Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
Thesis 2018:32

Bovine coronavirus in calves - experimental studies on virus shedding, transmission and whole genome sequencing

Bovint coronavirus hos kalv – virusutskilling, overføring og fullgenomsekvensering

Veslemøy Sunniva Oma

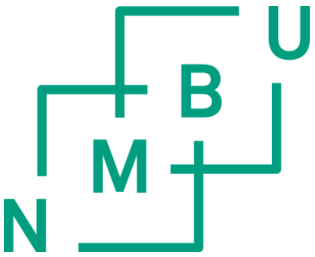
Bovine coronavirus in calves

- experimental studies on virus shedding, transmission and whole genome sequencing

Bovint coronavirus hos kalv – virusutskilling, overføring og fullgenomsekvensering

Philosophiae Doctor (PhD) Thesis

Veslemøy Sunniva Oma



Department of Production Animal Clinical Sciences

Faculty of Veterinary Medicine

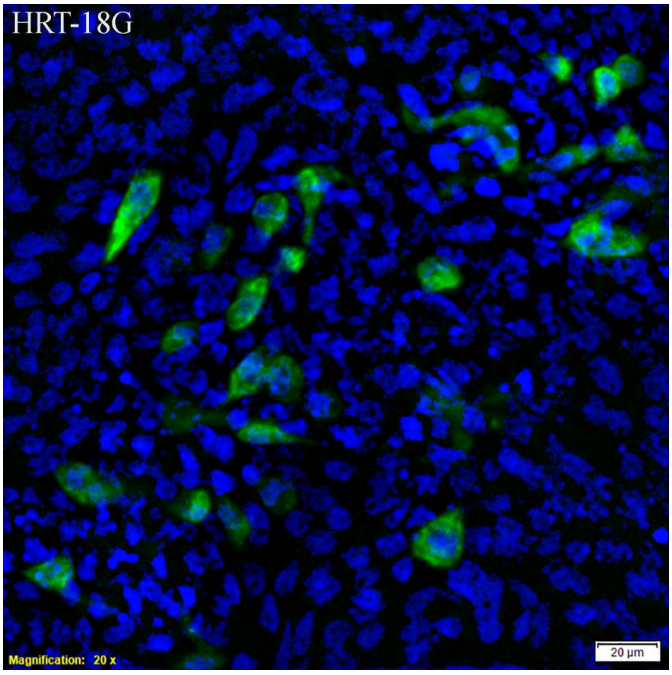
Norwegian University of Life Sciences

Adamstuen 2018

Thesis number 2018:32

ISSN 1894-6402

ISBN 978-82-575-1759-5



HRT-18G cells infected with bovine coronavirus from a nasal swab collected from a calf, six days after exposure. The cells are stained with anti-coronavirus antibodies labelled with fluorescein isothiocyanate and DAPI nuclear counterstain.

Photo by Hanne Haatveit

Table of contents

Acknowledgments

The work presented in this thesis was initiated at the Norwegian University of Life Sciences (NMBU) and conducted from 2013 to 2017 at NMBU and the Swedish University of Agricultural Sciences. The project was funded by the Research Council of Norway (NFR-project No 224771/E40), the Norwegian Research Funding for Agriculture and the Food Industry, and TINE Norwegian Dairies BA.

I wish to express my sincerest gratitude to my main supervisor, Maria, for her never-ending enthusiasm and support. Thank you for always having answers to my questions, and for answering them with engagement and a smile. Your positive attitude, knowledge and humor are a true inspiration. Furthermore, I wish to express my sincerest gratitude to my supervisor, Mette, for being my go-to person in the field of virology. Thank you for always being available and for sharing advice and wisdom. Your dedication and eye for detail have been essential for this work. I also want to thank my supervisor, Thea, for being a role model and for always caring for my well-being. Thank you for all the effort you have put into our work, and for always listening to my point of view.

Thank you, Ane Nødtvedt, for helping me complete my thesis and for always putting the welfare of the PhD students first.

Sincere gratitude to Madeleine Tråvén for arranging the animal experiments and for her faithful participation during the trials. Thank you, Stefan Alenius, for your enthusiastic contributions during the animal experiments and your belief in this project. I also want to thank Lena-Mari, Madde and the rest of the staff at IME, SLU for being wonderful colleagues and for taking good care of me during my stays in Uppsala. I also want to thank all the employees and students that swabbed their nostrils during the trials.

I want to thank Michael Berg and Anne-Lie Blomström at SLU for their friendly cooperation and advice during the sequencing studies.

I also want to thank my colleagues at Lindern for providing a good working environment and for helping me throughout my lab work. In particular, I want to thank Mamata, Stine, Elisabeth, Ingvild and Cristopher for their help and assistance during my studies.

I would like to thank all my fantastic colleagues in Building 4 for providing a good working environment and sharing knowledge and stories.

A big hug and thank you to my “work wife,” Ingrid Toftaker. Because of you, I’ve looked forward to going to work (almost) every day the past four years. Your endless support, social nature and hearty being have kept me going. I wonder how I will cope in life without sharing an office with you.

Finally, I would like to thank my family and friends for your love and encouragement, and Erling for being my biggest cheerleader and supporter throughout the PhD years. Together, we made it!

Abbreviations

BCoV	Bovine coronavirus
BHV-1	Bovine herpesvirus 1
BRSV	Bovine respiratory syncytial virus
BRD	Bovine respiratory disease
BVDV	Bovine viral diarrhea virus
BTM	Bulk tank milk
D	Day
ELISA	Enzyme-linked immunosorbent assay
GC	Genome copies
HCoV	Human coronavirus
MERS-CoV	Middle East respiratory syndrome coronavirus
MHV	Mouse hepatitis virus
NSP	Nonstructural protein
ORF	Open reading frame
PIV-3	Parainfluenza virus type 3
RdRp	RNA dependent RNA polymerase
RT-qPCR	Reverse transcriptase real-time quantitative PCR
SARS-CoV	Severe acute respiratory syndrome coronavirus
VRC	Viral RNA copies

List of papers

1. Oma, V.S., Tråvén, M., Alenius, S., Myrmel, M., Stokstad, M., 2016. Bovine coronavirus in naturally and experimentally exposed calves; viral shedding and the potential for transmission. Virol. J. 13, 100.

2. Oma, V.S., Klem, T., Tråvén, M., Alenius, S., Gjerset, B., Myrmel, M., Stokstad, M., 2018. Temporary carriage of bovine coronavirus and bovine respiratory syncytial virus by fomites and human nasal mucosa after exposure to infected calves. BMC Vet. Res. 14, 22.

3. Myrmel, M., Oma, V., Khatri, M., Hansen, H.H., Stokstad, M., Berg, M., Blomström, A.L., 2017. Single primer isothermal amplification (SPIA) combined with next generation sequencing provides complete bovine coronavirus genome coverage and higher sequence depth compared to sequence-independent single primer amplification (SISPA). PLoS ONE 12, e0187780.

Summary

Bovine coronavirus (BCoV) is a significant cause of respiratory disease and diarrhea in calves and of winter dysentery in adult cattle. These endemic diseases result in substantial economic losses and reduced animal welfare in cattle worldwide. Inter-herd transmission can occur directly, with contact between live animals, or indirectly, via contaminated personnel and equipment. This thesis aims to elucidate transmission of BCoV via animals, contaminated personnel and equipment, and to develop methods for tracing of the virus.

An animal experiment with BCoV infection in calves was conducted. Four BCoV-antibody-negative calves were commingled with six naturally infected calves originating from a herd experiencing a winter dysentery outbreak. Three weeks later, two naïve sentinel calves were introduced. Material was collected from the calves, equipment and nostrils of personnel after contact with the infected calves, and analyzed for presence of BCoV RNA by RT-qPCR. Selected samples were cultivated to detect infective BCoV. Results from a similar experiment with bovine respiratory syncytial virus (BRSV) were included in paper II.

The BCoV-infected calves showed mild general signs, and the most prominent signs were from the respiratory system (paper I). The overall clinical score corresponded well with the shedding of viral RNA the first three weeks after challenge. General depression and cough were the signs that correlated best with shedding of BCoV RNA, while peak respiratory rate and peak rectal temperature appeared more than a week later than the peak shedding. Nasal shedding preceded fecal shedding, and the calves had detectable amounts of viral RNA intermittently in feces through day 35 and in nasal secretions through day 28. However, virus isolation was unsuccessful from day six and day 18 from the two calves investigated. Viral RNA was not detected in blood, but was found in lymphatic tissue through day 42 after challenge. The sentinel calves that were introduced after three weeks were not infected. Even though calves shed viral RNA for many weeks, the studies in cell culture and live animals indicated a much shorter infectious period.

To investigate the mechanisms of indirect transmission, the presence of BCoV and BRSV RNA in human nostrils and clothes, boots and equipment after contact with infected calves were determined (paper II). For BCoV, 46% (n = 80) of the swabs from human nasal mucosa collected 30 min after exposure were positive by RT-qPCR. After two, four and six hours, 15%, 5% and 0% of the swabs were positive, respectively. Infective virions were not detected in mucosal swabs (n = 2). A high viral RNA load was detected on 97% (n = 44) of the coats,

boots, wristwatches and stethoscopes 24 h after exposure, and infective virions were detected in two of three samples. For BRSV, 35% (n = 26) of the human nasal mucosa swabs collected 30 min after exposure, were positive for BRSV RNA, but none were positive for infective virions. Of the boots and coats, 89% (n = 38) were positive for BRSV RNA 24 h after exposure, but all were negative for infective viruses. Altogether, the results from human nasal mucosa indicate short-lived carriage of virus RNA, no infective viruses and low importance for inter-herd transmission. Contaminated equipment on the other hand, appears to be of significance, particularly for BCoV. Based on the results from this study, herd-specific clothing and the disinfection of equipment transferred between farms are highly recommended to reduce the risk of inter-herd transmission.

The tracing of infections by molecular methods increases the opportunity to detect and rule out possible transmission routes. Two sequence-independent methods for amplification of viral RNA coupled with high throughput sequencing were compared regarding generation of the full-length genome of BCoV from a nasal swab (paper III). Both methods, single primer isothermal amplification (SPIA) and sequence-independent single primer amplification (SISPA), achieved high genome coverage (100% for SPIA and 99% for SISPA); however, there was a clear difference in the percentage of reads that mapped to BCoV. While approximately 45% of the SPIA reads mapped to BCoV (sequence depth of 169–284 944), only 0.07% of the SISPA reads (sequence depth of 0–249) mapped to the reference genome. The SPIA method represents a practical and efficient method for whole genome sequencing of BCoV from clinical samples.

In conclusion, the study provides information that is useful with regard to producing scientifically-based biosecurity advice, and enables detailed molecular studies of BCoV epidemiology that can be used to further explain dispersal patterns.

Norsk samandrag

Bovint coronavirus (BCoV) er ein viktig årsak til luftvegssjukdom og diaré hos kalvar og vinterdysenteri hos vaksne. Desse sjukdommane fører til store økonomiske tap og redusert dyrevelferd hos storfe over heile verda. Smitte mellom besetningar kan skje ved direkte kontakt mellom levande dyr eller indirekte, ved personar og utstyr som drar mellom besetningar. Denne avhandlinga tar for seg smitteoverføring av viruset, både mellom dyr og via forureina utstyr og personar, og metodar for å spore virusoverføringar.

Eit dyreforsøk med kalvar smitta med BCoV blei gjennomført. Fire BCoV-antistoff-negative kalvar vart oppstalla saman med seks naturleg infiserte kalvar. Desse seks kom frå ein besetning med akutt vinterdysenteri. Tre veker seinare blei to naive sentinelkalvar introdusert. Prøver vart samla inn frå kalvane, frå støvlar, frakk og utstyr og nasen til folk som hadde vore i kontakt med kalvane. Prøvematerialet blei analysert for viralt RNA med RT-qPCR og utvalgte prøver blei dyrka i cellekultur for å sjå etter infektive virus. Resultat frå eit liknande dyreforsøk med bovint respiratorisk syncytialvirus (BRSV) vart inkludert i artikkel II.

Dei BCoV-infiserte kalvane viste milde kliniske symptom, med dei tydelegaste symptoma frå luftvegane (artikkel I). Klinisk score korresponderte bra med utskiljinga av viralt RNA dei første tre vekene etter dyra vart smitta. Nedstemtheit og hoste var teikna som korresponderte best med utskiljinga av BCoV RNA, mens den raskaste respirasjonsraten og høgste temperaturen oppstod meir enn ei veke etter maks utskiljing. Enkelte av kalvane skilte ut viralt RNA frå nasen av og på i 28 dagar, og i avføring i 35 dagar, men viruset var ikkje mogleg å dyrke frå prøver tatt frå dag seks og frå dag 18 i dei to kalvane der dette blei undersøkt. Viralt RNA blei ikkje påvist i blod, men vart funne i lymfeknutar heilt til dag 42 etter eksponering. Sentinelkalvane som var introdusert etter tre veker blei ikkje smitta. Sjølv om kalvar kan skilje ut virus RNA i mange veker, viser studiane i cellekultur og med levande dyr, at den infeksiøse perioden er mykje kortare.

For å undersøke mekanismane bak indirekte smitte med personar og utsyr, vart førekomsten av BCoV og BRSV RNA på naseslimhinna til menneske og utstyr som hadde vore i kontakt med smitta kalvar, undersøkt. For BCoV var 46% (n=80) av svaberane frå menneskeslimhinne tatt 30 minutt etter kontakt med infiserte kalvar positive med RT-qPCR. Etter to, fire og seks timar var 15%, 5% og 0% av svaberane positive. Infektive virus vart ikkje funne. Mykje virus RNA vart påvist på 97% (n=44) av frakkane, støvlane, klokkene og

stetoskopa 24 timar etter kontakt med kalvane, og infektive virus vart funne i to av tre prøver. For BRSV var 35 % (n=26) av svaberane frå naseslimhinna hos menneske 30 minutt etter kontakt med infiserte kalvar positive, men ingen inneheldt infektive virus. Resultata viser at personar kan bere BCoV og BRSV RNA ein kort periode i nasen, men at det mest sannsynleg har lita tyding for smitteoverføring. Klede, støvlar og utstyr derimot, ser ut til å vere viktig for indirekte smitte, særleg for BCoV. Basert på desse resultata er det sterkt anbefalt å ha eige overtrekksklede og skotøy i kvar besetning, og desinfisere utstyr mellom bruk i ulike besetningar for å hindre overføring av BCoV og BRSV.

For å betre kunne spore BCoV smitte mellom besetningar, trengs ein metode for fullgenomsekvensering av viruset. To sekvensuavhengige metodar for oppformeiring av viralt RNA kopla med Illumina-sekvensering blei samanlikna med tanke på fullgenomsekvensering av BCoV frå ein nasesvaber (paper III). Begge metodane, med kortnamna SPIA og SISPA, gav tilnærma full dekning av virusgenomet (100% for SPIA og 99% for SISPA), men det var ein tydeleg forskjell i kor stor prosentdel av sekvensane som høyrte til BCoV. Mens ca. 45% av SPIA-sekvensane høyrte til BCoV, var det tilsvarande talet for SISPA berre 0,07%. Sekvensdjupet var 169-284 944 for SIPA og 0-249 for SISPA. Resultata viser at SPIA-metoden er ein god og praktisk måte å fullgenomsekvensere BCoV frå kliniske prøver.

Kort fortalt gir avhandlinga informasjon som er nyttig for å utforme evidensbaserte råd og kontrolltiltak mot smitteoverføring av BCoV. I tillegg skildrar vi ein metode som gjer detaljerte molekylære studiar av BCoV mogleg.

Introduction

Background

Bovine coronavirus (BCoV) was first described as the cause of neonatal calf diarrhea in the early seventies (Mebus et al., 1973; Stair et al., 1972). Later, it was recognized as the causative agent of winter dysentery (Saif, 1990), and there is now evidence of association with respiratory disease (Murray et al., 2016b; O'Neill et al., 2014; Saif, 2010; Storz et al., 2000a; Storz et al., 2000b). These diseases have severe negative impacts on animal welfare and performance, and are found with high prevalence in cattle all over the world. Diarrhea causes decreased growth rate and food utilization and increased mortality in calves (Boileau and Kapil, 2010). Winter dysentery, i.e. contagious diarrhea in adult cattle, has significant impact on milk production, growth rate, and animal welfare (Jactel et al., 1990; Toftaker et al., 2017). Bovine respiratory disease (BRD) is one of the major challenges for cattle welfare and efficient production worldwide, and the main cause of antibiotic treatment due to secondary bacterial infections (Edwards, 2010). Financial losses are due to mortality, reduced production, and growth performance, and the costs of additional handling and treatment.

The Norwegian cattle industry

Norwegian agriculture is undergoing structural changes. Production units are still mostly small and family-run; in 2016, dairy farms had on average 26 cows, while farms raising cattle for beef had 16 (Statistics Norway, 2017). However, there is a trend towards larger herds, with higher levels of specialization. The emergence of specialized beef production over the past decade is a result of beef shortages, due to higher milk yields from the dual-purpose Norwegian Red cow (Ruud et al., 2013). This has given rise to both suckler cow herds and fattening units for bull calves. The increase in herd sizes and decrease in the number of dairy herds is occurring across the developed world; this is increasing the potential for the spread of infectious diseases, as within-herd spread is facilitated by more animal-to-animal contact in larger herds, and between-herd spread increases with more animal movements between specialized herds (Barkema et al., 2015).

A high proportion of cattle producers are members of cooperative organizations, and more than 95% of Norwegian dairy and suckler cows are registered in either the Norwegian dairy herd recording system or the Norwegian beef cattle recording system (Animalia, 2017; Espetvedt et al., 2013). The use of vaccines is less common than in many other countries, as is metaphylaxis (mass antibiotic treatment), and all medical treatment must be initiated by a

veterinarian. Norway has eradicated several infectious diseases through control programs based on classification of closed herds and is, as a result, free of diseases like bovine virus diarrhea (BVD), bovine tuberculosis, and brucellosis (Hofshagen et al., 2017; Løken and Nyberg, 2013). Surveillance programs document the continued absence of these diseases, in addition to bovine rhinotracheitis and enzootic bovine leucosis (Hofshagen et al., 2017). Paratuberculosis is detected sporadically, while *Mycoplasma bovis* has never been detected (Hofshagen et al., 2017; Mørk et al., 2016).

Despite the absence of many common pathogens, diarrhea and BRD remain significant problems. These are the most frequent diseases of calves in Norway (Gulliksen et al., 2009d) and the main causes of calf mortality (Gulliksen et al., 2009c). Each year, epidemics of winter dysentery, BRD and calf diarrhea cause adverse effects on milk production, growth rate, and animal welfare, which in turn lead to severe financial losses for the cattle industry.

Bovine respiratory syncytial virus (BRSV) have been found to be the main etiologic agent behind outbreaks of BRD in Norway (Klem et al., 2014), and shares many characteristics with BCoV. The prevalence of both BCoV and BRSV is high in Norwegian cattle (Gulliksen et al., 2009b; Toftaker et al., 2016). The viruses are amongst the most important BRD pathogens in Norway, typically cause epidemics during the winter season, and it is likely that the two viruses share transmission routes and many risk factors. The present situation in Norway, with few known pathogens and transparent cooperative production systems, enables a proactive approach towards the most important pathogens.

Thesis background

Due to the substantial economic losses and welfare problems described above, a four-year-long research project financed by the Norwegian Research Funding for Agriculture and Food Industry was established in 2013, with the overall aim to establish the knowledge needed to choose the best control strategy for BCoV and BRSV in Norway. The project included two PhD students, one engineer and one researcher and the work presented in this thesis was produced within the research project.

In 2016, a national control program was launched in Norway as a joint initiative by all the main cattle organizations and the findings from the research project was implemented in the program.

The virus

BCoV belongs in the order *Nidovirales*, family *Coronaviridae*, genus *Betacoronavirus* and species *Betacoronavirus 1*. The *Betacoronavirus 1* species also includes human enteric coronavirus, human coronavirus-OC43, porcine hemagglutinating encephalomyelitis virus, equine coronavirus, and canine respiratory coronavirus (International Committee on Taxonomy of Viruses, 2017). Most domestic species and humans have significant diseases caused by coronaviruses, many of which are severe, with some being highly prevalent. An overview of some important coronaviruses, their host species, and the diseases they cause is presented in Table 1.

Table 1 – Overview of some important coronaviruses, their hosts and typical attendant diseases.

Host	Virus	Genus	Disease
Cat	Feline infectious peritonitis virus	α	Enteritis, Peritonitis
Swine	Transmissible gastroenteritis coronavirus	α	Gastroenteritis
Swine	Porcine respiratory coronavirus	α	Respiratory disease
Dog	Canine enteric coronavirus	α	Gastroenteritis
Human	Human coronaviruses 229E	α	Respiratory disease (common cold)
Swine	Porcine epidemic diarrhea virus	α	Enteritis
Dog	Canine respiratory coronavirus	β	Respiratory disease
Human	Human coronavirus OC43	β	Respiratory disease (common cold)
Swine	Hemagglutinating encephalitis virus	β	Vomiting and wasting disease
Cattle	Bovine coronavirus	β	Winter dysentery, enteritis, respiratory disease
Human	Severe acute respiratory syndrome coronavirus	β	Severe acute respiratory syndrome (SARS), gastroenteritis
Human	Middle East respiratory syndrome coronavirus	β	Middle East respiratory syndrome (MERS), pneumonia, gastroenteritis
Mouse	Murine hepatitis virus	β	Progressive demyelinating encephalitis, hepatitis
Chicken	Avian infectious bronchitis virus	δ	Bronchitis
Turkey	Turkey coronavirus	δ	Enteritis/Bluecomb disease

Since its discovery in the early seventies, BCoV has been described as an enveloped virus of approximately 100–150 nm in size (Stair et al., 1972). The BCoV genome consists of a single stranded non-segmented positive-sense RNA of approximately 31-kilo bases with 13 open reading frames (Figure 1). Coronaviruses contain the longest RNA genomes, and are almost twice the length of RNA genomes in viruses outside the order *Nidovirales* (Gorbalenya et al., 2006). Two-thirds of the genome in the 5' end is the open reading frame (ORF) 1, which encodes 16 mature proteins important for RNA synthesis (Denison et al., 2011). Most of these nonstructural proteins (nsps) are highly conserved in coronaviruses. Their function has

primarily been studied in severe acute respiratory syndrome coronavirus (SARS-CoV), and is shown in Table 2.

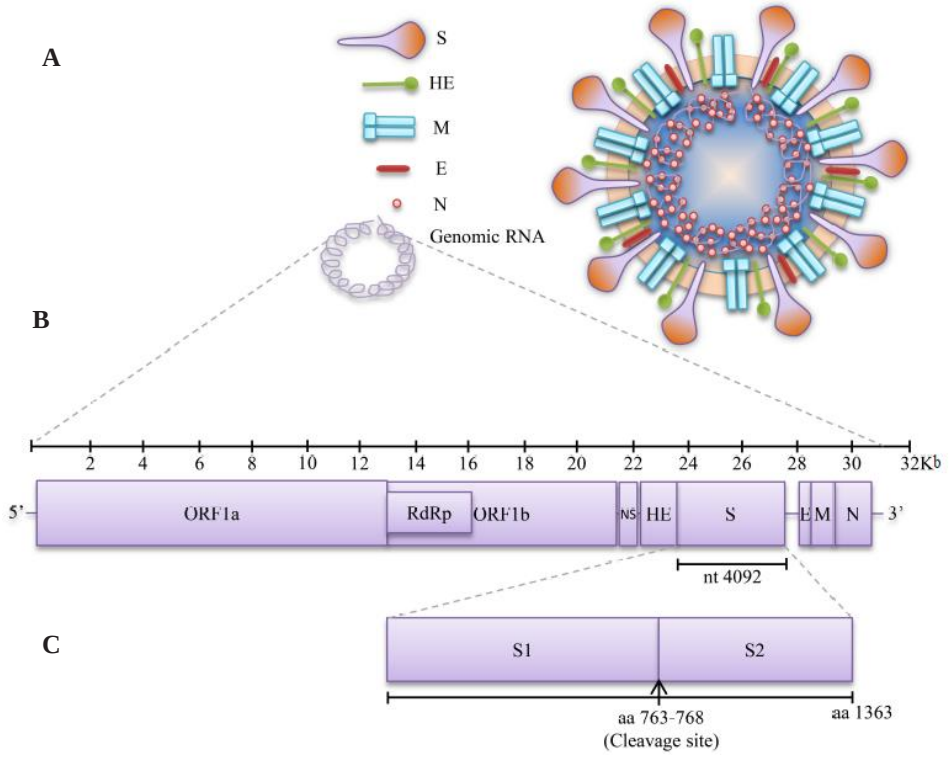


Figure 1 – Bovine coronavirus and its genome. A) Schematic structure of the virus particle; B) *Genome organization of bovine coronavirus;* and C) *Spike glycoprotein. Modified with permission from Bidokhti (2013).*

The genome also encodes five major structural proteins: the nucleoprotein (N), membrane glycoprotein (M), envelope protein (E), hemagglutinin-esterase glycoprotein (HE), and spike glycoprotein (S) (Lai, 1990; Saif, 2010; Siddell, 1995). The S protein has domains that mediate cell entry and agglutinate red blood cells. It consists of two subunits, S1 and S2. S1 binds the virus to the host cell receptors and is a target for neutralizing antibodies (Cavanagh, 1995). S2 mediates fusion of the viral envelope to the host cellular membranes (Cho and Yoon, 2014; Hansa et al., 2013).

Table 2 – Proposed functions of coronavirus non-structural proteins (nsps).

Name	Function
Nsp1	Suppressor of host protein synthesis, results in blocking innate immune response (Kamitani et al., 2009; Kamitani et al., 2006)
Nsp2	Unknown function (Fehr and Perlman, 2015; Graham et al., 2006)

Nsp3	Large, multi-domain transmembrane protein with several activities. ADP-ribose-1’’-phosphatase activity (Kuri et al., 2011). Papain-like proteases which cleaves nsp1, nsp2 and nsp3 of the replicase polyprotein (Fehr and Perlman, 2015; Neuman et al., 2008)
Nsp4	Important for organization and stability of double membrane vesicles (Gadlage et al., 2010)
Nsp5	Main protease, cleaving 11 sites of the replicase polyprotein (Fehr and Perlman, 2015; Lu et al., 1995; Thiel et al., 2003)
Nsp6	Potential transmembrane scaffold protein (Fehr and Perlman, 2015; Oostra et al., 2008)
Nsp7	Binds ssRNA. Forms complex with nsp8, may act as processivity clamp for RNA polymerase (Fehr and Perlman, 2015; Zhai et al., 2005)
Nsp8	Primase, produces RNA primers, enzyme important for polymerase initiation (Imbert et al., 2006; Perlman and Netland, 2009). Forms complex with nsp7, may act as processivity clamp for RNA polymerase (Fehr and Perlman, 2015; Zhai et al., 2005)
Nsp9	Binds RNA, interacts with nsp7 and nsp8 (Sutton et al., 2004; Zhai et al., 2005)
Nsp10	Cofactor for nsp16 and nsp14, promotes their activity (Bouvet et al., 2010; Smith et al., 2015)
Nsp11	Unknown function (Perlman and Netland, 2009)
Nsp12	RNA-dependent RNA polymerase (te Velthuis et al., 2010)
Nsp13	Helicase (Ivanov et al., 2004b; Seybert et al., 2000)
Nsp14	Exoribonuclease, important for proofreading (Eckerle et al., 2010; Eckerle et al., 2007; Minskaia et al., 2006)
Nsp15	Endoribonuclease (Bhardwaj et al., 2004; Bhardwaj et al., 2006; Ivanov et al., 2004a; Kang et al., 2007)
Nsp16	RNA methyltransferase, protects viral RNA from recognition and interferon induction (Decroly et al., 2008; Züst et al., 2011)

Replication fidelity

RNA viruses are known to have high rates of mutation and recombination. Their RNA dependent RNA polymerase (RdRp) lacks proofreading, which results in high frequencies of mutation (low fidelity). It has been proposed that multiple nsps in coronaviruses interact to perform RNA modifications in order to improve fidelity (Gorbalenya et al., 2006). The large genome size could otherwise make coronaviruses prone to fatal errors during replication (Smith and Denison, 2012). In particular, nsp14 has been shown to maintain high-level replication fidelity in several coronaviruses, and likely plays a role in RNA-dependent error recognition, prevention, and repair (Eckerle et al., 2010). Recombination is also common among coronaviruses and may be a way of generating diverse *and* functional RNA molecules (Lai, 1992).

Quasispecies

Like other RNA viruses, BCoV consists of quasispecies, namely a swarm of viruses similar but not identical to the main or "consensus" sequence (Eigen, 1993; Lauring and Andino, 2010). It is believed that BCoV evolves through quasispecies, and that some of the viruses are better suited for replication in, e.g. the respiratory rather than the enteric tract, and contribute to the genetic differences between viruses (Borucki et al., 2013; Saif, 2010; Zhang et al., 2007).

Molecular epidemiology and genetic variation

The molecular epidemiology of BCoV has been studied in many parts of the world, and most of the studies are based on the sequencing of parts of the S gene. Bok et al. (2015) found that seven Argentinean samples from diarrheic calves formed a cluster distantly related to the prototype strain Mebus, but belonging to the same serotype. Martinez et al. (2012) found that diarrheic samples from Cuba clustered together with US strains, suggesting a common origin. Lojkić et al. (2015) found that one strain of BCoV circulated for an extended period in one herd, indicating herd persistence for two years, while Liu et al. (2006) found that different strains circulated during four outbreaks in different years in the same herd, suggesting new introductions of the virus. These results show that sequencing BCoV can be a useful tool for understanding transmission dynamics and identifying transmission routes. As BCoV infections are endemic and highly prevalent, single gene sequencing may not provide sufficient resolution for distinguishing between closely related strains.

It is still uncertain as to whether different virus strains cause different clinical syndromes (Boileau and Kapil, 2010). Genetic differences have been found between so-called enteric and respiratory strains (Chouljenko et al., 1998; Fulton et al., 2013); however, even though some groups have found the same amino acid alterations in respiratory versus enteric strains (Chouljenko et al., 1998; Yoo and Deregt, 2001), others have not (Hasoksuz et al., 2002; Kanno et al., 2007). It is therefore likely that the differences are due to geographic and time variations and do not correspond solely to clinical differences (Hasoksuz et al., 1999). Kanno et al. (2007) found no specific genetic markers of pathogenicity and clinical signs in a 150 bp sequenced region of the S gene. In the same study, they found a high sequence identity of the HE gene in 55 samples from cattle with enteric or respiratory disease (Kanno et al., 2007). Bidokthi et al. (2012) found a high sequence identity regardless of clinical origin in samples from Sweden. These results show that further studies of a larger fraction of the BCoV genome are necessary to ascertain if genetic differences can be linked to clinical syndromes.

