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Original article

First detection of TBE virus in ticks and sero-reactivity in goats in a non-endemic region in the southern part of Switzerland (Canton of Ticino)

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ABSTRACT

In Switzerland, tick-borne encephalitis (TBE) is a notifiable human disease with an average of 210 cases per year in the last 10 years (2008–2017). A national surveillance conducted in 2009 reported a prevalence of 0.46% for tick-borne encephalitis virus (TBEV) detected in ticks, which is in accordance with the prevalences found in Europe from 0.1%–5%. The Canton of Ticino in the southern part of Switzerland, geographically separated from the rest of the national territory by the Alps, is considered a non-endemic region, as no autochthonous clinical cases and no TBEV presence in ticks have ever been reported. In order to understand the epidemiological situation in Ticino, we conducted a large study investigating the TBEV presence in field-collected *Ixodes ricinus* ticks and in goat and human sera. Goats and sheep were considered as sentinel hosts showing persistence of antibodies also after 28 months in the absence of symptoms; this longevity supports the data validity to characterize an area with the TBEV status. The goat sera collection was composed of a total of 662 samples from 37 flocks. The total seroprevalence was 14.6%. 39 (40%) of the 97 SNT-positive samples showed an antibody titer ≥ 1:120 which indicates recent infection and consequently the probable presence of active foci among the pastures frequented by the goats belonging to 10 flocks. In total, 51 owners participated in the study and all were TBEV antibody-free.

A total of 12’052 *I. ricinus* ticks (nymphs and adults) were collected and 1’371 pools were tested using quantitative real-time RT-PCR. Only one positive pool was reported with a prevalence of 0.35%. Metagenomic analysis revealed that the TBEV strain isolated from the ticks collected in Ticino is closely related to 2 strains coming from the Canton of Valais (99.1% and 98.7% identity, respectively), a neighbouring region of the Canton of Ticino. These two Cantons are close together but separated by high mountains (Alps) and we hypothesize that infected ticks were transported by wild animals from Valais into the Valle Maggia in Ticino where we found positive ticks. In conclusion, our data show for the first time the presence of TBEV in ticks and the related sero-reactivity in goats, confirming the presence of TBEV in the environment of the Canton of Ticino. Further surveillance studies will have to be conducted to follow the persistence of TBEV in this region.

1. Introduction

Ticks are considered the second most important vector for human diseases worldwide after the mosquitoes (Parola and Raoult, 2001). In Europe, the most widespread tick species is *Ixodes ricinus,* which may carry various pathogens with the potential to generate serious human and animal diseases. In Switzerland, the two most frequent tick-borne human diseases are Lyme borreliosis and tick-borne encephalitis (TBE). The first disease caused by the bacterial species complex *Borrelia burgdorferi* sensu lato is spread in Swiss forests up to an altitude of

