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Evaluation of Larger Test Portion Sizes for *Escherichia coli* Shiga Toxin Producer (STEC) on the Detection by Immunomagnetic Separation and Real-Time PCR in Meat and Vegetables

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Abstract

The aims of this study were (i) to evaluate the performance of the Assurance[®] GDS method combining immunomagnetic separation and real-time PCR for STEC detection in pooled samples (up to 375g) of vegetables and meat, and (ii) to compare its performances to that of the reference method ISO/TS-13136:2012 (25 g sample size) in artificially contaminated samples. The alternative Assurance[®] GDS method includes enrichment in proprietary broth at 41.5°C for 10 h followed by primary screening of TOP 7 STEC containing *eae*, *stx* genes and O157:H7 markers using MPX-Top 7 (IMS+PCR), secondary screening for serogroup identification using MPX-ID/EHEC-ID (IMS+PCR). And finally, cultural confirmation from same IMS beads on two selective agars is set up. For reference method, the enrichment was performed in BPW at 41.5°C for 18 h. A total of 120 samples of meat and vegetables, including 20 uninoculated and 100 samples spiked with stressed cells (<12 CFU/25 or 375g) of STEC, were analyzed using both methods. Our results showed that the Assurance[®] GDS method LOD₅₀ ranged from 0.12 CFU/375 to 0.522 CFU/375 g and the LOD₉₅ from 0.518 CFU/375 to 2.257 CFU/375 g. These data are similar to the LOD50 data of the reference method.

Keywords STEC detection · Pooling · meat · Vegetable · Performance data

Introduction

Pathogenic shiga toxin-producing *Escherichia coli* (STEC) are currently recognized as one of the most public health concern agent in Europe and all the world (EFSA 2016;

Marder et al. 2018). Pathogenic STEC are major foodborne zoonotic bacteria responsible of large outbreaks of bloody diarrhea and hemolytic and uremic syndrome (HUS) that can lead to death (Bruyand et al. 2018). Indeed, in Europe, in 2021, STEC were responsible 31 foodborne outbreaks, 6084 human infections, 901 hospitalizations and 18 deaths (Authority et European Centre for Disease Prevention and Control 2022). HUS is the leading cause of renal failure in children. Children and the elderly are the most likely to develop HUS. The estimated infectious dose is very low (Betts 2000).

The pathogenesis of STEC is also complex, generally involving three phases: (i) ingestion of contaminated food, (ii) colonization of the intestinal epithelium by STEC, and (iii) production of Shiga toxins (Stx) that disrupt normal cellular functions and damage cells. The complete list of virulence factors and mechanisms involved in STEC pathogenicity is not yet fully known. Nevertheless, Shiga Toxins (Stx) are the main virulence factors of STEC. They lead to the death of the target cells by stopping protein synthesis and induce lesions of the vascular endothelium, mainly intestinal, renal and cerebral, which explains the clinical

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manifestations with renal or neurological complications. The ability to adhere to the intestinal epithelium and colonize the intestine undeniably contributes to the pathogenesis process of STEC strains. Thus, the majority of STEC known to cause bloody diarrhea or HUS have one or more virulence factors that allow their adhesion to intestinal epithelial cells. The major adhesion factor of STEC is intimin, a protein encoded by the *eae* gene that resides on the locus of an enterocyte effacement pathogenicity island (EEL). The pathophysiology of strains possessing the *eae* gene is characterized by the development of enterocyte attachment-effacement (A/E) lesions, resulting from the combined action of proteins encoded by genes clustered on the EEA. These lesions are responsible for the diarrhea observed in the patient.

The combination of genes encoding virulence factors characterizes the potential pathogenicity of STEC isolates (FAO/WHO STEC EXPERT GROUP 2019). Nevertheless, some serotypes have been more frequently associated with human infections. Thus, in 2009, based on these parameters, USDA and EFSA identified STEC serogroups of concern for human health in the European Union (EU): these are *E. coli* strains belonging to the 5 STEC serogroups (O157, O26, O103, O111 and O145), also known as the “Top 5” and possessing the *eae* and *stx* genes for EFSA (Panel et al. 2020) (FAO; 2019). For the USDA, 2 serogroups are added to the top 5 (O121 and O45) (FSIS-GD-2014-0009 2014; FSIS-GD-2021-0007 2021).

Cattle are the main reservoir of STEC (Karmali, Gannon, et Sargeant 2010; Rhades et al. 2019; Salaheen et al. 2019). Infected cattle can carry the bacteria in their gastrointestinal tract without any symptoms of disease and shed them in their faeces (Brown et al. 1997; Chapman et al. 2001; Sarimehmetoglu et al. 2009). Detailed investigations have shown that without observance of appropriate cleaning steps and udder hygiene practices, fecal matter can contaminate the cow’s teats and udders, which in turn can contaminate the milk during the milking process (ruegg 2003). Carcasses may be also contaminated during certain operations carried out at the slaughterhouse. STEC contamination and outbreaks are also associated to fresh leafy vegetables (Gelting et al. 2011; Authority et European Centre for Disease Prevention and Control 2022). Vegetables can be contaminated with water, domestic and wild animals, workers and manure-based soil amendments (Islam et al. 2004; Jay-Russell et al. 2014).

