Risultati del 9° test inter-laboratorio nazionale per l'identificazione della presenza di ceppi di *E. coli* produttori di verocitotossina in campioni di semi di bietola - 2012

Il nono studio inter-laboratorio (*Proficiency test*, PT) sulla ricerca e l'identificazione dei ceppi di *E. coli* produttori di verocitotossina (VTEC) è stato organizzato nel 2012 dal Laboratorio Nazionale di Riferimento (LNR) per *E. coli* presso l'Istituto Superiore di Sanità ai fini della valutazione esterna di qualità dei laboratori coinvolti nel controllo ufficiale degli alimenti ed è stato dedicato alla ricerca di VTEC appartenenti ai cinque principali sierogruppi patogeni (O157, O26, O103, O111, O145) in campioni di semi di bietola. Poiché l'LNR per *E. coli* è anche Laboratorio Europeo di Riferimento (EU-RL) per questo patogeno, lo studio nazionale è stato condotto contestualmente a quello dedicato agli LNR per *E. coli* degli Stati Membri della UE, che ha visto la partecipazione di 37 LNR attivi nel settore della sanità pubblica veterinaria e della sicurezza alimentare, rappresentanti 26 Stati Membri dell'Unione Europea, la Norvegia, la Svizzera, la Turchia, la Russia e l'Egitto. Il report dello studio europeo è disponibile al sito web dell'EU-RL (http://www.iss.it/vtec/neww/cont.php?id=147&lang=2&tipo=15).

1. PARTECIPANTI

Allo studio nazionale hanno partecipato i 18 laboratori di seguito elencati:

- Centro Servizi Sanitari, Laboratorio di Sanità Pubblica, Trento
- IZS Abruzzo e Molise "G. Caporale", Reparto Igiene degli Alimenti, Teramo
- IZS Puglia e Basilicata, Ricerca e Sviluppo Scientifico, Foggia
- IZS Lombardia ed Emilia Romagna, Reparto di Microbiologia, Laboratorio Microbiologia, Brescia
- IZS Lombardia ed Emilia Romagna, Sezione di Bologna

- IZS Lombardia ed Emilia Romagna, Sezione di Piacenza, Gariga di Podenzano (PC)
- IZS Lazio e Toscana, Roma
- IZS Lazio e Toscana, Dir. Op. Controllo degli Alimenti, Roma
- IZS del Mezzogiorno, Sezione di Salerno, U.O. Microbiologia Alimentare, Fuorni (SA)
- IZS del Mezzogiorno, U.O.S."Biotecnologie applicate agli alimenti-OGM", Portici (Napoli)
- IZS della Sicilia, Area Microbiologia degli Alimenti, Palermo
- IZS della Sardegna, Laboratorio di Microbiologia e Terreni Colturali, Sassari
- IZS Piemonte, Liguria e Valle d'Aosta, Laboratorio Controllo Alimenti, Torino
- IZS Piemonte, Liguria e Valle d'Aosta, S.C. Biotecnologie, Torino
- IZS Umbria e Marche, Laboratorio Contaminanti Biologici PGCB, Perugia
- IZS Umbria e Marche, Laboratorio Controllo Alimenti, Sezione di Fermo
- IZS delle Venezie, Sezione Pordenone e Udine, Cordenons (PN)
- IZS delle Venezie, *OIE/National Reference Laboratory for Salmonellosis*, Legnaro (PD)

2. OBIETTIVI E STRUTTURA DEL TEST INTERLABORATORIO

La scelta dei semi destinati alla produzione di germogli come matrice per questo PT è dovuta al fatto che, negli ultimi anni, numerosi episodi epidemici di infezione da VTEC sono stati associati al consumo di germogli contaminati. In questi episodi, la contaminazione del prodotto finito e pronto per il consumo era frequentemente dovuta alla presenza del contaminante nei semi utilizzati per la produzione.

Gli obiettivi dello studio erano quindi: i) accrescere l'esperienza dei laboratori nell'uso del metodo molecolare standard per la ricerca dei VTEC; ii) valutare l'efficacia del metodo stesso su una matrice ad oggi non compresa nel suo campo di applicazione. Infatti, in assenza di un metodo standard specifico, il metodo sviluppato per la ricerca dei VTEC negli alimenti è stato adattato per l'analisi dei semi.

Ai laboratori partecipanti è stato richiesto di ricercare i VTEC appartenenti ai 5 sierogruppi maggiormente coinvolti nelle infezioni umane (O157, O111, O26, O103, and O145), utilizzando lo standard ISO/TS 13136: *Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups*. La procedura di laboratorio fornita ai laboratori, riportata in **Allegato,** includeva anche indicazioni per il trattamento dei campioni da effettuare prima dell'applicazione dello lo standard ISO/TS 13136.

3. MATERIALI E METODI

Il set inviato ai laboratori era costituito da 2 campioni di semi di bietola (campioni A e B, 50 g ciascuno) potenzialmente contaminati con VTEC appartenenti ai 5 sierogruppi maggiormente coinvolti nelle infezioni umane (O157, O111, O26, O103, and O145).

3.1. Preparazione dei campioni

La contaminazione artificiale dei campioni è stata ottenuta immergendo alcuni semi in una coltura liquida in fase esponenziale di uno stipite di VTEC O157 positivo per geni *vtx1* ed *eae*. I semi sono stati lasciati asciugare all'aria per 18 ore in una cabina a flusso laminare. Per costituire un campione positivo, un singolo seme contaminato è stato quindi aggiunto a 50 g di semi contenuti in un sacchetto da *stomacher*.

