Experimental Infection of Goats with Tick-Borne Encephalitis Virus and the Possibilities to Prevent Virus Transmission by Raw Goat Milk

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Abstract

Objectives: The aim of this work was to study the tick-borne encephalitis virus (TBEV) infection of goats and the possibilities to prevent human milk-borne infections either by immunizing animals or the heat treatment of milk. Methods: An experiment was conducted with 20 milking goats. Ten goats (half of them immunized) were challenged with live TBEV and 10 were left uninfected. Clinical signs and body temperatures of the animals were recorded and milk samples were collected daily. The presence of viral RNA and infectious virions in milk were detected by RT-PCR and intracerebral inoculation of suckling mice, respectively. Milk samples containing infectious virions were subjected to various heat treatment conditions and retested afterwards to assess the effect on infectivity. Results: The infected goats did not show any clinical signs or fever compared to uninfected ones. Infectious virions were detected for 8–19 days from the milk samples (genome for 3–18 days by PCR) of infected goats. Immunized goats did not shed the virus. After heat treatment of the milk, the inoculated mice survived. Conclusions: Goats shed the virus with their milk without showing any symptoms. Human milk-borne infections can be avoided both by immunizing goats and boiling/pasteurizing infected milk.

Key Words
Tick-borne encephalitis virus · Goat · Milk · Immunization · Alimentary infection

Introduction

Milk is often consumed raw among farm families because of taste and convenience [1]. There is also a small portion of the general population which also drinks raw milk because it is believed to be able to prevent and treat certain diseases. Raw-milk advocates suggest that boiled milk has a lower biological value; however, most of the beneficial nutrients in milk are heat stable or largely unaffected by pasteurization [2]. On the other hand, raw milk and cheeses and other dairy products made of milk can be contaminated with pathogens including Mycobacterium bovis, Salmonella, Campylobacter, Brucella, Listeria, Shigella, Shiga toxin-producing E. coli, Staphylococ-
coccus aureus, Streptococcus pyogenes, Yersinia enterocolitica, Coxiella burnetii and the tick-borne encephalitis virus (TBEV).

TBEV is a well-known zoonotic pathogen maintained in nature by a relationship of ticks, certain small rodent species and larger wild animals [3, 4]. Occasionally, humans are infected by ticks and the infection results mostly in benign flu-like symptoms, fever, headache and occasionally encephalitis. Since lasting paralysis only occurs in 6% of the cases and the case fatality rate is approximately 1–2% for the Western subtype of TBEV (0.2% in Hungary), the large majority of infections remain unobserved [5, 6]. In the past years, mostly sporadic human tick-borne encephalitis (TBE) cases were identified in Hungary; however, in 2007, 30 persons were hospitalized during 2 epidemics because of consuming raw goat milk that was infected with TBEV [7]. These outbreaks showed the importance of the alimentary route of TBEV infection.

The first reported milk-borne TBE epidemic occurred in Roznava, Slovakia, in 1951, where more than 600 people were infected, 271 of whom were hospitalized, after consuming contaminated milk, which was not pasteurized, from the local dairy [8]. Similar cases were observed in the European areas of the Soviet Union, where the disease was called 'biphasic milk fever'. A few years later it was confirmed by mouse experiments that TBEV can produce an infection when transmitted by consumption of food [9].

Belonging to the Flavivirus genus of the family Flaviviridae, TBEV is an enveloped RNA virus, which means that it is relatively sensitive to heat and detergents. However, it can still retain its infectivity in normal gastric juice (pH 1.49–1.80), as well as in gastric juice with reduced activity (pH 2.46–3.46) for up to 2 h. This stability in an acidic medium is similar to that of poliomyelitis and Coxsackie viruses [10]. Hydrochloric acid is secreted in the stomach between 45 and 60 min after consumption of the milk. Milk foods move out of the stomach quite quickly: the first milk consumed reaches the duodenum within minutes, and after 1.5–2 h there is no milk in the stomach [3]. Thus, TBEV can also reach the duodenum without losing its infectivity, where it probably binds to M cells of Peyer’s patches. M cells can transcytose pathogens into the intestinal lymphoid tissue, from where the bloodstream is accessible through the regional lymph nodes and the thoracic duct, and the blood can take pathogens to the place of secondary replication. Such a mechanism was described for enteric viruses that are known to use the alimentary tract as the portal of entry into a host, often causing asymptomatic or subclinical infection there, but afterwards becoming disseminated beyond the gastrointestinal tract and producing significant disease in distant tissues and organs, e.g. poliovirus infection of the central nervous system [11].

