Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Biomedical Sciences and Veterinary Public Health

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Prevalens av pestivirus hos renar

En studie av renpopulationer i Finland, Island, Norge och Sverige

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SUMMARY

Pestivirus includes four viral species, causing disease and major economic losses in animal production all over the world. The viruses can also infect a wide range of wild animal species, of which the clinical relevance of pestivirus infection is not much investigated. Reindeer kept for reindeer husbandry are of great importance for the indigenous people in Finland, Norway and Sweden, both economically and culturally. There is also a free-ranging feral population of reindeer in Iceland. The present study analysed sera from 217 reindeer in Finland, Iceland Norway and Sweden. Specific antibodies to pestivirus were analysed by an enzyme-linked immunosorbent assay (ELISA), and were found in samples from Iceland (8%), Norway (43.3%) and Sweden (49.3%). No seropositive animals were found in Finland. Negative samples from reindeer in populations where specific antibodies had been found were further analysed for pestivirus RNA by a real-time reverse transcription polymerase chain reaction (RT-PCR). This was done to find possible persistently infected animals. However, no pestivirus RNA was found. In addition, two samples from each population (in total 14 samples) with positive results from the ELISA, and one sample from Finland with a doubtful result, were further analysed by a virus neutralisation test (VNT). The VNT could confirm the positive ELISA results, and the sample with a doubtful result was shown to be negative in the VNT.

SAMMANFATTNING

Pestivirus inkluderar fyra virusarter som orsakar sjukdom och betydande ekonomiska förluster inom husdjursproduktionen världen över. Dessa virus har även förmågan att infektera ett stort antal vilda djurarter, där den kliniska betydelsen av infektion är mindre känd. Renar är mycket viktiga för ursprungsbefolkningarna i Finland, Norge och Sverige, både ekonomiskt och kulturellt. Det finns också en förvildad population av renar på Island. I den här studien analyserades serumprover från 217 renar från populationer i Finland, Island, Norge och Sverige. Specifika antikroppar mot pestivirus analyserades med en enzyme-linked immunosorbent assay (ELISA), och hittades i prover från Island (8 %), Norge (43,4 %) och Sverige (49,3 %). Inga seropositiva djur hittades i Finland. Negativa prover analyserades vidare med realtids reverse transcription polymerase chain reaction (RT-PCR), för att hitta pestivirus-RNA från möjliga persistent infekterade djur. Inget RNA från pestivirus kunde dock påvisas i proverna. Därutöver utfördes ett virusneutralisationstest (VNT) på två prover från varje population som hade ett positivt resultat i ELISA (totalt 14 prover), samt på ett prov från Finland med resultat i gråzonen mellan positivt och negativt. VNT-resultaten kunde konfirmera de positiva ELISA-resultaten, och provet med resultat i gråzonen visade sig vara negativt i VNT.

CONTENT

INTRODUCTION

Pestiviruses are causing health problems among animals and considerable economic losses for the livestock industry worldwide (Gavier-Widén *et al*., 2012). The International Committee on Taxonomy of Viruses (ICTV, 2017) has recognised four species of pestiviruses, which are Bovine viral disease virus type 1 and type 2 (BVDV-1 and BVDV-2), Border disease virus (BDV) and Classic swine fever virus (CSFV). The objective of this thesis was to study the prevalence of pestivirus in reindeer populations in different locations in Finland, Iceland, Norway and Sweden. Previous studies have reported seroprevalence of pestivirus in reindeer in Finland (Neuvonen *et al*., 1983), Norway (Stuen *et al*., 1993; Lillehaug *et al*., 2003; Tryland *et al*., 2005), and Sweden (Rehbinder *et al*., 1992; Kautto *et al*., 2012; Fahlander, 2017). There are, so far, no published data available on the pestivirus prevalence among reindeer in Iceland. The clinical relevance of pestivirus in reindeer is still unknown, but due to the apparent clinical signs of infection seen in other species, it is an interesting subject for research. More knowledge on the pestivirus prevalence in reindeer can be one step towards an understanding of its possible effects.

This study was performed within the project "Climate-change effects on the epidemiology of infectious diseases and the impacts on Northern Societies" (CLINF). CLINF is a Nordic Centre of Excellence (NCoE) within NordForsk's Joint Initiative on Arctic Research "Responsible Development of the Arctic, Opportunities and Challenges - Pathways to Action" (NordForsk, 2017). The NordForsk-CLINF project is investigating climate impacts on the health of humans and animals, and particularly the effects of climate sensitive infections (CSIs) on humans and animals (CLINF, 2017). CSIs are infections of which transmission is favoured by environmental alterations caused by climate change. There are several potential CSIs, and pestivirus belongs to the group "contact transmission and stress induced CSIs". In mild winters, when the snow is melting and freezing, or when there is rain-on-snow, a layer of ice is formed on the ground, preventing reindeer from reaching their feed (Rehbinder & Nikander, 1999; Forbes *et al*., 2016). Reindeer herders are then required to feed the animals supplementary to keep them from starving. The supplementary feeding keeps reindeer in close contact with each other, which increases the risk of infectious disease transmission between animals.

