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Insights into the assessment of highly pathogenic Shiga toxin-producing *Escherichia coli* in raw milk and raw milk cheeses by High Throughput Real-time PCR

Sabine Delannoy, Mai-Lan Tran, Patrick Fach

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1 **Insights into the assessment of highly pathogenic Shiga toxin-producing**
2 ***Escherichia coli* in raw milk and raw milk cheeses by High Throughput Real-**
3 **time PCR**

4

5

6 **Sabine Delannoy^a, Mai-Lan Tran^a and Patrick Fach^b**

7

8 Anses (The French Agency for Food, Environmental and Occupational Health &
9 Safety), Laboratory for Food Safety, Unit of 'Pathogenic *E. coli*' (COLiPATH) &
10 Genomics platform 'IdentyPath' (IDPA), 94701 Maisons-Alfort, France.

11

12 ^a Sabine Delannoy and Mai-Lan Tran contributed equally to this work.

13

14 ^b Address correspondence to Patrick Fach, patrick.fach@anses.fr

15

16

17 **Abstract**

18 Current methods for screening Enterohemorrhagic *Escherichia coli* (EHEC) O157
19 and non-O157 serogroups in raw milk products typically rely on the molecular
20 detection of *stx*, *eae*, and serogroup-specific *wzx* or *wzy* genes. As these genetic
21 markers can also be carried by non-EHEC strains, a number of 'false positive' results
22 are obtained during the screening step. The suitability of new EHEC markers (*espK*,
23 *espV*, *ureD*, *Z2098*, and CRISPR_{O26:H11}) were tested as candidates for a more
24 accurate screening of EHEC in dairy products. High-throughput PCR analysis of
25 1,451 DNA extracts from milk and raw milk cheeses positive for both *stx* and *eae*

26 demonstrated that addition of these new markers in the detection scheme resulted in
27 a higher selectivity with a systematic reduction of the number of presumptive positive
28 samples that require further O-group testing and confirmation by strain isolation. This
29 reduction is more important (26% to 52%, depending on the animal production
30 species) in the absence of prior IMS treatment of the **enriched culture** for the Top7
31 EHEC serotypes. However, even with prior treatment of the **enriched cultures** by IMS,
32 the reduction rate varied between 5% and >25%. Analysis of *eae*-subtype, *stx*-
33 subtypes indicated strong differences in the STEC (Shiga toxin producing *E. coli*)
34 flora between animal species (goat, sheep, and cow). This study also pointed toward
35 the possible presence of EHEC O80 (a new emerging EHEC serogroup in Europe) in
36 cow's raw milk cheeses, which warrants further investigations.

37

38

39

40 1. Introduction

41

42 Shiga toxin-producing *Escherichia coli* (STEC) are important zoonotic pathogens
43 comprising more than 400 serotypes (Beutin and Fach, 2014). It remains difficult to
44 fully define human pathogenic STEC or identify virulence factors for STEC that
45 absolutely predict the potential to cause human disease (EFSA and ECDC, 2021).
46 However, data reported worldwide on clinical STECs suggest that they carry most
47 often the intimin (*eae*) gene (EFSA and ECDC, 2021). The presence of intimin is an
48 aggravating factor, but other alternative factors of attachment like aggregative
49 adherence fimbriae (AAF) have been reported, during for example, the major
50 German outbreak with *E. coli* O104:H4 (Bielaszewska et al., 2011). AAFs are
51 regarded as the main adhesins in enteroaggregative *E. coli* (EAEC). The O104:H4
52 strain was a hybrid pathotype (EAEC-STEC) carrying the *stx2a* gene. Since the
53 German outbreak in 2011, this EAEC-STEC clone has declined but recently other
54 hetero-pathotypes like ExPEC-STEC (extra-intestinal pathogenic *E. coli* that acquired
55 *stx* genes) have emerged (Cointe et al., 2018). Thus, ExPEC-STEC of serotype
56 O80:H2 has been described for the first time in France (Soysal et al., 2016) and is
57 now reported as a major serotype associated with clinical outcomes in Europe
58 (Bruyand et al., 2019; EFSA and ECDC, 2021). ExPEC-STEC strains of serotype
59 O80:H2 carry the intimin gene *eae*-Xi, which is a variant very similar to the intimin
60 gene *eae*-Epsilon carried by STEC O103:H2, O45:H2 and O121:H19. The other
61 frequently recognized clinical STEC strains carrying intimin are STEC O157:H7 (*eae*-
62 Gamma), O145:H28 (*eae*-Gamma), O111 (*eae*-Theta) and O26 (*eae*-Beta) (Oswald
63 et al., 2000).

64 *E. coli* strains simultaneously possessing the *stx* and *eae* genes have a high
65 risk of causing STEC infections with HUS and diarrhea, they are termed
66 Enterohemorrhagic *E. coli* (EHEC). Both virulence factors are thus classically
67 targeted by the commercially available detection methods of EHEC O157 and non-
68 O157 in foods. The standardized EHEC detection procedures (ISO/TS 13136:2012;
69 USDA/FSIS MLG5C) also rely on the detection of these two markers (Anonymous,
70 2021; ISO, 2012). The reference methods are stepwise methods including an
71 enrichment step to get *E. coli* growing to a detectable level, a DNA extraction, a
72 sequential real-time PCR analysis for the detection of EHEC associated markers
73 (*stx1*, *stx2*, and *eae* genes) plus the selected O-group genetic markers in case of
74 detection of the *stx/eae* genes. A confirmation step, for suspect samples that gave
75 positive PCR reactions for *stx*, *eae* and at least one of the selected O-group relies on
76 isolation of *E. coli* strains and PCR confirmation of the pathogenicity traits on isolates
77 themselves (Anonymous, 2021; ISO, 2012). However, isolation procedures for EHEC
78 are laborious, time consuming and not always successful because of the lack of
79 biochemical features distinguishing EHEC from non-pathogenic *E. coli*.

80 Preliminary PCR-assays for detection of EHEC-associated genetic markers
81 must be specific enough to narrow down the number of samples that are subjected to
82 isolation. One of the main drawback of the current genetic markers used in the
83 reference methods (ISO/TS 13136:2012 and USDA/FSIS MLG5C) for screening
84 EHEC is the lack of selectivity of the first screening step that is based on detection of
85 the *stx* and *eae* genes (the two genes can be shared by non-EHEC strains).
86 Moreover, depending on the background flora of the food samples, the confirmation
87 step based on the current culture methods may lack the ability to isolate and confirm
88 some presumptive positive samples. Therefore, these detection methods generate a

89 large number of presumptive positives, which are never confirmed at the end during
90 the isolation step.

91 This represents major financial and logistical challenges for food manufacturers. It is
92 therefore important to improve the detection methods with the aim of increasing the
93 percentage of batches released at the end of the first screening step and reducing
94 the number of confirmations on isolated colonies while maintaining a high (or even
95 higher) level of product safety. We previously identified five genetic markers (*espK*,
96 *espV*, *Z2088*, *ureD* and CRISPR_{O26:H11}), which are preferentially associated with
97 typical EHEC (*E. coli* strains positive for the *stx* and *eae* genes, irrespective of their
98 serotype), and demonstrated that they could reduce, at the first screening step, the
99 number of presumptive positive samples in beef enrichments by 48.9% (Delannoy et
100 al., 2013a, Delannoy et al., 2013b, Delannoy et al., 2016). The objective of this
101 project was to assess the contribution of these genetic markers in the dairy sector in
102 the reduction of the number of presumptive positive samples after the first phase of
103 screening by *stx* and *eae*.

104

105 **2. Materials and methods**

106

107 ***2.1. Samples collected in the goat, sheep, and cow dairy sectors***

108 Raw milk and raw milk products collected in France from the goat, sheep, and cow
109 dairy sectors are routinely tested for *stx* and *eae* using the AFNOR approved
110 GeneDisc PCR array (Pall GeneDisc, Bruz, France) or the GENE-UP PCR
111 (bioMérieux, Marcy l'étoile, France), following the manufacturer's instructions.
112 Samples tested with the GENE-UP PCR can be either or not submitted to a prior
113 **VIDAS ESPT concentration** to catch the Top7 EHEC serotypes. In that case, the

114 37°C overnight-enriched cultures in Buffered Peptone Water (BPW) are processed
115 with the VIDAS® UP E. coli Serogroups - ESPT following the recommendation of
116 bioMérieux. Note that the VIDAS ESPT concentration which relies on the O-antigen
117 capture is primarily phage tail driven (a few antibodies are present according to the
118 manufacturer, bioMérieux) but here we refer to it as “IMS” as it provides a similar
119 enriched and concentrated *E. coli* population as immuno-magnetic separation (IMS)
120 does.

121 For this study, the CNIEL (French Dairy Inter-branch Organization), ANICAP
122 (Association Nationale Interprofessionnelle Caprine) and Confédération Générale de
123 Roquefort provided Anses (The French Agency for Food, Environmental and
124 Occupational Health & Safety) with selected DNA extracts issued from routine testing
125 of raw milk products for further analysis by high throughput qPCR with new
126 STEC/EHEC genetic markers, as described below. DNA samples received at Anses
127 were *stx-/eae-*, *stx+/eae-*, *stx-/eae+*, and *stx+/eae+*. Sampling was biased however to
128 get greater numbers of DNA samples positive for STEC, with a target of
129 approximately 1,500 *stx+/eae+*. Thus, this sampling scheme does not reflect the real
130 prevalence of STEC or EHEC in the French dairy sectors. However, it is well
131 designed to assess the contribution of the new genetic markers in the reduction in
132 the number of presumptive positive samples after the first phase of screening with *stx*
133 and *eae*. When samples positive for *stx* and *eae* were found positive for at least one
134 gene marker related to the Top5 EHEC serogroups using the GeneDisc array (Pall)
135 or the GENE-UP (bioMérieux), isolation of strains was attempted by external
136 laboratories for confirmation of the Top5 EHEC serotypes regulated in France (O157,
137 O145, O111, O103 and O26). Following the recommendation of the French ministry
138 of agriculture, the appropriate sanitary measures were taken in positive cases of

139 EHEC Top5. Unfortunately, this study was performed several months after the
140 samples were routinely tested by the external laboratories, and the EHEC strains
141 were not stored by these laboratories to be sent to Anses, but the data regarding the
142 isolation / confirmation of EHEC from presumptive positives were reported to Anses.

143

144 **2.2. High-throughput real-time PCR**

145 DNA extracts were obtained by the local routine laboratories following the protocols
146 recommended by the providers of the PCR kits for testing STEC: Pall GeneDisc
147 (Bruz, France) or GENE-UP PCR (bioMérieux, Marcy l'étoile, France). DNA preps
148 were stored at -20°C until transportation (at 0°C - 8°C) to Anses, within 2 to 3 days
149 (depending on the location of the routine laboratories). DNA extracts submitted to
150 freezing / thawing may have undergone potential DNA degradation by nucleases
151 during transportation to Anses. To overcome potential problem of DNA degradation,
152 data related to the stx/eae status was doubled checked by the local laboratories and
153 by Anses. Samples having the same stx/eae status before and after transportation to
154 Anses passed the 'quality control' and were selected for further analysis with
155 additional EHEC markers. DNA extracts when received in Anses were stored at -
156 20°C until used for pre-amplification. As the Fluidigm system relies on microfluidic
157 PCR, a pre-amplification is required by the manufacturer to guaranty the sensitivity of
158 the method (Michelet et al. 2014). For DNA pre-amplification, the TaqMan PreAmp
159 Master Mix (Fluidigm, USA) was used according to the manufacturer's instructions.
160 Primers were pooled combining equal volume of primers (200 nM final each). The
161 reaction was performed in a final volume of 5 µl containing 1µl TaqMan PreAmp
162 Master Mix, 1.25µl pooled primers mix, 1.25µl nuclease free water and 1.5µl DNA,
163 with one cycle at 95°C for 10 min, 14 cycles at 95°C for 15 s and 4 min at 60°C. At the

164 end of the cycling program, the reactions were diluted 1:10. Pre-amplified DNAs were
165 stored at -20°C until needed for high throughput PCR amplification.

