EU Reference Laboratory for *E.coli*

Department of Veterinary Public Health and Food Safety

Unit of Foodborne Zoonoses

Istituto Superiore di Sanità

EU Reference Laboratory (EU-RL) for *Escherichia coli*, including Verotoxigenic *E. coli* (VTEC)

Work Programme

1st January - 31st December, 2012

Introduction

The work programme of EU-RL for VTEC (EU-RL VTEC) for the year 2012 will consist of the following activities:

1. Consolidating the EU-RL structures

1.1. Staff

1.2. Accreditation of the laboratory

1.3. Administration and Reporting

2. Coordination of the NRLs network and provision of technical assistance and training

2.1. Annual workshop

2.2. Assistance to NRLs

2.3. Training

2.4. Characterization of VTEC strains isolated by the NRLs

3. Implementation of the EU-RL-VTEC web site

4. Co-operation with EC or other structures and projects related with food safety

4.1. Scientific and technical support to DG SANCO

4.2. The European Food Safety Authority (EFSA)

4.3. The European Committee for Standardization (CEN)

4.4 The Joint Research Centre (JRC), Ispra

4.5. The European Centre for Disease Control (ECDC)

4.6. The U.S. Department of Agriculture (USDA)

5. Reference materials

5.1. Plasmids carrying DNA targets for PCR-based diagnostic assays

5.2. Preparation of toxin-negative variants of VTEC O104:H4

6. Inter-laboratory comparison studies

6.1 Detection of VTEC in water samples

6.2. Detection of VTEC in seeds intended for sprout production

7. Research activities on VTEC

8. Missions

The objectives and the expected outputs of each action are indicated, as well as its duration, which will be either limited to 2012 or multi-annual (ongoing programme).

1. Consolidating the EU-RL structures

1.1. Staff

The permanent staff of ISS will continue to devote significant working time to the EU-RL's activities. Four persons, hired with EU-RL funds, will continue to work full time at the EU-RL-related activities with the status of "temporary staff employees".

Objective: *to appropriately cope with the EU-RL tasks.*

Expected output: *involvement of experienced and skilled staff in EU-RL activities.*

Duration: *ongoing*

1.2. Accreditation of the laboratory

The EU-RL VTEC is constantly improving the effectiveness of its management system through the use of the quality policy, according to its accreditation EN/ISO IEC 17025:2005 (N. 0779) obtained in 2007 from the Italian accreditation body (ACCREDIA). Beside the management of the laboratory, the accreditation covers the methods for detection and typing of VTEC related with EU-RL's tasks and activities. The possibility to submit additional methods for accreditation will be evaluated.

The EU-RL will continue to organize inter-laboratory comparison and proficiency studies according to the International Standard ISO/IEC 17043:2010 "*Conformity assessment – General requirements for proficiency testing*", which has recently been issued (see point 5). The process to get a formal accreditation ISO/IEC 17043:2010 as proficiency tests provider will continue, according to general policy of the ISS on this matter.

Objective: *continuous improvement of the organization of the EU-RL.*

Expected output: *maintaining the 17025 accreditation for a better quality of the services provided by the EU-RL.*

Duration: *ongoing*

1.3. Administration and reporting

The EU-RL will continue to manage the administration procedures related with the purchasing of materials, the shipment of reference materials and proficiency test samples, the missions of the staff. The general activity reports and those specific for the inter-laboratory studies will also be prepared.

Objective: *to improve the support to the EU-RL activities and the communication of the results obtained.*

Expected output: *more efficient organization of the EU-RL activities and high quality reports.*

Duration: *ongoing*

2. Coordination of the NRLs network and provision of technical assistance and training

2.1. Annual workshop with the NRLs

The 7th annual workshop will be held in the second half of 2012 in Rome. In alternative, upon agreement of DG SANCO, one of the NRLs could host the workshop at its own Institute. The results of the 2012 inter-laboratory studies will be presented and discussed. The training program for the benefit of NRLs will be discussed as well and plans for the following year will be established according to the NRLs needs. The program will also include updates on the surveillance and monitoring activities of VTEC infections carried out in the EU, information on new diagnostic tools, research results, recommendations, and exchange of experiences with presentations made from the NRL representatives. Representatives from the European Food Safety Authority (EFSA) and from the European Centre for Disease Control and Prevention (ECDC) will be invited. The level of satisfaction of the participants toward the workshop organization, the proposed topics, and the quality of presentations will be evaluated by a questionnaire. The results will be used for the continuous improvement of the organization.

