CONTROL OF PESTIVIRUS INFECTIONS IN THE MANAGEMENT OF WILDLIFE POPULATIONS

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CONTROL OF PESTIVIRUS INFECTIONS IN THE MANAGEMENT OF WILDLIFE POPULATIONS

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Artwork by Michael Marti

Infections with recognized and putative species of the genus pestivirus are not host-specific and are documented in many wildlife species. The study of pestivirus infections in wildlife species is important both to eradication programs and programs for maintaining the health and well-being of wildlife populations. Free-ranging wildlife species may act as reservoirs for pestiviruses that infect domestic species. For this reason, eradication efforts for classical swine fever virus include control of the virus in wild boar populations. The contribution of free-ranging species to the circulation of BVDV1, BVDV2, and BDV is less well understood. While substantial damage due to pestivirus infections has been demonstrated in a few specific wildlife populations, the impact of pestiviral infections on the well-being of most captive and free-ranging wildlife populations is largely unknown. The research topics summarizes our current understanding of pestiviral infections in wildlife and discusses the challenges in understanding and mediating their impact on captive and free ranging wildlife species.

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Table of Contents

Section 5: Pestiviruses in Rabbits and Zoological Collections

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EDITORIAL
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Editorial: Control of Pestivirus Infections in the Management of Wildlife Populations

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Keywords: bovine viral diarrhea virus, border disease virus, classical swine fever virus, emerging, pestivirus, wildlife

The Editorial on the Research Topic

Control of Pestivirus Infections in the Management of Wildlife Populations

The genus pestivirus in the Flavivirus family is comprised of single-stranded RNA viruses that infect domestic and wildlife hosts. The "classic" pestivirus species, bovine viral diarrhea virus type 1 (BVDV1), bovine viral diarrhea virus type 2 (BVDV2), border disease virus (BDV), and classical swine fever virus (CSFV) were first detected in domestic animals and early differentiation of pestivirus species was based on the domestic animal from which it was isolated. However, it became apparent that these four species were capable of infecting multiple domestic and free-ranging host species. In addition, emerging/putative species of pestivirus, such as pronghorn virus and giraffe virus, were isolated exclusively from wildlife species. The significant economic cost of the infection of domestic species with pestiviruses is well documented but the impact of pestivirus infections on wildlife species is less well studied.

Early reports of the isolation of pestiviruses from wildlife were treated as incidental findings. As eradication efforts for CSFV, BVDV1, and BVDV2 proceed around the world, concerns have been raised that wildlife species may act as reservoirs for pestiviruses. Data indicate that CSFV-infected Eurasian wild boar (*Sus scrofa*) pose a considerable threat to eradication programs in domestic swine. This has led to the implementation of CSFV control strategies in wild boar that combine detection, culling (with cooperation of hunters), and vaccination as detailed in the chapter by Moennig in this volume. Additionally, the chapter by Rossi et al. details the design and use of oral vaccines in wild boar populations.

The possibility of free-ranging cervids, small wild ruminants, and rabbits to be reservoirs for BVDV1 and BVDV2 is less clear. Viral detection and serological surveillance both indicate that multiple species of wild ruminants are susceptible to BVDV1 and BVDV2 infection as reported in the chapter by Wolff et al. Epidemiological data indicate that in North America, BVDV1 and BVDV2 infections have become established in wild ruminant populations, and that persistently infected animals are present in these populations as described for white-tailed deer in the chapter by Passler et al. However, to date, evidence demonstrating the introduction of pestiviruses into naïve cattle herds by exposure to wildlife is limited. In Europe, several free-ranging species have been investigated for the potential to be reservoir hosts for BVDV, and the chapters by Larska and Grant et al. discuss the potential for reindeer and rabbits as vectors for the reintroduction of BVDV1 into naive cattle herds. While questions regarding the role of wildlife species as reservoirs remain, it is becoming increasingly apparent that pestiviruses pose a threat to the health of captive and free-ranging wildlife.

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The potential of pestivirus infections to endanger wildlife species, such as chamois and big horn sheep should not be underestimated. The impact of BDV infections on reintroduced Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) demonstrate that pestivirus infections can contribute to the extinction of wild populations as detailed in the chapter by Serrano et al. While the population impact is less well understood, there is clear evidence for widespread circulation of BVDV in Rocky Mountain bighorn sheep (*Ovis canadensis, canadensis*), mountain goats (*Oreamnos americanum*), and mule deer (*Odocoileus hemionus*), and BVDV infection may contribute to pneumonia die-offs or reproductive disease as discussed by Wolff et al.

Captive wildlife species in zoological collections exist in a unique environment for disease transmission and circulation of pestiviruses in zoo and parks has been demonstrated as described in the chapter by Kottwitz and Ortiz. Among pestiviruses, BVDV 1 and 2 are of special concern in zoos due to the numerous exotic ruminant species these viruses can infect. As in cattle, persistently infected animals are central in the epidemiology of BVDV and have been described in many captive and free-ranging species, including captive mountain goats, as described by Nelson et al. An additional source for the introduction of BVDV into susceptible populations is embryo transfer, which is increasingly used in the propagation of at-risk wildlife populations. Therefore, it is important that screening of all reagents, particularly fetal bovine serum, for pestiviruses become a part of standard protocols.

The chapter by Ridpath and Neill discusses the challenges in detecting and determining the impact of pestivirus infections in wild populations. The surveillance for pestivirus infections in free-ranging wildlife species is hampered by the lack of species-specific reagents. In addition, surveillance of free-ranging species is hindered by the lack of regular surveillance programs and challenges in collective representative samples from wild populations. Estimation of the impact of pestivirus infections on population health rests on the development and institution of regular surveillance programs that focus on free-ranging and captive wildlife. Such programs will be highly dependent on a local veterinary support working closely with hunters, wildlife, and forestry agencies.

Two of the emerging pestivirus species, pronghorn virus and giraffe virus, have only been detected in wildlife species. The first detection of both species was incidental. It is probable that an organized surveillance of free ranging ruminants, particularly in the African continent, would detect other pestivirus species. Detection would, once again, be dependent on research reagents specific to wild ruminant species and representative samples of wild populations. The emergence of detection protocols based on techniques such as next generation sequencing may to some extent lessen the need for species specific reagents. However, the procurement of representative samples will remain a challenge.

The lack of host-specificity allow pestiviruses to infect domestic livestock as well as captive and free-ranging wildlife, posing unique challenges to different stakeholders. While current control measures for BVDV are focused only on cattle, increased attention on the status wildlife species is necessary, as is already done for CSFV control. The impact of pestiviruses on captive and free-ranging wildlife is less well understood; however, examples of substantial damage exist, necessitating increased research attention on the effects of pestiviruses on the health of heterologous hosts. As noted above, it is probable that there exist other pestivirus species in wildlife populations. Therefore, another research focus should be on the development and implementation of appropriate tools that allow detection of these novel pestiviruses. Although great advances have been made over the last decades, novel discoveries, such as the expansive role of heterologous hosts in the epidemiology of pestiviruses, continue to shape the understanding of the "classic" pestiviruses and require further vigorous research.

AUTHOR CONTRIBUTIONS

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PERSPECTIVE
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Challenges in Identifying and Determining the Impacts of Infection with Pestiviruses on the Herd Health of Free Ranging Cervid Populations

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Although most commonly associated with the infection of domestic livestock, the replication of pestiviruses, in particular the two species of bovine viral diarrhea virus (BVDV), occurs in a wide range of free ranging cervids including white-tailed deer, mule deer, fallow deer, elk, red deer, roe deer, eland and mousedeer. While virus isolation and serologic analyses indicate that pestiviruses are circulating in these populations, little is known regarding their impact. The lack of regular surveillance programs, challenges in sampling wild populations, and scarcity of tests and vaccines compound the difficulties in detecting and controlling pestivirus infections in wild cervids. Improved detection rests upon the development and validation of tests specific for use with cervid samples and development and validation of tests that reliably detect emerging pestiviruses. Estimation of impact of pestivirus infections on herd health will require the integration of several disciplines including epidemiology, cervid natural history, veterinary medicine, pathology and microbiology.

Keywords: pestivirus, cervids, wildlife diseases, surveillance, sampling

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INTRODUCTION

The recognized species of the Pestivirus genus include bovine viral diarrhea virus types 1 (BVDV1) and 2 (BVDV2), classical swine fever virus (CSFV), and border disease virus (BDV) (Simmonds et al., 2012). In addition to these four species, five putative species have been proposed; Bungowannah virus, giraffe virus, HoBi-like virus, pronghorn virus (PHV) and atypical porcine pestivirus. All four of the recognized species have been isolated from free ranging wildlife populations and two of the putative species, giraffe virus and PHV, have only been isolated from free ranging wildlife species (**Table 1**). Despite abundant evidence that pestiviruses currently circulate in wildlife populations, the full impact of exposure and prevalence of these infections are largely unknown. The limited information available regarding prevalence is mainly in the form of serological surveys (**Table 2**). Even though these studies have been limited and sporadic, they have demonstrated that a wide range of wildlife species havea wide range of wildlife species has been infected by pestiviruses. Further, controlled studies have shown that pestiviruses infect wild species and once infected they may transmit virus (Grondahl et al., 2003; Uttenthal et al., 2005, 2006; Duncan et al., 2008a; Nelson et al., 2008; Passler et al., 2010; USDA, 2010; Pruvot et al., 2014). While it is possible that positive serology results may be due to contact with domestic species, the high prevalence of seropositive samples within some isolated wild life populations without close contact with domestic species suggest that

pestiviruses are being maintained independently within wildlife populations. This is illustrated by a study in which the geographic location of BVDV antigen-positive cattle and BVDV-seropositive white-tailed deer were analyzed using the dual kernel density estimation method. An exploratory cluster analysis revealed 1 significant cluster of BVDV antigen-positive herds and 2 significant clusters of BVDV-seropositive deer. There was no spatial overlap between the clusters suggesting that BVDV is maintained independently in domestic livestock herds and in the white-tailed deer population.(Kirchgessner et al., 2013).

The purpose of this article is to review reports regarding pestivirus infections in wild cervids and to summarize some of the challenges involved in determining the impact of pathogens infecting free ranging cervids.

SURVEILLANCE BASED ON DETECTION OR ISOLATION OF PESTIVIRUSES

Pestiviruses, principally BVDV1 and BVDV2, have been detected in samples collected from free ranging cervid populations (**Table 1**). However, isolations or detection by PCR tend to be a rare event among the populations surveyed (for references see **Table 1**). Cattle may be acutely or persistently infected with BVDV (Evermann and Barrington, 2005). Similarly it has been demonstrated that, under experimental conditions, cervids may be acutely or persistently infected with pestiviruses such as BVDV1 or BVDV2 (Passler et al., 2007, 2009; Ridpath et al., 2007, 2008). Experimental infections with typical field strains of BVDV in immunocompetent cattle and white tailed deer tend to be mild or asymptomatic (Ridpath et al., 2007, 2013). The majority of the surveys conducted to date relied on serum or ear notch samples which, at least in cattle, are better for detecting persistent infections than acute infections (Ridpath et al., 2002; Liebler-Tenorio et al., 2004). Further, based on the pattern of viral antigen present in various tissues it appears that the pestivirus positive deer harvested from free ranging populations were probably persistently rather than acutely infected. In cattle, persistently infected animals make up less than one percent of the population at slaughter but have a significant impact on the health of cohorts (Hessman et al., 2009). The detection of persistently infected animals (PI) in any population, domestic or free ranging, is significant as PIs act as efficient vectors for keeping the virus in circulation. However, persistent infections in deer are only established if the fetus is infected in the first one third of pregnancy (Ridpath et al., 2008, 2012). Thus, infections of the fetus occurring during the final two thirds of pregnancy and all infections of animals post-birth result in acute infections rather than persistent infections. Failure to detect acutely infected animals will lead to underestimation of infection rate. Therefore, while detection of PIs yields significant information it cannot be used as a measure of prevalence of infection.

SEROLOGICAL SURVEYS

Antibodies against pestiviruses have been identified from serum collected from seven different families of free ranging wildlife; Antilocapridae, Bovidae, Giraffidae, Cervidae, Suidae, Camelidae, and Leporidae with the greatest number of wildlife host species in the Bovidae and Cervidae families (**Table 2**). In North America, the largest numbers of wild ruminants are found in the Cervidae family (Flather et al., 2009) with five species being represented: moose (*Alces alces*), elk/wapiti (*Cervus elaphus*), caribou/reindeer (*Rangifer tarandus*), mule deer (*Odocoileus hemionus*), and white-tailed deer (*Odocoileus virginianus*) (Conner et al., 2008). Pestivirus neutralizing antibodies have been detected in free ranging populations of all five species (**Table 2**). A limitation of serological surveys is that the level of antigenic cross reactivity between pestivirus species makes it difficult to absolutely identify the pestivirus species elicited the immune response (Dubovi, 2013).

Many serological surveillance studies in wildlife arise out of pestivirus control programs aimed at clearing a pestivirus species, such as BVDV1 and BVDV2, from domestic animal populations. The primary goal of many of these studies is to determine if wildlife species can serve as virus reservoirs for domestic species, not to determine the level of infection in wildlife populations. The significant problem with these serological surveillance studies is that the level of neutralizing antibodies is only determined against one of the four recognized pestivirus species and this may result in underestimation of infection with emerging pestivirus species. This was noted by the authors in one of the earliest large scale serology surveys of wildlife which used samples collected from free ranging ungulates residing in Africa (Hamblin and Hedger, 1979). This survey evaluated 3359 sera, collected from multiple species of wildlife in nine African countries, for neutralizing antibodies against BVDV. At that point in history, the BVDV2 species had not yet been identified. Thus, the laboratory reference strains used in this study only belonged to the BVDV1 species. Neutralizing antibodies were detected in sera from 17 different species. The authors noted that because pestiviruses are cross reactive it is possible that the serum neutralizing antibodies reported in their study, may be due to cross neutralization with "other viruses as yet unrecognized." It is also highly possible that antibodies against pestiviruses with limited cross reactivity with BVDV1 could have been missed in this and other studies.

Aside from an interest in pestiviruses that impact domestic species there are other reasons for the use of classic pestivirus strains in assays. Firstly, cytopathic reference strains from each of these species are readily available. This is not true of all emerging pestivirus species. To date only noncytopathic strains of the Giraffe and Pronghorn species are available. When cytopathic strains are used in virus neutralization (VN) tests, end points may be determined by observation of the cell monolayer. End point determination using noncytopathic strains requires secondary detection methods such as immunofluorescence, immunohistochemistry staining or polymerase chain reaction. Use of such secondary detection methods is time and cost prohibitive for large-scale surveillance projects.

Another consideration is that frequently emerging viruses, such as pronghorn virus (Vilcek et al., 2005) or atypical porcine pestivirus (Hause et al., 2015), do not initially grow well in cell

TABLE 1 | Detection of pestivirus species in samples collected from free ranging wildlife populations.

Pestivirus species	Family	Wildlife Population	Country	References
BVDV1 and BVDV2	Cervidae	White-tailed deer (*Odocoileus virginianus*)	US	Chase et al., 2008; Passler et al., 2008
BVDV1 BVDV2		Mule deer (*Odocoileus hemionus*)	US	Van Campen et al., 2001; Duncan et al., 2008b; Wolff et al., 2016
BVDV		Scottish red deer (*Cervus elaphus scoticus*)	Scotland	Nettleton et al., 1980
BVDV1		European roe deer (*Capreolus capreolus*)	Germany	Fischer et al., 1998
BVDV		Water deer (*Hydropotes inermis*)	South Korea	Kim et al., 2014
BVDV1		Sika deer (*Cervus nippon*)	China	Gao et al., 2011
BVDV1	Bovidae	Bighorn sheep (*Ovis canadensis*)	US	Wolff et al., 2016
BVDV1		Mountain goat (*Oreamnos americanus*)	US	Wolff et al., 2016
BDV		Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*)	Pyrenees (border between France and Spain)	Arnal et al., 2004
BVDV1		Canadian bison (*Bison bison bison*)	Canada	Deregt et al., 2005
CSFV	Suidae	Wild boar (*Sus scrofa*)	France	Simon et al., 2013
Giraffe	Giraffidae	Giraffe (*Giraffa camelopardalis*)	Kenya	Plowright, 1969
Pronghorn	Antilocapridae	Pronghorn (*Antilocapra americana*)	US	Vilcek et al., 2005

TABLE 2 | Free ranging species with reported titers against pestiviruses.

Family	Species	Geographic regions	References
Bovidae	Gemsbok (*Oryx gazella*), Roan antelope (*Hippotragus equinus*), Blue wildebeest (*Connachaetes taurinus*), Kudu (*Tragelaphus strepsiceros*), Eland (*Taurotragus oryx*), Buffalo (*Syncerus caffer*), Nyala (*Tragelaphus angasi*), Waterbuck (*Kobus ellipsiprymnus*), Defrassa waterbuck (*Kobus defrassa*), Lechwe (*Kobus leche*), Reedbuck (*Redunca arumdinum*), Sable antelope (*Hippotragus niger*), Oryx (*Oryx gazella*), Tsessebe (*Damaliscus lunatus*) Hartebeeste (*Alcelaphus buselaphus*), Wildebeeste (*Connachaetes taurinus*), Impala (*Aepyceros melampus*), Springbok (*Antidorcas marsupialis*), Duiker (*Sylvicapra grimmia*), Chamois (*Rupicapra pyrenaica pyrenaica*), Mouflon (*Ovis orientalis*), Bighorn sheep (*Ovis canadensis*), European bison, American bison	Africa, North America, Europe	Hamblin and Hedger, 1979; Depner et al., 1991; Marco et al., 2011; Wolff et al., 2016
Cervidae	Water deer (*Hydropotes inermis*), Reindeer/Caribou (*Rangifer tarandus*), Roe deer (Capreolus capreolus), Red deer (Cervus elaphus), Moose (Alces alces) Fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), Sika deer (*Cervus nippon*)	Asia, North America, Europe	McMartin et al., 1977; Lawman et al., 1978; ElAzhary et al., 1979; Couvillion et al., 1980; Van Campen et al., 2001; Lillehaug et al., 2003; Kim et al., 2014; Wolff et al., 2016
Giraffidae	Giraffe (*Giraffa camelopardalis*)	Africa	Hamblin and Hedger, 1979; Depner et al., 1991
Antilocapridae	Pronghorn antelope (*Antilocapra americana*)	North America	Barrett and Chalmers, 1975
Camelidae	Vicuna (*Vicugna vicugna*)	South America	Marcoppido et al., 2010
Suidae	Wild boar (*Sus scrofa*), Wart hog (*Phacochoerus aethoiopicus*)	Europe, Africa	Hamblin and Hedger, 1979
Leporidae	European rabbit (*Oryctolagus cuniculus*)	Europe	Frolich and Streich, 1998

lines commonly used in the laboratory (Vilcek et al., 2005). Finally, the pestivirus that the wild population was infected with may not yet have been isolated and characterized.

While there are valid reasons why serological surveys, based on VN tests, use reference strains from the four recognized species, it is highly probable that when these assays are used in

such surveys they miss titers resulting from exposure to emerging viruses that are genetically distant and antigenically distinct. The greater the genetic difference between pestiviruses, the lower the cross reactivity (Ridpath et al., 2010; Bauermann et al., 2012). For example, the emerging bovine pestivirus species known as HoBi-like virus, while distinct, is closer to the two BVDV species than to other emerging pestivirus such as pronghorn virus. In one study it was shown that a serum collected from a bovid infected by a HoBi-like viruses had a greater than 1/500 titer against a HoBi-like virus, averaged a greater than 1/300 titer against BVDV2 strains but did not neutralize the pronghorn virus (Bauermann et al., 2012).

While commercial ELISA kits are available for detecting antibodies against the classic pestiviruses, particularly BVDV, the limited cross reactivity that exists between emerging pestiviruses and classic pestiviruses make these tests unreliable for detecting antibodies resulting from infection by emerging pestiviruses (Bauermann et al., 2012). Further, these commercial tests are not designed to differentiate between antibodies raised against different pestivirus species.

While performing serology on a one time collection of samples from a population can give information on the occurrence and prevalence of exposure, it does not yield information on when the exposure occurred. To estimate time of exposure, multiple samples over time must be collected and archived.

CHALLENGES IN THE COLLECTION OF REPRESENTATIVE SAMPLES

Ideally samples should be representative of the population under study including biological, spatial, and temporal variables (Stallknecht, 2007). Further, samples must be collected while virus is present in tissues and tissues must be tested using technologies that maximize the probability of detecting the agent (Thurmond, 2003). Issues of access, cost and feasibility frequently preclude the gathering of such ideal samples.

If the goal is to detect a pestivirus the sample must be collected while the animal is still viremic. This not a problem with persistently infected cervids but is a problem with acutely infected cervids where the window of detectable viremia may be less than 5 days (Ridpath et al., 2007).

Both passive and active surveillance systems may be used to obtain cervid samples. Passive surveillance, which relies upon the observation and subsequent testing of an animal displaying clinical signs of disease or collection of samples from animals that have died of disease, is problematic for detecting infection with viruses, such as pestiviruses, which don't cause severe clinical disease. Passive surveillance tends to under estimate the impact of diseases that have significant mortality rates let alone those that result in subclinical disease. This is illustrated by an outbreak of hemorrhagic disease in white-tailed deer that occurred in Missouri. While it was estimated that the outbreak resulted in an 8% mortality rate, not one case of mortality or morbidity was reported by the public. The occurrence and extent of the outbreak were only noted because of surveillance conducted on 100 radio-monitored deer (Beringer et al., 2000). Some surveys for BVDV in deer have depended on getting samples from deer that were harvested by hunters (Duncan et al., 2008b; Passler et al., 2008). Hunting licenses usually require that the harvested animals are adults and most hunters desire to harvest healthy specimens. Thus, hunter harvested samples tend to represent healthy animals that have lived to sexual maturity, and based in studies in cattle, restricting surveys to healthy adults may result in underestimation of the incidence of persistent infection. In cattle it has been observed that animals persistently infected (PI) with BVDV are more frequently found among young stock than older stock because some (but not all) PI cattle succumb in the first year of life (Houe, 1992).

Even though hunter harvested samples may be skewed against including PI animals, BVDV PI animals have been detected in these samples (Van Campen et al., 2001; Chase et al., 2004; Duncan et al., 2008b; Passler et al., 2008) albeit at a low rate varying from 0.03 to 0.2%. The presence of PI deer indicates that BVDV circulates in these populations; however, their impact is difficult to assess.

The design of active surveillance systems requires an understanding of the social organization of the species to be studied. Unlike domestic livestock, wild deer do not confine their activities to large herd groups, cannot be rounded up without damaging ecosystems and social grouping, and are not amenable to handling. Populations are frequently divided into small breeding groups based on age and gender and contact between groups and make up within groups may change with the season. Neonates are frequently hidden rather than grazing with the herd.

The ideal surveillance program would include samples collected at multiple time points allowing retrospective analysis (Stallknecht, 2007). Archived samples are fundamental to estimating the introduction of a pathogen or detecting an increase in the incidence of infection.

ASSESSING THE IMPACT OF PESTIVIRUS INFECTIONS

It is easier to assess the impact of infection with high virulence pestivirus strains that result in clinically severe acute disease such as classic swine fever in swine or hemorrhagic syndrome in cattle. However, the impact of lower virulence pestiviruses is harder to assess, even in domesticated species. Previous studies using captive deer have demonstrated white-tailed deer infected by pestiviruses such as BVDV1, BVDV2, and PHV display very mild clinical signs even though they are undergoing significant immune suppression (Van Campen et al., 1997; Vilcek et al., 2005; Ridpath et al., 2007, 2008, 2012). While the immune suppression may lead to reduction in herd health and numbers, the contribution of pestivirus infections to the problem may be difficult to establish. The prevalence of BVDV persistent infection in cattle, while low, has significant impact on production. Lonergan et al. determined that while PI cattle represent only 0.3% of the cattle population on arrival in feedlots, they accounted for 2.6% of chronically ill cattle and 2.5% of cattle that died during the observation period (Loneragan et al.,

2005). Perhaps more importantly, exposure to PI animals has a significant impact on the health of cohorts. In the same study it was found that the risk of initial treatment for respiratory tract disease was 43% greater in cattle exposed to a PI animal, compared with those not exposed to a PI animal. Overall, 15.9% of initial respiratory tract disease events were attributable to exposure to a PI animal. In a subsequent study, Hessman et al. (2009) demonstrated that aside from overt disease, growth rates and feed conversion were negatively affected by the presence of PI cattle in feedlots. Comparing cattle lots with direct exposure to a PI with those without direct exposure revealed significant deficits in all performance outcomes associated with PI exposure. In the wild, where the rule is survival of the fittest, pestivirus infections which reduce efficiency in feed conversion and resistance to disease could be instrumental in a decline in animal numbers and population health.

CONCLUSIONS

The limited serologic surveillance that has been published focused on the levels of neutralizing antibodies against the recognized pestivirus species. Such studies may underestimate exposure to emerging pestiviruses. The value of serological studies is greatly enhanced if sequential testing of the same population over is conducted. Samples, collected from the same population, over time allows detection of changes in exposure patterns.

Many studies rely on samples generated from deer harvested by hunters. However, such samples may yield skewed data as the majority of hunter-generated samples come from healthy, primarily male adults. Further, the tests available are designed for detection of recognized pestiviruses in domestic species. The reagents used may not be appropriate for wild cervids or emerging pestiviruses that are only distantly related to the recognized pestivirus species. In particular, cell cultures derived from domestic species may not work for the propagation of viruses that are adapted to cervid hosts. In summary, the full impact of pestiviruses on cervid populations may not be recognized at this time.

Improved detection rests upon the development and validation of tests specific for use with cervid samples and the development and validation of tests that reliably detect emerging pestiviruses. Estimation of the impact of pestivirus infections will require the integration of several disciplines including epidemiology, cervid sociology, veterinary medicine, pathology and microbiology.

AUTHOR CONTRIBUTIONS

JR organized and drafted article. JN reviewed and amended article. Both authors agree to be accountable for this work.

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MINI REVIEW
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Pestivirus infection in reindeer (*Rangifer tarandus*)

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Reindeer species (*Rangifer tarandus,* Linnaeus, 1758) includes wild and semi-domesticated ruminants belonging to *Capreaolinae* subfamily of *Cervidae* family reared in Eurasia (reindeer subspecies) and North America (caribou subspecies). Herding of reindeer has a great historical, socio-economic and ecological importance, especially to indigenous ethnic minorities. Infectious disease threats may therefore impact not solely the animal population driving it to further extinction and irreversible alterations to the wild environments of northern hemisphere, but also add to cultural changes observed as negative impact of globalization. Introduction of new technologies to control of reindeer migration between dwindling pasture areas and intensification of reindeer husbandry may facilitate the intra- and interspecies transmission of pathogens. The role of the reindeer as a potential BVDV reservoir has been studied, however, the number of publications is rather limited. The observed seroprevalences of the virus varied significantly between different geographical regions with different epidemiological situation. Most frequently limited number of animals studied and the differences in the sensitivities and specificities of the diagnostic test used could have also influenced on the differences between the studies. No pestivirus has been ever detected in free-ranging reindeer, however, a putative pestivirus strain named V60-Krefeld has been isolated from reindeer kept at a German Zoo in the 1990's. The virus was characterized as border disease virus type 2 (BDV-2) closely related to German ovine strains. The cross-neutralization studies of the semi-domesticated reindeer sera from Sweden suggested infection with a strain related to BDV-1 or BDV-2. The available data indicates that reindeer might be infected by a endemic species-specific BDV-like strain. However, the interspecies transmission of BVDV from domestic animals should not be excluded, since the susceptibility of reindeer to BVDV-1 has been confirmed under experimental conditions.

Keywords: pestivirus, BVDV, BDV, reindeer, caribou

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REINDEER HUSBANDRY

Reindeer are cervids, which belong to *Rangifer tarandus* species (Linnaeus, 1758), subfamily *Capreaolinae* (New Word, telemetecarpal deer, Brookes, 1928). The worldwide population is estimated at more than four million animals of several main subspecies occupying different regions including Eurasian reindeer (*R. t. tarandus*) and caribou inhabiting Canada, U.S. (*R. t. caribou*) and Alaska (*R. t. granti*). Reindeer subspecies differ greatly in their size with the largest being the woodland caribou and the smallest being the wild Svalbard reindeer (*R. t. platyrhynchus*,

Vrolik, 1829) from Norwegian archipelago of Svalbard (with the largest island of Spitzbergen). The circumpolar distribution of reindeer determines their diet, migration potential and reproduction specificity. As the only mammals, they are able to survive winter by feeding only on lichen (*Cladonia rangiferina*). They breed seasonally, however, their reproduction cycle may adapt to climate changes and access to food. The greatest threat to reindeer are predators responsible for the high mortality in particular among the youngest animals (Tryland, 2012). The alterations of the climate, which may influence winter lichen pastures lead to the necessity of supplementary feeding. Additionally, fragmentation of pasture areas disrupts natural migration routes and increases the need of transport of reindeer by lorries and usage of motor vehicles such as four-wheel drive cars, snow scooters and helicopters in their herding (Tryland, 2012). Modernization, changes of reindeer management, gathering of animals for censusing or sorting for slaughter, ear tagging, and veterinary care increases animal density and stress, and finally can lead to increased risk of transmission of pathogens (Malmfors and Wiklund, 1996).

Reindeer were reported to suffer from foot-and-mouth disease (FMD), necrobacillosis, pasteurellosis, anthrax, brucellosis, paratuberculosis, infectious keratoconjunctivitis (IKC) and contagious ecthyma (Gavier-Widen et al., 2012; Tryland, 2012). Some of the pathogens are endemic in reindeer such as Cervid herpesvirus 2 (CvHV2; das Neves et al., 2010), CvHV3 and *R. tarandus* papillomaviruses 1 and 2 (RtPV1, RtPV2) associated with IKC (Smits et al., 2013). Despite the limited number of studies on pestivirus infections in reindeer, they provide a compelling evidence of wide spread of these viruses in many populations.

PESTIVIRUS EPIDEMIOLOGY IN REINDEER

Epidemiological data of pestivirus infections in the species of reindeer are limited to only a few studies, which were performed mostly at the end of last century (**Table 1**). Based on specific antibody presence, pestivirus infections were confirmed in the reindeer on two continents, Europe and North America. Since in all the serosurveys bovine viral diarrhea virus type 1 (BVDV-1) strains were used as a reference, the results indicated *de facto* BVDV seroprevalences. Only Kautto et al. (2012) have tried to establish the pestivirus strain responsible for the infections in reindeer in Sweden. The percentages of BVDV seropositive reindeer varied from 0% in the wild Svalbard reindeer of Norway (Stuen et al., 1993) and in the four populations of Swedish reindeer tested in the 1980's (Rehbinder et al., 1985) to nearly 70% in the migratory George River caribou from Canada (Elazhary et al., 1981). The seroprevalence has been associated with variables such as herd, age, and bovine herpesvirus type 1 (BoHV1) seropositivity (Rehbinder et al., 1992; Stuen et al., 1993; Lillehaug et al., 2003; Kautto et al., 2012). The reindeer or domestic ruminant densities have not been shown to explain the variation of herd seroprevalences. The differences in the herding techniques (extensive or intensive), management or the possible presence of persistently infected (PI) animals were more likely risk factors (Kautto et al., 2012). Similarly to many other infectious diseases, the risk of infection increased with the age, resulting in a significantly higher pestivirus seroprevalence in adult reindeer in comparison to calves (Stuen et al., 1993; Kautto et al., 2012). The possible interactions between pestiviruses and alphaherpesviruses has been observed in reindeer (Stuen et al., 1993; Tryland et al., 2005; Kautto et al., 2012), which by analogy to cattle (Kampa et al., 2009), could reflect the immunosuppressive nature of both virus groups.

The questions on the pestivirus strain and the source of infection in reindeer have been raised by some authors. Except for Kautto et al. (2012), only serological test based on BVDV-1 strains isolated from cattle were used. However, it is very likely that, as in case of alphaherpesviruses, the pestivirus circulating in reindeer is specific to the species (das Neves et al., 2010). The Swedish group (Kautto et al., 2012) have found that BVDV ELISA positive reindeer sera reacted with the highest titers to border disease virus 1 (BDV-1) 137/4 strain and with relatively lower titers to BDV-2 V-60 strain isolated from reindeer at German Zoo (Becher et al., 1999, 2003; Avalos-Ramirez et al., 2001). Some cross-reactivity toward BVDV-1 NADL strain was also observed, however, it was connected to the wider cross-reactivity of BVDV-1 (Becher et al., 2003). The study failed to demonstrate, which type was actually circulating in the reindeer population studied, however, it suggested BDV strain rather than a bovine pestivirus. Another convincing argument for independent infection process among reindeer and caribou was the lack of direct contact with domestic ruminants discussed by Elazhary et al. (1981) and Kautto et al. (2012), respectively. Rehbinder et al. (1992) concluded that closer contact of reindeer with domestic ruminants has not affected BVDV seroprevalence. The transmission of BVDV between cattle and reindeer by blood feeding flies has been suggested (Rehbinder et al., 1992). The virus was detected in the insects fed on PI calves (Tarry et al., 1991), however, the exposed BVDV naïve calves remained virus-free and did not seroconvert throughout the study (Chamorro et al., 2011). The transmission from cattle to reindeer was also found unlikely in Norway. The highest pestivirus seroprevalence was found in the extreme northern county of Finnmark where the density of domestic ruminants is very low (Stuen et al., 1993; Lillehaug et al., 2003). Additionally, most cattle in northern Scandinavia where reindeer are reared remains BVDV free after successful eradication plans implemented already in 1990's in Norway (Løken and Nyberg, 2013), Sweden (Hult and Lindberg, 2005), and Finland (Rikula et al., 2005).

PESTIVIRUS ISOLATION AND PATHOGENICITY IN REINDEER

The susceptibility of reindeer to BVDV-1 infection has been experimentally shown (Morton et al., 1990). The clinical signs included mucous and bloody diarrhea, transient laminitis, and coronitis, leucopenia, serous to mucopurulent nasal discharge. However, no pestivirus has been isolated from wild or semi-domesticated reindeer (Rehbinder et al., 1985;

TABLE 1 | A summary of the literature on pestivirus seroprevalence in the reindeer species (*Rangifer tarandus*).

Origin	Year	Reindeer subspecies	n/N*	Pestivirus seroprevalence	Serological test used**	Association	Reference
Svalbard, Norway Finnmark, Norway	1990 1991	*R. t. platyrhynchus* *R. t. tarandus*	0/40 54/326	0% 17%	VNT against NADL strain BVDV	•Age (calves: 6%; adults: 41%) •Herd (0–40%) •Concurrent seropositivity to BoHV1 (27% in adults; 1% in calves)	Stuen et al., 1993
Southern Norway	1993–2000	*R. t. tarandus*	34/810	4.2%	VNT against NADL and Norwegian MD2154/66 strains	•Population or area (0–51%)	Lillehaug et al., 2003
Finnmark, Norway	2000	*R. t. tarandus*	14/43	33%	VNT against NADL strain BVDV	•Concurrent seropositivity to BoHV1 (50%)	Tryland et al., 2005
Northern Sweden	1973–1982	*R. t. tarandus*	3/50	6%	In-house ELISA	•Herd (0-25%)	Rehbinder et al., 1992
Northern Sweden	1980	*R. t. tarandus*	0/26	0%	VNT against Ug-59 strain BVDV	No data	Rehbinder et al., 1985
Northern Sweden	2001–2002	*R. t. tarandus*	408/1158	35.2%	Commercial BVDV ELISA and VNT against six pestiviruses	•Age (calves: 19.2%; adults: 48.9%) •Concurrent seropositivity to BoHV1 (31%)	Kautto et al., 2012
Quebec, Canada	1978 1979	*R. t. caribou*	21/30 17/28	70% 60.7%	VNT against Oregon C24V strain BVDV	No data	Elazhary et al., 1981
Alaska, U.S.	1978–1981	*R. t. granti*	2/67	3%	VNT against Oregon C24V strain BVDV	No data	Zarnke, 1983

**number seropositive/number animals tested; **VNT, virus neutralization test.*

Rockborn et al., 1990; Kautto et al., 2012). The cases of conjunctivitis, ulcerative and necrotizing lesions of nose and oral mucosa in Swedish reindeer could not have been linked to BVDV infection (Rehbinder et al., 1985; Rockborn et al., 1990). The presence of pestivirus genome was also investigated in nearly 280 healthy slaughtered seronegative reindeer from the districts with up to 98% of virus seroprevalence using pan-pestivirus real-time RT-PCR (Kautto et al., 2012). No viral RNA was found in the sera of those animals.