Consequently, human infection is most often linked to the ingestion of contaminated food such as undercooked ground meat, raw milk cheese, or vegetables and water.

At present, there are no food criteria for STEC at the European level in the General Food Regulation (EC) 2073/2005 (except for sprouted seeds since 2013;

Commission Regulation (EU) No. 209/2013). This is mainly because the Committee concluded that, given the low prevalence of the pathogen, the application of a standard would probably not result in significant reductions in the associated risk to the consumer. Nevertheless, the same committee indicates that microbiological guidelines to reduce fecal contamination in the food chain can help reduce public health risks. The general regulation of the food legislation (EC) N° 178/2002 stipulates that producers are responsible for food safety throughout the food chain. As a result, the food industry needs fast, sensitive and complete methods for STEC detection to validate the STEC control measures implemented throughout the chain as well as for the release of the finished products.

The detection for highly pathogenic STEC can be performed using reference methods (e.g., ISO/TS-13136:2012, USDA). Rapid alternative methods validated and certified by third parties (AFNOR validation, Microval...), following international recognized standards (e.g., ISO 16140-2:2016) may be also applied for STEC detection. Even if the available tools have evolved, there are still some gaps in the tools as well as in some industrial practices. Particular attention must be paid to the analysis of STEC within the industry are still insufficiently informed or treated in a very variable manner from one company to another. Indeed, two challenges remain to be met. In one hand it appears that some industrialists tend to combine samples in order to reduce costs by increasing the sample size. To date, little data exists on the impact of test portion pooling (dry pooling) on the analysis result. Indeed, this pooling leads to a dilution of the target bacteria in the analyzed sample. It is essential to know the performance data of the detection protocols/kits for these different sample size and demonstrate no impact on results. On the other hand, the tools developed must also be able to detect pathogenic STEC strains in samples other than dairy and meat products, such as vegetables. These matrices are very particular since they have physico-chemical characteristics that could limit the detection of pathogenic strains.

To meet these two challenges, the alternative method Assurance® GDS for STEC detection (Millipore Sigma, Bellevue, USA) includes the use of short enrichment with a selective proprietary broth (m-EHEC) and the use of two technologies combining immuno-magnetic concentration (IMS) and real-time PCR to detect and identify STEC belonging to the top 7 serogroups (O157, O103, O111, O145, O26, O45, and O121).

The aims of this study were (i) to evaluate the performance of the Assurance® GDS method for the analysis of pooled samples of vegetables and raw meat with LOD50 data (ii) to compare its performances to that of the reference method ISO/TS-13136:2012 in artificially contaminated samples.

Materials and Methods

Shiga Toxin–Producing *E. coli* Strains

The strains used were from the French national reference laboratory collection. These strains have been isolated from humans or dairy products. The strains selected belong to the O111, O145 and O157 serogroups and possessed *eae* and *stx* virulence genes (Table 1). The strains were stored at -80° in cryoballs (bioMerieux, Marcy l'étoile, France). They were cultured in buffer peptone water (BPW) (bioMerieux, Marcy l'étoile, France) for 18/24h at 37°C . In order to inoculate the food matrices, series of dilutions, from the BWP tube, were carried out in tryptone salt (TS) (bioMerieux, Marcy l'étoile, France). In parallel, enumeration on TSA agar (bioMerieux, Marcy l'étoile, France) was performed to estimate the concentration of inoculated bacteria. Agar plates are incubated for 18/24 h at 37° .

Food Samples

The food matrices were purchased directly from a supermarket. Two vegetable food items (lettuce and spinach) and two meat food items (frozen beef and refrigerated beef with 20% fat) were considered.

Experimental Contaminations

For each food item, a total of 30 test portions were experimentally contaminated at three different levels as described in the standard ISO 16140-4:2020 (International Organization for Standardisation 2020). For level 0 (L0), 5 test portions were non spiked to confirm absence of bacteria in the samples. For level 1 (L1), 20 test portions were spiked with a concentration around one CFU/test portion to obtain fractional positives. Finally, for level 2 (L2), 5 tests portions were spiked around 5 CFU/test portion to obtain positive samples. In order to mimic the storage conditions of the matrices after inoculation, the food matrices were stored for 72 h at 4°C . At last, the volume of inoculum was adjusted so that it did not significantly influence the activity of the water (0.25ml for the 25g test sample and 3.75ml for 375g of food or 1%).”

Table 1 Characterization of the three strains evaluated in artificially contaminated study

| Name | Serotype | origin | <i>eae</i> | <i>stx1</i> | <i>stx2</i> | References |
|--------|----------|---------------|-----------------|-------------|-------------|-----------------------|
| EDL933 | O157:H7 | Human | <i>eae</i> -g1, | + | + | O'Brien et al. 1984 |
| 57724 | O111:H8 | Dairy Product | + | + | + | Kerangart et al. 2016 |
| 12803 | O145:H28 | Human | + | - | + | Kerangart et al. 2016 |

Enrichment

For the enrichment step protocol of the alternative method, 375g sample size were used. Test portions were diluted 1/5 in 1,500 ml of preheated proprietary broth at 41.5° (m-EHEC) (MilliporeMerck, Darmstadt, Germany). For large test sample, 375g of food are added to the bags, after which a portion of the enrichment broth is introduced into the bag. After, we homogenize the bag with our hands. We added the rest of the enrichment medium. Note that for the plants, before being introduced into the bags, they were cut with a sterile knife. The initial suspensions were incubated at 41.5° for 10 h.