Given that recombination events are common among coronaviruses, it is necessary to consider the full genome, or at least several genes, when studying strain differences (Lau et al., 2011; Pyrc et al., 2006). Although Kin et al. (2016) detected fewer recombinations between BCoVs than between HCoV-OC43s, recombination should be further studied to understand the evolution and dynamics of BCoV.

Next generation sequencing

Sanger sequencing, developed 40 years ago, is a robust technology for sequencing DNA fragments of up to 1000 nucleotides. This method has long been applied in research to characterize and classify viruses. Among its limitations is the fact that it requires sets of strain-specific primers for PCR amplification, which can be time-consuming and inefficient when sequencing large viral genomes, such as that of BCoV. Unknown or new sequences can be lost. Next generation sequencing (NGS) can be used to screen environmental and clinical samples for the presence of viral pathogens, without prior knowledge about which sequence to target. NGS could also be used to study quasispecies within single hosts and samples. However, to achieve coverage across large parts of the viral genome from clinical samples, pre-amplification is usually necessary. Such amplifications have in many instances been by propagation in cell culture (Chouljenko et al., 2001) or by specific primers (Borucki et al., 2013). Propagation in cell culture can alter the genome sequence (Borucki et al., 2013) and is not suitable for comparing nucleotide differences from clinical samples. Amplification using BCoV-specific primers can be cumbersome, and novel variants can be missed. There is a lack of reliable and efficient methods of whole genome sequencing of BCoV.

Epidemiology

Occurrence

Antibodies to BCoV have been detected all over the world, with a high prevalence (80–100%) (Boileau and Kapil, 2010; Ohlson et al., 2013; Paton et al., 1998). BCoV nucleic acid is also commonly detected in a variety of investigations, from both clinically affected and unaffected cattle. It was detected in 40% of nasal swabs from 1484 apparently healthy cattle before export from Australia (Moore et al., 2015) and 40% of nasal swabs from cattle in Quebec, without significant association with respiratory disease (Francoz et al., 2015). BCoV RNA was found in 22% of nasal swabs from cattle with BRD in an investigation in Brazil (Headley et al., 2018) and in 32% of diarrheic samples from calves in the US (Cho et al., 2013).

The prevalence of BCoV is also high in Norwegian cattle. In 2016, antibodies to BCoV were found in 84% of bulk tank milk samples from dairy herds in a national screening (to be published). The seroprevalence among calves was estimated to be 39% (Gulliksen et al., 2009a).

Persistence of infection

It is not clear how long the virus can persist in individuals and how the virus persists in populations. It has been proposed that BCoV causes persistent subclinical infections that could constitute an important virus source for other animals in a herd (Clark, 1993). This is primarily based on the findings that ten clinically normal cows were shedding BCoV-immune complexes in their feces for twelve weeks (Crouch et al., 1985), and adult cows were frequent shedding BCoV during the winter months in a dairy herd (Collins et al., 1987). However, it was not investigated whether the shedding occurred due to persistence or reinfection in the cows, and it was not determined if the detected antigen represented an infective virus. Kapil et al. (1991) documented viral antigens in the small and large intestines of infected calves three weeks post-inoculation, which indicated a long duration of infection. Again, infectivity was not determined. Another hypothesis is that the virus persists in populations by reinfections in cattle that occasionally transmit the virus to naïve animals (Boileau and Kapil, 2010; Saif, 2010).

Most investigations of BCoV shedding last for less than 21 days, and do not determine the infectivity of the shed virus (Cho et al., 2001a; El-Kanawati et al., 1996; Saif, 1987; Saif et al., 1986; Tsunemitsu et al., 1999). The question of persistent BCoV infections therefore remains unsolved. Investigations of prolonged shedding of BCoV should be in a closed environment, allowing for control of reinfections, and last for more than three weeks. It is also necessary to study the infectivity of the shed virus, in order to determine if BCoV creates persistent infections that pose an infection risk to other cattle. This is important, in order to choose efficient prevention strategies. If BCoV persists in individuals, preventing transmission between herds might be insufficient for controlling infections.

Host species

Although cattle are the main reservoirs for BCoV, the virus shows a broad host range and can infect turkeys (Ismail et al., 2001), dogs (Kaneshima et al., 2007), sheep (Tråvén et al., 1999), and wild and captive ruminants (Saif, 2010). Additionally, BCoV-like viruses found in camelids (Genova et al., 2008), horses (Barros et al., 2013; Guy et al., 2000), deer (Alekseev

et al., 2008), elks (Majhdi et al., 1997), giraffes (Hasoksuz et al., 2007), and water buffalos (Decaro et al., 2008d) may be able to infect cattle because of their resemblance to BCoV. Other species may therefore constitute a virus source for cattle and, from a biosecurity perspective, contact should be kept to a minimum. However, the importance of transmission from other animal species is most likely low compared to transmission from cattle.

Human enteric coronavirus (HECoV) 4408 that was isolated from a diarrheic child was closely related biologically and genetically to BCoV (Zhang et al., 1994), and was used to infect and induce seroconversion to BCoV in calves (Han et al., 2006). This strain may therefore be a BCoV that is able to infect humans (Saif, 2010). Phylogeny and molecular clock analyses show that HCoV OC-43 emerged during a zoonotic event from cattle to humans around 1890 (Vijgen et al., 2005). Although it is clear that species other than cattle can serve as reservoirs and vectors for BCoV, replication of BCoV in humans has not been explored.

Transmission

Within-herd transmission

The virus is highly contagious and rapidly spreads within herds (Alenius et al., 1991; Bidokhti et al., 2009). Transmission is possible through both the fecal–oral and the respiratory route (Heckert et al., 1991c; Saif et al., 1986). The predominant mode of transmission is through direct contact between animals. As there is a lack of experimental studies investigating virus shedding for longer than two weeks with sensitive detection methods, the duration of virus shedding and persistence in infected animals is uncertain. This knowledge gap hampers the prevention of infection, as guidance on biosecurity and animal movements is difficult to provide.

Related human coronaviruses are transmitted primarily through droplets, but there are also strong indications of airborne transmission, via aerosols (Booth et al., 2005; La Rosa et al., 2013). Thus, it is likely that airborne transmission can facilitate BCoV spread over short distances within a herd.

Transmission between herds

As BCoV is endemic, transmission through animal-to-animal contact due to livestock movements is an apparent risk of inter-herd transmission. Indirect transmission through fomites is also potentially important for the spread of BCoV between farms. Some descriptions of epizootics report that indirect transmission by humans or equipment was

suspected to be the cause of winter dysentery outbreaks (Hedström and Isaksson, 1951; Roberts, 1957), but little has been done to confirm these suspicions. Ohlson et al. found that not providing boots for visitors was associated with seropositivity for BCoV and BRSV in Swedish dairy farms (2010). Toftaker et al. found that a herd's bulk tank milk BCoV antibody status was influenced by the status of its neighboring herds (2016). These epidemiological studies point out the probable importance of indirect transmission, but it is yet to be explored in experimental studies. It is not known which passive vectors are important for transmission, e.g. humans, equipment, boots or clothing transferred between farms. Airborne transmission is most likely of minor importance for inter-herd transmission.

Stability in the environment

An important factor for indirect transmission is the ability of the virus to remain infective outside the host. Preservation of infectivity strongly depends on environmental factors like temperature, humidity, pH and UV-radiation. BCoV remains infective in solution at low temperatures for several weeks (Mullis et al., 2012) and related coronaviruses can survive after drying on surfaces for extended periods, even months (Otter et al., 2016). BCoV is resistant to acid pH as low as 4.6 (Panon et al., 1988) and is concentrated by clay and remains infective bound to the minerals *in vitro* (Clark et al., 1998). Infectivity in diarrheic feces may be better preserved than in normal feces, as has been found for SARS-CoV (Lai et al., 2005). The virus is thus most likely stable enough for indirect transmission to occur, but for how long remains to be investigated.

Pathogenesis

BCoV has a dual enteric and respiratory tropism. During experimental infections in seronegative animals, it has been suggested that the virus transmitted by aerosols starts replicating in the epithelium of the upper respiratory tract, whereas after oral inoculation, replication starts in the small intestine (Park et al., 2007; Saif et al., 1986). There is still uncertainty as to whether the virus spreads from the respiratory to the enteric tract through the swallowing of the virus, as proposed by some researchers (Clark, 1993; Saif, 2010), or through viremia, as found by others (Park et al., 2007).

In the respiratory tract of cattle, BCoV replicates in epithelial cells in the nasal turbinates, trachea and lungs, causing loss of cilia and potentially inducing interstitial pneumonia (Park et al., 2007). Enteric infection starts in the proximal small intestines and spreads throughout the small and large intestines (Clark, 1993). Replication destroys epithelial cells in the villi,

which are replaced by immature cells. This leads to fusion of adjacent villi, loss of microvilli, and atrophy of colonic ridges. The absorptive capacity is reduced, and the immature cells do not secrete normal digestive enzymes. Undigested food and lactose lead to increased microbial activity and osmotic imbalance, which draw more fluid to the gut lumen. The epithelial destruction can also lead to transudation of blood and hemorrhagic diarrhea (Boileau and Kapil, 2010).

Immune response

The immune response against BCoV infection has implications both for protection against disease and for diagnostics. The virus constitutes a single serotype with some antigenic variation between different strains (Clark, 1993; El-Ghorr et al., 1989). Acutely infected animals develop antibodies that persist for a long period, possibly for several years (Alenius et al., 1991; Lin et al., 2002; Tråvén et al., 2001). The protective immunity, however, is shorter. In two experimental studies, infected calves were not protected against reinfection with BCoV from different clinical origins, three weeks after the first challenge, but did not develop clinical signs (Cho et al., 2001a; El-Kanawati et al., 1996). In herds where BCoV-related disease is seen only in young individuals, cows are probably immune to the virus (Tråvén, 2000). Partial immunity may also be the reason for variable clinical signs of BCoV disease (Tråvén et al., 1993).

The mechanisms behind immunity against BCoV are still not fully understood. One animal experiment showed that high monoclonal antibody titers against HE and S proteins prevented attachment and infection in calf intestines (Deregt et al., 1989), and several field studies have shown that high titers of IgG antibodies to BCoV in serum are associated with protection against disease (Cho et al., 2001b; Heckert et al., 1991c). However, it is still uncertain whether protection is due to the serum antibodies themselves, or if the antibodies merely reflect previous exposure to BCoV (Saif, 2010). Heckert et al. found that passively derived maternal antibodies delayed and decreased systemic and mucosal antibody responses in calves, without hindering respiratory and enteric infections (1991a; 1991b).

Clinical signs

Infection with BCoV can be subclinical or result in severe disease. The severity of clinical signs depends on a wide range of factors, like the age of the animal, the amount of virus exposure, stress, coinfections, environmental conditions, and the immunologic status of the animal (Boileau and Kapil, 2010; Saif, 2010).

Respiratory disease caused by BCoV is usually mild, but mortality can be high in some outbreaks (Decaro et al., 2008a; Storz et al., 2000a). Clinical signs can occur in cattle of all ages and include discharge from the eyes and nose, fever, coughing, and increased respiratory rate (Saif, 2010). The respiratory signs can be accompanied by diarrhea. BCoV is, in addition to other viral, bacterial and mycoplasmal agents, implicated in multifactorial BRD and shipping fever. BRD develops in connection with stress due to weaning, shipping, commingling, dietary, and environmental changes. These factors favor the development of viral infections of the respiratory tract, which can be further complicated by bacterial infections. Other important viral pathogens in the development of BRD include bovine herpesvirus 1, bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV), and parainfluenza virus type 3 (PIV-3) (Boileau and Kapil, 2010; Lin et al., 2000; Murray et al., 2016b).

Diarrhea in calves caused by BCoV typically occurs between days 5 and 30 after birth, but can also occur earlier or later. This diarrhea is often more severe than rotavirus diarrhea, and is described as a yellow to blood-stained, mucus-containing diarrhea that can develop into profuse watery diarrhea (Bridger et al., 1978; Mebus et al., 1975; Saif et al., 1986). The majority of calves recover spontaneously, but disease can result in death if the diarrhea is severe and the animal is not treated (Izzo et al., 2015; Lewis and Phillips, 1978). Internationally, BCoV is recognized as one of the most important enteropathogens of acute diarrhea in young calves (Boileau and Kapil, 2010). There is little data on the impact of BCoV calf diarrhea in Norway, and the virus is seldom detected in diarrheic calves (Gulliksen et al., 2009a). As proposed by Tråvén, management could have an impact on the severity of the disease (2000).

Winter dysentery is characterized by acute diarrhea in multiple adult cows. The diarrhea may contain mucus and/or blood and is often preceded by pyrexia and loss of appetite, followed by a marked reduction in milk yield, which is still reduced four months after an outbreak (Macpherson, 1957; Toftaker et al., 2017). The disease has high morbidity (50–100%) and low mortality (1–2 %) (Van Kruiningen et al., 1985). Affected animals may also have nasolacrimal discharge and coughing. Often, calves and young stock are not affected, or have milder signs than lactating animals (Rollinson, 1948; Tråvén et al., 2001). In herds previously exposed to the virus, infections can be subclinical, whereas naïve herds often exhibit severe clinical signs. Outbreaks are mostly reported during the winter season, but also occur during the summer (Decaro et al., 2008c; Tråvén et al., 1993). Co-infections, corticosteroids, and

stressors—such as shipping and inclement weather—can exacerbate disease (Boileau and Kapil, 2010; Saif, 2010).

Diagnosis

All manifestations of BCoV disease require laboratory analyses for etiological diagnosis, as the clinical signs are non-specific. To diagnose acute infection, the most common methods currently used include detection of virus RNA in nasal swabs or feces, and detection of a titer rise of anti-BCoV antibodies in serum. In addition, screening of bulk tank milk or pooled milk samples for antibodies to BCoV can be used to screen large number of samples for previous exposure to the virus (Ohlson et al., 2013). For research purposes, the virus can be isolated in cell culture and a human cell-line (human rectal tumor cells, HRT-18) is most commonly used. RT-PCR (reverse transcription polymerase chain reaction) is a sensitive and routinely used method to detect virus nucleic acid in nasal and fecal specimens. Real-time RT-PCR (RT-qPCR) is a sensitive and specific detection method, and is less laborious than traditional gel-based RT-PCR (Decaro et al., 2008b). Samples for BCoV RNA detection should be collected at disease onset or shortly thereafter, and ideally transported on ice to a laboratory (Saif, 2010). On-farm antigen tests are available, but may have low sensitivity (Klein et al., 2009). Immunological methods for detecting specific antibodies to BCoV in milk or sera include virus neutralization test (VNT) (Fulton et al., 2013), hemagglutination inhibition (HI) (Lin et al., 2001) and, most commonly used, enzyme-linked immunosorbent assay (ELISA) (Alenius et al., 1991). As BCoV-specific IgG is maintained long after infection, a titer rise in paired samples is necessary for diagnosis of recent infection. Alternatively, BCoV IgM, which is present in serum for approximately one month after infection, could be used for diagnosis of acute infections (Tråvén et al., 2001).

Prevention

Given that disease development depends on several factors with regard to the host, agent, and environment, disease prevention does, too. By increasing immunity, decreasing pathogen exposure and optimizing environmental conditions, disease incidence can be reduced. Increased resistance and immunity of the animals can be achieved by improved calf health management: for instance, correct feeding of colostrum and reduced group size and age span. Internationally, vaccination, metaphylactic treatment with antimicrobials and management factors to improve calf health have been the traditional ways to prevent and handle disease (Murray et al., 2016a).

Few coronavirus vaccines are available, and it has proven difficult to develop safe vaccines that protect against disease, despite substantial efforts, e.g. against SARS-CoV (Tseng et al., 2012). No commercially available vaccine is labeled for prevention of winter dysentery or respiratory disease caused by BCoV. However, one modified live virus vaccine against BCoV and rotavirus (Calf-Guard, Zoetis) administered intranasally appeared to reduce the risk of treatment for BRD in a randomized clinical trial (Plummer et al., 2004). Another trivalent vaccine containing inactivated BCoV, rotavirus, and E. coli for immunizing pregnant cows and providing passive immunity against neonatal diarrhea in calves (Lactovac, Zoetis) increases antibody titers to BCoV in colostrum and serum, but the clinical effects against calf diarrhea are not well documented (Kohara et al., 1997; Waltner-Toews et al., 1985). Although there is only one serotype of BCoV, vaccine strains differ from circulating strains (Fulton et al., 2013; Gunn et al., 2015) and might not provide protection. Vaccination against bacterial and viral agents implicated in BRD is widely practiced, although the evidence of efficacy is often lacking (Murray et al., 2016a; Theurer et al., 2015).

The Norwegian control program

A national control program against BRSV and BCoV was launched in Norway in 2016. The program is carried out as a cooperation between all the major actors in the Norwegian cattle industry: TINE SA, the Norwegian Cattle Health Services, Nortura, the Norwegian Independent Meat and Poultry Association (KLF), Q Dairies, the Norwegian Meat and Poultry Research Centre (Animalia), the Norwegian Beef Breeders Association (Tyr), and Geno SA. For farmers, the program is voluntary. The goal is to reduce the prevalence of the two infections in the Norwegian cattle population. In brief, cattle herds are screened yearly for specific antibodies to BCoV and BRSV in bulk tank milk, milk from first parity cows, or serum from young stock older than five months of age. Herds are classified as either positive or negative based on the serology results. The positive herds are considered to have a higher risk of virus circulation than the negative herds. A key element of the program is to protect the negative herds against exposure to the viruses. This is done by strengthening biosecurity measures in negative herds and facilitating safe transport of livestock between negative herds, without contact with animals from positive herds. In addition, all farmers are encouraged to improve biosecurity routines that can reduce the probability of introducing the viruses into the herds, like providing boots and clothing for visitors, e.g. vets, technicians, advisors, and others. Outbreaks of diarrhea and BRD are reported by farmers and veterinarians to a notification hotline for infectious diseases, and the organizations put restrictions on animal

trade for three weeks after the recovery of all sick animals. In addition, extra hygiene and biosafety measures are implemented for at least two weeks after recovery, such as reduced number of visitors, extra use of disposable equipment, and collection of milk from unaffected herds before outbreak herds.

Animal experiments in BCoV research

Many experimental infection studies of bovine coronavirus in the natural host have been conducted. Some of the experiments have been performed to document different methods of diagnosis (Cho et al., 2001a), pathogenesis and pathology (Kapil et al., 1991; Park et al., 2007; Saif et al., 1986), clinical signs and antibody production (Tråvén et al., 2001), and cross-protection between different BCoV strains (Cho et al., 2001a; El-Kanawati et al., 1996; Reynolds et al., 1985). All animal experiments have ethical and welfare implications, and the rules and conventions regulating the use of animals have become stricter in recent decades. Also, respected scientific journals now require documentation that ethical guidelines have been followed. All experiments involving animals therefore need to be justified, and the 3 Rs (Replacement, Reduction, and Refinement) implemented. "Replacement" refers to replacing animal experiments with studies such as computer models or laboratory methods. This can be achieved by cultivating BCoV in cell culture instead of infecting cattle (Panon et al., 1988). However, *in vitro* testing can only partly replace *in vivo* infection, and *in vivo* studies are therefore sometimes required to answer research questions, e.g. the duration of BCoV shedding and clinical signs in the natural host. Field studies can partly replace the need of experimental studies, but in many instances a more controlled environment, e.g. with a known infection date, is necessary. "Reduction" means reducing the number of animals used in experiments, without the loss of information. Animal experiments with cattle are expensive and labor intensive and, as a consequence, the number of animals used is always relatively low, and seldom suitable for statistical analyses. The number of animals is therefore often selected based on practical and economic reasons. "Refinement" implies providing the best animal husbandry and experimental procedures to minimize stress, pain, and suffering for the animals involved in the experiment. As a consequence of stricter regulations and a growing awareness of animal welfare, it is becoming more and more common to implement the three Rs and consider procedures to secure the welfare of animals in experiments, e.g. by defining humane endpoints. There has also been an improvement in the reporting of animal experiments. The ARRIVE guidelines ("Animal Research: Reporting of *In Vivo*

Experiments") seek to improve the reporting of animal experiments and maximize the information published, thereby minimizing unnecessary studies (Kilkenny et al., 2010).

Knowledge gaps

BCoV causes respiratory disease, diarrhea in calves and winter dysentery in adult cattle: diseases that result in substantial economic losses and reduced animal welfare. A high seroprevalence on the herd level has been documented, but there is a lack of knowledge about how the virus is spread between farms. There is a need for more information about the duration of virus shedding that constitutes a risk to other animals. Long-lasting infection experiments are few, and the persistence of BCoV in cattle is still debated. Also, there are uncertainties about the pathogenesis of the disease and the reasons for different clinical signs in adult cattle and calves.

Another unexplored question is the extent to which humans may act as vectors for the virus, either by passive carriage or through infection. Also, the importance of equipment and clothing transferred between farms with regard to inter-herd transmission remains undetermined.

There is also a need for improved tracing of BCoV infections. Tracing the virus via molecular methods increases the opportunity to detect and rule out possible transmission routes. Too little variation is seen in the S protein coding region for molecular epidemiologic studies, and effective, reliable methods of amplification and whole genome sequencing of BCoV in clinical samples have not been established.

It is vital that Norwegian farmers and the control program are provided with evidence-based knowledge on how to prevent transmission of BCoV and BRSV between herds, in order to increase motivation and chance of success with regard to disease prevention.

Aims of Study

The overall objective was to generate knowledge necessary in order to control BCoV in the Norwegian cattle population by studying direct and indirect transmission, and facilitate molecular tracing of the virus.

Subgoals

a) Determine the duration and quantity of viral shedding in calves related to clinical signs, and study the presence of infective virus particles (paper I)

b) Document the presence of viremia and virus persistence in infected calves (paper I)

c) Determine the infectiousness of calves three weeks after infection by using sentinel calves (paper I)

d) Investigate the potential for transmission of BCoV via human nasal mucosa and equipment (paper II)

e) Establish a method for whole genome sequencing of BCoV (paper III)

Materials and methods

This section gives a brief overview of the material and methods used in this thesis; the details are provided in the papers. One animal experiment with BCoV was the basis for the main material for all three papers, while paper II also contains material from a BRSV experiment. An overview of the type of material and methods used in each paper is shown in Figure 2.

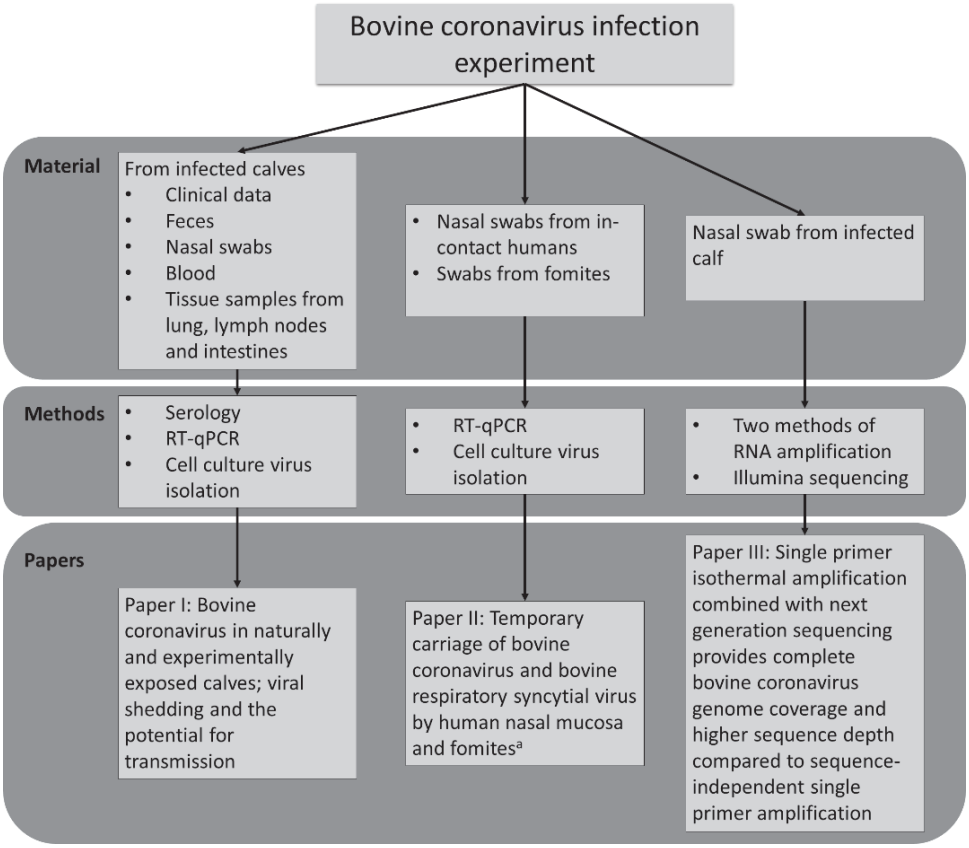


Figure 2 – Overview of material, methods and papers. [a]Paper II also included material from an experiment with bovine respiratory syncytial virus.

Study design

A live animal experiment with BCoV in the natural host was conducted at the Swedish University of Agricultural Sciences, and is described in detail in paper I. The experimental units were groups of calves and the intervention consisted of direct contact with BCoV-infected animals. The primary outcome was clinical signs, and the secondary outcome was presence of BCoV RNA, infective virus, and anti-BCoV antibodies. Three experimental

groups were included: one that was naturally infected with BCoV and two naïve groups. The naturally infected group originated from a herd that was in an early phase of a winter dysentery outbreak when the group was transported to the research facility.

The BRSV experiment took place at the Norwegian Veterinary Institute in 2015 (to be published). Briefly, six calves were infected after contact with two calves inoculated with a field isolate of BRSV, O4-4B/N-11 (Klem et al., 2014). The calves were housed in isolation units in groups of four, including one inoculated calf.

In order to test if human nasal mucosa and fomites could act as vectors for BCoV and BRSV, personnel and veterinary equipment were challenged by >10 min of contact with infected cattle. Swabs were taken from personnel before and at various time points up to 24 hours after challenge. Equipment was swabbed immediately after, and up to 24 hours after challenge.

In order to establish a method for whole genome sequencing of BCoV from clinical samples, two protocols for random amplification of RNA and cDNA synthesis were performed on a nasal swab sample from a calf from the BCoV experiment. The DNA was sequenced (Illumina) and the results compared.

Animals

Twelve seronegative weaned bull calves between six and twelve weeks of age were included in the BCoV experiment. They originated from two dairy herds that were negative for antibodies to BCoV in milk from primiparous cows when the experiment started.

Approximately 300 cattle were present in the dairy herd with winter dysentery.

In the BRSV experiment, eight seronegative weaned Norwegian Red calves between two and four months of age were included: six bulls and two heifers.

Collected material

Daily clinical data, paired blood samples (sera), EDTA-blood, nasal swabs, fecal samples, and tissue samples were collected from the calves.

Swabs from human nasal mucosa and fomites (boots, rubber coat, wristwatches, and stethoscopes) were collected after contact with infected animals.

Laboratory methods

Serology

Serum samples were analyzed for anti BCoV IgG by Svanovir BCV (Boehringer Ingelheim Svanova, Uppsala, Sweden) and a PP-value of <10 was regarded as negative.

RNA purification, RT-qPCR and quantification

RNA was purified from blood, tissue samples, feces and swabs and analyzed by RT-qPCR for BCoV, as described in the papers. The number of BCoV genome copies in the samples was estimated using a standard curve.

In the BRSV experiment, RNA was extracted from swabs and viral RNA quantified by droplet digital RT-PCR, as described in paper II.

Virus isolation

BCoV infectivity was determined by virus isolation in HRT-18 cells and fluorescent staining of virus antigen (paper I) or RT-qPCR before and after incubation on the cells (paper II).

BRSV infectivity was tested by isolation in bovine turbinate cells and fluorescent staining of virus antigen (paper II).

Sample preparation and high throughput sequencing

After initial centrifugation and pre-treatment of the swab material with RNase and DNase, RNA was prepared by phenol/chloroform phase separation and column purification. Two methods of random RNA pre-amplification were used for comparison: single primer isothermal amplification (SPIA) and sequence independent single primer amplification (SISPA). The SISPA protocol is described in paper III. The SPIA protocol was performed using NuGEN's Ovation RNA-Seq V2 kit (CA, USA). The kit provides a rapid method for preparing amplified cDNA from total RNA for downstream RNA-Seq applications. It employs the SPIA method to amplify total RNA into double stranded cDNA, as is shown schematically in Figure 3. Amplified cDNA samples were then purified and libraries created from both methods using Nextera Illumina kits at the sequencing facility. High throughput sequencing was performed on an Illumina MiSeq instrument.

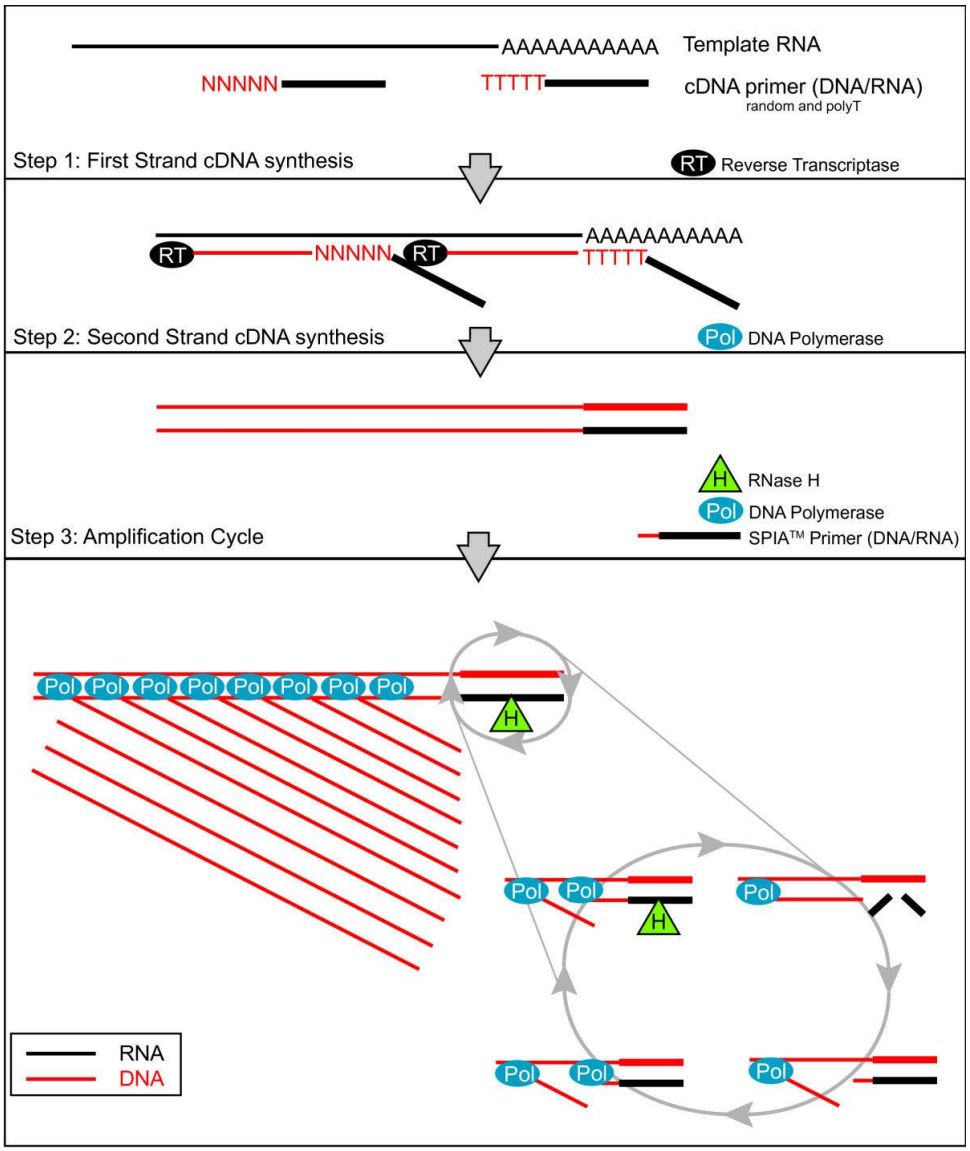


Figure 3 – Schematic representation of the single primer isothermal amplification (SPIA) process. Step 1: First strand cDNA is generated from template RNA using reverse transcriptase (RT) and two types of chimeric primers, random and oligo(dT), containing an RNA overhang. Step 2: DNA polymerase is added to the reaction to generate second strand cDNA. Step 3: ssDNA is amplified from the dsDNA template in a cycle in which a SPIA primer (DNA/RNA hybrid) anneals to the template, DNA polymerase begins duplicating the cDNA, and the RNA portion of the primer degraded by RNase H (which only degrades RNA when it is in a duplex with DNA), thus allowing another SPIA primer to bind to the template and restart the reaction. Figure courtesy of Watson et al. (2008).

