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Inventory of expertise, facilities, and activities on verocytotoxin-producing *Escherichia coli* (VTEC) within the National Reference Laboratories for *E. coli* of European Union Member States

CONTENT

1. INTRODUCTION

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) infections are a major public health concern, because of the severe illnesses that they can cause, such as hemorrhagic colitis and hemolytic uremic syndrome (HUS), especially among children and the elderly. The large number of outbreaks occurring all over the world underlines the importance of these pathogens and highlights the need for both mandatory disease notification and cooperation between laboratories, within and beyond state boundaries.

Due to this public health relevance, VTEC infections have been included in the Part A of Annex I (list of zoonoses that will receive priority in monitoring schemes) of the Directive 2003/99/EC, which lays down the Community system for monitoring and collection of information on zoonoses. The Commission of the European Communities (EC) has therefore established a "Community Reference Laboratory (CRL) for *Escherichia coli*, including Verocytotoxin-producing *E.coli* (VTEC)", which is located at the Istituto Superiore di Sanità in Rome (ISS, www.iss.it/vtec).

The EC has also asked EU Member States (MS) to establish their own National Reference Laboratory (NRL) for *E. coli* and VTEC, to create a European network of reference laboratories on this matter.

This document represents an inventory of the structures, expertise, facilities, and activities currently available within the European network of NRLs for *E. coli* that have been designated by MS. The aim is to provide the EC DG SANCO with a tool to evaluate the potential capacity existing in the different EU MS for analytical and monitoring activities as well as interventions in the case of emergency.

2. METHODS

The inventory was carried out by submitting a semi-structured questionnaire to the laboratories designated by the Member States as NRL. The questionnaire was also sent to the NRL of Norway. For data analysis, the questionnaires were entered in an EpiInfo version 6.04b database (Center for Disease Control and Prevention, Atlanta, Georgia).

3. RESULTS

Till March 2008, 28 NRLs had been designated by 25 MS+Norway. Four of the MS designated two NRLs with different fields of activity, e.g. food control and animal monitoring. Two MS, Luxembourg and Malta, indicated Laboratories located in other countries. Two other MS, Bulgaria, and the United Kingdom (UK) had not yet designated

their NRLs. Norway indicated its NRL, which was included in the inventory. Therefore, the data reported will refer to a total of 28 NRLs representing 24 countries.
As far as the legal status of the NRLs was concerned, 24 of the 28 NRLs were located in government institutions, and the remaining three in Veterinary University Faculties. Of the government institutions, 6 were food safety institutes, 12 veterinary institutes, and 6 public health institutes.
The questionnaire was sent to all the designated NRLs, and a reply was obtained from all of them (response rate 100%).

3.1. Quality assurance system

Twenty-five of the 28 NRL had a quality assurance system officially accredited according to the EN/ISO IEC 17025:2005, and the official accreditation procedure was in progress in 1 NRLs.

3.2. Staff personnel and analytical activities

The average number of staff personnel involved in VTEC-related activities per NRL was 2.6 scientists and 2.6 laboratory technicians. Most NRLs (26) were involved in food analysis, and 18 NRLs reported analytical activities on animal samples. Environmental samples were analyzed in 13 NRLs, and human samples in 4. During the last year, approximately a total of 7,300 food samples, 8,200 animal samples, 4,600 human samples and 2,000 environmental samples were investigated for the presence of VTEC by the NRLs.
Fourteen NRLs reported to act as reference laboratory for the confirmation and typing of VTEC strains isolated from peripheral laboratories in their countries. During the last year, a total of about 2,300 *E.coli* strains was received by those laboratories for confirmation and typing. Samples or strains submitted to the NRLs had been collected within both routine surveillance program and foodborne outbreak investigations.

3.3. Laboratory methods

3.3.1. VTEC identification

E.coli strains can be identified as VTEC by demonstrating either their capability to produce VT or the possession of *vtx* genes. Examination for VT production was carried out by a total of 8 NRLs: 2 had the availability of the Vero cell cytoxicity assay (VCA), 6 used immuno-detection commercial methods, and 1 both the methodologies.

Detection of *vtx* genes by PCR amplification techniques was performed by 18 NRLs, 4 of which also used hybridization with *vtx*-specific gene probes. The use of several different *vtx*-specific PCR primers was reported (Table 1). Summing up, 21 NRLs were able to identify the presence of *vtx* genes and/or VT production. Conversely, the methods to correctly perform the identification of an *E.coli* strain as a VTEC did not result available to 7 NRLs.