166 The Biomark™ real-time PCR system (Fluidigm, USA) was used for high-throughput
167 microfluidic real-time PCR amplification using the 192.24 dynamic array (Fluidigm).
168 This chip dispense 24 PCR mixes and 192 samples into individual wells, after which
169 on-chip microfluidics assemble PCR reactions in individual chambers prior to thermal
170 cycling resulting in 4608 individual reactions. Amplifications were performed using
171 either 6-carboxyfluorescein (FAM)- or Hexachloro-fluorescein (HEX)- and black hole
172 quencher (BHQ)-labeled TaqMan probes with PerfeCTa qPCR ToughMix, Low ROX
173 in accordance with manufacturer's instructions (QuantaBio, USA). A $4\mu\text{l}$ sample mix
174 was prepared per sample, containing $2\mu\text{l}$ PerfeCTa qPCR ToughMix, Low ROX, $0.2\mu\text{l}$
175 20X GE sample Loading Reagent (Fluidigm PN 85000735) and $1.8\mu\text{l}$ of diluted pre-
176 amplified DNA. A TaqMan® primer assay was prepared for each target, containing
177 $18\mu\text{M}$ of each primer and $4\mu\text{M}$ of probe. Three microliters of these primer assays
178 were mixed with equal volumes of Dynamic Array (DA) 2X assay loading reagent
179 (Fluidigm PN 85000736) to make assay mixes ($9\mu\text{M}$ primers and $2\mu\text{M}$ probe). Prior to
180 loading the samples and assay mixes into the inlets, the chip was primed in the IFC
181 Controller RX apparatus. Three μl of sample mixes, prepared as described, were
182 then loaded into each sample inlet of the dynamic array chip and $3\mu\text{l}$ of assay mixes
183 were loaded into assay inlets. The chip was then placed in the IFC Controller RX for
184 loading and mixing. After approximately 20 min the chip was ready for thermal cycling
185 and detection of the reaction products on the BioMark™ PCR System (Fluidigm).
186 PCR cycling comprised of 2 min at 50°C , 10 min at 95°C , followed by 40 cycles of 2-
187 step amplification of 15 s at 95°C , and 1 min at 60°C . Data were acquired on the
188 Biomark™ Real-Time PCR System and analyzed using the Fluidigm Real-time PCR

189 Analysis software to obtain crossing point (CP) values. The assays were performed
190 in duplicate and one negative water control was included per chip. *E. coli* gene
191 targets used for the real-time PCR amplification and all primers and probes that have
192 previously been described or which were designed for this study are reported in
193 Table 1. An inhibition control (IC) was performed on each sample to check for
194 potential inhibition of the PCR reaction due to intrinsic characteristics of the sample.
195 The IC is a recombinant pBluescript IISK+ plasmid containing the *dsb* gene from
196 *Ehrlichia canis* (Michelet et al., 2014). The plasmid was added to each sample at a
197 concentration of approximately 0.3 pg/μl. Primers and probe specific for the *E. canis*
198 *dsb* gene were used to detect the IC (Michelet et al., 2014).

199

200 **2.3. Presumptive positives in screening 1, screening 2, and EHEC** 201 **confirmation**

202 In screening 1, three methods were explored and are defined as followed: method A
203 (*stx*, *eae*, *espK/Z2098/CRISPR_{O26:H11}*), method B (*stx*, *eae*,
204 *espK/espV/CRISPR_{O26:H11}*) and method C (*stx*, *eae*, *espK/ureD/CRISPR_{O26:H11}*). A
205 presumptive positive for method A was a sample that tested *stx*⁺, *eae*⁺, and
206 *espK/Z2098/CRISPR_{O26:H11}*⁺ (meaning positive for at least one or more of the three
207 targets *espK*, *Z2098*, *CRISPR_{O26:H11}*). A presumptive positive for method B was a
208 sample that tested *stx*⁺, *eae*⁺, and *espK/espV/CRISPR_{O26:H11}*⁺ (meaning positive for
209 at least one or more of the three targets *espK*, *espV*, *CRISPR_{O26:H11}*). A presumptive
210 positive for method C was a sample that tested *stx*⁺, *eae*⁺, and
211 *espK/UreD/CRISPR_{O26:H11}*⁺ (meaning positive for at least one or more of the three
212 targets *espK*, *UresD*, *CRISPR_{O26:H11}*).

213 In screening 2, all DNA samples *stx+*/*eae+* were tested by PCR for the main EHEC
214 O-groups (O157, O145, O111, O103, O145, O121, O45 and O80) and their
215 corresponding *eae*-variant (*eae*-gamma, -beta, -epsilon, -theta subtypes) A
216 presumptive positive recorded in screening 2 was a sample *stx+*/*eae+* that tested
217 positive for the appropriate association O-group / *eae*-subtype.

218 Screening 1 with the new EHEC markers (methods A, B and C) reports the number
219 of presumptive positives for all EHEC serotypes and not only for the Top7 EHEC
220 serotypes. Screening 2, as based on the combination of the O-group / *eae* type
221 reports the number of presumptive positives for the Top7 EHEC serogroups plus
222 O80. Confirmation of EHEC from presumptive positives was definitely acquired after
223 isolation of EHEC strains by the routine testing laboratories and the information was
224 reported to Anses. Data presented in this study showed the number of presumptive
225 positives after the Screening 1 (New EHEC markers), the Screening 2 (O-groups /
226 *eae*-types), and the number of EHEC strains isolated during the confirmation step.

227

228 **2.4 Statistical analysis**

229 Statistical analyses were performed on R Studio version 1.2.5019. Two-proportions
230 z-tests (two-tailed or one-tailed) were used to compare proportions of presumptive
231 positive between the ISO method and the new alternate methods. The statistical tests
232 were performed with α of 5%. The null hypothesis was rejected when p-values
233 were < 0.05 .

234

235 **3. Results**

236

237 **3.1. Quality control, number of samples and PCR data points included in the**
238 **dataset**

239 DNA extracts from raw milk and raw milk cheeses were collected in a two-year period
240 to get significant results and to cover the diversity of the French dairy production. The
241 diversity of the samples made it possible to cover both the diversity of animal species
242 (cow, goat and sheep) and of dairy matrices (milk, cheese). Data related to the
243 *stx/eae* status as determined with conventional real-time PCR kits by the local
244 laboratories from the dairy sectors and with the Biomark™ chips (Fluidigm) by Anses
245 were compared. Samples having the same *stx/eae* status were selected for this study
246 to exclude the DNA extracts which could have undergone DNA degradation during
247 storage and transportation to Anses. The *stx/eae* status was corroborated for 4,691
248 DNA extracts, which were tested by both the local laboratories and Anses. These
249 samples having the same *stx/eae* status by conventional real-time PCR and by
250 microfluidics real-time PCR passed the 'quality control' and were used in the data
251 analysis. **Table 2** reports the 4,691 DNA extracts selected in this study and the
252 distribution of the samples according to the animal species (cow, goat and sheep),
253 the dairy matrices (milk, cheese) and the IMS pre-treatment of the **enriched culture**.
254 All DNA extracts received in Anses were tested in the Biomark™ chip-1 (Table 1),
255 which included in particular verification of the *stx/eae* status (*stx1*, *stx2*, *stx2a*
256 subtype, *eae*, *eae*-gamma, -beta, -epsilon, -theta subtypes) and five novel EHEC
257 molecular markers, *espK*, *espV*, *ureD*, *Z2098*, and CRISPR_{O26:H11} (i.e. 112,584 PCR
258 determinations). The number of *stx+/eae+* samples identified after the first screening
259 was 1,451 samples, which were further analyzed with the second chip (Biomark™
260 chip-2, Table 1) targeting in particular *stx1a* subtype, molecular markers of
261 serogroups O157 (*rfbEO157*, CRISPR_{O157:H7}), O145 (*wzyO145*, *fliCH28[O145]*), O121,

262 O103, O111, O26, O45, O80 (i.e. 34,824 PCR determinations). The study as a whole
263 represents 147,408 PCR determinations, which served to consolidate the results
264 presented in this manuscript.

265

266 **3.2. Screening raw milk and raw milk cheese samples for *stx1*, *stx2*, *stx1a* and** 267 ***stx2a* subtypes**

268 Among the 4,691 DNA extracts selected according to their *stx/eae* status, a total of
269 1,451 *stx+/eae+* samples, composed of goat milk (n=438), goat milk cheese (n=282),
270 sheep milk (n=86), sheep milk cheese (n=165), cow milk (n=127) and cow milk
271 cheese (n=353), were tested for *stx1*, *stx2*, and specific *stx1a* and *stx2a* subtypes.
272 Data showed that the distribution of the *stx1* and *stx2* genes is quite different
273 depending on the animal species (Figure 1). It is remarkable that almost 80% of the
274 *stx*-positive samples of sheep milk cheese are positive for *stx1* only. Subtype *stx1a*
275 represent 52% of these sheep milk cheese samples that are positive for *stx1* alone
276 (data not shown). The *stx2* gene was more prevalent in cow (71-76%) than in goat
277 (44-62%) and sheep (21-51%). The subtype *stx2a*, which is highly associated with
278 HUS cases, represents less than 5% of the *stx*-positive sheep samples, 15% to
279 18.5% of the *stx*-positive goat samples and about 33% of the *stx*-positive cow
280 samples (data not shown).

281

282 **3.3. Screening *stx*- and *eae*-positive raw milk products for *espK*, *espV*, Z2098,** 283 ***ureD* and CRISPR_{O26:H11}**

284

285 **Goat samples.**

286 720 *stx+/eae+* goat sample were submitted to Screening 1 (New EHEC markers).
287 These 720 *stx+/eae+* goat samples were composed of 408 samples (128 milk and
288 280 cheese) tested with a prior IMS treatment of the **enriched culture**, and 312
289 samples (310 milk and 2 cheese) which were not treated by a prior IMS. Samples
290 were investigated with methods A, B and C and the number of presumptive positives
291 was reported for each method.

292 For goat milk treated with IMS (n=128), the number of presumptive positives was 119
293 with methods A and B, 117 with method C. 116 samples were determined
294 presumptive positive with the three methods. Interestingly the CRISPR_{O26:H11} marker
295 was never detected (Figure 2).

296 For goat milk tested without IMS (n=310), the number of presumptive positives was
297 233 with method A, 228 with methods B and C. Forty-three *stx+/eae+* samples were
298 negative with the three methods (Figure 3).

299 For goat milk cheese (280 tested with IMS and two without IMS) we recorded one of
300 the two samples treated without IMS as negative, while the other was recorded
301 positive with method A and method C (data not shown). For goat milk cheese treated
302 with IMS (n=280), the number of presumptive positives was 268 with method A, 265
303 with method B and 261 with method C. Eleven samples were negative with the three
304 methods (Figure 4).

305

306 **Sheep samples.**

307 For sheep milk treated with IMS (n=83) 78 samples were presumptive positive
308 according to method A, 72 were presumptive positive according to method B, and 73
309 were presumptive positive according to method C. Four samples were negative with

310 the three methods (Figure 5). In addition, three sheep milk without IMS treatment
311 were analyzed and all were recorded presumptive positive with method A, method B,
312 and method C (data not shown).

313 For sheep milk cheese, 165 *stx+ / eae+* samples (five treated with IMS and 160
314 without IMS) were screened with methods A, B and C. Among the five samples
315 tested with IMS, two were found negative with all methods, one was presumptive
316 positive with method A, and two were presumptive positive with methods A, B, and C
317 (data not shown). The 160 samples that were not treated with IMS divided into 140
318 samples presumptive positive with method A, 73 samples presumptive positive with
319 method B, and 63 samples presumptive positive with method C. Twenty samples
320 were negative with all methods (Figure 6).

321

322 **Cow samples.**

323 Cow milk samples *stx+ / eae+* (n=127) were all treated with IMS and tested with
324 methods A, B and C. Presumptive positive cow milk samples divided into 107
325 presumptive positives with method A, 106 presumptive positives with method B, and
326 109 presumptive positive with method C. 17 samples were negative with all methods
327 (Figure 7).

328 For cow milk cheese, 353 *stx+ / eae+* samples (114 tested with IMS and 239 without
329 IMS) were tested with methods A, B and C. Among the 114 samples tested with IMS,
330 28 were negative with all methods, 81 were presumptive positive with method A, 83
331 were presumptive positive with method B, and 82 were presumptive positive with
332 method C (Figure 8). The 239 *stx+ / eae+* cow milk cheese which were not treated
333 with IMS divided into 172 presumptive positives with method A, 167 presumptive

334 positives with method B, 182 presumptive positives with method C, and 46 samples
335 that were negative with all methods (Figure 9).