Bioinformatics

Bioinformatic analyses included quality check, trimming, and genome alignment of BCoV sequences in CLC Genomics Workbench. Other sequences were annotated using Diamond and SortMeRNA.

Main results

Paper I

Bovine coronavirus in naturally and experimentally exposed calves – viral shedding and the potential for transmission

The BCoV-infected calves showed mild general signs, and the most prominent signs were from the respiratory system. The overall clinical score corresponded well with the shedding of viral RNA the first three weeks after commingling with infected calves. General depression and cough were the signs that correlated best with shedding of BCoV RNA, while peak respiratory rate and peak rectal temperature appeared more than a week later than the peak shedding. Nasal shedding preceded fecal shedding, and the calves had detectable amounts of viral RNA in their feces intermittently through day 35 and in nasal secretions through day 28. Virus infectivity was tested by virus isolation from nasal swabs from two calves between day 3 and day 28. The virus was isolated from day 3 in one of the calves and from days 3 to 13 in the other. Viral RNA was not detected in blood, but was found in lymphatic tissue through day 42 after challenge. The calves tested negative for antibodies to BCoV at the beginning of the trial and had seroconverted by day fourteen. Two naïve sentinel calves were commingled with BCoV-infected calves three weeks after exposure. Although the calves were shedding BCoV RNA 21 days after exposure, the sentinel animals did not get infected.

Paper II

Temporary carriage of bovine coronavirus and bovine respiratory syncytial virus by fomites and human nasal mucosa

Swabs were collected from the nasal mucosa of personnel after contact with calves shedding BCoV or BRSV. For BCoV, 46% (n=80) of the swabs collected 30 minutes after exposure were positive by RT-qPCR. After two, four, and six hours, 15%, 5% and 0% of the swabs were positive, respectively. Infective virions were not detected in mucosal swabs (n=2). For BRSV, 35% (n=26) of the nasal swabs collected 30 minutes after exposure were positive by RT-qPCR, but none were positive for infective virions.

Swabs were also collected from coats, boots and equipment after contact with virus-shedding calves. A high viral RNA load was detected on 97% (n=44) of the fomites 24 hours after exposure to BCoV-shedding calves, and infective virions were detected in two of three

swabs. Of the fomites exposed to BRSV, 89% (n=38) were positive for viral RNA 24 hours after exposure, but all were negative for infective viruses.

Paper III

Single primer isothermal amplification (SPIA) combined with next generation sequencing provides complete bovine coronavirus genome coverage and higher sequencing depth compared to sequence-independent single primer amplification (SISPA)

Two sequence-independent approaches coupled with high throughput sequencing were compared regarding generation of the full-length genome of BCoV from a nasal swab. Both methods achieved high genome coverage (100% for SPIA and 99% for SISPA); however, there was a clear difference in the percentage of reads that mapped to BCoV. While approximately 45% of the SPIA reads mapped to BCoV (sequence depth of 169–284 944), only 0.07% of the SISPA reads (sequence depth of 0–249) mapped to the reference genome. Although BCoV was the focus of the study, we also identified a bovine rhinitis B virus (BRBV) in the datasets. The trend for this virus was similar to that observed for BCoV regarding SPIA vs. SISPA, but with fewer sequences mapping to BRBV due to a lower amount of this virus. In summary, the SPIA approach used in this study produced coverage of the entire BCoV (high copy number) and BRBV (low copy number) and a high sequence/genome depth, compared to SISPA.

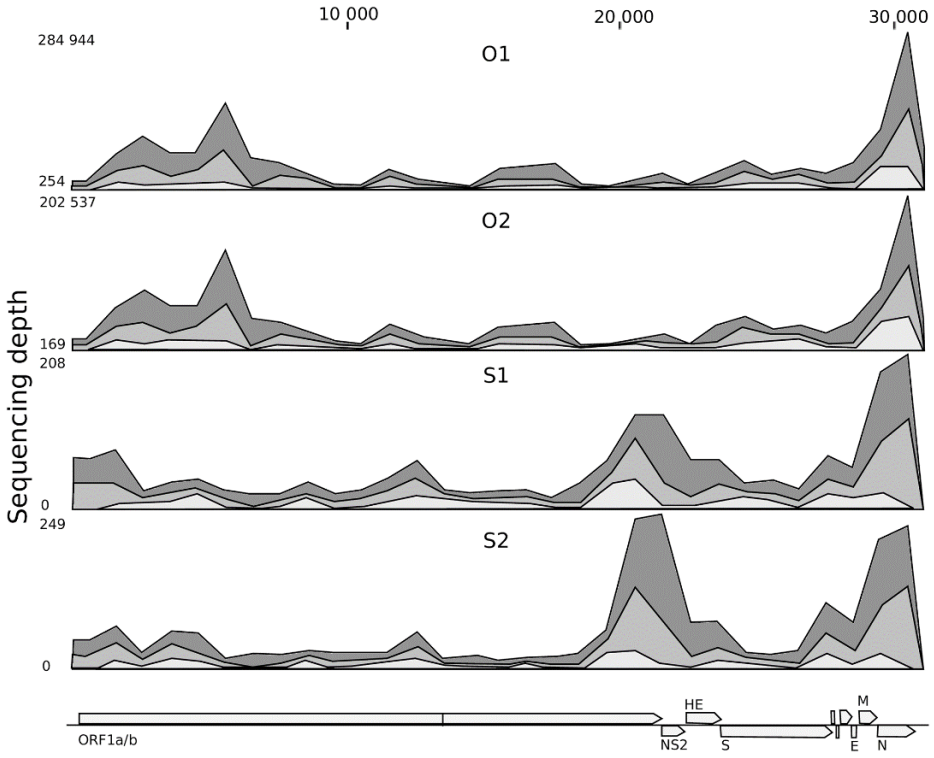


Figure 4 – Coverage and sequence depth of the samples; O1 and O2 (SPIA); S1 and S2 (SISPA). The lower part shows the annotation of the bovine coronavirus (strain Mebus). The different shading of grey shows the minimum, mean and maximum depth.

Discussion

BCoV causes substantial losses in the beef and dairy industries by causing diarrhea and respiratory disease in calves and adult cattle. Knowledge of clinical signs, viral shedding and transmission is important for preventing virus spread and minimizing disease. These aspects are addressed in paper I. The second part of the study (paper II) explores the possibilities for indirect transmission of BCoV and BRSV between herds. To aid the development of efficient methods for the tracing of BCoV infections, an efficient method for whole genome sequencing of the virus is presented in paper III. The results are discussed in detail in the individual papers. The main focus of this section is to discuss common questions for the study and matters that are less discussed in the papers.

Material and methodological considerations

Virus origin and experimental design

All BCoV material used in papers I–III originated from the same animal experiment described in paper I. The BCoV-infected calves originated from a herd experiencing a winter dysentery outbreak in Uppsala, in 2014. The advantage of using field cases of infected calves as a virus source is that infectivity and virulence will be more similar to the natural situation. Cell culture adapted and attenuated strains could have resulted in milder or different clinical signs, as described by Kapil et al. (1990); these authors reported zero to mild clinical signs after inoculating colostrum-deprived calves with a cell culture adapted BCoV strain. Calves inoculated with a virulent field isolate (derived from a calf with diarrhea) developed diarrhea, and, with increasing virus dose, also pneumonia (Kapil et al., 1990). A disadvantage of using a field strain of BCoV is that the strain has not previously been characterized or described.

The transmission model used was naturally infected calves shedding BCoV. In the majority of reported BCoV experiments, artificial inoculation of animals is used (Cho et al., 2001a; Reynolds et al., 1985); among the advantages of the model in our study are the close resemblance to how BCoV is transmitted in the natural situation. This was of particular importance for the study of clinical signs, shedding and infectiousness of the calves. On the other hand, the virus dose received by the experimental cattle could not be quantified, and could have varied among the animals in the trial; this in turn could have influenced the clinical signs and BCoV shedding. It was not possible to completely control against infections with other agents, and sequencing revealed co-infection with bovine rhinitis B virus (BRBV) in one of the calves (paper III). This could have influenced the clinical signs,

as BRBV was found to cause mild respiratory disease in gnotobiotic calves in an earlier study (Betts et al., 1971). However, few studies have been published in recent years about BRBVs impact and clinical signs (Hause et al., 2015). The virus was not significantly associated with BRD in a recent study (Ng et al., 2015). The number of BRBV genomes from the calf mucosa was much lower compared to BCoV (paper III), which indicates that BCoV was far more important with regard to the clinical signs.

The infection experiment included a limited number of animals and only calves, i.e. no adult animals. Calves are more commonly used to study BCoV than adult cattle; this is partly due to the expenses of purchase and housing and the challenges of finding BCoV-seronegative adults. Calves are also more commonly infected with BCoV than adult cattle, and are important sources of the virus. It is likely that adult cattle would have shown other clinical signs, and exhibited a different shedding pattern of BCoV than the calves in the present study. Care should therefore be taken when generalizing the findings to other age groups regarding the conclusions about clinical signs, shedding and pathogenesis.

Animal experiments, the three Rs and ethical considerations

An animal experiment with cattle is financially demanding, labor intensive and ethically challenging. Field studies or cell culture studies could be suggested as alternatives. It is possible to study viral shedding and transmission in a field study, if sufficient knowledge about an outbreak or active shedders is available. It requires fewer research animals, is cheaper, and is more similar to the natural situation. A major drawback, however, is the unknown starting point of the infection and the lack of control regarding feeding, treatment, housing and contact with other animals. In addition, the history of previous infections and disease is often unknown. These factors lower the reproducibility and reliability of the study. Replacement, for instance in the form of cell culture studies, is suited to the study of virus infectivity, e.g. after drying on surfaces (Sizun et al., 2000), but cannot be used to investigate virus shedding and actual transmission to susceptible animals. An experimental study was therefore chosen to meet the aims of the study. The natural host and only known animal model for BCoV is cattle, which are resource-demanding in purchase, housing, and husbandry, so the sample size is therefore limited in this and other studies (Kapil et al., 1991; Park et al., 2007; Tråvén et al., 2001). Refinement is important to limit negative welfare and reduce variability when few animals are used. This means minimizing the stress and suffering of the animals involved, and facilitating healthy, normal behavior. Refinement was pursued in the current experiment by housing the calves in small groups of even age, ad libitum

roughage feeding, close monitoring, and treatment of sick calves. The ARRIVE guidelines for improved reporting of animal experiments (Kilkenny et al., 2010) were followed to maximize the output of the study. In addition, samples and data from the experiment were utilized for multiple studies: the three papers presented here, genomic description of a bovine rhinitis B virus (Blomström et al., 2017), changes in immune cell gene expression during infection (to be published), and quasispecies and viral evolution during BCoV infections (to be published).

Validity

Although the collected material came from one animal infection experiment with one strain of BCoV, it can be argued that the study has good external validity. The infections occurred in the natural host, with a virulent field strain of BCoV and with natural exposure as intervention. In addition, the animals used were conventionally reared, of common dairy breeds, at the normal age for sale, transport and susceptibility to BCoV, relatively conventionally housed, and fed normal diets.

It is thus likely that many naïve field calves would react similarly with regard to virus shedding, antibody response and disease course. They might, however, have shown more severe signs if provided with a suboptimal environment. Other co-infections, less monitoring and treatment could have resulted in more severe disease, and possibly increased and longer-lasting shedding.

Although only one serotype of BCoV has been found (Hasoksuz et al., 1999), strain variations important for clinical signs and tropism have been discussed in international literature (Cho et al., 2001a; El-Kanawati et al., 1996; Tsunemitsu and Saif, 1995; Tsunemitsu et al., 1999), and other strains could have provided other results. Nevertheless, it is likely that many of the conclusions would also be valid for other strains of BCoV, particularly the conclusions from papers II and III.

Laboratory methods

Detection of BCoV by RT-qPCR and quantitation

Most of the virus detection in the study was done by RT-qPCR, which does not prove infectivity. To relate the amount of viral RNA detected to the number of infective virus particles, a “total to infective particles ratio” (T/I) was calculated (paper I). This was done for one nasal swab sampled in the acute stage of infection and gave a high T/I ratio. In paper I, various reasons for a high T/I ratio are discussed and, as found by Desmarets et al. (2016), the

ratio could change during the course of infection. Sub-genomic RNA could constitute a source of viral RNA detected by the RT-qPCR and cause an overestimation of the number of genome copies. This could happen when intracellular contents have been included in the RT-qPCR. Coronavirus replication includes a step of viral RNA synthesis from the 3' terminal, which forms a set of nested sub-genomic RNA encoding the BCoV structural proteins (Thiel et al., 2003). The RT-qPCR used in the papers targets the M protein gene near the 3' end of the genome; hence, it is possible that viral sub-genomic RNA was quantitated.

Virus isolation

It was important in our studies to estimate the duration of infective virus shedding. The sensitivity of virus detection can be low in cell culture, particularly for virus from clinical samples not adapted to grow in cell lines (Hodinka and Kaiser, 2013; Kapil et al., 1996). Thus, isolation does not necessarily reflect infectiousness *in vivo*. Experimental infection of naïve cattle will remain the gold standard for testing but, as discussed previously, it is challenging. RT-qPCR is more sensitive, less labor-intensive, and faster than viral culture and provides more accurate quantification of virus particles (van Elden et al., 2002). On the other hand, it cannot be used to detect infectivity like virus isolation does. The two methods therefore complement each other and should preferably be used in combination. A study of canine coronavirus in feces showed that infectivity fell to below the limit of detection after three months, even when stored at -70°C (Tennant et al., 1994). Even though our samples had been stored longer than three months, the finding that two out of three samples contained infective viruses shows that infectivity was not detrimentally affected by storage. However, this could still be the case with the BRSV samples, which had been stored for 1.5 years at -80°C before negative isolation in cell culture.

Preparation of samples and high throughput sequencing

To achieve good coverage across BCoV's large genome by high throughput sequencing (paper III), thorough purification and pre-amplification of RNA were necessary. Several steps of purification were performed to remove unwanted nucleic acids while retaining the viral RNA. The subsequent amplification of RNA with SISPA and SPIA techniques (described in paper III) increased the contents of viral nucleic acids considerably. As no amplification of nucleic acids is completely random, uneven sequencing depth across the BCoV genome was seen with both methods.

All available sequencing platforms produce sequencing errors. Even though Illumina is the most commonly used platform, knowledge about systematic errors in Illumina sequencing is

scarce. Schirmer et al. (2016) found that even by performing quality trimming and error correction, a substantial fraction of errors remains. Also, library preparation protocols can introduce bias through the processes of fragmentation, adapter ligation and PCR amplification (van Dijk et al., 2014). The problem of sequencing errors can partly be overcome by high sequencing depth (Schirmer et al., 2016). The consensus sequences obtained with the SPIA method described in paper III were identical for the technical replicates, which indicate that the method is reliable for whole genome sequencing of BCoV.

Interpretation of results

Paper I describes the clinical signs of BCoV infection in calves, and relates them to viral shedding and seroconversion. The finding that infection resulted in mild clinical signs in most of the calves, with the most pronounced signs from the respiratory system, is in accordance with some experimental studies of BCoV (Kapil et al., 1991; Tråvén et al., 2001), but differs from others where enteric signs were the most prominent (Cho et al., 2001a; Park et al., 2007). The reason for this might be differences between virus strains, environment, or calf management. Viral RNA was detected for four to five weeks in the infected calves, longer than previously reported from experimental trials. The long-lasting detectable RNA makes nasal swabs and RT-qPCR suitable for diagnosis. However, the importance of the shedding for transmission after approximately day 13 post-exposure, when virus isolation from the calves was no longer successful, is debatable. The decline of infectivity might have occurred simultaneously with the rise in specific antibodies to BCoV. As commingling with naïve calves three weeks post-exposure did not result in infection, it seems likely that the infectiousness of BCoV-infected calves declines between 14–21 days after exposure to BCoV. The BCoV shed after seroconversion was possibly bound to immune complexes (Crouch et al., 1985), which might have prevented infection in the naïve calves. Consequently, it should be possible to reduce the spread of BCoV by restricting the movement of cattle until three weeks after recovery from clinical disease, and this has also been implemented in the Norwegian control program. However, even when following such a general guideline, there is a considerable risk of infection spread during livestock trade. Many BCoV shedders have subclinical infections, and therefore still constitute a risk to other animals. Furthermore, with normal herd sizes being considerably larger than the number of animals in the experiment, the monitoring of clinical signs would most likely be less meticulous than during a trial, and mildly affected animals could be missed.

The strategy of the Norwegian control program is based on self-clearance of the virus in herds, which makes possible BCoV persistence an important topic. If the virus persists in individuals, herds might not be free of the virus even after strict biosecurity measures have been implemented. Further investigation is needed to determine if the finding of viral RNA persistence in lymphatic and intestinal tissues indicates that BCoV might become reactivated, for instance under stressful conditions. Previously, persistence of BRSV RNA in pulmonary lymph nodes has been found in calves for 71 days post-infection (Valarcher et al., 2001).

The second part of the study (paper II) explores the possibilities for indirect transmission of BCoV and BRSV between herds. The results indicated that humans can carry viral RNA in their nostrils for a short period after contact with infected animals, but the importance for transmission is most likely small, as infective BCoV or BRSV was not found in human nostrils. However, contaminated fomites appeared to represent a significant risk, as infective BCoV and high numbers of viral genome copies were found on fomites the day after contact. Isolation of BRSV from fomites was not successful, which could be due to a faster inactivation of BRSV compared to BCoV in the environment or inactivation during storage. The environmental inactivation rate for BCoV and BRSV has been sparsely studied, so comparisons are made with studies on evolutionary related viruses. However, as large differences in inactivation rate have been found for different strains of influenza viruses (Bean et al., 1982; Otter et al., 2016), comparisons should be interpreted with caution. The related human respiratory syncytial virus (HRSV) was inactivated after seven hours on contaminated countertops and after five hours on rubber gloves (Hall et al., 1980). On skin, the virus was inactivated in less than one hour (Hall et al., 1980), similar to the finding of few genome copies, and no infective virions in human nostrils after 30 minutes in our study. The rate of inactivation of viruses varies considerably between different studies, mainly due to differences in experimental conditions (Otter et al., 2016). Nevertheless, it seems like coronaviruses maintain infectivity for longer time periods than the respiratory syncytial viruses (Casanova et al., 2009; Hambling, 1964; Ijaz et al., 1985; Rechsteiner and Winkler, 1969); this is in concordance with the findings in paper II. The same guidelines and measures will, however, most likely be effective against both viruses. Measures should include the use of herd-specific boots, clothing, and other equipment. Objects that need to be transferred between farms should be thoroughly washed or disinfected between farms. Fomites that have been in contact with virus-shedding cattle constitute a greater risk for indirect transmission than the nostrils of in-contact personnel. This means that good hand-washing combined with

a change of clothes and equipment between herds is recommended to prevent inter-herd transmission.

The goal of the third part of the study (paper III) was to find a suitable method for whole genome sequencing of BCoV from clinical samples. A good method would provide close to full genome coverage, even sequencing depth across the genome and few sequencing artefacts, while being time efficient and affordable (Sims et al., 2014). As BCoV has a large RNA genome (31 kb), some form of amplification of the wanted nucleic acid is most often needed to achieve full genome coverage. This amplification could be done by proliferation of the virus in cell culture or by the use of specific primers targeting parts of the BCoV genome (Kin et al., 2016), or combinations of these (Chouljenko et al., 2001). Most of the sequencing of BCoV performed to date was done on material amplified with specific primers. One of the drawbacks of the method is that it can be cumbersome: Kin et al. used 42 different primers to cover less than 7000 nucleotides (2016) and Bidokhti et al. used 7 primer pairs to cover the 4000 bases of the S gene (2013). Another problem with specific primer amplification, or so-called amplicon sequencing, is that unknown sequences can be missed or result in poor coverage if the nucleotide changes are in the primer binding region. An advantage with amplicon sequencing is that it permits sequencing of low copy, difficult targets and might create an even coverage compared to random amplification. Amplicon sequencing can also allow for sequencing of a higher number of samples for the same price and sequencing depth, as only the targeted region is sequenced. An important pitfall of cell culture propagation is that it can introduce mutations due to virus adaptation to growth in cell culture (Borucki et al., 2013). The random amplification by the SPIA method described in paper III provided coverage across the full genome of BCoV, although the sequencing depth varied considerably throughout the genome, with peaks at the 3' end of the genome and at 6kb (ORF1a, within nsp1 coding region). These peaks were almost identical for the two technical replicates, but differed between the SPIA and the SISPA amplification method. This suggests that the uneven coverage is method specific and has less to do with secondary RNA structures, as proposed by Malbouef et al. (2013). Such uneven coverage distribution makes the method suboptimal for investigation of quasispecies and intra-host viral variants. It is also important to note that only one nasal swab was sequenced, which contained a high viral load. It needs to be determined how well the method performs in fecal samples and samples containing less virus. The method might not provide full genome coverage in all samples, and could possibly perform more poorly on fecal samples, for instance. On the other hand, as the SPIA method

provided two identical sequences of the whole genome of the two technical replicates and amplified the viral RNA considerably, it is likely to perform well enough on clinical samples from outbreaks to determine the consensus sequence. These sequences can be used for molecular epidemiology and the tracing of virus transmission. Such investigations are useful for developing further knowledge about BCoV spread between herds. For example, sequencing could aid in distinguishing reintroduction from persistence of BCoV within large herds, and identifying likely routes of inter-herd transmission.

Conclusions

- Experimental infection with BCoV in calves showed that prolonged shedding of BCoV RNA occurs; however, RNA detection does not imply infectiousness to other animals, and the infectious phase is most likely shorter than three weeks.
- BCoV that originated from a winter dysentery outbreak in adult cattle caused respiratory disease in calves.
- Infected calves harbored BCoV RNA in lymph nodes and intestinal tissue until day 42 post-exposure. Viremia was not detected, which suggests that the virus is transported from the airways to the intestines through swallowing of the virus.
- Humans in contact with infected calves can carry BCoV in their nostrils for a few hours, but the carriage seems short-lived and the transmission potential is likely limited.
- Fomites used in an environment with virus-shedding calves carried infective viruses for 24 hours, and represent a significant risk for indirect transmission of BCoV.
- Changing clothes and washing/disinfecting equipment in addition to thorough hand-washing is, in most instances, likely sufficient to prevent indirect transmission of BCoV between herds.
- An effective and reliable method for whole genome sequencing of BCoV from clinical samples is proposed. The method provided 100% genome coverage and a minimum sequencing depth of 169 across the genome. The method will be useful for the tracing of BCoV infections and assessment of virus spread.

Future perspectives

Additional studies on infectiousness 10 to 21 days post infection should be performed to further reduce the period of uncertain infectiousness in BCoV-infected calves. It should also be explored whether BCoV replication and shedding could be reactivated, e.g. under stressful conditions like transport and commingling of animals. These questions could be clarified by consecutive commingling of infected and naïve calves from ten days post-exposure.

Paper II showed that fomites can carry infective BCoV 24 hours after contact with a contaminated environment. Further knowledge of the duration of BCoV infectivity after storage under relevant conditions is necessary, to determine if fomites can transfer BCoV for even longer. Determination of infectivity in cell culture or exposure to naïve animals after storage under relevant conditions should be performed.

Whole genome sequencing (paper III) could be used to study the differences of BCoV genomes derived from the respiratory and enteric tract in individual animals. Also, BCoV genomes from typical winter dysentery outbreaks should be compared to the virus from respiratory outbreaks to determine if there are true differences related to clinical signs, or if differences are related to time and spatial origin.

In order to find the most important infection routes that lead to new introductions of BCoV into herds, whole genome sequencing from outbreaks of BCoV should be used. By implementing the method from paper III in combination with epidemiologic methods, transmission routes could be inferred between outbreaks of BCoV disease. Repeated sampling and sequencing from large herds, could indicate whether herds continuously contain BCoV-shedding animals or if infections are mainly caused by re-introduction of BCoV. This is important in order to determine if the biosecurity measures suggested in the control program are sufficient to control infection in large herds.

References

Alekseev, K.P., Vlasova, A.N., Jung, K., Hasoksuz, M., Zhang, X., Halpin, R., Wang, S., Ghedin, E., Spiro, D., Saif, L.J., 2008. Bovine-like coronaviruses isolated from four species of captive wild ruminants are homologous to bovine coronaviruses, based on complete genomic sequences. J. Virol. 82, 12422-12431.

Alenius, S., Niskanen, R., Juntti, N., Larsson, B., 1991. Bovine coronavirus as the causative agent of winter dysentery: serological evidence. Acta Vet. Scand. 32, 163-170.

Animalia, 2017. Storfekjøttkontrollen årsmelding 2016. Oslo.

Barkema, H.W., von Keyserlingk, M.A., Kastelic, J.P., Lam, T.J., Luby, C., Roy, J.P., LeBlanc, S.J., Keefe, G.P., Kelton, D.F., 2015. Invited review: Changes in the dairy industry affecting dairy cattle health and welfare. J. Dairy Sci. 98, 7426-7445.

Barros, I.N., Silva, S.O., Nogueira Neto, F.S., Asano, K.M., Souza, S.P., Richtzenhain, L.J., Brandao, P.E., 2013. A multigene approach for comparing genealogy of betacoronavirus from cattle and horses. Sci. World J. 2013, 349702.

Bean, B., Moore, B.M., Sterner, B., Peterson, L.R., Gerding, D.N., Balfour, H.H., Jr., 1982. Survival of influenza viruses on environmental surfaces. J. Infect. Dis. 146, 47-51.

Betts, A.O., Edington, N., Jennings, A.R., Reed, S.E., 1971. Studies on a rhinovirus (EC11) derived from a calf. II. Disease in calves. J. Comp. Pathol. 81, 41-48.

Bhardwaj, K., Guarino, L., Kao, C.C., 2004. The severe acute respiratory syndrome coronavirus Nsp15 protein is an endoribonuclease that prefers manganese as a cofactor. J. Virol. 78, 12218-12224.

Bhardwaj, K., Sun, J., Holzenburg, A., Guarino, L.A., Kao, C.C., 2006. RNA recognition and cleavage by the SARS coronavirus endoribonuclease. J. Mol. Biol. 361, 243-256.

Bidokhti, M.R., Tråvén, M., Fall, N., Emanuelson, U., Alenius, S., 2009. Reduced likelihood of bovine coronavirus and bovine respiratory syncytial virus infection on organic compared to conventional dairy farms. Vet. J. 182, 436-440.

Bidokhti, M.R., Tråvén, M., Krishna, N.K., Munir, M., Belak, S., Alenius, S., Cortey, M., 2013. Evolutionary dynamics of bovine coronaviruses: natural selection pattern of the spike gene implies adaptive evolution of the strains. J. Gen. Virol. 94, 2036-2049.

Bidokhti, M.R., Tråvén, M., Ohlson, A., Baule, C., Hakhverdyan, M., Belák, S., Liu, L., Alenius, S., 2012. Tracing the transmission of bovine coronavirus infections in cattle herds based on S gene diversity. Vet. J. 193, 386-390.

Blomström, A.-L., Oma, V., Khatri, M., Hansen, H.H., Stokstad, M., Berg, M., Myrmel, M., 2017. Genome sequence of a bovine rhinitis B virus identified in cattle in Sweden. Genome Announc. 5, e00172-17.

Boileau, M.J., Kapil, S., 2010. Bovine coronavirus associated syndromes. Vet. Clin. North Am. Food Anim. Pract. 26, 123-146.

Bok, M., Miño, S., Rodriguez, D., Badaracco, A., Nuñes, I., Souza, S.P., Bilbao, G., Louge Uriarte, E., Galarza, R., Vega, C., Odeon, A., Saif, L.J., Parreño, V., 2015. Molecular and antigenic characterization of bovine coronavirus circulating in Argentinean cattle during 1994-2010. Vet. Microbiol. 181, 221-229.

Booth, T.F., Kournikakis, B., Bastien, N., Ho, J., Kobasa, D., Stadnyk, L., Li, Y., Spence, M., Paton, S., Henry, B., Mederski, B., White, D., Low, D.E., McGeer, A., Simor, A., Vearncombe, M., Downey, J., Jamieson, F.B., Tang, P., Plummer, F., 2005. Detection of airborne severe acute respiratory syndrome (SARS) coronavirus and environmental contamination in SARS outbreak units. J. Infect. Dis. 191, 1472-1477.

Borucki, M.K., Allen, J.E., Chen-Harris, H., Zemla, A., Vanier, G., Mabery, S., Torres, C., Hullinger, P., Slezak, T., 2013. The role of viral population diversity in adaptation of bovine coronavirus to new host environments. PLoS One 8, e52752.

