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ORIGINAL ARTICLE

Quantitative isolation efficiency of O26, O103, O111, O145 and O157 STEC serotypes from artificially contaminated food and cattle faeces samples using a new isolation protocol

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Abstract

Aims: A range of new differential and confirmation plating media for some non-O157 Shiga toxin producing *Escherichia coli* (STEC) serotypes (O26, O103, O111, O145) and both sorbitol-positive and -negative O157 were evaluated using artificially contaminated samples.

Methods and Results: Dairy products (raw milk, cheese made from pasteurized milk and raw milk), meat (ground beef, fermented meat) and cattle faeces were artificially contaminated using clinical STEC strains. Isolation efficiency was 100%, 82·3%, 88·5%, 65·9%, 64·3% and 15·8%, respectively, for an inoculum size of ≤100 CFU 25 g^{-1}. The consecutive use of differential and confirmation media limited the incidence of false positive isolates from 0% for raw milk samples, cheese made from pasteurized milk and for fermented meat to 2·1% for cheese made from raw milk, and to 8·9% for ground beef.

Conclusions: Data presented in this paper indicated that the efficiency of the applied isolation method was dependent on sample-to-sample variation but not on the inoculum size.

Significance and Impact of Study: Data in this paper indicated that isolation of low levels of non-O157 and sorbitol-positive O157 STEC from food samples is possible.

Introduction

Since its identification as a food-borne pathogen in 1982, Shiga toxin producing *Escherichia coli* O157:H7 (STEC) has been identified as the cause of several outbreaks (Karmali 1989; Beutin *et al.* 1998; Paton and Paton 1998; Willshaw *et al.* 2001; Beutin *et al.* 2002). Domestic ruminants, mainly cattle, sheep and goats have been implicated as the principal reservoir (Blanco *et al.* 1996, 2003a, b, 2004). Typical O157:H7 STEC strains are not able to ferment sorbitol within 24 h. Based on this phenotypic feature, SMAC (Sorbitol MacConkey Agar) agar and modified SMAC media were developed to facilitate O157:H7 STEC isolation (March and Ratnam 1986; Fujisawa *et al.* 2000) and a specific International Organization for Standardization (ISO) method (ISO 2001) was developed for the detection of this pathogen in food and animal feeding stuffs. More recently, however, sorbitol-positive strains have been increasingly isolated from clinical cases (Gunzer *et al.* 1992; Bielaszewska *et al.* 1998; Karch and Bielaszewska 2001) and yet more than 100 other STEC serotypes have been implicated in cases of human disease. Among these other serotypes, O26, O103, O111 and O145 have been increasingly isolated from clinical cases (Eklund *et al.* 2001; Bettelheim 2003; Durso *et al.* 2004). Non-O157 STEC strains are a heterogeneous group which display a broad range of both phenotypic and genotypic differences (Schmidt *et al.* 1999; Bettelheim 2000, 2003; Eklund *et al.* 2001; Stephan and Schumacher 2001; Possé *et al.* 2007a).

Recently, new differential and confirmation agar media for O26, O103, O111, O145, sorbitol-negative O157 and sorbitol-positive O157 STEC were described (Possé *et al.* 2007b). In this paper, the selective isolation of STEC from artificially contaminated samples (meat, dairy, cattle faeces) using an optimized enrichment procedure and the new agar media is described.

Materials and methods

Sample preparation

The selective isolation method for non-O157 serotypes, sorbitol-negative O157 and sorbitol-positive O157 was evaluated on the following sample matrices: raw milk, cheese made from pasteurized milk, cheese made from raw milk, ground beef, fermented meat and cattle faeces. Upon arrival in the laboratory, all samples were stored at 4°C and were treated within 24 h. For all experiments, one or more subsamples of 25 g (or 25 ml for raw milk samples) were taken and diluted to a 1/10 ratio using Tryptone Soy Broth (TSB; Oxoid) with some modifications as described below. Diluted samples were homogenized for 2 min prior to artificial contamination.

From each sample, total colony count was determined by plating on Tryptone Soy Agar (TSA; Oxoid) and incubated for 24 h at 37°C; coliform counts were determined using violet red bile agar (VRB; Oxoid) using the same incubation conditions.

