Risultati del 7° test inter-laboratorio nazionale per l'identificazione della presenza di ceppi di *E. coli* produttori di verocitotossina in alimenti di origine vegetale - 2011

Il settimo studio inter-laboratorio (*Proficiency test*, PT) sulla ricerca e l'identificazione dei ceppi di *E. coli* produttori di verocitotossina (VTEC) è stato organizzato nel 2011 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 vegetali (spinaci). 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 ai LLNNRR per *E. coli* degli Stati Membri della UE, che ha visto la partecipazione di 30 LNR attivi nel settore della sanità pubblica veterinaria e della sicurezza alimentare, rappresentanti 21 Stati Membri dell'Unione Europea, la Norvegia, la Serbia, la Svizzera e la Turchia. Il report dello studio europeo è disponibile al sito web dell'EU-RL (http://www.iss.it/vtec/neww/index.php?lang=2&tipo=15&anno=2012).

1. PARTECIPANTI

Allo studio nazionale hanno partecipato 10 laboratori di 9 Istituti Zooprofilattici Sperimentali (IZS), di seguito elencati:

- IZS Lombardia e Emilia Romagna, Laboratorio di Microbiologia, Brescia
- IZS Lombardia e Emilia Romagna, Sezione di Bologna
- IZS del Mezzogiorno, Sezione di Salerno, Fuorni (NA)
- IZS Puglia e Basilicata, Laboratorio di Biotecnologie applicate agli alimenti e batteriologia speciale, Foggia
- IZS Abruzzo e Molise "G. Caporale", Reparto Igiene degli Alimenti, Teramo
- IZS delle Regioni Lazio e Toscana, Dir. Op. Controllo degli Alimenti, Centro di Rif. Reg. Enterobatteri Patogeni, Roma
- IZS delle Venezie, Sezione di Pordenone e Udine, Cordenons (PN)
- IZS Umbria e Marche, Laboratorio Contaminanti Biologici PGCB, Perugia
- IZS Piemonte, Liguria e Valle d'Aosta, Laboratorio Controllo Alimenti, Torino
- IZS della Sicilia, Area Microbiologia degli Alimenti, Palermo

2. OBIETTIVI E STRUTTURA DEL TEST INTERLABORATORIO

Il 3° e il 4° PT, condotti nel 2009 e nel 2010, sono stati dedicati alla ricerca dei cinque sierogruppi VTEC maggiormente coinvolti nelle infezioni umane (O157, O26, O103, O111 e O145), rispettivamente in tamponi superficiali di carcasse bovine e in campioni di latte bovino (report disponibili a www.iss.it/vtec).

Lo studio condotto nel 2011 (PT7) è stato dedicato alla ricerca di VTEC in alimenti di origine vegetale, visto il ruolo crescente di questi alimenti nella trasmissione delle infezioni da VTEC e da altri patogeni enterici.

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 il metodo che è stato raccomandato dall'EFSA nel 2009 ed è in procinto di essere pubblicato come standard ISO (ISO/WD TS 13136: "*Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method*").

Il metodo prevede lo screening delle colture di arricchimento per la presenza di geni di virulenza e sierogruppo-specifici mediante Real-Time PCR, seguito dall'isolamento dei ceppi VTEC dai campioni risultati positivi, mediante arricchimento per immuno-

concentrazione specifico per il sierogruppo identificato nella fase screening. Questo studio consisteva quindi di due fasi:

1. Screening delle colture di arricchimento per la presenza di geni di virulenza e sierogruppo-specifici mediante Real-Time PCR.
2. Isolamento e caratterizzazione dei ceppi VTEC dai campioni risultati positivi alla Real-time PCR, dopo ulteriore arricchimento mediante immuno-concentrazione specifico per il sierogruppo identificato nella fase screening.

La procedura di laboratorio inviata ai Laboratori è riportata come **Allegato 1**.

3- MATERIALI E METODI

Il set inviato ai laboratori era costituito da 3 campioni di spinaci (A, B e C) potenzialmente contaminati con VTEC appartenenti ai 5 sierogruppi maggiormente coinvolti nelle infezioni umane (O157, O111, O26, O103, and O145), e contenenti una flora microbica di *background*.