Bouvet, M., Debarnot, C., Imbert, I., Selisko, B., Snijder, E.J., Canard, B., Decroly, E., 2010. In vitro reconstitution of SARS-coronavirus mRNA cap methylation. PLoS Pathog. 6, e1000863.
Bridger, J.C., Woode, G.N., Meyling, A., 1978. Isolation of coronaviruses from neonatal calf diarrhoea in Great Britain and Denmark. Vet. Microbiol. 3, 101-113.
Casanova, L., Rutala, W.A., Weber, D.J., Sobsey, M.D., 2009. Survival of surrogate coronaviruses in water. Water research 43, 1893-1898.
Cavanagh, D., 1995. The coronavirus surface glycoprotein. In: Siddell, S. (Ed.), The Coronaviridae, Plenum Press, New York, pp. 73-113.
Cho, K.O., Hasoksuz, M., Nielsen, P.R., Chang, K.O., Lathrop, S., Saif, L.J., 2001a. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch. Virol. 146, 2401-2419.
Cho, K.O., Hoet, A.E., Loerch, S.C., Wittum, T.E., Saif, L.J., 2001b. Evaluation of concurrent shedding of bovine coronavirus via the respiratory tract and enteric route in feedlot cattle. Am. J. Vet. Res. 62, 1436-1441.
Cho, Y.I., Han, J.I., Wang, C., Cooper, V., Schwartz, K., Engelken, T., Yoon, K.J., 2013. Case-control study of microbiological etiology associated with calf diarrhea. Vet. Microbiol. 166, 375-385.
Cho, Y.I., Yoon, K.J., 2014. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. J. Vet. Sci. 15, 1-17.
Chouljenko, V.N., Kousoulas, K.G., Lin, X., Storz, J., 1998. Nucleotide and predicted amino acid sequences of all genes encoded by the 3' genomic portion (9.5 kb) of respiratory bovine coronaviruses and comparisons among respiratory and enteric coronaviruses. Virus Genes 17, 33-42.
Chouljenko, V.N., Lin, X.Q., Storz, J., Kousoulas, K.G., Gorbalenya, A.E., 2001. Comparison of genomic and predicted amino acid sequences of respiratory and enteric bovine coronaviruses isolated from the same animal with fatal shipping pneumonia. J. Gen. Virol. 82, 2927-2933.
Clark, K.J., Sarr, A.B., Grant, P.G., Phillips, T.D., Woode, G.N., 1998. In vitro studies on the use of clay, clay minerals and charcoal to adsorb bovine rotavirus and bovine coronavirus. Vet. Microbiol. 63, 137-146.
Clark, M.A., 1993. Bovine coronavirus. Br. Vet. J. 149, 51-70.
Collins, J.K., Riegel, C.A., Olson, J.D., Fountain, A., 1987. Shedding of enteric coronavirus in adult cattle. Am. J. Vet. Res. 48, 361-365.
Crouch, C.F., Bielefeldt Ohmann, H., Watts, T.C., Babiuk, L.A., 1985. Chronic shedding of bovine enteric coronavirus antigen-antibody complexes by clinically normal cows. J. Gen. Virol. 66, 1489-1500.
Decaro, N., Campolo, M., Desario, C., Cirone, F., D'Abramo, M., Lorusso, E., Greco, G., Mari, V., Colaianni, M.L., Elia, G., Martella, V., Buonavoglia, C., 2008a. Respiratory disease associated with bovine coronavirus infection in cattle herds in Southern Italy. J. Vet. Diagn. Invest. 20, 28-32.
Decaro, N., Elia, G., Campolo, M., Desario, C., Mari, V., Radogna, A., Colaianni, M.L., Cirone, F., Tempesta, M., Buonavoglia, C., 2008b. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J. Virol. Methods 151, 167-171.
Decaro, N., Mari, V., Desario, C., Campolo, M., Elia, G., Martella, V., Greco, G., Cirone, F., Colaianni, M.L., Cordioli, P., Buonavoglia, C., 2008c. Severe outbreak of bovine coronavirus infection in dairy cattle during the warmer season. Vet. Microbiol. 126, 30-39.

Decaro, N., Martella, V., Elia, G., Campolo, M., Mari, V., Desario, C., Lucente, M.S., Lorusso, A., Greco, G., Corrente, M., Tempesta, M., Buonavoglia, C., 2008d. Biological and genetic analysis of a bovine-like coronavirus isolated from water buffalo (Bubalus bubalis) calves. Virology 370, 213-222.

Decroly, E., Imbert, I., Coutard, B., Bouvet, M., Selisko, B., Alvarez, K., Gorbalenya, A.E., Snijder, E.J., Canard, B., 2008. Coronavirus nonstructural protein 16 is a cap-0 binding enzyme possessing (nucleoside-2'O)-methyltransferase activity. J. Virol. 82, 8071-8084.

Denison, M.R., Graham, R.L., Donaldson, E.F., Eckerle, L.D., Baric, R.S., 2011. Coronaviruses: an RNA proofreading machine regulates replication fidelity and diversity. RNA Biol. 8, 270-279.

Deregt, D., Gifford, G.A., Ijaz, M.K., Watts, T.C., Gilchrist, J.E., Haines, D.M., Babiuk, L.A., 1989. Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: demonstration of in vivo virus-neutralizing activity. J. Gen. Virol. 70, 993-998.

Desmarets, L.M.B., Vermeulen, B.L., Theuns, S., Conceição-Neto, N., Zeller, M., Roukaerts, I.D.M., Acar, D.D., Olyslaegers, D.A.J., Van Ranst, M., Matthijnssens, J., Nauwynck, H.J., 2016. Experimental feline enteric coronavirus infection reveals an aberrant infection pattern and shedding of mutants with impaired infectivity in enterocyte cultures. Sci. Rep. 6, 20022.

Eckerle, L.D., Becker, M.M., Halpin, R.A., Li, K., Venter, E., Lu, X., Scherbakova, S., Graham, R.L., Baric, R.S., Stockwell, T.B., Spiro, D.J., Denison, M.R., 2010. Infidelity of SARS-CoV Nsp14-exonuclease mutant virus replication is revealed by complete genome sequencing. PLoS Pathog. 6, e1000896.

Eckerle, L.D., Lu, X., Sperry, S.M., Choi, L., Denison, M.R., 2007. High fidelity of murine hepatitis virus replication is decreased in nsp14 exoribonuclease mutants. J. Virol. 81, 12135-12144.

Edwards, T.A., 2010. Control methods for bovine respiratory disease for feedlot cattle. Vet. Clin. North Am. Food Anim. Pract. 26, 273-284.

Eigen, M., 1993. Viral quasispecies. Sci. Am. 269, 42-49.

El-Ghorr, A.A., Snodgrass, D.R., Scott, F.M.M., Campbell, I., 1989. A serological comparison of bovine coronavirus strains. Arch. Virol. 104, 241-248.

El-Kanawati, Z.R., Tsunemitsu, H., Smith, D.R., Saif, L.J., 1996. Infection and cross-protection studies of winter dysentery and calf diarrhea bovine coronavirus strains in colostrum-deprived and gnotobiotic calves. Am. J. Vet. Res. 57, 48-53.

Espetvedt, M.N., Reksen, O., Rintakoski, S., Østerås, O., 2013. Data quality in the Norwegian dairy herd recording system: agreement between the national database and disease recording on farm. J. Dairy Sci. 96, 2271-2282.

Fehr, A.R., Perlman, S., 2015. Coronaviruses: an overview of their replication and pathogenesis. Methods Mol. Biol. 1282, 1-23.

Francoz, D., Buczinski, S., Belanger, A.M., Forte, G., Labrecque, O., Tremblay, D., Wellemans, V., Dubuc, J., 2015. Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. J. Vet. Intern. Med. 29, 381-387.

Fulton, R.W., Ridpath, J.F., Burge, L.J., 2013. Bovine coronaviruses from the respiratory tract: antigenic and genetic diversity. Vaccine 31, 886-892.

Gadlage, M.J., Sparks, J.S., Beachboard, D.C., Cox, R.G., Doyle, J.D., Stobart, C.C., Denison, M.R., 2010. Murine hepatitis virus nonstructural protein 4 regulates virus-induced membrane modifications and replication complex function. J. Virol. 84, 280-290.

Genova, S.G., Streeter, R.N., Simpson, K.M., Kapil, S., 2008. Detection of an antigenic group 2 coronavirus in an adult alpaca with enteritis. Clin. Vaccine Immunol. 15, 1629-1632.
Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., Snijder, E.J., 2006. Nidovirales: evolving the largest RNA virus genome. Virus Res. 117, 17-37.
Graham, R.L., Sims, A.C., Baric, R.S., Denison, M.R., 2006. The nsp2 proteins of mouse hepatitis virus and SARS coronavirus are dispensable for viral replication. Adv. Exp. Med. Biol. 581, 67-72.
Gulliksen, S.M., Jor, E., Lie, K.I., Hamnes, I.S., Løken, T., Åkerstedt, J., Østerås, O., 2009a. Enteropathogens and risk factors for diarrhea in Norwegian dairy calves. J. Dairy Sci. 92, 5057-5066.
Gulliksen, S.M., Jor, E., Lie, K.I., Løken, T., Åkerstedt, J., Østerås, O., 2009b. Respiratory infections in Norwegian dairy calves. J. Dairy Sci. 92, 5139-5146.
Gulliksen, S.M., Lie, K.I., Løken, T., Østerås, O., 2009c. Calf mortality in Norwegian dairy herds. J. Dairy Sci. 92, 2782-2795.
Gulliksen, S.M., Lie, K.I., Østerås, O., 2009d. Calf health monitoring in Norwegian dairy herds. J. Dairy Sci. 92, 1660-1669.
Gunn, L., Collins, P.J., O'Connell, M.J., O'Shea, H., 2015. Phylogenetic investigation of enteric bovine coronavirus in Ireland reveals partitioning between European and global strains. Ir. Vet. J. 68, 31.
Guy, J.S., Breslin, J.J., Breuhaus, B., Vivrette, S., Smith, L.G., 2000. Characterization of a coronavirus isolated from a diarrheic foal. J. Clin. Microbiol. 38, 4523-4526.
Hall, C.B., Douglas, R.G., Geiman, J.M., 1980. Possible transmission by fomites of respiratory syncytial virus. J. Infect. Dis. 141, 98-102.
Hambling, M.H., 1964. Survival of the respiratory syncytial virus during storage under various conditions. Br. J. Exp. Pathol. 45, 647-655.
Han, M.G., Cheon, D.S., Zhang, X., Saif, L.J., 2006. Cross-protection against a human enteric coronavirus and a virulent bovine enteric coronavirus in gnotobiotic calves. J. Virol. 80, 12350-12356.
Hansa, A., Rai, R.B., Dhama, K., Wani, M.Y., Saminathan, M., Ranganath, G.J., 2013. Isolation of bovine coronavirus (bcoV) in vero cell line and its confirmation by direct FAT and RT-PCR. Pak. J. Biol. Sci. 16, 1342-1347.
Hasoksuz, M., Alekseev, K., Vlasova, A., Zhang, X., Spiro, D., Halpin, R., Wang, S., Ghedin, E., Saif, L.J., 2007. Biologic, antigenic, and full-length genomic characterization of a bovine-like coronavirus isolated from a giraffe. J. Virol. 81, 4981-4990.
Hasoksuz, M., Lathrop, S.L., Al-dubaib, M.A., Lewis, P., Saif, L.J., 1999. Antigenic variation among bovine enteric coronaviruses (BECV) and bovine respiratory coronaviruses (BRCV) detected using monoclonal antibodies. Arch. Virol. 144, 2441-2447.
Hasoksuz, M., Sreevatsan, S., Cho, K.-O., Hoet, A.E., Saif, L.J., 2002. Molecular analysis of the S1 subunit of the spike glycoprotein of respiratory and enteric bovine coronavirus isolates. Virus Res. 84, 101-109.
Hause, B.M., Collin, E.A., Anderson, J., Hesse, R.A., Anderson, G., 2015. Bovine rhinitis viruses are common in U.S. cattle with bovine respiratory disease. PLoS One 10, e0121998.
Headley, S.A., Okano, W., Balbo, L.C., Marcasso, R.A., Oliveira, T.E., Alfieri, A.F., Negri Filho, L.C., Michelazzo, M.Z., Rodrigues, S.C., Baptista, A.L., Saut, J.P.E., Alfieri, A.A., 2018. Molecular survey of infectious agents associated with bovine respiratory disease in a beef cattle feedlot in southern Brazil. J. Vet. Diagn. Invest. 30, 249-251.

Heckert, R.A., Saif, L.J., Mengel, J.P., Myers, G.W., 1991a. Mucosal and systemic antibody responses to bovine coronavirus structural proteins in experimentally challenge-exposed calves fed low or high amounts of colostral antibodies. Am. J. Vet. Res. 52, 700-708.
Heckert, R.A., Saif, L.J., Myers, G.W., 1991b. Mucosal and systemic isotype-specific antibody responses to bovine coronavirus structural proteins in naturally infected dairy calves. Am. J. Vet. Res. 52, 852-857.
Heckert, R.A., Saif, L.J., Myers, G.W., Agnes, A.G., 1991c. Epidemiologic factors and isotype-specific antibody responses in serum and mucosal secretions of dairy calves with bovine coronavirus respiratory tract and enteric tract infections. Am. J. Vet. Res. 52, 845-851.
Hedström, H., Isaksson, A., 1951. Epizootic enteritis in cattle in Sweden. Cornell Vet. 41, 251-253.
Hodinka, R.L., Kaiser, L., 2013. Is the era of viral culture over in the clinical microbiology laboratory? J. Clin. Microbiol. 51, 2-8.
Hofshagen, M., Sviland, S., Gjevre, A.-G., Torp, M., 2017. Surveillance programmes - summary of results 2016. Norwegian Veterinary Institute, Oslo.
Ijaz, M.K., Brunner, A.H., Sattar, S.A., Nair, R.C., Johnson-Lussenburg, C.M., 1985. Survival characteristics of airborne human coronavirus 229E. J. Gen. Virol. 66, 2743-2748.
Imbert, I., Guillemot, J.C., Bourhis, J.M., Bussetta, C., Coutard, B., Egloff, M.P., Ferron, F., Gorbalenya, A.E., Canard, B., 2006. A second, non-canonical RNA-dependent RNA polymerase in SARS coronavirus. EMBO J. 25, 4933-4942.
International Committee on Taxonomy of Viruses, 2017. Virus Taxonomy: 2016 Release. Accessed: 17 Nov 2017, https://talk.ictvonline.org/taxonomy/
Ismail, M.M., Cho, K.O., Ward, L.A., Saif, L.J., Saif, Y.M., 2001. Experimental bovine coronavirus in turkey poults and young chickens. Avian Dis. 45, 157-163.
Ivanov, K.A., Hertzig, T., Rozanov, M., Bayer, S., Thiel, V., Gorbalenya, A.E., Ziebuhr, J., 2004a. Major genetic marker of nidoviruses encodes a replicative endoribonuclease. Proc. Natl. Acad. Sci. U. S. A. 101, 12694-12699.
Ivanov, K.A., Thiel, V., Dobbe, J.C., van der Meer, Y., Snijder, E.J., Ziebuhr, J., 2004b. Multiple enzymatic activities associated with severe acute respiratory syndrome coronavirus helicase. J. Virol. 78, 5619-5632.
Izzo, M., Gunn, A.A., House, J.K., 2015. Neonatal diarrhea. In: Smith, B.P. (Ed.), Large Animal Internal Medicine, 5th ed., Elsevier, St. Louis, Missouri, pp. 314-335.
Jactel, B., Espinasse, J., Viso, M., Valiergue, H., 1990. An epidemiological study of winter dysentery in fifteen herds in France. Vet. Res. Commun. 14, 367-379.
Kamitani, W., Huang, C., Narayanan, K., Lokugamage, K.G., Makino, S., 2009. A two-pronged strategy to suppress host protein synthesis by SARS coronavirus Nsp1 protein. Nat. Struct. Mol. Biol. 16, 1134-1140.
Kamitani, W., Narayanan, K., Huang, C., Lokugamage, K., Ikegami, T., Ito, N., Kubo, H., Makino, S., 2006. Severe acute respiratory syndrome coronavirus nsp1 protein suppresses host gene expression by promoting host mRNA degradation. Proc. Natl. Acad. Sci. U. S. A. 103, 12885-12890.
Kaneshima, T., Hohdatsu, T., Hagino, R., Hosoya, S., Nojiri, Y., Murata, M., Takano, T., Tanabe, M., Tsunemitsu, H., Koyama, H., 2007. The infectivity and pathogenicity of a group 2 bovine coronavirus in pups. J. Vet. Med. Sci. 69, 301-303.
Kang, H., Bhardwaj, K., Li, Y., Palaninathan, S., Sacchettini, J., Guarino, L., Leibowitz, J.L., Kao, C.C., 2007. Biochemical and genetic analyses of murine hepatitis virus Nsp15 endoribonuclease. J. Virol. 81, 13587-13597.

Kanno, T., Hatama, S., Ishihara, R., Uchida, I., 2007. Molecular analysis of the S glycoprotein gene of bovine coronaviruses isolated in Japan from 1999 to 2006. J. Gen. Virol. 88, 1218-1224.
Kapil, S., Pomeroy, K.A., Goyal, S.M., Trent, A.M., 1991. Experimental infection with a virulent pneumoenteric isolate of bovine coronavirus. J. Vet. Diagn. Invest. 3, 88-89.
Kapil, S., Richardson, K.L., Radi, C., Chard-Bergstrom, C., 1996. Factors affecting isolation and propagation of bovine coronavirus in human rectal tumor-18 cell line. J. Vet. Diagn. Invest. 8, 96-99.
Kapil, S., Trent, A.M., Goyal, S.M., 1990. Excretion and persistence of bovine coronavirus in neonatal calves. Arch. Virol. 115, 127-132.
Kilkenny, C., Browne, W.J., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 8, e1000412.
Kin, N., Miszczak, F., Diancourt, L., Caro, V., Moutou, F., Vabret, A., Ar Gouilh, M., 2016. Comparative molecular epidemiology of two closely related coronaviruses, bovine coronavirus (BCoV) and human coronavirus OC43 (HCoV-OC43), reveals a different evolutionary pattern. Infect. Genet. Evol. 40, 186-191.
Klein, D., Kern, A., Lapan, G., Benetka, V., Möstl, K., Hassl, A., Baumgartner, W., 2009. Evaluation of rapid assays for the detection of bovine coronavirus, rotavirus A and Cryptosporidium parvum in faecal samples of calves. Vet. J. 182, 484-486.
Klem, T.B., Kjæstad, H.P., Kummen, E., Holen, H., Stokstad, M., 2016. Bovine respiratory syncytial virus outbreak reduced bulls' weight gain and feed conversion for eight months in a Norwegian beef herd. Acta Vet. Scand. 58, 8.
Klem, T.B., Rimstad, E., Stokstad, M., 2014. Occurrence and phylogenetic analysis of bovine respiratory syncytial virus in outbreaks of respiratory disease in Norway. BMC Vet. Res. 10, 15.
Kohara, J., Hirai, T., Mori, K., Ishizaki, H., Tsunemitsu, H., 1997. Enhancement of passive immunity with maternal vaccine against newborn calf diarrhea. J. Vet. Med. Sci. 59, 1023-1025.
Kuri, T., Eriksson, K.K., Putics, A., Zust, R., Snijder, E.J., Davidson, A.D., Siddell, S.G., Thiel, V., Ziebuhr, J., Weber, F., 2011. The ADP-ribose-1"-monophosphatase domains of severe acute respiratory syndrome coronavirus and human coronavirus 229E mediate resistance to antiviral interferon responses. J. Gen. Virol. 92, 1899-1905.
La Rosa, G., Fratini, M., Della Libera, S., Iaconelli, M., Muscillo, M., 2013. Viral infections acquired indoors through airborne, droplet or contact transmission. Ann. Ist. Super. Sanita 49, 124-132.
Lai, M.M., 1990. Coronavirus: organization, replication and expression of genome. Annu. Rev. Microbiol. 44, 303-333.
Lai, M.M., 1992. RNA recombination in animal and plant viruses. Microbiol. Rev. 56, 61-79.
Lai, M.Y., Cheng, P.K., Lim, W.W., 2005. Survival of severe acute respiratory syndrome coronavirus. Clin. Infect. Dis. 41, e67-71.
Lau, S.K., Lee, P., Tsang, A.K., Yip, C.C., Tse, H., Lee, R.A., So, L.Y., Lau, Y.L., Chan, K.H., Woo, P.C., Yuen, K.Y., 2011. Molecular epidemiology of human coronavirus OC43 reveals evolution of different genotypes over time and recent emergence of a novel genotype due to natural recombination. J. Virol. 85, 11325-11337.
Lauring, A.S., Andino, R., 2010. Quasispecies theory and the behavior of RNA viruses. PLoS Pathog. 6.
Lewis, L.D., Phillips, R.W., 1978. Pathophysiologic changes due to coronavirus-induced diarrhea in the calf. J. Am. Vet. Med. Assoc. 173, 636-642.

Lin, X., O'Reilly, K.L., Burrell, M.L., Storz, J., 2001. Infectivity-neutralizing and hemagglutinin-inhibiting antibody responses to respiratory coronavirus infections of cattle in pathogenesis of shipping fever pneumonia. Clin. Diagn. Lab. Immunol. 8, 357-362.
Lin, X.Q., O'Reilly, K.L., Storz, J., 2002. Antibody responses of cattle with respiratory coronavirus infections during pathogenesis of shipping fever pneumonia are lower with antigens of enteric strains than with those of a respiratory strain. Clin. Diagn. Lab. Immunol. 9, 1010-1013.
Lin, X.Q., O'Reilly, K.L., Storz, J., Purdy, C.W., Loan, R.W., 2000. Antibody responses to respiratory coronavirus infections of cattle during shipping fever pathogenesis. Arch. Virol. 145, 2335-2349.
Liu, L., Hägglund, S., Hakhverdyan, M., Alenius, S., Larsen, L.E., Belák, S., 2006. Molecular epidemiology of bovine coronavirus on the basis of comparative analyses of the S gene. J. Clin. Microbiol. 44, 957-960.
Lojkić, I., Krešić, N., Šimić, I., Bedeković, T., 2015. Detection and molecular characterisation of bovine corona and toroviruses from Croatian cattle. BMC Vet. Res. 11, 202.
Lu, Y., Lu, X., Denison, M.R., 1995. Identification and characterization of a serine-like proteinase of the murine coronavirus MHV-A59. J. Virol. 69, 3554-3559.
Løken, T., Nyberg, O., 2013. Eradication of BVDV in cattle: the Norwegian project. Vet. Rec. 172, 661.
Macpherson, L.W., 1957. Bovine Virus Enteritis (winter dysentery). Can. J. Comp. Med. Vet. Sci. 21, 184-192.
Majhdi, F., Minocha, H.C., Kapil, S., 1997. Isolation and characterization of a coronavirus from elk calves with diarrhea. J. Clin. Microbiol. 35, 2937-2942.
Malboeuf, C.M., Yang, X., Charlebois, P., Qu, J., Berlin, A.M., Casali, M., Pesko, K.N., Boutwell, C.L., DeVincenzo, J.P., Ebel, G.D., Allen, T.M., Zody, M.C., Henn, M.R., Levin, J.Z., 2013. Complete viral RNA genome sequencing of ultra-low copy samples by sequence-independent amplification. Nucleic Acids Res. 41, e13.
Martinez, N., Brandão, P.E., de Souza, S.P., Barrera, M., Santana, N., de Arce, H.D., Perez, L.J., 2012. Molecular and phylogenetic analysis of bovine coronavirus based on the spike glycoprotein gene. Infect. Genet. Evol. 12, 1870-1878.
Mebus, C.A., Newman, L.E., Stair, E.L., Jr., 1975. Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf infected with calf diarrheal coronavirus. Am. J. Vet. Res. 36, 1719-1725.
Mebus, C.A., Stair, E.L., Rhodes, M.B., Twiehaus, M.J., 1973. Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. Vet. Pathol. 10, 45-64.
Minskaia, E., Hertzig, T., Gorbalenya, A.E., Campanacci, V., Cambillau, C., Canard, B., Ziebuhr, J., 2006. Discovery of an RNA virus 3'->5' exoribonuclease that is critically involved in coronavirus RNA synthesis. Proc. Natl. Acad. Sci. U. S. A. 103, 5108-5113.
Moore, S.J., O'Dea, M.A., Perkins, N., O'Hara, A.J., 2015. Estimation of nasal shedding and seroprevalence of organisms known to be associated with bovine respiratory disease in Australian live export cattle. J. Vet. Diagn. Invest. 27, 6-17.
Mullis, L., Saif, L.J., Zhang, Y., Zhang, X., Azevedo, M.S., 2012. Stability of bovine coronavirus on lettuce surfaces under household refrigeration conditions. Food Microbiol. 30, 180-186.
Murray, G.M., O'Neill, R.G., More, S.J., McElroy, M.C., Earley, B., Cassidy, J.P., 2016a. Evolving views on bovine respiratory disease: an appraisal of selected control measures - Part 2. Vet. J. 217, 78-82.

Murray, G.M., O'Neill, R.G., More, S.J., McElroy, M.C., Earley, B., Cassidy, J.P., 2016b. Evolving views on bovine respiratory disease: an appraisal of selected key pathogens - Part 1. Vet. J. 217, 95-102.
Mørk, T., Høgåsen, H.R., Sviland, S., 2016. En risikovurdering av import av levende storfe til Norge fra Sverige, Danmark, Nederland, Frankrike, Storbritannia, New Zealand og Canada. Norwegian Veterinary Institute, Oslo.
Neuman, B.W., Joseph, J.S., Saikatendu, K.S., Serrano, P., Chatterjee, A., Johnson, M.A., Liao, L., Klaus, J.P., Yates, J.R., Wuthrich, K., Stevens, R.C., Buchmeier, M.J., Kuhn, P., 2008. Proteomics analysis unravels the functional repertoire of coronavirus nonstructural protein 3. J. Virol. 82, 5279-5294.
Ng, T.F., Kondov, N.O., Deng, X., Van Eenennaam, A., Neibergs, H.L., Delwart, E., 2015. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J. Virol. 89, 5340-5349.
O'Neill, R., Mooney, J., Connaghan, E., Furphy, C., Graham, D.A., 2014. Patterns of detection of respiratory viruses in nasal swabs from calves in Ireland: a retrospective study. Vet. Rec. 175, 351.
Ohlson, A., Alenius, S., Tråvén, M., Emanuelson, U., 2013. A longitudinal study of the dynamics of bovine corona virus and respiratory syncytial virus infections in dairy herds. Vet. J. 197, 395-400.
Ohlson, A., Heuer, C., Lockhart, C., Tråvén, M., Emanuelson, U., Alenius, S., 2010. Risk factors for seropositivity to bovine coronavirus and bovine respiratory syncytial virus in dairy herds. Vet. Rec. 167, 201-206.
Oostra, M., Hagemeijer, M.C., van Gent, M., Bekker, C.P., te Lintelo, E.G., Rottier, P.J., de Haan, C.A., 2008. Topology and membrane anchoring of the coronavirus replication complex: not all hydrophobic domains of nsp3 and nsp6 are membrane spanning. J. Virol. 82, 12392-12405.
Otter, J.A., Donskey, C., Yezli, S., Douthwaite, S., Goldenberg, S.D., Weber, D.J., 2016. Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. J. Hosp. Infect. 92, 235-250.
Panon, G., Tache, S., Labie, C., 1988. Respective stability of rotavirus and coronavirus in bovine milk. Lait 68, 49-64.
Park, S.J., Kim, G.Y., Choy, H.E., Hong, Y.J., Saif, L.J., Jeong, J.H., Park, S.I., Kim, H.H., Kim, S.K., Shin, S.S., Kang, M.I., Cho, K.O., 2007. Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. Arch. Virol. 152, 1885-1900.
Paton, D.J., Christiansen, K.H., Alenius, S., Cranwell, M.P., Pritchard, G.C., Drew, T.W., 1998. Prevalence of antibodies to bovine virus diarrhoea virus and other viruses in bulk tank milk in England and Wales. Vet. Rec. 142, 385-391.
Perlman, S., Netland, J., 2009. Coronaviruses post-SARS: update on replication and pathogenesis. Nat. Rev. Microbiol. 7, 439-450.
Plummer, P.J., Rohrbach, B.W., Daugherty, R.A., Daugherty, R.A., Thomas, K.V., Wilkes, R.P., Duggan, F.E., Kennedy, M.A., 2004. Effect of intranasal vaccination against bovine enteric coronavirus on the occurrence of respiratory tract disease in a commercial backgrounding feedlot. J. Am. Vet. Med. Assoc. 225, 726-731.
Pyrc, K., Dijkman, R., Deng, L., Jebbink, M.F., Ross, H.A., Berkhout, B., van der Hoek, L., 2006. Mosaic structure of human coronavirus NL63, one thousand years of evolution. J. Mol. Biol. 364, 964-973.
Rechsteiner, J., Winkler, K.C., 1969. Inactivation of respiratory syncytial virus in aerosol. J. Gen. Virol. 5, 405-410.

