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Dairy milk from cow and goat as a sentinel for tick-borne encephalitis virus surveillance

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ABSTRACT

Tick-borne encephalitis (TBE) is one of the most severe human tick-borne diseases in Europe. It is caused by the tick-borne encephalitis virus (TBEV), which is transmitted to humans mainly via bites of *Ixodes ricinus* or *I. persulcatus* ticks. The geographical distribution and abundance of *I. ricinus* is expanding in Sweden as has the number of reported human TBE cases. In addition to tick bites, alimentary TBEV infection has also been reported after consumption of unpasteurized dairy products. So far, no alimentary TBEV infection has been reported in Sweden, but knowledge about its prevalence in Swedish ruminants is scarce. In the present study, a total of 122 bulk tank milk samples and 304 individual milk samples (including 8 colostrum samples) were collected from dairy farms (n = 102) in Sweden. All samples were analysed for the presence of TBEV antibodies by ELISA test and immunoblotting. Participating farmers received a questionnaire about milk production, pasteurization, tick prophylaxis used on animals, tick-borne diseases, and TBE vaccination status. We detected specific anti-TBEV antibodies, i.e., either positive (>126 Vienna Units per ml, VIEU/ml) or borderline (63–126 VIEU/ml) in bulk tank milk from 20 of the 102 farms. Individual milk samples (including colostrum samples) from these 20 farms were therefore collected for further analysis. Our results revealed important information for detection of emerging TBE risk areas. Factors such as consumption of unpasteurized milk, limited use of tick prophylaxis on animals and a moderate coverage of human TBE vaccination, may be risk factors for alimentary TBEV infection in Sweden.

1. Introduction

Tick-borne encephalitis (TBE) is one of the most severe human viral tick-borne diseases in Europe, and the infection may lead to serious neurological manifestations such as meningoencephalitis or myelitis [1]. TBE became a notifiable disease in humans within the European Union in 2012 [2] and since 2004 in Sweden where the cases are reported to the Regional Medical Officer and to the Swedish Public Health Agency [3]. The incidence of TBE in Sweden is increasing, from 174 notifiable cases in 2004–533 in 2021 [4]. Climate change has facilitated the expansion of ticks to higher latitudes and altitudes and increased the population density of ticks [5–7]. TBE is endemic in the coastal areas including the central regions of Södermanland, Uppsala, Stockholm and Östergötland and around the Lake Mälaren in Sweden.

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The virus has been gradually spreading west- and northwards to new regions and TBE cases are now reported from the southernmost region of Skåne to Gävleborg and Dalarna in the north (Fig. 1).

Tick-borne encephalitis, a zoonotic infection, is caused by the tick-borne encephalitis virus (TBEV), which belongs to the genus *Flavivirus* of the *Flaviviridae* family. TBE is usually transmitted to humans through tick bites, mainly *Ixodes* spp. The tick life cycle includes feeding on three vertebrate hosts [8], making ticks efficient vectors for transmitting infectious agents between humans, pets, livestock, and wildlife [7]. In Europe, *Ixodes ricinus* ticks are the most common TBEV vector [9]. *I. ricinus* usually transmits the European TBEV (TBEV-Eur) subtype [8], whereas *I. persulcatus* is the main vector for the Siberian (TBEV-Sib) and Far-Eastern (TBEV-FE) subtypes. TBEV-Sib and TBEV-FE are more virulent subtypes and cause more severe forms of the disease [10]. At present, these subtypes have not yet been identified in ticks in Sweden, but the geographical distribution and population density of *I. persulcatus* is increasing in northern Sweden [11].

The increase in abundance and geographic range of *I. ricinus* is the main factor for the expansion of endemic TBEV areas in Sweden [6]. Its spread into new areas is a severe emerging threat to human health in Europe and Asia [12,13]. Vaccination, if repeated according to the recommendations, is generally effective in preventing TBEV infection in humans [14]. The Public Health Agency of Sweden recommends vaccination for residents and frequent visitors of TBEV high-risk areas.

Apart from tick bites, another TBE risk factor is the consumption of milk and dairy products produced from unpasteurized milk [15]. Such alimentary infections have been reported in the Central and Eastern parts of Europe [16–24]. TBEV alimentary transmission has primarily occurred via either unpasteurized goat milk or cheese, but one TBE outbreak transmitted by unpasteurized cow milk was reported in Hungary, 2012 [21]. Additionally, a Norwegian study detected TBEV specific antibodies in sera from dairy cows and TBEV RNA in unpasteurized cow milk [25]. The Swedish Food Agency discourages consumption of unpasteurized milk particularly for children, pregnant women, elderly, or immunosuppressed individuals [26]. In general, most Swedish milk on the market is pasteurized or heat-treated in other ways with equivalent effect. To the best of our knowledge, no case of TBE due to consumption of unpasteurized milk or dairy products has been identified in Sweden.

Infected domestic ruminant animals do not usually display clinical symptoms, but they may develop a viraemia and excrete the virus via their milk, sometimes for more than three weeks after the first TBEV exposure [20,27]. Knowledge about the prevalence of TBEV infections in domestic ruminants in Sweden is limited. In a serological survey on TBEV in bulk tank milk sampled in 2013, serologically positive dairy herds were mainly found in southern and central Sweden, but also in two northern counties [28]. In Lithuania, 4.3 % and 4.5 % of the bulk tank milk samples from sheep and goats, respectively tested positive for TBEV in 2018 and 2019 [29]. To validate these results, ticks were flagged and collected in the surrounding area of the Lithuanian dairy milk farms in 2019 and the geographical distribution of the TBEV in the milk samples overlapped with the known TBE endemic areas and correlated with the incidence of TBE in humans in Lithuania. This study confirms that testing milk serves as a valuable tool for tracking the spatial distribution of TBEV. In addition, a way to identify new TBEV foci by detecting TBEV antibodies in unpasteurized sheep milk has been evaluated in Sweden [30].