Objectives: *i) to provide updates on the different aspects of VTEC infections; ii) to plan the training programs according to the NRLs needs; iii) to strengthen the relationships with and among the NRLs.*

Expected output: *consolidation of the NRL network.*
Duration*: 2012*

2.2. Assistance to NRLs

The EU-RL-VTEC will continue to assist the NRLs in the field of VTEC detection and typing, providing methods and standard operating procedures via the web site, and reference materials, in particular VTEC strains. Drafts of other standard operating procedures for detection of other pathogenic *Escherichia coli* in animals, food, and in other relevant matrices and for typing of the isolated strains will be developed and discussed with the NRLs. If needed, the EU-RL-VTEC will visit NRLs to help in solving problems.

Objectives**:** *to provide updated diagnostic tools and advice to the NRL Network.*
Expected output**:** *improved capability of the NRLs to detect and type VTEC.*
Duration: *ongoing*

2.3. Training

Upon request from NRLs within EU or from governmental institutions of third countries, the EU-RL will be available to receive visits of staff for individual training on specific topics related with detection and typing methods. Standard programs for short-term training visits on techniques for VTEC detection, identification, and typing will be updated, according to the needs of the NRLs and the evolution of the epidemiological picture of VTEC infections in the EU (see also point 2.4). The level of satisfaction of the trainees toward the organization, the program, and the quality of the stage will be evaluated by a questionnaire. The results will be used for the continuous improvement of the training program.

Objectives**:** *to provide specific training to the staff of the NRLs or other laboratories.*
Expected output**:** *improved capability to detect and type VTEC in EU Member States and other countries.*
Duration: *ongoing*

2.4. Characterization of VTEC strains isolated by the NRLs

The PFGE-typing, phage-typing and MLVA-typing techniques for subtyping VTEC O157 strains have been implemented and are available at the EU-RL VTEC. The corresponding standard operating procedure (SOP) will be published on the EU-RL VTEC website.

A repository containing the typing data for VTEC O157, including phagetypes and PFGE and MLVA profiles, has been created in the Bionumerics bioinformatic platform and presented at the 6th annual workshop. In a first phase, the molecular profiles of VTEC O157 isolates will be produced by the EU-RL, upon request of the NRLs who will send strains for typing. The strain characteristics will be uploaded in the repository and the bioinformatic analyses will be performed on the locally held database. Those NRLs who have the skills to produce molecular profiles will do the work by using the SOP published by the EU-RL and will send the profiles by E-mail for the uploading and bioinformatic analyses. A kick off meeting with representatives of these NRLs will be organized at the EU-RL to define and launch the repository. As a second step, the repository will be made available to the NRLs for direct uploading of the profiles and consultation *via* web. For the other NRLs, a specific training program on molecular typing will be carried out.

Objectives**:** *to provide specific training on VTEC O157 typing to the NRLs or other laboratories.*

Expected output**:** i) *improved capability to type VTEC O157; ii) repository of molecular profiles of VTEC O157 strains isolated from non-human sources, available for molecular epidemiology investigations.*

Duration: *2012*

3. Maintaining and implementing the EU-RL VTEC web site

The web site of the EU-RL VTEC (http://www.iss.it/vtec) will be maintained and updated on a regular basis with documents, methods, workshops and inter-laboratory studies reports, information on the NRLs, and links. The “Restricted Area” will be used for the on-line submission of the results of the inter-laboratory studies and of molecular typing of the VTEC O157 strains.

Objectives: *to continuously implement a tool for: i) the rapid communication of the*

EU-RL activities; ii) the collection of proficiency testing results and molecular typing data from the NRLs.

Expected output: *i) improved communication of the EU-RL activities; ii) improved collection of data from the NRLs.*

Duration: *ongoing*

4. Co-operation with EC structures or other structures and projects related with food safety

The EU-RL will continue the cooperation with EC structures or other structures and projects active in the field of human and animal health and food safety.

The following liaisons will be maintained and implemented:

4.1. Scientific and technical support to DG SANCO

The EU-RL VTEC will continue to provide scientific and technical support to DG SANCO for all the food safety issues related with VTEC or other groups of pathogenic *E. coli*.

