International Journal of Food Microbiology 291 (2019) 59–64

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Evaluation of PCR-based methods for the identification of enteroaggregative hemorrhagic *Escherichia coli* in sprouts☆

Luca Rotundo[a,b], Giulia Amagliani[b], Elisa Carloni[b], Enrica Omiccioli[c], Mauro Magnani[b], George Paoli[a,*]

[a] USDA, Agricultural Research Service, Eastern Regional Research Center, 600 E. Mermaid Lane, Wyndmoor, PA 19038, USA
[b] Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino "Carlo Bo", Urbino, (PU), Italy
[c] Diatheva srl Cartoceto, (PU), Italy

ARTICLE INFO

Keywords:
Escherichia coli O104
Multiplex real-time PCR
Virulence genes
Immunomagnetic separation
Enteroaggregative *E. coli*

ABSTRACT

In this study real-time PCR assays were evaluated for the detection of enteroaggregative hemorrhagic *Escherichia coli* (EAHEC) O104:H4 in artificially contaminated mung bean and/alfalfa sprouts inoculated with 1, 10, and 100 CFU of EAHEC O104:H4 per 25 g sample (20, 10, and 2 replicates respectively). After selective culture enrichment the samples were tested using commercial real-time PCR kits detecting *agg*R/*aai*C, *stx*/*eae*, and wzx_{O104}. Using the commercial real-time PCR kits, the artificially contaminated samples were detected in the range of 75–80% positive results when contaminated with approximately 1 CFU, and 100% at 10 and 100 CFU. Microbiological detection employing O104-specific immunomagnetic capture and plating onto chromogenic media (modified Rainbow Agar and CHROMagar STEC) and confirmation by latex agglutination and PCR gave similar results (Cohen's kappa value between 0.61 and 1). In addition, the real-time PCR assay targeting the *agg*R and *aai*C genes, indicative of enteroaggregative *Escherichia coli* (EAggEC), was tested against a panel of 60 bacterial strains and demonstrated 100% exclusivity (54 strains) and 100% inclusivity (6 strains). This study demonstrates the efficacy of the real-time PCR assays for the specific and sensitive detection of EAHEC from spouts.

1. Introduction

Enteroaggregative *Escherichia coli* (EAggEC) have been recognized as the most common bacterial cause of diarrhea in patients affected by HIV and infants in developing countries, as well as in travelers (Huang et al., 2004; Nataro et al., 2006). Members of this *E. coli* pathotype are characterized by their ability to adhere to intestinal cells in a distinct "stacked and brick-like" manner (Nataro et al., 1987). EAggEC are characterized by a large number of virulence factors (Okeke et al., 2010), several of which are encoded on the pAA2 virulence plasmid. The plasmid-borne virulence genes include the *astA* gene, encoding an enteroaggregative heat-stable toxin (EAST1), and *agg*R, encoding a transcriptional regulator that plays a main role in the pathogenic mechanism of EAggEC (Morin et al., 2013). The *agg*R gene has been considered a good target for the detection and identification of EAggEC (EFSA BIOHAZ Panel, 2013). To circumvent the possible loss of the plasmid, Taniuchi et al. (2012) suggested that the detection of the *aai*C gene, encoding a secreted protein and harbored by the chromosomally encoded *agg*R-activated pathogenicity island, could serve as a more stable target.

It has been observed that Shiga toxin-producing *Escherichia coli* (STEC) strains positive for *aai*C and *agg*R genes are associated with a higher risk of more severe illness than other STEC (EFSA BIOHAZ Panel, 2013). Indeed, in 2011 a hemorrhagic EAggEC (EAHEC) O104:H4 strain, having acquired a gene encoding Shiga toxin 2, caused an outbreak originating from contaminated fenugreek seeds in Germany, resulting in 2987 cases of gastroenteritis, 855 cases of haemolytic uremic syndrome and 53 deaths (Robert Koch Institute, 2011). Genome sequencing of the epidemic strain revealed the presence of virulence markers typical of both STEC (stx_2, *iha*, lpf_{O26}, lpf_{O113}) and EAggEC (*agg*A, *agg*R, *aai*C, *pic*, *aap*) pathotypes. Since then, the European Food Safety Authority Panel on Biological Hazards suggests STEC and

☆ Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

* Corresponding author at: Eastern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA.
E-mail address: george.paoli@ars.usda.gov (G. Paoli).

https://doi.org/10.1016/j.ijfoodmicro.2018.11.011
Received 2 August 2018; Received in revised form 9 November 2018; Accepted 9 November 2018
Available online 13 November 2018
0168-1605/ Published by Elsevier B.V.

EAggEC molecular detection criteria and zero tolerance for the pathogens in seeds and sprouts, respectively (EFSA BIOHAZ Panel, 2013). The European Commission also released an amendment to Regulation (EC) No 2073/2005 providing a microbiological criterion for sprouted seeds that calls for the absence of the top-5 STEC serogroups (O157, O26, O111, O103, O145) and O104:H4 in 25 g (Regulation (EC) No 209/2013).

Screening STEC for the presence of the additional virulence markers *aai*C or *agg*R genes is not routinely undertaken. However, the EFSA Panel on biological hazards (EFSA BIOHAZ Panel, 2013) published a series of recommendations relating to a molecular approach for the categorization of STEC strains that, in addition to testing for the presence of *stx* genes, included testing for the *aai*C and *agg*R genes.

Sprouts consumption has increased among consumers due to its nutritional benefits. Mung bean sprouts are the most widely consumed sprouts in the world, while alfalfa sprouts are among the most common sprout varieties consumed in the U.S. (Mueller, 2008; Oplinger et al., 1990). However, sprouts represent a food safety concern because they are processed under conditions (temperature, water activity, pH) that promote the growth of bacteria (including pathogens, if present) and they are often consumed raw. Therefore, outbreaks related to sprouts consumption have been recently reported in the United States (Dechet et al., 2014) and worldwide, also including those caused by pathogenic *E. coli* (EFSA, 2011; Gensheimer and Gubernot, 2016).