In the present study, we investigated the presence of TBEV-specific antibodies in bulk tank milk samples to identify and map emerging TBE risk areas in Sweden. We started with a nationwide serological screening of bulk tank milk samples collected from dairy farms. The serological analysis was complemented with a questionnaire to obtain information regarding milk production, pasteurization, tick prophylaxis used on animals, tick-borne diseases, and TBE vaccination. Farms positive for TBEV antibodies were recruited for monitoring and follow-up surveys, in which individual milk and colostrum samples were collected and analyzed.

2. Materials and methods

2.1. Study design

2.1.1. Survey

The present study started with a survey of bulk tank milk from dairy farms to detect TBEV-specific antibodies (Fig. 2). All cattle farms in the cattle database maintained by the Swedish Board of Agriculture (n = 10,211) were contacted by email. This database does not specify if the farm has dairy or meat production. However, all dairy cow farmers that received the information were invited to participate in the study. In addition, farmers with dairy animals could register on the Swedish National Veterinary Institute's website, and this link was also sent to members of the Swedish Goat Breeding Association and the Swedish Artisan Dairy producers. All farms that replied to the call were recruited for the study.

In this study, 102 farms from different parts of Sweden participated: dairy cattle (n = 79), goat (n = 18), dairy cow and goat (n = 3), sheep (n = 1), and water buffalo (n = 1; Fig. 3). Bulk tank milk samples were collected by the farmers from these farms between 5th and 14th of August 2019 and sent to the National Veterinary Institute (SVA), Uppsala, Sweden. At the three farms with both dairy cows and goats, the farmers collected one bulk tank milk sample from each species. All participating farmers received instructions on how to perform the sampling as well as materials for collecting and sending samples to SVA. The instruction provided to them is a standardized one used SVA when testing cell counts, bacteria, and antibodies in milk. In accordance with Swedish animal welfare legislation, the animals were on pasture during the grazing season [31,32].

2.1.2. Questionnaire to farms

All farms included in the survey (n = 102) were asked to fill in a questionnaire regarding milk production, pasteurization, tick prophylaxis used on animals, tick-borne diseases, and TBE vaccination (Appendix 1). Answers were processed in Microsoft Excel (version 2205) and presented by descriptive statistics.

2.1.3. Follow-up of farms with TBEV-specific antibodies

The 20 farms where TBEV-specific antibodies were detected, i.e., either positive above 126 Vienna Units per ml (VIEU/ml) or at borderline 63–126 VIEU/ml, were asked to send new bulk tank milk samples as well as individual milk samples. If possible, one bulk tank milk sample and up to 20 individual milk samples were collected from each farm. Both primiparous and multiparous cows were included. The samples were collected between 14th and 30th of October 2019.

2.1.4. Second follow-up

Farms which tested positive or borderline in the first survey and follow-up and were located in areas with notified human TBE cases (n = 5), were asked to provide new bulk tank milk samples and individual samples. Two farms submitted these second follow-up samples. Two bulk tank milk samples and 13 individual samples were collected between 15th of September and 15th of October 2020. Eight colostrum samples were also collected.

2.2. Detection of antibodies against tick-borne encephalitis virus in milk samples

2.2.1. Sample preparation

Bulk tank milk samples were taken directly from the milk tank of each farm, whereas individual samples were collected before milking individual animals. Samples were collected into 15-ml plastic test tubes, stored at − 20 °C at the National Veterinary Institute, Uppsala, Sweden, and transported frozen to Örebro University, Sweden. Upon arrival,

