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Development of a cell culture-based method for detecting infectious tick-borne encephalitis virus (TBEV) in milk products

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ABSTRACT

Tick-borne encephalitis outbreaks have been reported in Europe after consumption of raw milk products from infected animals. While molecular methods are commonly used in viral foodborne outbreak investigations due to their sensitivity, specificity and rapidity, there are very few methods to detect infectious tick-borne encephalitis virus (TBEV) in milk products for routine use/analyses. To address this gap, we developed a cell culture-based method to detect infectious TBEV in artificially contaminated raw goat milk and raw goat cheese, and evaluated the sensitivity of TBEV infectivity assays. Raw goat milk samples were spiked with TBEV to achieve inoculation levels ranging from 10^6 to 10^0 TCID₅₀/mL, and *Faisselle* and *Tomme* cheese samples were spiked so their TBEV concentrations ranged from 9.28×10^5 to 9.28×10^1 TCID₅₀ per 2.5g. To detect infectious TBEV, Vero cells were infected by raw goat milk. For cheese samples, after homogenisation and membrane filtration, Vero cells were infected with samples adsorbed on the filter (method A) or with samples eluted from the filter (method B). After 5 days, cytopathic effects (CPEs) were observed and TBEV replication in Vero cells was confirmed by an increase in the number of genome copies/mL that were detected in cell supernatant. Infected Vero cells exhibited CPEs for both milk and cheese samples. Infectious TBEV was detected to 10^3 TCID₅₀/mL in raw milk samples and to 9.28×10^1 TCID₅₀ from *Faisselle* samples using both methods A and B. For *Tomme* samples, method A was able to detect TBEV to 9.28×10^2 TCID₅₀/2.5g and method B to 9.28×10^3 TCID₅₀/2.5g. The number of positive samples detected was slightly higher with method A than with method B. To conclude, this qualitative cell culture-based method can detect infectious TBEV artificially inoculated into raw milk and cheese; it should be further evaluated during foodborne outbreak investigations to detect infectious TBEV from naturally contaminated milk and cheese.

1. Introduction

Tick-borne encephalitis (TBE) is the most frequent arbovirolosis in Europe, and affects the human central nervous system. The symptoms of TBE infections in humans include meningitis, encephalitis or meningo-encephalitis, with potentially fatal outcomes. Tick-borne encephalitis virus (TBEV), the causative agent, is an enveloped, positive-sense, single-stranded RNA virus belonging to the *Flavivirus* genus within the *Flaviviridae* family. TBEV is divided into five subtypes: the Far eastern, Siberian, Baikalian, Himalayan and European subtypes, the latter being the only prevalent subtype in Western Europe (Johnson et al., 2023). Although TBEV is primarily transmitted by *Ixodes* tick bites, cases of contamination have also been reported following the ingestion of contaminated dairy products. In Europe, 1% of human infections are associated with foodborne transmission of the virus (Kříž et al., 2009), and TBE outbreaks after consumption of raw milk products from infected animals have been reported in Central, Northern and Eastern Europe

(Balogh et al., 2010; Brockmann et al., 2018; Donchenko et al., 2005; Dorko et al., 2018; Holzmann et al., 2009; Hudopisk et al., 2013; Ilic et al., 2020; Kerlik et al., 2018; Kohl et al., 1996, Markovinović et al., 2016). Products derived from raw goat milk were generally the main source of contamination. The first foodborne outbreak in France occurred in 2020, in the Ain *département* (a *département* being a French administrative unit) in the eastern part of the country, with 43 confirmed human cases. All but one patient reported having consumed unpasteurised goat cheese from the same goat farm (Gonzalez et al., 2022). The molecular detection of TBEV in milk and cheese samples during outbreak investigations confirmed the foodborne transmission. In May 2021, the disease was included in the list of notifiable diseases, and between May 2021 and December 2022, food contamination was suspected to be at the origin of six out of 62 cases (12%) in the Auvergne-Rhône-Alpes region of France (Mailles et al., 2023).

Most foodborne viruses are currently difficult or impossible to cultivate and sensitive molecular methods are therefore used to detect

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viruses in food. Until now, molecular methods are often used to detect TBEV in milk and products derived from raw milk during the TBEV foodborne outbreaks investigation and infectious and non-infectious TBEV viral particles were detected in milk products.

The infectious dose of TBEV for humans after ingestion of contaminated products is still unknown at present. To ensure the safety of raw milk products and to improve the assessment of viral risk, a reliable method for detecting infectious viral particles in milk products is essential. Cell culture remains the gold standard method for detecting infectious viral particles and is currently used to isolate TBEV from ticks (Gonzalez et al., 2022). Previous to our study, no method based on cell culture had been developed for routinely detecting infectious TBEV in milk and cheese products. Indeed, it is challenging to detect infectious TBEV because of the composition of milk products, which could make it difficult to implement cell-based methods. The aim of this study was to develop and characterize a qualitative cell culture-based method for detecting infectious TBEV (i) in raw milk and (ii) in raw cheese. For this purpose, we used raw goat milk and raw goat cheese, as goat-derived milk products are commonly implicated in TBEV foodborne transmission in Europe.

