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

Report of the 5th inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC) identification and typing - 2010

1. INTRODUCTION

The duties of the EU Reference Laboratory for VTEC (EU-RL VTEC) include the organisation of proficiency tests (PT) on detection and typing methods for the designated National Reference Laboratories (NRLs) for *E. coli* in the EU Member States, EU Candidate Countries and certain third countries.

The 1st and 2nd rounds of PT (2007 and 2008, reports available here) on VTEC identification and typing among the NRLs for *E. coli* in the EU Member States aimed at the evaluation of the capability of the NRLs to identify an *E. coli* strain as a VTEC, and to identify the VTEC serogroups most involved in severe human infections. In the 1st PT, the NRLs were invited to participate using the methods currently in use in their laboratories to perform the tests. In the 2nd PT, the EU-RL VTEC provided a conventional PCR protocol for virulence genes. Moreover, the NRLs were invited to examine the same *E. coli* strains using a Real-Time PCR protocol developed for the detection of the main VTEC serogroup in foodstuffs presently adopted as international standard by the ISO as ISO/WD TS 13136.

Both the number of the participating NRLs and their performance were satisfactory and increased from the 1st to the 2nd PT. The aim of the 5th PT was to further assess the proficiency of the NRLs in the detection of the main VTEC virulence genes and to extend the range of pathogenic VTEC serogroups to be identified.

The results of this proficiency test have been discussed during the 5th Annual Workshop of the EU reference laboratories for *E. coli* held in Rome on 8 October 2010 (agenda and presentations available here).

This document represents the full evaluation report of the study.

2. OBJECTIVES AND DESIGN OF THE STUDY

The aim of the study was the assessment of the NRLs proficiency in the detection of the main VTEC virulence genes and in the identification of an extended range of pathogenic VTEC serogroups. The serogroups considered were 11, chosen among those most frequently reported as cause of human infections in Europe by the Food and Waterborne Diseases (FWD) surveillance network of the European Centre for Disease Control (ECDC), with the addition of O121, a serogroup epidemiologically important in North America.

The study consisted of 2 parts:

1. Identification and characterization of VTEC by conventional or Real-time PCR detection of the main virulence genes, using the protocols in use at the NRL.
2. Serogrouping: the strains belonged to serogroups included among the 11 most frequently reported as cause of human infections in Europe by the FWD surveillance network of the ECDC. The serogroups could be determined by either conventional or molecular serotyping, using the protocols in use at the NRL.

PCR procedures for detecting *vtx* and *eae* genes and serogroup-associated genes were made available in the EU-RL web site (link).

E. coli strains to be used as positive controls in the PCR or immunological assays were provided to the NRLs.

3. PARTECIPANTS

Thirty-three NRLs, representing 26 EU Member States, Norway, and Switzerland participated in the study. Each NRL received its own individual laboratory numerical code, which was reported in the result tables.

The NRLs participating in the study were:

- Austria, Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH
- Belgium, Scientific Institute of Public Health
- Belgium, Veterinary and Agrochemical Research Centre (CODA-CERVA), (also representing Luxembourg)
- Bulgaria, National Diagnostic and Research Veterinary Institute
- Cyprus, Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic. Veterinary Research Institute
- Denmark, National Food Institute, Technical University of Denmark
- Estonia, Veterinary and Food Laboratory

