montana and Utah) as defined by the United States Environmental Protection Agency Region Eight. Organizations thought to be in a position to detect significant health events in wild mammal populations were identified through agency listings, the internet and personal recommendations. Telephone or web-based interviews were conducted to determine the scope of the organization, the frequency of wild mammal interactions, procedures for dealing with wild mammals or related data and the role, or potential role, of the agency in wild mammal disease surveillance in the region. Data were analyzed using descriptive statistics and chi-square tests for comparing frequencies between categories.

Results

Wildlife Rehabilitators

Forty-three wildlife rehabilitators that met the inclusion criteria were identified including 30 in Colorado, eight in Utah, two in South Dakota and one in each of Wyoming, Montana, and North Dakota. Of these, interviews were conducted with at least one individual from 27 organizations. All groups were private and non-profit. The focus of all programs was individual animal rehabilitation; however, some facilities reported public education (19%) and population level wild animal health (7%) to be ancillary objectives.

Facility size, catchment area and record keeping information are presented in Table 1. Individuals interviewed reported that the number of mammals presented to the facility varied widely with season and from year to year. When data or records were used, it was most commonly for sharing with relevant groups including state wildlife agencies (82%), state public health agencies or other rehabilitators. Records were less frequently used to determine trends in presentation such that intake could be predicted, to look up treatment protocols used in the past, or for grant writing or summary reports to donors. The majority (90%) said they would be willing to share data with groups interested in regional wildlife disease trends.

Table 1: Facility size, radius of service and record keeping information for rehabilitation centers in the Rocky Mountain Region

Facility Case Load	**Percentage of interviewed facilities**
Small (<15 mammals/year)	30
Medium (15-50 mammals/year)	33
Large (50-100 mammals/year)	10
Very large (>100 mammals/year)	7
Radius of Service	
<50 km	17
50-250 km	53
>250 km	30
Record keeping	
Keep individual animal records (any format)	92
Spreadsheets or database	52
Paper records	43
Computer text documents	4
Animal information recorded*	
Species	100
Sex	96
Age	96
Body condition	91
Animal pick-up location**	87

*Other recorded information included treatment and outcomes, diet, duration at the facility, and contact information for submitter.

**Animal pick-up location was most often recorded by street address, but the format and accuracy was dependent on the discretion and knowledge of the individual submitting the animal

Only 30% of rehabilitators reported that they regularly test for disease and in most instances, disease testing was overseen by a consulting veterinarian. Mammal species tested most commonly included foxes, deer, raccoons, bats and coyotes. Diseases of concern included rabies, chronic wasting disease (CWD), plague, distemper, parvovirus, and various parasites. Eighty-eight percent of respondents had submitted an animal to a

veterinarian for necropsy; however, this was an infrequent occurrence. Rationale for having a necropsy performed included death of unknown causes, potential for human rabies exposure or concern about an infectious disease. Many animals present to rehabilitators with overt lesions consistent with trauma, in these cases post-mortem examination is rarely performed. No rehabilitators had an active, routine sampling scheme or saved potential diagnostic material for future use. Reasons for the infrequency of disease testing included expense, stress on the animal or lack of need.

Overall, 88% of respondents felt that rehabilitators had an important role in wildlife disease surveillance because these facilities see so many animals and represent a 'front line' of emerging disease issues. Other agencies perceived by rehabilitators to play a significant role in wildlife disease surveillance in their region are listed in table 2. Opinions on the quality of disease surveillance varied widely but a recurring comment was that disease surveillance was driven by public health and production limiting diseases.

Table 2: Agencies or organizations perceived by rehabilitators to be most involved in wildlife disease surveillance in the region (in descending order)

State Wildlife Agency
Health departments
Other rehabilitators
United States Fish and Wildlife Service
Veterinarians
Research groups and Universities
Humane societies
Animal control
Law enforcement
Center for Disease Control
Bureau of Land Management
United States Department of Agriculture

Zoos

Six zoos were identified within the geographic area of interest. All but one (83%) reported that they are occasionally presented with wild animals from the public, state wildlife agencies or police. Protocols for handling such animals vary by facility but a sick or injured animal may be rehabilitated by the zoo when they are approved for rehabilitation; if not, the animal is under the jurisdiction of state wildlife agencies. Disease testing in wild animals is at the discretion of zoo veterinarians, though some zoos collaborate routinely on infectious disease testing with the Association of Zoos and Aquariums. All respondents reported that they frequently receive phone calls from the public regarding sick or injured wild animals; zoos refer such calls to the state wildlife officials, rehabilitators, or the local humane society. When asked about concerns regarding diseases in wild animals, pathogens of significance to both wildlife and humans were cited including West Nile virus, rabies, chronic wasting disease, distemper and diseases foreign to their geographic area.

Outdoor Recreation Groups

Over 400 outdoor recreation groups were identified within the six states. A total of 137 groups were contacted and interviews were completed by 40 individuals. Interviews by category and state are presented in table 3; there was, however, considerable overlap between categories as many companies offered multiple types of activities.

Table 3: Number of outdoor recreation organizations within each group identified, contacted and response rate in the Rocky Mountain Region

Group type	Identified	Contacted	Response (%)
Hunting	328	52	21
Fishing	32	16	44
Outdoor Education	5	5	80
Hiking	19	19	42
River Guides	21	21	38
Wildlife Tours	16	16	13
Total	421	129	31*

*representing interviews from CO (12), MT (10), WY (8), UT (5), SD (3), and ND (2)

Individuals were asked about the types of mammals observed when on wilderness trips. Those reported, in order of decreasing frequency, were large ungulates, carnivores and small mammals. Seventy-nine percent of respondents said they had seen sick, injured, or dead animals. This number did not differ significantly by activity group ($p=0.55$), however, most guides from all groups (73%) reported that such findings were rare. Mortality events observed were most often attributed to road kill; however, respondents also noted having seen cases of presumed epizootic hemorrhagic disease in deer, predation, and gunshot deaths.

Directed actions taken by outdoor adventure personnel were influenced by the location of the observation and the presumed severity of what they had seen. Sixty-seven percent of responders reported that they would take some action based on their observations; this frequency did not vary by activity group (p=0.17). The most common action (71%) was reporting the observation of sick, dead or injured wildlife: the majority (64%) of individuals interviewed said they would report events to the state wildlife organization. Government land management agencies were also mentioned, as well as rehabilitators and highway patrol. Remaining individuals that would take action based on observations said they would either attempt to help or shoot a sick or severely injured animal.

When asked if they had concerns regarding infectious diseases in wild animal populations, 51% of total respondents reported that they were concerned. However, 32% of these people said that their concerns were only mild or specific to some diseases or species. Concerns regarding disease in wild populations did not differ by activity class (p=0.56). Reported disease issues were related to the health of wild populations in general but also to diseases with public health significance and the potential loss of income given the inherent relatedness of their profession to ecosystem health.

Private Veterinary Practitioners

The overall response rate was 4% and did not vary by state (p=0.50); survey responses by state and practice type are presented in table 4. Overall, veterinary clinics reported that they were presented with wild animals very rarely (56%) or never (21%). However, 21% of clinics reported that they saw wild animals on a monthly basis. The frequency with which wild animals were presented to clinics did not vary by state (p=0.69), although

wild animals were presented to small and mixed animal practices significantly more often than large animal practices (p=0.01). Wild animals were brought to veterinarians most often in the spring and summer. Rabbits, raccoons, skunks, bats and squirrels were the most commonly reported small or medium sized animals. Large animals included antelope, deer, elk, moose, bear and mountain lion. Turtles and a variety of wild fish species were reportedly presented to small and mixed animal clinics and the single aquatic animal veterinarian, respectively.

Table 4: Response rate and veterinary clinic type by state

State	**Surveys Mailed**	**Surveys Returned (%)**	**Clinic type (%)**
Colorado	576	21 (4)	Small animal predominantly (57) Mixed animal (29) Large animal predominantly (9) Other (5)
Utah	169	9 (5)	Small animal predominantly (33) Mixed animal (56) Other (11)
Wyoming	107	4 (4)	Small animal predominantly (75) Mixed animal (25)
North Dakota	98	4 (4)	Small animal predominantly (25) Mixed animal (50) Large animal predominantly (25)
South Dakota	180	6 (3)	Small animal predominantly (25) Mixed animal (75)
Montana	239	8 (3)	Small animal predominantly (60) Mixed animal (40)

Forty percent of the clinics had protocols in place for dealing with wild animals; this did not differ by type of clinic (p=0.52) or by state (p=0.77). Within clinics, protocols varied by species and health status of the animal, though if not euthanized, animals were most commonly (73%) transferred to a licensed rehabilitator or the state wildlife agency.

Procedures for transferring wild animals did not vary between state (p=0.57) or type of clinic (p=0.85).

Only 40% of responding veterinary clinics kept records on wildlife. Information collected and method of recording is presented in Table 5. Veterinary clinics reported that data were rarely used unless specifically requested by a wildlife health agency, which was reported to occur on a case by case basis: 35% of clinics reported that they had shared clinical information with a regulatory or wildlife health agency in the past. Sixty-one percent of clinics reported that they would be willing to share data with wildlife health related agencies and the remaining 39% were unsure. Many respondents commented that information sharing would depend on available time and resources. When veterinary clinics received phone calls regarding sick or injured wildlife they either referred the caller to state wildlife agencies, animal control officers, public health, rehabilitators, humane societies, or tried to answer the question themselves.

Table 5: Information and format of data collected on wild animals presented to veterinary clinics

Record keeping	**Percentage of Responding Clinics**
Species	96%
Age	60
Gender	70
Body condition	70
Location found	81
Clinical examination findings	89
Pathological findings	70
Paper records	93*
Spreadsheet or database	7*
Electronic medical records	30*

*information often recorded in multiple locations

Veterinarians were asked about the frequency of testing wildlife for specific diseases including rabies, West Nile virus, distemper, influenza, plague, tularemia or other agents. Forty-seven percent of respondents had tested for one or more of these or other diseases; most tests were sent out to state wildlife agencies, state diagnostic laboratories, state public health departments, and, rarely, private diagnostic laboratories. The rationale for testing included public health surveillance (77%), general disease awareness (20%), emerging disease surveillance (11%) and disease prevalence assessment (9%). Veterinarians felt that findings of diagnostic testing led to an active response in 58% of the cases. Responses reported included individual post exposure vaccination (rabies) and public health recommendations.

When asked about the role of private practice veterinarians in wildlife disease surveillance, 63% reported that veterinarians are central in the identification of important health events and can route information to the appropriate group, 14% said veterinary

clinics have little or no role, 10% believed that a veterinarian's role is individual animal care and diagnosis, 6% reported that veterinarians could play a role as needed and the remaining 5% felt that a veterinarian's role in wildlife disease is public service and education. Reported limitations to veterinary involvement included the lack of financial compensation for testing and shipping of samples, the lack of simple, standardized information reporting protocol and failure to obtain continuing education credits for any ancillary training in wildlife disease issues. When asked who they perceive to be most involved in regional wildlife disease surveillance, 74% of veterinary clinic respondents identified state wildlife agencies.

Veterinary Diagnostic Laboratories

Eight veterinary diagnostic laboratories within the geographic interest area were identified and interviewed. Two laboratories were private, international, fee-for-service companies that only conduct tests at the request of veterinarians and do no record keeping or disease reporting. The remaining laboratories represent fee-for-service veterinary diagnostic laboratories offering diagnostic services and participating in contract research; most were associated with a university or state animal health organization. A single lab is present within each state, though testing is not restricted to in-state animals.

The number of wild animal cases presented to diagnostic laboratories varied throughout the year but was usually weekly or monthly, except Wyoming, where a regular, daily caseload of wild animals was reported. Species represented was equally variable; however, many laboratories reported an apparent over-representation of mammals, species specifically known to harbor certain pathogens, or large, charismatic species.

Four of the six laboratories have specific personnel with wildlife interest. Diagnostic tests most commonly performed vary by region and species examined. At all laboratories, requested tests are sent out to other agencies if they cannot be performed in-house. Minimum duration of sample storage varies by laboratory; most laboratories hold fixed tissues, slides and paraffin blocks for 4-10 years while fresh/frozen tissue is routinely stored for 1-2 months. Cases of interest to the submitter, laboratory or relevant to any legal action can be held indefinitely; tissues from all wild animals submitted to Wyoming State Veterinary Laboratory are held indefinitely. Space was the most commonly cited limitation to tissue storage. All labs use a computer database, sometimes in conjunction with paper records; electronic records may be stored indefinitely. Test results are reported directly to the submitter and laboratory data is shared with relevant governmental agencies when deemed necessary, such as in the case of foreign animal diseases. Issues surrounding the sharing of wildlife disease information were focused on client confidentiality and logistics of information transfer.