For the enrichment step protocol of the ISO/TS-13136:2012 method, 25g samples sizes were diluted 1/10 in 225ml of BPW preheated to 41.5° in a stomacher bag (interscience, StNom, France). Instead of 37°C , initial suspensions were incubated for 18 h at 41.5°C , in order to have a better recovery of the bacteria in presence of high background flora.

The comparative study was considered unpaired study according ISO 16140-4:2020 (enrichments were performed in different test portions for each method) to simulate worst scenario comparing big sample size for the alternative method, against the validated sample size for the reference method.

Detection and Confirmation Step

Alternative Assurance GDS Method

The Alternative Assurance[®] GDS method is based on the use of three different kits. First kit targets all TOP 7 STEC (Assurance GDS[®] MPX-TOP 7). The other two others kits detect the specific serogroups in the enrichment broth (Assurance GDS[®] MPX-ID and Assurance GDS[®] EHEC-ID) (MilliporeMerck, Darmstadt, Germany). For each kit, 1 ml of enrichment was used to perform the specific immuno concentration (IMS), using the PickPen[®] tool following manufacturer's instructions. After 30 μ l of IMS product was directly introduced into the real time PCR mix and performed in Rotor-Gene Q themocycler (Qiagen, Courtaboeuf, France). Briefly the MPX-TOP 7 kit allowed the selective concentration and detection of TOP7 STEC, targeting *eae*, *stx*₁ and *stx*₂ genes and O157:H7 markers. For positive samples, MPX-ID kit was performed to obtain the specific STEC serogroup. In parallel, 10 μ l of the IMS suspension were isolated on two agar plates: ChromaAgar STEC (Chromagar, Paris, France) and TBX (MH-F, Oxoid, Wesel,

Germany). In addition, if the O157:H7 marker was also positive, EHEC-ID Kit was performed to confirm presence of the *E. coli* O157:H7, and 10 µl of IMS suspension were spread on two agars: ChromaAgar O157 (Chromagar, Paris, France) and TBX. In addition to PCR detection, direct isolation of 10 µl from m-EHEC enrichment broth was performed on ChromaAgar STEC for Top6 STEC and ChromaAgar O157 for O157 serogroup.

ISO/TS-13136:2012 method

DNA was extracted from 1 mL of enrichment culture using an automated method (EZ1-biorobot and DNA tissue card kit, Qiagen, Courtaboeuf, France). The *eae* and *stx* genes associated with the top seven serogroups markers were detected by real-time PCR according to the ISO/TS-13136:2012 and using primers and probes described previously (Fratamico et al. 2011; Lin et al. 2011; Perelle et al. 2004) (Table 2). Note that it is a simplex PCR with an internal control and TaqMan technology. For the PCR, the TaqMan gene expression master mix (Applied Biosystems, Foster City, CA) was used. For primers and probes, were used at the concentration recommended by the

manufacturer. Thermal cycling and detection were performed using a StepOne Plus system (Applied Biosystems). If the genes *eae*, *stx* and at least one of the top 7 serogroups markers were positive in the enrichment broth, there was a suspicion of pathogenic STEC bacteria. Thus, the confirmation step was implemented to isolate and identify the bacteria. For isolation, *E. coli* “Top7” IMS beads (Dynal, Oslo, Norway) procedure was performed using Dynabeads anti-“Top7” -E. (Dynal, Oslo, Norway) accordingly to the manufacturer instruction. More precisely, 50 µL of bead suspension (IMS) was streaked onto agar plates appropriate to the tested serogroup (Table 3). In parallel, a direct isolation of 10 µl of enrichment was performed on selective agar (Table 3).

For ISO/TS-13136:2012 and Assurance GDS methods, plates were incubated 18–24 h at 37°C. Presumptive-positive colonies were evaluated using a latex agglutination test with a phage protein targeting the top seven serogroups (bioMérieux, Marcy l'étoile, France).

The experiments carried out in this study are synthesized in Fig 1.

