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VALIDATION OF METHODS FOR THE DETECTION OF NEW EMERGING PATHOGENIC *ESCHERICHIA* COLI

"STECTRACK"

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G. DAUBE, L. DE ZUTTER.

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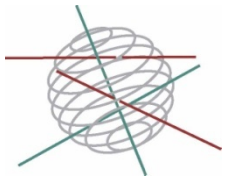

Agro-food

FINAL REPORT

" VALIDATION OF METHODS FOR THE DETECTION OF NEW EMERGING PATHOGENIC *ESCHERICHIA COLI*"

"STECTRACK"

SD/AF/06

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INDEX OF CONTENT

INDEX OF TABLES

INDEX OF FIGURES

1. Summary

Enterohaemorrhagic *Escherichia coli* (EHEC) are shigatoxin producing *E. coli* (STEC) that can cause serious disease to humans. These food-borne pathogens belong to the fifth most common zoonoses in Belgium, but due to their severe clinical symptoms in humans they are highly dreaded. They can cause a range of disease symptoms ranging from asymptomatically carriage over various diarrhoea symptoms to the life-threatening HUS (haemolytic uremic syndrome). Cattle are the main reservoir and infection of humans occurs through contact with faecal excretion material and consumption of contaminated food or water. A broad variety of serotypes is able to cause human infections, but the principal serotypes are O26, O103, O111, O145 and O157. These strains are denoted as new emerging pathogens by the WHO. The group of sorbitol non-fermenting (s-) O157:H7 strains are examined the most, because an ISO-method is available. For sorbitol fermenting (s+) O157 strains as well as for non-O157 STEC strains recently a new isolation method was developed in the Belspo project SD/AF/06A (Possé et al. 2008a).

The aim of the project was the optimization and the validation of the above-mentioned detection and isolation method for STEC in different matrices. In the first place immunomagnetic separation (IMS) was evaluated for the optimization of the STEC isolation method for cattle faeces (Ghent University, UGent). Second, molecular characterization of STEC strains was performed using a newly designed 33-mPCR as an alternative tool (University of Antwerp, VIB) and pulsed field gel electrophoresis (PFGE) (Institute for Agricultural and Fisheries Research, ILVO)**.** Also a smaller derived multiplex PCR (9-mPCR) was designed (VIB) and optimized for the screening of samples (ILVO). The third goal was the evaluation of different approaches for STEC isolation from human faecal samples (Universitair Ziekenhuis Brussel, UZ)**.** Finally the STEC detection and isolation method was validated by an in-house and an interlaboratory study which was based on the ISO 16140 guideline for the validation of alternative methods (University of Liège; UGent; ILVO).

For the optimization of the STEC isolation protocol for cattle faeces and the evaluation of the effect of IMS, cattle faecal samples were artificially inoculated with various numbers of STEC (10-100 and 100-1000 cfu/25g faeces) and isolated using the isolation protocol with 6h or 24h of enrichment followed by IMS and plating or direct plating on selective agars. Two types of IMS beads (Dynabeads and Captivate beads) were tested. Results showed that IMS (any of the two types of beads) had a highly positive effect on the isolation of serotype O157 (s-

and s+), whereas only a small or even a negative effect for non-O157 serotypes was found. This was largely clarified by results on pure broth suspensions of STEC, showing that high percentages were recovered from the IMS beads used in suspensions with the serotypes O157 (s- and s+), O26 and O103, but lower percentages were recovered for O111 and O145. Non-O157 STEC were often already efficiently isolated from faeces using only direct plating, whereas O157 (s- and s+) STEC were not. For the enrichment time, 24h generally gave higher isolation efficiencies than 6h. Finally for serotypes O157 (s- and s+), O26 and O103, a level of 10-100 cfu/25g was reliably detected, whereas for serotypes O111 and O145 only 100-1000 cfu/25g was reliably detected.

To accomplish the second task of the project, the Applied Molecular Genomics Group of the VIB Department of Molecular Genetics (UA-VIB) designed a proprietary 33-amplicon multiplex PCR (mPCR) assay combined with capillary electrophoresis. This mPCR assay contains the detection of 5 STEC serotypes (O26, O103, O111, O145, O157), the main virulence genes *VT1* with variants (*VT1ab*, *VT1c* and *VT1d*), *VT2* with six variants (*VT2b,c,d,e,f,g*) and consensus, *eae* with five variants *(eaeα1, eaeβ1, eaeγ1; eaeγ2; eaeε* and eaeζ), *ehx*, *tir, katP, saa, espP* and *FliC H2, H7, H8, H11* and *H28*. The assay was optimized and validated on a set of test strains representative for the priority amplicons. Next, this molecular technology was validated on a collection of 334 human clinical and animal strains from the Belgian STEC Reference Center (UZ).

This collection of human and animal strains was also characterized by performing the PulseNet Europe protocol for pulsed field gel electrophoresis (PFGE). This technique creates a fingerprint of a strain by means of rare cutter restriction enzyme cutting of DNA and gel electrophoresis. Analysis of the band patterns lead to clustering of strains according to similarity or relatedness. Then results of 33-mPCR and PFGE genotyping were combined to show eventual correlations between PFGE genotypes and virulence profiles. Also background information about the strains (date of isolation, human or animal source, clinical manifestation, outbreak information) was included to the analysis.

Combining mPCR and PFGE genotyping results, correlations were shown. In the first place STEC strains were clustered according to their serotype. Secondly a correlation occurred between virulence profile and PFGE clustering, concerning *VT* genes and other genes. Particularly for STEC O157, strains had very diverse *VT*-profiles, and strains with the same *VT*-profile clustered together. Concerning the clinical manifestation, 'asymptomatic' cases occurred more frequently for non-O157 than for O157 STEC, but besides this no correlation was shown between the PFGE clustering and the clinical manifestation or between the *VT*-profile and the clinical manifestation. Finally several case studies could be appointed based

on the PFGE dendrograms. In general the cases contained clones that persisted during several years, had similar virulence profiles and infected humans as well as animals.

As a part of the second task, the UA-VIB also designed a derived 9-amplicon multiplex PCR (9-mPCR) for fast sample screening. Using this 9-mPCR, a combination of serotypes (O26, O103, O111, O145, O157) and virulence genes (*VT1, VT2, eae* and *ehx*) is detected in one run and can be visualized using conventional gel electrophoresis.

Once the 9-mPCR was developed and tested on pure strains, an evaluation on samples was performed. Hereto ILVO (Institute for Agricultural and Fisheries Research) tested several methods to extract DNA from artificially inoculated samples. Methods were compared based on the ability to remove PCR inhibiting molecules and on the ability to isolate and purify DNA from STEC cells.

Out of four methods only two methods, in which no removal of sample debris was done, were suitable for sample preparation. The method using bead beating cell lysis described by Yu and Morrison (2004), was at least 10 times more sensitive than the method using the Qiagen Stool Mini Kit according to the manufacturer's instructions, and was therefore recommended. However, the method using bead beating cell lysis is much more time consuming than the Qiagen method and the use of a ribolyser is necessary.

As ILVO used the method employing the ribolyser in all following experiments, this method was used on artificially inoculated samples to determine its detection limit. All virulence marker genes and the serotype gene of strain MB3901 (serotype O157) could be detected in enriched minced beef and cheese from raw milk artificially inoculated with 2 cfu/25g sample. For cattle fecal samples the screening test was 10 times less sensitive; 21 cfu/25g feces could be detected.

Finally the influence of the volume of lysate used in the mPCR reaction mix was examined. An mPCR reaction containing 1 and 2µl of lysate DNA was performed, but no difference in detection was seen.

Testing of different clinical isolates of non-O157 STEC on the newly designed selective agars, showed that growth characteristics were generally as expected. However, more standardization of the preparation of the medium is needed to obtain more reproducible results. Some O103 isolates did not grow on the media prepared at UZ and the color of the colonies of O111 was often difficult to distinguish from O26.

Using artificially contaminated stool samples, the sensitivity of the STEC isolation protocol developed in a previous Belspo SPSD II project was similar to the protocol used routinely at UZ (10^3 and 10^4 cfu/5g). The sensitivity was about 10 times higher when using IMS. The method

performed well on frozen STEC positive samples, but this could only be tested on 14 samples, of which 11 with O157, 2 with O111 and one O26.

In-house validation of the STEC isolation protocol was performed to evaluate if the protocol is applicable for different types of food matrices. All samples used for this validation were artificially contaminated. Ten samples of minced beef, raw milk cheese and sprouted seeds were artificially inoculated with varying numbers (10-2000 cfu/25g) of non-stressed and stressed strains belonging to the serotypes O157 (s-) and (s+), O26, O103, O111 and O145. Cultured STEC strains were cold and freeze stressed by storing them for at least 5 days at respectively 2 and -18°C. Inoculated samples were pre-enriched in a weak selective medium for 6 hours followed by enrichment in a stronger selective medium for 18 hours. Direct plating on a selective medium was performed after each enrichment step. In a third pathway, an IMS (Dynabeads or Captivate beads) step was performed after 24h enrichment and prior to plating. Suspected colonies on the selective medium were purified and tentatively confirmed on a purification medium followed by a confirmation by a serotype PCR. Parallel to the classical isolation method, the 9-mPCR screening test was performed on the enrichment medium (after 24 hours enrichment). Results indicate that the isolation protocol as well as mPCR screening provide good detection of non-stressed and cold-stressed O26, O103, O157 (s+) and O145 in raw milk cheese and minced beef.
Detection of the other non-stressed and cold-stressed serotypes (O111 and O157 (s+)) in raw milk cheese and minced beef and of all serotypes under freeze stressed conditions in minced beef was low or almost zero.
Probably due to the high level of background flora, detection of any serotype in sprouted seeds was almost impossible even though inoculation numbers were as high as 2000 cfu/25g.

Finally the optimized STEC detection and isolation methods were validated by an interlaboratory study performed by national and international laboratories (twelve laboratories in total). First, a pre-trial experiment was organized to give the collaborative laboratories the possibility to become familiar with the isolation method. Secondly, the actual interlaboratory study was performed. Products necessary to prepare all culture media (in-house-prepared: IHP) and ready-to-use selective agar culture media (ready-to-use: RTU) were sent to the participating laboratories, as well as a questionnaire and a document to report the results. For each participating laboratory, 20 samples of 25g of minced beef were prepared: one sample for the temperature measurement upon arrival, one for the enumeration of the total count, Enterobacteriaceae and *E. coli*, two blank samples and sixteen samples inoculated

with single strains belonging to 4 serotypes at 2 levels of contamination in duplicate (30 cfu/g and 300 cfu/g). All strains were cold stressed. Samples were prepared the day of the shipment and had to be analyzed on a prefixed day. The University of Liège evaluated all results based on the recommendations of ISO 16140. Results showed no difference between RTU and IHP media. The arabinose test seemed difficult to be read, so the dulcitol test is now preferred for the confirmation of serotypes O103 and O111. Some mistakes were made during sample inoculation, like a wrong inoculation of four samples and no inoculation of one sample. If we do not take into account these mistakes, all four serotypes were detected with high sensitivity. In general it can be concluded that the laboratory performance is highly satisfactory.

2. Introduction

2.1. Context

Food borne shigatoxin-producing *E. coli* or STEC infection is a serious problem in human healthcare: these extremely severe pathogens can cause a range of disease symptoms ranging from asymptomatically carriage over various diarrhea symptoms to HUS (hemolytic uremic syndrome). Diagnosis of STEC is mostly directed to the serotype O157 because of the availability of a standard detection method exploiting the lack of sorbitol fermentation in the majority of the O157 strains. Due to the absence of routine detection methods for other serotypes, so-called non-O157 STEC, detailed epidemiological data on non-O157 infections are limited. However, recent data obtained in Belgium confirm WHO recommendations, identifying serotypes O26, O103, O111, O145 and sorbitol positive O157 as emerging pathogens, responsible for 80% of the hospitalized STEC infections and 23% of the HUS cases.

2.2. Objectives

Optimization and validation of STEC detection, isolation and identification methods:

A detection and isolation method for non-O157 STEC and sorbitol positive O157 STEC developed during the previous SPSD II project (Possé et al., 2008a) will be finally optimized resulting in a reliable, fast and robust method for different matrices including cattle feces for which IMS will be evaluated to increase the detection sensitivity.

Implementation of several molecular methods for fast sample screening and for strain characterization or confirmation. These will include a 33-amplicon multiplex PCR (33-mPCR) for a one-step virulence characterization and serotype identification of strains, a smaller multiplex PCR containing 9 amplicons (9-mPCR) for sample screening and confirmation of colonies and the PulseNet Pulsed Field Gel Electrophoresis (PFGE) protocol for strain typing.

Evaluation of several approaches for STEC detection and isolation from human fecal samples based on the previously developed protocols for screening samples, to assess feasibility for routine use in clinical laboratories.

Optimized STEC detection and isolation methods will be validated in-house on different food matrices, at different contamination levels and subsequently by an interlaboratory study for a selected matrix in both national and international laboratories, based on ISO guidelines.

2.3. Expected outcomes

Optimized detection and isolation method for STEC (including non-O157 strains) in cattle faeces.
Validated method for detection and isolation of STEC non-O157 and O157 (s+) strains in different food categories: meat, produce and dairy products
Molecular methods for STEC strain characterization (identification to serotype level as well as virulence and genetic typing) as alternative to conventional characterization: 33-mPCR assay for strain characterization.
Direct, quick and easy detection method for STEC in human faeces.