Per valutare la carica batterica, i singoli semi contaminati sono stati posti in 5 ml of PBS e sottoposti a vigorosa agitazione. Le cellule batteriche rilasciate sono state contate seminando diluizioni seriali della sospensione così ottenuta su piastre di agar MacConkey-Sorbitolo. Il livello medio di contaminazione riscontrato era di 4×10^6 CFU/seme.

Le caratteristiche dei campioni sono riportate nella Tabella 1 e sono state considerate come "*gold standard*".

Tabella 1. Caratteristiche dei campioni di semi inclusi nello studio

Contaminante	Campione A	Campione B
VTEC O157, *vtx1, eae*	8×10^4 CFU/g	-

3.2. Stabilità e omogeneità dei campioni

La stabilità e l'omogeneità dei campioni sono state verificate secondo quanto prescritto dalla norma ISO 17043:2010.

Per la verifica della stabilità, un gruppo di 5 campioni contaminati è stato preparato appositamente il 19 Settembre 2012 con le stesse procedure utilizzate successivamente per la preparazione dei campioni da impiegare nel test. I campioni sono stati conservati a temperatura ambiente e analizzati nei giorni 24 e 26 Settembre e 1, 3 e 4 Ottobre, sempre ottenendo i risultati attesi.

Per la verifica dell'omogeneità, i campioni da impiegare nello studio inter-laboratorio sono stati preparati tra il 13 e il 14 Novembre, e la loro omogeneità è stata verificata saggiando

12 campioni positivi e 12 campioni negativi, selezionati casualmente subito dopo la preparazione. I test sono stati effettuati il 15 Novembre e hanno prodotto i risultati attesi.

3.3. Invio dei campioni

I campioni, identificati con codici numerici a tre o quattro cifre assegnati casualmente e diversi per ogni laboratorio, sono stati mantenuti a temperatura ambiente fino al trasferimento in contenitori per la spedizione. Questa è stata effettuata il 19 Novembre mediante corriere. Ai laboratori è stato richiesto di iniziare le analisi entro 18 ore dall'arrivo stesso.

3.4. Raccolta ed elaborazione dei risultati

I laboratori hanno inviato i loro risultati direttamente via WEB, usando pagine dedicate accessibili attraverso la *Restricted Area* del sito web dell'EU-RL VTEC (www.iss.it/vtec), previa introduzione di *User ID* e *Password*, inviate a ogni laboratorio insieme al codice identificativo e alle istruzioni necessarie per il *log in*. Al termine del test, i partecipanti hanno avuto la possibilità di stampare direttamente il proprio *test-report* con i risultati inviati e quelli attesi.

3.5. Valutazione della *performance* dei laboratori

La *performance* è stata valutata in termini di:

- Concordanza (Kappa di Cohen)
- Sensibilità
- Specificità

Per il calcolo di tali parametri sono stati confrontati i risultati delle determinazioni analitiche ottenute dai laboratori con i valori reali (*gold standard*) dei campioni oggetto di analisi.

La valutazione del livello di concordanza è stata ottenuta attraverso il calcolo del Kappa di Cohen che permette di stimare l'accordo tra il risultato analitico e il valore gold standard, indipendentemente dalla componente imputabile al caso. Per la valutazione dell'accettabilità del valore Kappa è stata utilizzata la griglia di giudizio proposta da *Fleiss J.L. (Statistical methods for rates and proportions, 1981)* secondo la quale valori di $K \geq 0,75$ indicano livelli di concordanza eccellenti, valori $0,40 \leq K < 0,75$ buona concordanza e valori $K < 0,40$ livelli di concordanza scarsi. La sensibilità diagnostica è stata definita come la proporzione di campioni positivi correttamente identificati (presenza dei geni *vtx1*, *vtx2, eae* e sierogruppo specifici). La specificità diagnostica è stata definita

come la proporzione di campioni negativi identificati correttamente (assenza dei geni *vtx1*, *vtx2, eae* e sierogruppo specifici). Per tutti i parametri è stato calcolato il relativo intervallo di confidenza (95 % I.C.).

4. RISULTATI

I campioni sono stati inviati il 19 Novembre e sono stati recapitati regolarmente ai Laboratori partecipanti il giorno successivo, I risultati sono inviati via web entro la scadenza stabilita da 17 laboratori. Il laboratorio L29 non ha inviato i risultati.

Il primo *step* del metodo ISO/TS 13136 prevede lo screening delle colture di arricchimento per la presenza di geni di virulenza e sierogruppo-specifici mediante Real-Time PCR (Tabella 2).

Tabella 2. Ricerca dei geni di virulenza e sierogruppo-specifici nelle colture di arricchimento. Le caselle verdi evidenziano i risultati corretti, le caselle rosse i risultati sbagliati. Le caselle vuote indicano che il test corrispondente non è stato effettuato.