So far the only pestivirus isolated from *R. tarandus* species was V60-Krefeld (Reindeer-1) strain obtained from reindeer, which died with signs of severe diarrhea and anorexia at Duisburg Zoo in Germany in 1996 (Becher et al., 1999; Giangaspero et al., 2006). The outbreak affected all reindeer in the zoo herd as well as an European bison (*Bison bonasus*) from which another, closely related strain V65-Krefeld (Bison-1) was isolated (Becher et al., 1999; Giangaspero et al., 2006). Initially, the reindeer strain has been included as a separate novel species within the genus *Pestivirus* (Avalos-Ramirez et al., 2001). Further phylogenetic studies based on N^{Pro} and E2 coding sequences revealed that V60 and V65 strains were most closely related to border disease virus type 2 (BDV-2) strains isolated from German sheep in 1999 and 2000 (Becher et al., 1999, 2003). The V60 strain grew efficiently in Madin-Darby bovine kidney (MDBK) cells with titers reaching 10^6–10^7 $TCID_{50}$ after four consecutive passages (Giangaspero et al., 2006). The antiserum against V60 strain preferably neutralized BDV-like and classical swine fever virus (CSFV) strains, but did not react with BVDV-1 strains NY-1 and C86 (Avalos-Ramirez et al., 2001; Becher et al., 2003). If the pestivirus isolated from zoo reindeer is related to the virus circulating among free-living animals, which is very likely judging by the results of Kautto et al. (2012), the seroprevalences estimated using tests based on BVDV-1 strains may be underestimated.

In conclusion, the few reports on the distribution of pestivirus in reindeer has shown great variation of seroprevalences depending on the time of the study, geographical origin, animal age, health status, and epidemiological situation of other endemic infections. Pestivirus infection in free-ranging reindeer is more likely to be independent from domestic ruminants, mainly due to the circumpolar distribution of reindeer and therefore limited contact between the species, or pestivirus eradication in far animals as in Scandinavia. Some studies suggest that the pestivirus strain circulating in reindeer is probably a species-specific BDV-like virus.

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REVIEW
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Bovine Viral Diarrhea Virus (BVDV) in White-Tailed Deer (*Odocoileus virginianus*)

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Bovine viral diarrhea virus (BVDV) is the prototypic member of the genus *Pestivirus* in the family *Flaviviridae*. Infections with BVDV cause substantial economic losses to the cattle industries, prompting various organized control programs in several countries. In North America, these control programs are focused on the identification and removal of persistently infected (PI) cattle, enhancement of BVDV-specific immunity through vaccination, and the implementation of biosecure farming practices. To be successful, control measures must be based on complete knowledge of the epidemiology of BVDV, including the recognition of other potential sources of the virus. BVDV does not possess strict host-specificity, and infections of over 50 species in the mammalian order Artiodactyla have been reported. Over 50 years ago, serologic surveys first suggested the susceptibility of white-tailed deer (*Odocoileus virginianus*), the most abundant free-ranging ruminant in North America, to BVDV. However, susceptibility of white-tailed deer to BVDV infection does not alone imply a role in the epidemiology of the virus. To be a potential wildlife reservoir, white-tailed deer must: (1) be susceptible to BVDV, (2) shed BVDV, (3) maintain BVDV in the population, and (4) have sufficient contact with cattle that allow spillback infections. Based on the current literature, this review discusses the potential of white-tailed deer to be a reservoir for BVDV.

Keywords: bovine viral diarrhea virus, interspecific transmission, *Odocoileus virginianus*, wildlife reservoir, white-tailed deer

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INTRODUCTION

Since the first descriptions of bovine viral diarrhea virus (BVDV) in North American cattle in 1946 (Childs, 1946; Olafson et al., 1946), great strides have been made in understanding the virological, epidemiological, and pathophysiological features that have allowed BVDV to become one of the most important viral pathogens of cattle worldwide. The elucidation of the pathophysiology of persistently infected (PI) cattle and recognition of PI animals as the most important source for direct and indirect transmission of BVDV (McClurkin et al., 1984; Brownlie et al., 1987) has shaped current BVDV control measures to focus on eradication of PI cattle and prevention of *in utero* infections through vaccination and biosecurity measures. The development of molecular diagnostic techniques has allowed the classification of pestiviruses by genotypic diversity rather than by the mammalian host from which a virus was isolated and emphasized that pestiviruses lack strict host specificity (Nettleton, 1990; Giangaspero and Harasawa, 2007).

Interestingly, reports of apparent BVDV-associated disease outbreaks in heterologous hosts were published within a few years following the first description of BVDV in cattle (Richards et al., 1956; Brass et al., 1966). While the involvement of BVDV is uncertain or unlikely in some early reports of apparent heterologous infections, numerous studies have since demonstrated that BVDV infections are possible in many species of the mammalian order Artiodactyla, including domestic small ruminants, buffalo, swine, Old and New World camelids, and free-ranging and captive wildlife (Passler and Walz, 2010). The implications of heterologous BVDV infections including adverse effects on health and reproduction of affected species, ability to maintain BVDV in the population, and potential to become a reservoir host are still incompletely understood. To be a reservoir for BVDV and impede eradication efforts, a heterologous host species has to be: (1) susceptible to infection, (2) able to shed BVDV, (3) maintain the virus within individual hosts or the host-population, and (4) have sufficient contact with susceptible cattle herds. This review summarizes the current literature on BVDV infection in white-tailed deer and discusses whether this species has the potential to be a reservoir for BVDV.

SUSCEPTIBILITY OF WHITE-TAILED DEER TO INFECTION WITH BVDV

Serologic Evidence of Susceptibility

First evidence of susceptibility of white-tailed deer to BVDV infection was documented by Kahrs et al. (1964), who examined 200 sera from New York for presence of BVDV antibodies and detected a seroprevalence rate of 3% (Kahrs et al., 1964). Since then, other North American groups have investigated the presence of antibodies against BVDV in white-tailed deer and generally detected low seroprevalence rates (**Table 1**). Reported BVDV seroprevalence rates in white-tailed deer tend to be lower than those reported for other cervids such as mule deer (Stauber et al., 1977; Couvillion et al., 1980; Aguirre et al., 1995; Van Campen et al., 2001; Roug et al., 2012; Myers et al., 2015). Whether this difference reflects greater rates of contact between mule deer and cattle, or maintenance of BVDV in mule deer and transmission among conspecifics, is currently unknown. However, there are key differences in white-tailed and mule deer life history patterns that could explain these trends. Many mule deer populations exhibit migratory behavior, where they move elevationally between summer and winter ranges (Nicholson et al., 1997). Although most studies indicate that mule deer tend to avoid cattle (Stewart et al., 2002; Dohna et al., 2014), migratory movements could increase contact rates between mule deer and cattle due to direct contact around feeding sources, or indirect contact via use of resources that are partitioned temporally, particularly during periods when resources are limiting (Stewart et al., 2002). While white-tailed deer populations in northern regions with severe winter climates also migrate between summer and winter ranges (Nelson, 1998), cattle in these regions are generally confined to conventional production systems that include fenced enclosures and indoor containment facilities (Dohna et al., 2014), thus causing contact rates between wild ungulates and cattle to be less in these settings. Differences in vegetation patterns of cattle grazing lands across North America may also contribute to disparity between BVDV prevalence rates in mule and white-tailed deer. Whereas much cattle grazing east of the Rocky Mountains (excluding some areas such as southern Texas) is dominated by pastures of exotic or native grasses with minimal structural and vegetative diversity, much of the grazing land in western North America is on rangelands and has a greater preponderance of preferred deer browse species (Rickard et al., 1975; Loeser et al., 2007; Wagoner et al., 2013), thereby increasing the probability of contact between deer and cattle. Considering the ranges of white-tailed deer and mule deer, the overlap in dietary items on native range, and limited availability of water resources, it readily becomes apparent that this could be a contributing factor.

Two recent studies documented relatively high rates of BVDV antibody presence in white-tailed deer (Cantu et al., 2008; Wolf et al., 2008). A serosurvey conducted on 15 ranches in Northeastern Mexico, in which the overall rate of seropositive white-tailed deer was 63.5%, demonstrated that significantly greater seroprevalence rates were present on ranches where cattle were present, as compared to ranches without cattle. Other factors that were associated with increased prevalence rates of BVDV antibodies included the abundance of brush and exotic grasses, continuous grazing practices, and lower deer density (Cantu et al., 2008). In a study performed in Minnesota, a greater percentage (46%) of deer were seropositive in the southwestern study area that contained lower cattle densities, mostly composed of beef cattle herds. While the southeastern study area (seroprevalence in white-tailed deer: 25%) had greater cattle densities, the majority of these herds were composed of dairy cattle. The authors suggested that greater opportunities for contact between white-tailed deer and cattle exist on beef operations, where cattle are kept on pastures, rather than with dairy cattle that are largely

TABLE 1 | Reported seroprevalence rates in free-ranging white-tailed deer.

Location	Seroprevalence rate	Reference
New York State	3%	Kahrs et al., 1964
New York State, two locations	5.7 and 7.0%	Friend and Halterman, 1967
Maryland and Virginia, one wildlife refuge each	0/5 and 2/5 deer	Davidson and Crow, 1983
Florida, one location	0/6	Davidson et al., 1987
Quebec, one location	0%	Sadi et al., 1991
Colorado, one location	1/5 deer	Creekmore et al., 1999
Southern Minnesota, nine locations	25% (southeast) and 41% (southwest)	Wolf et al., 2008
Northeastern Mexico, 15 locations	63.5%	Cantu et al., 2008
Alabama, 23 locations	1.2%	Passler et al., 2008
Central New York State and four locations in Pennsylvania	6.01 and 0.34%, respectively	Kirchgessner et al., 2012
New York State	7.48%	Kirchgessner et al., 2013

confined (Wolf et al., 2008). A recent study documented that seroprevalence rates in New York (6.01%) were greater than those in Pennsylvania (0.34%), but beef cattle densities were similar in both sampling areas. However, the dairy cattle and total cow/calf densities were significantly greater in New York which may have contributed to increased rates of BVDV infection in white-tailed deer (Kirchgessner et al., 2012). Contact with cattle is the likely source for BVDV infections of white-tailed deer, as is further suggested by the absence of BVDV antibodies in a population of deer that had no direct or indirect contact with cattle in over 50 years (Sadi et al., 1991). However, maintenance of BVDV within deer populations independent of contact with cattle may also be possible, especially if PI deer are present.

Experimental Infection of White-Tailed Deer with BVDV

Susceptibility of white-tailed deer to infection with BVDV was first confirmed experimentally by Van Campen et al. (1997), who intranasally inoculated four mule deer fawns and one white-tailed deer fawn at 5–6 months of age with BVDV NY-1. In that study, infection with BVDV did not result in noticeable clinical signs or changes of white blood cell counts. However, of the five fawns, only one mule deer was seronegative to BVDV at the time of inoculation, which may have subdued the expression of clinical signs, but did not prevent viremia and seroconversion. Shedding of BVDV was demonstrated on nasal swab samples from three mule deer fawns, but not the white-tailed deer (Van Campen et al., 1997). In another study, BVDV-naïve white-tailed deer fawns were inoculated with BVDV 1b RO3-24272 or BVDV 2 RO3-20663 isolated from white-tailed deer carcasses in South Dakota (Ridpath et al., 2007b). All fawns became infected as indicated by seroconversion and/or viremia, and clinical signs including pyrexia, lethargy, and coughing were observed. On days 3 and 6 of the study, pronounced lymphopenia was observed in inoculated animals, and circulating lymphocyte counts were reduced by 50 and 60% in fawns inoculated with BVDV 1b and BVDV 2, respectively (Ridpath et al., 2007b). On day 3, following infection of these fawns, some leukocyte subpopulations were almost completely depleted but had recovered by day 9 of the study (Mark et al., 2005, 2006). Lymphoid depletion, apoptosis, and lymphoid necrosis were also detected in lymphoid tissues of four fawns inoculated with BVDV 1 544 WTD from a free-ranging white-tailed deer in Indiana (Raizman et al., 2009). Following inoculation, these fawns did not have clinical signs of BVDV-associated disease, but all were positive by virus isolation on tissues (Raizman et al., 2009). In a follow-up study using BVDV 1 544 WTD, infection of five white-tailed deer fawns similarly resulted in absence of clinical signs despite evidence of infection in all fawns, presence of viral RNA in buffy coat, nasal swab, and rectal swab samples, and marked lymphoid atrophy in the Peyer's patches (Negron et al., 2012).

The most severe clinical effects of BVDV in white-tailed deer may result from infection during pregnancy. Using BVDV 1b RO3-24272 or BVDV 2 RO3-20663 of white-tailed deer origin, Ridpath et al. (2008) investigated the outcome of infection of pregnant white-tailed deer (eight seronegative and two seropositive) that were inoculated in the first trimester at 6–7 weeks of gestation (Ridpath et al., 2008). BVDV infection resulted in clinical signs including depression, ill-thrift, and drooling within 7 days of inoculation. Four of the 10 inoculated deer died, and only three does gave birth to live fawns. The remaining pregnancies resulted in abortion, fetal resorption, and fetal mummification with evidence of transplacental BVDV infection in some fetal tissues. Three apparently healthy liveborn fawns were born to does seropositive at the time of inoculation, and these fawns were free from BVDV-infection and seronegative at birth. Two additional fawns were born by a seronegative doe. These fawns were determined to be seronegative at birth and positive for BVDV on virus isolation in buffy coat samples and antigen detection in skin samples (Ridpath et al., 2008). While the death of the fawns within 24 h of their birth prevented further confirmation of their persistent infection, the successful isolation of BVDV 163 days after inoculation strongly supports their PI status. In another study, nine pregnant does were inoculated with BVDV 1 BJ and BVDV PA131 at approximately 50 days of gestation (Passler et al., 2007). While clinical signs of BVDV infection were not observed during examinations from a distance, pregnancy losses occurred in 8/9 does. Whether these losses were BVDV-associated or caused by immobilization procedures during BVDV inoculation is uncertain; however, one of the fetuses of the doe that carried the pregnancy to term was delivered mummified, suggesting BVDV-associated reproductive failure. The fetal mummy was a twin to a liveborn, viable fawn that was hand-raised in an isolation facility. This fawn was confirmed to be PI with BVDV 2 PA131 based on virus isolation of serum, buffy coat, and nasal swab samples; RT-PCR of the serum and buffy coat; and detection of BVDV antigen in an ear notch sample by immunohistochemistry (Passler et al., 2007). The PI fawn remained free from clinical signs of disease and developed normally until it died suddenly at 5 months of age (**Figure 1**).

In contrast to the severe reproductive losses encountered in the above mentioned studies, the pregnancy of all does infected with BVDV by exposure to PI animals in two other studies advanced to term (Passler et al., 2009, 2010). In both studies, successful infection of all does was confirmed by demonstration of seroconversion, but with exception of two stillborn twins, all fawns were liveborn. Whether the observed differences in gestational viability in these studies were due to differences in the viral isolates used for inoculation or differences between the routes of exposure (intranasal inoculation vs. cohabitation with PI) is currently unknown. Previous studies in cattle (Brock and Cortese, 2001; Bielefeldt-Ohmann et al., 2008) and white-tailed deer (Passler et al., 2007) suggested that BVDV 2 isolates are able to cause transplacental infections more readily, and in pregnant goats, pregnancy losses were much more frequently associated with BVDV 2 PA131 than with BVDV 1b AU526 (Passler et al., 2014). Inoculation of pregnant deer during later gestation with BVDV 2 RO3-20663 resulted in pregnancy losses in 3/5 does inoculated during the second trimester and birth of apparently healthy, seropositive fawns from does infected in the

FIGURE 1 | Persistently infected fawn at approximately 4 months of age. The fawn was hand-raised in an isolation room and remained free from clinical signs of disease until sudden death at 5 months of age.

third trimester, confirming that BVDV infections of pregnant white-tailed deer are very similar to those of pregnant cattle (Ridpath et al., 2012).

Similar to the outcome of acute BVDV infection of white-tailed deer, the BVDV antigen distribution in PI white-tailed deer was recently demonstrated to be largely equivalent to that of PI cattle (Duncan et al., 2008a; Passler et al., 2012b). As in PI cattle, BVDV antigen was distributed broadly in many organ systems with greatest antigen staining in epithelial tissues. Skin samples were demonstrated to be a suitable sample for BVDV antigen detection in white-tailed deer. However, in lymphatic and alimentary tissues, which are commonly collected for BVDV diagnosis in cattle, BVDV antigen was detected at lower frequency and intensity, which may in part be due to moderate to severe lymphoid depletion in tissues of PI white-tailed deer (Duncan et al., 2008a; Passler et al., 2012b). Therefore, diagnosis of BVDV infections in white-tailed deer should not rely solely on lymphatic and alimentary tissues, but include samples from the hepatobiliary, integumentary, neurologic, and reproductive organs, which were demonstrated to contain the most pronounced BVDV antigen (Passler et al., 2012b).

Detection of BVDV in Free-Ranging White-Tailed Deer

The first isolation of BVDV from free-ranging white-tailed deer was made from two animals that were gunshot due to illness in two adjacent counties in South Dakota (Chase et al., 2004, 2008). BVDV was detected in multiple tissues of both deer by virus isolation and immunohistochemistry, and the BVDV antigen distribution in ear skin from both animals was consistent with the distribution in PI cattle. The authors also reported that following detection of the two positive deer, approximately 600 samples collected from white-tailed deer, elk, and mule deer in South Dakota were screened by immunohistochemistry, but all were negative for BVDV antigen (Chase et al., 2004, 2008). Similarly, in a recent study, ear notches from 367 hunter-harvested white-tailed deer were evaluated by antigen-capture ELISA, and BVDV antigen was not detected (Ilha et al., 2012). Three other surveys utilized immunohistochemistry or ELISA techniques to investigate the occurrence of BVDV in free-ranging cervids in the US. In Alabama, 1 of 406 ear notches (0.2%; 95% CI: 0–0.6%) was positive by immunohistochemistry, and the antigen distribution resembled that of PI cattle (Passler

et al., 2008). A survey that screened 5597 deer (including 141 white-tailed deer) for BVDV by immunohistochemistry, detected BVDV antigen in one mule deer from which BVDV 1 was subsequently isolated, but BVDV antigen was not detected in the white-tailed deer (Duncan et al., 2008b). The overall apparent prevalence for BVDV-infected deer in Colorado was 0.03% (95% CI: 0–0.1%; Duncan et al., 2008b). In Indiana, 2 of 745 (0.26%, 95% CI: 0.1–0.64) white-tailed deer were positive for BVDV by antigen capture ELISA, and subsequently a cytopathic and a non-cytopathic BVDV were isolated (Pogranichniy et al., 2008). During BVDV testing in cattle herds, acutely infected animals may occasionally cause positive results; however, skin biopsy (ear notch) testing by immunohistochemistry or antigen capture ELISA is considered to be specific for detection of PI c (Walz et al., 2010). To date, antigen detection assays have not been validated for use in white-tailed deer, but positive samples are assumed to have been collected from PI animals. In several experimental infection studies, the BVDV antigen distribution in ear notches of PI white-tailed deer as detected by immunohistochemistry was consistent with that of PI cattle (Passler et al., 2007, 2009, 2010; Ridpath et al., 2008); however, samples from acutely infected deer or deer infected with other pestiviruses have not been evaluated.

Surveys using samples from hunter-harvested deer potentially underestimate the true prevalence of PI animals as they contain an inherent bias regarding the classes of animals sampled. Deer harvests greatly underrepresent young of the year due to selectivity of hunters for adults and their "trophy" status (Ditchkoff et al., 2000), thus significantly reducing the probability of hunters harvesting PI animals, which may die early in life due to complications from BVDV infection. Additionally, deer that manifest symptoms associated with PI status may be less desirable for harvest due to previously reported occurrence of poor body condition, ill-thrift, and smaller body size (Chase et al., 2008). Furthermore, studies that screen for disease pathogens in wildlife often utilize simple random sampling methods that survey multiple, widespread populations across larger regions, as was performed in most studies that evaluated BVDV in white-tailed deer (Kahrs et al., 1964; Pogranichniy et al., 2007; Duncan et al., 2008b; Passler et al., 2008). These surveys may not adequately acknowledge social structures of deer populations and therefore miss evidence of BVDV hotspots as a result of intrapopulational maintenance. White-tailed deer exist in matrilineal groups in which female deer disperse only over small distances according to the rose petal hypothesis (Porter et al., 1991). Cantu et al. (2008) demonstrated that the overall prevalence of BVDV antibodies in captured white-tailed deer was 63.5%; however, large variations among the 15 different ranches were detected, and while the seroprevalence was as low as 11% on one farm, 100% of sampled deer were seropositive on another. The potential impact of biased data associated with the inclusion of specifically targeted animals should be considered during surveillance studies for BVDV. For example, surveillance programs for chronic wasting disease in white-tailed deer commonly include targeting animals that are "symptomatic" (Evans et al., 2014). While this approach may be beneficial for presence/absence surveillance or identification of "hot spots," inclusion of these data in prevalence studies could artificially inflate prevalence rates. These issues suggest that care should be taken when designing surveys for BVDV and other diseases that may manifest themselves in more clumped distributions (Nusser et al., 2008).

SHEDDING AND TRANSMISSION OF BVDV BY WHITE-TAILED DEER

In cattle, BVDV is shed in most excretions and secretions, including nasal discharge, saliva, tears, milk, urine, feces, and semen (Houe, 1995). While studies investigating the possible routes of BVDV transmission from infected white-tailed deer are sparse, the broad distribution of BVDV described in tissues of PI deer (Duncan et al., 2008a; Passler et al., 2012b) suggests that excretion of virus may be similar to cattle, and shedding was demonstrated following experimental acute infections and in PI white-tailed deer (Passler et al., 2007, 2009; Raizman et al., 2009; Ridpath et al., 2009; Negron et al., 2012). In a study using BVDV 1a 544 WTD for experimental infection of four seronegative fawns at approximately 3 weeks of age, BVDV was demonstrated on the nasal swab samples of two fawns and the rectal swab sample of one fawn by RT-PCR 7 days after infection. In contrast, BVDV was not detected in samples from the other two fawns on days 7 or 14 of the study (Raizman et al., 2009). Following inoculation of five seronegative fawns with BVDV 1a 544 WTD, BVDV RNA was detected in nasal, oral, and rectal swab samples of five, four, and five fawns, respectively, as early as 3 days after inoculation and for up to 18 days (Negron et al., 2012). Two days after inoculation, each fawn was cohabitated with 1–2 seronegative calves in an isolation room for 19 days. Direct contact with the infected fawns resulted in BVDV infection in four of six calves (Negron et al., 2012), demonstrating that acutely infected white-tailed deer can shed sufficient amounts of BVDV to transmit the virus to cattle that are in close contact. Another study investigated the potential for BVDV transmission from acutely infected white-tailed deer to seronegative calves by indirect contact (Ridpath et al., 2009). Fawns were inoculated with BVDV 2 RO3-20663 of white-tailed deer origin in isolation rooms that shared circulating air with rooms containing seronegative calves. To simulate opportunities of indirect contact between species, fawns and calves were bottle-fed using shared nipple bottles, and every second day, without prior cleaning, the calves were rotated into rooms that had been previously occupied by fawns. While BVDV infection was successful in all fawns, transmission of BVDV was documented in some, but not all calves, demonstrating that indirect contact may result in transmission of BVDV from deer to cattle (Ridpath et al., 2009).

During experimental cohabitation of pregnant white-tailed deer with two PI cattle in a 0.8 ha pen for 60 days, both species were observed to favor a common area in the pen enabling close interspecific contact (Passler et al., 2009). While direct interspecific contact was not noticed, deer were observed to use the feed trough shortly after the cattle. In that study, opportunity for direct and indirect BVDV transmission existed, and all does became infected with BVDV, resulting in the birth of PI fawns (Passler et al., 2009). In a follow-up study, one of the PI fawns

was cohabitated with six pregnant white-tailed deer during the first trimester of gestation (Passler et al., 2010). The deer shared feed and water sources in an approximately 2 ha pen throughout gestation. All does became infected as result of exposure to the PI fawn and evidence of transplacental infection was detected, suggesting that PI white-tailed deer can readily transmit BVDV to in-contact animals (Passler et al., 2010). To date, quantification of BVDV that is shed by PI white-tailed deer has been reported only from one deer that was born to a doe infected with BVDV 2 PA131 (Passler et al., 2007). Viral titration of nasal swab and serum samples collected from this fawn (**Table 2**) demonstrated that PI deer can continuously shed BVDV in quantities that are similar to PI cattle.

MAINTENANCE OF BVDV IN INDIVIDUAL HOSTS OR HOST-POPULATIONS

Maintenance of BVDV in some populations of white-tailed deer may result from continual viral input from cattle when there is sufficient interspecific contact. While acutely infected deer may also play a role in the transmission and maintenance of BVDV in white-tailed deer populations, the greatest likelihood of independent maintenance would result from the presence of PI deer during the first trimester of gestation, which is influenced by the viability of PI deer, level of dispersion of PI deer, and gestational age at which a new generation of PI deer could be generated. In experimental infection studies, the viability of PI white-tailed deer fawns was markedly shorter than that of uninfected fawns, and most PI fawns did not survive beyond 1 month of age (Ridpath et al., 2008; Passler et al., 2009, 2010). However, survival to 5 and 10 months of age was reported for two other PI white-tailed deer in experimental infection studies (Passler et al., 2007, 2010). Kirchgessner et al. (2013) recently suggested that in New York, where the critical gestational period for generation of a new PI deer would be between mid-January to mid-February, based on an assumed critical gestational age of 50–67 days, PI fawns would have to survive for at least 8 months. The viability of PI white-tailed deer in free-ranging populations is currently unknown; however, the detection of PI animals in surveys of hunter-harvested white-tailed deer (Passler et al., 2008; Pogranichniy et al., 2008), suggests that some PI deer survive into adulthood.

The gestational age chosen for infection in studies that sought to generate PI white-tailed deer was based on extrapolation of the critical gestational age in cattle considering the shorter gestation length in deer, and was reported to be approximately 50–67 days (Passler et al., 2007; Ridpath et al., 2008). Reported gestational ages at time of infection of pregnant white-tailed deer that gave birth to PI fawns were 43, 42–49, and 41 days, respectively (Passler et al., 2007, 2010; Ridpath et al., 2007a). All PI fawns in studies by this research group were born to does infected between 27 and 51 days of gestation (**Table 3**), indicating that the critical gestational age in deer may be earlier than suggested by extrapolations from cattle. Therefore, environmental or behavioral factors that increase the amount of contact of pregnant white-tailed deer with PI livestock or deer before 50 days of gestation would increase the likelihood of BVDV maintenance in deer populations.

A recent study conducted in New York analyzed whether areas with high BVDV seroprevalence rates in white-tailed deer were associated with greater rates of BVDV antigen-positive cattle and camelid herds, and identified three unique scenarios of BVDV epidemiology (Kirchgessner et al., 2013). In central New York, focal areas of elevated prevalence rates of BVDV antigen in livestock and BVDV antibodies in white-tailed deer were identified, indicating that cattle, camelids, and deer served together as a host community for BVDV. In contrast, in western New York, the greater rate of BVDV antigen prevalence in livestock was not associated with increased rates of seroprevalence in white-tailed deer. Interestingly, the western part of New York reportedly contained the greatest deer densities, indicating that the rate of BVDV transmission between cattle and deer is not dependent on deer densities (Kirchgessner et al., 2013), which was previously reported for white-tailed deer in Mexico (Cantu et al., 2008). In northern New York State, an area with low deer density, a cluster of high BVDV seroprevalence among white-tailed deer was detected. In that area of the state, the BVDV antigen prevalence in livestock was low, suggesting that BVDV was independently maintained in the white-tailed deer population. The authors suggested that rather than being a function of deer density, BVDV transmission among white-tailed deer is associated with deer behavior and migration patterns, including congregation in winter yards (Kirchgessner et al., 2013).

Deer wintering behavior in northern regions likely contributes to increased BVDV prevalence. Deer wintering areas are frequently characterized by very high densities of deer that are generally restricted to trail systems due to extreme snow depths. High contact rates between deer on these trail systems and at common food sources (Schmitt et al., 1997) could increase transmission rates, particularly if PI animals were present. Because deer wintering areas contain deer that may migrate from more than 30 miles away (Verme, 1973), rather than just containing deer that reside in close proximity to the wintering area, the potential exists in these habitats to expose deer populations to BVDV that reside in cattle-free areas. In contrast, more sedentary deer populations that are found where snow depths are not restrictive during winter may not have exposure rates as great, nor the potential for deer residing in cattle-free areas to be exposed. Additionally, the timing of congregation in wintering areas would likely increase the prevalence of PI

TABLE 2 | Titration of BVDV in serum and nasal swabs from a persistently infected fawn.

Day of sample collection	Serum virus isolation	Nasal swab virus isolation
8/25/06	6.2×10^5 $CCID_{50}$/ml	2×10^6 $CCID_{50}$/ml
9/25/06	6.2×10^5 $CCID_{50}$/ml	2×10^6 $CCID_{50}$/ml
10/23/06	6.2×10^5 $CCID_{50}$/ml	6.2×10^5 $CCID_{50}$/ml

Adapted from Passler (2010).

TABLE 3 | Gestational age and method of exposure in studies evaluating BVDV infection of white-tailed deer (Passler et al., 2007, 2009, 2010; Passler, 2010).

Fawn ID	Infection status	Date of birth	Method of exposure	Calculated age at exposure
GN	Persistently infected	8/25/2006	Intranasal	33
1	Seropositive	7/30/2007	PI cattle	56
2	Seropositive	7/30/2007	PI cattle	56
3	Seropositive	7/30/2007	PI cattle	56
4	Seropositive	7/30/2007	PI cattle	56
5	Persistently infected	8/4/2007	PI cattle	51
6	Persistently infected	8/15/2007	Intranasal	46
7	Persistently infected	8/21/2007	PI cattle	34
9	Seropositive	8/26/2007	PI cattle	29
10	Seropositive	8/26/2007	PI cattle	29
12	Persistently infected	8/28/2007	PI cattle	27
13	Seropositive	7/6/2008	PI deer	104
14	Seropositive	7/6/2008	PI deer	104
15	Seropositive	7/6/2008	PI deer	114
16	Seropositive	7/6/2008	PI deer	114
17	Seropositive	8/1/2008	PI deer	66
18	Seropositive	8/1/2008	PI deer	66
19	Seropositive	8/1/2008	PI deer	75
20	Seropositive	8/1/2008	PI deer	75
21	Persistently infected	8/13/2008	PI deer	41
22	Seropositive	8/16/2008	PI deer	63

Calculation of the gestational age at the time of infection was based on a 200-day gestation length.

deer. Because deer congregate in these areas during January–March (Ozoga and Gysel, 1972) and most pregnant does will be approaching the end of their first trimester in mid-January (Verme, 1977), the probability of producing PI fawns would be significantly elevated if PI animals were present. Finally, it is very common for supplemental food sources to be available to deer when in wintering areas. Local human residents often feed wintering deer to reduce overwinter mortality (Milner et al., 2014), and it is not uncommon for groups of 50–100 deer to be found at individual feed sites at the same time. The close proximity of these animals at, and sharing of, common food sources would significantly elevate exposure to BVDV if the virus was present in the wintering population. This scenario has led to high transmission rates of bovine tuberculosis in wintering populations of white-tailed deer in Michigan (Schmitt et al., 1997; Miller et al., 2003). More southerly deer populations would not experience this period of elevated exposure.

CONTACT OF WHITE-TAILED DEER WITH CATTLE

Factors that affect the transmission of BVDV in cattle populations include the duration of the infectious period, the presence of susceptible hosts that lack immunity necessary to prevent infection, infectiousness of the virus strain, and the number of adequate contacts between BVDV-infected and susceptible animals. The same factors likely also apply to maintenance of BVDV in populations of white-tailed deer and determine whether white-tailed deer can serve as a BVDV reservoir and cause spill-back infection to cattle. While shedding and transmission of BVDV was demonstrated in white-tailed deer, there is currently sparse information on how passage of BVDV through deer affects the infectivity of the virus for cattle and whether sufficient contact occurs between acutely infected or PI white-tailed deer and susceptible cattle. The occurrence of 'sufficient contacts' is key to the discussion of BVDV transmission from deer to cattle, and theoretically, both direct and indirect routes can result in transmission of BVDV between deer to cattle. A recent study that evaluated the co-occurrence of pathogens with either direct or indirect transmission route in cattle herds with or without exposure to elk determined that only indirectly transmitted pathogens co-occurred in both species (Pruvot et al., 2014).

There are many anecdotal reports of close contact between white-tailed deer and cattle in pastures and at feed and water sources that may promote direct interspecific transmission of BVDV. In a survey conducted by the United States Department of Agriculture, 49.3% of dairy operations reported deer or other members of the deer family had physical contact with dairy cattle or their feed, minerals, or water supply (United States Department of Agriculture, Animal Plant Health Inspections Service, and Veterinary Services, 2011). On operations on which contact of cattle with cervids occurred, 90.8% of farmers reported that cattle could possibly or sometimes have face-to-face contact with deer. In a similar survey of beef cattle producers, 72.6% of operations reported that cattle had physical contact with wild cervids (United States Department of Agriculture, Animal Plant Health Inspections Service, and Veterinary Services, 2011). A study in southwestern Manitoba reported that nearly 100% of

cattle producers had observed the presence of white-tailed deer on their farms (Brook et al., 2013). Of the interviewed farmers, 11 and 47% had observed direct or indirect contact, respectively, between white-tailed deer and cattle (Brook et al., 2013). In contrast to results of farmer surveys, sufficient direct contact of cattle and white-tailed deer was rarely reported in studies using visual observations or Global Positioning System (GPS) collars to study the spatial distribution of both species. In a 2-year-study in Michigan in which the number of contacts of white-tailed deer with other species were visually observed, only one direct contact and 273 indirect contacts between deer and cattle were recorded (Hill, 2005). Similarly, close contact of cattle and white-tailed deer was rare in a study in Texas, and deer tended to be displaced by cattle approaching at a distance of within 46 ± 5 m (Cooper et al., 2008). While cattle generally tolerate the presence of deer, deer tended to avoid cattle at distances lower than 50 yards (Krämer, 1973). The social relationships of cattle and deer are controversially discussed in the published literature (Krämer, 1973), and contact of both species is influenced by various factors including habitat type, season, presence and type of supplemental feed for cattle, and presence of barrier fencing at feed storage sites (Brook, 2010; Brook et al., 2013; Lavelle et al., 2015).

Indirect routes are likely to be more important for BVDV transmission than direct contact between deer and cattle; thus, virus survivability and distance from infected animals to susceptible animals are important factors contributing to indirect transmission of BVDV from deer to cattle. Since BVDV is an enveloped virus, the virus is unstable at low or high pH (Houe, 1995). In addition, temperature impacts the survivability of BVDV, which remained infective for greater than 6 weeks in manure slurry stored at 5°C but less than 2 weeks at 20°C (Botner and Belsham, 2012). Enhanced BVDV survivability at colder temperatures in combination with a greater potential for wild cervids and livestock to make indirect contact at common feed sources during winter months when forages are scarce (Brook et al., 2013), suggest a greater risk of indirect transmission during colder winter months. Food and water are aggregation points for cattle and deer, and BVDV can be transmitted horizontally via oral and nasopharyngeal secretions. Since oral and nasopharyngeal secretions contain mucus, survivability and infectivity were compared in fomites contaminated with BVDV in aqueous or mucus solution, and BVDV appeared to survive for longer periods of time on most fomites in the presence of mucus (Stevens et al., 2009). In addition, BVDV could be recovered at significantly greater levels and for longer periods of time in water in the presence of mucus than without mucus; however, this research was performed under laboratory conditions, and survivability could be potentially enhanced or reduced under different environmental and climatic conditions (Stevens et al., 2009). BVDV survives for up to 60 days in tissues of PI cattle, and the potential for BVDV transmission from carcasses of white-tailed deer to susceptible cattle was recently evaluated (Passler et al., 2012a).

