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Method for tick-borne encephalitis virus detection in raw milk products

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27

28 **Abstract**

29 The transmission of tick-borne encephalitis virus (TBEV) through food is rare, but can occur
30 through the consumption of raw milk products from animals infected by tick bites. In 2020,
31 France faced a TBEV outbreak linked to the consumption of unpasteurized goat cheese. The
32 aim of this study was to develop and characterize a molecular method for the detection of
33 TBEV in raw milk products based on the recent international standard PR ISO/DIS 16140-4.
34 The TBEV recovery rates varied with the inoculation level and settings. The LOD₅₀ and LOD₉₅
35 of TBEV were 6.40×10^3 genome copies per g or per mL and 2.84×10^4 genome copies per g or
36 per mL, respectively. The percentages of RT-qPCR inhibitions were lower than 75% and the
37 murine norovirus (MNV-1), used as process control, was detected in all samples with a
38 recovery rate higher than 1%, as recommended in ISO 15216. We conclude that the described
39 method is appropriate to detect TBEV in raw milk products for routine diagnosis, and to assess
40 potential health risks.

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49 **Keywords:** Tick-borne encephalitis virus; raw dairy products; RT-qPCR detection; process
50 control; RT-qPCR inhibition

51

52 **1. Introduction**

53 Tick-borne encephalitis (TBE) is an emerging tick-borne zoonotic disease affecting the central
54 nervous system of humans. The typical manifestations of TBE include meningitis, encephalitis
55 or meningoencephalitis, with potentially fatal outcomes. The causative agent, tick-borne
56 encephalitis virus (TBEV) is an enveloped, positive single-stranded RNA virus belonging to the
57 **Flavivirus genus within the Flaviviridae family. TBE virus** is divided into five subtypes: the Far
58 eastern, Siberian, **Baikalian, Himalayan** and European subtypes. The European subtype is the
59 **only prevalent subtype** in western Europe. TBE is endemic across Europe and has been a
60 notifiable disease in the European Union (EU) since 2012 (ECDC, 2019). TBE is an important
61 zoonotic infection for many countries in central, northern and eastern Europe. The EU
62 notification rate for TBE has fluctuated between 0.4 and 0.7 cases per 100000 population from
63 2015 to 2019. In France, between 4 and 25 confirmed cases per year were reported from 2015
64 to 2019 (ECDC, 2021).

65 TBEV is predominantly transmitted to humans by *Ixodes* tick bites in endemic areas.
66 Occasionally, the infection can also occur following the consumption of raw, unpasteurized
67 dairy products from infected animals such as goats, sheep and cows (Balogh et al., 2010;
68 Brockmann et al., 2018; Donchenko et al., 2005; Holzmann et al., 2009; Hudopisk et al., 2013;
69 Kohl et al., 1996; Markovinovic et al., 2016). Animals can excrete TBEV in milk for 3 to 14 days,
70 beginning as early as the second or third day post-infection (Beck et al., 2020; Gresikova et al.,
71 1975, 1958a, 1958b). After infection *via* the alimentary route, the incubation period,
72 estimated at 3–4 days, is shorter than following a tick bite (Fischer et al., 2020). From April to
73 June 2020, France recorded a human outbreak of TBEV infections through food
74 contamination. The outbreak took place in the Ain department, Rhône-Alpes region. A total
75 of 43 suspect TBEV cases were reported. The outbreak investigations carried out by the French
76 regional health agency and the National Center for Arboviruses showed that 41 positive
77 human samples were confirmed, and 40 people reported consuming unpasteurized goat's
78 cheese sourced from the same goat farm (Beck et al., 2020; Gonzalez et al., submitted)

79 A sensitive and reliable method for the detection of TBEV in dairy products is needed to ensure
80 the safety of raw milk products. The general strategy for the detection of viruses in food
81 samples consists of three steps: virus extraction, purification of viral RNA, and quantitative
82 molecular detection of purified viral RNA. The ISO 15216 standard proposes molecular

83 methods for the detection of enteric viruses (noroviruses and hepatitis A virus (HAV)) in high-
84 risk food categories, such as fruits, vegetables, water, and bivalve molluscs (Anonymous, 2017,
85 2019). But, a wide variety of foodstuffs including dairy products have also been implicated in
86 viral disease outbreaks, and standardized molecular methods are needed.