Table 2 Characteristics of PCRs used

| Target | Final concentration in the mix | | Cycle PCR |
|-------------|--------------------------------|--------|--------------|
| | Amorces | Sondes | |
| O26 | 500nM | 200nM | 50° : 2min |
| O103 | 500nM | | 95° : 10 min |
| O111 | 1µM | | 40 cycles : |
| O145 | 500nM | | 95°: 10min |
| O157 | 500nM | | 60° : 30s |
| <i>eae</i> | 800nM | | |
| <i>stx1</i> | 1µM | | 50° : 2min |
| <i>stx2</i> | 1µM | | 95° : 10 min |
| | | | 45 cycles : |
| | | | 95°: 10min |
| | | | 55° : 5s |
| | | | 60° : 25s |

Results Treatment

For each matrix category and for each method, the Level of Detection (LOD_{50}) was calculated (Wehling et al. 2011) using the Excel file downloadable from the ISO website (<http://standards.iso.org/iso/16140>). Then, the Relative Level of Detection ($RLOD_{50}$) was calculated as followed: $RLOD_{50} = LOD_{50}$ Assurance GDS/ LOD_{50} ISO/TS 13136:2012. Considering the unpaired study design, if the $RLOD$ value was less than 2.5, the two methods can be considered equivalent (International Organization for Standardisation 2020).

Results

All the twenty non-inoculated samples (L0) were negative with both methods. The enumeration data obtained showed that the spiked levels were between 0.1 CFU and 3 CFU

Table 3 Agar plates used according to the method and serogroups analyzed

| Serogroups | Methods | | |
|---------------------|--|---|---|
| | IMS | | Direct isolation |
| O26 | TBX (MH-F, Oxoid, Wesel, Germany) | RMAC (MH-F, Oxoid, Wesel, Germany) | RMAC-CT (MH-F, Oxoid, Wesel, Germany) |
| O103, O111 and O145 | | SMAC (MH-F, Oxoid, Wesel, Germany) | Bjorn Possé agar (26) |
| O157 | EHEC (bioMérieux, Marcy l'étoile, France) | SMAC-CT (bioMérieux, Marcy l'étoile, France) | EHEC-CT (bioMérieux, Marcy l'étoile, France) |

SMAC sorbitol MacConkey medium, CT cefixime and tellurite, TBX tryptone bile X-glucuronide medium, EHEC chromID enterohemorrhagic *E. coli* medium

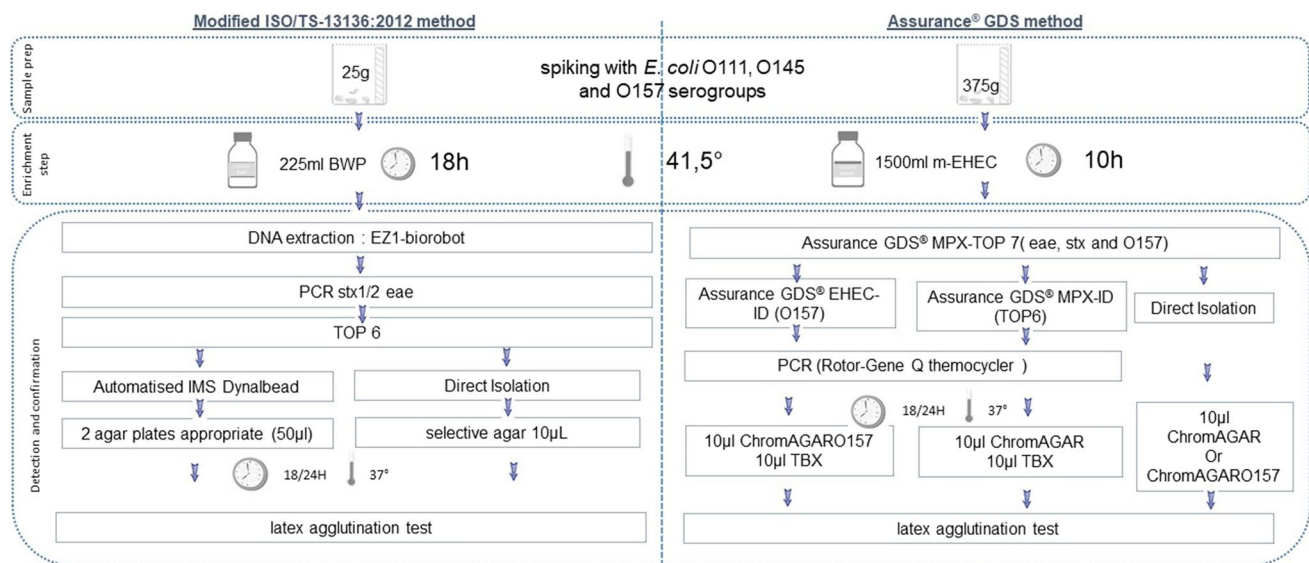


Fig. 1 Representation of the experiments implemented for the two methods

per test portion, for L1, and between 0.7 CFU and 12 CFU for L2. The inoculation levels were acceptable to obtain fractional results at L1 and positive results at L2 for both methods. In total during this study, 240 test portions were performed ($n=120$ for each method/sample size).

For both methods, all real-time PCR positive results were confirmed by isolation in culture media when using IMS.

The results obtained the ISO/TS-13136:2012 method and for the Assurance® GDS method are synthesized in the Table 4.