2. Materials and methods

2.1. Cell culture and virus stocks

The “Ain strain” TBEV was isolated in 2022 in France (Gonzalez et al., 2022) and was provided by the Virology Unit, Laboratory for Animal Health, ANSES (Maisons-Alfort, France).

A working viral stock was generated by infecting Vero cells (ATCC: CCL-81™) cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Thermo Fisher Scientific, United Kingdom), supplemented with 1% glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate and 5% foetal calf serum (FBS) (Eurobio Scientific, France). The virus titre was determined using a standard 96-well plate containing Vero cells and applying the median tissue culture infectious dose (TCID₅₀). Briefly, 10-fold dilutions of TBEV were prepared in DMEM then inoculated into the wells of plates containing a confluent monolayer of Vero cells. After incubating for 1h30 at 37 °C with 5% CO₂, the viral inoculum was removed and the cell monolayers were washed twice in Dulbecco’s Phosphate-Buffered Saline (DPBS) 1X (Thermo Fisher Scientific) before adding 100 µL DMEM and 2.5% FBS (Thermo Fisher Scientific). Following 5 days of incubation at 37 °C with 5% CO₂, the TBEV stock titre was 9.28×10^7 TCID₅₀/mL.

TBEV RNA genome copies per millilitre were quantified as described in Hennechart-Collette et al. (2022). Briefly, a standard curve of an in vitro transcribed TBEV-Hypr RNA molecule was used for comparison. Based on this method, the viral stock of TBEV had a titre of 1.27×10^9 genome copies/mL.

2.2. Viral infection

2.2.1. Selection of dairy goat milk samples

Three raw goat milk products were selected for this study: raw goat milk and two types of raw goat cheese (*Faisselle* and *Tomme de chèvre*). *Faisselle* is a type of fresh raw milk cheese named after the mold in which it is drained, and contains approximately 24% of fat content. *Tomme de chèvre*, on the other hand, is a white cheese crafted from raw goat’s milk, with a creamy texture. Following a slow curdling process lasting around 12 h, the fermented curd is delicately ladled into pots and allowed to drain briefly before it is packaged in trays. This cheese is matured for a period ranging from 60 to 120 days. *Tomme de chèvre*, distinguished by its uncooked pressed body and natural rind, contains approximately 23% of fat content.

Raw goat milk was provided by the experimental goat farm run by INRAE-UMR MoSAR (78850 Thiverval Grignon, France), while raw goat milk cheeses (*Faisselle* and *Tomme de chèvre*) were purchased from local

markets. The *Faisselle* was made by two different producers. The *Tomme de chèvre* was produced by the same farmer but came from different production batches.

2.2.2. Contamination of samples

Raw goat milk samples were spiked with TBEV to obtain inoculation levels ranging from 10⁶ to 10⁰ TCID₅₀/mL. Cheese samples (2.5 g) were spotted with 100 µL of TBEV prepared in DMEM using a micropipette to place drops on the cheese surface until concentrations reached from 9.28×10^5 to 9.28×10^1 TCID₅₀ per sample. One uninoculated food sample spiked with sterile water was used as a negative control during the entire sample processing and viral detection procedure.

2.2.3. Cell infection with spiked DMEM and goat milk samples

Vero cells were seeded onto 24-well plates at a density of 50,000 cells per well. One day post-seeding, each 24-well plate containing a confluent monolayer of Vero cells was washed with DPBS 1X. The preparation of samples for viral infection assays is presented in Fig. 1. Five Vero cell wells were inoculated with 100 µL of spiked raw goat milk and DMEM samples per each level of TBEV inoculation. In addition, tenfold-diluted samples of infected raw goat milk were prepared in DMEM supplemented with 1% glutamine, 1% penicillin-streptomycin, 1% sodium pyruvate and then inoculated into five Vero cell well samples per each level of TBEV inoculation.

After 1h30 at 37 °C with 5% CO₂, the samples were removed and cells were washed twice or three times with DPBS 1X to remove residual virus and matrix. One mL of medium supplemented with 2.5% FBS and 1% amphotericin B (Thermo Fisher Scientific) were added to the cell monolayers. Phenotypical cell appearance and cytopathic effects (CPEs) were observed daily and photographed under the light microscope (40X) (Nikon eclipse TS100) on the 5th day post infection (dpi) at 37 °C with 5% CO₂. To confirm TBEV replication in Vero cells, the cell culture supernatant was harvested for molecular analysis as described below. Each experiment, from the spiking to the observation of CPEs in wells on 5 dpi, was performed twice. An increase of the viral genomic titres in cell culture supernatant on 5 dpi confirm a productive viral infection.

2.2.4. Cell infection with spiked cheese samples

For detecting infectious TBEV from spiked cheese, Vero cells were seeded onto 60-mm-diameter Petri dishes at a density of 300,000 cells per dish. One day post-seeding, plates containing a confluent monolayer of Vero cells were washed in DPBS 1X.