Artificial contamination

All STEC strains used for artificial contamination have been described (Possé *et al.* 2007a); they belonged to serotypes O26, O103, O111, O145, sorbitol-positive (S+) O157 and sorbitol-negative (S−) O157 (Table 1). Strains were stored at −80°C using Pro-Lab Microbank cryovials (Ontario, Canada) according to the manufacturer's instructions. Strains were cultured on TSA at 37°C for 24 h. One colony was transferred into TSB and incubated at 37°C for 24 h to reach a stationary phase culture (10^9 CFU ml^{-1}). Ten-fold serial dilutions were made in Buffered Pepton Water (BPW; Oxoid) and an appropriate volume of the diluted bacterial culture was added to the 1/10 diluted and homogenized sample to obtain different contamination levels. Artificial inoculations were performed using single STEC strains. One hundred microlitre\ of the diluted bacterial culture was also streaked onto duplicate TSA plates and incubated for 24 h at 37°C for total colony count. Only samples artificially contaminated with ≥1 CFU 25 g^{-1} of sample were taken into account for this study. From each sample, a blank (not artificially contaminated sub sample) was also evaluated.

Table 1 Virulence profiles of clinical STEC strains used for artificial sample contamination

Strains	Serotype	Source	*stx1*	*stx2*
PH11, PH25	O26:H−	Belgium§	+	−
PH09, PH14, PH15, PH16	O26:H11	Belgium§	+	−
PH02, PH05, PH23	O103:H2	Belgium§	+	−
PH29, PH37	O111:H−	Belgium§	+	−
PH31, PH32	O111ac	Belgium§	+	−
PH01, PH27	O145:H−	Belgium§	−	+
PH06, PH08	O145:H−	Belgium§	+	−
PH54, PH55, PH56	O157:H7 (s−)*	Belgium‡	−	+
PH69, PH73	O157:H7 (s−)*	Belgium§	−	+
PH70	O157:H7 (s+)*	Belgium§	+	+
PH74, PH79, PH80	O157:H7 (s+)*	Germany†	−	+

*(s+) indicates a group of strains belonging to serotype O157 able to ferment sorbitol within 24 h. (s−) indicates strains unable to ferment sorbitol within 24 h.

†Strains kindly donated by H. Karch, Germany.

‡Veterinary and agrochemical research centre, Ukkel, Belgium

§Strains kindly donated by the Belgian national VTEC reference laboratory (by D. Piérard).

Pre-enrichment and selective enrichment

After artificial contamination of the 1/10 diluted and homogenized sample (except for cattle faeces), 8 mg l^{-1} novobiocine and 16 mg l^{-1} vancomycin were added to the TSB enrichment medium. After 6 h of pre-enrichment at 37°C, 2 mg l^{-1} rifampicine, 1·5 g l^{-1} bile salts and 1·0 mg l^{-1} potassium tellurite were added to the enrichment medium and incubated further for 18 h at 42°C (selective enrichment). Cattle faeces samples were directly enriched during 24 h at 42°C in TSB modified with 8 mg l^{-1} novobiocine, 16 mg l^{-1} vancomycin, 2 mg l^{-1} rifampicine, 1·5 g l^{-1} bile salts and 1·0 mg l^{-1} potassium tellurite.

Selective isolation and purification

After the two sample enrichment stages (pre-enrichment and selective enrichment) or the single selective enrichment for the cattle faeces, 100 μl of a decimal dilution (10^{-3} to 10^{-5}) of the enriched sample was plated onto the selective differential medium for non-O157 STEC or O157 STEC which has been described (Possé *et al.* 2007b). Briefly, these media were composed of MacConkey agar base (BD Biosciences, Franklin Lakes, NJ) 40 g l^{-1}, bile salts No. 3 (Sigma) 3·5 g l^{-1}, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, Glycosynth, Warrington, UK) 0·05 g l^{-1}, isopropyl-β-D-thiogalactopyranoside (IPTG, Glycosynth) 0·05 g l^{-1}, novobiocin (Sigma) 8·0 mg l^{-1}, potassium tellurite (Sigma) 2·5 mg l^{-1}. The differential medium for

non-O157 was supplemented with 6·0 g l^{-1} sucrose and sorbose (Sigma). The differential medium for O157 was supplemented with sorbitol (Sigma) 10·0 g l^{-1}. On the former medium, O26 strains produce purple colonies. O103 and O111 strains produced bluish-coloured colonies and O145 strains produced green colonies. All colonies showed dark-coloured centres as a result of X-gal hydrolysation (Possé *et al.* 2007b). The selective differential medium for O157 strains producedtwo 'suspected' colours: purple colonies indicate O157 sorbitol-positive strains, while bluish-green colonies indicate O157 sorbitol-negative strains.

