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Abstract

Influenza D virus, IDV, is a member of the *Orthomyxoviridae* family and was discovered in North America, 2011. The virus was first found in swine but has since then been found in a broad range of hosts. Studies have shown that cattle appear to be the main reservoir. The epidemiology of IDV is not fully understood, but it appears to be transmitted through direct-contact and aerosols in cattle. Experimental studies on calves have shown that IDV can infect both the upper- and lower respiratory tract but only cause mild respiratory clinical signs. Histological examinations following experimental infection have shown that IDV mainly cases cause epithelial neutrophil infiltration and mild epithelial attenuation. Immunologically there is evidence suggesting that IDV can inhibit the immune response in the host. The threat posed by IDV is the link between it and bovine respiratory disease (BRD). Studies have found that IDV is often prevalent in cattle suffering from BRD and it has been suggested that IDV infections can promote the development of BRD.

This study aimed to continue the surveillance and mapping of Influenza D among dairy cattle in Sweden where a former study left off to further explore the prevalence and dynamics of IDV in Sweden. To assess the situation, 338 bulk tank milk samples were collected and analysed with an in-house indirect ELISA.

The results from the study suggest that the seroprevalence of IDV in Swedish dairy herds has increased since 2019. It also appears as the seroprevalence for IDV is much higher in southern Sweden. Based on the statistical analysis performed, larger herds seem to be more likely to be positive. However, the difference in mean PP-values was not significantly different between each herd size category. This could be an indication that there are certain age groups in the herds that have elevated antibody levels.

Keywords: Influenza D virus, IDV, BRD, seroprevalence, Sweden, ELISA, dairy cattle, cows

Sammanfattning

Influensa D virus, IDV, är en av medlemmarna i familjen *Orthomyxoviridae* och upptäcktes i Nordamerika under 2011. Viruset hittades först i svin men har sedan dess återfunnits i ett brett spektrum av värddjur. Studier har visat att nötkreatur verkar vara reservoar. Epidemiologin för IDV är inte helt klarlagd, men det verkar överföras genom direktkontakt och aerosoler hos nötkreatur. Experimentella studier på kalvar har visat att IDV kan infektera både övre- och nedre luftvägarna, men att det i de flesta fall endast orsakar milda respiratoriska kliniska tecken. Histologiska undersökningar efter experimentell infektion har visat att IDV huvudsakligen försvagar epitelet i luftvägarna och orsakar epitelial neutrofilinfiltration. Immunologiskt finns det bevis som tyder på att IDV kan hämma immunförsvaret i värddjuret. Studier har visat att IDV ofta förekommer hos nötkreatur som drabbats av bovine respiratory disease (BRD) och det har föreslagits att IDV-infektioner kan främja utvecklingen av BRD.

Syftet med denna studie var att fortsätta övervakningen och kartläggningen av seroprevalensen för IDV i svenska mjölkbesättningar. En första studie på detta utfördes 2019 och tanken var att studierna skulle kunna jämföras för att påvisa förändrad förekomst och eventuella dynamiker. För att bedöma situationen samlades det in 338 mjölktanksprover och dessa analyserades med en intern indirekt ELISA.

Resultaten från studien tyder på att seroprevalensen för IDV har ökat hos svenska mjölkbesättningar sedan 2019. Det verkar även som att seroprevalensen är mycket högre i södra Sverige. Baserat på den utförda statistiska analysen verkar större besättningar vara mer benägna att vara positiva för IDV. Skillnaden i genomsnittliga PP-värden skilde sig dock inte signifikant mellan storlekskategorierna. Detta skulle kunna vara en indikation på att förhöjda nivåer av IDV-antikroppar förekommer i en specifik åldersgrupp, inte hela besättningar.

Nyckelord: Influensa D virus, IDV, seroprevalens, Sverige, ELISA, mjölkkor, nötkreatur

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Abbreviations

BRD	Bovine respiratory disease
BTM	Bulk tank milk
BVD	Bovine viral diarrhoea
ELISA	Enzyme-linked immunosorbent assay
HA	Hemagglutinin
HEF	Hemagglutinin-esterase-fusion
HI	Hemagglutination inhibition assay
IAV	Influenza A virus
IBV	Influenza B virus
ICV	Influenza C virus
IDV	Influenza D virus
NA	Neuraminidase
PCR	Polymerase Chain Reaction
SLU	Swedish University of Agricultural Sciences
SVA	Swedish National Veterinary Institute

1. Introduction

In 2011 a novel virus was discovered in North America amongst pigs which exhibited influenza-like clinical signs (Hause *et al.* 2013). The virus, characterized as influenza D virus D (IDV), was then to be identified in cattle with signs of respiratory disease. Since then the virus has been shown to be widely spread around the world through virus- or IDV-specific antibody detection in both cattle and swine (Hause *et al.* 2014), but also in other species including humans in which antibodies have been detected (White *et al.* 2016; Trombetta *et al.* 2019). In 2019, a first serological study was conducted and the results showed that IDV is present amongst cattle in Sweden (Ahlgren 2019).

Even though IDV only caused mild to moderate clinical signs in experimental infection studies in calves (Ferguson *et al.* 2016; Salem *et al.* 2019), it has been suggested that the virus could predispose cattle to other infections associated with BRD (Ng *et al.* 2015; Mitra *et al.* 2016).

Bovine respiratory disease (BRD) is one of the major disease complexes that cause suffering and severe economic losses in the cattle industry (Griffin 1997). Extensive research has been done and is still ongoing to be able to understand and prevent BRD.

This study aimed to continue where the former study left off in the surveillance and mapping of Influenza D among dairy cattle in Sweden to further explore the prevalence and dynamics of IDV in Sweden. For this, bulk tank milk samples collected from the BVDV program were analysed by an in-house indirect ELISA developed by SVA and SLU (Chiapponi *et al.* 2020).

2. Literature Review

2.1. Influenza D virus

2.1.1. Classification

Orthomyxoviridae is a family of segmented, negative-sense RNA-viruses. Infections by these viruses are associated with causing disease in both humans and animals around the world. Orthomyxoviridae is divided into seven genera: *Alfainfluenzavirus*, *Betainfluenzavirus*, *Gammainfluenzavirus*, *Deltainfluenza-virus*, *Thogotovirus*, *Quaranjaviru*s, and *Isavirus* (ICTV 2019). Members of the *Orthomyxoviridae* family are enveloped, and the genome is formed by 6 to 8 linear segments of single negative-stranded RNA. Each influenza genus contains a single designated species; Influenza A virus (IAV) for *Alfainfluenzavirus*, Influenza B virus (IBV) for *Betainfluenzavirus*, Influenza C virus (ICV) for *Gammainfluenzavirus*, and Influenza D virus (IDV) for *Deltainfluenzavirus*. On the envelope glycosylated and non-glycosylated viral proteins are expressed which the virus uses to infect the host cell. IAV and IBV have two types: hemagglutinin (HA) and neuraminidase (NA) while ICV and IDV express only one type: hemagglutinin-esterase-fusion (HEF). The major cause of disease in animals is the IAV in contrast to humans where influenza A, B, and C cause disease (Quinn *et al.* 2011).

