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Report of the 1st inter-laboratory study on Verocytotoxin-producing *E. coli* (VTEC) identification and typing - 2007

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 has been conducted in 2007. The results of this proficiency test have been discussed during the 2nd Annual Workshop of the EU reference laboratories for *E.coli* held in Rome on 23 November 2007 (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 aim of the study was to verify and improve the capability of the NRL to correctly identify an *E.coli* strain as a VTEC, and then to identify the VTEC serogroups most involved in severe human infections. Some VTEC strains were distributed in the blind to the NRL who wish to participate, and the study consisted of 3 parts:

1. Identification of the VTEC status of the strain: this could be performed by showing either the production of VT by phenotypic assays (Vero cells, immunologic kits) or the presence of VT-coding genes (PCR, gene probes).
2. Detection of the main VTEC virulence genes. The strains possessed at least one of the following VTEC virulence genes: the genes coding VT1 and /or VT2 and the intimin-coding *eae* gene.
3. Serogrouping: the strains expressed O antigens commonly occurring among VTEC strains pathogenic to human beings.

The NRLs were invited participate in any combination of the 3 parts, using the methods currently in use in their lab to perform the tests.
PCR procedures for detecting *vtx* and *eae* genes and *E.coli* strains to be used as positive controls in the PCR assays were made available by the CRL for those NRLs who made the request.

3. PARTECIPANTS

Twenty-one NRLs from 16 EU Member States and from Norway participated in the study. Each NRL received its own laboratory code together with the evaluation of its individual results. The NRLs participating in the study were:

- Belgium (1): University of Liege, Faculty of Veterinary Medicine, National Reference Laboratory in food microbiology (LNR-MDA)
- Belgium (2): Veterinary and Agrochemical Research Centre
- Czech Republic: Veterinary Research Institute - Výzkumný ústav veterinárního lékarství
- Denmark: The Danish Institute for Food and Veterinary Research (DFVF)
- Estonia: Veterinary and Food Laboratory
- Finland (1): Finnish Food Safety Authority Evira, Helsinki
- Finland (2): Finnish Food Safety Authority Evira, Kuopio
- France: Ecole Nationale Vétérinaire de Lyon (ENVL)
- Germany: Bundesinstitut für Risikobewertung, (BfR)
- Hungary (1): Central Agricultural Office - Directorate Food and Feed Safety, Central Food Microbiological Laboratory
- Hungary (2): Central Agricultural Office - Directorate Food and Feed Safety, Central Feed Investigation Laboratory
- Ireland: Department of Agriculture & Food Laboratories
- Norway: National Veterinary Institute
- Poland: Panstwowy Instytut Weterynaryjny – Panstwowy Instytut Badawczy Pulawach, Zaklad Higieny Zywnosci Pochodzenia Zwierzecego
- Romania: Institute for Hygiene and Veterinary Public Health
- Slovakia: State Veterinary and Food Institute Dolný Kubín
- Slovenia: University of Ljubljana, Veterinary Faculty, National Veterinary Institute
- Spain: Centro Nacional de Alimentación. Agencia Española de Seguridad Alimentaria y Nutrición (AESAN)

- Sweden (1): Statens Veterinärmedicinska Anstalt (SVA)
- Sweden (2): Livsmedelsverk (SLV)
- The Netherlands: Laboratory of the Food and Consumer Product Safety Authority

4. MATERIALS AND METHODS

The sample sent to the NRLs was constituted by 5 strains of *E.coli*. NRLs were requested to identify the serogroup (O antigen), capability to produce VT, 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
A07	O111	-	-	-	+
B07	O103	-	-	+ (*)	+
C07	O157	+	+	+	+
D07	O26	+	+	+	+
E07	O145	+	+	-	+

* Strain B07 possessed a *vtx2* modified gene, due to the presence of an insertion sequence in the DNA segment coding for the B subunit of the toxin. It can provide either positive or negative results depending on the PCR primers used.

For strain B07, the results provided by the participating NRLs were considered correct or wrong according to the PCR primers used: primers designed on the B subunit had to provide a negative result, while primers designed on the A subunit had to provide a positive reaction.