Insects, especially those requiring blood meals such as mosquitoes and tabanid flies may also have the potential to serve as a source of indirect BVDV transmission (Tarry et al., 1991). Horse and deer flies are tabanid flies, of which there are an estimated 4,300 different species worldwide. The female flies are aggressive blood feeders, and are capable of feeding on many different types of mammals. Horse flies were able to transmit BVDV to susceptible cattle after feeding on a PI steer (Tarry et al., 1991); however, the ability to transmit from PI deer to cattle, while conceptually possible, has not been demonstrated. Many species of mosquitos lack mammalian host specificity, and thus could also be a potential source of mechanical transmission between wildlife and livestock. Although insects could serve as an indirect route of transmission, no epidemiologic data are available to estimate the risk of arthropods as transmitters of BVDV infection from deer to cattle or vice versa.

SUMMARY

Bovine viral diarrhea virus is a ubiquitous pathogen capable of infecting more than one host species. A key issue in the design of BVDV control measures is to determine if heterologous hosts constitute an infection reservoir. In order to serve as an infection reservoir, four key requirements must be met including: (1) susceptibility to BVDV, (2) BVDV maintenance, (3) BVDV transmission, and (4) sufficient contact that allow spillback infections. With respect to susceptibility to BVDV infection, experimental infection studies which are corroborated by epidemiologic investigations provide strong evidence that BVDV infection occur in white-tailed deer, including transplacental infections and birth of PI offspring. Furthermore, BVDV can be maintained in white-tailed deer populations as strongly evidenced by epidemiologic data indicating high seroprevalence rates. Persistently infected deer are described, and these deer are capable of shedding BVDV at levels consistent with PI cattle. Some PI deer were described through epidemiologic investigations to survive into adulthood providing strong evidence that deer can be important sources of BVDV for susceptible animals. Finally, evidence that PI deer and naïve cattle make sufficient contact to result in spillback infections to cattle is weak. While data exist that indicate deer and cattle make direct contact and that potential indirect contact exists at food and water aggregation points, the low prevalence of PI deer along and scarcity of sufficient contacts between PI deer and naïve cattle suggest a low risk for white-tailed deer as an important reservoir of BVDV in most environments. BVDV infections should be considered a threat to the health and reproductive success of deer, but the greatest risk for BVDV infection in cattle likely resides in PI cattle.

AUTHOR CONTRIBUTIONS

TP, SD, and PW contributed to the preparation, review, and revision of the manuscript.

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The control of classical swine fever in wild boar

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Classical swine fever (CSF) is a viral disease with severe economic consequences for domestic pigs. Natural hosts for the CSF virus (CSFV) are members of the family *Suidae*, i.e., Eurasian wild boar (*sus scrofa*) are also susceptible. CSF in wild boar poses a serious threat to domestic pigs. CSFV is an enveloped RNA virus belonging to the pestivirus genus of the *Flaviviridae* family. Transmission of the infection is usually by direct contact or by feeding of contaminated meat products. In recent decades CSF has been successfully eradicated from Australia, North America, and the European Union. In areas with dense wild boar populations CSF tends to become endemic whereas it is often self-limiting in small, less dense populations. In recent decades eradication strategies of CSF in wild boar have been improved considerably. The reduction of the number of susceptible animals to a threshold level where the basic reproductive number is $R_0 < 1$ is the major goal of all control efforts. Depending on the epidemiological situation, hunting measures combined with strict hygiene may be effective in areas with a relatively low density of wild boar. Oral immunization was shown to be highly effective in endemic situations in areas with a high density of wild boar.

Keywords: classical swine fever, wild boar, oral vaccination, control, wildlife diseases

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INTRODUCTION

Classical swine fever (CSF) is an acute viral infection of pigs that causes major economic losses especially in countries with dense populations of domestic pigs. A series of outbreaks occurred in the European Union (EU) after the introduction of the non-vaccination policy in 1990. The economic losses caused by an outbreak in The Netherlands in 1997 were as high as 2.3 billion US$ and more than 11 million pigs had to be destroyed. Only a fraction of these pigs were actually infected or suspect of being infected. The rest had to be killed for preventive measures or welfare reasons in areas that have been under movement restrictions for prolonged periods. Other European countries were also affected by serious outbreaks between 1990 and 2001, e.g., Belgium, Czech Republic, Germany, Italy, and Spain (Meuwissen et al., 1999). Except for Australia, North America, and the EU the infection is endemic in most other parts of the world where pigs are kept. The CSF virus (CSFV) is an enveloped particle containing a single stranded RNA with positive polarity. Taxonomically, it belongs to the genus Pestivirus in the family *Flaviviridae*. It is readily inactivated by common disinfectants and detergents, however, in moist environments, e.g., ham, salami type sausages, fresh pork and excretions of infected pigs it can survive for weeks or even months (Edwards, 2000; Kaden et al., 2004). Contaminated meat and meat products are dangerous sources for the spread of CSF or the fresh introduction of the infection into CSF-free regions, respectively. Related agents are bovine viral diarrhea virus, border disease virus of sheep and a number of other pestiviruses that have recently been detected in wild ruminants. Ruminant

pestiviruses occasionally infect pigs subclinically and cross-reacting antibodies of the resulting immune response may pose some problems for the serological diagnosis of CSF. The Eurasian wild boar (*sus scrofa*) is equally susceptible to infection with CSFV. In areas with domestic pigs and wild boar the infection is frequently transmitted from domestic pigs to wild boar and vice versa. CSFV has no known reservoir or animal vector other than swine. The virus does not infect humans, however, it can be transmitted experimentally to ruminants and rabbits.

CLINICAL SIGNS

Domestic pigs as well as wild boar are highly susceptible to CSFV infection. Clinical signs and pathogenesis of the infection have been extensively studied in domestic pigs. Although, there are fewer data available for wild boar it is safe to assume that there are no significant differences between domestic pigs and wild boar (Kaden et al., 2004). There are three different clinical courses: Acute CSF, chronic and late onset CSF. The latter is the result of prenatal infection (Artois et al., 2002; Moennig et al., 2003).

The acute infection lasts less than 4 weeks and animals either recover or die within this period. When infected with CSFV strains that recently circulated in Europe piglets are getting severely ill and up to 90% of them die within 4 weeks post infection. Pyrexia with temperatures higher than 40°C is a characteristic sign in juvenile animals. Early symptoms are lethargy, conjunctivitis, huddling together, respiratory signs, conjunctivitis, constipation followed by diarrhea and anorexia. Central nervous symptoms are frequent, e.g., convulsions, weakness of hind legs, staggering gait, and incoordination of movement. Immunosuppression and severe leukopenia facilitate secondary infections of the gastrointestinal and/or respiratory tract. Skin and internal organs often display petechial to ecchymotic bleedings. Infected animals are shedding virus through all secretions and excretions. With increasing age of the infected pigs, clinical signs become milder, less specific and most adult pigs recover. First virus-neutralizing antibodies are detectable 2 weeks post infection and convalescent animals have a stable lifelong immunity against CSFV, which is predominantly humoral.

In domestic pigs the chronic form of the disease, which is always fatal, plays an important role, since infected animals shed large amounts of virus until their death. Chronic CSF develops in a few juvenile animals, which fail to respond efficiently to the infection. Affected pigs are not able to clear the virus and the disease lasts longer than 4 weeks. Early symptoms resemble those of the acute infection. During the course of the disease clinical signs become weaker and less specific, including chronic enteritis, wasting and undulating fever. Sick animals shed virus for the rest of their lives and they die between 2 and 4 months post infection. Antibodies may be produced but they are often not detectable since they are complexed by circulating virus. Neither field nor experimental data on this form of CSF in wild boar are available (Artois et al., 2002). It is questionable whether chronically sick wild boar have a survival chance in their natural habitat.

In infected pregnant pigs CSFV—like other pestiviruses—is able to cross the placenta and to infect fetuses. Depending on the stage of gestation and viral virulence the infection has different outcomes in pigs. After intrauterine infection during early pregnancy a number of disorders occur, e.g., stillbirths, abortions, and mummified fetuses. Infection of sows around 80–90 days of pregnancy may lead to the birth of persistently viremic piglets, which can survive for up to 11 months. Often these piglets are not readily recognized, because they appear clinically normal at the time of birth. Occasionally congenital tremor is observed. After birth, their condition deteriorates and they usually show poor growth ("runt") and wasting. This course of CSF is always fatal and it is called "late onset CSF." In domestic pig populations these viremic piglets are dangerous virus reservoirs because they shed large quantities of virus during their lifetime. In pregnant wild boar sows intrauterine infection under laboratory conditions also yields persistently viremic piglets (Depner et al., 1995), however, these animals apparently do not play an important role for the perpetuation of CSF in wild boar populations since their survival time likely to be short (Kaden et al., 2005b).

DIAGNOSIS

In domestic pigs first suspicion for an outbreak of CSF is usually raised by the clinical picture, especially when the severe acute form of disease is observed. However, due to the rather unspecific symptoms, a long list of other infectious diseases has to be considered as differential diagnosis, e.g., African swine fever, Erysipelas, porcine reproductive, and respiratory syndrome, purpura hemorrhagica, porcine circovirus 2 infections, and other infections with high fever not responding to antibiotic treatment. In wild boar typical indicators for a disease outbreak may be an unusual number of pigs found dead or the observation of sick animals with atypical behavior.

Any clinical suspicion of CSF in domestic pigs and wild boar must be verified using laboratory diagnostic methods. Depending on the state of the samples collected from dead or freshly shot wild boar and the technical capabilities of the laboratories involved a number of laboratory techniques can be applied. The methods for viral and serological detection of CSFV infections are well established and there are detailed descriptions in the "Manual of Standards" of the OIE (Anonymous, 2015) and in the "Diagnostic Manual" attached to "Decision 2002/106/EC" issued by the EU Commission (Anonymous, 2002; Greiser-Wilke et al., 2007).

The tissues recommended for the detection of virus are tonsil, lymph-nodes, spleen, ileum, and kidney. For serological tests it should be attempted to collect tissue fluids from shot wild boar.

Standard techniques for the isolation of virus are based on the use of susceptible porcine cell cultures. Cells infected by CSFV are not lysed and therefore the infection must be visualized using indirect methods, e.g., fixing cells and staining viral antigen using mono- or polyclonal antibodies conjugated with enzymes or fluorescent dyes. Although, virus isolation is time consuming and not as sensitive as polymerase chain reaction after reverse transcription (RT-PCR) it is used for the establishment of virus collections and it allows further analysis of the isolate, e.g., genotyping or analysis of viral virulence in animal experiments. Although, CSFV presents no hazard for humans,

there is the risk that the virus escapes from the laboratory and might infect susceptible pigs. Therefore, any work involving live virus including its propagation should be carried out in BSL-3 (agricultural) facilities.

For quick results direct antigen detection can be carried out using immunofluorescence or peroxidase staining with polyclonal or monoclonal antibody conjugates on fixed cryosections of organ material. However, the sensitivity of this method is limited and a negative result does not rule out CSF in case of a clinical suspicion. The interpretation of test results is not trivial and it requires experienced laboratory personnel.

In recent years RT-PCR has become the method of choice, since it yields quick results and it is highly sensitive. It can be used for individual as well as pooled samples (Depner et al., 2006).

Serological diagnosis of CSF is performed using either virus-neutralization assay or a commercially available enzyme-linked immunosorbent assay (ELSA). The former is still considered to be the gold standard, however, it is slow, labor intensive, and often the fluids retrieved from wild boar carcasses are not suitable for use in tissue culture based tests.

VACCINES

Several types of CSF vaccines have been developed for use in domestic pigs. The efficacy of old inactivated preparations was poor, while more recently developed modified live vaccines (MLV) are highly efficacious and have an excellent safety record in pigs of all ages, e.g., the GPE—and a number of variants of the lapinized Chinese strain (C-strain) of CSFV (Bognar and Meszaros, 1963). Currently MLVs are being used worldwide for the prophylactic vaccination of domestic pigs. They are suitable tools for limiting the severe economic effects of CSF in countries with endemic infection and, when properly used in systematic control programs, their use often was and still is a first step toward eradication of the infection (Terpstra, 1991). Once countries are free from CSF vaccination is usually prohibited.

For oral immunization of wild boar several variants of the C-strain have been used. The efficacy of the vaccine virus after oral administration was tested in domestic pigs and wild boar piglets. Whereas, after parenteral immunization protection is already achieved 2–3 days post vaccination, oral vaccination yields a slightly delayed protection against challenge with virulent CSFV about 4 days after application of the vaccine virus. Neutralizing antibodies were demonstrable after 10 days (Kaden and Lange, 2001). In addition the C-strain virus was tested in non-target species, e.g., cattle, goat, sheep, foxes, hares, rabbits, and mice. In none of these species a clinical reaction was observed (Chenut et al., 1999; Kaden et al., 2010).

In early experiments with oral CSF vaccines, e.g., in Romania, C-strain virus was injected into hen eggs which were then used for oral immunization. Success with these baits was variable. Outbreaks in the early 1990s in Germany have prompted another attempt to develop a new generation of baits, partly based on the experiences made with baits used for oral immunization of foxes against rabies. These baits consisted of corn flour, fat, and almond flavor. A plastic blister with 2 ml aliquots of 10^6 protective doses$_{50}$ of C-strain virus "Riems" was incorporated in the baits (Kaden et al., 2000, 2005a). Since the original baits were too large for uptake by young animals experiments were made to reduce bait size and replace the liquid vaccine formulation by freeze-dried virus (Brauer et al., 2006). On the genetic level differentiation of vaccine virus from field virus can be made using real-time RT-PCR for the detection of sequence variations (Beer et al., 2007).

However, the general disadvantage of conventional MLV is that vaccinated animals cannot be distinguished serologically from convalescent pigs. In order to overcome this impediment the live DIVA (Differentiating Infected from Vaccinated Animals) vaccine CP7_E2alf has been developed. It is based on a bovine virus diarrhea virus backbone containing the major envelope protein (E2) of CSFV strain Alfort. CP7_E2alf induces a solid immunity in wild boar after oral immunization and it did not induce any clinical signs in non-target species after oral inoculation, e.g., calves, young goats, lambs, and rabbits. Neither fever nor leukopenia was registered in the inoculated animals and virus could not be isolated from purified white blood cells or from nasal or fecal excretions. In another experiment it was shown that the vaccine was also innocuous for the target species: no clinical signs, transmission, or shedding of the vaccine virus was observed (Tignon et al., 2010; König et al., 2011; Gabriel et al., 2012). This novel vaccine has been licensed by the European Medicines Agency in 2014 and has the potential to replace conventional MLV in oral immunization of wild boar. Other live DIVA vaccines based on porcine adenovirus as vector for the E2 glycoprotein of CSFV have been developed earlier, however, it is not clear whether they were intended to be used in the control of CSF in wild boar (Hammond et al., 2003).

EPIDEMIOLOGY

Transmission routes of CSFV are comparable in wild boar and domestic pigs: Virus is mainly transmitted by direct contact with infected animals. Indirect spread by infected feces, food, and carcasses also plays a role. Naïve populations usually get infected accidentally by indirect and sometimes direct contact with infected domestic pigs or by wild boar feeding on garbage on landfills or rest areas where contaminated food had been dumped. There are well documented reports that CSFV may spill over directly or indirectly from wild boar to domestic pigs, e.g., in Germany 92 primary outbreaks of the infection in holdings of domestic pigs in the years 1993–1997 occurred in areas with endemic CSF in wild boar. It was proven that 60% of these cases were caused by direct or indirect contact with wild boar (Teuffert et al., 1997; Fritzemeier et al., 2000).

Until the early 1990s, little was known concerning the significance of wild boar in the epidemiology of CSF and unfortunately there are hardly any published records of historic CSF outbreaks in wild boar. Earlier observations indicated that CSF outbreaks in wild boar were self-limiting (Terpstra, 1987), probably due to high virulence of viral strains circulating at that time.

However, in the last 25 years ample evidence has accumulated in Europe that wild boar may become a dangerous virus reservoir, although many of today's CSF outbreaks with strains of moderate

virulence currently circulating in the field will still die out spontaneously. This seems to be true for small populations of about 2000 wild boar or less where the infection seems to be cleared within 1 year. In contrast CSFV tends to persist and become endemic for years in larger populations (Rossi et al., 2005). In addition to population size, host animal density plays a role in virus persistence, since the high turnover in a dense population provides a quicker renewal of young susceptible pigs, thereby increasing the chance of the virus to persist in the population. Thus, population size and density are crucial factors for viral survival in wild boar populations (Artois et al., 2002).

The course of a CSF outbreak in wild boar largely depends on the threshold, i.e., the number of susceptible animals in an affected population in a defined area. The threshold criterion for each infectious disease in wildlife is the basic reproductive number (R_0). $R_0 > 1$ means that there are enough susceptible animals to allow the number of secondary infections caused by the first case exceed one, and as a consequence the infectious disease will perpetuate and ultimately become endemic. Below the threshold it is expected $R_0 < 1$ (Hone et al., 1992; Lloyd-Smith et al., 2005), i.e., the absolute number of susceptible animals is so low that the infection is likely to come to an end. When an epidemic is caused by highly virulent variants of CSFV this number has been estimated to be 1.4 susceptible pigs per km^2 (Hone et al., 1992). Since the threshold number is significantly influenced by the mortality rate due to the infection this number is lower in endemic areas with low virulent variants of CSFV. It has been estimated to be one susceptible pig per km^2 (Anonymous, 1999). Artois et al. (2002) estimated the threshold value to be approximately 200 susceptible pigs in an area of about 220 km^2. With more than 200 susceptible animals in the area the R_0 allows an infected pig to infect one or more other susceptible pigs, i.e., $R_0 > 1$. Consequently the main goal of any effort to control CSF in wild boar is to stop transmission of the virus by reducing the number of susceptible individuals in the infected area until the threshold is reached (Artois et al., 2002; Anonymous, 2009). In endemic situations the majority of adult animals have survived infection and they are immune for the rest of their lives thereby reducing the number of susceptible animals, while juvenile pigs without maternal antibodies are susceptible and they serve as reservoir for CSFV.

Long-term observations suggest that during the last decades populations of wild boar have increased in density and that the species has occupied new territories (Acevedo et al., 2007, 2014; Ruiz-Fons et al., 2008). Main reasons for this expansion are changing biological and ecological parameters, e.g., relatively mild winters due to a gradual climate change leading to longer mating and delivery seasons and improvement of the feed basis due to more intense farming and/or more shelter available in rural areas that have been abandoned (Acevedo et al., 2007). In addition the flexibility of the species to colonize a wide range of new habitats, including even urban areas, intentional introduction for hunting purposes and a decrease or absence of predators have significantly contributed to this expansion. Together with the high reproduction rate these factors have led to the current situation where wild boar is the most widespread and also the most abundant wild ungulate species in Europe. In case of CSF outbreaks in these populations there is a high probability that the virus will persist for a longer period as has been observed in several outbreaks in Germany and France during the last decade of the last century (Laddomada, 2000; Pol et al., 2008). Once introduced CSFV spreads according to the social and spatial structure of the affected populations, i.e., virus transmission within a social group and between groups. Within social groups, the virus is transmitted very efficiently and with high frequency by direct and indirect contact, especially between piglets. In contrast contacts between social groups are limited and virus is mainly spread indirectly by excretions and carcasses of infected animals. Direct transmission between groups during the rutting season through male dispersers or after vigorous drive hunting resulting in the disruption of social order may also occur, or when new social groups are being established. The high reproduction rate of wild boar provides a constant supply of young animals serving as reservoir for CSFV.

CONTROL MEASURES

In the EU, CSF control in domestic pigs is based on a stamping out strategy, i.e., outbreaks in domestic pigs are eradicated by culling of infected and suspected animals. The measures are accompanied by temporary animal movement restrictions. Prophylactic vaccination is banned. However, when there is the danger of uncontrollable spread of CSF the EU Commission may approve emergency vaccination. This applies for domestic pigs as well as wild boar.

For obvious reasons programs for the control and eradication of CSF in wild boar have to be different from those applied in domestic pigs. When the suspicion of a CSF outbreak in a wild boar population is confirmed, hunting should be banned temporarily in order not to disperse infected animals into uninfected areas. When designing a control program it is essential for epidemiologists and wild life managers to have information about structure and density of the affected animal populations. Stakeholder, e.g., veterinary officers, hunters, and farmers should take part in the planning. The program should address the following issues (Laddomada, 2000):

- A clear delineation of the infected risk area and the definition of a surrounding surveillance area
- Description of measures to be applied to detect infection in wild boar
- Nomination of organizations and persons involved in control measures and establishment of a clear chain of command
- Measures to be taken to control the infection (see below)
- Epidemiological investigations
- Virological and serological controls on animals shot or found dead, according to standard statistical methods.
- Destruction of infected carcasses and strict hygienic measures when carcasses are eviscerated
- Rules for the use of inspected wild boar meat from CSFV-negative animals

- Movement restrictions for domestic pigs in the designated areas
- Criteria for lifting control measures

Environmental factors, in particular natural barriers have to be taken into account when infected and surveillance areas are defined, e.g., rivers, high mountains and major freeways. In addition movement of wild boar and population densities should be taken into consideration. The structure of the local wild boar population and its subpopulations has to be recorded. Animal density should be estimated as accurately as possible and epidemiological data on virological and serological prevalence should be taken into account. However, it is difficult to estimate the density of wild boar populations because these animals prefer dense vegetation and they have a nocturnal activity pattern. Therefore, indirect methods rather than direct counts of pigs are used for the estimation of population density and abundance. A relatively simple method which can be applied in emergency situations, e.g., CSF outbreaks, is hunting bag analysis if possible over several years (Acevedo et al., 2007; Bosch et al., 2012). Other more complex methods are pellet counts (Acevedo et al., 2007) and capture—recapture—for instance, in combination with non-invasive genetic sampling (Hebeisen et al., 2008; Ebert et al., 2010).

With these data at hand a decision on control measures should be made. Depending on geographical conditions and the size and structure of the affected wild boar population these measures may be either "minimal intervention" in the case of small and isolated populations, feeding, fencing, hunting, trapping, oral immunization, or a combination of the above when larger and more complex populations in wide open areas are affected.

Public Awareness and Education

Measures to control CSF in wild boar are complex and may be long-lasting. For the success of the program it is therefore very important to first launch an awareness campaign addressing all groups affected or involved in control measures, e.g., veterinary authorities, hunters, farmers, and the local public. Especially, hunters are a critical group because their cooperation is essential and because control programs, e.g., hunting ban or depopulation efforts targeting piglets, are not compatible with their goals and hunting traditions. Many of them do not consider CSF a problem, and therefore educational measures should precede or accompany each control program highlighting the impact of CSF on sports hunting and the general economy. Especially, for vaccination campaigns and targeted shooting of piglets awareness and cooperation of hunters is crucial. Compensatory measures such as bonuses for each piglet shot could be considered to stimulate co-operation of hunters.

Surveillance and Monitoring

In most cases samples for surveillance and monitoring are provided by hunters. Quality of samples is often poor since it depends on the time of sampling after the killing of the animal, ambient temperature, and time elapsing until delivery to the laboratory. Samples should be accompanied by information on the location where the sample has been taken and sex and age of the animal (Anonymous, 2009).

In disease free times serological surveys are an inexpensive and convenient tool to detect a fresh CSF outbreak. Specific antibodies persist lifelong in convalescent animals and only a limited number of samples is necessary to detect e.g., an expected prevalence of 5% with a 95% confidence. In contrast virological surveys would require a much higher number of samples to detect a similar low level of prevalence (Artois et al., 2002).

During a control program progress should be monitored very closely by investigating virologically and serologically the highest number of killed animals possible. Results of laboratory tests will yield an accurate account of control progress.

Minimal Intervention

In small isolated wild boar populations CSF outbreaks can be self-limiting as was observed in an outbreak in the southern part of Switzerland (Canton of Ticino). When the outbreak was notified the infected area was declared a "risk zone" and the surrounding area the "surveillance zone." All hunting activities in the risk zone were temporarily banned, but hunting continued in the surveillance zone. Hunting was again allowed in the risk zone after 7 months and juvenile pigs were the major targets. Diagnostic and biological data of all 1294 wild boar found dead or shot between May 1998 and January 2000 were recorded and analyzed. Of 528 animals from the risk zone 179 were virus-positive and 167 seropositive, whereas only one animal was seropositive from the surveillance zone. After another year no more virus-positive animals were found. Seropositive animals were found in all age groups during the first hunting campaign. However, after 12 more months seropositive animals were all older than 1 year (Schnyder et al., 2002). Similar observations were made in neighboring Italian territories with a similarly structured wild boar population (Zanardi et al., 2003).

Feeding

Feeding of wild boar is primarily used to facilitate trapping, shooting, or distraction of wild boar from crops (Massei et al., 2011). However, the latter is sometimes difficult or impossible, since additional feed is only accepted when feed in the natural environment is scarce. In order to achieve optimal results experienced hunters should decide on density and location of feeding stations. The common practice of artificial feeding in winter is not unambiguous, since it may contribute to survival and improved reproduction and thereby to population growth (Geisser and Reyer, 2005; Gamelon et al., 2011).

The usefulness of feeding for the control of CSF is also somewhat controversial, because—depending on the circumstances—it may promote or hamper the spread of CSFV. Feeding prevents local wild boar including infected animals from migrating to distant food sources thereby restricting the long distance spread of CSFV. On the other hand excessive artificial feeding could be an incentive for neighboring uninfected wild boar to move into the infected area. This may result in the spread of the virus into susceptible animals from formerly uninfected areas. However, cautious and limited use of feeding can be a

useful tool to keep infected animals in a defined area and to limit spread of the virus (Artois et al., 2001).

Fencing

Wild boar-proof fences have been described and they are being used on a small scale mainly to protect crops or certain ecological environments. Fencing could be also a method to effectively restrict wild boar movement in larger areas and thereby prevent the spread of CSFV. The efficiency of fencing is depending on the fencing system used and its intactness, since long fences of dozens of kilometers are vulnerable to destruction by wildlife and other influences. In addition the practical feasibility and public acceptance of implementing fencing in emergency situations in larger areas is limited. In case fencing is considered as an element of a control program, suitable areas for fencing must be identified taking into consideration the epidemiological situation and the spatial distribution of wild boar populations (Anonymous, 2014).

Hunting

Several programs for controlling infectious diseases of wildlife have attempted the reduction of host population size in order to lower the density of both infected and susceptible individuals in a population. The goal was to achieve a low probability of transmission of infection between animals and to reach the specific threshold density of susceptible animals. Population reduction has been used in programs to control bovine tuberculosis in badgers in the United Kingdom, fox rabies throughout mainland Europe and CSF in wild boar in France, Germany, and Italy. In all cases population control was attempted by culling using different methods, e.g., hunting, gassing (foxes and badgers), trapping, and poisoning. In Europe there would be little or no public acceptance for the latter method since it is considered inhumane and non-target species may be affected. In addition there are no toxicants registered for this purpose in Europe or North America. For a number of years poisoning of wild boar was successfully applied in New Zealand and Australia (Massei et al., 2011). Another theoretical method to mitigate wild boar populations would be fertility control by feeding contraceptives in baits. However, the lack of long-acting contraceptives, possible uptake of baits by non-target species and the fact that wild boar meat is for human consumption prevented the use of this method. Modern immunocontraceptives are also unsuitable, since they have to be administered parenterally, i.e., using a remote delivery system for injecting each individual pig. A drawback of most campaigns for reduction of host animal populations is the lack of control of the target level of animal population decrease. Results of these campaigns so far were not sustainable (Aubert, 1999; Donnelly et al., 2003; Massei et al., 2011), partly because attempts to reduce population size by culling were often compensated by a higher reproduction activity and immigration from neighboring populations. If depopulation attempts do not reach the threshold density the infection will persist, probably at a lower level.

At first sight hunting seemed to be the method of choice for the reduction of the number of susceptible wild boar. However, in most countries wild boar are a major target species for sports hunting, which tends to maintain pig populations close to 50% of the level of the carrying capacity, thereby maximizing the production of new-born animals. This partly explains that—despite steadily increasing hunting bags all over Europe—wild boar populations have not decreased; in contrast they have increased over the last decades (Keuling et al., 2013). There is an obvious conflict between the goals of hunters and the goals of CSF control, which might impede control efforts considerably. Another problem is hunters' tradition not to shoot piglets or to hunt using artificial light. However, an effective hunting plan as part of a CSF control program must involve the preservation of adult animals that can be considered to be immune and the shooting of juvenile animals which are most likely susceptible to the virus. Targeting of piglets and young female pigs will have the most noticeable effect on the wild boar population (Bieber and Ruf, 2005; Toigo et al., 2007).

Considering the fast replication of wild boar the theoretically necessary reduction of >70–80% of the population could only be achieved through professional hunting campaigns with very high killing rates. In practice however, these rates are rarely if ever reached and in addition it must be considered that breeding rates following such a drastic reduction of population will be very high (Bieber and Ruf, 2005; Gamelon et al., 2011).

Therefore, hunting measures alone are not considered to be efficient for CSF control, but despite these limitations hunting can be useful as a complementary control measure (Zanardi et al., 2003) and necessary for collecting samples for laboratory diagnosis (Anonymous, 2009).

Trapping

In smaller areas trapping can be used as an additional method for the reduction of susceptible wild boar. Different types of traps have been developed, which can be used to trap single wild boar or larger groups of pigs (Massei et al., 2011). The efficiency of the method was recently demonstrated in a forest area of 25 km^2 in Bulgaria where a CSF outbreak had occurred. The density of wild boar was estimated to be 6 pigs/km^2. Of a total of 156 animals 119 were removed by trapping within 3 months (Alexandrov et al., 2011). As a result the CSF infection chain was interrupted and the area became CSFV-free. The advantages of trapping are that it can be used in defined areas including residential areas, and (young) age classes can be removed selectively. The social disturbance in the population will be low compared to hunting. The disadvantages are that trapping is labor-intensive and it only works, when naturally occurring feed is scarce. Since it requires euthanasia, there might be little public acceptance and traps might be tampered with by adversaries.

Hunting and trapping not only leads to increased reproduction but it also might influence wild boar behavior with respect to an increase of their home range size and increased nightly activity (Scillitani et al., 2010).

Oral Immunization

Oral immunization of wild life species has proven to be very efficacious in the control of rabies. MLV or recombinant live vaccines were being used for that purpose. Oral vaccination of

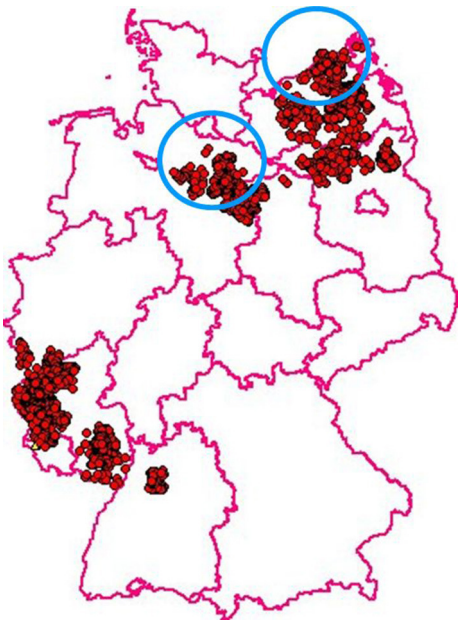

FIGURE 1 | CSF in wild boar in Germany: Between 1994 and 2008 3049 virus positive cases were recorded. Blue circles indicate the regions where the two primary outbreaks were located in 1992. (Courtesy of Friedrich-Loeffler-Institute, Animal Disease Information System, TSN, 2015).

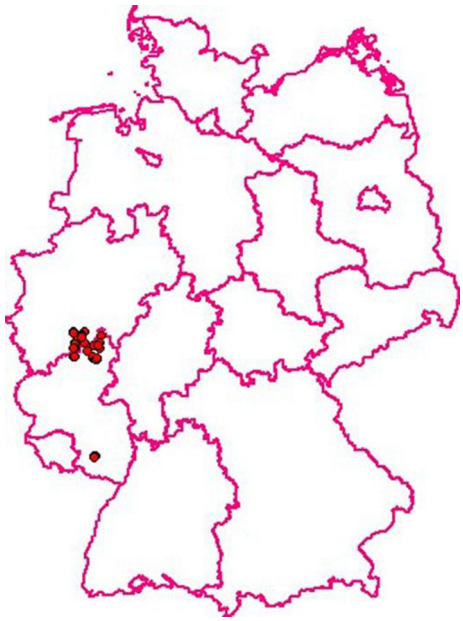

FIGURE 2 | CSF in wild boar in Germany: 52 cases were recorded between January and July 2009. The last case was detected July, 29th 2009. Oral immunization was continued until spring of 2012. (Courtesy of Friedrich-Loeffler-Institute, Animal Disease Information System, TSN, 2015).

wild boar against CSF can also be used as a method to decrease the number of susceptible animals in an infected population.

In 1992 two different variants of CSFV were introduced into the wild boar population by primary outbreaks in Northern Germany. The affected populations were dense and like the rest of the country they had been free from CSF. Conventional control measures, i.e., increased hunting pressure, hygienic measures, and the establishment of risk and surveillance zones failed to yield sustainable results and the infection spread and became endemic and—most likely by human interference—spilled over into more distant areas of the country. Between 1994 and 2008 a total of 3049 CSF cases were recorded in Germany (**Figures 1**, **2**). In this critical situation the option of oral immunization was revisited and first experimental vaccination campaigns were started in 1994. After a few years the outcome of these experiments resulted in a protocol that can be summarized as follows:

The decision to use oral vaccination in the control of CSF should be made on the following criteria: (a) high infectious pressure when there are holdings of domestic pigs in the area; (b) CSF is endemic; (c) high density of wild boar and a high probability that a fresh CSF outbreak becomes endemic; (d) CSF outbreaks in natural reserves with restricted hunting.

The mode of bait distribution differs from oral vaccination of foxes and has to take into account the feeding characteristics of wild boar. Depending on the population structure 0.5–1 feeding place of about 200 m^2 per 100 ha of hunting area is established. Wild boar should be attracted to the feeding place by laying out corn approximately 10 days prior to the distribution of baits. Thirty to forty baits are laid out by hand and covered with soil. Two to four weeks later an identical second vaccination is performed. Aerial distribution of baits is possible and can be applied in dense forests or difficult terrains where manual distribution would be difficult. After each vaccination hunting should be temporarily banned for at least 5 days and after the second vaccination feeding places should be inspected for residual baits in order to assess uptake rates. Usually, bait uptake is high and varies between 80 and 90%. Only when feeding places are not well accepted by the animals uptake rates can be lower (Kaden et al., 2002). The efficacy of oral vaccination should be assessed using serological monitoring. An extended ban of hunting in the vaccinated areas could help to maintain a stable population of predominantly immune animals for as long as possible. In case limited hunting activities are allowed they should concentrate on the shooting of piglets, because they cannot be immunized by oral immunization and they are the reservoir for the virus.

Initially two campaigns with two vaccinations each (double-vaccination) were used per year, i.e., in spring and autumn. However, the disadvantage was that only 20–30% of the young age class turned seropositive. Due to the natural behavior of wild boar adult animals are the first to pick up baits at the expense of the most important target animals, i.e., juvenile adults and weaned piglets. Apart from the competition with adult pigs these age groups can only be reached from an age of 4 months and older depending on bait size. Consequently frequent campaigns are necessary and only after introduction of a third double-vaccination campaign in summer piglets born in spring can be reached at the age of 5 months and more than 50% of young animals shot at the age of approximately 6 months can be found seropositive.