87 A method based on the ISO 15216 procedure has been developed to detect noroviruses in
88 milk products (Hennechart-Collette et al., 2017). Due to the complexity of the methods and
89 the presence of substances that can inhibit PCR amplification, a comprehensive set of controls
90 is used, including controls for RT-qPCR inhibition and extraction efficiency (Lee et al., 2012;
91 Maunula et al., 2013). This method has not yet been validated for the detection of TBEV in
92 raw milk products.

93 The aim of this study was to characterize a RT-qPCR method for the detection of TBEV in raw
94 milk products based on the recent international standard PR ISO/DIS 16140-4:2018
95 (Microbiology of the food chain – Method validation-Part 4: Protocol for method validation in
96 a single laboratory).

97

98 **2. Materials and methods**

99 **2.1. Viruses and cells**

100 The TBEV Hypr strain was provided by the Laboratory for Animal Health, Anses (Maisons-
101 Alfort, France). The strain was isolated in 1953 in the Czech Republic from the blood of a 10-
102 year-old child. The complete genome sequence is available in GenBank (accession number:
103 U39292.1). A working viral stock was generated on Vero NK cells (ATCC: CCL-81TM) cultured in
104 Dulbecco's Modified Eagle's medium (DMEM) (ThermoFisher), supplemented with 1%
105 penicillin-streptomycin, 1% sodium pyruvate, and 2.5% fetal calf serum.

106 Virus titer was determined by standard plaque assay on Vero NK cells (ATCC: CCL81TM) and
107 expressed as plaque forming units per milliliter (PFU/mL). Briefly, 10-fold dilutions of TBEV
108 were prepared (from 10⁻¹ to 10⁻⁸) and inoculated into wells of 12-well plates containing a
109 confluent monolayer of Vero cells. After a 1h30 incubation at 37°C with 5% CO₂, the viral
110 inoculum was removed and the cell monolayers were washed twice in Dulbecco's Phosphate-
111 Buffered Saline (DPBS) 1X (ThermoFisher) and overlaid with Minimum Essential Media (MEM)
112 2X (ThermoFisher)-Avicel 2.4% (volume:volume). Following a 5-day incubation time at 37°C
113 with 5% CO₂, the infected monolayers were washed three times with DPBS 1X before fixation

114 with 4% paraformaldehyde and staining with 0.4% crystal violet. The TBEV stock titer was
115 6.30×10^8 TCID₅₀/mL.

116 Quantification of TBEV RNA genome copies per millilitres was performed against a standard
117 curve of an *in-vitro* transcribed TBEV-Hypr RNA molecule. Based on this method, the viral stock
118 of TBEV had a titer of 5.19×10^8 genome copies/mL.

119 The murine norovirus MNV-1 (CW1 strain) was provided by the Fougères Laboratory - Anses
120 (Fougères, France) (strain obtained from Dr. H. Virgin from Washington University (Saint Louis,
121 MO, USA)), and was propagated on a mouse leukemic monocyte macrophage cell line (RAW
122 264.7, ATCC TIB-71™) (Cannon et al., 2006). RAW 264.7 was grown at 37°C in an atmosphere
123 containing 5% CO₂ in DMEM supplemented with GlutaMAX™, 1% non-essential amino acids,
124 and 10% fetal bovine serum (Life Technologies, Saint-Aubin, France). The production stock of
125 MNV-1 had a titer of approximately 3.09×10^7 TCID₅₀/mL. The number of MNV-1 RNA copies
126 was estimated by RT-qPCR using the standard curve obtained with ten-fold dilutions of the *in*
127 *vitro* RNA transcripts as previously described (Fraise et al., 2017). Based on this method, the
128 production stock of MNV-1 had a titer of approximately 9.00×10^{11} genome copies/mL.

129

130 **2.2. Experimental design and artificial contamination of raw dairy products**

131 The experimental design described in PR ISO/DIS 16140-4:2018 was used. A total of 14 raw
132 dairy product types (including cow and goat raw milk and/or cow, goat and sheep raw cheese)
133 were purchased from a local supermarket.

134 Table 1 describes the raw milk dairy samples. A ten-fold serial dilution of TBEV inocula was
135 prepared in DMEM with concentrations ranging from 5.19×10^2 to 5.19×10^5 genome
136 copies/sample.