The results of the latex tests to confirm presumptive colonies isolated ($n = 210$) on the agar plates after IMS and after direct plating are synthesized in Table 5. In 91% of the cases (191 out of 210), the first colony tested was confirmed. For 7% of the cases (15 out of 210), it was necessary to test 2 or 3 colonies to confirm the presumptive colony. In particular, more than one colony was required to confirm the serogroup for 1 raw beef meat sample inoculated with *E. coli* O111, 3 samples of frozen minced meat spiked with *E. coli* O145 and 11 samples of spinach spiked with *E. coli* O157. For 4 samples (2%) of spinach spiked with *E. coli* O157, the latex tests on isolated colonies from direct streaked samples on the ChromaAgarO157 plates were not confirmed. In addition, one sample showing several non-isolated bacteria was analyzed with latex test, in order to ensure the absence of target bacteria on these four types of agars.

The data obtained allowed the performance comparison between IMS and direct isolation. The IMS allowed STEC isolation and confirmation for 100% of the samples ($n=140$) compared with 94% of the samples ($n=66$) for direct isolation. When comparing the number of colonies required to obtain the positive result, 92% of the samples analyzed

after IMS (129 out of 140) required only one colony compared with 88% (62 out of 70) for direct plating. In addition, 4 spinach samples were confirmed when using IMS and negative when spreading the broth directly on ChromAgarO157. Globally, the agar plates with direct plating from spinach enrichment showed more background bacteria than the plates with the spread of the three other matrices. The addition of the IMS step for this matrix, allows a very strong reduction of the presence of background flora compared to direct plating. For serogroups O145 and O111, the performance of IMS and direct plating were equivalent. In addition, our study showed that after IMS, the performances of TBX and Chromagar were similar. Indeed for 70 samples, the colonies were identified positively with latex tests, with TBX agar (70 samples) and with both ChromAgar media (55 samples with ChromAgarSTEC, and 15 samples with ChromAgarO157).

For the confirmation step, latex tests were performed on each agar (Table 5). For the majority of the samples (92.5%), only one colony was required to obtain positive results with the latex test. For 11 samples (5.9%), it was necessary to perform between 1 and 9 latex tests to identify the target bacteria. For these 11 samples, 10 were from raw beef meat and 1 from lettuce both spiked with *E. coli* O111 strain. Finally, for 1 sample (0.5%), several non-isolated bacteria were tested and no target bacteria was confirmed.

The IMS and direct isolation comparison showed that, for the serogroups O145 and O157, IMS and direct plating were equivalent. For 2 samples of lettuce and Fresh ground meat (1.1%) inoculated with O111 STEC, no growth was observed after direct isolation of the broth on Bjorn agar. The O111 STEC were more difficult to isolate and identify among the

Table 4 Data obtained with the LNR method adapted from TS13136 and the gds method

| Matrix | Serotype | Method | Test sample | Level | CFU/test sample | Number of repetitions | Detection | Confirmation | LOD ₅₀ | LOD ₉₅ | LOD : LOD ₅₀ alternative /LOD ₅₀ reference |
|--------------------|-----------------------|---------------------------------|-------------|-------|-----------------|-----------------------|-----------|--------------|-------------------|-------------------|--|
| lettuce | O111:H8 | LNR method adapted from TS13136 | 25g | 0 | 0 | 5 | 0/5 | 0/5 | 0,42 | 1,81 | 0,742 |
| | | | | 1 | 0,683 | 10 | 6/10 | 6/10 | | | |
| | | | | 2 | 2,73 | 3 | 3/3 | 3/3 | | | |
| | | | | 1 | 0,813 | 10 | 8/10 | 8/10 | | | |
| | | | | 2 | 3,252 | 2 | 2/2 | 2/2 | | | |
| | | | | 0 | 0 | 5 | 0/5 | 0/5 | 0,31 | 1,339 | |
| Frozen Minced Meat | O145:H28 | LNR method adapted from TS13136 | 25g | 0 | 0 | 5 | 0/5 | 0/5 | 0,54 | 2,35 | 0,35 |
| | | | | 1 | 3,001 | 10 | 10/10 | 10/10 | | | |
| | | | | 2 | 12,049 | 3 | 3/3 | 3/3 | | | |
| | | | | 1 | 0,378 | 10 | 1/10 | 1/10 | | | |
| | | | | 2 | 1,971 | 2 | 1/2 | 1/2 | | | |
| | | | | 0 | 0 | 5 | 0/5 | 0/5 | 0,12 | 0,518 | |
| Spinach | O157:H7 | LNR method adapted from TS13136 | 25g | 0 | 0 | 5 | 0/5 | 0/5 | 0,52 | 2,22 | 0,96 |
| | | | | 1 | 0,547 | 10 | 4/10 | 4/10 | | | |
| | | | | 2 | 2,734 | 3 | 3/3 | 3/3 | | | |
| | | | | 1 | 0,484 | 10 | 4/10 | 4/10 | | | |
| | | | | 2 | 2,42 | 2 | 1/2 | 1/2 | | | |
| | | | | 0 | 0 | 5 | 0/5 | 0/5 | 0,52 | 2,26 | |
| Spinach | Assurance® GDS method | 375g | 0 | 0 | 5 | 0/5 | 0/5 | 0,52 | 2,26 | | |
| | | | 1 | 0,547 | 10 | 6/10 | 6/10 | | | | |
| | | | 2 | 2,734 | 3 | 3/3 | 3/3 | | | | |
| | | | 1 | 0,484 | 10 | 4/10 | 4/10 | | | | |
| | | | 2 | 2,42 | 2 | 1/2 | 1/2 | | | | |
| | | | 0 | 0 | 5 | 0/5 | 0/5 | 0,52 | 2,26 | | |