The elution of TBEV from cheese samples for viral infection assays is presented in Fig. 1. Cheese samples (2.5 g) were transferred to a 50 mL tube containing lysing matrix D ceramic beads (Fisher Scientific) with 7.5 mL of DMEM and were homogenised using FastPrep®-24 tissue cell homogeniser for 2×20 s at 6 ms^{-1} (MP Biochemicals). The sample was then transferred to a new 50 mL tube and ceramic beads were washed twice with 5 mL of DMEM. After centrifugation at 10,000 g for 30 min at 4 °C to remove debris, the supernatant was centrifuged again at 10,000 g for 10 min at 4 °C before 0.22 µm filtration to remove bacteria. A half dilution with DMEM was performed when filtration proved difficult.

Virus particles were concentrated by membrane filtration under a vacuum with a 47-mm-diameter Durapore uncharged membrane filter (hydrophilic polyvinylidene difluoride, hereinafter noted H-PVDF, with a pore size of 0.45 µm (Sigma Aldrich, HVLV 04700)). The filter membrane was then either directly placed in a 60-mm-diameter Petri dish (method A) or was transferred to a 50 mL tube containing 5 mL of DMEM to elute viruses (method B).

A flowchart showing methods A and B for cheese sample analysis is presented in Fig. 1.

For method A, the protocol for Vero cell infection consisted in directly placing the filter on the cells. Briefly, 1 mL of infection medium supplemented with 1% glutamax, 1% penicillin-streptomycin and 1% sodium pyruvate was added onto the cells then the filter membrane with its adsorbed viruses was placed directly on the cells.

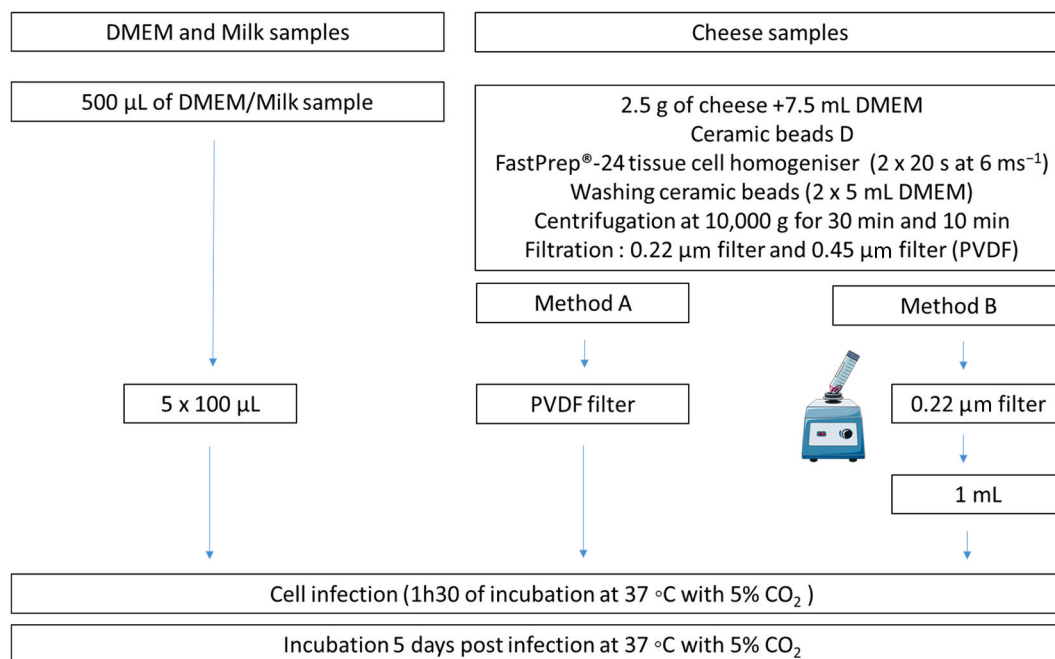


Fig. 1. Flowchart of infection protocols with DMEM, goat milk and cheese samples (methods A and B).

For method B, Vero cells were infected with viruses eluted from the H-PVDF filter. To elute viruses from the filter, it was placed in a 50-mL falcon tube with 5 mL of infection medium supplemented with 1% glutamax, 1% penicillin-streptomycin and 1% sodium pyruvate; the side of the filter containing the sample and adsorbed viruses faced inwards. The tube was vortexed three times (10 s) and the filter was soaked for 10 min at room temperature with constant agitation (about 60 rpm) to elute as many virus particles as possible from the filter. After being vortexed three more times (10 s) and filtered through a 0.22-µm filter to remove filter debris, 1 mL of the eluate was placed in a Petri dish.