336

337 **3.4. Screening *stx*- and *eae*-positive raw milk products for *eae* subtypes**
338 **gamma, beta, epsilon, and theta and correlation with the serogroups**
339 **O157, O145, O121, O103, O111, O26, O45, and O80**

340

341 Following the ISO/TS 13136:2012 reference method (ISO, 2012), samples that tested
342 positive for both *stx* and *eae*, must be subjected to a second screening step for
343 specific serogroup determination. Confirmation of EHEC in the sample is definitely
344 acquired after isolation of EHEC strains from the sample. All *stx*+/*eae*+ samples
345 recorded in this study (n=1,451) were tested for *eae*-subtype, serogroup
346 determination and EHEC strain isolation was reported based on the information
347 collected by the routine testing laboratories. Figures 2-9 showed the number of
348 presumptive positive as determined according to the correlation between the *eae*-
349 subtypes and the serogroup. The following associations were considered to identify
350 the presumptive positives: *eae*-gamma with O157 and O145, *eae*-beta with O26,
351 *eae*-theta with O111 and *eae*-epsilon with O121, O103 and O45 (note that the PCR
352 test used for *eae*-epsilon is cross-reacting with the very similar *eae*-Xi subtype which
353 is associated with EHEC O80:H2). **These combinations of *eae*-subtypes and**
354 **serogroups are the most common ones among the Top7 EHEC serogroups.**
355 **However, other rare combinations exist (STEC O103:H11 and *eae*-beta, STEC**
356 **O103:H25 and *eae*-theta. Also, *eae*-beta can be found in certain STEC O111 and**
357 **STEC O145). Such rare associations were not considered in the analysis of the data**

358 presented in this study, potentially underestimating slightly the true number of
359 potential positives.

360 The relationship with the information of strain isolation is reported for each of the
361 eight sectors as assigned in the Venn diagrams of Figures 2-9 to appreciate the
362 pertinence of the three methods A, B and C. As a whole, 297 EHEC strains were
363 isolated during the confirmation step. Most of them were isolated from samples
364 treated by IMS. All EHEC strains belonged to the Top5 EHEC serogroups (O157 [n =
365 51], O26 [n = 83], O103 [n = 88], O145 [n = 69], O111 [n = 6]). No O80 strain could
366 be isolated in the absence of specific IMS reagent for this serogroup, which has
367 emerged recently in Europe. A high correlation was observed regarding the
368 CRISPR_{O157} positive signals (targeting EHEC O157:H7) in dairy samples and the
369 data related to the isolation of the 51 EHEC O157 strains (data not shown). In
370 addition, we showed that the marker *fliC*_{H28[O145]} could be used with success to
371 increase the specificity of screening for EHEC O145:H28 (data not shown). This
372 marker has been designed previously to detect EHEC O145:H28 that harbor a
373 characteristic *fliC*_{H28} allele (Beutin et al. 2015).

374

375 **Goat samples.**

376 Data obtained on the 720 *stx+*/*eae+* goat samples showed clearly that presumptive
377 positive samples for the Top7 and O80 EHEC serogroups were mostly concentrated
378 in sector 3 (i.e. samples recorded positive with the methods A, B, and C). There are
379 239 EHEC strains isolated from goat's samples, and 236 strains out of 239 (98.75%)
380 were derived from samples positive by all three methods (sector 3) as reported in
381 Figures 2, 3 and 4. Only three EHEC strains were isolated out of sector 3 (Figure 4):
382 two strains of serogroup O103 isolated from goat milk cheese that were presumptive

383 positive respectively with method A only (sector 1) and methods A and B (sector 2),
384 and one O157 strain isolated from a goat milk cheese that was negative by all
385 methods (sector 8).

386

387 **Sheep samples.**

388 Data obtained on the 251 *stx+/eae+* sheep samples gave a clear evidence that the
389 presumptive positive for the Top7 and O80 EHEC serogroups were mostly
390 concentrated in sector 3 (i.e. samples recorded presumptive positive with methods A,
391 B, and C) as reported in Figures 5 and 6. Two EHEC strains of serogroups O26 and
392 O103 were isolated (Figure 6). The two strains derived from sheep milk cheese that
393 were recorded presumptive positive with all methods (sector 3).

394

395 **Cow samples.**

396 Data obtained on the 480 *stx+/eae+* cow samples indicated that presumptive
397 positives for the Top7 and O80 EHEC serogroups were highly associated with sector
398 3 (Figures 7, 8 and 9). These findings were corroborated with the isolation of 56
399 EHEC strains, all of which, except one, derived from cow samples recorded in sector
400 3. The unique strain of serogroup O26 isolated out of sector 3 was related to one
401 sample that was positive for CRISPR_{O26:H11} alone. Therefore, all EHEC strains
402 isolated from cow were derived from samples recorded presumptive positive with
403 methods A, B and C.

404

405 **3.5 Comparison of methods A, B, and C for an efficient pre-screening of raw** 406 **milk and raw milk cheeses**

407 As a whole, 1,451 samples were tested positive for both *stx* and *eae*, and must be
408 subjected to a second screening targeting the O-group gene markers according to
409 the ISO/TS 13136:2012 reference method (ISO, 2012). Alternate methods of pre-
410 screening (methods A, B, and C) include in addition to *stx/eae*, the detection of five
411 novel markers (*espK*, *espV*, *Z2098*, *ureD*, and CRISPR_{O26:H11}) to refine the first
412 screening step. Based on 1) the data obtained with the pre-screening methods A, B
413 and C, 2) the data on the correlation between the presence of the O-antigen markers
414 and the *eae*-subtypes, and 3) the information related to the isolation of EHEC strains
415 (n=297), one may consider that the three methods are quite equivalent. However, the
416 method A provided a higher number of presumptive positive after the first screening
417 step. This observation was clearly evidenced in sheep milk cheese that were treated
418 without a prior IMS treatment of the **enriched culture**. For these samples, the number
419 of presumptive positive was 140 with method A, 73 with method B, and 63 with
420 method C. The significant number of extra presumptive positives recorded with
421 method A with regard to the other two methods (**one-tailed two-proportions z-tests, p-**
422 **values equal 2.214e-07 and 1.656e-09 for methods B and C respectively**) was not
423 corroborated by the second screening step with the O-antigen markers and the *eae*
424 subtype, nor with the isolation step. Thus, methods B and C were more selective than
425 method A. Overall, methods B and C recorded 1,113 and 1,115 presumptive
426 positives respectively, while the conventional ISO/TS 13136:2012 reference method
427 recorded 1,451 presumptive positives. **The reduction in the number of presumptive**
428 **positives recorded with methods B and C over the ISO method is significant (one-**
429 **tailed two-proportions z-tests, p-values equal 2.434e-15 and 3.568e-15 for methods**
430 **B and C respectively with regard to the ISO method)**. Refinement of the first *stx/eae*
431 screening step with these two alternate methods B and C was remarkable (23.3%

432 and 23.15% reduction of presumptive positives respectively with regard to the ISO
433 reference method). As illustrated in Tables 3 and 4, the alternate method B (the most
434 selective method) provided a significant reduction of the number of samples that
435 necessitate further screening for the EHEC serogroups determination (second step of
436 the ISO reference method). Table 3 showed that the reduction in the number of
437 presumptive positives was modest when the samples were subjected to a prior IMS
438 treatment, except for cow milk cheese samples where a significant reduction (27.2%)
439 was achieved (one-tailed two-proportions z-test, p-value=0.003612). Reductions in
440 the number of presumptive positive was however significant (one-tailed two-
441 proportions z-tests, p-values <0.05) for all samples without a prior IMS treatment:
442 26.5% reduction for goat milk, 29.7% reduction for cow milk cheese, and up to 51.9%
443 for sheep milk cheese (Table 4).

444

445

446

447 **4. Discussion**

448

449 *E. coli* strains simultaneously possessing the *stx* and *eae* genes have a higher risk of
450 causing STEC infections with HUS and diarrhea. Both virulence factors are thus
451 targeted in a first screening step by detection methods of highly pathogenic STEC in
452 foods. This first screening step is followed by specific detection of genes related to
453 EHEC serogroups, in a second screening step (Anonymous, 2021; ISO, 2012). This
454 approach generates many presumptive positives, which must then be confirmed by
455 isolation and then phenotypic and genotypic characterization of the isolated strain in
456 order to confirm the presence of all virulence factors in a single strain. Thus, the

457 current methods present major financial and logistical challenges for food
458 manufacturers. It is therefore important to improve the detection methods with the
459 aim of increasing the percentage of batches released at the end of the first screening
460 step and of reducing the number of confirmations on isolated colonies while
461 maintaining a high (or even higher) level of product safety. We previously identified
462 four genetic markers (*espK*, *espV*, *Z2088*, *ureD*), which are preferentially associated
463 with typical EHEC (*E. coli* strains positive for the *stx* and *eae* genes, irrespective of
464 their serotype) (Delannoy et al., 2013a, 2013b). We demonstrated that, in association
465 with CRISPR_{O26:H11}, a marker characterizing a specific clone of EHEC O26 that is
466 negative for these four genetic markers (Delannoy et al. 2015), one could reduce at
467 the first screening step the number of presumptive positive samples in beef
468 enrichments by 48.9% (Delannoy et al. 2016). The objective of this project was to
469 assess by high throughput microfluidic PCR the contribution of these five genetic
470 markers in the dairy sector in the reduction of the number of presumptive positive
471 samples after the first phase of screening. We used DNA extracts that were tested
472 either with the AFNOR approved GeneDisc PCR array (Pall GeneDisc, Bruz, France)
473 or the GENE-UP PCR (bioMérieux, Marcy l'étoile, France), and showed that the data
474 correlate well with the Fluidigm microfluidic real-time PCR approach developed in this
475 study. That way, we evidenced on natural samples that the sensitivity of the Fluidigm
476 microfluidic real-time PCR is equivalent to that of the approved conventional real-time
477 PCRs. As acknowledged in the literature (Olwagen et al, 2019), the sensitivity of the
478 Fluidigm microfluidic real-time PCR is equivalent to that of the conventional real-time
479 PCR when a pre-amplification step is used.

480 Validation of the pertinence of such a new approach including these five markers was
481 carried out on 1,451 samples that tested positive for both *stx* and *eae*. Half of these

482 1,451 samples derived from samples submitted to an IMS treatment, which induced a
483 bias in the selection of specific serogroups (regulated EHEC serogroups). We
484 focused first on the *stx* status of these dairy samples with regard to the data reported
485 in the Tessy database, which collected the clinical cases associated with STEC in
486 Europe (EFSA, 2020). This database showed that about 93% of HUS are associated
487 with *stx/eae* positive strains and that 97% of HUS are associated with strains carrying
488 at least one gene of the Stx2 family. In addition, about 84% of HUS reported in the
489 Tessy database are associated with strains carrying at least the *stx2a* subtype
490 (EFSA, 2020). In this study on dairy products, we showed significant differences of
491 the *stx* status of the samples, according to the animal species (goat, sheep, and cow)
492 and the matrices (raw milk, raw milk cheese). Thus, it is remarkable that *stx2* was
493 highly dominant in cow samples, while it is much less prevalent in sheep samples
494 (surprisingly *stx1* was highly prevalent in sheep samples with about 80% of sheep
495 raw milk cheese positive only for *stx1*). The *stx2a* subtype (significantly associated
496 with HUS) was recorded at a higher occurrence in cow samples (33% of the *stx*-
497 positive cow samples). However, in terms of public health significance, these
498 differences in the distribution of the *stx* types and sub-types in dairy products remains
499 questionable. Therefore, additional biomarkers should be tested in complement to
500 the *stx* genes to get a more precise risk assessment.

501 A more significant refinement of the first EHEC screening step could be achieved by
502 including CRISPR_{O26:H11}, *espK*, and *espV*, *ureD*, or *Z2098* in the detection scheme.
503 Introduction of these markers provided a significant reduction of the number of
504 *stx+/eae+* samples that require a second screening step for serogroup determination
505 according to the ISO/TS 13136:2012 reference method (ISO, 2012). Thus, the more
506 specific results were obtained with method B (*stx* / *eae* / *espK* / *espV* /

507 CRISPR_{O26:H11}) and method C (*stx* / *eae* / *espK* / *ureD* / CRISPR_{O26:H11}), which
508 provided a reduction rate of about 23%. Given the additional information on the
509 association of the Top7 EHEC serogroups and the *eae*-subtypes, we determined
510 these two methods as the best approach to narrow down the EHEC screening step in
511 dairy samples. Nevertheless, method A (*stx* / *eae* / *espK* / *Z2098* / CRISPR_{O26:H11})
512 also provided accurate results in all samples, except for sheep milk cheese that were
513 treated without a prior IMS treatment of the **enriched culture**. Further investigation
514 would be required to identify why method A is less selective than the other methods
515 in that specific case.

