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Tick-borne encephalitis virus in cows and unpasteurized cow milk from Norway

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Abstract

Tick-borne encephalitis virus (TBEV) is recognized as the most important zoonotic tick-transmitted virus in Europe. TBEV is mainly transmitted to humans through bites from TBEV-infected ticks (*Ixodes ricinus* and *Ixodes persulcatus*). However, alimentary infection after consumption of unpasteurized milk and cheese from domestic ruminants has been reported. There is little information about TBEV in ruminants in Norway. The objectives of this study were to analyse unpasteurized cow milk for TBEV RNA and to study the presence of IgG antibodies to TBEV in the same animals. A total of 112 milk and blood samples were collected from cows from five different farms spread from southern to northern Norway. The milk samples were analysed by an in-house reverse transcription (RT) real-time polymerase chain reaction and confirmed by pyrosequencing. Serum samples were screened by a commercial enzyme-linked immunosorbent assay and verified by a TBEV-specific serum neutralization test. We found TBEV RNA in unpasteurized milk collected from farms in the municipalities of Mandal, Skedsmo and Brønnøy in 5.4% of the tested animals. Specific antibodies to TBEV were only detected in Arendal, where 88.2% of the tested animals were positive. Further studies on milk containing TBEV RNA should be performed to conclude if TBEV found in unpasteurized milk in Norway is infectious, which could be of great importance in a One Health perspective.

KEYWORDS
domestic ruminants, real-time PCR, serum neutralization test, tick-borne encephalitis virus, unpasteurized milk

1 | INTRODUCTION

Tick-borne encephalitis virus (TBEV) is the causative agent of tick-borne encephalitis (TBE) in humans, and it is the most important zoonotic tick-transmitted virus in Europe from a medical perspective (Suss, 2011). TBEV is a positive-sense, single-stranded RNA virus in the *Flaviviridae* family, within the *Flavivirus* genus. TBEV is occasionally transmitted to humans through bites from TBEV-infected *Ixodes ricinus* or *Ixodes persulcatus* ticks, which are recognized as both vectors and reservoirs (Lindquist & Vapalahti, 2008). TBEV infection in humans often results in unspecific transient febrile symptoms, but the clinical outcome ranges from asymptomatic to severe infection

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in the central nervous system (Kaiser, 2012). The virus is traditionally divided into three main subtypes, the European, the Siberian and the Far Eastern (Ecker, Allison, Meixner, & Heinz, 1999). At least two additional subtypes are known at present, the Baikalian and the Himalayan subtypes (Dai, Shang, Lu, Yang, & Xu, 2018; Kovalev & Mukhacheva, 2017). In Norway, only the European subtype is found (Andreassen et al., 2012; Paulsen et al., 2015; Soleng et al., 2018). The closely related Louping ill virus (LIV) has been detected in Norway. However, no outbreaks have been reported since the last outbreak in 1991, reported by the Norwegian Veterinary Institute (Gao et al., 1993; Ulvund, Vik, & Krogsrud, 1983). The disease caused by the different subtypes of TBEV varies in severity and mortality; the European and Siberian subtypes generally have fatality rates of approximately 1%–3%, while the Far Eastern subtype fatality rate might be as high as 20%–40% (Dorrbecker, Dobler, Spiegel, & Hufert, 2010; Gritsun, Frolova, et al., 2003a).

Consumption of raw milk and other dairy product seems to be an increasing trend both in Norway as well as in other European countries due to the alleged health benefits and better taste of natural products. Outbreaks of *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter* spp. and TBEV have been reported after consumption of unpasteurized milk in Europe (Bogovic & Strle, 2015; Costard, Espejo, Groenendaal, & Zagmutt, 2017; Willis et al., 2018). Alimentary TBEV infection following consumption of unpasteurized milk and cheese from domestic ruminants has been reported in approximately 1% of all TBE cases (Kriz, Benes, & Daniel, 2009). However, this number may differ significantly. The highest occurrence of alimentary TBE is known from Slovakia, where up to 17% of TBE cases are caused by consumption of infected milk (Kerlik et al., 2018). For other countries, the available information is limited. The largest reported outbreak of alimentary TBE occurred in Slovakia in 1951–1952, where at least 660 people became infected (Kerlik et al., 2018).

