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The role of European Union Reference Laboratories in food safety crisis: the experience of the EU-RL for *Escherichia coli* during the recent outbreak of *E. coli* O104:H4 infections

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The EU-RL for *Escherichia coli* was thoroughly involved in the recent outbreak of *E. coli* O104:H4 infections occurred in Europe. Based on the activities carried out in the previous year, a method for the detection of the outbreak strain in food was rapidly developed and distributed to the NRL network, together with appropriate reference materials. The EU-RL also performed laboratory testing of different types of samples and organized an inter-laboratory study on the detection of VTEC in seeds used for the production of sprouts. During the entire period of the crisis, it provided continuous scientific and technical support to DG SANCO. Moreover, the EU-RL participated in the inspection mission to Egypt carried out by the Food and Veterinary Office and took part in several working groups and initiatives established by EFSA and/or ECDC. The experience of the *E. coli* O104:H4 outbreak confirmed that the activities of EU-RLs can provide an important contribution to the EC preparedness to face food safety crisis.

The EU Reference Laboratories (EU-RLs) are established and financed by the Directorate General for Health and Consumers (DG Sanco) to support the European Commission (EC) in facing specific food and feed hazards or specific animal diseases, according to the Regulation (EC) No. 882/2004 on official controls. When an EU-RL is established, the Member States (MS) have to designate their own National Reference Laboratory (NRL) on the same hazard, in order to create an EU laboratory network on that topic. The duties and functions of EU-RLs and NRLs are detailed in the Articles 32 and 33 of Reg. (EC) No. 882/2004, respectively. In practical terms, the EU-RLs duties involve: (1) developing reference analytical methods; (2) coordinating the application of such methods by the NRLs, in particular by organizing proficiency tests (PTs); (3) coordinating the network of NRLs, by providing them information on the advances in the field, reference materials, and specific training on analytical methods; and (4) providing scientific and technical assistance to the Commission, in particular to the DG Sanco, and the European Food Safety Authority (EFSA). The NRLs duties include the collaboration with the respective EU-RL, the coordination of the activities of the laboratories involved in official controls in their own country, including the organization of PTs, the dissemination of the information received from the EU-RL, and the scientific and technical assistance to their Authorities. If the resulting laboratory networks comply with those duties, the analytical methods, the reference materials, and the PTs are transferred by the EU-RLs to the NRLs and by the NRIs to the local laboratories involved in official controls. The final aim of these activities is that any foodstuff produced or imported in any MS of the EU is tested using the same state-of-art methods and with comparable levels of proficiency. The added value of such networks can be particularly important in food safety crisis involving different MS, when testing food with standardized, rapid and reliable methods is essential to provide the competent authorities with the data needed to plan appropriate control measures and to inform correctly the consumers.

The aim of this note is to describe the experience of the EU-RL for *Escherichia coli* during the recent outbreak of *E. coli* O104:H4 infections occurred in Europe and to discuss on how the activities carried out by our network of *E. coli* reference laboratories in the previous years may have contributed in terms of EU preparedness to face the crisis.

The EU-RL for *E. coli*

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), and the large number of outbreaks occurring all over the world (Caprioli *et al.,* 2005). Due to this public health relevance, VTEC infections have been included in the list of zoonoses that will receive priority in monitoring schemes according to the Directive 2003/99/EC, and the EC established an EU-RL for VTEC in 2006.

The EU-RL VTEC is housed within the Istituto Superiore di Sanità (ISS) in Rome and its characteristics and activities have already been described in this Journal (Caprioli *et al.,* 2010). The network of NRLs coordinated by the EU-RL VTEC includes all

* The views or positions expressed in this text correspond to the authors and are not, and cannot be regarded as representing the position, the views or the policy of the European Commission.

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the EU Member States and also laboratories of other European countries (www.iss.it/vtec).