Lab	Identificazione dei geni di virulenza e dei geni associati al sierogruppo in:															
	Campione A								Campione B							
	vtx1	*vtx2*	*eae*	O26	O157	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O26	O157	O103	O111	O145
Valore atteso	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
L04	+	-	+	-	+	-	-	-	-	-	-					
L16	+	-	+	-	+	-	-	-	-	-	-					
L19	+	+	+	-	+	-	-	-	-	-	-					
L21	+	+	+	-	+	-	-	-	-	-	-					
L23	+	-	+	-	+	-	-	-	-	-	-					
L28	+	-	+	-	+	-	-	-	-	-	-					
L45	+	-	+						-	-	-					
L51	+	-	+	-	+	-	-	-	-	-	-					
L55	+	-	+	-	+	-	-	-	-	-	-					
L57	+	-	+	-	+	-	-	-	-	-	-					
L75	+	-	+	-	+	-	-	-	-	-	-					
L78	+	-	+	-	+	-	-	-	-	-	-					
L88	+	-	+	-	-	-	-	-	-	-	-					
L99	+	-	+	-	+	-	-	-	-	-	-					
L113	+	+	+	-	+	-	-	-	-	-	-					
L114	+	-	+	-	+	-	-	-	-	-	-					
L116	+	-	+	-	+	-	-	-	-	-	-					

Questa parte dello studio è stata effettuata correttamente da 13 laboratori che, hanno quindi ottenuto valori individuali di sensibilità e specificità del 100% e valori di concordanza, valutati tramite il Kappa di Cohen, pari a 1, con limiti di confidenza pari a 0,41 – 1.

Tre laboratori (L19, L21, L113) hanno utilizzato kit commerciali che individuano la presenza dei geni *vtx* ma non sono in grado di distinguere i geni *vtx1* da quelli *vtx2*. Questi laboratori hanno quindi riportato correttamente la presenza di geni *vtx* nel campione A, segnalando però la presenza di entrambe le varianti geniche. Per questi laboratori la sensibilità era pari al 100%, la specificità all' 87,5% e il valore di K era 0,79, con limiti di confidenza pari a 0,21 - 1.

Un laboratorio (L45) non ha effettuato la ricerca dei geni sierogruppo-specifici, ottenendo sensibilità e specificità del 100% e un valore di K pari a 1, con limiti di confidenza pari a 0,21 - 1, visto il minor numero di test effettuati.

La successiva fase dell'isolamento dei ceppi VTEC dai campioni PCR-positivi è stata condotta da 15 laboratori, mentre I laboratori L45 e L113 non hanno inviato i risultati relativi all'isolamento.Questi 15 laboratori, dopo aver identificato correttamente la presenza del gene associato al sierogruppo O157 nelle colture di arricchimento, hanno anche isolato e geno-tipizzato correttamente il ceppo VTEC O157 dal campione A, con l'eccezione del laboratorio L21, che ha segnalato la presenza di entrambe le varianti dei geni *vtx.* L'errore era ancora dovuto all'utilizzo di kit commerciali non in grado di distinguere i geni *vtx1* da quelli *vtx2*.

Tabella 3. Isolamento e geno-tipizzazione dei ceppi VTEC dalle colture di arricchimento Real Time PCR-positive. Le caselle verdi evidenziano i risultati corretti, le caselle rosse i risultati sbagliati. Le caselle vuote indicano che il test corrispondente non è stato effettuato.

Laboratorio	Isolamento e geno-tipizzazione dei ceppi VTEC dalle colture di arricchimento							
	Campione A				Campione B			
	VTEC	Genotipo			VTEC	Genotipo		
Valore atteso	O157	*vtx1*	*vtx2*	*eae*	Nessuno	*vtx1*	*vtx2*	*eae*
		+	-	+		-	-	-
L04	O157	+	-	+				
L16	O157	+	-	+				
L19	O157	+	-	+				
L21	O157	+	+	+				
L23	O157	+	-	+				
L28	O157	+	-	+				
L51	O157	+	-	+				
L55	O157	+	-	+				
L57	O157	+	-	+				
L75	O157	+	-	+				
L78	O157	+	-	+				
L88	O157	+	-	+				
L99	O157	+	-	+				
L114	O157	+	-	+				
L116	O157	+	-	+				

5. Considerazioni

- Lo studio è stato condotto a termine da 17 laboratori coinvolti nel controllo ufficiale degli alimenti, che includevano 9 Istituti Zooprofilattici Sperimentali.
- Tutti i laboratori hanno effettuato la fase di screening molecolare delle colture di arricchimento mediante Real-time PCR, identificando correttamente il campione contaminato da VTEC.
- Quindici laboratori hanno effettuato la fase dell'isolamento del ceppo di VTEC O157 dalla coltura PCR-positive, riportando una performance analitica eccellente.
- Questo studio ha confermato che il metodo ISO/TS 13136 basato sullo screening mediante Real-time PCR delle colture di arricchimento rappresenta uno strumento robusto per la ricerca dei VTEC anche quando applicato a matrici non strettamente comprese nel suo campo di applicazione.

ALLEGATO

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9th inter-laboratory study on the detection of Verocytotoxin-producing *E. coli* (VTEC) belonging to the serogroups most involved in human infections (O157, O111, O26, O103, and O145) in samples of seeds intended for sprout production

Laboratory Guideline

Introduction

In the absence of specific international standards for the detection of VTEC in seeds, the method developed for the detection of VTEC in foodstuffs, CEN/ISO TS 13136 "Microbiology of food and animal feed -- Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens -- Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 serogroups", has been adapted to this matrix. In particular, the following aspects have been considered:

1. Seeds are generally contaminated at very low levels. Nonetheless, the sprouting process is characterized by conditions (humidity, heat) favoring the pathogen's enrichment. Therefore, 50 gr of seeds are analyzed instead of the usual 25 gr of food items, in order to increase the sensitivity of the assay.

– Seeds are generally dried. Therefore, the contaminating pathogens are supposed to be stressed.

– The contamination may occur on the surface of the seeds as well as inside their body.

