

PREVALENCE OF TICK-BORNE ENCEPHALITIS VIRUS (TBEV) IN SAMPLES OF RAW MILK TAKEN RANDOMLY FROM COWS, GOATS AND SHEEP IN EASTERN POLAND

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Cisak E, Wójcik-Fatla A, Zając V, Sroka J, Buczek A, Dutkiewicz J: Prevalence of tick-borne encephalitis virus (TBEV) in samples of raw milk taken randomly from cows, goats and sheep in eastern Poland. *Ann Agric Environ Med* 2010, **17**, 283–286.

Abstract: A total of 119 unpasteurized milk samples taken from 63 cows, 29 goats and 27 sheep bred on 8 farms situated on the territory of the Lublin province (eastern Poland), an area of risk of tick-borne encephalitis (TBE), were examined for the presence of RNA of tick-borne encephalitis virus (TBEV) by the nested RT-PCR method. Milk samples were also tested for the presence of anti-TBEV antibodies by ELISA test. By RT-PCR, the greatest prevalence of TBE virus was found in the milk of sheep (22.2%), followed by milk of goats (20.7%) and cows (11.1%). By ELISA, the greatest prevalence of anti-TBEV antibodies was found also in the milk of sheep (14.8%), followed by milk of cows (3.2%) and goats (0%). The results suggest a potential risk of infection with TBEV by drinking raw milk on endemic areas of TBE, and indicate a need for milk pasteurization before consumption.

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Key words: tick-borne encephalitis virus, raw milk, RT-PCR, antibodies, epidemiology.

INTRODUCTION

Tick-borne encephalitis (TBE) is a zoonotic infection endemic to Eastern and Central Europe and Russia, caused by a tick-borne encephalitis virus (TBEV). In European countries, TBE is regarded as one of the most important and potentially fatal human infections of the central nervous system, causing annually thousands of cases. TBE presents as meningitis in about 50% of patients, as meningoencephalitis in 40%, and as meningoencephalomyelitis in 10% [5, 7, 9, 15, 20, 23, 24, 25].

TBEV is mainly transmitted to humans by tick bites, but alimentary infections due to consumption of raw (unpasteurized) milk occur in certain regions [7, 9, 19, 23]. According to published reports, these infections are primarily due to con-

sumption of goat milk and/or cheese [1, 2, 7, 8, 10, 11, 12, 13, 16, 17, 22], less commonly to consumption of sheep and cow milk and/or cheese [6, 12, 13, 14, 22, 26, 27]. According to Kriz *et al.* [12], out of a total of 7,288 TBE cases reported in the years 1997–2008 in the Czech Republic, 64 cases (0.9%) were food-borne, due to consumption of raw milk or cheese. In Slovakia, Labuda *et al.* [13] reported during 5 years 33 cases of TBE food-borne infections after drinking raw milk, which accounted for circa 9% of the total cases.

The risk of infection with TBEV by milk consumption was assessed hitherto by serological examinations of animals suspected to be a source of milk-borne infections in humans [2, 21, 26, 27]. To the best of our knowledge, no epidemiological examinations of milk samples taken from milk-giving animals in endemic areas have been carried



out. To fill this gap and to assess a real risk of human infection by drinking raw milk, we examined the randomly selected samples of unpasteurized milk for the presence of TBEV, using the RT-PCR method. The samples were taken from cows, sheep and goats living on TBE risk area in Lublin province (eastern Poland).

MATERIALS AND METHODS

Milk samples. A total of 119 milk samples were taken from 63 cows, 29 goats and 27 sheep bred on 8 farms situated on the territory of the Lublin province (eastern Poland). The samples were collected directly after milking into Falcon test tubes and processed according to Blackwell *et al.* [4]. Milk fractions were prepared by centrifugation of whole milk at $1,000 \times g$ for 10 min. A syringe needle was placed beneath the cream layer in the tube, and the skim milk then was drawn up by a syringe and transferred to another tube. Samples of skim milk were stored at -80°C before PCR and ELISA assays.