Reynolds, D.J., Debney, T.G., Hall, G.A., Thomas, L.H., Parsons, K.R., 1985. Studies on the relationship between coronaviruses from the intestinal and respiratory tracts of calves. Arch. Virol. 85, 71-83.
Roberts, S.J., 1957. Winter dysentery in dairy cattle. Cornell Vet. 47, 372-388.
Rollinson, D.H., 1948. Infectious diarrhoea of dairy cows. Vet. Rec. 60, 191.
Ruud, T.A., Nafstad, O., Wittussen, H.T., Juul-Hansen, B.-O., Mellby, J.O., Røhnebæk, E., Aass, L., Rustad, L.J., Anderssen, Å.M.F., 2013. Økt storfekjøttproduksjon i Norge. Animalia, Oslo.
Saif, L.J., 1987. Development of nasal, fecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. Vet. Immunol. Immunopathol. 17, 425-439.
Saif, L.J., 1990. A review of evidence implicating bovine coronavirus in the etiology of winter dysentery in cows: an enigma resolved? Cornell Vet. 80, 303-311.
Saif, L.J., 2010. Bovine respiratory coronavirus. Vet. Clin. North Am. Food Anim. Pract. 26, 349-364.
Saif, L.J., Redman, D.R., Moorhead, P.D., Theil, K.W., 1986. Experimentally induced coronavirus infections in calves: viral replication in the respiratory and intestinal tracts. Am. J. Vet. Res. 47, 1426-1432.
Schirmer, M., D'Amore, R., Ijaz, U.Z., Hall, N., Quince, C., 2016. Illumina error profiles: resolving fine-scale variation in metagenomic sequencing data. BMC Bioinformatics 17, 125.
Seybert, A., Hegyi, A., Siddell, S.G., Ziebuhr, J., 2000. The human coronavirus 229E superfamily 1 helicase has RNA and DNA duplex-unwinding activities with 5'-to-3' polarity. RNA 6, 1056-1068.
Siddell, S.G., 1995. The coronaviridae: an introduction. In: Siddell, S.G. (Ed.), The Coronaviridae, Plenum Press, New York, pp. 1-10.
Sims, D., Sudbery, I., Ilott, N.E., Heger, A., Ponting, C.P., 2014. Sequencing depth and coverage: key considerations in genomic analyses. Nat. Rev. Genet. 15, 121-132.
Sizun, J., Yu, M.W., Talbot, P.J., 2000. Survival of human coronaviruses 229E and OC43 in suspension and after drying on surfaces: a possible source of hospital-acquired infections. J. Hosp. Infect. 46, 55-60.
Smith, E.C., Case, J.B., Blanc, H., Isakov, O., Shomron, N., Vignuzzi, M., Denison, M.R., 2015. Mutations in coronavirus nonstructural protein 10 decrease virus replication fidelity. J. Virol. 89, 6418-6426.
Smith, E.C., Denison, M.R., 2012. Implications of altered replication fidelity on the evolution and pathogenesis of coronaviruses. Curr. Opin. Virol. 2, 519-524.
Stair, E.L., Rhodes, M.B., White, R.G., Mebus, C.A., 1972. Neonatal calf diarrhea: purification and electron microscopy of a coronavirus-like agent. Am. J. Vet. Res. 33, 1147-1156.
Statistics Norway, 2017. Livestock husbandry. Accessed: 19 Jan 18, https://www.ssb.no/jord-skog-jakt-og-fiskeri/statistikker/jordhus/aar
Storz, J., Lin, X., Purdy, C.W., Chouljenko, V.N., Kousoulas, K.G., Enright, F.M., Gilmore, W.C., Briggs, R.E., Loan, R.W., 2000a. Coronavirus and Pasteurella infections in bovine shipping fever pneumonia and Evans' criteria for causation. J. Clin. Microbiol. 38, 3291-3298.
Storz, J., Purdy, C.W., Lin, X., Burrell, M., Truax, R.E., Briggs, R.E., Frank, G.H., Loan, R.W., 2000b. Isolation of respiratory bovine coronavirus, other cytocidal viruses, and Pasteurella spp from cattle involved in two natural outbreaks of shipping fever. J. Am. Vet. Med. Assoc. 216, 1599-1604.

Sutton, G., Fry, E., Carter, L., Sainsbury, S., Walter, T., Nettleship, J., Berrow, N., Owens, R., Gilbert, R., Davidson, A., Siddell, S., Poon, L.L., Diprose, J., Alderton, D., Walsh, M., Grimes, J.M., Stuart, D.I., 2004. The nsp9 replicase protein of SARS-coronavirus, structure and functional insights. Structure 12, 341-353.

te Velthuis, A.J., Arnold, J.J., Cameron, C.E., van den Worm, S.H., Snijder, E.J., 2010. The RNA polymerase activity of SARS-coronavirus nsp12 is primer dependent. Nucleic Acids Res. 38, 203-214.

Tennant, B.J., Gaskell, R.M., Gaskell, C.J., 1994. Studies on the survival of canine coronavirus under different environmental conditions. Vet. Microbiol. 42, 255-259.

Theurer, M.E., Larson, R.L., White, B.J., 2015. Systematic review and meta-analysis of the effectiveness of commercially available vaccines against bovine herpesvirus, bovine viral diarrhea virus, bovine respiratory syncytial virus, and parainfluenza type 3 virus for mitigation of bovine respiratory disease complex in cattle. J. Am. Vet. Med. Assoc. 246, 126-142.

Thiel, V., Ivanov, K.A., Putics, A., Hertzig, T., Schelle, B., Bayer, S., Weissbrich, B., Snijder, E.J., Rabenau, H., Doerr, H.W., Gorbalenya, A.E., Ziebuhr, J., 2003. Mechanisms and enzymes involved in SARS coronavirus genome expression. J. Gen. Virol. 84, 2305-2315.

Toftaker, I., Holmøy, I., Nødtvedt, A., Østerås, O., Stokstad, M., 2017. A cohort study of the effect of winter dysentery on herd-level milk production. J. Dairy Sci. 100, 6483-6493.

Toftaker, I., Sanchez, J., Stokstad, M., Nødtvedt, A., 2016. Bovine respiratory syncytial virus and bovine coronavirus antibodies in bulk tank milk - risk factors and spatial analysis. Prev. Vet. Med. 133, 73-83.

Tråvén, M., 2000. Winter dysentery caused by bovine coronavirus; no rule without an exception : diagnostics, clinical picture, epidemiology and herd immunology. Dissertation, Swedish University of Agricultural Science, Uppsala.

Tråvén, M., Carlsson, U., Lundén, A., Larsson, B., 1999. Serum antibodies to bovine coronavirus in Swedish sheep. Acta Vet. Scand. 40, 69-74.

Tråvén, M., Näslund, K., Linde, N., Linde, B., Silván, A., Fossum, C., Hedlund, K.O., Larsson, B., 2001. Experimental reproduction of winter dysentery in lactating cows using BCV -- comparison with BCV infection in milk-fed calves. Vet. Microbiol. 81, 127-151.

Tråvén, M., Sundberg, J., Larsson, B., Niskanen, R., 1993. Winter dysentery diagnosed by farmers in dairy herds in central Sweden: incidence, clinical signs and protective immunity. Vet. Rec. 133, 315-318.

Tseng, C.-T., Sbrana, E., Iwata-Yoshikawa, N., Newman, P.C., Garron, T., Atmar, R.L., Peters, C.J., Couch, R.B., 2012. Immunization with SARS Coronavirus Vaccines Leads to Pulmonary Immunopathology on Challenge with the SARS Virus. PLoS One 7, e35421.

Tsunemitsu, H., Saif, L.J., 1995. Antigenic and biological comparisons of bovine coronaviruses derived from neonatal calf diarrhea and winter dysentery of adult cattle. Arch. Virol. 140, 1303-1311.

Tsunemitsu, H., Smith, D.R., Saif, L.J., 1999. Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. Arch. Virol. 144, 167-175.

Valarcher, J.F., Bourhy, H., Lavenu, A., Bourges-Abella, N., Roth, M., Andreoletti, O., Ave, P., Schelcher, F., 2001. Persistent infection of B lymphocytes by bovine respiratory syncytial virus. Virology 291, 55-67.

van Dijk, E.L., Jaszczyszyn, Y., Thermes, C., 2014. Library preparation methods for next-generation sequencing: tone down the bias. Exp. Cell Res. 322, 12-20.
van Elden, L.J., van Kraaij, M.G., Nijhuis, M., Hendriksen, K.A., Dekker, A.W., Rozenberg-Arska, M., van Loon, A.M., 2002. Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia. Clin. Infect. Dis. 34, 177-183.
Van Kruiningen, H.J., Hiestand, L., Hill, D.L., Tilton, R.C., Ryan, R.W., 1985. Winter dysentery in dairy cattle: recent findings. Compend. Contin. Educ. Pract. Vet., S591-S596.
Vijgen, L., Keyaerts, E., Moes, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A.M., Van Ranst, M., 2005. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J. Virol. 79, 1595-1604.
Waltner-Toews, D., Martin, S.W., Meek, A.H., McMillan, I., Crouch, C.F., 1985. A field trial to evaluate the efficacy of a combined rotavirus-coronavirus/Escherichia coli vaccine in dairy cattle. Can. J. Comp. Med. 49, 1-9.
Watson, J.D., Wang, S., Von Stetina, S.E., Spencer, W.C., Levy, S., Dexheimer, P.J., Kurn, N., Heath, J.D., Miller, D.M., 2008. Complementary RNA amplification methods enhance microarray identification of transcripts expressed in the C. elegans nervous system. BMC Genomics 9, 84.
Yoo, D., Deregt, D., 2001. A single amino acid change within antigenic domain II of the spike protein of bovine coronavirus confers resistance to virus neutralization. Clin. Diagn. Lab. Immunol. 8, 297-302.
Zhai, Y., Sun, F., Li, X., Pang, H., Xu, X., Bartlam, M., Rao, Z., 2005. Insights into SARS-CoV transcription and replication from the structure of the nsp7-nsp8 hexadecamer. Nat. Struct. Mol. Biol. 12, 980-986.
Zhang, X., Hasoksuz, M., Spiro, D., Halpin, R., Wang, S., Vlasova, A., Janies, D., Jones, L.R., Ghedin, E., Saif, L.J., 2007. Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation. Virology 363, 1-10.
Zhang, X.M., Herbst, W., Kousoulas, K.G., Storz, J., 1994. Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. J. Med. Virol. 44, 152-161.
Züst, R., Cervantes-Barragan, L., Habjan, M., Maier, R., Neuman, B.W., Ziebuhr, J., Szretter, K.J., Baker, S.C., Barchet, W., Diamond, M.S., Siddell, S.G., Ludewig, B., Thiel, V., 2011. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. Nat. Immunol. 12, 137-143.

Papers

Oma et al. Virology Journal (2016) 13:100
DOI 10.1186/s12985-016-0555-x

Virology Journal

RESEARCH Open Access

Bovine coronavirus in naturally and experimentally exposed calves; viral shedding and the potential for transmission

Veslemøy Sunniva Oma[1*], Madeleine Tråvén[2], Stefan Alenius[2], Mette Myrmel[3] and Maria Stokstad[1]

Abstract

Background: Bovine coronavirus (BCoV) is a widely distributed pathogen, causing disease and economic losses in the cattle industry worldwide. Prevention of virus spread is impeded by a lack of basic knowledge concerning viral shedding and transmission potential in individual animals. The aims of the study were to investigate the duration and quantity of BCoV shedding in feces and nasal secretions related to clinical signs, the presence of virus in blood and tissues and to test the hypothesis that seropositive calves are not infectious to naïve in-contact calves three weeks after BCoV infection.

Methods: A live animal experiment was conducted, with direct contact between animal groups for 24 h as challenge procedure. Four naïve calves were commingled with a group of six naturally infected calves and sequentially euthanized. Two naïve sentinel calves were commingled with the experimentally exposed group three weeks after exposure. Nasal swabs, feces, blood and tissue samples were analyzed for viral RNA by RT-qPCR, and virus isolation was performed on nasal swabs. Serum was analyzed for BCoV antibodies.

Results: The calves showed mild general signs, and the most prominent signs were from the respiratory system. The overall clinical score corresponded well with the shedding of viral RNA the first three weeks after challenge. General depression and cough were the signs that correlated best with shedding of BCoV RNA, while peak respiratory rate and peak rectal temperature appeared more than a week later than the peak shedding. Nasal shedding preceded fecal shedding, and the calves had detectable amounts of viral RNA intermittently in feces through day 35 and in nasal secretions through day 28, however virus isolation was unsuccessful from day six and day 18 from the two calves investigated. Viral RNA was not detected in blood, but was found in lymphatic tissue through day 42 after challenge. Although the calves were shedding BCoV RNA 21 days after infection the sentinel animals were not infected.

Conclusions: Prolonged shedding of BCoV RNA can occur, but detection of viral RNA does not necessarily indicate a transmission potential. The study provides valuable information with regard to producing scientifically based biosecurity advices.

Keywords: BCoV, BCV, Experimental infection, Clinical signs, Virus shedding, Transmission potential, Viremia, Biosecurity, Virus quantification, RT-qPCR, Virus isolation

* Correspondence: Veslemoy.Oma@nmbu.no
[1]Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Ullevålsvegen 72, 0454 Oslo, Norway
Full list of author information is available at the end of the article

Background

Bovine coronavirus (BCoV) is an important livestock pathogen with a high prevalence worldwide. The virus causes respiratory disease and diarrhea in calves and winter dysentery in adult cattle. These diseases result in substantial economic losses and reduced animal welfare [1]. One way of reducing the negative consequences of this virus is to prevent virus transmission between herds. Inter-herd transmission is possible either directly via transfer of live animals [2, 3], or indirectly via contaminated personnel or equipment [4]. Measures to prevent virus spread between herds must be based upon knowledge of viral shedding, the potential for transmission to susceptible animals and the role of protective immunity. Several observational studies have been published on BCoV shedding in feces of diarrheic calves and after transportation to feedlots [3, 5–10]. However, relatively few studies on BCoV pathogenesis with emphasis on transmission potential under controlled conditions have been published.

BCoV belongs to the genus *Betacoronavirus* within the family *Coronaviridae,* also including the closely related HCoV-OC43, which causes respiratory infections in humans, and the human pathogens SARS-CoV and MERS-CoV [11–13].

BCoV consists of one serotype with some antigenic variation between different strains [14, 15]. Acutely infected animals develop antibodies that persist for a long period, possibly for several years [16–18]. However, the protective immunity is shorter and incomplete. In two experimental studies, infected calves were not protected against reinfection with a different BCoV strain three weeks after the first challenge, but did not develop clinical signs [19, 20].

BCoV is transmitted via the fecal-oral or respiratory route [15]. It infects epithelial cells in the respiratory tract and the intestines; the nasal turbinates, trachea and lungs and the villi and crypts of the small and large intestine, respectively [21, 22]. Replication leads to shedding of virus in nasal secretions and in feces. Important factors for the pathogenesis are still not fully explored, such as how the virus infects enterocytes shortly after introduction to an animal. Viremia has been detected in one study by Park et al. [21]. Clinical signs range from none to severe, and include fever, respiratory signs and diarrhea with or without blood [1, 15]. As the time of infection is usually unknown and laboratory diagnostics are usually not performed, occurrence of clinical signs is the most relevant parameter to relate to viral shedding. The majority of experimental studies have used BCoV inoculation as challenge procedure, which may influence clinical signs and viral shedding, and thereby the transmission potential compared to natural infection. It has been hypothesized that BCoV can cause chronic subclinical infections which could be an important virus source [15]. Kapil et al. documented viral antigen in the small and large intestines of infected calves three weeks post inoculation [23]. Crouch et al. found that ten cows were shedding BCoV-immune complexes in the feces for 12 weeks [24]. It is, however, difficult to establish whether there is true persistence of virus, or reinfection of partially immune animals and whether these animals represent a risk to other animals. There is a lack of experimental studies investigating viral shedding pattern for longer periods than two weeks, with sensitive detection methods. Viral load and infectivity also needs to be determined. This is of high practical relevance, since the farmers need guidance on biosecurity in trade and transport of live animals.

The current study was conducted to fill prevailing gaps in the knowledge on fundamental aspects of BCoV infection. The specific aims were to:

1. study the duration and quantity of BCoV shedding in feces and nasal secretions, related to clinical signs in calves.
2. study the presence of viremia and persistence of virus in lymphatic, intestinal and lung tissue.
3. test the hypothesis that seropositive calves are not infectious to naïve in-contact calves three weeks after BCoV infection.

Methods

Study design

A live animal experiment with the natural host was conducted. The experimental units were groups of calves and the intervention consisted of direct contact with BCoV-infected animals. The primary outcome was clinical signs, and the secondary outcome was presence of BCoV RNA and BCoV antibodies. Three experimental groups were included; the Field group (FG, $n = 6$) that was naturally infected with BCoV, the naïve Exposed group (EG, $n = 4$) and the naïve Sentinel group (SG, $n = 2$). An overview of the study design is shown in Fig. 1.

Animals, housing and husbandry

Animals

Twelve BCoV seronegative weaned bull calves between six and twelve weeks of age were included, seven were Swedish red and white, four were Swedish Holstein and one Swedish mountain breed. They originated from two dairy herds, initially negative for antibodies to BCoV in milk from primiparous cows. The calves were allocated to groups according to herd of origin and day of arrival. The sequence of euthanasia of the EG and SG calves was random, determined by drawing of lots.

Natural outbreak of winter dysentery

FG originated from a herd that was in an early phase of a winter dysentery outbreak. When FG was transported

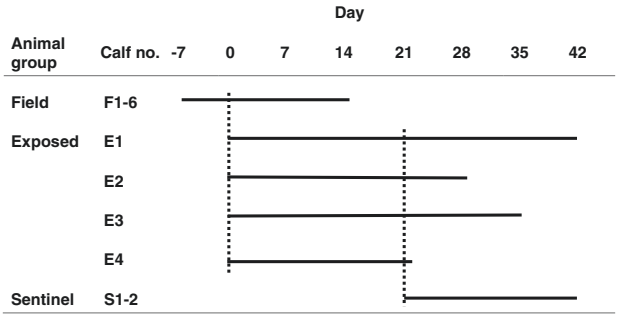


Fig. 1 Timeline of the experiment. The solid lines symbolize the timespan when the calves participated in the experiment. The dashed lines symbolize commingling of the indicated animals for 24 h; e.g. the field group arrived at the research facility on day −7, commingled with the exposed group day 0 and left the research facility day 14. The calves in the exposed group were sequentially euthanized from day 22 to day 42. The Sentinel group arrived at the research facility day 21 and commingled with the Exposed group the following 24 hours

to the research facility, the calves showed mild signs of respiratory disease. Two days later, a severe outbreak confirmed by RT-PCR and serology to be caused by BCoV with bloody diarrhea and reduction in milk production, took place in the herd.

Research facility

The experiment was conducted at the stationary clinic at the Department of Clinical Sciences at the Swedish University of Agricultural Sciences. The facility was closed for other animals during the experiment, and had restricted admission for people. Personnel used designated clothing, and had no contact with other cattle the same day. Each group was housed in separate pens within the same room. Due to the type of facility and design of the study, acclimatization period was not possible for any of the groups. Clinical examinations and sampling were consistently done in the order SG, EG and FG.

Challenge procedure

To mimic standard managerial conditions, direct contact was chosen as challenge procedure for both EG and SG. The commingling was done by moving EG into the other two groups' pens for 24 h.

Refinement and treatment procedures

Efforts were made to minimize the stress and discomfort for the animals involved. The calves were kept groupwise in pens with straw bedding, were fed a commercial calf concentrate twice daily and had access to haylage ad libitum. The animals were monitored by a trained animal technician and a veterinarian at least three times a day. Indications for antibiotic treatment (30 000 IU procaine benzyl penicillin/kg bodyweight/day i.m. for five consecutive days) were abnormal sounds on lung auscultation or prolonged high temperature. Indication for treatment with a non-steroidal anti-inflammatory drug (Metacam vet, Boehringer Ingelheim Vetmedica, Germany) was severe depression, and oral fluid with electrolytes was to be given to moderately dehydrated animals. Euthanasia was achieved by i.v. injection of pentobarbital (Euthasol vet., Le Vet, Netherlands).

Clinical score

Daily clinical examinations were performed by a veterinarian and clinical signs were scored as presented in Table 1 (modified after Hägglund et al. [25, 26] and Silverlås et al. [27]). A score above two on three consecutive days was categorized as mild clinical disease; a

Table 1 Clinical scoring system

Score	Respiratory rate (breaths/min)	Fever	Cough	Nasal discharge	Demeanor	Fecal consistency
0	≤49	≤39,5	No cough observed	Normal	Bright, alert	Normal
1	50–54	39,6–39,9	Sporadic cough	Serous or mucous	Mildly depressed	Pasty
2	55–64	40–40,4	More than one sporadic cough every 10 min of observation	Mucopurulent or purulent	Moderately depressed	Runny
3	65–74	>40,5	–	–	Severely depressed	Watery
4	75–85	–	–	–	–	Runny or watery with blood

The score from each category was added to give a daily clinical score for each of the calves in the experiment

score above six on three consecutive days as moderate disease and a score above eleven was categorized as severe clinical disease.

Collection of material

Nasal swab specimens and fecal samples from FG were collected approximately every third day from day −4 (D-4) to D14. From EG, nasal swabs and fecal samples were collected every day from D0 to D25 and then every third day until D35. Nasal swabs from SG were collected D24, D27 and D29. The nasal specimens were collected by rotating a flocked ESwab™ (Copan, Brescia, Italy) approximately five cm inside one of the calf's nostrils. The specimens were frozen and stored at −70 °C before further processing. Blood was drawn from the jugular vein upon arrival and D1, D2, D3, D5, D7, D9, D11, D14, D21, D35 and D41 using sterile evacuated tubes with and without EDTA-anticoagulant. The EDTA-blood was centrifuged and the cell fractions were stored separately at −80 °C before further processing. Sera were stored at −20 °C until analyzed. Tissue samples from lung, medial retropharyngeal and mesenteric lymph nodes, ileum, and colon were stored in RNA-later at −20 °C.

Antibody ELISA

Serum samples were analyzed for anti BCoV IgG by Svanovir BCV-Ab (Boehringer Ingelheim Svanova, Uppsala, Sweden) according to the manufacturer's instructions. Samples from SG were also tested for antibodies to bovine respiratory syncytial virus (BRSV) by Svanovir BRSV-Ab (Boehringer Ingelheim). The optical density (OD) at 450 nm was measured and corrected by subtracting the OD for the negative control. Percent positivity (PP) was calculated as (sample OD/positive control OD) × 100, and a PP-value of <10 was regarded as negative.

Extraction of RNA and RT-qPCR

Fecal samples (diluted 1:10 in PBS) and nasal swab specimens were centrifuged at 9700 x *g* for 10 min. RNA was extracted from 140 μl supernatant and 140 μl plasma by QIAamp Viral RNA Mini QIAcube kit (Qiagen, Hilden, Germany), eluted in 50 μl and frozen at −80 °C. RNA from blood cell fractions from calf E4 on D5 and calf E3 on D7 was extracted with Qiazol (Qiagen) and chloroform phase separation mixed with 70 % ethanol (1:1) and purified using RNeasy Mini Kit column (Qiagen), while RNA was extracted from 30–50 mg tissue samples, using RNeasy Plus Universal Mini Kit (Qiagen). RT-qPCR was performed using RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, MA, USA). Two microliters of RNA was added to a 18 μl reaction volume containing 200 nM each of forward and reverse primers and 250 nM TaqMan probe [28]. The thermal profile included an RT step with 30 min at 55 °C followed by 95 °C for 2 min. Thereafter, 40 cycles with 15 s at 95 °C and 60 s at 60 °C were conducted. The RT-qPCR was performed on a Stratagene Mx3005p™ (Agilent Technologies, CA, USA) and a positive and a negative control were included in each run. In order to evaluate inhibition of the RT-qPCR, RNA extract from some fecal samples were diluted 1:10 and compared to undiluted RNA. The Ct-values in these samples suggested negligible levels of inhibitors. Inhibitors in plasma and cell extracts were evaluated by spiking with mengovirus RNA. Comparison of Ct-values showed that plasma had no negative effect, while the cell fractions had an inhibitory effect, giving an increase of one Ct-value.

Virus quantitation

In order to estimate the number of BCoV viral RNA copies (VRC) in the clinical samples, a standard curve was prepared using tenfold dilutions of a plasmid containing the BCoV target sequence. Aliquoted BCoV RNA was used as a calibrator and included in every RT-qPCR plate to adjust for inter plate variation. The number of VRC in the clinical samples was calculated using the formula:

$$Q_S = Q_C * 10^{\frac{Ct_S - Ct_C}{m}}$$

Where Q_s = *viral RNA copies in sample,* Q_c = *viral RNA copies of calibrator,* Ct_s = *Ct value of sample,* Ct_c = *Ct value of calibrator* and m = *slope of the standard curve.*

The standard curve covered the range from 10.8 to 1.08×10^{10} plasmid copies, and showed a strong linear relationship with a high coefficient of determination ($R^2 = 0.996$) and a high amplification efficiency (96.5 %). The limit of quantification (LOQ) for the plasmid was 10.8 copies which represented 3.6 $\log_{10}$ BCoV VRC per nasal swab and ml plasma, 4.6 $\log_{10}$ VRC/g feces and 4.2 $\log_{10}$ VRC/g tissue.

Virus isolation

Virus infectivity was tested by virus isolation from nasal swabs from E1 and E3 between D3 and D28 (D3, D6, D7, D8, D10, D13, D18, D23 and D28). The swab supernatants were diluted 1:25 in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Paisley, Scotland), filtered through a 0.8 μm filter (Sartorius Stedim Biotech, Goettingen, Germany) and added to a monolayer of 4-days-old human rectal tumor cells (HRT-18G, ATTCC CRL-11663) in a 24-well plate. In addition, infective virus was titrated from one nasal swab supernatant using two-fold endpoint dilutions in a 96-well plate. After 1 h incubation at 37 °C, the inoculum was replaced with DMEM with 1 % fetal calf serum and antibiotics (5000 IU penicillin and 5 mg streptocillin/ml). After two days at 37 °C and 5 % CO_2, the cells were

fixed with Intracellular Fixation buffer (eBiosience, CA, USA) and stained with 1:80 dilution of monoclonal mouse anti-coronavirus antibody labelled with fluorescein isothiocynate (BioX Diagnostics, Rochefort, Belgium) and DAPI nuclear counterstain (Thermo Fischer Scientific). The wells were observed under a fluorescent microscope for antigen positive cells.

Results

Clinical outcome

An overview of clinical signs in all groups is presented in Table 2. Five out of six FG calves showed mild clinical disease. EG's daily clinical scores are shown in Fig. 2. Three out of four EG calves showed mild disease, and one calf moderate clinical disease. SG did not develop clinical signs that were categorized as disease in the clinical scoring system. However, both calves had some days with intermittent nasal discharge and sporadic cough and S1 had a few days with intermittently runny feces. Blood-tinged diarrhea or nasal discharge was not observed in any of the groups.

Serology

All calves tested negative for antibodies to BCoV at the beginning of the trial. At D14 all calves in FG and EG had seroconverted (Additional file 1: Table S1). The SG was still seronegative to BCoV D42 and did not show an increase in titer for antibodies to BRSV.

Viral RNA in blood

BCoV RNA was not detected in any of the blood samples analyzed.

Nasal shedding of viral RNA

The nasal shedding of BCoV RNA from FG and EG is presented in Fig. 3a, and Fig. 2 shows EG calves' individual shedding. Briefly, FG was shedding BCoV RNA D-4 through D11, and in EG all swabs were positive from D1 through D12, and at least one out of four calves was positive through D28 (Fig. 3a). Two calves were positive in nasal swabs with a concentration of 5.4 $\log_{10}$ and 4.0 $\log_{10}$ VRC/swab the day of commingling with SG. None of the nasal swabs from SG were positive.

Fecal shedding of viral RNA

Fecal shedding of BCoV RNA in FG and EG is shown in Fig. 3b, and the individual shedding from EG in Fig. 2. Viral RNA was detected in fecal samples from FG between D-4 and D14. Fecal samples from EG were negative D0 and D1. At least two out of four calves were positive every day from D2 through D17 and BCoV RNA was intermittently detected through D35. After D14, three calves had a period of four to six days with negative results, before they again started shedding BCoV RNA for three to five days (Fig. 2).

Association between PCR positivity and clinical signs

The association between BCoV PCR results and selected clinical signs is shown in Figs. 4 and 5. The overall clinical score showed good correlation with detection of BCoV RNA. General depression and cough were the individual scores that showed the best association with BCoV RNA shedding. The highest mean respiratory rate and rectal temperature appeared more than a week later than the peak shedding.

Table 2 Key clinical signs and treatment during an experiment with BCoV infected calves

Animal group	Calf no.	Peak rt[a] (°C)	Number of days with				Peak clinical score	Days with clinical score >6	Day of treatment initiation[d]
			depression	diarrhea[b]	nasal discharge[c]	respiratory rate ≥65			
Field	F1	40,3	0	3	1	0	5	0	−1
	F2	39,8	3	2	1	0	8	2	3
	F3	40,1	2	0	7	0	7	1	−5[e]
	F4	40,6	1	0	4	1	7	2	2
	F5	39,7	2	13	1	2	6	0	-
	F6	39,2	1	0	4	0	5	0	-
Exposed	E1	39,5	7	0	2	3	7	1	-
	E2	39,8	4	1	7	1	8	3	7
	E3	40,2	8	1	19	2	9	5	5 and 18
	E4	39,9	6	1	8	0	8	4	-
Sentinel	S1	39,2	1	4	3	0	5	0	-
	S2	39,4	2	0	3	0	4	0	-

The calves were exposed to BCoV in the field (F1-6), were exposed to F-animals (E1-4) or exposed to E-animals (S1-2). [a]Peak rectal temperature (rt) [b]Runny to watery stools were considered diarrheic. [c] Mucopurulent or purulent nasal discharge (nasal discharge score =2). [d] Five days of i.m. treatment with 30 000 IU procaine benzylpenicillin was initiated on indicated day. [e] Calf F3 was treated for six days

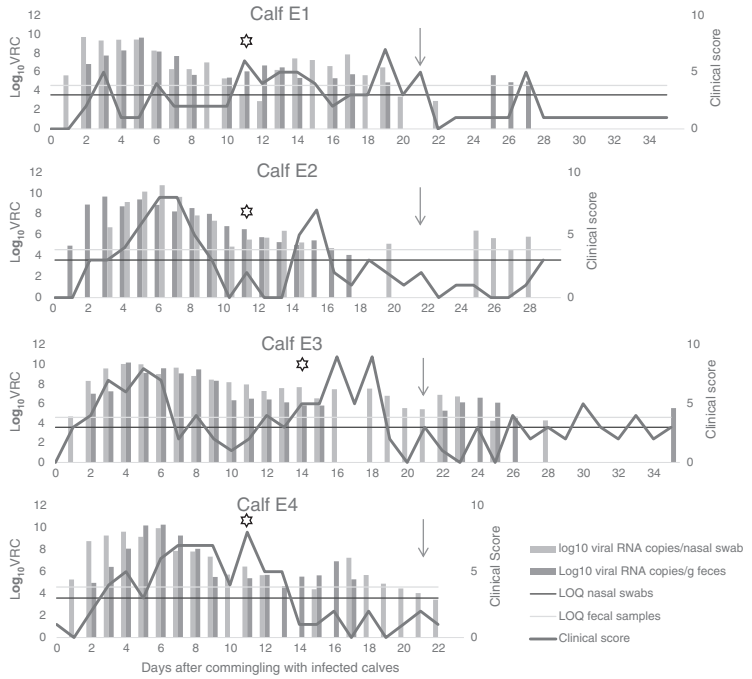


Fig. 2 Clinical score and viral shedding in BCoV infected calves. Number of BCoV viral RNA copies (VRC) per nasal swab and per gram of feces collected from calves in the Exposed group (EG) from day 0 (the day of commingling with the Field group) through day 35. Limit of quantification (LOQ) in nasal swab specimens and fecal samples is indicated with horizontal lines. The values under LOQ are extrapolated and less accurate. A star indicates the day of seroconversion (percent positivity > 10) to BCoV and the arrow indicates the day of commingling with the Sentinel group (day 21). The clinical scores are calculated based on daily registrations of clinical signs from the calves in EG

Viral RNA in tissues

Viral RNA was detected in lymph nodes from the EG calves euthanized three, four, five and six weeks after infection (Table 3). Viral RNA was also detected in ileum and colon from the animals euthanized five and six weeks after infection, but not in lung tissue.