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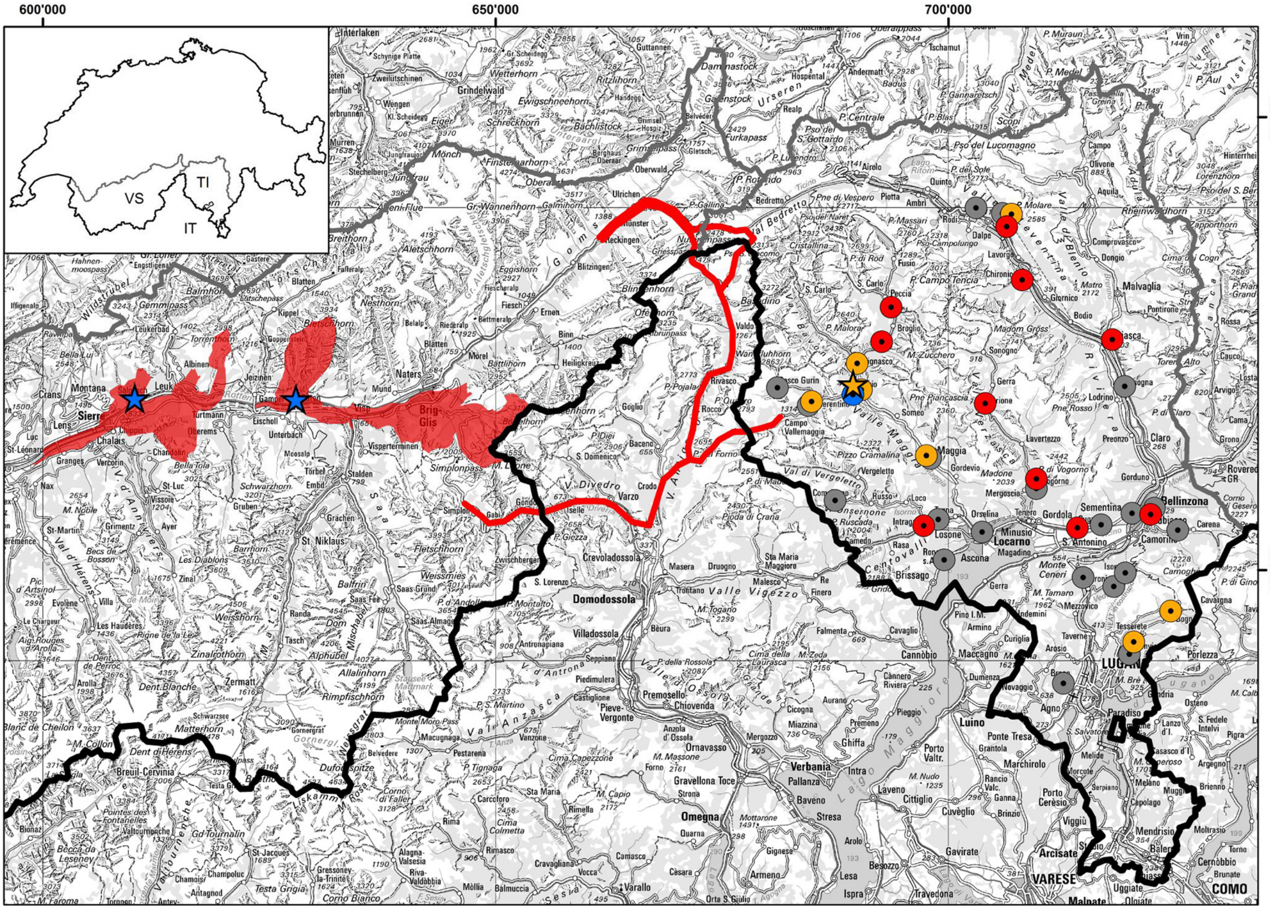

Fig. 1. Distribution of the positive tick pool (orange star), the seropositive flocks with an antibody titer ≥ 1:120 (active foci, red dot), the seropositive flocks (past infection, orange dot) and the seronegative flocks (grey dot) in Canton of Ticino. The TBEV strains were isolated in two endemic areas in Canton of Valais (blue star) and in a pool of ticks in Valle Maggia (orange star). The Canton of Ticino and the Canton of Valais are separated by high mountains (Alps) partly situated in Italy. The red line shows the game ways between the two Cantons supporting the transfer of the TBEV from Valais to Ticino.

1’500 m (Medlock et al., 2013) overlapping the distribution of *I. ricinus*. In contrast, the tick-borne encephalitis virus (TBEV) shows an irregular distribution over a large geographical range with a patchy occurrence in restricted foci of limited size (Dobler et al., 2011), where the TBEV circulates among tick and vertebrate populations (Charrel et al., 2004; Dumpis et al., 1999).