In most of the alimentary TBE cases, the virus is transmitted through unpasteurized milk and cheese from goat, but TBEV infection through consumption of unpasteurized milk from cow and sheep has also been reported (Balogh et al., 2010; Caini et al., 2012; Gresikova, Sekeyova, Stupalova, & Necas, 1975; Holzmann et al., 2009; Hudopisk et al., 2013; Markovinovic et al., 2016). Infected domestic ruminants do not display clinical symptoms, but they may develop a viremia with a duration of approximately 1 week (Balogh et al., 2012; Van Tongeren, 1955). In milk samples from infected ruminants, TBEV has been detected for up to 19 days post-infection (Balogh et al., 2012). Detectable antibodies in ruminants have been found for at least 28 months after infection (Klaus, Ziegler, Kalthoff, Hoffmann, & Beer, 2014). The clinical manifestation of alimentary TBE in humans may differ from TBE after tick bites. The alimentary TBE is biphasic, similar to infection caused by the European subtype of TBEV, with some observed differences. Alimentary-transmitted TBE has a shorter incubation period compared to TBEV infection through tick bite (3–4 days and 7–14 days, respectively). While the biphasic form is dominant for alimentary TBE, the biphasic form represents about 20%–30% of all TBEV infections after tick bite. Non-severe meningoencephalitis is observed for alimentary TBE, while clinical manifestations of tick-associated TBE may be more severe with "aseptic" meningitis, meningoencephalitis and meningomyeloencephalitis (Gritsun, Nuttall, & Gould, 2003b; Ruzek, Dobler, & Donoso Mantke, 2010).

Impacts

- This is the first report of tick-borne encephalitis virus (TBEV) in unpasteurized milk in Norway.
- The study provides updated information on TBEV distribution in Norway.
- Norwegian authorities are currently considering to allow the sale of unpasteurized dairy products. Our study indicates that a risk assessment may be suitable to evaluate the consequences of consuming unpasteurized dairy products from domestic ruminants grazing in areas of Norway where TBEV is detected.

There is limited knowledge of the presence of TBEV in domestic ruminants in Norway. A study from 1973 detected a seroprevalence of 17.7% in bovine sera in western Norway (Traavik, 1973). According to the Norwegian Surveillance System of Communicable Diseases (MSIS), the incidence of human TBE is low in Norway, with a total of 143 reported cases since 1997, of which 16 were reported in 2017. The human cases are limited to southern Norway (Norwegian Institute of Public Health, 2018). However, *I. ricinus* ticks carrying TBEV have been detected in coastal areas from the county of Østfold in the southeast up to Brønnøy located in the county of Nordland in northern Norway. This indicates that the virus is more widespread in Norway than the reported human cases may suggest (Andreassen et al., 2012; Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018). Outbreaks of alimentary TBEV infections can be prevented by pasteurizing milk before consumption or by vaccination against TBEV (Hudopisk et al., 2013; Offerdahl, Clancy, & Bloom, 2016). For instance, immunization of goats has been demonstrated as an effective method of preventing TBEV infection from unpasteurized milk (Balogh et al., 2012).

The objectives of this study were to analyse unpasteurized milk samples from cows for TBEV RNA and to study the presence of antibodies to TBEV in the same animals.

2 | MATERIALS AND METHODS

2.1 | Collection of milk and blood samples

A total of 112 milk samples were collected from grazing dairy cows between June 2014 and September 2017 on farms located in the municipalities of Skedsmo, Arendal, Mandal, Finnøy and Brønnøy in Norway. The Skedsmo samples were collected in June, the Brønnøy and Finnøy samples were collected in September, and the Mandal

and Arendal samples were collected in October (Figure 1, Table 1). All cows included in the study have been grazing close to their respective farms during daytime for more than 1 year. Blood samples were taken from the same animals and separated to serum. In addition to the milk samples from individual cows, a total of five samples from bulk milk tanks at the farms were tested from Skedsmo, Mandal (two samples), Finnøy and Brønnøy. The samples were stored in sterile tubes at −80°C until further processing. All farms, except the farm in Skedsmo, are situated in areas where ticks are known to be abundant. Of the five municipalities in this study, Mandal and Arendal are the only two where human cases of TBE have been reported in Norway (Norwegian Institute of Public Health, 2018).