When deciding where to vaccinate there are several possible scenarios: Vaccination in the infected area is applied in order to increase population immunity in order to reach the threshold of $R_0 < 1$. In addition the surrounding surveillance area may be vaccinated simultaneously in order to prevent virus spread outside the infected area. Alternatively, only the surrounding area may be vaccinated as a prophylactic measure in order to stop spread of the virus outside the infected area (*cordon sanitaire*). In the latter case no particular measures are taken in the infected area when there is reason to believe that the infection will fade out over time without intervention (Kaden and Lange, 2001).

Assuming that the threshold for $R_0 < 1$ is reached at a level of about 200 seronegative pigs in an area of approximately 220 km^2, this value can be reached when 500 wild boar living in that area show a seroconversion of 60%. With higher numbers of wild boar seroconversion rates must increase accordingly (Artois et al., 2002).

In Germany results of oral immunization of wild boar varied considerably. In four federal states maximum seroconversion rates after three vaccination campaigns ranged between 37 and 72%. A distinction between antibodies against field virus and vaccine virus was not possible, but it was clearly shown that seroconversion levels rose after each oral vaccination campaign. Virus prevalence was highest in pigs <1 year (79–88%), in the age class 1–2 years prevalence varied between 9 and 19%, whereas adult animals >2 years were rarely found virus-positive (Kaden et al., 2002). Control programs should last at least 2 years and the vaccination area has to be large enough to include animal movements (Kaden et al., 2002). After introduction of oral vaccination all outbreaks of endemic CSF in wild boar in Germany were eradicated within a few years.

A direct assessment of the efficacy of hunting measures alone vs. oral vaccination combined with hunting was carried out in the German federal state of Rhineland-Palatinate from 1999 to 2005. For 3 years after notification of the CSF outbreak in wild boar the control was based on increased hunting, in particular juvenile pigs. General hygiene measures were part of the control plan. Both measures had no noticeable effect on the endemic persistence of CSF. From 2002 until the end of the study the strategy was changed and oral immunization was started as a new major control tool (von Rüden et al., 2008). In parallel wild boar found dead and shot pigs in the restriction area, totaling over 110,000 animals, were tested virologically and serologically for CSF. The laboratory records contained information about geographical origin, gender, and age of the pigs. About 82% of all virologically positive animals were piglets, thus clearly demonstrating that these animals were the virus reservoir and responsible for perpetuating the epidemic/endemic. When the hunting bag was analyzed it became clear that during the whole control program older animals were overrepresented and that not enough young pigs had been shot. This was a clear proof that despite all awareness programs local hunters did not fully support the control program. In piglets the virological prevalence was higher and the serological prevalence was lower compared to adult pigs and yearlings before the start of oral immunization. These differences were significant. After the start of the oral immunization campaign in February 2002 virus prevalence decreased markedly and seroprevalence increased considerably all over the age classes. The last virus-positive wild boar was recorded in July 2009 (von Rüden et al., 2008). In retrospect it is safe to assume that the introduction of oral immunization of wild boar against CSF was a most crucial factor for the eradication of the infection from the German wild boar population (**Figures 1**, **2**).

Criteria for the lifting of restrictions are the last virologically positive case and the serological status of juvenile animals. When all young wild boar, after waning of maternal immunity, are seronegative and the last virus-positive animal was found more than a year ago it can be assumed that the infection has faded out.

OUTLOOK

The control of CSF in wild boar has significantly improved during the last three decades, and a number of tools for the control of CSF are available and strategies have been developed to eradicate the infection in dense wild boar populations. However, there are several details worth amending: In order to enhance sensitivity of virus isolation from organ samples from wild boar, the use of RNA transfection could be introduced routinely (Meyer et al., 2015). This might also minimize bacterial contamination problems often associated with field samples from hunters.

Vaccination plays a major role in the inventory of control measures (Rossi et al., 2010). Two major problems are still associated with oral vaccination: Due to the hierarchical structure of wild boar families old animals tend to eat most baits at feeding places. This could only be solved by devising mechanical barriers that can only be bypassed by small piglets. In addition bait size hinders the acceptance by piglets. Smaller baits might lower the age of pigs that can be reached with oral vaccination. Since present vaccines are based on conventional MLVs there is no distinction possible between infected and vaccinated animals. For future vaccination campaigns it would be desirable to have a DIVA vaccine for oral vaccination available. The serological distinction of vaccinated pigs would greatly facilitate monitoring of progress of control programs.

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REVIEW
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Controlling of CSFV in European wild boar using oral vaccination: a review

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Classical swine fever (CSF) is among the most detrimental diseases for the swine industry worldwide. Infected wild boar populations can play a crucial role in CSF epidemiology and controlling wild reservoirs is of utmost importance for preventing domestic outbreaks. Oral mass vaccination (OMV) has been implemented to control CSF in wild boars and limit the spill over to domestic pigs. This retrospective overview of vaccination experiences illustrates the potential for that option. The C-strain live vaccine was confirmed to be highly efficacious and palatable baits were developed for oral delivery in free ranging wild boars. The first field trials were performed in Germany in the 1990's and allowed deploying oral baits at a large scale. The delivery process was further improved during the 2000's among different European countries. Optimal deployment has to be early regarding disease emergence and correctly designed regarding the landscape structure and the natural food sources that can compete with oral baits. OMV deployment is also highly dependent on a local veterinary support working closely with hunters, wildlife and forestry agencies. Vaccination has been the most efficient strategy for CSF control in free ranging wild boar when vaccination is wide spread and lasting for a sufficient period of time. Alternative disease control strategies such as intensified hunting or creating physical boundaries such as fences have been, in contrast, seldom satisfactory and reliable. However, monitoring outbreaks has been challenging during and after vaccination deployment since OMV results in a low probability to detect virus-positive animals and the live-vaccine currently available does not allow serological differentiation of infected from vaccinated animals. The development of a new marker vaccine and companion test is thus a promising option for better monitoring outbreaks during OMV deployment as well as help to better determine when to stop vaccination efforts. After rabies in red fox, the use of OMV against CSF in European wild boar can be considered as a second example of successful disease control in wildlife. The 30 years of disease control experience included in this review may provide options for improving future disease management within wild populations.

Keywords: ***Pestivirus*, wildlife, diseases, management, surveillance, *Sus scrofa***

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INTRODUCTION

Classical swine fever (CSF) is a major threat to commercial pig production worldwide (Edwards et al., 2000). This multi-systemic disease can affect both domestic pigs and wild boar such that outbreaks among wild boar can significantly impact commercial pig farms. CSF outbreaks among wild boars present a constant threat of introduction into domestic pigs. In Germany during the 1990's, approximately two thirds of primary outbreaks among domestic pigs were attributed to direct or indirect contact with CSF infected wild boar (Fritzemeier et al., 2000).

Generally, control of wildlife reservoirs is a significant challenge (Delahay et al., 2009; Gortázar et al., 2015). To combat infectious diseases, vaccination is often used to decrease the proportion of susceptible animals in a population below a threshold needed for disease maintenance among that population (Rupprecht et al., 2003; Blancou et al., 2009).

Different vaccination approaches for wild boar have been developed and tested, some of them directly under field conditions (e.g., lyophilized vaccines in Russia), others under experimental conditions (Kaden et al., 2000). Under experimental conditions, live attenuated vaccines showed high efficacy and complete safety upon oral immunization of individual animals (Kaden et al., 2000). To deliver the vaccines on a larger scale, oral bait formulations were subsequently developed and tested by Kaden et al. (2000) during the 1990's. These baits were suitable for oral mass vaccination (OMV) and in the following years the approach was considered as a satisfactory option for improving CSF virus (CSFV) control in wild boar in Western Europe (EFSA, 2008; von Rüden et al., 2008; Rossi et al., 2010).

However, upon implementation of large scale oral CSF vaccination, it was discovered that the vaccination process and design need further improvement and was subsequently revisited. As a consequence, adaptations were introduced in all areas of CSF control in wild boar including the baiting strategy, population management, and surveillance design (Rossi et al., 2014). This review addresses and summarizes multiple aspects of oral vaccination of wild boar including its successes and failures, its drawbacks and advantages.

VACCINATION TOOLS

Vaccines

Several CSFV vaccines are available and have been used successfully to control the disease in multiple countries worldwide (van Oirschot, 2003; Greiser-Wilke and Moennig, 2004; Blome et al., 2006; Luo et al., 2014). The most widely used vaccines are conventional live attenuated vaccines including the well-known lapinised "Chinese" C-strain or its derivatives, and the Thiverval strain. These vaccines have shown outstanding efficacy and safety, but do not allow serological differentiation of infected animals from vaccinated ones; for this reason, vaccinated animals are subject to trade restrictions. To overcome these limitations, marker vaccines have been developed based on different vector platforms and expression systems (for review see Beer et al., 2007; Dong and Chen, 2007; Blome et al., 2013). These approaches allow differentiation for field detection of virus infection versus vaccination (DIVA; van Oirschot, 2003; Leifer et al., 2009).

Live Attenuated Vaccines

These traditional live attenuated vaccines have been used worldwide in eradication campaigns both intramuscularly (IM) in domestic pigs and in oral bait formulations in wild boar (Kaden and Lange, 2001). IM application of these vaccines confers protection a few days after immunization (van Oirschot, 2003), before neutralizing antibodies are detected. Antibody detection is typically possible within 2 weeks after vaccination (Kaden and Lange, 2001; Vandeputte et al., 2001). Upon oral immunization, protection is usually conferred within 2 weeks or less (Kaden and Lange, 2001; Blome et al., 2012; Renson et al., 2013), depending on the virulence the pathogenic strain the individual is exposed to. Duration of immunity is at least 6–10 months regardless of the route of administration (intramuscular or oral; Kaden and Lange, 2001). Indications exist that immunity might be even life-long. In the European Union (EU), oral vaccination of wild boar has proven to be very effective for the eradication of the virus (EFSA, 2008). The major drawback of live vaccines is that it is impossible to differentiate antibodies induced by field virus infections from antibodies induced by vaccination.

Marker Vaccines

Baculovirus-expressed E2 recombinant protein subunit vaccines were the first generation of non-replicative marker vaccines for CSF. The efficacy of these two available E2 sub unit vaccines was extensively studied and was determined to be lower than the efficacy of classic C-strain vaccines (Uttenthal et al., 2001). Vaccination could not prevent the "carrier sow syndrome" and subsequently the late onset of CSF (Depner et al., 2001). An additional drawback of this vaccine is that it cannot be used for oral vaccination in baits. In recent years, new approaches have been used to develop marker vaccines that allow a DIVA principle while having the advantages of live vaccines (Beer et al., 2007; Blome et al., 2013). Two promising candidates, *pestivirus* chimera "CP7_E2alf" and flc11, were then compared within an EU-funded research project to decide which would be followed up for licensing. Based on the comparative trial and pre-existing data on safety and efficacy, "CP7_E2alf" was chosen for further assessment and marketing (Blome et al., 2012).

Regarding the BVDV/CSFV chimera "CP7_E2alf," which carries the CSFV E2 and a BVDV backbone (Reimann et al., 2004), immunization and challenge trials showed that after a single intramuscular or oral vaccination, the antibody titers were stable for a minimum of 6 months and full protection from lethal challenge infection was observed. In follow-up experiments, this vaccine proved to be safe and efficacious against challenge with CSFV strains of different genotypes and virulence. The vaccine preparation for intra-muscular use has been recently registered in the EU (Suvaxyn CSF Marker, Zoetis), and there is supportive data showing potential for oral vaccination development.

Diagnostic Tools

For the diagnosis of CSF in wild boar and monitoring following oral vaccination, all methods used for domestic pigs may be used (Blome et al., 2006). These techniques include both direct (virus isolation, antigen detection, genome detection) and indirect (antibody) test systems.The commercial E2-ELISA displays a sensitivity that is in general quite similar to the virus neutralization test (VNT). The specificity is usually high, between 98 and >99.5%. However, cross-reactions may occur with ruminant pestiviruses, especially BDV. Moreover, poor quality of samples derived from wild boar can lead to false positive and negative reactions, especially in ELISA (EFSA, 2008). In recent years, a combination of commercial E2 antibody ELISAs and CSFV specific real-time RT-PCRs has been used to monitor wild boar populations. As CSFV does not present different serotypes, no problems in detecting antibodies against different strains are anticipated (for testing different genotypes see Schroeder et al., 2012). Suitable sample matrices are blood or serum, different organs and even swab samples (Anonymous, 2002; Petrov et al., 2014). Virus isolation in susceptible cell cultures and neutralization tests have been employed as confirmatory assays for CSFV and CSFV specific neutralizing antibodies, respectively. For C-strain vaccination scenarios, sampling and testing strategies have been developed that allow targeted testing (Kaden et al., 2006). Live attenuated vaccine strains such as the C-strain or CP7_E2alf show a very limited replication even in the target host (Koenig et al., 2007). However, highly sensitive detection techniques such as real-time RT-PCR can lead to vaccine virus detection in blood and organs from wild boars that have received oral vaccination (Blome et al., 2011). To rapidly differentiate these detections from field virus infection (genetic DIVA), specific real-time RT-PCR systems have been developed for different C-strain variants and marker vaccine CP7_E2alf (Li et al., 2007; Huang et al., 2009; Leifer et al., 2009). While traditional live attenuated vaccines do not allow a serological DIVA concept, CP7_E2alf has a marker system that is based on the detection of CSFV Erns antibodies. Animals vaccinated with CP7_E2alf will carry CSFV E2 but not CSFV Erns antibodies while field virus infected animals will also show CSFV Erns responses. At present, one Erns ELISA is commercially available (PrioCHECK CSFV Erns, Thermofisher) and additional approaches are currently under development based on either ELISA or Luminex technology (e.g., Aebischer et al., 2013; Xia et al., 2015).

Baits

For a feasible oral immunization scheme, a suitable delivery vehicle in the form of bait is needed. Such baits need to fulfill a wide range of requirements. The most obvious requirement is the acceptance of the bait by the target species. Bait detectability (odor, color), palatability (taste), and uptake must all be considered. Wild boars are omnivores and consume a wide range of foods, but can have very clear preferences for certain food items such as acorns (Brandt et al., 2006; Ballesteros et al., 2011). During initial bait studies with wild boars kept in enclosures, no clear preference were observed between different aromas tested (e.g., apple, corn, almond, hazelnut, truffle, potatoes). This was also confirmed during subsequent field studies with free-ranging wild boars (Schuster, 1996). The animals tend to prefer baits containing plant-derived compounds, especially corn meal, over animal-derived compounds (Schuster, 1996). Based on these studies, the present commercial bait matrix that accompanies the Riemser Schweinepestoralvakzine (IDT Biologika, former Riemser Arzneimittel) consists of corn meal, paraffin wax, milk powder, aroma (almond), and hardened coconut oil. To assess bait uptake, bait markers can be incorporated in the bait matrix or the blister. During initial field trials, tetracycline was used (Kaden et al., 2000). However, bait markers efficient in this species (i.e., tetracycline, iophenoxic acid, rhodamine) are supposed at risk for human health, since wild boar are hunted and consumed by people (Ballesteros et al., 2013; Sage et al., 2013) and subsequently increase the overall cost of vaccination, which compromises the use of chemical markers at a large scale in natural populations (EFSA, 2008; Anses, 2012). Beef tallow which was used in the original bait, was removed from the bait matrix because regulatory requirements limited the use of certain bait materials (e.g., products derived from terrestrial animals; tissues that may transmit spongiform encephalopathy). The bait has a relatively low melting point (30°C) and is therefore not suitable for distribution in areas during periods with high elevated temperatures. To protect the liquid vaccine against environmental factors, including the bait matrix, the formulated vaccine (1.6 ml) is filled in a vaccine container after vaccine production. Subsequently, the PVC vaccine container (20 mm × 20 mm × 7 mm) is sealed with an aluminum foil and incorporated into the bait matrix (40 mm × 40 mm × 15 mm). For CSFV it is important that the vaccine is released in the oral cavity so that it can be taken up by the tonsils to initiate the immune response. Therefore, to release the vaccine in the oral cavity of the wild boar, the animal needs to perforate the vaccine container with its teeth. If baits are too small, it could be swallowed without chewing and the vaccine blister will not be perforated. However, the present bait may be too big for piglets (<4 months of age) to consume. Faust (2007) observed that piglets only played with the baits and showed an incomplete uptake. In this case, the vaccine was not released into the oral cavity, resulting in a failed vaccination attempt in juvenile wild boar. Also, shape and texture can influence bait handling and possibly result in increased vaccine spillage (e.g., dripping on the ground). Several baits composition and shapes were tested in piglets during a former European collaborative project but did not result in a better uptake in that age class in continental European countries. This may be due to the low palatability of baits in comparison with the natural food available during spring and summer when juvenile wild boars are still piglets (Sage et al., 2011; see An Adapted Bait Delivery Process). A field trial for wild boar vaccination in Italy with the new live marker vaccine "CP7_E2alf" and the classic IDT® bait gave results similar to oral vaccination campaigns with C-strain which is encouraging for future vaccination applications even though the commercial version of the oral marker vaccine is not yet available (Feliziani et al., 2014).

DEPLOYMENT CHALLENGES

A Short History of Oral Mass Vaccination

There is always a big gap between the development of vaccination tools by researchers and the deployment of vaccination in the field (van Oirschot, 2003). First, research results have to be translated into industrial products. Then, field trials are needed to assess the efficacy of the bait delivery process (see An Adapted Bait Delivery Process and Assessing Vaccination Efficacy) and allowing an official vaccine registration (OIE [World Organisation for Animal Health], 2012). After that, the process has to be adjusted to the specific local environmental conditions (An Adapted Bait Delivery Process). Regarding the C-strain, the only available oral vaccine currently available on the market, the industrial production and field trials were mainly implemented in Germany during the 1990's and early 2000's by Kaden (1998), Kaden et al. (2000, 2002, 2003, 2004, 2005) and the IDT® company. Once the strategy is officially adopted, many practical problems must be solved before deployment including prerequisite and exhaustive census of the vaccination grounds, organizing the logistics for frozen or cold transportation and storage of several thousands of vaccine-baits within isolated areas, delivering the technical information to hunters and controlling bait distribution and consumption in the field. Stop-hunting 1 week before and during bait distributions has been implemented to avoid animal disturbance and to limit the risk of false PCR-positive results (Louguet et al., 2005). Vaccine-baits alone are relatively cheap (around 1 euro per bait) so the cost of treating one square kilometer of forest averages 400–500 euro per year. However, significant secondary costs are associated with the management of endemically infected areas such as testing of hunter killed animals and incidentally discovered carcasses for CSF serology and virology, the compensation for carcass destruction (CSF positive carcasses to CSF virology are destroyed), the control of carcass identification and trade. During the 2000's in France the total cost of CSF management in wild boar was estimated around 1500 euro per square kilometer of treated forest and per year. Since the early 2000's, the use of OMV is officially supported by the European communities (Council Directive 2001/89/EC) and has been adopted in many countries as part of their emergency plan with an important proportion of success including Germany, Luxembourg, France, Slovakia, Bulgaria, and Latvia (EFSA, 2008; Pol et al., 2008) (**Table 1**). However, many challenges have to be still addressed for improving the baits delivery process, monitoring and efficacy (see further sections).

An Adapted Bait Delivery Process

Currently, bait distribution is provided by hunters (i.e., by hand delivery) on feeding grounds. Attempts to distribute baits by aircraft were completed (Kaden et al., 2002), but are not generally used, possibly due to high costs (EFSA, 2008). Furthermore, several field studies confirmed that wild boar are omnivorous and opportunistic animals that need to be pre-baited before vaccine delivery in order to limit bait uptake by non-target species (e.g., red fox, badgers, martens, birds, etc; Sage et al., 2011; Ballesteros et al., 2013). The C-strain vaccine bait has been classical delivered under ground to target wild boar specifically (Kaden et al., 2002) and to protect live-vaccine against damage due to hot temperatures and consecutive efficacy loss. However, recent behavioral studies using camera traps and different delivery process demonstrated that baits put under ground may decrease wild boar uptake (especially in juvenile boar) while not effectively preventing the consumption by non-target species (Sage et al., 2011). Low bait uptake in piglets less than 6 months old has been a constant problem in previous vaccination attempts (Sage et al., 2011) and the consecutive low vaccination rates in that age class (Rossi et al., 2011; Calenge and Rossi, 2014) have been a well know factor decreasing vaccination efficacy in both human and animal populations (Anderson and May, 1990; see Retrospective Analyses based on Hunting Data). Interestingly, using specific feeders for excluding big animals did not improved the bait uptake in piglets (Sage et al., 2010) and small baits that were efficiently consumed by piglets in Spain (Ballesteros et al., 2009) were poorly consumed in continental European areas, possibly as a result of different food availability between continental and Mediterranean ecosystems during summertime (Sage et al., 2010, 2011). The current vaccination process is based on three double campaigns in spring, summer, and autumn; each campaign comprising two vaccine-baits-distribution spaced by 28 days, aiming at maximizing antibody titters (by booster vaccination) and the proportion of vaccinated juvenile wild boars (Kaden et al., 2004; EFSA, 2008). However, recent retrospective studies, taking into account wild boar demography and spatial structure, confirmed that bait uptake in juvenile wild boar less than 1 year is always very low in summer (∼5%) and autumn (<30%) compared to spring (40–70%). This explains why 1 year is necessary for reaching a maximum seroprevalence in wild boar populations within these areas (Calenge and Rossi, 2014). The classic vaccination process corresponds to the delivery of about 40 baits per vaccination ground and a density of one to two vaccination ground per square kilometer of treated forest (EFSA, 2008). The vaccination effort and the percentage of vaccinated wild boar are correlated until an optimum (i.e., 1.25 baiting places per km^2 in North-eastern France) but vaccination efficacy is strongly influenced by the season and year in relation to natural food competing with feed stations and baits (Calenge and Rossi, 2014). Thus, it is probable that uncontrolled factors (i.e., temperature, rain fall, population dynamics, etc) generate huge variations in the vaccination success even though the baiting process is conserved or intensified from year to year. Finally, vaccination success relies on the delimitation of the vaccinated area, which we further discuss in Sections "Assessing Vaccination Efficacy and Alternative or Complementary Strategies."

Monitoring CSF within Vaccinated Areas

Monitoring CSF outbreaks when vaccinating with the C-strain vaccine has been challenging during the past deployment attempts, since the non-marker vaccine (C-strain) strongly impacts the performance and significance of the diagnostic tools. First, antibodies targeting the C-strain and the wild CSFV strains cannot be differentiated using serological tests (Beer et al., 2007; Dong and Chen, 2007; Blome et al., 2013). Therefore,

TABLE 1 | Documented classical swine fever (CSF) outbreaks in wild boar in Europe and management measures including oral mass vaccination (OMV).

Period	Country and region	Reference	Infected area (max)	Vaccinated area (max)	Outbreak period	Vaccination period	Restriction period	Vaccination treatment
1992–2002	Germany, Lower Saxony	Kaden et al., 2000, 2002 FLI	6278 km^2	1300 km^2 (1993–1994) 5736 km^2 (1997–2004)	12/1992 13.06.2002	10/1993 08/2004	12/1992 12/2004	Field trials Two campaigns a year
1999–2002	Germany, Saxony Anhalt	FLI	709 km^2	3365 km^2	12.10.1999 19.09.2000	12/1999 11/2001	12.10.1999 31.12.2002	Field trials Two campaigns a year
2001–2002	Germany, Saarland	FLI	275 km^2	645 km^2	26.01.2001 13.06.2002	03/2002 10/2003	01/2001 06/2004	Field trials Two campaigns a year
2002	Germany, Northrhine-Westphalia	EURL CSF-DB	759 km^2	1531 km^2	22.04.2002 14.10.2002	08/2002 10/2004	08/2002 09/2004	Field trials Two campaigns a year
2005–2007	Germany, Northrhine-Westphalia	EURL CSF-DB	1993 km^2	1993 km^2	07.10.2005 04.05.2007	12/2005 03/2010	10/2005 03/2010	Three campaigns a year
1999–2002	Germany, M-W Pomerania	Kaden et al., 2004 FLI	12928 km^2	13942 km^2	01.03.1993 21.07.2000	12/1994 06/2002	01.03.1993 31.12.2002	Field trials Two campaigns a year
1995–1997	Germany, Brandenburg	Kern and Lahrmann, 2000 FLI	5059 km^2	9173 km^2	14.03.1995 26.04.2000	04/1995 04/2001	14.03.1995 31.12.2002	Field trials Two campaigns a year
1999–2001	Germany, Baden-Württemberg	Köppel et al., 2007 FLI	703 km^2	1291 km^2	30.09.1998 19.11.1999	08/1999 10/2001	30.09.1998 31.12.2002	Three campaigns a year
1997–2002	Italy, Varese	Zanardi et al., 2003	370 km^2	None	05/1997 12/2000	–	05/1997 02/2002	No OMV limited collective hunting
1985–1990	Italy, Tuscany South	Rutili et al., 1998 OIE	3800 km^2	None	10/1985 11/1990	–	10/1985 11/1990	No OMV "Intensified" hunting
1992–1995	Italy, Tuscany North	Rutili et al., 1998 OIE	304 km^2	None	01/04/1992 01/08/1992	–	01/04/1992 12/1995	No OMV limited collective hunting
1995–1996	Italy, Piacenza	Rutili et al., 1998 OIE	75 km^2	None	09/1995 01/1996	–	Not documented	No OMV limited collective hunting
1998–2000	Swiss, Ticino	Schnyder et al., 2002, OIE	166 km^2 (risk area)	No vaccination done	05/1998 01/2000	–	05/1998 01/2001 (OIE)	No OMV limited collective hunting
1999–2003	Germany, Rhineland-Palatinate, Eifel	von Rüden et al., 2008 EURL CSF-DB	8568 km^2	8600 km^2	05.01.1999 24.03.2003	02/2002 03/2005	01/1999 03/2008	Three vaccination campaigns a year
2002–2004	Germany Rhineland-Palatinate, Palatinate	von Rüden et al., 2008 EURL CSF-DB	4833 km^2	4300 km^2	23.10.1998 12.11.2004	01/2003 02/2006	06/2005 02/2008	Three vaccination campaigns a year
2009	Germany, Right-Side of the Rhine	EURL CSF-DB	5038 km^2	5038 km^2	01/2009 07/2009	02/2009 04/2010	01/2009 06/2012	Three vaccination campaigns a year
2009	Germany, Rhineland-Palatinate, Palatinate	EURL CSF-DB	862 km^2	862 km^2	02.03.2009 30.04.2009	03/2009 04/2010	02/2009 06/2012	Three vaccination campaigns a year
1992–1997	France, Vosges du Nord	Rossi et al., 2005a,b		No OMV	01/1992 01/1997	–	01/1992 12/2000	No OMV normal collective hunting

(Continued)

TABLE 1 | Contineud

Period	Country and region	Reference	Infected area (max)	Vaccinated area (max)	Outbreak period	Vaccination period	Restriction period	Vaccination treatment
2002–2003	Luxembourg, whole country	SANCO 10257/2003, Brauer et al., 2006 EURL CSF-DB	2592 km^2	2592 km^2	11/2001 08/2002	03/2003 09/2005	11/2002 09/2005	Three vaccination campaigns a year
2003	France Thionville	Pol et al., 2008	200 km^2	No OMV	04/2002 07/2002	–	04/2003 03/2005	No OMV limited collective hunting
2003–2007	France, Vosges du Nord	Pol et al., 2008; Rossi et al., 2010; Calenge and Rossi, 2014 EURL CSF-DB	2890 km^2	2890 km^2 (1250)	14/04/2003 01/05/2007	08/2004 06/2010	09/2004 11/2011	Three vaccination campaigns a year
2005–2008	Slovakia	EURL CSF-DB ADNS SCoFCAH	9897 km^2	9897 km^2	07/2004 05/2008	02/2005 11/2010	07/2004 06/2011	Three vaccination campaigns a year
2004–2009	Bulgaria,	EURL CSF-DB ADNS, WAHID SCoFCAH	35887 km^2	35887 km^2	05/2004 09/2009	07/2005 To date	05/2004 To date	Three vaccination campaigns a year
2007–2009	Hungary	ADNS SCoFCAH	∼4500 km^2		01/2007 10/2009		01/2007 09/2012	No OMV
2006–2007	Romania	ADNS SCoFCAH	63247 km^2	63247 km^2	01/2006 11/2007	05/2007 12/2011	01/2006 09/2012	Three vaccination campaigns a year
2002	Belgium	EURL CSF-DB ADNS	743 km^2	–	11/2002 11/2002	–	11/2002 01/2004	No OMV
2012–to date	Latvia	ADNS SCoFCAH	∼9000 km^2	∼5000 km^2	16.11.2012–to date (last reported case 26.03.2015)	05/2013–to date	16.11.2012–to date	Three vaccination campaigns a year

FLI, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Germany; EURL CSF-DB, European database for Classical swine fever; OIE, world organization for animal health; SANCO, European Commission Directorate-General for Health and Consumers; ADNS, European Animal Disease Notification System; SCoFCAH, European Standing Committee on the Food Chain and Animal Health.

during OMV, seroprevalence is indicative of an average level of population immunity but not of CSF circulation (Kaden et al., 2006; Calenge and Rossi, 2014). Second, the proportion of viropositive individuals is very low in vaccinated populations, which compromises the probability of virus detection even though hunting bags are exhaustively examined within infected areas, (i.e., representing several thousands samples per year; van Oirschot, 2003; Rossi et al., 2010) using highly sensitive PCR tools (Blome et al., 2006). Additionally, among sparse PCR-positive results, false positive may occur after several vaccination campaigns, corresponding to C-train genome traces (in spleen samples), which interfere also with monitoring efficacy and justified the development of DIVA-PCR we yet described in Section "Diagnostic Tools" (Blome et al., 2011). After the completion of vaccination, seroprevalence remains high for some years, so that CSF circulation cannot be correctly monitored; surveillance has thus to be maintained for at least 3 years after OMV completion even though no more cases are detected (Kaden et al., 2006; Rossi et al., 2014; Saubusse et al., accepted). The longitudinal monitoring of capture-marked-recaptured wild boar may help in better interpreting wild boar immune response, but is also spatially limited and time consuming (Rossi et al., 2011; Saubusse et al., accepted). The future development of a new marker vaccine would help to improve outbreak monitoring within vaccinated areas, since antibodies from vaccinated and infected animals could be differentiated using companion serological tests.

ASSESSING VACCINATION EFFICACY

Retrospective Analyses based on Hunting Data

At the Outbreak Level

Vaccination success was determined through retrospective studies based on field hunting data collected in Germany, Luxembourg, and France, which showed evidence of a significant increase of seroprevalence up to 60% and a quick decrease of viroprevalence under 1% within vaccinated areas within 1–6 years (Kaden et al., 2002, 2003; von Rüden et al., 2008; Rossi et al., 2010). Nevertheless, vaccination success has not been complete as CSFV has been spreading in spite of vaccination in continuous forested areas (Kaden et al., 2002) and CSFV may re-emerge after disappearance of the virus for several years (EFSA, 2008). Such problems possibly arose because (i) vaccination areas were too small compared to the actual area at risk of disease spread (i.e., the whole connected forested areas), (ii) juvenile were not correctly immunized during the critical average age

of infection, (iii) vaccination was not maintained for enough time (EFSA, 2008). Retrospective analyses performed in France highlighted that, vaccination is not necessarily preventing CSF spread within connected forested areas, due to the fact emergency vaccination is not effective enough to break the chain of transmission immediately. Nevertheless, proactive vaccination, when performed within a 24 km width buffer vaccination area surrounding the virus wave front (i.e., corresponding to 1 year virus spreading average distance), is able to limit further disease persistence possibly by preventing the re-invasion between neighboring sub-populations (Rossi et al., 2010). These results are logical since a maximum seroprevalence is ultimately reached after 1 year of deployment (i.e., after a complete cycle of three double vaccine distributions was achieved; Rossi et al., 2010). An accurate delimitation of infected and vaccinated areas, according to landscape/forest structure and existing barriers, is thus considered as a critical step for controlling CSFV in wild boar using vaccination (EFSA, 2008).

At the European Level

As previously discussed by Rossi et al. (2005a) and Kramer-Schadt et al. (2009), the dimension of the risk areas (in square km^2), which depends on forest extend and structure, has been the main factor influencing outbreaks duration from 1985 up to 2009 ($R^2 = 0.46$, $p < 0.001$, **Table 1** and **Figure 1**), When OMV was performed three times a year, the average outbreaks duration decreased ($OMV_{effect} = -13$ months, ± 10.8, $p = 0.22$), but at the same time, the average delay between the last viropositive result and the end of restrictions measures increased ($OMV_{effect} = +12.9$, ± 5.1, $p = 0.02$); possibly as a result of the confusing effect of OMV on serological and PCR results (see Deployment Challenges). Thus, OMV has not reduced the cost of CSF management, but it has been the only strategy preventing outbreaks re-emergence in large connected forested areas in Europe [e.g., Palatinate (Ge) and Vosges du Nord (Fr)] (EFSA, 2008; **Table 1**).

Modeling Efficacy

The epidemiological modeling of wildlife diseases is a tool used to support disease control and mitigation measures. Mathematical models from population ecology demonstrate the principle of OMV in wildlife (Anderson et al., 1981). The approach focuses the estimation of a minimum population proportions that should be protected against infection during an OMV program to halt the spread of CSF. Along with early field trials of OMV such models proposed an average 40–50% population level immunity as sufficient to stop CSF spread (Hone et al., 1992; Guberti et al., 1998). However, the models relied on critical simplifications that may have led to an underestimation of the threshold population immunity. The infectious period may differ between infected wild boar individuals because immunocompetency varies according to age and body condition, and the occurrence of rare chronic (i.e., long-lasting) infection is a critical factor regarding CSF dynamics (Kramer-Schadt et al., 2009) and vaccination efficacy (Lange et al., 2012), which could not be caught by average simplest models. Moreover, most of the wild boar populations subjected to OMV are big and distributed over large connected areal (Rossi et al., 2005a). Thus, the assumption of sufficiently contact within the complete population on the temporal scale of an individual CSF infection was not biologically reasonable and spatially explicit models were required for better understanding the persistence patterns of CSF in the wild. Next step to support OMV planning was the application of stochastic meta-population modeling that suggested a useful population level immunity of 60% (EFSA, 2008) in line with field estimates from vaccination areas. More recent research, implementing individual-based models of wild-boars moving and getting infected in a spatially explicit habitat landscape, were finally implemented for testing different vaccination strategies (Lange et al., 2012). These last models highlighted that the probability of CSFV eradication particularly relied on the implementation of preventive vaccination and the maintenance of vaccination effort for at least 5 years (Lange et al., 2012). A possible next modeling step could be to take into account the temporal and spatial variation of vaccination efficacy (Calenge and Rossi, 2014).

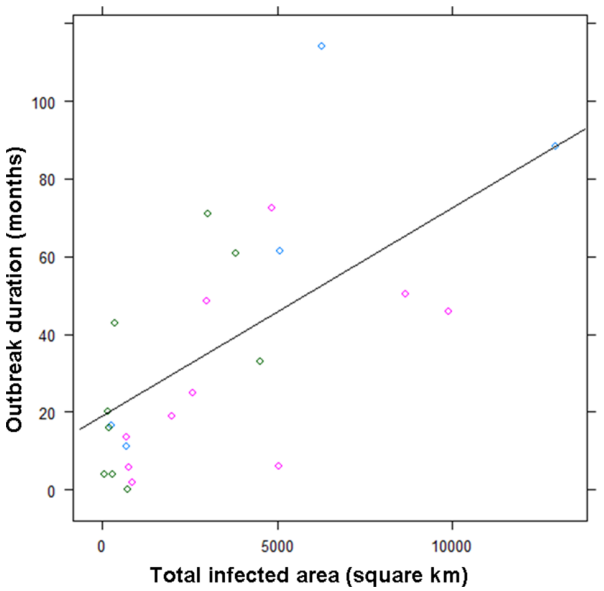

FIGURE 1 | Duration of outbreak (number of months with viropositive results) as a function of infected areas and of the vaccination treatment over 24 "fade out" outbreaks (1985 to 2009). Green circles correspond to non-vaccinated areas, blue circles correspond to primary field trials using two simple or double campaigns a year (before 2002), pink circle correspond to the current oral mass vaccination (OMV) scheme using three double campaigns a year (mainly after 2002). The line is representing the average linear regression linking the duration of outbreak to infected areas.