3.1. Preparazione dei campioni

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

Tabella 1: Caratteristiche dei campioni di spinaci inclusi nello studio

Contaminante	Campione A	Campione B	Campione C
VTEC O157, *vtx1, vtx2, eae*	40 CFU/ml	0	0
VTEC O145, *vtx1, eae*	0	40 CFU/ml	0

Per ogni sospensione batterica utilizzata per contaminare i campioni, è stata calcolata l'incertezza di misura associata all'inoculo, secondo quanto prescritto dalla ISO TS 19036:2006, ottenendo i seguenti valori:

VTEC O157: 0.27 log cfu/ml
VTEC O145: 0.37 log cfu/ml

3.2. Stabilità e omogeneità dei campioni

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

Per la verifica della stabilità, un gruppo di campioni è stato preparato appositamente il 5 Maggio 2011 con le stesse procedure utilizzate successivamente per la preparazione dei campioni da impiegare nel test. Questi campioni sono stati conservati a 5°C +/- 3°C (ISO 7218:2007) e analizzati periodicamente secondo il protocollo dello studio inter-laboratorio. Le analisi sono state effettuate nei giorni 6, 7 e 11 Maggio, sempre ottenendo i risultati attesi.
I campioni da impiegare nello studio inter-laboratorio sono stati preparati il 19 Maggio e la loro omogeneità è stata verificata saggiando 5 set di campioni selezionati casualmente subito dopo la preparazione. I test sono iniziati il giorno stesso della preparazione e hanno prodotto i risultati attesi.

3.3. Invio dei campioni

I campioni, identificati con codici numerici a tre cifre assegnati casualmente e diversi per ogni laboratorio, sono stati mantenuti a 5°C +/- 3°C fino al trasferimento in contenitori refrigerati per la spedizione. Questa è stata effettuata il 23 Maggio mediante corriere. Ai laboratori è stato richiesto di registrare la data e l'ora di arrivo dei campioni e la loro temperatura, e 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 presentazione di *user ID* e *password*, inviate ad 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* di un laboratorio è stata valutata "soddisfacente" quando è stata riscontrata correttamente la presenza/assenza di VTEC in tutti e 3 i campioni.
Le caratteristiche di *performance* del metodo sono state valutate, limitatamente alla fase di screening mediante Real-Time PCR, 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 23 Maggio e sono stati recapitati ai Laboratori partecipanti il giorno successivo, con l'eccezione del laboratorio L17, che li ha ricevuti il giorno stesso, e del laboratorio, L39 che li ha ricevuti il 25 Maggio. Per i nove laboratori che hanno riportato l'informazione, la temperatura alla ricezione era inferiore a 8 °C (tra 0,8 e 7°C) per 4 laboratori; per 4 laboratori la temperatura era compresa tra 10 e 14 °C. Il laboratorio che ha ricevuto il campione in ritardo (L39) ha riportato una temperatura di 20 °C.
I risultati sono inviati via web da 9 Laboratori entro la scadenza stabilita. Un Laboratorio (L59) non ha inviato i risultati.

Il primo *step* del metodo ISO/WD TS 13136 prevede lo screening delle colture di pre-arricchimento per la presenza di geni di virulenza e sierogruppo-specifici mediante Real-Time PCR. Questa parte dello studio è stata effettuata correttamente da tutti i 9 laboratori che hanno inviato i risultati (100%). Questi laboratori hanno riportato correttamente la presenza/assenza di tutti i geni target nelle colture di pre-arricchimento dei tre campioni di spinaci. I risultati sono riportati nella 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.

Lab	Identificazione dei geni di virulenza e dei geni associati al sierogruppo in:																							
	Campione A								Campione B								Campione C							
	vtx1	*vtx2*	*eae*	O157	O26	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O157	O26	O103	O111	O145	*vtx1*	*vtx2*	*eae*	O157	O26	O103	O111	O145
Valore Atteso	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L07	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L13	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L17	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L38	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L39	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L49	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L79	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L93	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-
L96	+	+	+	+	-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-

I risultati relativi all'isolamento e alla geno-tipizzazione dei ceppi VTEC dalle colture di arricchimento Real Time PCR-positive sono riportati in Tabella 3.