Besides the European legislation requiring control of STEC in sprouted seeds (Regulation (EC) No 209/2013), mentioned above, the U.S. Food and Drug Administration very recently published standards for commercial sprout production (US FDA, 2017). Thus, effective methods for pathogen detection in vegetables and fresh produce, including sprouts, are needed to guarantee consumer safety and several studies have been conducted to find effective culture enrichments or real-time PCR-based methods to provide rapid and sensitive methods for detection of STEC and EAggEC in sprouts or vegetables (Amagliani et al., 2018; Baranzoni et al., 2014; Barbau-Piednoir et al., 2018; Barletta et al., 2013; Feng et al., 2010; Jinneman et al., 2012; Kuwayama et al., 2011; Taniuchi et al., 2012; Tzschoppe et al., 2012; Weagant and Bound, 2001).

Taking into account BIOHAZARD EFSA Panel recommendations and epidemiological data, the purpose of this study was to evaluate the performance of real-time PCR-based methods for the specific and sensitive detection of EAggEC/EAHEC in sprout samples. To this end, Diatheva FLUO Real-Time PCR assays (Diatheva srl, Cartoceto, Italy) were used for detection and characterization of an O104:H4 EAHEC strain from artificially contaminated mung bean and alfalfa sprouts. The Diatheva FLUO kits are a set of triplex real-time PCR assays targeting *stx*1/*stx*2 and *eae* (STEC FLUO Detection kit; catalogue code MBK0068), O-serogroup-specific targets (*E.coli* O104 FLUO kit; for information contact Diatheva via their web-page at https://www.diatheva.com/contact-us), and *agg*R and *aai*C genes (Enteroaggregative *E.coli* kit; for information contact Diatheva via their web-page at https://www.diatheva.com/contact-us). While the STEC FLUO and Serotype FLUO detection kits have been evaluated previously (Amagliani et al., 2018; Rotundo et al., 2018), this is the first study to report an evaluation of the Enteroaggregative *E.coli* kit. Microbiological detection, using selective chromogenic media, and isolate confirmation, by immunological and alternate real-time PCR methods, were conducted on the same samples.

2. Materials and methods

2.1. Bacterial strains and specificity testing

The bacterial strains used in this study are listed in Table 1. In addition, DNA extracted from an O104:H4 strain from the 2011 outbreak in Germany, kindly provided by Alexander Mellmann University of Munster, was tested. A total of 60 bacterial strains were used to assess the specificity of the Enteroaggregative *E.coli* kit. Fifty-four bacterial strains, including 13 species from 12 genera were used for exclusivity testing (Table 1). The exclusivity set included 40 strains of *E. coli* that were negative for the *agg*R and *aai*C gene targets. Twenty-nine of the *E. coli* strains had gene(s) for production of Shiga toxin (11 strains with *stx*1, 21 strains with *stx*2, and 3 strains with both *stx*1 and *stx*2) and 16 strains had a gene encoding intimin (10 strains with both *eae* and *stx*1 or *stx*2 and 6 strains with *eae* but no *stx* gene). Six *agg*R- and *aai*C-positive strains of *E. coli* were used for inclusivity testing, 4 of which had a *stx*2 gene. A more complete description of relevant characteristics of individual strains is available in Supplementary Table 1.

Table 1
Bacterial strains used and results of EAggEC PCR specificity testing.

Bacterial strains*	Number of strains tested	Number of *stx*-positive strains	Number of *eae*-positive strains	Number of *aggR*- & *aaiC*-positive strains
Exclusivity				
Citrobacter freundii	1	0	0	0
Enterobacter cloacae	1	0	0	0
Enterococcus faecalis	1	0	0	0
Escherichia coli	40	29	16	0
Listeria innocua	1	0	0	0
Listeria monocytogenes	1	0	0	0
Klebsiella pneumoniae	1	0	0	0
Proteus vulgaris	1	0	0	0
Pseudomonas aeruginosa	1	0	0	0
Salmonella enterica	2	0	0	0
Shigella flexneri	1	0	0	0
Staphylococcus aureus	2	0	0	0
Yersinia pseudotuberculosis	1	0	0	0
Inclusivity				
Escherichia coli	6	4	6	6

* Stains used for exclusivity testing: *C. freundii* ATCC 8090; *E. cloacae* ATCC13047; *E. faecalis* UU 4421; *E. coli* strains ATCC 25922 (O6), UU1, UU2, UU3, D2435 (O48), D2587 (O174), D3435 (O73), D3509 (O2), D3522 (O8), D3546 (O128), D3602 (O174), D3648 (O139), ED495 (O113), ED513 (O128), ED546 (O159), ED585 (O111), ED600 (O26), ED603 (O121), ED643 (O26), ED645 (O145), ED654 (O26), EF292 (O145), EF299 (O145), EF333 (O26), EF334 (O26), EF335 (O26), EF337 (O26), ATCC 35150 (O157:H7), [RM8799, RM9387, RM1037, 9.0124, 6.0830, and EQA6 3744 (O104:H7)], [94-3024, TW04909 (O104:H21)], and 1.2673 (O104:H12); *K. pneumoniae* ATCC13883; *L. innocua* ATCC 33090; *L. monocytogenes* ATCC 9525; *P. vulgaris* UU1; *P. aeruginosa* ATCC10145; *S. enterica* (UU7 serotype Enteritidis and UU2 serotype Newport); *S. flexneri* ATCC 12022; *S. aureus* [ATCC 6538 and ATCC 25923]; and *Y. pseudotuberculosis* R852; Strains used for inclusivity: *E. coli* O104:H4 strains 2011C-3493, 2009EL-2050, 2009EL-207, EQA4 2710 and DNA from an EAggEC O104:H4 strain involved in the 2011 German outbreak (kindly provided by Dr. Helge Karch (University of Münster Germany)); and strain UU O42 (O42). A more complete description of relevant characteristics of individual strains, including the source of the strains, is available in Supplementary Table 1.