137 The 14 dairy samples were randomly allocated to four different settings (R1, R2, R3 and R4).
138 Four different dairy products were allocated in random order to each setting (R1 to R4).
139 Emmental de Savoie cheese and Goat's raw milk were included in two settings. Table 2
140 describes the allocation of the food matrices to the different settings, along with inoculum
141 levels of TBEV used for artificial contamination. In short, four settings (R1 to R4) with four
142 TBEV inoculation levels were tested, corresponding to 16 analyses in total.

143 Each sample was co-inoculated with 9.00×10^8 genome copies of MNV-1, used as an internal
144 control virus, just before adding elution buffer. One un-inoculated food sample was used as
145 negative control.

146 **2.3. Sample processing**

147 The method used to recover TBEV was previously adapted from the ISO 15216 (Anonymous,
148 2019, 2017) shellfish method, as described for the detection of noroviruses in raw milk
149 products (Hennechart-Collette et al. 2017). Briefly, milk and cheese (2.5 mL or 2.5 g) were
150 homogenized in 7.5 mL of 100 mM Tris-HCl, 50 mM glycine, and 1% beef extract, pH 9.5 (TGBE)
151 by quickly inverting the tube several times, before addition of 1.5 mg of proteinase K (0.2 mg
152 of proteinase K/mL) (Roche Diagnostics). Following an incubation of 15 min at 37°C, proteinase
153 K was cold-inactivated by placing the sample in an ice bath for 15 min. The food sample was
154 then centrifuged at $8,500 \times g$ for 15 min at 4°C to pellet debris, and 1 mL of the clarified sample
155 was processed for nucleic acid extraction.

156 **2.4. Viral RNA extraction**

157 Total nucleic acid extraction was carried out using the NucliSENS® easyMag™ platform with
158 the “off-board Specific A” protocol, according to the manufacturer's instructions (bioMérieux).
159 Nucleic acids were eluted in 100 µL of elution buffer and stored at -80°C.

160 **2.5. Primers and probes**

161 Primers and probes used to detect TBEV have already been described in the literature by
162 Gondard et al. (2018). The primers and the TaqMan® probe targeting the ORF1 polyprotein of
163 the murine norovirus (MNV-1), which were designed using Beacon Designer software (Bio-
164 Rad, Marnes-la-Coquette, France), have also been described in the literature (Martin-Latil et
165 al., 2012b). The TBEV and MNV-1 probes were labeled with the FAM reporter dyes at the 5'-
166 end, and a BHQ1 at the 3'-end. The sequence of the primer pairs and the TaqMan probes used
167 were as follows: for TBEV, the sense primer (TBE-Euro-F) was: 5'-TCCTTG AGCTTGACA AGA
168 CAG-3', the antisense primer (TBE-Euro-R) was: 5'-TGTTTC CAT GGC AGAGCCAG-3' and the
169 TaqMan probe (TBE-Euro-P) was: 5'-FAM-GGAACACCTTCCAACGGCTTGGCA-BHQ1-3'. For
170 MNV-1, the sense primer (MNV-3193-F) was 5'-CCGCCATGGTCCTGGAGAATG-3', the antisense
171 primer (MNV-3308-R) was 5'-GCACAACGGCACTACCAATCTTG-3' and the TaqMan probe (MNV-

172 3227-P) was 5'-FAM-CGTCGTCGCCTCGGTCCTTGTC AA-BHQ1-3'. All the primers and probes
173 were purchased from Eurofins MWG Operon (Les Ulis, France).