Inoculated spread plates were incubated for 24 h at 37°C. All colonies present on the differential medium, suspected as well as nonsuspected were counted. Five to 15 suspected colonies were transferred to one or more confirmation media according to the scheme shown in Fig. 1, which was adopted from a previously published paper (Possé *et al.* 2007b). Briefly, confirmation media were composed of phenol red broth base (Sigma) supplemented with a specific carbohydrate and agar. Confirmation media were incubated for 24 h at 37°C. For O157 sorbitol-positive and O157 sorbitol-negative strains, no additional confirmation media were applied during experiments presented in this paper.

Thirty-one experiments made use of immunomagnetic separation (IMS) using Dynabeads coated with specific antibodies for O26, O103, O111, O145 or O157 (Dynal, Oslo, Norway), according to the manufacturer's instructions. Briefly, 1 ml of each enriched culture was added to 20 μl of *E. coli* O26, O103, O111, O145 or O157 specific Dynabeads depending on the serotype targeted during the experiment. The suspension was mixed for 10 min and placed on a magnetic particle concentrator (Dynal) prior to removing the supernatant. The beads were washed three times using 1 ml of 10 mmol l^{-1} phosphate-buffered saline containing 0·05% (v/v) Tween 20 (PBS-T; Sigma-Aldrich, Poole, UK) and resuspended in 100 μl PBS-T. This suspension was then plated onto the selective isolation medium and incubated for 24 h at 37°C.

Confirmation and identification

All colonies from the confirmation media or 5 to 15 suspected colonies from the O157-differential medium were subsequently confirmed using conventional *stx*-PCR analysis as described before (Possé *et al.* 2007a). PCR-based confirmation of colonies expressing suspected morphology and colour on both differential and confirmation media indicated successful isolation of the strain used for artificial sample contamination. Colonies with suspected phenotype on the differential medium or on both the differential and confirmation media, which appeared *stx*-PCR negative were indicated as 'false positive'.

Nonsuspected colonies as well as colonies which could not be confirmed were identified using API20 (Biomérieux, Marcy l'Etoile France) tests according to the manufacturer's instructions.

Results

Dairy samples

The total colony count and the amount of coliforms present in dairy products varied significantly among sample types, as indicated in Table S1. No coliforms were present in raw milk samples, except for one batch of samples with $1{\cdot}0 \times 10^4$ CFU ml^{-1} coliforms, while total colony count

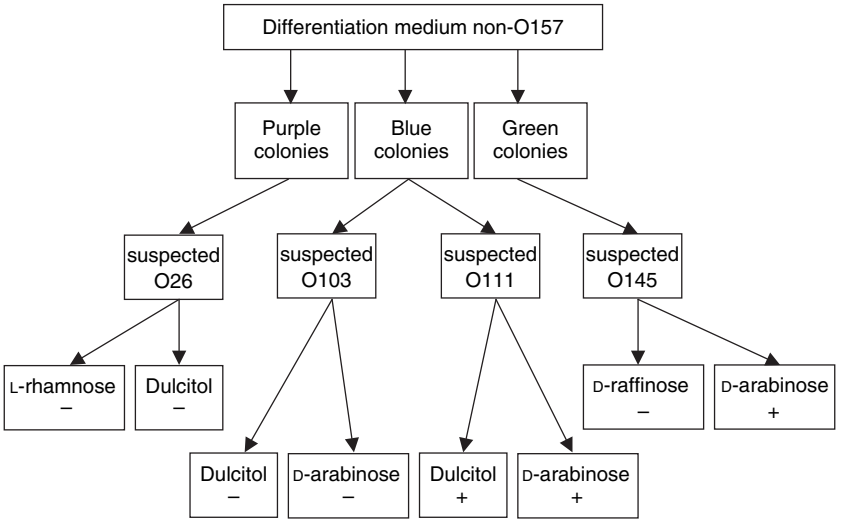

Figure 1 Use of differential and confirmation media for the isolation of O26, O103, O111 and O145 STEC strains.

Table 2 Isolation efficiency of clinical STEC from various artificially contaminated matrices at the indicated inoculation levels without using IMS