All strains were grown overnight in a tube containing 10 mL of Tryptic Soy Broth medium (TSB) in a New Brunswick Innova 4200 incubator (Eppendorf, Hauppauge, NY) at 37 °C with shaking (180 rpm) or streaked onto Tryptic Soy Agar (TSA) at 37 °C for 18 h. To prepare DNA for specificity testing, a single colony was collected with a sterile loop, picked into 50 µl of nuclease free water and the DNA was extracted by heating the sample at 95 °C for 10 min. Five microliters of the boiled extract was used to provide template DNA for the PCR assay. The thermocycling conditions consist of 95 °C for 10 min, 40 cycles at 95 °C 15 s and 60 °C for 1 min using the ABI7500 FAST instrument. The amplification signal was acquired in the green, red, and yellow channels, as described in the manufacturer's instruction.

The *E. coli* O104:H4 2011C-3493 strain was chosen for the contamination of alfalfa (*Medicago sativa*) and mung bean sprouts (*Vigna radiata*) because this strain carries both *agg*R and *aai*C gene targets as

Table 2
Primer and probe sequences used for the real-time PCR confirmation of EAggEC O104:H4 colonies.

Primer/probe	Nucleotide sequence	Final Conc. (μM)	Reference
stx 1/2-F	TTT GTY ACT GTS ACA GCWGAA GCY TTA CG	1.25	Wasilenko et al. (2012)
stx 1/2-R	CCC CAG TTC ARWGTR AGR TCM ACR TC	1.25	Wasilenko et al. (2012)
stx 2-P	/56FAM/ TCG TCA GGC /ZEN/ ACT GTC TGA AAC TGC TCC /3IAbkFQ/	0.25	Wasilenko et al. (2012)
aggR-333f	CAG CGA TAC ATT AAG ACG CCT AAA G	1	Hidaka et al. (2009)
aggR-448r	CGT CAG CAT CAG CTA CAA TTA TTC C	1	Hidaka et al. (2009)
aggR-pro	/56-TAMN/ AGA TGC TTG CAG TTG TCC GAA TTG GTC /3BHQ_2/	0.2	Baranzoni et al. (2014)
wzx_{O104} F	TGT CGC GCA AAG AAT TTC AAC	1	Bugarel et al. (2010)
wzx_{O104} R	AAA ATC CTT TAA ACT ATA CGC CC	1	Bugarel et al. (2010)
wzx_{O104} P	/5Cy5/ TTG GTT TTT TTG TAT TAG CAA TAA GTG GTG TC /3BHQ_2/	0.2	Baranzoni et al. (2014)

well as the *stx2* gene.

2.2. Preparation of inoculum, contamination and enrichment procedure

E. coli strain 2011C-3493 was grown on modified Rainbow Agar (mRBA, Biolog, Hayward, CA, USA) and incubated at 37 °C for 16 h. A single colony from the overnight culture was transferred into a tube containing 10 ml of TSB and incubated in a New Brunswick Innova 4200 incubator (Eppendorf) overnight at 37 °C with shaking (180 rpm). Ten-fold serial dilutions were made in phosphate-buffered saline (PBS, Sigma-Aldrich) up to 10^{-9} and 1 ml portions of the dilutions corresponding to ~1, 10, and 100 CFU/ml were used to inoculate food matrices. One hundred microliters of appropriate dilutions were plated on TSA and, after overnight incubation at 37 °C, colonies were counted to determine the experimental cell concentrations.

Alfalfa and mung bean sprouts were purchased from a local retail market. Test portions of 25 g were placed into sterile filter bags and were contaminated with approximately 1, 10, and 100 CFU of *E. coli* O104:H4 (10 bags, 5 bags, and 1 bag, respectively) and stored at +4 °C for 48 h. Afterward, samples were homogenized in 225 ml of modified Buffered Peptone Water with pyruvate (mBPWp, Acumedia, Neogen Corporation, Lansing, MI) and pummeled using a Stomacher Lab Blender 400 (Seward Laboratory System, Bohemia, NY, USA) for 60 s. The enrichments were incubated for 5 h at 37 °C, at which time acriflavin hydrochloride (10 mg/l) (Sigma Aldrich, St. Louis, MO), cefsulodin sodium salt (10 mg/l) (A. G. Scientific, Inc., San Diego, CA), and vancomycin hydrochloride (8 mg/l) (Sigma Aldrich) (ACV) were added to the samples and incubated for 18 h at 42 °C, as recommended by the FDA-BAM method. One uninoculated sample was included in the experiment as negative control. The experiment was repeated so that a total of 20, 10, and 2 samples of the ~1, 10, and 100 CFU inocula, respectively, were tested.

The level of background microbial population at time 0 and after 48 h at 4 °C was examined using three types of Petrifilm plates (Petrifilm™ AC, 3 M, St. Paul, MN, USA): Aerobic Count Plate (ACP), *E.coli*/Coliform Count Plate (*E.coli*/CCP), and *Enterobacteriaceae* Count Plate (ECP).