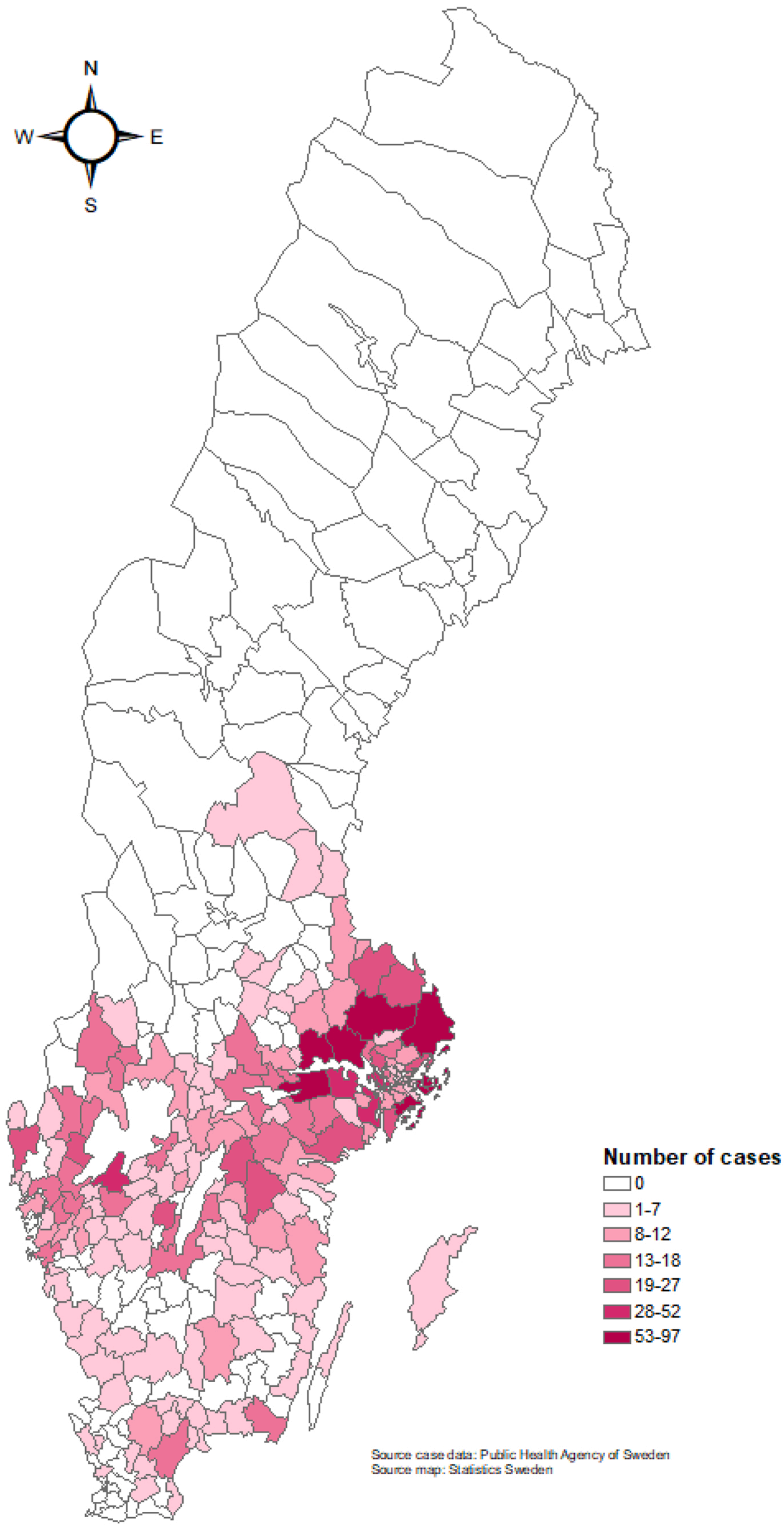

Fig. 1. Geographical distribution of human TBE cases in Sweden 2016–2021.

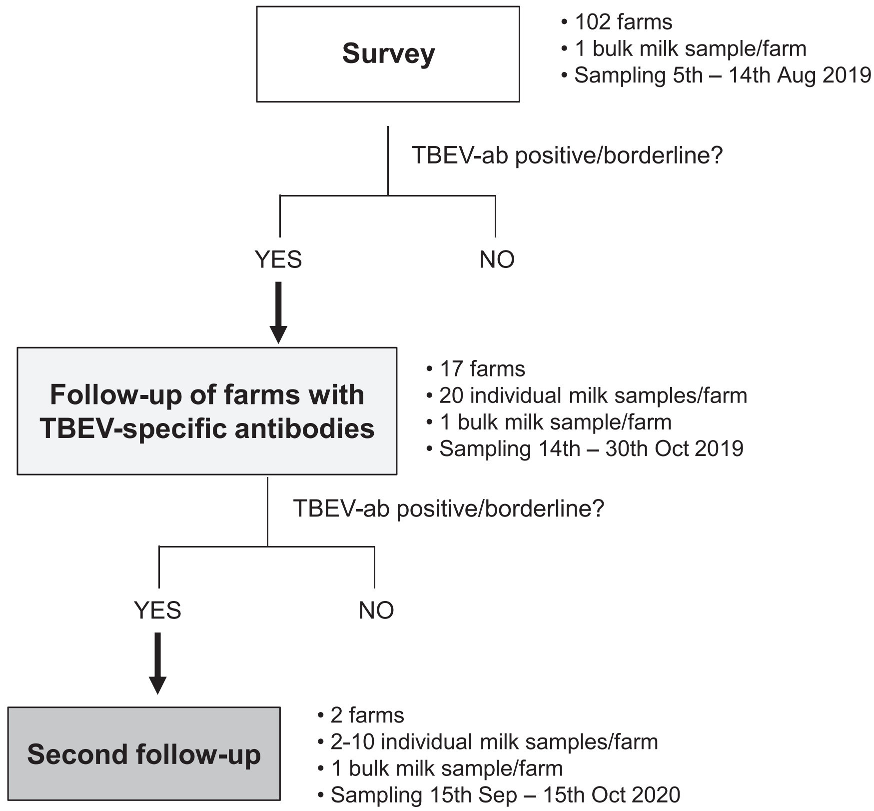

Fig. 2. Study design: 1) Survey of bulk tank milk in Sweden, 2) Follow-up of farms with bulk tank milk testing positive or borderline for TBEV- specific antibodies (TBEV-ab), 3) Positive or borderline farms from the first survey and second sampling round and located in areas with notified TBEV cases. These were further asked to provide new bulk tank milk samples and individual samples.

samples were thawed at room temperature and dispensed into 1.5 ml microtubes (Sarstedt, Nürnbrecht, Germany) for centrifugation at 16,100× *g* for 10 min. A syringe needle was placed beneath the cream layer to draw up the skim milk and transfer it to new microtubes. The skimmed milk samples were stored at − 80 °C until analyses.

2.2.2. ELISA

A commercially available enzyme-linked immunosorbent assay (ELISA, Immunozym FSME IgG All Species Progen Biotechnik GMBH, Heidelberg, Germany) was used for detection of anti-TBEV-specific antibodies in the milk samples as described previously [30]. The assay was optimized for milk samples and samples were analysed according to the manufacturer's protocol. The optical density was measured at 450 nm using a Multiskan-Ascent spectrophotometer (Lab Systems, Thermo Fisher Scientific, Waltham, MA, USA). Using standard curves, sample concentrations were read in VIEU/ml. Empirical cut-off values [33] for TBEV Immunoglobulin G antibodies were compared with those based on the Yonden Index and were assessed as follows: negative (<63 VIEU/ml), borderline (63–126 VIEU/ml), and positive (>126 VIEU/ml). Samples with borderline or positive titers were further analysed by Immunoblotting. All samples were run in duplicates.

2.2.3. Cells and transfection

Baby hamster kidney (BHK-21) cells were maintained at 37 °C and 5 % CO_2 in Dulbeccos's modified Eagle's medium (DMEM, Sigma-Alrich, St. Louis, MO, USA), supplemented with 5% fetal bovine serum and 1 % each of penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA). The cells were grown in T25 culture flasks to reach 70–90 % confluence. After trypsinization, 10^6 cells were transfected with 4 μg of TBEV-ME pCAG plasmid expressing a fused membrane-envelope (ME) protein of TBEV using Nucleofector kit L on a Nucleodector II Device (Lonza, Colonge, Germany), as per manufacturer's instructions. The transfected cells were incubated for 96 h at 37 °C and 5 % CO_2, then lysed in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA).