– The enrichment cultures of seeds may contain inhibitors of the DNA polymerase used for the PCR screening of the samples.

The procedure comprises the following sequential steps:

- Smashing of the seeds to allow the release of possibly internalized bacteria;
- Transfer of the sample to the enrichment medium;
- Microbial enrichment;

- Nucleic acid extraction;
- Detection of virulence genes;
- Detection of serogroup-associated genes;
- Isolation from positive samples.

1. Treatment of the seed samples

The samples are constituted by 50 gr of seeds placed in a stomacher bag.

1. The seeds are smashed directly in the stomacher bag, using a mortar with pestel or other similar tools, before adding the enrichment broth. It is advisable to put the bag with the sample into another sterile stomacher bag, to limit the possibility of spill over due to cuts in the bag.
2. The smashed seeds are added with 450 ml BPW and incubated for 24 hrs at 37°C (either static or in agitation). Check carefully for the integrity of the stomacher bag after smashing the seeds. In case of damages evidence transfer the smashed seeds aseptically to a sterile container (flask or a new stomacher bag) before adding the culture medium. This operation must be done under a Biohazard laminar flow hood.
3. A 5 ml aliquot of the enrichment culture is taken, mixed by vortex (in order to detach as much as possible the bacteria possibly adhering to seeds), centrifuged at 500 X g 1 min to sediment the seeds' debris.
4. One ml aliquot of the supernatant is taken at this stage and used for DNA preparation.

2. Nucleic acid extraction, detection of virulence and serogroup-associated genes, and isolation of VTEC

This procedure is described in the **Annex 1**. Briefly, it is based on a Real-time PCR screening of enrichment cultures to detect the presence of virulence genes (*vtx*1 and *vtx*2, and *eae*) and serogroup-specific genes for O26, O103, O111 and O145, followed by the isolation of VTEC from PCR-positive samples, accomplished by an immuno-concentration enrichment step specific for the serogroups identified in the PCR step.

One ml aliquot of the enrichment culture performed and treated as described in the previous paragraph is used for DNA extraction and purification, accomplished according to the ISO 20837 "Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of foodborne pathogens - Requirements for sample preparation for qualitative detection". The remaining culture shall be stored at 4°C for the isolation steps that will follow a positive PCR result.

To perform the Real-time PCR, the DNA sample is diluted 1:10 before use. In the case of absence of amplification of the internal amplification control (IAC), the DNA template is used at the dilution of 1:30.

The method is sequential:

Step 1: Detection of the genes *vtx1*, *vtx2* and *eae.*

Step 2: Samples positive for both *vtx* and *eae* are tested for the serogroup-associated genes (molecular serogrouping).

Step 3: Isolation of the VTEC strain; samples positive at the same time for *vtx*, *eae*, and at least one of the serogroup-associated genes are submitted to a further step aimed at isolation of the VTEC strain. This requires serogroup-specific enrichment based on IMS or other immuno-capture suitable approaches. A guideline for the isolation of the different VTEC serogroups is also included in the **Annex 1**.

Step 4: Characterization of the isolate i.e. identification, detection of *vtx* genes, the *eae* gene and the serogroup gene.

Annex 1

Laboratory procedure for the detection of Shiga toxin (Verocytotoxin)-producing *Escherichia coli* (STEC/VTEC) belonging to O157, O111, O26, O103 and O145 serogroups in seed samples - Qualitative Method

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) cause severe disease in humans such as haemorrhagic colitis and haemolitic uraemic syndrome (HUS). Although STEC may belong to a large number of serogroups, those that have been firmly associated with severe human disease, in particular HUS, belong to O157, O26, O111, O103, O145 (1), and represent the targets of this Laboratory Procedure.

In this Laboratory Procedure, the wording Shiga toxin (Stx) is synonymous of Verocytotoxin (Vtx).
The following nomenclature has been adopted throughout the text:
stx: Shiga toxin genes (synonymous of *vtx)*
Stx: Shiga toxin (synonymous of Vtx)
STEC: Shiga toxin-producing *Escherichia coli (*synonymous of VTEC: Verocytotoxin-producing *Escherichia coli).*

1. Scope

This Laboratory Procedure describes a method for the detection of (i) the major virulence genes of STEC (2,3), and (ii) the genes associated with the serogoups O157, O111, O26, O103 and O145 (3,4).
In the case of detection of these genes, the isolation of the strain is attempted, to confirm the simultaneous presence of the genes in the same live bacterial cell.

The analytical approach is based on the use of the Real-time PCR..

This Laboratory Procedure is applicable to enrichment cultures from seed samples.

2. Normative references

ISO/DIS 7218

General requirements and guidance for microbiological examinations

ISO/DIS 20837

Requirements for sample preparation for qualitative detection

ISO/DIS 20838

Requirements for amplification and detection of qualitative methods

ISO/DIS 22174

General method specific requirements

3. Terms and definitions

3.1 Shiga toxin-producing *Escherichia coli* (STEC): Microrganism possessing the Stx-coding genes

3.2 Shiga toxin-producing *Escherichia coli* (STEC) potentially pathogenic to humans: Microrganism possessing the Stx-coding genes and the intimin-coding gene *eae*.

3.3 Shiga toxin-producing *Escherichia coli* (STEC) highly pathogenic to humans: Microrganism possessing the Stx-coding genes, the intimin-coding gene *eae* and belonging to one of the serogroups in the scope of the present Laboratory Procedure.

