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Report of the 2nd inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC) identification and typing - 2008

1. INTRODUCTION

The duties of the Community Reference Laboratory for VTEC (CRL-VTEC) include the organisation of proficiency tests 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 first inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC) identification and typing among the NRLs for *E.coli* in the EU Member States was conducted in 2007, aiming 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. The NRLs were invited to participate using the methods currently in use in their laboratories to perform the tests. The NRL performance was in general good (report available at http://www.iss.it/vtec/atti/cont.php?id=71&lang=2&tipo=2) and, based on this successful result, the aim of the 2008 study was the harmonization of the PCR methods used for the detection of the main VTEC virulence genes. Therefore, the conventional PCR protocol to be used was provided by CRL-VTEC, and the NRL performance was evaluated through a proficiency test.

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 and presently under discussion at the ISO as a forthcoming international standard.

The results of this proficiency test have been discussed during the 3rd Annual Workshop of the EU reference laboratories for *E.coli* held in Rome on 5 December 2008 (agenda and presentations available at www.iss.it/vtec in the 'Events' session).

This document represents the full evaluation report of the study.

2. OBJECTIVES AND DESIGN OF THE STUDY

The aims of the study were:

1. The harmonization of the conventional PCR methods used for the detection of the main VTEC virulence genes through the use of a PCR protocol provided by CRL-VTEC.
2. To involve the NRLs in the use of a Real-Time PCR protocol developed for the detection of the main VTEC serogroup in foodstuffs and presently under discussion at the ISO as an international standard. The application of the protocol on isolated strains was considered as a first step to introduce this forthcoming international standard for food analysis to the NRLs.

A set of VTEC strains was distributed in the blind to the NRL who accepted to participate, together with the conventional PCR method to be used for the detection of the main VTEC virulence genes, and the Real-Time PCR protocol developed for the detection of the main VTEC virulence genes and serogroups. Both methods are available for downloading at http://www.iss.it/vtec/docu/cont.php?id=109&lang=2&tipo=4.

Therefore, the study consisted of 4 parts:

1. Identification and characterization of VTEC by conventional PCR detection of the main virulence genes using the protocol provided by CRL-VTEC.
2. Serogrouping: the strains expressed O antigens commonly occurring among VTEC strains pathogenic to human beings. The serogroups could be determined by either conventional or molecular serotyping.
3. **Facultative**: VT production by phenotypic assays (Vero cells, immunologic kits).
4. **Facultative**: Real-Time PCR identification and characterization of the VTEC strains using the primers and probes described in the guideline provided by CRL-VTEC.

The NRLs were provided with the *E.coli* strains to be used as positive controls in the PCR assays.

3. PARTECIPANTS

Twenty-eight NRLs from 22 EU Member States plus Norway participated in the study. Each NRL received its own individual laboratory numerical code, which has been reported in the following tables.

The NRLs participating in the study were:

- Austria - Österreichische Agentur für Gesundheit und Ernährungssicherheit Institut für medizinische Mikrobiologie und Hygiene

- Belgium - University of Liege, Faculty of Veterinary Medicine, National Reference Laboratory in food microbiology
- Belgium - Veterinary and Agrochemical Research Centre
- Cyprus - Laboratory for the Control of Food of Animal Origin (LCFAO)
- Czech Republic - Veterinary Research Institute
- Denmark - Department of Microbiology and Risk Assessment, 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 *
  - * Different laboratories of the Finnish NRL
- France - Ecole Nationale Vétérinaire de Lyon
- Germany - Federal Institute for Risk Assessment BfR
- Hungary - Central Agricultural Office Directorate Food and Feed Safety, Central Food Microbiological Laboratory
- Hungary - Central Agricultural Office Directorate Food and Feed Safety, Central Feed Investigation Laboratory
- Ireland - Department of Agriculture & Food Laboratories Backweston
- Italy - Istituto Superiore di Sanità
- Latvia - National Diagnostic Centre - Laboratory of Food and Environmental Investigations (LFEI)
- Lithuania - National Veterinary Laboratory
- Norway - National Veterinary Institute
- Poland - National Veterinary Research Institute, Pulawy
- Portugal - Laboratório Nacional de Investigação Veterinária
- Slovakia - National Reference Centre of Environmental Microbiology - Public Health Authority of Slovak Republic
- Slovakia - State Veterinary and Food Institute, Dolný Kubín
- Slovenia - National Veterinary Institute, Veterinary Faculty, University of Ljubljana
- Spain - Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición AESAN
- Spain - Laboratorio Central de Sanidad Animal