RNA isolation. Total RNA was extracted from the 200 μl volume of defatted milk, using Qiamp Viral Mini Kit (Qiagen, USA) according to producer instruction. The amounts of extracted RNA measured with NanoDrop ND1000 spectrophotometer (USA) were in the range of 19.8–47.8 $\text{ng}/\mu\text{l}$ in each probe. The viral RNA was stored at -80°C until further analysis.

Reverse transcription-polymerase chain reaction (RT-PCR). The RT-PCR reaction was performed using QuantiTect Reverse Transcription Kit (Qiagen, USA) according to producer instruction. The kit procedure comprises two main steps: elimination of genomic DNA and reverse transcription. The RT-PCR reaction was carried out in a 20 μl reaction volume which contained the following mix of reagents: gDNA Wipeout Buffer 7 \times , RNase-free water, Quantiscript Reverse Transcriptase, RT primer mix, Quantiscript RT Buffer 5 \times , and template RNA in a volume of 2 μl (from 40–98 ng). The reaction was performed in MJ Research Thermal Cycler (PTC-150, MJ Research Inc., Waltham, USA), under the following conditions: 2 min at 42°C for gDNA elimination, 30 min at 42°C and 3 min at 95°C for reverse transcription.

First PCR reaction. The first PCR reaction was performed in the volume of 25 μl which contained the following mix of reagents: 1 \times PCR buffer with 1.5 mM MgCl_2 , 0.625 U of Ampli Taq polymerase (all from Qiagen, USA), 2 mM dNTP in a final concentration of 0.1 μM (Fermentas, ABO, Poland), primers Pp1: 5'-GCG TTT GCT TCG GAC AGC ATT AGC and Pm1: 5'-GCG TCT TCG TTG CGG TCT CTT TCG in a final concentration of 7.5 pmol each (Eurogentec, Seraing, Belgium), nuclease-free water, and 1 μl of cDNA. The reaction was performed in MJ Research Thermal Cycler (PTC-150, an MJ Research Inc., Waltham, USA), under the following conditions: 2 min at

94°C for denaturation as the initial step, followed by 40 cycles of PCR, each of 30 sec at 94°C , 30 sec at 55°C and 1 min at 72°C . The final extension was performed in 5 min at 72°C . Positive control was obtained from the low pathogenic TBEV strain Langat (kindly provided by Dr. J. Süß, Friedrich Löffler Institute, Jena, Germany). RNase-free water was used as a negative control.

Precautions to exclude contamination were as follows: One-use tips with filters (PCR-clean/dualfilter/sterile) and one-use Eppendorf test tubes (PCR-clean, free of detectable human DNA, DNase, RNase, PCR inhibitor) were used. The glass and plastic surfaces were treated with RNase Zap (Ambion, the RNA company, USA) which completely removes contamination with RNase.

Nested PCR. The second amplification was carried out with 2 μl of the first amplification product diluted in nuclease-free water. The 50 μl of total reaction volume contained the following components: 1 \times PCR buffer with 1.5 mM MgCl_2 , 1.25 U of Ampli Taq polymerase (all from Qiagen, USA), 2 mM dNTP in final concentration of 0.1 μM (Fermentas, ABO, Poland), primers Pp2: 5'-TCG GAC AGC ATT AGC AGC GGT TGG and Pm2: 5'-TGC GGT CTC TTT CGA CAC TCG TCG in final concentration of 15 pmol each (Eurogentec, Seraing, Belgium), and nuclease-free water. The reaction was performed in MJ Research Thermal Cycler (PTC-150, MJ Research Inc., Waltham, USA), under the same conditions as the first amplification, followed by 30 cycles.

The PCR products were detected in UV light as ethidium bromide-stained 178-bp bands electrophoresed with a marker on 3% agarose gel.

DNA sequencing. DNA sequencing was performed with 30% of randomly selected positive samples with the ABI PRISM 310 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) using ABI Prism Big Dye Terminator v. 3.1 Cycle Sequencing Kits and Big Dye X Terminator Purification Kit (Applied Biosystems). The results were compared with published sequences in the GenBank database using the BLAST server at the National Center for Biotechnology Information (Bethesda, Maryland, USA).

