Comparison of two methods for the detection of verotoxin-producing *E. coli* in human faecal samples during an outbreak of HUS in Apulia, Southern Italy

Rosa Monno[1], Antonio Parisi[2], Cosimo Montagna[2], Nicoletta Annamaria Addante[2], Carmen Capolongo[1], Elena Baglivo[1], Giuseppe Miragliotta[1]

[1]Faculty of Medicine, University of Bari, Italy;
[2]Istituto Zooprofilattico della Puglia e della Basilicata, Putignano, Italy

SUMMARY

This study evaluated the diagnostic performances of an ELISA method and a molecular method for the detection of verotoxin in faecal samples during an outbreak of haemolytic-uraemic syndrome (HUS) occurring in Apulia, Southern Italy. Two of the 16 faecal samples were positive for verotoxin when analysed by ELISA and resulted PCR positive for stx1, stx2, eaeA and serogroup O26. The other 14 faecal samples resulted negative with both tests. The detection of verotoxin in faecal samples by ELISA is a simple, sensitive, specific and rapid method (2 hours) of considerable utility for routine clinical testing laboratories without access to more specialized diagnostic procedures.

KEY WORDS: VTEC, HUS, ELISA.

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Enterohaemorrhagic *E. coli* (EHEC) is a microorganism that can cause severe food-borne disease. EHEC is able to produce two powerful toxins called verotoxin (VT) or shiga-like toxins (SLT), and is therefore also reported as verotoxigenic *E. coli* (VTEC). These toxins inhibit the protein synthesis of the endothelial cells (Müthing *et al.,* 2009). EHEC is responsible for haemorrhagic colitis and haemolytic-uraemic syndrome (HUS), a severe syndrome active especially in children under five years of age occurring in 5-10% of EHEC infections (Karmali M.A. *et al.,* 2010). HUS is characterized by acute renal failure, haemolytic anaemia and thrombocytopenia (Webster *et al.,* 2014). In Europe, VTEC infection is reported with variable frequency and several serotypes are involved. In the past the serovar of *E. coli* capable of producing the toxins was thought to be only O157:H7. However, recently, other serovars such as O26, O111, O103, O145, O91 have been found to produce one or both of the verotoxins stx1 and stx2 (Gould *et al.,* 2013; Frank *et al.,* 2011). Primary sources of VTEC infections are raw or undercooked ground-meat products, raw milk, dairy products, vegetables and sprouts contaminated by manure or water used for irrigation. VTEC infections are more frequent in the warmer months (Gyle, 2007). In Italy a HUS Surveillance System is actively coordinated by the National Laboratory at the Istituto Superiore di Sanità (ISS), Rome, Italy. This Surveillance System normally finds 2-5 cases/year in Apulia, Southern Italy. However in the period June-September 2013, 21 cases of HUS were notified to the ISS by the Italian Society of Pediatric Nephrology of Apulia. Studies carried out at the National Laboratory of the ISS for *E. coli* revealed that 19 of these 21 patients (11 months - 15 years old; M/F: 12/7) had

Corresponding author
Rosa Monno
Department of Internal Medicine
and Public Health Hygiene Section
University of Bari Policlinico
Piazza G.Cesare 11 - 70124 Bari, Italy
E-mail: r.monno@igiene-seconda.uniba.it

microbiological and/or serological evidence of VTEC O26 infection (Dr Alfredo Caprioli, personal communication).

The purpose of this work is to compare the diagnostic performances of an ELISA method and a molecular method for the detection of verotoxin in human faecal samples.

In the period 20 July 2013 - 10 September 2013 the Microbiology and Virology Laboratory of the University Hospital Policlinico of Bari in Southern Italy received a total of 16 faecal samples from several units of the same Hospital. Seven were from children with suspected HUS, eight from adults suffering from dysentery and there was one adult case of suspected HUS. In addition further 20 faecal samples collected from patients with enteritis (10) and from an asymptomatic subject (10) were analysed.

The detection of *E. coli* verotoxin was performed by an ELISA method (E. coli Vero (Shiga) Toxin Stool Antigen Detection assay, IVD Research Inc. Alifax, Padova, Italy). The assay was carried out according to manufacturer's instructions.

In addition, all the faecal samples tested by ELISA assay were sent to be analysed by molecular methods at the Istituto Zooprofilattico della Puglia e Basilicata, Putignano, Italy (a public veterinary Institute which conducts prevention, control and research activities in animal health and welfare, food safety and environmental protection). For molecular analysis, faecal samples were inoculated in 10 ml of modified Tryptone Soy Broth (m-TSB, Oxoid, UK) supplemented with novobiocin 20 mg/ml and incubated for 18-24 h at 42°C. After incubation, all the cultures were tested by real-time PCR for the Shiga toxin genes (Sxt1 and Sxt2) and eaeA. DNA extracts were obtained using Chelex 100 according to manufacturer's instructions (Bio-Rad, Milan, Italy). Real-time PCR was performed according to ISO13136-2012 (Anonymous, 2012). Samples positive for sxt genes were screened by real-time PCR for the detection of O157, O111, O26, O103 and O145 serogroup-specific genes. Samples yielding positive results were enriched using specific serogroup Dynabeads immunomagnetic separation and streaked on Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC, Oxoid, UK). Plates were incubated at 37°C for 18-24 h. Up to 50 colonies were selected and tested by real-time PCR for the presence of the serogroup and virulence specific genes. After isolation, the selected positive colonies were biochemically identified using API 20E system (BioMérieux, Italy) and then subjected to slide agglutination with Dryspot *E. coli* Serocheck, Oxoid, Milan, Italy.