Objectives: *to support DG SANCO in managing any food safety issues related with E. coli.*

Expected output: *scientific and technical support to DG SANCO*

Duration: *ongoing*

4.2. The European Food Safety Authority (EFSA)

The EU-RL will contribute as reference laboratory (scientific and technical advice, elaboration of methods, sub-typing of VTEC O157, etc.) to any EFSA initiative in the field of VTEC. These could include either the evaluation of specific issues or the implementation of monitoring programs by the EFSA Task Force on Zoonoses Data Collection, according to the document "*Technical specifications for the monitoring and reporting of VTEC on animals and food on request of EFSA*" (*EFSA Journal; 7(11): 1366*).

Objectives: *to provide scientific and technical advice to EFSA on food safety issues related with E. coli.*

Expected output: *mutual exchange of information and opinions on VTEC.*

Duration: *ongoing*

4.3 The European Committee for Standardization (CEN), Technical Committee 275 – Food analysis – Horizontal methods, WG 6 – Microbial contamination.

The validation study of the method EN/ISO 16654 for E. coli O157, which will involve 14 NRLs for *E. coli* that have been already enrolled, will begin as soon as the formal agreement between CEN and EU-RL VTEC will be signed and funding will be available.

Objectives: *to complete the validation of the method EN/ISO 16654 for E. coli O157, according to the EC* Mandate *for standardization* M/381 *addressed to CEN.*

Expected output: *validation report for the method EN/ISO 16654 for E. coli O157*

Duration: *2012-2013*

4.4 The Institute for Health and Consumers Protection of the Joint Research Centre (JRC), Ispra.

The collaboration with the Institute for Health and Consumers Protection of the Joint Research Centre (JRC) in Ispra (VA, Italy) for the validation of the CEN ISO TS 13136 method for the detection of VTEC in food will continue. Following the publication of the preliminary study of the validation of the Real Time PCR screening step, a collaborative study needed for the full validation will be designed, based on the requirements of the ISO 16140 standard. The study, which should involve the NRLs network, could be carried out in 2013, upon availability of a dedicated grant.

Objectives: *to complete the validation of the method CEN ISO TS 13136 for the detection of VTEC in food.*

Expected output: *experimental design of the validation project for of the method CEN ISO TS 13136*

Duration: *2012*

4.5. The European Centre for Disease Control (ECDC) Food- and Waterborne Diseases (FWD) Program

The EU-RL will continue to take part into the Coordination Group of the ECDC FWD Program, with the aim of ensuring connection and activity harmonization between

this network and the network of Reference Laboratories in the veterinary and food safety fields (Regulation (EC) No. 882/2004). The EU-RL-VTEC will also continue the liaison with the ECDC reference laboratory for VTEC infections (the WHO International *Escherichia* and *Klebsiella* Centre of the Statens Serum Institut, Copenhagen), which is in charge of the external quality assurance activities for the network. This will allow the harmonization of the identification and typing schemes, making the respective monitoring programs and databases compatible for comparison of human and non-human data. In particular, the PCR-based method for the characterization of the different *vtx* genes subtypes will be adjusted, according to the results of the joint inter-laboratory study with the NRLs conducted in 2011.

Another collaboration will involve a study aiming at the collection of the strains of VTEC O104:H4 that have been isolated before the 2011 outbreak occurred in Germany, to investigate the relationships among the strains and the evolution of this clonal lineage.

Objectives: *i) to harmonize the identification and typing schemes for VTEC used in the monitoring programs carried out by the medical and veterinary networks of NRLs; ii) to continue the scientific collaboration on VTEC O104:H4.*

Expected output: *i) common microbiological database for comparison of human and non-human isolates of VTEC; ii) revised method for vtx genes sub-typing.*

Duration: *2012*

4.6. The U.S. Department of Agriculture (USDA)

The collaboration with the Eastern Regional Research Center of USDA (Wyndmoor, PA, USA) will be maintained and implemented. The collaboration, so far focused on the identification of genetic markers that could represent candidate targets for the detection of VTEC in food, will be extended to other aspects of VTEC biology, including the mechanisms of emergence of new pathogenic clones, such as VTEC O104:H4. A joint research programme, started in 2011 and aimed at studying the VTEC sero-pathotypes most associated with severe human disease by means of whole genome automated sequencing, will be continued (see also point 6).