2.2.4. Immunoblotting

The BHK-21 lysate containing TBEV ME protein was separated by gel electrophoresis gels (NuPAGE 4–12 % Bis-Tris gels, Invitrogen, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane (iBlot transfer stacks, Thermo Fischer Scientific, Waltham, MA, USA). The membranes were incubated in a blocking solution containing Tris buffered saline with 0.1 % Tween 20 (TBS-T) and 5 % w/v non-fat dried milk for 1 h at room temperature. Milk samples that tested positive or borderline for TBEV-specific antibodies by ELISA were diluted in TBS-T (1:1). After three washes with TBS-T, the membranes were incubated with the diluted milk samples overnight at 4 °C. Following another three washes with TBS-T, the membranes were incubated with a rabbit polyclonal secondary antibody (anti-bovine (H+L) HRP, Invitrogen, Carlsbad, CA, USA) diluted in the blocking solution (1:1000) for 1 h at room temperature. The protein bands were visualized using enhanced chemiluminescene substrate (Thermo Fischer Scientific, Waltham, MA, USA) and a ChemiDoc MP imaging system (BioRad, Hercules, CA, USA).

3. Results

3.1. Detection of antibodies against tick-borne encephalitis virus in milk samples

3.1.1. Survey

A total of 105 bulk tank milk samples from the sampled herds were analyzed and screened for anti-TBEV-IgG antibodies using a commercial ELISA. Antibodies were detected in 20 samples of which 2.9 % (n = 3; 95 % Confidence Interval (CI) 0.6–8.3) of the samples were positive and

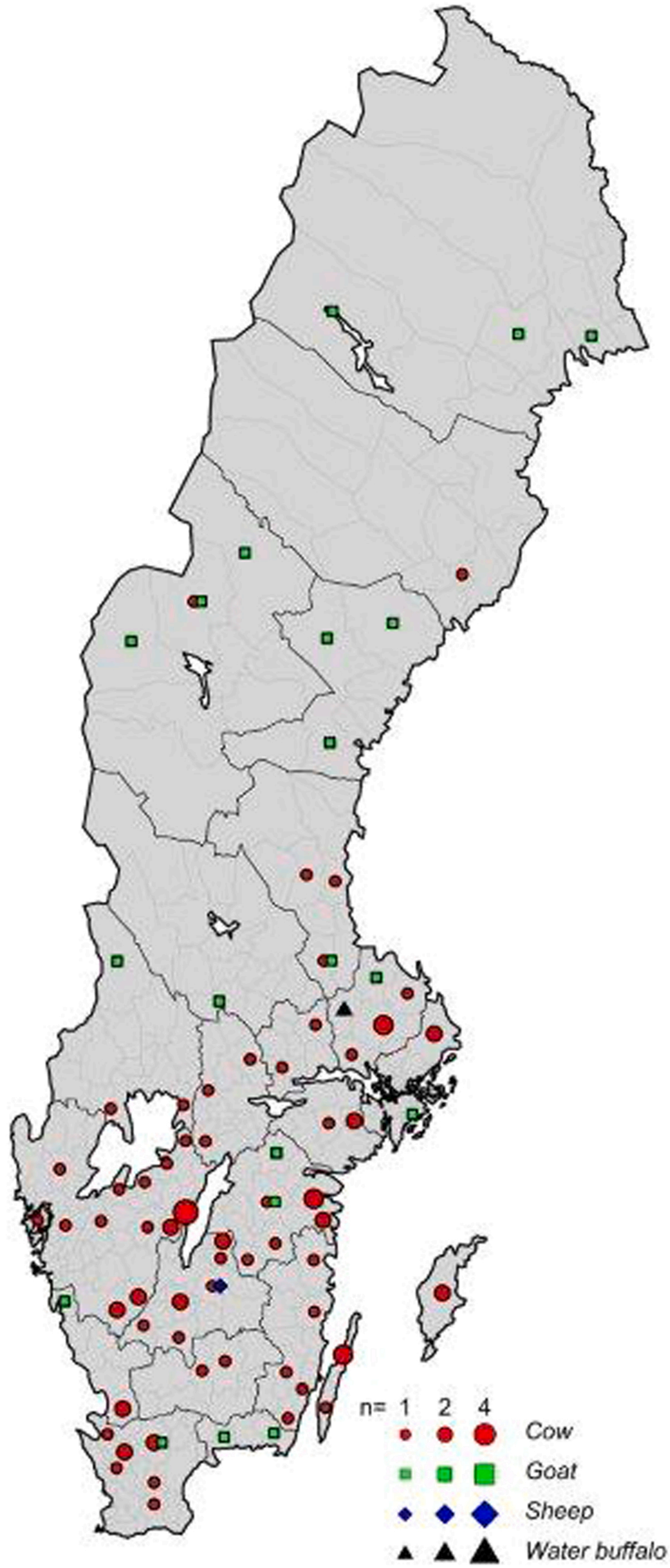

Fig. 3. Location of the participating farms in the survey. Animal species indicated are: cow (red), goat (green), sheep (blue) and water buffalo (black). The size of the symbol indicates the number (n) of participating farms in each municipality (1, 2 or 4).

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16.2 % (n = 17; 95 % CI 9.4–25.9) were putatively positive with borderline levels (Table 1). The positive samples came from dairy cow farms in the Swedish counties of Gotland, Örebro and Värmland.

3.1.2. Follow-up of farms with TBEV-specific antibodies

Dairy cow farms that tested positive or borderline in the screening study were asked to participate in a second sampling. Out of these 20 farms, 17 farms responded, and individual samples (n = 186), along with bulk tank milk samples (n = 15), were collected. All bulk tank milk samples tested negative. Antibodies to TBEV were detected in three individual samples from two farms (Table 2). All individual samples (n = 183) tested at the remaining 12 farms were negative.

3.1.3. Second follow-up

Of the five farms that were contacted one year later for a second follow-up, two could provide samples. Farm #13, which had borderline samples in the first follow-up, provided both individual milk (n = 4) and colostrum samples (n = 6). Farm #31, which had positive samples in the first survey, but negative ones in the first follow-up, also provided both individual milk (n = 1) and colostrum samples (n = 2) for analysis. The latter samples had been stored at − 20 °C, since the timepoint of milking in the early spring (March-April 2020). In farm #13, one milk and one colostrum sample showed borderline levels of anti-TBEV-IgG. At farm #31, both colostrum samples displayed positive levels of TBEV antibodies (Table 3). As a comparison, the level of anti-TBEV-IgG in colostrum from the second follow-up differed considerably from the levels in the positive bulk tank milk and individual samples in the survey and first follow-up. The levels were almost four times as high (Fig. 4).