2.3. DNA isolation and real-time PCR detection from artificially contaminated sprouts

One aliquot (1 ml) of alfalfa or mung bean sprout enrichment culture was recovered and the genomic DNA was extracted using the PrepSEQ Rapid Spin Sample Preparation kit (Life technologies, Foster City, CA, USA) following the manufacturer's instruction. The genomic DNA was tested using the STEC FLUO detection kit (MBK0068) for *stx* and *eae* detection, the *E. coli* O104 FLUO kit (MBK0085) for the identification of the O104 serogroup, and the Enteroaggregative *E. coli* kit (MBK0084) for detection of *agg*R and *aai*C targets. These PCR assays provide a ready to use PCR mastermix containing primers, probes, polymerase, positive controls, and Internal Amplification Control (IAC), manufactured by Diatheva srl (Italy). The thermocycling conditions were the same as described above in Section 2.1.

2.4. Microbiological, immunological and real-time PCR confirmation of E. coli O104 isolates

Enrichment culture samples were also tested using chromogenic media. One-ml aliquot of alfalfa or mung bean sprout enrichment cultures was collected and mixed with 20 μl of *E. coli* O104:H4 immunomagnetic beads (IMBs) (Abraxis, Warminster, PA, USA) and incubated for 10 min with agitation (14 rpm) on a rotating mixer (Fisher Scientific). Subsequently, the tubes were placed into a magnetic rack for 3 min, the supernatant was discarded and the IMBs were washed three times with phosphate buffered saline plus 0.05% Tween 20 (PBST, Sigma Aldrich). The IMBs were resuspended in 100 μl of PBST, gently vortexed, split into two 50 μl-aliquots and plated onto mRBA supplemented with 0.05 mg/l cefixime trihydrate, 5 mg/l novobiocin sodium salt, and 0.15 mg/l potassium tellurite hydrate (Sigma Aldrich) and Chromagar STEC (CHROMagar, Paris, France) using a sterile swab or loop. The plates were incubated at 37 °C for 18 h in the dark. After overnight growth, presumptive *E. coli* O104 colonies from both mRBA and CHROMagar were tested using the *E. coli* O104:H4 Latex test kit (Abraxis, Warminster, PA, USA) following the manufacturer's instruction. Colonies yielding a positive latex agglutination test were confirmed by further real-time PCR using primers and probes for identification of *stx*2, *agg*R, and wzx_{O104} (Table 2).

The genomic DNA was extracted as described above in Section 2.1 and the PCR assay was performed using 20 μl of Environmental Master Mix (Life Technologies) and 5 μl of genomic DNA in the presence of IAC. Primer and probe concentrations are listed in Table 2. Multiplex real-time PCR was performed with the ABI 7500 FAST under the following cycle condition: denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 59 for 1 min.

3. Results and discussion

Shiga toxin-producing *Escherichia coli* (STEC) are responsible of severe foodborne outbreaks worldwide and the O157, O26, O103, O111, and O145 serogroups are considered the most epidemiologically relevant (EFSA and ECDC, 2017). Also an *E. coli* O104:H4 strain caused severe illness in Germany in 2011 (Robert Koch Institute, 2011) and represents an uncommon pathotype due to the presence of both STEC and EAggEC genetic characteristics, hence this strain is referred to as hemorrhagic EAggEC (EAHEC). Several official methods such as ISO13136:2012, ISO16654:2001 and the FDA-BAM have been developed and issued from regulatory agencies to enhance food control and food safety, and recently the EFSA Panel on Biological Hazards suggested the screening of STEC for the presence of *aai*C and *agg*R genes as part of their surveillance plan (EFSA BIOHAZ Panel, 2013).

The PCR-based methods developed by Diatheva are rapid and sensitive real-time PCR assays for the detection of *stx-eae*, O-group, and

*agg*R-*aai*C target genes in foodstuffs. In previous studies (Amagliani et al., 2018; Rotundo et al., 2018) the performance of the STEC FLUO detection and *E.coli* O104 FLUO kits was assessed using different enrichments, food matrices and real-time PCR assays. The aim of this study was to evaluate the Diatheva kits for detection of EAHEC in sprout samples artificially contaminated with *E. coli* O104:H4. The results were compared with those obtained using the microbiological detection: selective culture enrichment, IMS, plating on selective chromogenic media, and confirmation by latex agglutination and real-time PCR.

3.1. Specificity testing

The inclusivity and exclusivity of the Enteroaggregative *E.coli* kit was tested using a panel of 60 bacterial strains (Table 1). Genomic DNA from six strains of *E. coli* serotype O104:H4 carrying the *agg*R and *aai*C targets were tested for the inclusivity, and 54 strains lacking the target genes were tested for the exclusivity. The results (Table 1) show 100% specificity: all the bacterial strains positive for the *agg*R and *aai*C targets were amplified (6/6) and no false positives were obtained from the non-EAggEC samples (0/54) or from the negative control. The IAC was amplified in all samples. The inclusivity and exclusivity of the STEC FLUO and Serotype FLUO kits was reported previously (Amagliani et al., 2018) but the specificity of these assays was confirmed in the present study.

3.2. Microbiota examination of sprouts

The native bacterial communities associated with the mung bean and alfalfa sprout samples were enumerated and characterized in uninoculated samples before and after 48 h storage at 4 °C by plating on ACP, *E.coli*/CCP, and ECP Petrifilms. The total aerobic bacteria from mung bean sprouts enumerated on ACP increased ~16-fold during the 48 h of storage (from 7.3×10^7 to 1.2×10^8 CFU/g), while the number of *Enterobacteriaceae* on ECP increased by 10-fold (from 1.3×10^6 to 1.3×10^7) and the *E.coli*/Coliform on the *E.coli*/CCP increased by ~2.6-fold (from 3.4×10^5 to 8.8×10^5) (Table 3). The alfalfa sprout samples tested before and after the incubation enumerated on ACP, ECP, and *E.coli*/CCP plates show an increase of 3.7, 3.22, and 2.07-fold, respectively, from starting concentrations of 4.6×10^8, 5.55×10^7, and 2.9×10^7, respectively (Table 3).

