

Detection of *Escherichia coli* O157 in raw and cooked meat: comparison of conventional direct culture method and Enzyme Linked Fluorescent Assay (ELFA)

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Abstract

Background: Verocytotoxin *Escherichia coli* is a frequent and important cause of diarrhea and haemolytic uremic syndrome all over the world. Consumption of ground beef, lettuce, and other kinds of food have been associated with outbreaks,

The aim of this study was to detect the presence of *E. coli* O157 in meat products collected from hospital food catering services in Rome, using a rapid detection method in comparison with a standard culture method to verify the effectiveness of HACCP system.

Methods: Three hundred and ten food samples (80 of cooked and 230 of raw meat) were screened for *E.coli* O157 by ISO culture method and by enzyme-linked-fluorescent-assay (ELFA)-based methods (VIDAS®system, bioMérieux). All isolates obtained were tested for VT1 and VT2 genes by PCR. The statistical analysis considered absolute frequencies and percentages. The K statistic was applied to assess agreement between direct culture method and the VIDAS system.

Results: A total of 6 (1,9%) *E.coli* O157 isolates were recovered from raw meat samples by the culture method; of these only four were identified by PCR as VTEC producers. A total of 9 (2,9%) *E.coli* O157 isolates were recovered from raw meat samples by the VIDAS® system. No *E.coli* O157 was detected in cooked products. All comparisons between the direct culture method and the VIDAS system were statistically significant ($K= 0,795$; $p<0.001$).

Conclusions: The present study showed how ELFA-based methods are highly specific and rapid for the detection of *E.coli* O157 in food samples compared with the direct culture method. ELFA method is useful to verify the effectiveness of the HACCP system in the risk management of potential contaminating hazards during the preparation of foods for susceptible persons.

Key words: VTEC O157, ELFA, PCR, hospital food service

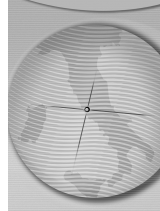
Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC) O157 is a globally important pathogen frequently associated with haemolytic uremic syndrome (HUS) requiring dialysis and transfusion, chronic kidney failure or neurological impairment [1].

E. coli O157 is also associated with diarrhoeal diseases together with other pathogens like *Campylobacter* and *Norovirus*, but it has not yet been included in large epidemiological surveillance programmes and could be implicated in the significant increase of diarrhoeal diseases recorded over recent years in Italy [2]. Nevertheless, in recent years, though the European Union has taken giant steps with regards to food safety through the significant evolution of the EU Food Law [3],

foodborne diseases remain a major problem and one of public health concern. Epidemiological data show an increasing incidence of infectious diarrhea in Italy from 1993 to 2006 (from 2.32 per population of 100,000 in 1993 to 5.01 per population of 100,000 in 2006) [4,5].

E. coli O157 belongs to the group of enterohaemorrhagic *E.coli* (EHEC) that expresses its pathogenicity with the production of one or more Shiga-like toxins, also called verocytotoxins (VT1, VT2), and with the presence of several accessory virulence genes acquired by horizontal gene transfer. VTEC that cause disease in humans belong to a restricted number of serogroups: O157:H7 is the most prevalent group followed by a few



other serogroups, such as O26, O111, O103, O145 and O121 [2,6].

Surveillance data indicate that the VTEC O157 infection is evolving [7]. Since the first description of this illness in the USA in 1982 [8], the geographic range of the organism is growing and the pattern of disease transmission is changing. As a matter of fact, outbreaks have been reported in Croatia [9], Turkey [10], Italy [11], Scotland [12], Wales [13], Japan [14] and recently, in Europe [7], in Canada [15], in Scotland [16], one other in Japan [17] and two outbreaks in USA [18].

In Italy, a national surveillance system for HUS in pediatric patients has been operating since 1988, with a total of 439 HUS cases from 1988 to 2004 [19]. The mean annual incidence of HUS in persons aged between 0 and 14 years from 1988 to 2006 was 0.3 per 100,000 population [20], lower than in other European countries [21- 23]. Between 2005 and 2008, 158 HUS clinical cases were confirmed in pediatric patients (age range from 4 months to 15 years) from 18 Italian Regions [24].