174 **2.6. RT-PCR conditions**

175 One-step quantitative real-time RT-PCR amplifications were performed in duplicate on the
176 CFX96™ real-time PCR detection system (Bio-Rad). Reactions were performed in a 25 µL
177 reaction mixture containing 1X RNA UltraSense™ master mix and 1.25 µL of RNA UltraSense™
178 enzyme mix, which are components of the RNA UltraSense™ One-Step Quantitative RT-PCR
179 System (Life Technologies), 2 U RNase inhibitor (Life Technologies), 1.25 µg of bovine serum
180 albumin (Life Technologies), 500 nM of forward primer, 900 nM of reverse primer, 250 nM of
181 probe and 5 µL of RNA extract. Positive controls containing RNA extracted from virus
182 suspensions and a negative control containing all the reagents but the RNA template, were
183 included with each set of reaction mixtures. The one-step quantitative real-time RT-PCR
184 program involved 60 min of reverse transcription of RNA at 55°C, followed by a 5 min
185 denaturation step at 95°C, and finally 45 cycles of 15 s at 95°C, 1 min at 60°C and 1 min at
186 65°C. Fluorescence was recorded by the apparatus at the end of the elongation steps (1 min
187 at 65°C) for each amplification cycle. All the samples were characterized by corresponding
188 cycle threshold (Ct) values. Negative samples yielded no Ct value. A standard curve for each
189 viral target was generated with RNA extracts resulting from the serial dilution of viral stock
190 suspension in distilled water. The slopes (S) of the regression lines were used to calculate the
191 amplification efficiency (E) of the quantitative real-time RT-PCR reactions according to the
192 formula $E=10^{|1/S|}-1$, to determine the performance of quantitative real-time RT-PCR assays.
193 TBEV and MNV-1 recovery rate percentages from spiked samples were calculated by using the
194 standard curves obtained with viral inoculum dilution and the following formula: quantity of
195 virus recovered after spiking experiments x (volume of elution buffer) / quantity of viral
196 inoculum x 100.

197 The RNA extracted from TBEV was used as an external amplification control (EAC) to monitor
198 RT-PCR inhibition in samples. This approach is described in ISO 15216, where an external
199 control RNA (i.e. an RNA species carrying the target sequence of interest) is added to an
200 aliquot of RNA sample. The percentage of RT-PCR inhibition in each tested sample is calculated
201 by comparing these results with the results of EAC RNA in the absence of sample RNA (i.e. in

202 water). Percentages of RT-qPCR inhibition in extracted RNA were calculated using the
203 following formula: $100 - (\text{quantity of external control RNA detected in sample} / \text{quantity of}$
204 $\text{external control RNA detected in ultrapure water}) \times 100$.

205 **2.7. Statistical analysis**

206 All statistical analyses were performed using Statgraphics Centurion XVII software
207 (Statgraphics Centurion Version 17.1.04). The effect of virus (TBEV and MNV-1) on virus
208 recovery rates was first evaluated by using one-way analysis of variance (ANOVA). The result
209 of the ANOVA is a p -value associated with the hypothesis that the mean recovery rates of all
210 groups were the same.

211 The influence of additional factors on TBEV extraction yields was studied by using one-way
212 ANOVA: (1) dilution of TBEV RNA extracts (pure vs. 10-fold diluted), (2) the inoculated level of
213 TBEV, and (3) setting experiments (R1 to R4). Because the extraction yields were statistically
214 different according to the inoculated level of TBEV and setting experiments (ANOVA, $p < 0.05$),
215 a multiple-comparison procedure (Fisher's least-significant-differences (LSD)) was applied to
216 determine which inoculated level of TBEV or which setting provided the highest extraction
217 yields.

218 Graphs plotting the mean and its standard error for each group illustrate the multiple
219 comparison procedure. When confidence intervals for means do not overlap, the difference
220 between two groups of a factor is significant.

221 The influence of setting experiments on MNV-1 extraction yields was studied by using one-
222 way ANOVA.

223 The limits of detection (LOD) which correspond respectively to 50% (LOD₅₀) and 95% (LOD₉₅)
224 of the probability of virus detection in water were calculated with the method for estimating
225 POD (probability of detection) function and the LOD (limit of detection) of a qualitative
226 microbiological measurement method, as described by Wilrich et al. (2009). POD-LOD
227 calculation software was used (version 9, dated 2017-09-23) (Wilrich et al., 2009). This
228 program can be freely downloaded from [www.wiwiss.fu-](http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html)
229 [berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html](http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/professoren/wilrich/index.html).