LITERATURE REVIEW

Reindeer husbandry

Reindeer husbandry is practised on large areas in the north. The reindeer husbandry area of Sweden covers about half of the total land area, ca 240'000 km^2 (Sametinget, 2016). In Finland, the reindeer husbandry area is about 36% of the total area, or 122'936 km^2 (Reindeer Herder's Association, 2017), and in Norway reindeer husbandry is practiced in about 40% of the land area (Norwegian Government, 2017), which is around 130'000 km^2 (total land area is 323'878 km^2, according to Utrikespolitiska institutet, 2017).

All reindeer and caribou belong to the same species, which is divided into seven subspecies (Røed, 2007). The Eurasian tundra reindeer subspecies, *Rangifer tarandus tarandus* L., is present in northern Scandinavia and Russia. There are about 250'000 semi-domesticated reindeer in Norway (Norwegian Government, 2017), and Sweden (Sametinget, 2017) respectively, and 200'000 in Finland (Reindeer Herder's Association, 2017). In the end of the 18th century, reindeer were imported to Iceland from Norway (Thórisson, 1984). The intention was to teach Icelandic farmers reindeer husbandry, but that was never accomplished. Today there is a free-ranging feral reindeer population in eastern Iceland, estimated to be around 6400 animals in the summer of 2017, before the annual hunt (Thórisson & Thórarinsdóttir, 2017).

Reindeer herding in Scandinavia has developed from the hunting of wild reindeer, and has long traditions among the indigenous people, the Sámi (Bjørklund, 2013). The reindeer husbandry is of great importance for the Sámi, both for livelihoods and for the cultural values it keeps. In Norway and Sweden, reindeer husbandry is by law reserved only for the Sámi (Reindriftsloven 2007; SFS 1971:437), while in Finland any EU citizen can own and herd reindeer (Renskötsellag 848/1990. 14.9.1990).

The year of reindeer herding

Reindeer are seasonal breeders with mating in the autumn and calving in the spring (Ropstad, 2000). Females get in heat from the end of September and are pregnant for about 225 days. Most calves are born in May, and females usually give birth to one calf at a time, with few rare exceptions (Rehbinder & Nikander, 1999). The traditional way of reindeer herding in a mountainous Sámi village can be divided into eight seasons (figure 1) (Karlsson & Constenius, 2005; Samiskt informationscentrum, 2017). In the early summer, when pastures are getting lush, the reindeer start to recover from their body loss that they have achieved during winter. This is also an important time for calves, since they have good access to nutrition to let them grow fast. When the summer weather gets warmer and insects start to bother the animals, they usually migrate up to the mountains, to protect themselves. The pastures are better in the valleys and marshlands, so in the cooler evenings the reindeer move there to graze. Summer is also the time for ear-marking of calves, and for that purpose they are gathered by the reindeer herders. Every reindeer owner has their own specific marking, by which they can be recognised. When summer turns into autumn the weather gets cooler, and the reindeer spend more time in valleys and marshlands, grazing to build up fat reserves for the winter. Autumn is the time for slaughter of bulls, and the animals are once again gathered by the reindeer herders. In late autumn, when snow start to cover the pastures, the reindeer are also gathered, and then divided into smaller groups to be moved into winter pastures. Winter can be tough, with limited access to feed.

Reindeer mostly feed on lichens which they dig up from under the snow. When the snow starts to melt, it is time for reindeer herders to move their animals back to the lands where the females will give birth to new calves in spring.