4. Principle

4.1 General

The detection of STEC and of the 5 serogroups comprises the following sequential steps:

1. Microbial enrichment
2. Nucleic acid extraction
3. Detection of virulence genes
4. Detection of serogroup-associated genes in samples positive to point 3
5. Isolation from samples positive to points 3 and 4.

A flow-diagram of the whole procedure is given in Annex A.

4.1.1 Microbial enrichment

The number of STEC cells to be detected is increased by incubating the test portion in the non-selective liquid nutrient medium Buffered peptone water (BPW).

The BPW is to be used to analyse the seeds samples since they are supposed to contain stressed target bacteria (dryed matrix).

4.1.2 Nucleic acid extraction

Bacterial cells are separated from the enrichment medium and lysed. The nucleic acid is then purified according to any protocol allowing the production of template DNA suitable for Real Time PCR.

4.1.3 Target genes

The purified nucleic acid is used for the detection of the following target genes:

- the main virulence genes for STEC: *stx* genes, encoding the Shiga toxins and the *eae* gene, encoding a 90KDa protein, the intimin, involved in the attaching and effacing mechanism of adhestion, a typical feature of the pathogenic STEC strains.
- the *rfbE (O157), wbdI(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.1.4 Detection

The Real-time PCR products are detected by light emission in a 5' nuclease PCR assay.

4.1.5 Isolation

Once a STEC is detected and the serogroup identified, in order to isolate the STEC strain, a serogroup-specific enrichment is performed followed by plating onto the agar Trpytone-bile-glucuronic medium (TBX) or onto a specific selective medium where available (see note in Annex A).

5. Diluent, culture media and reagents

5.1 Culture media

5.1.1 Buffered peptone water (BPW)

Peptone	10	g
Sodium chloride	5.0	g
Disodium phosphate (Na_2HPO_4)	3.5	g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5	g
Water	to 1000	ml

pH 7.2 ± 0.2

Preparation

Dissolve the components or the dehydrated powder in the water. Adjust pH with a pH-meter pH 7.4 +/- 0.2 at 25°C (6.8) and sterilize by autoclaving at 121°C 15 min (6.9).

5.2 Reagents for nucleic acid extraction

The reagents to be used for nucleic acid extraction are not listed being dependent on the method adopted (9.3)

5.3 Reagents for PCR

See ISO 20838.

5.3.1 Oligonucleotides (primers) and detection probes

Primers and probes for specific detection of the target gene sequences by Real time PCR are listed in Annex E.

6. Equipment

Usual microbiological laboratory equipment (see ISO 7218) and, in particular, the following:

6.1 Water bath up to 100° C

6.2 Incubator according to ISO 7218, 37°± 1°C

6.3 Appatarus for nucleic acid extraction

Appropriate equipment according to the method adopted. If needed.

6.4 Pipettes for volumes between 1µl and 1000 µl

6.5 Thin walled Real-Time PCR microtubes (0,2 ml /0,5 ml reaction tubes), multi-well PCR microplates or other suitable light transparent disposable plasticware.

6.6 Thermal cycler Several brands of apparatus are available and can be chosen according to the laboratory policies.

6.7 Apparatus for detection of the PCR product

Light emission following 5' nuclease PCR assay is detected by the Real-time PCR apparatus

6.8 pH-meter capable of measuring to an accuracy of +/- 0.05 pH units and its resolution shall be 0.01 pH units

6.9 Autoclave according to ISO 7218

6.10 Stomacher peristaltic blender with sterile bags possibly with a device for adjustin speed and time

7. Sampling

In the absence of specific International Standards dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8. Preparation of test sample

Prepare the test sample in accordance with the specific International Standard dealing with the product concerned where available, or other suitable guidelines.

9. Procedure

9.1 Test portion and initial suspension

9.1.1 General

Use the necessary quantity of enrichment medium to yield a final dilution of 1/10 of the original test portion (50 gr seeds + 450 ml BPW).

9.2 Enrichment

9.2.1 Incubation

Incubate the stomacher bag or the tube/bottle (9.1.2) at 37°C ± 1°C for 16 h to18 h.

9.2.2 Process control (for Real-time PCR)

Perform process control according to ISO 22174.
Guidance on internal amplification control (IAC) and process control are given in Annex D.

9.3 Nucleic acid preparation

Use an appropriate nucleic acid extraction procedure for Gram-negative bacteria. A comprehensive collection of methods can be found in (7). Alternatively, commercial kits may be used according to the manufacturers' instructions.

9.4 Real-time PCR amplification

9.4.1 General

Follow all requirements for the PCR amplification as described in ISO 20838 "Microbiology of food and animal feeding stuffs – Polymerase chain reaction for the detection of food pathogens – amplification and detection".
Primers and detection probes for conducting the real-time PCR are described in Annex E.

9.4.2 PCR controls

In accordance with ISO 22174, examples of PCR controls are given in Annex D**.**

9.4.3 Detection of PCR products

Light emission is captured by the apparatus once generated during the amplification.

9.4.4 Interpretation of PCR results

The PCR results obtained, including the controls specified in ISO 22174 and in Annex D, are interpreted by the software linked to the apparatus. During amplification, the Software monitors 5'-nuclease PCR amplification by analysing fluorescence emissions (*Rn*) of the reporter dye for each sample. ΔRn was *Rn* minus the baseline reporter dye intensity established in the first few cycles. At the end of the PCR, a reaction was considered positive if its ΔRn curve exceeded the threshold, defined as 10 times the standard deviation of the mean baseline emission calculated between the first few cycles. The cycle threshold (*Ct*) was defined as the cycle number at which a sample's ΔRn fluorescence crossed the determined threshold value.