The role of regional diagnostic laboratories in wildlife disease surveillance or outbreak investigation is largely to provide diagnostic support to lead agencies coordinating the effort; this involves collaboration to determine the most appropriate approach to meet the objectives of the program. Individuals interviewed identified state wildlife agencies as the most important group involved in regional wildlife disease surveillance. Other agencies noted included the federal wildlife groups and state agriculture agencies.

State Public Health Departments

For each state a single individual was identified for interview. All interviewees were within the state public health department; however, only two states, North Dakota and Wyoming, have a veterinarian employed to work on health issues affecting animals and humans. The mandate of all state public health departments focuses on humans; wildlife only factors in when it is identified as a potential risk to humans, specifically as an infectious disease reservoir. All states monitor diseases found in wild animal populations, however, all respondents reported that their involvement in identification, diagnosis and information management pertaining to these diseases was minimal. While two states reported that the public health agency will harvest tissues for diagnostic testing specific to reportable diseases, remaining states had sampling done at veterinary diagnostic laboratories. Information pertaining to disease in wild mammals was primarily managed by state wildlife agencies and provided to the public health department when needed. Many individuals interviewed identified this duplication of records as redundant.

Conclusion

Of the groups surveyed within the Rocky Mountain Region, wildlife rehabilitators have the most contact with sick or injured wildlife and thus may serve as a good source for information and diagnostic material. However, there are overt limitations to the usefulness of this passive data source. In general, young animals and certain species are over-represented for rehabilitation and facilities are not uniformly distributed, resulting in biased samples. While the majority of rehabilitators interviewed were willing to share information and participate in disease surveillance programs, the paucity of diagnostic

testing and largely unsearchable nature of individual animal health records restricts the usefulness of this data for surveillance purposes. Conditions impacting willingness to participate included time and resource investment and agreement on the use of data; those respondents unwilling to share data from their facilities cited distrust of recipients and concerns that it would require too much effort. Many rehabilitators cited insufficient funding and veterinary support as limitations to the amount of disease testing that could be done. These limitations have been observed in other studies that also identified rehabilitators as an important resource for wildlife health information;[7] tools to overcome these obstacles are required before this data can be aggregated for emerging disease trend identification.

Zoological parks have recently been identified as possible sentinels for emerging disease events given their diverse animal populations with variable susceptibilities, close observation and increased ease of handling, serial sampling opportunities and archived samples.[8,9] The six zoos identified within the Rocky Mountain region worked closely with veterinarians and kept detailed records and sample banks on collection animals, however, the frequency with which they dealt with free-ranging, wild mammals was rare. All zoos reportedly worked closely with state wildlife agencies and therefore information pertaining to health events in free-ranging animal populations would be conveyed to the state level quickly.

The role of private veterinary clinics in wildlife disease surveillance is negligible. Over three-quarters of the clinics responded that they very rarely or never see sick or injured

wild animals and of those that did, only 40% kept records, making veterinary clinics poor sources for cases or information. Clinics referred wild animal cases to rehabilitators and state wildlife agencies, suggesting that these groups represent a more efficient target for surveillance efforts. These results are consistent with other studies where over 70% of veterinarians surveyed reported that they had limited involvement with wild animals and that most re-directed cases to wildlife rehabilitators.[7] The response rate of veterinarians in this survey was low. Opportunistic follow-up with local practitioners suggested that many individuals have so little involvement with wild animals that they felt their responses would be worthless; therefore the findings of this study may actually over-represent the involvement of veterinarians in wildlife disease surveillance. While many respondents reported that they believed veterinarians are important in wildlife health events in general, lack of funding, lack of information on diseases harbored in free ranging animals, difficulty in sampling and shipping and insufficient data collection protocols were cited as limitations on their involvement.

Outdoor recreation personnel expressed an interest and concern for diseases in wild animal populations; however, the frequency with which they encounter sick, injured or dead animals is low and tends to be skewed towards larger, charismatic species. While they were beyond the scope of this project, other individuals who may be in a similar position as outdoor recreation personnel to observe wildlife health events would be rural landowners and farmers. Such individuals often have a great deal of local ecology knowledge and may identify changes in animal patterns. The identification of any potentially significant health events by this group would be reported to the state wildlife

agency. Likewise, state public health agencies and veterinary diagnostic laboratories play an important role in wildlife disease surveillance by conducting diagnostic testing; however, these laboratories largely provide support to state wildlife agencies and thus data from the laboratory infrastructure is most likely captured by state wildlife officials.

Overall, state wildlife agencies were the most commonly cited groups for aggregation of wildlife health related information in the Rocky Mountain Region and should therefore play a key role in the identification of events that may represent a threat to public health, domestic animals or biodiversity. These agencies must therefore be equipped with, or have access to, the personnel and resources necessary to quickly and effectively respond to wildlife health events and tools to analyze state wildlife health information for surveillance purposes. Post-mortem examination of dead animals has long been a mainstay of wildlife disease surveillance; it is an inexpensive means to gain insight into specific causes of mortality, however, it requires that individuals have extensive training in wildlife pathology.[6,10] Investigation into wildlife health events are further complicated by the fact that veterinary diagnostic modalities are rarely tested or validated in wild animals and interpretation of findings can be challenging. While an evaluation of state wildlife groups was beyond the scope of this project, cursory review of staff listings identified veterinarians within state wildlife agencies in only three states and no reference to individuals with specialty training in pathology. Most diagnostic laboratories interviewed expressed a strong interest in working with state wildlife agencies; however, this can be logistically difficult given geographic separation of some groups. Wyoming

State Veterinary Laboratory is an exception, as state wildlife personnel are housed in the same location fostering regular communication and collaboration.

Identification of cases within a passive system is influenced by the willingness of individuals to participate, awareness or detection pressure for the disease, clinical manifestation and fatality rates, knowledge and education, and the availability of a diagnostic lab to diagnose or confirm cases. It is logistically difficult to accurately recover sick and dead wild animals, as has been exemplified in experimental studies evaluating carcass recovery and mortality estimates.[11] Mass mortality events are likely to be detected and reported by biologists or the public; however, these events represent only a fraction of mortality in wild animals.[6] Given the difficulty of identifying health related events in small and inconspicuous species, systematic trapping or other techniques would need to be employed. Information reporting and submissions to laboratories are increased when infrastructure facilitating delivery of the animal or tissue is optimized. In conclusion, within the Rocky Mountain Region, state wildlife agencies appear to be the key node for opportunistic surveillance data. Should state-level passive surveillance programs for the identification of emerging health events in wild animal populations remain the primary tool for detection of emerging infectious diseases, wildlife health agencies require sufficient resources to support and train personnel. These agencies should be evaluated to determine the effectiveness of data collection, analysis, interpretation, dissemination and collaboration of this information.

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Chapter 4: Histopathologic and immunohistochemical findings in two white-tailed deer fawns persistently infected with Bovine viral diarrhea virus

Introduction

Bovine viral diarrhea virus (BVDV), family Flaviviridae, genus *Pestivirus*, is a significant production disease of domestic cattle. The clinical spectrum of BVDV-associated disease is broad and dependent on both host and viral characteristics. Infection of pregnant cattle with noncytopathic BVDV between approximately 45–125 days of gestation results in fetal infection and strain-specific immunotolerance. These fetuses are unable to clear the virus and remain persistently infected (PI).[1] Calves born PI may be stunted, weak, or clinically normal.[1,2] Typically, PI cattle shed more virus than acutely infected individuals and shed it over the entire course of their life, acting as a constant viral challenge to other susceptible animals.[1,3] As such, PI individuals are central to the maintenance of BVDV in cattle populations.

Evidence for BVDV infections has been found in a wide range of wild ruminant species. Persistent pestivirus infection has been documented in a mouse deer (*Tragulus javanicus*) at the Copenhagen Zoo. A buck, doe, and female sibling were identified as PI following multiple BVDV isolations.[4] Persistent infection has also been documented in an eland (*Taurotragus oryx*) in Zimbabwe following isolation of virus on separate occasions.[5] Reports of persistent infection in wild animals are largely opportunistic findings and little is known about the pathogenesis of disease in these species. Recently, persistent BVDV

infection in white-tailed deer (*Odocoileus virginianus*) has been achieved under experimental conditions.[6] The objective of the present study was to characterize the histopathologic lesions and distribution of BVDV antigen in the tissues of PI white-tailed deer fawns.

Materials and methods

Virus propagation, titration and isolation and determination of serum neutralizing titers

The virus, R03-20663, used in the present study was isolated from deer carcasses submitted to South Dakota State University for diagnostic testing.[7] This virus was noncytopathic, determined by lack of cytopathic effect in cultured Madin Darby bovine kidney (MDBK) cells[8] and belonged to the BVDV2 species based on phylogenetic analysis of 5' UTR sequences as described previously.[9] Viruses were propagated, titrated and reisolated from buffy coat samples using protocols described in a previous paper.[10] Viral-neutralizing titers in serum were determined using previously described techniques.[11]

Housing, inoculation, and sampling of doe and fawns

Handling and treatment of the doe and fawns complied with the Animal Welfare Act as Amended (7 USC, 2131–2156). The pregnant doe was purchased from a commercial breeder and tested for antibodies against BVDV by serum neutralization and presence of replicating BVDV by virus isolation from buffy coat samples. Based on date of contact with buck, it was estimated to be between 6 and 7 weeks pregnant. The doe was housed

in a climate-controlled barn, operated at a BL2 containment level, for the duration of the experiment, and observed a minimum of twice daily.

On the day of inoculation, blood was drawn from the doe to determine pregnancy status and level of serum-neutralizing antibodies. A second blood sample was drawn at day 32 postinoculation. The limited number of blood samples collected was done to minimize stress on the doe as this might negatively impact pregnancy. The doe was inoculated with 5 ml of 1.0×10^6 tissue culture infectious dose (TCID)/ml of R03-20663 by the oral/nasal route (viral titer determined as described above). This dose was similar to that used in previous studies in white tailed deer fawns.[12]

Pregnancy was confirmed at time of inoculation and 32 days postinoculation using an enzyme-linked immunosorbent assay (ELISA) test that measures the presence of pregnancy-specific protein B (PSPB) in serum.[a] Serum samples were shipped to, and run at, the commercial laboratory that produces the test.[b] Blood and ear notch samples were collected from live fawns. Buffy coat samples were isolated and tested by virus isolation and serum samples were tested for neutralizing titers against BVDV as described above. Ear notch samples were tested for the presence of BVDV antigen using a commercial antigen capture ELISA tested as per the manufacturer's protocol for testing bovine ear notches.

RNA was prepared from viruses isolated from buffy coat samples and nucleotide sequence determined as described.[12] The sequence of the BVDV isolated from fawns

was compared to the inoculum virus given to the doe by phylogenetic analysis as described previously.[12]

Histopathology and immunohistochemistry

Samples were collected from multiple fawn tissues including lymphoid, endocrine, urogenital and nervous tissue, the gastrointestinal tract, respiratory tract, and skin. Tissues were fixed in 10% buffered formalin for 24 hrs and then transferred to alcohol for one day before processing. Paraffin-embedded tissue blocks were sectioned at 5 μm and stained with hematoxylin and eosin.

BVDV immunohistochemical staining was done on a Ventana Benchmark Auto Immunostainer.[c] The primary antibody was 15.c.5 BVDV Mab[d] used at a 1:3,000 dilution. Sections were counterstained with hematoxylin. Ear skin from a PI and non-PI bovine were included on all slides as positive and negative tissue controls, respectively.

To characterize the lymphocytes present in tissue, sections were stained using CD3[e] and CD79a/mb-1[f] antibodies as T- and B-cell markers, respectively. The CD3 was used at a dilution of 1:75 and the CD79a/mb-1 antibody at 1:20. Reactions were conducted in a Ventana NEXES Auto Immunostainer[c] with a hematoxylin counterstain. Positive tissue control slides were made using a section of lymph node. Negative control slides were sections of test tissue with the primary antibody removed and replaced with an irrelevant, isotype similar, mouse antibody.[c]

BVDV immunohistochemical staining was evaluated using a semi-quantitative intensity scoring system similar to that previously reported, where 0 = no detectible antigen; + = weak, faint or minimal antigen in the cytoplasm; ++ = moderate, uniformly distributed cytoplasmic staining; and +++ = intense cytoplasmic staining.[13]

Results

The doe did not have detectable antibodies against BVDV at the time of inoculation. At 32 days postinoculation, the doe's serum antibodies had a 7.1$\log_2$ titer against type 2 BVDV, but no titer against type 1 BVDV. Two fawns, one male and one female, were delivered live, 163 days after inoculation. Both fawns were undersized but appeared otherwise normal. These two fawns were positive for BVDV by virus isolation from buffy coat, positive for BVDV antigen by antigen capture ELISA (ACE) test, and negative for BVDV antibodies based on serum-neutralizing titers. Based on phylogenetic analysis the viruses isolated from these two fawns were identical and matched the inoculum virus. During the first night after birth, the doe apparently killed the two fawns.