Virus isolation

Virus was isolated from nasal swabs from calf E1 on D3 and from E3 in the period D3 to D13. A photograph of infected cells is shown in Fig. 6. The titer of infective BCoV in the nasal swab was 2560 per 50 μl swab medium (1 ml in total) corresponding to 4.7 $\log_{10}$ infective particles in a swab containing 9.8 $\log_{10}$ VRC, giving a total to infective particles ratio (T/I) of 5 $\log_{10}$.

Discussion

The present study showed that calves infected with BCoV shed viral RNA for five weeks, and harbored viral RNA in intestinal tissues and lymph nodes even longer. Interestingly, contact with these calves three weeks after challenge, when the clinical condition had improved and the calves had seroconverted, did not lead to infection in sentinel calves and virus isolation was not possible from calves shedding viral RNA at this time point.

In concordance with other studies [18, 29], all EG calves became BCoV positive shortly after contact with infected calves and shed viral RNA continuously for two weeks. This supports that introduction of BCoV into a naïve population leads to a high basic reproduction number (R_0). R_0 depends on the duration of the infectious period, the number of exposed susceptible individuals and the probability of a susceptible individual to be infected. In herds and transportation systems where cattle from different herds are commingled, the risk of virus transmission is high.

The detection of BCoV RNA in nasal swabs from naïve calves in EG shortly after exposure might be due to passive inhalation of virus excreted by the FG, or to virus replication in the respiratory tract. Since the viral load in the nasal swabs from EG exceeded that of FG at D2, the study confirms that BCoV replicated massively in the airways of EG calves already at D2. Fecal shedding

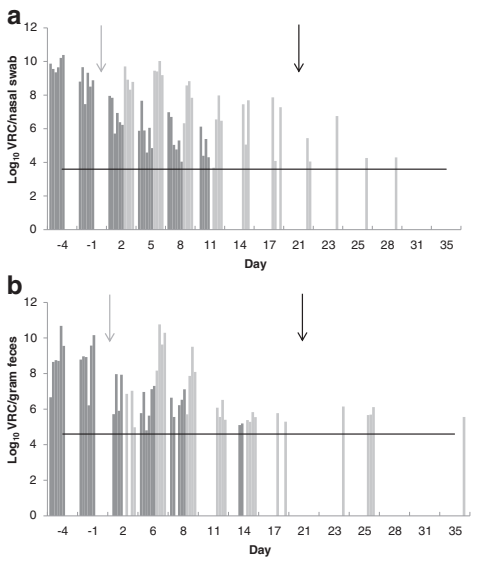


Fig. 3 Log_{10} viral RNA copies (VRC) of BCoV per nasal swab (**a**) and gram feces (**b**). Shedding of BCoV from calves in the Field group (FG) (dark grey) and in the Exposed group (EG) (light grey). Grey arrow; day of EG and FG commingling. Black arrow; day of Sentinel group and EG commingling. The horizontal lines show the limit of quantification of VRC

started later than nasal shedding which is in concurrence with other studies [30]. Saif and colleagues found that when inoculating calves intranasally, BCoV was first detected in nasal epithelial cells and secondly in feces. In contrast, in calves inoculated orally, fecal detection of BCoV preceded detection in nasal swab specimens. They concluded that the infection route could determine the sequence of infection of the respiratory and intestinal tract [22]. The present study supports that the respiratory route is the most common infection route when calves are naturally infected by direct contact. With indirect virus spread, the fecal-oral route could be more common.

Nasal swabs were more often positive for BCoV than fecal samples in this trial, most likely due to a higher limit of detection for BCoV in feces than in nasal swabs. For diagnostic purposes, nasal swab specimens therefore seem advantageous to fecal samples for virus detection in calves with suspected BCoV related disease.

Moving and commingling are associated with stress, which has been found to affect the intestinal immune system [31]. It is possible that stress increased the BCoV RNA shedding observed in the EG calves after introduction of the sentinel calves. Buying and selling of calves often involve extended transportation and commingling with susceptible cattle. The stress response, and a possible increased fecal shedding of virus, would probably be higher under field conditions.

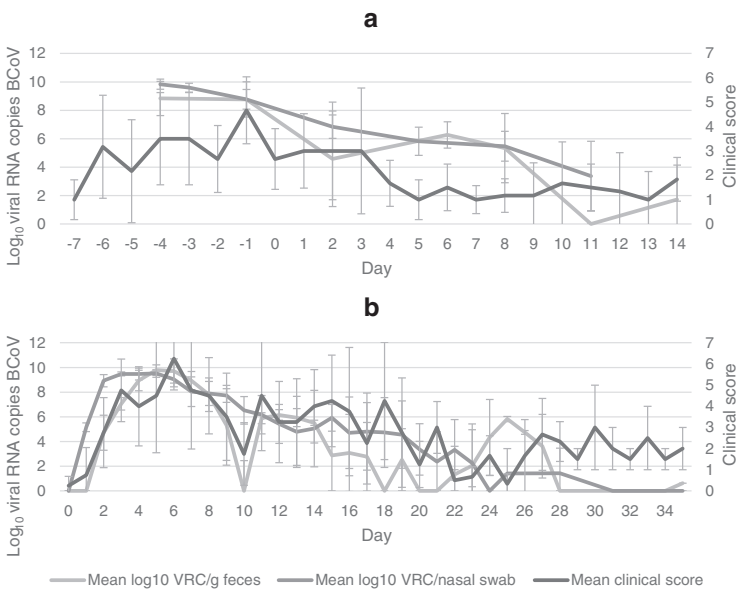


Fig. 4 Clinical score and viral shedding in calves exposed to BCoV. Mean clinical score and mean log_{10} viral RNA copies (VRC) of BCoV per nasal swab and gram feces from calves in the Field group (FG) (**a**) and Exposed group (**b**)

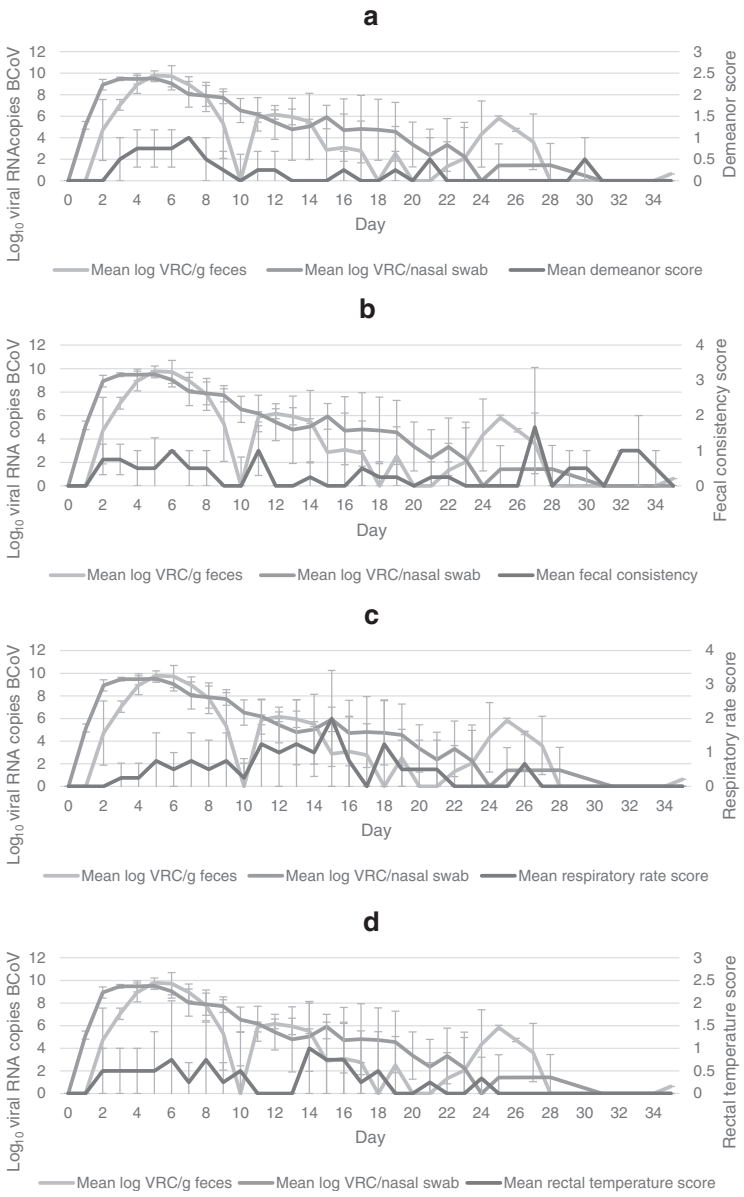


Fig. 5 Association between viral shedding and scoring of clinical signs. Mean daily shedding of BCoV and scoring of demeanor (**a**), fecal consistency (**b**), respiratory rate (**c**) and rectal temperature (**d**) of calves in the Exposed group after exposure to BCoV infected calves. The shedding is shown as mean $\log_{10}$ viral RNA copies (VRC) of BCoV per nasal swab and gram feces

In the acute stage of the infection, the agreement between positive PCR results and clinical score was relatively high. Three weeks after exposure to BCoV, the clinical signs and detection of viral RNA varied more independently. In an experiment with porcine deltacoronavirus, the severity of the clinical signs did not correlate with the

Table 3 Log_{10} viral RNA copies of BCoV per gram tissue

Days post exposure	Calf	Medial retropharyngeal lymph node	Mesenteric lymph node	Lung	Ileum	Colon
22	E4	6.9	6.3	Not done	Not done	Not done
28	E2	6.7	Negative	Not done	Not done	Not done
35	E3	Negative	5.0	Negative	6.0	5.2
42	E1	6.2	7.4	Negative	7.0	6.0

Tissue samples from lymph nodes, lung, Ileum and Colon were harvested from exposed group calves euthanized at the indicated number of days after exposure to field group calves. The number of viral RNA copies (VRC) of BCoV was quantified with RT-qPCR and the limit of quantification was 4.2 log_{10} VRC/g tissue

shedding of virus in conventionally reared piglets, only in gnotobiotic piglets [32]. This indicates that secondary pathogens and changes in microbiota are important for disease development and clinical signs. The present study supports that after the acute stage of disease other factors than virus replication are important for clinical signs; for instance secondary bacterial infections.

Although the sentinel calves did not get infected with BCoV, they showed sporadic unspecific signs during the trial, but below the mildest category "mild disease" in the clinical scoring system. Since acclimatization was not possible, the calves changed environment including feeding routines when enrolled in the experiment, which could cause the signs observed. Other infectious agents could also have been present, and if so, most likely less virulent pathogens. Bovine virus diarrhea virus and bovine herpesvirus 1 are not present in Sweden [33], and the sentinel calves showed no serologic response to BRSV. Co-infection between BCoV and other agents is likewise possible in FG and EG, as is the case under field conditions.

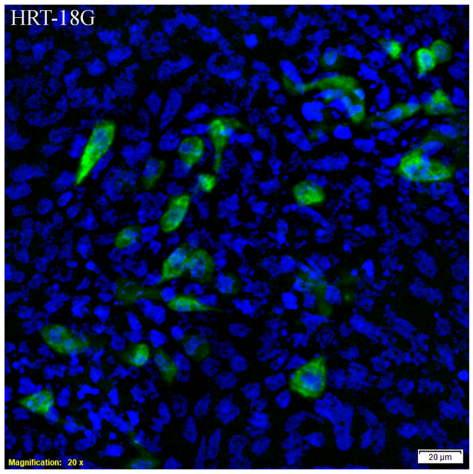


Fig. 6 HRT-18G cells infected with BCoV from a nasal swab. The cells were infected with supernatant from a nasal swab taken from calf E3 six days after exposure to BCoV. The cells are stained with anti-coronavirus antibodies labelled with fluorescein isothiocynate and DAPI nuclear counterstain

Unlike most enteric viruses, BCoV is enveloped and therefore susceptible to environmental inactivation [1]. One might expect that the conditions in the forestomaches and abomasum would inactivate BCoV and one possibility is that BCoV is transported from the oronasal cavity to the small intestines through the bloodstream. However, viremia was not detected in the present study, and transport of the virus to the intestines appears to have been through the digestive tract. Park and colleagues [21] detected BCoV RNA in serum samples from calves infected with a winter dysentery strain between day three and eight post inoculation. They used nested PCR for detection, which is generally a more sensitive method than RT-qPCR, but also more vulnerable for contamination [34]. Short viremic period or intake of a lower virus dose in naturally infected calves could also explain the negative results in the present study. Inhibition of the RT-qPCR by plasma components was tested and ruled out. Despite the absence of detectable viremia in the present study, BCoV RNA was found in mesenteric lymph nodes at late stages of the infection. Viral RNA must have been transferred in low concentrations in blood or lymph to the draining lymph node, by antigen presenting cells or as free virus particles.

The finding of BCoV RNA in lymph nodes, ileum and colon six weeks after infection indicates coronavirus persistence in calves, however, the importance of this persistence for virus transmission is uncertain. Other coronaviruses are known to create persistent or chronic infections in mice and cats [35, 36]. MERS-CoV is shown to be excreted for more than a month in humans [37] and human coronavirus 229E creates persistent infections in vitro [38]. Although fecal shedding of BCoV RNA was detected five weeks post infection in the present study, the transmission potential at this stage is most likely negligible, as at three weeks post infection.

BCoV VRC were quantified by RT-qPCR, which does not give information on the number of infective particles. The ratio of total to infective particles (T/I) is challenging to establish for BCoV due to difficulties in cultivating virus from clinical samples. In the present study, virus titration showed a T/I ratio of approximately

5 $\log_{10}$. With this high T/I ratio it is not surprising that virus isolation was unsuccessful after D13, when the VRC numbers are decreasing. It also agrees with the sentinel calves not getting infected D21. In contrast, roughly 8.8 $\log_{10}$ VRC were detected per nasal swab and gram feces from the seronegative FG calves that infected the EG calves. With a T/I ratio of 5 $\log_{10}$, each nasal swab and gram of feces contained more than 3.8 $\log_{10}$ infective virus particles.

The high T/I ratio and the failure of virus isolation after D13 could be due to either few infective particles or low sensitivity of the isolation method. Low levels of infective particles could be caused either by high production of defective particles or by neutralizing effect of antibodies. Low sensitivity could be caused by suboptimal conditions in cell culture compared to in vivo (particularly for virus from clinical samples not adapted to cell culture growth), dilution of viral content in the swab, and freezing and thawing of the material. For feline enteric coronavirus, the T/I increased from 3–4 $\log_{10}$ during the first week after infection, to up to 8 $\log_{10}$ 28 days post infection [39], the increase possibly caused by the antibody response.

Few methods are available for studying transmission potential apart from live animal experiments, although ethically challenging and resource demanding. Existing literature is based on experimental studies examining BCoV shedding for 14 [20, 22, 40] to 21 [19, 41] days. To the authors' knowledge, the present study is the first to study the shedding for as long as six weeks under experimental conditions. In addition, it is also the first to study the impact of this shedding using sentinel calves. Although a low number of calves were used, the results indicate that calves are not infectious three weeks after exposure to BCoV. This information is important and relevant in order to produce scientific based advices on how to avoid introduction of BCoV into herds. Further investigation of calves at different stages of disease is recommended to verify and corroborate these findings. The effect of stress related to transport on viral shedding and infectivity should also be considered.

In the present study, the virus that caused winter dysentery in adult cattle primarily gave respiratory disease in calves. Niskanen et al. also found that BCoV derived from an outbreak of winter dysentery caused mainly respiratory disease in weaned calves [29], supporting that BCoV is an important cause of respiratory disease in calves [42, 43] and winter dysentery in adults [17]. The economic and welfare consequences of BCoV therefore include the combined effects of neonatal enteritis, respiratory disease in young cattle and winter dysentery in adults. Also considering the high prevalence worldwide, BCoV is an important loss-inflicting factor in the cattle industry.

Conclusions

The current study shows that calves infected with BCoV are RT-qPCR positive in nasal and fecal specimens for a longer period than earlier recognized. However, contact with naïve calves three weeks after exposure did not lead to infection. A low level of infective particles could be due to either production of a high level of defective particles and/or production of neutralizing antibodies. The study provides highly relevant information when designing biosecurity advice regarding animal trade and coronaviral disease in cattle.

Additional file

Additional file 1: Table S1. Antibodies to BCoV. (DOCX 14 kb)

Acknowledgements

We thank Kerstin Mover Berglund, Dennis Larsson and Bengt Norén for taking good care of the calves during the trial, Mamata Khatri for performing the virus isolation and Hanne Haatveit for preparing the picture of the stained cells.

Funding

This project was funded by The Research Council of Norway (NFR-project No 224771/E40), The Norwegian Research Funding for Agriculture and Food Industry, and TINE Norwegian Dairies BA.

Authors' contributions

MS was the project leader; VSO, MT, SA, MM and MS conceived and designed the experiment; VSO, SA and MT performed the experiment; VSO and MM planned and performed the lab analyses. All authors wrote, read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The trial was conducted in line with national and international guidelines for the care and use of animals and approval was given by the Ethics Committee for Animal Experiments, Uppsala, Sweden [protocol number C45/14].

Author details

[1]Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Ullevålsvegen 72, 0454 Oslo, Norway. [2]Department of Clinical Sciences, Swedish University of Agricultural Sciences, 75007 Uppsala, Sweden. [3]Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, Ullevålsvegen 72, 0454 Oslo, Norway.

Received: 18 February 2016 Accepted: 3 June 2016
Published online: 13 June 2016

References

1. Boileau MJ, Kapil S. Bovine Coronavirus Associated Syndromes. Vet Clin North Am Food Anim Pract. 2010;26:123–46.
2. Decaro N, Mari V, Desario C, Campolo M, Elia G, Martella V, Greco G, Cirone F, Colaianni ML, Cordioli P, Buonavoglia C. Severe outbreak of bovine coronavirus infection in dairy cattle during the warmer season. Vet Microbiol. 2008;126:30–9.
3. Fulton RW, Step DL, Wahrmund J, Burge LJ, Payton ME, Cook BJ, Burken D, Richards CJ, Confer AW. Bovine coronavirus (BCV) infections in transported commingled beef cattle and sole-source ranch calves. Can J Vet Res. 2011;75:191–9.
4. Mee JF, Geraghty T, O'Neill R, More SJ. Bioexclusion of diseases from dairy and beef farms: risks of introducing infectious agents and risk reduction strategies. Vet J. 2012;194:143–50.

5. Cho KO, Hoet AE, Loerch SC, Wittum TE, Saif LJ. Evaluation of concurrent shedding of bovine coronavirus via the respiratory tract and enteric route in feedlot cattle. Am J Vet Res. 2001;62:1436–41.
6. da Silva MR, O'Reilly KL, Lin X, Stine L, Storz J. Sensitivity comparison for detection of respiratory bovine coronaviruses in nasal samples from feedlot cattle by ELISA and isolation with the G clone of HRT-18 cells. J Vet Diagn Invest. 1999;11:15–9.
7. Hasoksuz M, Lathrop SL, Gadfield KL, Saif LJ. Isolation of bovine respiratory coronaviruses from feedlot cattle and comparison of their biological and antigenic properties with bovine enteric coronaviruses. Am J Vet Res. 1999;60:1227–33.
8. Lathrop SL, Wittum TE, Loerch SC, Perino LJ, Saif LJ. Antibody titers against bovine coronavirus and shedding of the virus via the respiratory tract in feedlot cattle. Am J Vet Res. 2000;61:1057–61.
9. Lin XQ, Oe KL. Storz J, Purdy CW, Loan RW. Antibody responses to respiratory coronavirus infections of cattle during shipping fever pathogenesis. Arch Virol. 2000;145:2335–49.
10. Hasoksuz M, Hoet AE, Loerch SC, Wittum TE, Nielsen PR, Saif LJ. Detection of respiratory and enteric shedding of bovine coronaviruses in cattle in an Ohio feedlot. J Vet Diagn Invest. 2002;14:308–13.
11. Vijgen L, Keyaerts E, Lemey P, Maes P, Van Reeth K, Nauwynck H, Pensaert M, Van Ranst M. Evolutionary history of the closely related group 2 coronaviruses: porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, and human coronavirus OC43. J Virol. 2006;80:7270–4.
12. Perlman S, Netland J. Coronaviruses post-SARS: update on replication and pathogenesis. Nat Rev Microbiol. 2009;7:439–50.
13. Yang D, Leibowitz JL. The structure and functions of coronavirus genomic 3′ and 5′ ends. Virus Res. 2015;206:120–33.
14. El-Ghorr AA, Snodgrass DR, Scott FMM, Campbell I. A serological comparison of bovine coronavirus strains. Arch Virol. 1989;104:241–8.
15. Clark MA. Bovine coronavirus. Br Vet J. 1993;149:51–70.
16. Lin XQ, O'Reilly KL, Storz J. Antibody responses of cattle with respiratory coronavirus infections during pathogenesis of shipping fever pneumonia are lower with antigens of enteric strains than with those of a respiratory strain. Clin Diagn Lab Immunol. 2002;9:1010–3.
17. Alenius S, Niskanen R, Juntti N, Larsson B. Bovine coronavirus as the causative agent of winter dysentery: serological evidence. Acta Vet Scand. 1991;32:163–70.
18. Tråvén M, Näslund K, Linde N, Linde B, Silván A, Fossum C, Hedlund KO, Larsson B. Experimental reproduction of winter dysentery in lactating cows using BCV – comparison with BCV infection in milk-fed calves. Vet Microbiol. 2001;81:127–51.
19. Cho KO, Hasoksuz M, Nielsen PR, Chang KO, Lathrop S, Saif LJ. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. Arch Virol. 2001;146:2401–19.
20. El-Kanawati ZR, Tsunemitsu H, Smith DR, Saif LJ. Infection and cross-protection studies of winter dysentery and calf diarrhea bovine coronavirus strains in colostrum-deprived and gnotobiotic calves. Am J Vet Res. 1996;57:48–53.
21. Park SJ, Kim GY, Choy HE, Hong YJ, Saif LJ, Jeong JH, Park SI, Kim HH, Kim SK, Shin SS, et al. Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. Arch Virol. 2007;152:1885–900.
22. Saif LJ, Redman DR, Moorhead PD, Theil KW. Experimentally induced coronavirus infections in calves: viral replication in the respiratory and intestinal tracts. Am J Vet Res. 1986;47:1426–32.
23. Kapil S, Trent AM, Goyal SM. Excretion and persistence of bovine coronavirus in neonatal calves. Arch Virol. 1990;115:127–32.
24. Crouch CF, Bielefeldt Ohmann H, Watts TC, Babiuk LA. Chronic shedding of bovine enteric coronavirus antigen-antibody complexes by clinically normal cows. J Gen Virol. 1985;66:1489–500.
25. Hägglund S, Hu K, Vargmar K, Poré L, Olofson AS, Blodörn K, Anderson J, Ahooghalandari P, Pringle J, Taylor G, Valarcher JF. Bovine respiratory syncytial virus ISCOMs-Immunity, protection and safety in young conventional calves. Vaccine. 2011;29:8719–30.
26. Hägglund S, Hu KF, Larsen LE, Hakhverdyan M, Valarcher JF, Taylor G, Morein B, Belák S, Alenius S. Bovine respiratory syncytial virus ISCOMs–protection in the presence of maternal antibodies. Vaccine. 2004;23:646–55.
27. Silverlås C, Näslund K, Björkman C, Mattsson JG. Molecular characterisation of Cryptosporidium isolates from Swedish dairy cattle in relation to age, diarrhoea and region. Vet Parasitol. 2010;169:289–95.
28. Decaro N, Elia G, Campolo M, Desario C, Mari V, Radogna A, Colaianni ML, Cirone F, Tempesta M, Buonavoglia C. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J Virol Methods. 2008;151:167–71.
29. Niskanen R, Lindberg A, Tråvén M. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. Vet J. 2002;163:251–9.
30. Thomas CJ, Hoet AE, Sreevatsan S, Wittum TE, Briggs RE, Duff VRC, Saif LJ. Transmission of bovine coronavirus and serologic responses in feedlot calves under field conditions. Am J Vet Res. 2006;67:1412–20.
31. Campos-Rodriguez R, Godinez-Victoria M, Abarca-Rojano E, Pacheco-Yepez J, Reyna-Garfias H, Barbosa-Cabrera RE, Drago-Serrano ME. Stress modulates intestinal secretory immunoglobulin A. Front Integr Neurosci. 2013;7:86.
32. Ma Y, Zhang Y, Liang X, Lou F, Oglesbee M, Krakowka S, Li J. Origin, evolution, and virulence of porcine deltacoronaviruses in the United States. MBio. 2015;6:e00064.
33. Surveillance of infectious diseases in animals and humans in Sweden 2014. In SVA's rapportserie 31 (Elvander M, Lahti E, Rosendal T eds.). Uppsala, Sweden: National Veterinary Institute (SVA)
34. Homan WL, Vercammen M, De Braekeleer J, Verschueren H. Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR1. Int J Parasitol. 2000;30:69–75.
35. Siddell S, Wege H, Ter Meulen V. The Biology of Coronaviruses. J Gen Virol. 1983;64:761–76.
36. Addie DD, Schaap IAT, Nicolson L, Jarrett O. Persistence and transmission of natural type I feline coronavirus infection. J Gen Virol. 2003;84:2735–44.
37. Poissy J, Goffard A, Parmentier-Decrucq E, Favory R, Kauv M, Kipnis E, Mathieu D, van der Werf S, Guery B. Kinetics and pattern of viral excretion in biological specimens of two MERS-CoV cases. J Clin Virol. 2014;61:275–8.
38. Arbour N, Ekande S, Cote G, Lachance C, Chagnon F, Tardieu M, Cashman NR, Talbot PJ. Persistent infection of human oligodendrocytic and neuroglial cell lines by human coronavirus 229E. J Virol. 1999;73:3326–37.
39. Desmarets LMB, Vermeulen BL, Theuns S, Conceição-Neto N, Zeller M, Roukaerts IDM, Acar DD, Olyslaegers DAJ, Van Ranst M, Matthijnssens J, Nauwynck HJ. Experimental feline enteric coronavirus infection reveals an aberrant infection pattern and shedding of mutants with impaired infectivity in enterocyte cultures. Sci Rep. 2016;6:20022.
40. Saif LJ. Development of nasal, fecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. Vet Immunol Immunopathol. 1987;17:425–39.
41. Tsunemitsu H, Smith DR, Saif LJ. Experimental inoculation of adult dairy cows with bovine coronavirus and detection of coronavirus in feces by RT-PCR. Arch Virol. 1999;144:167–75.
42. Saif LJ. Bovine respiratory coronavirus. Vet Clin North Am Food Anim Pract. 2010;26:349–64.
43. Gulliksen SM, Jor E, Lie KI, Løken T, Akerstedt J, Østerås O. Respiratory infections in Norwegian dairy calves. J Dairy Sci. 2009;92:5139–46.

Oma et al. BMC Veterinary Research (2018) 14:22
DOI 10.1186/s12917-018-1335-1

BMC Veterinary Research

RESEARCH ARTICLE Open Access

Temporary carriage of bovine coronavirus and bovine respiratory syncytial virus by fomites and human nasal mucosa after exposure to infected calves

Veslemøy Sunniva Oma[1*], Thea Klem[1], Madeleine Tråvén[2], Stefan Alenius[2], Britt Gjerset[3], Mette Myrmel[4] and Maria Stokstad[1]

Abstract

Background: In order to prevent spread of the endemic pathogens bovine coronavirus (BCoV) and bovine respiratory syncytial virus (BRSV) between herds, knowledge of indirect transmission by personnel and fomites is fundamental. The aims of the study were to determine the duration of viral RNA carriage and the infectivity of viral particles on fomites and human nasal mucosa after exposure to BCoV and BRSV. During two animal infection experiments, swabs were collected from personnel (nasal mucosa) and their clothes, boots and equipment after contact with calves shedding either virus. Viral RNA was quantified by RT-qPCR or droplet digital RT-PCR (RT-ddPCR), and selected samples with high levels of viral RNA were tested by cell culture for infectivity.

Results: For BCoV, 46% ($n = 80$) of the swabs from human nasal mucosa collected 30 min after exposure were positive by RT-qPCR. After two, four and six hours, 15%, 5% and 0% of the swabs were positive, respectively. Infective virions were not detected in mucosal swabs ($n = 2$). A high viral RNA load was detected on 97% ($n = 44$) of the fomites 24 h after exposure, and infective virions were detected in two of three swabs. For BRSV, 35% ($n = 26$) of the human nasal mucosa swabs collected 30 min after exposure, were positive by RT-ddPCR, but none were positive for infective virions. Of the fomites, 89% ($n = 38$) were positive for BRSV RNA 24 h after exposure, but all were negative for infective viruses.

Conclusions: The results indicate that human nasal mucosa can carry both BCoV and BRSV RNA after exposure to virus shedding calves, but the carriage seems short-lived and the transmission potential is likely limited. High viral loads on contaminates fomites 24 h after exposure to infected animals, and detection of infective BCoV, indicate that contaminated fomites represent a significant risk for indirect transmission between herds.

Keywords: Indirect transmission, Virus infectivity, Biosecurity, Bovine respiratory disease, Human nasal mucosa, Cattle

Background

Bovine coronavirus (BCoV) and bovine respiratory syncytial virus (BRSV) are contagious pathogens detrimentally affecting production and animal welfare in the cattle industry. The viruses are part of the bovine respiratory disease complex and are endemic worldwide. BRSV and BCoV can cause epidemics of respiratory disease and additionally BCoV cause diarrhea in calves and adult cattle (winter dysentery) [1–4]. The traditional way of handling and preventing these diseases is through metaphylactic antibiotic treatment, use of vaccines, or changes in management to improve calf health in herds [5]. The within-herd prevalence and morbidity of BCoV and BRSV infections are high [6, 7] and once the virus enters a herd, circulation is difficult to mitigate. An additional preventive strategy is therefore to reduce inter-herd transmission of virus. Movement of live animals between herds is an important transmission route [8]. If this risk is under control,

* Correspondence: Veslemoy.Oma@nmbu.no
[1]Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, P.O. Box 8146 Dep, 0033 Oslo, Norway
Full list of author information is available at the end of the article

the next question concerns the contribution of indirect spread of virus between herds. Indirect spread can occur via e.g. personnel travelling between herds, their clothes or equipment.

Important risk factors for indirect spread are the level of virus contamination of relevant surfaces and the infectivity of the viruses. Enveloped respiratory viruses like BCoV and BRSV are generally fragile outside the host [9]. However, as related viruses like human respiratory syncytial virus (HRSV) and human coronavirus 229E remain infective for several hours on contaminated surfaces like countertops and surgical gloves [10, 11], there is a potential for indirect transmission. Epidemiological studies also point out the importance of indirect transmission; Ohlson et al. found that lack of boot provision for visitors was a risk factor for infections with both viruses [12] and Toftaker et al. found that a herd's BCoV and BRSV antibody status was influenced by the status of its neighboring herds [8].