NRL performance was evaluated by calculating the following parameters:

- Agreement (*Cohen's kappa*)
- Sensibility
- 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.

Sensibility was defined as the proportion of positive samples correctly identified.

For serogroup identification, a positive result was defined as the correct identification.

Specificity was defined as the as the proportion of negative samples correctly identified.

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

Information about the reagents, PCR primers and methods used by the NRL was requested.

5. RESULTS

As a whole, 403 single tests (19.2 tests per NRL on average) were performed.

Table 2 shows the number of test performed by the NRLs.

Table 2 – Number of tests performed by the NRLs

no. of test	*no. of Labs*	*%*
25 (all)	5	24%
20-24	8	38%
15-19	6	29%
10-14	1	5%
5-9	1	5%

Five NRLs performed all the 25 tests required. All the NRLs but one performed the tests for molecular characterisation of the strains, e.g. the identification of *vtx1*, *vtx2*, and *eae* genes. The identification of the O serogroup was performed by 17 NRLs, but for one of them it was restricted to O157. The capability to produce VT was analysed by 7 NRLs.

5.1 Laboratories

The *overall agreement (K)* of the results obtained by the participating NRLs is reported in Figure 1.

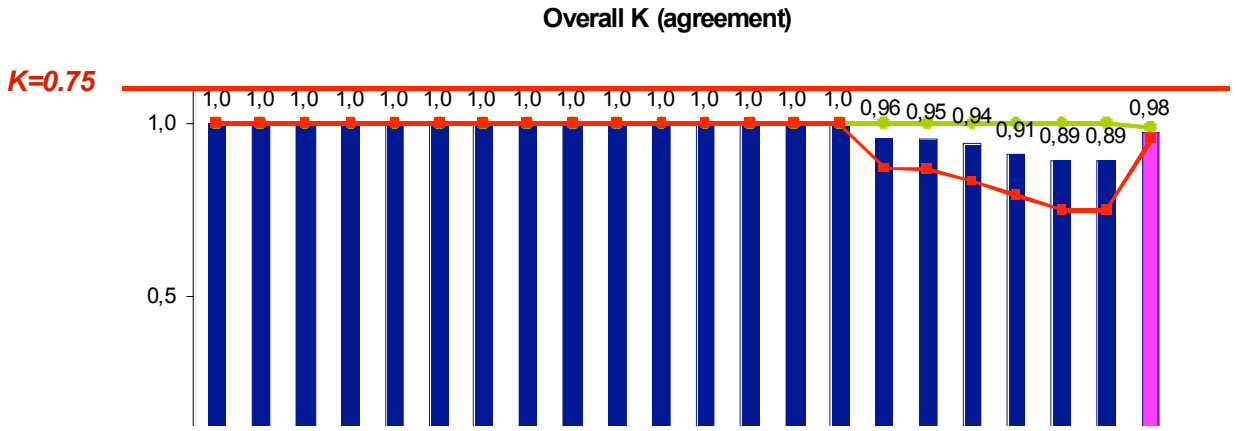

Figure 1. Overall agreement (K) for each NRL. Pink bar represents the NRL altogether. Green and red lines refer to upper and lower 95% confidence limits.

All the NRLs had excellent agreement with the gold standard, and 15 NRLs had a perfect agreement. The overall sensibility was 0.97 (95% CI: 0.93 – 0.99). Individual NRL values are reported in Figure 2. Sensibility ranged from 1.0% (16 NRLs) to 0.89 (1 NRL).