Table 1. PCR primers used by the NRLs for *vtx1* and *vtx2* genes detection

Primers used:	VT subunit target	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

3.3.2. VTEC typing

Serotyping can be used for the characterization of strains previously identified as VTEC but also for the presumptive identification of VTEC belonging to the most common serogroups, in particular *E.coli* O157. In this case, the strains are isolated on the basis of

their O antigen and should subsequently be examined for VT production or the presence of *vtx* genes.

Determination of the O antigen (serogroup) was performed by 25 NRLs, but for 11 of them it was restricted to O157 only. Antisera to *E.coli* O26 (14 NRLs), O111 (13), O103 (12), and O128 (9) were the most frequently available.

H antigen serotyping was performed in 13 NRLs, and in 11 of them it was restricted to H7. H antigen genotyping (*fliC* genes) was available in 5 NRLs.

None of the NRLs performed phage-typing of *E.coli* O157.

Typing of *vtx* genes by PCR-based methods was performed in 14 NRLs, with the use of several different PCR primers and protocols.

PCR amplification of accessory virulence genes was reported by 17 NRLs, all of them looking for the presence of the intimin-coding *eae* gene using the primer listed in table 2.

As far as molecular typing techniques were concerned, pulsed field gel electrophoresis (PFGE) was available in 13 NRLs.

Table 2. 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

3.3.3. Methods for VTEC detection in different matrices

VTEC detection in the different matrices (feces, food, environmental samples) can be based on two different approaches: i) the detection of any VTEC strains present in the sample by seeking its capability to produce VT, and/or the presence of *vtx* genes; ii) a detection strategy specifically aiming at the VTEC serogroups most commonly associated with human disease, in particular *E.coli* O157. The second approach utilizes serogroup-specific enrichment procedures. These are based on immuno-separation techniques,

which use serogroup-specific LPS-antibodies coated to magnetic beads (immuno-magnetic separation, IMS) or other supports. Originally developed for *E.coli* O157, these reagents are now available for other main VTEC serogroups.

3.3.3.1. Isolation of VTEC from foodstuffs

Sensitive methods for the isolation of VTEC O157, based on O157-specific enrichment procedures using immuno-separation techniques, were in place in a total of 22 NRLs. Twenty NRLS used standard methods based on the IMS procedure: the EN/ISO 16654:2001 horizontal method for the detection of *Escherichia* coli O157 in food and animal feeding stuffs or the very similar method published with the same purpose by the Nordic Committee on Food Analysis, the method NMKL No 164, 2. Ed. 2005. Six NRLs used the bioMérieux VIDAS® ICE, validated by AFNOR certification. Four NRLs had availability of both the VIDAS® and the IMS techniques.

Searching for VTEC non-O157 was performed by 10 NRLs using different enrichment and isolation media, followed by the identification of colonies as described in the previous paragraphs 3.3.1 and 3.3.2. Four of these NRLs used IMS enrichment steps specific for all of for some of the following VTEC serogroups: O26, O103, O111, and O145.

3.3.3.2. Isolation of VTEC from feces

There is no internationally recognized standard method for the isolation of VTEC from feces. However, for the detection of *E.coli* O157 the methods based on the immuno-separation enrichment mentioned before can be adapted to feces and have been used in many studies published in the literature. Eleven NRLs used such methods for analyzing animal or human feces.

Isolation of VTEC non-O157 from feces was performed by 10 NRLs using different isolation media, followed by the identification of colonies as described in the previous paragraphs 3.3.1 and 3.3.2. Five of these NRLs used IMS enrichment steps specific for all of for some of the following serogroups: O26, O103, O111, and O145.

3.3.3.3. Direct detection of the presence of VTEC in different matrices

Food and feces samples can be screened for the presence of VTEC by rapid methods revealing the presence of their phenotypic or genotypic markers. As far as food samples are concerned, detection methods specific for *E.coli* O157 were used by 20 NRLs: 10 used PCR-based methods, 6 immuno-detection methods, mainly the VIDAS® ECO, and 5 used both types of methods. The same *E.coli* O157-specific methods were used for feces by 12 (PCR) and 1 (VIDAS) NRLs, respectively.