Outbreaks have been associated mainly with the consumption of ground beef [6] but also with a wide variety of foods including unpasteurized milk [25], unrefrigerated sandwiches [26], potatoes [27], spinach [28], lettuce [29], unpasteurized apple cider [30], drinking water [31] and bovine and broil meat and products thereof [7]. Person-to-person transmission was documented too [32]. An incident involving tap water, including well water was recently notified as the origin of four European outbreaks [7].

Data from investigations in the United States suggest that foodborne outbreaks from 1982 to 2002 occurred most frequently in communities (29%), restaurants (28%) and schools (9%) [33], but reports of outbreaks of VTEC infection in hospital settings are unusual [34,35]. Outbreaks also occurred in Canada both in the community and in day care [36]. A foodborne outbreak caused by VTEC O157 in a nursing home occurred in Scotland in 1996 and involved 501 cases, with 27 HUS cases and 21 fatal cases [37]. The majority of haemolytic uremic syndrome cases were in children 0-4 years old and were mostly associated with serogroup O157 [38]. In Italy too, outbreaks were reported in 1992 (nine cases caused by serotype O111), in 1993 (fifteen cases determined by serotype O157) [11] and in 1997 (three cases caused by serotype O26) [19]. Between 1988 and the 2004, 17 family clusters of infection with VTEC have been recovered [19].

In 2007, fourteen European Union Member States reported a total of 65 food-borne outbreaks of human pathogenic *E. coli*, which constituted

1.2% of the total number of reported food-borne outbreaks in the EU and which increased by 38.3% compared to 2006 [7].

The largest number of human cases (39.4%) originated from outbreaks associated to the catering services or restaurants. In 2007, France and Germany accounted for 44.6% of pathogenic *E. coli* outbreaks. The overall reporting rate in the EU was 0.02 per population of 100,000, ranging from 0.01 per 100,000 in Poland and Spain to 0.49 per 100,000 in Malta. The Settings where outbreaks were caused by pathogenic *E. coli* in Europe mainly involved households, catering services and restaurants and one outbreak was described in a hospital setting also [7].

Even today, *Escherichia coli* O157 remains a Public Health concern as indicated by the alert and the information contained in RASFF portal 2009 and, recently, the information related to the presence of this pathogen in beef hamburgers from Austria, in September 2010 [39].

The aim of this study was to determine the presence and prevalence of VTEC O157 in raw and cooked meat product samples coming from a hospital conventionalised hospital food catering service in Rome using an enzyme-linked-fluorescent-assay (ELFA)-based method in comparison with the standard culture method, in order to verify the risk management of VTEC using the Hazard Analysis and Critical Control Point (HACCP) system implemented and applied to the hospital conventionalised food catering service.

Materials and methods

Sample collection

During the period 2002 - 2004, 310 meat products (raw and cooked meat) were collected from a hospital conventionalised food catering service in Rome that produces 1600 meals a day (no. 1600/day products).

Samples of raw meat, of about 100 grams each, were collected from the refrigerators of the butcher department that served the hospital and samples of cooked meat were collected from the hospital's kitchen just after cooking. All samples (94 of raw minced, 56 of raw hamburger, 48 of raw meatball, 32 of raw meatloaf, 48 of cooked meatball and 32 of cooked meatloaf) were transported to the laboratory at 4°C and tested for *E.coli* O157.

The meatball and meatloaf samples were analyzed before and after cooking. A detailed list of the investigated samples is presented in Table 1.

All samples were analysed for *E. coli* O157 by ISO direct culture method and by enzyme-linked-fluorescent-assay (ELFA)-based methods (VIDAS®system, bioMérieux).

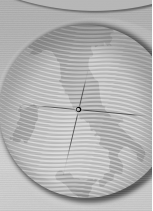


Table 1. Sample origin.

<i>Specimen</i>	<i>Number</i>	<i>%</i>
Raw minced	94	30,32
Raw hamburger	56	18,06
Raw meatball	48	15,48
Raw meatloaf	32	10,32
Cooked meatball	48	15,50
Cooked meatloaf	32	10,32
Total	310	100,00

All isolates obtained were tested for VT1 and VT2 genes by PCR method.