Histopathology

Samples of thirty-four tissues were collected from one or both fawns. Tissues from the female fawn showed evidence of mild autolysis, most prominent in the brain, while remaining sections were in good postmortem condition. Sections of the gastrointestinal tract evaluated included oral mucosa, salivary gland, esophagus, rumen, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, and liver. No microscopic lesions were observed in the oral mucosa, salivary gland, esophagus, cecum, colon, rectum, and adjacent anal gland. Lymphoid tissue throughout the small intestine was reduced and

present as a diffuse band within the lamina propria, most prominent in the ileum. Enterocytes of the jejunum were diffusely vacuolated. The ruminal papillae and pancreatic islet cells were morphologically consistent with fetal or perinatal tissue. Hepatocytes often contained a single intracytoplasmic glycogen-containing vacuole, and there was a moderate amount of extramedullary hematopoiesis within sinusoids.

Representative sections of the respiratory tract including the nasal orifice, nasal turbinates, trachea, and lung were collected. In both fawns, the pulmonary parenchyma was incompletely inflated. The nasal orifice, nasal turbinates, and trachea were histologically unremarkable. Lymphoid organs evaluated included the mesenteric, submandibular, retropharyngeal, ileocecal and parotid lymph nodes, tonsil, spleen, and thymus. There was a paucity of lymphocytes within all lymph nodes and medullary rays were prominent; evidence of necrosis or apoptosis was absent. Lymphoid depletion was most prominent within the germinal centers of follicles (Fig. 1C). Follicles were also markedly depleted in the tonsil and spleen. Splenic lymphocytes were most common in periarteriolar lymphoid sheath. The cortex of the thymus appeared thin in some areas. The amount of extra medullary hematopoiesis in the spleen was adequate for a young animal.

Sections of cerebrum were taken from the middle of the parietal lobe, including a portion of thalamus. Vacuolation of rare individual neurons was noted in the brain of the male fawn. Purkinje cells were occasionally observed within the granular cell layer of the cerebellum of both fawns; remaining cells were normally placed. Sections from the

female reproductive tract examined included ovarian tissue, fallopian tube, fimbriae, and broad ligament. No microscopic lesions were observed in the ovary, which was comprised of many primordial follicles and stroma. The testes were immature but did not contain any microscopic lesions. The kidney of one fawn was mildly congested.

Myocardial cells of both animals were diffusely thin. There were no microscopic lesions in the skin or skeletal muscle of the diaphragm. Adipocytes adjacent to other organ tissue were moderate to markedly hypoplastic in both fawns. Diffuse vacuolation of follicular cells was present in the thyroid gland of both fawns. The adrenal glands were histologically unremarkable.

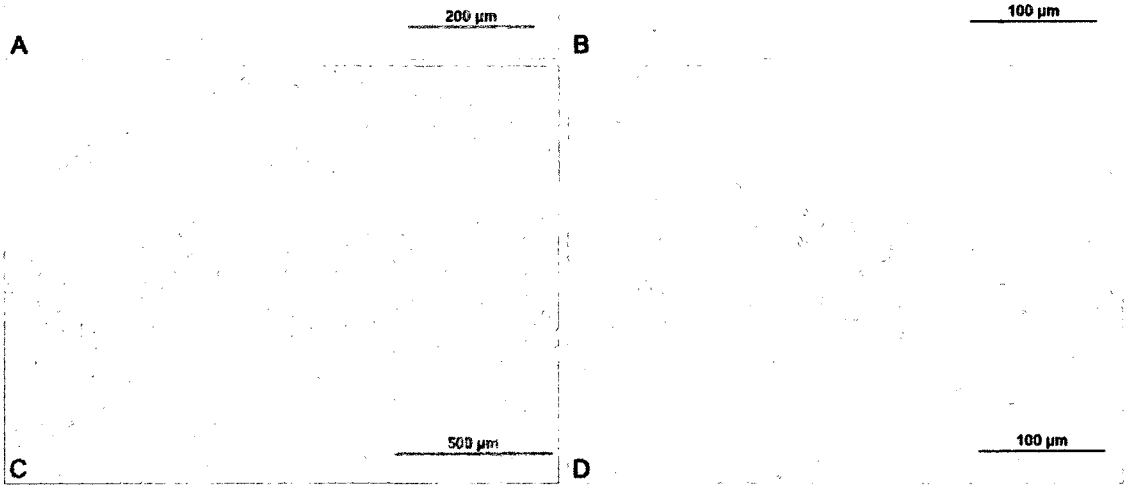

Figure 1. A, Skin, white-tailed deer. Antigen present in epithelial cells of the skin and adnexal structures. Bovine viral diarrhea virus immunohistochemistry (BVDV IHC). Bar = 200 μm **B,** Brain, white-tailed deer. Antigen present in neurons, endothelial cells and rare glial cells. BVDV IHC. Bar = 100 μm. **C,** Retropharyngeal lymph node, white-tailed deer. Marked follicular atrophy. Hematoxylin and eosin. Bar = 500 μm. **D,** Retropharyngeal lymph node, white tailed deer. Antigen present in only rare histiocytic cells. BVDV IHC. Bar = 100 μm.

Immunohistochemistry

BVDV antigen. Positive staining for BVDV antigen was found in nearly every tissue but most consistently present in epithelial cells and vascular endothelium of numerous organs. Cutaneous, follicular, and glandular epithelium of both haired and non-haired skin was consistently positive (++) in both animals (Fig. 1A). Endothelial cells of blood vessels in the skin were weakly positive (+) and fibrovascular connective tissue failed to take up stain in either animal. Skeletal muscle was occasionally positive (+) in the lip of the male fawn but no staining was apparent in the same section taken from the female. Dermal leukocytes and myocytes present in sections of haired skin were negative in both animals.

Details on antigen distribution throughout the cardiovascular system are presented in Table 1. Antigen was present throughout the respiratory tract, most prominently in the respiratory epithelium but also in glandular and supporting structures. Cardiac myocytes were variably positive in both animals.

Table 1. Distribution of bovine viral diarrhea virus antigen in the cardiovascular system by organ and cell type.*

Tissue	Cell type	Male	Female
Heart	Myocytes	+	++
	Endothelium	++	++
	Connective tissue	0	0
Nasal turbinates	Respiratory epithelium	++	+
	Vessel	+	+
	Bowman's glands	+	+
	Bone or cartilage	0	0
Trachea	Epithelium	+++	+++
	Glands	+	+
	Cartilage	0	0
Lung	Pneumocytes	++	+
	Interstitium	++	+
	Alveolar macrophages	+	+
	Bronchi/bronchiole epithelium	+++	++
	Bronchi/bronchiole glands	++	+
	Bronchi/bronchiole cartilage	+	NA
	Bronchi/bronchiole smooth muscle	0	0

* 0 = no detectible antigen; + = weak, faint or minimal antigen in the cytoplasm; ++ = moderate, uniformly distributed cytoplasmic staining; and +++ = intense cytoplasmic staining. NA = not applicable; tissue or cell type was not present in examined sections.

Neurons of both the cerebellum and cerebrum (Fig. 1B) were diffusely positive; however, more intense staining was noted in the neuronal cell bodies of the cerebrum (male: ++, female: +++) relative to the cerebellum (Purkinje and granule cells, both animals: +). Only rare staining was observed in the macroglia or microglia of either animal. Strong antigen staining (+++) was present in the endothelial cells of vessels in both the cerebrum and cerebellum of both animals. Additionally, vasculature in the meninges overlying the

cerebrum (+) and cerebellum (+++) was antigen positive in both animals. No antigen was identified within the neuropil.

The distribution of antigen in the gastrointestinal tract, by location and cell type, is presented in Table 2. Weak staining for BVDV was present in the epithelium throughout the gastrointestinal tract of both fawns. Antigen was rarely detected in blood vessels, leukocytes, or myocytes of the intestine. Hepatocytes were strongly and diffusely antigen-positive and hepatic vasculature exhibited more positive staining than blood vessels in other tissues. Kupffer cells, randomly distributed throughout the liver, were the most intensely positive cells in the liver of both animals.

Table 2. Distribution of bovine viral diarrhea virus antigen in the gastrointestinal tract by organ and cell type.*

Tissue	Cell type	Male	Female
Oral cavity	Epithelium	+++	+
	Connective tissue	0	0
	Blood vessel	+	0
Salivary gland	Ductular epithelium	+	0
Esophagus	Epithelium	+	+
	Ct, muscle, bv, lymphocytes	0	0
Rumen	Epithelium	++	++
	Ct, muscle, bv, lymphocytes	0	0
Duodenum	Epithelium	+	+
	Ct, muscle, bv, lymphocytes	0	0
Jejunum	Epithelium	+	0
	Ct, muscle, bv, lymphocytes	0	0
Ileum	Epithelium	+++	+
	Connective tissue	0	0
	Muscle layers	+	0
	Blood vessels	+	0
	Lymphocytes	+	0
Cecum	Epithelium	+	+
	Ct, muscle, bv, lymphocytes	0	0
Colon	Epithelium	+	+
	Ct, muscle, bv, lymphocytes	0	0
Rectum	Epithelium	+	0
	Ct, muscle, bv, lymphocytes	0	0
	Muscle layers	+	0
Pancreas	Pancreatic acini and islets	0	0
Liver	Hepatocytes	+	+
	Bile Ducts	0	0
	Kupffer cells	+++	+++
	Endothelium	+	+

* Ct = connective tissue; bv = blood vessel; 0 = no detectible antigen; + = weak, faint or minimal antigen in the cytoplasm; ++ = moderate, uniformly distributed cytoplasmic staining; and +++ = intense cytoplasmic staining.

Both follicular (male: ++, female: +) and parafollicular cells (male: ++, female: +) of the thyroid gland stained positive for BVDV antigen. Adrenal cortical secretory cells and medullary cells were positive (+) in the male fawn; however, no antigen was detected in the adrenal gland of the female fawn. The urogenital system showed mild staining in both animals. Renal tubular epithelium and vascular endothelium was weakly positive (+) in both fawns. Rare epithelial cells of the fallopian tube were positive (+) in the female while no positive staining was noted in the male reproductive system.

Distribution of viral antigen in lymphoid tissue is presented in Table 3. In lymph nodes, only rare cells stained positively for BVDV antigen (Fig. 1D). These positive cells may represent lymphocytes or macrophages. In the tonsil, rare round cells were positive while surrounding epithelium was diffuse and strongly positive. Epithelial cells and rare round cells were positive in the thymus.

Table 3. Distribution of bovine viral diarrhea virus antigen in lymphoid tissue by organ and cell type.*

Tissue	Cell type	Male	Female
Mesenteric LN	Lymphocytes	0	0
	Non-lymphocyte leukocytes	+	+
Mandibular LN	Lymphocytes	0	0
	Non-lymphocyte leukocytes	+	+
Retropharyngeal LN	Lymphocytes	0	0
	Non-lymphocyte leukocytes	+	+
Parotid LN	Lymphocytes	NA	0
	Non-lymphocyte leukocytes	NA	+
ICE LN	Lymphocytes	NA	0
	Non-lymphocyte leukocytes	NA	+
Tonsil	Lymphocytes	+	+
	Crypt epithelium	+++	++
Spleen	Red pulp (macrophages)	+++	+++
	Smooth muscle, lymphocytes	0	0
Thymus	Lymphocytes	+	+
	Epithelium	++	++

* 0 = no detectible antigen; + = weak, faint or minimal antigen in the cytoplasm; ++ = moderate, uniformly distributed cytoplasmic staining; and +++ = intense cytoplasmic staining. NA = not applicable; tissue or cell type was not present in examined sections.

T- and B-cell staining. Immunohistochemical staining for T- and B-cells was performed on all slides containing lymphoid tissue. Depletion of B-lymphocytes was observed in all lymph nodes examined including the mesenteric, submandibular, retropharyngeal, parotid, and ileocecal. The tonsils of both animals lacked distinct lymphoid follicles and were comprised almost exclusively of T-lymphocytes with rare B-cells distributed throughout the section. Rare B-lymphocytes were present randomly throughout the thymus. Within periarteriolar lymphoid sheaths of the spleen, T-cells were prominent but

B-cells were rare. Rare B-cell follicles were present in the spleen. Sections of jejunum, ileum, and colon present on slides were also evaluated. Within the jejunum and ileum the majority of lymphocytes within the lamina propria were T-cells with perivascular accumulations of B-cells. No lymphoid tissue was identified in the examined section of colon.

Discussion

The two fawns were classified as persistently infected given the stage of gestation at the time of inoculation, isolation of virus identical to the inoculum strain, and presence of antigen in skin and serum in the absence of BVDV antibodies. While a temporal pattern of viremia could not be documented in these animal given their premature death, diagnostic test results are consistent with previously reported experimental induced persistent infection in white-tailed deer[6] and cattle.