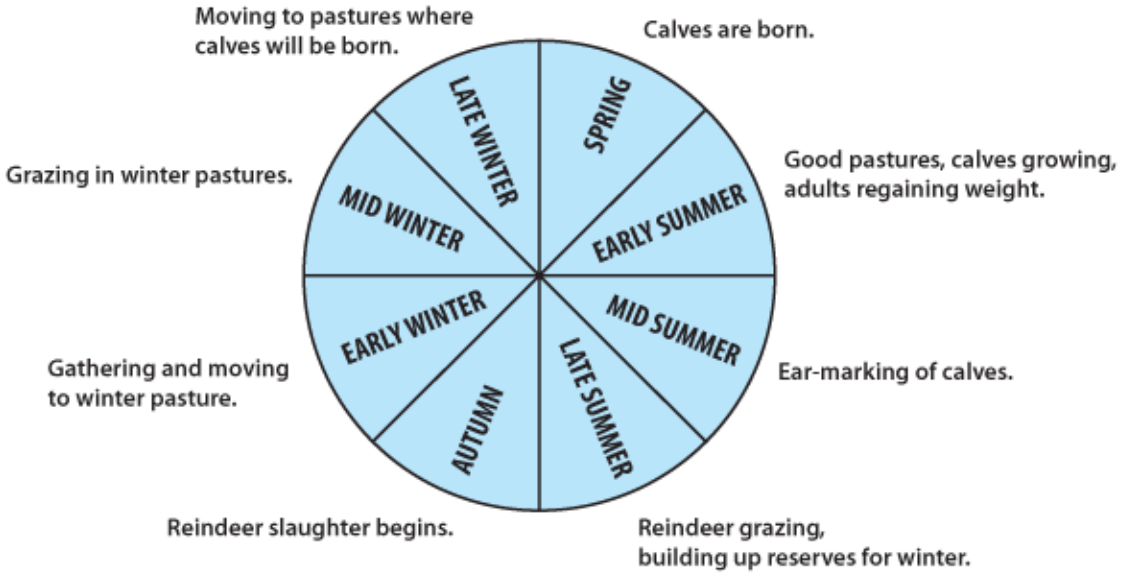

Figure 1. *Schematic drawing of the reindeer year*.

Difficulties for the reindeer husbandry

There are several factors with potential to affect reindeer husbandry in a negative way, such as predation of calves and conflicting interests claiming land from the reindeer herding area (Pape & Löffler, 2012). Another such factor is alterations in weather and environment. When the winters are mild, and the snow is melting and freezing, a layer of ice is formed on the ground, keeping reindeer from reaching the feed (Rehbinder & Nikander, 1999; Forbes *et al*., 2016). This calls for a need of supplementary feeding, either by keeping reindeer in enclosures or by gathering them every day for feeding. Both strategies keep the animals in close contact with each other, which is a risk factor for infectious disease transmission (Åhman, 2002).

Pestivirus

Pestiviruses are single stranded positive-sense RNA viruses (Gavier-Widén *et al*., 2012). The *Pestivirus* genus belongs to the *Flaviviridae* family and there are now four viral species of pestivirus accepted by the International Committee on Taxonomy of Virus (ICTV); Bovine viral diarrhoea virus type 1 and type 2 (BVDV-1 and BVDV-2), Border disease virus (BDV) and Classical swine fever virus (CSFV) (ICTV, 2017). However, there are proposals of including several new pestivirus species to the genus (Smith *et al*., 2017).

Pestiviruses of ruminants can be divided into two different biotypes; cytopathic (CP) and non-cytopathic (NCP) viruses (Gavier-Widén *et al*., 2012). The CP pestiviruses cause cell lysis during replication in tissue cell cultures, while NCP pestiviruses can replicate without damaging the target cells. Only NCP pestiviruses can infect the foetus *in utero* by crossing the

placenta (Nettleton & Entrican, 1995). If a foetus is infected by a NCP pestivirus before immunocompetence is developed, the foetus develops immunotolerance to the virus and are born persistently infected (PI). A PI individual will shed virus and transmit infection to other animals but will not develop specific antibodies to the virus. Also, these calves may lack behind concerning growth and development compared to non-infected of the same age (Brownlie *et al*., 1998).

Bovine viral diarrhoea

BVDV is mostly associated with disease in cattle, but can infect a wide range of domestic and wild ungulates (Passler & Walz, 2009; Nelson *et al*., 2016). In cattle, the virus may cause different clinical signs depending on the host's immune status, age and the virulence of the virus strain. Acute BVDV infection of immunologically naïve animals usually give rise to mild clinical signs, including leukopenia, fever and diarrhoea (Lanyon *et al*., 2014). The immunosuppression may predispose for other infectious diseases. A female, who is infected during pregnancy, may have different outcomes depending on the age of the foetus (Brownlie *et al*., 1998). Infection in the first trimester of pregnancy may result in a PI calf, but may also cause abortion or give teratogenic effects in the foetus. If the infection occurs after the foetus have developed immunocompetence, its immune system will be able to recognise the virus and clear the infection. Mucosal disease is seen in PI animals, when the NCP BVDV that is causing the persistent infection mutates into CP BVDV (Lanyon *et al*., 2014). Clinical signs of mucosal disease are erosions of epithelium, diarrhoea and ultimately death.

There are two genotypes of BVDV, namely BVDV-1 and BVDV-2, and a group of atypical pestiviruses tentatively designated BVDV-3 (Liu *et al*., 2009). Finland, Norway and Sweden are free from BVDV in cattle, after accomplishment of successful control programmes, and are keeping the free status by BVDV surveillance programmes (Evira, 2016; Norwegian Veterinary Institute, 2017; Swedish National Veterinary Institute, 2017). Iceland has never had a confirmed case of BVDV in cattle (Valsson *et al*., 2001; Icelandic Food and Veterinary Authority, 2017).