2.4. Research team

During the project frequent contacts between different partners occurred in order to organize the laboratory work of the partners involved in the relevant tasks. As foreseen in the technical specification of the project, all partners involved in the different tasks were working synergetic so that the same work was not done in duplicate.
For task 2 a good collaboration was developed between partner 2, 3 and 4 to develop the 33- and 9-mPCR: which genes to include, delivery of a collection of human isolates by partner 4 to partner 2 and of DNA from the strains by partner 2 to partner 3. Partner 3 implemented the 33-mPCR and confirmed the presence of nine genes detected by partner 2 by other primers (confirmation tests). The scientist of partner 2 followed the practical work done in the laboratory of the coordinator and evaluated the obtained results with the promoters of both partners (C and P2). Also decisions about the type and number of samples to be examined in Task 4.1 (in house validation) were made in consensus between the coordinator and partner 2. Partner 2 delivered all technical information to partner 4 so that the latter was able to start with this task 3. Since partner 4 had at the start problems with the performance of the isolation protocol using the selective medium, partner 4 followed a short technical training in the laboratory of the coordinator.
For the preparation of the organization of the international ring-trial a first meeting was organized between the coordinator, partner 2 and partner 5. Then a technical workshop was organized for the participating laboratories before sending out the samples. Also other details (conditions for sending out the samples, time schedule,…) of the trial were discussed. The ring-trial was organized by the coordinator in cooperation with partner 5. Samples were prepared by technical staff of the coordinator and partner 5, whereas partner 5 was responsible for the sending of the samples, analysis of the laboratory results and the reporting of the ring-trial.

3. Results

3.1. Task 1: Evaluation of IMS and optimization of STEC detection in cattle faeces

- Partners:
    - Ghent University

- Objective:

The purpose of this study was to evaluate the technique of immunomagnetic separation (IMS) and to optimize a method for isolation of non-O157 shiga toxin-producing *Escherichia coli* (STEC) serotypes O26, O103, O111, O145 and sorbitol positive (s+) O157 from cattle faeces using artificially contaminated faecal samples.

- Materials and Methods:
    - STEC isolation from cattle faeces

Cattle faecal samples were artificially inoculated with varying numbers (10-100 cfu/25g and 100-1000 cfu/25g) of clinical STEC strains and consequently recovered using a selective enrichment step of 6 or 24h, followed by plating on selective agars (Possé et al., 2008a) preceded by IMS or not. Two types of IMS beads, Dynabeads (Dynal, Oslo, Norway) and Captivate beads (Lab M, Bury, UK), were implemented, each on one set of four faecal samples. Isolated strains were confirmed using serotype-specific PCR (O26: DebRoy et al., 2004; O145: Feng et al., 2005; O103: Fratamico et al., 2005; O111 and O157: Paton and Paton, 1998).

    - Evaluation of IMS beads on pure suspensions

Evaluation of Dynabeads and Captivate beads was performed on pure suspensions of STEC. Overnight broth cultures of each serotype, were serially diluted in phosphate buffered saline (PBS) to 10-50, 50-250, 250-500 and >500 cells/ml and concentrations were determined by counting on Tryptone Soy Agar (TSA). In a first experiment detection limits and recovery percentages were determined for both types of beads by performing IMS on every dilution and plating the resulting solution on TSA+YE plate (TSA supplemented with 0.6% yeast extract (YE); LP0021; Oxoid). This was repeated up to seven times per dilution. In a second experiment loss-making factors in the IMS (Dynabeads) procedure were determined. IMS was performed on two dilutions of every strain (50-250 and 750-1250 CFU/ml) in duplicate and all waste and end solutions were streaked on TSA+YE. The waste solutions originated from the aspiration of

the sample matrix and the washing of the bead-bacteria complexes when immobilized by the magnet.

- Results
    - STEC isolation from cattle faeces

Results using direct plating showed that serotype O145 was isolated more efficiently after 6h of enrichment, whereas for serotypes O103 and O111 24h of enrichment was more efficiently (results not shown). Isolation efficiencies of serotypes O26, O157 (s+) and O157 (s-) were similar for both enrichment times (**Table 1**). Isolation efficiencies were higher for non-O157 serotypes than serotype O157 and higher if samples were inoculated in a higher level.

Secondly, results showed that IMS was most beneficial for the isolation of the serotype O157 (s- and s+) with an increase of 4 up to 7 positive samples. For non-O157 serotypes, IMS was less useful, because direct plating generally already gave high isolation efficiencies and the effect of IMS often was variable (Results for O157 (s-), O26 in Table 1; Results for O157 (s+), O103, O111, O145 not shown).

Concerning the detection limits, the level of 10-100 cfu/25g was reliably isolated using direct plating for the serotype O103 and by using IMS and plating also for O157 (s- and s+) and O26 (Table 1). For serotypes O145 and O111 the level of 100-1000 cfu/25g was reliably isolated using direct plating only.

Table 1. Isolation efficiencies of STEC O157 (s-) and O26 from (artificially inoculated) cattle faeces samples.

Inoculation level	10-100 cfu/25g							
Enrichment time	6h		24h		Combination 6h/24h			
Method	Direct plating	IMS** and plating	Direct plating	IMS and plating	Direct plating	IMS and plating	Effect IMS	Combination of methods
O157 s-	1/8	7/8	0/8	8/8	1/8	8/8	+7	8/8
O26	5/8	6/8	5/8	6/8	5/8	7/8	+2	7/8

*x=number of positive experiments / y= total performed experiments

** use of Dynabeads or Captivate beads

- Evaluation of IMS beads on pure suspensions

Recovery efficiency was variable from serotype to serotype. STEC was efficiently recovered from the pure suspension using both types of IMS beads, except for some serotypes in dilution 10-50 cells/ml (O103, O111 Captivate beads) and 50-250 cells/ml (O111, O145 Dynabeads) for which some negative results were obtained (results not shown).

Recovery percentages of serotypes O157 (s- and s+), O26 and O103 were relatively high for both types of beads, with higher percentages using Dynabeads than using Captivate beads (**Table 2**). For serotypes O145 and O111 recovery percentages were moderate only when using Captivate beads, but very low when using Dynabeads.

Table 2. Recovery percentage of Dynabeads and Captivate beads IMS for the used STEC serotypes.

Used beads	Dynabeads	Captivate beads
O157 s-	145%	62%
O157 s+	57%	35%
O26	70%	62%
O103	84%	19%
O111	3%	32%
O145	6%	26%

Results on loss-making factors in the IMS procedure using Dynabeads showed that for the well recovered serotypes O157 (s- and s+), O26 and O103 no loss-making factors could be determined in the procedure, whereas for the weakly recovered serotypes O111 and O145, it was found that respectively the aspiration of the sample matrix and the washing of the bacteria-beads complexes are responsible for a great loss of cells.

- Conclusions

In conclusion, the positive effect of IMS on the isolation efficiency of STEC O157 (s- and s+) in faeces, was not always observed for non-O157 STEC. This could largely be clarified by results on pure broth suspensions of STEC, showing that the bead affinities were high for O157 (s- and s+), O26 and O103, but weaker for O111 and O145. Also non-O157 STEC were often already efficiently isolated from faeces using direct plating whereas O157 (s- and s+) STEC were not. Finally, concerning the enrichment time, 24h gave the highest or similar isolation efficiencies compared to 6h for most of the serotypes.

3.2. Task 2: Implementation of multiplex PCR and Pulsenet Europe PFGE protocol

3.2.1. 33-mPCR and PFGE

- Partners:
    - University of Antwerp: Development of the 33-mPCR and characterization of strains
    - ILVO: Implementation of PulseNet Europe (PNE) PFGE protocol on strains and characterization of the collection of strains

- Objective:

The purpose of this study is to develop a 33-mPCR as a molecular tool for strain characterization. Secondly, this mPCR and the PNE PFGE protocol were implemented to characterize a collection of ca. 300 human clinical and animal STEC strains.

- Results
    - Development of the 33-mPCR assay

The Applied Molecular Genomics (AMG) group of the VIB Department of Molecular Genetics (UA-VIB) designed a proprietary 33-mPCR (mPCR) assay. This 33 amplicon mPCR assay contains 5 STEC serotypes (O111, O157, O103, O26 and O145) and the main virulence genes *VT1* with three variants (*VT1ab*, *VT1c* and *VT1d*), *VT2* with six variants (*VT2 b,c,d,e,f,g*) and consensus, *eae* with five variants *(eae α1, eaeβ1, eaeγ1; eaeγ2; eaeε* and eae*ζ*), *ehx*, *tir, katP, saa, espP, FliC H2, H7, H8, H11* and *H28* (Nielsen et al., 2003; Blanco et al., 2004; Persson et al., 2007).

To accomplish this task successfully the VIB collected all publicly available sequences using BLAST analysis with the 33 reference sequences described above as query sequences (**Figure 1**). Data clean-up and manual curating resulted in a total of 856 similar sequences for further analysis. Next these sequences were properly aligned (Muscle) and organized in a tree (clearcut) structure (**Figure 2**). The obtained trees were manually verified and groups assigned. These groups, reflecting the 33 reference sequences, served as starting point for multiplex primer design. Hereto, a consensus sequence is generated from each of the 33 alignments taking into account ambiguity bases which can't be included in the subsequent primers. Furthermore, to reduce the possibility of false positive amplifications the VIB designed an

algorithm that allows verification of the generated primers for a specific group for the absence of annealing possibilities on sequences for all the other groups. The resulting list of group specific sequences is subsequently fed into our MultiPCR algorithm for the multiplex PCR assay.

The assay(s) were optimized and validated on a set of test strains representative for the priority amplicons. Next, this molecular technology was validated on a collection of 334 human and animal strains from the Belgian STEC Reference Center (UZ).

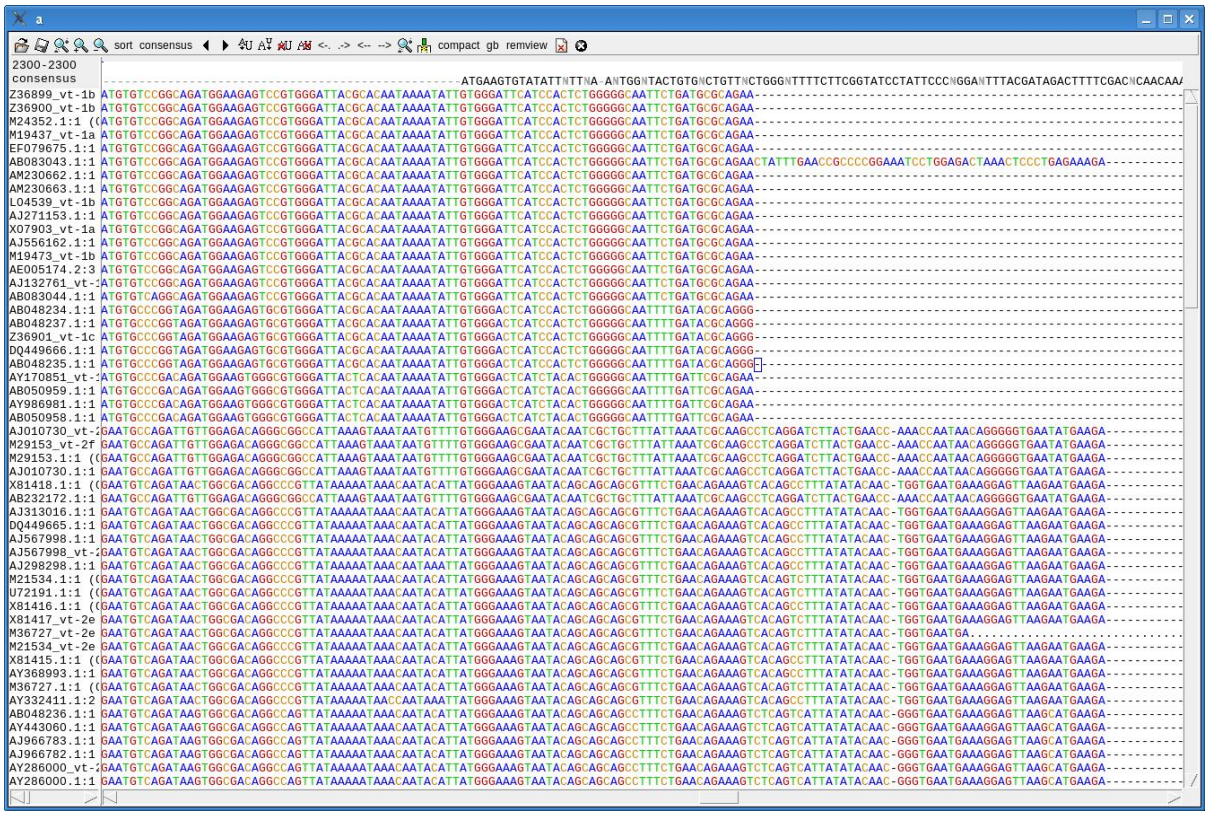

Figure 1 . Screenshot of the aligned sequences that are similar to STEC reference sequences, obtained by BLAST analysis.