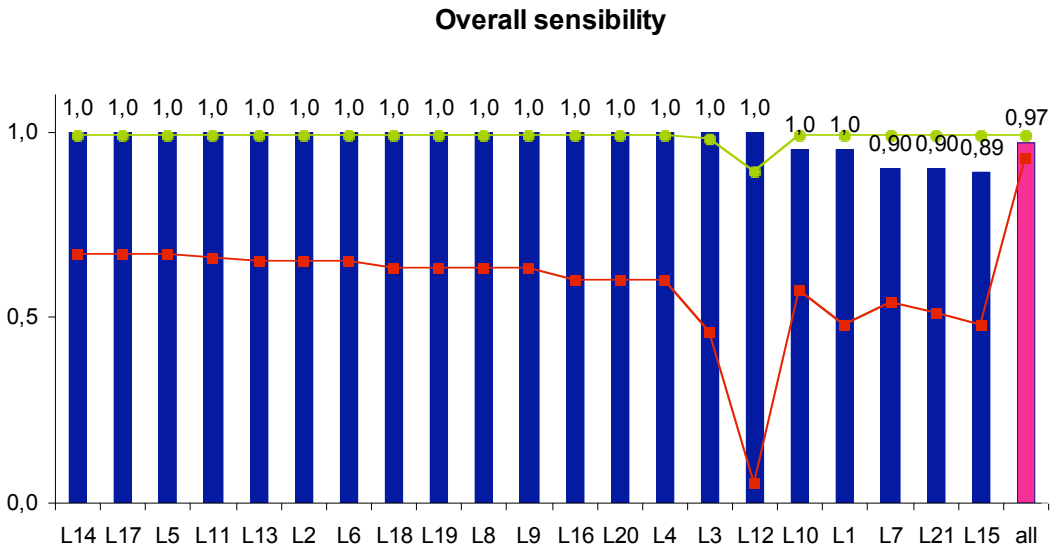

Figure 2. Overall sensibility for each NRL. Pink bar represents the NRL altogether. Green and red lines refer to upper and lower 95% confidence limits.

The overall specificity was 0.99 (95% CI: 0.97 – 1.00). Individual NRL values are reported in Figure 3. Sensibility ranged from 1.0% (16 NRLs) to 0.89 (1 NRL). Specificity ranged from 1.00 (15 NRLs) to 0.90 (1 NRL).

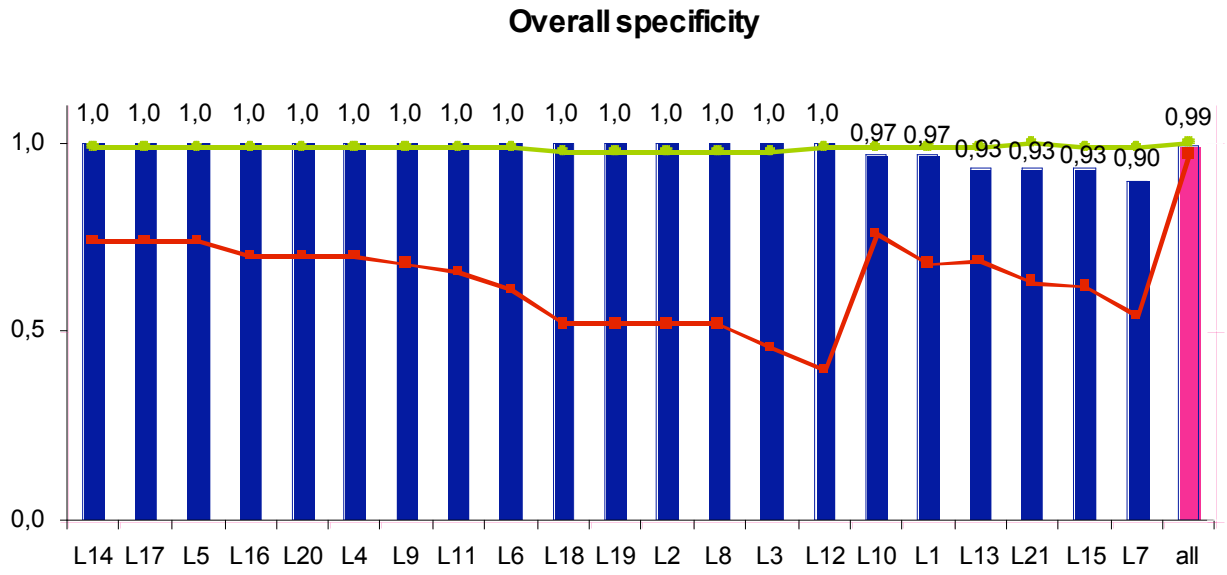

Figure 3. Overall Specificity for each NRL. Pink bar represents the NRL altogether. Green and red lines refer to upper and lower 95% confidence limits.