	All serotypes	O26%*	O103%*	O111%*	O145%*	O157 S− %*	O157 S+ %*
Raw milk							
All inocula	100·0 (24/24)	100·0 (12/12)	100·0 (5/5)	100·0 (5/5)	100·0 (2/2)	ND	ND
≤100 CFU 25 g^{-1}	100·0 (14/14)	100·0(4/4)	100·0 (4/4)	100·0 (4/4)	100·0 (2/2)	ND	ND
Cheese made from pasteurized milk							
All inocula	82·0 (41/50)	81·8 (18/22)	16·7 (1/6)	100·0 (4/4)	100·0 (10/10)	100·0 (4/4)	100·0 (4/4)
≤ 100 CFU 25 g^{-1}	82·3 (28/34)	77·8 (14/18)	0·0 (0/2)	100·0 (4/4)	100·0 (6/6)	ND	100·0 (4/4)
Cheese made from raw milk							
All inocula	86·8 (33/38)	87·5 (7/8)	100·0 (4/4)	100·0 (2/2)	100·0 (2/2)	80·0 (8/10)	83·3 (10/12)
≤100 CFU 25 g^{-1}	88·5 (23/26)	75·0 (3/4)	100·0 (2/2)	100·0 (2/2)	100·0 (2/2)	80·0 (8/10)	87·5 (7/8)
Ground beef							
All inocula	67·2 (41/61)	47·4 (9/19)	66·7 (6/9)	50·0 (5/10)	90·0 (18/20)	100·0 (3/3)	ND
≤100 CFU 25 g^{-1}	65·9 (27/41)	40·0 (4/10)	66·7 (6/9)	50·0 (5/10)	100·0 (11/11)	100·0 /1/1)	ND
Fermented meat							
All inocula	62·2 (23/37)	50 (2/4)	50 (5/10)	33·3 (2/6)	84·6 (11/13)	75·0 (3/4)	ND
≤ 100 CFU 25 g^{-1}	64·3 (18/28)	66·7 (2/3)	42·9 (3/7)	40·0 (2/5)	83·3 (10/12)	100·0 (1/1)	ND
Cattle faeces							
All inocula	28·9 (13/45)	ND	17·6 (3/17)	0·0 (0/13)	20·0 (1/5)	100·0 (5/5)	80·0 (4/5)
≤100 CFU 25 g^{-1}	13·6 (3/22)	ND	0·0 (0/5)	0·0 (0/9)	0·0 (0/5)	100·0 (1/1)	100·0 (2/2)
All matrices							
All inocula		73·8 (48/65)	47·1 (24/51)	45·0 (18/40)	84·6 (44/52)	88·5 (23/26)	85·7 (18/21)
≤100 CFU 25 g^{-1}		71·1 (27/38)	51·7 (15/29)	50·0 (17/34)	83·8 (31/37)	84·6 (11/13)	92·9 (13/14)

ND, Not done.
*Total values for each sample matrix are presented as % of confirmed/total number of experiments.

ranged between $1{\cdot}60 \times 10^4$ and $4{\cdot}60 \times 10^7$ ml^{-1}. Coliform counts between <10·0 and $2{\cdot}20 \times 10^6$ g^{-1} and total colony counts between $2{\cdot}05 \times 10^6$ and $2{\cdot}30 \times 10^8$ g^{-1} were found for cheese made from pasteurized milk. Coliform counts between $9{\cdot}60 \times 10^6$ and $2{\cdot}54 \times 10^7$ g^{-1} and total colony counts between $1{\cdot}85 \times 10^7$ and $5{\cdot}82 \times 10^7$ g^{-1} were found for cheese made from raw milk.

Table S1 indicates the strains used for artificial inoculation, the inoculum size, sample matrix and the results of the experiment: confirmed STEC isolate, false positives and the nonsuspected/suspected ratio on the differential medium. A summarized version of these data is show in Table 2. Overall, 24 samples of raw milk were artificially contaminated with single strains belonging to different serotypes. All the experiments (24/24) resulted in PCR confirmed isolates: all of the samples inoculated with high levels (≥100 CFU 25 ml^{-1}) as well as with low levels (≤ 100 CFU 25 ml^{-1}) yielded confirmed isolates. No nonsuspected colonies were found on the differential media. Table 3 indicates the number of false positive isolates (suspected colonies but PCR negative) based solely on the non-O157 differential medium and the amount of false positives based on the combination of the differential medium and confirmation media. Only one out of 95 suspected isolates (1·0%) on the differential medium could not be confirmed by PCR as the clinical strain added to the sample; subsequent evaluation of this strain on the confirmation media identified this strain as 'nonsuspected'. API20E test identified this isolate as an *E. coli*.