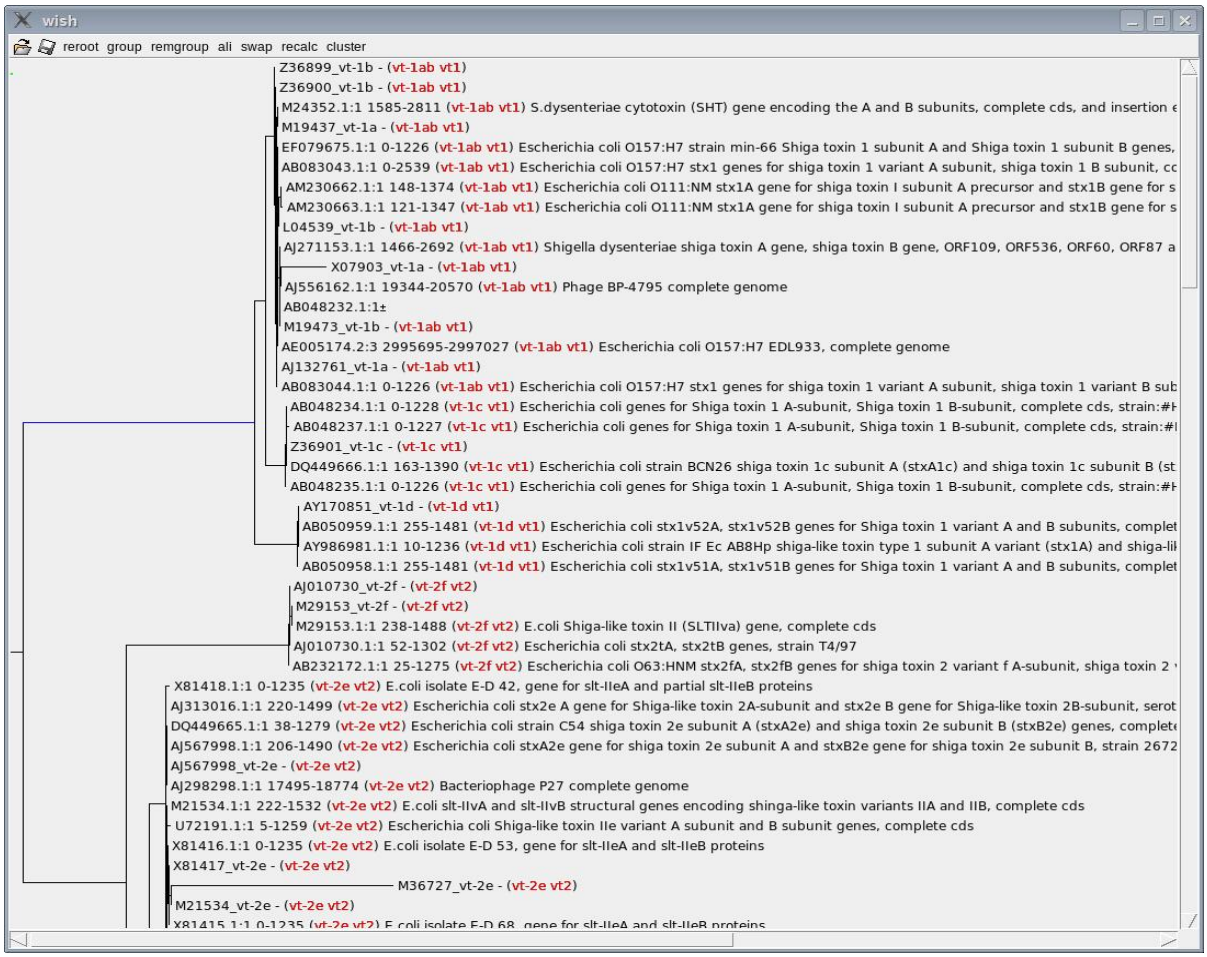

Figure 2. Screenshot of the organization of similar STEC sequences in a tree structure**.**

- Characterization of a STEC collection

Using the 33-mPCR, 321 strains of the STEC collection (334 strains) were characterized. 62% of the typed strains were of serotype O157, 10% of O26, 5% of O103, 3% of O111 and 3% of O145. Similar percentages were shown for the FliC types; 63% H7, 10% H11, 6% O103, 3% H8 and 3% H28. Concerning the main virulence genes, 39% of the strains carried *VT1*; of which 89% subtype *vtx1a* or *b*, 9% vtx1c and 1% vtx1d. 71% of the strains carried any variant of *VT2*, of which 53% carried subtype *vtx2*, 39% carried *vtx2c* or *d,* 6% *vtx2b*, only one strain subtype *vtx2e* in a combination with *vtx2g* or *h.* 88% of the strains carried *eae*, of which 65% subtype *γ1*, 11% *β1*, 5% *ε*, 4% *γ2* and 4% *ζ* and 92% of the strains carried *ehx.* Other virulence genes like *EspP* was carried by 78% of the strains, *KatP* by 76%, *Tir* by 11% and *Saa* by 3% of the strains.

In a second place PNE protocol for PFGE was performed on the STEC collection, leading to fingerprints of all strains (**Figure 3**).The PNE protocol with the restriction enzyme XbaI was used (http://www.pulsenet-europe.org)

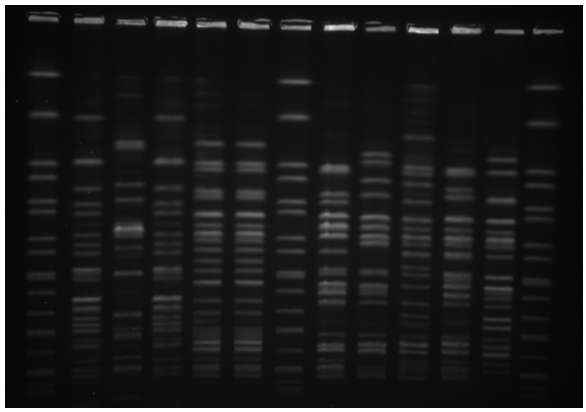

Figure 3 PFGE fingerprints of 10 STEC strains and three times the standard strain *Salmonella* Braenderup (H9812) obtained after restriction with the enzyme *Xba I* and the PNE protocol for PFGE.

Using Bionumerics, PFGE patterns were analyzed and clustered based on similarity. Then results of mPCR and PFGE genotyping were combined to screen for eventual correlations between relatedness and virulence profiles. Also background information about the strains (date of isolation, human or animal source, clinical manifestation, outbreak information) was included to the analysis.

PFGE clustering of serotypes

In the first place STEC strains were clustered according to their serotype. This was shown in the tree-structure (**Figure 4**) and in the three-dimensional preview of the collection (**Figure 5**) in which strains of the same serotype are grouped together.

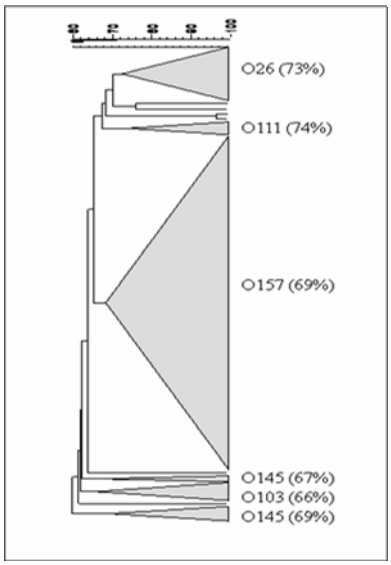

Figure 4. Tree-structure of STEC strains based on PFGE DNA fingerprints**.**

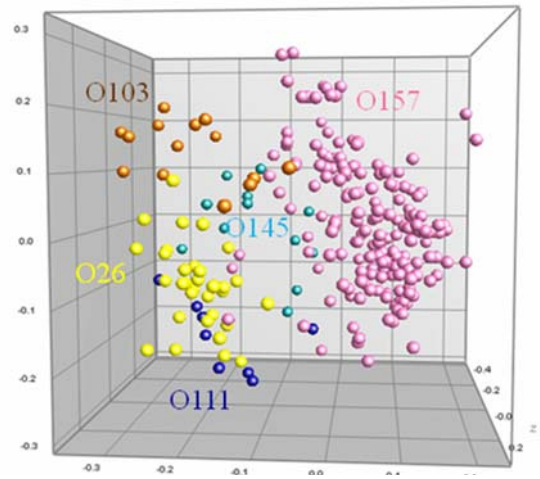

Figure 5. Three-dimensional preview of STEC strains based on PFGE DNA fingerprints. Colour according to serotype; O157, pink; O103, brown; O145, green; O26, yellow; O111, blue.

Correlation between virulence profile and PFGE clustering

VT-profile

Concerning the *VT*-profile, most strains belonging to the serotype O26, O103 and O111 carried only *VT1.* For O145 the main *VT*-profile was *vtx2* (**Table 3**). For O157 the *VT*-profile was diverse with two prominent profiles *vtx2* and *vtx2c or d* and three profiles combining these genes with *VT1*.

For serotype O157, results showed that the *VT*-profile is correlated to the PFGE clustering or strains carrying the same *VT* genes were more related. This is illustrated in a two-dimensional view (**Figure 6**) of the strains positioned according to their relatedness and coloured according to *VT*-profile.

Table 3 *VT*-profiles of strains belonging to serotypes O26, O103, O111, O145 and O157.

Serotype	*VT*-profile	Percentage
O26	*VT1*	86% (25/29)
	vtx2	7% (2/29)
	vtx2 + vtx2c or d	3% (1/29)
O103	*VT1*	85% (11/13)
	VT1 + vtx2c or d	15% (2/13)
O111	*VT1*	63% (5/8)
	VT1 + vtx2	37% (3/8)
O145	*vtx2*	58% (7/12)
	VT1	33% (4/12)
	vtx2 + vtx2c or d	8% (1/12)
O157	*vtx2c or d*	35% (63/178)
	vtx2	32% (56/178)
	vtx2c or d + vtx2	17% (30/178)
	VT1 + vtx2c or d	9% (16/178)
	VT1 + vtx2	6% (10/178)
	VT1 + vtx2 + vtx2c or d	1% (2/178)

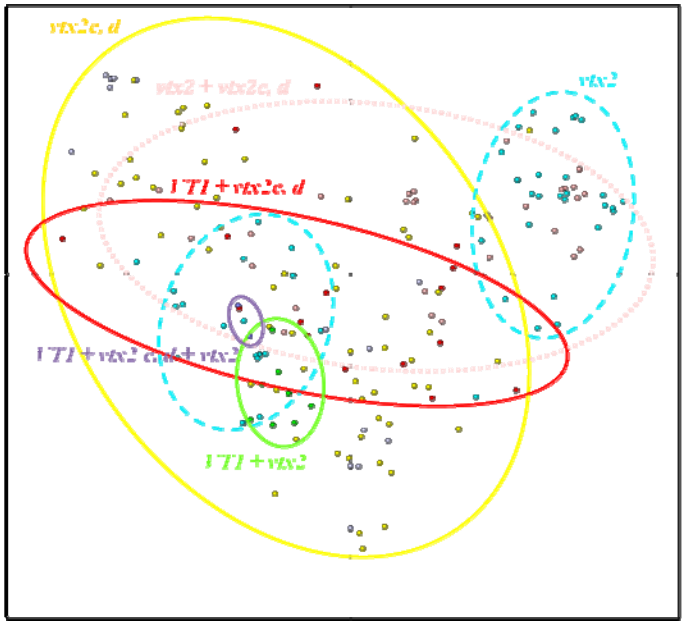

Figure 6 . Two-dimensional view of PFGE clustering for serotype O157, with coloring according to the *VT*-profile; *vtx2c* or *d*, yellow; *vtx2*, blue; *vtx2c* or *d* + *vtx2*, pink; *VT1* + *vtx2c* or *d*, red; *VT1* + *vtx2*, green; *VT1* + *vtx2c* or *d* + *vtx2*, purple.

For non-O157 serotypes no analysis concerning a correlation between *VT*-profile and PFGE clustering was performed, because mainly only one *VT*-profile overruled and the amount of strains was low.

Other genes

Also the presence of other genes, like *katP, Tir* and *EspP* was correlated to the PFGE clustering (**Figure 7**). The gene *saa* was carried by only ten strains of the collection.

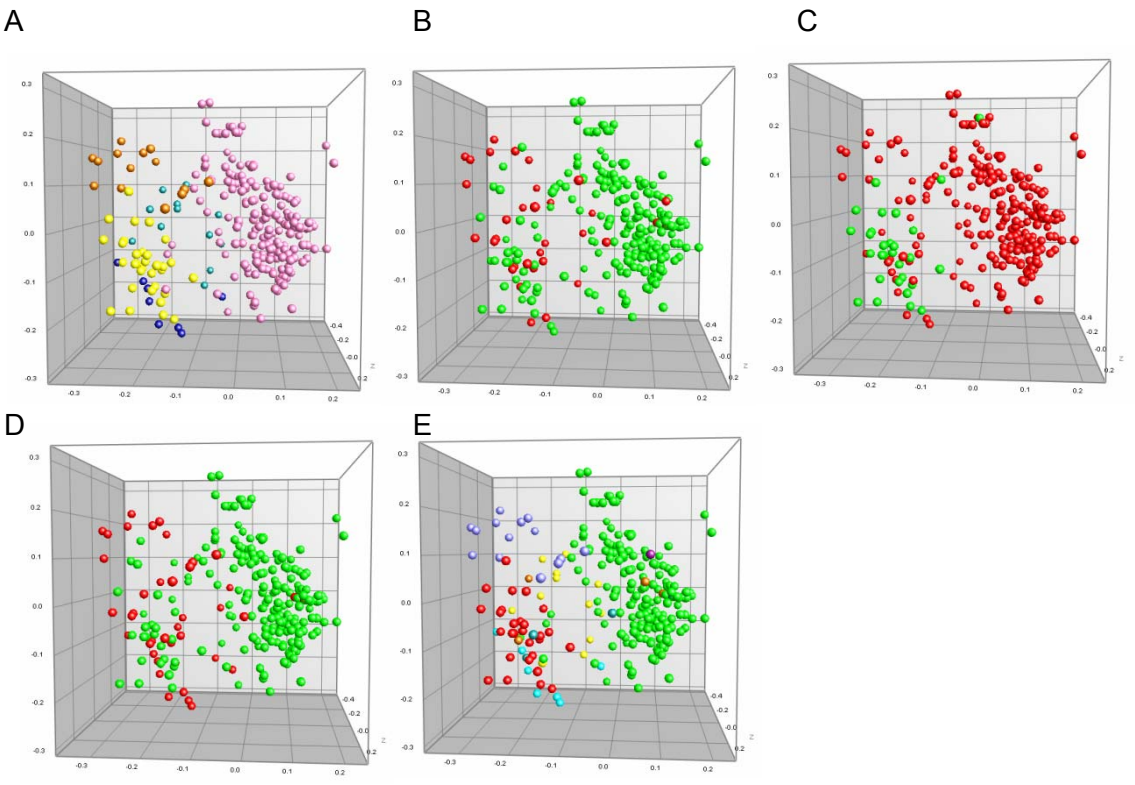

Figure 7. Three-dimensional view of STEC collection, with indication of *katP*, *Tir* or *EspP* detection and FliC type. (A. Colour according to serotype; O157= pink; O103= brown; O145= green; O26= yellow; O111= blue. B. *katP* detection; present= red; absent= green. C. *Tir* detection; present= red; absent= green. D. *EspP* detection; present= red; absent= green. E. FliC types; H7= green, H11= red; H2= purple; H28= yellow; H8= blue; H4= lilac)

Correlation between virulence profile, PFGE clustering and clinical manifestation

The clinical manifestation 'asymptomatic' occurs more frequently for non-O157 than for O157 STEC (**Figure 8**).