Human nasal mucosa might also be a vector for inter-herd virus transmission, as traffic of personnel between herds is common. Carriage of BCoV and BRSV in human nostrils has not been studied. Generally, there are few studies on indirect transmission of these viruses, and no experimental studies have been performed. Molecular methods and virus isolation in cell culture can be used to study the level of virus carriage and infectivity, which are determinants for virus transmission. Combined, these methods provide sensitive quantification of viral genomes and assessment of virus infectivity.

Consequently, the aim of the present study was to investigate whether personnel (nostrils) and fomites carry viral RNA and infective viruses after exposure to BCoV or BRSV infected animals.

Methods

Study design and animal experiments

The present study was performed during two animal experiments, one with BCoV and one with BRSV, and during a field outbreak of winter dysentery. Swabs were rubbed in the nostrils of personnel and on their coats, boots, wristwatches and stethoscopes at different time points after animal contact, and examined for viral RNA and infective viruses.

The BCoV experiment was conducted in 2014 at the Swedish University of Agricultural Sciences (SLU) as described by Oma et al. [13]. A total of ten bull calves between six and twelve weeks of age were included, six were Swedish red and white, three were Swedish Holstein and one Swedish mountain breed. Briefly, four calves at SLU were exposed to a group of six calves brought in from SLU's research farm that experienced an outbreak of winter dysentery. The field outbreak was confirmed by RT-qPCR and serology to have been caused by BCoV. After comingling for 24 h, the calves were housed in the isolation unit within their original groups of four and six animals. As six of the calves were naturally exposed to BCoV in the field, the dates of infection were unknown. The presented contamination study was conducted within a three week period while the calves showed signs of disease and shed virus as detected by RT-qPCR. The number of BCoV RNA copies in nasal swabs from the ten calves varied between $\log_{10}$ 2.9 and 10.4 (mean of $\log_{10}$ 6.9) during the study period.

The BRSV experiment took place at the Norwegian Veterinary Institute in 2015 (to be published). A total of eight Norwegian Red calves between two and four months of age were included, six bulls and two heifers. Briefly, six of the calves were infected after contact with two calves inoculated with a field isolate of BRSV, O4-4B/N-11 [14]. The calves were housed in isolation units in groups of four including one inoculated calf. The contamination study was conducted on three different days within one week while the calves showed signs of respiratory disease and shed virus. The number of BRSV RNA copies in nasal swabs from the calves varied between $\log_{10}$ 2.7 and 8.1 (mean of $\log_{10}$ 5.6) during the study period.

Both experiments were conducted in line with the ARRIVE guidelines for planning and reporting in vivo experiments and the concept of the 3R's (Reduction, Replacement and Refinement) [15, 16]. In both experiments, efforts were made to minimize the stress and discomfort for the animals. The animals were closely monitored and medical treatment were administered in line with national Norwegian and Swedish recommendations for treatment of pneumonia and diarrhea in calves.

Exposure procedure and sampling schemes

Table 1 presents an overview of exposed personnel and fomites. During ten minutes, the personnel handled and examined animals that showed clinical signs and shed either BCoV or BRSV. In the BCoV experiment, swabs were collected from human nostrils prior to and 0.5, 2, 4 and 6 h after exposure to the animals. The BRSV experiment included only a single time point (0.5 h), as viral RNA was not detected in nasal swabs collected during a BRSV pilot study.

Clean boots, coats, wristwatches and stethoscopes were used. After exposure to the animals, boots were rinsed in lukewarm water until visually clean and left to dry. All fomites were stored at 16–18 °C, in a room separate from the animals.

Sampling procedure

A detailed protocol was developed for collection of material from fomites and human nostrils. The same person collected all the material from fomites in each

Table 1 Overview of personnel and fomites that were sampled after exposure to virus shedding animals

	BCoV[a] experiment	BRSV[b] experiment	Winter dysentery outbreak in dairy herd
No. of animals	10	8	300
No. of persons	16	12	19
Personnel			
No. of challenges	86	26	19
Hours between exposure and sampling	−0.5, 0.5, 2, 4 and 6	0.5	0.5, 2, 4
Fomites			
No. of challenges	44	38	–
No. and types	12 rubber coats, 16 rubber boots, 8 stethoscopes, 8 wrist watches	19 rubber coats, 19 rubber boots	–
Hours between exposure and sampling	0, 2 and 24	2 and 24	–

Sample collection was performed during two animal experiments and one outbreak of winter dysentery (caused by BCoV). [a]BCoV – bovine coronavirus, [b]BRSV - bovine respiratory syncytial virus

experiment, and instructed the personnel that took part in the human mucosa trial. Specimens were collected with ESwab™ (Copan, Brescia, Italy) and stored in 1 ml of Liquid Amies medium. Gloves were used throughout the experiments.

Specimens from human nasal mucosa were collected by rotating a swab inside one nostril for a couple of seconds. When a person was sampled more than once, the left and right nostrils were sampled alternately. Sampling of fomites was performed by moistening the tip of the swab with Amies medium before lightly rubbing a defined area (5 cm × 10 cm of coats and boots) without visible contamination. For wristwatches and stethoscopes, the area was approximately 3 cm × 3 cm and 2 cm × 5 cm, respectively. At later time points, new areas were sampled. After sample collection, swabs were stored at 4 °C for no more than two hours and thereafter at −70 °C until use.

RNA extraction and quantification of viral genomes

BCoV

RNA was extracted from 140 μl of the Amies medium by the QIAamp Viral RNA Mini QIAcube kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, eluted in 50 μl buffer and stored at −80 °C. RT-qPCR was performed, in duplicates for nasal swabs, using RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen, MA, USA) and the target was an 85 bp fragment of the M protein gene [17]. Two μl of RNA was used in a total volume of 20 ul containing 200 nM each of forward and reverse primer and 250 nM TaqMan probe. The thermal profile included an RT step at 55 °C for 30 min followed by 95 °C for 2 min and thereafter 40 cycles of 15 s at 95 °C and 1 min at 60 °C. RT-qPCR was run in a Stratagene Mx3005p™ (Agilent Technologies, CA, USA) and each run included a positive (RNA from the nostril of a BCoV positive trial calf) and negative control (water).

Positive swabs from human nasal mucosa were subjected to Kaplan-Meier survival analysis in Stata (Stata SE/14, Stata Corp., College Station, TX, USA). The function shows the cumulative survival, i.e. carriage of BCoV RNA over time, which descends as personnel turns BCoV RNA negative. As the exact time-point a person turned negative was unknown, the mid-point between the last positive and the first negative sample was used in the analysis [18].

In order to estimate the number of BCoV RNA genome copies (GC), a standard curve was prepared using tenfold dilutions of a plasmid containing the BCoV target sequence. The BCoV RNA positive control was aliquoted and included in every RT-qPCR plate as a calibrator to adjust for inter-plate variation. The number of GC in clinical samples was calculated using the formula from Livak and Schmittgen [19]:

$$Q_s = Q_c{}^*(1+E)^{-(Ct_s - Ct_c)}$$

Where Q_s = *sample RNA copy number*, Q_c = *calibrator RNA copy number*, Ct_s = *sample Ct value*, Ct_c = *calibrator Ct value* and E = *efficiency of target amplification.*

The standard curve covered the range from 10.8 to 1.08×10^{10} copies. The curve showed a strong linear relationship with a high coefficient of determination ($R^2 = 0.996$) and a high amplification efficiency (E = 0.965). The limit of quantification represented $\log_{10}$ 3.6 BCoV GC per swab from human nasal mucosa and fomites.

BRSV

RNA was extracted from 200 μl of Amies medium, using the automated NucliSens easyMAG protocol (Biomérieux, Marcy l'Etoile, France), according to the manufacturer's instructions. Quantification of BRSV genomes was

conducted in duplicate, with Bio-Rad's QX200 ddPCR System (droplet digital PCR). Each run included a positive (RNA from the nostril of a BRSV positive trial calf) and negative control (water). Droplet generation and transfer of droplets were as described by the manufacturer. The One-Step RT-ddPCR Advanced Kit for Probes (BioRad, CA, USA) and 2 μl RNA were used. The sequence of primers and probe (5'FAM and BHQ1 as quencher) was as described [20], targeting a 123 bp region of the BRSV N gene. Primers and probe concentrations were as recommended by the kit manufacturer and with the following cycling conditions; 50 °C for 60 min, 95 °C for 10 min and 40 cycles of 95 °C for 30 s and 60 °C for 1 min. The ramp rate was set to 2 °C/s. Data processing and absolute quantification of BRSV genomes per μl RNA was performed with QuantaSoft Version 1.7 (BioRad).

Half-life calculation for BRSV RNA carriage was unattainable due to single sampling.

Testing of virus infectivity

BCoV

Virus infectivity was tested in five samples with the highest level of BCoV RNA, using integrated cell culture RT-qPCR; swabs from a wristwatch, a stethoscope and a coat collected 24 h after exposure, and from two human nostrils, collected 30 min after exposure. The swab medium was diluted 1:10 in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, DE, USA) and added, in duplicate wells, to monolayers of 4-days-old human rectal tumor cells (HRT-18, ATCC CRL-11663) in a 24-well plate. Positive control (cultivated BCoV from a calf in the experiment), positive control in Amies medium and negative controls (cells only) were included. After incubation at 37 °C for one hour, the inoculum was removed, the cells washed and DMEM with 1% fetal calf serum (FCS) and antibiotics (5000 IU penicillin and 5 mg streptomycin/ml) was added. Simultaneously, cells were harvested from one parallel well of each sample as a time zero replication control. After three days incubation at 37 °C in 5% CO_2, cells were harvested from the remaining wells and RNA extracted with Qiazol (Qiagen), chloroform phase separation (mixed 1:1 with 70% ethanol), and RNeasy Mini Kit column (Qiagen). The amount of RNA was measured using Nanodrop (Thermo Fisher Scientific) and equal amounts analyzed by BCoV RT-qPCR run in duplicates as described. Relative quantification of target RNA from incubated and time zero replication control cells was performed using the standard curve.

BRSV

BRSV infectivity was tested in ten swabs showing the highest level of viral RNA by RT-ddPCR; eight swabs from coats and two from human nostrils, collected 24 h and 30 min after exposure, respectively. Fetal bovine turbinate cells (courtesy of Swedish Veterinary Institute) propagated in Eagle's minimal essential medium (BioWhittaker, Belgium) in 96 well plates were incubated with 50 μl filtered swab samples for 30 min. Medium with 2% FCS was added, the plates were incubated at 37 °C in 5% CO_2 and the supernatant passaged after seven days. Samples were cultivated in duplicates with positive (cultivated BRSV from a calf in the experiment) and negative controls (cells only). The cells were observed for cytopathic effect (CPE) and infection visualized by direct immunofluorescence test using FITC Moab a-BRSV (Bio-X Diagnostics, Rochefort, Belgium). Culture supernatants were harvested and tested by the BRSV RT-ddPCR as described.

Results

Viral RNA in human nasal mucosa

The positive controls were consistently positive throughout the analyses, and all negative controls were negative. No viral RNA was detected in human nasal mucosa that was sampled prior to exposure to animals, however, positives were found among samples collected after exposure (Table 2). The number of BCoV GC per swab is shown in Fig. 1. Estimated half-life of BCoV RNA carriage was less than 90 min and the estimated longest persistence was five hours (Fig. 2). The positive BRSV swabs contained between $\log_{10}$ 1.3 to 3.3 genome copies.

Viral RNA on fomites

The positive controls were consistently positive throughout the analyses, and all negative controls were negative. BCoV RNA was detected on all boots, coats and stethoscopes, and on seven out of eight wristwatches, 24 h after exposure. The eighth watch was positive for BCoV RNA 15 min and two hours after exposure. The copy numbers of BCoV RNA 24 h after exposure are presented in Fig. 3. BRSV RNA was detected on 18 out of 19 boots sampled after two hours and 16 out of 19 boots after 24 h. For the coats, 17 out of 19 were positive two hours after exposure, and 18 out of 19 were positive after 24 h. There were minor differences in BRSV RNA copy numbers between samples collected 2 and 24 h after exposure and no tendency of reduction in copy numbers (Fig. 4).

Virus infectivity

BCoV

RT-qPCR results from cells inoculated with swab material from a wristwatch or a stethoscope indicated a 1000-fold increase in the number of RNA-copies after three days of incubation. Cells inoculated with swab material from human nostrils and from a rubber coat showed no increase in viral RNA during incubation. Positive virus controls were positive, and the Amies medium showed no inhibition of virus replication. No BCoV RNA was detected in negative control wells.

Table 2 BCoV and BRSV RNA in human nasal swabs

	BCoV[a] experiment		BRSV[b] experiment		Winter dysentery outbreak	
Hours between exposure and sampling	Total no. of swabs	No. of positive swabs (%)	Total no. of swabs	No. of positive swabs (%)	Total no. of swabs	No. of positive swabs (%)
−0.5	67	0	ND[c]	ND	ND	ND
0.5	80	37 (46%)	26	9 (35%)	7	1 (14%)
2	68	10 (15%)	ND	ND	1	0
4	38	2 (5%)	ND	ND	12	0
6	28	0	ND	ND	ND	ND
24	11	0	ND	ND	ND	ND
Total	292	49 (17%)	26	9 (35%)	20	1 (5%)

RT-qPCR and droplet digital RT-PCR results in swabs from the nasal cavity of personnel before and after exposure to BCoV or BRSV infected calves
[a]BCoV – bovine coronavirus
[b]BRSV – bovine respiratory syncytial virus
[c]ND = Not done

BRSV

No CPE was seen in cells incubated with swab material or with passaged material and RT-ddPCR results did not indicate any virus replication after two passages in the cells. Positive control wells were positive, and negative control wells were negative.

Discussion

This is the first time BCoV and BRSV contamination of personnel and fomites has been described. The PCR results indicate that fomites (like clothes, boots, wristwatches and stethoscopes) exposed to virus pose an infection risk to cattle. For BCoV in particular, fomites seem to represent a high risk, as virus isolation detected infective viruses after 24 h. Consequently, measures to prevent inter-herd transmission should include actions against indirect spread of virus.

As high copy numbers of viral RNA on fomites indicated a transmission potential, further investigations were performed in order to assess whether the detected RNA could represent infective viruses. Although infectivity ideally should be studied in live animals, cell culture was used due to practical, ethical and economic reasons. Virus isolation in cell culture may have a low sensitivity [21], but the use of integrated cell culture RT-qPCR increases the possibility of detecting infective viruses [22]. Using this method, we showed that visually clean surfaces of fomites can carry infective BCoV for at least 24 h after exposure to infected animals.

As reviewed by La Rosa et al., related coronaviruses and HRSV can be transmitted by fomites in addition to direct transmission through droplets and aerosols [23]. It is therefore plausible that BCoV and BRSV could be transmitted between farms via personnel and fomites. Even if protective clothing is used and changed between herds, personnel might constitute a risk of virus transmission as human nasal mucosa could be a potential hideaway for infective viruses. In addition, BCoV has been isolated from a diarrheic child and is most likely

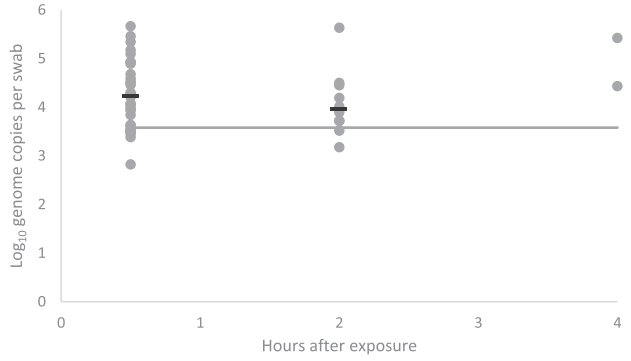


Fig. 1 Bovine coronavirus (BCoV) contaminated human nasal mucosa. Log_{10} genome copies of BCoV per positive swab. Personnel had close contact for at least ten minutes with calves shedding BCoV. Swabs were taken from human nostrils at different time points after exposure to the calves. The grey line shows the limit of quantification and the black short lines indicate median genome copies per positive swab

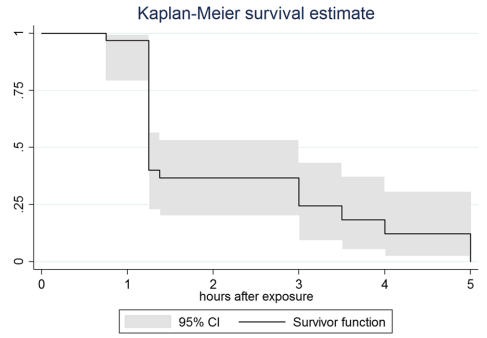


Fig. 2 Kaplan-Meier survival function for BCoV RNA carriage in human nasal mucosa

the ancestor of a related human pathogen [24–26], thus the ability to replicate in cells in the human nasal mucosa cannot be excluded. Human nasal mucosa was therefore studied in place of skin, oral mucosa or hair that could also act as passive vectors for the viruses.

Sellers et al. have shown that human nasal mucosa is a possible vehicle for foot-and-mouth-disease virus even when a high level of biosecurity is implemented [27]. This was refuted by Amass et al. who found a low risk of virus transmission by personnel after hand wash and change of outerwear [28]. Wright et al. found a low risk of prolonged human nasal carriage of the virus [29]. In the present study, we aimed to study whether human nasal mucosa is a possible vehicle for transmission of BCoV and BRSV. Based on our results, this is a possibility, but the low level of viral RNA and the failure to detect infective virus after a few hours, indicate a low risk of virus transmission from human nasal mucosa.

In the present study, personnel was sampled during an experimental setting and during an outbreak of winter dysentery in the field. The results showed that nasal carriage of BCoV in humans was less common in the outbreak situation than during the animal experiment. Factors that could have influenced the amount of virus in the two settings were differences in virus exposure, degree of contact between animals and personnel and environmental conditions. Other factors could be repeated swabbing of the same nostril, nose touching and nose blowing.

The finding that neither BCoV nor BRSV could be cultivated from human nasal swabs resembles the rapid inactivation on skin for respiratory syncytial virus [11] and human coronavirus 229E [30]. This could be due to substances or microorganisms in the mucosa that neutralize or inactivate the virus. Although there is a chance of underestimating the risk, due to e.g. freezing and thawing, dilution and filtering of the samples, the virus transmission potential of mucosa is probably low. There were no sign of BCoV replicating in human nasal mucosa, as the amount of BCoV RNA found were low and declining over time.

Despite the general view that enveloped viruses are fragile outside the host, several coronaviruses remain infective after drying on surfaces for more than 24 h as reviewed by Otter et al. [31]. The present study indicates that BCoV has a similar property. Infective BRSV, on the other hand, was not detected in any of the samples, which was similar to HRSV after drying on surfaces for seven hours [11]. Studies of HRSV survival in cell culture medium and aerosols also showed a higher inactivation rate compared to coronaviruses [32–35]. This suggests that BRSV is more susceptible than BCoV to degradation by environmental factors, and that the importance of indirect BRSV transmission after 24 h, is probably low. As demonstrated by Mullis et al. [36], viral infectivity is more rapidly lost than viral RNA.

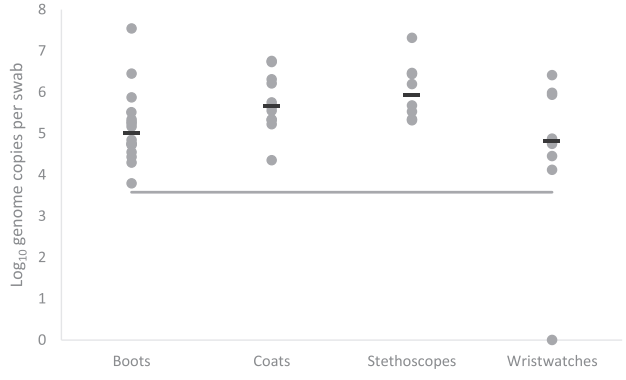


Fig. 3 Bovine coronavirus (BCoV) contaminated fomites. Log_{10} numbers of genome copies per swab taken 24 h after exposure to BCoV-infected calves. The grey line shows the limit of quantification and the black short lines indicate median genome copies per swab

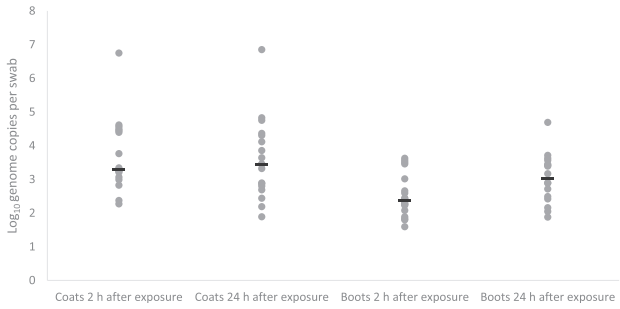


Fig. 4 Bovine respiratory syncytial virus (BRSV) contaminated fomites. Log_{10} numbers of genome copies in positive swabs taken after exposure to BRSV-infected calves. The black short lines indicate median genome copies per swab

For both viruses, the viral RNA level recovered from boots was lower than from coats, possibly due to the rinsing with water. However, as high genome copy numbers remained, rinsing might not be sufficient to prevent virus transmission. This is supported by epidemiologic data that show an increased risk of seropositivity for BRSV and BCoV in herds that do not provide boots to visitors [37]. The present BCoV experiment indicated that also stethoscopes and wristwatches could serve as vehicles. These items are often brought between farms without cleaning/disinfection, and can carry infective virus particles for at least 24 h after exposure to infected cattle.

Conclusions

Personnel pose a risk in inter-herd transmission of BRSV and BCoV when bringing fomites between herds. In order to control the spread of these viruses, biosecurity measures should be implemented, including herd-specific clothing and equipment and washing/disinfection of fomites. Although personnel may carry the viruses intra-nasally for shorter periods of time, the relative importance of contaminated mucosa for indirect transmission is less than that of contaminated fomites.

Abbreviations

BCoV: Bovine coronavirus; BRSV: Bovine respiratory syncytial virus; CPE: Cytopathic effect; Ct: Cycle threshold; DMEM: Dulbecco's Modified Eagle Medium; FCS: Fetal calf serum; GC: Genome copies; HRSV: Human respiratory syncytial virus; HRT: Human rectal tumor cells; RT-ddPCR: Reverse transcription droplet digital polymerase chain reaction; RT-qPCR: Quantitative reverse transcriptase polymerase chain reaction; SLU: Swedish University of Agricultural Sciences; μl: Microliter

Acknowledgements

The authors would like to acknowledge Mamata Khatri, Ingvild Berg Nyman and Elisabeth Furuseth Hansen at the Department of Food Safety and Infection Biology, Norwegian University of Life Sciences for skillful technical assistance.

Funding

This study was supported by the Research Council of Norway (NFR-project No 224771/E40), the Norwegian Research Funding for Agriculture and Food Industry and TINE Norwegian Dairies BA. The funders had no role in the design of the study, data collection, analyses, decision to publish or preparation of the manuscript.

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

VSO, MT, SA, MM and MS designed and performed the BCoV experiment. TK, BG, MM and MS designed and performed the BRSV experiment. All authors wrote, read and approved the manuscript.

Ethics approval and consent to participate

The animal experiments were conducted in agreement with national and international guidelines for the care and use of animals. Approval for the BCoV experiment was given by the Ethics Committee for Animal Experiments, Uppsala, Sweden (protocol no. C45/14) and by the Norwegian Animal Research Authority (approval no. 7468) for the BRSV experiment. Verbal consent was obtained from the personnel participating in the study. Written consent was deemed not necessary, as the aim of the study was purely animal health related, and the Southeastern Regional Committee for Medical and Health Research Ethics in Norway exempted the study from application (reference no. 2016/2012 A).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

Author details

[1]Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, P.O. Box 8146 Dep, 0033 Oslo, Norway. [2]Department of Clinical Sciences, Swedish University of Agricultural Sciences, 75007 Uppsala, Sweden. [3]Norwegian Veterinary Institute, P.O. Box 750 Sentrum, 0106 Oslo, Norway. [4]Department of Food Safety and Infection Biology, Norwegian University of Life Sciences, P.O. Box 8146 Dep, 0033 Oslo, Norway.

Received: 14 July 2017 Accepted: 4 January 2018
Published online: 22 January 2018

References

1. Tråvén M, Näslund K, Linde N, Linde B, Silván A, Fossum C, Hedlund KO, Larsson B. Experimental reproduction of winter dysentery in lactating cows using BCV – comparison with BCV infection in milk-fed calves. Vet Microbiol. 2001;81:127–51.
2. Brodersen BW. Bovine respiratory syncytial virus. Vet Clin North Am Food Anim Pract. 2010;26:323–33.
3. Boileau MJ, Kapil S. Bovine coronavirus associated syndromes. Vet Clin North Am Food Anim Pract. 2010;26:123–46.
4. Su S, Wong G, Shi W, Liu J, Lai AC, Zhou J, Liu W, Bi Y, Gao GF. Epidemiology, genetic recombination, and pathogenesis of coronaviruses. Trends Microbiol. 2016;24:490–502.
5. Murray GM, O'Neill RG, More SJ, McElroy MC, Earley B, Cassidy JP. Evolving views on bovine respiratory disease: an appraisal of selected control measures - part 2. Vet J. 2016;217:78–82.
6. Klem TB, Gulliksen SM, Lie KI, Løken T, Østerås O, Stokstad M. Bovine respiratory syncytial virus: infection dynamics within and between herds. Vet Rec. 2013;173:476.
7. Hägglund S, Svensson C, Emanuelson U, Valarcher JF, Alenius S. Dynamics of virus infections involved in the bovine respiratory disease complex in Swedish dairy herds. Vet J. 2006;172:320–8.
8. Toftaker I, Sanchez J, Stokstad M, Nødtvedt A. Bovine respiratory syncytial virus and bovine coronavirus antibodies in bulk tank milk - risk factors and spatial analysis. Prev Vet Med. 2016;133:73–83.
9. Boone SA, Gerba CP. Significance of fomites in the spread of respiratory and enteric viral disease. Appl Environ Microbiol. 2007;73:1687–96.
10. Sizun J, MW Y, Talbot PJ. Survival of human coronaviruses 229E and OC43 in suspension and after drying on surfaces: a possible source of hospital-acquired infections. J Hosp Infect. 2000;46:55–60.
11. Hall CB, Douglas RG, Geiman JM. Possible transmission by fomites of respiratory syncytial virus. J Infect Dis. 1980;141:98–102.
12. Beaudeau F, Ohlson A, Emanuelson U. Associations between bovine coronavirus and bovine respiratory syncytial virus infections and animal performance in Swedish dairy herds. J Dairy Sci. 2010;93:1523–33.
13. Oma VS, Tråvén M, Alenius S, Myrmel M, Stokstad M. Bovine coronavirus in naturally and experimentally exposed calves; viral shedding and the potential for transmission. Virol J. 2016;13:100.
14. Klem TB, Rimstad E, Stokstad M. Occurrence and phylogenetic analysis of bovine respiratory syncytial virus in outbreaks of respiratory disease in Norway. BMC Vet Res. 2014;10:15.
15. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol. 2010;8:e1000412.
16. Russell WMS. The increase of humanity in experimentation: replacement, reduction and refinement. Laboratory Animals Bureau, Collected Papers. 1957;6:23–5.
17. Decaro N, Elia G, Campolo M, Desario C, Mari V, Radogna A, Colaianni ML, Cirone F, Tempesta M, Buonavoglia C. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J Virol Methods. 2008;151:167–71.
18. Dohoo I, Martin W, Stryhn H. Modelling survival data. In: Veterinary epidemiologic research. 2nd ed. Charlottetown, Prince Edward Island, Canada: VER Inc.; 2009. p. 467–522.
19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 2001;25:402–8.
20. Boxus M, Letellier C, Kerkhofs P. Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. J Virol Methods. 2005;125:125–30.
21. Kapil S, Richardson KL, Radi C, Chard-Bergstrom C. Factors affecting isolation and propagation of bovine coronavirus in human rectal tumor-18 cell line. J Vet Diagn Investig. 1996;8:96–9.
22. Reynolds KA, Gerba CP, Pepper IL. Detection of infectious enteroviruses by an integrated cell culture-PCR procedure. Appl Environ Microbiol. 1996;62:1424–7.
23. La Rosa G, Fratini M, Della Libera S, Iaconelli M, Muscillo M. Viral infections acquired indoors through airborne, droplet or contact transmission. Ann Ist Super Sanita. 2013;49:124–32.
24. Zhang XM, Herbst W, Kousoulas KG, Storz J. Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. J Med Virol. 1994;44:152–61.
25. Han MG, Cheon DS, Zhang X, Saif LJ. Cross-protection against a human enteric coronavirus and a virulent bovine enteric coronavirus in gnotobiotic calves. J Virol. 2006;80:12350–6.
26. Vijgen L, Keyaerts E, Moes E, Thoelen I, Wollants E, Lemey P, Vandamme AM, Van Ranst M. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J Virol. 2005;79:1595–604.
27. Sellers RF, Herniman KAJ, Mann JA. Transfer of foot-and-mouth disease virus in the nose of man from infected to non-infected animals. Vet Rec. 1971;89:447–9.
28. Amass SF, Mason PW, Pacheco JM, Miller CA, Ramirez A, Clark LK, Ragland D, Schneider JL, Kenyon SJ. Procedures for preventing transmission of foot-and-mouth disease virus (O/TAW/97) by people. Vet Microbiol. 2004;103:143–9.
29. Wright CF, Gloster J, Mazelet L, Paton DJ, Ryan ED. Short-lived carriage of foot-and-mouth disease virus in human nasal cavities after exposure to infected animals. Vet Rec. 2010;167:928–31.
30. Wolff MH, Sattar SA, Adegbunrin O, Tetro J. Environmental survival and microbicide inactivation of coronaviruses. In: Schmidt A, Wolff MH, Weber O, editors. Coronaviruses with special emphasis on first insights concerning SARS. Basel: Birkhäuser Verlag; 2005. p. 201–12.
31. Otter JA, Donskey C, Yezli S, Douthwaite S, Goldenberg SD, Weber DJ. Transmission of SARS and MERS coronaviruses and influenza virus in healthcare settings: the possible role of dry surface contamination. J Hosp Infect. 2016;92:235–50.
32. Hambling MH. Survival of the respiratory syncytial virus during storage under various conditions. Br J Exp Pathol. 1964;45:647–55.
33. Rechsteiner J, Winkler KC. Inactivation of respiratory syncytial virus in aerosol. J Gen Virol. 1969;5:405–10.
34. Ijaz MK, Brunner AH, Sattar SA, Nair RC, Johnson-Lussenburg CM. Survival characteristics of airborne human coronavirus 229E. J Gen Virol. 1985;66:2743–8.
35. Rabenau HF, Cinatl J, Morgenstern B, Bauer G, Preiser W, Doerr HW. Stability and inactivation of SARS coronavirus. Med Microbiol Immunol. 2005;194:1–6.
36. Mullis L, Saif LJ, Zhang Y, Zhang X, Azevedo MS. Stability of bovine coronavirus on lettuce surfaces under household refrigeration conditions. Food Microbiol. 2012;30:180–6.
37. Ohlson A, Emanuelson U, Tråvén M, Alenius S. The relationship between antibody status to bovine corona virus and bovine respiratory syncytial virus and disease incidence, reproduction and herd characteristics in dairy herds. Acta Vet Scand. 2010;52:37.