The histopathologic and immunohistochemical changes observed in the two PI fawns examined are similar to those seen in PI cattle with some subtle differences. Lymphoid tissue of the fawns contained less antigen than is commonly reported in cattle; lymphocytes and macrophages have been strongly positive in bovine studies.[14,15] The spleen of both fawns had abundant, strongly positive cells present in the red pulp; similar cells were observed in lymph nodes. Morphologically these cells were consistent with macrophages as has been reported previously; however, the paucity of staining of lymphocytes in the deer fawns conflicts with previous reports in cattle.[16]

In the gastrointestinal tract, faint staining was observed in the epithelial cells from the oral cavity to the rectum. Blood vessels were rarely positive for viral antigen. In PI calves and adult cattle, BVDV antigen has been detected in epithelial cells and rare mesenchymal cells in the gingiva, tongue, esophagus, abomasum, omasum, rumen, and ileum; however, no positive staining was noted below the ileocecal orifice.[16] Strong positive staining was observed in the hepatocytes and Kupffer cells of the fawns consistent with reports in cattle.[17]

Viral antigen was present in neuronal cell bodies of the cerebellum and cerebral cortex consistent with that reported in cattle.[18,19] In contrast to reports in cattle,[19] however, antigen was identified in blood vessels and meninges along with Purkinje and granule cells of these two fawns. The cerebral cortex and hippocampus have been proposed as predilection sites for BVDV antigen in cattle with approximately 90% of neurons staining positively.[19] Mapping of viral distribution within the brain was not done in these fawns so predilection sites within the central nervous system remain unknown.

In bovines, the distribution of BVDV antigen in the respiratory tract has been extensively studied and virus has been identified in epithelium, glands, vessels, circulating leukocytes, and chondrocytes.[13] A similar pattern was observed within the deer fawns; however, staining was only rarely noted in chondrocytes and lymphocytes.

The most prominent histologic finding in the two fawns was lymphoid depletion. While healthy, age matched controls were not available for inclusion in this study, lymphoid

tissue examined contained significantly fewer lymphocytes than deer fawns previously examined by the authors. In clinically normal PI cattle, histologic lesions are also rare.[16,19] Lymphoid depletion is present in calves with mucosal disease[20] but is not commonly reported in healthy PI animals. Lymphoid depletion observed in these two fawns was consistent with a paucity of B-cells. These findings suggest that there may be a difference in the clinical expression of persistent infection between cattle and deer.

Tissue sections available for evaluation may not fully represent the distribution of virus within all PI deer; however, findings suggest that antigen is broadly distributed throughout many organs and cell populations consistent with the pathogenesis in cattle. Overall, epithelial cells were most often positive and immunohistochemical staining of skin biopsies, as is commonly done with cattle, may be an effective test for persistent infection in white-tailed deer.

The prevalence of BVDV in wild populations and subsequent risk to domestic livestock is unknown. Seroprevalence studies of numerous North American wild cervid populations including mule deer (*Odocoileus hemionus*),[21,22] elk (*Cervus canadensis*),[21,23] moose (*Alces alces*),[24,25] American bison (*Bison bison*),[26] caribou (*Rangifer tarandus caribou*),[27] and pronghorn (*Antilocapra americana*)[28] suggest a history of exposure to BVDV and subsequent seroconversion. The virus has been isolated from white-tailed deer in South Dakota with mucosal disease and strong positive BVDV immunohistochemical staining in skin and other tissues.[7] BVDV has also been isolated from mule deer in North America[29] and other cervids worldwide.[30] Experimental

infection of mule and white-tailed deer with BVDV revealed that they are susceptible to infection and can shed virus following inoculation without showing signs of clinical disease.[31] Likewise, infected elk showed no clinical signs, but all animals became infected and transmission to non-inoculated, in-contact animals was observed.[32]

Given the similarity of pathologic lesions observed in PI deer relative to cattle it may be hypothesized that PI deer also represent a significant risk of disease transmission. Persistently infected mouse deer have been shown to infect cattle without direct contact.[33,34] The role of wildlife in the epidemiology of BVDV should be considered if BVDV is to be effectively managed in cattle populations.

Sources and manufacturers

a. BioPRYN™, Biotracking LLC, Moscow, ID.

b. Biotracking LLC, Moscow, ID.

c. Ventana Medical Systems Inc., Tucson, AZ.

d. IDEXX, Westbrook, ME.

e. Dako North America, Inc., Carpinteria, CA.

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Chapter 5: Persistent Bovine viral diarrhea virus infection in wild cervids of Colorado.

Introduction

Bovine viral diarrhea virus (BVDV) is a clinically significant production virus within the family *Flaviviridae*, genus *Pestivirus*. In cattle, the pathogenesis is complex, and the outcome of exposure to the virus is influenced by many host and agent variables. Epidemiologically, the most significant factor of BVDV infection within herds is the presence of persistently infected (PI) individuals; these animals are the primary source of new infections within a cattle population.[1] Persistent infection occurs when a fetus is exposed to a noncytopathic BVDV and develops immunotolerance specific to that BVDV strain. The result is that the fetus is unable to clear the virus and therefore is PI.[2] Persistently infected cattle shed more virus than acutely infected individuals and provide constant challenge doses to contact animals.[1,2] Calves born PI may be weak or small, but others may be clinically normal.[2,3]

Seroprevalence studies of wild ungulate populations worldwide provide evidence of exposure to BVDV and subsequent seroconversion. In North America, when positive titers are observed, antibody prevalence ranges from 4% in pronghorn (*Antilocapra americana*) of Alberta, Canada,[4] to 70% in caribou (*Rangifer tarandus*) of Quebec, Canada.[5] Other species with evidence of exposure include mule deer (*Odocoileus hemionus*),[6,7] elk (*Cervus elaphus*),[6] moose (*Alces alces*),[8,9] and bison (*Bison bison*).[10]

The virus has been isolated from mule deer[11] and white-tailed deer (*O. virginianus*; Ludwig J, McClurkin A: 1981, BVD in Minnesota white-tailed deer. Proceedings of the Wildlife Disease Association Conference, p. 38) in North America, and other cervids worldwide.[12] Several pestiviruses distinct from BVDV have been isolated from wildlife, including a pestivirus isolated from pronghorn antelope in Wyoming that was classified as a new pestivirus genotype.[13] Experimental infection of mule deer and white-tailed deer with BVDV revealed that they are susceptible to infection and can shed virus following inoculation without showing signs of clinical disease.[14] Likewise, infected elk showed no clinical signs, but all animals became infected and transmission to noninoculated, in-contact animals was observed.[15]

Persistent infections are not restricted to cattle. In other countries, captive mousedeer (*Tragulus javanicus*)[16] and an eland (*Taurotragus oryx*)[17] have been shown to be PI with BVDV following repeated virus isolation. Experimental infection of a pregnant white-tailed deer resulted in the birth of a PI fawn.[18] Bovine viral diarrhea virus was isolated from two white-tailed deer from eastern South Dakota with clinical presentations of mucosal disease.[19] A type 1 virus was isolated from one deer and a type 2 virus was isolated from the other deer; the deer were located within 15 miles of each other. The two white-tailed deer in South Dakota were BVDV immunohistochemistry (IHC)-positive in skin and other tissues, suggesting that both animals were PI.[19] In Alabama, a single white-tailed deer out of 406 samples collected from hunter-harvested animals was positive for BVDV using IHC, an apparent prevalence of 0.2% (95% confidence interval [CI]: 0–0.6%).[20] Reports of persistent infection in species other than cattle are largely

opportunistic findings or experimental infection; little is known about the prevalence or survivorship of PI animals in the wild. The objective of the current study was to determine the prevalence and distribution of PI cervids in Colorado.

Materials and Methods

Samples were collected during the 2005–2006 hunting season from deer, elk, and moose presented to the Colorado Division of Wildlife for chronic wasting disease (CWD) testing. A full thickness, 3-cm^2 section was collected from the dorsal ear, marked with a unique identification number, and stored at –20°C until testing. Information obtained from the hunter at the time of sample collection included species, geographic location (Universal Transverse Mercator (UTM)) where the animal was harvested), sex, and date of harvest.

Following collection of all samples, tissues were fixed in 10% buffered formalin for 24–48 hr, paraffin-embedded, and sectioned at 5 μm. Immunohistochemical staining BVDV antigen was performed using a Ventana Benchmark Auto Immunostainer.[a] The primary antibody was 15.c.5 BVDV monoclonal antibody (mAb)[b] used at a 1:3,000 dilution. Sections were counterstained with hematoxylin. Skin from a PI and non-PI bovine were included within each staining run as positive and negative tissue controls, respectively.

The location of animals tested and results of the BVDV IHC were evaluated visually using geographic information system (GIS) software. Prevalence of BVDV IHC-positive animals by species within regions was calculated by converting point source information

to polygon data with the unit of analysis being the Colorado Division of Wildlife data analysis unit (DAU); these units are biologically significant and presumed to estimate the geographic range and density of animals within that region. At the time of sampling, there were 55 deer, 47 elk, and 4 moose DAUs within the state. Confidence intervals were calculated from a beta posterior distribution assuming a noninformative prior.

Results

Samples were collected and processed from 5,951 animals; 5,895 of these were shot in the state of Colorado. Of the in-state submissions, individual spatial location and animal information was available on 5,597 animals comprised of 2,934 mule deer, 2,516 elk, 141 white-tailed deer, and 6 moose. These samples represented a sampling fraction of 0.5% for deer and moose, and 1.0% of elk, based on statewide, preharvest estimates (Colorado Division of Wildlife, unpublished data). Sample proportions by DAU varied from 0–5.4% for deer, 0–3.7% for elk, and 0–0.9% for moose. The distribution of IHC-positive and -negative animals within the state is shown in Figure 1.

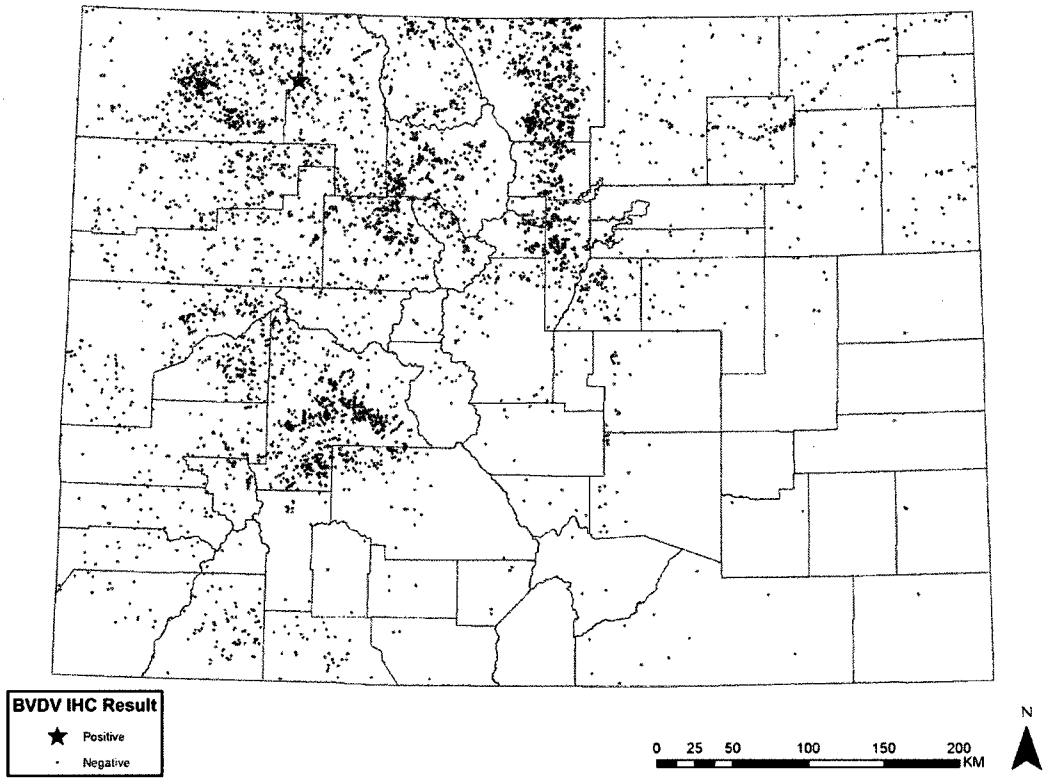

Figure 1. Geographic distribution and results of cervid samples tested for *Bovine viral diarrhea virus* (BVDV) using immunohistochemistry (IHC).