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.

Lab	Isolamento e geno-tipizzazione dei ceppi VTEC dalle colture di arricchimento											
	Campione A				Campione B				Campione C			
	VTEC	Genotype			VTEC	Genotype			VTEC	Genotype		
		vtx1	*vtx2*	*eae*		*vtx1*	*vtx2*	*eae*		*vtx1*	*vtx2*	*Eae*
Valore Atteso	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**Nessuno**	**-**	**-**	**-**
L07	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L13	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L17	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L38	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L39	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L49	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L79	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L93	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**
L96	**O157**	**+**	**+**	**+**	**O145**	**+**	**-**	**+**	**-**	**-**	**-**	**-**

Tutti i 9 laboratori, dopo aver identificato correttamente la presenza dei gene associati ai sierogruppi O157 e O145 nelle colture di arricchimento, hanno anche isolato e tipizzato correttamente i ceppi VTEC dai campioni A e B.

Le caratteristiche di *performance* nelle due fasi del metodo (screening mediante Real-Time PCR e isolamento e geno-tipizzazione dei ceppi VTEC) sono state considerate separatamente.

I valori di concordanza sono stati valutati tramite il Kappa di Cohen, che è risultato pari a 1 sia come valore complessivo che per i singoli laboratori. Anche i valori di sensibilità e specificità complessivi e dei singoli laboratori sono stati del 100%.

5. Considerazioni

- Nove dei 10 laboratori che hanno partecipato al PT hanno inviato i risultati e hanno effettuato correttamente tutte le analisi richieste, riportando una performance analitica eccellente (K=1)
- L'invio diretto dei dati via web ha favorito la raccolta e l'elaborazione dei risultati.
- Questo studio ha confermato che il metodo ISO/WD TS 13136 basato sullo screening mediante Real-time PCR delle colture di pre-arricchimento rappresenta uno strumento robusto per la ricerca dei VTEC negli alimenti.

Allegato 1

European Union Reference Laboratory for *E. coli*
Department of Veterinary Public Health and Food Safety
Unit of Foodborne Zoonoses and Veterinary Epidemiology
Istituto Superiore di Sanità

7th inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC): detection and identification in spinach samples

Laboratory procedure

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 Technical Specification.

In this Technical Specification, the wording Shiga toxin (Stx) is synonymous of Verocytotoxin (Vtx). The following nomenclature has been adopted in this Technical Specification:
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 Technical Specification describes a horizontal method for the detection of (i) the major virulence genes of STEC (2,3), and (ii) the genes associated with the serogroups 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 TS has been developed based on the use of the Real-time PCR as reference technology for the detection of the virulence and serogroup-associated genes. Therefore a Real-time PCR protocol is described in detail.

This Technical Specification is applicable to:

- products intended for human consumption and the feeding of animals,
- environmental samples in the area of food production and food handling,
- environmental samples in the area of primary production.

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 Technical Specification.

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
5. Isolation from positive samples.

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 a non-selective liquid nutrient medium, either:

(a) Tryptone-soy broth (TSB) supplemented with 1.5 gr/l bile salts n. 3 and 16 mg/l of novobiocin (mTSB+N).

(b) Buffered peptone water (BPW)

c) Tryptone-soy broth (TSB) supplemented with 1.5 gr/l bile salts n. 3 and 12 mg/l of acriflavin (mTSB+A) for analysis of dairy products.

The broth mTSB is to be used when analysing matrices suspected to contain high levels of contaminating microflora. Novobiocin and acriflavin inhibit the growth of Gram-positive bacteria and promotes the growth of Gram-negative cells including STEC. The broth BPW is to be used to analyse samples which are supposed to contain stressed target bacteria (such as frozen products), to resuscitate stressed STEC cells, and expected lower levels of contaminating microflora than in fresh samples.