Comparing the two food matrices, alfalfa sprouts contained a higher concentration of total aerobic bacteria than mung bean sprouts (Table 3). Jinneman et al. (2012), Kim et al. (2009), and Matos et al. (2002) obtained results similar to the results reported herein (an average of 10^7–10^8 CFU/g, 10^8 CFU/g, and 7×10^6 CFU/g, respectively) when enumerating the microbiota on mixed sprouts or alfalfa sprouts using ACP. Moreover, Tzschoppe et al. (2012) counted *Enterobacteriaceae* in average of 10^6 CFU/g in ready to eat vegetables, while under storage condition (6 °C for 72 h) the total aerobic bacteria counted were similar to those observed in the present study.

Although the population of *E. coli*/coliform and the *Enterobacteriaceae* both increased during storage, the total aerobic bacteria started off at higher levels and increased at a greater rate than the two indicator populations. These observations show that natural bacterial populations on sprouts are able to grow during storage and, though this could lead to a higher risk of infection due to potential pathogen growth, the increase in the number of total aerobic bacteria might also hinder pathogen detection.

3.3. Detection of E. coli O104:H4 in sprouts using Diatheva kits

Alfalfa and mung bean sprout samples artificially contaminated with ~1, 10, and 100 CFU/sample and enriched using mBPWp + ACV were tested by real-time PCR using the STEC FLUO (for *stx* and *eae* amplification), the *E.coli* O104 FLUO (for O104-group identification), and the Enteroaggregative *E.coli* (for *agg*R and *aai*C amplification) kits, provided by Diatheva srl. The results show that the three molecular assays were able to detect all the replicates inoculated with 100 and 10 CFU (2/2 and 10/10 positive results, respectively) on both mung bean and alfalfa sprout samples. However, the ~1 CFU contaminated samples gave positive results with 90% (18/20) for mung bean sprout samples and 75% (15/20) for alfalfa sprout samples (Table 4). The uninoculated samples gave negative results with both food matrices. Low level inoculation (approaching 1 CFU/sample) as conducted in the present study is expected to yield positive results for only a fraction of the artificially contaminated samples due to the difficulty in ensuring that a single viable cell is delivered to each sample. In addition, the viable inoculum could be stressed and injured during the storage condition at 4 °C. Similar fractional positive results were obtained by Amagliani et al. (2018), Baranzoni et al. (2014), and Tzschoppe et al. (2012) using an inoculum level < 10 CFU per sample. Moreover, Hara-Kudo et al. (2016) and Tozzoli et al. (2018) carried out an inter-laboratory test using *E. coli* O157:H7 and the top five STEC strains as inoculum for 25 g of sprout samples obtaining 75% positive results using inocula of 10 CFU/g, and positive percentages in the range of 66.7% and 91.7% using 0.2 CFU/g, respectively.

It is important underline that, though not all of the low inoculum samples yielded a positive result, each of Diatheva PCR-positive samples was positive for all the three Diatheva PCR kits (*stx/eae*, O104, and *agg*R/*aai*C). This result enhances the reliability of the molecular assays and demonstrates that using the three kits in combination allow the reliable detection of EAggEC serogroup O104.

3.4. Microbiological, immunological and real-time PCR confirmation of E. coli O104 isolates

Microbiological detection of EAggEC O104 in mung bean and alfalfa sprouts was also carried out on every sample. Selective enrichment cultures were subjected to *E.coli* O104-selective IMS, plating onto CHROMagar and mRBA selective media, immunological and molecular confirmation of presumptive-positive colonies by anti-O104 latex agglutination and real-time PCR for *stx*2, *agg*R, and O104 (*wzx*$_{O104}$) target genes (Table 2). The results (Table 4) suggest that, of the two selective chromogenic media tested, mRBA may perform better than CHROMagar STEC for the isolation and detection of the O104:H4 strain used in this study, especially at low levels of contamination (1 or 10 CFU).

CHROMagar STEC and mRBA used for testing mung bean sprout samples contaminated with ~1 CFU were able to reveal EAggEC O104 in 15/20 (75%) and 16/20 (80%) samples, respectively, and all the

Table 3
Background microbial community associated with retail sprouts at the time of purchase and after 48 h stored at 4 °C.

Sprouts	Microbiota on sprouts at the time of purchase (CFU/g)			Microbiota on sprouts after 48 h (CFU/g)		
	ACP*	*E.coli*/CCP*	ECP*	ACP*	*E.coli*/CCP*	ECP*
Mung bean	7.30×10^6	3.40×10^5	1.30×10^6	1.20×10^8	8.80×10^5	1.30×10^7
Alfalfa	4.60×10^8	5.55×10^7	2.90×10^7	1.70×10^9	1.15×10^8	9.35×10^7

* Aerobic Count Plate (ACP); *E.coli*/Coliform Count Plate (*E.coli*/CCP); *Enterobacteriaceae* Count Plate (ECP).

Table 4
Real-time PCR and microbiological detection of EAggEC O104:H4 from mung bean and alfalfa sprout samples. Sprout samples were artificially contaminated with an O104:H4 strain at three different levels of inoculation. The EAggEC O104:H4 was detected by real-time PCR assays (Diatheva FLUO kits[b]) and microbiological methods.