After 1h30 at 37 °C with 5% CO₂, the culture medium and the filter for method A or eluate for method B were removed and 1 mL was harvested for molecular analysis as described below. Two mL of infection medium supplemented with 2.5% FBS and 1% Amphotericin B were then added onto the cell monolayers. Following incubation for 5 day at 37 °C with 5% CO₂, cells and CPEs were observed and photographed under the light microscope (40X). The morphology of infected and mock-infected cells was observed. To confirm TBEV replication in Vero cells, its viral genome was detected by RT-qPCR assays with RNA extracted from 1 mL of cell supernatant 1h30 post infection (pi) and on 5 dpi. If on 5 dpi the viral genome had increased in supernatants by at least 2 log₁₀, CPEs were confirmed and the sample was considered TBEV (+).

Each experiment, from the spiking to the observation of CPEs on 5 dpi, was performed three times.

2.2.5. Molecular detection of TBEV to confirm CPEs

Nucleic acid was extracted using a NucliSens® easyMAG™ platform according to the manufacturer's instructions (bioMérieux, Marcy l'Etoile, France). Nucleic acid was eluted in 100 µL of elution buffer and stored at -80 °C.

One-step quantitative RT-qPCR amplifications were performed in duplicate on the CFX96™ real-time qPCR detection system (Bio-Rad) as described in Hennechart-Collette et al. (2022).

2.2.6. Statistical analysis

To study the effect of two methods (A and B) used to detect infectious TBEV from cheese samples, a Chi-square test was applied using the Excel function (Windows 10 Professional Version 21H2), which gives the *p*-value. If the *p*-value is lower than the chosen level of significance of error

(5%), then the relationship between the two variables is statistically significant. For this analysis, the effects of (1) the method used, (2) the nature of the cheese sample and (3) the TBEV inoculum level for detecting infectious TBEV from raw goat cheese were tested.

3. Results

3.1. Infectivity assays for TBEV from DMEM and raw goat milk

3.1.1. TBEV replication in vero cells

To monitor the replication of TBEV from the contaminated dairy product samples, the advent of CPEs and the phenotypic appearance of the cells were checked daily until 5 dpi. Phase contrast images showed that dead cells are larger and are not refractive. By comparison, CPE in TBEV infected cells are represented by refractive cells and in chains of at least 3–4 cells. Molecular analysis are applied to confirm CPE from microscopic observation and an increase of 3 log₁₀ in the culture supernatant on 5 dpi confirm a productive viral infection. Fig. 2 shows TBEV-induced CPEs from spiked DMEM samples and raw milk products. Microscopic observation on 5 dpi with uninoculated DMEM and raw goat milk samples showed that Vero cells cultured in DMEM or in goat milk had a similar phenotypic appearance, demonstrating the absence of cytotoxicity due to milk components.

3.1.2. Sensitivity of TBEV infectivity assays

To assess the sensitivity of TBEV infectivity assays, DMEM and milk samples were spiked at different inoculation levels. Table 1 shows the results of qualitative detection of infectious TBEV from spiked DMEM and raw milk samples according to the quantity of TBEV inoculated. CPEs were visible in Vero cells infected with DMEM spiked with TBEV at levels ranging from 10⁶ to 10 TCID₅₀/mL. Undiluted milk samples spiked with TBEV ranging from 10⁶ to 10³ TCID₅₀/mL and ten-fold dilution of milk samples spiked with TBEV ranging from 10⁶ to 10⁴ TCID₅₀/mL showed visible CPEs. Infectivity was detected in 50% of diluted milk samples at a contamination level of 10³ TCID₅₀/mL, while no cell infection was observed below this level of TBEV contamination. No CPEs were observed with DMEM infected with 10⁰ TCID₅₀/mL of TBEV or with milk samples infected with TBEV within a range from 10² to 10⁰ TCID₅₀/mL. No viral genome or CPEs were detected in