NOTE: The addition of novobiocin is controversial and has been investigated by several authors. It has been observed that the Minimum Inhibitory Concentration (MIC) of the antibiotic for non-O157 STEC is lower than for O157 strains (5). The addition of novobiocin in the enrichment broth mTSB at the usual concentration of 20 mg/l, as specified in the ISO 16654:2001 standard, seems to inhibit the growth of about one third of non-O157 strains (6) increasing the risk of false negative results. The 16 mg/l concentration represents the most balanced option to inhibit the growth of the contaminating background microflora expected in enrichment cultures of food samples, while still allowing the growth of STEC cells.

4.1.2 Nucleic acid extraction

Bacterial cells are separated from the enrichment medium and lysed. The nucleic acid is then purified according to the requirements of the adopted detection system.

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 *stx* genes encode a family of toxins including two main types: stx1 and stx2. The latter comprises seven recognized variants (from stx2a to stx2g). Only the variants stx2a, stx2b, and stx2c have been found to be produced by the STEC strains included in the field of application of this TS, and therefore constitute the target stx-coding genes of the proposed TS. The GenBank accession numbers corresponding to the stx2 variants-coding genes are:
  stx2a: X07865
  stx2b: AF043672
  stx2c: M59432
- the *rfbE (O157), wbdl(O111), wzx(O26), ihp1(O145)* and *wzx(O103)* genes, to identify the corresponding serogroups.

4.1.4 Detection

The detection of the target genes is performed accoding to the adopted detection system. 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 modified Tryptone- Soy broth (mTSB)

5.1.1.1.1 Basic medium

Composition

Casein peptone	17	g
Soy peptone 3	g	
D(+) Glucose	2.5	g
Sodium chloride	5	g
Dipotassium hydrogen phosphate (K_2HPO_4)	4	g
Bile salts no. 3	1.5	g
Distilled water	to 1	L

pH 7.4 ± 0.2

Preparation

Dissolve the components or the dehydrated medium in 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).

5.1.1.1.2 Novobiocin solution

Composition

Novobiocin	0,16	g
Water	10	ml

Preparation

Dissolve the novobiocin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.1.1.3 Acriflavin solution

Composition

Acriflavin	0,12	g
Water	10	ml

Preparation

Dissolve the acriflavin in the water and sterilize by membrane filtration.

Prepare on the day of use.

5.1.1.1.4 Preparation of the complete medium

Immediately before use, add 1 ml of novobiocin (5.1.1.1.2) or acriflavin solution (5.1.1.1.3) to 1000 ml of cooled mTSB (5.1.1.1.1)

The final concentration of novobiocin is 16 mg per litre of mTSB.

The final concentration of Acriflavin is 12 mg per litre of mTSB

5.1.2 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.

6.4 Pipettes for volumes between 1µl and 100 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

It is important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport or storage. Sampling is not part of the method specified in this Technical Specificiation. See the specific International Standard dealing with the product concerned. If there is no specific International Standard 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. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject.

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.

9.1.2 For matrix sample supposed to contain high level of annex floras

For solid matrices, aseptically transfer a test portion of sample to a stomacher bag containing the appropriate amount of mTSB added with novobiocin or acriflavin (5.1.1.1.4). Bags with filters should be preferred.

Homogenise in a stomacher (see ISO 7218) (6.10).

For liquid matrices, transfer the test portion of liquid sample, using a sterile pipette, directly into the base of the tube/bottle containing the enrichment broth mTSB added with novobiocin or acriflavin (5.1.1.1.4).

9.1.3 For matrix sample supposed to contain stressed target bacteria

In the case of frozen products, allow them to thaw at room temperature, then transfer the test portion to a stomacher bag containing the appropriate volume of BPW (5.1.2) and proceed as above.

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 PCR amplification (for Real-time PCR)

9.4.1 General

The PCR amplification approach described is based on real-time PCR.

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.

The method is sequential (flow in Annex A):

- Step 1: detection of the Stx-coding genes and the *eae* gene (PCR A in Annex E);
- Step 2: samples positive at the first step are tested for the molecular serogrouping (PCR B in Annex E);
- Step 3: samples positives at both steps are subjected to strain isolation (flow in the Annex B).

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.