516 Identification of additional gene markers i.e. *espK*, *espV* and CRISPR_{O26:H11} to better
517 distinguish typical EHEC from other *E. coli* pathogroups would substantially enhance
518 the power of EHEC test systems providing a significant reduction of 'presumptive
519 positive' in dairy samples. As expected, samples tested with a prior IMS treatment to
520 select the Top7 EHEC serogroups provided a lower reduction rate of presumptive
521 positives (5% to 27% reduction depending on the matrices / species) compared with
522 those which were not subjected to an IMS treatment (26% to 52% reduction rate
523 depending on the matrices / species). One could notice that some dairy samples
524 could be potentially contaminated by more than one EHEC serogroup. This was
525 clearly evidenced by samples giving a positive reaction for both CRISPR_{O26:H11} and
526 one or more of the other markers *espK*, *espV*, *Z2098*, *UreD* and O-group associated
527 genes. Data on the correlation between the presence of the O-antigen markers and
528 the *eae*-subtypes, together with the information related to the isolation of 297 EHEC
529 strains evidenced the reliability of these EHEC markers. Strains of EHEC isolated by
530 the routine testing laboratories during the confirmation step belonged to the Top5
531 EHEC serogroups (O157 [n = 51], O26 [n = 83], O103 [n = 88], O145 [n = 69], O111

532 [n = 6]). Given the fact that >99% of these strains were isolated from samples tested
533 positive with the new EHEC markers we showed that these markers can be used with
534 high confidence in the dairy industry. These data corroborate previous studies
535 showing that all EHEC strains isolated from dairy products were detected with these
536 genetic markers (Delannoy et al., 2013a, 2013b, 2015). We observed very few misses
537 (3 strains out of 297). In goat milk cheese treated with IMS one EHEC O157 isolate
538 (sector 8) was missed by all methods. We hypothesized here a probable alteration of
539 the DNA during transportation to Anses or a simple clerical error on the part of the
540 cooperating labs, given the large number of samples involved. Otherwise, in goat
541 milk cheese with IMS, one EHEC O103 isolate (sector 1) was missed by methods B
542 and C and not by method A. Another EHEC O103 isolate (sector 2) was missed by
543 method C and not by methods A and B. Unfortunately, the routine testing laboratories
544 performing the confirmation step did not conserve these strains. It was thus
545 impossible to perform additional studies to understand why these strains were not
546 detected.

547 In this study, we showed that the introduction of the new EHEC markers in the
548 detection scheme (at the first screening step on enriched culture) would certainly
549 provide the dairy industry a selective and reliable method for tracking the main EHEC
550 serogroups in raw milk products. This study also pointed toward the possible
551 presence of EHEC O80 (an emerging pathogen in Europe) in cow raw milk cheeses,
552 which warrants further investigations. This work should be considered with interest to
553 define a new, more selective approach for detecting highly pathogenic STEC in raw
554 milk products.

555

556 **Declaration of competing interest**

557 The authors declare that they have no known competing financial interests or
558 personal relationships that could have appeared to influence the work reported in this
559 paper.

560

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567

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666

667 **Legends of Tables and Figures**

668

669 **Table 1.** Primers and probes used in this study. ^a F, forward primer; R, reverse
670 primer; P, probe. ^b Probes were labeled with either 6-HEX or 6-FAM and BHQ1
671 (Black Hole Quencher). ^c Oligonucleotide described by Perelle et al. (2004), ^d
672 Oligonucleotide described by Nielsen and Andersen (2003), ^e Oligonucleotide
673 described by Delannoy et al. (2013a), ^f Oligonucleotide described by Delannoy et al.
674 (2013b), ^g Oligonucleotide described by Delannoy et al. (2012), ^h Oligonucleotide
675 described by Delannoy et al. (2015), ⁱ Oligonucleotide described by Perelle et al.
676 (2005), ^j Oligonucleotide described by Bugarel et al. (2010), ^k Oligonucleotide
677 described by Fratamico et al. (2009), ^l Oligonucleotide described by Beutin et al.
678 (2015).

679

680 **Table 2.** Dairy samples (n=4,691) selected in this study. Distribution of the samples
681 according to the animal species (cow, goat and sheep), the dairy matrices (milk,
682 cheese) and the IMS pre-treatment of the **enriched culture**.

683

684 **Table 3.** Dairy samples treated with IMS and tested *stx+/eae+* (n=737). Reduction of
685 the number of presumptive positives by introducing new EHEC markers (method B)
686 in the first screening phase of the ISO/TS 13136 reference method. ^a **two-tailed two-**
687 **proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with method B**
688 **was significantly different from the ISO/TS 13136 reference method when p-value <**
689 **0.05. ^b one-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive**
690 **positives with the ISO/TS 13136 reference method was significantly greater than**
691 **method B when p-value < 0.05. N/A for not applicable.**

692

693 **Table 4.** Dairy samples treated without IMS and tested *stx+*/*eae+* (n=714). Reduction
694 of the number of presumptive positives by introducing new EHEC markers (method
695 B) in the first screening phase of the ISO/TS 13136 reference method. ^a **two-tailed**
696 **two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with method**
697 **B was significantly different from the ISO/TS 13136 reference method when p-value**
698 **< 0.05. ^b one-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive**
699 **positives with the ISO/TS 13136 reference method was significantly greater than**
700 **method B when p-value < 0.05. N/A for not applicable.**

701

702

703 **Figure 1.** Proportion (percentage) of the *stx1* and *stx2* genes in 1,451 *stx+*/*eae+*
704 dairy samples. Distribution according to each dairy category sample.

705

706 **Figure 2.** Goat milk treated with IMS. Screening 1 (New EHEC marker): comparison
707 of methods A, B and C on 128 samples positive for both *stx* and *eae* genes. *Stx*⁺ for
708 samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples giving a
709 positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for *espK*
710 and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or *espV*,
711 *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*, *espK/Z2098*
712 **for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for samples**
713 **giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a negative**
714 **result for *espK* and *ureD*. Sector ① (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ② (*stx*⁺, *eae*⁺,**
715 ***espK/Z2098*⁺, *espK/espV*⁺), sector ③ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺,**
716 ***espK/ureD*⁺), sector ④ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector ⑤ (*stx*⁺, *eae*⁺,**
717 ***espK/espV*⁺), sector ⑥ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector ⑦ (*stx*⁺, *eae*⁺,**

718 *espK/ureD*⁺) and sector ⑧ (*stx*⁺, *eae*⁺). N=x is the total number of samples per
719 sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for each sector the
720 number of presumptive positives based on the serogroup/ *eae* variant correlation. ‘-’
721 means negative. Note that some samples may be presumptive positive for more than
722 one EHEC serogroup. Confirmation: the number of EHEC strains (n=x) isolated
723 during the confirmation step is reported for each sector.

724

725 **Figure 3.** Goat milk treated without IMS. Screening 1 (New EHEC marker):
726 comparison of methods A, B and C on 310 samples positive for both *stx* and *eae*
727 genes. *Stx*⁺ for samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples
728 giving a positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for
729 *espK* and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or
730 *espV*, *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*,
731 *espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for
732 samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a
733 negative result for *espK* and *ureD*. Sector ① (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ②
734 (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺), sector ③ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺,
735 *espK/espV*⁺, *espK/ureD*⁺), sector ④ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector
736 ⑤ (*stx*⁺, *eae*⁺, *espK/espV*⁺), sector ⑥ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector
737 ⑦ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ⑧ (*stx*⁺, *eae*⁺). [n=x] is the number of
738 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
739 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. N=x is the total number of
740 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for
741 each sector the number of presumptive positives based on the serogroup/ *eae*
742 variant correlation. ‘-’ means negative. Note that some samples may be presumptive

743 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
744 strains (n=x) isolated during the confirmation step is reported for each sector.

745

746 **Figure 4.** Goat milk cheese treated with IMS. Screening 1 (New EHEC marker):
747 comparison of methods A, B and C on 280 samples positive for both *stx* and *eae*
748 genes. *Stx*⁺ for samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples
749 giving a positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for
750 *espK* and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or
751 *espV*, *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*,
752 *espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for
753 samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a
754 negative result for *espK* and *ureD*. Sector ❶ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ❷
755 (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺), sector ❸ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺,
756 *espK/espV*⁺, *espK/ureD*⁺), sector ❹ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector
757 ❺ (*stx*⁺, *eae*⁺, *espK/espV*⁺), sector ❻ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector
758 ❼ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ❽ (*stx*⁺, *eae*⁺). [*n=x*] is the number of
759 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
760 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. N=x is the total number of
761 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for
762 each sector the number of presumptive positives based on the serogroup/ *eae*
763 variant correlation. '-' means negative. Note that some samples may be presumptive
764 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
765 strains (n=x) isolated during the confirmation step is reported for each sector.

766

767 **Figure 5.** Sheep milk treated with IMS. Screening 1 (New EHEC marker):
768 comparison of methods A, B and C on 83 samples positive for both *stx* and *eae*
769 genes. *Stx*⁺ for samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples
770 giving a positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for
771 *espK* and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or
772 *espV*, *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*,
773 *espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for
774 samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a
775 negative result for *espK* and *ureD*. Sector ❶ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ❷
776 (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺), sector ❸ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺,
777 *espK/espV*⁺, *espK/ureD*⁺), sector ❹ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector
778 ❺ (*stx*⁺, *eae*⁺, *espK/espV*⁺), sector ❻ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector
779 ❼ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ❽ (*stx*⁺, *eae*⁺). [*n=x*] is the number of
780 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
781 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. N=x is the total number of
782 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for
783 each sector the number of presumptive positives based on the serogroup/ *eae*
784 variant correlation. '-' means negative. Note that some samples may be presumptive
785 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
786 strains (n=x) isolated during the confirmation step is reported for each sector.

787

788 **Figure 6.** Sheep milk cheese treated without IMS. Screening 1 (New EHEC marker):
789 comparison of methods A, B and C on 160 samples positive for both *stx* and *eae*
790 genes. *Stx*⁺ for samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples
791 giving a positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for

792 *espK* and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or
793 *espV*, *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*,
794 *espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for
795 samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a
796 negative result for *espK* and *ureD*. Sector ❶ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ❷
797 (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺), sector ❸ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺,
798 *espK/espV*⁺, *espK/ureD*⁺), sector ❹ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector
799 ❺ (*stx*⁺, *eae*⁺, *espK/espV*⁺), sector ❻ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector
800 ❼ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ❽ (*stx*⁺, *eae*⁺). [*n=x*] is the number of
801 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
802 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. *N=x* is the total number of
803 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for
804 each sector the number of presumptive positives based on the serogroup/ *eae*
805 variant correlation. '-' means negative. Note that some samples may be presumptive
806 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
807 strains (*n=x*) isolated during the confirmation step is reported for each sector.

808

809 **Figure 7.** Cow milk treated with IMS. Screening 1 (New EHEC marker): comparison
810 of methods A, B and C on 127 samples positive for both *stx* and *eae* genes. *Stx*⁺ for
811 samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples giving a
812 positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for *espK*
813 and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or *espV*,
814 *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*, *espK/Z2098*⁻
815 for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for samples
816 giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a negative

817 **result for *espK* and *ureD*.** Sector ❶ (stx^+ , eae^+ , $espK/Z2098^+$), sector ❷ (stx^+ , eae^+ ,
818 $espK/Z2098^+$, $espV^+$), sector ❸ (stx^+ , eae^+ , $espK/Z2098^+$, $espK/espV^+$, $espK/ureD^+$),
819 sector ❹ (stx^+ , eae^+ , $espK/Z2098^+$, $espK/ureD^+$), sector ❺ (stx^+ , eae^+ , $espK/espV^+$),
820 sector ❻ (stx^+ , eae^+ , $espK/espV^+$, $espK/ureD^+$), sector ❼ (stx^+ , eae^+ , $espK/ureD^+$)
821 and sector ❽ (stx^+ , eae^+). [$n=x$] is the number of samples that tested positive for the
822 CRISPR_{O26:H11} PCR assay detecting the O26 strains negative for both *espK*, *espV*,
823 *Z2098* and *ureD*. $N=x$ is the total number of samples per sector. Screening 2 (O-
824 groups / *eae*-subtypes): the table indicates for each sector the number of
825 presumptive positives based on the serogroup/ *eae* variant correlation. ‘-’ means
826 **negative**. Note that some samples may be presumptive positive for more than one
827 EHEC serogroup. Confirmation: the number of EHEC strains ($n=x$) isolated during
828 the confirmation step is reported for each sector.

829

830 **Figure 8.** Cow milk cheese treated with IMS. Screening 1 (New EHEC marker):
831 comparison of methods A, B and C on 114 samples positive for both *stx* and *eae*
832 genes. Stx^+ for samples giving a positive result for *stx1* and/or *stx2*, eae^+ for samples
833 giving a positive result for *eae*, $espK/Z2098^+$ for samples giving a positive result for
834 *espK* and/or *Z2098*, $espK/espV^+$ for samples giving a positive result for *espK* and/or
835 *espV*, $espK/ureD^+$ for samples giving a positive result for *espK* and/or *ureD*,
836 ***espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for**
837 **samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a**
838 **negative result for *espK* and *ureD*.** Sector ❶ (stx^+ , eae^+ , $espK/Z2098^+$), sector ❷
839 (stx^+ , eae^+ , $espK/Z2098^+$, $espK/espV^+$), sector ❸ (stx^+ , eae^+ , $espK/Z2098^+$,
840 $espK/espV^+$, $espK/ureD^+$), sector ❹ (stx^+ , eae^+ , $espK/Z2098^+$, $espK/ureD^+$), sector
841 ❺ (stx^+ , eae^+ , $espK/espV^+$), sector ❻ (stx^+ , eae^+ , $espK/espV^+$, $espK/ureD^+$), sector

842 ⑦ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ⑧ (*stx*⁺, *eae*⁺). [*n=x*] is the number of
843 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
844 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. N=*x* is the total number of
845 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for
846 each sector the number of presumptive positives based on the serogroup/ *eae*
847 variant correlation. '-' means negative. Note that some samples may be presumptive
848 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
849 strains (*n=x*) isolated during the confirmation step is reported for each sector.