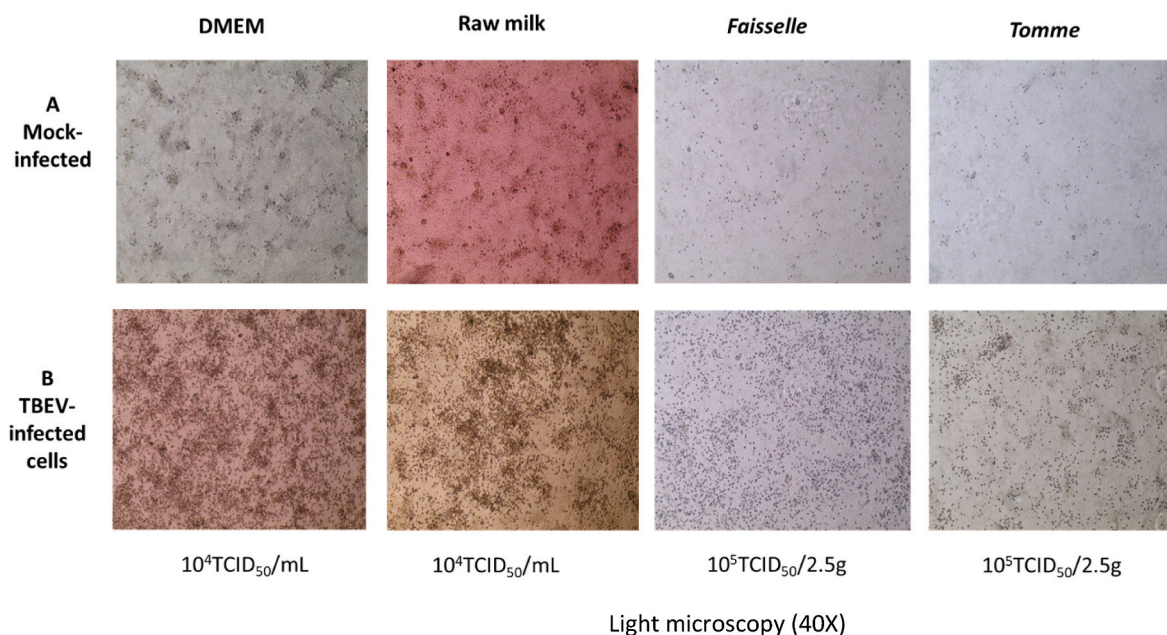


Fig. 2. Cytopathic effects (CPEs) represented by refractive cells and chains of at least 3–4 cells induced by TBEV in Vero cells. Phase contrast images of mock-infected (A) and TBEV-infected cells (B) on 5 dpi for DMEM, milk and cheese samples. The quantities of virus indicated under the photos are the doses inoculated into the matrices.

Table 1

Number of infectious samples detected from spiked DMEM and raw milk according to the levels of TBEV inoculated and dilution of inoculated samples.

TBEV (TCID ₅₀ /mL)	DMEM (500 µL) Pure	Milk (500 µL)	
		Pure	10-fold
10 ⁶	2/2	2/2	2/2
10 ⁵	2/2	2/2	2/2
10 ⁴	2/2	2/2	2/2
10 ³	2/2	2/2	1/2
10 ²	2/2	nd/2	nd/2
10	2/2	nd/2	nd/2
1	nd/2	nd/2	nd/2
0	nd/2	nd/2	/

Two experiments were performed from DMEM and milk according to the inoculation levels. Five Vero cell wells were infected with 100 µL of raw goat milk and DMEM samples. If CPEs were observed and confirmed on one out of the five wells, TBEV was considered to have been detected in the DMEM or milk samples. “x/2”: Number of infectious samples detected in the two experiments carried out.

nd: virus not detected: no CPEs were observed and no TBEV was detected in wells using a molecular approach.

uninoculated cells on 5 dpi.

To confirm TBEV replication in Vero cells, RT-qPCR assays were performed with RNA extracted from the culture supernatant on 5 dpi. RT-qPCR results showed a mean of $8.30 \pm 8.10 \log_{10}$ genome copies/mL, which confirmed virus-dependent CPEs observed whatever the inoculation level of TBEV in DMEM and milk.

During analysis of DMEM and milk spiked with 10^6 TCID₅₀/mL of TBEV, the maximum amount of residual genomic material observed in the third washing buffer was approximately $2.00 \times 10^4 \pm 1.00 \times 10^4$ genome copies/mL for DMEM and $2.00 \times 10^5 \pm 1.00 \times 10^5$ genome copies/mL for milk. The mean increases of 3–4 \log_{10} between the viral genomic titres in cell culture supernatant and the maximum amount of residual genomic material observed in the third washing buffer confirmed that the detection of TBEV from culture supernatants on 5 dpi did not originate from the inoculum’s viral genome. These results confirmed that the detection of TBEV from culture supernatants on 5 dpi

did not originate from the inoculum’s viral genome.

3.2. Infectivity assays for TBEV from raw cheese

3.2.1. TBEV replication in vero cells

To monitor the replication of TBEV from the cheese samples in cell culture, methods A and B were evaluated for detecting infectious TBEV from two raw goat cheeses (*Faisselle* and *Tomme de chèvre*). The impact of the matrix on Vero cells and the presence of CPE were first assessed from microscopic observation (Fig. 2). The microscopic observation of Vero cells treated with uninoculated *Faisselle* and *Tomme de chèvre* on 5 dpi showed no cytotoxic effect of cheese residue on Vero cells. The microscopic observation did not allow to make difference between TBEV replication in cell culture according to methods A and method B. But, in any cases, phase contrast images showed that the dead cells are larger and are not refractive. In comparison, CPEs in TBEV-infected cells are represented by refractive cells and by chains of at least 3 to 4 cells. Regardless, microscopic observation (Fig. 2) is an indicative step to identify a CPE, but the result should always be confirmed by RT-qPCR both to ensure specificity and viral replication.

To confirm TBEV replication, RT-qPCR assays were performed using RNA extracted from the cell supernatant harvested at 1h30 post infection (pi) and on 5 dpi. The results of TBEV replication in Vero cells for cheese samples are presented in Table 2. The viral genome titres in the culture supernatant on 5 dpi reached 10^6 to 10^8 genome copies/mL for *Faisselle* and 10^5 to 10^7 genome copies/mL for *Tomme de chèvre* according to the TBEV inoculation level. For cheese samples, the mean increases of $3.32 \pm 1.72 \log_{10}$ between the viral genomic titres in cell culture supernatant between 1h30 pi and 5 dpi confirm a productive viral infection.