In the *Orthomyxoviridae* family, IDV is most closely related to ICV. They share about 50% of their complete amino acid sequence, and both are composed of 7 segments; PB1, PB2, P3, HEF, NP, M, and NS. IDV also expresses its M1 proteins differently from other influenza viruses (Hause *et al.* 2014). It has also been shown that IDV is extremely resistant to acidic environments and high temperatures in comparison to IAV, IBV and ICV. Of the four viruses, only IDV retained its infectivity after being exposed to +53°C solution for 60 minutes and even remained infective after another 60 minutes. When treated with pH 3.0 for 30 minutes IDV lost approximately 20% of its infectivity while the other viruses were inactivated. The authors of the study found that the pH- and temperature resistance probably originated from IDV's HEF by using a reverse genetic system to remove the NA

and HA glycoproteins from an IAV and replace it with a HEF from IDV. The modified IAV exhibited the same resistance to acidity and heat as IDV when challenged (Yu *et al.* 2017).

It has also been discovered that IDV HEF utilizes an open receptor-binding cavity to infect host cells which makes the virus able to accommodate a wider range of glycan moieties. This could be an explanation for the broad host range of IDV according to the authors (Song *et al.* 2016).

2.1.2. Discovery

IDV was discovered back in April of 2011 in a group of 15-week-old swine (Hause *et al.* 2013). The pigs were suffering from influenza-like symptoms. Nasal swabs were collected from these animals and were sent to Newport Laboratories in Worthington, Minnesota, for virus isolation. The samples were negative for influenza A virus by real-time reverse transcription PCR, but when the samples were inoculated in porcine testis cell, a cytopathic-effect characteristic for influenza virus was observed. Further investigation, including deep-genome sequencing and enzymatic assays, were performed. The preliminary data suggested that the virus most likely was a member of the *Orthomyxoviridae* family and that its enzymatic activity profile was related to the influenza C virus. The genome sequencing supported that this virus was most closely related to the human ICV, in contrast to IAV and IBV, and therefore it was preliminary named C/swine/Oklahoma/1334/2011 (C/OK) (Hause *et al.* 2013).

After the discovery of C/OK in swine, a study on the prevalence in cattle was performed (Hause *et al.* 2014). By using reverse transcription PCR (RT-PCR) and targeting the PB1 gene from the swine C/OK, the authors tested samples submitted for microbiological diagnostics from cattle exhibiting respiratory disease. A total of 45 samples from six different states in the U.S. were analysed, and eight samples (18%) were positive, all originating from Minnesota and Oklahoma. Three of the bovine samples were examined with deep genome sequencing and showed that all seven segments from the three viruses were over 96% identical to C/OK. Phylogenic analysis between C/OK and other members of the *Orthomyxoviridae* family was performed with the three viruses to understand the evolution of C/OK. The samples were most closely related to C/OK and secondly to human ICV. The authors suggested that C/OK had diverged from the human ICV during evolution since the bovine samples were highly homologous to C/OK and showed no signs of reassortment with human ICV. To further test this the authors conducted in vitro reassortment tests between human ICV and C/OK from swine and cattle. The results showed that human ICV and C/OK were unable to perform reassortment which, according to McCauley *et al.* (2012), meant that they were not part of the

same genus. Furthermore, the cross-reactivity between human ICV- and C/OK antibodies were tested with agar gel immunodiffusion and showed that there was no cross-recognition of the antibodies between the C/OK virus and human ICV. Due to novel genetic and epidemiological characteristics, the authors concluded the study with a suggestion to introduce a new genus in the *Orthomyxoviridae* family, influenza D virus (McCauley *et al.* 2012; Hause *et al.* 2014).

2.1.3. Affected species

IDV circulates among cattle and swine and can replicate and transmit disease in these hosts. Studies have shown that cattle express almost double mean geometric IDV-specific antibody titers than swine. The virus is widely spread in cattle, suggesting that cattle are a reservoir for the virus (Hause *et al.* 2013, 2014). However, IDV-specific antibodies have also been detected in small ruminants, horses, camelids, and wild boars (Quast *et al.* 2015; Ferguson *et al.* 2018; Nedland *et al.* 2018; Murakami *et al.* 2019). Additionally, mice, guinea pigs, and ferrets are susceptible to infection by IDV through experimental studies (Hause *et al.* 2013; Sreenivasan *et al.* 2015; Oliva *et al.* 2020). Experimentally infected ferrets could transmit the disease by direct contact to other ferrets. Ferrets are often used as animal models for the transmission studies of influenza A virus in humans, and these results could support the possibility that IDV can infect humans (Hause *et al.* 2013).

This hypothesis was further explored when White *et al.* (2016) conducted a serological study on humans by comparing the IDV serological status of adults exposed to cattle or not in the northern-central parts of California. The study included 46 participants, among which 35 were regularly exposed to cattle, and the remaining 11 were not. Both hemagglutination inhibition assay (HI) and micro-neutralization assay (MN) were used to detect IDV antibodies. However, due to the limited amounts of serum samples, HI could be run only on some samples. The results from MN showed that 97% of the exposed group were positive for IDV and 18% in the non-exposed group. In the HI-results, 91% were positive in the exposed group and 75% in the non-exposed. The high values from the HI on the non-exposed group were explained as a result of a small sample size. These results could indicate that humans working with cattle have greater exposure to IDV and thus, a more considerable risk of developing the disease if the virus is zoonotic (White *et al.* 2016).

Trombetta *et al.* (2019) has also conducted a serological study for the prevalence of IDV in humans living in Italy but on a much larger scale. They assessed 1281 serum samples from humans living in central and southern Italy collected between the years of 2005 and 2017 with HI- and virus neutralization assays. The results showed a low prevalence of 5.1-9.8% between 2005-2007, followed by a sharp

increase in 2008. The highest levels were achieved in 2008, 2009, 2010, 2013, 2014, and 2016 ranging from 33.9-46.0%, and the lowest in 2011, 2012, 2015, and 2017 ranging from 11.9-25.7%. The authors argued that the peaks in seroprevalence in humans probably were linked with IDV outbreaks in cattle and swine and that the virus spilled over to humans. Another support for that theory was that the sero-prevalence in humans rapidly declined some of the following years; when there were not any outbreaks in animals the seroprevalence in humans declined. IDV seems to be able to infect humans, but if it actually can cause disease in humans and therefore, be classified as a zoonotic is still unknown (Trombetta *et al.* 2019).