Food	Inoculum CFU/25 g ± SD	Microbiological detection & confirmation[c]				Diatheva RT-PCR[b, c]
		CHROMOGENIC media		Latex agglutination	Multiplex RT-PCR[a]	*stx/(eae) aggR/aaiC*, O104[b]
		CHROM- agar STEC	mRBA O104:H4			
Mung bean sprouts	1.4 ± 0.19	15/20	16/20	16/20	16/20	18/20
	14.0 ± 1.9	8/10	10/10	10/10	10/10	10/10
	140.0 ± 19	2/2	2/2	2/2	2/2	2/2
Alfalfa sprouts	1.8 ± 0.31	15/20	17/20	17/20	17/20	15/20
	18.0 ± 3.1	10/10	10/10	10/10	10/10	10/10
	180.0 ± 31	2/2	2/2	2/2	2/2	2/2

[a] Presumptive EAggEC O104:H4 colonies identified on Chromogenic media were further confirmed by latex agglutination and real-time multiplex PCR for *stx*, *agg*R, and O-group target genes using primer described in Table 2.
[b] Total genomic DNA was extracted from enrichment cultures and used for real-time PCR using the Diatheva STEC FLUO kit for *stx/eae* detection, Enteroaggregative *E.coli* kit for *agg*R/*aai*C detection, and *E.coli* O104 FLUO kit.
[c] Number of positive samples/number of inoculated samples.

presumptive colonies were confirmed by latex agglutination and real-time PCR. When inoculated at ~10 CFU, 8/10 (80%) samples were positive using CHROMagar, whereas the same samples streaked onto mRBA gave 100% positive results (10/10). The 2011C-3493 strain was isolated using both chromogenic media 100% of the time when the mung bean samples were inoculated with 100 CFU. On the other hand, the EAggEC strain was isolated from 15/20 (75%) and 17/20 (85%) alfalfa sprouts contaminated with ~1 CFU on CHROMagar STEC and mRBA, respectively. Using 10 or 100 CFU as inoculum the EAggEC O104:H4 strain was isolated from all samples (10/10 and 2/2 replicates, respectively) using both chromogenic media (Table 4). Uninoculated samples were plated onto CHROMagar and mRBA and no presumptive EAggEC colonies were isolated.

Microbiological methods, often relying on selective culture enrichment and selective chromogenic media, are considered 'gold standard' methods for detection of foodborne pathogens due to their sensitivity and specificity. CHROMagar and mRBA are designed to recover a wide range of STEC from different sources (FDA-BAM, appendix 1.09, Jinneman et al., 2012), however complex microbiota in foodstuff such as sprouts, as well as the nutritional and supplement compositions of the media, may reduce their efficiency. To overcome this issue IMS is widely used before plating to reduce the background microbiota and enhance the recovery of specific STEC O-serogroups (Fratamico et al., 2011; Jinneman et al., 2012; Wasilenko et al., 2012). However, LeJeune et al. (2006) and Tutenel et al. (2003) suggest that samples with high background microbiota or long enrichment time (24 h instead of 6 h) are a challenge for IMS due to the increased competition of non-target microbiota that may hinder pathogen detection.

3.5. Comparison between Diatheva assays and microbiological methods

A comparison of the Diatheva real-time PCR methods and the microbiological isolation and confirmation showed perfect agreement with 100% accuracy for replicates contaminated with 100 and 10 CFU (Cohen's kappa = 1). At low contaminations levels (~1 CFU/sample) the molecular and microbiological methods present good agreement (Cohen's kappa = 0.62); specifically, the Diatheva kits yielded more positive results (18/20) than either plating on CHROMagar (15/20) or mRBA (16/20) (Table 4). Alfalfa sprout samples contaminated with 100 or 10 CFU also showed perfect agreement (Cohen's kappa = 1), with 100% of positive results (2/2 and 10/10, respectively) when tested with both chromogenic media and Diatheva assays. At a contamination level near ~1 CFU in alfalfa sprouts 15/20 samples yielded EAggEC O104 isolates using CHROMagar as well as by Diatheva assays, and 17/20 samples were confirmed using mRBA, showing good agreement between the two methods (Cohen's kappa = 0.61).

Not all the samples positive by the Diatheva PCR assays were subsequently isolated and confirmed on chromogenic media. Indeed at the low inoculum level (~1 CFU/sample) two mung bean samples that tested positive using the Diatheva kits but did not reveal putative STEC on either mRBA and CHROMagar. On the other hand, one each of the mung bean sprout and alfalfa sprout samples contaminated with ~1 CFU tested positive for mRBA but negative on CHROMagar and with Diatheva assays and one sample was negative for Diatheva kits but positive for CHROMagar and mRBA, with a single STEC colony on each media. To further evaluate these contradictory results, a new DNA extraction was carried out on frozen cells harvested from the culture enrichment samples previously tested by Diatheva kits, amplifying them using both the Diatheva assays and the primer and probes described in Table 2. The same results were obtained, suggesting that no cross reaction or cross contamination occurred during the first analysis. Hara-Kudo et al. (2016) also reported some samples that tested positive for the real-time PCR were negative by IMS and plating. This may occur because, although high levels of background flora can obscure detection of the target organism on chromogenic media, the sensitivity and specificity of PCR is less affected by an excess of non-target DNA.

In conclusion the real-time PCR procedure for the detection of EAHEC is able to detect the O104:H4 strain in mung bean and alfalfa sprouts at a contamination level of ~1 CFU/25 g after 18 h enrichment, showing good agreement with the IMS and microbiological plating method. Using the Enteroaggregative *E.coli* kit in combination with the STEC and *E.coli* O104 FLUO kits guarantees the maximum coverage of EAggEC strains providing important information relevant to pathogenic potential. Because the real-time PCR technique is more rapid than microbiological methods, the Diatheva FLUO kits assays could be a suitable platform for the food industry and regulatory agency to detect EAggEC pathogens in food, in order to improve consumer safety and providing data that could be used to better explain the real epidemiological distribution of these virulence factors and related *E. coli* pathotypes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.11.011.