Fifty samples of cheese made from pasteurized milk were artificially contaminated with single strains belonging to different serotypes (Table S1). Overall isolation efficiency of the inoculated STEC strain was 82·0% (41/50) with inoculation of ≤100 CFU 25 g^{-1} resulting in an isolation efficiency of 82·3% (28/34). One hundered per cent of the samples yielded PCR confirmed isolates after artificial contamination (inoculum size between 25 and 475 CFU 25 g^{-1}) with O111, O145, O157 sorbitol-positive or O157 sorbitol-negative strains; 81·8% (18/22) of O26 contaminated samples yielded confirmed isolates. One sample of cheese made from pasteurized milk presented a coliform count of $5{\cdot}00 \times 10^5$ g^{-1}: all four experiments using this sample failed in isolating the inoculated STEC because of overgrowth by *Hafnia alvei, Enterobacter cloacae, E. coli, Klebsiella pneumoniae* and *Kluyvera spp*. Only one out of six O103 contaminated samples (16·7%) was successful in yielding the inoculated STEC strain: both experiments with an O103 inoculum size of 44 CFU 25 g^{-1} were unsuccessful and showed no growth on the differential medium. Other samples inoculated with O103 STEC showed growth of nonsuspected background bacteria: API20E results identified these colonies as *Proteus vulgaris*. Table 3 indicates eight false positives that occurred out of 135 colonies were evaluated (5·9%) to

Table 3 Incidence of false positive isolates on non-O157 differential medium and confirmation media from artificially contaminated samples without using IMS

	Total %*	O26%*	O103%*	O111%*	O145%*
Raw milk	1·0 (1/95)	0·0 (0/56)	5·6 (1/18)	0·0 (0/13)	0·0 (0/8)
Cheese made from pasteurized milk	5·9 (8/135)	9·2 (8/87)	0·0 (0/13)	0·0 (0/12)	0·0 (0/23)
Cheese made from raw milk	2·1 (1/47)	0·0 (0/19)	0·0 (0/12)	12·5 (1/8)	0·0 (0/8)
Ground beef	13·0 (36/276)	18·2 (12/66)	22·2 (10/45)	0·0 (0/41)	11·3 (14/124)
Fermented meat	2·9 (3/104)	0 (0/4)	10·0 (3/30)	0·0 (0/8)	0·0 (0/62)
Cattle faeces	87·5 (140/160)	ND	74·7 (59/79)	100 (78/78)	100·0 (3/3)
Differential medium		8·6 (20/232)	37·1 (73/197)	49·4 (79/160)	7·5 (17/228)
Raw milk	0·0 (0/92)	0·0 (0/56)	0·0 (0/16)	0·0 (0/12)	0·0 (0/8)
Cheese made from pasteurized milk	0·0 (0/117)	0·0 (0/79)	0·0 (0/3)	0·0 (0/12)	0·0 (0/23)
Cheese made from raw milk	2·1 (1/47)	0·0 (0/19)	0·0 (0/12)	12·5 (1/8)	0·0 (0/8)
Ground beef	8·9 (23/257)	11·5 (7/61)	22·2 (10/45)	0·0 (0/41)	5·5 (6/110)
Fermented meat	0·0 (0/101)	0 (0/4)	0·0 (0/27)	0·0 (0/8)	0·0 (0/62)
Cattle faeces	77·3 (68/88)	ND	0·0 (0/20)	100 (67/67)	100·0 (1/1)
Differential and confirmation media		3·2 (7/219)	8·1 (10/123)	46·2 (68/148)	3·3 (7/212)

ND, Not done.
*Total amount of false positive isolates is represented as % of number of false positive isolates/total number of isolates.

be originating from the non-O157 differential medium. Subsequent use of the confirmation media reduced this number to 0% for isolates originating from O26 inoculated experiments. Four false positives out of 19 colonies evaluated (21·1%) were isolated from the O157 differential medium (Table 4); all were subsequently identified with API20 as *E. coli* (Table 4).

Thirty-eight samples of cheese made from raw milk were artificially contaminated with single strains belonging to different serotypes: overall isolation efficiency of the inoculated strain was 86·8% (33/38) with inoculation of ≤100 CFU 25 g^{-1} resulting in an isolation efficiency of 88·5% (23/26). All samples contaminated with O103, O111 or O145 strains resulted in confirmed isolates, while only 87·5% (7/8), 80% (8/10) and 83·3% (10/12) of samples resulted in confirmed isolates after contamination with O26, O157 sorbitol-negative and O157 sorbitol-positive strains, respectively. Both experiments using a sample inoculated with 25 CFU of strain PH73 (O157 S−) were negative: plates were overgrown with *E. coli* and *H. alvei*. Table 3 indicates an incidence of false positive isolates on the non-O157 differential medium of 2·1% (1/47): this isolate remained 'suspected' after subsequent plating on the confirmation media and was identified using API20E as an *E. coli*.