PLOS ONE

RESEARCH ARTICLE

Single primer isothermal amplification (SPIA) combined with next generation sequencing provides complete bovine coronavirus genome coverage and higher sequence depth compared to sequence-independent single primer amplification (SISPA)

Mette Myrmel[1☯]*, Veslemøy Oma[2‡], Mamata Khatri[1‡], Hanne H. Hansen[3], Maria Stokstad[2], Mikael Berg[4], Anne-Lie Blomström[4☯]

1 Department for Food Safety and Infection Biology, Norwegian University of Life Sciences, Oslo, Norway, **2** Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Oslo, Norway, **3** CIGENE, Norwegian University of Life Sciences, Ås, Norway, **4** Department of Biomedical Sciences and Veterinary Public Health, Section of Virology, Swedish University of Agricultural Sciences, Uppsala, Sweden

☯ These authors contributed equally to this work.
‡ These authors also contributed equally to this work.
* mette.myrmel@nmbu.no

OPEN ACCESS

Citation: Myrmel M, Oma V, Khatri M, Hansen HH, Stokstad M, Berg M, et al. (2017) Single primer isothermal amplification (SPIA) combined with next generation sequencing provides complete bovine coronavirus genome coverage and higher sequence depth compared to sequence-independent single primer amplification (SISPA). PLoS ONE 12(11): e0187780. https://doi.org/10.1371/journal.pone.0187780

Editor: Ulrich Melcher, Oklahoma State University, UNITED STATES

Received: August 23, 2017

Accepted: October 25, 2017

Published: November 7, 2017

Data Availability Statement: The NGS data is deposited in SRA (NCBI); accession number SRP075933. The GenBank accession number for the bovine rhinitis B virus is KY432299.1.

Funding: This work was supported by Norges forskningsråd, No 224771/E40, https://www.forskningsradet.no/no/Forsiden/1173185591033; Swedish research council, Formas-2012-586,

Abstract

Coronaviruses are of major importance for both animal and human health. With the emergence of novel coronaviruses such as SARS and MERS, the need for fast genome characterisation is ever so important. Further, in order to understand the influence of quasispecies of these viruses in relation to biology, techniques for deep-sequence and full-length viral genome analysis are needed. In the present study, we compared the efficiency of two sequence-independent approaches [sequence-independent single primer amplification (SISPA) and single primer isothermal amplification (SPIA, represented by the Ovation kit)] coupled with high-throughput sequencing to generate the full-length genome of bovine coronavirus (BCoV) from a nasal swab. Both methods achieved high genome coverage (100% for SPIA and 99% for SISPA), however, there was a clear difference in the percentage of reads that mapped to BCoV. While approximately 45% of the Ovation reads mapped to BCoV (sequence depth of 169–284 944), only 0.07% of the SISPA reads (sequence depth of 0–249) mapped to the reference genome. Although BCoV was the focus of the study we also identified a bovine rhinitis B virus (BRBV) in the data sets. The trend for this virus was similar to that observed for BCoV regarding Ovation vs. SISPA, but with fewer sequences mapping to BRBV due to a lower amount of this virus. In summary, the SPIA approach used in this study produced coverage of the entire BCoV (high copy number) and BRBV (low copy number) and a high sequence/genome depth compared to SISPA. Although this is a limited study, the results indicate that the Ovation method could be a preferred approach for full genome sequencing if a low copy number of viral RNA is expected and if high sequence depth is desired.

www.formas.se. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Next generation sequencing (NGS) has become a valuable tool in virology studies and has the power to generate whole genome sequences (WGS) from small amounts of virus, rapidly and at a relatively low cost [1]. Although direct WGS of the foot-and-mouth disease virus (FMDV) has been reported [2], viral RNA amounts in clinical samples are usually too low for direct WGS. Amplification of the nucleic acid is usually required and sequence-dependent and independent methods are available. A common method for amplifying viral RNA is RT-PCR using virus specific primers. This method is, however, cumbersome due to the requirement of multiple sets of primers to cover the full viral genome. Sequence-independent methods are attractive in that they allow sequencing of highly divergent viruses, but efficient reduction of background nucleic acids is required in order to get good coverage and depth (number of times each nucleotide is sequenced) of the viral genomes [3]. Using standard Sanger sequencing only the dominating strain(s) is(are) sequenced. In contrast, NGS yields deep sequencing meaning that each nucleotide in the sample is sequenced several times. While standard sequencing misses allelic variants with a frequency below 20% in a population [4], NGS can detect less abundant variants depending on sequencing depth. This provides an increased opportunity for viral metagenomics (study of the total viral community), for epidemiologic surveillance of viruses, and for obtaining insight into viral evolution and fitness. Infectious agents, and especially RNA viruses, can rapidly change and adapt to their host and produce many variants (quasispecies) [1, 5–8]. In order to identify these variants, thousands of sequences are needed, which makes NGS particularly useful. An RNA virus family that has gained specific interest, through the emergence of SARS and MERS as well as through containing a number of animal pathogens, is *Coronaviridae* [9]. Coronavirus have high mutation rates and are prone to recombination [10]. Virus quasispecies composition with negative and positive interactions among mutants, is the source of virus evolution during "group selection" and influences the biological behaviour of a viral population [5, 11]. Therefore, it is important not only to have techniques that can rapidly sequence new emerging coronaviruses but also those that investigate the quasispecies population in order to understand the biology of these important viruses.

The aim of the present study was to evaluate two different sequence-independent methods for coronavirus RNA/cDNA amplification. Sequence-independent single primer amplification (SISPA) and NuGEN's Ovation RNA-seq system v2 (single primer isothermal amplification-SPIA) were tested in order to obtain complete genomes of a bovine coronavirus (BCoV) from a nasal swab using Illumina for sequencing. Focus was put on total reads, total BCoV reads, read depth and genome coverage.

Materials and methods

Sampling, extraction of RNA and RT-qPCR

A nostril specimen was collected from a calf naturally exposed to BCoV during a transmission study [12]. Briefly, a flocked eSwab™ (Copan Diagnostics, CA, USA) was swept inside the calf's nostril and kept frozen in 1 ml transport medium at -80˚C until analysis. The specimen was thawed on ice and the swab medium centrifuged at 9700 x g for 10 min. Two technical replicates were processed individually until library preparation and NGS. The supernatant (140 μl) was treated with 2.8 μg RNase A (Sigma-Aldrich, MO, USA) and 6 Units Turbo DNase (Ambion, MA, USA) for 30 min at 37˚C. Thereafter RNA was extracted with QIAzol (Qiagen, Hilden, Germany) and chloroform phase separation. The RNA containing aqueous phase was mixed with 70% ethanol (1:1) and added to an RNeasy Mini Kit column (Qiagen), according to the manufacturer. The RNA concentration (3–5 ng/μl) and purity were measured by

Nanodrop (Thermo Scientific, DE, USA) and the RNA was kept at -80˚C until further use. Estimation of the BCoV copy number (4,1 x 10^5 genome copies used) was done with RT-qPCR using primers and probe as described by Decaro and colleagues [13]. A tenfold dilution series of a plasmid containing the BCoV amplicon was used as a quantification standard. Quantification of a bovine rhinitis virus (700 genome copies used) that was incidentally found in the nasal sample, was performed in an identical way after establishing a rhinitis virus RT-qPCR and production of a plasmid containing the rhinitis virus target.

SISPA—Sequence independent single primer amplification (sample S1 and S2)

A variant of the SISPA protocol was followed [14]. Ten μl RNA was reverse transcribed and tagged using 10 μM primer FRoV26-N (GCC GGA GCT CTG CAG ATA TCN NNN NN) [15] and Superscript III (Invitrogen, MA, USA) according to manufacturer's instructions. Second strand was made by adding 0.5 μl of Klenow fragment (3' -> 5' exo-) (New England Biolabs, England) to the cDNA and incubation at 37˚C for 60 min and 75˚C for 10 min. Double stranded (ds) DNA was kept at -20˚C prior to PCR amplification.

Three aliquots of 6 μl tagged dsDNA were amplified using 0.8 μM of primer FR20 (GCC GGA GCT CTG CAG ATA TC), 1 U KOD DNA polymerase (Merck Millipore, Darmstadt, Germany), 1 mM $MgCl_2$ and 0.2 mM dNTP. The amplification steps were 95˚C for 20 s followed by 30 cycles at 95˚C for 30 s, 58˚C for 30 s, 72˚C for 90 s and completed at 72˚C for 10 min. The PCR products were purified with the NucleoSpin® Gel and PCR clean-up kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol for products in solution, and eluted in 45 μl buffer. Primer sequences were cleaved off using 30 U EcoRV (Promega, WI, USA) and incubation at 37˚C for 60 min, before pooling of aliquots and purification using NucleoSpin® Gel and PCR clean up kit with a final elution volume of 65 μl. The DNA concentration was measured using Nanodrop (50–52 ng/μl).

SPIA—Single primer isothermal amplification (Ovation, sample O1 and O2)

Five μl RNA was subjected to cDNA synthesis and amplification using the Ovation RNA-Seq System V2 (NuGen, CA, USA) following the manufacturer's instructions. In short, cDNA was produced using oligo dT and random hexamers. DsDNA was generated, purified using Agencourt RNAClean XP beads (Beckman Coulter, CA, USA) and amplified on beads using single primer isothermal amplification (SPIA) [16]. The SPIA reaction was 4˚C for 1 min, 47˚C for 60 min, 80˚C for 20 min and hold at 4˚C. After removing the beads, 40 μl of amplified dsDNA was purified with Qiaquick PCR purification Kit (Qiagen) and eluted in 30 μl of buffer. The DNA concentration was measured by Nanodrop (104–108 ng/μl).

Library preparation and sequencing

Four libraries (representing S1-2 and O1-2) were prepared for sequencing using Illumina Nextera XT DNA Library Preparation Kit (Illumina, CA, USA). In order to (i) achieve a high concentration of nucleic acid for sequencing and (ii) remove primer-dimers from the PCR product, slight modifications to the manufacturer's protocol were made; (i) using 1,5 ng total input DNA and (ii) an extra clean-up cycle after the PCR step, with 1.5 x ratio Agencourt AMPure XP Beads (Beckman Coulter). Libraries were validated by quantification using Qubit® 2.0 Fluorometer and Qubit® dsDNA HS Assay kit (Invitrogen) and size analysis using a 2100 BioAnalyzer system with the DNA High Sensitivity kit (Agilent Technologies, CA,

USA). The mean library concentration and length were 1.5 ng/μl and 740 bp, respectively. For normalization, the libraries were diluted to 2 nM using the equation

$$Molarity = \frac{\text{ng}/\mu\text{l x } 10^6}{660 \text{ x Average library fragment length}}$$

Finally equal amounts of each library were pooled. Sequencing was performed using a MiSeq (Illumina) and 600 Cycles MiSeq Reagent Kit v3 in a paired-end mode.

Data analysis

Raw data from the MiSeq run were quality checked and trimmed (Q≥30; max number of ambiguities = 2) using CLC Genomic workbench (v7.5.3) (Qiagen) in order to remove poor data. Reads that passed the quality criteria were mapped against a reference BCoV genome available in GenBank (strain Mebus, accession number U00735.2) using the reference mapping tool (default values) in the CLC Genomic workbench. Sequences not mapping to BCoV were annotated through blastx analysis using Diamond [17]. The Diamond was run in sensitive mode and the blastx was performed against the nr-database (NCBI) using an e-value cut-off at 0.0001. SortMeRNA [18] was used to characterise the ribosomal RNA (rRNA) composition of each dataset and was run against the following databases: rfam 5.8s, rfam 5s, silva arc 16s, silva arc 23s, silva bac 16s, silva bac 23s, silva euk 18s and silva euk 28s.

Results

Sequence data

The majority of the reads from all four datasets remained after quality trimming although the ends were trimmed making the average read length 175–184 nt (Table 1). Mapping of the reads to the BCoV reference revealed a clear difference between the datasets originating from the two amplification methods, as only 0.07% of the reads from the SISPA datasets mapped to BCoV compared to 42–47% of the reads from the Ovation datasets (Table 1).

Regarding the non-BCoV sequences, for both methods the majority (87–89%) could be annotated through blastx as eukaryotic and 10–13% were of bacterial origin. SortMeRNA classified approximately 11% of all the reads from each of the Ovation datasets as rRNA and of these eukaryotic 18s and 28s were most abundant. In the SISPA datasets, nearly 8% were

Table 1. Sequence data output and results of the bovine coronavirus mapping.

Reads	Data sets			
Raw	**O1**	**O2**	**S1**	**S2**
Total reads	9 231 412	6 353 426	6 930 526	6 271 024
Average length (nt)	216,4	228,6	246,5	256,6
Trimmed				
Total reads	9 190 660	6 325 015	6 907 172	6 258 362
Average length (nt)	175,0	183,6	175,8	182,2
Mapped to BCoV				
Total reads	3 891 314	3 027 739	4 554	4 654
% mapped reads	42,34	47,87	0,07	0,07
Range of depth	254–288 944	164–202 537	0–208	0–249

One nasal swab from an infected calf was processed in duplicate with the Ovation (O) and the SISPA (S) protocols.

https://doi.org/10.1371/journal.pone.0187780.t001

Table 2. Distribution of annotated sequences. Reads were classified through bovine coronavirus mapping and blastx. The table shows percentage of reads from the ovation (O) and SISPA (S) samples that mapped to bacteria, archaea, eukarya and virus.

	Data sets			
	O1	O2	S1	S2
Bacteria	2,45	2,25	11,94	12,93
Archaea	0,0005	0,0011	0,0002	0
Eukarya	21,81	19,20	88,03	87,03
Virus	75,72	78,55	0,02	0,01

https://doi.org/10.1371/journal.pone.0187780.t002

classified as rRNA with half of the sequences mapping to the rfma 5s database and the other half to the 18s and 28s eukaryotic databases.

Interestingly, apart from a low number of phage and retrovirus sequences, another +ssRNA virus was identified in all samples—a bovine rhinitis B virus (BRBV). In O1 and O2 between 9 000–11 000 reads were identified covering most of the BRBV genome, while in S1 and S2, only 3 and 10 sequences, respectively, were identified. The BRBV identified has been genetically characterised [19]. The difference in overall viral content between the data sets generated with the two amplification methods was obvious and Table 2 shows the combined classification of the reads annotated through BCoV mapping and blastx.

BCoV coverage and sequence depth

For the SISPA samples (S1 and S2), coverage of the BCoV genome was 99% and the 5' and 3' untranslated regions (UTRs) were not complete. Also, in the S2 assembly a number of short regions towards the middle of the genome lacked coverage. Unlike the SISPA method, BCoV coverage for the Ovation samples (O1 and O2) was complete, including both UTRs (Fig 1). The consensus sequences of BCoV in O1 and O2 were identical.

When comparing data from the two amplification approaches, there was a clear difference between number of reads mapping to the reference BCoV genome and sequence depth, despite the high similarity in genome coverage and the total number of reads (Table 1). For both SISPA sets, only 0.07% of reads mapped to the reference genome and the sequence depth varied between 0–208 for S1 and 0–249 for S2. In contrast, 42.34% (O1) and 47.87% (O2) of the reads from samples prepared by the Ovation protocol mapped to the BCoV genome. Sequencing of the Ovation samples was, therefore, much deeper and displayed a depth of 254–284 944 (S1) and 169–202 537 (S2). Hence, the lowest sequence depth for the Ovation samples was comparable to the highest depth for SISPA. All data sets displayed a peak at the 3´end of the genome (N-gene) (Fig 1). For the Ovation samples a peak showed at 6 kb (ORF1a), while in the SISPA samples there was a peak just after 20 000 bp (end of ORF1/b and covering the NS2 gene). Also towards the 5´end of all samples, an increased sequence depth was found compared to the middle part of the genome.

Discussion

High-throughput sequencing has provided the possibility of sequencing not only the consensus sequence of a virus but the viral cloud (quasispecies) that exists in a particular sample [7]. For RNA viruses this is of extra importance as these viruses have a high mutation rate and exist, in an individual, as a population of related viruses which may affect the viral fitness, host specificity and pathogenesis [5, 11]. Coronaviruses are the largest RNA viruses known (27–32 kb positive sense RNA) and are considered emerging pathogens in both humans and animals [9]. In order to understand the biology of coronaviruses, it is important to have tools that can

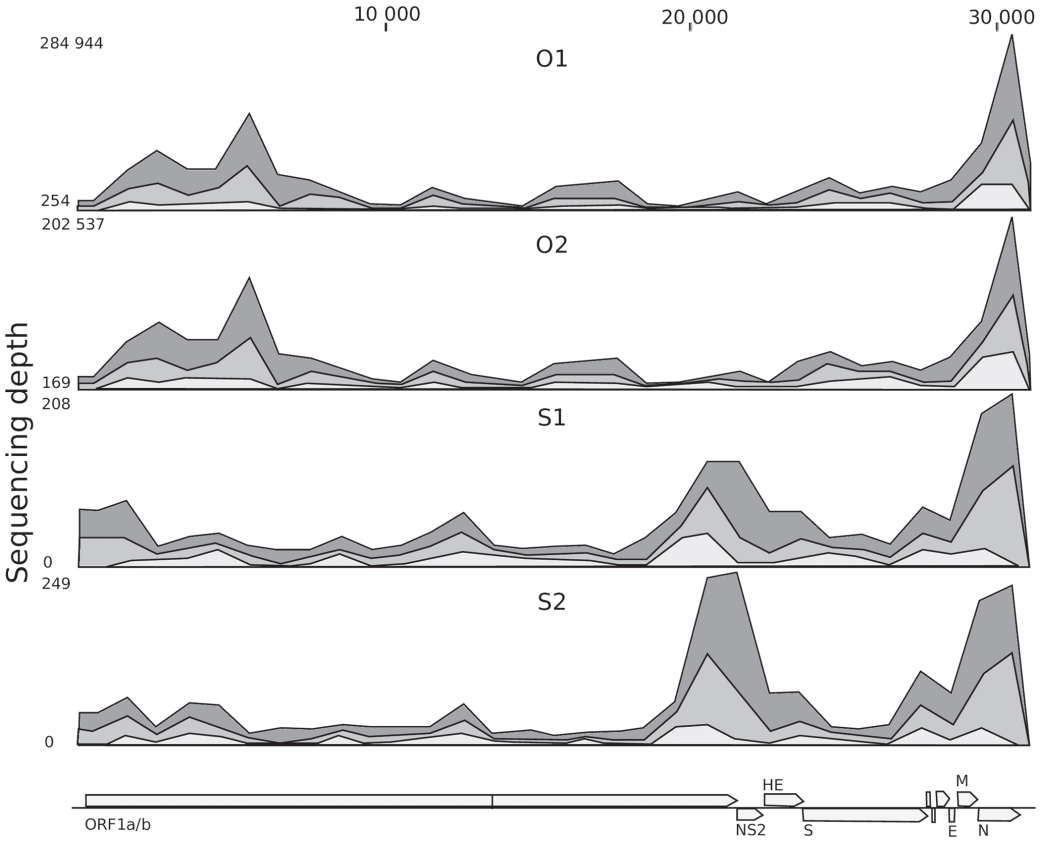


Fig 1. Coverage and sequence depth of the samples; O1 and O2 (ovation); S1 and S2 (SISPA). The lower part shows the annotation of the bovine coronavirus (strain Mebus) used in the mapping. The different shading of grey shows the minimum, mean and maximum depth values over a 1000 bp region.

https://doi.org/10.1371/journal.pone.0187780.g001

generate full-length genome sequences of emerging coronaviruses as well as provide information on the quasispecies population. Direct WGS of the FMDV has been performed on samples containing $\geq$ 1 x 10^7 genome copies per ul RNA [2]. However, as BCoV has a larger RNA genome (32 kb compared to 8.5 kb for FMDV) and our clinical samples contained less viral RNA, pre-amplification of the RNA was considered a necessity.

The present study compared two methods, SISPA and Ovation, for sequence-independent amplification of BCoV RNA. SISPA was chosen as it is a commonly used method for virus detection and has also been used for full-length viral genome sequencing [20]. The method includes tagged random primers to produce/label cDNA/DNA prior to PCR targeting the tag-sequence [15]. Ovation, on the other hand, is based on single-primer isothermal linear amplification (Ribo-SPIA) [21] and has been shown to generate full-length genomes of HIV, respiratory syncytial and West Nile virus from as little as 100 viral RNA copies [22].

In the present study, the two amplification methods gave approximately the same total number of reads and a very high coverage of the BCoV genome. While Ovation gave complete coverage, the SISPA approach missed parts of the UTRs as well as short regions in the middle of the genome. Although both methods produced sequences that covered all or most of the BCoV genome, there was a clear difference in the percentage of reads mapping to the reference genome. While less than 1% of the reads from the SISPA data sets mapped to BCoV, around 45% of the Ovation reads did. As most of the total SISPA reads (> 99%) originated from eukaryotes and bacteria and only 8% were classified as rRNA, filtration of the sample as well as an additional DNase treatment of the RNA could have resulted in more viral reads using the SISPA protocol. However, the much higher ratio of BCoV reads (approx. 45%) from the Ovation data sets compared to 0,07% still indicates a significant advantage of this amplification method compared to the SISPA protocol. The benefit of the Ovation method is not restricted to deep sequencing of BCoV as this protocol also gave roughly 1000 times more BRBV reads than the SISPA protocol.

The large difference in number of reads that mapped the BCoV reference genome resulted in a significant difference in sequence depth between the methods. The SISPA dataset had a maximum depth of 249 compared to 284 944 for the Ovation method. This difference was also found for BRBV as Ovation gave a sequence depth of 0–1000 covering the majority of the genome, while 3 and 10 sequences in total were present in the two SISPA data sets. The lower sequencing depth of BRBV was probably due to the lower number of BRBV genomes in the sample (1 to 600 compared to BCoV genomes).

The pattern of sequence depth was almost identical for the technical replicates O1 and O2 and was very similar for replicates S1 and S2 (Fig 1). An uneven depth was seen across the genome for both methods with a sequence depth peaking at the 3' end of the genome. For quasispecies analysis, a difference in sequence depth across the genome could pose a problem, as the possibility to compare variation in different regions will be reduced. The Ovation kit includes poly-T primers in addition to random (6N) primers, this could cause the 3'end peak, but does not explain the peak in the SISPA dataset. Fragmentation is a factor that may influence the variation of sequence coverage. However, in a study by Knierim et al. (2011) three different fragmentation methods (nebulization, sonication and enzymatic) gave similar coverage patterns [23]. Rosseel et al. (2013) got similar results, as they did not observe any differences when comparing fragmented to unfragmented samples. According to Malboeuf et al. (2012) depth variability across a genome could be due to secondary RNA structures [22], but this was not confirmed by Rosseel et al. who found biased annealing of the random primer, caused by the tag sequence, to influence SISPA results the most [24]. Extending the random sequence (from 6N to 12 N) and including more than one primer tag, may reduce the bias, for both methods.

Although the present study is limited, the results on two technical replicates show a clear difference between the two methods regarding efficient amplification of the RNA genome from two different viruses. Also, as amplification of nucleic acids is prone to introduction of errors in the nucleotide sequence, analysis of two technical replicates enabled some control of the sequencing quality. Focus was put on the consensus sequences, as these are anticipated to be identical in technical replicates. This was also the result for O1 and O2 replicates and indicates that Ovation is a feasible method for reconstructing the genome haplotype of a 32 kd BCoV.

In summary, the high amounts of BCoV and BRBV reads using the Ovation system indicate a high efficiency of this method for amplification of viral RNA from high and low copy number samples, compared to the SISPA protocol.

Disclaimer

None of the authors are connected to Nugen. The material contained in this study has not been sponsored by Nugen.

Author Contributions

Conceptualization: Mette Myrmel, Maria Stokstad, Mikael Berg, Anne-Lie Blomström.

Formal analysis: Mette Myrmel, Anne-Lie Blomström.

Funding acquisition: Mette Myrmel, Maria Stokstad.

Methodology: Mette Myrmel, Veslemøy Oma, Mamata Khatri, Hanne H. Hansen.

Supervision: Mette Myrmel.

Writing – original draft: Mette Myrmel, Veslemøy Oma, Hanne H. Hansen, Mikael Berg, Anne-Lie Blomström.

References

1. Quinones-Mateu ME, Avila S, Reyes-Teran G, Martinez MA. Deep sequencing: becoming a critical tool in clinical virology. J Clin Virol. 2014; 61(1):9–19. https://doi.org/10.1016/j.jcv.2014.06.013 PMID: 24998424.
2. Logan G, Freimanis GL, King DJ, Valdazo-Gonzalez B, Bachanek-Bankowska K, Sanderson ND, et al. A universal protocol to generate consensus level genome sequences for foot-and-mouth disease virus and other positive-sense polyadenylated RNA viruses using the Illumina MiSeq. Bmc Genomics. 2014; 15.
3. Delwart EL. Viral metagenomics. Rev Med Virol. 2007; 17(2):115–31. https://doi.org/10.1002/rmv.532 PMID: 17295196.
4. Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, et al. Multiple, linked human immunodeficiency virus type 1 drug resistance mutations in treatment-experienced patients are missed by standard genotype analysis. J Clin Microbiol. 2005; 43(1):406–13. https://doi.org/10.1128/JCM.43.1.406-413.2005 PMID: 15635002.
5. Domingo E, Sheldon J, Perales C. Viral quasispecies evolution. Microbiol Mol Biol Rev. 2012; 76 (2):159–216. https://doi.org/10.1128/MMBR.05023-11 PMID: 22688811.
6. Bexfield N, Kellam P. Metagenomics and the molecular identification of novel viruses. Vet J. 2011; 190 (2):191–8. https://doi.org/10.1016/j.tvjl.2010.10.014 PMID: 21111643.
7. McElroy K, Thomas T, Luciani F. Deep sequencing of evolving pathogen populations: applications, errors, and bioinformatic solutions. Microb Inform Exp. 2014; 4(1):1. https://doi.org/10.1186/2042-5783-4-1 PMID: 24428920.
8. Blomström AL. Viral metagenomics as an emerging and powerful tool in veterinary medicine. Vet Q. 2011; 31(3):107–14. https://doi.org/10.1080/01652176.2011.604971 PMID: 22029881.
9. Weiss SR, Navas-Martin S. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol Mol Biol Rev. 2005; 69(4):635–64. https://doi.org/10.1128/MMBR.69.4.635-664.2005 PMID: 16339739.
10. Lai MMC. Recombination in large RNA viruses: Coronaviruses. Semin Virol. 1996; 7(6):381–8.
11. Andino R, Domingo E. Viral quasispecies. Virology. 2015; 479–480:46–51. https://doi.org/10.1016/j.virol.2015.03.022 PMID: 25824477.
12. Oma VS, Traven M, Alenius S, Myrmel M, Stokstad M. Bovine coronavirus in naturally and experimentally exposed calves; viral shedding and the potential for transmission. Virol J. 2016; 13:100. https://doi.org/10.1186/s12985-016-0555-x PMID: 27296861.
13. Decaro N, Elia G, Campolo M, Desario C, Mari V, Radogna A, et al. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J Virol Methods. 2008; 151(2):167–71. https://doi.org/10.1016/j.jviromet.2008.05.016 PMID: 18579223.
14. Blomström AL, Widen F, Hammer AS, Belak S, Berg M. Detection of a novel astrovirus in brain tissue of mink suffering from shaking mink syndrome by use of viral metagenomics. J Clin Microbiol. 2010; 48 (12):4392–6. https://doi.org/10.1128/JCM.01040-10 PMID: 20926705.

15. Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005; 102 (36):12891–6. https://doi.org/10.1073/pnas.0504666102 PMID: 16118271.

16. Kurn N, Chen PC, Heath JD, Kopf-Sill A, Stephens KM, Wang SL. Novel isothermal, linear nucleic acid amplification systems for highly multiplexed applications. Clin Chem. 2005; 51(10):1973–81. https://doi.org/10.1373/clinchem.2005.053694 PMID: 16123149

17. Buchfink B, Xie C, Huson DH. Fast and sensitive protein alignment using DIAMOND. Nat Methods. 2015; 12(1):59–60. https://doi.org/10.1038/nmeth.3176 PMID: 25402007.

18. Kopylova E, Noe L, Touzet H. SortMeRNA: fast and accurate filtering of ribosomal RNAs in metatranscriptomic data. Bioinformatics. 2012; 28(24):3211–7. https://doi.org/10.1093/bioinformatics/bts611 PMID: 23071270

19. Blomstrom AL, Oma V, Khatri M, Hansen HH, Stokstad M, Berg M, et al. Genome Sequence of a Bovine Rhinitis B Virus Identified in Cattle in Sweden. Genome Announc. 2017; 5(19). https://doi.org/10.1128/genomeA.00172-17 PMID: 28495761.

20. Djikeng A, Halpin R, Kuzmickas R, Depasse J, Feldblyum J, Sengamalay N, et al. Viral genome sequencing by random priming methods. BMC Genomics. 2008; 9:5. https://doi.org/10.1186/1471-2164-9-5 PMID: 18179705.

21. Dafforn A, Chen P, Deng G, Herrler M, Iglehart D, Koritala S, et al. Linear mRNA amplification from as little as 5 ng total RNA for global gene expression analysis. Biotechniques. 2004; 37(5):854–7. PMID: 15560142.

22. Malboeuf CM, Yang X, Charlebois P, Qu J, Berlin AM, Casali M, et al. Complete viral RNA genome sequencing of ultra-low copy samples by sequence-independent amplification. Nucleic Acids Res. 2013; 41(1):e13. https://doi.org/10.1093/nar/gks794 PMID: 22962364.

23. Knierim E, Lucke B, Schwarz JM, Schuelke M, Seelow D. Systematic comparison of three methods for fragmentation of long-range PCR products for next generation sequencing. PLoS One. 2011; 6(11): e28240. https://doi.org/10.1371/journal.pone.0028240 PMID: 22140562.

24. Rosseel T, Van Borm S, Vandenbussche F, Hoffmann B, van den Berg T, Beer M, et al. The origin of biased sequence depth in sequence-independent nucleic acid amplification and optimization for efficient massive parallel sequencing. PLoS One. 2013; 8(9):e76144. https://doi.org/10.1371/journal.pone.0076144 PMID: 24086702.

ISBN: 978-82-575-1759-5
ISSN: 1894-6402

Norwegian University
of Life Sciences

Postboks 5003
NO-1432 Ås, Norway
+47 67 23 00 00
www.nmbu.no

101472 / ANDVORDGRAFISK.NO