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FINAL REPORT SUMMARY

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«STECTRACK»

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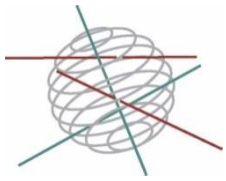

Agro-food

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"STECTRACK"

SD/AF/06

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K. Verstraete, K. De Reu, J. Robyn, L. Herman, M. Heyndrickx, J. Del-Favero, D. Piérard, G. Daube, L. De Zutter. **"Validation of methods for the detection of new emerging pathogenic *Escherichia coli* – *STECTRACK*".** Final Report Summary. Brussels : Belgian Science Policy 2009 –7 p. (Research Programme Science for a Sustainable Development)

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.