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A new integrated approach to analyze bulk tank milk and raw milk filters for the presence of the *E. coli* serogroups frequently associated with VTEC status

Francesca Albonico, Claudia Gusmara, Theo Gugliotta, Monica Loiacono, Michele Mortarino, Alfonso Zecconi

Department of Veterinary Medicine, Università degli Studi di Milano, via Celoria 10, 20133 Milan, Italy

Corresponding author: Alfonso Zecconi

Department of Veterinary Medicine

Università degli Studi di Milano Via Celoria 10, 20133 Milan, Italy

Phone: +390250318073

Fax: +390250318079

Mail: alfonso.zecconi@unimi.it

Abstract

We optimized a combination of microbiological and molecular methods to quickly identify the presence of the O157 and the six non-O157 serogroups (O26, O45, O103, O111, O121 and O145) most frequently associated with VTEC status, at herd level. The lower detection limit of this methodology is 10^1 CFU/ml for each of the serogroups tested.

We tested 67 bulk tank milk (BTM) and raw milk filters (RMF) derived from dairy herds located in Lombardy and Trentino Alto Adige. We identified 3 positive samples and 20 positive samples out of 67 respectively in the BTM and RMF. Interestingly, several samples showed positivity for more than one serogroups at the same time. We also identified the presence of *E.coli* O45 and O121 for the first time in raw milk and raw milk filters. Once screened the seven serogroups of interest in our

samples, we evaluated the real pathogenicity of our positive, non-O157 samples through two parallel molecular biology methods: virulence gene research by PCR, and HRMA and sequencing. The most frequently isolated serogroups in milk were O157 (2.64%), O103 (2.11%), and O145 (1.06%), while in RMF the frequencies were, respectively 14.92%, 4.48%, and 2.98%. Moreover, this is the first published report in Italy of positive recovery of O45 and O121 serogroups in milk and milk filters. The new diagnostic approach proposed investigate the presence of the O157 and big six non-O157 serogroups at farm level and not to identify VTEC hazard only once the product is processed and/or is ready to be consumed.

1. Introduction

Shiga toxins- or verotoxin- producing *E. coli* (VTEC), are a group *E. coli* causing severe illnesses in humans, and acquired significance for the food industry during the last 30 years (EFSA 2015; Caprioli et al., 2014; Pexara et al., 2012). Typically, the easy transmission and its very low infectious dose (<10 cells), emphasize the importance of this bacterium as a foodborne pathogen that has been correlated with human diseases (Etcheverría and Padola, 2013; Pearce et al., 2004; Willshaw et al., 1994).

More than 600 VTEC serotypes have been identified (Pexara et al., 2012); nevertheless, only a small number of serotypes are more often correlated with severe disease in humans. Besides O157:H7, the most well-known and widespread VTEC, Centers for Disease Control and Prevention (CDC) has identified other six O groups recognized as a growing concern for public health and responsible for 71% of all illnesses caused by VTEC: O26, O45, O103, O111, O121 and O145 (Brooks et al., 2005; Rangel et al., 2005; Wasilenko et al., 2012).

The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks showed as the most commonly reported VTEC serogroup in 2014 was O157, (46.3% of cases with known serogroup) followed by O26, O103 and O145 serogroups.

Outbreaks are generally ascribed to the consuming of contaminated meat or milk and dairy products, in particular those derived from cattle. Moreover, the direct contact with ruminant in farms and/or the consumption of water, unpasteurized drinks and vegetables contaminated with ruminant faeces are often involved in outbreaks (Armstrong et al., 1996; Caprioli et al., 2005; Cody et al., 1999; Hilborn et al., 1999; Olsen et al., 2002).

VTEC strains are the only group of pathogenic *E. coli* described with zoonotic potential, and ruminants are recognized as the principal reservoir for human infections. Cattle, in particular, have been identified as the most important source of human infections, harbouring the organisms as a transient member of their normal gut micro flora, at anorectal junction level (Caprioli et al., 2014; Caprioli et al., 2005; Ferens and Hovde, 2011). The faecal excretion of VTEC by ruminants appears to be sporadic, but in some cases, it can persist for several months (Geue et al, 2009; Gyles, 2007; Heuvelink et al, 1999). The excretion amount varies for several reasons, including season (Berry and Wells, 2010), age (Hussein and Sakuma, 2005), nutrition, type of housing (Jacob et al., 2009), stress (Rostagno, 2009), the number of animals in a farm (Erilsson et al, 2005), animal health status (Byrne et al, 2003) and the geographical area in which the farm is located (LeJeune and Wetzel, 2007). Finally, among the same herd, excretion varies on individual bases and some cows can be considered "strong excretory" for VTEC (Chase-Topping et al., 2008).