2.1.4. Epidemiology

Despite multiple studies on IDV, the epidemiology of the virus is not fully understood. In an experimental study by Ferguson *et al*. (2016), it was shown that IDV-seronegative calves could seroconvert and develop the disease when experi-mentally inoculated. The authors also showed that the inoculated calves could transmit the disease to naive calves in the same pen which was not inoculated. Faecal samples and rectal swabs from this study were not positive for IDV, which could imply that the virus is not transmitted in faeces (Ferguson *et al.* 2016).

Salem *et al*. (2019) also found that seronegative calves, when challenged with IDV, could seroconvert and develop the disease. Furthermore, the authors showed that seronegative calves that were being held three meters from the challenged animals could develop the disease. Additionally, the authors found the IDV genome in air samples taking during their trial, suggesting that IDV can be transmitted by aerosol (Salem *et al.* 2019).

To summarize, the current knowledge obtained through experimental studies suggests that IDV is transmitted through direct contact and aerosol. The virus is probably not transmitted by feces since IVD has not been found in faecal samples or rectal swabs (Ferguson *et al.* 2016; Salem *et al.* 2019).

As previously reported, the virus infects both cows and pigs broadly, and multiple species exhibit seroprevalence. However, the prevalence and seroprevalence of IDV are much higher in cattle than in pigs. Therefore it has been suggested that the former can be considered as reservoir species for the virus (Hause *et al.* 2014).

Furthermore, the virus has also been proposed to be involved as one of the causes in combination with other pathogens associated with bovine respiratory disease (BRD). Ng *et al.* (2015) performed a study on 50 young dairy cattle calves suffering from BRD and 50 healthy calves of similar age acting as controls to study which viruses are associated with BRD. Nasal swabs were taken from each animal, and

the samples were enriched and sequenced to identify the involved viruses. In total, the virus was detected in 68% of the BRD-affected calves and 16% in controls through real-time PCR. The authors found that the viruses significantly associated with BRD in this study were bovine adenovirus 3, bovine rhinitis A virus, and IDV. 38% of the sick animals and 8% of the controls exhibited co-infections with multiple respiratory viruses. IDV was present in eight (14%) of the sick animals and none of the controls. In seven of the eight cases, the calf was suffering from co-infection with either bovine adenovirus 3 or bovine papillomavirus 2 (Ng *et al.* 2015).

Mitra *et al.* (2016) conducted a study on young cattle in feedlots in Mexico and Kansas in the U.S. The authors took nasal swab samples from animals exhibiting acute respiratory clinical signs linked to BRD and animals without clinical signs from the same pens. A total of 93 samples were collected, 47 samples from diseased animals, and 46 from asymptomatic animals at the time of the sample collection. Through high throughput sequencing, the researchers identified which viruses were present in the nasal secretions. By comparing the results obtains between the two groups of animals, IDV seemed to be more significantly detected in animals with signs of respiratory disease than in those without clinical signs (Mitra *et al.* 2016).

Olivia *et al.* (2019) performed a study on the seroprevalence of IDV in cattle and small ruminants in France. In the study, 5373 serum samples from cattle and 625 serum samples from small ruminants were examined with HI. The results showed differences in seroprevalence between the regions in the cattle samples. The region with the highest seroprevalence (70%), Pays de la Loire, had a much higher proportion of fattening herds. Other regions with larger proportions of dairy- and beef herds had much lower seroprevalences, ranging from 31-48.2%. The authors argued that the greater exchange and introduction of young animals in fattening herds could be the explanation. (Oliva *et al.* 2019). This could indicate that some production forms are more predisposed to IDV than others.

2.1.5. Clinical signs and Pathogenesis

Since the discovery of this virus, a limited number of experimental infections and field studies have been carried out to evaluate the pathogenicity of IDV and the associated clinical signs following infection by this virus. Ferguson *et al.* (2016) performed an experimental infection study with IDV in nine 4-month old, sero-negative, dairy cattle calves. Three calves were inoculated intranasally using a catheter to administrate 10^7 $TCID_{50}$ of D/bovine/C00046N/Mississippi/2014 in a volume of 10 ml. The six remaining calves were inoculated with sterile PBS and split into a contact group and control group with three calves in each group. Each of the calves in the contact group was paired with an infected calf and placed in the

same pens, and the control calves were kept separate. In controls, no clinical signs were observed; however, in the infected and contact group, mild respiratory symptoms were observed. The most observed was nasal discharge, observed in one infected calf and three contact calves. Three individuals exhibited either serous ocular discharge, depression dry coughing. The level of segmented neutrophils from blood samples differed significantly between the exposed and unexposed groups. Higher levels of segmented neutrophils were observed in the exposed groups. However, no significant statistical difference could be observed between both groups regarding clinical parameters such as rectal temperature, heart rate, respiratory rate, or hematological parameters such as total white cell count, and their subpopulation, excluding neutrophils (Ferguson *et al.* 2016).

In another study, Salem *et al.* (2019) included 14 colostrum deprived calves born in a bovine herpesvirus 1 (BOHV-1)- and bovine viral diarrhea (BVDV)-free experimental station, and two additional calves with maternal antibodies against IDV were added to the control group for the sake of statistical significance. The calves were transferred to the experimental setting at the age of 3-7 days to avoid early IDV infection and were reared for 2-6 weeks before the challenge. Before inoculation, all calves were tested negative by PCR on nasal secretions for IDV, BRSV, BCoV, BPI3, *Mycobacterium bovis, Histophilus somni, Pasteurella multocida, and Mannheimia haemolytica.* Additionally, the colostrum-deprived calves were also checked before the test to ensure they were seronegative for IDV. Eight colostrum-deprived calves were inoculated with 10 ml of viral suspension corresponding to 10^7 $TCID_{50}$ of IDV from the strain D/bovine/France/5920/2014. The viral suspension was administrated through aerosol inhalation using a compressor and a mask covering the nose and mouth of the inoculated calves. The control group was inoculated with the same procedure but with 10 ml of swine testis supernatant cells and three colostrum-deprived calves, acting as aerosol sentinels did not receive any treatment. The animals were then divided and placed into two separate pens. In the first pen, the control group was placed, containing three colostrum-deprived calves and the two seropositive calves. The second pen was divided into two part by steel panels at three meters apart. In the first section, the eight infected calves were housed, and in the second the three aerosol sentinels. During the experiment, two inoculated calves and one aerosol sentinel showed mild clinical signs, and three inoculated calves exhibited moderate clinical signs. Mild clinical signs were defined as infrequent spontaneous coughing and slight tachypnea (35-40/min). Moderate clinical signs consisted of repeated spontaneous coughing, abdominal dyspnea with increased respiratory rate (35-60/min), and wheezing lung sounds with no effect on general state or appetite. None of the animals in the aerosol or challenge group exhibited hyperthermia or any signs of lowered appetite or general

state during the test. No clinical signs were observed in the control group except for one animal suffering from arthritis with no respiratory signs (Salem *et al.* 2019).