Tissue from a single animal harvested in the northwest portion of the state was classified as positive; abundant antigenic staining was present in the following: cytoplasm of epithelial cells of the epidermis, sebaceous glands, and hair follicles; rare dermal mononuclear cells; vascular endothelial cells; myocytes; and rare chondrocytes. This animal was an adult male mule deer, negative for chronic wasting disease. No unusual conditions were noted by the hunter or by the individual sampling the animal. A section of retropharyngeal lymph node, collected for CWD surveillance, was also positive on BVDV IHC. Polymerase chain reaction (PCR)[21] was performed on a sample of fresh ear

and lymph node from the positive deer, tissue from a PI bovine was used as a positive control. Both tissues were PCR positive, and the amplified product was consistent with a type 1 strain.

The positive deer was shot in DAU D-2; the apparent prevalence for that DAU was 0.52% (95% CI: 0–1.06). For all remaining DAUs, the apparent prevalence was 0, although sample sizes were small for many units, and the upper limit of the 95% confidence intervals for individual DAUs ranged from 1.2 to 95%. Overall, apparent prevalence among all Colorado deer was 0.03%; the 95% confidence interval on the overall prevalence for PI deer was 0–0.10%. The apparent prevalence of BVDV IHC-positive elk and moose in the state and all individual DAUs was 0. The upper limit of the 95% confidence interval for state level prevalence in elk was 0.12%; for individual DAUs, the upper limit ranged from 0.67 to 95%. For moose, the 95% confidence interval on state prevalence was 0–34%.

Discussion

In recent years, the role of wildlife in the epidemiology of infectious diseases of livestock has been under increased scrutiny. Ecologic changes altering the natural environment of wildlife hosts and the expansion of people, with their animals, into previously undeveloped areas has increased the probability of contact among humans, domestic animals, and wild species. Globally, wild animals may serve as reservoirs for infectious diseases of substantial economic and public health significance; likewise, pathogens of domestic animals can result in significant morbidity and mortality in wild populations. For these reasons, it is important to understand the potential for interspecies transmission

of agents, techniques for identification of spill-over events, and tools to evaluate the significance of this occurrence.

In the current study, the statewide prevalence of persistent BVDV infection was extremely low. The prevalence of PI cattle varies significantly with production type, management, geographic location, and age. In chronically ill and dead feedlot cattle, the prevalence may exceed 2%;[22] however, estimates of PI in United States beef calves is <0.5%.[23] The prevalence of PI cattle in Colorado is unknown. Given that the virus is assumed to have spilled over from cattle, and that cervid densities tend to be less aggregated than cattle in general, the low prevalence identified in the present study is not surprising. The sampling protocol employed is influenced by both the allocation of hunting permits at the state level as well as individual animal selection and submission by hunters. Adult animals are significantly overrepresented in a sample collected from hunters. Persistently infected calves are often unthrifty, and it is conceivable that similar immunosuppression occurs in cervids and that these animals do not live to be hunted. Alternatively, unthrifty looking animals may be avoided by hunters. Such biases may underestimate the true prevalence of disease in free-ranging populations.

Diagnosis of a PI animal requires repeated isolation of virus over a period of time; in cattle, however, IHC has been shown to be an extremely effective technique for differentiating between PI and acutely infected individuals.[24] Immunohistochemistry staining in one experimentally infected PI deer showed an identical staining pattern to cattle.[18] Given the nature of the sampling frame of this cross-sectional study, follow-up

testing was impossible; however, the strong antigenic staining in the skin in combination with the positive PCR result on both skin and lymph node suggests that this animal was truly positive.

While the current study had a large sample size, some geographic areas lacked sufficient samples to detect the virus even if the virus was present at a very high level. The opportunistic nature of available samples meant that sampling intensity was not uniform and hunter harvest samples submitted for CWD testing likely came from different areas than would be targeted in a study designed to look specifically for the presence of BVDV in cervids. Prevalence surveys for infectious disease in most wild populations are inherently more challenging than such activities in domestic livestock; common population parameters used in the design of sampling strategies are often unknown and collection of tissues from live animals can be expensive and logistically difficult. For these reasons, opportunistic sampling strategies are often employed. Such protocols can be useful for the detection of disease, but are often of limited epidemiological value.

The current study suggests that the prevalence of BVDV PI cervids in Colorado is low, but that natural infection does happen. Identification of a PI deer suggests that there is more transient infection occurring within the state, an assumption supported by previous studies that have identified a high seroprevalence in mule deer in parts of Colorado (Myers EP: 2001, Assessing the role of selected infectious disease agents in neonatal mule deer fawn mortality on the Uncompahgre Plateau of western Colorado. Thesis, Colorado State University Department of Microbiology Immunology and Pathology, Fort

Collins, CO). Further studies are required to assess the significance of this finding for both domestic livestock and wild animal populations.

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Chapter 6: Pooled samples for the diagnosis of BVDV in wild cervids

Introduction

Identification of bovine viral diarrhea virus in non-bovid species has prompted increased investigation into the distribution of the virus across different species and geographic regions worldwide. The prevalence of persistent infection appears to be very low; published estimates range from 0.03% in Colorado[1] to 0.2% in Alabama.[2] As such, the number of animals needed to test in order to identify a positive animal within a region is high and may be cost prohibitive in some areas.

Herd level testing is the application of a diagnostic scheme to population of animals sharing a common risk factor. Such approaches are commonly employed in both epidemiologic investigations and production animal medicine; the motivation for the testing may vary from identification of disease in a population, estimating prevalence or demonstrating disease freedom. The objectives of the herd test dictate the method to be employed; for some populations and diseases minimizing false negatives or false positives is the most significant concern, while in other situations cost-benefit ratios are the most important factor in the selection of a specific testing approach. In the case of free-ranging wildlife, financial resources are often limited; minimizing costs while obtaining meaningful information is therefore very important.

Recently the use of RT-PCR on pooled bovine ear notch supernatant has been identified as a sensitive, specific and economically efficient diagnostic test for identifying BVDV antigen in a large number of cattle.[3,4] When a positive pool is identified, follow up tests are used to identify the positive individual within the group so that appropriate control measures can be taken. In free ranging populations however, individual animals are not identified so the use of pooled samples would be to detect the virus within the pooled population. The objective of this study was to determine the maximum pool size for deer skin samples that could be effectively used for detecting persistent BVDV infection in free ranging cervids within a region.

Materials and Methods

This study used an experimental approach; supernatant from BVDV positive and negative mule deer (*Odocoileus hemionus*) were combined to identify the minimal amount of supernatant from a positive individual that could be detected using the PCR. Positive ear tissue was obtained from a previous study in which a single male mule deer was determined to be persistently infected with BVDV based on immunohistochemical staining in the skin and BVDV PCR.[1] The tissue had been stored in a -20 degree Celsius freezer for approximately one year prior to immunohistochemical staining and PCR, then a further 6 months at -70 degrees Celsius before it was used in this study. Positive supernatant was obtained by placing a 250mg section of ear in a 5ml round bottom tube with 1ml of PBS, vortexed and allowed to stand for 10 minutes. The supernatant was removed from the tube containing the tissue and used for pooling. Negative supernatant was obtained by placing 10 250mg ear samples identified as negative on IHC, in 10ml of

PBS for 10 minutes, vortexed and removed from the tissue tube. Negative supernatant was made using tissue from 10 different mule deer each day the samples were combined.

Serial dilutions were done, titrating the positive supernatant with the negative supernatant. These dilutions were 1:1, 1:4, 1:9, 1:49, 1:99, 1:499, 1:999, 1:9,999 and a sample of negative supernatant alone. For each extraction a total of 200ul of supernatant was used. RNA extraction was done using a Qiagen mini kit[b]. The polymerase chain reaction (PCR) protocol has been published previously.[5] Included in each run was a sample from a known PI bovine, an extraction water control and a PCR water control; in some runs non-dilute supernatant from the positive deer sample was also included. To compare the sensitivity and specificity for each sample dilution, a receiver operator curve was employed.

Results

A total of 215 PCR reactions were done in 7 sessions. Four of these sessions, including 129 PCR reactions, were considered invalid. In three of four invalid sessions, all known positive samples, including positive controls, were negative. In the fourth invalid session, the RNA extraction kit was found to be contaminated with BVDV RNA. In the three valid sessions, there were 91 PCR reactions including controls. Of these, 62 were known positive pooled samples. Results are reported in Table 1. In each session, all negative pools were negative on PCR (specificity 100%) and a receiver operator curve (ROC) could not be drawn. By December 10, 2007, the single positive deer tissue sample was no longer positive by PCR.

[b] Qiagen, USA

Table 1: Dilution, number or sample pools, PCR results and sensitivity of positive pools

Dilution	Pools	Positive	Negative	Sensitivity	95% CI
1:1	4	2	2	50%	1-99%
1:4	6	5	1	83%	54-100
1:9	6	5	1	83%	54-100
1:49	6	3	3	50%	10-90
1:99	10	6	4	60%	30-90
1:499	10	2	8	20%	0-45
1:999	10	2	8	20%	0-45
1:9,999	10	3	7	43%	2-58

Discussion

This study was influenced by variables common to molecular diagnostics and sample handling; these variables complicated the interpretation of a routinely used test in a new species and the pooling of samples. In one of the sessions there was contamination of the RNA extraction kit with BVDV RNA; this resulted in all samples, including the negative controls, to be falsely positive. Contamination is a commonly cited problem in PCR because of the high sensitivity of many assays.[6,7] Sampling handling techniques and clean working conditions are imperative to minimizing cross contamination; negative extraction and PCR controls are important to include in every run to detect contamination and aid in the identification of the step in which contamination occurred. In this case the source of contamination was traced back to the buffer solution in the extraction kit. In the remaining sample runs where the results were considered invalid, the positive internal controls were not positive. Reasons for this may include failure to complete any step in the PCR process resulting in lack of amplification of the target product. The loss of

potential information from the invalid PCR reactions resulted in extremely wide confidence intervals around the estimates of sensitivity for the remaining tests.

The apparent sensitivity did not change predictably with serial dilutions; this result was likely influenced by the small sample size. Based on the premise that there would be less RNA present in the sample with subsequent dilutions, it was hypothesized that the sensitivity of the test would decrease as the pool size was increased. Although there was a general, decreasing trend in the confidence interval, the apparent sensitivity for each test showed no apparent pattern. The paucity of samples available for testing at some dilutions resulted in extremely wide confidence intervals and the apparent sensitivity is likely not meaningful in these cases. All supernatant pools from animals negative on BVDV IHC were negative on PCR resulting in a specificity of 100%.

Unfortunately, increasing the number of tests done to narrow the confidence interval was not possible because the single sample of BVDV positive deer skin became falsely negative. The sample had been collected almost 24 months prior to the experiment and was frozen at both -20 and -70 degrees Celsius. As this sample was the only tissue available for experimentation, it was removed from the freezer and handled on multiple occasions. Both the protracted nature of the freezing and the freeze-thaw-refreeze process may have resulted in false negative results as RNA viruses tend to be fragile. In house diagnostic test evaluation revealed that desiccation, protracted emersion in PBS and heating of the ear notch samples resulted in false negatives on BVDV RT-PCR (Ushijima, A., unpublished). Although this study did not investigate the impact of

different freezing temperatures, duration or freeze-thaw on the test, it may be hypothesized that similar handling could impact the sensitivity of the assay.

In cattle, pooled PCR for BVDV has been reported to have a sensitivity of 100% (95% CI, 85-100%) and a specificity of 98% (95% CI, 93-99). This study utilized field samples that may have been subjected to adverse handling conditions, however, when at the laboratory tissue was not frozen for the duration of the single deer sample used in this study; this difference may partially account for the decreased sensitivity of the PCR on mule deer tissue. Alternatively, differences in sensitivity may be related to differences between deer and cattle that influence the PCR which was developed for use in bovines. It is unlikely that the virus itself was the source of variation between species as the virus identified in the mule deer was consistent with BVDV type I commonly identified in domestic cattle of Colorado.[1]

If sample pooling is to be used for identification of BVDV within wild animal populations, it would be optimal if the test had a high sensitivity. A high sensitivity is needed so that any population of animals with a positive test result could be investigated further to verify BVDV infection in the group (identify false positives) and estimate prevalence. Prevalence of disease within the population does not affect the sensitivity or specificity of a test, but it can influence the positive and negative predictive values. For a disease with a low prevalence, the negative predictive value is high but the positive predictive value is low. In general, this means that most test negatives are truly disease negative, however the probability of a sample that tests positive to truly be positive is

low. In the case of pooled BVDV PCR on deer ear supernatant however, the test is 100% specific and therefore the positive predictive value would also be 100% and follow-up testing would not be necessary to confirm a positive result on pooled samples.

This study was markedly limited by having tissue from only a single positive deer, and only a small section of skin from that animal. Persistently infected cattle have been shown to have abundant viral antigen in their skin, however antigen has also been demonstrated in the skin of acutely infected individuals suggesting that assays that identify viral antigen may also be used to identify an acutely infected individual.[8] Applying this test in a new species necessitates the evaluation of the ability of the PCR to differentiate between a true PI and an acutely infected individual. Similarly, it may be that not all PI deer, like cattle, have a uniform quantity of virus per area of skin and connective tissue. For this reason further evaluation of the pooled supernatant technique is required using tissue from multiple persistently and acutely infected animals.