Table 4 (continued)

| Matrix | Serotype | Method | Test sample | Level | CFU/test sample | Number of repetitions | Detection | Confirmation | LOD ₅₀ | LOD ₉₅ | RLOD : LOD ₅₀ alternative/LOD ₅₀ reference |
|--------------------------------|-----------------------|---------------------------------|-------------|-------|-----------------|-----------------------|-----------|--------------|-------------------|-------------------|--|
| Fresh ground meat with 20% fat | O111:H8 | LNR method adapted from TS13136 | 25g | 0 | 0 | 5 | 0/5 | 0/5 | 0,34 | 1,48 | 0,726 |
| | | | | 1 | 1,195 | 10 | 6/10 | 6/10 | | | |
| | | | | 2 | 5,973 | 3 | 3/3 | 3/3 | | | |
| | | | | 1 | 0,125 | 10 | 7/10 | 7/10 | | | |
| | | | | 2 | 0,708 | 2 | 0/2 | 0/2 | | | |
| | Assurance® GDS method | | 375g | 0 | 0 | 5 | 0/5 | 0/5 | 0,37 | 1,62 | |
| | | | | 1 | 1,195 | 10 | 9/10 | 9/10 | | | |
| | | | | 2 | 5,973 | 3 | 3/3 | 3/3 | | | |
| | | | | 1 | 0,125 | 10 | 1/10 | 1/10 | | | |
| | | | | 2 | 0,708 | 2 | 2/2 | 2/2 | | | |

lettuce’s microbiota, but also on fresh ground meat with 20% fat. Except for the Frozen Minced Meat matrix, on SMAC, TBX EHEC and EHEC-CT agars, our data showed that it is difficult to differentiate the target bacteria from the other bacteria present on the agars, whatever the agar used. On the other hand, for the O157 :H7 strain, CT-SMAC agar appeared to be very selective for background flora. After incubation, on plates, the majority of colonies were identifiable.

The LOD50 for the reference method (ISO/TS 13136) ranged from 0.343 CFU/25 to 0.544 CFU/25 g and the LOD95 ranged from 1.483 CFU/25 to 2.351 CFU/25g. For the alternative Assurance® GDS method, LOD50 were between 0.120 CFU/375 g and 0.522 CFU/375 g and LOD95 were between 0.518 CFU/375 g and 2.257 CFU/375 g (see Table 4).

The RLOD50 (=LOD50 Assurance GDS in 375 g/LOD50 ISO/TS 13136:2012 in 25g) is between 0.35 and 0.96. These values are not only below 2.5, the maximum ratio acceptable according ISO 16140-4:2020, but also below 1, showing a slightly better performance for the alternative method in 375 g compared with the reference method at 25g. The use of a larger sample size (up to 375 g) for STEC detection in meat and vegetables can be considered validated against the reference method.

Discussion

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens implicated into human infection that can be acquired through the ingestion of critical foods such as undercooked meat and fresh vegetables. One of our objectives was to evaluate the alternative method Assurance® GDS performances to detect top 7 STEC serogroups. The objective of our work was to evaluate the alternative method Assurance® GDS to detect top 7 STEC serogroups. Our work has particularly sought to answer two challenges: First, fill the gap associated to the limited data showing the impact of dry pooling on pathogen detection. Indeed, many industries use pooling but do not know its impact on the performance of analytical tools. And second, demonstrate the ability of the analytical methods to detect pathogenic STEC strains in other samples than dairy and meat products, like vegetables. These food matrices have physico-chemical properties that could limit the detection of bacteria.

The data obtained for both methods showed that equivalent results were obtained (70% and 62% of the experimentally contaminated samples) when comparing detection and confirmation steps using Assurance® GDS method and ISO/TS-13136:2012 method.

For some samples, results were negative after the enrichment step for both methods. These results are not surprising since fractional recovery were intended and contamination rates were very low. Based on statistical distribution, some bags were probably not contaminated (Amagliani et al.

Table 5 Number of positive latex tests out of the total number of latex tests performed on positive suspected colonies for both methods

| Method | Number of positive latex tests out of the number of latex tests performed | | 1/1 | Between 1/2 and 1/9 | 0/Scratch | No colony on the agar |
|-----------------------------------|---|---------------|-----|---------------------|-----------|-----------------------|
| LNR method adapted from TS13136 | SMAC | IMS | 55 | 6* | 1* | 0 |
| | TBX | IMS | 47 | 3* | 0 | 0 |
| | EHEC | IMS | 12 | 0 | 0 | 0 |
| | Bjorn Possé ¹ | direct spread | 46 | 2 [†] | 0 | 2 [‡] |
| | EHEC-CT | direct spread | 12 | 0 | 0 | 0 |
| Assurance [®] GDS method | ChromAgarSTEC | IMS | 55 | 0 | 0 | 0 |
| | | direct spread | 51 | 4 [§] | 0 | 0 |
| | ChromAgarO157 | IMS | 6 | 9** | 0 | 0 |
| | | direct spread | 11 | 0 | 4** | 0 |
| | TBX | IMS | 68 | 2** | 0 | 0 |