Screening of food and feces samples for VTEC non-O157 was performed by PCR in 14 and 12 NRLs, respectively, while 3 NRLs used the Vero cell assay to detect VT production in both the matrices. For both VTEC O157 and non-O157 screening, the PCR targets used in all the NRLs were the *vtx* genes, in most cases coupled with the intimin-coding *eae* gene.

3.4. Monitoring activities

Monitoring activities on food were carried out by 19 NRLs (14 for VTEC O157 only), on animals by 16 NRLs (8 for VTEC O157 only), on environmental samples by 9 NRLs (6 for VTEC O157 only). Five NRLs were involved in surveillance of human, 4 of which considering all VTEC. Four of these NRLs were hosted by institutions representing the national reference laboratory for VTEC in the Enter-net surveillance network.

3.5. Culture collections and data-bases

Culture collections of VTEC strains isolated from food were maintained at 15 NRLs, for a total of approximately 750 strains of VTEC O157 and 1,300 of VTEC non-O157. Strains from animals sources were collected by 16 NRLs, for a total of approximately 3,400 strains of VTEC O157 and 2,700 of VTEC non-O157. Around 900 human strains of VTEC O157 and 1,500 of VTEC non-O157 were maintained at 7 NRLs. Four NRLs had also collections of environmental strains (1,000 VTEC O157 and 280 VTEC non-O157).

In most cases the culture collections were linked with databases reporting the strain characteristics (serogroup, *vtx* gene type, presence of *eae* genes) and information on the source of isolation.

4. CONCLUDING REMARKS

Most NRLs for *E.coli* (18 out of 28) are located in food safety or veterinary institutions. The great majority of the 28 NRLs that responded to the questionnaire have a quality assurance system officially accredited according to the EN/ISO IEC 17025:2005.

Half of the NRLs act as reference laboratory for the confirmation and typing of VTEC strains isolated from peripheral laboratories, and 21 NRLs are able to identify an *E.coli* strain as a VTEC by detecting the presence of *vtx* genes and/or VT production. However, the use of several different *vtx* PCR primers and protocols was reported, indicating the need for consensus procedures for both *vtx* genes detection and typing.

Methods to detect *vtx* genes and/or VT production were not available to 7 NRLs. However, 5 of those laboratories could make a presumptive identification of *E.coli* O157, the most important VTEC serotype, based on the identification of the serogroup. These results prompted the CRL-VTEC to focus the first inter-laboratory comparison studyon the identification and typing of VTEC. The study was conducted in 2007 with encouraging results (full report available at www.iss.it/vtec) and will be followed by a second one in 2008.The goal is having NRLs able to correctly identify an *E.coli* strain as a VTEC in every EU MS.

As far as food analysis is concerned, 21 NRLs use standard (EN/ISO, NMKL, AFNOR) methods for the isolation of VTEC O157, based on specific immuno-separation enrichment procedures. This warrants homogeneity in food controls performed for this pathogen in most EU MS, at least at the laboratory level. Experience in detection of VTEC non-O157 in food was reported by a limited number of NRLs, and this is likely due to the lack of standard analytical methods for these organisms.

Monitoring activities for VTEC O157 on animal populations are carried out by many NRLs, and this could represent the basis for developing monitoring schemes in the framework of Directive 2003/99/EC.

Culture collections of VTEC strains isolated from food and animal sources are maintained at many NRLs and in most cases there are databases reporting basic information on the characteristics of the strains and the sources of isolation. This indicates that there is the possibility to build up a European database on the microbiologic characteristics of VTEC isolated from food and animal sources. Such a database will be proposed by CRL-VTEC and will collect the data on the strains isolated by NRLs. It will be harmonized with the Enter-net network database for human isolates.

In conclusion, this inventory of the current activities indicates that an acceptable level of expertise on VTEC O157 does exist in most of the European NRLs, warranting a sufficient homogeneity in food controls. Conversely, the inventory revealed that the skills in detecting VTEC non-O157 need to be improved. These results have been already used by the CRL-VTEC to calibrate its capacity building program towards NRLs. Therefore, due to the problems and lacks emerged for some NRLs in the correct identification of *E.coli* strains as VTEC, the first inter-laboratory comparison study has been focused on VTEC identification and typing.