Grazing ruminants frequently come into contact with ticks [12], and the goat species is particularly vulnerable as they are grazed on meadows and their main forage is grass and bushes. Ticks may infect these milking animals with various pathogens, including TBEV. During the viremic phase of the infection, TBEV is secreted with the milk of goats, and it is hypothesized that immunosuppression of the animals due to Anaplasma phagocytophilum infection (a pathogen also spread by ticks) could help TBEV to get into the milk [13]. When a goat was experimentally infected, TBE virus was detectable from its milk by inoculation of embryonated eggs and adult mice in the period of 3–8 days postinfection, while the animal showed no clinical signs or fever [14]. In a later study, TBEV was isolated repeatedly from the milk of infected goats for 5–25 days after infection and infectivity was maintained in various milk products such as yoghurt, cheese and butter [3]. It is reported that TBEV has been demonstrated to be virulent for up to 8 days after infection in the milk of cows, goats and sheep [15]. However, pasteurization was confirmed to prevent milk-borne TBEV infection [16].

The cases of human TBE caused by raw goat milk and the lack of recent experimental data suggested the design of an experiment on milking goats to study – using classical and modern virological methods – the possible clinical signs of TBE in goats, the virus spread in milk and the options to prevent human TBEV infection.

Materials and Methods

Experimental Design

Twenty milking goats with 3-week-old suckling kids were divided into 4 groups with 5 mothers in each. The 4 groups were kept in isolated rooms, bedded on straw and fed with 3–4 kg of grass hay and 0.2 kg of maize per goat per day.

Two groups were immunized with inactivated human TBEV vaccine (FSME-IMMUN CC; Baxter Vaccine AG, Vienna, Austria), administered subcutaneously in the neck of the animals 2 times with 16-day intervals. The vaccine doses were the same for all goats since all the animals had a similar body weight. Seroconversion of the immunized animals was checked from serum samples by indirect immunofluorescence assay and hemagglutination inhibition assay.

Five immunized and 5 naïve goats were challenged with live TBEV strain Kem I [17, 18] by subcutaneous administration of 500 µl ca. 10³ TCID₅₀ brain suspension of TBEV-inoculated suckling mice. Infected goats were milked daily for 2 weeks, while

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clinical signs and body temperatures were recorded daily for both infected and control groups (fig. 1).

The experimental procedure was authorized by the Ethical Committee for Animal Care of the National Center for Epidemiology and was in accordance with national and international rules regarding animal experiments.

Animal Samples
Blood samples were collected from immunized goats to determine their immunological state. Sera were stored at +4° until immunofluorescent-antibody assay, and at –20° afterwards. Milk samples were collected daily from 10 TBEV-infected goats, 5 of which had been immunized previously against TBEV. All milk samples were stored at –20° until examination.

Serological Examination of Goat Sera to Determine Immune State
The quality of these assays was assessed by an external quality assurance program of the European Network for the Diagnostics of ‘Imported’ Viral Diseases [19]. The laboratory performed well both in anti-TBEV IgM and anti-TBEV IgG detection from human samples.

Indirect Immunofluorescence Assay
TBEV strain Kem I was used as antigen, propagated on a Vero cell line for 5 days. The harvested cells were washed in PBS, dried to immunofluorescence slides and fixed by –20° acetone. Serum dilutions (1:5–1:640) were dropped onto the dried cells and incubated for 1 h at 37°. After washing with PBS, the cells were incubated with 1:20 dilution of FITC-conjugated anti-goat IgG antibody (anti-goat IgG-FITC conjugate, Calbiochem, San Diego, Calif.), and the slides were covered with a cover slip using a drop of PBS/glycerol 1:1. Fluorescence of positive cells was examined under a fluorescent light microscope (Axiostar Plus, Carl Zeiss, Oberkochen, Germany).