- Finland, Finnish Food Safety Authority Evira, Helsinki
- Finland, Finnish Food Safety Authority Evira, Kuopio
- France, VetAgro Sup Campus Vétérinaire de Lyon
- Germany, Federal Institute for Risk Assessment (BfR)
- Greece, Central Public Health Laboratory and National School of Public Health
- Hungary, Central Agricultural Office, Food and Feed Safety Directorate
- Hungary, Central Agricultural Office, Food and Feed Safety Directorate
- Ireland, Central Veterinary Research Laboratory
- Italy, Istituto Superiore di Sanità
- Lithuania, National Food and Veterinary Risk Assessment Institute
- Netherlands, Food and Consumer Product Safety Authority
- Norway, National Veterinary Institute
- Poland, National Institute of Hygiene
- Poland, National Veterinary Research Institute
- Portugal, Laboratório Nacional de Investigação Veterinária
- Romania, Institute for Hygiene and Veterinary Public Health
- Slovakia, Public Health Authority, UVSZR
- Slovakia, State Veterinary and Food Institute Dolný Kubín
- Slovenia, National Veterinary Institute
- Spain, Agencia Española de Seguridad Alimentaria y Nutrición, AESAN
- Spain, Laboratorio Central de Sanidad Animal
- Sweden, National Food Administration, SLV
- Sweden, National Veterinary Institute, SVA
- Switzerland, University of Zurich
- UK, Health Protection Agency Centre for Infections, (also representing Malta).

4. MATERIALS AND METHODS

The test material sent to the NRLs was constituted by five strains of *E. coli* (samples 1 to 5). NRLs were requested to detect the presence of *vtx1* (group), *vtx2* (group) and *eae* genes and to identify the serogroup (O antigen or serogroup-associated genes) by either conventional or molecular serotyping.

4.1. Sample preparation

The characteristics of the strains are reported in Table 1 and were considered as the gold standard.

Table 1: Characteristics of the *E. coli* strains included in the study

Strain	Serogroup	*vtx1* gene (group)	*vtx2* gene (group)	*eae* (intimin) gene
1	**O121**	**-**	**+**	**+**
2	**O91**	**+**	**-**	**-**
3	**O113**	**+**	**+**	**-**
4	**O145**	**+**	**-**	**+**
5	**O111**	**-**	**-**	**+**

4.2. Assessment of the stability and homogeneity of the samples

The test materials consisted of bacterial cultures seeded by stabbing into soft nutrient agar (0,3%) in borosilicate glass vials. The test strains were selected among those present in the EU RL reference collection and checked for the presence of all the required genetic and phenotypic features.

A specific stability assessment was not performed, since previous experiences supported the assumption that the time range between the preparation of the samples and the deadline for submission of results by NRLs would have been short enough to assure the detection of all the strain characteristics.

The test samples were prepared on 12 May, incubated 18 hrs at 37°C +/-1°C and labelled with randomly generated numerical codes (3 digits), different for each NRL.

The homogeneity of the samples was evaluated according to the requirements of ISO 17043:2010. Three sets of samples were randomly selected and tested for the required characteristics. All the tests gave the expected results.

The test samples were shipped on 17 May by courier. The NRLs were requested to record the date and time of sample delivery, and date and time the analyses start.

4.3. Collection and elaboration of the NRL results

The results were submitted directly through an on-line system, using a dedicated page in the “Restricted Area” of the EU-RL web site. The NRLs received their own user ID and password for the log-in procedure and a step-by-step procedure for the submission of the results. After the log-in, they had access to an *Entry form* to confirm their participation in

the study and to a dedicated section for submitting the test results. This section also contained a *Shipment form* with the list of the samples to be analysed and the fields to collect the information on the arrival date, temperature and quality of the sample, and the possibility to write notes to specify any problem with the sample delivery/packaging. Before uploading the serotyping results, the NRL were requested to indicate the serogroups that they were able to identify either by immunological or molecular methods.
At the end of the study, the participants could print their own individual reports directly from the web site.

4.4. Evaluation of the NRL performance

The NRL performance was evaluated by calculating the following indicators:

- Agreement (*Cohen's kappa*)
- Sensitivity
- Specificity

Cohen's kappa refers to the level of association between two measurements of the same item. K test estimates the level of agreement beyond chance. The threshold values described by Fleiss J.L. (*Statistical methods for rates and proportions, 1981*) were used for the evaluation of the value of Kappa. Therefore Kappa values > 0.75 were considered as “excellent” agreement, values between 0.4 and 0.75 as “good” agreement and values <0.45 as “poor” agreement.
Sensitivity was defined as the proportion of positive samples correctly identified. Specificity was defined as the proportion of negative samples correctly identified. The 95% confidence interval (95%CI) was calculated for all the above-mentioned indicators.
The performances of each NRL in identifying the serogroup of the strains were evaluated according to the indication of their capability to identify that specific serogroup (see section 4.3). Therefore a failure in the identification of a specific serogroup was evaluated as an error only if the NRL had indicated to be able to identify that specific serogroup.