ELISA test. The commercial test Immunozytm FSME IgG All Species (Progen Biotechnik GMBH, Heidelberg, Germany) was used for detection of the specific anti-TBEV antibodies in milk samples. Based on the paper by Bex *et al.* [3], the milk samples were diluted before testing with 0.1 M Tris/HCl buffer (pH=7.4) in the ratio 1:1. Results were read spectrophotometrically at OD=450 nm.

RESULTS

The results of the RT-PCR examination of milk samples for the presence of TBEV are presented in Table 1. The greatest prevalence of TBE virus was found in the milk of

Table 1. Prevalence of TBEV in milk samples from various animal species, determined by RT-PCR.

Animal species	Number of examined animals	Total positive	%
Cows	63	7	11.1
Goats	29	6	20.7
Sheep	27	6	22.2
Total	119	19	16.0

sheep (22.2%), followed by milk of goats (20.7%) and cows (11.1%). The sequence analysis of positive samples obtained from milk confirmed that the amplified products were identical with the 96–100% homology with TBEV sequences; accession numbers are as follows: GQ266392 (TBEV strain AS33), FJ572210 (TBEV strain Salem), U39292 (TBEV strain Hypr), U27495 (TBEV strain Neudoerfl).

By ELISA, the greatest prevalence of anti-TBEV antibodies was found also in the milk of sheep (14.8%), followed by milk of cows (3.2%) and goats (0%) (Tab. 2). The results obtained by ELISA were not correlated with those obtained by RT-PCR ($\chi^2=0.25$, $p>0.05$).

DISCUSSION

The RT-PCR examination for the presence of TBEV in randomly selected raw milk samples collected on the risk-area of TBE in eastern Poland revealed a considerable prevalence of infection, mostly in sheep and goat milk. These results are noteworthy for public health, the more so as the hitherto published papers report the occurrence of TBEV in raw milk samples associated with clinical cases in humans, while the epidemiological examinations of randomly selected milk samples, with no links to human clinical cases, are lacking.

The occurrence of TBEV in goat milk and/or cheese, associated with clinical cases in humans, has been reported by numerous authors [1, 2, 7, 8, 10, 11, 12, 13, 16, 17, 22]. A lesser numbers of authors have reported the occurrence of TBEV in milk and/or cheese from sheep [6, 12, 13, 27] and cows [12, 14, 22, 26].

Because of common consumption of cow milk, demonstrating of the presence of TBEV in over 10 percent of the raw cow milk samples is of particular epidemiological significance. In this respect, our results are in accord with those reported by Leonov *et al.* [14] and Vereta *et al.* [26] who described the significance of cow milk as the source of TBEV in Siberia. Leonov *et al.* [14] isolated TBEV from the milk of 1 cow out of 27 examined.

The results of the examination of milk samples with ELISA test for the presence of specific anti-TBEV antibodies showed no correlation with those obtained by RT-PCR. This may suggest that the ELISA test is less appropriate for detecting the presence of TBEV in milk, compared to RT-PCR.

Table 2. Prevalence of anti-TBEV antibodies in milk samples from various animal species, determined by ELISA test.

Animal species	Number of examined animals	Total positive	%
Cows	63	2	3.2
Goats	29	0	0
Sheep	27	4	14.8
Total	119	6	5.0

In conclusion, our results indicate that the consumption of raw milk from sheep, goats and cows may be associated with risk of infection with TBEV. Thus, pasteurization of milk before consumption is strongly recommended [18], in particular in the localities where grazing goats, sheep and cows are exposed to tick bite.

Acknowledgements

This study was supported by the Polish Ministry of Science and Higher Education (Grant: N N404 029435). The authors are grateful to Dr J. Süß from the Friedrich Löffler Institute in Jena, Germany, for kind providing of the TBEV positive control, and to Dr hab. Anna Szymanowska from the University of Life Sciences in Lublin, Poland, for kind providing the milk samples from sheep.

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