Hemagglutination Inhibition Assay
The hemagglutination inhibition test was carried out using a modified Takatsy micromethod. Aspecific inhibitors and hemagglutinins were removed from the sera with kaolin and goose erythrocytes, respectively. The total hemagglutinating antibody content of sera was measured using goose erythrocytes and TBE virus prepared in-house from the first Hungarian isolate Kem I as published [20]. An inhibition observed in 1:10 or higher final dilution was evaluated as reactive.

Detection of Infectious Particles from Milk by Mouse Inoculation
Twenty microliters of the goat milk samples was inoculated into the frontal brain lobe of 1- to 2-day-old NMRI suckling mice, each sample to a litter containing 10–12 animals. The mice were monitored daily for 10 days and clinical signs (paralysis of legs, weakness, tremors) were recorded, the occurrence of which was considered as proof for the presence of infectious virus in the sample.

Detection of Viral RNA from Milk by Nested RT-PCR
After collection, the milk samples were stored at –20°. Before nucleic acid preparation, the samples were thawed and vortexed. Nucleic acids were extracted from 140 μl of milk samples with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer’s instructions. Viral RNA was eluted in 60 μl of elution buffer.

Reverse transcription (RT) was done in 20 μl volume by a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, Calif., USA) with 2 μl RNA, and the rest was stored at –70°.

For nested PCR assay, the primers targeting the NS5 region of the virus genome [21, 22] were applied, with a slightly modified PCR protocol. The first round of PCR was prepared with 2 μl of the RT mixture in a solution of GeneAmp 10X PCR Buffer containing 15 mM MgCl₂, 4 μl of 25 mM MgCl₂, 10 mM of each dNTP, 50 pM of each of the first-round primers and AmpliTaq DNA Polymerase (Roche Molecular Systems Inc., Branchburg, N.J., USA) in a final volume of 42 μl. Thirty-five amplification cycles were performed (30 s at 95°, 30 s at 55°, 30 s at 72°) in an Eppendorf Mastercycler. Then 2 μl of this amplification mixture was subjected to 30 cycles of a nested PCR with 20 pM of the internal primers. After the PCR was completed, 10 μl of the amplified product was analyzed in 2% agarose gel and visualized under UV light.

To determine the relative virus load of the chosen samples, 5 μl of the eluted RNA was measured in a one-step real-time RT-PCR reaction using SuperScript™ III Platinum™ One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, Calif, USA) according to a modified version of the protocol [23] in a LightCycler 2.0 instrument (Roche Diagnostics, Mannheim, Germany). The results from the samples were compared to the titers of the in-house virus dilution series measured by microneutralization reaction. Fluorescence results were analyzed in LightCycler Software 4.0.

Heat Treatment of Pooled Milk Samples Containing Infectious Virus Particles
To examine the effect of heat treatment (heating or boiling) on the infected milk, 2 goats were selected: one with a markedly high virus content in its milk according to real-time PCR results and another with lower virus content (No. 76967 and No. 77702). Aliquots of pooled positive milk samples of both infected goats were subjected to heat treatment as follows: at room temperature for 3, 6, 12, 24 and 48 h; at 65° for 5 min, 15 min and 30 min; and at 100° for 3 min. Heat treatment at 65° was performed using a Heto water bath (model IBN 18/HWT 100, Heto-Holten A/S, Allerod, Denmark); for 100° heat treatment, tubes were immersed in boiling water. Treated milk was inoculated to suckling mice and animals were monitored twice a day for 10 days.

Statistical Analysis of Body Temperature Data
Temperature data were analyzed using the Minitab® 15.1.1.0. software (Minitab Inc., State College, Penn., USA).