To summarize, an infection of solely IDV in the experimental setting merely seems to be able to cause mild upper and lower respiratory clinical signs in young cattle in the upper and lower respiratory tract.

2.1.6. Histology

Histological examination is a valuable tool to understand the pathogenesis of a disease. Ferguson *et al.* (2016) examined tissue samples from the nasal cavities, trachea, bronchus, and lungs from the calves included in their study. No gross lesions were reported, but they found that inoculated calves exhibited a significantly higher level of tracheal inflammation. The lesions were characterized as multifocal areas of epithelial neutrophil infiltration and mild epithelial attenuation. The level of neutrophil infiltration in the tracheal tissue was also significantly higher in inoculated calves compared to the controls. No microscopic lesion was found in the lungs in either controls or challenged animals (Ferguson *et al.* 2016).

In the study by Salem *et al.* (2019) three inoculated calves, euthanized eight days after exposure, showed gross lesion. These animals exhibited macroscopic lung lesions in the cranial right lung lobe. The lesions appeared to be patchy dark red areas of atelectasis. No gross lesion was found in the nasal cavities, larynx, or trachea in these animals. To confirm the presence of IDV in the lower respiratory tract, samples from these animals were analysed with RT-PCR. RNA from IDV was found in the nasal cavities, trachea, cranial- and caudal lobes of the lungs, and in the mediastinal- and tracheal lymph nodes At the end of the experiment, 22-23 days after exposure, all animals were euthanized, and no gross lesions caused by IDV could be found in the remaining animals in either group. Histological examinations were performed on all calves on the olfactory bulb, nasal mucosae, trachea, mediastinal, and tracheal lymph nodes, and lung tissue sections with gross lesions. Microscopic lesions were found in the nasal mucosae, and right cranial lobe in two of the calves euthanized at day eight and in the right cranial- and accessory lobe in two inoculated calves euthanized at the end of the experiment. The lesions in the upper respiratory tract showed infiltration of *lamina propria* by mononuclear cells in the nasal epithelium, a characteristic of subacute rhinitis. In the lower respiratory tract, there were neutrophils in the bronchial lumens, neutrophilic and macrophagic alveolitis, and peribronchial and septal lymphoplasmacytic infiltration in the lung typical for subacute bronchointerstitial pneumonia (Salem *et al.* 2019).

2.1.7. Immunology

The immunological response to IDV has been studied by Salem *et al.* (2019). To examine the seroconversion in the host, serum samples were collected from each calf and analysed with HI. All inoculated calves in the study that lived past day eight postexposure had seroconverted by day 15. Additionally, one of the aerosol sentinels underwent seroconversion by day 22. None of the controls showed any elevated levels of IDV-antibodies. ELISAs targeting IDV-specific IgG1 and IgG2 were used on serum samples from five inoculated calves to characterize the IgG expression. IgG1 antibodies were present in all animals by the end of the experiment. Only a minor response to IgG2 was found in two samples at the end of the study. The authors also used bronchoalveolar lavage specimens collected during their study to examine the host's immunological response in the lower respiratory tract to IDV. BAL samples from day zero, before exposure, and on day two, seven, and 14, post-exposure, were analyzed using microfluidic qPCR to quantify the transcriptomic response molecules in the host. The results showed significant over-expression of the genes coding for pathogen recognition receptors and proinflammatory chemokines in inoculated calves. There was no significant difference in the expression of the IFN-α gene, genes involved in the type I interferon pathway (IRF1, IRF3, IRF7, and STAT2), or interferon antiviral-induced molecule ISG15. Also, there was an overexpressing of SOCS1 and SOCS3, genes involved in down-regulation of the immune response (Salem *et al.* 2019).

2.1.8. Diagnostics

The diagnostics of IDV is based on direct detection of the virus by RT-PCR or indirectly by searching for specific antibodies following infection. For molecular detection, real-time RT-PCR has been primarily used since IDV was discovered in 2011. According to Risk assessment for influenza D in Europe published by EFSA in 2020, the preferred PCR methods are either the one described by Hause *et al.* (2013) or Faccini *et al.* (2017). The Hause *et al.* method uses a real-time RT PCR targeting the PB1 gene sequence from the first discovered IDV virus, D/OK (Hause *et al.* 2013). The RT-PCR described by Faccini *et al.* also targets the PB1 region, but the authors used several PB1 sequences from several IDV strains when designing the primers and the probe (Faccini *et al.* 2017). In the report from EFSA both described methods are deemed equally sensitive and specific (Chiapponi *et al.* 2020)

For serological detection of IDV-antibodies enzyme-linked immunosorbent assay (ELISA) or HI are primarily used. ELISA is a serological method used to quantify the amount of specific antigens or antibodies in samples. The main idea is that enzyme-linked specific antibodies targeting either antigens or antibodies are added to a solution containing a sample. When the antibodies have had sufficient time to

bind the sample, substrate is added to the solution, and the enzymes on the specific antibodies start degrading the substrate. The products from the reaction colour the test solution in proportion to the amount of bound specific antibodies and the optical density of the solution can then be assessed for quantification of the target antigen or antibody (Engvall & Perlmann 1972).

The HI is another way to detect specific antibodies from samples. The test utilizes hemagglutination, which is viral or bacterial surface protein's ability to bind to red blood cells (RBC), creating a network of bound antigens and RBC. In a solution where hemagglutination fully occurs, no blood can be seen; instead, the solution is slightly tinted red. On the other hand, if there is not enough virus or bacteria to cause hemagglutination, blood will be found at the bottom of the wells. When performing the HI, samples containing antibodies are added to wells in increased rates of dilution. A set amount of specific antigens is then added to each well and are allowed to react with the antibodies. Lastly, blood is added to each well to allow hemagglutination. The concentration of antibodies is then expressed as the highest antibody dilution where hemagglutination does not occur (Hirst 1942; Chiapponi *et al.* 2020).

