The European Union Reference Laboratory for Verocytotoxin (VT)-producing Escherichia coli (VTEC), hosted by the Istituto Superiore di Sanità, Rome


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 (Caprioli et al., 2005; Karmali et al., 2003). The large number of outbreaks around 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. Consequently in Directive 2003/99/EC (Part A of Annex I), which lays down the European system for monitoring and collection of information on zoonoses, VTEC infections have been included in the list of zoonoses with priority for monitoring schemes. The European Commission (EC) decided therefore to appoint a European Union Reference Laboratory (EU-RL) for VTEC and, following a public call for candidates, the Istituto Superiore di Sanità (ISS), was given a 5-year mandate, starting on the 1st of July, 2006. Article 32 of Regulation (EC) No. 882/2004 describes the functions of Reference Laboratories for feed and food. In practical terms, these duties involve: (1) developing reference methods for detection, identification and typing of VTEC; (2) coordinating the application of such methods by the NRLs, in particular by organising inter-laboratory proficiency testing; (3) coordinating the network of European NRLs, by providing them with information on advances in the field, reference materials, and specific training in analytical methods; and (4) providing scientific and technical assistance to the Commission, in particular to the Directorate general for health and consumers (DG Sanco) and the European food safety authority (EFSA). DG Sanco finances the activities of the European Union Reference Laboratory.

The laboratory is housed within the Department of Veterinary Public Health and Food Safety of the Istituto Superiore di Sanità, and has a staff of 5 researchers, 3 technicians, 1 technical management assistant and 1 PhD student. It has been awarded certification for the ISO/IEC 17025 international standard by the Italian accreditation organisation (SINAL). Specific facilities are dedicated to different types of detection and typing methods, including microbiological and serological assays, conventional and Real Time (RT) PCR, pulsed field gel electrophoresis, and gene sequencing.

Coordination of the network of NRLs

The European Union Reference Laboratory for VTEC coordinates a network of 32 NRLs for E. coli, designated by the 27 Member States. The NRLs indicated by the competent authorities of Switzerland, Norway, Serbia, the Republic of Macedonia, and Turkey are also invited to participate. Annual workshops are organized for the NRLs with the aim: 1) of providing an overview of the surveillance and monitoring activities of VTEC infections carried out in the EU; 2) of disseminating information on new diagnostic tools, research results, and recommendations of the EU-RL; 3) of discussing the results of the inter-laboratory proficiency tests (ILPT) carried out; 4) of exchanging experiences, with presentations on the NRLs activities. Representatives from DG Sanco, EFSA, and from the European Centre for Disease Control (ECDC) regularly take part in the workshops. The number of NRLs participating in the workshops increased from 23 in 2006 to 30 in 2008. The laboratory’s web site (www.iss.it/vtec) is also used to disseminate information to the NRL network.

Cooperation with other reference laboratories

The European Union Reference Laboratory actively cooperates with the WHO International Escherichia and Klebsiella Centre (directed by Dr. Flemming Scheutz), hosted by the Statens Serum Institut, Copenhagen, which operates as Reference Laboratory for VTEC infections for the network of European NRLs in the medical field, that are coordinated by the ECDC. The main objective of this cooperation is to standardise identification and typing schemes and related external...
Focus on a laboratory

quality assurance programmes, to ensure that the respective monitoring programmes and databases are comparable for human and veterinary data.

Assistance to NRLs of EU Member States and third countries

The laboratory of the Istituto Superiore di Sanità maintains and distributes reference materials, such as *E. coli* reference strains, DNA extracts and antigens for serological assays. In particular, *E. coli* strains to be used as positive controls in the PCR assays included in the PTs were distributed to all the NRLs. Standard operating procedures (SOP) for the detection, identification and typing of VTEC have been drafted and distributed. They can be consulted at www.iss.it/vtec.

It also hosts personnel from the NRLs and other laboratories for training courses. Until now, 18 scientists have visited the EU-RL for periods ranging from one week to four months. They came from 8 NRLs and 4 universities of 7 Member States, and from 4 laboratories of third countries (Chile, Croatia, Egypt, Nigeria).

Figure 1 shows visiting scientists from the NRL of Poland and from the Central Veterinary Institute of Croatia during their training course.

Development of methods and organisation of inter-laboratory PTs

Identification and typing of VTEC strains

The first requirement in VTEC bacteriology is to be able to distinguish VTEC from the commensal, non-pathogenic *E. coli* that are usually present in faecal or food samples. At the current time, the main approach is the detection of VT-coding genes (vtx) by PCR techniques. The next step is the typing of the isolated strains to identify VTEC strains belonging to the restricted number of clonal lineages most often associated with severe human diseases. This can be done by determining the serogroup of the strains by serological or molecular methods, and detecting additional virulence genes, such as the intimin-coding eae gene. Consequently, the EU-RL VTEC drafted an SOP for identification and typing and organised 4 rounds of proficiency testing, so that all the NRLs would ultimately be able:

- to correctly identify an *E. coli* strain as a VTEC by PCR detection of virulence genes;
- to correctly identify *E. coli* O157 and the non-O157 VTEC serogroups mainly associated with severe human disease, according to the data collected and published by the ECDC (EFSA-ECDC, 2010).