If the results are ambiguous, check the emission curves. Positive samples give a curve with a clear increase in fluorescence, starting from a number of cycles corresponding to the *Ct*.

If the controls yield unexpected results, repeat the procedure.

9.5 Strain isolation

The pattern of virulence genes, exhibited by STEC which are considered to be pathogenic to humans, is complex and it is possible that different strains presenting only part of the virulence gene pattern may be present in the food sample at the same time. Therefore, the isolation of the STEC strains is required to confirm that the positive PCR signals are generated from genes that are simultaneously present in the same live bacterial cell.

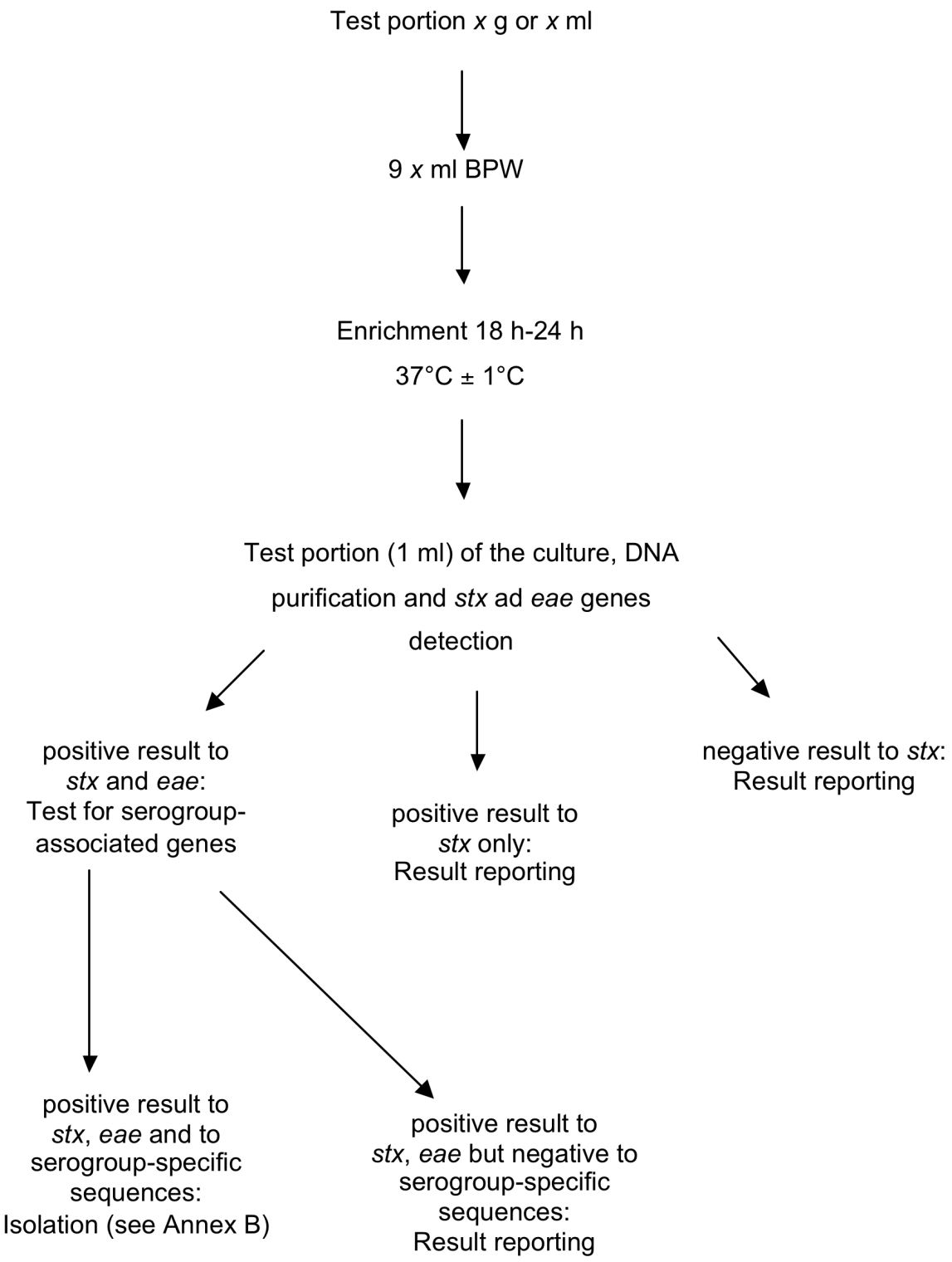
A serogroup-specific enrichment followed by direct plating onto suitable solid media and screening of the colonies for the presence of the virulence genesis are required.

The real-time PCR protocol described in this Laboratory Procedure may be used in order to confirm the presence of the virulence genes in the isolated colonies. In alternative other equivalent PCR protocols can be downloaded from the EU RL VTEC website (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3).

