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Report of an inter-laboratory study on the detection of VTEC in seeds intended for sprout production

1. INTRODUCTION AND OBJECTIVES OF THE STUDY

Sprouts have been implicated as the source of the outbreak of VTEC O104:H4 infections occurring in Europe. Despite the strong epidemiological evidences on their role, the outbreak strain has never been isolated form neither the sprouts nor the seeds used for their production. This has stressed the importance of laboratory testing of seeds, which have represented the source of sprout contamination in most of the epidemic episodes described in the literature.

The tracing back of the different types of seeds used to generate the sprouts involved in the German outbreak and in the following French episode originated RASFFs involving different Member States. The NRLs of those MS had to perform the laboratory testing, and DG SANCO asked the EU Reference Laboratory for VTEC (EU-RL VTEC) to provide them technical assistance.

During the investigations on seed samples collected in Italy, the EU-RL VTEC detected contamination with VTEC other than O104 in some seed lots. Those samples were used to organize an inter-laboratory study on naturally contaminated seeds for the benefit of the NRLs of the MS most involved in the crisis.

Naturally contaminated seeds represent a valuable material for this type of studies, since artificially contaminated samples could not mimic what really happens in nature (e.g. possible internal contamination of seeds).

The objectives of the study were:

1. To better harmonize the laboratory procedures in place in the different laboratories.
2. To contribute to a better understanding of the nature of the natural contamination of seeds with VTEC.

2. DESIGN OF THE STUDY

During the laboratory analyses that followed the tracing back investigations on the seed lots possibly involved in the outbreak, the EU-RL VTEC analyzed 10 lots of beet seeds (50 g test samples) on 12 June 2011 and found that three of them were positive for *stx2* and *e-hly*, and negative for *stx1* and *eae* at the Real Time PCR screening. VTEC strains with the same genotype (and negative for enteroaggregative adhesion markers) were isolated from two of these 3 samples. The strains belonged to serogroup O74.

One of the 2 samples from which the VTEC strains had been isolated and two of the 7 negative samples were chosen for the inter-laboratory study. Fifty grams portions were taken from the total 500 g aliquot available at ISS and tested again on 5 July. The PCR screening confirmed the results of the first analysis and the samples were sent to the participating laboratories between 8 and 12 July.

Participants were asked to detect the *vtx1* and *vtx2* genes in the enrichment cultures and perform the isolation of the VTEC strain from the positive samples. Testing the presence of *eae* and *e-hly* genes was facultative.

The participants could use either the method released by the EU-RL VTEC or other methods in use their laboratories.

Table 1 shows the codes assigned to the samples sent to the participant laboratories.

Table 1. Random codes assigned to the samples for each laboratory.

ID Lab	Sample A	Sample B	Sample C
EC_1	208	506	371
EC_2	934	266	524
EC_3	654	720	245
EC_4	541	125	684
EC_5	335	651	512
EC_6	463	213	662
EC_7	771	346	852
EC_8	999	426	312

The Expected results of the real time PCR screening are reported in table 2.

Table 2. Expected results of the real time PCR screening for the seed samples used in the study

Sample A	Sample B	Sample B
vtx2+; vtx1-, ehly+, eae-	negative	negative

3. PARTICIPANTS

The NRLs of the EU Member States involved in the seed RASFFs (DE, FR, IT, NL, UK) participated in the study, together with laboratories particularly involved in seed testing and in the discussion developed at the EFSA level: the NRLs of Belgium and Denmark and the ANSES. The 8 participating laboratories are listed below:

- Belgium - Institute of Public Health, National Reference Laboratory in food microbiology
- Denmark - National Food Institute, Technical University of Denmark
- France - VetAgro Sup Campus Vétérinaire de Lyon
- France - ANSES - Laboratoire de Securite des Aliments, Pole HQSA, Maisons-Alfort
- Germany - Federal Institute for Risk Assessment (BfR)
- Italy - Istituto Superiore di Sanità
- Netherlands – RIVM, Bilthoven
- UK - Health Protection Agency

3. RESULTS

All the eight participants used the method released by the EU-RL VTEC with some of them adopting minor adjustments. The results of the Real time PCR screening performed on enrichment cultures are summarized in table 3.