Results

Body Temperature of Goats after Infection
Ten of 20 milking goats – 5 immunized and 5 naïve – were subcutaneously infected with 500 μl suspension of live Kem I strain TBEV (ca. 10⁴ TCID₅₀). After infection,
the rectal body temperature of the goats was taken every day for 10 days to identify a possible febrile state. To be used as control data, the body temperature of noninfected naïve and immunized goats was also recorded for the appropriate time periods. A two-way ANOVA analysis (p < 0.05) showed that there is no significant difference between the mean temperatures of the groups: 38.75 °C for the infected, 38.89 °C for the immunized-infected, 38.99 °C for the immunized control and 38.94 °C for the naïve control. The mean temperatures of the individual days were also compared, but no significant difference was found (p < 0.05).

Serological Results of Immunized Goats
On the 24th and 34th day following TBEV immunization, whole blood samples of 3 of 5 goats were taken to assess the animals’ immune state. Serum samples were tested by indirect immunofluorescence assay, and if positive were confirmed by hemagglutination inhibition assay for the presence of anti-TBEV IgG antibodies. Twenty-four days after immunization, none of the 3 goats was positive for anti-TBEV antibodies. Ten days later, another set of blood samples were taken from the 3 goats and examined. This time all the tested sera showed immune reaction with specific IgG detectable by both indirect immunofluorescence and hemagglutination inhibition. IIF antibody titers were 1:40 for all 3 goats and the confirmatory HA1 titers were 1:20, 1:20 and 1:160. Thus, this date was chosen for the virus challenge of the immunized animals.

Detection of TBEV from Milk of Infected Goats by Mouse Inoculation and RT-PCR
Milk samples of 5 naïve goats were tested daily between the 2nd and 9th day postinfection for the presence of TBEV by mouse inoculation and RT-PCR. Two additional samples from each goat from the 13th and 23rd day postinfection were also tested. TBEV was detectable from the milk of all 5 goats. Table 1 shows the results on each day following infection starting from the 2nd day by

Table 1. Body temperatures and milk sample results of 5 TBEV-infected goats examined by suckling mouse inoculation and specific RT-PCR and Ct values of samples chosen for real-time RT-PCR

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Goat No. 76110</th>
<th>Goat No. 76967</th>
<th>Goat No. 77447</th>
<th>Goat No. 77450</th>
<th>Goat No. 77702</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (°)</td>
<td>VI</td>
<td>PCR</td>
<td>Ct</td>
<td>VI</td>
<td>PCR</td>
</tr>
<tr>
<td>2</td>
<td>37.9</td>
<td>N</td>
<td>n.d.</td>
<td>38.4</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>38.6</td>
<td>N</td>
<td>n.d.</td>
<td>38.2</td>
<td>P</td>
</tr>
<tr>
<td>4</td>
<td>38.8</td>
<td>N</td>
<td>n.d.</td>
<td>38.1</td>
<td>P</td>
</tr>
<tr>
<td>5</td>
<td>38.9</td>
<td>N</td>
<td>n.d.</td>
<td>38.8</td>
<td>P</td>
</tr>
<tr>
<td>6</td>
<td>39.2</td>
<td>P</td>
<td>N</td>
<td>39.6</td>
<td>P</td>
</tr>
<tr>
<td>7</td>
<td>38.7</td>
<td>P</td>
<td>N</td>
<td>40.1</td>
<td>P</td>
</tr>
<tr>
<td>8</td>
<td>38.6</td>
<td>N</td>
<td>n.d.</td>
<td>39.6</td>
<td>P</td>
</tr>
<tr>
<td>13</td>
<td>38.8</td>
<td>P</td>
<td>N</td>
<td>38.8</td>
<td>P</td>
</tr>
</tbody>
</table>

Positive results are highlighted with grey color. The standard used in the real-time RT-PCR reaction was a 1:1,000 dilution of the in-house prepared virus suspension (Ct = 22.01). VI = Virus isolation; N = negative; P = positive results; n.d. = not done and no data.
mouse inoculation tests and RT-PCR. On the 6th day after infection, milk samples of all goats were positive by mouse inoculation, and TBEV could be detected as early as the 2nd day postinfection for 1 animal. On the 13th day, virus excretion was still detected from the milk samples of all goats by mouse inoculation, but not from all by RT-PCR. This may be the result of the difference between the virus loads of the milk samples of different animals. On the 23rd day, milk samples were available from 4 of 5 goats, of which 1 was positive for TBEV by both methods.