Regarding which test to use, the authors of the EFSA report argued that since HI-test is more subjective, ELISA should be preferred when performing serological testing for IDV antibodies (Chiapponi *et al.* 2020).

2.2. Cattle production in Sweden

In 2019 there were in total 305 570 dairy cows in Sweden, organized into 3253 herds (Statistics Sweden 2020). The mean dairy herd cow number was 94 animals. The general trend in Sweden is that the herds are getting fewer, the cow population is declining, but herd sizes are increasing. Indeed, back in 1999, there were 448 520 dairy cows and 13 963 herds with a mean size of 32 cows (Statistics Sweden 2020)

The Swedish Official Milk Recording Scheme at Växa Sverige enrolled 70% of the Swedish dairy herds and 68% of the Swedish dairy cows in 2019. Out of the affiliated herds, 94% were conventional, and 6 % organic. Around 33% of the herds used automatic milking systems. Approximately 44 % were tie stalls, and the remaining herds were free stalls, out of which 75 % were heated systems and 25 % cold stables (Växa Sverige 2020).

In 2019 there were 210 086 suckler cows and 10 266 suckler herds in Sweden, with a mean herd size of 21 (Statistics Sweden 2020). About 60% of the meat production

in Sweden comes from dairy herds and the rest from smaller, more specialized suckler herds (The Swedish Board of Agriculture 2020).

Sweden has a long tradition of disease prevention by implementing eradication and control programs. Regarding the implementation of biosecurity measures among the herds in Sweden, there are a couple of compulsory and voluntary programs. The bovine viral diarrhoea virus (BVDV) program was launched in 1993. The aim was to eradicate BVDV from Sweden by detecting affected herds and eliminate the infection. When the program started the estimated prevalence of infected herds was 40%. After 11 years the prevalence was down to 0.9%, and since 2014 Sweden has declared itself BVDV-free. The program is still ongoing and from 2002 compulsory to prevent eventual new outbreaks of the disease. Today, surveillance of dairy herds is performed by sampling bulk tank milk in conjunction with milk quality testing, and beef herds are performed by blood sampling at slaughter. It is also synchronized with the surveillance of bovine leucosis and infectious bovine rhinotracheitis which also has been eradicated similarly (Hult & Lindberg 2005; SVA 2020).

An example of a voluntary program is the Smittsäkrad besättning. The aim is to increase the overall biosecurity in cattle herds, by enhancing the farmers' knowledge of infectious diseases and to provide tools for implementing on-farm biosecurity routines. The program consists of three levels, at the first level there is a theoretical course in biosecurity and a risk assessment inquiry, the second level includes a veterinary visit with biosecurity and hygiene checkpoints in combination with advisory on biosecurity and the third level adds an on-farm practical course in biosecurity. Herds enrolled in the program are entitled to higher economic compensation from the Board of Agriculture in case of salmonellosis. There are regulations regarding animal contacts that apply to all levels in the program (Växa Sverige 2020).

Through surveillance and control programs, diseases can be detected at an early stage and proper actions can be taken to minimize and prevent the spread of diseases. Understanding the transmission and prevalence of diseases is necessary when developing these programs. Involving the farmers and different actors in the industry through education and economic incentives can lead to improved sustainability in the cattle industry.

2.3. Respiratory disease in Swedish cattle

Since IDV is not routinely searched for in Sweden its role in causing airway related disease is still unknown. BRD is regularly detected in Sweden, but it is difficult to estimate the frequency. In the animal health data from cattle during 2019, curtesy

to Kajsna Olnéus on the Swedish Board of Agriculture for the data, approximately 10000 airway-related diagnoses were recorded for individuals, groups, and herds in Sweden. In total during this period, 271 445 diagnoses were recorded, meaning that roughly 4% were respiratory. However, since all diagnoses, such as prophylactic measures and healthy, are included in these data, the percentage of airway-related disease among diseased animals is probably higher (Djurhälsodata 2019, SJV).

According to Svensson *et al*. (2003) heifers, less than 90 days of age are most commonly affected by diarrhoea followed by airway infection (Svensson *et al.* 2003).

According to SVA and Hägglund *et al.* (2016), the primary agents causing respiretory disease in Sweden among cattle is mainly viruses and parasites. The most common agents include bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCoV), bovine parainfluenza virus 3 (BPIV3), and lungworm, *Dictyocaulus viviparus*. Bacterial infections are most commonly seen as secondary infections where *Pasteurella multocida* is the most common, followed by *Mannheimia haemolytica* and *Hisophilus somni* (Hägglund *et al.* 2006; SVA 2020).

3. Material and methods

3.1. Study population and sampling

Bulk tank milk (BTM) samples from 338 herds were obtained from the Swedish BVDV control program. The BVD surveillance is based on a risk-based design where herds are individually categorized based on animal movement during the preceding 12-month period. All Swedish dairy herds are enrolled in the BVD-program, and a total of approximately 2600 BTM samples are collected each year. The sampling was performed in conjunction with milk quality testing at Eurofins laboratory in Jönköping and sent to the National Veterinary Institute (SVA) in Uppsala for analysis.

Out of the samples that arrived for BVD analysis at SVA during February and March in 2020, 338 samples were randomly selected. The samples were sent to SLU and kept at +4°C until analysis.

The BVD-samples are coded using bar code labels. After analysis, the samples were decoded regarding county and herd size at Växa Sverige.

3.2. Analysis of milk

In the study, an in-house indirect ELISA was used to detect and analyze the presence of IgG antibodies specific for Influenza D virus in BTM samples. The ELISA was developed by SVA och SLU to screen cattle samples and was used in Salem *et al.* (2019) and Alvarez *et al.* (2020). The sensitivity and specificity were examined by Alvarez *et al.* (2020) during their screening for IDV in Argentina using the method with serum samples. To validate the method, they used 300 confirmed IDV-negative bovine samples and 37 IDV-positive bovine samples. Their results showed that the ELISA detected 31/37 positive samples and 300/300 negative samples. This corresponds to a sensitivity of 87% and a specificity of 100% (Alvarez *et al.* 2020).

The IDV antigens used for the assay comes from the supernatant of a detergent treated cell-lysis solution of IDV (virus strain: D/Bovine/France/5929/2014) grown in porcine testis cells (ATCC CRL-17469). Infected cells are treated with 10% Triton x-100 which solubilizes the proteins, lipo- and glycoproteins from the virus and cells. The material was then centrifuged to remove non-solvable materials, and the supernatant was used as an ELISA antigen. Through this process, the virus becomes inactive since the protein coat is destroyed.