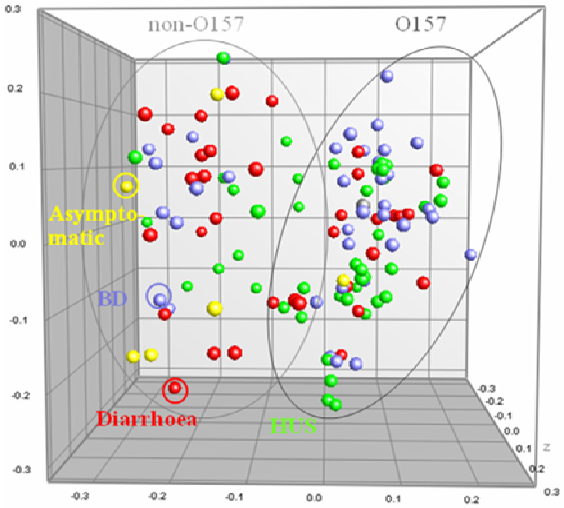

Figure 8 . Three-dimensional view of human clinical STEC strains, with colouring according to clinical manifestation. Yellow, asymptomatic; purple, bloody diarrhoea; red, diarrhoea; green, HUS.

No correlation was shown between the *VT*-profile and the clinical manifestation, and between the PFGE clustering and the clinical manifestation (**Figure 9**).

A B

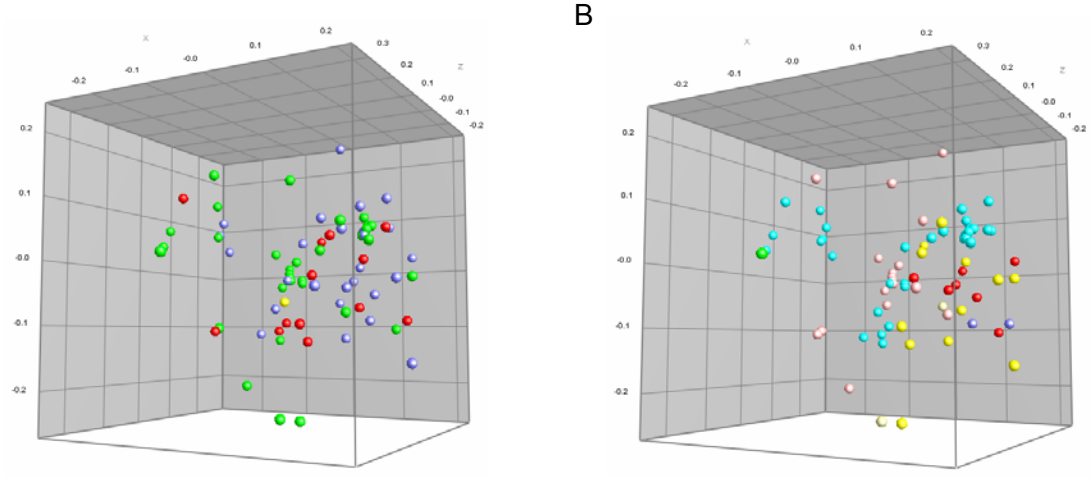

Figure 9 Three-dimensional view of human clinical STEC O157 strains. A. Colour according to clinical manifestation; yellow, asymptomatic; purple, bloody diarrhoea; red, diarrhoea; green, HUS. B. Colour according to *VT*-profile; *vtx2c* or *d*, yellow; *vtx2*, blue; *vtx2c* or *d* + *vtx2*, pink; *VT1* + *vtx2c* or *d*, red; *VT1* + *vtx2*, green; *VT1* + *vtx2c* or *d* + *vtx2*, purple.

Case studies

Results showed an outbreak that occurred in August 2006, which infected three people of which one developed HUS. At the same time the same clone was isolated from cattle as well. These 5 strains were part of a bigger cluster of 14 strains with nearly identical PFGE patterns (97% similarity) and similar virulence profile. During several years this clone was isolated from animals and humans, causing a variety of clinical manifestations.

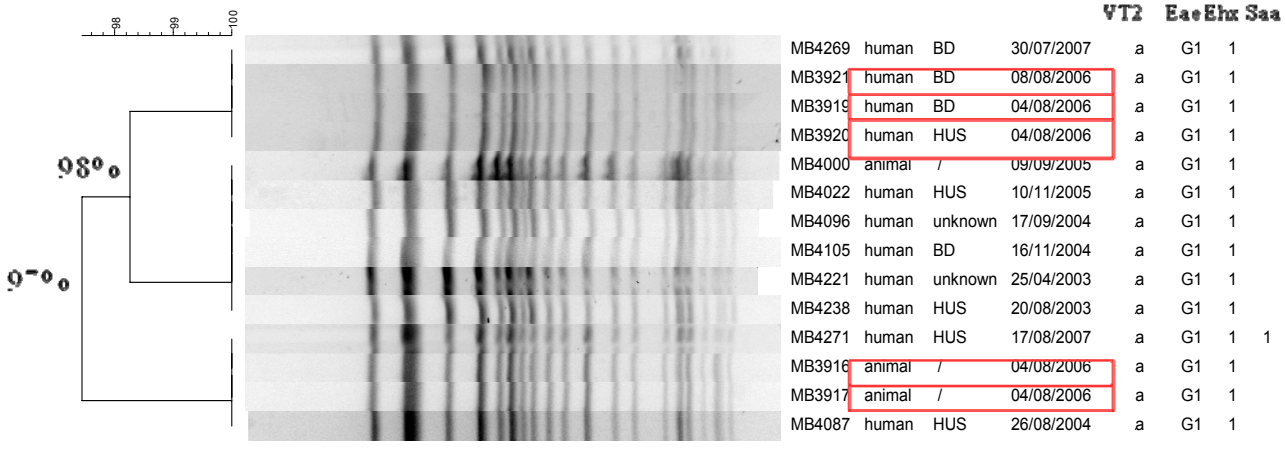

Figure 10 . PFGE clustering of a persisting clone causing an outbreak in August 2006.

- Conclusions

A 33-mPCR was developed by the VIB-UA and implemented on a collection of human clinical and animal STEC strains. As a result virulence profiles were created for all strains in the collection. Also the PulseNet Europe protocol for PFGE was performed, creating fingerprints that leaded to clustering of the strains according to similarity or relatedness.

Combining mPCR and PFGE genotyping results, correlations between relatedness and virulence profiles were shown. Also background information about the strains (date of isolation, human or animal source, clinical manifestation, outbreak information) was included in the analysis. In the first place STEC strains were clustered according to their serotype. Secondly a correlation occurred between virulence profile and PFGE clustering, concerning *VT* genes and other genes. Particularly for STEC O157, strains had very diverse *VT*-profiles, and strains of the same *VT*-profile clustered together. Concerning the clinical manifestation, 'asymptomatic' cases occurred more frequently for non-O157 than for O157 STEC, but beside this no correlation was shown between the PFGE clustering and the clinical manifestation or between the *VT*-profile and the clinical manifestation. Finally several case studies could be appointed based on the PFGE dendrograms, that in general contained clones that persisted during several years, had similar virulence profiles and infected as well humans as animals.

3.2.2. Derived multiplex PCR for sample screening

- Partners:
  - University of Antwerp: 9-mPCR design
  - ILVO: optimization screening method

- Objective:

The VIB has designed a 9-mPCR for fast sample screening. Using this mPCR, a combination of serotypes (O26, O103, O111, O145, O157) and virulence genes (*VT1, VT2, eae* and *ehx*) is detected in one run and can be visualized using conventional gel electrophoresis. In the first year of the project, the mPCR mix and protocol for sample screening were developed.

- Materials and Methods:

The VIB tested the 9-mPCR on the collection of 334 human and animal STEC strains from the Belgian STEC Reference Center (UZ). Results were compared to typing results obtained by

ILVO and UZ on the same strains by means of individual PCR's; the comparison is listed in **Table 4**.

Table 4 PCR typing results on 334 human and animal STEC strains from the different partners (Percentages represent the amount of STEC strains found positive for the target gene).

Partner	*VT1*	*VT2*	*eae*	*ehx*	O157	O26	O103	O111	O145
VIB	39%	70%	88%	91%	62%	9%	5%	3%	3%
ILVO	39%	71%	87%	91%	63%	10%	7%	4%	6%
UZ	39%	71%	88%	91%	62%	10%	5%	3%	3%

Typing results of the collection of human and animal STEC strains were very similar for the three partners (Table 6).

Once the 9-mPCR was developed and tested on pure strains, an evaluation on artificially contaminated samples was performed. Therefore ILVO tested several methods to extract DNA from these samples, as an efficient DNA extraction is indispensable for sensitive mPCR detection. Methods were compared based on the ability to remove PCR inhibiting molecules and on the ability to isolate and purify DNA from STEC cells.

In a first stage the inhibitory effect of matrix components was tested for different extraction methods. Samples of cattle feces, minced beef and cheese from raw milk were enriched during 24h followed by DNA extraction of the background flora. Then a series of dilutions of STEC target DNA was added to the lysate prior to the implementation of 9-mPCR. Four DNA extraction protocols were tested. Each protocol started with removing sample debris by centrifugation or filtration. In protocol 1A, 1B and 3: one mL of enrichment broth culture was centrifuged at 3000g for 1 minute, then the supernatant was transferred to a new tube and centrifuged at 13000g for 5 minutes to pellet the bacteria (Osek et al., 2002). In protocol 1A the pellet was resuspended in 100µl lysis solution (0.05 M NaOH, 0.125% SDS) and heated at 90°C for 17 minutes, followed by vortexing and centrifugation at 13.000g for 1 minute. In protocol 1B the pellet was washed two times with PBS (phosphate buffered saline) prior to heat lysis. In protocol 3 the pellet was resuspended in lysis buffer and DNA extraction was performed according to the method described by Yu and Morrison (2004) using bead beating and purification by QiaAMP columns (Qiagen, Düsseldorf, Germany). In protocol 2: five mL of enrichment broth culture was filtered through a Whatman No. 4 filter (Whatman, Kent, UK) and filtrate was centrifuged for 5 minutes at 13000 g to pellet the bacteria (Rijpens et al., 1998). The pellet was dissolved in 100µl lysis solution (0.05 M NaOH, 0.125% SDS) and heated at 90°C for 17 minutes, followed by vortexing and centrifugation at 13.000g for 1 minute.

PCR was performed in a final volume of 25 µl, containing 10µL reaction mix (VIB, Antwerp, Belgium), 0.125 µl Titanium Taq polymerase, 15 µl double-distilled water (ddH2O), 1 µl lysate and target DNA with a concentration of 300, 30, 3, 0.3 or 0.03 ng per 25µl reaction mix (strain MB3901 with serotype O157).

The PCR program consists of a denaturation step at 98°C for 3 minutes followed by 30 cycles which each consist of a denaturation step at 95°C for 45 seconds, a primer annealing step for 45 seconds at 58°C and an elongation at 72°C for 2 minutes. After 30 cycles an additional elongation at 72°C for 10 minutes is performed.

Electrophoresis was performed on a 2.5% agarose gel using SeaKem LE agarose (Lonza, USA)

- Results:

The inhibitory effect of the three matrices was compared for the four DNA extraction methods (**Figure 11**).

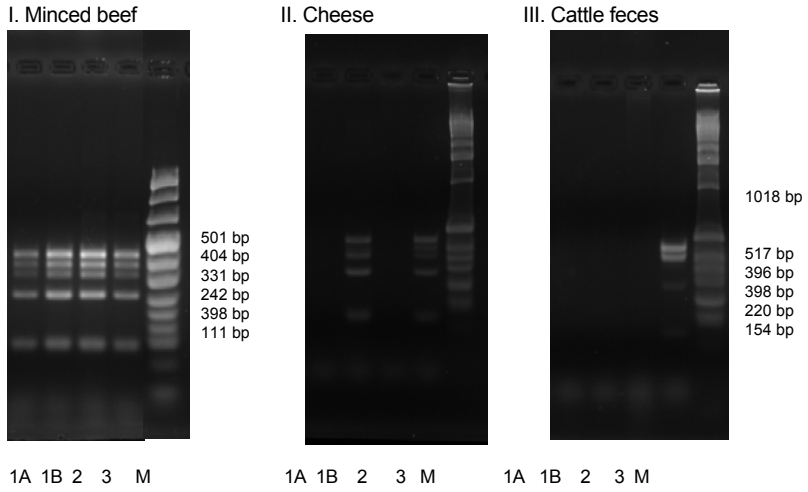

Figure 11 Inhibitory effect of matrix components on 9-mPCR for 4 DNA extraction methods using a target DNA dilution and 3 matrices (I, II & III) . Methods: 1A: heat lysis of bacteria in pellet; 1B: heat lysis of bacteria in pellet after washing; 2: heat lysis of filtrate; 3: bead beating lysis and purification by QiaAMP column of the precipitated DNA. Target DNA (strain MB3901) added to the PCR reaction: 0.3 ng/25µl reaction mix. Amplicon length target genes: O157, 101bp; *eae,* 264bp; *VT1,* 352bp; *VT2,* 399bp; *ehx,* 442bp. M: pUC mix 8/ 1 kb DNA Molecular Weight Marker (Invitrogen, USA).

The 10^{-3} dilution of the target DNA solution (resulting in 0.3 ng per 25µl reaction mix) added to the mPCR reaction mix could be detected in all three sample matrices (Figure 11). For minced beef all extraction methods successfully removed inhibitory components. For cheese, methods 1B (heat lysis after washing of bacteria in pellet) and 3 (bead beating lysis and purification by QiaAMP column) were successful. For cattle feces only method 3 succeeded in removing inhibitory components.

In the next experiment the efficiency of cell lysis and DNA isolation of methods 1B and 3 was tested. Samples of minced beef, cheese and feces were enriched and subsequently artificially inoculated with high concentrations of STEC O157 (strain MB3901). Results were disappointing because $6*10^4$ STEC bacteria /ml could not be detected in any matrix using any method. The stage of the protocol in which most cells were lost, was identified by plate counting the amount of STEC bacteria present in every stage of the protocol. Results indicated that 95% of the STEC cells were washed away in the early step removing sample debris.