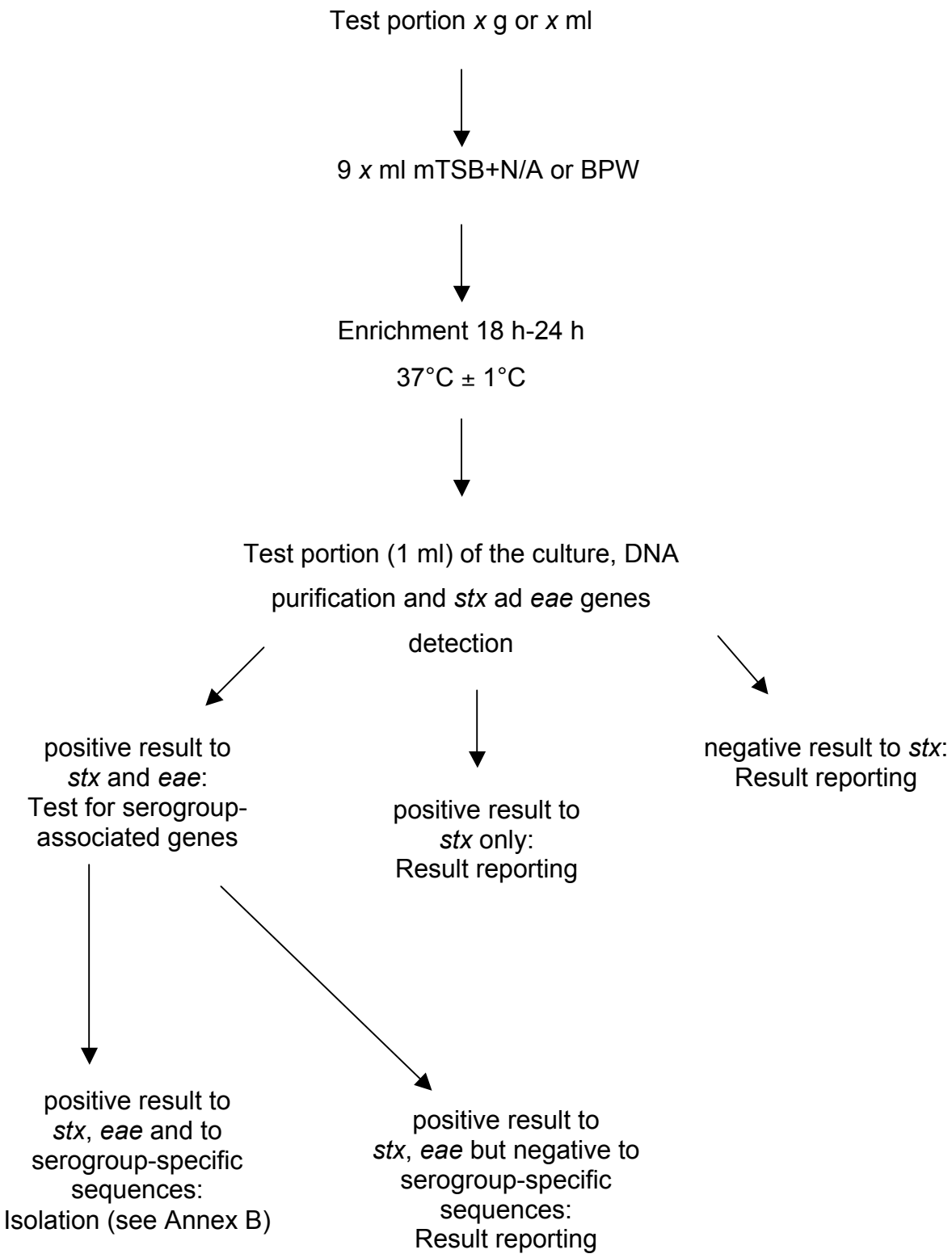
The real-time PCR protocol in Annex E or any other equivalent PCR protocol (http://www.iss.it/vtec/work/cont.php?id=152&lang=2&tipo=3) may be used in order to confirm the presence of the virulence genes in the isolated colonies.

STEC isolation is described in the flow chart of Annex B.

> **NOTE:** In the absence of positive results for the presence of *eae* gene, and generally in absence of positivity to serogroup-associated genes isolation is not attempted.