5.2 Analytical tests

5.2.1 Identification of the O serogroup

The identification of the serogroup has been performed by 17 NRLs (Figure 4).

test	Strain / Labs	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
serotyping	A07 O111	t	-	-	t	t	t	t	-	t	t	t	t	-	t	t	t	t	-	-	t	t
	B07 O103	t	-	-	t	t	-	t	-	t	false	t	t	t	t	false	t	t	-	-	t	t
	C07 O157	t	-	-	t	t	t	t	-	t	t	t	t	t	t	t	t	t	-	t	t	t
	D07 O26	t	-	-	t	t	t	t	-	t	t	t	t	t	t	t	t	t	-	-	t	t
	E07 O145	t	-	-	t	t	-	t	-	t	t	t	t	t	t	false	t	t	-	-	t	t

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

Figure 4. Results of serogroup identification for each NRL.

Fifteen NRLs made the test on all the strains, and one limited the identification to serogroup O157. The methods used were slide agglutination with commercial antisera or in house made antisera, or PCR amplification of serogroup-associated genes. All the NRLs who did the test, correctly identified serogroups O157 (17 NRLs), O26 (16 NRLs) and O111 (15 NRLs). Two NRLs failed to identify O103, and one O145. The overall agreement (K) was 0.95 (CI: 0.86 – 1). The Sensibility was 0.96 (CI: 0.86 – 1), and the

specificity 0.99 (CI: 0.96 – 1). Figure 5 reports the sensibility and specificity for the single serogroups.