3.2.2. Efficiency of infectivity assays for TBEV from spiked raw cheese

To assess the sensitivity of TBEV infectivity assays, cheese samples were spiked at different inoculation levels. The results of infectious TBEV detection for each method according to the level of inoculation are presented in Table 2. The number of positive samples detected was slightly higher with method A (13/24) than with method B (8/24). In detail, 8/12 and 5/12 infectious *Faisselle* samples were detected with

Table 2
Qualitative detection of TBEV from raw cheese samples according to the level of inoculation and the cell culture-based methods.

Methods	Cheese		TBEV (TCID ₅₀ /2.5g of sample)				
			9.28x10 ⁵	9.28x10 ³	9.28x10 ²	9.28x10 ¹	
A	<i>Faisselle</i>	Genome copies/sample at T0 (1h30)	$3.97 \times 10^3 \pm 3.91 \times 10^3$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	
		Genome copies/sample at T1 (5 dpi)	$2.25 \times 10^3 \pm 3.60 \times 10^8$	$7.22 \times 10^5 \pm 8.96 \times 10^6$	$3.35 \times 10^5 \pm 1.30 \times 10^8$	$3.16 \times 10^6 \pm 5.66 \times 10^6$	
		TBEV replication in Vero cells Log ₁₀ (T1-T0)	+4.75	+4.85	> +4.52	> +4.50	
	<i>Tomme</i>	No. of infectious samples	3/3	3/3	1/3	1/3	
		Genome copies/sample at T0 (1h30)	$1.42 \times 10^4 \pm 1.18 \times 10^4$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	
		Genome copies/sample at T1 (5 dpi)	$1.67 \times 10^7 \pm 2.8 \times 10^5$	$2.33 \times 10^5 \pm 1.48 \times 10^5$	$1.74 \times 10^5 \pm 8.11 \times 10^4$	$<4.00 \times 10^2$	
	B	<i>Faisselle</i>	TBEV replication in Vero cells Log ₁₀ (T1-T0)	+3.07	> +3.36	> +3.24	< +0.60
			No. of infectious samples	1/3	2/3	2/3	nd/3
			Genome copies/sample at T0 (1h30)	$4.94 \times 10^2 \pm 1.75 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$
		<i>Tomme</i>	Genome copies/sample at T1 (5 dpi)	$9.35 \times 10^6 \pm 9.90 \times 10^6$	$1.36 \times 10^7 \pm 4.29 \times 10^6$	$<4.00 \times 10^2$	$2.99 \times 10^6 \pm 2.83 \times 10^4$
TBEV replication in Vero cells Log ₁₀ (T1-T0)			+4.27	> +5.13	< +0.60	> +4.47	
No. of infectious samples			2/3	2/3	nd/3	1/3	
<i>Faisselle</i>		Genome copies/sample at T0 (1h30)	$1.23 \times 10^3 \pm 1.18 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	$<1.00 \times 10^2$	
		Genome copies/sample at T1 (5 dpi)	$2.44 \times 10^7 \pm 2.85 \times 10^7$	$1.60 \times 10^6 \pm 1.07 \times 10^5$	$<4.00 \times 10^2$	$<4.00 \times 10^2$	
		TBEV replication in Vero cells Log ₁₀ (T1-T0)	+4.38	> +4.20	< +0.60	< +0.60	
		No. of infectious samples	2/3	1/3	nd/3	nd/3	

Three experiments were performed using cheese products according to the inoculation levels.

“x/3”: Number of infectious samples detected in the three experiments carried out.

Nd: not detected.

The limit of TBEV detection by RT-qPCR is 10² TBEV genome copies/mL.

methods A and B, respectively, whereas 5/12 and 3/12 infectious *Tomme* samples were detected with methods A and B, respectively.

Methods A and B were both able to detect 9.28×10^1 TCID₅₀ from *Faisselle* samples. For *Tomme* samples, method A was able to detect 9.28×10^2 TCID₅₀ and method B 9.28×10^3 TCID₅₀. Method A was able to detect 2 more samples with 9.28×10^3 TCID₅₀ and 3 more samples with 9.28×10^2 TCID₅₀ inoculation levels than method B.

Overall, for all samples of *Faisselle* and *Tomme* inoculated with various levels of TBEV, the number of infectious samples was slightly —though not significantly— higher with method A than method B (X^2 test, $X = 3.84$, p -value = 0.145). With both methods (A and B), 13/24 (54.2%) the proportion of positive samples was slightly higher for *Faisselle* (13/24, 54.2%) than for *Tomme* (8/24, 33.3%), but the difference was not statistically significant (X^2 test, $X = 3.84$, p -value = 0.145). The number of positive samples varied significantly with the virus inoculum levels (X^2 test, $X = 7.82$, p -values = 0.015). The percentage of detection for 9.28×10^5 (8/12) and 9.28×10^3 TCID₅₀ (8/12) of TBEV per sample was higher than with other TBEV inoculum levels (3/12 for 9.28×10^2 TCID₅₀ and 2/12 for 9.28×10^1 TCID₅₀).