- Sweden - National Veterinary Institute, SVA
- Sweden – Livsmedelsverk, SLV
- The Netherlands - Laboratory of the Food and Consumer Product Safety Authority, VWA

4. MATERIALS AND METHODS

The panel of samples sent to the NRLs was constituted by 7 strains of *E.coli*. NRLs were requested to identify the serogroup (O antigen), capability to produce VT (not mandatory), the presence of *vtx1*, *vtx2* and *eae* genes. 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*	*VT*	*vtx1*	*vtx2*	*eae*
A08	**O145**	**+**	**+**	**-**	**+**
B08	**O121**	**+**	**-**	**+**	**+**
C08	**O111**	**+**	**+**	**+**	**+**
D08	**O157**	**+**	**-**	**+**	**+**
E08	**O103**	**+**	**+**	**-**	**+**
F08	**O91**	**+**	**+**	**-**	**-**
G08	**O26**	**-**	**-**	**-**	**+**

NRLs were requested to identify the serogroup of the strains using methods currently available in the laboratory, while the presence of virulence genes had to be assessed by a PCR protocol provided by the CRL (available at: http://www.iss.it/vtec/docu/cont.php?id=109&lang=2&tipo=4). The NRL performance was evaluated by calculating the following parameters:

- 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. For serogroup identification, a positive result was defined as the correct identification of the strain. Specificity was defined as the proportion of negative samples correctly identified.

95% confidence interval (95%CI) was calculated for all the above mentioned parameters.

5. RESULTS

5.1 Laboratories

5.1.1 Samples examined

As a whole, 732 single tests were performed. Table 2 shows the number of test performed by the NRLs.

No. of results provided	*NRLs*	*%*
all (35)	3	11%
30-34	4	14%
25-29	16	57%
20-25	2	7%
< 20	3	11%

Table 2. Number of tests performed by the NRLs

Three NRLs performed all the 35 tests requested by Parts 1, 2 and 3. Twenty-five NRLs performed the tests for molecular characterization of the strains, e.g. the identification of *vtx1*, *vtx2*, and *eae* genes. The identification of the O serogroup was performed by 27 NRLs. The capability to produce VT was analyzed by 8 NRLs.

5.1.2. Mandatory tests

The overall number of the tests requested as mandatory was 784 and the number of those effectively performed was 676 (86,2%). Four NRLs performed all the tests requested.

5.2 Analytical tests

5.2.1 Identification of the O serogroup (mandatory)

The identification of the serogroup was performed by 27 NRLs (Figure 4).

strain / lab		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28
A08	O145	t	t	t	t	na	t	t	t	t	na	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na
B08	O121	na	na	na	na	na	t	false	na	t	false	na	na	na	t	na	false	na	false	t	na	t	na	t	na	na	t	na	na
C08	O111	t	t	t	t	na	t	t	t	t	t	t	t	na	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
D08	O157	t	t	t	t	na	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t
E08	O103	t	t	t	t	na	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	false
F08	O91	na	na	na	na	na	na	t	na	t	t	t	na	na	t	t	na	t	na	t	na	t	t	t	na	na	t	t	na
G08	O26	t	t	t	t	na	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	false

t = correct result; false = not correct identification of the serogroup; na - result not provided.

Figure 4. Results of serogroup identification, for each NRL

Seven NRLs made the test on all the strains, while the proportion of laboratories which performed serotyping for the 5 most frequent VTEC serogroups (O145, O111, O157, O103, O26) was 86% (24 out of 28). All but one the NRLs performed the O157 serogrouping, with 100% agreement.

The methods used were slide agglutination with commercial antisera or in house prepared antisera, or PCR amplification of serogroup-associated genes.

All the NRLs who did the test correctly identified serogroups O157 (27 NRLs), O111 (25 NRLs), O145 (24 NRLs), O91 (13 NRLs). One NRL failed to identify the O103 and O26 serogroups. O121 was the serogroup most hardly identified, with 17 NRLs not providing the result and 4 NRLs mis-identifying the O121 strain as O103.

The overall agreement (K) was 0.94 (CI: 0.91 – 1). The sensitivity was 0.96 (CI: 0.98 – 1), and the specificity 0.99 (CI: 0.91 – 1).

Figure 5 reports the sensitivity and specificity for the single serogroups.