Beyond parameters inherent to the test procedure itself, the usefulness of pooled testing for BVDV in free ranging deer would be influenced by the selection for individuals within a herd. Only by collecting samples from a group of animals that share common features, like a geographic range, is it possible to begin to understand the ecology of the disease. Identification of regions with positive animals relative to those without would allow for the investigation into risk factors for disease in wild animals.

In cattle there are numerous diagnostic assays for BVDV exposure and infection, many of these tests could be used in, or modified for use in, other species. As with cattle however, the selection of the appropriate test necessitates a solid understanding of the pathogenesis of BVDV infection in the host along with characteristics of the tests themselves. Identification of persistently infected individuals represents the 'tip of the iceberg' as the prevalence of persistent infection is much lower than acute infection. In cattle however, identification of PI calves is considered the key risk factor for BVDV within a herd[9]; as such it is an important measure of virus within the herd.

With further research, techniques for pooling supernatant from deer skin may make the application of this test in wild animals useful; however the acquisition of tissue for use in testing may limit the application of this test. If tissues collected for other, routine testing could be used for BVDV diagnostics this would make initiation of such a testing program more financially, and logistically, appealing. In states in which chronic wasting disease surveillance is mandatory or recommended, lymphoid tissue is harvested from thousands of animals each season. The positive deer sample used in this study was also positive on PCR of the retropharyngeal lymph node; however nothing is known about the sensitivity of specificity of this tissue for use in BVDV surveillance programs.

Although this study was limited by available material for testing, preliminary results suggest that supernatant from a section of deer skin, positive for BVDV on immunohistochemistry, may be combined with abundant negative supernatant and often be detected. This suggests that pooling of samples from multiple animals sharing

biologically significant characteristics such as location, may be used to identify areas in which BVDV is present in free ranging animals. By targeting areas known to contain positive animals, research efforts can be focused such that maximal information regarding this interspecies transmission is obtained.

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Chapter 7: Bovine Viral Diarrhea virus in wild cervids of Colorado: Estimation of risk to domestic cattle using a stochastic simulation modeling approach

Introduction

With growing frequency, infectious disease agents previously thought to be restricted to a single host are being recognized in new species. Increased identification of interspecies transmission may be a function of more testing and better diagnostic modalities. However escalating aggregation and interaction of animal species may also play an important role in increased recognition of interspecies transmission. One particular area of concern is transmission of diseases between free ranging and domestic animal species. So called 'interface' diseases are bi-directional and can affect both livestock and wildlife populations[1]. Much of the research on interface diseases focuses on agents of regulatory concern like tuberculosis[2] or brucellosis[3]. Non-regulated disease agents can also be transmitted; given the lack of standardized testing and reporting protocols, the magnitude and significance of this transmission is often difficult to discern.

Bovine viral diarrhea virus (BVDV) is an economically significant pestivirus that, in cattle, can manifest a variety of ways; the outcome of infection is dictated by both host and agent factors and ranges from subclinical infection to severe disease. For maintenance of the virus within a herd, the creation of persistently infected (PI) calves following the infection of a cow in her first trimester of pregnancy is the most important

factor; these PI animals shed abundant virus for life[4]. Pestiviruses have poor host specificity and serological evidence suggests a broad range of wild animal species that have been exposed to and infected with BVDV[5]. Recently, experimental studies have confirmed that persistently infected cattle cohabited with pregnant deer will result in persistently infected fawns[6]. The presence of a wildlife reservoir for BVDV could markedly impede disease control programs.

In Colorado, surveys of BVDV titers in deer have identified a >60% seroprevalence in some areas[7] along with naturally occurring persistent infection[8]. Although the prevalence of persistent infection is low, the significance of this finding is unclear. Challenges inherent to the investigation of infectious disease in free ranging animal populations include logistical difficulties in procuring samples and sufficient funding to conduct testing, particularly when a disease or agent is uncommon and a large sample size is needed. As such, targeted sampling can be used to increase the likelihood of obtaining sufficient samples to begin to look for risk factors. Risk analysis and simulation modeling are systematic approaches that can be employed to identify a quantitative or qualitative risk related to an action or event[9]. In the case of infectious disease investigation, such techniques may be employed to focus on a specific subset of the population or area when resources are limited. The objectives of this study were to develop a stochastic, risk assessment model to identify areas of Colorado in which BVDV in cervids poses the greatest risk to cattle, identify variables that may influence the risk of interspecies BVDV transmission, and validate model output by comparing model-identified high risk areas with regional prevalence of BVDV in cattle.

Materials and Methods

Overview

A stochastic simulation model was created to estimate the number of PI beef calves on a monthly and regional basis as a result of contact between a pregnant cow or heifer and a PI cervid. The model used regional data on cervid and cattle populations, and diagnostic data on the prevalence of persistently infected cervids in Colorado. For each iteration of the model, a region of Colorado and month was selected. The model stochastically simulated the number of PI cervids present, the number of cows or heifers that were likely to contact those PI cervids, the number of these cows or heifers that were pregnant, and then the number that were in their first trimester.

Data

Wildlife population density

The Colorado Division of Wildlife has statewide animal population estimates recorded for each data analysis unit (DAU). The DAUs are biologically significant and presumed to estimate the geographic range and density of animals within that specific geographical area. At the time of sampling for BVDV testing, there were 55 deer, 47 elk and 4 moose DAUs within the state; the location and area of a DAU varies by species. Because persistent infection has only been identified in deer of Colorado[8], deer DAUs were selected as the spatial unit of analysis. To standardize the regional populations of each species such that this data could be included in the model, population estimates for elk and moose were recalculated for each deer DAU. This was done in ArcGIS® using the

union function to create many smaller polygons, calculating the percentages of original species DAU and population of that DAU then re-summing them together for the area of the deer DAU using the dissolve function. The result was a population estimate per species for each deer DAU.

Bovine population density

Bovine populations for Colorado were taken from the National Agricultural Statistics Service 2002 Census of Agriculture[c]. Animal numbers are reported at the county level; these were converted to the deer DAU areas using a similar technique as described for other cervids. In this analysis only beef cows and heifers, excluding those on feed, were included.

Prevalence of persistent BVDV infection in wild cervids of Colorado

Data on the prevalence and distribution of persistent BVDV infection in free-ranging cervids was reported previously[8]. Briefly, animals hunted in the state of Colorado in the 2005-2006 hunting season and submitted for CWD testing to the Colorado Division of Wildlife were eligible for inclusion in the study. Testing was conducted using immunohistochemistry for BVDV antigen in the skin and results were reported by species and DAU.

[c] http://www.agcensus.usda.gov/

Model

The model was developed using a commercially-available spreadsheet package[d] and stochastic simulation add-in software[e]. The model was run for 10,000 iterations. An overview of the model is shown in Figure 1.

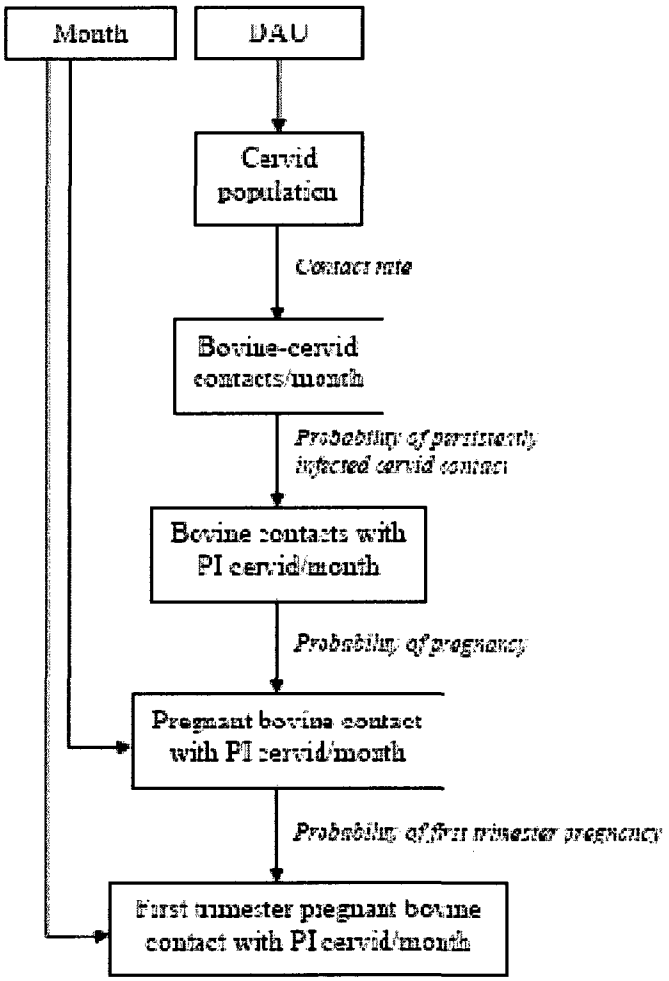

Figure 1. Simulation model overview

[d] Excel 2007, Microsoft Corporation, Redmond WA
[e] @Risk 4.5, Palisade Corporation

Model parameterization

Selection of month

Interspecies interactions and bovine pregnancy status are not uniformly distributed throughout the year. To allow for this variation, each iteration simulated a single month m, which was selected from a Uniform(1,12) distribution, with 1=January and 12=December. Pregnancy probability p_m and first-trimester probability f_m differ according to month.

Selection of region

Bovine and cervid population sizes vary according to region. To allow for this variation, each iteration simulated a single region r, which was selected from a Uniform(1,54) distribution, corresponding to the 54 deer DAU regions in the state of Colorado. Bovine and cervid population sizes, b_r and c_r, respectively, varied according to region.

Contact rates

Adequate contact rates t_m, were fixed at 0.005 (1 contact per 200 animals) based on previous studies using GPS to estimate contact between deer and cattle on pasture[10]. All adequate contacts are assumed to involve a cow or heifer that is susceptible to BVDV.

Bovine pregnancy parameters

The model simulated the seasonal breeding cycle and pregnancy status of Colorado beef herds. For each breeding month m there was probability of being pregnant p_m and a probability of pregnant animals being in their first trimester f_m. Pregnancy parameters

were based on a 63 day breeding period starting in June where 62% of the herd would be bred in the first cycle, 24% in the second cycle and 9% in the third cycle with 5% of the cow herd remaining open[11]. Probability of being pregnant was lowest (5%) at the end of the calving season in May, and highest (95%) after the breeding season in August-February. The model assumed that all beef herds in Colorado began breeding in June, no pregnancy loss occurred, and all pregnancies lasted 280 days. Among pregnant cows and heifers, probability of being in the first trimester ranged from 0 in December-May to 1 in July-September.

Cervid population size

For each region r, cervid population size c_r equaled the sum of the estimated deer d_r, moose o_r and elk e_r population sizes. The deer population d_r was the Colorado Division of Wildlife deer population estimate for the selected region, whereas o_r, and e_r were estimates calculated by redistributing moose, and elk population data from their species-specific DAU polygons to deer DAU polygons as described previously. In doing so, the moose, and elk populations were assumed to be evenly distributed within their species-specific DAUs.

Beef cow population sizes

Similarly, for each region r, the beef cow and heifer population size b_r was an estimate based on redistributing beef cow and heifer data from county polygons to deer DAU polygons. In doing so, the beef cow and heifer populations were assumed to be evenly distributed within county.

Prevalence of persistently-infected cervids

Prevalence of persistently-infected deer was assumed to be similar throughout Colorado. When all cervids were included in the model, the prevalence was assumed to be similar in moose, and elk to that of deer. Prevalence data was based on hunter-submitted deer samples as previously described.

Model structure

For each iteration i, the model selects a month m_i and region r_i. The model then selects the corresponding cervid population c_r and contact rate (bovine contacts per cervid per month) t_m. The number of cows or heifers k_i with adequate cervid contact is sampled from Binomial(c_r,t_m) if c_r is <32,000, and from a normal distribution with mean = c_r*t_m and standard deviation = t_m*(1-t_m) if $c_r \geq 32{,}000$; two distributions were used as the number of contacts with adequate cervid contact was a negative value when the population exceeded 32,000. The prevalence of PI cervids v_i is sampled from Beta(positive samples + 1, submitted samples – positive samples + 1)[9]. The number of cows or heifers l_i with PI cervid contact is sampled from Binomial(k_i,v_i). The number of cows or heifers s_i that are pregnant when they have contact with a PI cervid is sampled from Binomial(l_i,p_m). The number of pregnant cows or heifers n_i that are in their first trimester when they have contact with a PI cervid is sampled from Binomial(s_i,f_m). Model outputs include region r_i, month m_i, and number of pregnant cows or heifers n_i that are in their first trimester and have adequate contact with a PI cervid. The model was first run using only the deer population and re-run using the population of all cervids in the DAU.