4. Discussion

TBE outbreaks after consumption of raw milk and cheese from infected goats are well documented in Europe (Balogh et al., 2010; Wójcik-Fatá et al., 2023). The consumption of cow milk (Caini et al., 2012) or sheep milk (Dobler et al., 2012; Gresfková et al., 1975; Zeman et al., 2004) has also been described in TBEV transmission but less frequently. In France, the molecular detection of TBEV in goat milk products, the seroprevalence surveys demonstrating animal seroconversions, the detection of TBEV in ticks and the answers to patient questionnaires all confirmed contamination through food in 2020. In Germany, a study by Brockmann et al. (2018) on tick-borne encephalitis transmitted by unpasteurised goat milk and cheese revealed heterogeneous distribution of TBEV in cheese samples, and highlighted the viability of TBEV in cheese.

Until now, TBEV detection in milk and products derived from raw milk during the investigation of foodborne TBEV outbreaks has primarily relied on molecular methods, but they do not distinguish between infectious and non-infectious viral particles in milk products.

Cell culture remains the gold standard for detecting infectious viral particles. Vero cells which are currently used to cultivate and isolate TBEV from ticks (Gonzalez et al., 2022) were applied in this study to

detect infectious TBEV particles from artificially contaminated goat dairy products. Advantages of cell culture are to detect the infectious virus, to contribute to the assessment of the viral risk linked to TBEV in food hygiene and to isolate the strain of TBEV involved in the event of food poisoning. To our knowledge, very few studies have described methods for detecting infectious TBEV in raw dairy products. Vero cell infection has been described to detect TBEV from naturally contaminated cheese during a foodborne outbreak, but few details have been provided regarding the methods used to limit cell cytotoxicity from the food samples (Brockmann et al., 2018). Another study has evaluated the viability of TBEV particles (Hypr strain) in skimmed and microfiltered raw cow milk using a stable porcine kidney cell line (Saier et al., 2015). In addition to the cell culture methods, infectious TBEV particles can be detected by inoculating mice brains with infected milk samples (Rónai and Egyed, 2020) but this approach is time-consuming and requires permission for animal testing.

The present study optimized a qualitative cell culture-based method for detecting infectious TBEV from artificially contaminated raw milk samples and the detection limit obtained with our cellular based methods for detecting infectious TBEV was 10³ TCID₅₀/mL in raw milk samples and 9.28×10^3 TCID₅₀ in *Faisselle* samples using both methods A and B. The limits of detection obtained during the characterization of the molecular methods for tick-borne encephalitis virus detection in raw dairy products have been previously published. The LOD₅₀ and LOD₉₅ for TBEV were 6.40×10^3 genome copies per g or per mL and 2.84×10^4 genome copies per g or per mL, respectively (Hennechart-Collette et al., 2022).

The protocols of our methods were optimized to detect infectious TBEV in 500 µL of raw goat's milk and in TBEV eluted from 2.5 g of cheese. The test portion of goat's milk samples had to be compatible with conducting NSB3 tests (waste management), and the 2.5 g of cheese sample corresponds to the test portion previously described in the characterization of molecular methods for tick-borne encephalitis virus detection in raw dairy (Hennechart-Collette et al., 2022). Other studies could be carried out to evaluate a larger sample size.

The impact of the matrix on Vero cells and the presence of CPE were first assessed from microscopic observation. The microscopic observation of Vero cells treated with uninoculated raw dairy products (milk and cheese samples) on 5 dpi showed no cytotoxic effect of dairy products residue on Vero cells and corroborated that the method used in the infection is harmless to the cells.

Bacteria and fungal flora present in raw milk as well as rennet and

salt added to make cheese could affect the application of the cell-based methods for detecting infectious TBEV. In this study, to avoid the contamination of the Vero cells by the growth of yeast and mold and to prevent cell cytotoxicity, adapted protocols were applied. Our optimized protocols for milk and cheese products provide to add antibiotic in the cells infection medium, to remove milk samples after cell infection and washing cells to avoid residual matrix. For cheese samples, a pre-processing step is needed before using cell culture-based assays. Indeed, to prevent cell cytotoxicity from cheese components, we used a filtration method to concentrate TBEV and to eliminate as much cheese matrix residue as possible from eluates.

The data obtained with spiked samples revealed a limit of detection of 10^2 TCID₅₀/mL of TBEV in DMEM and 10^3 TCID₅₀/mL TBEV in milk. Milk components, which belong to diverse classes of organic chemical structures, may impact virus infectivity and modify virus-receptor interactions, potentially explaining the difference in limit of detection observed according to the matrices. For instance, one study has shown that milk globule membrane isolated from buttermilk or whey cream and their lipid components inhibit infectivity of rotavirus in vitro (Fuller et al., 2013).