850

851 **Figure 9.** Cow milk cheese treated without IMS. Screening 1 (New EHEC marker):
852 comparison of methods A, B and C on 239 samples positive for both *stx* and *eae*
853 genes. *Stx*⁺ for samples giving a positive result for *stx1* and/or *stx2*, *eae*⁺ for samples
854 giving a positive result for *eae*, *espK/Z2098*⁺ for samples giving a positive result for
855 *espK* and/or *Z2098*, *espK/espV*⁺ for samples giving a positive result for *espK* and/or
856 *espV*, *espK/ureD*⁺ for samples giving a positive result for *espK* and/or *ureD*,
857 *espK/Z2098*⁻ for samples giving a negative result for *espK* and *Z2098*, *espK/espV*⁻ for
858 samples giving a negative result for *espK* and *espV*, *espK/ureD*⁻ for samples giving a
859 negative result for *espK* and *ureD*. Sector ① (*stx*⁺, *eae*⁺, *espK/Z2098*⁺), sector ②
860 (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/espV*⁺), sector ③ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺,
861 *espK/espV*⁺, *espK/ureD*⁺), sector ④ (*stx*⁺, *eae*⁺, *espK/Z2098*⁺, *espK/ureD*⁺), sector
862 ⑤ (*stx*⁺, *eae*⁺, *espK/espV*⁺), sector ⑥ (*stx*⁺, *eae*⁺, *espK/espV*⁺, *espK/ureD*⁺), sector
863 ⑦ (*stx*⁺, *eae*⁺, *espK/ureD*⁺) and sector ⑧ (*stx*⁺, *eae*⁺). [*n=x*] is the number of
864 samples that tested positive for the CRISPR_{O26:H11} PCR assay detecting the O26
865 strains negative for both *espK*, *espV*, *Z2098* and *ureD*. N=*x* is the total number of
866 samples per sector. Screening 2 (O-groups / *eae*-subtypes): the table indicates for

867 each sector the number of presumptive positives based on the serogroup/ *eae*
868 variant correlation. '-' means negative. Note that some samples may be presumptive
869 positive for more than one EHEC serogroup. Confirmation: the number of EHEC
870 strains (n=x) isolated during the confirmation step is reported for each sector.

871

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874

Figure 1. Proportion (percentage) of the *stx1* and *stx2* genes in 1,451 *stx+/eae+* dairy samples. Distribution according to each dairy category sample

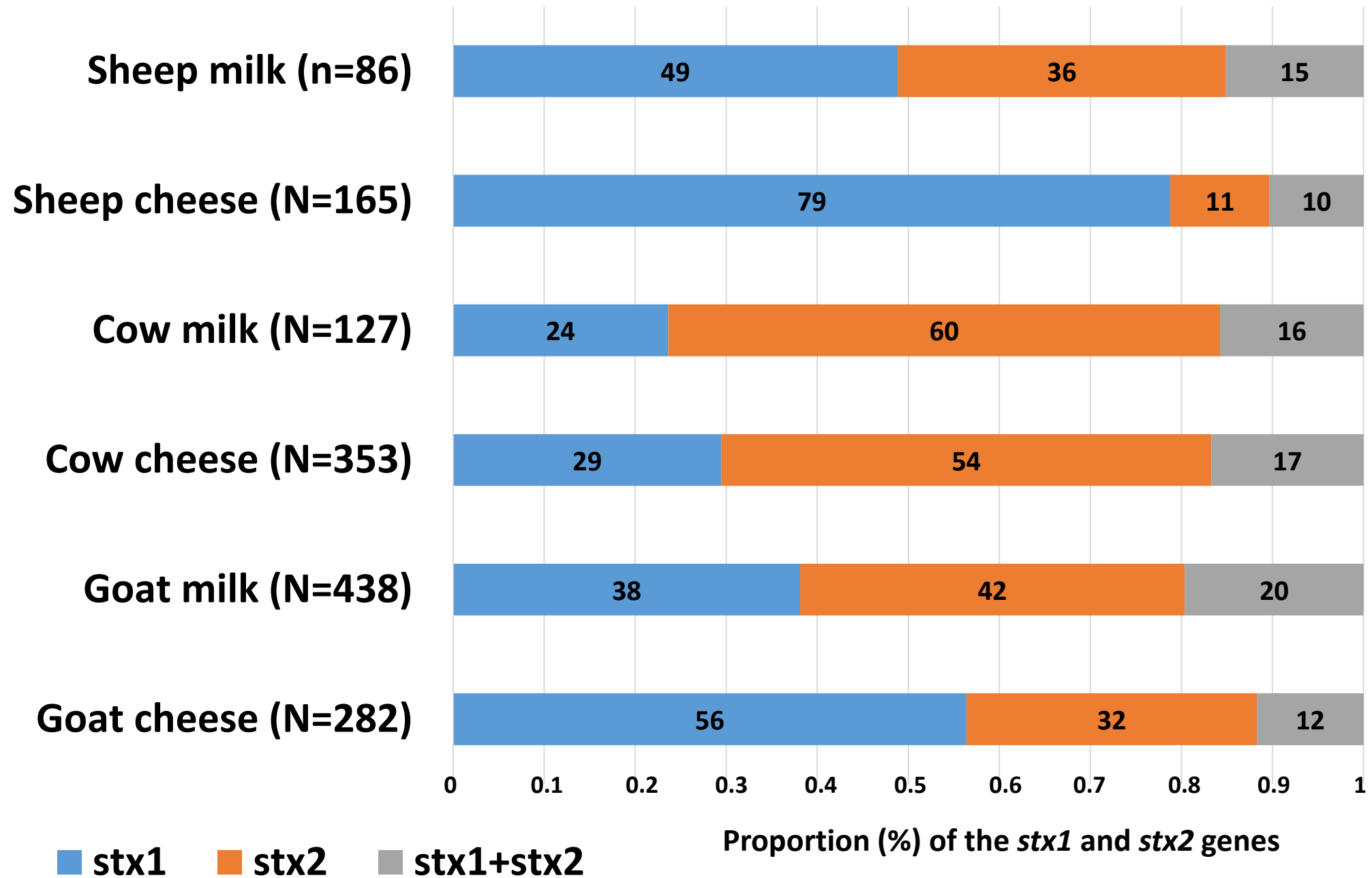
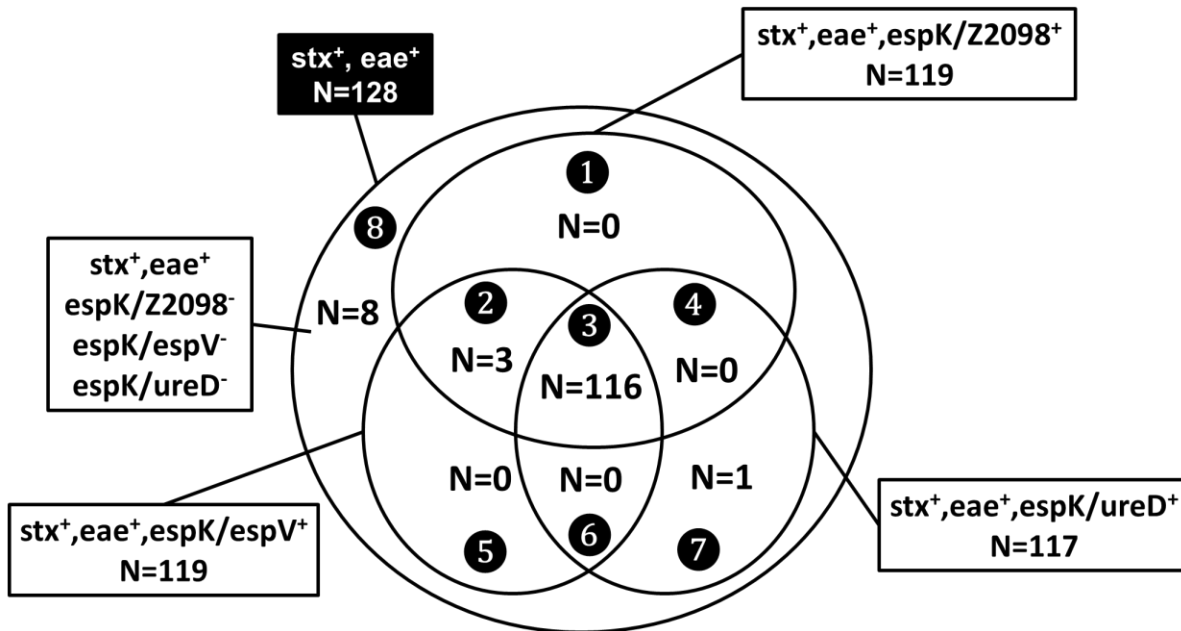


Figure 2. Goat milk / With IMS treatment

**Screening 1:
New EHEC markers**



**Screening 2:
O-groups / eae-types**

Sector	Presumptive positive for EHEC serogroups
①	-
②	O26 (n=1)
③	O45 (n=4), O121 (n=1), O111 (n=5), O145 (n=4), O103 (n=41), O26 (n=29), O157 (n=45)
④	-
⑤	-
⑥	-
⑦	-
⑧	O80 (n=1)

**Confirmation:
EHEC strains isolated**

EHEC strains (n=72) isolated from sector ③

- O157 stx+ eae+ (n=28)
- O26 stx+ eae+ (n=22)
- O103 stx+ eae+ (n=20)
- O145 stx+ eae+ (n=2)

Number of Presumptive Positives for each method :

Method A (stx+, eae+, espK/Z2098/CRISPR_{O26:H11}+) : 119

Method B (stx+, eae+, espK/espV/CRISPR_{O26:H11}+) : 119

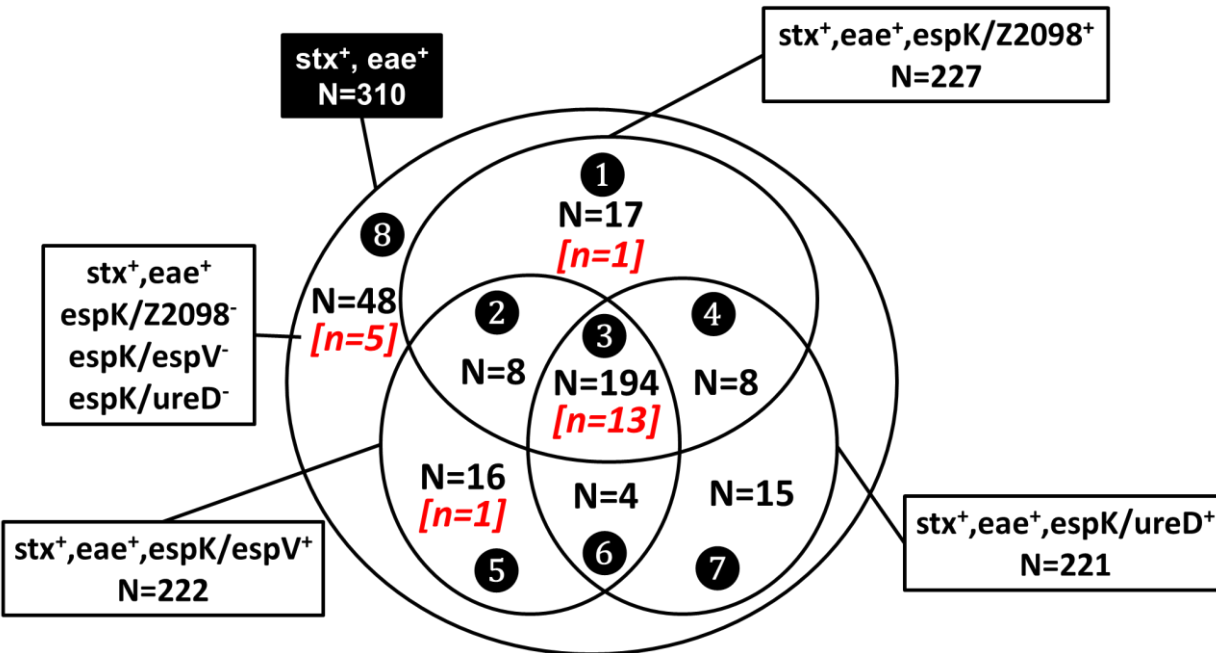
Method C (stx+, eae+, espK/ureD/CRISPR_{O26:H11}+) : 117