Validation

Diagnostic testing for BVDV in cattle

In attempt to identify areas of heavy BVDV infection, or areas where sufficient testing had been done to report a low prevalence with any confidence, the Colorado State University Veterinary Diagnostic Laboratory database was searched to identify all BVDV results and tests between July 1, 2001 and June 30, 2005. The database was searched by codes that correspond with diagnostic tests including BVDV capture ELISA, immunohistochemistry, BVDV FA test, and BVDV PCR. Initial review of the requested tests had an animal owner name and geographic location on less than 10% of the submissions. Given the lack of geospatial referencing available, all of the testing was analyzed at the level of the submitting veterinarian.

Veterinary clinic addresses were obtained through the CSU VDL database and recorded as point data in ArcGIS; individual BVDV related tests and results were associated with each individual veterinarian. To allocate a geographical region and associated bovine population to individual veterinarians, thiessen polygons were created. Thiessen polygons are polygons created from point data (location of veterinarians) by the perpendicular bisectors of the lines between all points. The resulting shape has boundaries contain only one point and define the area that is closest to each individual point relative to all other points. Diagnostic laboratory data on BVDV testing in cattle was then converted to the deer DAU polygons using techniques described for cattle and non-deer wildlife. Patterns of BVDV testing and infection were evaluated in relation to BVDV infection identified in cervids and in the model.

Results

Disease model

The model converged, with less than 1.5% change in parameter values per iteration, in 2900 iterations. For a given month and DAU, the number of PI calves resulting from contact with a PI deer ranged from 0 to 2. No PI calves occurred 98.9% of the time with 1 PI occurring 1.0% of the time and 2 PIs occurring 0.1% of the time. The creation of PI calves was significantly different across months (X^2 0.01499, 11 df, $p<0.0001$). Figure 2 shows the number of PI calves infected by month; there is a strong seasonal trend coinciding with the breeding period and bovine gestation.

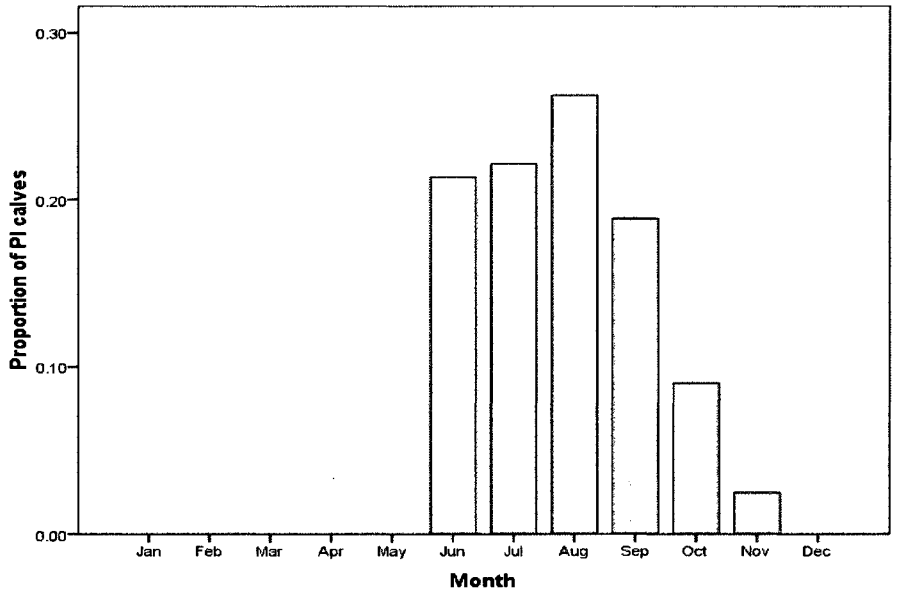

Figure 2: Histogram of PI calves resulting from bovine contact with PI deer by month.

The presence and number of PI calves was significantly different across DAUs (X^2 0.0274, 53df, $p<0.0001$). Sixteen DAUs (30%) had no PIs, 35 (65%) had 5% or less of the total number of PIs and 3 DAUs had from 6-19% of all PI calves resulting from

10,000 iterations of the model. The location of DAUs and the percentage of PIs is shown in Figure 3. When the deer population was replaced with the sum of all cervids (deer, elk, moose) within the deer DAU polygons, a similar pattern was observed with DAUs 2, 7 and 19 having the greatest numbers of PI calves created following exposure to a PI cervid.

Validation

There were 138 vets submitting 60,092 BVDV tests during the time period. The majority of tests were ELISAs, followed by FA, PCR and IHC. Distribution of diagnostic tests alone and adjusted by bovine population were evaluated visually for any spatial relationship with the expected distribution of PI cattle following contact with deer in the model. Frequencies of BVDV diagnostic tests conducted per head are reported at the level of deer DAUs in figure 4, the frequency of positive BVDV tests per head are reported in figure 5. Although there are more positive test results in areas where more testing is conducted, there was no apparent spatial relationship between any testing or results in cattle and model predictions of PI cattle resulting from contact with PI deer.

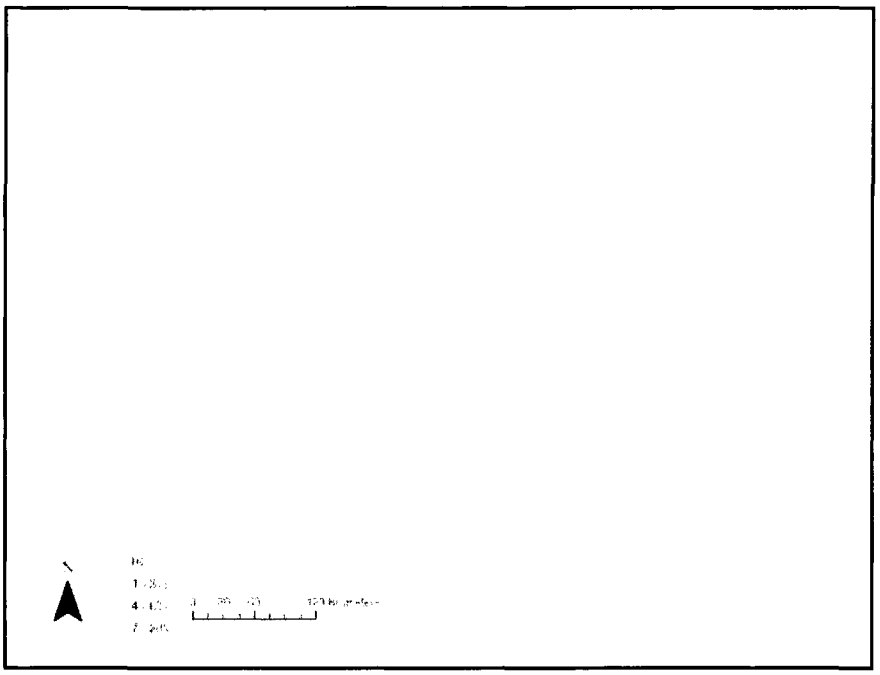

Figure 3: Percentage of total PI calves resulting from deer contact within deer DAUs of Colorado

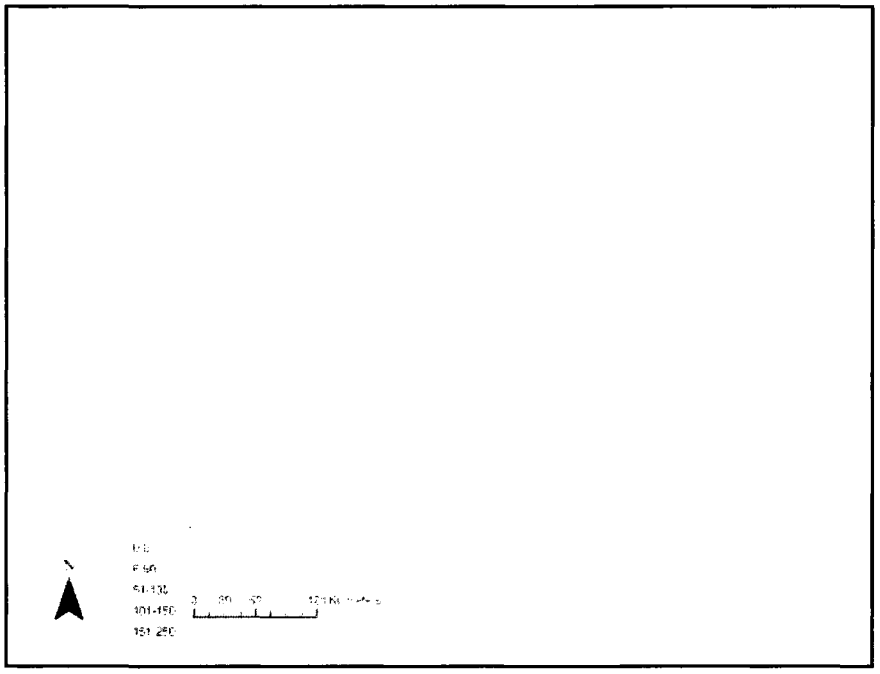

Figure 4: Number of BVDV related diagnostic tests requested per 1000 cows by DAU

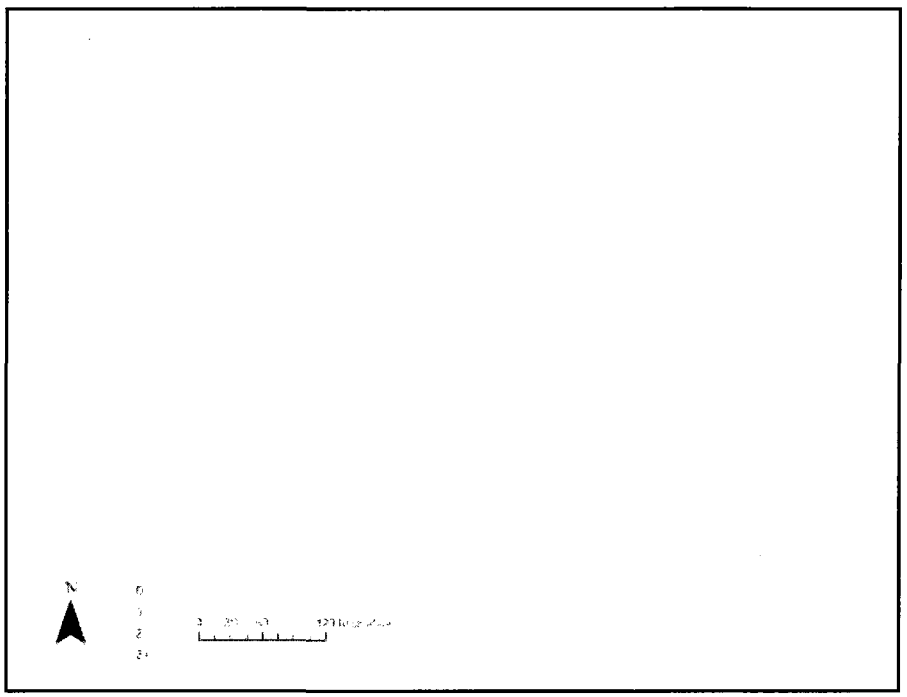

Figure 5: Positive BVDV tests per 1000 cows by deer DAU

Discussion

The objective of this study was to identify areas of Colorado where BVDV infection in cervids may pose a risk to domestic cattle using a basic, stochastic simulation model. Thirty five percent of the PI calves resulting from maternal contact with PI deer were located in only three DAUs. The areas with the greatest number of PI calves, D-7 and D-2, are adjacent to each other in the northwest corner of the state and D-2 was the site of a single IHC positive and presumed PI deer identified through a cross sectional survey for BVDV in CO cervids[8]. The area with the third highest number of simulated PIs occurring in a month, D-19, is located in Uncompahgre Plateau where a high seroprevalance to BVDV has been identified in deer[7] and two dead deer fawns, both positive for BVDV by PCR, were found within two days of each other[12]. These three

DAUs also have the highest number of deer and, in this model, the greatest number of contacts with cattle and therefore opportunities to yield persistently infected calves. When the sum of all cervids was used as the wildlife population input parameter, the same DAUs were the site of the most PI calves, these areas also remained the top three regions of the state with the most cervids.

There was a strong seasonal pattern of the months in which PI bovines would result from interspecies contact. This pattern is not surprising given the assumptions of the model, the breeding period and pasture season of beef cattle and the narrow window for infection of pregnant cows to yield a persistently infected calf. The number of PIs is not consistent during the high risk months; transmission peaks in August and then declines. This temporal pattern suggests that the highest risk period for cattle could be targeted for intervention such as decreasing contact between wild and domestic animals.