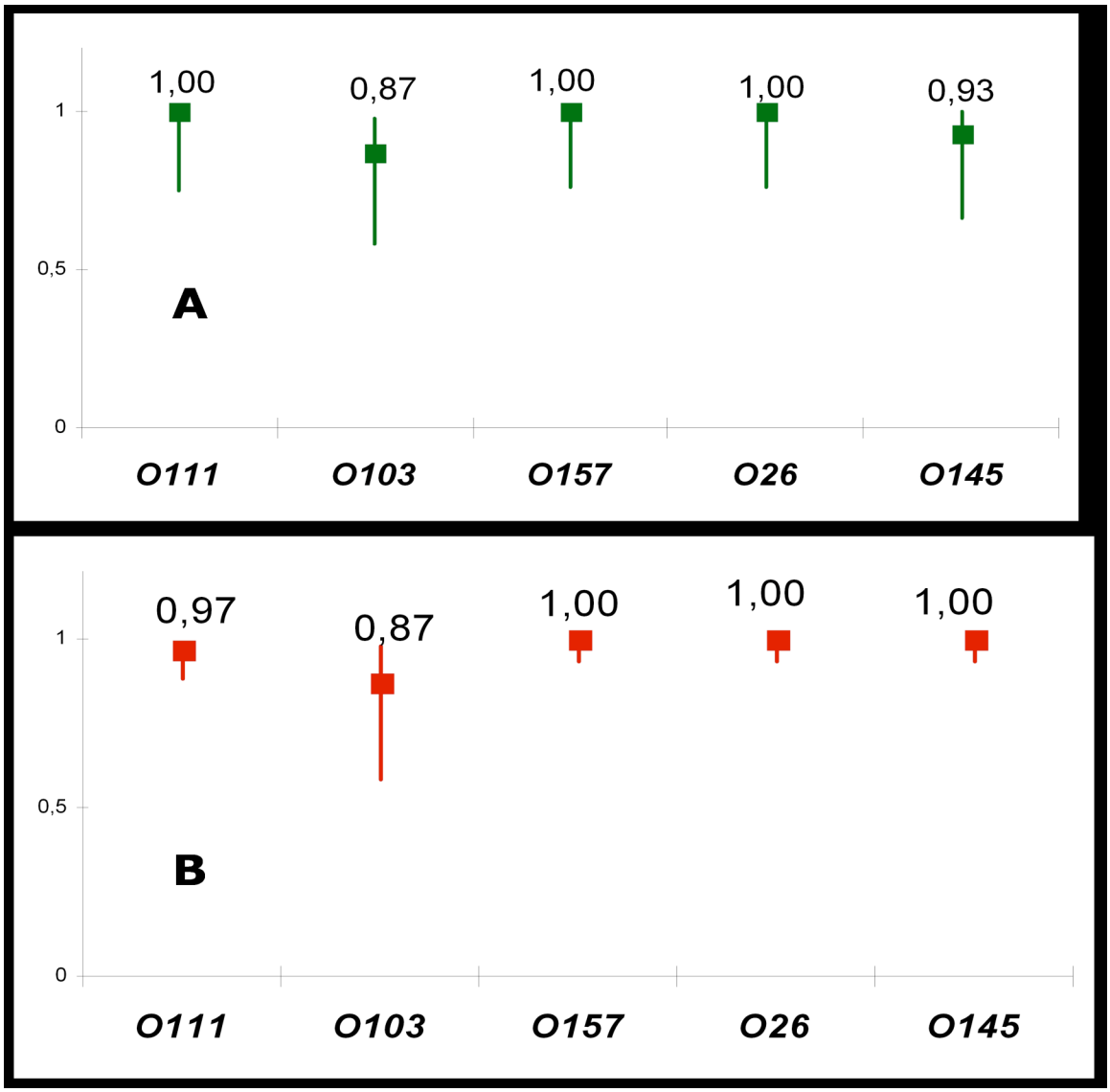

Figure 5 – Overall sensibility (panel A) and specificity (panel B) for serogroup identification

5.2.2 Identification of the capability of the strains to produce VT

VT production was tested by 7 NRLs (Figure 6). The methods used were the Vero cell cytotoxicity assay (VCA) by 2 NRLs, immuno-enzymatic commercial kits by 4 NRLs, and latex commercial kits by 2 NRLs; one NRL used two methods.

test	Strain / Labs	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
VT production	A07 VT-	t	-	-	-	t	-	-	-	-	t	-	-	t	t	-	-	t	-	-	-	t
	B07 VT-	false	-	-	-	t	-	-	-	-	t	-	-	t	t	-	-	t	-	-	-	t
	C07 VT+	t	-	-	-	t	-	-	-	-	t	-	-	t	t	-	-	t	-	-	-	t
	D07 VT+	t	-	-	-	t	-	-	-	-	t	-	-	t	t	-	-	t	-	-	-	t
	E07 VT+	t	-	-	-	t	-	-	-	-	t	-	-	t	t	-	-	t	-	-	-	t

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

Figure 6. Detection of VT production for each NRL.

All the NRLs who did the test identified correctly the 3 VT-positive strain. One NRL using a latex commercial kit provided a wrong result for one of the VT-negative strains. The overall agreement (K) was 0.93 (CI: 0.79 – 1). The Sensibility was 1.00 (CI: 0.83 – 1), and the specificity 0.90 (CI: 0.54 – 0.99).

5.2.3 PCR detection of virulence genes

PCR detection of virulence genes was performed by 20 NRLs: 19 performed the tests for *vtx1, vtx2* and *eae* genes, and one NRL for the *vtx* genes only. The PCR primers used by the NRLs are listed in Table 3 and Table 4.

Table 3. PCR primers used by the NRLs for *vtx1* and *vtx2* genes detection

Primers used:	**VT subunit**	**Reference**
KS7/KS8	1B subunit	(Russmann et al. 1995)
Stx1F/Stx1R	1A subunit	(Paton and Paton 1998)
B54/B55	1A subunit	(China et al. 1996)
VT1-F/VT1-R/VT1-P	1A subunit	(Nielsen and Andersen 2003)
VT1fp/VT1bp	1A subunit	(Pass et al. 2000)
5'I/3'I	1A subunit	(Brian et al. 1992)
Stx1comF/Stx1comR	1 B subunit	(Beutin et al. 2007)
LP30/LP31	1A subunit	(Cebula et al., 1995)
VT1-F/VT1-R/VT1-Probe	1B subunit	No reference given
Vtx1-F/vtx1-R	1 A subunit	No reference given
MK1/MK2	1A/2A subunit	(Karch and Meyer 1989)
LP43/LP44	2A subunit	(Cebula et al. 1995)
GK3/GK4	2B subunit	(Russmann et al. 1995)
Stx2F/Stx2R	2A subunit	(Paton and Paton 1998)
B56/B57	2A subunit	(China et al. 1996)
VT2F/VT2R/VT2P	2A subunit	(Nielsen and Andersen 2003)
VT2fp/VT2bp	2A subunit	(Pass et al. 2000)
5'II/3'II	2A subunit	(Brian et al. 1992)
VT2cm/VT2f	2B subunit	(Pierard et al. 1998)
VT2-F/VT-2R/VT2-Probe	2A subunit	No reference given
Vtx2-F/vtx1-R	2A subunit	No reference given