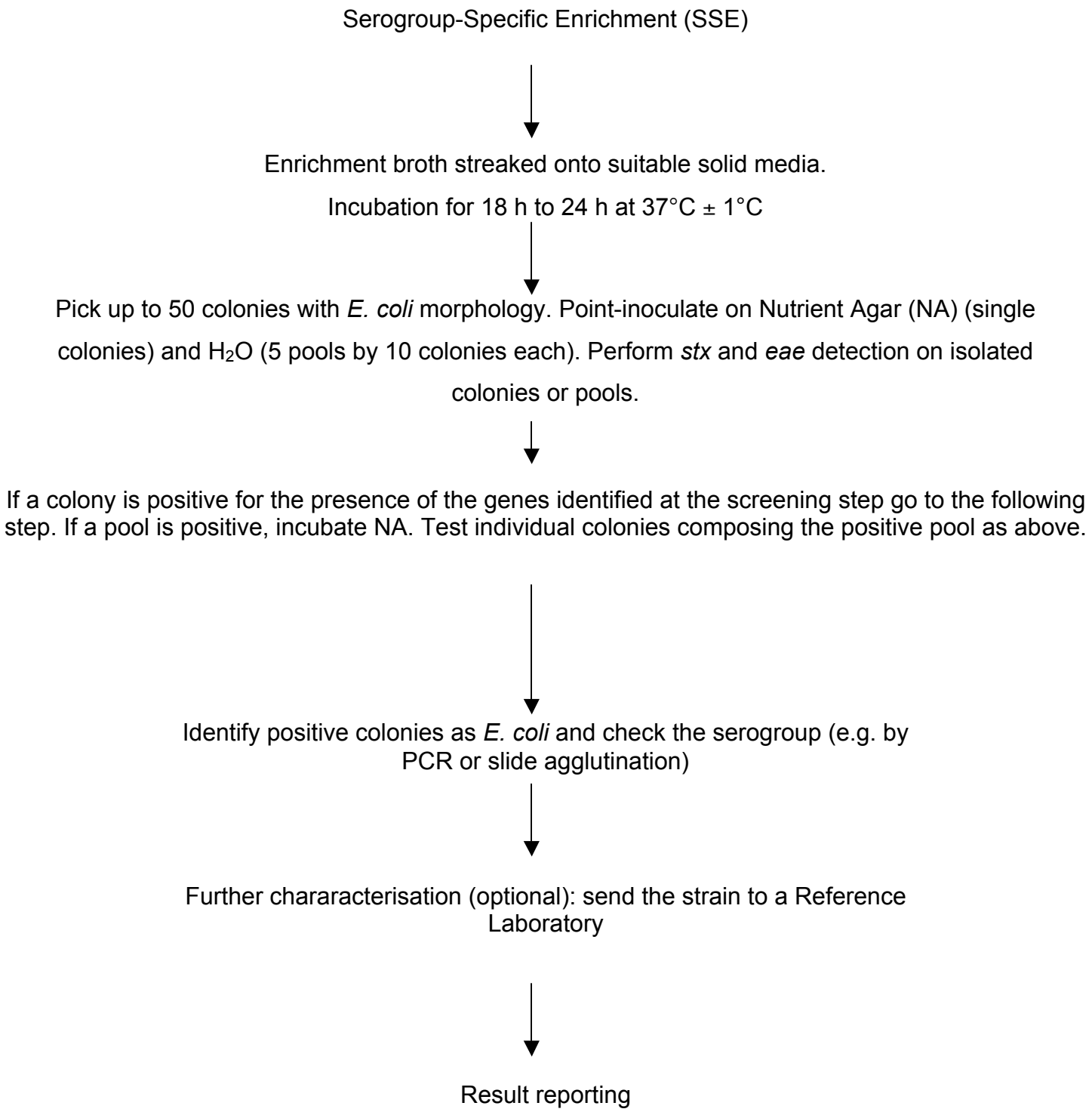
Annex A

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**TGAATCGCCAGCGGCATCAGC ACCTTGTCGCCTTGCGTATA**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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