Direct culture method

E. coli O157 detection and identification was determined by the reference method described by International Organization for Standardization ISO 16654:2001 [40]. Briefly, 25 g of each sample was diluted in 225 ml of Modified Tryptone Soya broth (mTSB - Oxoid, UK) added with Novobiocin, homogenized for 2 min at 260 rpm using a Stomacher (Model 400 circulator, Seward, Norfolk, England) and incubated for 18-24h at 41.5°C according to ISO method, as well as the remaining steps. After enrichment and immunomagnetic concentration steps, the selective and differential isolation of enterohemorrhagic *E. coli* O157 was carried out on MacConkey Agar with Sorbitol, Cefixime, and Tellurite (CT-SMAC - Oxoid, UK).

Enzyme Linked Fluorescent Assay (ELFA)

After 6-7 h of incubation, one milliliter of mTSB was added to 9 ml of MacConkey broth (Oxoid, UK) containing cefixime (0,05 mg l⁻¹) and potassium tellurite (2,5 mg l⁻¹) and incubated at 35-37°C for 18 hours. This enrichment step constituted the first stage of the VIDAS®system procedures ELFA-based methods. All of the other steps were performed automatically by VIDAS®system instrument, as described by the manufacturer's instructions (bioMérieux, France) [41].

PCR assay

One ml of an 18-h mTSB culture was used for the DNA template prepared by the boiling method [42]. DNA samples were amplified by PCR for the genes encoding verotoxins 1 and 2 as previously described by Pollard DR *et al.* [43]. The primer's sequences were:

VT1a 5'-GAAGAGTCCGTGGGATTACG -3' (location within the gene: 1191-1210, 130 bp); VT1b 5'-AGCGATGCAGCTATTAATAA-3' (location within the gene: 1301-1320, 130 bp); VT2a 5'-TTAACCACACCCACGGCAGT-3' (location within the gene: 426-445, 346 bp); VT2b 5'-GCTCTGGATGCATCTCTGGT-3' (location within the gene: 752-771, 346 bp).

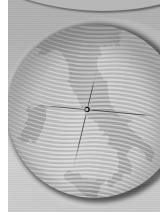
PCR detection was performed with the GeneAmp PCR System 9600 (Perkin-Elmer-Applied Biosystems GmbH, Weiterstadt, Germany). The PCR products were analyzed by ethidium bromide-stained agarose gels. Molecular size markers (100-bp ladder; GIBCO-BRL) were included in each gel.

A reagent blank containing all the components of the reaction mixture, with the exception of template nucleic acid (which was substituted with sterile distilled water), was included in every PCR procedure. The specificity of the PCR protocol was evaluated using the positive control strain (*E. coli* O157:H7 - EDL 933; VT1+, VT2+, *eae*+) provided by the Italian National Institute of Health-ISS of Rome-Italy.

Statistical analysis

Considering a total of 1.752.000 meals (1600 meals a day, for three years, for 365 days every year), and a set level of significance at 5%, we defined an expected prevalence of VTEC O157 in raw and cooked meat of 2% (with the worst acceptable result being 0.4%), and in order to ensure an adequate collection of data, the sample size calculation indicated that we had to collect 294 samples. Considering scientific evidence [2] about the low prevalence of *E. coli* O157 in food, we extended the sampling to 5%.

The aim was to describe the results obtained by the two different detection methods applied for *E. coli* O157, the direct culture and VIDAS®system



ELFA-based method, which detect absolute frequencies and percentages.

The Kappa statistic was applied to measure the agreement for two qualitative variables, in particular the agreement between the direct culture method versus VIDAS®system ELFA-based method, and the interpretation of the coefficient is evaluated using Landis and Koch's table [44]. The level of significance was set at $p < 0.05$. Analyses were performed using the statistical software SPSS12.00 for Windows.

Results

No *E. coli* O157 were detected from the eighty cooked meat samples both with direct culture and VIDAS®system ELFA-based method.