3.1.4. Immunoblotting

Selected positive or borderline TBEV-IgG samples were further analyzed for the presence of TBEV antibodies by target-specific immunoblotting. The milk samples were used as the source of primary antibodies to detect the envelope (E) protein of TBEV. Immunoblotting confirmed that TBEV antibodies were detectable in colostrum samples in which concentrations of anti-TBEV-IgG were high, as indicated by an intense band at 50 kDa representing the E protein (Fig. 5). Bulk tank milk samples tested positive by ELISA could not be confirmed by immunoblotting. The same applied to positive individual milk samples in the follow-up.

3.2. Questionnaire

The questionnaire (Appendix 1) was sent out to all participating farms (n = 102) in the survey to gain information about milk production and management, as well as tick-related issues. A total of 99 questionnaires were completed. Of those, 76 were from dairy cow farms, 18 from dairy goat, 3 with both dairy cows and goat, and 1 each from sheep and water buffalo farm. The number of milking animals ranged from 2 to 620 (on average 76) in dairy cow farms and from 1 to 114 (on average 36) in the goat farms. The dairy sheep and water buffalo farms had 4 respective 13 milking animals.

3.2.1. Sales of unpasteurized milk from the farms

Thirty-six percent (n = 36) of the 99 farms reported increased milk production during the previous five years, 6 % (n = 6) a decrease, and 57 % (n = 58) no change. The milk from most of the farms (69 %; n = 68) was transported to a dairy plant. Of these, 68 % (n = 67) were dairy cow and 1% (n = 1) was goat. Twenty-one percent of the farms sold the milk on the farm (as milk, cheese, or other milk products), and 16 of these 21 farms were dairy goat farms. Five percent reported that the produced milk was partly transported to a dairy plant and partly sold on the farm.

Of the 21 farms selling all the milk on the farm, six reported that no milk or milk products were pasteurized prior to marketing, seven responded that all milk or milk products were pasteurized, and another seven pasteurized some. One farm (1 %) did not respond.

3.2.2. Pasture types

Fifty-one percent of the farms (n = 50) reported their animals grazed on different types of pastures e.g., cultivated grassland, meadows with bushes and trees, and shoreline meadows or wetlands. About one fourth (26 %; n = 26) used cultivated grass land exclusively and 23 % (n = 23) only meadows with bushes and trees. Of the respondents, 47 % (n = 47) reported no surface water on the pasture, 29 % (n = 29) had flowing creeks and/or rivers, 11 % (n = 11) had ponds and/or lakes with stagnant water, and 12 % (n = 12) had a combination of flowing creeks and/or rivers, and ponds and/or lakes with stagnant water.

3.2.3. Low use of tick prophylaxis

Fifty-three percent of the farms (n = 52) used no tick prophylaxis on their production or companion animals; 6 % (n = 6) solely on their companion animals, 13 % (n = 13) solely on their production animals, and 7 % (n = 7) on both groups of their animals. One farm (1 %) did not respond. Interestingly, 18 of the 21 farms (87 %) with dairy goats did not use tick prophylaxis on either animal categories.

3.2.4. Observation of ticks

Thirty-one percent of the farms (n = 31) reported an increase in number of ticks the previous five years, 42 % (n = 42) did not observe an increase, and seven reported a decrease. About one fifth (19 %; n = 19) of the farms answered that they did not usually observe ticks or observed them rarely.

3.2.5. Tick-borne diseases in animals

Thirty-two percent of the farms (n = 32) had experienced increasing problems with tick-borne diseases, such as anaplasmosis and babesiosis, in their herds during the previous five years. All of these, except one farm (1 %) with water buffalos, were farms with dairy cows. Sixty-six percent of the farms (n = 65) reported no increasing problems in their herd and 2 % (n = 2) did not respond to the question. Eighty-one percent of the farms (n = 80) reported no increasing problems with stillbirths or abortions. Eighteen percent of the farms (n = 18), all with dairy cows, stated increasing problems and 2 % farms (n = 2) did not respond.

3.2.6. Vaccination

More than half of the farms (54 %; n = 53) answered that no persons on the farm were vaccinated against TBE. Nineteen percent (n = 10) of these farms were from the northern areas in Sweden where no indigenous TBE cases have been detected so far. Fourteen percent of the farms

Table 1
Detection by ELISA of tick-borne encephalitis virus antibodies in bulk tank milk samples surveyed between the 5th and 14th of August 2019.

Animal species	No. of samples	No. of positive samples	% of positive samples	No. of borderline samples	% of borderline samples
Dairy cow[a]	82	3	3.7	17	20.7
Goat[a]	21	0	0	0	0
Sheep	1	0	0	0	0
Water buffalo	1	0	0	0	0
Total	105	3	2.9	17	16.2

[a] Three farms kept both dairy cows and goats.

Table 2
Detection by ELISA of tick-borne encephalitis virus antibodies in bulk tank milk and individual milk samples from dairy cows at farms with samples above the cut-off or at borderline. Samples collected between the 14th and 30th of October 2019.

Farm ID	County	Sample type	No. of samples	No. positive samples	% of positive samples	No. borderline samples	% of borderline samples
# 27	Västra Götaland	Bulk tank milk	1	0	0	0	0
		Individual milk	20	0	0	1	5.0
# 28	Västmanland	Bulk tank milk	1	0	0	0	0
		Individual milk	20	0	0	4	20.0
# 30	Småland	Bulk tank milk	1	0	0	0	0
		Individual milk	20	0	0	2	10.0
# 35	Skåne	Bulk tank milk	1	0	0	0	0
		Individual milk	20	1	5.0	0	0
# 48	Västmanland	Bulk tank milk	1	0	0	0	0
		Individual milk	20	2	10.0	0	0

Table 3
Detection by ELISA of tick-borne encephalitis virus antibodies in individual milk and colostrum samples from dairy cows at two farms. Samples were collected between the 15th of September and 15th of October 2020.