Virus Evolution under Vaccination Pressure

Classical swine fever virus can be assigned to three genotypes with three to four sub-genotypes each. These genotypes do not translate into serotypes that pose a problem in diagnostics or vaccination. Over the last decade, mainly strains of genotype 2, especially 2.1 and 2.3 were circulating in Europe. In the wild boar, only subtype 2.3 was prevalent (for review see Beer

et al., 2015). In general, CSFV is exceptionally stable for an RNA virus (Vanderhallen et al., 1999), and mass application of the C-strain did not induce a detectable evolution of the virus in the wild (see below). Indeed, due to this observed genetic stability, even a single point mutation could be considered relevant for molecular epidemiological studies of CSF outbreaks. The evidence of separate evolution of the two outbreaks in the 1990–2000's traced one outbreak to the strain Rostock and the other to the strain Uelzen in France and Germany. This has shown that environmental factors including absence of a forest continuum between two regions have a real contribution to containment of the disease (Pol et al., 2008; Simon et al., 2013). More recently, full genome sequencing has been carried out to investigate the evolution of the CSFV during a long-term outbreak within the wild boar population in the Vosges du Nord mountains region. The samples were chosen based on the results of partial sequencing (Simon et al., 2013) and the availability of temporal and spatial data in relation to the application of the C strain vaccine. It was demonstrated that the identified clusters were associated with the presence of barriers including roads, rivers, or railways rather than to a viral strategy to escape to the vaccine immune response.

ALTERNATIVE OR COMPLEMENTARY STRATEGIES

What about the Depopulation Option?

During the 1980's and early 1990's, the "pre-vaccination era," wild boar density was considered to be the main factor favoring virus emergence and endemic persistence; reaching a threshold value of about 1 wild boar per km^2 was recommended for achieving virus eradication according to a pure density-dependent argument and assuming a high virulence of virus strains (Hone et al., 1992; Guberti et al., 1998). At that time, CSF control in wild boar was supposed to be achieved through depopulation only, such as used in domestic pigs, and depopulation was expected to be performed by increasing hunting pressure and/or destroying trap-captured animals (EFSA, 2008). Such depopulation strategy was even recommended by the European experts and the former EU legislation to the member states faced with CSF in wild boar (Alexandrov et al., 2011; 91/685/CEE, Art. 6, par. 5, letter e). In practice, the depopulation strategy has never been satisfactory for controlling outbreaks in wild populations, and was even considered as an aggravating factor for CSF spread and persistence by some authors (Laddomada, 2000; Artois et al., 2002; Schnyder et al., 2002). Many reasons could explain the failure of the depopulation strategy, even if the density-dependent approach had been effective, including (i) wild boar density at which the virus could fade out was probably lower than that which could be achieved through hunting, (ii) the exact population size and density of the involved wild boar population were rarely known, and (iii) the low acceptability of depopulation among hunters, especially when targeting females and very young piglets (EFSA, 2008). In the field, it is likely that the infected populations were managed according to typical hunting strategies, focused on maintaining or increasing a populations' size, with moderate hunting pressure on reproducing females (Gamelon et al., 2012; Keuling et al., 2013). One may even fear that the actual hunting pressure during the early stage of the outbreaks was actually lower than before CSFV emergence due to the lethality induced by the virus and the difficulty of hunting sparse animals (Rossi et al., 2005a). Additional "depopulation tools" such as trap-capture or poisoning, were sometimes carried out in the field in Europe (Alexandrov et al., 2011), but trapping is not cost-effective for the large-scale management of wild boar and poisoning has been considered unacceptable for both animal welfare and human safety in Europe (EFSA, 2008). Finally, more recent studies suggested that the density-dependent approach was not effective for eradication of CSF given that: (i) wild boar density is not the main factor driving CSF persistence which rather relys on landscape structure (related to the total population size at risk) and the moderate virulence of virus strains involved in wild outbreaks (Rossi et al., 2005a; Kramer-Schadt et al., 2009), (ii) increasing hunting pressure might increase population turnover and increase the risk of disease persistence in naïve piglets (EFSA, 2008), (iii) hunting is known to increase home range size and could thus contribute to increasing the mixing and disease transmission between social groups or subpopulations (Keuling et al., 2008; Saïd et al., 2012). It is notable that the depopulation strategy was again addressed by the European communities regarding the management of African Swine Fever (ASF) recently emerging in the European wild boar; depopulation was not considered as an suitable option given its lack of efficacy, in spite of a lack of available vaccine (EFSA, 2014; Gavier-Widén et al., 2015).

Restraining Wild Boar Movements and CSF Spread

The intrinsic spreading of CSF within natural wild boar populations relies mainly on the forest structure and the presence of physical barriers. Due to the forest habitat of the species, the main factor influencing CSF spread (and persistence) within wild boar populations is the connectivity (∼distance) between neighbor forest patches (Rossi et al., 2005a, 2010). Physical barriers may also participate in limiting animal movements, especially fenced motorways and major rivers or lakes (Laddomada, 2000; Schnyder et al., 2002; Rossi et al., 2010). However, the efficacy of barriers for preventing animal movements depends on their nature and/or the combination with the forest structure (Martin et al., 2013). The reliability and practicability of erecting fences for preventing disease spread in wild boar has been addressed regarding both CSF and ASF control in Europe (EFSA, 2008, 2014). In theory, this solution is attractive especially when OMV is not possible, but in practice it has been found poorly satisfactory (e.g., the recent spread of ASF in Lithuania in spite of huge fencing efforts, EFSA, 2014). The main problems with using fences include that: (i) it is costly, (ii) it takes time to build during which diseases may spread further, (iii) our knowledge about the exact position of the wave front of a wildlife disease at time t is not always accurate, (iv) wild

boar are very good at damaging fences and fences must be regularly checked and fixed, which is costly and seldom achieved. As discussed previously, collective hunting is expected to increase animal home range and dispersal, thus hunting bans or banning of hunting dogs during collective hunting has been implemented around physical barriers to limit the risk disease spread out of infected areas (Louguet et al., 2005; Rossi et al., 2011). Nevertheless, hunting restrictions do not prevent natural seasonal movements of wild boars, which are often unrelated to human activities (Siat et al., 2010). Finally, a main aspect for control of CSF spread in wild boar is the prevention of direct and indirect contacts between wild boar and domestic pigs, which relies essentially on (i) biosecurity measures and swill feeding control at pig farms (ii) control of wild boar feeding, carcass trade and viscera releases (Laddomada, 2000; EFSA, 2008).

CONCLUSION

Wild boar vaccination against CSF has been applied for more than 15 years in the EU using a highly efficient live attenuated vaccine, the C strain-Riems, delivered in baits. While intensifying hunting or erecting fences has not been adequate for preventing disease spread or persistence, OMV has proved to be effective in maintaining herd immunity and achieving CSF control; it is the only available method for CSF eradication in large forested areas. On the other hand, CSF may also be quickly eradicated without vaccination in small forested areas ($<$1000 km^2) well delimited by physical barriers by establishing hunting restrictions to avoid disease spread (e.g., Thionville in France and Ticino in Switzerland). Obviously, CSF control is also dependant on the precautionary measures taken for carcasses control and pig farm biosecurity. An integrated strategy is preferred to a single one to maximize the chance of success and also combining other strategies with vaccination should be considered. It is interesting to note that intensified hunting, feeding bans and fencing were recently re-evaluated as possible management measures for controlling ASF in Europe and were not considered to be adequate since now, given past experience during CSF outbreaks (EFSA, 2008, 2014).

The current OMV method relies on multiple bait distributions per year which represents a huge collective effort. Thus, it relies on the involvement of the stakeholders including hunters, wildlife agencies, local, and central veterinary services, and local and reference laboratories. Efforts must be coordinated between neighboring regions or countries when sharing the same forested areas, wild boar populations and outbreaks. Management success relies not only on baiting intensity or the number of vaccination campaigns. First, the landscape structure (forest and barriers) has to be considered for determination of the infected areas and development of a monitoring scheme. This enables quick and proactive deployment of OMV (24 km buffer area). Second, a multiple-year application of OMV is necessary to prevent CSF re-emergence. Furthermore, OMV does not generate genetic evolution of the virus strains.

Oral mass vaccination is costly during and after OMV deployment; carcass monitoring and restrictions last for several years after vaccination due to the confounding effect of non-marker vaccine on surveillance hunting data. Diagnostic tools have to be reliable and adapted for this purpose. Future outbreaks could be addressed and controlled more rapidly using oral marker vaccine (which is validated but not yet commercially available) and companion serological tools (which have to be validated). Even if the youngest piglets cannot eat the baits, experience of the last years of vaccination showed that they were protected by maternally derived antibodies. Therefore, applying repeated vaccination for adult females could avoid this possible failure in vaccination programs.

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REVIEW
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Persistent *Bovine Viral Diarrhea Virus* Infection in Domestic and Wild Small Ruminants and Camelids Including the Mountain Goat (*Oreamnos americanus*)

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Bovine viral diarrhea virus (BVDV) is a pestivirus best known for causing a variety of disease syndromes in cattle, including gastrointestinal disease, reproductive insufficiency, immunosuppression, mucosal disease, and hemorrhagic syndrome. The virus can be spread by transiently infected individuals and by persistently infected animals that may be asymptomatic while shedding large amounts of virus throughout their lifetime. BVDV has been reported in over 40 domestic and free-ranging species, and persistent infection has been described in eight of those species: white-tailed deer, mule deer, eland, mousedeer, mountain goats, alpacas, sheep, and domestic swine. This paper reviews the various aspects of BVDV transmission, disease syndromes, diagnosis, control, and prevention, as well as examines BVDV infection in domestic and wild small ruminants and camelids including mountain goats (*Oreamnos americanus*).

Keywords: ***bovine viral diarrhea virus,*** **mountain goats (*Oreamnos americanus*), small ruminants, persistent infection, wildlife diseases, goats**

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INTRODUCTION

Bovine Viral Diarrhea Virus (BVDV), an RNA virus, is a pestivirus in the family Flaviviridae. Other pestiviruses include *Border Disease Virus* (BDV) in sheep and *Classical Swine Fever Virus* (CSFV) in swine, and more new pestiviruses are being discovered. Though the preponderance of BVDV research has been focused on the primary host, domestic cattle (*Bos taurus),* there is increasing evidence that the virus infects and causes persistent infection in a wider range of species, including mountain goats (Nelson et al., 2008) and domestic goats (Bachofen et al., 2013). This paper aims to review reports of non-bovine persistently infected (PI) animals, including the mountain goat (*Oreamnos americanus*), and evaluate the implications of wildlife reservoirs of BVDV infection and its impact on the cattle industry.

TRANSMISSION

There are multiple methods of BVDV transmission; the virus can spread horizontally within a herd as well as transmit vertically from cow to calf. Horizontal transmission can occur via transiently

infected (TI) animals that shed virus during acute infection. Horizontal transmission can also occur due to PI animals that shed virus throughout their lifespan in all bodily secretions (nasal and ocular discharges, milk/colostrum, semen, urine, and feces; Van Campen and Frolich, 2001). Experiments show that BVDV environmental survival is dependent upon temperature and moisture levels with a maximum survival in bovine farm slurry at 5°C for 3 weeks and at 20°C for 3 days (Botner and Belsham, 2012). There are anecdotal and experimental reports of indirect BVDV transmission from contaminated pens, rectal examination gloves, hypodermic needles, nose tongs, and ambient air (Niskanen and Lindberg, 2003). Experimental vector transmission from PI animals has been successful using horse flies *(Haematopota pluvialis)* and stable flies *(Stomoxys calcitrans),* but not horn flies *(Haematobia irritans,* Chamorro et al., 2011). Since studies of indirect transmission can be difficult to adequately control, repeated studies with strict controls are necessary to determine and confirm the many possible indirect routes of BVDV transmission.

A PI animal occurs when the fetus is exposed to BVDV in the first or second trimester (45–125 days), prior to maturation of its immune system. In these feti, the virus is recognized as self, resulting in an immune-tolerant state and persistent viremia without seroconversion. However, if a different strain of the virus infects the PI animal (superinfection), they can immunologically respond, resulting in seropositivity (Walz et al., 2010). Vertical transmission may occur from a PI dam *in utero* to her offspring. In vertical transmission, the outcome of infection is determined by the stage of fetal maturation when exposed to the virus *in utero*. If the fetus is infected in the first trimester, it will likely abort, mummify, or show a variety of congenital defects. Infection during the second trimester results in a PI animal, as previously discussed. By the third trimester of gestation (>180 days), the fetus is immune-competent and will mount an immune response that may result in abortion, or the birth of a healthy or weak and seropositive calf. BVDV virus can be transmitted from PI or TI animals through direct contact, shared feed and water sources, environmental contamination, frozen embryos or semen, *in utero,* or fomites (Thurmond, 2005).

Nettleton (1990) stated that "the probability exists, therefore, that pestiviruses have evolved along with their own host species. Interspecies transmission is achieved easily experimentally and it is prudent to believe that it will occur readily in domestic and free-living ruminants when permitted to do so by new husbandry practices or changes in population dynamics." The pestiviruses are known to cross animal species from both experimental and natural studies (Van Campen and Frolich, 2001). The question that has emerged is how pathogenic are the viruses when they spill over to another animal host (DeFilippis and Villarreal, 2000)? Once interspecies transmission occurs is there intraspecies spread, which propagates the infection in the spillover population?

In the majority of cases involving llamas and alpacas there has been some commingling with cattle, sheep, or goats. The consensus has been that there is spillover of pestiviruses, primarily BVDV, from cattle to llamas and alpacas (Belknap et al., 2000). Levels of BVDV in viremic cattle that are PI are very high, $>10^4$ $TCID_{50}$/0.1 mL (Brownlie et al., 2000). This would make them prime candidates for shedding to susceptible llamas and alpacas. However, if the infecting virus did not replicate well, or the immune response was elevated, then further intrahost spread would not likely occur. This latter observation appears the best explanation to date. However, serologic data from camels (Evermann, 2006), and wildlife, including roe deer (Fischer et al., 1998), strongly suggest that unique pestiviruses are infecting these species independent of cattle, sheep and goats. This would indicate that there are several host clusters (**Figure 1A**) in which strains of pestivirus are circulating within the cluster. Given optimum conditions such as BVDV PI animals, commingling stress, temporarily immunosuppressed pregnant animals, pestivirus naïve animals, and virulent pestivirus strains, then pestivirus transmission may occur between clusters (**Figure 1B**). Following infection, disease may occur, but rarely would an epidemic develop since intrahost spread would likely be negligible (Mattson, 1994; DeFilippis and Villarreal, 2000).

DISEASE SYNDROMES

Bovine viral diarrhea virus is known for causing a variety of disease presentations in cattle and other ungulates. There are two genotypes of the virus: BVDV-1 and BVDV-2, both of which have also been isolated from non-bovine species. The genotypes are further divided into cytopathic (CP) and non-cytopathic (NCP) subtypes. CP BVDV arises from rare mutations of the NCP strains. NCP viruses are associated with the majority of BVDV infections (90%) and cause mild transient infection as well as persistent infection. CP biotypes cause severe acute and peracute transient disease as well as mucosal disease in superinfected PI animals (Walz et al., 2010). In general, transient BVDV infections can be divided into five categories: acute, severe acute, hemorrhagic infection, bovine respiratory disease, and immunosuppression-only. In addition to these five syndromes, BVDV can also cause chronic disease and mucosal disease in PI animals (Evermann and Barrington, 2005). PI animals may be subclinically infected or may be runted with ill thrift. The importance of acute (transient) infections in the transmission and maintenance of BVDV within a population of animals (domestic and wild) should not be underestimated. These TI animals are responsible for up to 93% of all *in utero* infections that result in the birth of PI calves (Wittum et al., 2001).

WHAT IS THE ROLE OF BVDV PERSISTENT INFECTION IN WILD UNGULATES?

Although BVDV is named for its primary host, its prevalence in non-bovine species has become increasingly recognized. To date, the virus has been isolated in over 40 species and serological evidence indicates that most wild ruminants are susceptible to BVDV infection. In addition to wildlife, multiple domestic non-bovid species have also been reported to carry and spread the disease, including sheep, goats, new world camelids, and swine. There is evidence of transient infection

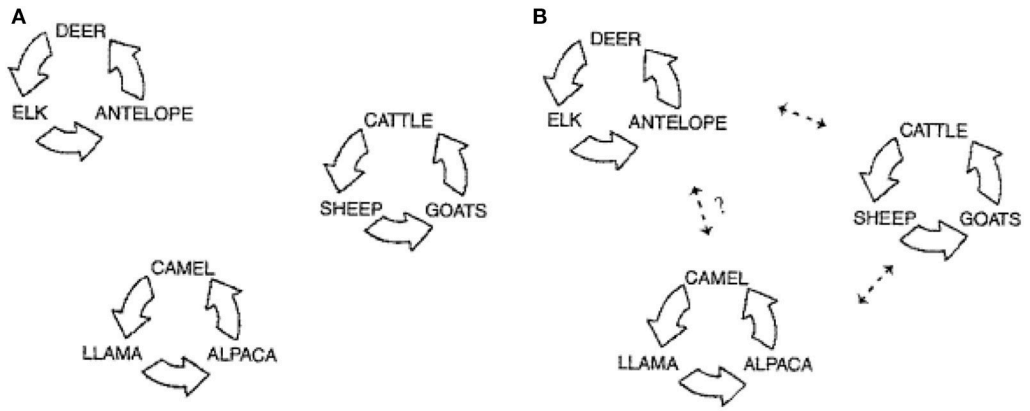

FIGURE 1 | Schematic depicting the three population groupings for pestivirus infections. (A) Represents the circulation of the virus infection within three distinct main host clusters: wildlife, domestic livestock, and camelids. **(B)** Represents the documented spread of virus between these clusters, and the potential for transmission between the camelid cluster and wildlife cluster (modified from Evermann, 2006).

within most of these species, resulting in the familiar BVDV syndromes of reproductive insufficiency, respiratory disease, and immunosuppression (Carman et al., 2005; Vilcek and Nettleton, 2006; Ames, 2008; Nelson et al., 2008). However, a select few have been proven to become PI with the virus and act as a significant transmission source to other susceptible species. Natural or experimental persistent infection has been reported in mountain goats (Nelson et al., 2008) and domestic goats (Bachofen et al., 2013), domestic sheep (Scherer et al., 2001), swine (Terpstra and Wensvoort, 1997), alpaca (Mattson et al., 2006), eland, mule deer, white-tailed deer, and mousedeer (Duncan et al., 2008). Since PI animals represent the greatest risk for disease transmission, the identification of PI wildlife species is cause for concern.

In most cases of infection with BVDV in non-bovid species, the primary source of virus is unknown, though the virus exposure is assumed to stem from initial spillover from cattle. This spillover can occur through multiple routes, including direct contact, aerosol, environmental contamination, or fomite transmission, such as shared feed and water sources or shared equipment (Ames, 2008). Direct contact and shared environment are important sources for cattle producers to consider when attempting to eliminate BVDV-associated disease from their herds, while shared equipment is a more important consideration in captive wildlife collections.

In a USDA agricultural census, over 60% of dairy and over 70% of beef producers reported direct contact of their stock to wild cervids (USDA National Agriculture Statistics Service, 2007). Of the potential PI wildlife species, white-tailed deer *(Odocoileus virginianus)* likely present the greatest threat to livestock producers due to their wide range and adaptability to dairy or ranching management systems. Deer are commonly in close contact to cattle, often sharing feed, water, and lounging sites. This environmental interaction between species serves as a source of BVDV transmission. Multiple studies have examined the interactions between white-tailed deer and cattle and how the virus transmits between the two species (Passler et al., 2007; Duncan et al., 2008; Passler and Walz, 2009). One experiment housed seven pregnant white-tailed deer with two known PI cattle to test whether the virus would transmit through cohabitation. Though the does and cows were not observed in physical contact, feed and water sources as well as lounging areas were shared between species. Of the nine live fawns born, all three singlet fawns were born PI (virus positive, antibody negative), and all twin fawns cleared the infection and were born virus negative and seropositive (Passler and Walz, 2009).

Another North American cervid known to have the potential for persistent infection is mule deer *(Odocoileus hemionus)*. Thus, in a routine survey of tissues from hunter-harvested deer in Colorado for chronic wasting disease, BVDV was added to the testing protocol. A single adult male mule deer was positive on skin immunohistochemistry (IHC), which is a consistent finding in BVDV PI cattle. The animal showed no signs of illness, though the virus was identified in both the submitted ear and lymph node, and these findings are suggestive of persistent infection. PCR was performed to further characterize the virus as BVDV-1. The source of infection in this case was unknown, but is assumed to be spillover from cattle (Duncan et al., 2008).

In 2000, a survey was performed on 1539 eland *(Taurotragus oryx)* in Zimbabwe to assess the number of animals infected with BVDV in a high density cattle area, and 32% of eland sampled were antibody positive on ELISA. Three animals were found to have NCP BVDV. Two of these seroconverted on subsequent sampling dates, but one young female remained viremic over time and was determined to be persistently affected. The PI animal eventually lost condition and died following an episode of febrile illness (Vilcek et al., 2000). Presumably domestic cattle served as the primary viral source.

There are multiple reports of PI domestic species including new world camelids. Two PI crias were identified on a Pennsylvania breeding farm. Both presented for stunting and immunosuppression. The first affected cria was determined to be PI following repeated virus isolation in the absence of seroconversion. Euthanasia was elected to protect the breeding herd. The second cria was euthanized after initial virus isolation

at 6 weeks of age. On the same day, blood was submitted from the 15 adults on the property. 14/15 were seropositive and one additional male was transiently viremic (Mattson et al., 2006).

A second report in alpacas chronicled a farm in eastern Ontario, Canada, where a chronically ill cria was evaluated at necropsy. Upon reviewing the herd records, it was discovered that the cria's dam had traveled to four different breeding farms during her pregnancy, two of which had experienced numerous abortions and stillbirths. The Ontario herd experienced vague herd illness following the birth of the PI cria, characterized by anorexia, lethargy, and several abortions (all of which were positive for BVDV). Out of 20 animals tested, 17 were positive for antibody to both BVDV strains. Each of the 13 crias born after the initial abortion were tested at birth for BVDV using RT-PCR. Only one of these crias was positive, euthanasia was elected. The euthanized cria was positive for virus at necropsy using IHC (Carman et al., 2005).

Bovine viral diarrhea virus infection has also been reported in domestic sheep. Ewes were experimentally infected with NCP BVDV-2 at three different stages of gestation. Of 19 ewes infected at 50–60 days of gestation, there was a 77% fetal death loss. The lambs that were born alive were positive for BVDV at birth and were negative for antibody after maternal antibody waned, which confirms PI status. Of the ewes infected at 65–70 days gestation, the death loss was 66.6%, and the live lambs were virus negative and antibody positive, demonstrating an appropriate immune response to viremia *in utero*. Ewes infected with BVDV at 120–125 days of gestation gave birth to healthy virus negative, antibody positive lambs (Scherer et al., 2001).

Domestic swine can also become PI. Terpstra and Wensvoort published a case in which a litter of piglets became infected with BVDV. Of the 13 pigs in the litter, seven died within 2–4 weeks of birth. Three of the remaining six were euthanized due to wasting and deep ulcers of the jaw and extremities. Three of the remaining pigs survived until slaughter, including one PI boar, one seropositive boar, and one intersex pig. The PI boar remained viremic and immunotolerant until slaughter at 26 weeks of age. The viremic boar shed the virus in oropharyngeal fluid, urine, and semen and was leukopenic from 3 months onward (Terpstra and Wensvoort, 1997). Since persistent infection can occur in domestic swine, feral swine can potentially become sources for disease transmission.

In 2005, routine quarantine at the Copenhagen zoo revealed a BVDV PI Lesser Malayan mousedeer *(Tragulus kanchil),* prompting a trace back to be performed on the deer's lineage. The trace back identified 10 PI animals in two generations all of which could be traced back to a single PI female. All other animals in contact with these PI mousedeer were found to be antibody positive and virus negative. The PI mousedeer was asymptomatic throughout the testing, but was viremic over multiple testing dates without evidence of seroconversion (Uttenthal et al., 2006). This is the first report of the existence of mature PI animals, other than cattle, that were able to reproduce and produce PI offspring.

Domestic goats can be infected with BVDV with reproductive disease as the most common disease manifestation. PI cattle are considered the main source of infection, as this occurs under natural and experimental conditions. Pregnant goats in direct contact with a PI calf aborted or produced PI kids, and pregnant goats in contact with PI kids produced additional PI kids (Bachofen et al., 2013). Another similar study with pregnant goats exposed to PI heifers with BVDV-2a resulted in abortion and stillbirth but not PI kids suggesting that the development of PI kids is relatively rare (Broaddus et al., 2007). Another study, produced similar results with intranasal inoculation of pregnant goats with BVDV-1 or BVDV-2 resulting in reproductive loss and, less commonly, PI kids further suggesting that BVDV may be maintained in goat populations (Passler et al., 2014). While a native Korean goat developed diarrhea due to BVDV-2 infection (Kim et al., 2006), reports of goats with non-reproductive disease associated with BVDV infection are rare.

There is also evidence for BVDV infection in wild goats. Antibodies to BVDV have been detected in serosurveys of wild mountain goats in Canada (Garde et al., 2005), and wild Alpine and Iberian ibex in Europe (Fernandez-Sirera et al., 2011). There is also direct evidence for BVDV-1 and BVDV-2 infection in mountain goats. Mountain goats in Nevada experienced an all age bacterial bronchopneumonia die off during the winter of 2009–2010 and three sampled mountain goats from this outbreak were seropositive for BVDV-1 and BVDV-2 on virus neutralization. In 2011, one mountain goat kid from the same area also died of bronchopneumonia with suppurative mural enteritis and suppurative serositis suggesting secondary septicemia. Necrotizing mesenteric lymphadenitis prompted testing for BVDV infection. Though BVDV IHC was negative, virus isolation of spleen was positive for BVDV1a confirming current natural infection in a free-living mountain goat (Wolff et al., in press).

To the authors' knowledge, mountain goats are the only wild goat species with definitive evidence of persistent pestivirus infection. Two captive mountain goats from a zoological collection in Idaho were proven to be infected with BVDV-2. While one goat had evidence of systemic BVDV-2 infection by IHC, virus isolation, and PCR with sequencing, the histological lesions indicated that suppurative enteritis with bacterial septicemia was a major factor in the cause of death. Longitudinal evidence of prolonged BVDV infection was not possible in this goat, but persistent infection was considered probable due to prolonged seronegativity and widespread virus distribution without associated necrosis. The second goat from the same premises had suppurative bronchopneumonia and suppurative hepatitis indicating bacterial septicemia was again the likely cause of death. This second goat had repeated longitudinal evidence of BVDV-2 infection by virus isolation and PCR with sequencing yet was seronegative over time providing definitive proof of persistent infection (Nelson et al., 2008).

The epidemiology and spectrum of disease syndromes due to bovine viral diarrhea infection in mountain goats is currently not well understood. Serosurvey of the Idaho zoological collection cohorts (including domestic sheep, domestic goats, mule deer, and whitetail deer in the same pen) suggested there may have been transmission between these wild caught mountain goats and the other ruminants, but the origin of this virus was not determined (Nelson et al., 2008). Evidence of BVDV infection in domestic cattle and free-living bighorn sheep, mountain goats, and mule deer sharing the same range in Nevada demonstrated interspecies transmission in wild settings (Wolff et al., in

press). Since pestivirus infection causes immunosuppression with increased susceptibility to bacterial infection, BVDV likely played this indirect role in these mountain goats with septicemia. BVDV infection in mountain goats likely affects reproductive rates as seen in domestic goats and may cause diarrhea as seen in Korean goats (Kim et al., 2006), but this has not been proven. The difficulty of access and limited numbers of these high mountain dwellers will limit further investigations into the incidence, epidemiology, and full characterization of natural disease.

CONTROL AND PREVENTION

Control and prevention of BVDV is based on three elements: elimination of PI animals, biosecurity, and early detection. Many Scandinavian countries are considered BVDV-free following widespread eradication programs in the 1990s based on these elements (Stahl and Alenius, 2012). The methods used to eradicate the disease included identification of positive herds, implementation of quarantine protocols, elimination through rigorous test and cull strategies, and prevention of BVDV introduction into non-infected herds.

Considering that PI animals provide a significant source for virus transmission, the key factor in BVDV control is identification and elimination of PIs. The Swedish eradication program identified PIs by performing serology on virus positive herds to find seronegative animals. Once seronegative animals were detected, virus isolation was performed. If an animal was found to be seronegative and virus positive, it was declared a PI and was eliminated from the herd (Stahl and Alenius, 2012).

Once BVDV is eliminated from the herd, rigorous biosecurity programs should be established to prevent re-introduction of the virus. All incoming animals, including purchased calves, replacement heifers, cows, and bulls, should be tested using one of the methods described above. Three week quarantine practices should also be implemented before introducing newly acquired animals into a disease free herd (Walz et al., 2010). Semen and embryos being used in breeding programs should also be considered as a source for herd infection and only be acquired from BVDV-free sources.

Vaccination may have a role in preventing disease, but efficacy in field conditions is not well documented and practicality is likely limited. Vaccination in domestic livestock is recommended in cases where the risk of re-introduction is high, but should always be used in conjunction with other control methods (Stahl and Alenius, 2012). When a vaccination program is considered, it is important to remember that antibodies associated with vaccination may complicate diagnosis of PI animals. The goal of vaccination is to limit transmission and severity of clinical disease in affected animals, rather than true prevention of BVDV infection. Vaccine use is commonly targeted to prevent the development of PI offspring (Walz et al., 2010).

IMPLICATIONS FOR DOMESTIC LIVESTOCK PRODUCERS

In a 2013 study, seroprevalence of BVDV in cattle was compared to that of white-tailed deer in the state of New York (Kirchgessner et al., 2013). Seroprevalence in cattle herds was found to closely mirror seroprevalence in hunter-harvested white-tailed deer. Given these findings, it is likely that there is an element of spillover/spillback that maintains the disease between these two species. This may also be true of the other species discussed in this paper. In many of the reports (white tail deer, alpaca), the PI animals had known exposure to BVDV positive herds during their gestation. According to a recent USDA survey, over 60% of dairy producers and 70% of beef ranchers report direct contact between their stock and wild cervids (USDA National Agriculture Statistics Service, 2007). Reduced contact between cattle and wildlife can be achieved with non-lethal methods such as high or electrified fencing, livestock protection dogs, enclosing stored feedstuffs, reduction of wasted feed, and elimination of baiting and winter feeding practices (Van Campen and Rhyan, 2009; VerCauteren et al., 2012).

Producers with multiple domestic species on the same premises should be aware of the potential for disease spread among their animals. As discussed earlier, BVDV PIs have been found in sheep, alpacas, and swine. Even if the species are not in direct contact, these PI animals increase the potential for BVDV spread through environmental contamination or use of shared equipment. Biosecurity measures should be implemented for control of disease on mixed-species farms, including decontamination of shared equipment, separation of shared feed or water sources, and reduction of disease spread by personnel tending to multiple species (change clothes, footbaths, hand washing between species). Care should be taken to isolate animals returning from mixed-species exhibitions (fairs, rodeos, shows) upon their return to the breeding herd.

IMPLICATIONS FOR WILDLIFE MANAGEMENT

As in cattle herds, factors such as population density, adequate habitat/forage, and herd behavior contribute to the number of seropositive susceptible wildlife species in a given area. Though it is an uncommon disease of wildlife, BVDV should be viewed as a threat to the health of wildlife populations and measures should be considered to reduce transmission of the disease within the ecosystem.

For mountain goats, remote high mountain habitat has likely historically minimized BVDV transmission, though as this habitat is increasingly encroached upon by domestic livestock grazing and human development, opportunities for disease transmission are likely increasing over time. Since multiple wildlife species have been shown to be capable of persistent and transient infection, interactions with other wild species such as deer and bighorn sheep also increase the opportunity for disease transmission. Assuming mountain goats are affected similar to domestic goats, BVDV infection likely causes significant reductions in the reproductive rate which could pose additional challenges for this wild species.

Increased surveillance is an important factor in the control and understanding of BVDV infection in wildlife. Implementation of BVDV testing on hunter-harvested samples may be a good way to track disease progression within an

ecosystem. The antigen capture ELISA (ACE) test, once validated, could be performed on blood or ear notch samples collected at carcass inspection. It would be a sensitive, specific, and inexpensive way to monitor disease prevalence. PCR could also be performed on pooled samples, as is commonly preferred for diagnosis in cattle herds. Vaccination may eventually become an important consideration for control of BVDV in areas with high disease prevalence in wildlife species.

IMPLICATIONS FOR ZOOLOGICAL COLLECTIONS

The implications of BVDV in zoological collections are similar to those in the domestic livestock industry. Increased biosecurity practices should be implemented to prevent fomite transmission of the disease between species. Quarantine and testing of new arrivals is also an important consideration for disease control captive collections. Disease transmission should be a consideration when mixed species exhibits are being planned and the animals should be tested accordingly.

CONCLUDING REMARKS

Bovine viral diarrhea virus is a disease with significant economic and health implications for positive herds. It causes economic losses to producers through loss of production, increased susceptibility to infection, and reproductive insufficiency. Considerable effort has gone into control and eradication of the disease through identification and elimination of PI cattle, but because wildlife and non-bovid have the potential for persistent infection, they must also be considered as an integral part of any eradication effort. Additionally, increased monitoring is an essential part of disease control and identification of new host species. Surveillance for BVDV in wild animal populations is increasing in areas with high seroprevalence and will likely continue to improve. Cattle producers, wildlife conservationists, and zoological staff members all need to consider the role of nonstandard BVDV hosts when attempting to control or eliminate the disease within a population.

AUTHOR CONTRIBUTIONS

DN is the first author and had the greatest contribution to the research, editing, and writing of this paper. JD is the second author and significantly contributed to the research and writing of this paper. PW is the third author and significantly contributed to the research and writing of this paper. JE is the final author and mentored and editing this paper with some primary writing contribution.

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Border Disease Virus: An Exceptional Driver of Chamois Populations Among Other Threats

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Though it is accepted that emerging infectious diseases are a threat to planet biodiversity, little information exists about their role as drivers of species extinction. Populations are also affected by natural catastrophes and other pathogens, making it difficult to estimate the particular impact of emerging infectious diseases. Border disease virus genogroup 4 (BDV-4) caused a previously unreported decrease in populations of Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) in Spain. Using a population viability analysis, we compared probabilities of extinction of a virtual chamois population affected by winter conditions, density dependence, keratoconjunctivitis, sarcoptic mange, and BD outbreaks. BD-affected populations showed double risk of becoming extinct in 50 years, confirming the exceptional ability of this virus to drive chamois populations.

Keywords: emerging diseases, extinction risk, pestivirus, population viability analysis, keratoconjunctivitis, *Rupicapra*, sarcoptic mange, VORTEX

INTRODUCTION

In the early 21st century, infectious diseases are considered a substantial threat to planet biodiversity (Daszak et al., 2000). Habitat loss, overexploitation, invasive species, and climate change are the best known drivers of species extinction by far, in part due to the lack of information on the role of pathogens in species extinction (Smith et al., 2009). While the importance of pathogens in species conservation is common knowledge, few people understand the power of diseases to drive extinction, or cause important economic losses. An example of this potential role of pathogens occurred in the Central and Eastern Pyrenees in 2001, when a border disease virus was responsible for a dramatic decrease (over 80%) of several Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*) populations (Marco et al., 2009). The etiological agent of these epidemics was classified into the Border disease virus genogroup 4 (BDV-4; Arnal et al., 2004), which had been present in the Pyrenees for at least two decades (Marco et al., 2011). The reasons for the emergence of the disease are still unclear (Marco et al., 2015). Pyrenean chamois is a flagship species that supports rural economies by attracting ecotourists and hunters from around the world. The epidemics caused a local cessation in game activities and the decline of hunting revenues.