**Virus Concentration of Selected Milk Samples**

Based on virus detection results, samples from the 6th and 7th day were chosen for measuring viral load by real-time RT-PCR. The titers were related to standards that included 4 dilutions of TBEV suspension previously titrated by microneutralization. Table 1 shows the Ct values of the samples. The concentrations showed great variance for the 6th day, the crossing points differing in 22 cycles between the samples detected the earliest (Ct = 13.71) and the latest (Ct = 35.98). On the 7th day, concentrations were less varying, with the Ct values ranging from 25.64 to 31.67; however, for goat No. 76967, the suspected positivity on the 7th day detected by nested RT-PCR was not confirmed by real-time PCR. The highest concentration was measured for the sample of goat No. 76967 on the 6th day, corresponding in order of magnitude to the 1:10 dilution of the reference-infected suckling mouse brain suspension.

**Detection of TBEV from Milk of Previously Immunized Infected Goats by Mouse Inoculation and RT-PCR**

Milk samples of 5 immunized, TBEV-challenged goats were collected daily after infection and were tested by suckling mouse inoculation to find out whether infectious TBEV was secreted with milk in spite of immunity. Animals were monitored twice a day for 10 days and death occurred only in 1 group, inoculated with milk from the 2nd day after the infection of goats. However, the following days’ milk samples from the same goat caused no symptoms and PCR examination of the brains of the ill mice gave negative results. Thus, the death of these animals was probably due to aspecific (maybe bacterial) infection.

**Detection of TBEV from Infectious Milk after Heat Treatment by Mouse Inoculation**

Table 2 summarizes the results of the animal experiments. Milk samples of 2 goats were examined: one with the highest virus content in its milk (No. 76967) and another with a lower virus concentration (No. 77702).

For goat No. 77702, the untreated infectious milk pool killed suckling mice on the 4th day after inoculation. After incubation at room temperature, the milk still proved infectious, as all mice were killed regardless of the duration of incubation. The virus was capable of infection even after 2 days at room temperature, though symptoms appeared a little later than in the case of the control. However, after heat treatment of the milk at 65°C, the animals did not become infected even after the shortest incubation time (5 min). Since, in general, the heat treatment at 65°C is not sufficient for inactivation of TBEV, this result might be related to the small test samples used. Regarding incubation at 100°C, a 3-min treatment was sufficient to prevent the infection of the inoculated suckling mice.

When using the milk with higher virus content, incubation at room temperature brought similar results: the virus remained infectious after 2 days, but symptoms occurred a little later after a longer incubation time (12–48 h). Remarkably, treatment at 65°C could not prevent the infection in this case since after incubation for 30 min the milk remained infectious, though symptoms occurred a
little later than for the untreated control. Three minutes at $100^\circ$ was as effective as for the other milk pool, all mice survived without symptoms.

Discussion

TBE spread by raw milk is a disease that occurs almost every year in Hungary and in most TBEV-endemic countries. To our knowledge, goats, cows and sheep that are infected with TBEV do not become ill and shed the virus with their milk without showing any symptoms of the infection. However, publications studying the physiological signs of TBEV infection of goats are rather rare.

In the present study, 10 goats were infected experimentally with live TBE virus and their body temperatures were recorded over the following days. Five of them were naïve animals and 5 were immunized previously with 2 doses of human vaccine to see if vaccination prevents viremia. Body temperatures of 10 uninfected goats (5 naïve and 5 immunized) were also recorded and used as a control in the data analysis. The statistical analysis of the temperature data (two-way ANOVA method) showed that neither the infection nor the vaccination caused significant increase in the body temperature of the animals at $p < 0.05$. Thus, a febrile state could not be detected, and the goats came through the infection without showing visible symptoms in different experimental conditions.