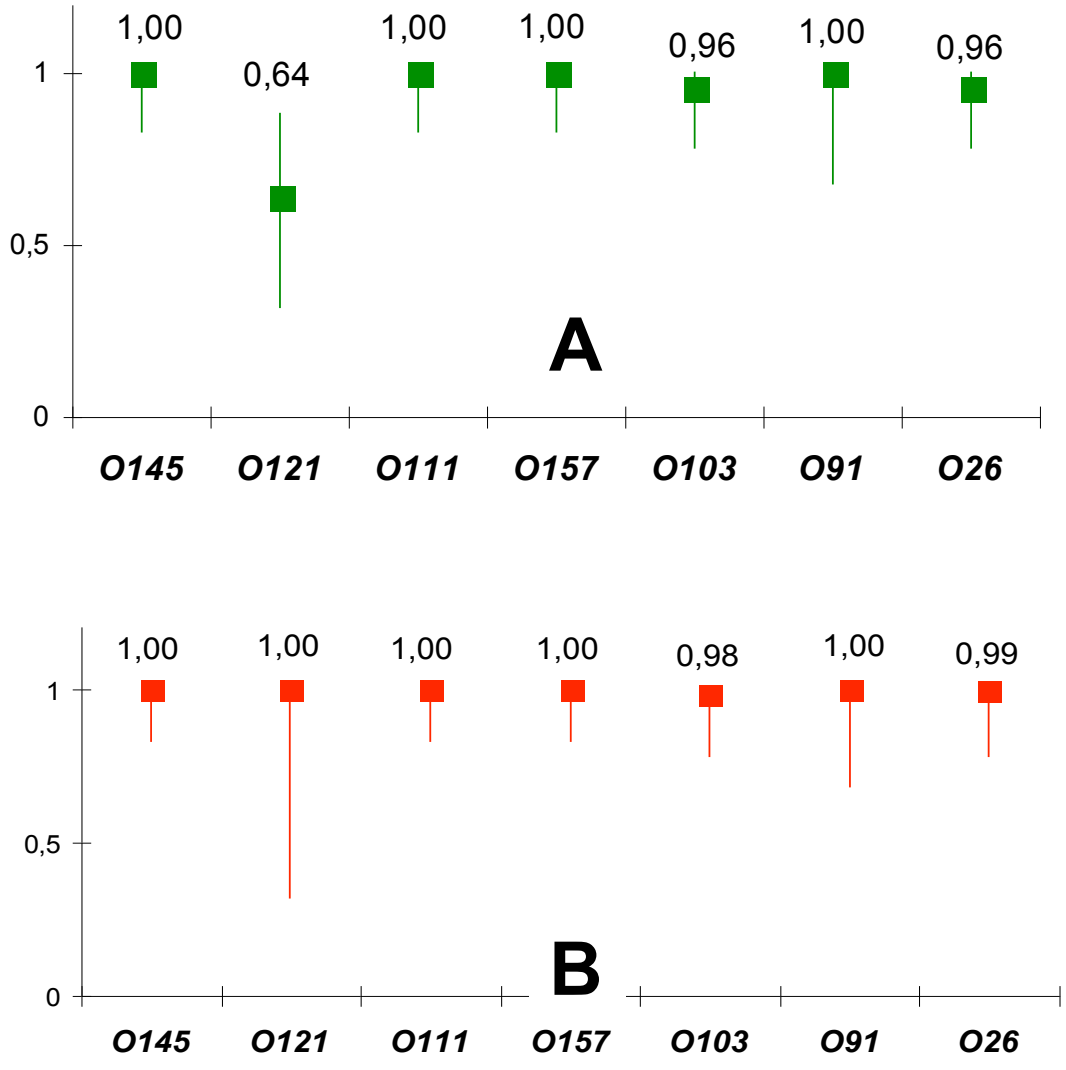

Figure 5 – Overall sensitivity (panel A) and specificity (panel B) for serogroup identification

5.2.2 Identification of the capability of the strains to produce VT (facultative)

VT production was tested by 8 NRLs (Figure 6). The methods used were the Vero cell cytotoxicity assay (3 NRLs) and antibody-based commercial kits (5 NRLs).

strain		L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28
A08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
B08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
C08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
D08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
E08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
F08	VT+	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na
G08	VT -	na	na	na	na	na	t	na	na	t	na	na	na	na	na	t	t	na	na	t	na	na	t	t	na	na	t	na	na

t = correct result; false = not correct identification of the serogroup; na = result not provided.

Figure 6. Detection of VT production for each NRL

All the NRLs who did the test correctly identified both VT-positive and VT-negative strains. The overall agreement (K) was 1.00. The sensitivity was 1.00 (CI: 0.81 – 0.91), and the specificity 1.00 (CI: 0.91 – 0.98).