Table 4 Incidence of false positive isolates on O157 differential medium from artificially contaminated samples without using IMS

	Total %*	O157 S− %*	O157 S+ %*
Raw milk	ND	ND	ND
Cheese made from pasteurized milk	21·1 (4/19)	0·0 (0/7)	33·3 (4/12)
Cheese made from raw milk	17·8 (8/45)	12·5 (2/16)	20·7 (6/29)
Ground beef	9·1 (1/11)	9·1 (1/11)	ND
Fermented meat	41·7 (10/24)	0·0 (0/9)	66·7 (10/15)
Cattle faeces	0·0 (0/25)	0·0 (0/15)	0·0 (0/10)
Differential medium		5·2 (3/58)	30·3 (20/66)

ND, Not done.
*Total amount of false positive isolates is represented as % of number of false positive isolates / total number of isolates.

Meat samples

As shown in Table S1, some samples of ground beef presented coliform loads up to $2{\cdot}5 \times 10^3$ g^{-1}, while none of the fermented salami sausage samples evaluated had detectable levels of coliforms (<10 CFU). Total colony counts were between $1{\cdot}40 \times 10^3$ and $3{\cdot}85 \times 10^6$ g^{-1} for ground beef samples and between $7{\cdot}65 \times 10^5$ and $4{\cdot}95 \times 10^8$ g^{-1} for fermented meat samples.

Overall, artificially contaminated ground beef samples were analysed during 84 experiments with inoculum sizes between 1 and 6400 CFU 25 g^{-1} of sample. In 61 experiments, no IMS (immunomagnetic separation) was applied. As indicated in Table 2, experiments without IMS resulted in 47·4% (9/19), 66·7% (6/9), 50·0% (5/10), 90·0% (18/20) and 100% (3/3) isolation efficiencies for O26, O103, O111, O145 and O157 S− contaminated samples, respectively. Overall isolation efficiency of the inoculated strain without application of IMS was 67·2% (41/61) with inoculation of ≤100 CFU 25 g^{-1} resulting in an isolation efficiency of 65·9% (28/42). From the 61 contaminated samples with no IMS treatment applied, 276 colonies were isolated from the non-O157 differential

Table 5 Isolation efficiency of clinical STEC from various matrices with the use of serotype-specific IMS

Serotype	Strain*	Total colony count	Coliforms	Inoculation level CFU 25 g^{-1}	Confirmed isolate(s)†	False positive on diff. medium	False positive on diff and conf media
Raw milk							
O103	PH02	4·60E+07	1·00E+04	11875	+	–	–
O111	PH29	4·60E+07	1·00E+04	2250	+	–	–
Ground beef							
O26	PH11	3·80E+03	<10·0	10	+	–	–
O26	PH11	4·50E+03	<10·0	10	4/4	0/4	0/4
O26	PH11	3·80E+03	<10·0	300	2/2	0/2	0/2
O26	PH11	4·50E+03	<10·0	300	4/4	0/4	0/4
O103	PH05	4·50E+03	<10·0	10	2/2	0/2	0/2
O103	PH05	4·50E+03	<10·0	300	+	–	–
O111	PH32	4·50E+03	<10·0	10	2/2	0/2	0/2
O111	PH32	4·50E+03	<10·0	300	2/2	0/2	0/2
O145	PH14	4·50E+03	<10·0	10	2/2	0/2	0/2
O145	PH14	4·50E+03	<10·0	300	1/2	0/2	0/2
Fermented meat							
O26	PH11	8·80E+06	<10·0	10	2/2	0/2	0/2
O26	PH11	8·80E+06	<10·0	300	+	–	–
Cattle faeces							
O26	PH09	5·00E+05	1·20E+05	3200	+	–	–
O103	PH02	5·00E+05	1·20E+05	8000	+	+	–
O111	PH29	5·00E+05	1·20E+05	4350	+	–	–
O145	PH01	5·00E+05	1·20E+05	4850	+	+	+
O157 s–	PH54	5·00E+05	1·20E+05	4050	+	+	ND

Diff. medium: differential medium (non-O157 or O157); Conf medium: confirmation media; ND, not done.

*'PH' numbers indicate clinical strains used for artificial contamination. Details on strains are shown in Table 1.