Farm ID	County	Sample type	No. of samples	No. positive samples	% of positive samples	No. borderline samples	% of borderline samples
# 13	Värmland	Individual milk	4	0	0	1	25.0
		Colostrum	6	0	0	1	16.7
# 31	Västra Götaland	Individual milk	1	0	0	0	0
		Colostrum	2	2	100	0	0

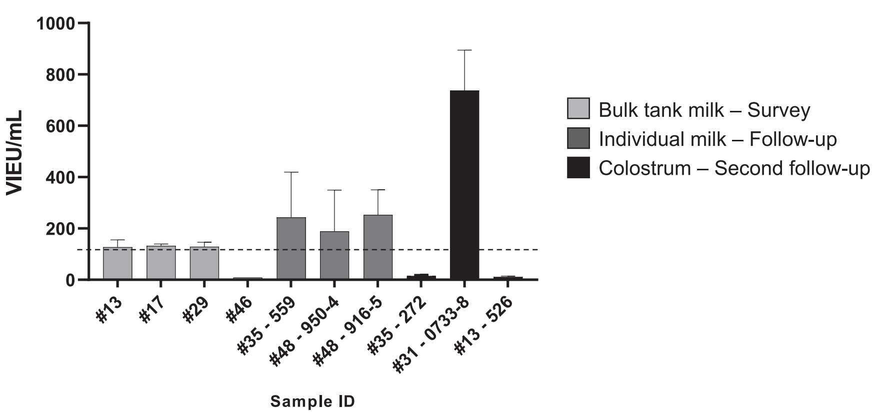

Fig. 4. Comparison of anti-TBEV-IgG levels (in VIEU/ml) in different types of milk. The level of anti-TBEV-IgG differs considerably between bulk tank milk samples (survey), individual milk samples (follow-up) and colostrum samples (second follow-up). Antibody titers > 126 VIEU/ml (dotted line) were considered positive according to manufacturer's instructions. Sample are indicated as Farm#, and the individual samples show identification of the animal. Bulk tank milk: farm #13, 17, 29, 46. Individual milk: farm #35, 48. Colostrum: farm #35, 31, 13. The level of anti-TBEV-IgG is the mean of two samples (n = 2) and error bars represent standard deviation (SD) of the mean.

(n = 14), all situated in TBEV high-risk areas, answered that all persons on the farm were vaccinated. Around one-third of the farms (32 %; n = 32), all of which except 6 % (n = 2) were in TBEV high-risk areas, reported that some persons on the farm were vaccinated against TBE.

4. Discussion

As ticks expand their geographical distribution in Sweden [11,34], new risk areas for TBE are being detected [4]. An improved surveillance system is pivotal for control of endemic and emerging diseases. Although human TBE is a notifiable disease in Sweden, classifying risk areas based on human cases is problematic, as many infected cases are never detected. Attempts to identify new geographical areas endemic for TBEV have been made using serological studies of blood or milk or investigating the presence of virus or antibodies against TBEV in different vertebrate animals, birds, and ticks [28,30,35,36]. Samples from wildlife, e.g., blood samples collected during hunting, have been used as a surveillance method [35], but some wild animal species move over wider areas, thus giving unspecific indications about TBEV risk areas. Individual blood samples from farm animals can be an effective way when searching for new TBEV hot spots and monitoring the known areas [36], but this method requires veterinarians to obtain several samples from each farm, thus increasing the cost. Unless sampling is done at slaughter or in conjunction with other surveillance or surveys, it may also be an animal welfare issue.

The approach presented here, i.e., the use of bulk tank milk samples from dairy farms, offers numerous advantages for studying the prevalence of TBEV in countries where the animals are on pasture during those parts of the year when tick density is highest. Bulk tank milk samples from dairy cow farms are routinely collected in national control programs for surveillance of diseases related to animal health, and TBEV antibody detection could be integrated in these surveillance programs despite being mainly a human disease. In 2021, there were 2 955 dairy cow farms in Sweden, with 301,850 dairy cows and heifers [37]. The dairy farms are spread widely, from the south to the north, which further improves the possibility to cover emerging areas for TBE by surveying bulk tank milk. A report from the Public Health Agency of Sweden [4] shows that TBE cases in humans are expanding northwards and westwards, which corresponds to the pattern in the rest of Europe. The Swedish animal welfare legalization [31,32] requires all dairy animals be on pasture during the grazing season. This period also correlates with the seasonal distribution of reported TBE cases in Sweden. The tick season ranges from April to October with minor variations from year to year and area in Sweden. Our study was conducted from August to October, to cover the end of the grazing season and the vector period. This maximized the possible length to exposure for TBE, and this period also correlates with the highest number of reported TBE human cases [4].

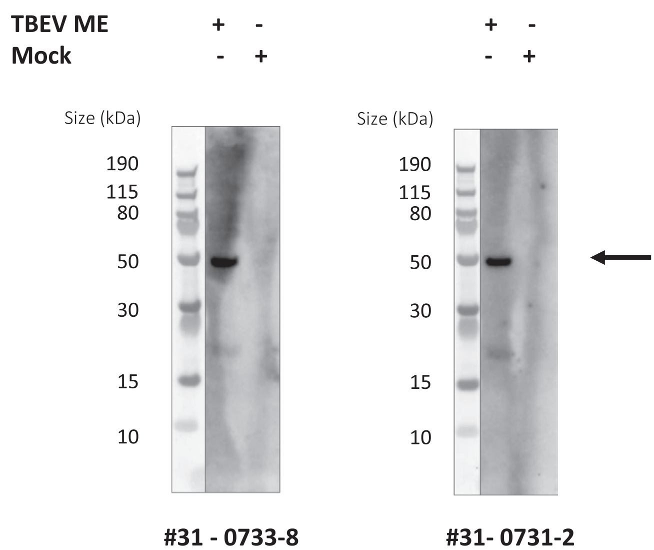

Fig. 5. Immunoblotting of colostrum samples. TBEV membrane (M) and envelope (E) proteins expressed in BHK-21 cells by transfecting the cells with a plasmid encoding these proteins. TBEV-E protein (≈ 50 kDa) is indicated by an arrow and non-transfected cells are denoted as mock. Sample IDs are indicated as farm number and identification number of the animal.