Objectives: *i) to identify genetic markers that could represent candidate targets for the detection of VTEC in food; ii) to compare the EU and US laboratory approaches*

for the detection of VTEC in food.

Expected output: *i) scientific publications; ii) consolidation of the collaboration; iii) publication of whole genome sequences of VTEC strain belonging to the main sero-pathotypes causing severe human disease.*

Duration: *2012*

5. Reference materials

5.1. Plasmids carrying DNA targets for PCR-based diagnostic assays

A collection of plasmids carrying the DNA targets for the real time PCR screening step of the ISO TS 13136 has been prepared in collaboration with the JRC (Ispra) and is available for distribution to NRLs since April 2011. In the framework of the present workprogramme, the collection will be integrated with other target genes, such as those needed for the detection and identification of the VTEC O104:H4 strain associated with the large 2011 outbreak. The new set of reference materials will include the target DNA sequences for the enteroaggregative adhesion, a key feature of VTEC O104, as well as those identifying the O104 and H4-coding genes. Plasmid-based reference materials will be an important resource for implementing the PCR-based methodologies for VTEC detection, with the added value of a safe and easy distribution to and handling by the NRLs.

- ***Objectives:*** *to implement and update the collection of reference materials for VTEC detection and typing.*
- ***Expected output:*** *i) extended collection of reference plasmids for PCR-based VTEC detection and typing; ii) non-class 3 reference materials, safe and easy to distribute and handle.*

  Duration: *2012*

5.2. Preparation of toxin-negative variants of VTEC O104:H4

The production of a toxin-negative variant of VTEC O104:H4 outbreak strain will be attempted. The excision of the bacteriophage carrying the Shiga-toxin genes will be induced by protocols based on the use of the antimicrobial mytomicin or physical agents such as the UV light. The cured clones obtained will be then stabilized and tested for all the phenotypic characteristics of the original strain (antimicrobial

resistance, growth on selective media, entero-aggregative adhesion, biofilm formation). Obtaining such an Stx-phage defective enteroaggregative O104:H4 strain would allow a safe distribution of a reference strain to the NRLs and other third countries laboratories. Such a reference strain will be of great usefulness in setting up methods for the isolation of this emerging pathogen and for studying its biology (e.g. biofilm formation, capability to colonize farm animals).

Objectives: *to implement and update the collection of reference materials for VTEC detection and typing*.

Expected output: *non-toxigenic variant of VTEC O104:H4 to be used as non-class 3 reference strain.*

Duration: *2012*

6. Inter-laboratory comparison studies

Two studies are planned for 2012: i) a study on the detection of VTEC in water samples; ii) a study on the detection of VTEC in seeds intended for the production of sprouts. The latter will be confirmed and better defined after the release of the EFSA Scientific Opinion on "*The risk posed by Shiga toxin producing E. coli (STEC) and other pathogenic bacteria in seeds and sprouted seeds*", expected before the end of 2011.

A third study for the validation of the method EN/ISO 16654:2001 for the detection of *E. coli O157* in food will be carried out upon availability of CEN funding (see point 4.3).

6.1. Detection of VTEC in water samples

Recent epidemic episodes have brought to the attention the role of water in the transmission of VTEC infections. Irrigation water has been often reported as the source of VTEC contamination of fresh produce involved in outbreaks. Other epidemic episodes have been linked to recreational water. Finally, water is largely used in the production of sprouted seeds, and testing spent irrigation water has been indicated as an alternative or a complement to seed testing. Testing water samples of different origin for the presence of VTEC represents therefore an important challenge for food and public health laboratories. In the absence of specific

international standards, the method developed for the detection of VTEC in foodstuffs can be adapted to this particular matrix.

In particular, the proposed study will consist on the examination of artificially contaminated water samples for the presence of VTEC by using the CEN ISO TS 13136 “Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method”.

Objectives: *i) to further train the NRLs in the use of the international standard for VTEC detection; ii) to test the effectiveness of the detection method when applied to a matrix not included in its field of application*

Expected output: *improved capability of the NRLs to detect and VTEC.*

Duration*: 2012*

6.2. Detection of VTEC in seeds intended for sprout production

Sprouts have been implicated as the source of the large outbreak of VTEC O104:H4 infections recently occurred in Europe. Despite the strong epidemiological evidences on their role, the outbreak strain has never been isolated form neither the sprouts nor the seeds used for their production. The same situation has occurred in other outbreaks, and confirms the difficulties of detecting STEC in seeds.