Minimum inhibitory concentrations (MICs) were determined using the E-test methodology (AB Biodisk, Solna, Sweden), commercially distributed by Biomérieux (Biomérieux, Florence, Italy) and for some antibiotics by disk diffusion method.

By ELISA 14 of the 16 faecal samples tested negative for *E. coli* verotoxin whereas two samples resulted positive. These two samples were from children with confirmed HUS. The further 20 faecal samples analysed were negative with both tests.

The two VTEC positive samples by ELISA also resulted positive for Stx2, eaeA and for serogroup O26 by molecular methods. From these two PCR positive samples, *E. coli* O26, the same serotype responsible for the outbreak of HUS, was isolated by a serogroup-specific enrichment protocol. All the faecal samples found to be negative by ELISA also tested negative by molecular methods, except for one sample that was positive only for the eaeA gene, suggesting the presence of an enteropathogen *E. coli* (EPEC). Therefore the sensitivity and specificity of ELISA assay was 100%.

The susceptibility test, performed by E-Test on the two *E. coli* O26 isolates, showed the following MIC values expressed as mg/L for strain 1 and strain 2 respectively: amoxicillin 6 and 4; amoxicillin-clavulanate 4 and 3; Piperacillin-tazobactam 0.75 and 0.75; Ceftriaxone 0.064 and 0.032; Imipenem 0.125 and 0.19; Meropenem 0.023 and 0.023; Ciprofloxacin 0.023 and 0.032; Levofloxacin 0.023 and 0.032; Amikacin 2 and 1.5; Gentamicin 0.38 and 0.38; Tigecycline 0.064 and 0.094; Colistin 0.25 and 0.125; Fosfomycin 0.25 and 0.38; Trimethroprim-sulfamethoxazole 0.64 and 0.47; Piperacillin-tazobactam 1.5 and 1.5; Gentamicin 1.5 and 0.5.

The susceptibility tests for cefoxitin, cefpodoxime, ceftazidime, aztreonam and chloranphenicol were performed using the disk diffusion method and the two strains of *E. coli* O26 were

fully susceptible to all the antibiotics tested with this method according to EUCAST 2014. Both strains exhibited high MIC values only to rifampicin (12 mg/L and 16 mg/L) and tetracycline (8 mg/L and 4 mg/L).

The pathogenicity of VTEC is enhanced by plasmid-encoded virulence factors and a pathogenicity island with more than 40 genes known as the locus of enterocyte effacement. In particular, the eae gene (*E. coli* attaching and effacing) encoding for the adherence factor intimin is localized on this pathogenicity island and is often found in highly pathogenic EHEC strains (Ho N.K. *et al.,* 2013). This gene was found in the two strains of *E. coli* O26 isolated during the outbreak occurring in our area in the period July-September 2014, confirming their pathogenicity.

In Apulia, Southern Italy, 2-5 cases/year of HUS have been reported (ISS, personal communication). During the last outbreak of HUS, no source of the *E. coli* responsible for the cases of HUS was identified, even though extensive searches were carried out for the bacterium in various foods and water environments. Recently, in our area of Southern Italy, *E. coli* O26 was detected in 0.6% of the samples of raw water-buffalo milk examined (Lorusso *et al.,* 2009). Susceptibility tests have shown that the two strains of *E. coli* O26 were fully susceptible to all the antibiotics tested. High MIC values were found for tetracycline and rifampicin, but EUCAST does not report the relative breakpoint.

Several molecular methods have been proposed for the rapid detection of *E. coli* O26 VTEC from foods and for the rapid identification and characterization of both clinical and environmental isolates (Lorusso *et al.,* 2011; Burgos, 2012).

Even if referring to a very small number of patients, verotoxin detection on faecal samples performed by ELISA is a simple, sensitive (100%), specific (100%) and cost effective tool for a rapid diagnosis (2 h) of VTEC infection. This assay is of considerable utility for routine clinical laboratories without access to more specialized diagnostic procedures, particularly for the detection of non O157 VTEC. However molecular methods, which are labour intensive and require highly skilled staff, might be used for further information on the type, presence of other virulence genes, and serogroups of VTEC present in the faecal samples, especially during outbreaks. Prompt reporting to the health authorities of any suspected VTEC/EHEC following positive ELISA screening is essential for the diagnosis of infections caused by this microorganism.

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