The regional administration was powerless in the face of the epidemics, and border disease gained notoriety among hunters, veterinarians, wildlife biologists, and the local population. Strict regulations shut down hunting in areas showing mortality or low fertility or limited hunting bags in game states with the presence of the virus. While these measures aimed to minimize chamois mortality in the Pyrenees, the effects of the epidemics remain (Marco et al., 2015). However, it is unclear if the attention paid to BDV is groundless given that other older diseases (e.g., infectious keratoconjunctivitis, IKC or sarcoptic mange, SM) have also caused population collapses of chamois throughout Europe. In this perspective article, we aim to elucidate this question of whether BDV does in fact threaten the population viability of Pyrenean chamois, and whether its impact is more important than the risk associated with other/previous epidemics. We used a stochastic simulation of the risk of extinction in a fictitious chamois population regulated by density dependent processes, climate events, and the effect of old and emerging infectious disease, namely border disease (BD). Moreover, we briefly review the natural history of the affected host (*Rupicapra* spp.) and the epidemiology of these three diseases.

A SHORT BIOSKETCH OF CHAMOIS

Pyrenean chamois (*R. p. pyrenaica*) is by far the species most vulnerable to BDV infection. The northern chamois (*R. rupicapra*), however, has been suggested to act as a spillover, but no outbreak has been recorded to date (Martin et al., 2011; Fernández-Sirera et al., 2012b). This short biosketch summarizes the details of both species that are determinant for our modelling purposes.

Rupicapra is a long-lived (life expectancy 21 years; Gonzalez and Crampe, 2001), medium-sized, mountain-dwelling mammal inhabiting central and southern Europe. These caprinae species are nearly monomorphic with males about 20–33% larger than females (Pépin et al., 1996; Garel et al., 2009; Rughetti and Festa-Bianchet, 2011). This mammal follows a capital breeder strategy showing compensatory feeding in advance of breeding attempts (Houston et al., 2007). In fact, males gain much more mass (40% heavier) than females from spring to autumn in anticipation of the rutting period (November–December), and this difference decreases reaching a minimum in early spring (4%, Rughetti and Festa-Bianchet, 2011). Female chamois are basically monotocous (170 days gestation period, 1 offspring per year, and rarely twins) with a moderate degree of polygyny (Loison et al., 1999b), e.g., about four females for a given male and year (Corlatti et al., 2013). Though the female chamois is sexually mature at 18 months of age (Couturier, 1938), it rarely contributes to population demography before 3 years of age. In colonizing populations, two-year-old females can contribute significantly to recruitment (63.3–95%, Houssin et al., 1993; Loison et al., 2002). As the density increases, age at primiparity shifts from two to three years old with a proportion of reproducing females varying from 80% (Storch, 1989) to more than 90% (Houssin et al., 1993; Pérez-Barbería et al., 1998; Loison et al., 2002). Overall, in early summer more than 80% of prime-aged females (3–8 years) are accompanied by a kid (Houssin et al., 1993; Pérez-Barbería et al., 1998; Loison et al., 2002).

In females, reproductive success is stable until at least 10 or 14 years of age but begins to decrease between 12 and 16 years of age (Crampe et al., 2006; Tettamanti et al., 2015). In males, reproductive success has not been properly assessed, but field observations suggest that only fully adult males ($\geq$6 years) hold the largest harems and copulate most often (Lovari and Locati, 1991; Corlatti et al., 2013, 2015). Thus, longevity appears to be the main determinant of lifetime reproductive success in chamois. There is a slight but detectable cost of reproduction in males during the mating season (28% decrease in body mass; Mason et al., 2011) that is unappreciable in females (Garel et al., 2011a). Recruitment rates (proportion of offspring surviving through the winter per female) are lower for young (3–4 years) females (0.15–0.22) than for prime-aged females (0.41 per year for 5- to 16-year-old females, Crampe et al., 2006). Generation time varies from 5 to 8 years (Crampe et al., 2006).

The annual survival rate is normally low in kids (<1-year-old, 58%; Loison et al., 1994) and high in maturing (91%, for 1.5–3.5 years old) and adult individuals (96%; Loison et al., 1999a; Gonzalez and Crampe, 2001; Corlatti et al., 2012). Mortality of kids (<1 year) is higher (42%) and fluctuates more than in the other age classes (Crampe et al., 2002). Interestingly, there are no sexual differences in mortality rates (Loison et al., 1999a; Gonzalez and Crampe, 2001; Bocci et al., 2010; Corlatti et al., 2012).

DENSITY DEPENDENCE

The growth rate of chamois populations is affected by density at a time lag of 1 year (Willisch et al., 2013), i.e., animal numbers in 1 year negatively influence population growth in the following year. The fertility rate (kid/female ratio) is the main trait affected by delayed density dependence. Other authors (Capurro et al., 1997) observed that such delayed effects of density (2-year lag) did not affect birth rates but rather that total mortality rates of both kids and adults increased by up to 72% or 19–21%, respectively.

ENVIRONMENTAL DEPENDENCE

The role of exceptional snowy winters as stochastic factors regulating chamois populations (Schröder, 1971) has long been recognized. Seasonal snow cover limits locomotion and access to forage, and low temperatures increase thermoregulatory cost. Though the behavior of chamois aims to compensate for food shortages caused by wintertime, prolonged snow cover, and avalanches shape chamois populations (Jonas et al., 2008). In fact severe snowfalls (e.g., 165–590 cm of cumulative snowfall) can increase mortality by more than twice that recorded in normal winters (Crampe et al., 2002; Rughetti et al., 2011). The impact of extreme snow falls is especially severe for kids (Willisch et al., 2013) and adult age classes (>10 years; Rughetti et al., 2011), without a strong impact on reproduction. Though

winter cumulative snowfalls in these ecosystems show great interannual variation, episodes of heavy snow falls affecting chamois populations tend to occur at least once every 10 years (Capurro et al., 1997; Rughetti et al., 2011; Willisch et al., 2013). In addition, early summer conditions determine chamois population dynamics through their effect on diet quality (Gálvez-Cerón et al., 2013; Villamuelas et al., 2015), body growth, reproductive success, and survival (Garel et al., 2011b).

THE IMPACT OF OLD INFECTIOUS DISEASES

The influence of diseases on chamois populations had been reported by the early 20th century. IKC and SM are two of the best known infectious diseases with relevance for the viability of chamois populations.

IKC caused by *Mycoplasma conjunctivae* affects domestic and wild caprinae worldwide (Giacometti et al., 2002). The infection produces unilateral or bilateral inflammation of the conjunctiva and in advanced stages results in corneal opacity and transient blindness. Recovery from the disease is possible, but the ocular lesion may progress to corneal ulceration and perforation, or a non-healing lesion that leads to death due to starvation or accident. Consequently, the impact of IKC in populations of chamois is often critical. In the wild, the number of sick individuals peaks in summer (Loison et al., 1996; Arnal et al., 2013) since flies are suspected to contribute to spread the disease (Giacometti et al., 2002). The first reported outbreak of IKC in wild ungulates, chamois in the Austrian Alps, dates to 1916. Since then, IKC outbreaks are commonly reported in chamois populations from the Alps and Pyrenees (Giacometti et al., 2002; Arnal et al., 2013). These IKC outbreaks are characterized by a short duration of 1–2 years (Loison et al., 1996; Arnal et al., 2013), high morbidity, low mortality, and spontaneous recovery (Loison et al., 1996). In fact, individuals that overcome the infection can show lower infection susceptibility in subsequent epizootic episodes. However, IKC is sometimes associated with high mortalities (>30%; Loison et al., 1996; Giacometti et al., 2002), with the reasons for extreme events largely unknown.

Females and juveniles are especially affected by IKC with the number of affected adult males usually being low (Arnal et al., 2013). Sexual segregation between males and females during the summer could be related to this sex-biased susceptibility. In other cases, the age-class distribution of cases attributable to an IKC outbreak appears proportional to the initial population structure (Arnal et al., 2013). After an IKC epizootic episode, fertility of female chamois (number of kids/adult females) experiences a slight decrease (10–19%; Loison et al., 1996; Arnal et al., 2013) and begins to recover 1 year after the outbreak. This decline in reproductive index during the early post-epidemic periods may have resulted from a low neonatal survival. Occasionally IKC infection in chamois becomes endemic and outbreaks with mild consequences are observed every 3–4 years (Gauthier, 1994).

SM epizootics caused by the burrowing mite *Sarcoptes scabiei* also have a recognized impact on wildlife conservation (Pence and Ueckermann, 2002). Infected animals typically suffer from severe dermatitis, becoming dehydrated, emaciated and eventually dying from the infection. Amongst caprinae hosts, scabies-induced mortality of chamois populations has been reported for slightly over a century in the Alps (Onderscheka, 1982; Rossi et al., 1995), and for more than a decade in the Cantabrian Mountains, northwestern Spain (Fernández-Morán et al., 1997). No sex or age class has been shown to have higher susceptibility to scabies, and the potential effect of SM on either fertility or recruitment of females in diseased populations has not been determined. The number of chamois with visible scabies lesions peaks from late winter (March; Rossi et al., 2007) to late spring (May; Fernández-Morán et al., 1997). Rare cases are observed in summer and autumn. Demographic decline due to SM is highly variable. The epidemic cycle is characterized by an initial peak of infection associated with high mortality (>80%) followed by successive epidemic waves with lower impact (10–25%; Lunelli, 2010). Though the initial growth rates of some populations recover 2 years after the initial outbreak (Fernández-Morán et al., 1997), the impact of SM peaks from 4 to 6 years after the first scabietic animals are observed (Rossi et al., 2007; Turchetto et al., 2014).

Pneumonia caused by Pasteurellaceae species (e.g., *Mannheimia haemolytica, M. glucosidal*, *or Bibersteinia trehalosi*), *Mycoplasma* spp. or respiratory viruses are another cause of acute die-off of chamois populations (Citterio et al., 2003; Posautz et al., 2014). Unfortunately, demographic data describing the impact of pneumonia outbreaks on chamois populations is scarce and incomplete. Thus, this polymicrobial disease was not included in our population viability analysis.

BDV: A KEY POPULATION DRIVER

Border disease virus belongs to the Pestivirus genus (Flaviridae family), is distributed worldwide and can cross the species barrier. The virus can be transmitted horizontally, by direct contact, and vertically *in utero* resulting in abortion of the fetus or in the birth of a persistently infected (PI) individual, depending on the period of gestation, with a short life expectancy (Schweizer and Peterhans, 2014). BD causes important economic losses on farms and virulent strains can cause systematic reproductive failure (Nettleton et al., 1999) and high mortalities in sheep (Chappuis et al., 1986; Vega et al., 2015).

In chamois, BD infection has severe consequences causing mortality in individuals of all ages, being considered an emerging disease for chamois populations in the Pyrenees. Clinical signs in naturally infected chamois include emaciation, alopecia, and neurological depression, the latest associated with non-suppurative encephalitis (Marco et al., 2007). Abortion has been also described under experimental conditions (Martin et al., 2013). Mortality rates vary enormously among populations (Fernández-Sirera et al., 2012a). In fact, while most populations are severely affected by successive outbreaks, others appear to coexist with the virus without consequence (Marco et al., 2015). An age-structured dependent infection (Pioz

TABLE 1 | Summary of parameter input base values used in the population viability analysis (PVA) of Chamois (*Rupicapra*).

Simulation input	Base value	Source
Reproductive system and rates		
Breeding system	Polygyny	Loison et al., 1999b
Age of first reproducing females	2–3	Loison et al., 2002
Age of first reproducing males	5–6	Corlatti et al., 2013, 2015
Maximum age of female reproduction	16	Crampe et al., 2006
Maximum age of male reproduction	16	NA
Maximum number of litter per year	1	Loison et al., 1999c
Maximum number of young per year	1	
Sex ratio at birth	1:1	Crampe et al., 2006
Breeding at low density (%)	70 for 2 years old females 90 for ≥3 years old females	Houssin et al., 1993; Pérez-Barbería et al., 1998; Loison et al., 2002
% Adult females breeding	88 (7)	Couturier, 1938; Storch, 1989; Houssin et al., 1993; Pérez-Barbería et al., 1998; Loison et al., 2002
% Adult females having one litter per year	100	
Average litter size	1	
Maximum litter size	1	
Mortality rates		
% Mortality from age 0–1 years	42 (37)	Loison et al., 1999a; Crampe et al., 2002; Loison et al., 2002; Rughetti et al., 2011
% Mortality from age 1–2 years	19 (17)	
% Mortality from age 3–10 years	18 (17)	
% Mortality for 10 years old	74 (28)	
Catastrophes		
Severe snow fall	At least once a decade	Crampe et al., 2002; Jonas et al., 2008; Rughetti et al., 2011
Mortality rates due to disease outbreak		
Keratoconjunctivitis outbreak (2 years)	6% kids (13) and 70% yearlings (18), 20% females (13) and 9% males (9)	Loison et al., 1996; Giacometti et al., 2002; Arnal et al., 2013
Sarcoptic mange outbreak (5 years)	10.5% kids (18) and 14% yearlings (6.5), 52.5% females (26.5) and 60% males (18)	Rossi et al., 1995, 2007; Fernández-Morán et al., 1997
Border disease outbreak (5 years)	50.5% kids (58.5), 51.8% yearlings (75.7), 45.7% females (86.8), and 47% males (19.5)	Marco et al., 2007, 2009; Fernández-Sirera et al., 2012a; Annual counts of the Catalan Department of Agriculture, Livestock, Game, Fishery, and Food

The numbers in the column "Source" correspond to the references used for a given base value. Data in parenthesis is the standard deviation of the mean base value due to environmental variation. We assume no sexual differences in mortality rates. NA indicates no information available.

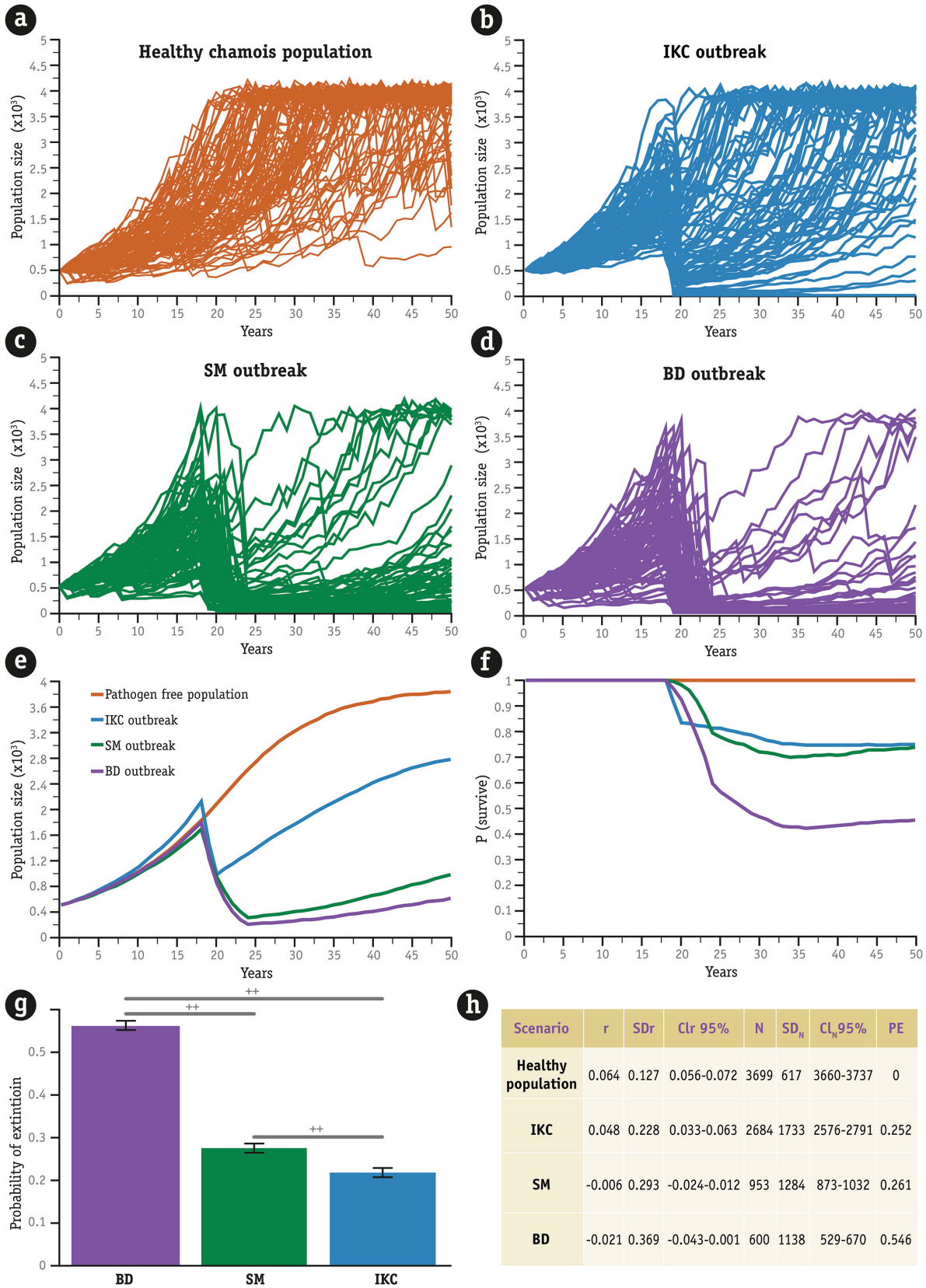
et al., 2007) and a seasonal spread of the virus (Beaunée et al., 2015) have been suggested for chamois populations. Viral mutation, host factors, climatic variation, and other ecological conditions may be playing an unknown but important role in explaining these different epidemiological scenarios.

STOCHASTIC SIMULATION OF POPULATION EXTINCTION

An assessment of the risk of extinction is often required for conservation and management plans. The most realistic models incorporate causes of fluctuations in population size to predict probabilities of extinction (Boyce, 1992). In fact, many life history traits are in essence stochastic. Population viability analysis (PVA) is a method of quantitative analysis to determine the probability of extinction of a given population (Boyce, 1992). VORTEX (Lacy, 1993) is a powerful software for stochastic simulation of the extinction process under a broad range of situations (e.g., harvesting rates, age-specific reproduction rates, fixed or random catastrophes, and among others).

BASIC SIMULATION INPUT

We used VORTEX 10.0.7.0 (Lacy et al., 2015) to estimate viability of a population of an initial size of 500 chamois in a hypothetical scenario with a carrying capacity of 4000 individuals. Extinction was reached when population numbers were reduced to 30 individuals, the minimum viable population size recommended for a successful reintroduction of chamois (Lovari et al., 2010). Each population was simulated for 50 years and 1000 iterations. Neither inbreeding depression (i.e., reduction of a first-year survival among inbred individuals) nor concordance of reproduction and survival were considered in our PVA. Though dispersal has been reported in chamois



Scenario	r	SDr	Clr 95%	N	SD_N	Cl_N95%	PE
Healthy population	0.064	0.127	0.056-0.072	3699	617	3660-3737	0
IKC	0.048	0.228	0.033-0.063	2684	1733	2576-2791	0.252
SM	-0.006	0.293	-0.024-0.012	953	1284	873-1032	0.261
BD	-0.021	0.369	-0.043-0.001	600	1138	529-670	0.546

FIGURE 1 | Continued

FIGURE 1 | Continued

Stochastic simulations of the extinction process in a fictitious population of 500 chamois for 50 years. The hypothetical scenario has a carrying capacity of 4000 individuals and suffered the demographic consequences of five heavy winters. **(a)** A pathogen-free population of chamois only driven by density dependence and climate severity. **(b)** The consequences of a severe 2-year outbreak of infectious keratoconjunctivitis (IKC) in year 20. **(c,d)** The simulation of 5-year sarcoptic mange (SM) and border disease (BD) outbreaks, respectively, are shown, also in year 20. The number of populations that went extinct, represented by lines perpendicular to the *X* axis in **(b–d)** is greater for the population affected by BDV than for the other two (summarized in **e**). In any case, extinction was reached when the population number was reduced to 30 individuals. We performed 1000 simulations in each case, but **(a–d)** plots show only the output of the first 100 simulations. Along the same line, **(f)** shows the lower probability of survival after a disease outbreak. Information presented in plots **(e)** and **(f)** is based on 1000 simulations. The bar plot in **(g)** summarizes the results of ANOVA aimed at testing differences between probabilities of extinction over 50 years caused by the three diseases. These probabilities were calculated for 60 simulations (20 for each pathogen), with outbreaks occurring at different population sizes (from 600 to 1550, 50 simulations for each case). Whiskers represent the standard deviation and the horizontal lines the results of a *post hoc* Tukey's HSD test. Statistically significant differences, at $\alpha = 0.05$, are indicated by crosses. Statistical summary is shown in table **(h)**. Mean stochastic growth rate of the population (r), Mean final population size (N), associated standard deviations (SD_r, SD_N) and confidence intervals at 95% (CI_r, CI_N), and mean probability of extinction (PE) after our 1000 simulations.

populations (Loison et al., 1999c; Crampe et al., 2007), our theoretical population was considered closed. Data on the reproductive system, reproductive rates, and mortality rates are summarized in **Table 1**. Concerning catastrophes, our population had a yearly probability of 0.1 of experiencing an exceptionally snowy winter (at least five heavy snow falls over the study period). The impact of this extreme environmental variation on chamois survival is summarized in **Table 1**. No impacts on reproduction are expected after such catastrophes, but after a severe winter the carrying capacity of the ecosystem will increase by 10%. In fact, after severely snowy winters the availability of nutritious plants in the Alpine pastures increases the following summer (Pettorelli et al., 2005), favoring body weight gains and hence survival (Garel et al., 2011b). Finally, although we did not consider density-dependence effects on reproduction rates of adult females, the potential effects of overcrowding on mortality of young age classes was considered to be increasing by 10% of the standard deviation of mortality rates.

DISEASE SIMULATION INPUT

We used a PVA to compare the impact of IKC and SM outbreaks with the effect of BD epidemics on stochastic population growth rate (r), mean population size (N), standard deviations (SD_r, SD_N) and confidence intervals (95%) of a simulated population of chamois. We modeled four populations: one pathogen-free, a second affected by IKC, another by SM and the last by BD. The length of the epidemics was estimated by averaging the mean number of years that chamois population is affected by the disease after the first outbreak (i.e., clinical cases are detected and/or population parameters differ from the pre-epidemic period): 2 years for IKC (Loison et al., 1996; Giacometti et al., 2002; Arnal et al., 2013), 5 years for SM (Fernández-Morán et al., 1997; Rossi et al., 2007), and 5 years for BD (Fernández-Sirera et al., 2012a).

The effect of diseases on the host was modeled as sex and age-specific harvesting rates (i.e., extra increase in chamois mortality during the epidemic). To compare probability of extinctions due to the effect of diseases, we ran 60 simulations (20 for each pathogen) with outbreaks occurring in different population sizes (from 600 to 1550, about 50 each). Results were compared by ANOVA and a Tukey's HSD *post hoc* test. Details about our scenario settings are summarized in **Table 1**.

THE GOOD, THE BAD, AND THE UGLY

Despite having suffered the consequences of five severe winters, our pathogen-free population of chamois grew from 500 to 3699 chamois in 50 years (**Figures 1a,h**). Consequently, the stochastic growth rate was positive (Stoch-$r = 0.064$, **Figure 1h**) and no extinction process occurred during the simulation. However, growth rate of the healthy population and hence the probability of extinction was seriously affected by the three infectious agents ($F_{2,27} = 358.8$, $p < 0.01$, **Figures 1f,g**).

Summarizing the impact of the three pathogens and inspired by the prominent film of the master Sergio Leone, we can imagine that IKC (**Figure 1b**) plays the "good" character, SM the "ugly" (**Figure 1c**), with the border disease virus surely cast in the role of the "bad" (**Figure 1d**, but see **Figures 1e,f** for a multiple comparison). After our simulated outbreaks, growth rate of the affected population decreased from 0.048 (IKC) to -0.021 units (BD), whereas probability of extinction ranged between 0.25 (IKC) and 0.55 (BD). Probability of extinction for a healthy population was 0 (**Figure 1h**, table). Though the impact of SM was between IKC and BD, the probability of extinction caused by each pathogen was statistically different (**Figure 1**, Tukey's HSD test at $\alpha = 0.05$). It is interesting to note that the relative standard deviation of the mean extant populations was greater after the BD outbreak ($100 \times SD/\text{Mean} = 189\%$) than after the other outbreaks (134% for the SM outbreak and 64% for IKC; **Table 1**). This result agrees with the variety of epidemiological scenarios of BD in the populations of Pyrenean chamois (Fernández-Sirera et al., 2012b)

CONCLUDING REMARKS

Though our population modeling is not free of limitations (e.g., assumes a fixed length for the epizootics, only one epizootic at a time, no previous hunting-harvesting, lack if recovery due to

herd immunity), it is able to illustrate quite well the impact of BD on the population dynamics of chamois. The IKC is characterized by short (1–2 years) and female-biased outbreaks (Arnal et al., 2013), affecting young age classes more, whereas SM shows longer outbreaks (from 2 to 6 years; Rossi et al., 2007), affecting all age and sex classes. BD epidemics, however, are the only of the three resulting in long outbreaks (>10 years in some populations) with abortions, neonatal and adult mortality. Moreover, the existence of persistent infected individuals can lead to the circulation of BDV among individuals over long periods of time which is an exceptional peculiarity of this pathogen. That peculiarity has at least been proven in domestic flocks (Schweizer and Peterhans, 2014), and in theoretical models for BD epidemics in chamois populations (Beaunée et al., 2015). To date, however, there are few evidences for the existence of PI in chamois populations (Marco et al., 2015). Alternatively, we cannot rule out the possibility of chronic shedding (non-PI individuals by definition) for explaining viral maintenance in chamois (Cabezón et al., 2011; Martin et al., 2013). In any case, both mechanisms (PI and/or long-lasting viraemia) would contribute for viral persistence of BDV in chamois populations.

Comparing the impact of several diseases from field data is not straightforward, as epidemics occur in different populations, at different stages of colonization and in contrasting environments. The simulation approach allowed us to compare the impact of the three studied pathogens considering similar situations and taking into account stochastic processes. Among the three pathogens under study, BDV showed the highest probability of extinction over 50 years: this probability reached values >50%, and thus even large host populations may go extinct under the pressure of intense epidemics. Given this high probability of host extinction, we argue that BDV is an exceptional driver of chamois populations and entails specific extinction risk. Further research should be oriented to illustrate more realistic scenarios, e.g., combining the impacts of more than one epidemics for a given period (IKC + BD) or including the impact of hunting-harvesting. Management actions designed to limit the impact of the virus should be evaluated and implemented, as the natural host-pathogen dynamics may not reach equilibrium in a near future.

AUTHOR CONTRIBUTIONS

AC-C and ES performed the literature review. ES analyzed the data. All authors contributed to conceiving the project and participated in the interpretation of results and final preparation of the paper.

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PERSPECTIVE
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Evidence of *Bovine viral diarrhea virus* Infection in Three Species of Sympatric Wild Ungulates in Nevada: Life History Strategies May Maintain Endemic Infections in Wild Populations

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Evidence for *bovine viral diarrhea virus* (BVDV) infection was detected in 2009–2010 while investigating a pneumonia die-off in Rocky Mountain bighorn sheep (*Ovis canadensis, canadensis)*, and sympatric mountain goats (*Oreamnos americanum*) in adjacent mountain ranges in Elko County, Nevada. Seroprevalence to BVDV-1 was 81% ($N = 32$) in the bighorns and 100% ($N = 3$) in the mountain goats. Serosurveillance from 2011 to 2015 of surviving bighorns and mountain goats as well as sympatric mule deer (*Odocoileus hemionus*), indicated a prevalence of 72% ($N = 45$), 45% ($N = 51$), and 51% ($N = 342$) respectively. All species had antibody titers to BVDV1 and BVDV2. BVDV1 was isolated in cell culture from three bighorn sheep and a mountain goat kid. BVDV2 was isolated from two mule deer. Six deer ($N = 96$) sampled in 2013 were positive for BVDV by antigen-capture ELISA on a single ear notch. Wild ungulates and cattle concurrently graze public and private lands in these two mountain ranges, thus providing potential for interspecies viral transmission. Like cattle, mule deer, mountain goats, and bighorn sheep can be infected with BVDV and can develop clinical disease including immunosuppression. Winter migration patterns that increase densities and species interaction during the first and second trimester of gestation may contribute to the long term maintenance of the virus in these wild ungulates. More studies are needed to determine the population level impacts of BVDV infection on these three species.

Keywords: *bovine viral diarrhea virus*, bighorn sheep, mountain goat, mule deer, Nevada, *Odocoileus hemionus*, *Oreamnos americanum*, *Ovis canadensis*

INTRODUCTION

The pestivirus *bovine viral diarrhea virus* (BVDV) is considered an important disease of cattle, and infection also occurs in other domestic and wild ruminants (Passler and Walz, 2010). BVDV infection has been documented through serosurveillance and virus isolation in a number of captive and free ranging North American ungulate species including, Rocky Mountain bighorn sheep (*Ovis canadensis, canadensis*; Van Campen et al., 2003) mountain goats (*Oreamnos americanum*; Nelson et al., 2008), white-tail deer (*Odocoileus virginianus*; Pogranichniy et al., 2008; Wolf et al., 2008; Kirchgessner et al., 2013) mule deer (*Odocoileus hemionus*; Van Campen et al., 2001; Roug et al., 2012), elk (*Cervus elaphus*; Tessaro et al., 1999), moose (*Alces alces*; Kocan et al., 1986), bison (*Bison bison*; Taylor et al., 1997) pronghorn (*Antilocapra americanum*; Dubay et al., 2006) and caribou (*Rangifer tarandus*; Morton et al., 1990).

Bovine viral diarrhea virus can cause clinical disease including gastrointestinal and respiratory disease, reproductive loss, and lymphoid depletion causing immunosuppression in susceptible ungulates. Infection of pregnant females during the first trimester of pregnancy may also produce persistently infected (PI) young. Immunotolerant to the virus, PI animals are life-long and efficient shedders and are the primary transmitters of virus to cohorts, although transiently infected (TI) animals may also play a significant role in virus transmission (Thurmond, 2005). PI individuals have been reported in free-ranging mule (Duncan et al., 2008) and white-tail deer (Chase et al., 2008) and in captive mountain goats (Nelson et al., 2008). PI white-tail deer fawns were produced when dams were exposed to PI cattle (Passler et al., 2009) and white-tail deer (Passler et al., 2010), or experimentally infected (Passler et al., 2007; Ridpath et al., 2008) during the first trimester of gestation. Although contact with domestic cattle is considered the likely source of introduction of BVDV into free-ranging ruminant populations (Kocan et al., 1986; Nielsen et al., 2000), the virus can be maintained and is likely endemic in some North American wildlife populations.

We identified BVDV infection over time in sympatric Rocky Mountain bighorn sheep, mountain goats, and mule deer on adjacent mountain ranges [East Humboldt range (EHR) and Ruby Mountains (RMs)] in Elko County, Nevada. We propose that the virus has become endemic within all three mountain ungulate species. The timing of movement to and residence on winter range, which occurs during the first two trimesters of pregnancy in all species, results in increased animal densities and species overlap. Increased contact between and within species could potentiate transmission and perpetuate virus maintenance within these populations. Impacts of BVDV infection on population health and annual recruitment could not be quantified in this study but bears further investigation.

ANIMAL HANDLING

All capture, handling and disease surveillance activities were approved and conducted under the direction of the Nevada Department of Wildlife (NDOW). Live animal sampling was conducted in January and February following helicopter net gun capture. In addition 20 bighorn sheep were captured via ground darting (Pneu-dart, Williamsport, PA, USA) using BAM^{TM} (Wildlife Pharmaceuticals, Inc., Windsor, CO, USA) as previously described (Wolfe and Miller, 2009). Blood samples were collected using routine jugular venipuncture. Ear notch samples were taken in a standard manner utilizing a v-cut ear notcher, producing a base cut of 8 mm with 10 mm to tip (Nasco, Salida, CA, USA).

LABORATORY ANALYSIS

Serum virus neutralization (SN) for BVDV1 antibody titers was conducted at the Washington Animal Disease Diagnostic Laboratory, College of Veterinary Medicine, Pullman, Washington (WADDL) utilizing Singer strain as previously described (World Organization for Animal Health, 2008) and BVDV2 utilizing strain 125 at Oregon State University, Veterinary Diagnostic Laboratory in Corvallis, Oregon (OSU-VDL) as previously described by (Montrose et al., 2015). All histopathology, immunohistochemistry for pestivirus and PCR for BVDV on fresh and archived tissues blocks was performed at WADDL as previously described (Nelson et al., 2008). Virus isolation was conducted on fresh tissue and whole blood and pestivirus typing by PCR on serum at USDA–ARS as previously described by (Ridpath et al., 2008). Ear notches submitted to USDA-ARS were screened for BVDV using the antigen-capture ELISA (ACE; Herdchek®, Idexx Laboratories, Westbrook, ME, USA) as previously described (Ridpath et al., 2008).

BVDV IN ROCKY MOUNTAIN BIGHORN SHEEP

Rocky Mountain bighorn sheep were introduced into the RM in 1989–1990 and the EHR in 1992. In the winter of 2009–2010, approximately 91% (population estimate 175) and 95% (population estimate 140) of the herds respectively, were lost due to an all age bacterial pneumonia die-off. During the disease investigation VN titers were detected to BVDV1 in 81% ($N = 32$) of the sheep with 77% having titers $\geq$ 1:512. Fifteen also had a seroprevalence for BVDV2 of 93% (titers ranged from 1:8 to 1:256). Histologic lesions from mortalities recovered during the pneumonia die-off were consistent with bacterial pneumonia and non-specific for pestivirus disease. Archived samples from the 2009 to 2010 die-off were submitted to USDA-ARS including serum from six animals for PCR and tissues from three mortalities for virus isolation. Four of the six sera were positive by PCR for BVDV2. Two of these animals were seronegative to BVDV1 at the time of capture but no serum was submitted for BVD2 and the others had VN antibody titer to BVDV1 of 1:128 and 1:512 and to BVDV2 of 1:16 and 1:64 respectively. BVDV1 was isolated from tissue in these three mortalities. Only one of these animals, an adult ram, was sampled prior to death and was seronegative for BVDV1 and no serum was submitted for VN

for BVDV2. Paraffin-embedded splenic tissue from this ram was negative for BVDV by PCR but positive for pestivirus on immunohistochemistry.

Subsets of the survivors and their offspring have been sampled annually since 2012. Seroprevalence to BVDV1 was 80% ($N = 26$) in 2012, 33% ($N = 3$) in 2013, 57% ($N = 7$) in 2014, and 28% ($N = 7$) in 2015 (**Figure 1A**). In 2013 and 2014 a total of 10 non-paired ear notch samples were tested by ACE. All samples were negative.

BVDV IN MOUNTAIN GOATS

Mountain goats were introduced into the RM in 1964 and '67 and into the EHR in 1981. In the winter of 2009–2010 approximately 30% (population estimate 220) and 13% (population estimate 130) of the herds respectively, were lost to an all age bacterial pneumonia die-off. During the disease investigation three mountain goats in the EHR were sampled and all were seropositive to BVDV1, with two having BVDV1 titers of ≥1:512, and all having titers to BVDV2 of 1:32. In 2011 an approximately 8 weeks-old, male kid was found in the RM. The animal was surrendered to the NDOW but died 36 h later. Histopathology revealed a bacterial bronchopneumonia, and necrotizing mesenteric lymphadenitis suggested the possibility of BVDV infection. Though immunohistochemistry on paraffin embedded tissue blocks of intestine, lung and lymph node was negative, virus isolation on archived splenic tissue was positive for BVDV1. The VN titer for BVDV1 was 1:32; no serum was submitted for VN to BVDV2.