1 : 9 samples inoculated with O111 into Fresh ground meat at level 2 and 1 sample inoculated with O111 into Fresh ground meat at level 3 **2** : 1 sample inoculated with O111 into Fresh ground meat at level 2 and 1 sample inoculated with O111 into lettuce at level 3 **3** : 1 sample inoculated with O111 into Fresh ground meat at level 2 and 1 sample inoculated with O111 into lettuce at level 2. **4** : 3 samples inoculated with O145 into frozen minced meat at level 2 and 1 sample inoculated with O111 into Fresh ground meat at level 3 **5** : 16 samples inoculated with O157 into spinach at level 2 and 3

2018; Bouvier et al. 2017; Koyama et al. 2017). In addition, a work developed by Tzschoppe et al. 2012, showed that strains experimentally inoculated in vegetable decrease significantly after a cold storage of 72h (Tzschoppe, Martin, et Beutin 2012). Thus, the strains inoculated in vegetables may have died before the beginning of the enrichment step.

Finally, the data obtained allowed us to calculate the detection limit of the whole method, including molecular detection and cultural isolation. Indeed, it is mandatory to obtain fractional results in order to compare both methods and sample size by estimating respective LOD₅₀ (Wehling et al. 2011). The fractional recovery at level 1 (L1), has to be between 25% ($n=5$) and 75% ($n=15$) of the number of samples tested. Note that for the lettuce matrix for the Assurance[®] GDS method, the number of positives at L2 was 16. However, we considered this value as acceptable.

The calculated RLOD₅₀ were between 0.35 and 0.96. It is therefore lower than 2.5 (AL limit for an unpaired study design), The use of 375-g portions can be successfully applied for STEC detection in vegetables and meat according to ISO 16140-2:2016. These results are particularly encouraging and indicate two things:

(1) That the methods evaluated implemented on a large sample size are performing well for meat and vegetables. (2) The methods are able to detect and correctly confirm a very small amount of target strains in 375g sample size.

In addition, both methods also allowed a good detection and confirmation of the strains in challenging matrices considering the physicochemical composition and the background flora. The results are aligned with the previous study (Costa et al. 2019), showing good performance of the ISO/TS 13136 and Assurance[®] GDS method in vegetables in the validated 25 g sample size (Costa et al. 2019).

Regarding the enrichment step, both media used (mEHEC and BPW) allowed the growth of STEC. Kang et al. 2021, showed that *E. coli* achieved similar counts in mEHEC selective media compared to non-selective BPW media (Kang et al. 2021).

The application of a higher enrichment temperature (41.5°C) seems very effective. Indeed, it allowed a sufficiently important development of the bacteria experimentally inoculated at low rate so that they could be detected. We can also hypothesize that an increase in temperature limits the growth of certain bacteria, which translates into a decrease in the number of bacteria on the agar medium. Finally, the temperature 41.5°C was chosen because it is recommended for the collection of STEC O157:H7 in ISO 16 654 and it will also be applied in the future ISO 13 136 as recommended by the working group 463.

The enrichment is usually done in a static way, the addition of a stirring step followed by a reduction of the enrichment time could be an improvement. Indeed, it would reduce the development of *Clostridium* and promote the growth of STEC (Hallewell et al. 2017; Kang et al. 2021; Verhaegen et al. 2015; Zhou et al. 2011).

For the confirmation step, many studies demonstrated difficulties to detect and confirm STEC in vegetables compared to meat (Amagliani et al. 2018; Costa et al. 2019; Delbeke et al. 2015; Hara-Kudo et al. 2016; Margot et al. 2015; Tzschoppe, Martin, et Beutin 2012). However, our results showed that Assurance[®] GDS method (using m-Ehec) and ISO/TS13136 (using BPW) allowed sufficient growth of STEC to allow detection and confirmation in both vegetable and meat, with respectively 375 g and 25 g sample size tested. The results obtained with Assurance[®] GDS and the ISO/TS13136 method are consistent with previously for the

detection of STEC serogroups in vegetables (Costa et al. 2019; Feldsine et al. 2016).

For both methods (Assurance[®] GDS and ISO/TS 13136), all samples positive at the detection step (PCR) were confirmed after IMS. These results confirm that IMS is an effective method to concentrate STEC in complex matrices. Hara-kudo et al. 2016 showed that the sensitivity of IMS plating detections was the same or lower than that of real-time PCR assays. (Hara-Kudo et al. 2016). Despite these good results, differences were observed at the IMS confirmation step between the two methods. Indeed, after the ISO/TS13136 method, it was more difficult to isolate O111 STEC. Two hypotheses can explain this result : (1) the use of a non-selective medium, such as BPW, induces higher levels of back-growing microbiota in the enrichment broth (Gelting et al. 2011). This may result in greater difficulty in isolating target bacteria from others on the agar. (2) A few authors have also described differences between serogroups isolation (Feldsine et al. 2016; Fratamico et al. 2011; Hallewell et al. 2017).