Therefore, in a next experiment, the Qiagen Stool kit (method 4) and the bead beating extraction method with subsequent DNA purification by QiaAmp column (method 3bis) were implemented directly on the enrichment broth culture without the removal of sample debris. Samples of minced beef, cattle feces and cheese from raw milk were enriched and subsequently artificially inoculated with a series of dilutions of STEC O157 (strain MB3901). Using method 3, $4*10^3$ cfu/ml could be detected in minced beef and $4*10^4$ cfu/ml in cheese and cattle feces (**Figure 12A**). In **Figure 12B** the detection sensitivity of STEC in feces could be compared using the QiaAMP Stool kit (method 4) and using the bead beating extraction method with subsequent DNA purification by QiaAmp column (method 3bis) on the same fecal sample. An exact detection limit for both methods could not be determined, because the inoculated fecal sample was already naturally contaminated with STEC. But the results in Figure 12 did show that the bead beating method with subsequent QiaAMP DNA purification (method 3bis) is at least 10 times more sensitive than the QiaAMP stool protocol (method 4). More concrete, using method 4, $1,8*10^5$ cfu/ml could only just be detected , whereas using method 3, $1,8*10^4$ cfu/ml could still easily be detected.

A. Method 3.

B. Method 3 versus Method 4

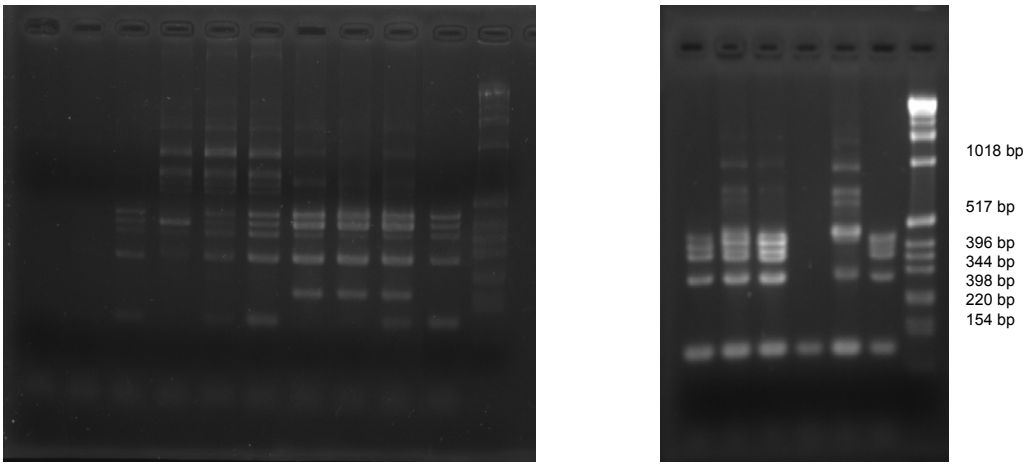

Figure 12 Sensitivity of 9-mPCR in detecting virulence marker and serotype genes of STEC O157 in cattle faeces using two DNA extraction methods (3bis and 4, see text). Matrices: lanes 1-3: cheese from raw milk; lanes 4-6: minced beef; lanes 7-9, 12-16: cattle feces. A. Enriched sample inoculated with STEC O157 (strain MB3901) in a series of dilutions and DNA extracted using method 3. Target STEC cell concentration: $4*10^2$ (lanes 1, 4, 7); $4*10^3$ (lanes 2, 5, 8); $4*10^4$ cfu/ml (lanes 3, 6, 9). B. Enriched sample inoculated with STEC O157 (strain MB3901) and DNA extracted using methods 3 (lane 13, 14, 16) and 4 (12-15). Target STEC cell concentration: $1,8*10^4$ cfu/ml (lane 13); $1,8*10^5$ (lanes 12,14). Blanks: lane 15, 16. Positive controle: lane 10, 17: 0,5 ng/µl target DNA of STEC O157 (strain MB3901). 1 kb DNA Molecular Weight Marker: lanes 11, 18. Amplicon length target genes: O157, 101bp; *eae,* 264bp; *VT1,* 352bp; *VT2, 3*99bp; *ehx,* 442bp.

In a following experiment the detection limit of the 9-mPCR sample screening test using method 3bis on samples inoculated with STEC O157 (MB3901) was determined. All virulence marker genes and the serotype gene of strain MB3901 could be detected in enriched minced beef and cheese from raw milk artificially inoculated with 2 cfu/25g sample (**Figure 13**). For cattle fecal samples the screening test was 10 times less sensitive; 21 cfu/25g feces could be detected (**Figure 13**).

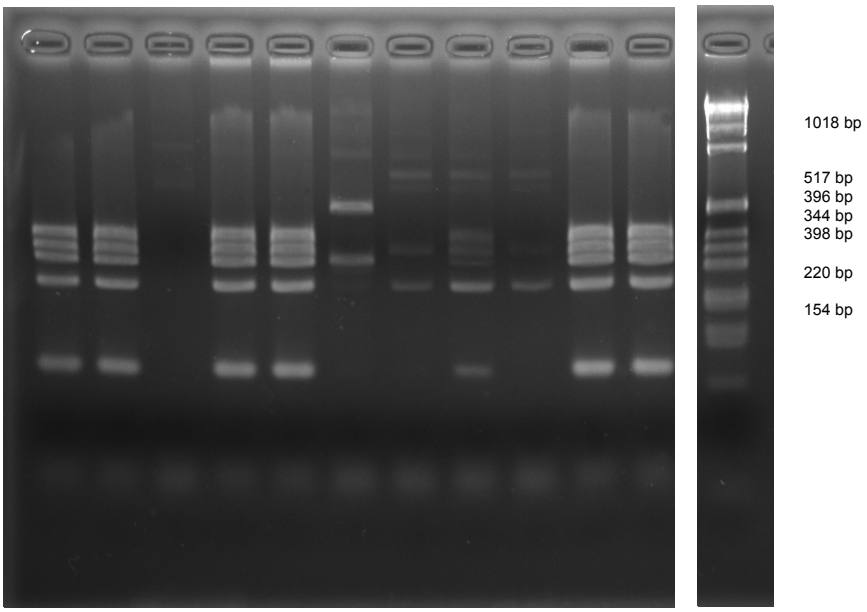

Figure 13 . Detection limit of 9-mPCR screening test. Matrix: Cheese from raw milk (lanes 1-3); Minced beef (lanes 4-6); Cattle feces (lanes 7-9); No matrix (lanes 10,11). Samples inoculated with STEC O157 (strain MB3901) prior to enrichment in two concentrations: 2 cfu/ml (lanes 1,4, 7, and 10) and 21 cfu/ml (lanes 2, 5, 8 and 11). Blanks: lanes 3, 6, 9. Amplicon length target genes: O157, 101bp; *eae,* 264bp; *VT1,* 352bp; *VT2,* 399bp; *ehx,* 442bp. 1 kb DNA Molecular Weight Marker: lane 12.

Finally the influence of the volume of lysate used on the 9-mPCR reaction was examined. An mPCR reaction containing 1µl and 2µl of lysate DNA was performed, but no difference in detection was seen.

- Conclusions:

Method 3 and 4, without the removal of sample debris, were the only two methods suitable for sample preparation. Method 3, using bead beating cell lysis, is at least 10 times more sensitive than method 4 and therefore recommended. However, method 3 is much more time consuming than method 4 and secondly, if a laboratory does not have a ribolyser (which is needed for the bead beating lysis), the use of method 4 (Qiagen Stool kit) is advised. The detection limit for method 3 was ascertained as being 2 cfu/25g or less in raw milk cheese and minced beef but 21 cfu/25g in cattle feces.

For the mPCR screening test the ILVO used the DNA extraction method using bead beating and QiaAMP column purification (method 3) for task 4.1 and the UZ used the Qiagen Stool kit protocol (method 4)(task 3).

3.3. Task 3 Evaluation of the detection and isolation method on human clinical samples

- Partners:
    - Universitair Ziekenhuis Brussel

- Objective:

To evaluate the optimized detection and isolation method for STEC developed in this project on human samples.

- Methods and Materials:

1. Preliminary tests: The differentiation- and confirmation media developed in the previous SPSDII project for non-O157 were tested on a collection of STEC clinical isolates that were different from those used for the initial development of these media. For this purpose, pure cultures of the isolates were cultivated on media prepared following the instructions for preparation.
2. Direct extraction of DNA from stools followed by PCR detection of STEC: the Qiagen stools kit was used for this purpose, as no ribolyser was available in the lab. The extracted DNA was then tested using the 9-mPCR developed in this project.
3. Detection of STEC in artificially contaminated stool samples:
   Routine stool samples submitted to the laboratory and tested negative for STEC were contaminated with dilutions of suspensions of known STEC isolates obtained from fresh cultures on agar. Counting of the number of colony forming units was performed by subculturing dilutions of the original suspension and counting the colonies.
   The artificially contaminated stool samples were tested as follows:
    - Direct plating on sorbitol-MacConkey agar (SMAC) followed by a multiple PCR targeting *VT1*, *VT2* and *VT2f* variant (routine protocol at UZ) performed first on mixture of colonies, then on individual colonies.
    - Direct plating on the selective medium for non-O157 (B1), followed by the 9-mPCR protocol developed in this project performed first on mixture of colonies, then on individual colonies.
    - IMS with Captivate beads for the different serogroups expected in these samples followed by plating on B1, followed by the 9-mPCR protocol

developed in this project performed first on mixture of colonies, then on individual colonies.

- Finally, the mTSB enrichment broth used for IMS was also used directly after extraction by Qiagen DNA extraction kit.

4. Detection of STEC in naturally contaminated stool samples:
   Only a limited number of naturally contaminated samples were available, most of them being positive for O157 (s-). They were tested using the same techniques as the artificially contaminated samples.

- Results:

1. Preliminary tests: the preparation of the differentiation media was difficult. A number of unsuccessful attempts had to be made before obtaining interpretable results. However, it should be underlined that we did not obtain even clear-cut colorations of colonies as those presented with media obtained from the coordinator or even with those prepared with products provided by the coordinator. In general, the growth characteristics of isolates from the 4 non-O157 serogroups on differentiation medium for non-O157 were as expected, but we made the following observations:
   a. O26: Of the 9 tested isolates, only 8 grew as the expected purple colonies; the last one grew as a light purple colony. This isolate was atypical since it did not ferment sorbitol.
   b. O103: Of the 8 tested isolates, 3 did not grow on the selective medium for non-O157, one grew only as very small colonies. The 4 other isolates grew as blue colonies, as expected.
   c. O111: only 1 of 7 isolates grew as clearly blue colonies, the other 6 grew as light purple colonies, difficult to distinguish from O26.
   d. O145: all 8 isolates grew as green colonies as expected. The only point worth to mention is that one isolate formed rather small colonies.

   Only one O157 (s+) isolate was available for testing. Growth characteristics on B1 were as expected.

   On the purification media, all isolates grew as expected, with the exception of one O103 isolate that did ferment dulcitol when O103 is expected not to ferment this sugar.
2. Direct extraction of DNA from stools followed by PCR detection of STEC: very deceiving results were obtained in a few preliminary tests and this technique was abandoned.
3. Combined results of several experiments are listed in **Table 5**.

Table 5 Isolation efficiencies of STEC O26, O103, O111 and O145, inoculate in different levels to 4 to 6 human faecal samples, using several isolation methods.

Level of inoculum (range)	2-6 10^4 cfu/5g			2-6 10^3 cfu/5g		
Method	Direct plating[1]	Direct plating[2]	IMS[2]	Direct plating[1]	Direct plating[2]	IMS[2]
Selective medium	SMAC	B1	B1	SMAC	B1	B1
O26	5/6*	6/6	6/6	3/6	4/6	4/6
O103	6/6	6/6	6/6	4/6	6/6	6/6
O111	6/6	6/6	6/6	2/6	2/6	4/6
O145	3/4	3/4	4/4	4/4	2/4	4/4

Level of inoculum (range)	2-6 10^2 cfu/5g			2-6 10^1 cfu/5g		
Method	Direct plating[1]	Direct plating[2]	IMS[2]	Direct plating[1]	Direct plating[2]	IMS[2]
Selective medium	SMAC	B1	B1	SMAC	B1	B1
O26	1/6	0/6	3/6	0/4	0/4	1/4
O103	2/6	3/6	5/6	0/6	0/6	4/6
O111	1/6	1/6	4/6	0/6	0/6	2/6
O145	1/4	1/4	2/4	0/4	0/4	0/4

1: tested by using routine PCR protocol (consensus primers for all VT variants)
2: tested by multiplex PCR developed in this project.
*x=number of positive experiments / y= total performed experiments

In addition, the multiplex PCR was tested directly on mTSB enrichment broth, after extraction by Qiagen DNA extraction kit, with the following results: at levels of 2-6 10^3 cfu/5 g, all 6 samples were positive by the multiplex PCR developed in this project, at levels of 2-6 10^3 cfu/5 g, 5/6 samples were positive, at levels of 2-6 10^2 cfu/5 g, 3/6 samples were positive and at levels of 2-6 10^1 cfu/5 g, 1/6 samples was positive.

4. Results on detection of STEC in naturally contaminated stool samples are listed in **Table 6**.

Table 6 Isolation efficiencies of STEC O26, O103 and O111, from naturally contaminated human faecal samples, using several isolation methods.

Method	PCR on mTSB enrich-ment	Direct plating B1		Direct plating SMAC		IMS B1		IMS SMAC	
		Positi-ve Colo-nies	9-mPCR	Positi-ve Colo-nies	9-mPCR	Positi-ve Colo-nies	9-mPCR	Positi-ve Colo-nies	9-mPCR
O157	7/11*	8/11	8/11	10/11	11/11	11/11	11/11	10/11	11/11
O111	2/2	2/2	2/2	-	2/2	1/2	1/2	-	2/2
O26	1/1	1/1	1/1	-	1/1	1/1	1/1	-	1/1

*x=number of positive experiments / y= total performed experiments

The PCR performed directly on mTSB broth after extraction of DNA by Qiagen DNA extraction kit was less sensitive than the test performed on colonies (Table 7).
In general, the performance of both solid media was similar on naturally contaminated samples.