2.2 | Detection of tick-borne encephalitis virus in raw milk from cows

Fat from all milk samples was removed by centrifuging at 6,000 *g* for 10 min according to Cisak et al. (2010). Viral RNA from skimmed milk was then extracted using QIAamp® Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. The elution volume was 60 µl. Directly after extraction, the RNA was reversely transcribed to cDNA with random primers (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster city, CA, USA), followed by detection of TBEV RNA with in-house RT real-time polymerase chain reaction (PCR) and pyrosequencing assays according to Andreassen et al. (2012). The real-time PCR amplifies a 54 base pair fragment located on the envelope gene of TBEV, specific to the Norwegian TBEV strain (Andreassen et al., 2012; Skarpaas et al., 2006). All real-time PCR positive samples were further analysed by pyrosequencing according to the manufacturer's manual for sequence analysis (SQA) on the BioTage system (Pyromark ID, QIAGEN, GmbH, Hilden, Germany). A positive control ("Soukup") was used in the real-time PCR, and further in the pyrosequencing analysis, to compare and confirm the sequences revealed from the positive samples (Andreassen et al., 2012).

FIGURE 1 Geographical locations of the five farms included in the study. A total of 112 cow milk and serum samples were collected from Skedsmo (Akershus county), Arendal (Aust-Agder county), Mandal (Vest-Agder county) and Finnøy (Rogaland county). Map from Kartverket (Creative Commons Attribution ShareAlike 3.0) [Colour figure can be viewed at wileyonlinelibrary.com]

2.3 | Detection of IgG antibodies against tick-borne encephalitis virus in cow serum

Serum samples from cows were screened for IgG antibodies against TBEV by a commercial enzyme-linked immunosorbent assay (ELISA, Enzygnost® Anti-TBE virus IgG, Siemens Healthcare, GmbH, Marburg, Germany) according to the manufacturer's protocol, with one modification: the conjugate was changed to peroxidase-labelled antibody to bovine IgG diluted 1:30,000 (KLP, Gaithersburg, USA). The conjugate was diluted in IgG Conjugate Buffer Microbiol (Enzygnost® Anti-Rubella Virus IgG, Siemens Healthcare, GmbH, Marburg, Germany). Serum from calves vaccinated against TBE with the TicoVac-vaccine (Pfizer Ltd, Ramsgate Road, Sandwich, Kent, CT13 9 NJ, UK) was used as positive controls and serum from calves which never had been exposed to ticks as negative controls.

Positive samples from the ELISA were re-tested in a TBEV-specific serum neutralization test (SNT) at the Center for Virology of the Medical University of Vienna, as described by Stiasny, Holzmann, and Heinz (2009). Briefly, serial dilutions of heat-inactivated samples were incubated with TBEV (strain Neudoerfl) for 1 hr at 37°C. Baby hamster kidney (BHK-21) cells were added, and incubation was continued for 3 days. The presence of virus in the cell culture supernatant was assessed by ELISA. The virus neutralization titre was defined as the reciprocal of the sample dilution that showed a 90% reduction in the absorbance readout compared to the control without antibody. Samples with titres equal to 10 and higher were defined as TBE seropositive.

3 | RESULTS

3.1 | Tick-borne encephalitis virus in raw milk from cows

Tick-borne encephalitis virus RNA was detected by RT real-time PCR and pyrosequencing in six of the 112 (5.4%) analysed raw milk samples from cows. The positive samples originated from Mandal, Skedsmo and Brønnøy, in 28.6%, 13.6% and 2.1% of the animals, respectively. In Mandal, two of the three real-time PCR positive samples were confirmed by pyrosequencing. Five positive samples from

TABLE 1 Prevalence of tick-borne encephalitis virus in 112 unpasteurized milk samples collected from cows at five farms in Norway