†Confirmed isolate(s): This indicates the number of isolation experiments yielding one or more isolates confirmed as the strain used for artificial sample inoculation compared with the total number of isolation experiments conducted. If only one experiment is presented, result is indicated by '+' for experiments yielding one or more confirmed isolates or '–' if this was not the case. Total values for each sample matrix are presented as % of confirmed/total number of experiments.

medium. As shown in Table 3, 36 out of these 276 colonies (13·0%) were identified as false positive on the differential medium: 18·2% (12/66), 22·2% (10/45), 0·0% (0/41) and 11·3% (14/124) originated from O26, O103, O111 and O145 contaminated samples, respectively. After subsequent streaking on the confirmation media, 23 out of 257 (8·9%) colonies with suspected morphology remained false positives: 11·5% (7/61), 22·2% (10/45), 0·0% (0/41) and 5·5% (6/110) of presumed O26, O103, O111 and O145 isolates failed PCR confirmation, respectively. All false positive isolates were confirmed as *E. coli* using API20E. Table 4 indicates that one false positive isolate was found on the O157-differential medium; this isolate was identified as *E. coli*. As shown in Table 5, 23 out of 84 experiments were subjected to IMS treatment. All experiments conducted with the application of IMS resulted in the successful isolation of the strain used for sample inoculation. No false positive colonies were identified during experiments using IMS.

Thirty-seven experiments were conducted using fermented meat as sample matrix, without application of IMS treatment: 28 had an inoculation size equal or below 100 CFU 25 g^{-1}. Overall isolation efficiency of the inoculated strain was 62·2% (23/37) with 64·3% (18/28) isolation efficiency based on inoculation levels ≤100 CFU 25 g^{-1}. Isolation efficiency was 50·0% (2/4), 50·0% (5/10), 33·0% (2/6), 84·6% (11/13) and 75·0% (3/4) for experiments using strains belonging to O26, O103, O111, O145 and O157 S–, respectively (Tables 2 and S1). Out of a total of 104 suspected isolates on the non-O157 differential medium, three (2·9%) appeared as false positives (Table 3). No false positives were isolated from O26, O111, O145 or O157 S– contaminated samples. After subsequent streaking on the confirmation media, the overall ratio of false positives dropped to 0·0% (0/101), as all three isolates from O103 inoculated experiments appeared as nonsuspected on the confirmation media. Isolation of O157 strains from fermented meat samples yielded an incidence of false positives of 41·7%, all originating from O157 sorbitol-positive contaminated samples. During two experiments IMS treatment was successfully applied (Table 5) .

Cattle faeces

During 50 experiments, cattle faeces were artificially contaminated using a pathogenic strain belonging to serogroup O26, O103, O111, O145 or O157 (S−/S+): in 45 out of 50 experiments, no IMS was applied. Coliform counts were between $3{\cdot}00 \times 10^4$ and $1{\cdot}7 \times 10^7$ g^{-1}, total colony counts were between $1{\cdot}0 \times 10^5$ and $1{\cdot}90 \times 10^7$ g^{-1}, as indicated in Table S1. Overall isolation efficiency was 28·9% (13/45) with inoculation levels between 1 and $5{\cdot}0 \times 10^4$ CFU 25 g^{-1}. Inoculation of ≤100 CFU 25 g^{-1} resulted in an even lower isolation efficiency of 13·6% (3/22) (Table 2). Serotype specific isolation efficiency was 17·6% (O103), 0·0% (O111), 20·0% (O145), 100% (O157 S−) and 80% (O157 S+). No non-O157 contaminated samples with an inoculum size below 100 CFU 25 g^{-1} yielded confirmed isolates because of overgrowth with *E. coli,* while all three samples contaminated with ≤100 CFU 25 g^{-1} of O157 strains (both sorbitol-positive and negative) were successful. Table 3 indicates that 160 isolates from the non-O157 differential medium were evaluated: 87·5% (140/160) appeared as false positives, with 59 and 78 isolates originating from O103 and O111 contaminated samples. False positive ratio remained high at 77·3% (68/88) after streaking of the isolates on the confirmation media: 67 false positive isolates originated from O111 contaminated samples. Of 79 isolates from O103 contaminated experiments with suspected morphology on the differential medium, 20 showed suspected morphology on the confirmation media: all were subsequently confirmed by PCR. Table 5 indicates five experiments where IMS treatment was applied after enrichment of artificially contaminated cattle faeces. Inoculum size was between $3{\cdot}20 \times 10^3$ and $8{\cdot}00 \times 10^3$ CFU 25 g^{-1}. All five experiments using IMS allowed isolation and confirmation of the inoculated strain.

Discussion

This report describes the recovery of strains belonging to a selection of STEC serotypes from different sample matrices. As STEC infections have already occured at low infectious dose (Willshaw *et al.* 1994; Tilden *et al.* 1996) sensitive methods for isolation of STEC from food products are crucial. Artificially inoculating samples with a known concentration of pathogenic strains in this study allowed a quantitative data analysis which is more difficult to perform with naturally contaminated samples. Artificially contaminated samples also reduce the amount of samples to be screened for quantitative data analysis and thus vastly reduce laboratory resources required.