Border disease

Border disease virus was first observed in sheep but has also been detected in several other ruminant species (Nettleton *et al*., 1998; Becher *et al*., 1999; Strong *et al*., 2010) and pigs (Kawanishi *et al*., 2014). Border disease in non-pregnant sheep is generally subclinical, or with mild clinical signs (Nettleton *et al*., 1998). Infection in pregnant sheep may cause different signs depending on the stage of pregnancy and the virulence of the infecting strain. Resorption or abortion of the foetus is a common outcome of infection. Lambs born alive may be weak and small. BDV can also cause central nervous signs and growth of abnormal fleece, which has given Border disease its alternative name, Hairy shaker disease. BDV has been associated with outbreaks of highly fatal disease among Pyrenean chamois (*Rupicapra pyrenaica*), a wild goat-like species living in the Pyrenean mountains (Marco *et al*., 2006). BDV currently comprises seven genotypes, and an additional eighth genotype has been described by Peletto *et al*. (2016).

Pestivirus infection in reindeer

Seropositivity for pestivirus-specific antibodies has previously been confirmed in reindeer populations of Finland, Norway and Sweden (table 1). In Finland, a study of 300 reindeer from

two populations, found a BVDV seroprevalence of 58% (Neuovonen *et al*., 1983). In Norway, Stuen *et al*. (1993) analysed 326 serum samples from reindeer in seven districts in Finnmark, northern Norway, and reported a seroprevalence of 17%. Tryland *et al.* (2005) found a seroprevalence of 33% out of 48 reindeer, sampled from carcasses collected in winter pastures in four different districts in Finnmark in 2000. Another Norwegian study (Lillehaug *et al*., 2003) tested 810 serum samples taken from free-ranging reindeer in seven districts of southern Norway in 1999 and 2000. They found an average seroprevalence of 4.2%, but the difference between the populations with lowest and highest seroprevalence were ranging from 0% to 51%. In a study by Kautto *et al.* (2012) specific antibodies to pestivirus was found in 35% of 1158 serum samples, which were sampled in 13 different reindeer herding districts in Sweden, in 2000 to 2002. A retrospective study of 50 Swedish reindeer from four different herds sampled in 1973 to 1975, 1977 and 1982, found antibody titres against BVDV in 6% of the animals (Rehbinder *et al*., 1992). Prevalence of pestivirus among reindeer in Iceland has not yet been reported.

Table 1. Previously published data on seroprevalence of pestivirus in reindeer from Finland, Norway and Sweden

Study	Country	Year (s) of sampling	No. of sampling sites	Total no. of samples	Average prevalence	Analysis method (s)
Neuvonen *et al*. (1983)	Finland	1983	2	300	58%	no data available
Stuen *et al*. (1993)	Norway	1991	7	326	17%	VNT
Lillehaug *et al*. (2003)	southern Norway	1999–2000	7	810	4.2%	VNT
Tryland *et al*. (2005)	northern Norway	2000	4	48	33%	VNT
Rehbinder *et al*. (1992)	Sweden	1973–1982	4	50	6%	ELISA
Kautto *et al*. (2012)	Sweden	2000–2002	13	1158	35%	ELISA, VNT

Since reindeer are often free-ranging, it can be difficult to monitor clinical signs of disease. It is not yet known which species of pestivirus the reindeer are infected by and clinical signs of natural infection have not been much investigated. Becher *et al*. (1999) reported the isolation of a pestivirus from a reindeer in a German zoo. Genetic analysis showed that the strain, named Reindeer-1, was more similar to BDV than to other established pestivirus species. The pestivirus infection was linked to abortion in the reindeer. In a study by Morton *et al.* (1990) two reindeer were experimentally infected by a cytopathic strain of BVDV called Singer strain. Both reindeer had clinical signs from the infection, consisting of gastrointestinal disease with loose faeces containing blood and mucus, a transient laminitis or coronitis, and a relative leukopenia. One reindeer also had serous nasal discharge which turned mucopurulent and then dry and crusty. These findings show that there are pestivirus strains that can cause clinical signs in reindeer.

MATERIAL AND METHODS

Animals

In total, 217 serum samples from reindeer in different populations of Norway, Sweden, Finland and Iceland were included in the study (table 2). Samples had been collected beforehand in November and December 2016, and in January 2017 by researchers within the NordForsk-CLINF project, and the blood had been sampled either at the time of slaughter or from animals after being shot during hunting. Samples from Finland, Norway and Sweden were taken from three different populations in each country, in a north to south gradient.