5.2.3 Detection of virulence genes by conventional PCR (mandatory)

PCR detection of virulence genes with the method provided by the CRL was performed by 25 NRLs. The other 3 laboratories (n. 19, 20 and 27) analyzed the strains by the real Time-PCR protocol only. The results of the PCR tests performed for the detection of virulence genes are shown in Figure 7.

strain	lab	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28
A08	vtx1+	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
B08	vtx1-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
C08	vtx1+	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
D08	vtx1-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	false	t	t	na	na	t	t	t	t	t	t	na	t
E08	vtx1+	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
F08	vtx1+	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	false	t	t	na	na	t	t	t	t	t	t	na	t
G08	vtx1-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
A08	vtx2-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
B08	vtx2 +	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
C08	vtx2 +	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
D08	vtx2 +	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
E08	vtx2-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
F08	vtx2-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
G08	vtx2-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t

strain	lab	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21	L22	L23	L24	L25	L26	L27	L28
A08	eae +	t	t	t	t	t	t	t	t	t	t	t	t	false	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
B08	eae +	t	t	t	t	t	t	t	t	t	t	false	t	false	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
C08	eae +	t	t	t	t	t	t	t	t	t	t	t	t	false	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
D08	eae +	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
E08	eae +	t	t	t	false	t	t	t	t	t	t	t	t	false	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
F08	eae-	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	na	na	t	t	t	t	t	t	na	t
G08	eae +	t	t	t	t	t	t	t	t	t	t	t	t	t	t	t	false	t	t	na	na	t	t	t	t	t	t	na	t

t = correct result; false = not correct identification of the serogroup; na = result not provided.

Figure 7. PCR detection of virulence genes.

The identification of the *vtx1* gene was performed correctly in all the strains by 24 of the 25 NRLs who did the test. The remaining laboratory provided two wrong responses (one false negative and one false positive). The overall agreement (K) was 0.97 (CI: 0.94 – 1). The sensitivity was 0.99 (CI: 0.94 – 1), and the specificity 1.00 (CI: 0.92 – 1.00).

The identification of the *vtx2* gene was performed correctly in all the strains by all the 25 NRLs who did the test. Therefore overall agreement (K) was 1.00 (CI: 0.98 – 1). The sensitivity was 1.00 (CI: 0.94 – 1.00), and the specificity 1.00 (CI: 0.95 – 1.00).

The identification of the *eae* gene was performed correctly in all the strains by 21 of the 25 NRLs who did the test. All the *eae*-negative strains were correctly identified while 7 false negative results were reported. The overall agreement (K) was 0.85 (CI: 0.81 – 0.95). The sensitivity was 0.86 (CI: 0.81 – 0.91), and the specificity 0.96 (CI: 0.91 – 0.98).

5.2.4 Detection of virulence and serogroup-associated genes by Real-Time PCR (facultative)

The detection of VTEC virulence genes and/or serogroup-associated genes by Real-Time PCR was performed by 16 NRLs. Of these, 12 NRLs performed all the analytical tests requested.

The results are shown in Figure 8.

Of the 13 NRLs which performed the molecular serogrouping, 12 correctly identified all the O serogroups. One laboratory incorrectly identified an O121 strain as O103. In addition to the screening panel provided in the CRL guidelines, one NRL included also the O91 serogroup and correctly identified the strain.

All the 15 NRLs that tested the samples for the presence of *vtx2* and *eae* genes identified correctly all the strains. The presence of the *vtx1* gene was not correctly identified by 2 NRLs.

6. REMARKS

- 27 out of the 31 NRLs (87%) designated till now participated in inter-laboratory study, against the 20 who participated in the 2007 study.
- The percentage of NRLs who performed serogrouping increased from 80% to 97%;
- The NRLs performance was generally satisfactory, in particular for the identification of the most frequent VTEC serogroups and for PCR identification of *vtx* genes;

- A full characterization (detection of serogroup and virulence factors) was performed for a high proportion (68%) of the strains included in the study;
- Only 3 NRLs reported a *poor* agreement (Kappa value < 0.45) with the gold standard results for serogrouping, *vtx1* gene detection and *eae* gene detection, respectively;
- One laboratory did not perform serotyping, even for VTEC O157;
- A good accuracy of the tests was obtained in terms of sensitivity and specificity;
- An effective, harmonized conventional PCR method for VTEC identification is now available in the VTEC-NRL network;
- The Real-Time PCR protocol was effective in detecting virulence and serogroup-associated genes. It was used by 16 NRLs, and represents a first step toward their acquisition of the real-Time PCR-based method for the detection of VTEC non-O157 in food and animal samples, as specified in the forthcoming international standard.