5. RESULTS

The samples were sent to the 33 NRLs that had accepted to participate in the study on 17 May. Twenty-seven NRLs received the samples on 18 May and the remaining six on 19 may. After receiving the samples, one of the NRLs (L51) communicated that it was not longer available to perform the tests and was not included in the result tables.

After starting the analyses, some NRLs communicated they were having problems with two of the test samples, reporting the concomitant presence of two different VTEC strains. Further analyses showed that a few laboratories participating in the study received strains that were cross-contaminated during the sample preparations, despite the favourable results obtained in the homogeneity assessment tests performed before sending the samples. The cross contamination involved the test samples 1 and 2, containing VTEC O121 and VTEC O91. It occurred at a low rate and was not revealed by the homogeneity testing, performed on three randomly selected sets of test samples. The laboratories that received 1 or 2 contaminated strains obtained PCR results that were a combination of the genotypes of the two strains. Therefore, samples 1 and 2 were excluded from the result evaluation for all the NRLs.

5.1. Detection of virulence genes by PCR

PCR detection of virulence genes was performed by all the 32 NRLs. The results are reported in Table 2.

Twenty-eight NRLs (87%) correctly identified the presence/absence of all the target genes in the test samples. Three NRLs (L02, L09 and L21) made errors in detecting the *vtx1* gene. Two (L02 and L21) failed to detect the gene in Sample 3 and one (L09) wrongly reported a false positive in Sample 5.

Finally, one NRL (L22) detected the *eae* gene in the *eae*-negative O113 VTEC strain (Sample 3).

Table 2. PCR detection of virulence genes. The green boxes indicates the correct results, the red boxes the wrong results.

NRL	Detection of genes in:								
	Sample 3			Sample 4			Sample 5		
	vtx1	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*	*vtx1*	*vtx2*	*eae*
True value	+	+	-	+	-	+	-	-	+
L01	+	+	-	+	-	+	-	-	+
L02	-	+	-	+	-	+	-	-	+
L03	+	+	-	+	-	+	-	-	+
L04	+	+	-	+	-	+	-	-	+
L05	+	+	-	+	-	+	-	-	+
L09	+	+	-	+	-	+	+	-	+
L10	+	+	-	+	-	+	-	-	+
L12	+	+	-	+	-	+	-	-	+
L14	+	+	-	+	-	+	-	-	+
L15	+	+	-	+	-	+	-	-	+
L17	+	+	-	+	-	+	-	-	+
L19	+	+	-	+	-	+	-	-	+
L20	+	+	-	+	-	+	-	-	+
L21	-	+	-	+	-	+	-	-	+
L22	+	+	+	+	-	+	-	-	+
L23	+	+	-	+	-	+	-	-	+
L24	+	+	-	+	-	+	-	-	+
L25	+	+	-	+	-	+	-	-	+
L27	+	+	-	+	-	+	-	-	+
L28	+	+	-	+	-	+	-	-	+
L29	+	+	-	+	-	+	-	-	+
L31	+	+	-	+	-	+	-	-	+
L34	+	+	-	+	-	+	-	-	+
L36	+	+	-	+	-	+	-	-	+
L37	+	+	-	+	-	+	-	-	+
L41	+	+	-	+	-	+	-	-	+
L42	+	+	-	+	-	+	-	-	+
L44	+	+	-	+	-	+	-	-	+
L45	+	+	-	+	-	+	-	-	+
L46	+	+	-	+	-	+	-	-	+
L49	+	+	-	+	-	+	-	-	+
L50	+	+	-	+	-	+	-	-	+

5.2. Identification of the O serogroup

Information on the availability of reagents for serogroup identification was provided by all the 32 NRLs and is summarized in Table 3. The methods included slide or tube agglutination with commercial or in house prepared antisera, or PCR amplification of serogroup-associated genes.