The test essentially consists of 9 steps: coating the plates, washing, blocking the plates, adding the samples, washing to remove the excess of the sample, adding conjugate, washing to remove the excess of the conjugate, adding the substrate, stopping the reaction, and then measuring the results with spectrometry in 450 nm.
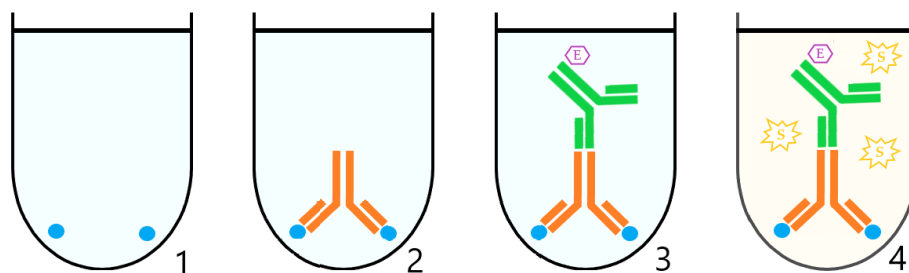

Figure 1. Illustration of the indirect ELISA. In the first picture blue dots represents IDV-antigen. In the second the orange figure represents an antibody from the BTM-samples binding to the IDV antigens. In the third a green figure with a purple box represents an anti-bovine mouse antibody conjugated with horseradish peroxidase binding to the IDV-antibody. In the last picture substrate, illustrated as yellow figures, is added to the solution and the horseradish peroxidase starts degrading substrate. The resulting products tints the solution yellow.

The test was run in 96-well plates (Nunc polysorp). The whole virus antigen was first diluted 1:1000 in a 0.05 M sodium carbonate-bicarbonate buffer containing 4.29 g/l of Na_2CO_3 $10H_2O$, and 2.93 g/l of $NaHCO_3$ (coating buffer, pH 9.6). Afterward, the plates were coated with 100 µl/well of whole virus antigen from the virus strain D/Bovine/France/5929/2014 and incubated overnight at +4°C.

After incubation, the plates were washed twice with 300µl/well of PBS-T. PBS-T is a washing and dilution buffer consisting of PBS with 0.05% Tween and is made by mixing the concentrate of the Svanova washing buffer with deionized water. The plates are then blocked with an additional 300µl/well of PBS-T and incubated at room temperature for one hour.

Previously known IDV-IgG-positive, low positive, and negative control sera were diluted 1:50 with PBS-T in low binding U-shaped plates and were then mixed, and 100 µl of each sample was added in 4 wells for each control sera in column 11 and 12. The last four wells in columns 11 and 12 were filled with 100 µl of PBS-T. The milk samples were mixed, and 100 µl of each sample was added to the remaining 80 wells on each plate and then incubated at +37°C for one hour. Afterward, the plates were washed with 300 µl/well of PBS-T, three times.

S	S	S	S	S	S	S	S	S	S	B	B
S	S	S	S	S	S	S	S	S	S	Pos	Pos
S	S	S	S	S	S	S	S	S	S	(+)	(+)
S	S	S	S	S	S	S	S	S	S	Neg	Neg
S	S	S	S	S	S	S	S	S	S	Pos	Pos
S	S	S	S	S	S	S	S	S	S	(+)	(+)
S	S	S	S	S	S	S	S	S	S	Neg	Neg
S	S	S	S	S	S	S	S	S	S	B	B

Figure 2. Plate layout for each 96-slot plate. S = Sample, B = PBS-T, Pos = positive, (+) = low positive, Neg = negative.

The conjugate used was a mouse anti-bovine IgG1 (clone 2:2) conjugated with horseradish peroxidase from the Svanova BRSV kit. The conjugated antibody was diluted 1:2 with PBS-T and 100 µl of the solution was added to each well, and the plates were then incubated at +37°C for one hour. Following the incubation, the plates were washed four times with 300 µl PBS-T in each well.

After washing the plates, 100 µl of Tetramethylbenzidine substrate (Svanova), was added to the wells starting a reaction with the horseradish peroxidase which colours the solution in correlation to the amount of specific bovine IgG antibodies present in the sample. Afterward, the plates were incubated at room temperature for 10 minutes. 50 µl of the stop solution, 10% H_2SO_4, was then added to stop the reaction and the plates were measured by spectrometry at 450 nm to determine the optical density of each sample.

The positive percent (PP) values were calculated for each sample using the following formulas:

1. $OD_{Virus\ antigen} - OD_{Control\ antigen} = $ Corrected OD (COD)
2. $(COD_{Sample} / COD_{Positive\ control}) * 100 = $ PP-value

The cut-off value for positive samples was set to 10 by multiplying the highest negative control value (5), times 2.

3.3. GIS

Maps displaying Sweden and its different counties were created by using the software ArcGIS (ArcGis Desktop version 10.7.1) to give an overview of the results from the sampling and regarding other relevant information such as herd intensity in each county.

3.4. Statistical analysis

For statistical analysis, the software Stata (Stata version 16) was used. Sampled herds were organized into five different herd size groups. The number of IDV-positive cows in each category were not normally distributed and was, therefore, log-transformed. To determine if the difference in PP-value for positive samples in each group were significant linear regression was performed. To determine if the odds ratio for IDV differed based on herd size logistic regression was performed. The significance level was set to $P < 0.05$ for both tests.

4. Results

During the spring of 2020, 338 BTM samples were collected from 20 counties in Sweden from dairy herds and assessed for the prevalence of anti-IDV IgG antibodies. 135 out of the 338 sampled herds were positive. In 12 of the 20 sampled counties positive herd samples were found. Herd sizes ranged from 10 to over 500 cows in the samples collected, and the sample sizes varied a lot from county to county. Fourteen of the herds included in the study had unknown herd size. The largest sample size was from Kalmar with 61 samples, and the lowest was from Jönköping with only one sample. An unfortunate effect of the randomization of the study resulted in no samples being collected from Kronoberg county (Table 1).

The results showed an increased positive percentage in southern Sweden, excluding Jönköping and Östergötland (Figure 3). From the height of Södermanland and further north, the positive percentages in samples for each county sharply declined.
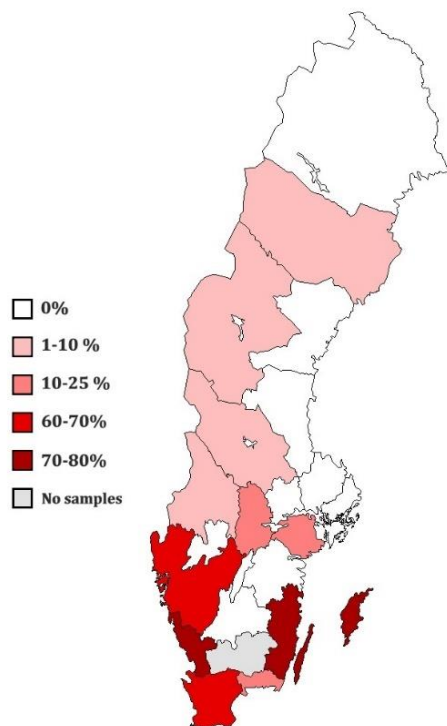

Figure 3. Map of Sweden displaying the positive percentage of collected samples in each county. Map image (ArcGis, 2020) is the intellectual property of Esri and is used herein under license. Copyright © 2020 Esri and its licensors. All rights reserved.