In Switzerland, natural foci are defined by registering the numbers of autochthonous human cases and/or detection of the virus in ticks and are reported on a map published on the website of the Swiss Federal Office of Public Health (www.bag.admin.ch). This map indicates the areas where vaccination against TBEV is recommended. A national study on the prevalence of TBEV in ticks reported a mean virus prevalence of 0.46% (Gäumann et al., 2010), which is in accordance with the prevalence of 0.1%–5% found in Europe (Dumpis et al., 1999; Oehme et al., 2002; Randolph, 2001). Another recent study showed the absence of TBEV in questing *I. ricinus* ticks collected in urban and suburban areas in Switzerland (Oechslin et al., 2017). The low prevalence, the spotted distribution and the irregular circulation of TBEV makes the detection of new foci very expensive and time-consuming (Klaus et al., 2010a). In addition, it must be considered that areas with TBEV-positive ticks do not always coincide with areas defined by the human TBE incidence as shown in a country-wide surveillance study in Switzerland in 2009 with a total of 62’343 *I. ricinus* ticks collected in 145 places (Gäumann et al., 2010).

Many aspects remain inexplicable being confronted with a complex eco-epidemiological system, characterized by an intricate interplay between virus, ticks and tick hosts, human exposure (Kunze and ISW, 2018), and environmental and ecological conditions. Small rodents considered as amplifier hosts play an important role in the enzootic transmission cycle of TBEV (Randolph et al., 1999). However, these hosts develop a short viraemic phase (2-3 days) resulting in a low viral transmission potential to ticks. An essential element for virus maintenance is the non-viraemic transmission between infected nymphs and non-infected larvae co-feeding on the same host without viraemia (Randolph, 2001). Small animals develop an immune response which consists in the development of persistent TBEV-specific antibodies (Tonteri et al., 2016). Therefore, an alternative and complementary way to detect TBE foci is the serological approach as demonstrated by Burri who tested rodents captured at four endemic sites in Switzerland (Burri et al., 2012). Other animals like goats and sheep show a regular persistence of antibodies after subclinical infection. These animals live in specific areas over prolonged periods where they eventually acquire TBEV through repetitive tick bites (Klaus et al., 2012). Therefore, the flocks are considered as sentinels for the presence of TBEV in a specific geographic area, thus helping to better describe the epidemiological situation (Klaus et al., 2010b).

In Switzerland, TBE is a notifiable human disease with an average of 210 cases per year in the last 10 years (2008–2017) with a peak of 257 cases in 2017 (UFSP, 2017). The southern part of Switzerland, precisely the Canton of Ticino, separated from the rest of the national territory by the Alps, is considered a non-endemic region, as no autochthonous clinical cases have ever been reported. The present study is the first report of the presence of TBEV in *I. ricinus* ticks in this region. As the Canton Ticino was omitted in the already mentioned first national tick surveillance study in 2009, we conducted a canton-wide study in 2014 to elucidate the prevalence of TBEV in *I. ricinus* ticks in this region. The

Table 1
TBEV-specific antibodies in goats collected in 2014, 2015 and 2016 in 6 districts of the Canton of Ticino.