Table 2. *North-south* l*ocations, numbers of animals sampled, year of sampling and whether samples were taken at slaughter or hunt*

Location	No. of samples	Year of sampling	Slaughter/hunt
Finland, north	19	2016	slaughter
Finland, mid	21	2017	slaughter
Finland, south	21	2017	slaughter
Iceland	25	2017	hunt
Norway, north	20	2016	slaughter
Norway, mid	20	2017	slaughter
Norway, south	20	2017	slaughter
Sweden, north	19	2016	slaughter
Sweden, mid	32	2016	slaughter
Sweden, south	20	2016	slaughter

Study design

All 217 serum samples were analysed for pestivirus antibodies by a blocking ELISA (figure 2). Samples with seronegative results from populations in which also seropositive or doubtful results were obtained (thus, potentially PI animals), were further analysed for pestivirus RNA by real-time reverse transcription polymerase chain reaction (RT-PCR), and so were samples with doubtful results. The Swedish samples had already been analysed by a blocking enzyme-linked immunosorbent assay (blocking ELISA) and samples with negative or doubtful outcome from the test had been further analysed by real-time RT-PCR (Eklund, 2017). A total of 15 samples with seropositive or doubtful results from blocking ELISA were further analysed by a virus neutralization test (VNT). Selection of samples for VNT was based on the two samples with highest measured optical density (OD) from each sampling site.

Figure 2. *All 217 serum samples were analysed by blocking ELISA. Fifteen selected samples with a positive or doubtful result from the ELISA were analysed by VNT, and 112 samples with negative or doubtful results from the ELISA were analysed by real-time RT-PCR.*

Blocking ELISA

All 217 serum samples were analysed with SERELISA BVD p80 Ab Mono Blocking kit (Synbiotics Corporation, Lyon, France), using the manufacturers procedure for ovine and caprine serum samples. All samples were diluted 1:5. The kit is designed to detect specific antibodies to the non-structural protein p80/125, which is common to all strains of BVDV and BDV (Martin *et al*., 2011).

All serum samples were tested in duplicates, incubated in wells coated with BVD/BD p80/125 antigen, binding specific antibodies from the serum to the antigen. Thereafter the wells were washed and an anti-BVD/BD p80/125 monoclonal antibody peroxidase conjugate was added, binding to free antigenic sites in the wells. A second wash step was performed to eliminate excess conjugate, and a substrate was added to each well. This reveals enzyme linked to the antigen-anti-BVD/BD p80/125 peroxidase complex by transformation of the substrate to a coloured product. At last the optical density (OD) was measured in each well at a wavelength of 450 nm, and a competition percentage of each sample were calculated. Each sample with a competition percentage of more than 40 % was considered positive. Samples with competition percentage between 20 and 40 % were considered as “doubtful”.

RNA extraction and real-time RT-PCR

Nucleic acids from a total of 112 serum samples were extracted and analysed by real-time RT-PCR for pestivirus RNA. The purpose of the analysis was to find samples from potentially persistently infected animals, i.e. samples with pestivirus RNA but without specific antibodies to the virus.

RNA was extracted by using a Bullet Stool kit (Diasorin, Stillwater, MN, USA) and Magnatrix 8000+ extraction robot (Magnetic Biosolutions, Stockholm, Sweden) using magnetic beads for isolation and purification of viral nucleic acids. Serum samples were added to separate wells on a plate together with ≥800 U/ml proteinase K (Sigma-Aldrich, Saint Louis, MO, USA), and run in the extraction robot on an in-house protocol used by the Swedish National Veterinary Institute. Included in each extraction were also positive and negative controls, which were also used as controls in the real-time RT-PCR. The extractions were run over night, and the robot was keeping the samples in a cooling block at 10 ºC, until the morning when they were

transferred to a refrigerator. All samples were analysed by real-time RT-PCR the day after extraction.

For the real-time RT-PCR analysis, an AgPath-ID One Step RT-PCR kit (Applied Biosystems, ThermoFisher Scientific) was used. Primer and probes used (Hoffman *et al*., 2006) were:

- forward primer BVD190-F: 5'-GRAGTCGTCARTGGTTCGAC-3',
- reverse primer V326: 5'-TCAACTCCATGTGCCATGTAC-3', and
- probe TQ-Pesti: 5'-TGC YAY GTG GAC GAG GGC ATG C-3'.

Two µl of extracted nucleic acids and 13 µl mastermix were used per well. The mastermix included 7.5 µl AgPath 2X RT-PCR buffer, 0.6 µl forward primer [10 µM], 0.6 µl reverse primer [10 µM], 0.2 µl probe [10 µM], 0.6 µl AgPath 25X RT-PCR enzyme mix and 3.5 µl nuclease-free H_2O.

The PCR program was as follows: reverse transcription at 45 °C for 10 minutes, initial activation at 95 °C for 10 minutes, and 48 two-step amplification cycles at 95 °C for 15 seconds and 60 °C for 45 seconds.

Virus neutralisation test

The principle of virus neutralisation test (VNT) is to incubate serum together with virus and susceptible cells. If the serum contains specific neutralising antibodies to the virus, the virus will be neutralised and not able to cause cytopathic effect (CPE).