Table 4. PCR primers used by the NRLs for *eae* gene detection

Primers used:	Reference
SK1/SK2	(Karch et al. 1993)
B52/B53	(China et al. 1996)
eaeAF/eaeAR	(Paton and Paton 1998)
eae F2/eae R/eae P	(Nielsen and Andersen 2003)
eaeA fp/eaeA bp	(Pass et al. 2000)
eaeF/eaeR	(Vidal et al., 2004)
Int-Fc/Int-Rc	(Batchelor et al. 1999)
Eae-F/eae-R	No reference given
EAE-F/EAE-R/EAE-Probe	No reference given

The results of the PCR tests performed for the detection of virulence genes are shown in Figure 7.

test	Strain / Labs	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
vtx1 detection	A07 vtx1-	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	B07 vtx1-	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	C07 vtx1+	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	D07 vtx1+	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	E07 vtx1+	t	t	t	t	t	t	false	t	t	t	t	-	t	t	t	t	t	t	t	t	t
test	Strain / Labs	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
vtx2 detection	A07 vtx2-	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	B07 vtx2+ (*)	t	t	t	t	t	t	false	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	C07 vtx2+	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	D07 vtx2+	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	E07 vtx2-	t	t	t	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
test	Strain / Labs	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	L17	L18	L19	L20	L21
eae detection	A07 eae+	-	t	-	t	t	t	t	t	t	t	t	-	false	t	t	t	t	t	t	t	t
	B07 eae+	-	t	-	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	false
	C07 eae+	-	t	-	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t
	D07 eae+	-	t	-	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	false
	E07 eae+	-	t	-	t	t	t	t	t	t	t	t	-	t	t	t	t	t	t	t	t	t

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

Figure 7. PCR detection of virulence genes.

The identification of the *vtx1* gene was performed correctly in all the strains by 19 of the 20 NRLs who did the test. The remaining laboratory provided a wrong response (false negative). The overall agreement (K) was 0.98 (CI: 0.93 – 1). The sensibility was 0.98 (CI: 0.90 – 1), and the specificity 1.00 (CI: 0.89 – 1.00).

The identification of the *vtx2* gene was performed correctly in all the strains by 19 of the 20 NRLs who did the test. The remaining laboratory provided a wrong response (false negative, according to the primers used) for strain B07. The overall agreement (K) was 0.98 (CI: 0.93 – 1). The sensibility was 0.98 (CI: 0.97 – 1.00), and the specificity 1.00 (CI: 0.79 – 1.00).

The identification of the *eae* gene was performed correctly in all the strains by 16 of the 18 NRLs who did the test. The remaining laboratories provided a wrong response (false negative) for 1 and 2 strains, respectively. The overall agreement (K) and the specificity could not be evaluated, due to the absence of negative strains in the sample. The sensibility was 0.96 (CI: 0.86 – 1).

6. REMARKS

- 21 out of the 31 NRLs (67.7%) designated till now participated in the study;
- the NRLs performance was in general satisfactory, especially for PCR identification of virulence factors;
- an excellent overall (0.98) agreement between results from the NRLs and the true values (Gold Standard) was obtained;
- a good accuracy of the tests was obtained in terms of sensibility and specificity;
- a relevant number of NRLs did not participate in the study;
- a wide spectrum of PCR protocols was uses for the identification of virulence genes;
- 4 NRLs did not perform serotyping, even for *E.coli* O157;

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