A total of six out of 230 raw meat samples (2,61 %) were positive for *E. coli* O157 with the direct culture method and serologically confirmed by Italian National Institute of Health (ISS) of Rome-Italy. The presence of *E. coli* O157 in these samples was also detected by VIDAS®system

ELFA-based method (Table 2).

A total of nine out of 230 raw meat samples (3,91%) were *E. coli* O157 positives by VIDAS®system ELFA-based method (Table 2).

The comparative efficacy of two methods (ISO direct culture and ELFA methods) for recovery of *E. coli* O157 from raw and cooked meat is shown in Table 2. The majority of *E. coli* O157 were recovered by VIDAS®system ELFA-based method (sensitivity 100% and specificity 99%, in comparison to ISO direct culture).

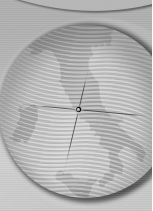
The agreement in the total sample and in the subgroups between the direct culture method and VIDAS®system ELFA-based method is summarized in Table 2. All comparisons were significant ($p < 0.001$) and elevated ($k > 0.60$), in particular with data that referred to "raw hamburger" samples where $K=1$.

The identification of VT1/VT2 *E. coli* O157 producers by PCR was performed on all positive VTEC samples obtained by direct culture method. Only four out of six *E. coli* O157 were identified

Table 2. Comparative isolation of *E. coli* O157 by different methods.

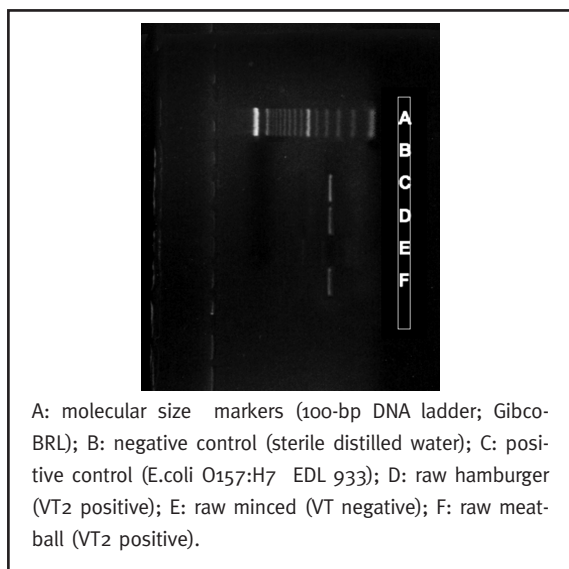
Specimen	No.	<i>E. coli</i> O157 positive (%)		K-test		
		Culture	VIDAS	K	p	Landis and Koch's interpretation
Raw minced	94	2 ^a (2,12)	4 (4,25)	0.657	<0.001	Substantial agreement
Raw hamburger	56	2 (3,57)	2 (3,57)	1.000	<0.001	Almost perfect agreement
Raw meatball	48	2 ^b (4,16)	3 (6,25)	0,789	<0.001	Substantial agreement
Cooked meatball	48	0 (0,00)	0 (0,00)	n.c.		
Meatball	96	2 (2,08)	3(3,13)	0.795	<0.001	Substantial agreement
Raw meatloaf	32	0 (0,00)	0 (0,00)	n.c.		
Cooked meatloaf	32	0 (0,00)	0 (0,00)	n.c.		
Meatloaf	64	0 (0,00)	0 (0,00)	n.c.		
All raw meat	230	6 (2,61)	9 (3,91)	0.794	<0.001	Substantial agreement
Total	310	6 (1,93)	9 (2,90)	0.795	<0.001	Substantial agreement

^aPCR VT2 positive; ^bPCR VT1 positive; n.c. The Kappa-test is not computable in this subgroup because the culture or/and VIDAS are constant.



by PCR as VTEC producers of which two VT1 positives from raw meatballs and two VT2 positives from raw mince (Table 2 and Figure 1).

Figure 1. Amplification products obtained by PCR of VT2 gene analyzed by agarose (2%) gel electrophoresis.