Location (municipality, county, date)	No. of milk samples	Positive by real-time polymerase chain reaction	Confirmed by pyrosequencing (prevalence; %)
Skedsmo, Akershus, 02.06.2014	22	5	3 (13.6)
Arendal, Aust-Agder, 05.10.2015	17	0	0 (0)
Mandal, Vest-Agder, 01.10.2014	7	3	2 (28.6)
Finnøy, Rogaland, 11.09.2017	19	0	0 (0)
Brønnøy, Nordland, 02.09.2015	47	1	1 (2.1)
Total	112	9	6 (5.4)

Skedsmo were detected by real-time PCR, and three of them were confirmed by pyrosequencing. At Brønnøy, one sample was positive by both real-time PCR and pyrosequencing (Table 1).

Both bulk tank milk samples from Mandal were positive by real-time PCR and confirmed by pyrosequencing, while the remaining tank samples were negative (data not shown).

3.2 | Antibodies against tick-borne encephalitis virus in cow serum

IgG antibodies against TBEV were detected by ELISA in 16 of the 112 tested animals. However, positive samples by SNT were only detected in Arendal, where 15 out of 17 (88.2%) samples were positive. The titres in the SNT ranged from 15 to 1,280. ELISA-positive samples from Skedsmo and Finnøy were negative by SNT (Table 2, Supporting Information Table S1).

None of the cows with TBEV-positive milk by RT real-time PCR and pyrosequencing had detectable TBE-antibodies in serum by SNT (Tables 1 and 2).

4 | DISCUSSION

This is the first study to report TBEV in unpasteurized cow milk in Norway. TBEV RNA was detected in raw milk collected from areas both with and without reported human TBE cases.

A study from TBE-endemic areas in Poland found, by RT-PCR, an overall TBEV prevalence of 11.1% in unpasteurized milk from cows (Cisak et al., 2010). The overall prevalence was lower in the current study (5.4%). Regions with no previously reported TBE cases were included in our study, which is in contrast to the Polish study, where only high-endemic areas were included (Cisak et al., 2010). In Norway, TBEV has a wider geographical distribution than the human cases reported by MSIS, as recently documented by studies on ticks (*I. ricinus*) and healthy blood donors (Larsen et al., 2014; Paulsen et al., 2015; Soleng et al., 2018). In Brønnøy in Nordland county, Soleng et al. (2018) detected a TBEV prevalence of up to 3% and 9% in *I. ricinus* nymphs and adults, respectively. Our data from one positive milk sample in Brønnøy support the suspicion that TBEV is circulating close to the northern border of *I. ricinus* ticks' geographical distribution in Norway (Soleng et al., 2018).

The TBEV RNA-positive milk samples from Skedsmo were somewhat unexpected, because ticks only sporadically are found on livestock in this area (personal communication by the farmer). However, *I. ricinus* ticks may appear outside their normal range via transportation by mammals and migratory birds, which also may play an important role in transmission and distribution of TBEV (Labuda & Nuttall, 2004). Large mammals may facilitate short to medium range transportation of ticks, while birds may transport ticks over long distances and across geographical barriers (Hasle et al., 2009).

Seroprevalence studies on TBE in domestic animals in Europe have demonstrated that animals may serve as useful sentinels for detection of TBEV risk areas (Klauset al., 2010, 2012; Rieille, Klaus, Hoffmann, Peter, & Voordouw, 2017; Salat, Mihalca, Mihaiu, Modry, & Ruzek, 2017). In the present study, a total of 112 serum samples from five farms were analysed. TBEV-specific antibodies were detected in cattle in Arendal only. The fact that TBE-antibodies were detected in cows from Arendal is in accordance with the reported

TABLE 2 Seroprevalence of IgG antibodies against tick-borne encephalitis virus in 112 serum samples collected from cows at five farms in Norway

Location (municipality, county, date)	No. of serum samples	Positive by enzyme-linked immunosorbent assay	Confirmed by serum neutralization test (seroprevalence; %)
Skedsmo, Akershus, 02.06.2014	22	1	0
Arendal, Aust-Agder, 05.10.2015	17	14	15 (88.2)
Mandal, Vest-Agder, 01.10.2014	7	0	0
Finnøy, Rogaland, 11.09.2017	19	1	0
Brønnøy, Nordland, 02.09.2015	47	0	0
Total	112	16	15 (13.4)

human TBEV cases, as this region has the highest number of reported TBE cases annually (Norwegian Institute of Public Health, 2018).