Table 3. Cumulative results of the Real Time PCR screening performed on enrichment cultures

Sample *(expected results)*	N. of positive results / N. of tests, for the following genes:			
	vtx1	**vtx2**	***Eae***	***E-hly***
Sample A *(vtx2+, e-hly+)*	**0/8**	**0/8**	**2/6**	**0/4**
Sample B *(negative)*	**0/8**	**1/8**	**0/6**	**0/4**
Sample C/negative	**0/8**	**0/8**	**0/6**	**0/4**

The isolation of *E. coli* strains responsible for the PCR positive tests was obtained for the two *eae*-positive samples but not for the only *vtx2*-positive sample.

4. DISCUSSION

All the laboratories returned results that did not match those expected on the basis of the previous tests performed on the samples at the EU-RL.

However, this lack of correspondence is not completely surprising.

As a matter of fact, seed samples are constituted by particles, which may be singularly contaminated and non-homogeneously distributed within the lots. Moreover, according to the few data available in the literature, the contamination of seeds frequently occurs at very low levels, in terms of cfu/gr.

The results obtained could therefore reflect a non-homogeneous, low-level contamination of the lots from which the test samples were taken.

This hypothesis is supported by the results of the tests performed at ISS. A total of three portions of the seed lots used in the study have been tested at ISS in two different dates, before sending the samples and during the study itself, with the results shown in table 4.

Table 4. Real Time PCR results obtained at ISS by repeating the analyses on 3 seed portions of the lots used in the study.

Sample	Positivity to Real time PCR (Ct value), by date of testing											
	1st test - 12 June 2011				2nd test - 5 July 2011				3rd test - 14 July 2011			
	vtx1	*vtx2*	*eae*	*e-hly*	*vtx1*	*vtx2*	*eae*	*e-hly*	*vtx1*	*vtx2*	*eae*	*e-hly*
Sample A	-	+ (Ct=20)	-	+ (Ct=20)	-	+ (Ct=31)	-	+ (Ct=31)	-	-	-	-
Sample B	-	-	-	-	-	-	-	-	-	+ (Ct=37)	-	-
Sample C	-	-	-	-	-	-	-	-	-	-	-	-

Additional testing was also performed on the other seed lot from which a VTEC strain had been isolated at the first analysis.

Eight 50 g portions were prepared and tested on 15 July. The results for the *vtx2* gene detection are shown in table 5, in comparison with those obtained in the initial test made on 12 June.

Table 5. Real Time PCR results obtained by analysing 8 portions of a seed lot from which a VTEC strain had been isolated at the first analysis.

Date of testing	Positivity to *vtx2* Real time PCR (Ct value) per test portion								
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
12 June, 2011	**+ (Ct=23)***								
14 July, 2011		**+ (Ct=36)**	**-**	**-**	**+ (Ct=37)**	**-**	**-**	**-**	**-**

These data support the view that the apparently not congruent results of the inter-laboratory study were due to a non-homogeneous, low-level contamination of the lots from which the test samples were taken.

It cannot be excluded that the level of contamination has decreased by time during the

study period. However, the available data seem to indicate that seed contamination with enteropathogens could persist for much longer periods (www.fda.gov/food/foodsafety/product-specificinformation/fruitsvegetablesjuices/ucm078789.htm).

Moreover, two laboratories reported the presence of the *eae* gene and were also able to isolate the corresponding *E. coli* strain from a sample that had proved negative at the first test performed at the EU-RL one month earlier.

As a whole, the results suggest that:

1. The method developed during the discussion held with different laboratories during the outbreak crisis and released by the EU-RL VTEC, represents a useful tool for the detection of VTEC in seed samples;
2. A negative laboratory test does not necessarily prove the absence of a pathogen in a seed lot.

In conclusion, the major issue for the identification of the presence of VTEC in seed lots appears to be the sampling strategy, which could not be adequate to the extent of the contamination that may be encountered in seed lots.

Therefore, the approach of testing single seed samples seems to be not effective in identifying positive lots and a two-stage sampling strategy (n=5 and C=0) could be more appropriate.