230

231

232 **3. Results**

233 **3.1. Virus recoveries and limit of TBEV detection in raw dairy products**

234 To characterize the detection of TBEV in raw milk products, the extraction yields of TBEV and
235 MNV-1 used as process control, and the limits of detection (LOD) of TBEV were determined
236 from artificially contaminated raw dairy products. Table 3 shows the mean extraction yields
237 obtained for TBEV according to the inoculum level and experiment settings (R1 to R4) and the
238 mean extraction yields obtained for MNV-1 used as a process control virus. The average of
239 viral recoveries calculated from pure RNA extracts ranged from 2.23% to 100.00% for TBEV
240 and between 51.29% and 81.35% for MNV-1. MNV-1 was detected in all RNA extracts
241 analysed. The mean recovery rates obtained with pure RNA extracts for TBEV and MNV-1 were
242 not significantly different (one-way ANOVA; p -value=0.2195). As expected, no viral RNA TBEV
243 was detected in the un-inoculated samples.

244 The TBEV limits of detection (LOD) for the method were calculated by the Wilrich approach in
245 each setting with all samples. The TBEV LOD₅₀ and LOD₉₅ values (which correspond to 50% and
246 95% probability of a positive TBEV result, respectively) are shown in Table 4. The TBEV LOD₅₀
247 and LOD₉₅ values for all four settings were 6.40×10^3 genome copies per g or per mL and
248 2.84×10^4 genome copies per g or per mL, respectively.

249

250 **3.2. Influence of experimental factors on extraction yield**

251 The influence of the different experimental factors on TBEV and MNV-1 were statistically
252 analysed. First, we assessed RT-qPCR inhibition in dairy products. The RT-qPCR inhibitions
253 ranged from 0.00% and 1.52% for all raw dairy products samples analysed with EAC (data not
254 shown). The absence of RT-qPCR inhibition from raw milk products was confirmed by
255 statistical analysis (one-way ANOVA; p -value=0.8048). Diluting TBEV RNA extracts did not
256 increase viral extraction yields, as shown by the F factors ranging from 0.01 to 1.53.

257 To assess the influence of the different experimental factors on the TBEV extraction from raw
258 milk products, we compared the mean virus recovery rates from spiked samples. The TBEV
259 recovery rates with pure RNA varied with the inoculation level (one-way ANOVA; p -
260 value=0.0176) and with the experiment settings from R1 to R4 (one-way ANOVA; p -
261 value=0.0399). More specifically, the multiple-comparison tests showed that inoculations

262 with 5.19×10^3 genome copies of TBEV differed significantly from 5.19×10^4 genome copies of
263 TBEV per sample. The R1 setting was significantly different from the R3 and R4 settings, but
264 did not differ significantly from R2. Mean TBEV recoveries from allocated food settings R1 and
265 R2 were higher than other allocated food settings.

266 The statistical analysis also revealed that the allocated food settings (R1 to R4) did not
267 influence the recovery rates of MNV-1 (one-way ANOVA; p -value=0.8597).

268

269 **4. Discussion**

270 The consumption of raw milk, raw cheese products, and artisanal cheese made from raw milk
271 is popular in France. Although food-borne transmission of TBEV is rare, TBE outbreaks linked
272 to the consumption of dairy products are well-documented (Balogh et al., 2010; Brockmann
273 et al., 2018; Donchenko et al., 2005; Holzmann et al., 2009; Hudopisk et al., 2013; Kohl et al.,
274 1996; Markovinovic et al., 2016). Importantly, TBEV excretion in the milk of infected goats,
275 sheep and cows occurs soon after tick-bite infection and starts 3 to 4 days post-infection.
276 (Fisher et al., 2020). Humans may acquire TBEV infection through the consumption of
277 contaminated raw dairy products. The recent cluster of human TBEV infections in France
278 shows that the development of a method for TBEV detection in raw milk products is needed
279 to ensure the safety of these products.

280 The ISO 15216 procedure has been applied for the detection and quantification of HAV and
281 norovirus in different food matrices such as bottled water, food surfaces, vegetables, and
282 shellfishes (Anonymous, 2019, 2017). Recently, methods have been developed to recover
283 norovirus, hepatitis E virus and hepatitis A virus from dairy products (Battistini et al., 2020;
284 Fumian et al., 2009; Hennechart-Collette et al., 2017; Sayed et al., 2020). However, to our
285 knowledge, no publications have characterized the validation of a method to detect TBEV from
286 raw milk and raw cheese products.