Molecular analysis are applied to confirm specific TBEV replication in Vero cells culture supernatant. The cellular method depends on the molecular approach in the sense that RT-qPCR makes it possible to verify viral replication (linked to the increase in TBEV genomes in the supernatant) and to ensure the specificity of the observed CPE. Additionally, a new plaque assay using the infected cells and their supernatant to corroborate the data obtained by real-time PCR could be performed. In the event of foodborne outbreaks, this additional step would make it possible to isolate the TBEV strain from food samples and then characterize the strain by sequencing.

For cheese samples, the microscopic observation of CPE did not allow to make difference between TBEV replication in cell culture media after methods A and method B treatments. The viral genome titers in the culture supernatant on 5 dpi reached respectively 10^6 to 10^8 and 10^5 to 10^7 genome copies/mL. In the same way, by testing spiked milk samples, 10^8 genome copies of TBEV/mL were obtained in culture supernatant on 5 dpi. These results showed that the culture-based method is not dependent on the molecular method in terms of limit of TBEV detection due to the fact that the limit of TBEV detection determined by RT-qPCR is 10^2 genome copies/mL.

The cell culture-based method was developed for detecting TBEV in artificially contaminated dairy products samples. The spiked food samples are currently used to optimize the methods of detection/quantification and determine the LOD of pathogens in food microbiology. Naturally contaminated samples are difficult to find but the method developed on artificially contaminated samples can then be applied to samples from food poisoning or surveillance studies, which will allow us to confirm its contribution in terms of viral risk assessment.

Goat milk contains many somatic cells that could be infected with TBEV. Assuming that TBEV could be present in somatic cells in naturally contaminated milk samples, an efficient grinding step could play an important role in releasing TBEV from cells. In this study, milk and cheese products were artificially contaminated and we chose the strategy of mechanical cell disruption. The FastPrep®-24 homogeniser keeps viral particles intact for further cell-based analysis and could break up the somatic cells present in naturally contaminated cheese samples. Several strategies of mechanical cell disruption have previously been described, e.g. the use of a vortex mixer or stomacher, which is part of the standard equipment in many laboratories. Nevertheless, neither a stomacher nor a vortex mixer could be used for homogenising hard cheeses such as *Tomme de chèvre* because of their texture, thus preventing the release of TBEV from cheese samples (Data not shown).

In this study, we tested the sensitivity of two qualitative cell methods, A and B, for detecting infectious TBEV from cheese samples. The number of infectious results obtained with method B was lower than with method A, although not statistically different. This result could be

explained by the loss of TBEV during the analytical process, which appeared higher with method B than with method A. With method B, the loss of virus could be compensated for by adding a step to concentrate the virus eluted from the filter, such as an ultra-filtration step. Unlike method A, method B (when the virus is eluted from a filter) could be further used for quantitative detection of infectious TBEV in cheese products by using the TCID₅₀ titration method.

Methods A and B were applied in this study to analyse infectious TBEV from two different types of raw goat cheese (*Faisselle* and *Tomme de chèvre*). Our study showed a small but not statistically significant impact of the type of cheese on the sensitivity of infectious TBEV detection. The number of positive assays obtained for *Faisselle* (13/24) was higher than that obtained for *Tomme* samples (8/24). These results could be explained by the fact that cheeses analysed in this study differ in terms of composition, manufacturing process, maturation time and texture. It would be useful to evaluate the developed methods for assessing TBEV viability in raw milk products according to the animal species producing the milk, and the different technological cheese-making processes. Indeed, the constituents of milk, subject during the cheese manufacturing process to the action of ferments, salt and temperature, could affect the infectivity of the virus and its stability in finished products. The experiments carried out by Rónai and Egyed (2020) showed that TBEV survived in artificially contaminated unsalted goat cheese, but salt treatment made goat milk and cheese safe to consume.

In this study, we developed a qualitative method to detect infectious TBEV in dairy products using artificially contaminated samples. It would be now be useful to apply our method for assessing TBEV viability in naturally contaminated milk products and various types of cheese from different dairy ruminants. To conclude, in the event of a foodborne illness outbreak, RT-qPCR, which provides rapid results within one day, is proposed for initial screening to quickly identify the presence of viral RNA without differentiating between infectious and non-infectious viral particles. Confirmatory testing using cell-based methods, which takes approximately one week, is applied to confirm the presence of infectious viruses. By combining these methods, we can ensure a comprehensive approach to managing foodborne illness outbreaks. The rapid RT-qPCR screening allows for quick identification and initial response, while the cell-based methods provide a detailed understanding of the infectious risk, ensuring that appropriate measures can be taken to protect public health.

CRediT authorship contribution statement

Catherine Hennechart-Collette: Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. **Laure Mathews-Martin:** Visualization, Writing – review & editing. **Lisa Fourniol:** Methodology. **Audrey Fraisse:** Writing – review & editing. **Sandra Martin-Latil:** Writing – review & editing. **Laure Bournez:** Writing – review & editing, Funding acquisition. **Gaëlle Gonzalez:** Writing – review & editing, Methodology, Funding acquisition. **Sylvie Perelle:** Writing – review & editing, Funding acquisition, Conceptualization.

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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