The outbreak of *E. coli* O104:H4 and its possible sources

Between May and June 2011, a severe outbreak of bloody diarrhea and HUS associated with infection with an unusual VTEC serotype (*E. coli* O104:H4) occurred in Germany. Numerous cases were reported in other European countries, but most of them had acquired the infection by traveling to Germany. According to the European Centre for Disease Control and Prevention (ECDC), there were more than 4,000 cases, with 46 deaths and 786 cases of HUS (http://ecdc.europa.eu/en/activities/sciadvice/Pages/Epidemiological_Updates.aspx).
The first epidemiologic studies indicated the consumption of mixed salads as the main risk factor for acquiring the infection, and preliminary microbiologic analyses indicated cucumbers imported from Spain as the possible source. However, further analyses did not confirm the presence of the outbreak strain in the cucumbers. At the beginning of June, further epidemiologic studies indicated that the cause of the outbreak was the consumption of sprouts produced by a single German producer. However, all the microbiologic investigations conducted by the German authorities on the sprouts, the seeds used for sprouting, and the plant structures failed to isolate the outbreak strain (Frank *et al.* 2011).
On 24 June, a distinct outbreak due to a VTEC O104:H4 isolate similar to the German epidemic strain was reported in France (Gault *et al.* 2011). The episode involved 16 adult cases and no direct connection with Germany was detected. However, patients had participated in a school party where they ate raw sprouts grown in the school itself by the children. This second outbreak strongly supported the hypothesis that sprouts were the source of the outbreak. The only sprout species grown by both the French school and the German producer was fenugreek, and tracing back analyses coordinated by EFSA indicated an Egyptian farm as the common origin of the seeds (EFSA, 2011a).

The outbreak *E. coli* strain

The outbreak strain belonged to the uncommon serotype O104:H4 and, surprisingly, did not possess the *eae* gene, a genetic marker of the "attaching-effacing" mechanism of adhesion to the intestinal mucosa which is present in the VTEC serogroups most associated with severe human infections. However, VTEC O104:H4 showed the enteroaggregative adhesion, typical of another group of diarrheagenic *E. coli* (Scheutz. *et al.* 2011). The genome sequencing confirmed that the outbreak strain was an enteroaggregative *E. coli* (EAEC) that had acquired a VT converting phage (Mellmann *et al.* 2011). This unusual combination of virulence genes had already been described in a strain of VTEC O111 isolated from an outbreak of HUS (Morabito *et al.* 1998) and could explain the high virulence of VTEC O104:H4.

The role of the EU-RL for *E. coli*

Since 2007, the EU-RL has coordinated the development of an international standard on the detection of VTEC in food and animal feed. The method is based on the Real Time PCR screening of food enrichment cultures and aims at the detection of the VTEC serogroups most associated with severe human infections. It is about to be published as CEN ISO TDS 13136 "Horizontal method for the detection of Shiga toxin-producing *Escherichia coli* (STEC) belonging to O157, O111, O26, O103 and O145 serogroups - Qualitative Method" and has been recommended by EFSA for the detection of VTEC non-O157 in food samples within monitoring programs (EFSA, 2009). The method targets both virulence genes (*vtx*1 and *vtx*2, and *eae*) and serogroup-specific genes, and the EU-RL organized four rounds of PT on its use. Thirty-one NRLs participated in the PTs and received the reference control strains. Reports are available at the EU-RL web site (www.iss.it/vtec).
The first PCR screening step of the CEN ISO TDS 13136 targets a *vtx*2 gene which is also present in the VTEC O104:H4 outbreak strain. Therefore, when the outbreak occurred and testing food for the presence of VTEC O104:H4 was urgently required, nearly all the NRLs already had a method suitable for screening food and excluding the presence of any type of VTEC. However, the following sequential steps of the method could not be applied to *vtx*-positive samples, since the VTEC O104:H4 outbreak strain lacked the *eae* gene, which represents the second target of the method.
Using data present in the literature (Bugarel *et al.* 2010) and DNA sequences available in the gene bank, the EU-RL developed a standard operating procedure specific for the detection of the VTEC O104:H4 outbreak strain in food samples and for the presumptive typing of the isolated strains. According to this procedure, *vtx*-positive samples are subjected to PCRs to identify the genes associated with the O104 (*wzx*O104) and H4 (*fliCH4*) antigens. The method also included detailed protocols for the isolation and the complete characterization of VTEC O104:H4 from the PCR-positive samples. A first draft version of the procedure was released through the EU-RL web site on 27 May, 3 days after the occurrence of the outbreak had been communicated. The method was evaluated on the 1st of June, when a reference VTEC O104:H4 outbreak strain was obtained from the Robert Koch Institute, and a second revision was released on 2 June, after a thorough exchange of ideas with several NRLs.
Using the reference strain, the EU-RL prepared and distributed DNA samples to be used as positive control in the molecular assays for the detection of VTEC O104:H4. Between 3 June and 6 June, these reference DNA preparations were sent to 14 NRLs of EU Member States. In the two following weeks, the samples were provided to other 4 NRLs, to 19 other European laboratories, and to 5 laboratories outside Europe. Moreover, scientists from the NRL of Bulgaria, the NRL of Poland, and from the University of Kafkas, Turkey, visited the EU-RL VTEC in July, to make hands on training in the analytical procedures.
The involvement of sprouts as the possible source of the outbreak raised the need of testing the seeds used for the production of sprouts to be consumed raw. Since seed testing poses technical issues, the EU-RL updated the laboratory procedure for VTEC O104 detection by including an annex specifically dedicated to this issue. The updated procedure was released on 14 June in the EU-RL web site. Moreover, to further support the NRLs of the MS involved in the RASFFs on seeds and to harmonize the laboratory procedures in place in the different laboratories, the EU-RL organized an inter-laboratory study on the detection of VTEC in seeds. The study was conducted in July, it included 8 laboratories and the results are available at the EU-RL website.
During the entire period of the crisis, the EU-RL VTEC provided scientific and technical support to DG SANCO. In particular,