The VNT was carried out in 96-well cell culture plates. One plate was used as a control plate (figure 3), with virus control, cell control, and controls with known positive and negative serum samples (i.e. with or without specific antibodies to the virus). The positive control used was a commercial BVD antiserum (AHVLA, Surrey, UK) and the negative control came from a BVDV negative bovine serum sample. Each serum sample was also tested as serum control in row A on the test plates (serum and cells in the wells), to see if the serum itself would have cytopathic effect on the cells. All serum samples were heat inactivated in a water bath of 56 ºC for 30 minutes, and were tested in duplicates.

	1	2	3	4	5	6	7	8	9	10	11	12
	Work. dilution	**Work. dilution * 10^{-1}**	**Work. dilution * 10^{-2}**	**Work. dilution * 10^{-3}**	**Cell control**		**Positive serum control**		**Negative serum control**		**Serum sample 1a**	
A	virus cells	virus cells	virus cells	virus cells	cells	cells	serum cells	serum cells	serum cells	serum cells	serum cells	serum cells
B	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
C	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
D	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
E	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
F	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
G	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells
H	virus cells	virus cells	virus cells	virus cells	cells	cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells	serum virus cells

Figure 3. *The distribution of serum, virus and cells on the control plate. Column 1-4 are virus controls (blue fields), column 5-6 are cell control (grey fields), column 7-8 is positive control (green fields) and column 9-10 is negative control (red fields). Column 11-12 (yellow fields) are representing how the tested serum samples (15 in total) were distributed on the test plates, with row A used for serum controls.*

Serum samples were tested in a five-fold serial dilution, from 1:5 to 1:78125, diluted in Eagle's minimal essential medium (EMEM) with tricine (SVA, Uppsala, Sweden). The virus used in this test was the cytopathic BVDV-1 strain Borgen diluted to a 50 % tissue culture infective dose ($TCID_{50}$), which means that the virus at that dilution will cause CPE in 50 % of the infected tissue culture cells. The $TCID_{50}$ was based on previous VNT protocols where this strain had been used. Three additional virus dilutions were made with 10^{-1}, 10^{-2} and 10^{-3} of the working dilution. The four viral dilutions were added to 8 wells each on the control plate (figure 3), and the working dilution was added to all wells with test serum, except for serum controls. When serum and virus were added, the plates were incubated in 37 ºC with 5 % CO_2 for 60 minutes. Then bovine turbinate cells (TB cells) in EMEM with 20% foetal bovine serum (Gibco, ThermoFisher Scientific, Carlsbad, CA, USA) were added to all wells and the plates were incubated in 37 ºC with 5 % CO_2 for 5 days. After 5 days, the results were read by light microscopy. Samples were considered positive if there was inhibition of CPE in at least one well. Antibody titre was determined for each sample by using a template developed at the Swedish National Veterinary Institute, based on the Kärber method.

RESULTS

Blocking ELISA

Specific antibodies to pestivirus were found in 8% of samples from Iceland, 43.3% from Norway and 49.3% from Sweden (denoted as "positive" in table 3). No specific antibodies to pestivirus were found in samples from Finland, but one sample was considered as "doubtful", with a competition percentage of 27.9% (i.e. between 20% and 40%).

Table 3. *Number of positive, negative and doubtful samples, and seroprevalence with a 95% confidence interval (CI), per country*

Country	Positive	Negative	Doubtful	Total no. of samples	Seroprevalence, %, [95% CI]
Finland	0	60	1	61	0 [0-5.9%*]
Iceland	2	23	0	25	8 [0-18.6]
Norway	26	33	1	60	43.3 [30.8-55.9]
Sweden	35	29	7	71	49.3 [37.7-60.9]

*using a one-sided 97.5% confidence interval

An overview of the seroprevalence of the sampled animals per country can be seen in figure 4.