- Conclusions:

The preliminary tests on differentiation and confirmation media, showed that adaptation of the instructions for the preparation of the differentiation media were needed. The growth on these media have also to be interpreted with caution, taking into account the problems of standardisation. The performance of the selective medium for non-O157 (B1) has to be further investigated with more O103 STEC isolates.
For the artificially contaminated stool samples: similar results were obtained by direct plating on SMAC and B1. The sensitivity of the method was enhanced by use of IMS. This effect was seen for the 4 serogroups, but more pronounced for O26.
The sensitivity of the new media was not higher than SMAC medium from which randomly selected colonies were selected. For O157 (s-) positive samples, this can be explained by better performance of SMAC medium while for the three non-O157 samples available, the high concentration of STEC explains that both methods performed well. With the artificially contaminated samples, IMS enrichment yielded some enhancement of the sensitivity, especially with serogroup O26.
This method could be useful when studying HUS patients, presenting several days (generally one week) after the start of the acute diarrhoea, when concentrations of STEC have dropped.

3.4. Task 4. Validation of the STEC isolation studies

3.4.1. In-house validation of STEC isolation method

- Partners:
  - Ghent University: culture-based isolation of STEC in food samples
  - ILVO: mPCR screening of the food samples

- Objective:

The STEC isolation protocol developed in a previous SPSD II project (Possé et al., 2008a) is intended to be applicable for different types of food matrices and for samples from the primary

production stage. Therefore the protocol was firstly be validated in-house followed by a validation by an interlaboratory study

- Methods and Materials:

All samples used for this validation were artificially contaminated. Ten samples of minced beef, cheese from raw milk and sprouted seeds were artificially inoculated with varying numbers (10-2000 cfu/25g) of non-stressed and stressed strains belonging to the serotypes O157 (s-) and (s+), O26, O103, O111 and O145. Cultured STEC strains were cold and freeze stressed by storing them for at least 5 days at respectively 2°C and -18°C. Inoculated samples were enriched in a weak selective medium for 6 hours followed by enrichment in a stronger selective medium for 18 hours. Direct plating on a selective medium (B1) was performed after each enrichment step. In a third pathway, an IMS (Dynal or Captivate) step was performed after 24h enrichment and prior to plating. Suspected colonies on B1 were purified and tentatively confirmed on a purification medium (B2) followed by a confirmation by a serotype PCR. Parallel to the classical isolation method, the mPCR screening test has been performed on the enrichment medium (after 24 hours enrichment). DNA is extracted from the enrichment medium using a method described by Yu and Morrison, 2004 (method 3bis, in task 3.2.2).

- Results:

Results of the isolation of STEC using the plating method are listed in **Table 7.**

Table 7 Isolation efficiency of STEC O157 (s-) and (s+), O26, O103, O111 and O145 in minced beef (A), cheese from raw milk (B) and sprouted seeds (C) using a protocol with 6 or 24 hours of selective enrichment followed by direct plating or 24h of selective enrichment followed by IMS (Dynal or Captivate) prior to plating.IMS kits implemented: Dynal (O157 (s-) and (s+), O26, O103), Captivate (O111, O145).

A.

Minced beef	Non-stressed cells 10-30 cfu/25g			Stressed cells (2°C 7 days) 10-30 cfu/25g			Stressed cells (-18°C 14 days) 10-60 cfu/25g		
Enrich-ment	6h	24h	24h	6h	24h	24h	6h	24h	24h
Plating	Direct plating	Direct plating	IMS + plating	Direct plating	Direct plating	IMS + plating	Direct plating	Direct plating	IMS + plating
O157 (s-)	2/5*	6/10	7/10	1/9	4/10	9/10	0/10	2/10	6/10
O157 (s+)	2/10	2/10	7/10	2/10	2/10	5/10	0/10	0/10	0/10
O26	3/5	10/10	9/10	1/9	10/10	10/10	0/10	5/10	6/10
O103	4/5	10/10	9/10	0/9	10/10	10/10	0/10	9/10	10/10
O111	2/10	3/10	6/10	0/10	3/10	3/10	0/10	5/10	7/10
O145	2/5	10/10	9/10	0/9	9/10	9/10	0/10	7/10	9/10

B.

Cheese raw milk	Non-stressed cells 10-30 cfu/25g			Stressed cells (2°C 7 days) 10-30 cfu/25g		
Enrich-ment	6h	24h	24h	6h	24h	24h
Plating	Direct plating	Direct plating	IMS + plating	Direct plating	Direct plating	IMS + plating
O157 (s-)	0/10	9/10	10/10	1/10	10/10	10/10
O157 (s+)	0/10	1/10	4/10	0/10	2/10	4/10
O26	8/10	10/10	10/10	6/10	10/10	10/10
O103	6/10	9/10	10/10	4/10	10/10	10/10
O111	3/10	10/10	10/10	2/10	10/10	10/10
O145	3/10	10/10	10/10	6/10	10/10	10/10

C.

Sprouted seeds	Non-stressed cells 10-30 cfu/25g			Non-stressed cells 600-2000 cfu/25g			Stressed cells (2°C 7 days) 10-30 cfu/25g		
Enrich-ment	6h	24h	24h	6h	24h	24h	6h	24h	24h
Plating	Direct plating	Direct plating	IMS + plating	Direct plating	Direct plating	IMS + plating	Direct plating	Direct plating	IMS + plating
O157 (s-)	0/10	0/10	0/10	0/5	0/5	1/5	0/10	0/10	1/10
O157 (s+)	1/10	0/10	0/10	0/5	0/5	0/5	0/10	0/10	0/10
O26	0/10	0/10	0/10	2/5	1/5	1/5	0/10	0/10	0/10
O103	0/10	0/10	0/10	1/5	1/5	2/5	0/10	0/10	0/10
O111	0/10	0/10	0/10	0/5	0/5	0/5	0/10	0/10	0/10
O145	0/10	0/10	0/10	0/5	0/5	0/5	0/10	0/10	0/10

*x/y: x=number of positive experiments; y= total performed experiments

In general, for minced beef samples inoculated with non-stressed and cold stressed cells, serotypes O26, O103 and O145 were very well isolated using direct plating after 24h, O157 (s-) was efficiently isolated using IMS, and detection rates of O157 (s+) also increased using IMS. Non-stressed O111 was isolated insufficient by direct plating and detection rates increased using IMS. Recovery rate remained low for cold stressed O111 cells, even when using IMS. For freeze stressed cells, detection rates were low for each serotype and for all tested methods in a first inoculation experiment (results not shown), but this could be due to the low inoculation level (1-5 cfu/25g). Inoculating minced beef samples with a higher number of freeze stressed cells resulted in high detection rates for O103, O26, O111, O145 and O157(s-). Recovery rates for O103 using direct plating or IMS followed by direct plating after 24h were very high. For O111, O145, O26 and O157 (s-) recovery rates increased using IMS followed by plating after 24h. For O157 (s+) higher inoculation did not result in a satisfying detection with any method tested.
In concrete, for minced beef samples inoculated with non-stressed or cold stressed cells (10-30 cfu/25g), detection rates of the serotypes O26, O103 and O145 were high to maximal (90-100%) using the protocol of 24 h enrichment followed by direct plating or IMS followed by plating resulting in a reliable (≥75%) detection of 10-30 cfu/25 g. Detection rates of these serotypes were lower when 6h enrichment was followed by direct plating. For the serotype O157 (s-) detection rates increased using IMS after 24h of enrichment from 40-60% to 70-90% for cold stressed and non-stressed cells. Detection rates were lower when 6h enrichment was followed by direct plating. The implementation of IMS for the isolation of STEC O157 (s+) resulted in the detection rates to increase from 20% to 50-70% for non-stressed and cold-stressed cells. Using direct plating after 6h of enrichment STEC O157 (s+) was detected in only 20% of the samples. Non-stressed and cold stressed STEC O111 was isolated from 0-30% of the samples using direct plating (after 6h and 24h of enrichment) and 30-60% of the samples using IMS followed by plating. In conclusion, for the serotypes O157 (s+) and O111 10-30 cfu/25g were not reliably detected. For freeze stressed cells, detection rates did not reach 50% for O157(s+) using any method. For all serotypes detection rates were 0% using direct plating after 6h enrichment. Detection rates for freeze stressed O103 and O145 were high to maximal (70-100%) using direct plating or IMS followed by direct plating after 24h and a detection limit of 10-30 cfu/25g was reached. For O157(s-), O26 and O111 detection rates were 20-50% using direct plating and 60-70% using IMS. A detection limit of 10-30 cfu/25g was not reached for O157(s-), O26 and O111 (**Table 7A**).

For cheese from raw milk inoculated with non-stressed and cold stressed cells, detection rates of the serotypes O157 (s-), O26, O103, O111 and O145 were high to maximal (90-100%) using the protocol of 24 h of enrichment followed by direct plating or IMS followed by plating resulting

in a reliable detection of 10-30 cfu/25g. For samples inoculated with non-stressed and cold stressed STEC O157 (s+) cells, detection rates were below 50% (10-40%) using the protocol of 24h enrichment followed by direct plating or IMS followed by plating. Using the protocol with 6h enrichment, non-stressed and cold stressed STEC cells were detected in 0-10% of the samples for O157 (s-) and (s+), 60-80% for O26, 40-60% for O103, and 20-30% for O111 and 30-60% for O145 (**Table 7B**).

For sprouted seeds detection rates of STEC were low for all serotypes and all methods. Only STEC O157 (s-) and (s+), O26 and O103 could be detected (10-40%). A detection limit of 10-30 cfu/25g sprouted seeds was reached for none of the serotypes. Experiments to determine the detection limit by inoculating non-stressed cells up to 2000 cfu/25g were carried out in a second stage. Detection rates of STEC again stayed low for all serotypes and methods. Only 40% of O103 and 20% of O26 and O157 (s-) could be detected. Detection limit for all serotypes must be >2000 cfu/25g (**Table 7C**).

Parallel to the classic isolation method an mPCR (Task 3.2.2) was performed on the 24h enrichment media. **Table 8** presents the expected amplification products for the different strains used in the inoculation experiments. The mPCR reaction mix includes 9 primerpairs; 5 serotype specific (O111, O145, O103, O26 & O157) and 4 virulence gene specific (eae, ehx, VT1 & VT2). The mPCR reaction amplifies 4 sequences in every strain, one serotype specific and three virulence specific sequences.

Table 8 List of possible mPCR amplification products for the different strains used for inoculation and the respective lengths of these fragments.

strain	serotype	O26	ehx	VT2	VT1	O145	eae	O111	O103	O157
PH4	O103	-	+	-	+	-	+	-	+	-
PH25	O26	+	+	-	+	-	+	-	-	-
PH27	O145	-	+	+	-	+	+	-	-	-
PH29	O111	-	+	-	+	-	+	+	-	-
PH59	O157 s-	-	+	+	-	-	+	-	-	+
PH76	O157 s+	-	+	+	-	-	+	-	-	+
bp		492	442	399	352	303	264	214	160	101

During the implementation of the mPCR on the DNA extracted from the enrichment media some samples had a shortage of amplification products. It differed between samples from which fragments did not become amplified. Most samples also showed aspecific amplification. All aspecific fragments were of bigger size than the fragments of interest, implicating no

interference between aspecific amplification fragments and fragments necessary for identification of the strains. (**Figure.14**)

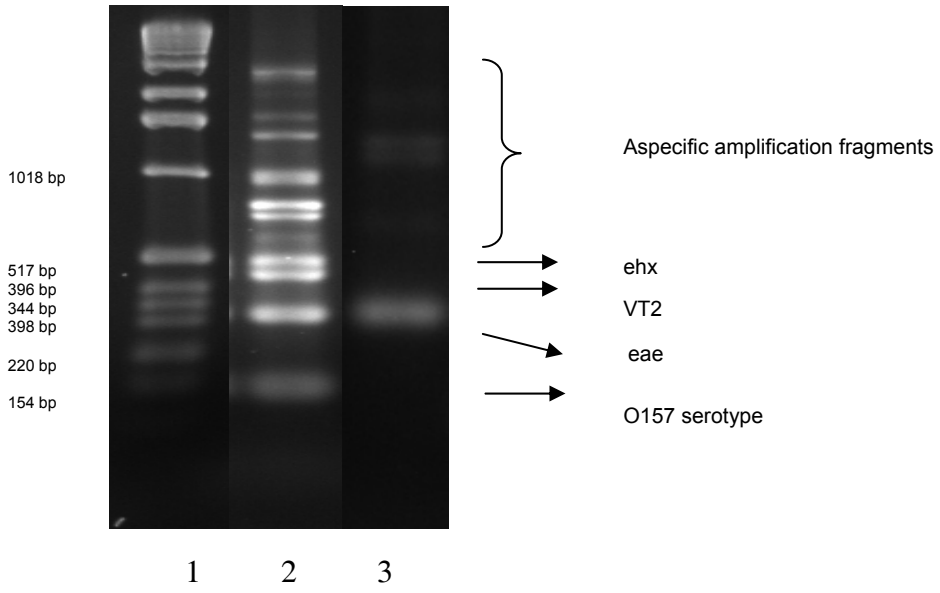

Figure 14 Representation of 9-mPCR on DNA extracted from raw milk cheese artificially contaminated with *E. coli* O157 (1 kb DNA Molecular Weight Marker: lane 1. Cheese from raw milk sample inoculated with STEC O157 showing *ehx, eae, VT2* and O157 serotype and aspecific amplification: lane 2. Cheese from raw milk sample inoculated with STEC O157 showing only *eae* amplification).

Results of the detection of STEC using the small mPCR on DNA extracted from enrichment media are shown in **Table 9** for minced beef. A distinction has been made between samples for which all target genes were amplified (4 / 4) and samples with a shortage of amplified genes (1 to 3 / 4).

Table 9 Efficiency for detecting STEC O157 (s-) and (s+), O26, O103, O111 and O145 under different conditions in minced beef using mPCR. (A) Results with 4 out of 4 expected fragments amplified. (B) Results with 1 to 3 out of 4 expected fragments amplified. (C) Results showing amplification of 1 or more fragments: (C) = (A) + (B).

A.

Minced beef		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g	Stressed cells (-18°C 14 days) 10-60 cfu/25g
mPCR	O103	9/10*	8/10	3/10
	O26	9/10	9/10	2/10
4/4	O145	8/10	7/10	1/10
Amplification	O111	0/10	0/10	1/10
products	O157(s-)	8/10	9/10	3/10
	O157(s+)	5/10	6/10	1/10

B.