A previous study has shown that goats and sheep have a measurable antibody response for at least 28 months after primary infection (Balogh et al., 2012). The antibody response seems to vary between species, and it is unclear if all animals exposed to the virus develop an immune response (Klaus et al., 2010, 2012). This might explain why the samples taken from Skedsmo, Mandal and Brønnøy had detectable TBEV RNA in the milk, but all animals tested negative for neutralizing antibodies to TBEV in the serum. Balogh et al. (2012) showed that infected goats had measurable virus in the milk for up to 19 days post-infection, but immunized goats did not shed TBEV through the milk. The positive serum samples from Arendal were sampled in October. If these cows were infected, and thereby immunized in the spring or summer, there will be no virus left in the milk samples taken in the fall, but the IgG will remain detectable for several months. This might be the reason why we did not detect TBEV RNA in the cow milk from this area, where 15 out of 17 animals had TBEV-specific antibodies. The sampling from Skedsmo occurred in June, and three milk samples were found to be positive for TBEV RNA. It is not known if these animals developed neutralizing antibodies to TBEV post-infection. While the age of the cows and introduction of animals to new areas may affect the results, all animals in the present study were adults and had been grazing in the same area for more than one season.

Sera testing positive for TBEV-specific IgG antibodies by ELISA may cross-react to the closely related LIV. For this reason, a neutralization test is recommended to confirm the ELISA results (Rieille et al., 2017). However, TBEV and LIV are so closely related that antibodies to either virus may also react in a neutralization test. On the other hand, LIV has not been detected in Arendal previously, and Louping ill cases have not been reported in Norway since the last report from the Norwegian Veterinary Institute in 1991 (Gao et al., 1993; Ulvund et al., 1983; Ytrehus, Vainio, Dudman, Gilray, & Willoughby, 2013). Furthermore, 7,615 ticks collected from all over Norway have been analysed for LIV, and all were found to be LIV negative (Paulsen et al., 2017).

A study by Cisak et al. (2010) found no correlation between milk samples tested by ELISA for presence of specific antibodies against TBEV with those obtained by RT-PCR, and they suggested that the ELISA is less appropriate for detecting the presence of TBEV-specific antibodies in milk. For this reason, ELISA on our collected milk samples was not performed.

Detection of TBEV RNA in unpasteurized milk alone cannot prove that TBEV is endemic in an area, but detection of TBEV RNA in ticks and results from serological assays may indicate that the virus is circulating in an area. The presence of TBEV may therefore pose a direct risk for the human population living in, or visiting these areas, even without any reported TBE cases. In this study, we found no relationship between TBEV RNA in milk and TBE IgG in serum. However, TBEV RNA in ticks has been detected in all areas, except for Skedsmo, where, to our knowledge, no ticks have been examined for TBEV.

Further studies on TBEV in milk should be performed to conclude if TBEV found in unpasteurized milk in Norway is infectious. These results, as others reported in Europe, may point to the importance of considering a precautionary principle when consuming unpasteurized milk products in areas where TBEV is distributed (Balogh et al., 2012; Offerdahl et al., 2016). Previous studies have demonstrated that pasteurization of milk is an effective method to prevent TBEV infections from dairy products (Hudopisk et al., 2013; Offerdahl et al., 2016). Further seroprevalence studies in domestic animal populations with broader geographical coverage and greater sample size should be carried out, accompanied by risk assessments to evaluate both the prevalence of TBEV RNA in milk and the consequences of consuming unpasteurized milk from ruminants in Norway. Furthermore, a seroprevalence study on people working in close contact with these animals could provide important epidemiological data for risk evaluation, as they could have been exposed to ticks or may be infected via unpasteurized milk.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICAL APPROVAL

All blood samples and milk samples were considered diagnostic and were collected by trained veterinarians. Vaccination of calves, to serve as positive controls, was authorized by the Norwegian Food Safety Authority (FOTS ID 8135).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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