Subsets of die-off survivors and their offspring have been captured annually since 2012 for marking and disease surveillance. Seroprevalence by VN to BVDV1 in 2012 was 88% ($N = 9$), 46% ($N = 15$) in 2013, 41% ($N = 24$) in 2014, and 60% ($N = 15$) in 2015 (**Figure 1B**). In 2013 and 2014 a total of 30 non-paired ear notch samples were tested by ACE; all samples were negative. In 2015, three mountain goat kid mortalities were recovered from the EHR. Two died from bacterial bronchopneumonia at 8–10 weeks of age and one was a perinatal death. Splenic tissues ($N = 2$) and lung ($N = 1$) were tested for BVDV by PCR and were negative. Histopathology lesions were non-specific for BVDV in all three kids.

BVDV IN MULE DEER

Mule deer are native to Nevada. The population of deer in the management units which include the RM and EHR from 2011 to 2015 was estimated at 20,000 (NDOW, unpublished data). A migration study conducted between 2011 and 2015 involved the capture and sampling of 342 deer (236 adults and 106 fawns). All deer received radio collars which emit a mortality signal if no movement is detected from the animal for 8 h and all mortalities were investigated; however, none were recovered within a timeframe to determine if infectious disease was the cause of death.

Seroprevalence to BVDV1 across all age classes was 35% ($N = 101$) in 2011; 52% ($N = 108$) in 2012; 48% ($N = 117$) in 2013; 55% ($N = 20$) in 2014; and 80% ($N = 15$) in 2015 (**Figure 1C**). Seroprevalence to BVDV1 for fawns (estimated age 7 and 8 mos.) across all sample years was 7.5% ($N = 106$) with 62% ($N = 8$) having titers of ≥1:512. Serum samples from 33 deer with BVDV1 titers ≥ than 1:512 were submitted for endpoint titers as well as VN titers for BVDV2. Twenty four percent of deer had endpoint titers of 1:2048 and 12% had endpoint titers of 1:1024 to BVDV1 and from 1:32 to 1:1024 for BVDV2.

In 2013, 84 non-paired ear notch samples were tested by ACE. Six animals were positive, two adult females and four fawns. One fawn had a titer to BVDV1 of ≥1:512 the rest of the deer were seronegative. BVDV2 was isolated from the ear notch of another fawn. Three of four fawns died 3–4 months after capture of trauma, predation, or unknown cause. One fawn was confirmed alive at 11 months post-capture but dropped his collar and was lost to follow up. One adult female died of trauma and one was presumed predated 7 and 17 mos. post-capture respectively. BVDV2 was isolated from whole blood from another doe whose single ear notch sample was ACE negative. This doe was VN negative for BVDV1 and BVDV2. In 2014, 19 non-paired ear notch samples collected from adult does were tested by ACE; all were negative.

DISCUSSION

Infection with BVDV1 and BVDV2 was detected over a 5 year period based on serology, serum and tissue PCR, antigen-capture ELISA, and virus isolation from three wild ungulate species in adjacent mountain ranges in northeastern Nevada. These findings are consistent with endemic infection in these sympatric populations.

Previous studies have found a high prevalence of VN titers in cervid populations (Van Campen et al., 2001; Lillehaug et al., 2003; Passler et al., 2008) and in other wild ungulates (Passler and Walz, 2010) suggests endemic infection. In this study, repeated serosurveillance from 2010 to 2015 indicated a high percentage of VN titers to BVDV1 in the bighorn sheep (28–80%), mountain goats (25–50%), and mule deer (35–80%) and to BVDV2 in tested animals: bighorn sheep 93% ($N = 15$), mountain goats 100% ($N = 3$), and mule deer 100% ($N = 33$). Seroprevalence to BVDV1 in lambs was 0% ($N = 4$), kids 25% ($N = 4$), and fawns 7.5% ($N = 106$). It is unknown when maternal antibodies to BVDV wane, so positive titers may represent maternally derived antibodies or postnatally acquired infection, but either scenario suggests that BVDV1 and BVDV2 are circulating in these populations.

BVDV1 and BVDV2 were detected by virus isolation, PCR, or ACE in all three species. BVDV1 was isolated from bighorn sheep and a mountain goat kid. Two of four sheep that were PCR positive for BVDV2 were seronegative for BVDV1 with no serum submitted for BVDV2, and the other PCR-positive sheep were seropositive for both. Cross reaction can occur in serologic assays between strain antibodies which could account for the titers to BVDV1 in these sheep. Alternatively, the BVDV1 titers were due to a previous transient infection. BVDV2 was also isolated from a doe and a fawn. The doe was ACE negative and the fawn ACE

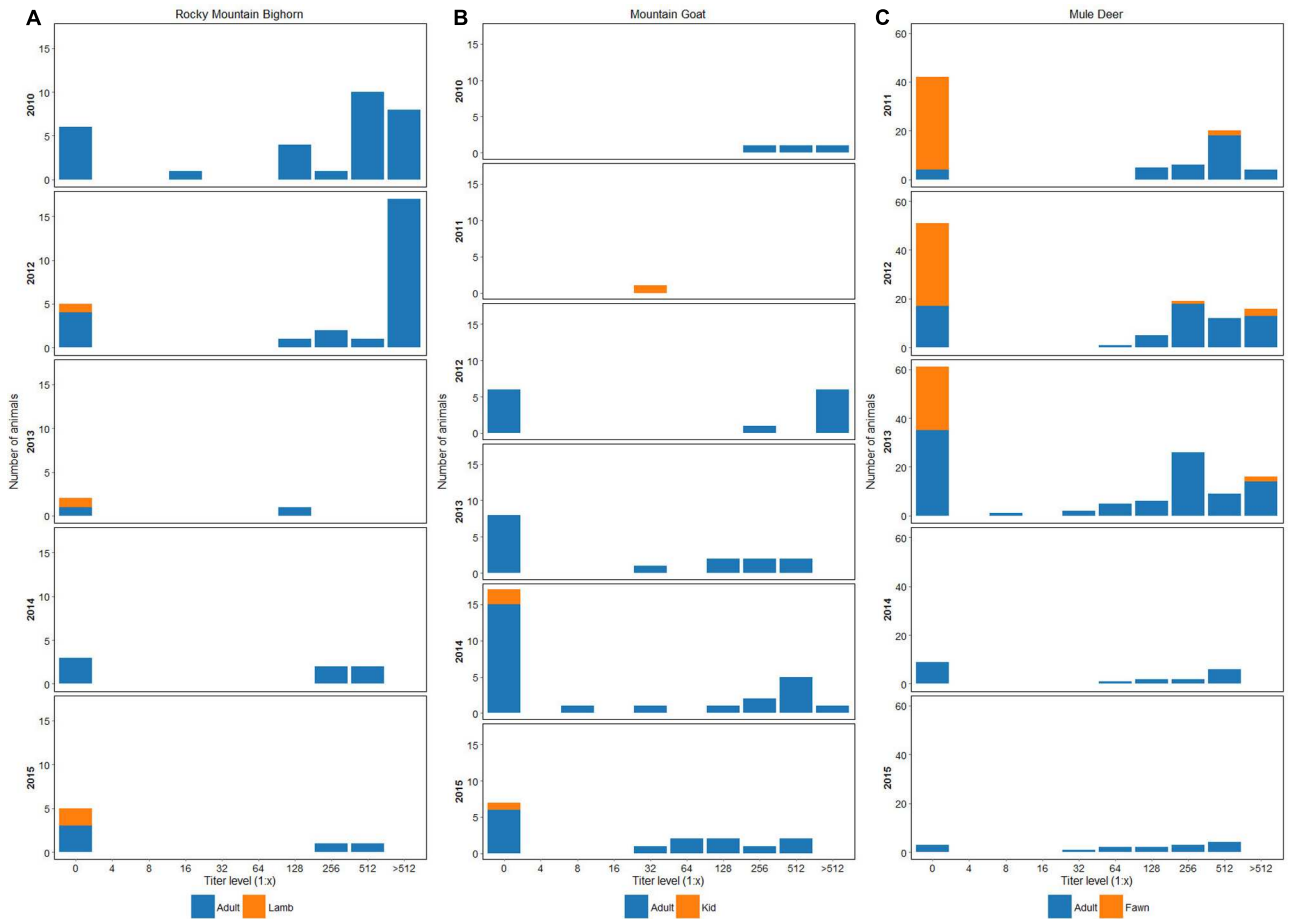

FIGURE 1 | (A) Seroprevalence and antibody titers to BVDV1 in bighorn sheep sampled in the RM and EHR from 2010 to 2015. **(B)** Seroprevalence and antibody titers to BVDV1 in mountain goats sampled in the EHR and RM from 2010 to 2015. **(C)** Seroprevalence and antibody titers to BVDV1 in mule deer from 2010 to 2015. In years where fawns were sampled, 1–2 each year have had virus neutralization titers of 1:256 or greater to BVDV-1. Fawns were 7–8 months-old at capture and titers could represent maternal antibody or naturally acquired infection.

positive; both were VN seronegative for BVDV1 and BVDV2. The ACE is considered to be 90–95% specific for identifying PI in cattle, but is less sensitive for detection of acute or transient infection; and ACE has not been validated in wild ruminants. Most likely the fawn was PI, and the doe may have been acutely infected or PI with BVDV2. Five other mule deer were also positive by ACE. One fawn had a BVDV1 titer of $\geq$1:512 which may indicate that he was PI with BVDV2 and was either acutely infected with, or had maternal antibodies to, BVDV1. Without repeated tests, it is not possible to definitively determine whether these individuals that had direct evidence of BVDV infection are TI or PI. However, our data suggests both conditions exist in these sympatric populations providing the means for maintaining the virus over time.

The history of BVDV infection within susceptible domestic and wild ungulate populations in this region has not been documented; thus a domestic origin of the virus has not been definitively determined. The EHR and RM encompass 3 billion hectares, and are comprised of public and private land, and Federal livestock grazing allotments cover 60–70 and 95% of these mountain ranges, respectively. Private ranches surround both mountains and are the primary source of the cattle which graze the allotments (**Figure 2A**). Radio collar data and visual observations from aerial and ground surveys confirm that temporal and spatial overlap occurs between wild ungulates and domestic cattle and between these wildlife species. Although domestic cattle have grazed these mountains for decades, infection with BVDV was first detected in 2010 in bighorn sheep and mountain goats and 2011 in mule deer. Prior serosurveillance for BVDV was not conducted, so it is unknown when spill-over from cattle to wildlife or transmission between wildlife species may have occurred.

Seasonal migration and reproductive timing likely play important roles in transmission and potential maintenance of the virus in these ungulate populations. Seasonal migration results in congregation of these animals on winter range increasing animal densities and the chance for intra- or interspecies viral transmission (Van Campen et al., 2001; Wolf et al., 2008; Passler et al., 2010). After rut, which peaks in mid to late November for all three species, global positioning system (GPS) collar data

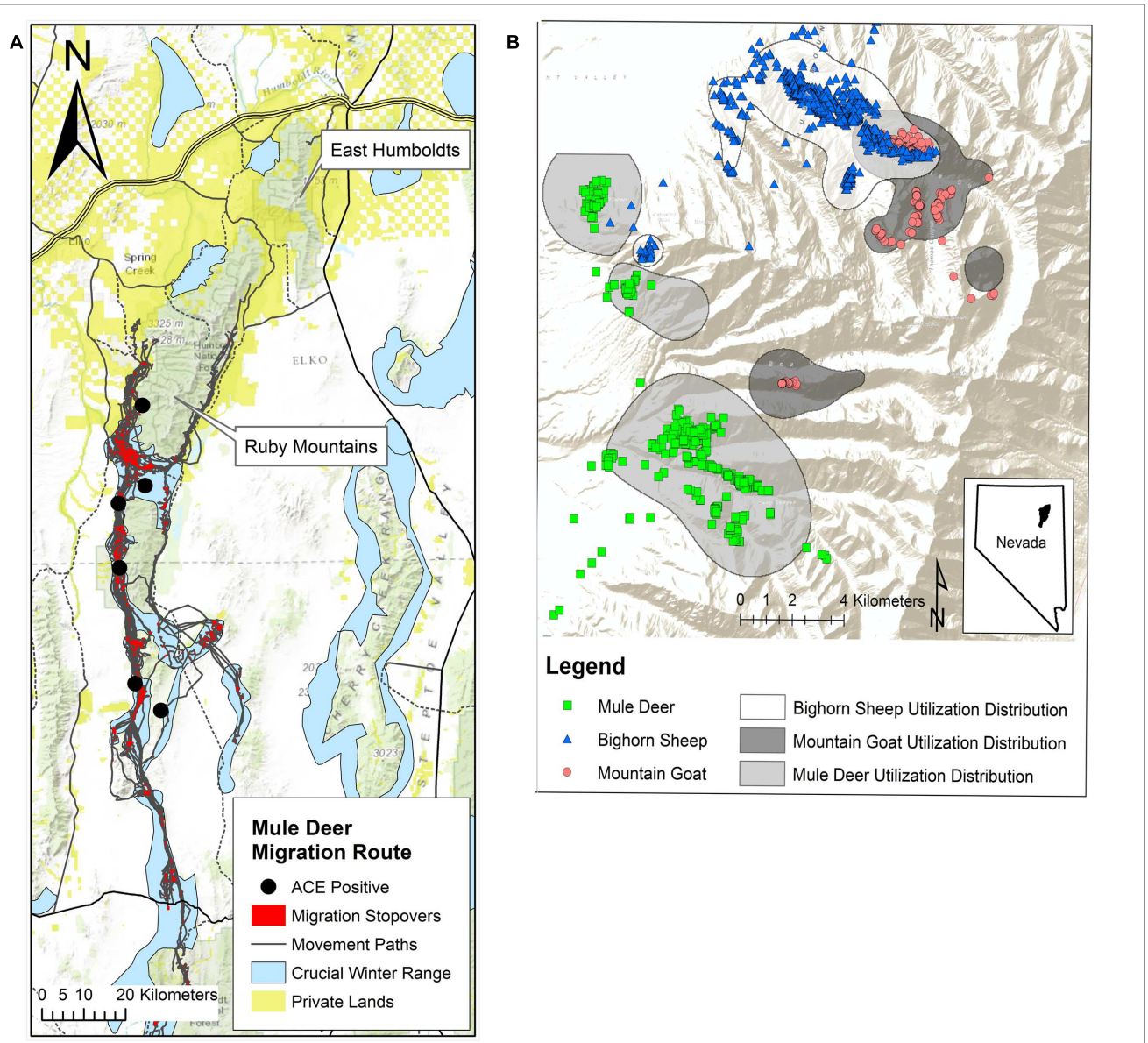

FIGURE 2 | (A) Population-level migration route (gray) and stopover sites (red) estimated for the Ruby Mountain (RM) mule deer herd, 2012–2013 (Sawyer and Brittell, 2014) population estimate of 20,000. Approximately 80% of this herd exhibits a long distance migratory strategy to winter range (blue) while the remaining 20% are considered resident deer exhibiting only altitudinal movement. Autumn migration on average begins around October 15 and can last for up to 90 days. In spring deer begin to migrate back from winter range starting mid-March to mid-April. Migrating deer may spend 90% of their time at these stopover sites along the migration route and GPS collar data (12 locations/day for 18 months) for two mule deer indicated that individuals can remain in these stopover areas for 1–2 months. This migration pattern increases densities during the first 2 months of gestation increasing the risk that naïve does could be exposed to BVDV. Gold squares represent private land holdings, primarily cattle ranches that run along the base of the mountain providing an opportunity for interspecies contact. Black circles indicate capture locations of the six deer that were antigen-capture ELISA positive on a single ear notch sample taken in 2013. **(B)** Global positioning system (GPS) multiple daily location collar data from December 1, 2012 to February 15, 2013, for mountain goats (887 location points, $N = 5$) and bighorn sheep (2622 location points, $N = 7$) in the northern portion of the RMs. Blue triangles and red circles illustrate winter range overlap between the two species. Population densities are increased during the first trimester of pregnancy when animals move to and congregate on winter range thus increasing the risk that naïve ewes and nannies could be exposed to BVDV. There is also limited overlap (green squares/blue triangles) occurring between bighorn sheep and mule deer (1480 location points, $N = 8$).

confirmed that each species moves to, or are on, their respective winter range through the first trimester of gestation: 0–60 days in bighorn sheep (Lawson and Johnson, 1987) and mountain goats (Wigal and Coggins, 1987) and 0–66 days in mule deer (Mackie et al., 1987). Infection of a naïve dam during gestation can produce PI offspring, as experimentally proven in white-tail deer (Passler et al., 2010). Thus increased densities along migration routes, converging winter ranges and reproductive timing in these species likely provides an ideal environment for virus transmission and maintenance in a population (**Figures 2A,B**).

The importance of BVDV infection with regard to morbidity and mortality is not known in these populations. Whether BVDV infection in the bighorn sheep or mountain goats played a significant role in the 2009–2010 pneumonia die-offs is also not clear. The 2009–2010 disease event in the EHR and the RM was attributed to pneumonia caused by *Mycoplasma ovipneumoniae* and secondary infection with *Pasteurellaceae* sp. (Besser et al., 2012; Shanthalingam et al., 2014) consistent with the majority of all-age, die-offs that have been reported in bighorn sheep in the western United States since 1980 (Cox and Carlsen, 2012). Possibly immunosuppression resulting from BVDV infection may have been a predisposing factor for the 2009–2010 pneumonia events. Two free-ranging bighorn sheep (Van Campen et al., 2003) as well as two captive mountain goats (Nelson et al., 2008) that presented with bacterial pneumonia had concurrent BVDV infection. In contrast, pneumonia die-offs were documented in two additional bighorn sheep herds in Nevada in 2011, with no serologic evidence of BVDV infection (NDOW, unpublished data). Experimental infection of adult and young mule deer (Van Campen et al., 1997), white-tail deer (Ridpath et al., 2008, 2012; Raizman et al., 2009; Passler et al., 2010) and elk (Tessaro et al., 1999) with BVDV caused subclinical to severe clinical disease and immunosuppression. Although, mule deer mortalities were investigated; infectious disease could not be confirmed as contributing to the death. We have not directly associated clinical disease with BVDV infection in this study, However, our findings strongly support that further testing for BVDV, should be included when investigating cases of respiratory disease in at risk wildlife species.

The importance of BVDV-induced reproductive disease in these wild ungulate species is unknown. Reproductive loss including fetal resorption, fetal mummification, abortion, weak fawns, and PI fawns has been documented in white-tail deer infected during the first and second trimesters of gestation (Ridpath et al., 2008, 2012; Passler et al., 2009, 2010), however, experimental infection of white-tail does during the third trimester of gestation did not affect reproduction (Ridpath et al., 2012). Autumn aerial surveys of mule deer between 1998 and 2008 indicated a significant drop in fawn recruitment which could not be fully explained by typical population drivers such carrying capacity, climatic conditions or predation. Recent surveys indicate fawn recruitment has slightly increased (NDOW unpublished data), but bighorn and mountain goat populations have not recovered after the 2009–2010 die-off. On-going annual losses of lambs at approximately 4–12 weeks of age from bacterial pneumonia is an epidemiologic feature in some bighorn sheep die-offs associated with mixed infections of *M. ovipneumoniae* and *Pasteurellaceae* sp. (Cox and Carlsen, 2012; Cassirer et al., 2013). In the EHR we noted a similar pattern in mountain goats with kids developing clinical signs and dying of bacterial pneumonia between 8 and 12 weeks of age (NDOW unpublished data) with no evidence of active BVDV infection. Further investigation to determine if infection with BVDV is affecting fawn, lamb and kid production, early survival or recruitment in these populations is warranted.

CONCLUSION

Wild ungulates and cattle concurrently graze public and private lands in the EHR and RM creating potential for interspecies BVDV transmission. Ideal conditions for viral transmission such as the production of PI animals from pregnant naïve animals or a virulent strain spillover (Thurmond, 2005; Evermann, 2006) likely occurred perpetuating infection in three previously naïve species. Winter range overlap between bighorn sheep and mountain goats and philopatric mule deer migration patterns and timing may have further contributed to the transmission and potential maintenance of the virus in these populations. The impacts of BVDV infections on the health and recruitment in these three species are unknown creating an unpredictable variable confounding management of wild ungulate populations in Nevada.

AUTHOR CONTRIBUTIONS

Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work: PW, CS, CM, MC, JR, JE, DN. Drafting the work or revising it critically for important intellectual content PW, CS, CM, MC, JR, JE, DN. Final approval of the version to be published: PW, CS, CM, MC, JR, JE, DN. Agreement to be accountable for all aspects of the work PW, CS, CM, MC, JR, JE, DN.

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Assessment of the rabbit as a wildlife reservoir of bovine viral diarrhea virus: serological analysis and generation of trans-placentally infected offspring

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Eradication of bovine viral diarrhea virus (BVDV) is ongoing in many European countries and is based on removal of persistently infected (PI) cattle. In this context, low-level risks, including alternative reservoirs of infection, may become more important as the number of BVDV-free herds increases. Alternative reservoirs include livestock, such as sheep and goats, as well as wildlife, including deer and rabbits. Due to the extensive nature of the beef industry in Scotland, where an eradication program started in 2010, contact between cattle and alternative reservoir hosts is common. Seroprevalence to BVDV in rabbit populations can be high. In addition, rabbits can be infected with BVDV by natural routes, indicating that they could be a wildlife reservoir of infection. We analyzed the potential risk to livestock from rabbit populations in the UK by two approaches. First, ∼260 serum samples from free-ranging wild rabbits in Scotland and northern England were tested for BVDV-specific antibodies by ELISA. Only three samples exhibited low level BVDV-specific reactivity, suggesting that BVDV infection of rabbits was not frequent. Second, rabbits were challenged with BVDV at day 7 or 12 of pregnancy. This did not lead to any clinical signs in the infected animals or obvious increases in abortion or stillbirth in the infected dams. Samples from the dams, placental material and ∼130 offspring were tested by BVDV-specific RT-PCR and antibody ELISA. Positive PCR results in the placentas and in the tissues and body fluids of rabbits up to 10 days old showed that trans-placental infection of rabbits with BVDV had occurred. Many of the offspring had BVDV-specific antibodies. These data support the view that a wildlife reservoir of BVDV in rabbit poses a small but non-zero risk of re-infection for BVDV-free cattle herds. Rabbits are susceptible to infection with BVDV but only a small proportion of free-living rabbits in the UK appear to have been infected.

Keywords: pestivirus, persistent infection, disease reservoir, wildlife diseases, livestock diseases

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Introduction

Bovine viral diarrhea (BVD) is an endemic disease, caused by bovine viral diarrhea virus (BVDV), with a significant impact on cattle production and health due to the abortifacient and immunosuppressive effects of infection. Maintenance in the UK herd is driven by persistently infected (PI) animals that were infected *in utero*, so that they tolerate BVDV infection and shed virus continuously.

Bovine viral diarrhea virus PI animals may show no clinical signs as calves, however, they often have a reduced growth rate and productivity and their life-expectancy is significantly reduced. Herds with BVDV generally have reduced reproductive performance and a higher rate of diseases such as diarrhea and pneumonia (Evermann and Faris, 1981) as a consequence of BVDV related immunsuppresion. Because of the economic losses due to BVDV infection, many European countries have undertaken eradication programs. Pioneered by Scandinavian countries, national compulsory eradication programs are ongoing in Austria, Switzerland, Germany, Ireland, and Scotland and are based on detection and removal of PI animals with or without vaccination of uninfected animals in the herd (Stahl and Alenius, 2012). In several other countries, regional and voluntary programs exist.

Scotland has a BVD eradication program based on the identification of PI cattle and the restriction of their sale or movement. However, flaws in the design or implementation of control programs and potential spread from wildlife reservoirs may impact Scotland's ability to become and remain BVD-free.

Bovine viral diarrhea virus can cross the species barrier relatively easily, particularly into sheep, where it causes a disease clinically indistinguishable from that caused by Border Disease Virus (Carlsson, 1991). Antibodies against BVDV have been detected in a wide range of wild and domesticated ruminant and porcine species (Doyle and Heuschele, 1983; Becher et al., 1997; Scherer et al., 2001; Van Campen et al., 2001) and persistent infection has been demonstrated in sheep, goats, pigs, alpaca, white-tailed deer, eland, mouse deer, and American mountain goats (Terpstra and Wensvoort, 1997; Vilcek et al., 2000; Scherer et al., 2001; Carman et al., 2005; Uttenthal et al., 2005; Passler et al., 2010; Bachofen et al., 2013a). In the early years of BVDV research, a wide range of non-artiodactyls such as horses, cats, dogs, several small laboratory animal species (guinea pig, mouse, rabbit) and embryonated chicken eggs were inoculated with the virus in order to determine the host range (Baker et al., 1954). The only non-artiodactyl animal in which virus could be propagated upon intravenous inoculation was the rabbit. These authors reported (Baker et al., 1954) that calves inoculated with spleen homogenate from rabbits that had been infected with BVDV 5 days earlier showed clinical signs typical of transient BVDV infection. Furthermore, BVDV could be serially passaged, both within rabbits and between rabbits and cattle, using lymphoid cell suspensions (Baker et al., 1954). More recently, a serological survey in Germany showed that 40% of sera sampled from 100 wild rabbits exhibited low neutralizing antibody titres against BVDV (Frolich and Streich, 1998). However, only a third of the positive results could be confirmed by ELISA and no virus could be isolated from any rabbit. A recent experimental study has demonstrated that rabbits can be infected with BVDV by both parenteral and natural routes but shedding of virus was not detected (Bachofen et al., 2014). Thus, there are indications that rabbits could be a natural wildlife reservoir for BVDV. Since rabbits are abundant in countries such as the United Kingdom and Ireland, often living on or near livestock pastures, a BVDV reservoir in rabbits could have significant consequences for BVDV eradication campaigns in these countries, especially toward the end of an eradication scheme. In this study we have used a serological survey of free-ranging wild rabbit populations and experimental infection of pregnant rabbits to determine whether BVDV infected rabbits pose a risk to in-contact livestock.

Materials and Methods

Ethics Statement

This study was conducted in the UK in compliance with the Home Office of Great Britain and Northern Ireland 'Animals (Scientific Procedures) Act 1986' and with the approval of the Moredun Research Institute Experiments and Ethical Review Committee (E53/14).

Virus

The BVDV isolate (MRI103) used for the experimental exposures was isolated from the serum of a Scottish PI bovid which was free of maternal antibodies, and passaged six times on bovine turbinate (BT) cells. After three passages, the virus was titrated on BT cells and a multiplicity of infection (MOI) of 0.01 was used for the following passages as previously described (Frolich and Streich, 1998). Medium from the sixth passage, containing BVDV at a titre of 10^6 TCID50/mL, was clarified by centrifugation at 4000 $\times$ *g* for 30 min and stored in aliquots at −80°C before use. All cells, tissue culture medium (Iscove's modified Dulbecco's medium, IMDM; Sigma–Aldrich, Dorset, UK) and foetal bovine serum (FBS) used were tested free of pestivirus and antibodies against pestivirus. The 5′UTR and N_{pro} coding region of the isolate were sequenced for phylogenetic typing as previously described (Bachofen et al., 2013b) and MRI103 was determined to be a BVDV-1a virus.

Animals and Treatments

Twenty mated female New Zealand White rabbits were purchased from a certified breeder with an 80% likelihood of pregnancy, for delivery on estimated day 5 of gestation. The rabbits were acclimatized for 2 days prior to being assigned randomly into two groups of eight animals and one group of four animals that were housed in individual boxes, with each group in a separate room. In cattle, BVDV infection during the first 120 days of pregnancy is thought to result in persistent infection of the fetus (Charleston et al., 2001). Therefore, in challenging pregnant rabbits we used two time points that were within the same portion of the rabbit gestation period (up to day 13). The two groups of eight rabbits were exposed to BVDV intravenously on day 7 (Group 1) or day 12 (Group 2) of gestation via the ear vein with 1ml of virus (10^6 TCID50) whilst the remaining four rabbits (Group 3) were mock

infected with 1 ml of IMDM. A pre-infection blood sample was also collected from each animal. The inoculum of 10^6 TCID50 had previously been used to induce transient infection in rabbits (Bachofen et al., 2014). One animal from Group 2 and one animal from Group 3 had to be withdrawn from the experiment due to issues with subsequent sampling. The body temperature of each animal was monitored daily by a subcutaneous microchip placed in the neck region (idENTICHIP; Animalcare, York, UK). The animals were observed twice daily until the delivery of the first offspring after which observations were made four times a day. Nesting material was included in all boxes and any live offspring found outside the nest were recovered to it. Any dead offspring or placental tissues found in the boxes were collected and frozen for later analysis. All remaining animals were euthanized at the end of the study (approximately day 10 after birth of the offspring).

At post-mortem examination, samples of lung, heart, liver, spleen, kidney, ileum (sacculus rotundus) and appendix were placed into neutral buffered formal saline, processed routinely through graded alcohols prior to being embedded in paraffin wax and stored at 4°C until required. For detection of BVDV viral RNA, tissue samples were collected into RNAlater (Life Technologies, Paisley, UK). Blood samples were collected post mortem by cardiac puncture and were allowed to clot before drawing off serum, while urine samples were taken directly from the bladder. Samples of serum and urine were frozen under aseptic conditions and stored at −80°C until required.

RNA Isolation and BVDV Real-Time RT-PCR

RNA isolation from blood or body fluid samples was performed using a viral RNA mini kit (Qiagen, Manchester, UK) according to the manufacturer's instructions. For tissue samples, homogenisation of about 30 mg of frozen tissue by ceramic beads in RLT buffer (Qiagen) using the Precellys 24 tissue homogenizer was followed by RNA isolation using the RNeasy mini kit (Qiagen).

Buffy coats from blood samples were isolated using a commercial red cell lysis buffer (Promega, Southampton, UK). Subsequent RNA isolation was performed using QIAShredder columns and the RNeasy mini kit (Qiagen).

For simultaneous detection of the viral genome and host beta-actin RNA, an established BVDV-1 specific real time RT-PCR (Willoughby et al., 2006) was used with a generic actin assay (Crook et al., 2012) in a duplex assay on an ABI 7500 sequence detection system (Applied Biosystems-Life Technology, Paisley, UK). Virus-positive RNA samples ($Ct < 40$) were retested to confirm the result. Additionally, end-point PCR amplification of BVDV RNA directly from serum or urine was performed as described previously (Bachofen et al., 2013b) and the PCR products were characterized by capillary electrophoresis in a commercial instrument (Qiaxcel, Qiagen).

Sample Collection from Wild Rabbits

Serum samples were obtained from wild rabbits shot at three locations in the UK as described previously (Boag et al., 2001, 2013). The majority of samples came from a 400 ha site in Perthshire, Scotland (182; 2008–2011, **Figure 1**), while others were obtained in North Yorkshire, England (31; 2004–2009) and the island of Coll, Scotland (45; 1985–2014). Serum samples were stored at −20°C until required for analysis of serological responses to BVDV antigens.

ELISA for Detection of BVDV Antibodies

A biphasic, indirect antibody capture ELISA was used to detect BVDV antibodies in serum samples. The test was used essentially as described previously (Bachofen et al., 2014). Briefly, alternate columns of a 96-well ELISA plate (high binding, Greiner Bio-One, Gloucestershire, UK) were coated with antigen from Igepal treated BVDV (isolate C24V) infected cells or with an equivalent antigen preparation from uninfected cells. Prior to usage, plates were blocked for 45 min at room temperature with a solution of 4% milk powder in phosphate buffered saline (PBS) containing 0.05% Tween20 (PBST). The rabbit serum samples were diluted 1:50 in PBST containing 2% milk powder and added in quadruplicates to the plate. After incubation (1 h) and washing, the horseradish peroxidase (HRP) conjugated anti-rabbit Ig antibody was added (P0448; Goat anti-rabbit immunoglobulins/HRP, diluted at 1:1000; Dako UK, Cambridgeshire, UK). Following a further 1 h incubation and a wash step, bound antibody was visualized by adding tetramethylbenzidine substrate (SureBlue, KPL Inc., Gaithersburg, USA). The reaction was stopped after 5 min by addition of 0.18 M sulphuric acid and absorbance at 450 nm was measured in an ELISA plate reader (Dynex MRX_{II}, Dynex Technologies, West Sussex, UK). Aliquots of positive terminal serum from BVDV-infected rabbits from a previous experiment were used as positive control, while serum from mock-infected rabbits was used as the negative control (Bachofen et al., 2014). Plate to plate variation was normalized by calculation of the sample to positive (S/P) ratio for each sample relative to the positive and negative control serum on each plate. The following formula was used to calculate S/P values, where the corrected OD is the mean OD of positive antigen wells minus the mean OD of the negative antigen wells inoculated with the same sample:

$$S/P = \frac{(corrected\ OD\ of\ sample - \ corrected\ OD\ of\ negative\ control)}{(corrected\ OD\ of\ positive\ control \ - \ corrected\ OD\ of\ negative\ control)}$$

For each ELISA sample set, a cut-off for positive samples was calculated based on all samples (free-ranging rabbits) or on the negative control Group 3 (experimentally infected rabbits) as the arithmetic mean plus 3 standard deviations.

Histopathology and Immunohistochemistry (IHC)

Paraffin-wax embedded tissue sections were cut (5 μm), mounted on glass microscope slides and stained with haematoxylin and eosin (H&E) prior to evaluation by light microscopy. Selected tissue sections from offspring that were PCR positive for BVDV and for which samples were available were subjected to IHC for BVDV as described previously (Bachofen et al., 2014).

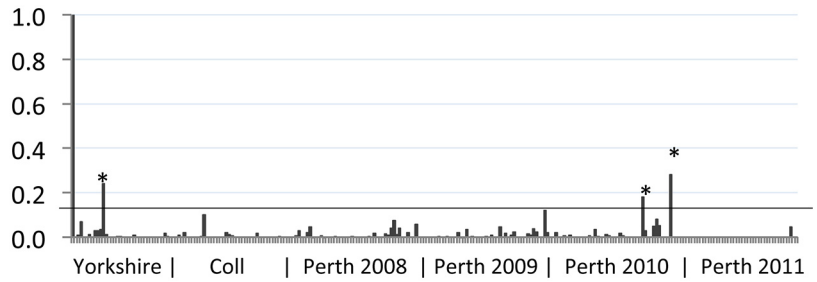

FIGURE 1 | Reactivity of wild rabbit serum to bovine viral diarrhea (BVD) virus antigen. The sample to positive (S/P) value (vertical axis) for each sample tested was plotted. Control positive serum (leftmost sample 1) has an S/P value of 1 while control negative serum (sample 2) has a value of 0. The geographic source of samples is indicated beneath the chart, with year of collection for Perth samples. Samples from Yorkshire were collected between 2007 and 2014; samples from Coll were collected between 2008 and 2012. The position of the S/P value cut-off for these samples (0.13) is indicated by a horizontal line. Samples that gave S/P values >0.13 are indicated by asterisks (*).

Statistical Analysis

Analysis of BVDV-specific antibody responses between the two groups of infected rabbits was by Student's *t*-test (two-tailed, assuming equal variance between the two datasets), while comparisons of infection levels between groups was by Fisher's exact test (two tailed; Fisher, 1922). Seroprevalence estimates were made using Epitools epidemiological calculators (Sergeant, 2015; http://epitools.ausvet.com.au.) and confidence intervals (CIs) were calculated by the Binomial (Clopper–Pearson) 'exact' method within Epitools.

Results

Serological Analysis of Wild Rabbit Samples

Rabbit serum samples were tested for BVDV-specific antibodies using an indirect ELISA modified for use with rabbit serum, as described previously (Bachofen et al., 2014). The results of this serosurvey are summarized in **Figure 1**. We have previously shown that rabbits infected by non-parenteral routes developed BVDV-specific antibody responses with S/P values ranging from 0.1 to 1.2 (Bachofen et al., 2014). Among the free-ranging wild rabbit samples tested, the mean S/P value was 0.01 and a cut-off of 0.13 was used to identify potential positive samples. Eleven samples with S/P values greater than 0.1 were retested and of these, four samples remained above 0.1 and three samples had S/P values above 0.13 in both tests. Positive samples originated from Yorkshire ($n = 1$) and Perthshire ($n = 2$). From this analysis the frequency of BVDV-seropositive rabbits in the areas surveyed is estimated at 3.2% (95% CI 0.1–16.7%) for Yorkshire; 0% (95% CI 0.0–7.9%) for Coll; and 1.1% (95% CI 0.1–3.9%) for Perthshire. Although the three sampled areas are geographically distinct, there is no significant difference between regional prevalence estimates and the overall prevalence estimate was 1.2% (95% CI 0.2–3.4%).