Table 1. Results from the indirect ELISA regarding sample sizes, number of positive samples and positive percentages, and average positive PP-value with standard deviation for each county.

County	Sample Size	Number of positive samples (%)	Average positive PP-value and standard deviation
Stockholm	7	0	-
Uppsala	12	0	-
Södermanland	15	2 (13)	62 ± 8
Östergötland	6	0	-
Jönköping	1	0	-
Kronoberg	0	-	-
Kalmar	61	46 (75)	48 ± 10
Gotland	7	5 (71)	43 ± 17
Blekinge	7	1 (14)	13
Skåne	40	25 (63)	60 ± 21
Halland	13	10 (77)	64 ± 10
Västra Götaland	57	39 (68)	46 ± 16
Värmland	10	1 (10)	44
Örebro	13	3 (23)	49 ± 26
Västmanland	3	0	-
Dalarna	12	1 (8)	16
Gävleborg	7	0	-
Västernorrland	6	0	-
Jämtland	14	1 (7)	11
Västerbotten	41	1 (2)	14
Norrbotten	6	0	-
Total	338	135 (40)	

The sampled herds were sorted by herd size to examine if there was a correlation between herd size and IDV-prevalence (Table 2). There was no significant statistical difference of positive PP-values between herd sizes. However, the odds ratio for a herd being positive for IDV increased as the herd size grew larger (Table 3). This suggests that larger herds are more susceptible to being positive for IDV, but at the same time have similar levels of IDV-specific antibodies in comparison to smaller herds.

Table 2. Results from the indirect ELISA based on herd size presenting sample size, positive samples, positive percentage of the samples included in the category, and average positive OD-value with standard deviation.

Herd size	Number of herds included in the study	Number of herds with IDV-specific antibodies in BTM (%)	Average positive PP-value and standard deviation
1-50	103	20 (19)	48 ± 21
51-100	102	35 (34)	49 ± 20
101-150	53	28 (53)	54 ± 16
151-200	29	18 (62)	52 ± 10
>200	37	30 (81)	50 ± 16
Unknown	14	4 (29)	27 ± 22
Total	338	135 (46)	

Table 3. Odds ratio regarding positive samples for each herd size category in comparison to the herd size category 1-50.

Herd Size	Odds ratio	Z	OR 95% confidence interval	p-value
51-100	2,04	2,22	1,086 to 3,829	0,027
101-150	4,37	4,01	2,125 to 8,999	0,000
151-200	7,42	4,35	3,006 to 18,310	0,000
>200	24,99	5,97	8,681 to 71,945	0,000

5. Discussion

The results of this study show that IDV is still present in Sweden. Not all counties had positive samples, and the largest percentage of positive samples were found in the south. This is probably connected to the higher cow density in the south. There are, however, inconsistencies in the data. When studying figure 3 there are no positive samples in the county of Jönköping or Östergötland which contradicts the hypothesis. According to Figure 4, both counties have a high density of cows/km^2 as most of the southern counties in Sweden. Arguably the most probable cause that no positive samples were found in these counties would be due to the low sample size (1 and 6 respectively). Due to the samples being coded, no adjustments for sample sizes in proportion to cow-density in each county could be made. On the other hand, these counties might not follow the trend seen in southern Sweden. To evaluate this, a larger study with more samples included would be required.
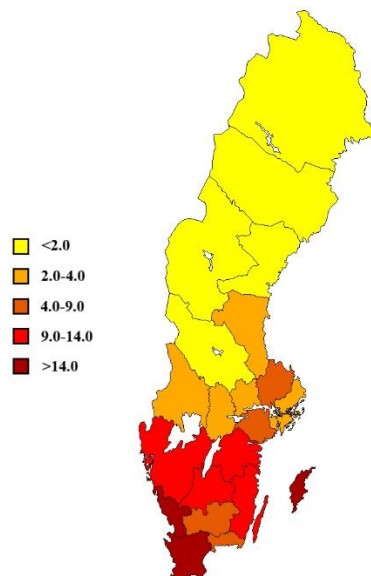

Figure 4. Map of Sweden displaying the total number of cattle per km^2 in each county. Map image (ArcGis, 2020) is the intellectual property of Esri and is used herein under license. Copyright © 2020 Esri and its licensors. All rights reserved.

The first study of IDV prevalence in Sweden was performed by Ahlgren (2019). The author performed a retrospective analysis on 1093 bovine serum samples collected from the IBR and BVDV surveillance programs between 2016 and 2019. 472 randomized BTM samples collected during 2019 were also analyzed to determine the current prevalence. Both serum- and BTM samples were analyzed with the same indirect-ELISA described in this study. The results from the serum samples showed a decreasing prevalence of IDV from 2016 to 2019. 147 (31%) of the BTM-samples were positive during 2019. In this study 135 BTM-samples (40%) were positive. The results from the BTM-samples performed by Ahlgren (2019) are displayed in figure 5. Positive samples were found in the counties of Västra götaland, Halland, Kalmar, Skåne Gotland, and Örebro. The positive percentage in each county was respectively 13%, 6%, 4%, 3%, 3%, and 1%. When compared to the results of this study (Figure 3) it appears that IDV has spread to more counties. This could be a result of different sample sizes between the studies in each county, and since no data regarding sampling size is available from Ahlgren (2019) it cannot be excluded. On the other hand, the results of this study could be an indication that the seroprevalence for IDV is increasing among dairy cows in Sweden. Trombetta *et al.* (2019), described in their study peaks and lows in human seroprevalence for IDV in Italy. These peaks closely followed outbreaks of IDV in cattle and pigs (Trombetta *et al.* 2019). At least in Italy, the seroprevalence for IDV appears to be dynamic; it increases or declines from year to year, and there is probably a similar dynamic in Sweden. When hosts become immune to a pathogen it circulates less and when the hosts start becoming seronegative the pathogen starts circulating again. In the results by Ahlgren (2019) positive serum samples were highest in 2016, 49%, and then steadily decline in the following years down to 9% in 2019. The results of this study could be an indication that IDV is starting to spread again after a few years of decline. To further explore this notion, continuous and targeted studies on the seroprevalence would be required in the following years to determine if the apparent increase were due to different sampling or an actual increase.