Village (flock)	District	Collection period	Total number of sera	Total SNT-positive sera	SNT Seroprevalence (%)	Number of SNT samples with an AB titre ≥ 1:120*
Bellinzona	Bellinzona	2015	20	7	35	5
Bellinzona	Bellinzona	2016	4	0	0	0
Gudo	Bellinzona	2014	12	0	0	0
Isone	Bellinzona	2016	3	0	0	0
Medeglia	Bellinzona	2015	3	0	0	0
Monte Carasso	Bellinzona	2016	2	0	0	0
Rivera	Bellinzona	2015	17	0	0	0
S. Antonio	Bellinzona	2016	5	0	0	0
Campello	Leventina	2016	20	0	0	0
Chironico	Leventina	2016	14	5	36	3
Molare	Leventina	2016	22	2	9	0
Osco	Leventina	2015	20	0	0	0
Rossura	Leventina	2015	20	2	10	1
Berzona	Locarno	2016	10	0	0	0
Brione Verzasca	Locarno	2016	20	1	5	1
Cavigliano	Locarno	2016	21	0	0	0
Cugnasco	Locarno	2015	20	11	55	8
Intragna	Locarno	2015	20	6	30	3
Ronco s/Ascona	Locarno	2016	20	0	0	0
Solduno	Locarno	2016	4	0	0	0
Spruga	Locarno	2016	19	0	0	0
Vogorno	Locarno	2015	20	17	85	4
Cademario	Lugano	2016	7	1	14	0
Cadro	Lugano	2015	4	0	0	0
Maglio di Colla	Lugano	2016	8	1	12	0
Sonvico	Lugano	2015	10	1	10	0
Biasca	Riviera	2014	37	3	8	3
Osogna	Riviera	2016	5	0	0	0
Bosco Gurin	Valle Maggia	2014	34	0	0	0
Cavergno	Valle Maggia	2016	23	8	35	0
Cerentino (1)	Valle Maggia	2014	15	2	13	0
Cerentino (2)	Valle Maggia	2015	35	0	0	0
Cevio	Valle Maggia	2014	38	1	3	0
Maggia (1)	Valle Maggia	2014	39	2	5	0
Maggia (2)	Valle Maggia	2016	27	0	0	0
Menzonio	Valle Maggia	2014	40	20	50	8*
Prato Sornico	Valle Maggia	2016	24	7	29	3*
Total	**Valle Maggia**		**275**	**40**	**14.5**	**11**
Total	**5 districts**		**387**	**57**	**14.7**	**28**
Total	**all districts**		**662**	**97**	**14.6**	**39**

* SNT antibody titre ≥ 1:120 shows a very recent infection.

unexpected PCR-positive result for the presence of TBEV in ticks required a further investigation using a metagenomics and a serological approach. The TBEV was isolated and characterized and the sera of goats and humans within the TBEV-positive region were analysed in order to reflect the epidemiological situation in Ticino.

2. Materials and methods

2.1. Ticks collection and analysis

The ticks were collected by flagging the low vegetation between May 4 and 19, 2014 at sixty collection points within the Canton of Ticino. The points were chosen to obtain a representative geographic distribution over the whole territory. The minimal collection size per collection point was set to 400 ticks within four hours of collection. Collected ticks were frozen at – 80 °C until use. For each sampling site, ticks were identified and separated according to species, sex, and developmental stages. Five adult ticks or 10 nymphs were pooled for DNA/RNA extraction and processed by real-time RT-PCR according to the methods described by Gäumann et al. (2010).

2.2. Serum collection from goats and analysis

Goat sera were collected by the Cantonal Veterinary Service of the Canton Ticino in the context of the national survey on epizootic diseases. The flocks were randomly chosen. Of these sera, 662 from the years 2014–2016 were stored at -20 °C for retrospective analysis. All samples were analyzed using the ELISA Kit Immunozym FSME IgM and IgG (Progen GmbH, Heidelberg, DE) following the protocol described by Klaus et al. (2010b). Samples positive in the ELISA were confirmed using the serum neutralization test (SNT) (Klaus et al., 2010b).

2.3. Human serum collection and serological analysis

In total, 69 goat owners living in Valle Maggia were invited to participate in the TBEV seroprevalence study authorized by the Ethics Committees on research involving humans of the Canton Ticino (n° 2017 – 00430). The farmers received a letter including information on the study and were asked to complete a questionnaire and to sign a Patient Informed Consent form to participate. The questionnaire provided information on demographics, TBE vaccination status of the local population, previous trips to TBEV endemic areas in the last 5 years, possible exposures to ticks and noticed bites in the last 2 years and consumption of raw milk from goats. Sera were stored at -20 °C until use. All samples were analyzed by ELISA and the positive samples were confirmed by SNT (Ackermann-Gäumann et al., 2018).