Meat products and cattle faeces presented a more competitive natural bacterial population compared to dairy products with important sample to sample variations, decreasing overall isolation efficiency to 65·6% (ground beef), 62·2% (fermented meat) and 28·9% (cattle faeces) compared to 87·5% (98/112) for all dairy sample matrices. To our knowledge, no publications are available which evaluate the isolation efficiency of isolation methods for non-O157 STEC serotypes from food and faeces. However, studies have been published dealing with O157 STEC isolation, naturally contaminated samples or samples which were inoculated and plated on selective media without an enrichment procedure being applied. Moreover, many reports included an immunological or molecular approach, which inhibits correct comparison to data described in this paper.

Fermented meat samples were characterized by a low pH (4·8–5·0); this has possibly affected growth conditions during enrichment, which may partially explain some samples where no growth was observed after plating on the differential medium. Additional buffering of the enrichment media can possibly overcome this problem. Some dairy samples presented highly competitive natural bacterial populations (nonsuspected background bacteria, Table S1) limiting the growth potential of the inoculated pathogenic STEC strain. One sample of cheese made from pasteurized milk showed overgrowth of the inoculated strain by *E. coli, H. alvei* and *Ent. cloacae,* resulting in experiments with negative isolation result. Data indicated that overall isolation efficiency is lowered because of the sporadic occurrence of highly competitive natural populations. This may be indicated by the levels of naturally occurring coliforms in the sample, but also by nonsuspected (noncoliform) naturally occurring bacteria, highlighting the importance of sample to sample variation for isolation success.

Results in Table S1 and Table 2 demonstrates that except for cattle faeces, little or no difference in isolation efficiency was found after inoculation with low numbers (≤100 CFU 25 g^{-1}) or high numbers (≥100 CFU 25 g^{-1}). This indicated that inoculum size does not limit the potential of the isolation procedure. Results presented in Table 5 suggest that the isolation efficiency for all sample matrices may be vastly improved by application of serotype-specific IMS, but this approach needs further research. Furthermore, incorporation of IMS in the procedure increases the cost considerably.

The incidence of false positives in the differential medium or in the combination of differential and confirmation media is correlated to the sample matrix and the serotype of the strain used for sample inoculation. In total, 1640 isolates from the differential media were analysed: 1483 isolates originated from non-IMS treated experiments and 157 isolates were taken from experiments using IMS treatment. Of 1483 isolates from non-IMS

treated experiments, 939 were identified as 'suspected' on the differential media: 835 remained 'suspected' after subsequent streaking on the appropriate confirmation media. Out of 835, 114 isolates were identified as false positives while 721 isolates were confirmed using PCR. As indicated by the incidence of false positives from O111-contaminated samples, isolation of strains belonging to this serotype remains problematic because of the lack of specificity of the confirmation media; a moderate decrease from 49·4% to 46·2% was observed because of the consecutive application of both the differential and the confirmation media. On the contrary isolation of O26, O103 and O145 strains resulted in an incidence of false positive isolates well below the 10% limit after application of differential and confirmation media. Isolates from both inoculated and blank samples with suspected morphology on the differential and/or confirmation media which could not be confirmed by *stx*-PCR were regarded as false positives in this study. However, no serotype-specific PCR was applied to these false positives: possibly some *stx*-negative strains belonging to serotype O26, O103, O111, O145 or O157 were identified as 'false positive' during the experiments described in this study.

Overall, the application of the isolation procedure described in this study (pre-enrichment, selective enrichment, differential media and confirmation media) resulted in confirmed isolates in 100·0% of the experiments using inoculated raw milk samples, 82·0% of the experiments using inoculated cheese made from pasteurized milk, 86·8% of the experiments using inoculated cheese made from raw milk, 67·2% of the experiments using inoculated ground beef, 62·2% of the experiments using inoculated salami sausages and 28·9% of the experiments using inoculated cattle faeces. This method requires further validation using a large-scale study of naturally contaminated samples. Results presented in this paper indicated that isolation efficiency was not correlated to inoculum size (except for cattle faeces), however, preliminary data indicates the potential beneficial use of IMS after enrichment for ground beef, fermented meat (salami) and cattle faeces.

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Supplementary material

The following supplementary material is available for this article online:

Table S1 Isolation efficiency of clinical STEC from various artificially contaminated matrices at the indicated inoculation levels, without using IMS.

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