A number of assumptions were made during the development of this model; such assumptions were necessary due to the lack of quantitative data required to address the objective. Central in the analysis of herd health issues is an understanding of the population in question. For free-ranging wildlife basic population parameters are often lacking because of the marked expense of collecting such data. For this analysis, population estimates were obtained from the state wildlife agency that estimates these numbers so that hunter harvest quotas can be allocated. In Colorado the spatial areas allocated to populations is thought to represent biologically significant boundaries; however these can be difficult to evaluate. By treating DAUs as discrete units in the

model and polygons in GIS, the assumption is made that the number of animals is homogenously distributed across this area which is inherently incorrect. Deer DAU's were selected for the spatial unit of analysis because deer were the only species found to have PI in a recent survey[8]. While recalculation of the population of elk and moose was necessary to standardize the spatial unit, demolition of the species specific DAUs means a loss of any species specific clustering and movement information.

Likewise there are limitations in the information available regarding cattle. For this analysis beef cattle on range were selected for inclusion because they are presumed to be the most likely group of animals to contact wild cervids. The NASS survey provides county level population data, however it is difficult to tease out information of interest of this project as animals are classified by production type and not specifically management such as pasture grazing. The NASS data is also tied to the location of the producer and not necessarily the animals themselves; in the case of beef cattle on pasture the animals may be put on pasture hundreds of miles from the location of the surveyed owner and therefore would be incorrectly georeferenced in this analysis.

A significant gap in knowledge is the frequency of contact between wild and domestic animals. Contact rates can be assumed to vary significantly by region, species involved and season; little quantifiable information is available for Colorado. In this study a contact frequency of five deer in 1000 (0.005) have a spatial relationship with a bovine such that virus could be transmitted. When this contact rate was compared to studies that measured bovine and deer interactions using GPS collars[10] the number of contacts in the

model was consistent with the observed deer and cattle interactions. In this model however, this contact rate did not account for the regional bovine population. Given the significance of contact frequency in disease transmission a better understanding and quantification of this parameter is required before more refined estimates of disease transmission can be made.

There was no discernable relationship between the output of the model and the distribution of BVDV testing or positive results in cattle. This may be because no relationship exists or because the study lacked the sensitivity to identify such a relationship. BVDV testing is done at the discretion of the animal owner, usually following recommendation from a consulting veterinarian and testing is therefore biased. Cattle within different production systems and stages of production likely have different criteria upon which diagnostic testing would be sought; sufficient information to control for these biases cannot be obtained retrospectively from the diagnostic laboratory database. Likewise producers and veterinarians can send samples to any diagnostic laboratory that provides the test of interest, it is therefore possible that a significant number of diagnostics for CO cattle have been performed in other laboratories and the results are not available for inclusion in this study. Finally, for BVDV, results of diagnostic testing must be considered with the animal history. Individual animals may have an antibody titer and thus be deemed 'positive' for BVDV, however that result may reflect vaccination or infection. It is also difficult to retrospectively discern which animals were acutely infected versus persistently infected through the database search

methods employed. Inconsistencies associated with testing for BVDV make the use of available diagnostic lab data challenging at the state level.

Similar to the problems associated with not knowing the exact physical location of cattle, the diagnostic test information for BVDV was neither associated with a specific animal location or owner location; in this analysis it had to be considered with the location of the submitting veterinarian. This limitation to the use of diagnostic laboratory data is not restricted to BVDV and must be considered when undertaking any analysis of this nature when standardized disease and animal information is mandated. To circumvent this problem, thiessen polygons were created to allocate a particular area, and associated animals, to a veterinarian. This approach provided an estimate of testing within an area, however it is limited by the fact that veterinarians tend to be clustered in towns and that animal owners may do herd level testing through a veterinarian that is geographically very removed from where the animals are if samples (i.e. earnotches for BVDV) can be collected by farm staff during routine processing.

Simulation models for BVDV infection in cattle have been used to evaluate control strategies[13] and risk factors[14,15]. Most of this work has been done in dairy herds with limited application to a pasture situation however recent work in beef cattle suggest that these techniques may assist in management decisions[16]. Beef cattle on pasture have significantly different opportunities for exposure to wild animals than dairy cattle and this information also needs to be incorporated into models. The model created in this study was limited to the infections resulting from a wild animal and transmission of the virus

within the cattle population was not considered. Future work should involve integration of this information.

Within Colorado, a basic simulation model was used to identify geographic areas, and months, in which transmission of BVDV from cervids to cattle is most likely to occur. These areas corresponded with previous studies on BVDV in deer and represent the greatest numbers of deer within the state but do not appear to be related to regions where BVDV has been identified in bovine populations. While this study may be used to identify regions in the state in which to focus research or control strategies, of equal importance is the identification of important information that is unavailable and therefore hampers the investigation of disease transmission between wildlife and domestic animals. Accurate population estimates and inter-species contact rates are essential for evaluation of disease spread and risk analysis; these parameters may be applied to many different diseases.

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Chapter 8: Infectious disease investigations in free-ranging wildlife: lessons learned from BVDV

The aim of this dissertation was to evaluate techniques for investigation into health related events at wildlife/livestock interface using bovine viral diarrhea as a model. In chapter 2, general principles of disease surveillance were reviewed with respect to implementing such system in free ranging wildlife. Design principles of disease surveillance systems are similar regardless of the species and disease in question. There is, however, marked variation in the logistics and difficulty surrounding the implementation of such systems in different classes of animals. Wildlife disease surveillance requires unique adaptations to traditional protocols and is often challenging to conduct in an unbiased manner; interdisciplinary teams should be employed to optimize the quality of data obtained.

Given these logistical challenges, opportunistic case identification has been widely used for detection of disease events in wild animals. In chapter 3, the role of different agencies and organizations in the Rocky Mountain Region of the United States were reviewed to identify significant wildlife health events or aggregate information from multiple sources. Overall wildlife rehabilitators were in contact with the greatest number of animals; however, the data from these groups, in its current state, is insufficient for surveillance purposes. This data source could be improved by providing infrastructure for standardizing and recording animal information, providing training on disease issues, facilitating the submission of samples for diagnostic evaluation and developing long-term working relationships that benefit all parties involved. Wild animal data from all survey

groups aggregated at the level of state wildlife organizations; these agencies are therefore central in this type of surveillance activity and require sufficient resources to ensure that appropriate testing is conducted and that data and samples are being managed in such a way that they can be used in the future as necessary.

Aggregation of wild animal, health related data alone does not constitute surveillance; the synthesis, analysis and communication of findings is required to provide meaningful information to invested groups. Opportunistic case reporting may fail to identify diseases of low prevalence, or diseases that cause minimal morbidity and mortality in the wild host. Bovine viral diarrhea virus (BVDV) is an important pathogen of domestic cattle. Serological, experimental, and individual case studies have explored the presence and pathogenesis of the virus in wild ungulates; however, there remain large gaps in knowledge regarding BVDV infection in non-bovine species. The virus is assumed to have been transmitted from cattle, but a better understanding of the role of free ranging animals in the epidemiology of BVDV could aid management strategies in cattle.

Bovine viral diarrhea virus has been isolated from deer and experimental infections have provided information on acute BVDV infections in non-bovid species; however more information regarding the pathogenesis and manifestation of persistent infection in deer was required before large scale testing of wild cervids could be implemented. In chapter 4, the histopathology and immunohistochemical findings of two experimentally, persistently infected white-tailed deer (*Odocoileus virginianus*) fawns are described. Histologic lesions were minimal, but BVDV antigen was distributed widely throughout

many tissues and cell types, most notably epithelium and vascular endothelium, consistent with that reported in cattle. These findings provided information to support the use of IHC on skin ('ear notch') samples from wild cervids for the identification of persistently infected deer.

In chapter 5, results of a cross-sectional study to determine the prevalence and distribution of Colorado deer, elk, and moose persistently infected with BVDV are reported. Full-thickness ear tissue samples collected from animals presented to the Colorado Division of Wildlife for chronic wasting disease surveillance in the 2005–2006 hunting season were used; tissue from 5,597 harvested animals (2,934 mule deer, 2,516 elk, 141 white-tailed deer, and 6 moose) was paraffin-embedded and stained for BVDV using immunohistochemistry. A single adult male mule deer had BVDV antigen in the skin; staining distribution was consistent with that seen in PI cattle. Skin and lymph node were also positive for viral RNA by polymerase chain reaction, and the virus was determined to be a type 1. The prevalence of BVDV PI cervids in Colorado is very low; however, the identification of a naturally infected adult PI animal in the wild confirms the presence of the virus in free-ranging populations.

The low prevalence of infection in Colorado resulted in a costly investigation into the distribution of the virus within the state. In chapter 6, the use of pooling supernatant from ear notch samples for use in PCR is discussed. BVDV antigen could be detected in pools as dilute as 1:9999, however the pooled sensitivity was low, unreliable and insufficient positive tissue was available for testing resulting in wide confidence

intervals. Further investigation into the used of pooled diagnostics is required before this approach should be employed in a surveillance program.

Using the data collected in related projects, a basic simulation model is presented in chapter 7. This risk analysis model is designed to identify regions of Colorado in which persistently infected calves are most likely to be born to cattle exposed to BVDV from a free-ranging deer. Outputs of the model were consistent with previous studies of the virus within the area and also with regional deer population estimates. In an attempt to identify correlation with BVDV testing and diagnoses in cattle, a review of Colorado State University Veterinary Diagnostic Laboratory was conducted for the period prior to the collection of prevalence estimates from deer population. There was no spatial relationship between testing in cattle and the results of the risk model or other published results on the distribution of BVDV in deer; however the available data for cattle has biases that limit its usefulness in this type of study. Given the growing concern of diseases that may be transmitted between domestic and wild animals it is imperative that information is collected and maintained in a format such that it can be used to evaluate interspecies disease spread.

Taken together, results of these studies suggest that BVDV infection is present in Colorado wildlife, but the prevalence is low. By investigating the presence of this virus in cervids, a number of gaps were identified that limit the study of diseases that may be transmitted between wild and domestic animals. The paucity of freely available, sufficiently detailed information on basic wild animal parameters and wildlife/livestock

interactions was highlighted. Irrespective of the disease in question, lack of information regarding contact rates and population distributions will hamper investigations into interspecies transmission. Very detailed wildlife population information is available for some species in specific regions; however other species and geographic locations have very little background information. Even where detailed data exists, this information may have been collected by a particular individual or group for a directed purpose and is therefore not easily available. While there are inherent problems with the centralization of detailed biological data, and the range of species and locations is too vast to even begin to list, it would be prudent to select a subset of species and locations where free ranging animals may pose a risk to animals or humans and begin to collate this information. Many diseases may be transferred between cattle and cervids; as some of these agents are of regulatory concern, baseline population data should be centralized, or at minimum inventoried, so that this data is quickly available if it was needed in a disease intervention program.

By studying the pathways through which animal health information is transmitted, it is apparent that groups who would benefit from collaboration often do not communicate. The difficulty of procuring samples or having facilities and equipment to conduct specific testing limits the power of many wildlife studies; improved coordination of research efforts between groups would result in more efficient use of resources and increased opportunities. Communication of wildlife health information was mandated in some cases, but in others it was influenced by location, accessibility and resources. Working to

improve communication in areas where social networks are more limited and sharing information and resources would aid in the investigation of all health related events.

Based on the research to date, it is not well understood if BVDV is identified in wild animals following multiple contacts (re-introductions) of deer with BVDV positive cattle, or if the virus is circulating in the wild population. This question is fundamental should BVDV eradication, or marked reduction, ever become goal of producers and agricultural managers. If the virus is maintained in wildlife, control efforts need to include wild animals. However if infection in cervids is the result of repeated spill-over then control efforts should focus on reducing the prevalence of infection in cattle. Further research into the maintenance of BVDV within a region, with consideration given to any species that may be involved in the epidemiology, will elucidate important information for control efforts.

Diagnostic tests are central in any disease investigation and many control programs. Continued investigation into the sensitivity and specificity of individual animal and pooled tests in non-bovid species is important to ensure that maximal information may be gleaned from the limited number of samples that get to a laboratory.

Much of the funding and concern regarding infectious diseases in cervids focuses on the economic impact this may have on the agricultural industry. Although these concerns are valid, it is important to also consider the wildlife population themselves. Our ability to identify morbidity and mortality in these animals is limited and varies by species,

location and awareness. In some populations it would take a marked decrease in the population to draw the attention of people and an investigation into the change. If a change in population is noted it is likely to have been the result of multiple causes including both infectious and environmental; communication between biologists and epidemiologists is therefore essential to investigate causation.

It is often more logistically challenging to study disease in free-ranging, wild animals, but an open mind to alternative approaches for sample and data acquisition and interdisciplinary research teams can facilitate the means to answer important health related questions. Challenges should not supersede investigation into disease issues where a better understanding of the wild animal component could improve health for all species involved.