287 Different methods have been developed to recover TBEV from milk products. Because TBEV
288 can be excreted in the milk of infected animals (Balogh et al., 2012; Gresikova, 1958b), the
289 detection of TBEV antibodies in milk using ELISA tests, followed by validation with western
290 blot assays, has been applied (Wallenhammar et al., 2020). Molecular methods after thawing
291 milk samples and nucleic acid extraction have also been used (Balogh et al., 2012). Unlike for

292 milk samples, methods to detect TBEV from cheese samples have not been described. Herein,
293 we propose to detect TBEV in milk products using a molecular method that employs
294 proteinase K treatment and that was adapted from the ISO 15216 (Anonymous, 2019, 2017)
295 method for the detection of HAV and noroviruses in shellfish. A total of 14 different raw milk
296 products sourced from goats, cows and sheep were selected in this study. The experimental
297 design from PR ISO/DIS 16140-4:2018 was applied to characterize the method used to detect
298 TBEV in these raw milk products. Using this design, we were able to validate the detection
299 method for a wide of raw milk products, representative of samples analyzed during different
300 TBE food-borne outbreaks (Balogh et al., 2012; Beck et al., 2020; Brockmann et al., 2018;
301 Dorko et al., 2018; Ilic et al., 2020).

302 The composition of food products can affect virus extraction (Blaise-Boisseau, et al., 2010;
303 Summa et al., 2012) and the main obstacles for detection of viruses in food are the presence
304 of inhibitory substances in the samples. During the analysis of dairy products, milk
305 components such as casein, whey proteins, lactose, and fat could interfere with virus recovery
306 (Yavarmanesh et al., 2013, 2010). Because of this, the implementation of different controls
307 such as the virus process control and the External Amplification Control (EAC), which are
308 described in the ISO 15216 procedure, are mandatory to validate the results. The use of a virus
309 as a process control is essential for validating viral detection in food, and according to the
310 recommendations in ISO 15216, extraction yields of the process control must be higher than
311 1% to validate the assay (Hennechart-Collette et al., 2015, 2014, Martin-Latil et al., 2014,
312 2012a, 2012b; Stals et al. 2011a, 2011b). In this study, MNV-1 was detected in all RNA extracts
313 and was recovered with an efficiency that ranged from 51.29% to 81.35% in raw milk products.
314 These results are consistent with a previous study where MNV-1 was used as a process control
315 for the detection of norovirus in milk and cottage cheese (Hennechart-Collette et al., 2017).
316 The second control, the EAC is a RNA carrying the target sequence of interest that is added
317 prior to the RT-PCR detection step to monitor RT-PCR inhibition in samples. According to the
318 recommendations in ISO 15216, the inhibition rates for RNA extracted from food samples
319 must be lower than 75% to validate the assay. The RT-qPCR inhibitions for dairy products
320 analysed ranged from 0.00% and 1.52%. These data confirm what other studies have reported
321 about inhibition of real-time RT-PCR assays used to quantify viruses in milk and cottage cheese
322 (Hennechart-Collette et al., 2017). The inhibition rates for RNA extracted from milk products
323 seemed to be lower than for other samples, such as vegetables, where the presence of PCR

324 inhibitors appeared to be more pronounced (Coudray-Meunier et al., 2013; Fraisse et al.,
325 2017; Plante et al., 2011).

326 The LOD is based on detection/non detection criteria and describes an assay's ability to detect
327 the target sequence at low levels. Evaluating the limits of detection allows to accurately assess
328 the relative technical quality and performance of the method to detect virus from dairy
329 products. In this study, for all raw dairy products analysed, the LOD₉₅ value of TBEV was
330 2.84×10^4 genome copies per g or per mL. Using the proteinase K method, the detection limit
331 of norovirus in milk and cottage cheese was in agreement with our data and was 10^5 genome
332 copies per g or mL (Hennechart-Collette et al., 2017).

333 TBEV excreted in milk and present in raw milk dairy products can be infectious. The risk of
334 infection depends largely on the stability of the virus in milk and cheese. Survival of TBEV in
335 milk or cheese products would depend on milk treatment or cheese characteristics, such as
336 the quantity of salt in the final product (Ronai and Egyed, 2020). It has been demonstrated
337 previously that pasteurization destroys viable TBEV particles in milk (Offerdahl et al., 2016;
338 Ronai and Egyed, 2020), but a simulation of the thermal regime utilized for cheese production
339 did not completely inactivate the Langkat virus (another tick-borne flavivirus, BSL-2 model for
340 TBEV) in milk (Offerdahl et al., 2016). Thus, TBEV virus could survive in milk used to make
341 cheese products and further studies should be performed to assess the infectious risk
342 associated to milk and cheese products.