Figure 5. Map displaying IDV-positive BTM-samples in Swedish counties from the indirect-ELISA performed by Ahlgren, 2019.

The results from the statistical analysis suggested that larger herds had an increased prevalence of IDV (Table 3), but also showed that despite this preposition the average positive PP-values were not significantly different between each herd size. These results suggest that regardless of herd size, the proportion of affected animals in each positive herd remains the same. One explanation for this could be that certain age categories express larger quantities of antibodies against IDV. The study by Oliva *et al.* (2019) showed that fattening herds, in which there are a larger proportion of younger animals compared to other production forms, exhibits higher seroprevalence to IDV (Oliva *et al.* 2019). One hypothesis could be that the younger animals in dairy herds such as first-calvers would have more recently, in comparison to older cows, undergone seroconversion and thus have higher titers of antibodies. This would mean that while younger, more newly recruited animals, would increase the concentration of specific anti-IDV IgG in the BTM, the older cows would dilute it and therefore steady the concentration. If the proportion of each age group and the recruitment level is roughly the same in Swedish dairy herds this could be an explanation of why the average positive PP-values between herd sizes don't differ significantly.

Even though the epidemiology of IDV is still not fully understood, studies suggest that it is spread through aerosols and direct contact in cattle (Ferguson *et al.* 2016; Salem *et al.* 2019). There are limited data regarding indirect transmission by IDV. It is more resistant to acidity and heat than other influenza types, but studies examining the persistence of IDV in the environment are required (Yu *et al.* 2017).

IDV has been suggested as one of the pathogens involved in causing BRD. Studies in feedlots have found that IDV is more prevalent in animals suffering from BRD than in healthy animals in the same setting. It has also been shown to be prevalent as a co-infection with other pathogens when animals are suffering from BRD. However, since IDV is not routinely analysed in Sweden the impact of IDV on BRD is still unknown.

The symptoms exhibited in experimental studies on calves on IDV has been mild. Since BTM samples were used in this study, we can't tell if the IDV positive herds had respiratory signs or not. It seems like IDV can infect both the upper and the lower respiratory tract. The elicited response histologically primarily showed affection in the upper respiratory tract causing weakening of the epithelia and attracting neutrophils. When the immune response in calves was studied it was shown that that the expression of SOCS1 and SOCS3 were upregulated which weakens the immune response in the host by inhibiting cytokine production. To summarize, an infection by solely IDV does not seem to be harmful, but further investigation is needed to evaluate the pathogenic impact of this virus as a cause of

BRD. However, due to its properties, it seems like it can effectively spread in herds and predispose the animals to secondary infections contributing to BRD.

In conclusion, IDV is present in Sweden and the virus prevalence seems to be increasing. IDV might contribute to an increasing onset of BRD in cattle and it would be interesting to study the prevalence in other production forms to see if the prevalence differs. Analysing outbreaks of BRD in Swedish cattle for IDV could prove a valuable tool to understand the Swedish situation. Further studies are needed to characterize the role and impact this virus has in Swedish cattle production.

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Populärvetenskaplig sammanfattning

Influensa D virus (IDV) är ett relativt nytt virus, som upptäcktes i Nordamerika under 2011. Det finns totalt fyra influensavirus, Influensa A, B, C och D. Samtliga hör till virusfamiljen *Orthomyxoviridae*. I vardagligt språk pratas det ofta om influensa som en och samma sjukdom, men i själv verket så har olika influensavirus olika preferenser och egenskaper.

IDV påvisades för första gången i grisar med influensaliknande symptom. När forskare först började studera viruset såg de att det var mest likt influensa C virus (ICV) i sina egenskaper och genomiska utseende. Detta gjorde att IDV inledningsvis blev kallat för ett ICV. Efterföljande studier på viruset kom så småningom fram till att det måste vara en ny typ av influensavirus. Detta baserade de på bland annat att ICV och det nya viruset inte utbytte egenskaper, såsom influensavirus av samma typ gör, samt att antikroppar som var riktade mot virusen inte reagerade på det andra viruset. Det nya namnet blev därmed influensa D virus.

IDV-förekomsten verkar vara störst hos nötkreatur, men det infekterar även grisar och virusantikroppar har påvisats i hästar, får, getter, kameldjur, vildsvin och människor. IDV har påvisats i djur i Nord- och Sydamerika, Asien, Europa och Afrika.

Det finns fortfarande mycket att studera runt hur viruset sprids mellan värdar. Experimentella studier på kalvar har visat att IDV åtminstone sprids via direktkontakt och aerosol. Nötkreatur som utsatts för experimentella studier med IDV har främst fått lindriga symptom från övre- och nedre luftvägarna. IDV verkar inte kunna orsaka kraftig sjukdom ensamt utan har istället i studier visats bereda väg för andra bakterier och virus. Detta gör IDV genom att försvaga skyddet i luftvägarna och skapa goda förutsättningar för andra bakterier och virus att kunna tillväxa. En studie har även visat att IDV verkar kunna hämma immunförsvarets aktivering genom att bryta signalering. Detta skulle teoretiskt kunna få till följd att värdens immunförsvar inte svarar tillräckligt när den utsetts för efterföljande infektioner efter IDV.

I den här studien följde vi upp den första provtagningen för IDV från 2019 för att kunna fortsätta övervaka förekomsten av IDV i svenska mjölkbesättningar och se

om förekomsten har förändrats. Prover samlades in från 338 svenska mjölkbesättningars mjölktankar och analyserades efter förekomsten av antikroppar riktade mot IDV. Analysen utfördes med en indirekt ELISA som har utvecklats av SLU och SVA.

Resultaten från studien visade att IDV fortfarande finns i Sverige. I jämförelse med studien från 2019 hade förekomsten ökat från sex län till tolv. Andelen positiva besättningar var som störst i södra Sverige. Vi såg även att risken för en besättning att ha IDV ökade i proportion med besättningsstorlek. Gårdar med få djur var betydligt mindre benägna att ha IDV i jämförelse med de stora gårdarna. Trots detta låg antikroppsnivåerna på ungefär samma nivå i alla positiva besättningar. En förklaring till detta skulle kunna vara att det bara är vissa ålderskategorier i besättningarna som faktiskt har förhöjda antikroppsnivåer. Eftersom andelen djur i varje åldersgrupp antagligen är snarlik i svenska besättningar skulle detta kunna resultera i liknande antikroppsnivåer.