Infection of Pregnant Rabbits

To determine whether rabbits could be infected by BVDV *in utero*, pregnant rabbits were challenged with a BVDV type 1a strain that had previously been shown to induce transient infection of rabbits by parenteral and natural routes (Bachofen et al., 2014). Details of the Groups, litter sizes and survival are given in **Table 1**. One dam in Group 2 did not produce a litter, while three offspring in Group 1, three in Group 2 and one in Group 3 were stillborn. Due to the potential for resorption or abortion of offspring caused by the stress of transport, handling, treatments and sampling, the pregnant dams and their live offspring were not sampled until the end of the experiment (10 days after the birth of the first litters). At this point all surviving animals were euthanized and subject to post-mortem examination. About half of the live-born offspring appeared to be killed by the dams and some were partially eaten (**Table 1**). All dead animals and placentas were removed when they were detected and tissue samples were collected where possible.

Serology

Terminal serum samples from all dams were tested by BVDV-specific ELISA to demonstrate seroconversion (compared to seronegative pre-infection samples), which was used as indicator of successful infection. All offspring, where a terminal serum sample could be obtained, were tested in the same way to demonstrate presence of BVDV-specific antibodies, without distinction between maternal antibodies or antibodies generated by the offspring. The S/P values of the terminal samples are shown in **Figure 2** and summarized in **Table 2**. A cut-off for samples to be considered positive was calculated from Group 3 as the mean S/P value plus three standard deviations (0.2). The data show that dams in Groups 1 and 2 responded to BVDV infection with a mean S/P value of 0.57. The timing of infection did not have a significant effect on the terminal titre, although the Group 1 dams (infected at day 7 after mating) appeared to have a lower mean S/P value (0.40) than dams in Group 2 (0.75) that were infected 5 days later.

Notably, not all of the dams appeared to respond strongly to infection in challenge Groups 1 and 2. Several had low S/P values (e.g., KPXD, KRRH; **Table 2**), suggesting that the challenge or the immune response may have been sub-optimal in some animals. However, most of these animals had other

TABLE 1 | Details of groups and number of animals.

Group (*n*)[a]	Challenged[b]	Litters[c]	Days[d]	Total offspring	Stillborn	Survived[e]
1 (8)	Day 7	8	30–32	67	3	41 (61%)
2 (7)	Day 12	6	32	44	3	18 (41%)
3 (3)	Day 12 (Mock)	3	30–31	20	1	10 (50%)

[a]*Group number and number of animals (n) in each study group from which results were obtained.*
[b]*Day (post-mating) on which challenged. Mock-challenged animals received culture medium only.*
[c]*Number of animals that delivered litters.*
[d]*Day on which litters delivered (days post mating).*
[e]*Number of offspring (and percentage of group total) that survived until the end of the experiment (at least 10 days after birth).*

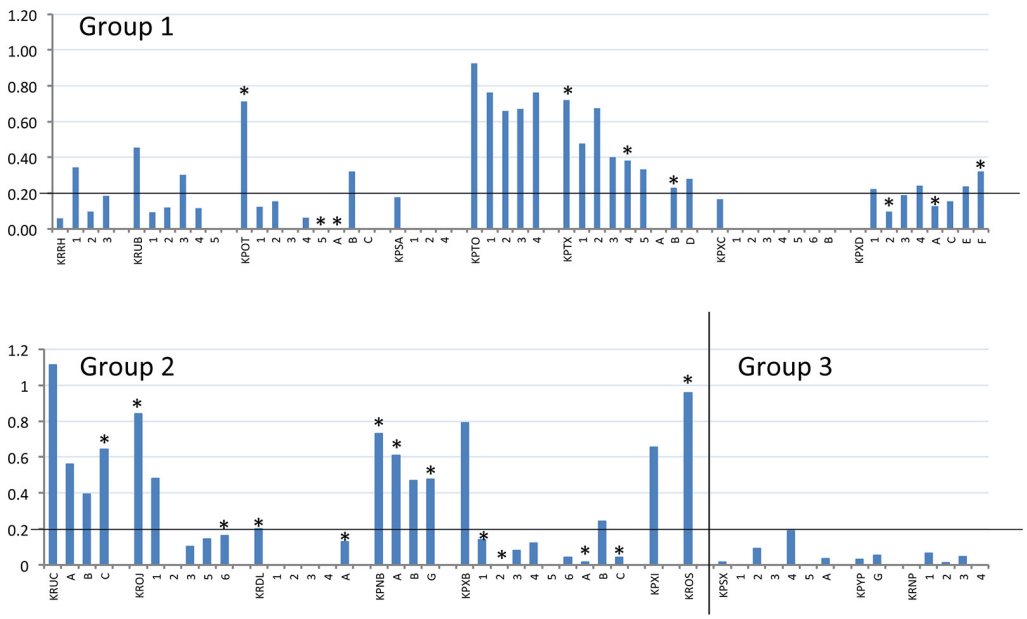

FIGURE 2 | Reactivity of BVDV-infected rabbits and their offspring to BVDV antigen. S/P values (vertical axis) of terminal serum samples from all tested dams and offspring (horizontal axis) are plotted. Dams are identified by their four-letter ear-tag codes, while the tested offspring follow the respective dams, identified by single letters or numbers depending on their survival, as described in **Table 2**. Experimental group is indicated within each chart. The position of the S/P value cut-off for these samples (0.2) is indicated by a horizontal line on each chart. All offspring tested here by ELISA were also tested by RT-PCR and positive RT-PCR results are indicated by an asterisk above the relevant column (*).

evidence of infection, such as BVDV RNA detected in placenta or offspring.

Pathology in Infected Rabbits

No lesions suggestive of BVDV infection were present on examination of H&E sections of any dams or available offspring.

Immunohistochemistry

Fixed tissues were only available from the dams and those offspring that survived to the end of the experiment (numbered offspring, **Table 2**) and of these, animals KPOT-5, KPTX-4 and KPXB-2 were found to be positive in tissues for BVDV by RT-PCR, whilst additional animals KPXD-2, KROJ-6, and KPXB-1 were RT-PCR positive in serum (**Figure 2**). Sections of all fixed tissues from these six animals only were tested for the presence of pestivirus-specific antigen as described previously (Bachofen et al., 2014). Repeated attempts at IHC gave inconsistent results in the negative control rabbit preparations making interpretation meaningless. Positive and negative controls using BVDV-infected cattle tissue sections gave unequivocal staining patterns, suggesting that the problems were due to the rabbit-specific secondary reagent.

Detection of Viral RNA

RNA was extracted from placentas recovered from three rabbits in Group 1, six rabbits in Group 2 and two rabbits in Group 3. These samples were assayed for the presence of BVDV-specific RNA by real-time RT-PCR. The results of these assays are summarized in **Table 2** and showed that two animals from Group 1 and four animals from Group 2 shed placentas that contained BVDV RNA. There was no clear association between the detection of BVDV-specific RNA and the terminal BVDV-specific antibody titre in the dams.

Serum and tissues from most offspring were tested for the presence of BVDV RNA by RT-PCR. Where available, serum

TABLE 2 | Outcome of diagnostic analysis.

Animal[a] (group)	Placenta[b]	Offspring[c]	Offspring BVDV RT-PCR tissue[d]		Offspring BVDV RT-PCR serum (urine)[e]		Serology (offspring)[f]
			POS	NEG	POS	NEG	
KRRH (1)	–	A,B; 1–3	A	B,1,2,3		A,(B),1,2,3	0.06 (+)
KRUB (1)	–	A–C; 1–5	B	1,4,5	(B)	(A),1,2,3,4,5	0.46 (+)
KPOT (1)	29	A,C; 1–7	5	A,1,2,3,4,6	A	C,1,2,3,4,5,6	0.71 (+)
KPSA (1)	–	A-D; 1–6		1,2		A,B,C,1,2,3	0.18 (–)
KPTO (1)	NEG	A,B; 1–4		1,2		B,1,2,3,4	0.93 (+)
KPTX (1)	28	A–D; 1–6	B,4	C,D,1,2,3,5	B	A,(C),D,1,2,3,4,5	0.72 (+)
KPXC (1)	–	A–D; 1–6	A,C	1,2,5,6		B,1,2,3,4,5,6	0.16 (–)
KPXD (1)	–	A,B,C,E,F; 1–4	A,D,F	1,2,3,4	A,F,2	B,C,E,1,3,4	0.00 (+)
KRUC (2)	NEG	A–H		A,B	C	A,B,D,(E,G,H)	1.11 (+)
KROJ (2)	31	A; 1–6		1,2,3,4,5,6	6	(A),1,2,3,4,5,6	0.84 (+)
KRDL (2)	26	A; 1–6	A	1,2,3,4	(A)	1,2,3,4	0.20 (–)
KPNB (2)	31	A–G	E,G	A,B,C,D,F	A,(E),G	B,(C,D),F	0.73 (+)
KPXB (2)	NEG	A–C; 1–6	A,C,2	1,3,4,5,6	A,C,1,2	B,3,4,5,6	0.79 (+)
KPXI (2)	–	No litter					0.65 (0)
KROS (2)	29	A–F	B	A,C,D,E,F		(A,C,D,E,F)	0.96 (0)
KRNP (3)	–	A,B; 1–4		A,B,3,4		1,2,3,4	0.00 (–)
KPYP (3)	NEG	A-G		A,B		(A,B,C,D,E,F),G	0.03 (–)
KPSX (3)	NEG	A; 1–6		1,2		A,1,2,3,4,5	0.02 (–)

[a]*Animal identifier for each pregnant dam sourced, with experimental group in parenthesis.*
[b]*Detection of BVDV-specific RNA in recovered placentas by real-time RT-PCR. Numbers indicate threshold cycle (Ct) values for detection of BVDV; NEG indicates that no specific amplification was found; – indicates that no tissue was available for analysis.*
[c]*Offspring which died before day 8 are given as letters, while offspring that survived until the end of the experiment are denoted by numbers.*
[d]*Tissue from at least two offspring in each litter were assayed for BVDV-specific RNA by real-time RT-PCR (appendix in all tested except KROS-B and KPOT-A, from which kidney was used). POS indicates detection of BVDV with Ct of 22–25; NEG indicates that no specific amplification was found for the listed offspring.*
[e]*detection of BVDV-specific RNA in serum of offspring by direct end-point RT-PCR or by BVDV-specific real-time RT-PCR. POS indicates detection of BVDV-specific amplicon; NEG indicates that no specific amplification was found for the listed offspring. Where no serum was available, urine was tested by the same method and these results are given in parenthesis. All of the adults were negative for BVDV-specific RT-PCR in serum collected before infection and at port-mortem examination.*
[f]*Bovine viral diarrhea-specific antibody responses were calculated for each animal from a post mortem blood sample (**Figure 2**). The S/P value for each dam is given, while the detection of at least one offspring sample with BVDV-specific antibodies (S/P > 0.2) is indicated by (+). Where all tested offspring were BVDV antibody negative, this is shown as (–). A lack of testable samples is indicated by (0).*

(or urine) from each animal was tested in a direct end-point PCR assay as described previously (Bachofen et al., 2013b) and these results were repeated where possible by real-time RT-PCR analysis of RNA extracted from serum or urine. Of 117 samples tested, 23 samples were found to be positive (**Table 2**). To confirm these results, RNA was prepared from tissues of at least two offspring from each litter, including all of the offspring found to be potentially positive by direct RT-PCR. RNA was prepared from appendix where available and from kidney in two cases where appendix could not be recovered. These RNA samples were tested by real-time RT-PCR and showed that the majority of RNA samples from tissues of offspring with positively testing serum or urine were also positive by real-time RT-PCR, with Ct values of between 21 and 23; while a small number of real-time RT-PCR positive samples were from tissues of offspring that were BVDV-negative by direct PCR.

It is notable that most of the samples with detectable BVDV RNA came from offspring that were killed by their mothers prior to day 8 after birth. Within the challenged groups, 16 of 52 killed offspring had RT-PCR evidence of BVDV infection while only seven positive samples were found among the 59 offspring that survived to the end of the experiment ($p < 0.02$).

All of the offspring that could be tested for serum antibodies against BVD antigens were also tested by RT-PCR for the presence of viral RNA (**Figure 2**). RT-PCR-negative serum samples had S/P values ranging from zero to 0.76; while RT-PCR-positive samples had S/P values from zero to 0.64. There was no clear correlation between the presence of detectable viral RNA in serum and the antibody response.

Discussion

The aim of this study was to gather evidence to address the possibility that BVDV-infected rabbits could form a wildlife reservoir and be a risk of re-infection for cattle herds which were free of BVDV and unvaccinated. Serological analysis of more than 250 samples from Yorkshire, Coll and Perth showed that only three samples had S/P values that may represent BVDV-specific

antibody responses (**Figure 1**). These samples, however, exhibited high non-specific binding of the negative control antigen in the ELISA and could therefore be false positives. Positive samples could be the result of infection with BVDV, although cross reactivity with other pestiviruses due to antigenic relatedness is also formally possible (Ridpath, 2013). The low frequency of positive samples (1.2%) suggests that BVDV is not established as an endemic infection of rabbits in the UK regions tested and is therefore likely to present a small risk of infection to in-contact livestock.

We have previously demonstrated that rabbits can be productively infected with BVDV type 1a (Bachofen et al., 2014) with virus propagation detected in gut-associated lymphoid tissue and with the development of virus-specific and virus-neutralizing antibody responses. In this study, BVDV viral RNA was detected in rabbits at day 5 after infection but not 3 weeks later, when the animals had seroconverted. To investigate the possibility that infection of pregnant rabbits might lead to the generation of BVDV PI offspring, two groups of rabbits were infected with BVDV1a on day 7 and on day 12 after mating. Following previous results (Bachofen et al., 2014) we would expect the period of potential virus shedding from the dams to have ceased by the end of gestation (23 days after day 7 and 18 days after day 12). Thus the detection of BVDV RNA in the offspring of 80% of infected rabbits is most likely to be the result of trans-placental infection. However the definition of persistent infection by BVDV is based on immune tolerance of the virus and its presence in multiple tissues and body fluids. While BVDV RNA was detected in serum (or urine) of some offspring, the presence of BVDV-specific antibodies in several RT-PCR-positive sera (**Figure 2**) may be the result of maternal transfer or of the immune response by offspring to intra-uterine infection, suggestive of possible transient infection. Further work is required to clarify this.

Of the 15 animals challenged, 14 produced litters, suggesting minimal ill-effects of the transport and infection of the animals. Indeed, the frequency of litters (17 of 18 dams delivered litters with an average litter size of 7.3) was higher than the supplier's predicted level of 80% and the frequency of stillbirth among the litters was unaffected by group (**Table 1**). However, about half of the offspring (47%) were killed by the dams in the period up to day 8 after birth, after which all remaining offspring survived to the end of the experiment. In Group 1, 39% of the offspring were killed, compared to 59% of Group 2 offspring and 50% of the control group, and this difference was significant between Groups 1 and 2 ($p < 0.04$). This implies that the handling and/or challenge procedures at day 12 of gestation had a significant negative association with survival of the offspring. This appears to be a higher rate of neonatal mortality than in a comparable commercial system (individually housed dams), which showed average litter size of 9.6 and pre-weaning mortality of 15% (Szendro and McNitt, 2012).

It was also notable that the surviving offspring included significantly fewer animals that were RT-PCR positive after testing of serum and tissue samples than the offspring that were found dead ($p < 0.02$). This may suggest that infected offspring are preferentially killed or, alternatively, that transplacental BVDV infection of neonatal rabbits is cleared within the first 10 days after birth, i.e., only in those animals that survived long enough. There was, however, no correlation between the level of BVDV-specific antibodies in the offspring and the detection of viral RNA, suggesting that circulating antibodies in the offspring did not protect them from viraemia, although they may contribute to the resolution of BVDV infection.

It was unclear in this study whether the BVDV-specific antibodies detected in the offspring were the result of maternal transfer or were generated in the offspring following *in utero* infection. Rabbits are immunocompetent at birth but have a restricted antibody repetoire, which continues to diversify up to about 8 weeks of life (Knight and Winstead, 1997). However, it is notable that the two infected dams with the lowest S/P values (KRRH and KPXD; 0 and 0.06, respectively; **Table 2**) delivered offspring with higher S/P values, while all of the other infected dams had higher S/P values than their offspring (**Figure 2**). These distinct patterns suggest that maternal transfer of antibodies may not be the only method by which the offspring gain BVDV-specific antibodies.

The immunohistochemical analysis performed on tissue samples from RT-PCR positive offspring was inconclusive. Greater binding of the labeled secondary antibody to the cytoplasm of epithelia of the appendices and renal medullae was observed in negative control preparations suggesting that goat derived antibodies bound to these rabbit tissues in a non-BVDV antigen mediated manner. Further work is required to optimize these methods for use in rabbits.

The results of this study suggest that while trans-placental infection of BVDV can occur in rabbits, relatively few of the offspring (21%) have evidence of infection from RT-PCR of tissue or body fluids. This supports the serological data that BVDV infection of free-ranging wild rabbits in the UK is infrequent. However, it is not clear from these data whether the infected rabbits were PI in the manner understood for livestock, as this would require further testing at multiple time points. It would also be beneficial to perform longer-term studies on rabbits trans-placentally infected with BVDV to determine whether they shed virus or generate a BVDV-specific antibody response. Although the proportion of infected offspring appears lower in rabbits than in cattle, the proportion of births resulting in infected offspring was high, particularly for challenge at day 12 of gestation, which led to the birth of infected offspring in every litter. This could contribute to maintaining presence of the virus in the rabbit population. The reduced survival of virus-positive offspring compared to virus-negative offspring, however, would limit the opportunity for transmission. The overall seroprevalence of BVDV in rabbits was low but, if the seropositive results represent true positives, 1% of the rabbit population would still translate into a large number of animals, suggesting a non-zero risk of transmission. Furthermore, confidence intervals for prevalence estimates

in geographically distinct areas covered a wide range and it is conceivable that seroprevalence may be high in specific areas, as previously suggested by Frolich and Streich (1998) based on field studies in Germany. In summary, there is a non-zero risk of BVDV infection in rabbits and although this is unlikely to be of epidemiological relevance for most control scenarios, it may theoretically play a role in the tail end of an eradication campaign, particularly in agricultural systems with a high likelihood of contact between cattle and rabbits.

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Bovine Viral Diarrhea Virus in Zoos: A Perspective from the Veterinary Team

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The many different species in close proximity make zoological collections a unique environment for disease transmission. Bovine Viral Diarrhea Virus (BVDV) is of special concern with zoos due to the numerous exotic ruminant species that this virus can infect. BVDV occurs as both a non-cytopathic and a cytopathic strain both of which are capable of infecting exotic ruminants. The cytopathic strain causes mucosal disease (MD) and death. Infection with the non-cytopathic strain may produce persistently infected (PI) animals. PI individuals may show vague clinical signs, including abortion. Management of BVDV in zoos should focus on identification of PI individuals and prevention of infection of other animals of the collection. Variability makes serological testing as the sole method of screening for BVDV infection undesirable in exotic ruminants. Combination testing provides a definitive answer, especially in sensitive wildlife. Use of a combination of antigen-capture ELISA (ACE) with haired skin, Real Time-PCR (RT-PCR) on whole blood, and antibody detection via serum neutralization has the greatest potential to identify PI animals. An animal that is positive on both ACE and RT-PCR, but is negative on serology should be considered highly suspicious of being a PI, and should be isolated and undergo repeat testing 4–6 weeks later to confirm positive status. This testing methodology also allows screening of pregnant and newborn animals. Isolation or culling may need to be considered in animals determined to be positive via combination testing. These decisions should only be made after careful consideration and evaluation, especially with endangered species.

Keywords: bovine viral diarrhea virus, exotic ruminant, zoo, persistent infection, abortion

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INTRODUCTION

Pestiviruses, within the family Flaviviridae, are viruses that may persist undiagnosed in exotic animals and zoological collections. To understand how this happens, and the unique concerns when addressing zoo animals, one must first understand some of the nuances of zoos and zoo collection management. There are few places in the natural world that rival the species density and diversity as modern zoos and wild animal parks. Interactions may occur in species that have overlapping natural environments, but rarely encounter each other in the wild. Modern zoos also serve as breeding facilities for endangered wildlife, and as a result may unintentionally provide perpetuating sources of infection or reservoir hosts for pathogens due to attempts to breed from very limited founder populations (Mohamed, 2015).

Most public zoos in America and Europe have experienced a positive shift over the last 30 years away from traditional bare, concrete floored, and chain-link fence enclosures. These traditional

displays allowed easy viewing by zoo patrons but may have further perpetuated the spread of diseases by increasing animal stress, which may result in weakened immune function, along with exposure to feces and other contaminants that can serve as sources of infection. Unfortunately, there are still privately owned facilities where animals exist in these conditions. The mixing of species and space limitations in a zoo can create a unique environment for transmission of diseases. Traditional mixed species displays, such as an "African Savannah" or "Asian Rainforest," combining different species can inadvertently serve as sources of infection to novel hosts. Newer management practices, such as rotational displays designed to allow movement between separate, but shared display areas through the day have maximized space and environmental enrichment for zoo animals, but may also result in direct and indirect mixing of species in a manner that results in exposure to novel diseases (Nolen, 2002; Coe, 2003).

Zoo veterinarians must remain vigilant for disease causing organisms capable of infecting multiple hosts. Obviously, the viral and bacterial diseases usually receiving the most attention for routine surveillance and prevention are those capable of causing zoonotic infection in humans. Infection of any zoo animal with a reportable disease having public health importance strikes fear in all associated zoo veterinarians and zoo administration (National Notifiable Diseases Surveillance System, 2015; Backues et al., 2011). Traditional estimates of disease occurrence have attributed as much as 75% of human emerging infectious diseases to zoonotic pathogens obtained from exposure to animals and wildlife (Taylor et al., 2001). Zoonotic diseases in zoos are local headline news stories even if there is no risk of zoo patron exposure and become national news if there is a human infection (Science on NBC News.com, 2005; Ganucheau, 2015). The American Association of Zoo Veterinarians (AAZV) and Association of Zoos and Aquariums and similar organizations in Canada, Europe, and Australia maintain quarantine guidelines for the introduction of new animals, but those quarantine guidelines are designed to be flexible, allowing for nuances of individual collections (Backues et al., 2011; Association of Zoos and Aquariums, 2015). These organizations also maintain specific guidelines for human interaction with zoo animals, to prevent human exposure (Backues et al., 2011; Association of Zoos and Aquariums, 2015).

The intervention of public health authorities, including veterinarians, for management of a disease outbreak reportable to government agencies may alleviate some of the pressure on zoo management for animal care decisions. However, this may further complicate the management of a disease outbreak in a zoo because normal protocols for isolation and eradication of infection in domestic animals, such as dramatic depopulation for an avian influenza outbreak in a broiler chicken farm, may be unreasonable for a zoo displaying rare, or endangered animals. This delicate balance of surveillance and identification of reportable diseases, combined with the ever looming presence of public perception of appropriate zoo management, means that diseases may go undetected for long periods of time, slowly brewing in the zoo collection for years, before being properly identified. Despite these concerns, general goals of all zoo health programs should involve steps that insure: (1) protecting the visiting public from disease exposure while protecting the animals from exposure from humans (2) protecting the collection from disease exposure from each other and (3) preventing the introduction of disease into regions around the zoo.

PESTIVIRUSES

Pestiviruses have been historically classified into four phylogenetic groups: bovine viral diarrhea virus serovar 1 (BVDV-1), bovine viral diarrhea virus serovar 2 (BVDV-2), classical swine fever virus (CSFV), and border disease virus (BDV) of sheep (Vilcek et al., 2000). The identification of novel strains of BVDV, including giraffe-1 and reindeer-1 have occurred within the last 15 years, however much remains unknown about these viruses (Avalos-Ramirez et al., 2001). While classical swine fever virus has been eradicated from many developed countries, CSFV remains a disease immediately reportable in many countries due to its potential economic impact (Classical Swine Fever Surviellence Plan USDA, 2007). Border disease has been recognized in most sheep-rearing areas of the world. However, it along with BVDV, remain diseases that may not be immediately reportable to government agencies or other authorities (Vilcek et al., 2000; Classical Swine Fever Surviellence Plan USDA, 2007).

BVDV is of concern to zoos because of the variety of species in the Order Artiodactyla, and suborder Ruminatia, including *Cervidae*, *Antilocapridae*, and *Bovidae,* with confirmed infections. Specifically, BVDV has been documented in wild and captive populations of mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), elk (*Cervus elaphus*), Japanese serow (*Capricornis crispus*), Canadian bison (*Bison bison bison*), water buffalo (*Bubalis bubalis*), roe deer (*Capreolus capreolus*), mouse deer (*Tragulus javanicus*), red deer (*Cervus elaphus*), bongo (*Tragelaphus eurycerus*), eland (*Taurotragus oryx*), wildebeest (*Connochaetes sp.*), nilgai (*Boselaphus tragocamelus*), axis deer (*Axis axis*), and barasingha deer (*Cervus duvaucelii*) (Doyle and Heuschele, 1983; Becher et al., 1999; Tessaro et al., 1999; Deregt et al., 2005; Craig et al., 2008). BVDV has also been isolated from animals frequently found in petting zoos including domestic cattle, alpaca, sheep, and goats (Pratelli et al., 2001; Scherer et al., 2001; Mattson et al., 2006; Mishra et al., 2007). All of these species are commonly found in modern zoos, may act as a reservoir for infection of other captive ruminants by BVDV.

BVDV IN ZOO ANIMALS

Because BVDV infections in domestic cattle herds can cause significant economic losses, this virus has been the subject of intense research in domestic cattle. The pathogenesis of BVDV appears to be similar in domestic and wild ruminants, however specifics of individual species infection requires additional research. In domestic cattle, two viral genotypes (BVDV-1 and BVDV-2) have been identified, and cytopathic and non-cytopathic biotypes are described according to their effects in cell culture (Gamlen et al., 2005; Vilcek et al., 2005). The

non-cytopathic biotype of BVDV is capable of establishing persistent infections (PI) in a fetus when the dam is infected during a specific window of gestation. This occurs when an immunologically naıve cow is infected with a non-cytopathic viral strain between 45 and 125 days of gestation (Brock, 2003; Gamlen et al., 2005; Peterhans et al., 2010). It is presumed that infected wild ruminants will have a similar infective window during the first one-third of gestation. However, those points in time have not yet been clearly defined experimentally in any species other than white tailed deer, where infection occurring between days 45 and 52 of gestation result in a PI fawn (Passler et al., 2007). This specific window of time predates immune system maturation, allowing the virus to remain in the affected fetus. Such fetuses may develop normally, and can be born apparently healthy, but will remain infected with the virus for life (Brock, 2003; Passler et al., 2007; Peterhans et al., 2010).

In addition to domestic cattle, persistent BVDV infection has been identified in domestic sheep and alpaca, which are common inhabitants of petting zoos (Carman et al., 2005; Mattson et al., 2006). There are also reports of definitive natural and experimental persistent infection in zoo animals and wildlife, including mouse deer, white tailed deer, North American elk, mountain goats, and eland (Tessaro et al., 1999; Vilcek et al., 2000; Uttenthal et al., 2005; Passler et al., 2007, 2009, 2010; Nelson et al., 2008). Animals infected with non-cytopathic BVD strains are considered immunotolerant to the infecting viral strain, and as a result are unable to clear the virus (Brock, 2003; Passler et al., 2007). PI neonates will heavily shed virus throughout their lives, creating a significant reservoir for infection, especially in a closed herd (Brock, 2003; Shoemaker et al., 2009). The PI infected animal should be of greatest concern within zoological collections because they may appear healthy while concurrently exposing multiple animals within the zoo to the virus. Continual viral shedding of a PI animal, combined with the possibility of minimal outward signs, makes it difficult to detect the source of infection in exotic species. This may also allow infection of hosts this virus has never before encountered. Persistent infections are often associated with decreased fertility, immunosuppression, stunted growth, and secondary infections (Potgieter, 1995; Brock, 2003). Decreased fertility is of extreme concern in zoological collections attempting to breed endangered wildlife because of the limited number of available breeding stock. Simply put, endangered species cannot afford to lose breeding specimens to viral diseases that may be preventable.

The cytopathic BVDV biotype can also be isolated from PI animals. Typically infection with cytopathic strains alone cause an acute phase disease, with rapid onset of clinical signs, patient debilitation, and death. Cytopathic strains are characterized by unrestricted viral replication, producing a large amount of virus that enters the environment, but that may be self-limiting due to resultant mortality (Peterhans et al., 2010). The cytopathic biotype develops from mutations of the non-cytopathic strain, include recombination with host cell mRNA, gene translocation and duplication, and point mutation (Brock, 2003; Peterhans et al., 2010). Cytopathic BVD viruses usually fail to establish chains of infection due to death of the infected animal, and are generally considered unable to cause persistent infection, despite isolation from PI animals (Brock, 2003). In a zoo, disease conditions like diarrhea will often lead to isolation of the ill animal from herd members not showing signs of disease as diagnostic testing is performed by veterinary staff. Assuming that isolation is prompt, this may reduce risk of exposure of other animals to cytopathic virus strains until a diagnosis is achieved.

Superinfection of PI animals with a cytopathic strain may trigger mucosal disease (MD) (Brock, 2003; Nelson et al., 2008). In a domestic cattle herd, MD is characterized by a relatively low morbidity and high case fatality between the age of 6 months and 2 years. It is unknown if exotic ruminants will develop MD with the same associated clinical signs as domestic animals. Typical gross lesions in domestic species include extensive mucosal ulceration primarily within the gastrointestinal tract, with resultant diarrhea, weight loss, and wasting (Brock, 2003; Nelson et al., 2008). The variability of clinical signs for BVDV in exotic ruminants complicates diagnosis and may result in infections being overlooked because of the lack of pathognomonic or any clinical signs.

CONTROL OF BVDV INFECTION

In a zoo, the clinical signs of diarrhea and weight loss associated with MD will result in animals being held off displays, quarantined, and diagnostic testing performed to determine the cause. Unfortunately, by the time clinical signs are observed, the virus has already contaminated the environment. To reduce possible transmission, all incoming animals should be quarantined appropriately for their species and tested for BVDV. The identification of asymptomatic PI individuals that are new additions, as well as those existing within the collection will help to control new sources of infection. Asymptomatic PI individuals present the greatest threat to zoo collections, especially to mixed species collections or those utilizing rotational exhibits. The focus for management of BVDV in zoos should be determining infected, especially PI, individuals and taking appropriate steps to prevent and control the spread of the virus. While vaccination is utilized to prevent fetal infections in domestic cattle, vaccination for BVDV has not been well studied and remains unproven in exotic species. In addition, the diagnostic tests used for domestic cattle likely have not been validated in exotic ruminants, and as said before, there is no pathognomonic histopathologic lesion described for BVDV (BVD, 2013). Definitive diagnosis can only be reliably made based on virus isolation or demonstration of the virus within tissues (BVD, 2013).

Serological surveys have been performed in wildlife and zoological collections in an attempt to screen for disease conditions. A recent study, evaluating the seroprevalence for BVDV in a zoo in Kuwait, had an overall prevalence of 5.3% in the bovids and cervids evaluated, with prevalence as high as 60% in Sitatunga (*Tragelaphus spekii*). In that same study Axis deer, Barbary sheep, Water deer, Dorcas and Fallow deer showed no evidence of antibodies in blood sera (Uttenthal et al., 2005). A similar 2011 study of 163 animals, composed of 39 Cameroon sheep (*Ovis ammon aries*), 11 Barbary sheep, (*Ammotragus lervia*), 57 pygmy goats (*Capra hircus*), nine Angora goats (*Capra hircus*), 21 mountain goats

(*Capra aegagrus-aegagrus*), seven llamas (*Lama glama*), eight Persian goitred gazelle (*Gazella subgutturosa subgutturosa*), seven Caspian red deer (*Cervus elaphus maral*), two fallow deer (*Dama dama*), and two camels (*Camelus dromedarius*) in two Turkish zoos showed negative serum antibodies to BVDV (Yeşilbag et al., 2011). A third 2011 study of archived and fresh samples from eight different European zoos utilizing cell culture and antibody enzyme-linked immunosorbent assay (ELISA) evaluations demonstrated detection of BVDV antibodies in 23.3% (21/90) of the animals evaluated (Probst et al., 2011). Because PI individuals occur due to failure of the immune system to respond appropriately to infection by BVDV, serology has its place for survey of populations, but also has substantial shortcomings in identifying all infected individuals in a closed herd in an attempt to eradicate the virus. PI individuals may have no detectable antibody titer and thus will not be identified if this is the sole form of screening utilized in a zoo. Not only can PI individuals screened serologically remain undetected, they serve as a nidus of infection unless they are identified.

Serologic testing for domestic animals is relatively easy, however simply obtaining a blood sample for serology from most exotic ruminants generally involves the use of special restraint chutes and/or general anesthesia which present the additional risks of chute trauma or death due to anesthesia complications. Combination testing may be considered more reliable for sensitive wildlife that can only be sampled on one or limited occasions due to these risks. Use of a combination of antigen-capture ELISA (ACE) on haired skin with Real Time-PCR (RT-PCR) on whole blood (buffy coat, collected in EDTA) and antibody detection via serum neutralization has the greatest likelihood of identifying PI specimens and those that may be transiently infected (Brock, 2003; Walz et al., 2010; BVD, 2013). Haired skin should ideally be taken from the ear or caudal tail fold, however the structure of the animal being evaluated and public perception of potential permanent marks, as from an ear sample, must be considered with zoo animals (Walz et al., 2010; BVD, 2013). An animal that is positive on both ACE on haired skin, as well as RT-PCR on whole blood, but is negative on serology is considered highly suspicious of being a PI and should be isolated and undergo repeat testing 4–6 weeks later to confirm positive status (Brock, 2003; Walz et al., 2010; BVD, 2013). This follow up testing may prove to be problematic with especially sensitive zoo species, but is essential for identification of PI specimens. The risk of anesthesia must be weighed against the benefits of identifying potential disease exposure for the entire collection. Animals that are positive on RT-PCR and have positive serum titers may be considered transiently infected. These individuals need to be isolated and monitored closely for the development of clinical signs.

Pregnant females with a screening serum antibody titer to BVDV may have been exposed to the virus within the first trimester of pregnancy and as a result be carrying a PI fetus. These animals should be quarantined until the offspring is born then thoroughly screened for BVDV. The offspring should be tested for persistent infection via whole blood RT-PCR in combination with ACE or immunohistochemistry on a haired skin sample (Walz et al., 2010; BVD, 2013). Because this methodology detects actual viral antigen, not antibodies, the presence of virus can be determined in the presence of maternal antibodies if the offspring is sampled after ingestion of colostrum (Walz et al., 2010; BVD, 2013). It is also important to remember that BVDV has been isolated from commercial fetal calf plasma. If plasma is utilized in place of colostrum for neonates, or serum is used in reproduction techniques in adults, it can serve as a source of infection (Brock, 2003).

Because of risk of transmission, it is not wise to introduce any viremic animals to others that may be in the first trimester of gestation (BVD, 2013). Depending on the species, culling or complete isolation with assisted reproduction or artificial insemination techniques followed by thorough screening of offspring in the event of an endangered species, may be the most practical means of preventing the spread of the virus.

CONCLUSION

BVDV is a virus capable of infecting exotic ruminants, many of which are commonly housed in zoos. Animals persistently infected with BVDV present the greatest danger to a zoological collection. Because there is no cure for BVDV, management practices in zoo collections must focus on detection of PI individuals and the prevention of the spread of the virus. Veterinarians must realize that while convenient and economical, serologic testing alone is not sufficient to rule out BVDV infection in zoo animals. Combination testing utilizing ACE from haired skin with RT-PCR has the greatest likelihood of identifying PI specimens and those that may be transiently infected. This should serve as the current "Gold Standard" for thorough survey of zoological collections.

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