Minced Beef		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g	Stressed cells (-18°C 14 days) 10-60 cfu/25g
mPCR	O103	1/10*	1/10	2/10
	O26	0/10	0/10	1/10
1 – 3 / 4	O145	0/10	2/10	2/10
Amplification	O111	2/10	0/10	1/10
products	O157(s-)	2/10	0/10	0/10
	O157(s+)	0/10	3/10	1/10

C.

Minced Beef		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g	Stressed cells (-18°C 14 days) 10-60 cfu/25g
mPCR	O103	10/10*	9/10	5/10
	O26	9/10	9/10	3/10
1 – 4 / 4	O145	8/10	9/10	3/10
Amplification	O111	2/10	0/10	2/10
products	O157(s-)	10/10	9/10	3/10
	O157(s+)	5/10	9/10	2/10

*x/y: x=number of positive experiments; y= total performed experiments

For all serotypes except O111 the number of samples where all expected amplification fragments are detected is higher than the amount of samples where detection of 1 to 3 instead of 4 amplification fragments occurs (**Table 9 A & B**). Results of mPCR on DNA of non-stressed and cold stressed cells (2°C 7 days) show good recovery rates for O103, O26, O145 and O157 (s-). 80 to 100% of the samples inoculated with the four mentioned serotypes showed at least one of the expected amplification products and 10-30 cfu/25g was reliably detected (**Table 9C**). The mPCR detection rate for O111 under non-stressed and both stressed conditions, in contrast, does not exceed 20% and in none of the samples mPCR fragments were detected (**Table 9A,B & C**). mPCR detection rates of cold stressed O157 (s+) increased compared to non stressed O157 (s+).With the classical isolation protocol (**Table 6**) this phenomenon was not found. 90% of the samples inoculated with the cold stressed O157 (s+) cells showed 1 or more mPCR fragments after separation on gel, compared to only 50% of the samples inoculated with non-stressed O157(s+) cells. The inoculation with 10-30 cfu/25g was only detected reliable using cold stressed cells (**Table 9C**). Detection rates of all serotypes stressed at -18°C for 14

days were low. In only 20 to 50% of the samples amplification fragments were detected, which implicates that detection limit is >10-60 cfu/25g (**Table 9C**).

Table 10 Comparison of detection with mPCR and the classic isolation method of STEC O157 (S-) and (S+), O26, O103, O111 and O145 under different conditions in minced beef. Samples are considered positive if at least one of the performed methods (plating after 6h, plating after 24h or IMS followed by plating after 24h) detected the inoculated strain.

Minced beef	Non-stressed cells 10-30 cfu/25g		Stressed cells (2°C 7 days) 10-30 cfu/25g		Stressed cells (-18°C 14 days) 10-60 cfu/25g	
	mPCR	plating	mPCR	plating	mPCR	plating
O103	10/10*	10/10	9/10	10/10	5/10	10/10
O26	9/10	10/10	9/10	10/10	3/10	6/10
O145	8/10	10/10	9/10	9/10	3/10	9/10
O111	2/10	6/10	0/10	4/10	2/10	7/10
O157(s-)	10/10	9/10	9/10	10/10	3/10	6/10
O157(s+)	5/10	6/10	9/10	7/10	2/10	0/10

*x/y: x=number of positive experiments; y= total performed experiments

The results in **Table 10** shows that efficiency of detecting non-stressed and cold stressed O103, O26, O145 and O157(S-) with mPCR or plating is comparable for both methods (around 80-100%). Detection rates for non-stressed O111 is respectively 60% with plating and only 20% with mPCR. For cold stressed O111 no STEC cells are detected when mPCR is applied and only 40% using plating. STEC O157(s+) has a higher detection rate using mPCR when cells are cold stressed and freeze stressed, but lower when non-stressed. For all freeze stressed STEC serotypes, exceptO157 (s+), detection is better using the classic plating method.

Results of the detection of STEC in raw milk cheese using the small mPCR on DNA extracted from the enrichment media are shown in **Table 11**. As for mPCR on DNA from minced beef enrichment media a distinction has been made between samples for which all target genes were amplified (4 / 4) and samples which showed amplification of less fragments than expected (1 to 3 / 4).

Table 11. mPCR efficiency for detecting STEC O157 (s-) and (s+), O26, O103, O111 and O145 under different conditions in raw milk cheese. (A) Results with all expected fragments amplified by mPCR. (B) Results with 1 to 3 out of 4 expected fragment amplified by mPCR. (C) Results showing amplification of 1 or more fragments: (C) = (A) + (B).

A.

Cheese raw milk		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g
mPCR	O103	7/10*	10/10
	O26	9/10	9/10
4/4	O145	1/10	0/10
Amplification	O111	3/10	5/10
Products	O157s-	8/10	10/10
	O157s+	1/10	1/10

B.

Cheese raw milk		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g
mPCR	O103	1/10*	0/10
	O26	0/10	0/10
1 - 3 / 4	O145	7/10	10/10
Amplification	O111	4/10	2/10
Products	O157s-	0/10	0/10
	O157s+	0/10	0/10

C.

Cheese raw milk		Non-stressed cells 10-30 cfu/25g	Stressed cells (2°C 7 days) 10-30 cfu/25g
mPCR	O103	8/10*	10/10
	O26	9/10	9/10
1 - 4 / 4	O145	8/10	10/10
Amplification	O111	7/10	7/10
Products	O157s-	8/10	10/10
	O157s+	1/10	1/10

*x/y: x=number of positive experiments; y= total performed experiments

In most raw milk cheese samples all expected amplification fragments are detected on an electrophoresis gel following mPCR, except for samples inoculated with STEC O145. This is mostly due to non amplification of the *ehx* fragment of the STEC O145 strain, in which case only 3 out of 4 amplification products are visualized on gel. For STEC O111 the amount of samples showing 1-3 or exactly 4 amplification products is almost the same (**Table 11 A & B**).

For serotypes O26, O103, O145 and O157 (s-) good recovery rates (at least 1 out of 4 fragments amplified) for non-stressed and stressed cells are acquired ranging from 80-90% and 90-100% respectively. For these serotypes under both conditions 10-30 cfu/25g is detected reliably. O111 has a recovery rate of 70% under both conditions whereas recovery rates of non- and cold stressed O157 (s+) do not exceed 10%. As a result 10-30 cfu/25g is not not reliably detected for both serotypes (**Table 11 C**).

Table 12 Comparison of detection with mPCR and classic isolation method of STEC O157 (s-) and (s+), O26, O103, O111 and O145 under different conditions in raw milk cheese. Samples are considered positive if at least one of the performed methods (plating after 6h, plating after 24h or IMS followed by plating after 24h) detected the inoculated strain.

Cheese raw milk	Non-stressed cells 10-30 cfu/25g		Stressed cells (2°C 7 days) 10-30 cfu/25g	
	mPCR	plating	mPCR	plating
O103	8/10*	10/10	10/10	10/10
O26	9/10	10/10	9/10	10/10
O145	8/10	10/10	10/10	10/10
O111	7/10	10/10	7/10	10/10
O157(s-)	8/10	10/10	10/10	10/10
O157(s+)	1/10	4/10	1/10	4/10

*x/y: x=number of positive experiments; y= total performed experiments

Comparing the efficiency of the mPCR or the classic plating method for detecting STEC serotypes in a raw milk cheese under non-stressed conditions it's clear that plating method has slightly better results, as for serotypes O103, O26, O145, O111 and O157 (s-) recovery using the classical plating method is 100%, whereas recovery using mPCR is respectively 80%, 90%, 80%, 70% and 80% for the previously mentioned serotypes. Only for STEC O157 (s+) recovery is low using both methods: 10% using mPCR and 40% using plating.
For stressed STEC cells the difference in recovery is small. For 70% of samples inoculated with O111 mPCR detects 1 or more of the 4 possible STEC genes, whereas detection is 100% when using plates. O26 cells are detected in 9 and 10 samples by mPCR and plating method respectively, while for O157 (s+) both methods are not able to detect STEC cells in more than 4 out of 10 (**Table 12**).

mPCR was also conducted on DNA from a sprouted seeds enrichment matrix inoculated with the STEC serotypes. mPCR was only performed on DNA originating from samples which already tested positive using direct plating or IMS following direct plating, both performed after 24h of enrichment. None of these samples showed amplification of any of the target

sequences. Detection rates were 0% and the detection limit is above 600-2000 cfu/25g for all serotypes.

- Conclusions:

Minced beef:

- Plating method:

Detection of non-stressed or cold stressed O26, O103 and O145, inoculated at a level of 10-30 cfu/25g, is around 90-100% with or without the use of IMS. Implementation of IMS for detection of non-stressed O111 as well as for non-stressed and cold stressed sorbitol fermenting and sorbitol non-fermenting O157 results in a better recovery rate. The recovery rate for cold stressed O111 is low with and without use of IMS.
Except for O103 which is isolated from 90% (without IMS) -100% (with IMS) of all samples, detection rates are generally lower for freeze stressed serotypes (10-60 cfu/25g): 0% for O157 (s+), 50% (direct) -60% (IMS) for O26, 70% (direct) -90% (IMS) for O145 and 50% (direct) to 70% (IMS) for O111. Using IMS on freeze stressed O157 (s-) increases the amount of positive samples from 20% to 60%.

- mPCR on enriched samples:

Detection of 1 or more amplified fragments for non-stressed or cold stressed O145, O103, O26 and O157 (s-) ranges from 80% to 100%. O111 is detected in a limited amount of samples and in none of the O111 containing samples all amplification fragments were detected.
Detection of the expected mPCR targets is very low when screening freeze stressed STEC cells.

- mPCR vs plating method:

The efficiency of detecting non-stressed and cold stressed O103, O26, O145 and O157 (s-) with mPCR or plating is comparable for both methods (around 80-100%). When comparing plating method and mPCR for O111, the number of positive samples found is higher when employing the classic plating method. The same conclusion applies to the detection of freeze

stressed serotypes except for O157 (s+). Detection rates of this serotype under freezing conditions are very low for both methods.

Raw milk cheese:

- Plating method:

For all serotypes except O157 (S+) detection rates approach 100%. This conclusion only applies to non- and cold stressed serotypes. The number of O157 (S+) inoculated samples found positive with or without using IMS is lower than 50%.

- mPCR on enriched samples:

For all serotypes except O157 (s+) detection rates ranged from 70% to 100%. Almost no samples which were inoculated with O157 (s+) STEC cells, tested positive when subjected to mPCR. Samples inoculated with freeze stressed STEC cells were not subjected to a mPCR test because of aforementioned reasons.

- mPCR vs plating method:

For the serotypes O26, O103, O111, O145 and O157 (s-), it was clear that the plating method gave as well for non-stressed as for cold stressed conditions a slightly better result (100%) compared to the mPCR method (70-100%). Detection of O157 (s+) in raw milk cheese samples was low (<50%) for mPCR and plating method, with the use of the classical isolation again resulting in a higher detection.

Sprouted seeds:

As a result of a high number of background organisms, the total amount of samples which tested positive for any of the serotypes under different testing conditions using any of the possible plating methods was extremely low. Using the mPCR method on the samples positive on plate, no positive results were found.

3.4.2. Validation of STEC isolation method using interlaboratory study

- Partners:
  - University of Liège
  - Ghent University

- Objective:

In the interlaboratory study 12 collaborative laboratories (national and international) participated. In order to be a candidate the laboratory must have at least experience in the isolation of *E. coli* O157.

The following Belgian laboratories were involved:

- the laboratories of the partners (except VIB) of the project,
- WIV (food microbiological laboratory) (K. Dierick)
- University of Ghent (Laboratory of Food Microbiology and Preservation) (M. Uyttendaele)

Also laboratories from other European countries participated such as :

- Food and Consumer Product Safety Authority, Zutphen, The Netherlands (A. Heuvelink)
- AFSAA-LERQAP, Maisons-Alfort, France (P. Fach)
- ADRIA development, France (M. Ranou)
- Ecole Vétérinaire de Lyon, France (C. Rozand)
- Danish Institute for Food and Veterinary Research, Copenhagen, Denmark (M. Jeppe Boel)
- National Veterinary Institute, Oslo, Norway (M. Cudjoe)

Since STEC are classified as risk class 3 organisms (but with a limited risk for workers since they are not normally infectious by the airborne route) (www.biosafety.be), transportation of these strains required specific precautions, which are only available by some delivery services. An import permit was required for the international shipment of Category A substances as defined by European legislation UN2814 and packing instructions 602 and 650 were to be respected at all times; for further details http://unece.org/trans/danger/publi/adr/adr_e.html. Shipping samples, strains and required products as category A products resulted in a higher cost per package compared to standard delivery.

The collaborative study was divided in 2 parts, namely a pre-trial and the ring-trial.

1/ Pre-trial experiment

A standard operating procedure for the isolation of the target organisms O26, O103, O111 and O145 from minced beef was developed by partner 5 in cooperation with the coordinator and partner 2. The following items were sent to all participating laboratories: the standard operating procedure for the isolation of the target organisms, the necessary products to prepare the media and the strains which will be used in the interlaboratory study. These shipments were organized by partner 5 in cooperation with the coordinator.

The pre-trial gave the collaborative laboratories the possibility to become familiar with the isolation method to be used and especially with the colony aspect of the different serotypes on the different selective agar plates.

2/ Interlaboratory study

- Methods and Materials:

Based on the comments received from the laboratories during the pre-trial the protocol was adapted by partner 5 in order to avoid misunderstandings during the ring-trial.

Prior to the ring-trial, the products necessary to prepare all culture media (IHP) and already prepared selective agar culture media (RTU) were sent to the participating laboratories. These last media were also sent in order to evaluate the effect of the preparation of the culture media on the obtained final results.

Also a questionnaire and a document to report the results were developed by partner 5. As food matrix minced beef was used. For the preparation of all samples a large batch of minced beef was purchased from a local butcher shop.

For each participating laboratory, 20 samples of 25g of minced beef were prepared in stomacher bags :

- 1 sample for the measurement of the temperature at arrival in the laboratory (sample T)
- 1 sample for the enumeration of the total count, *Enterobacteriaceae* and *E. coli* (sample C)
- 2 blank samples
- 16 inoculated samples: 4 serotypes at 2 levels of contamination in duplicate (30 cfu/g and 300 cfu/g)

For the inoculation all strains were cold stressed as mentioned in the in-house validation study.