Acknowledgements

This research was supported in part by an appointment to the Agricultural Research Service (ARS) Research Participation Program administrated by the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy (DOE) and the U.S. Department of Agriculture

(USDA). ORISE is managed by ORAU under contract number DE-SC0014664. All opinions expressed in this paper are the author's and do not necessarily reflect the polices and views of USDA, ARS, DOE, or ORAU/ORISE.

Conflict of interest

Author Rotundo, author Amagliani, author Carloni, and author Paoli have no conflict of interest; author Magnani (Professor at University of Urbino) hold shares of Diatheva srl, and Enrica Omiccioli is an employee of Diatheva srl. Diatheva srl provided some materials needed for the study; however, Diatheva srl had no influence on results generated in this work.

References

Amagliani, G., Rotundo, L., Carloni, E., Omiccioli, E., Magnani, M., Brandi, G., Fratamico, P., 2018. Detection of Shiga toxin-producing *Escherichia coli* (STEC) in ground beef and bean sprouts: evaluation of culture enrichment conditions. Food Res. Int. 103, 398–405.

Baranzoni, G.M., Fratamico, P.M., Rubio, F., Glaze, T., Bagi, L.K., Albonetti, S., 2014. Detection and isolation of Shiga toxin-producing *Escherichia coli* (STEC) O104 from sprouts. Int. J. Food Microbiol. 173, 99–104.

Barbau-Piednoir, E., Denayer, S., Botteldoorn, N., Dierick, K., De Keersmaecker, S.C., Roosens, N.H., 2018. Detection and discrimination of five *E. coli* pathotypes using a combinatory SYBR® green qPCR screening system. Appl. Microbiol. Biotechnol. 102, 3267–3285. https://doi.org/10.1007/s00253-018-8820-0. (Accessed 01 July 2018).

Barletta, F., Ochoa, T.J., Cleary, T.G., 2013. Multiplex real-time PCR (MRT-PCR) for Diarrheagenic. In: Wilks, M. (Ed.), PCR Detection of Microbial Pathogens. Methods in Molecular Biology (Methods and Protocols) Vol. 943. Humana Press, Totowa, NJ, pp. 307–314. https://doi.org/10.1007/978-1-60327-353-4_21. (Accessed 01 July 2018).

Bugarel, M., Beutin, L., Martin, A., Gill, A., Fach, P., 2010. Micro-array for the identification of Shiga toxin-producing *Escherichia coli* (STEC) seropathotypes associated with hemorrhagic colitis and hemolytic uremic syndrome in humans. Int. J. Food Microbiol. 142, 318–329.

Dechet, A.M., Herman, K.M., Chen Parker, C., Taormina, P., Johanson, J., Tauxe, R.V., Mahon, B.E., 2014. Outbreaks caused by sprouts, United States, 1998–2010: lessons learned and solutions needed. Foodborne Pathog. Dis. 11, 635–644.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2013. Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. EFSA J. 11, 3138. https://doi.org/10.2903/j.efsa.2013.3138. (Accessed 01 July 2018).

European Food Safety Authority (EFSA), 2011. Shiga toxin-producing *E. coli* (STEC) O104:H4 2011 outbreaks in Europe: taking stock. EFSA J. 9, 2390. https://doi.org/10.2903/j.efsa.2011.2390. (Accessed 01 July 2018).

European Food Safety Authority (EFSA) and European Center for Disease Prevention and Control (ECDC), 2017. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA J. e05077, 15.

Feng, P., Weagant, S.D., Jinneman, K.G., 2010. Diarrheagenic *Escherichia coli*. In: Bacteriological Analytical Manual, 8th edition. U.S. Food and Drug Administration (Revised, Chapter 4A). http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm (Accessed 01 July 2018).

Fratamico, P.M., Bagi, L.K., Cray Jr., W.C., Narang, N., Yan, X., Medina, M., Liu, Y., 2011. Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin–producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in ground beef. Foodborne Pathog. Dis. 8, 601–607.

Gensheimer, K., Gubernot, D., 2016. 20 years of sprout-related outbreaks: FDA's investigative efforts. In: Pen Forum Infectious Diseases. Vol. 3 Oxford University Press. https://doi.org/10.1093/ofid/ofw172.1140. No. suppl_1. (Accessed 01 July 2018).

Hara-Kudo, Y., Konishi, N., Ohtsuka, K., Iwabuchi, K., Kikuchi, R., Isobe, J., Tanouchi, A., 2016. An interlaboratory study on efficient detection of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O121, O145, and O157 in food using real-time PCR assay and chromogenic agar. Int. J. Food Microbiol. 230, 81–88.

Hidaka, A., Hokyo, T., Arikawa, K., Fujihara, S., Ogasawara, J., Hase, A., Hara-Kudo, Y., Nishikawa, Y., 2009. Multiplex real-time PCR for exhaustive detection of diarrhoeagenic *Escherichia coli*. J. Appl. Microbiol. 106, 410–420.

Huang, D.B., Okhuysen, P.C., Jiang, Z.D., DuPont, H.L., 2004. Enteroaggregative *Escherichia coli*: an emerging enteric pathogen. Am. J. Gastroenterol. 99, 383.

Jinneman, K.C., Waite-Cusic, J.G., Yoshitomi, K.J., 2012. Evaluation of Shiga toxin producing *Escherichia coli* (STEC) method for the detection and identification of STEC O104 strains from sprouts. Food Microbiol. 30, 321–328.

Kim, H., Lee, Y., Beuchat, L.R., Yoon, B.J., Ryu, J.H., 2009. Microbiological examination of vegetable seed sprouts in Korea. J. Food Prot. 72, 856–859.