2.4. Virus isolation and shotgun metagenomics analysis of TBEV

To determine the whole genome of the TBEV strain TI Cevio,

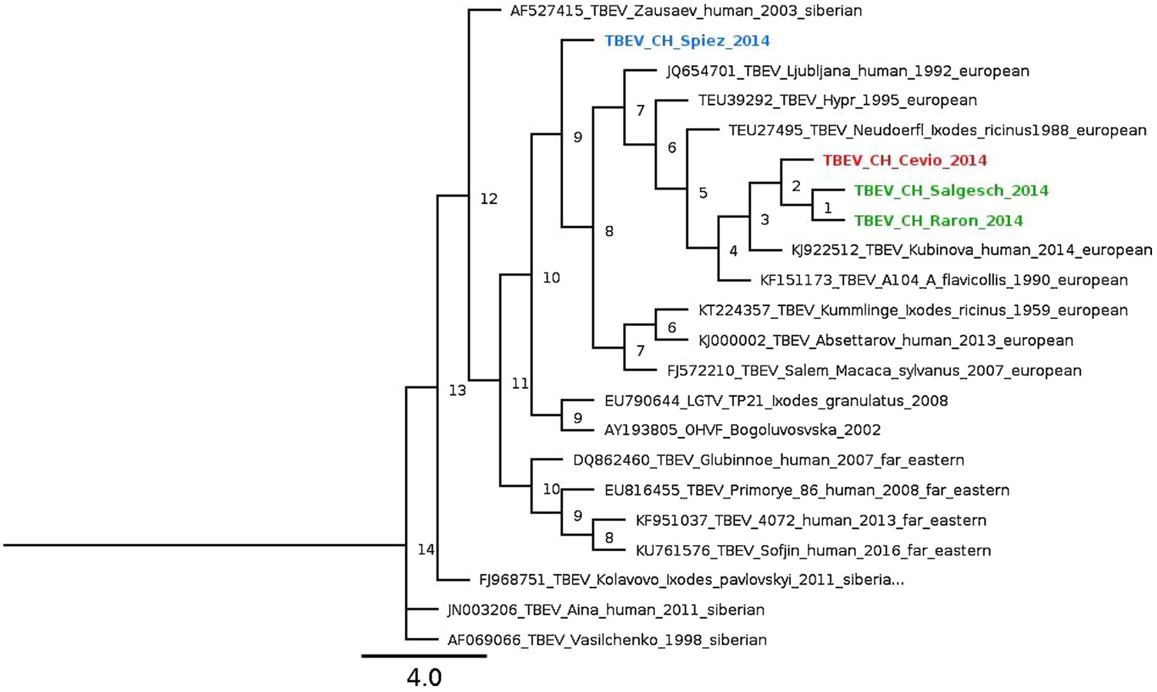

Fig. 2. The phylogenetic analysis shows that the TBEV strain (TBEV_CH_Cevio_2014) isolated from the ticks collected in Canton of Ticino is closely related to 2 strains from the Canton of Valais (99.1% to TBEV_CH_Raron_2014 and 98.7% to TBEV_CH_Salgesch_2014).

porcine kidney stable (PS) cells were inoculated with 100 µl of PCR-positive tick homogenate for virus propagation in a BSL-3 facility (Gäumann et al., 2010). Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) for subsequent NGS analysis according to the manufacturer's instructions. The RNA was reverse transcribed using the Super Script III First-Strand (Thermo Fisher Scientific, Carlsbad, CA, USA) and the cDNA was quantified with the Qubit dsDNA HS Assay Kit 100 on a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Whole genome amplification was performed using the PicoPlex WGA kit (Takara Bio Inc.Japan) and amplification products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany). The cDNA was fragmented in Micro tubes using a Covaris M220 focused ultrasonicator (60 s, 20 °C, Duty Factor 20, Cycles/Burst 200 and Peak Power 50) targeting peak fragments with a mean length of 400 bp. The fragmented cDNA was used for library preparation using the Ion Plus Fragment Library Kit on the AB library builder system (Thermo Fisher Scientific, Carlsbad, CA, USA). A ratio of 0.5x AMPure XP (Beckmann Coulter, United States) to sample was used to purify DNA fragment length of > 400 bd. DNA Library mean lengths and quality were checked using High Sensitivity DNA reagents and chips on a Bio Agilent 2100 Bioanalyzer (Agilent Technologies, United States). Emulsion PCR, enrichment and chip loading were carried out using the Ion 530 Chip kit and the Ion 510™ & 520™ & 530™ Kit-Chef on the Ion Chef system (Thermo Fisher Scientific, Carlsbad, CA, USA). The library was sequenced on the Ion S5 system (Thermo Fisher Scientific, Carlsbad, CA, USA).