Discussion

In this study, two detection methods were comparatively evaluated for the identification of VTEC O157 in meat samples: the direct culture (ISO) method and the enzyme-linked-fluorescent-assay (ELFA)-based method [35]. A molecular PCR method was also applied to the isolates in order to differentiate the *E. coli* O157 VT1 e VT2 producers.

E. coli O157 was isolated by the VIDAS® system ELFA-based method from nine samples (9/310 = 2,9%) compared with six confirmed by culture positives samples (6/310 = 1,9%) with a sensitivity of 100% and specificity of 99% and all comparisons were significant ($p < 0.001$) and elevated ($k > 0.60$). Our low percentage of *E. coli* O157 recovery is consistent with other Italian reports [45465-47] and European studies [4849-50], while the prevalence of *E. coli* O157 reported in USA is higher than other countries [33].

The VIDAS® system ELFA-based method had already shown, in other studies, a higher efficiency and rapidity in recovering *E. coli* O157 in food samples compared to ISO direct culture detection [5123-54].

However, as already highlighted by one investigation [55], standard microbiological methods used to control the putative presence of pathogenic bacteria require a long period to be performed (up to 72 hours) and many materials and equipment. Moreover, a small amount of food samples can be analysed (about 0.1 g) that may

not be sufficient to represent the actual extent of bacterial contamination of food. Therefore, the development of rapid, reliable, sensitive and easy-to-use methods for counting bacteria in food samples should be desirable.

It is our opinion that the VIDAS® system ELFA-based method proves to be a reliable alternative to conventional direct culture ISO methods for screening a large number of food samples received through hospital food catering services and a valuable tool for the management of VTEC risk, even if it is more expensive and gives positive results that have to be confirmed by culture.

The absence of *E. coli* O157 in all cooked meat, especially those derived from contaminated raw meat, also gives evidence of the Good Manufacturing Practice as well as the effectiveness of internal HACCP plan to prevent risk of *E. coli* O157 contamination.

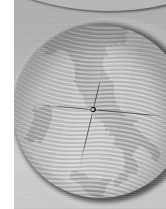
The VIDAS® system ELFA-based method and direct culture method used in this study to detect *E. coli* O157 strains in foods are not amenable for the detection of verocytotoxin producers, while primers used for amplification of VT1 and VT2 genes allow us to differentiate the VTEC strains. In addition, the PCR technology allows large-scale screening of many bacterial colonies isolated from contaminated foods. Our data, together with other published reports [56, 57], indicates that handled raw meat is a critical foodstuff, especially in hospital conventionalised food catering services, and a critical control point for possible cross-contaminations.

In Italy, since 1997 the HACCP method is a mandatory legal preventive method [58] in all food industries, hospital catering services included and, recently, reinforced by Reg. 852/2004/EC [59] which underlines and confirms the importance of the HACCP strategy in primary production too.

A risk analysis through the rapid detection of foodborne pathogens is needed to verify the efficacy of the HACCP system.

The identification of VTEC is troublesome since they are phenotypically indistinguishable from commensal *E. coli* strains, and the demonstration of the verocytotoxin production is not sufficient to assess its pathogenic potential. The most common approach for the detection of this pathogen relies on the simultaneous detection of a number of different genetic determinants including either virulence genes or serogroup associated genes, usually by real time PCR involving the Taqman technology [60].

The future of epidemiology and prevention of VTEC includes: a) improving the surveillance



by transferring serotyping techniques of VTEC to the public health and clinical laboratories; b) developing an Integrated Disease Surveillance system; c) using PCR methods for rapid detection of VTEC pathogen strains in foods and clinical specimens because of their high sensitivity and the presumption that an infectious dose lower than 10 colony forming unit for gram of contaminated food has been described for VTEC; d) developing farm and slaughterhouse-

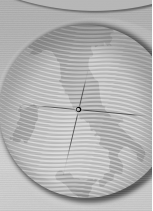
based methods to decrease cross-contamination of meat; e) encouraging the application and implementation of HACCP methods to educate community (schools, care centres etc.), catering services and household consumers to prevent cross-contamination, to cook meat thoroughly and to respect the hot-hold link; f) involving clinical laboratories to screen for O157, O26, O111, O103 also and for other emerging serotypes.

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