Table 3. Availability of reagents for serogroup identification, based on the NRLs' declaration in the entry form.

Number (%) of NRLs potentially able to identify the serogroup:										
O157	**O26**	**O103**	**O111**	**O145**	**O91**	**O121**	**O113**	**O128**	**O55**	**O146**
31	31	31	31	31	24	21	20	19	18	13
(97%)	(97%)	(97%)	(97%)	(97%)	(75%)	(66%)	(62%)	(59%)	(56%)	(41%)

Samples 4 and 5 contained VTEC strains belonging to one of the top five serogroups commonly causing severe disease in humans (O145 and O111). Sample 3 contained a VTEC O113 strain, a serogroup comprised in the extended list of VTEC serogroups involved in human infections, according to the ECDC surveillance data.

The identification of the serogroup was performed by all the 32 NRLs and the results are shown in Table 4.

As a whole, the samples 4 and 5 were correctly identified by all the NRLs that had declared to be able to identify the corresponding serogroups, with the exception of L05, which failed to identify the O145 serogroup in sample 4.

Sample 3 was correctly recognised as a VTEC O113 by 17 out of the 20 NRLs that had declared to be able to identify this serogroup. Three of those NRLs (L10, L15 and L49) failed to identify the serogroup.

Table 4. Results of serogroup identification, by NRL. The green boxes indicates the correct results, the red boxes the wrong results, and the yellow boxes the results not provided following the declaration of not being able to identify the serogroup in the entry form.

	Sample 3	Sample 4	Sample 5
True value	**O113**	**O145**	**O111**
L01	O113	O145	O111
L02	O N.T.	O145	O111
L03	O N.T.	O145	O111
L04	O113	O145	O111
L05	O113	O N.T.	O111
L09	O N.T.	O N.T.	O N.T.
L10	O N.T.	O145	O111
L12	O113	O145	O111
L14	O113	O145	O111
L15	O N.T.	O145	O111
L17	O113	O145	O111
L19	O113	O145	O111
L20	O N.T.	O145	O111
L21	O N.T.	O145	O111
L22	O N.T.	O145	O111
L23	O N.T.	O145	O111
L24	O113	O145	O111
L25	O N.T.	O145	O111
L27	O N.T.	O145	O111
L28	O N.T.	O145	O111
L29	O113	O145	O111
L31	O113	O145	O111
L34	O113	O145	O111
L36	O113	O145	O111
L37	O N.T.	O145	O111
L41	O113	O145	O111
L42	O113	O145	O111
L44	O113	O145	O111
L45	O113	O145	O111
L46	O113	O145	O111
L49	O N.T.	O145	O111
L50	O N.T.	O145	O111

5.3 Overview of the analytical performances

The analytical performances of the participating NRLs were evaluated in terms of agreement, sensitivity and specificity considering together the results of genotyping and serogroup identification, only for samples 3, 4 and 5 (Table 5).

Table 5. Genotyping and serogroup identification of the *E. coli* strains (samples 3, 4 and 4): agreement (Cohen's Kappa) between the results obtained by the NRLs and the true values of the samples. The green boxes highlight the values of Kappa >0.75 (excellent agreement); the yellow boxes the values between 0.45 and 0.75 (good agreement), and the red boxes the values < 0.45 (poor agreement

NRL	K values		
	K value	95% lower limit	95% upper limit
L01	1	0.70	1
L02	0.89	0.49	1
L03	1	0.64	1
L04	1	0.69	1
L05	0.91	0.59	1
L09	0.77	0.13	1
L10	0.91	0.57	1
L12	1	0.70	1
L14	1	0.67	1
L15	0.91	0.60	1
L17	1	0.70	1
L19	1	0.65	1
L20	1	0.60	1
L21	0.89	0.49	1
L22	0.91	0.56	1
L23	1	0.66	1
L24	1	0.70	1
L25	1	0.60	1
L27	1	0.60	1
L28	1	0.60	1
L29	1	0.70	1
L31	1	0.70	1
L34	1	0.70	1
L36	1	0.70	1
L37	1	0.66	1
L41	1	0.69	1
L42	1	0.67	1
L44	1	0.70	1
L45	1	0.69	1
L46	1	0.70	1
L49	0.92	0.62	1
L50	1	0.60	1