Figure 3. Goat milk / Without IMS treatment

**Screening 1:
New EHEC markers**

**Screening 2:
O-groups / eae-types**

**Confirmation:
EHEC strains isolated**



[CRISPR_{O26:H11}]

Number of Presumptive Positives for each method :

Method A (stx⁺, eae⁺, espK/Z2098/CRISPR_{O26:H11}⁺) : 233

Method B (stx⁺, eae⁺, espK/espV/CRISPR_{O26:H11}⁺) : 228

Method C (stx⁺, eae⁺, espK/ureD/CRISPR_{O26:H11}⁺) : 228

Sector	Presumptive positive for EHEC serogroups
1	O26 (n=1), O157 (n=1)
2	O26 (n=1)
3	O80 (n=8), O45 (n=14), O121 (n=3), O145 (n=16), O103 (n=23), O26 (n=47), O157 (n=18)
4	-
5	O45 (n=1), O103 (n=2)
6	O121 (n=1), O103 (n=1)
7	O26 (n=1)
8	O26 (n=2)

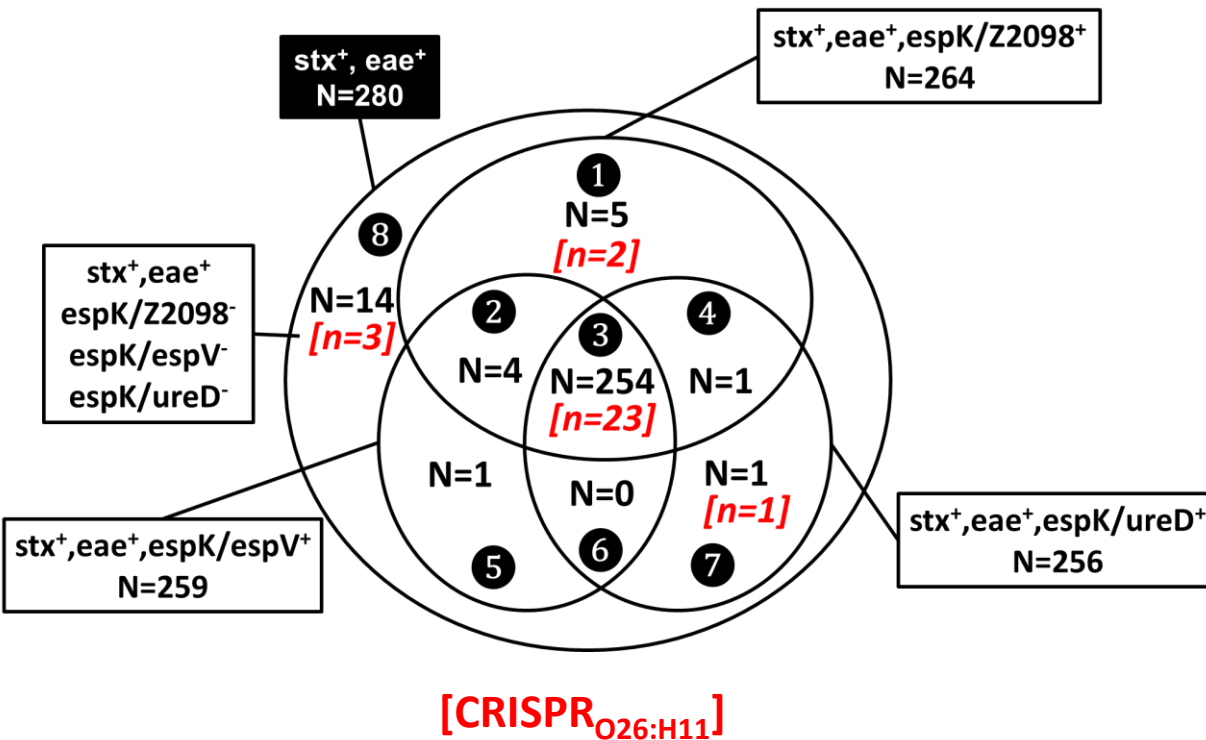
EHEC strains (n=2) isolated from sector 3

O157 stx⁺ eae⁺ (n=1)

O103 stx⁺ eae⁺ (n=1)

Figure 4. Goat milk cheese / With IMS treatment

**Screening 1:
New EHEC markers**



Number of Presumptive Positives for each method :

Method A (stx+, eae+, espK/Z2098/CRISPR_{O26:H11}+) : 268

Method B (stx+, eae+, espK/espV/CRISPR_{O26:H11}+) : 265

Method C (stx+, eae+, espK/ureD/CRISPR_{O26:H11}+) : 261

**Screening 2:
O-groups / eae-types**

Sector	Presumptive positive for EHEC serogroups
①	O103 (n=1), O26 (n=2)
②	O103 (n=1)
③	O80 (n=1), O45 (n=21), O121 (n=3), O111 (n=1), O145 (n=93), O103 (n=95), O26 (n=68), O157 (n=19)
④	-
⑤	-
⑥	-
⑦	O26 (n=1)
⑧	O26 (n=6), O157 (n=1)

**Confirmation:
EHEC strains isolated**

EHEC strains (n=162) isolated from sector ③

O157 stx+ eae+ (n=16)

O26 stx+ eae+ (n=32)

O103 stx+ eae+ (n=49)

O145 stx+ eae+ (n=64)

O111 stx+ eae+ (n=1)

EHEC strain (n=1) isolated from sector ①

O103 stx+ eae+ (n=1)

EHEC strain (n=1) isolated from sector ②

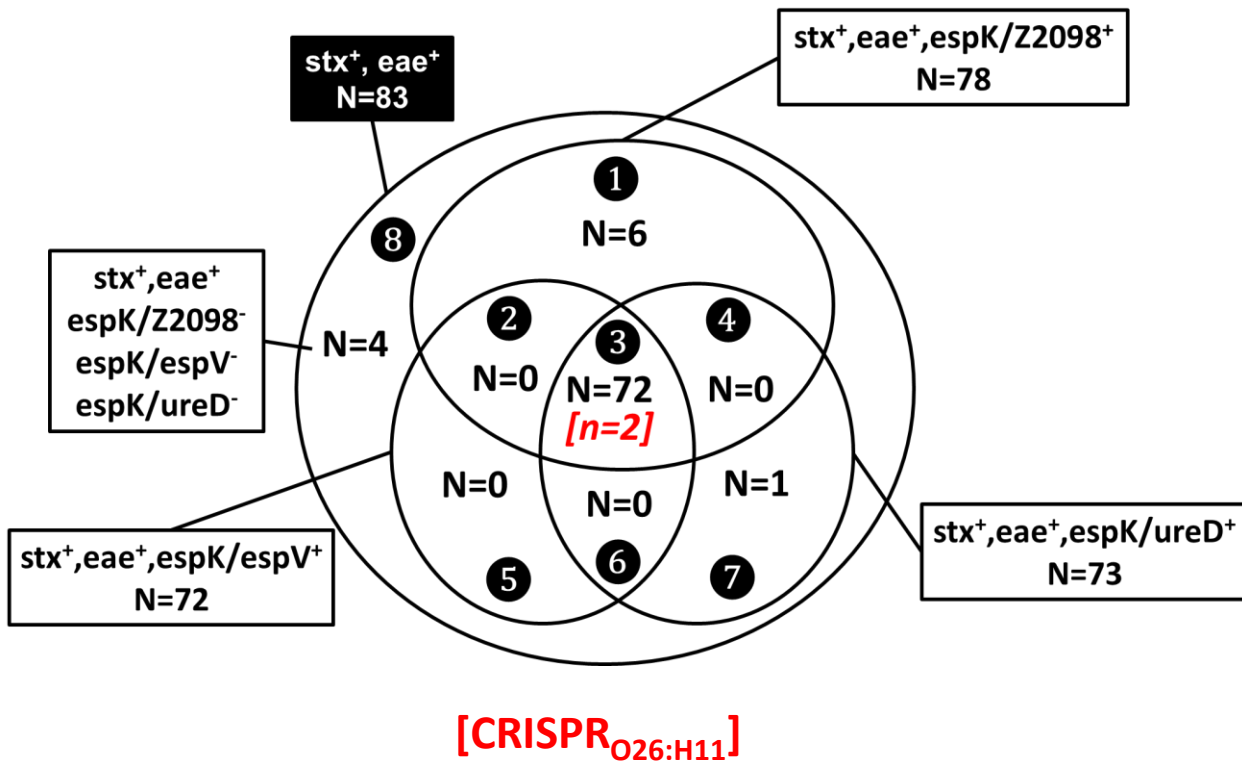
O103 stx+ eae+ (n=1)

EHEC strain (n=1) isolated from sector ⑧

O157 stx+ eae+ (n=1)

Figure 5. Sheep milk / With IMS treatment

**Screening 1:
New EHEC markers**



Number of Presumptive Positives for each method :

Method A (stx+, eae+, espK/Z2098+/CRISPR_{O26:H11}+) : 78

Method B (stx+, eae+, espK/espV+/CRISPR_{O26:H11}+) : 72

Method C (stx+, eae+, espK/ureD+/CRISPR_{O26:H11}+) : 73

**Screening 2:
O-groups / eae-types**

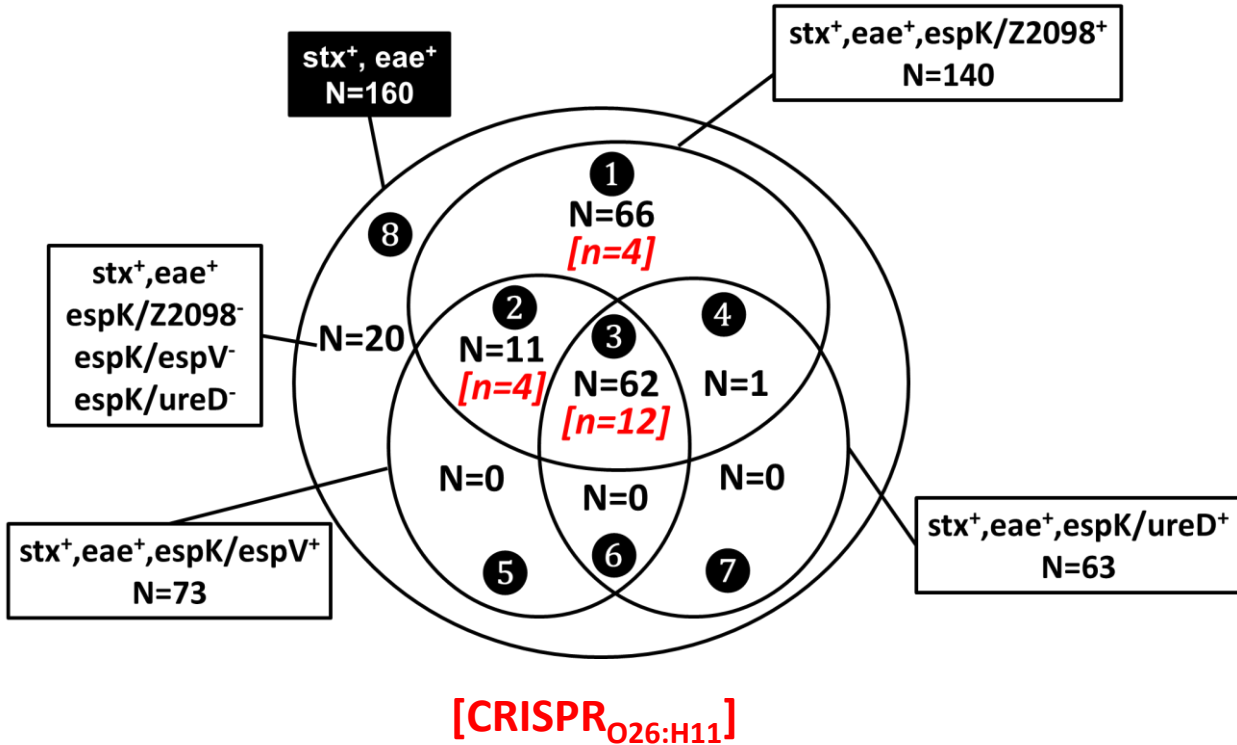
Sector	Presumptive positive for EHEC serogroups
①	-
②	-
③	O45 (n=2), O121 (n=2), O145 (n=5), O103 (n=7), O26 (n=27), O157 (n=25)
④	-
⑤	-
⑥	-
⑦	-
⑧	-