All samples were prepared the day of the shipment (17th November 2008). The samples were packaged in such a way that the temperature in the package would not exceed 7°C during the foreseen transportation time of 1 day. The shipment included also the questionnaire and the document for reporting the results, the RTU media and products necessary to prepare some culture media. The laboratories had to measure the temperature of the extra sample (T) included in the package and to record this temperature on the questionnaire. All laboratories were asked to analyse the samples on a prefixed day (19th November 2008). In the laboratories the analysis started respectively 1 and 2 days after the foreseen starting date since the transport time to these laboratories took more time than foreseen. The analysis had to be carried out according to the standard operating procedure. For selective plating ready-to-use (RTU) media (obligatory) and on laboratory-made media (in-house-preparation: IHP, voluntary) had to be used. Deviations and special observations during analysis were noted on the questionnaire. All results were noted on the document for reporting the results.

All laboratories had to send their results and the questionnaire within a fixed period to partner 5 (before the 12th December 2009).

This partner evaluated their own results and those from the collaborating laboratories based on the recommendations of ISO 16140. A test report was send to each laboratory by the end of February 2009.

- Results:
  - There was no difference in results between RTU and IHP media.
  - The arabinose test seems to be difficult to read. Therefore only the dulcitol test is proposed to be used in the confirmation test for the differentiation of strains belonging to the serotypes O103 and O111.
  - When the wrong inoculation of some samples (4) or the lack of inoculation for one sample are not taken into account, all isolated strains corresponded to the STEC strains inoculated to the samplesamples were correctly

The incorrect inoculation of two samples with the wrong serotype was proven by performing PFGE on the isolated strains and the strains used for inoculation. Identical fingerprints confirmed that wrong strains were used for inoculation.

- Conclusions:

In conclusion, the laboratory performance was in general satisfactory

3.5. Task 5 Validation by workshops, meetings and publications

For the participating laboratories of the ring-trial, a workshop was organized the 17th of September 2008. During the workshop the developed classical method was presented, including a practical demonstration. A second workshop was organized the 20th of April 2009. The participating laboratories of the ring-trial, stakeholders and government were invited. During the workshop guest speakers cited the STEC problems, and results on the STECTRACK project and the ring-trial were presented.

Four meetings of the follow-up committee were organized in which the members could contribute to the research project. Interesting discussions were held and recommendations were formulated.

Publications are listed in chapter 6.

4. Adjustments made to original project planning

Schedule of the initial planning (art. 3 from annex 1 of the contract) is used as a reference for the actual planning.

Task 1.	Months	Man-months year 1		Man-months year 2	
	Partner	1 to 6	7 to 12	13 to 18	19 to 24
Task 1.1: cattle feces	C	6	1		
Task 1.2: simplification	C		2		

The optimization of the STEC isolation protocol for cattle feces took 12 man-months instead of 7 man-months in year 1 (Task 1: C). Since the mPCR for the screening of enrichment cultures was not available at the moment that these experiments were performed this screening was not carried out. In addition to what was described in the technical description of the project extra IMS experiments were carried out using coated beads from a different supplier. These additional experiments were caused by the fact that using IMS beads from a first supplier did not give satisfying results. Also recovery experiments with both types of beads (Dynal and Captivate) were done. A publication on the results was prepared in year 2.

Task 2.	Months	Man-months year 1		Man-months year 2	
	Partners	1 to 6	7 to 12	13 to 18	19 to 24
Task 2.1: full scale PCR	P2		4		
	P3		5		
Task 2.2: small scale PCR	P2	6			

A collection of 334 human and animal STEC strains from the Belgian STEC Reference Center (UZ) was delivered to ILVO and DNA was extracted (Task 2.1: P2). PFGE was carried out on the collection (Task 2.1: P2). During the first year the VIB designed a full scale and a small scale PCR (Task 2.1: P3). The ILVO designed a sample screening test using the small scale PCR (Task 2.2: P2). Typing results obtained by PFGE and the full scale PCR were linked in year 2.

Task 3.	Months	Man-months year 1		Man-months year 2	
	Partner	1 to 6	7 to 12	13 to 18	19 to 24
Task 3: Evaluation	P4			6.3	

The UZ started experiments to optimize the STEC isolation protocol for human feces in year 2.

Task 4.	Months	Man-months year 1		Man-months year 2	
	Partners	1 to 6	7 to 12	13 to 18	19 to 24
Task 4.1: In house	C		2	6	
	P2			6	2
Task 4.2: interlaboratory study	C				4
	P2				1
	P4				1
	P5			1.3	6

The in-house validation of the STEC isolation protocol started for both partners at the beginning of year 2. The classical plating method (Task 4.1: C) was performed in parallel to the sample mPCR screening test (Task 4.1: P2) for STEC detection. Meetings for the organization of the ring trial were held at the University of Ghent in May and June 2008 (Task 4.2). In August the University of Liège contacted the participating laboratories. A first workshop for the participating laboratories was organized in September 2008, to present and demonstrate the developed method. The ring-trial was organized in week 47 of 2008. Results were gathered and analyzed by the University of Liège University and made a report of the ring-trial in collaboration with the coordinator.

Task 5.		Man-months year 1		Man-months year 2	
	Months	1 to 6	7 to 12	13 to 18	19 to 24
Task 5.1: Follow-up meetings	C		1		1
	P2		1		1
	P3		0,1		0,1
	P4		0,1		0,1
	P5		0,1		0,1
Task 5.2: Workshop/reports/publications	C				1
	P2		1		2
	P3				0,1
	P4				0,1
	P5				0,1

In April 2009, a final workshop was organized, for the participating laboratories, stakeholders and government, to present the results of the STECTRACK project with special focus on the results of the ring-trial.

Follow-up meetings have been held the 22nd of May 2007, the 23rd of January 2008, the 8th of October 2008 and the 20th of April 2009.

A first publication on the evaluation of IMS and optimization of the isolation protocol for cattle feces, was prepared in year 2. (Verstraete et al., submitted). A second publication on the interlaboratory study to validate the isolation method for STEC in food, is in preparation. (Verstraete et al., in preparation)

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6. Publications

A publication "Effect of immunomagnetic separation on the detection of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O145 and sorbitol positive O157 from artificially inoculated cattle faeces" is prepared and submitted. (Verstraete et al., submitted).
A publication "Validation of the isolation method of Shiga toxin-producing *Escherichia coli* O26, O103, O111, O145 from artificially inoculated minced beef in an interlaboratory study" is in preparation. (Verstraete et al., in preparation)

Other publications were an article in a newsletter on dairy food and poster presentations on congresses.

VERSTRAETE, K., DE REU, K. "Kinderen ziek na eten hoeve-ijsje besmet met STEC" Nieuwsbrief TAD Zuivel – ILVO-T&V, jg. 3, nr. 1, februari 2008, p.5.

VERSTRAETE, K., DE ZUTTER, L., HERMAN, L., HEYNDRICKX, M., DE REU, K. "Optimization of a detection and isolation method for VTEC O26, O103, O111, O145 and sorbitol positive O157 in cattle faeces."
Poster with abstract on PEN congress about "*E. coli*: Pathogenicity, Virulence and Emerging Pathogenic Strains", Rome, Italy, 5th –8th of March 2008, poster abstracts p. 35.

VERSTRAETE, K., DE ZUTTER, L., HERMAN, L., HEYNDRICKX, M., DE REU, K.
"Optimization of a detection and isolation method for VTEC O26, O103, O111, O145 and sorbitol positive O157 in cattle faeces."
Poster with abstract on Thirteenth Conference on Food Microbiology, Ghent University, 11 – 12 September 2008, Conference Book p 116.

VERSTRAETE, K., DE ZUTTER, L., HERMAN, L., HEYNDRICKX, M., DE REU, K.
"Optimization of a detection and isolation method for VTEC O26, O103, O111, O145 and sorbitol positive O157 in cattle faeces." Poster and abstract on 21st International ICFMH Symposium - Food Micro 2008, Aberdeen, United Kingdom, 1 – 4 September 2008, Abstract book, p 377.

VERSTRAETE, K., ROBYN, J., DE ZUTTER, L., HERMAN, L., HEYNDRICKX, M., DE REU K. "Validation of a detection and isolation method for shiga toxin-producing Escherichia coli O26, O103, O111, O145 and sorbitol positive O157 in food."

Poster met abstract op "Fourteenth Conference on Food Microbiology", Liège University, 18 - 19 juni 2009, Proceedings of the Fourteenth Conference on Food Microbiology p 126.

Verstraete K., Robyn J., De Zutter L., Herman L., Heyndrickx M., De Reu K. Validation of a detection and isolation method for shiga toxin-producing *Escherichia coli* O26, O103, O111, O145 and sorbitol positive O157 in food. 6 th Balkan Congress of Microbiology, Ohrid, Macedonia, 28-31 oktober 2009 – Book of abstracts, Session Molecular Methods. Abstract of poster 8.10P, p. 191.

7. Presentations / Lectures

An oral presentation was given by Prof. L. De Zutter on the technical workshop for the participating laboratories of the ring-trial, the 17th of September 2008.

Oral presentations on the STEC workshop in Brussels on 20th of April 2009.

K. Verstraete, Presentation of research results of the STECTRACK project:

- Evaluation of immunomagnetic separation (IMS) and the optimization of an isolation method for STEC in cattle faeces.
- Molecular characterization of a collection of human clinical and animal STEC strains by using 33-mPCR and PFGE

J. Robyn, Presentation of research results of the STECTRACK project:

- In-house validation of STEC isolation methods on food

B. Taminiau, Presentation of research results of the STECTRACK project:

- Validation of STEC isolation method using interlaboratory study

8. General conclusions

This study had the aim to investigate if 1/ the isolation method for STEC in cattle faeces could be optimized by using IMS, 2/ to implement multiplex PCR as a molecular typing tool and as a screening method, 3/ to evaluate different approaches of the STEC isolation method for STEC in human clinical samples, and 4/ to validate the detection and isolation method for STEC in food, in-house and using an international collaborative study.

In conclusion, IMS did not have the desired positive effect on the isolation efficiency of non-O157 serotypes from faeces as it had for serotype O157. Results on pure broth suspensions of STEC showed that IMS beads had only low affinities for non-O157 serotypes compared to O157. For further optimization of the method, alternative techniques to IMS should be looked for.

A 33-mPCR for strain characterization was designed and implemented on a STEC collection creating virulence profiles of all strains. Fingerprints of the strains were obtained using the Pulsenet Europe protocol for PFGE. Results of both genotyping techniques were combined giving insight in relatedness and virulence of strains. Results showed that strains were clustered according to their serotype and in a second level according to their virulence profile, particularly for the diverse *VT*-profiles of O157 strains. No correlation was denoted between the virulence profile or the PFGE clustering and the clinical manifestation. Case studies could be appointed based on the PFGE dendrograms. In general they contained clones that persisted during several years and that infected humans as well as animals.

A smaller multiplex PCR was developed and optimized for use in a STEC screening method for food and cattle faeces. Once the mPCR was developed and tested on pure strains, an evaluation on samples was performed. Methods were compared based on the ability to remove PCR inhibiting molecules and on the ability to isolate and purify DNA from STEC cells. The method described by Yu and Morrison (2004) employing a ribolyser gave the best results. A method based on the Qiagen Stool Kit, which was less sensitive, can also be used if no ribolyser is present. All virulence marker genes and the serotype gene could be detected in enriched minced beef and cheese from raw milk artificially inoculated when the method described by Yu and Morrison (2004) was used to purify DNA.

Clinical isolates of non-O157 STEC were tested by evaluating growth characteristics on the media. Results were generally as expected. However, some strains did not grow well or gave deviating morphology, so more standardization of the preparation and possible optimization of the medium is still needed.
Using artificially contaminated stool samples, the sensitivity of the STEC isolation and detection protocol developed in a previous Belspo SPSD II project was similar to the protocol used routinely in the UZ . Between 10^3 and 10^4 cfu/5g was reliable detected when no IMS was performed and a ten times lower detection was obtained by inclusion of IMS.
The method performed well on frozen samples naturally contaminated with STEC, but this could only be tested on 14 samples, of which 11 with O157, 2 with O111 and one O26.

The optimized STEC isolation protocol (Possé et al., 2008a) was intended to be applicable for different types of food matrices. Therefore the protocol was validated in-house. Ten samples of minced beef, raw milk cheese and sprouted seeds were artificially inoculated with varying numbers (10-2000 cfu/25g) of non-stressed and stressed (cold and freeze) strains belonging to the serotypes O157 (s- and s+), O26, O103, O111 and O145. These samples were tested with the tested STEC isolation protocol as well as with the derived 9-mPCR. Detection of non-stressed or cold stressed O103, O26, O145 and O157 (s-) in minced beef was high and comparable when using both methods. Detection of O111, O157 (s+) or freeze stressed STEC cells in the mentioned matrix was more difficult. When detecting non-stressed or cold stressed STEC in raw milk cheese with either of both methods, only detection of O157 (s+) was lower. Due to the high level of background flora, detection of any serotype in sprouted seeds was almost impossible.

In an interlaboratory study in which 12 laboratories (both national and international) participated the optimized STEC detection and isolation methods were validated for a selected food matrix. A pre-trial experiment preceded the actual interlaboratory study. In the interlaboratory study 20 samples of 25g of minced beef were sent to every laboratory. Sixteen samples were inoculated with single strains belonging to the 4 serotypes O26, O103, O111 and O145 at 2 levels of contamination in duplicate (30 cfu/g and 300 cfu/g). All strains were cold stressed. Results were evaluated based on the recommendations of ISO 16140. The laboratory performance was in general very satisfactory.

9. Recommendations in the frame of policy

A reliable and sensitive method to isolate non-O157 and O157 (s+) STEC from different matrices has become available. Using the method, STEC can be detected in a wide range of domains: in the primary production stage, the food chain and human clinical cases. Epidemiologic data, generated by prevalence studies, can be used for the monitoring of STEC and to formulate intervention measures. An international interlaboratory study also proved its wide applicability in different labs, and therefore it will be presented on international meetings (including ISO, CEN,...). Molecular methods are now available for detailed strain characterization as alternative to conventional methods.

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