Milk samples were collected daily from the experimentally infected goats to determine the time range when TBE virus is shed with milk and whether immunization prevents the virus secretion. Samples were examined by classical (animal inoculation) and modern (PCR) virological methods. In the case of naïve-infected goats, it was determined, since there was 1 animal that had TBEV in its milk at the end of the experiment, 23 days postinfection. The shortest shedding time was 2 days (6th and 7th day postinfection), although for the animal in question, infectious virus was detected again on the 13th day by animal inoculation. The longest shedding could not be determined, since there was 1 animal that had TBEV in its milk at the end of the experiment, 23 days postinfection. The findings of the animal inoculations were confirmed by a TBEV-specific RT-PCR.

Regarding previously immunized infected goats, infectious TBEV was not detectable from the milk samples by mouse inoculation suggesting that the immunization prevented the development of virus infection and viremia. This means that the human vaccine that was used for the immunization is effective for goats as well.

A group of milk samples were selected based on the day when TBEV was detectable from the most samples to measure virus concentration by real-time RT-PCR. The results show that the amount of virus secreted in milk varies highly between the individual animals. Relative concentrations were compared to the members of a virus dilution series, and it was found that the sample with the highest virus content originated from the goat that had a remarkable peak in its body temperature the following day, although this increase in temperature was not statistically significant. This may suggest that the higher concentration of infectious virus particles could induce a slight elevation in temperature for this animal. Since no such observations have been reported so far, and according to current views, goats show no symptoms of TBEV infection, the question arises whether this was the result of the high virus content of the inoculum.

On the other hand, based on the real-time RT-PCR results, most of the examined milk samples contained much less virus than the mentioned sample with the highest virus content. In 1 case, the amount of virus detected by mouse inoculation was below the sensitivity limits of the real-time RT-PCR method, although all animals received the same dose of infectious virus. The great individual differences between the time ranges of virus shedding and between the amounts of secreted virus in milk point out that many physiological factors have influence on the virus spread in a naïve host organism’s body and on the secretion of virus with milk.

In the 2nd part of the experiment, the infectious milk samples of 2 goats were pooled (separately) and aliquots were subjected to heat treatment at various temperatures for different time intervals. To represent household practices, 3 temperatures were chosen: room temperature to simulate the state of raw milk left without refrigeration (using it for production of cheese or cottage cheese); $65^\circ$ to simulate heating, but not boiling or pasteurizing the milk; and $100^\circ$ to simulate boiling. When intracranially inoculated to suckling mice, the control milk pool with higher virus content caused the death of the animals by the 3rd day after inoculation, while the other pool with lower virus content killed the mice by the 4th day. Incubation at room temperature of the milk pools did not prevent the lethal infection of suckling mice, although after a longer incubation time (more than 12 h) the death of the animals occurred somewhat later, probably because the live virus content decreased during incubation. This could be observed for both milk pools. Incubation at $65^\circ$, symbolizing heating (but not boiling) the milk, brought different results for the 2 pools. The pool with lower virus content
lost its infectivity after treatment at 65° for 5 min or more; however, the pool with higher virus content retained its infectivity even after heating for 30 min. Treatment at 100° for 3 min eliminated the infectivity of both pools.

The findings of the present study underline that consumption of raw goat milk should be avoided because of the risk of virus infection, and among common household conditions, the simplest and safest way to avoid milk-borne TBE is to boil the milk before drinking or processing since the possible infectious virus content might survive plain heating. If the consumer insists on drinking the milk unboiled, another option would be the immunization of goats in TBEV-endemic areas, because the results show that the milk of the immunized animals was not infectious. Further studies are needed to determine how long this immunity lasts in goats and also to test whether natural immunity lasts for a lifetime, because goat vaccination is the most promising measure to prevent TBE infection in endemic areas for consumers of raw-milk products.

In Hungary, goat husbandry is not widespread; thus, specific goat vaccines against TBEV are not available. Vaccination is merely optional—even for humans. However, for those who consume unboiled milk, this would be a highly recommendable preventive measure. According to a study performed among farmers, those who know about the risks are less likely to drink raw milk, so informing the public should also be a high priority.

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References