**Confirmation:
EHEC strains isolated**

No EHEC strains where isolated during the confirmation step

Figure 6. Sheep milk cheese/ Without IMS treatment

**Screening 1:
New EHEC markers**



Number of Presumptive Positives for each method :

Method A (stx+, eae+, espK/Z2098+/CRISPR_{O26:H11}+) : 140

Method B (stx+, eae+, espK/espV+/CRISPR_{O26:H11}+) : 73

Method C (stx+, eae+, espK/ureD+/CRISPR_{O26:H11}+) : 63

**Screening 2:
O-groups / eae-types**

Sector	Presumptive positive for EHEC serogroups
①	O26 (n=4)
②	O26 (n=5)
③	O80 (n=3), O121 (n=1), O145 (n=2), O103 (n=5), O26 (n=38), O157 (n=2)
④	-
⑤	-
⑥	-
⑦	-
⑧	O26 (n=1)

**Confirmation:
EHEC strains isolated**

EHEC strains (n=2) isolated from sector ③

O26 stx+ eae+ (n=1)

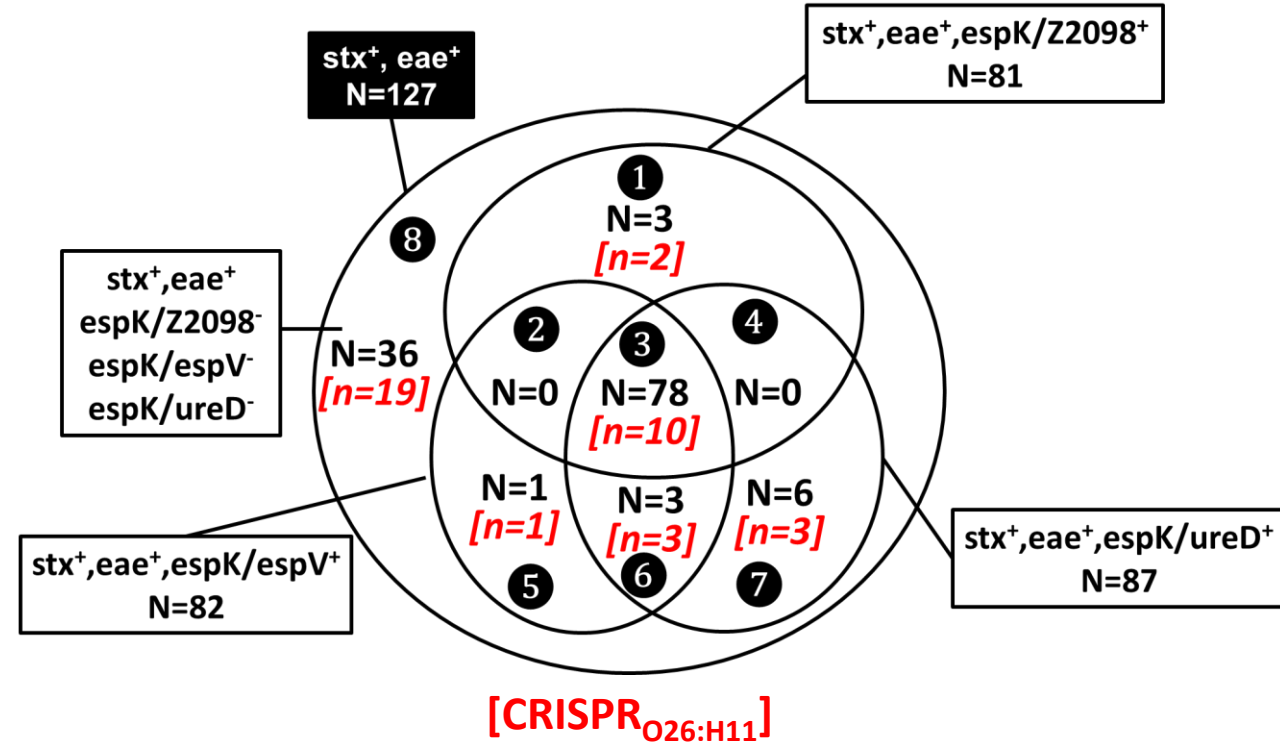
O103 stx+ eae+ (n=1)

Figure 7. Cow milk / With IMS treatment

**Screening 1:
New EHEC markers**

**Screening 2:
O-groups / eae-types**

**Confirmation:
EHEC strains isolated**



Number of Presumptive Positives for each method :

Method A (stx+, eae+, espK/Z2098/CRISPR_{O26:H11}+) : 107

Method B (stx+, eae+, espK/espV/CRISPR_{O26:H11}+) : 106

Method C (stx+, eae+, espK/ureD/CRISPR_{O26:H11}+) : 109

Sector	Presumptive positive for EHEC serogroups
①	O26 (n=1)
②	-
③	O80 (n=16), O45 (n=9), O121 (n=4), O111 (n=8), O145 (n=6), O103 (n=12), O26 (n=22), O157 (n=3)
④	-
⑤	-
⑥	O26 (n=3)
⑦	O26 (n=3)
⑧	O26 (n=14), O157 (n=1))

EHEC strains (n=17) isolated from secteur ③

O157 stx+ eae+ (n=1)

O26 stx+ eae+ (n=7)

O103 stx+ eae+ (n=3)

O145 stx+ eae+ (n=2)

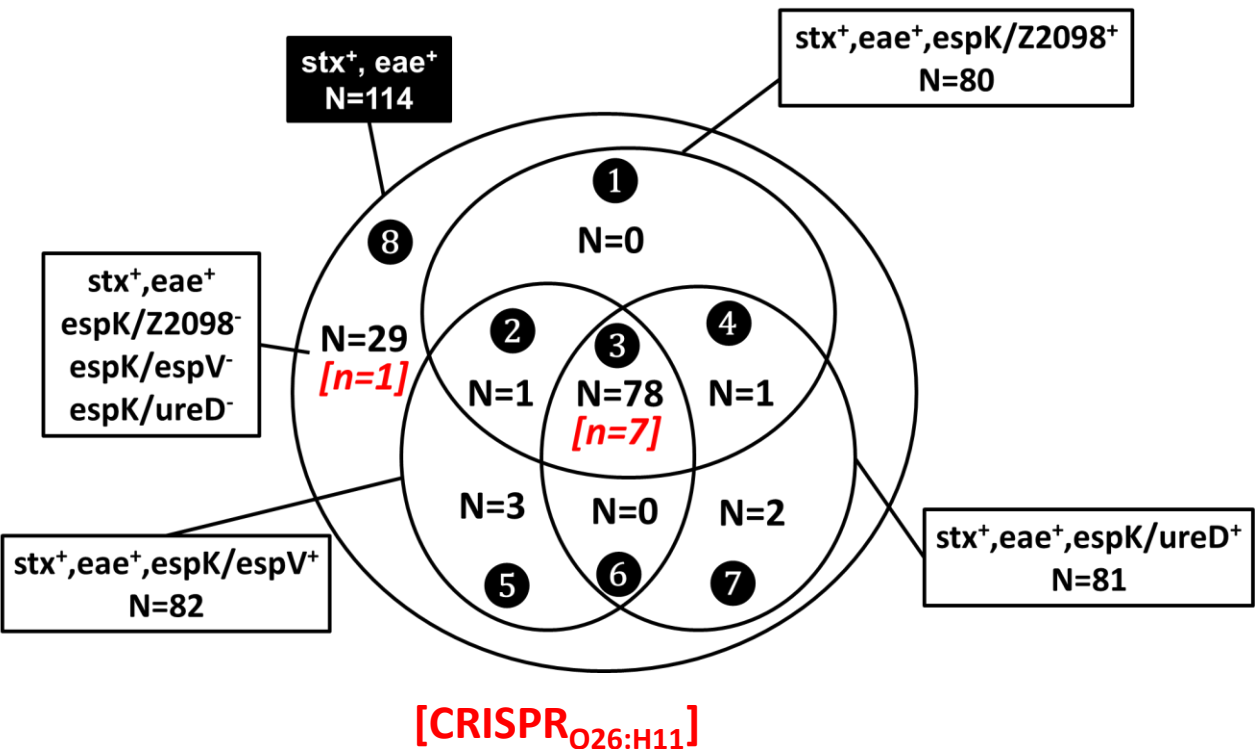
O111 stx+ eae+ (n=4)

Figure 8. Cow milk Cheese / With IMS treatment

**Screening 1:
New EHEC markers**

**Screening 2:
O-groups / eae-types**

**Confirmation:
EHEC strains isolated**



Sector	Presumptive positive for EHEC serogroups
1	-
2	-
3	O80 (n=11), O45 (n=4), O121 (n=7), O145 (n=2), O103 (n=23), O26 (n=30), O157 (n=5)
4	O111 (n=1)
5	O26 (n=1)
6	-
7	O157 (n=1)
8	O80 (n=3), O111 (n=1), O103 (n=1), O26 (n=5)

EHEC strains (n=30) isolated from sector 3

O157 stx+ eae+ (n=2)

O26 stx+ eae+ (n=17)

O103 stx+ eae+ (n=11)

Number of Presumptive Positives for each method :

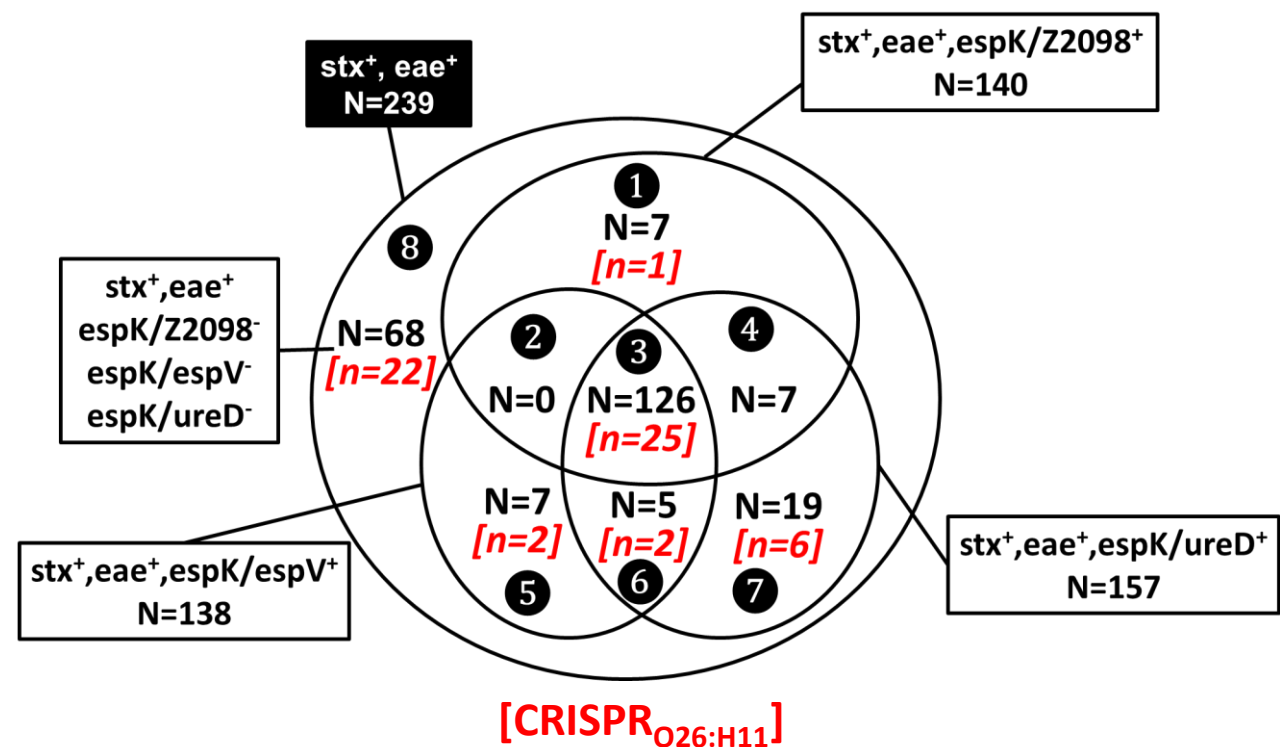
Method A (stx+, eae+, espK/Z2098+/CRISPR_{O26:H11}+) : 81

Method B (stx+, eae+, espK/espV+/CRISPR_{O26:H11}+) : 83

Method C (stx+, eae+, espK/ureD+/CRISPR_{O26:H11}+) : 82

Figure 9. Cow milk Cheese / Without IMS treatment

Screening 1: New EHEC markers



Number of Presumptive Positives for each method :

Method A (stx⁺, eae⁺, espK/Z2098/CRISPR_{O26:H11}⁺) : 172

Method B (stx⁺, eae⁺, espK/espV/CRISPR_{O26:H11}⁺) : 167

Method C (stx⁺, eae⁺, espK/ureD/CRISPR_{O26:H11}⁺) : 182

Screening 2: O-groups / eae-types

Sector	Presumptive positive for EHEC serogroups
①	O26 (n=1), O157 (n=1)
②	-
③	O80 (n=46), O45 (n=20), O121 (n=8), O111 (n=4), O145 (n=4), O103 (n=22), O26 (n=37), O157 (n=4)
④	O45 (n=2), O103 (n=2), O26 (n=2)
⑤	O26 (n=2)
⑥	O26 (n=2)
⑦	O80 (n=2), O45 (n=2), O121 (n=1), O103 (n=2), O26 (n=6)
⑧	O80 (n=3), O45 (n=2), O121 (n=1), O103 (n=2), O26 (n=24)

Confirmation: EHEC strains isolated

EHEC strains (n=8) isolated from sector ③

O157 stx⁺ eae⁺ (n=2)

O26 stx⁺ eae⁺ (n=3)

O103 stx⁺ eae⁺ (n=1)

O145 stx⁺ eae⁺ (n=1)

O111 stx⁺ eae⁺ (n=1)

EHEC strains (n=1) isolated from secteur ⑦

O26 stx⁺ eae⁺ (n=1)

Table 1. Primer and probe sequences used in this study.