Seed samples are constituted by particles, which may be singularly contaminated and non-homogeneously distributed within the lots. Moreover, according to the few data available in the literature, the contamination of seeds frequently occurs at very low levels, in terms of cfu/gr. In addition, bacteria on/within seeds may be stressed and therefore exhibit low culturability during the enrichment phase that precedes the PCR tests.

Nonetheless, seeds are considered to be the source of sprout contamination in most of the epidemic episodes described in the literature, and this underlines the importance of laboratory testing of this matrix.

In July 2011, the EU-RL organized a pilot study involving 8 laboratories, using a lot of beet seeds naturally contaminated with a VTEC O74 strain and identified during the investigations on seed samples related with the of VTEC O104:H4 outbreak. Naturally contaminated seeds represent a valuable material for this type of studies,

since artificially contaminated samples could not mimic what really happens in nature (e.g. possible internal contamination of seeds).

However, all the laboratories, despite using the method released by the EU-RL, returned results that did not match those expected on the basis of the previous tests performed at the EU-RL itself. This lack of correspondence likely depended on a low level contamination not homogeneously distributed within the lot. This hypothesis was supported by the results of additional tests performed at the EU-RL and included in the report of the study (at http://www.iss.it/binary/vtec/cont/PT6_Report.pdf).

Based on these results, the option to use the same lots of seed in a larger scale study was discarded and the study would be carried out with artificially contaminated seed samples. In any case, the opportunity to carry out such a study will be considered in the light of the conclusions of the EFSA Scientific Opinion on "*The risk posed by Shiga toxin producing E. coli (STEC) and other pathogenic bacteria in seeds and sprouted seeds*", which is expected to be released before the end of 2011.

7. Research activities on VTEC

The ongoing research studies on VTEC pathogenicity, phylogenesis and molecular epidemiology will be continued in the framework of the numerous scientific collaborations established by the EU-RL VTEC with other EU and non-EU institutions (see point 4). They will represent the basis for improving the detection of these pathogens in their reservoirs and vehicles, as well as for a better understanding of the epidemiology of the infections.

Due to the public health impact of the VTEC O104:H4 outbreak, research will be carried out with the aim of understanding the way this new VTEC clone emerged and made its appearance in Europe. A collaboration with the WHO International *Escherichia* and *Klebsiella* Centre of the Statens Serum Institut in Copenhagen will aim at the collection of the strains of VTEC O104:H4 that have been isolated before the occurrence of the 2011 outbreak, to investigate the relationships among the strains and the evolution of this clonal lineage.

The mechanisms of acquisition of Stx-converting phages by *E. coli* strains belonging to pathogroups other than VTEC, in particular the enteroaggregative *E. coli* (EAEC) and the atypical EPEC (aEPEC), will also be investigated.

Objectives: *to improve the knowledge of: i) the pathogenetic mechanisms of VTEC; ii) the biological bases of the emergence of new pathogenic E. coli clones; iii) to identify candidate molecular targets for the identification and typing of VTEC and other pathogenic E. coli.*

Expected output: *i) scientific publications; ii) development of innovative methodologies for detection and typing of pathogenic E. coli.*

Duration*: ongoing*

8. Missions

The following missions may be needed in 2012:

- Participation of a EU-RL representative in 2 meetings of the Coordination Group of the ECDC Food- and Waterborne Diseases and Zoonoses (FWD) Surveillance Network, presumably in Stockholm.
- Participation of a scientist in the 19th CEN/TC275 WG6 annual plenary meeting (location and dates to be defined).
- Participation of a scientist in the 8th *International VTEC Symposium (VTEC 2012)* that will be held in Amsterdam in May 2012 (http://vtec2012.org), to present the activity of the EU-RL in the field of detection methods.
- A visit to one NRL can be planned for 2012, upon agreement with the EC and the interested country.

Objectives: *to maintain and strengthen the institutional and scientific relationships of the EU-RL.*

Expected output: *i) scientific networking; ii) visibility of the EU-RL activities.*

Duration*: 2012*

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