Kuwayama, M., Shigemoto, N., Oohara, S., Tanizawa, Y., Yamada, H., Takeda, Y., Matsuo, T., Fukuda, S., 2011. Simultaneous detection of virulence factors from a colony in diarrheagenic *Escherichia coli* by a multiplex PCR assay with Alexa Fluor-labeled primers. J. Microbiol. Methods 86, 119–120.

LeJeune, J.T., Hancock, D.D., Besser, T.E., 2006. Sensitivity of *Escherichia coli* O157 detection in bovine feces assessed by broth enrichment followed by immunomagnetic separation and direct plating methodologies. J. Clin. Microbiol. 44, 872–875.

Matos, A., Garland, J.L., Fett, W.F., 2002. Composition and physiological profiling of sprout-associated microbial communities. J. Food Prot. 65, 1903–1908.

Morin, N., Santiago, A.E., Ernst, R.K., Guillot, S.J., Nataro, J.P., 2013. Characterization of the AggR regulon in enteroaggregative *Escherichia coli*. Infect. Immun. 81, 122–132.

Mueller, S.C., 2008. In: Summers, C.G., Putnam, D.H. (Eds.), Irrigated alfalfa Management in Mediterranean and Desert zones, Chapter 22. University of California Agriculture and Natural Resources, Report No. 8308, . http://alfalfa.ucdavis.edu/IrrigatedAlfalfa/pdfs/UCAlfalfa8308SeedProd_free.pdf (Accessed 01 July 2018).

Nataro, J.P., Kaper, J.B., Robins-Browne, R., Prado, V., Vial, P., Levine, M.M., 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. Pediatr. Infect. Dis. J. 6, 829–831.

Nataro, J.P., Mai, V., Johnson, J., Blackwelder, W.C., Heimer, R., Tirrell, S., Tirrell, S., Edberg, S.C., Braden, C.R., Morris Jr., Glenn, Hirshon, J.M., 2006. Diarrheagenic *Escherichia coli* infection in Baltimore, Maryland, and New Haven, Connecticut. Clin. Infect. Dis. 43, 402–407.

Okeke, I.N., Wallace-Gadsden, F., Simons, H.R., Matthews, N., Labar, A.S., Hwang, J., Wain, J., 2010. Multi-locus sequence typing of enteroaggregative *Escherichia coli* isolates from Nigerian children uncovers multiple lineages. PLoS One 5, e14093.

Oplinger, E., Hardman, L., Kaminski, A., Combs, S., Doll, J., 1990. Mungbean. In: Alternative Feed Crop Manual. University of Wisconsin, Cooperative Extension Services, Madison, WI. http://col.st/ZkAlA (Accessed 01 July 2018).

Robert Koch Institute, 2011. Report: Final Presentation and Evaluation of Epidemiological Findings in the EHEC O104:H4 Outbreak, Germany 2011. http://www.rki.de/EN/Content/infections/epidemiology/outbreaks/EHEC_O104/EHEC_final_report.pdf?_blob=publicationFile (Accessed 01 July 2018).

Rotundo, L., Fratamico, P.F., Amagliani, G., Carloni, E., Omiccioli, E., Magnani, M., 2018. Comparison of the Diatheva STEC FLUO with BAX system kits for detection of O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) in ground beef and bean sprout samples using different enrichment protocols. Food Anal. Methods. https://doi.org/10.1007/s12161-018-1269-z. (Accessed 01 July 2018).

Taniuchi, M., Walters, C.C., Gratz, J., Maro, A., Kumburu, H., Serichantalergs, O., Berkeley, L., 2012. Development of a multiplex polymerase chain reaction assay for diarrheagenic *Escherichia coli* and *Shigella* spp. and its evaluation on colonies, culture broths, and stool. Diagn. Microbiol. Infect. Dis. 73, 121–128.

Tozzoli, R., Maugliani, A., Michelacci, V., Minelli, F., Caprioli, A., Morabito, S., 2018. Validation on milk and sprouts of EN ISO 16654: 2001-Microbiology of food and animal feeding stuffs-Horizontal method for the detection of *Escherichia coli* O157. Int. J. Food Microbiol. https://doi.org/10.1016/j.ijfoodmicro.2018.05.005.

Tutenel, A.V., Pierard, D., Vandekerchove, D., Van Hoof, J., De Zutter, L., 2003. Sensitivity of methods for the isolation of *Escherichia coli* O157 from naturally infected bovine faeces. Vet. Microbiol. 94, 341–346.

Tzschoppe, M., Martin, A., Beutin, L., 2012. A rapid procedure for the detection and isolation of enterohaemorrhagic *Escherichia coli* (EHEC) serogroup O26, O103, O111, O118, O121, O145 and O157 strains and the aggregative EHEC O104: H4 strain from ready-to-eat vegetables. Int. J. Food Microbiol. 152, 19–30.

US Food and Drug Administration (FDA), 2017. Compliance with and recommendations for implementation of the standards for the growing, harvesting, packing, and holding of produce for human consumption for sprout operations: guidance for industry. Fed. Regist. 82, 7751–7753. https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/ucm510578.htm (Accessed 01 July 2018).

Wasilenko, J.L., Fratamico, P.M., Narang, N., Tillman, G.E., Ladely, S., Simmons, M., Cray Jr., W.C., 2012. Influence of primer sequences and DNA extraction method on detection of non-O157 Shiga toxin–producing *Escherichia coli* in ground beef by real-time PCR targeting the *eae*, *stx*, and serogroup-specific genes. J. Food Prot. 75, 1939–1950.

Weagant, S.D., Bound, A.J., 2001. Evaluation of techniques for enrichment and isolation of *Escherichia coli* O157:H7 from artificially contaminated sprouts. Int. J. Food Microbiol. 71, 87–92.