Primer or probe ^a	Sequence 5' → 3' ^b	Chip code
<i>stx1</i> _F ^c	TTTGTYACTGTSACAGCWGAAGCYTTACG	1
<i>stx1</i> _R ^c	CCCCAGTTCARWGTRAGRTC MACRTC	1
<i>stx1</i> _P ^c	CTGGATGATCTCAGTGGGCGTTCTTATGTAA	1
<i>stx2</i> _F ^c	TTTGTYACTGTSACAGCWGAAGCYTTACG	1
<i>stx2</i> _R ^c	CCCCAGTTCARWGTRAGRTC MACRTC	1
<i>stx2</i> _P ^c	TCGTCAGGCACTGTCTGAAACTGCTCC	1
<i>eae</i> _F ^d	CATTGATCAGGATTTTTCTGGTGATA	1
<i>eae</i> _R ^d	CTCATGCGGAAATAGCCGTTA	1
<i>eae</i> _P ^d	ATAGTCTCGCCAGTATTCGCCACCAATACC	1
<i>eae</i> -beta_F ^d	GGTGATAATCAGAGTGCGACATACA	1
<i>eae</i> -beta_R ^d	GGCATCAAATACGTA ACTCGAGTAT	1
<i>eae</i> -beta_P ^d	CCACAGCAATTACAATACTACCCGGTGCA	1
<i>eae</i> -gamma_F ^d	GACTGTTAGTGCGACAGTCAGTGA	1
<i>eae</i> -gamma_R ^d	TTGTTGTCAATTTTCAGTTCATCAA	1
<i>eae</i> -gamma_P ^d	TGACCTCAGTCGCTTTAACCTCAGCC	1
<i>eae</i> -epsilon_F ^d	ATACCCAAATTGTGAAAACGGATA	1
<i>eae</i> -epsilon_R ^d	CACTAACAACAGCATTACCTGCAA	1
<i>eae</i> -epsilon_P ^d	CCAGATGTCAGTTTTACCGTAGCCCTACCA	1
<i>eae</i> -theta_F ^d	TGTTAAAGCACCTGAGGTTACATTTT	1
<i>eae</i> -theta_R ^d	TCACCAGTAACGTTCTTACCAAGAA	1
<i>eae</i> -theta_P ^d	TCAACCTTGTTGTCAATTTTCAGTCCATCA	1
<i>espK</i> _F ^e	GCAGRCATCAAAGCGAAATCACACC	1
<i>espK</i> _R ^e	TCGTTTGGTAACTGTGGCAGATACTC	1
<i>espK</i> _P ^e	ATTCAGATAGAAGAAGCGCGGGCCAG	1
<i>espV</i> _F ^e	TCAGGTTCTCGTCTGATGCCGC	1
<i>espV</i> _R ^e	CTGGTTCAGGCCTGGAGCAGTCC	1
<i>espV</i> _P ^e	CTTGCAACACGTTACGCTGCCGAGTATT	1
Z2098_F ^f	CTGAAAAGAGCCAGAACGTGC	1
Z2098_R ^f	TGCCTAAGATCATTACCCGGAC	1

<i>Z2098_P^f</i>	TAACTGCTATACCTCCGCGCCG	1
<i>ureD_F^e</i>	GCAATAATTGACTCTGATTGCC	1
<i>ureD_R^e</i>	GCTGCTGCGGTAAAATTTACT	1
<i>ureD_P^e</i>	TACGCTGATCACCATGCCTGGTGC	1
CRISPR _{O26:H11_F^g}	AAACCGATCTCCTCATCCTC	1
CRISPR _{O26:H11_R^h}	ATCAACATGCAGCGCGAACG	1
CRISPR _{O26:H11_P^g}	CCAGCTACCGACAGTAGTGTGTTCC	1
<i>Stx2a_F</i>	TTCTGTTAATGCAATGGCGGCG	1
<i>Stx2a_R</i>	CCAGTATTCTTTCCCGTCAACCTTC	1
<i>Stx2a_P</i>	AATGTGTCATCCTCATTATACTTGG	1
<i>rfbE_{O157}-F^c</i>	TTTCACACTTATTGGATGGTCTCAA	2
<i>rfbE_{O157}-R^c</i>	CGATGAGTTTATCTGCAAGGTGAT	2
<i>rfbE_{O157}-P^c</i>	AGGACCGCAGAGGAAAGAGAGGAATTAAGG	2
<i>wzx_{O26}-F^c</i>	CGCGACGGCAGAGAAAATT	2
<i>wzx_{O26}-R^c</i>	AGCAGGCTTTTATATTCTCCAACCTT	2
<i>wzx_{O26}-P^c</i>	CCCCGTAAATCAATACTATTTACAGAGGTTGA	2
<i>wzx_{O103}-Fⁱ</i>	CAAGGTGATTACGAAAATGCATGT	2
<i>wzx_{O103}-Rⁱ</i>	GAAAAAAGCACCCCCGTACTTAT	2
<i>wzx_{O103}-Pⁱ</i>	CATAGCCTGTTGTTTTAT	2
<i>wbd_{O111}-F^c</i>	CGAGGCAACACATTATATAGTGCTTT	2
<i>wbd_{O111}-R^c</i>	TTTTTGAATAGTTATGAACATCTTGTTTAGC	2
<i>wbd_{O111}-P^c</i>	TTGAATCTCCCAGATGATCAACATCGTGAA	2
<i>wzx_{O121}-F^j</i>	TGGTCTCTTAGACTTAGGGC	2
<i>wzx_{O121}-R^j</i>	TTAGCAATTTTCTGTAGTCCAGC	2
<i>wzx_{O121}-P^j</i>	TCCAACAATTGGTCGTGAAACAGCTCG	2
<i>wzx_{O45}-F^j</i>	TACGTCTGGCTGCAGGG	2
<i>wzx_{O45}-R^j</i>	ACTTGCAGCAAAAAATCCCC	2
<i>wzx_{O45}-P^j</i>	TTCGTTGCGTTGTGCATGGTGGC	2
<i>wzy_{O145}-F^k</i>	ATATTGGGCTGCCACTGATGGGAT	2
<i>wzy_{O145}-R^k</i>	TATGGCGTACAATGCACCGCAAAC	2
<i>wzy_{O145}-P^k</i>	AGCAGTGGTTCGCGCACAGCATGGT	2
<i>wzx_{O80}-F</i>	CAGTTATACCGATCCTTAATTTACAAGGA	2
<i>wzx_{O80}-R</i>	GCTTACAAAAGACACTGGAATTATAATTCC	2

wZX080-P	CGCAGGGTTATCGATTTTGGGTGCTACT	2
fliC H28 _[O145] -F ^l	AATCATTGTAGCTTTATTGTAGGTGTAGTCT	2
fliC H28 _[O145] -R ^l	ATGGTGCTGTTGTTAATGCTAGCA	2
fliC H28 _[O145] -P ^l	AGCTGCTGCACCAAACCGTTGGAA	2
CRISPR _{O157:H7} B_F ^g	GGGAACACAAACCGAAACACA	2
CRISPR _{O157:H7} B_R ^g	CTTAGTGTGTTCCCCGCGC	2
CRISPR _{O157:H7} B_P ^g	CGATCAATCCGAATATGAGCGGT	2
CRISPR _{O157:H7} C_F ^g	GAACACTTTGGTGACAGTTTTTGT	2
CRISPR _{O157:H7} C_R ^g	CTTAGTGTGTTCCCCGCGC	2
CRISPR _{O157:H7} C_P ^g	CACTGTTTTGGTGACGGTTTATCC	2
<i>Stx1a</i> _F	ATGGACAAGACTCTGTTTCGTGTA	2
<i>Stx1a</i> _R	AATTCAGTATTAATGCCACGCTT	2
<i>Stx1a</i> _P	CCAGAATTGCATTAATGCTTCCAAAAGAA	2

^a F, forward primer; R, reverse primer; P, probe.

^b All probes were labeled with 6-HEX or 6-FAM and BHQ1 (Black Hole Quencher).

^c Oligonucleotide described by Perelle et al. (2004),

^d Oligonucleotide described by Nielsen and Andersen (2003),

^e Oligonucleotide described by Delannoy et al. (2013a),

^f Oligonucleotide described by Delannoy et al. (2013b),

^g Oligonucleotide described by Delannoy et al. (2012a),

^h Oligonucleotide described by Delannoy et al. (2015a),

ⁱ Oligonucleotide described by Perelle et al. (2005),

^j Oligonucleotide described by Bugarel et al. (2010),

^k Oligonucleotide described by Fratamico et al. (2009).

^l Oligonucleotide described by Beutin et al. (2015).

Table 2. Dairy samples (n=4,691) selected in this study. Distribution of the number of samples according to the animal species (cow, goat and sheep), the dairy matrices (milk, cheese) and the IMS pre-treatment of the enriched culture.

Animal species	Dairy matrice	With IMS pre-treatment	Without IMS pre-treatment
Goat samples (N=1,601)	Milk	165	1025
	Cheese	408	3
Sheep samples (N=965)	Milk	331	7
	Cheese	13	614
Cow samples (N=2,125)	Milk	926	77
	Cheese	304	818

Table 3. Dairy samples treated with IMS and tested *stx+/eae+* (n=737). Reduction of the number of presumptive positives by introducing new EHEC markers (method B) in the first screening phase of the ISO/TS 13136 reference method. ^a two-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with method B was significantly different from the ISO/TS 13136 reference method when p -value < 0.05. ^b one-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with the ISO/TS 13136 reference method was significantly greater than method B when p -value < 0.05. N/A for not applicable.

Animal species	Dairy matrice	ISO Method (<i>stx+</i> , <i>eae+</i>)	Method B (<i>stx+</i> , <i>eae+</i> , <i>espK/espV/CRISPR</i> _{O26:H11+})	p -value ^a	Reduction of the number of presumptive positives	p -value ^b
Goat samples (N=408)	Milk	128	119	0.2535	7.03%	N/A
	Cheese	280	265	0.2649	5.35%	N/A
Sheep samples (N=88)	Milk	83	72	0.3127	13.25%	N/A
	Cheese	5	3	N/A	N/A	N/A
Cow samples (N=241)	Milk	127	106	0.1412	16.5%	N/A
	Cheese	114	83	0.007224	27.2%	0.003612

Table 4. Dairy samples treated without IMS and tested *stx+*/*eae+* (n=714). Reduction of the number of presumptive positives by introducing new EHEC markers (method B) in the first screening phase of the ISO/TS 13136 reference method. ^a two-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with method B was significantly different from the ISO/TS 13136 reference method when p -value < 0.05. ^b one-tailed two-proportions z-test, $\alpha = 0.05$. The proportion of presumptive positives with the ISO/TS 13136 reference method was significantly greater than method B when p -value < 0.05. N/A for not applicable.

Animal species	Dairy matrice	ISO Method (<i>stx+</i> , <i>eae+</i>)	Method B (<i>stx+</i> , <i>eae+</i> , <i>espK/espV/CRISPR</i> _{O26:H11+})	p -value ^a	Reduction of the number of presumptive positives	p -value ^b
Goat samples (N=312)	Milk	310	228	3.847e-05	26.5%	1.924e-05
	Cheese	2	0	N/A	N/A	N/A
Sheep samples (N=163)	Milk	3	3	N/A	N/A	N/A
	Cheese	160	77	2.423e-10	51.9%	1.211e-10
Cow samples (N=239)	Milk	0	0	N/A	N/A	N/A
	Cheese	239	167	3.771e-05	29.7%	1.886e-05