343 To conclude, the molecular method characterized in this study according to the ISO 15216
344 recommendations in terms of controls (process control and EAC) successfully detected TBEV
345 in raw milk products. This method for detecting TBEV in contaminated raw milk samples is
346 essential both in routine diagnostic laboratories and to enable potential health risks to be
347 assessed.

348

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Table 1: Raw milk and cheese samples

		Dairy products
Cheese	Raw cow's milk cheese	Tomme de Savoie
		Emmental de Savoie Raclette de Savoie Saint Nectaire Tomme fermière
	Raw goat's milk cheese	Selle sur Cher
		Rigotte de Condrieu Mâconnais Valençay Le Chevrot Crottin de Chavignol
Raw sheep's milk cheese	Pérail la houlette	
Milk	Raw milk	Goat's raw milk
		Cow's raw milk

Table 2: Allocation of the food matrices to the different settings, along with inoculum levels of TBEV used for artificial contamination.

		Settings			
		R1	R2	R3	R4
TBEV contamination levels	High	Tomme de Savoie	Raclette de Savoie	Goat's raw milk	Tomme fermière
	Medium	Selle sur Cher	Mâconnais	Emmental de Savoie	Goat's raw milk
	Low	Emmental de Savoie	Valençay	Le Chevrot	Crottin de Chavignol
	Very low	Rigotte de Condrieu	Pérail la houlette	Saint Nectaire	Cow's raw milk

Table 3: Means of TBEV recoveries calculated for four inoculum levels of TBEV in the presence of MNV-1

Virus	Number of genome copies	RNA extracts	Repeated experiment R1 (%±SD)	Repeated experiment R2 (%±SD)	Repeated experiment R3 (%±SD)	Repeated experiment R4 (%±SD)
TBEV	5.19x10 ⁵	pure	85.13±3.75 (2/2)	100.00 (2/2)	13.76±2.00 (2/2)	2.23±0.03 (2/2)
		10 fold-diluted	100.003 (2/2)	100.00 (2/2)	21.08±0.34 (2/2)	0.38±0.11 (2/2)
		Factor (F) (diluted/undiluted)	1.17	1.00	1.53	0.17
	5.19x10 ⁴	pure	4.77 (1/2)	12.24±2.21 (2/2)	27.29±0.85 (2/2)	16.15±1.44 (2/2)
		10 fold-diluted	nd	nd	7.48±6.30 (2/2)	0.20±0.14 (2/2)
		Factor (F) (diluted/undiluted)	/	/	0.27	0.01
	5.19x10 ³	pure	100.00 (2/2)	nd	nd	nd
		10 fold-diluted	nd	nd	nd	nd
		Factor (F) (diluted/undiluted)	/	/	/	/
	5.19x10 ²	pure	nd	nd	nd	nd
		10 fold-diluted	nd	nd	nd	nd
		Factor (F) (diluted/undiluted)	/	/	/	/
MNV-1	9.00 x10 ⁸		81.35±18.72	51.29±32.23	60.13±39.90	74.02±38.26
	Total samples with recovery rates >1%			16/16		

The mean of replicates was used for each inoculation level sample. Results are expressed as means of viral extraction recovery (%)±standard deviation (SD).

The number of positive Ct determinations is given in parentheses for TBEV.

The ratio between the mean of extraction yields obtained with undiluted RNA extracts and the mean obtained with 10-fold diluted RNA extracts was calculated to determine whether the dilution of RNA extracts enhanced mean extraction yields (F).

nd: not detected

Table 4: LOD₅₀ and LOD₉₅ calculated by the Wilrich approach for TBEV

Target virus	/g or /mL	R1	R2	R3	R4	All settings
TBEV	LOD ₅₀	6.800x10 ³	6.40x10 ³	6.400x10 ³	6.400x10 ³	6.40x10 ³
	LOD ₉₅	3.00x10 ⁴	2.68x10 ⁴	2.68x10 ⁴	2.68x10 ⁴	2.84x10 ⁴