2.5. Bioinformatics

Reads were quality checked using the Ion Torrent Suite software version 5.8.0 and trimming was performed with Trimmomatic V0.35 to a minimum DNA fragment length of 100 bp (Bolger et al., 2014). Reference alignment of trimmed reads to 19 TBEV genomes belonging to all three subtypes (European, Siberian and Far Eastern) was performed with Bowtie2 (Langmead et al., 2009). Taxonomic classification of trimmed reads was performed on read level using Kraken2 version 2.0.7 (Wood and Salzberg, 2014). Kraken2 classification reports were visualized with the web application PAVIAN (Breitwieser and Salzberg, 2016). Trimmed reads were also assembled using SPAdes version 3.12.0 (Bankevich et al., 2012) under the "careful" and "iontorrent" parameter and a k-mer range of 21, 33, 55, 77, 99 and 127. Assembled contigs were back-mapped with Bowtie2 to the most related TBEV strain to TBEV strain TI Cevio. Phylogenetic analysis was performed with RAxML V8.2.7 (Geneious R11, GTR + Γ substitution model with 1000 bootstrap replicates) (Stamatakis, 2014).

2.6. Statistical analysis

Statistical analysis was performed on the global set of data sampled from three collection periods: 2014, 2015 and 2016. The null hypothesis of no difference among the observed frequencies of categorical variables (Total SNT-positive sera; Number of SNT samples with an AB titre $\geq$ 1:120) between the groups (Ticino vs. Valle Maggia) was tested with the Pearson's Chi-square test, setting the significance level at $\alpha = 0.05$. The confidence intervals on reported frequencies were calculated at a level of 95%. Data analysis was performed using R language v.3.3.2.

3. Results

3.1. Ticks

A total of 12'052 *I. ricinus* ticks (nymphs and adults) were collected at only 43 out of 60 established points due to adverse weather conditions. In total, 1'371 pools were tested using quantitative real-time RT-PCR. Of these, one positive pool of nymphs was reported at the site near Cevio located in Valle Maggia resulting in a prevalence of 0.35% (Fig. 1).

3.2. Goat sera

The goat sera collection was composed of a total of 662 samples from 37 different flocks, 215 sera from 2014, 189 sera from 2015 and 258 from 2016. Of these sera, 139 (21%) were positive according to the

ELISA test and 97 (97/662, 14.6%) were confirmed by SNT. A total of 19 flocks (19/37, 51%) reacted TBEV seronegative (ELISA or SNT tests; Fig. 1). Of the 97 SNT-positive samples, 39 (39/97, 40%) showed an antibody titer ≥ 1:120 which indicates a very recent infection, thus the probable presence of active foci into the pastures frequented by the goats belonging to the 10 flocks (10/37, 27%; Table 1 and Fig. 1). The other SNT-positive samples coming from 8 different flocks (21%) showed lower antibody titers, which indicate a past infection (last 5 years) (Fig. 1). In Table 1, we reported the goat seroprevalence for the Valle Maggia region (14.5%), and for the other districts of the Canton Ticino (14.7%; Bellinzona, Leventina, Locarno, Lugano, Riviera). The total seroprevalence considering all regions including the Valle Maggia was 14.6%.