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the EU-RL took part in a working group that also included the SANCO Units C/3 and G/4, EWRS contact points, RASFF contact points, and representatives of ECDC, EFSA, and WHO EURO. The objectives of the group were to provide a regular update of the epidemiological situation and the investigations in the MS and discuss all the possible issues related with the outbreak. The group met regularly by audio-conferences in the period between 26 May and 30 June. Scientists of the EU-RL also participated in the following working groups and initiatives established of EFSA and/or ECDC:

- preparation of the scientific report "Urgent advice on the public health risk of Shiga-toxin producing *Escherichia coli* in fresh vegetables", available at http://www.efsa.europa.eu/en/efsajournal/doc/2274.pdf;
- EFSA taskforce set up to coordinate the tracing back of the seeds involved in the outbreak. The taskforce produced the technical report "Tracing seeds [with a particular emphasis on fenugreek (Trigonella foenum-graecum)] in relation to the O104:H4 Shiga-toxin producing *E. coli* 2011 Outbreak(s)" (EFSA, 2011a);
- joint EFSA/ECDC assessment of the VTEC O104 outbreak, with preparation of a document intended to serve as a general overview and a reference for risk management measures and entitled "STEC O104:H4 2011 Outbreaks in Europe: Taking Stock" (EFSA, 2011b);
- EFSA "*ad hoc*" working group to produce a Scientific Opinion on "The risk posed by Shiga-toxin producing *E. coli* (STEC) and other pathogenic bacteria in seeds and sprouted seeds". The opinion will be published by the end of November 2011.

The EU-RL also participated in an inspection mission to Egypt carried out by the Food and Veterinary Office (FVO) to complete the traceback exercise of the imported lots of fenugreek seeds and to assess the production systems in place, including the laboratory controls. The mission was carried out in August and the report is available at http://ec.europa.eu/food/fvo/index_en.cfm?reptoshow=3.

Finally, the EU-RL was requested to perform laboratory testing of food samples and bacterial cultures from Germany and of samples of seeds from Egypt.

Reflections on the events

The VTEC O104:H4 outbreak strain presented an unusual combination of virulence genes, conferring an elevated degree of virulence. This confirms that recombination events among enteric bacteria can generate new phenotypes that, once introduced in a susceptible population, may pose serious public health problems.

The episode confirmed that the contamination of vegetal food chains with VTEC may result in large, severe outbreaks.

Beside the human costs, this outbreak caused a dramatic reduction of the consumer confidence in vegetable consumption, a drop of the vegetable market as a whole, and trade disputes between the EU and third countries, such us the Russian Federation and Egypt for the export and the import of vegetables, respectively.

When the outbreak occurred and testing food was urgently required, the EU network of *E. coli* reference laboratories already had a suitable screening method, tested by several rounds of PT. The method was rapidly modified to become specific for VTEC O104:H4 and the related reference materials were distributed to the NRLs.

The capacity of a laboratory network to provide prompt and effective response to emergencies strictly depends on the activities carried out before they may occur, in particular sharing methods and reference materials and participating in inter-laboratory studies.

The activities of EU-RLs can provide an important contribution to the EC preparedness to face food safety crisis.

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