STEC isolation is described in the flow chart of Annex B

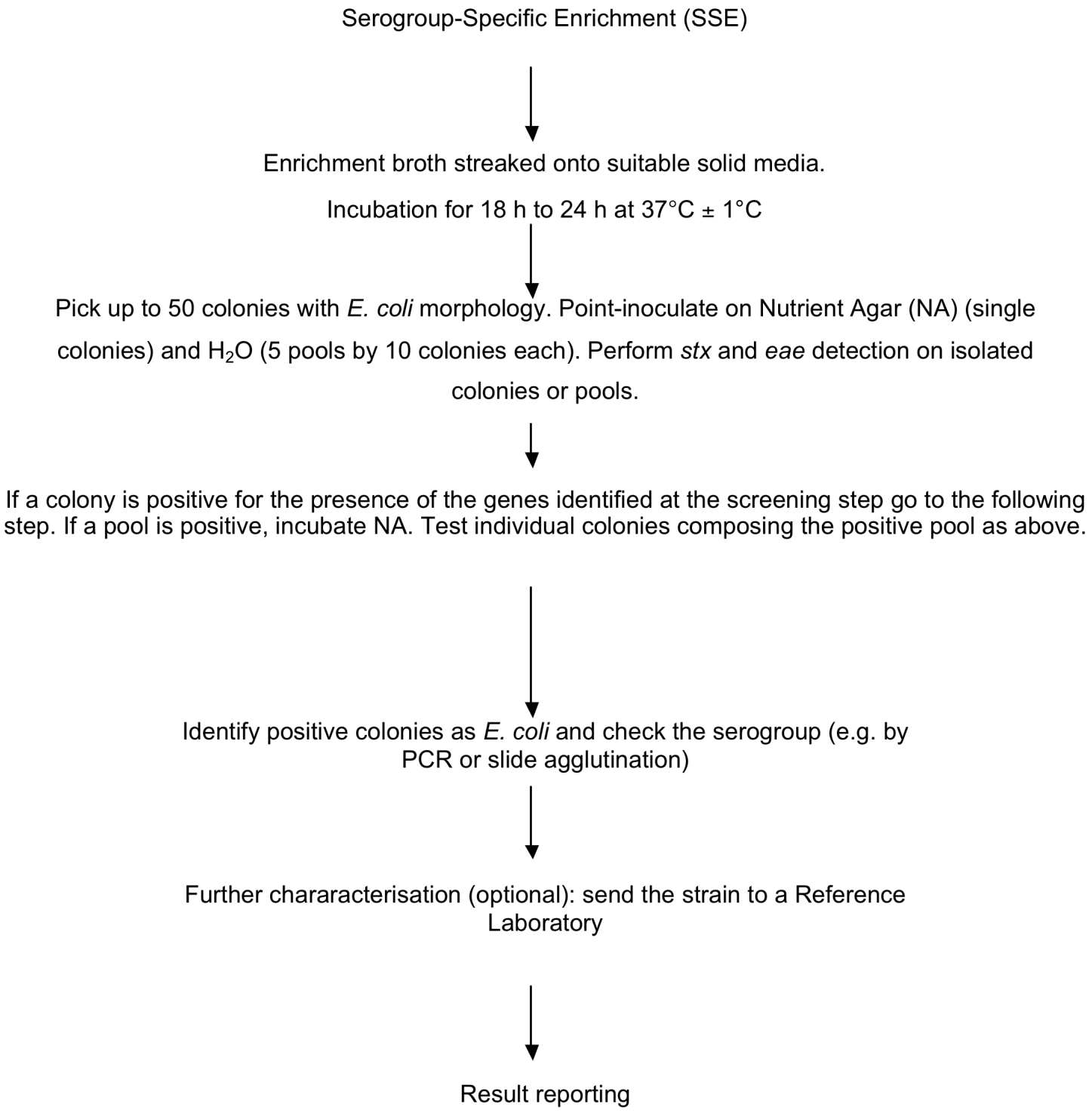
Annex A

Flow-diagram of the screening procedure



Annex B

Flow-diagram of the isolation procedure



Annex D
Internal Amplification Control

Three different internal amplification controls (IACs) can alternatively be used in Real-time PCR:

- A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems, Foster City, CA, USA). The reagent kit include all reagent necessary (primers, a Vic™probe, IAC target DNA and blocking solution). The IAC target DNA must be diluted 10 times to achieve a copy number of approximately 100 per PCR reaction. The PCR product length is not declared to the customer.
- The open formula pUC 19 based internal amplification control IAC developed by Fricker et al. (11). Approximately 100 copies of target DNA (pUC 19) should be used per PCR reaction. The size of the IAC was 119 bp.
- A recombinant plasmid (named pIAC-STEC) can be used in the *stx*-specific real-time PCR assay (Auvray *et al*. personal communication). This IAC contains the following DNA fragment cloned into the *Eco*RI site of pUC19:5'-ATT**TTTGTTACTGTGACAGCTGAAGCTTTACG**TGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATA**GATGTTGATCTTACATTGAACTGGGG**AATT-3' (bold letters: stx1/stx2 forward and reverse primers binding sites sequences; underlined sequence: IAC-probe binding site). The IAC is co-amplified with *stx* genes using the same primers as *stx* (Annex E), under the same conditions and in the same PCR tube (12). It is detected by a specific DNA probe (5' [Red640]-CAAGGCGACAAGGTGCTGATGCCG-[BHQ2] 3') included in the PCR mix at a concentration identical to that of the *stx1* and *stx2* DNA probes (both labelled with [Fluorescein] and [BHQ1] at their 5' and 3' ends, respectively). 64 copies of the IAC should be used per PCR reaction. The IAC PCR product is 96 bp-long. The performance of the resulting *stx*-IAC real-time PCR assay has been shown using artificially and naturally contaminated food samples (Auvray *et al*. personal communication).

The last two systems may be also used as an extraction control by adding 100 copies of the pUC 19 plasmid or of the pIAC-STEC to the sample aliquot prior to the DNA purification step.