3.3. Human sera

In total, 51 of 69 (74%) goat owners participated in the study. None of them reported a vaccination against TBEV or a previous history of TBE, 4 individuals had a previous diagnosis of Lyme borreliosis. One human serum sample was positive in the ELISA test, but could not be confirmed by SNT. A history of yellow fever vaccination could account for the false positive result of this sample.

The target population chosen seems to be suitable for the study, since all participants (100%) had contact with ticks in 2016 and 77% also in 2017. In addition, 42% indicated to consume raw goat's milk, which represents an alternative – although rare – route of infection. All owners were TBEV antibody free.

3.4. TBEV shotgun sequencing analysis

3.4.1. Virus propagation

TBEV propagation on PS cells was successful; Cq-values of RT-qPCR analysis showed an increase of Cq 32.9 (tick homogenate) to a Cq 12.4 within five days.

3.4.2. Shotgun sequencing

Running a 530 Chip on an Ion S5 system yielded 13'813'274 raw reads with a mean read length of 215 bp. Applying a trimming of adapters and a filtering to a minimum fragment length of 100 bp resulted in 8'403'544 trimmed reads.

3.4.3. Reference alignment

Bowtie2 aligned 3.54% of all trimmed reads to 19 TBEV genomes, 6'735 reads were aligned one time and 252'346 reads > 1 time. The highest number of reads (68'180) were aligned to TBEV strain Kubinova (KJ922512). Alignment analysis of all trimmed reads to KJ922512 resulted in 314'682 aligned reads, 249'241 (2.97%) reads with a mean length of 211 bp remained after removal of duplicates. An average cover of 4'151 (lowest at 16 and highest at 28'278) was obtained for the genome of KJ922512.

3.4.4. De novo assembly and back-mapping

De novo assembly using SPAdes resulted in 17'545 contigs (k-mer 127), of which 469 could be back-mapped (Bowtie2) to the reference TBEV strain Kubinova (KJ922512).

3.4.5. Phylogenetic analysis

The TBEV strain isolated from ticks collected in the Cevio area is closely related to 2 strains isolated in the Canton of Valais (99.1% to TBEV_CH_Raron_2014 and 98.7% to TBEV_CH_Salgesch_2014, Fig. 2), a neighbouring Canton of the Canton Ticino (Fig. 1). These 3 strains (southern group; Fig. 2) show a close relationship with the reference TBEV strain Kubinova (KJ922512). However, TBEV strains from the north of Switzerland (represented by TBEV strain Spiez; Fig. 2) cluster to TBEV strain Absettarov (AF091005).

3.5. Statistical analysis

No statistical difference was observed between the two groups (Ticino vs Valle Maggia, data not shown). The measured frequencies (SNT seroprevalence and the presence of active foci) were similar and indicate that the risk is not greater in one area than the other.

4. Discussion

The sampling of ticks was very successful with a total of 12’004 of the species *I. ricinus*. This high number of ticks collected is essential to obtain a current and complete epidemiological picture of TBEV in the Canton of Ticino. Molecular analysis showed for the first time the presence of TBEV in one tick pool collected near the village of Cevio in Valle Maggia. The result is surprising, since no autochthonous human TBEV infection had ever been reported in Ticino, designated so far as a TBEV-free region. This TBEV focus shows a prevalence of 0.35%, which is below the national mean prevalence of 0.46% (Gäumann et al., 2010).

To better understand the virus distribution in Valle Maggia, we screened goat sera for the presence of TBEV specific antibodies and obtained a seroprevalence of 14.5% (Table 1). The presence of high antibody titres (> 1:120) in goat sera in this area might indicate the presence of two active and recent foci (Menzonio and Prato Sornico, Table 1). In a large serosurveillance in Germany, the authors concluded that positive sera indicate unexpected TBEV foci in regions currently defined as TBEV non-risk areas (Klaus et al., 2012). In a next step, the possible contact of humans with virus-infected ticks was investigated taking the goat owners living in Valle Maggia as target group. This group was chosen based on frequent contact to livestock animals, ticks and vegetation (pastures). In this case, the human seroprevalence was 0% pointing out once again the complex interaction between vector-virus-host.