In this study, the occurrence of TBEV antibodies in bulk tank milk from dairy cows was 3.7 % and an additional 20.7 % were considered borderline. This percentage is lower than that detected in serum samples from individual dairy cows (13.4 % seropositivity) in Norway [25], which may be due to the dilution effect of pooling, and because there are lower levels of antibodies in cow milk compared to serum. However, Blomqvist et al. [28] showed a reasonable predictive correlation between antibody levels in bulk tank milk and mean serum levels within a herd and concluded that the serological examination may be useful as a sentinel surveillance method to identify geographical risk areas for TBE.

To confirm our results from the survey, individual milk and colostrum samples from anti TBEV-IgG positive farms were collected and analyzed. Colostrum supplies the immunological naïve newborn calf with antibodies against many infections. As it is rich in antibodies, we hypothesized that colostrum would display higher TBEV antibody titers as well. As expected, we found that the positive colostrum samples did display higher levels than both milk from individual animals and bulk tank milk from herds. The TBEV-antibodies in colostrum were also confirmed by our target-specific immunoblotting assay. The levels of anti-TBEV-IgG in the tested bulk tank milk and individual milk samples seemed to be too low for detection by the immunoblotting assay, even though the ELISA assay confirmed the presence of antibodies.

To our knowledge, no case of alimentary TBE has been reported in Sweden. A recent study in Lithuania detected 4.3% and 4.5% TBEV positive milk samples from systematically selected goat and sheep farms, respectively [29]. This highlights the potential for alimentary TBE infections also in Sweden. Cases of alimentary TBE could be unreported, due to lack of awareness, both by the public and health professionals, of this potential way of transmission. Patients may have been bitten by ticks in close connection to the onset of illness and therefore, did not suspect that the TBE transmission could have been alimentary.

Historical data show improved public health due to pasteurization [38]. The Swedish Food Agency [26] generally discourages consumption of unpasteurized milk in particularly for children, pregnant women, older or immunosuppressed individuals. Pasteurization of milk is an effective way to inactivate TBEV, thus eliminating the risk of alimentary TBE. Pasteurization also prevents other food-borne pathogens, such as *Campylobacter*, *Escherichia coli*, *Salmonella* and *Listeria monocytogenes*. However, there are still many advocates for unpasteurized products. Clays et al. [38] found no scientifically viable arguments for unpasteurized products except the change in taste, but other factors, such as culture and tradition, can lie behind choosing unpasteurized products. In Sweden, only milk pasteurized or treated in other ways with equivalent effect can be sold to consumers. However, farms are allowed to sell a maximum of 70 l of unpasteurized milk per week directly to the customer if the consumer is informed that the milk may contain pathogenic microbes and that it is recommended to heat treat the milk before consumption. Based on the results from the questionnaire in our study, surprisingly many farms sold unpasteurized milk and/or milk products or consumed milk directly at the farms. Since this implicates a considerable risk for public health, including possible transmission of TBEV, this trading should be further studied. Unfortunately, the questionnaire did not reveal if proper written information about the product was provided to the customer.

During the second follow-up, we found one dairy cow farm (# 31) where colostrum samples tested positive for anti-TBEV-IgG antibodies by both ELISA and immunoblotting. We examined the number of reported human TBE cases in the region close to the farm. During 2010–2021, 16 human cases were reported in the surroundings of Lake Skagern, situated on the border of Västra Götaland, Värmland and Örebro counties, according to the Public Health Agency of Sweden [4]. We identified that the farm (# 31) was situated in the vicinity of several human TBE cases reported during this time period (Fig. 6). Further, colostrum samples from two sheep farms located close to the south-eastern shore of Lake Skagern, tested positive for anti-TBEV-IgG [30]. Together, these suggest a TBEV hot spot in close proximity to farm #31 (Fig. 6). Similar geographical distribution patterns in Lithuania were observed for TBEV-positive milk samples, where TBEV presence could be correlated to human TBE cases and TBE hot spots [29]. These findings support that testing of milk for TBEV-antibodies or TBEV is a promising strategy for identifying new endemic risk areas.

The method for analysis of TBEV antibody levels in bulk tank milk needs further evaluation. The reference values provided by the manufacturer for the ELISA-kit used in this study are based on individual serum samples and using these reference values for bulk milk samples may lead to false negative results. These cut-off values should be adjusted and complemented by Immunoblotting.

Another consideration is dilution effect of sampling bulk tank milk. The sampling reflected the number of animals in each herd, which ranged from large-scale production farms with several hundred animals to hobby farming with only a few. Blomqvist et al. [28] confirms that the dilution effect - resulting from both the ratio of seropositive and seronegative animals and the individual antibody levels of seropositive animals - needs to be considered in antibody analyses of bulk tank milk. Even though the bulk tank milk tested negative for TBEV antibodies in the follow-up, individual milk samples could display high levels of TBEV antibodies. These discrepancies are also discussed by Blomqvist et al. [28] who found individual positive samples in herds where the bulk tank milk was seronegative.

A putative limitation with the current method is risk of cross-reactivity with antibodies against other flaviviruses circulating within the study area. To our knowledge, TBEV is the only autochthonous flavivirus in Sweden. The closely related Louping ill virus (LIV) is mainly present in the United Kingdom and Ireland, but has also been identified in Norway [39] and Denmark [40]. However, we consider cross-reactivity in the milk samples analyzed in this study is highly unlikely because no LIV infections have been reported to date in Sweden. However, this method should be used with caution in areas where LIV or other closely related flaviviruses are present.