The number and performance of the NRLs participating in the different PTs on virulence gene detection and strain serotyping are given in figure 2 and figure 3, respectively. Both the number and the performance of the participating NRLs have since increased. In particular, it is important to note that all the NRLs are now able to correctly identify VTEC strains belonging to serogroup O157, and most of them those belonging to the other four serogroups principally involved in human infections: O26, O103, O111, O145.

![Figure 1: Visiting scientists.](image)

![Figure 2: Proficiency tests organised by the reference laboratory on the detection of virulence genes in VTEC strains by PCR. For each gene, blue bars represent the number of NRLs which obtained correct results for all the strains included in the test.](image)

[Graph showing proficiency tests results for virulence genes vx1, vx2, and eae across years 2007 to 2010.]

Figure 2: Proficiency tests organised by the reference laboratory on the detection of virulence genes in VTEC strains by PCR. For each gene, blue bars represent the number of NRLs which obtained correct results for all the strains included in the test.
Detection of VTEC in food samples
In 2007, since standard methods were only available for VTEC O157, the European Committee for Standardization (CEN) (Technical Committee 275 – Food analysis – Horizontal methods, WG 6 – Microbial contamination) designated the European Union Reference Laboratory for VTEC as the coordinator of an ad hoc working group assigned to develop a standardised, Real Time PCR-based horizontal method for the detection of the VTEC serogroup most associated with severe human infections. Since then, the working group has developed a method that is now being issued as an ISO-CEN Technical Specification named “Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method” (ISO-TS13136). The method targets both virulence genes (\(vtx1\) and \(vtx2\), and \(eae\)) and serogroup-specific genes and can be used for screening samples. Samples which test positive at the same time for \(vtx\), \(eae\), and serogroup-specific genes are submitted to a further step in order to isolate the VTEC strain responsible for the positive PCR reactions. The method has been recommended by EFSA for the detection of VTEC non-O157 in food samples within monitoring programmes (EFSA, 2009) and, after discussion with the NRLs, the laboratory of the Istituto Superiore di Sanità organised three rounds of PT on its use (Figure 4). Since the method is based on the use of RT-PCR, which is the state-of-the-art technology, the first round was carried out on a range of isolated VTEC strains, in order to familiarise the laboratories with the molecular screening approach. Sixteen NRLs participated in the study, and 10 correctly identified all the genes in all the strains. The following two rounds involved the use of contaminated matrices requiring application of the complete detection method. The second round concerned the detection of VTEC O157 and non-O157 in carcass swabs. Sixteen NRLs participated in the study: 14 correctly performed the RT-PCR screening step and 13 the isolation step as well. The third round concerned the detection of VTEC non-O157 in milk. Twenty-nine NRLs participated: 25 correctly performed the PCR screening step and 24 the isolation step as well.

![Figure 3. Proficiency tests organised by the reference laboratory on the identification of the VTEC serogroups most involved in human disease in Europe.](image)

**Figure 3. Proficiency tests organised by the reference laboratory on the identification of the VTEC serogroups most involved in human disease in Europe.** For each serogroup, blue bars represent the number of NRLs that correctly identified the serogroup. The red bars indicate the NRLs that obtained incorrect results or did not perform the assay.

![Figure 4. Proficiency tests organised by the reference laboratory on the detection of the VTEC serogroups most involved in human disease, using the Real Time PCR-based ISO-CEN Technical Specification recommended by EFSA (EFSA, 2009).](image)

**Figure 4. Proficiency tests organised by the reference laboratory on the detection of the VTEC serogroups most involved in human disease, using the Real Time PCR-based ISO-CEN Technical Specification recommended by EFSA (EFSA, 2009).** Blue bars represent the number of NRLs that participated in each PT and green bars the number of NRLs that correctly detected all the target genes included in the method.
Detection of VTEC O157 in animal samples
There is as yet no ISO method for detecting VTEC O157:H7 in animal samples. However, the ISO 16654:2001 method for detection in food and feed, based on specific immunomagnetic separation after an enrichment step, has been adapted for the examination of animal feces in many investigations and has been recommended by EFSA for the detection of VTEC O157 in animal samples within monitoring programmes (EFSA, 2009). Consequently, the laboratory organised a PT on its use for the isolation of VTEC O157 from carcass swabs. Twenty-nine NRLs participated in the study and 27 correctly identified the presence/absence of VTEC O157 in the samples.

Conclusions
A large variety of VTEC can be found in the food-producing animal populations (Caprioli et al., 2005), but only a limited number of seropathotypes have been consistently associated with human infections (Karmali et al., 2003; EFSA, 2007; EFSA-ECDC, 2010). This is a particular challenge to develop specific detection methods for the most pathogenic types (EFSA, 2007; EFSA, 2009) that will require a network of skilled and trained laboratories throughout the EU for their detection in the infection vectors. The European Union Reference Laboratory for VTEC is working to consolidate such a network, in order: (1) to increase knowledge of the epidemiology of VTEC infections in Europe; (2) to collect standardised data on the prevalence of these pathogens in food samples finalised according to the definition of microbiological criteria for VTEC; (3) to provide the EC with more standardised operative structures and tools for dealing with possible food safety emergencies.

References
European Food Safety Authority. 2009. Technical specifications for the monitoring and reporting of verotoxigenic Escherichia coli (VTEC) on animals and food (VTEC surveys on animals and food) on request of EFSA. EFSA Journal; 7(11):1366.