A recent study performed in a non-endemic area in southeast Norway with a TBEV prevalence of 0.14% in ticks showed a very low positive seroprevalence of 0.65% in blood donors (Larsen et al., 2014). In contrast, the seroprevalence in a highly endemic region in eastern Poland was 19.8% in forestry workers and 32% in farmers (Cisak et al., 1998). We hypothesise that the presence of TBEV in Ticino, emphasized by a seroprevalence of 14.6% in goats - could be too recent to have caused human disease. Our data confirm the local circulation of TBEV in the Canton of Ticino with the presence of active foci (10), not only in Valle Maggia, as evidenced by the high antibody titres. No statistical difference was observed between the two groups where positive ticks were found, the first within the Valle Maggia and the second within other districts of the Canton Ticino.

Goats were also used as sentinel animals in a study performed in the Canton of Valais (located in the southwest of Switzerland) to identify new risk areas for TBE with a SNT seroprevalence of 1.7% (Rieille et al., 2017). It is interesting to observe that this region has a similar epidemiological history as the Ticino: a national tick survey in 2009 identified new TBE foci in Valais, known to be non-endemic (Gäumann et al., 2011). In the following years, human TBE cases were reported and tick surveys confirmed the presence of TBEV (Rieille et al., 2014). As both Cantons are close to each other, although separated by the Swiss alps, we suppose that infected ticks were transported from Valais into the Valle Maggia in Ticino by wild animals. In fact, the hunting guard of this area confirmed that wild animals cross between the two cantons (Fig. 1). In addition, phylogenetic analysis showed that the TBE strain isolated in Ticino is closely related to those present in Valais (respectively 98.7 and 99.1%). Considering that all the SNT-positive goats - except two - were born in Ticino and have never left the region (personal communication), this result led to the assumption that the origins of the TBEV virus Ticino could be in the Canton of Valais.

A limitation of our study is the random assortment of the goat sera, which is due to the retrospective analysis of the sera available in our veterinary department. Indeed all flocks were analysed only once over the 3 years and smaller and bigger flocks (2–20 goats) were compared. Moreover, antibody detection applied does not provide any information on the time and place of infection. However, our data show the presence of active TBEV foci (high antibody titres) through serum samples collected in 2014, 2015 and 2016, emphasizing the long lasting presence of active foci. Making abstraction of the temporal concept, we still have the confirmation of TBEV presence in Ticino. In addition, TBEV specific antibodies in goats and in sheep are still detectable after 28 months; this longevity supports the validity to characterize an area by the TBEV status (Klaus et al., 2014).

An important point that emerged was the consumption of raw goat milk by the owners (41.6%), representing an alternative route of TBEV transmission. In fact, the virus is secreted in low concentrations in goat milk during the viraemic phase (Balogh et al., 2010); TBEV could be detected for 8–19 days in milk samples in Hungary (Balogh et al., 2012). Although rare, a few milk-borne cases are described in the literature in endemic areas, for example in Slovenia (Hudopisk et al., 2013), in Croazia (Markovinovic et al., 2016), in Hungary (Balogh et al., 2010), in Germany (Brockmann et al., 2018) and in Slovakia (Kerlik et al., 2018). In the Canton of Ticino, this transmission route is not known so far, but our data on the local goat seroprevalence impose an accurate and specific information for farmers to underline the importance of pasteurization processes.

In conclusion, our data show for the first time the presence of TBEV at two levels in the Canton of Ticino, once in ticks and second by the sero-reactivity in goats confirming its diffusion in the environment. Currently, no human TBE cases have been reported and the Canton of Ticino is still defined as non-endemic area. Further epidemiological surveillance studies have to be planned to follow more closely the spread of TBEV in the environment.

Conflict of interest

All of the authors declare no conflicts of interest related to this article.

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