Vaccination of humans, if repeated according to the recommendations, is an effective way to prevent TBE and should be encouraged in risk areas [14]. The Public Health Agency of Sweden recommends vaccination for residents or frequent visitors to TBE high risk areas in Sweden. However, vaccination against TBE is not included in the national vaccination program in Sweden and thus is not free of charge, except for children up to the age of 18 years in the regions of

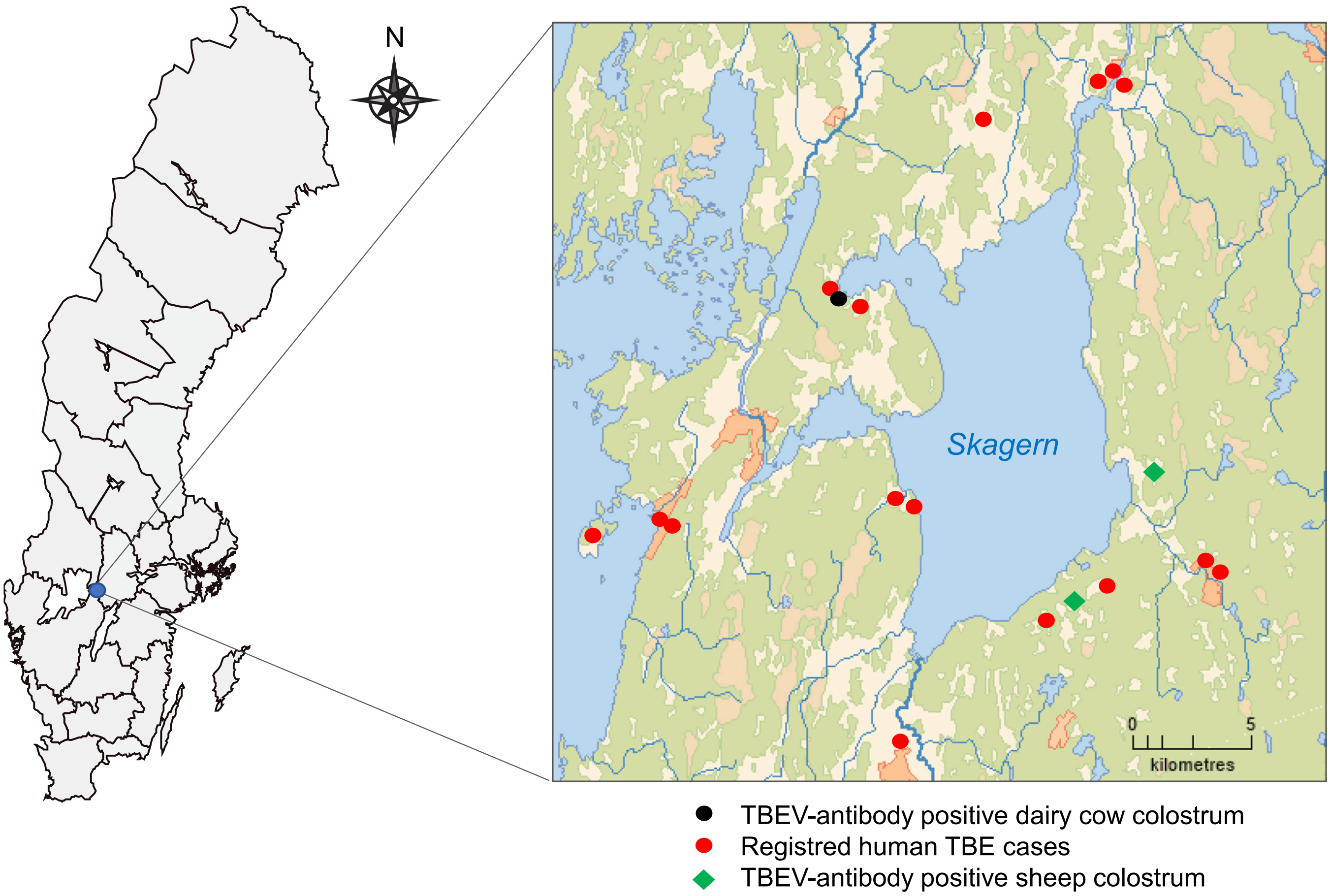

Fig. 6. Geographical locations of registered human TBE cases in the area of Lake Skagern, Sweden (red dots). Location of TBEV antibody-positive colostrum samples from dairy cows are indicated by a black dot and from the sheep farms by green squares [30].

Södermanland, Uppsala, and Östergötland. The cost might affect the willingness to get vaccinated. Our questionnaire revealed that the TBE vaccination status of employees and persons living at the participating farms was moderate. In total, 46 farms, all except two of them situated in TBE high-risk areas, answered that all or some persons on the farm were vaccinated.

The use of tick prophylaxis on production and companion animals on the farms in this survey was low, especially on the dairy goat farms where 18 of 21 did not use it. In total, 46 farms used prophylaxis for either their companion animals and/or production animals. However, use of repellents may reduce tick bites on the animals but is not sufficient for avoiding viraemia and transfer of the virus via milk.

In conclusion, there is a potential risk for transmission of alimentary TBEV via unpasteurized milk in Sweden. In Sweden it is compulsory to have dairy animals out on pasture, which increases the exposure to ticks. In addition, the low vaccine coverage among the farmers may be due to lack of knowledge about alimentary TBEV infections and the risks associated with TBE. Bulk tank milk can be used as a sentinel for detecting emerging TBE risk areas. This method is simple and non-invasive as the farmers can easily take the samples themselves. The results from such surveys are important for monitoring of TBE and improve preventive strategies against it.

Ethical approval

Bulk tank milk and individual milk samples were collected and handled by the farmers. The sampling was conducted by ordinary milking routines at the farm and the study was not classified as an animal experiment.

The National Veterinary Institute (SVA), Uppsala, Sweden, processed personal data to complete the survey. All farmers who submitted the questionnaire gave their personal data consent for processing the data in accordance with the Data Protection Regulation (EU) 2016/679. The farmers were informed that SVA reserves the right to use submitted questionnaire responses and the results of the survey for research purposes within the framework of SVA's activities. Processing and possible publication have been anonymized to the extent possible under Swedish law. Publication of research is done without reference to individual survey responses unless required by Swedish law.

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CRediT authorship contribution statement

Anna Omazic: Conceptualization, Data curation, Project administration, Supervision, Validation, Visualization, Writing – original draft. **Amélie Wallenhammar:** Conceptualization, Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft. **Elina Lahti** Conceptualization, Supervision, Validation, Writing – review & editing. **Naveed Asghar:** Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Alexander Hanberger:** Data curation, Formal analysis, Writing – original draft. **Marika Hjertqvist:** Validation, Visualization, Writing – review & editing. **Magnus Johansson:** Conceptualization, Funding acquisition, Methodology, Resources, Validation, Writing – review & editing. **Ann Albihn:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2023.101958.

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