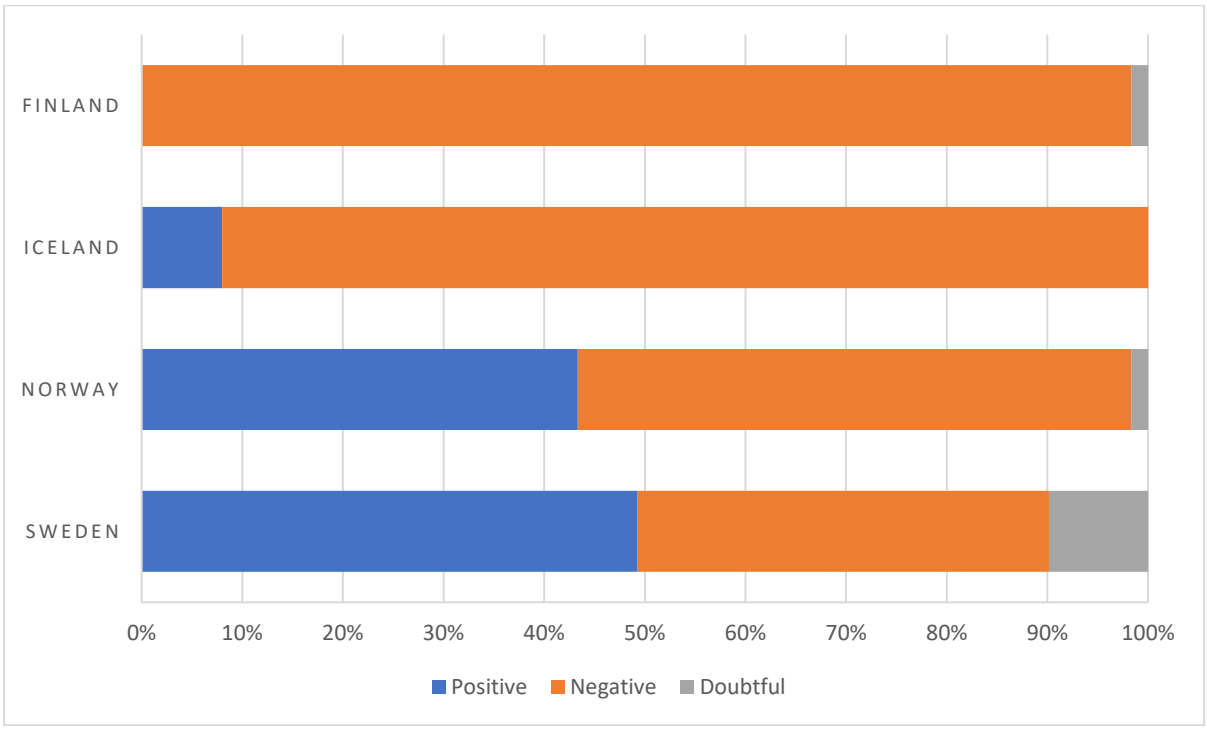

Figure 4. *Proportions of positive, negative and doubtful results from each sampled country.*

When the sampled animals were divided into two age groups, "calves" for animals ≤1 year and "adults" for animals >1 year, the total seroprevalence for the age group "adults" was 34.5%, and for "calves" 17.5% (table 4). The total seroprevalence for all sampled reindeer was 29%.

Table 4. *Results from blocking ELISA divided into age groups "calves" (≤ 1 year) and "adults" (> 1 year) shown as number of positive results out of the total number of animals from each sampling site, and as a percentage*

Sampling site	**Calves**		**Adults**		**Total**	
	positive/total	%	positive/total	%	positive/total	%
Finland, north	0/10	0	0/9	0	0/19	0
Finland, mid	0/14	0	0/7	0	0/21	0
Finland, south	0/10	0	0/11	0	0/21	0
Iceland	0/1	0	2/23	8.7	2/25	8
Norway, north	1/10	10	4/10	40	5/20	25
Norway, south	3/10	30	8/10	80	11/20	55
Norway, mid	2/10	20	8/10	80	10/20	50
Sweden, south	0/10	0	7/10	70	7/20	35
Sweden, mid	8/13	61.5	4/10	40	20/32*	62.5
Sweden, north	3/9	33.3	5/10	50	8/19	42.1
Total	17/97	17.5	38/110	34.5	63/217	29

* Nine of the sampled animals were of unknown age, and eight of them were positive in the test.

Real-time RT-PCR

All 112 samples tested by real-time RT-PCR analysis were negative for pestivirus nucleic acids. The additional control samples from the RNA extraction were found positive and negative, respectively.

Virus neutralisation test

VNT could confirm the positive results from blocking ELISA in samples from Norway and Sweden, with antibody titres within the interval 1:25-1:1400 (table 5). The two Icelandic serum samples caused CPE in the serum controls, but for sample I1a there were wells without CPE with serum concentration 1:125 and lower, indicating it was positive for neutralizing antibodies. For sample I1b all wells had CPE. The sample from Finland with doubtful result in blocking ELISA, F1a, were found negative in VNT.

Table 5. *Samples included in VNT with positive/negative results, antibody titre results and previously obtained competition percentages from blocking ELISA*

Sample	Positive/negative, VNT	Antibody titre result, VNT	Competition %, ELISA
Finland, F1a	negative	<1:5	27.9
Iceland, I1a	positive*	-	55.9
Iceland, I1b	-*	-	50.4
Norway, N1a	positive	1:625	102.3
Norway, N1b	positive	1:125	99.3
Norway, N2a	positive	1:25	101.4
Norway, N2b	positive	1:125	97.5
Norway, N3a	positive	1:280	98.1
Norway, N3b	positive	1:1400	98.9
Sweden, S1a	positive	1:280	87.5
Sweden, S1b	positive	1:625	77.0
Sweden, S2a	positive	1:56	93.0
Sweden, S2b	positive	1:280	88.5
Sweden, S3a	positive	1:280	82.5
Sweden, S3b	positive	1:625	86.0

* The sera from Iceland caused CPE in the serum controls.