Conversely, for Assurance[®] GDS method, it was more complex to identify *E. coli* O157 among spinach microbiota enrichment broth on the agar plates. Indeed, after direct plating, some of the O157 STEC were not isolated. Moreover, we note that for this serogroup associated with the spinach matrix, it was necessary to perform more latex tests in order to identify the target bacteria. On the ChromAgar O157 plates from the spinach enrichment, we observed (1) the presence of either high numbers of indigenous microbiota and (2) the presence of background microbiota with a morphotype close to O157:H7. This could explain the difficulties encountered in isolating O157 in the spinach matrix.

For ISO/TS 13136, the IMS steps was performed in addition to the PCR step and each serogroup required the use of different capture beads. Conversely, the Assurance[®] GDS method, IMS was performed using one single solution containing the 7 antibodies targeting the top 7 and used for both PCR detection and cultural isolation. Our results showed that on large test sample (375g of matrix), bead pooling does not seem to affect the performance of the IMS. Nevertheless, it is important to remember that the beads used for the two methods are different and can show slightly different performances. Noll et al., 2016 showed that simultaneous IMS did not affect the sensitivity of target cell detection (Noll et al. 2016). In case of suspicion of contamination by several serogroups, pooling allows to perform less IMS and therefore to save time to results and resources.

For 6 samples, the direct spread of the enrichment broth (compared to IMS), did not allow to isolate the target bacteria. For two samples inoculated with an O111 strain, the Bjorn Possé medium appeared too selective and did not allowed the growth of the target bacteria. For the other samples, the

presence of either high indigenous microbiota from spinach on the ChromAgarO157 plates, compared to the plates from the IMS, may explain these results. It is important to highlight that the Chromagar O157 medium could be improved in selectivity and specificity, by the addition of solution of Potassium Tellurite (final concentration of 2.5 mg/L).

Similar results to ours have been described in other studies (Delbeke et al. 2015; Tzschoppe, Martin, et Beutin 2012). However, the opposite phenomenon has also been observed (Delbeke et al. 2015; Verstraete et al. 2012). These data may be dependent on the strains isolated as well as the matrix tested. This is why we recommend systematically to perform IMS and direct spreading in parallel. Nevertheless, the results obtained with the IMS as well as with a direct spread of the enrichment on agar medium showed that the choice of agars is a real challenge. Although our study showed equivalent performance between TBX and CHROMAgar STEC and CHROMAgar O157, we consider the use in parallel, of one selective agar and one non-selective agar can bring benefits to the cultural isolation step (Hirvonen, Siitonen, et Kaukoranta 2012; Jenkins et al. 2020; Verhaegen et al. 2015). Indeed, it has been demonstrated that ChromAgar agars were selective and allowed to reduce considerably the annexed flora especially for plants, which allows to save time on latex test (Delbeke et al. 2015; Gouali et al. 2013; Kalule, Keddy, et Nicol 2018; Kanki, Seto, et Kumeda 2014; Lewis, Cernicchiaro, et Moxley 2020; Tzschoppe, Martin, et Beutin 2012).

Our study showed that either after an IMS or a direct spread of the broth on agar medium, the screening using latex tests targeting serogroups can limit the number of additional PCR tests used to identify and characterize STEC strains.

The data obtained for the new implementation are very satisfactory. Nevertheless, our study highlighted the fact that it would be wise to better understand and know the behavior of the target strains as well as the microbiota of the tested food during the enrichment step. In fact, the enrichment of STEC is a balance between the growth of the food microbiota and target bacteria. This growth will also have an impact on the confirmation step on agar media.

Also, for STEC detection is crucial to improve the performance of agar plates. Indeed, it would be interesting to have chromogenic agar plates allowing to better differentiate STEC from the total flora, but also the serogroups between them.

Conclusion

Our study showed that (1) the modified protocol described in ISO/TS13136 (using 41.5°C instead 37°C for preenrichment step) and implemented in the French National Reference Laboratory is capable of detecting low levels of STEC contamination

in complex foods such as vegetables and vegetables. (2) Assurance[®] GDS method (including enrichment of 375 g sample size with 1/5 ratio in m-EHEC) at 41.5°C followed by IMS, detection and confirmation is a viable and sensitive tool for the detection of STEC in big sample size samples.

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Author Contribution M. BOUVIER and D. THEVENOT SERGENTET, built in collaboration the experimental protocol. The experiments were implemented by M. BOUVIER and M. CANIZARES. B. HAMADOU and M. GUENSER also contributed to acquisition of data. The article was written by M. BOUVIER. All authors have critically reviewed the manuscript.

Data Availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Conflict of Interest M. Bouvier declares no conflict of interest. M. Canizares declares no conflict of interest. B. Hamadou declares no conflict of interest. M. Guenser declares no conflict of interest. D. Thevenot Sergentet declares no conflict of interest.

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