The level of agreement between the results of each NRL and the true values was evaluated by the Cohen's Kappa values. The overall agreement (K) was 0.98 (CI: 0.92 – 1.0). All the participating NRLs reported an "excellent" agreement with the true value of the samples (K > 0.75). However, considering the limited number of samples and assays available for the evaluation of K, the lower limit of the 95% C.I. could not exceed the threshold of "good" agreement (0.40 < K < 0.75) for all the NRLs.

The overall sensitivity of the participating NRLs in identifying the virulence genes and the serogroup of the *E. coli* strains was 97.5% (95% CI: 95.5% – 99,4%) and the overall specificity was 99.7% (95% CI 99.4% – 100%). The sensitivity and specificity values for each NRL are reported in Table 6.

Table 6. Genotyping and serogroup identification of *E. coli* strains (samples 3, 4 and 5): Sensitivity and Specificity for each NRL. The red boxes highlight values < 100%.

	Sensitivity (Se) and Specificity (Sp) for NRL										
NRL	L01	L02	L03	L04	L05	L09	L10	L12	L14	L15	L17
Se	100%	85.8%	100%	100%	87.5%	100%	87.5%	100%	100%	87.5%	100%
Sp	100%	100%	100%	100%	100%	75.0%	100%	100%	100%	100%	100%
NRL	L19	L20	L21	L22	L23	L24	L25	L27	L28	L29	L31
Se	100%	100%	85.7%	100%	100%	100%	100%	100%	100%	100%	100%
Sp	100%	100%	100%	95.7%	100%	100%	100%	73%	64%	100%	100%
NRL	L34	L36	L37	L41	L42	L44	L45	L46	L49	L50	
Se	100%	100%	100%	100%	100%	100%	67%	100%	87.5%	100%	
Sp	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	

Twenty-five of the 32 NRLs obtained 100% values for both sensitivity and specificity

7. REMARKS

1. Thirty-two NRLs representing 25 EU Member States participated in the study, together with the NRLs of Norway and Switzerland.
2. Twenty-eight of the 32 NRLs (87%) correctly identified the presence/absence of all the virulence genes in the test samples considered.
3. Thirty NRLs (94%) were able to identify correctly the two strains comprised among the top five serogroups (O111 and O145). Twenty out of the 32 NRLs,

claimed to be able to identify the less common O113 VTEC serogroup and 17 correctly typed the strain in sample 3.

4. The set of test strains included two other less common VTEC serogroups: O91 and O121. Unfortunately, these samples could not be considered for analysis due to the cross-contamination that affected a few of the sample replicates during preparation. However, 20 and 21 NRLs claimed to be able to identify serogroups O91 and O121, respectively, and most of the NRLs receiving the non-contaminated replicates actually identified correctly these serogroups.
5. Considering altogether the results of genotyping and serotyping, an excellent agreement (K value>0.75) of the results with the true values was observed for all the participating NRLs. A good accuracy was also obtained in terms of sensitivity and specificity.
6. Measures to minimize the possibility of cross-contamination of the test samples during preparation have to be implemented. These will include the preparation of the vials for each strain in different days/spaces and an increase in the number of tests to be performed for the homogeneity assessment.
7. The web site section implemented for the submission of the results of the inter-laboratory studies proved to be a valuable tool for gathering and managing the results. It will be further improved, based on the comments collected from the users.
8. In conclusion, most NRLs proved able to correctly identify an *E. coli* strain as a VTEC, and all but one were also able to properly identify the top five serogroups most involved in severe human disease. About 60% of the NRLs were also able to identify the less common VTEC serogroups O91, O113 and O121. The increase of the number of NRLs able to identify the extended the range of VTEC serogroups associated with human disease will be one of the major goals of the next PT programs.