DISCUSSION

Results from blocking ELISA analysis revealed presence of specific antibodies to pestivirus in all sampled populations in Iceland, Norway and Sweden. This study did not find any seropositive sample from Finland, in contrast to the previous Finnish study (Neuvonen *et al*., 1983) where a seroprevalence of 58% was seen. The samples of the present study came from other regions of Finland than the samples from the previous study. It would be interesting to include samples from reindeer in the same regions, as were studied in 1983, in a future study on pestivirus prevalence. However, there are more than 30 years between these studies and the pestivirus infection may have been cleared from the reindeer population. Also, Finland has eradicated BVDV from the cattle population after the prior study (Evira, 2016). The impact of BVDV freedom in cattle on the prevalence of pestivirus in reindeer should probably not be overestimated though, since the other countries in this study are also free from BVDV (Norwegian Veterinary Institute, 2017; Swedish National Veterinary Institute, 2017; Valsson *et al*., 2001; Icelandic Food and Veterinary Authority, 2017), but still have prevalence of pestivirus in reindeer.

If there are no presence of antibodies in calves of a population, there is likely no pestivirus circulating in that population. Seropositive calves were found in all but one population in the present study. When interpreting these results, a potential presence of colostral maternal antibodies should be considered. According to Løken (1995), maternal antibodies to BDV may remain in lambs for up to six months after birth. If the same time span of remaining maternal antibodies applies to pestivirus-specific antibodies in reindeer, maternal antibodies could possibly affect the results. No birth data are available for the animals, but given that most reindeer calves are born in May, and the sampling were carried out from November to January, some of the animals may have been about six months of age at the time of sampling.

No pestivirus RNA was found in real-time RT-PCR analysis of samples with negative or doubtful results from blocking ELISA. This corresponds to unpublished results from a Next-generation sequencing (NGS) analysis carried out by researchers at the Swedish National Veterinary Institute in 2017, on blood, buffy coat, and swabs from nasal cavity, eye and rectum, from a selection of the reindeer individuals as sera were taken from for this study. NGS is a method for sequencing whole genomes and, with use of bioinformatics, study from which organisms the genomes origin (Blomström, 2011). The method can be used as a diagnostic tool for finding unknown pathogens in a sample.

The selection of sampled individuals may give a false picture of the pestivirus prevalence in the populations. Most of the samples come from animals in normal slaughter, which have been examined and approved before slaughter. These animals are expected to be healthy. The remaining part of the material in this study comes from animals sampled after being shot during hunt. It is possible that the seroprevalence would be more accurate if randomly selected live animals had been sampled. To find possible PI reindeer, the sampling should probably be aiming for young animals with clinical signs, e.g. retarded growth rate, if PI reindeer show signs like PI cattle. There are reports of PI white-tailed deer (Duncan *et al*., 2008), which is of the same *Capreolinae* subfamily as reindeer. However, there are still no evidence for the existence of PI reindeer.

The original plan for the VNT in this study was to include multiple pestivirus strains and see which strain the pestivirus antibodies could neutralise to the highest degree. However, due to practicalities and time limits, this plan was not possible to fulfil, and just one strain of pestivirus was used. Therefore, the VNT could only confirm the positive results, which were already obtained in the ELISA, and show that the Finnish sample with doubtful ELISA result was negative. The VNT results for the Icelandic samples are partially uncertain, since the cells of the serum controls were damaged by the sera itself. One of the samples though, still had wells with virus and intact cells where the serum concentration was low, which indicates a positive result. The Icelandic samples were as mentioned, in contrast to samples from other countries, taken during hunt, and the time between death of the animal and sampling of blood could sometimes be several hours. This may be a factor affecting the VNT serum control cells. The blood samples taken in connection to slaughter were taken immediately after death.

One weakness of this study is that the number of samples from each population is just a small fraction of the population in total. So, a positive result is best interpreted as that pestivirus is prevalent in the specific population, and a negative result cannot rule out the possibility of pestivirus prevalence. The seroprevalence with a 95% confidence interval is for Iceland 8% (0-18.6%), for Norway 43.4% (30.8-55.8%) and for Sweden 49.3% (37.7-60.9%). For Finland, where no seroprevalence was found, a one-sided 97.5% confidence interval was calculated to be 0-5.8%. The confidence intervals would have been narrower, and the prevalence percentage more accurate, if more animals had been sampled. Another weakness is that the samples are from healthy animals. For future research on pestivirus in reindeer, it would be interesting to go for animals with suspected clinical signs, and specifically search for PI calves. This could be done in populations with high seroprevalence among calves, where it is most likely to find possible PI reindeer. It is a challenging task, because of the free-ranging nature of reindeer herding, but it could bring a lot of interesting information to be used for reducing infection pressure in the reindeer populations. Also, it could bring us closer to an answer of the question whether pestivirus infection in reindeer is of clinical relevance, or not.

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