ANNEX E

The Real-time PCR protocol described is based on the use of the following primers and probes which shall be considered as refererence reagents. However, other primers and probes may be used provided that they have been recognised equivalent to those indicated in the tables E.1 and E.2 according to the ISO 16140 rules.

Primers and probes for the PCR assays

Tables E.1 and E.2 provides respectively the primers and probes sequences for:

- the detection of *stx* and eae genes by real-time-PCR (PCR A);
- the detection of serogroup-related genes genes using real-time-PCR (PCR B).

In these tables , the chemistry of the reporter and quencher phluorophores is not indicated, being largely dependent on the real time PCR instruments available in each laboratory.

Table E.1: Degenerate primers and TaqMan probes used for 5' nuclease PCR assays. ([§]3 and [*]2)

Target gene	Forward primer, reverse primer and probe sequences (5'-3')[a]	Amplicon size (bp)	Location within sequence	GenBank accession number
stx1[§]	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	878–906 983–1008 941–971	M16625
stx2[§ b]	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC **Probe-**TCGTCAGGCACTGTCTGAAACTGCTCC	128	785–813 785–813 838–864	X07865
*eae**	CAT TGA TCA GGA TTT TTC TGG TGA TA CTC ATG CGG AAA TAG CCG TTA **Probe-**ATAGTCTCGCCAGTATTCGCCACCAATACC	102	899-924 1000-979 966-936	Z11541

[a] In the sequence Y is (C, T), S is (C, G), W is (A, T), R is (A, G), M is (A, C).

[b] This combination of primer/probe recognises all the *stx2* variants but the *stx2f*

Table E.2. Primers and probes used for amplification of O antigen specific genes in 5' nuclease PCR assays. ([§]3 and [*]4)

Target gene (serogroup)	Forward primer, reverse primer and probe sequences (5'-3')	Amplicon size (bp)	Location within sequence	GenBank accession number
[§]*rfbE* (O157)	TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT **Probe-**AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	348–372 412–435 381–410	AF163329
[§]*wbdI* (O111)	CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC **Probe-**TTGAATCTCCCAGATGATCAACATCGTGAA	146	3464–3489 3579–3609 3519–3548	AF078736
[§]*wzx* (O26)	CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAACTTT **Probe-**CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	5648–5666 5757–5782 5692–5724	AF529080
[§]*ihp1* (O145)	CGATAATATTTACCCCACCAGTACAG GCCGCCGCAATGCTT **Probe-**CCGCCATTCAGAATGCACACAATATCG	132	1383–1408 1500–1514 1472–1498	AF531429
[*]*wzx* (O103)	CAAGGTGATTACGAAAATGCATGT GAAAAAAGCACCCCCGTACTTAT **Probe-**CATAGCCTGTTGTTTTAT	99	4299–4323 4397–4375 4356–4373	AY532664

Annex F

Isolation of STEC strains

Follow the procedure described below to isolate STEC strains from real time PCR positive samples:

1) Perform a serogroup-specific enrichment (SSE) on the remaining enrichment culture (see Note 1)
2) Streak SSE onto TBX or other suitable medium (see note 2). Incubate for 18 to 24 hours at 37°C
3) Pick up 10 to 50 colonies with *E. coli* morphology or with characteristic aspect (see Note 5) and point-inoculate on nutrient agar (NA) (see Note 3) and H_2O (the colonies may be pooled in water up to a number of ten per pool).
4) Perform the detection of the *stx*-coding gene and the *eae* gene on the isolated colonies or the H_2O pools (see Note 4).
5) If a pool is positive, go back to NA and assay the individual colonies forming the positive pool in order to select one single positive colony.
6) Identify the colonies as *E. coli* and confirm the serogroup the sample was positive to in the screening PCR assay (e.g. by PCR B in the Annex E), see Note 5.
7) Isolates may be sent to the a Reference Laboratory for further characterization.

NOTE 1: Serogroup-specific enrichment may be achieved by using immunocapture systems such as immuno-magnetic separation (IMS) or equivalent. Generally, refer to the instruction supplied by the manufacturer.
For O157 positive samples, use ISO 16654 or alternative methods validated according to ISO 16140.

NOTE 2: For O157 positive samples, use ISO 16654 or alternative methods validated according to ISO 16140. Sorbitol-fermenting *E. coli* O157 are susceptible to tellurite contained in the CT SMAC medium indicated in ISO 16654. Therefore the use of a second SMAC isolation plate without antibiotics is recommended. In the absence of Sorbitol-negative colonies on the plates, the screening of Sorbitol-positive colonies is suggested.
For STEC O26 isolation, a differential solid media (MacConkey) containing Rhamnose instead of lactose is commercially available (RMAC). It is very effective in distinguishing STEC O26 strains, which do not ferment Rhamnose, from other *E. coli*.

NOTE 3: There are several types of nutrient agar media commercially available either ready to use plates or prepared in house from dehydrated powders. Every type of non-

selective nutrient agar media (e.g. TSA) is suitable for the purpose of maintaining the colonies for further characterisation. Enterohaemolysin Agar, can also be used. It gives the advantage to detect the Enterohaemolysin production, which is a common feature of STEC pathogenic to humans.

NOTE 4: The Real Time PCR described in this protocol may be adopted to confirm the presence of the *stx* and *eae* in the isolated strains. Conventional PCR may be used as an alternative (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3).

NOTE 5: Colony confirmation as *E. coli* may be achieved by using any commercial biochemical multi-assay or by assessing the indole production. Confirmation of the serogroup may be achieved either by PCR or by agglutination with commercial antisera.

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