Raw milk and raw milk-products, are among the main food sources of VTEC infection in humans; therefore, identification of pathogens at herd level is of primary importance in public health. Pathogen detection can be performed on bulk tank milk (BTM) and raw milk filters (RMF). These latter ones are components of milking machines aimed to catch debris as well as faeces particles. In the RMF, the dimension of the pore, usually 100-150 μm, are too big to prevent pathogenic bacteria to be retained by the filter. For this reason, positive raw milk filter indicates that the raw bulk tank milk were contaminated, and raw milk filter analysis to identify pathogens has been reported in several investigations (Giacometti et al., 2012; Murphy et al., 2005; Van Kessel et al., 2011).

Serotype O157: H7 is the most common cause of diseases and epidemics VTEC in humans, which is why the methods of detection and isolation, both microbiological and biomolecular, are well developed and widely available for routine analysis (Osek 2001; West et al., 2003). However, the increasing importance of the not-O157 serogroups as the cause of outbreaks in humans induced the development of several selective microbiological and molecular biological methods to investigate VTEC presence in food, even if they are seldom applied to identify the presence of VTEC at the primary production level.

Indeed, if the identification of *E.coli* O157:H7 is relatively easy with the available microbiological techniques, the identification of other serogroups is not equally easy, and even more difficult when VTEC concentration is low.

In the present study, a combination of microbiological and molecular methods was optimized for the identification, at herd level, of the O157 and the most important non-O157 serogroups (O26, O45, O103, O111, O121, and O145) in BTM and RMF as well as the assessment of their VTEC status.

2. Materials and methods

2.1 Sampling collection

Sixtyseven BTM samples, and the corresponding 67 RMF samples, have been collected. These samples were from dairy herd located in Lombardy (n=47), and in Trentino Alto Adige (n=20). The BTM samples were collected at the end of the milking, while RMF were obtained at the end of milking session just before the cleaning and disinfection procedures. All the samples were stored at 4°C and delivered refrigerated (4°C) to Department of Veterinary Medicine of University of Milan within eight hours from collection. Once arrived in the laboratory, the samples were frozen at -80°C until processed.

2.2. *E. coli* VTEC reference strains

Strains of *E. coli* serogroups O157, O26, O45, O103, O111, O121, and O145, provided from European Union Reference Laboratory VTEC (ISS Rome, Italy), were cultured and used in the subsequent analysis as positive controls, to determine the low level of sensitivity of the analytical protocol.

2.3 *E. coli* isolation with VIDAS® UP ESPT

To isolate *E. coli* strains belonging to the serotypes of our interest the VIDAS® and VIDAS® UP ESPT kit tools were utilized. These tools enable us to perform an enzyme immunoassay using a cocktail of phage proteins directed against *E. coli* O157, O26, O45, O103, O111, O121, and O145 strains. The VIDAS® ESPT kit allows the use of two protocols: ESPT1 and ESPT2. We used the ESPT2 protocol, that allows to obtain colonies that are still alive and therefore usable for a further step of isolation using culture methods. We utilized the ESPT2 protocol as suggested by the manufacturer with the addition of some minor modifications, as described below, in order to obtain a sufficient number of purified bacteria, necessary for the subsequent molecular analysis.

2.3.1 Bulk milk samples

To determine the low level of sensitivity of the analytical protocol in milk samples, five milk samples previously tested negative for *E. coli*, were inoculated with low amounts (10^1 and 10^2 CFU/sample) of each of the *E. coli* serogroups O157, O26, O45, O103, O111, O121 and O145, and processed as described below for the BTM samples.

The BTM samples, once defrosted, were incubated at 37°C in buffered peptone water (Oxoid, Milan, ITA) for 18-24 hours by adding of acriflavine (10mg/l) (Sigma, Missouri, USA) to inhibit the growth of Gram positive, as recommended by the manufacturer. Eight hundreds µl of the obtained suspension were loaded in the first well of the instrument's cartridge and the protocol ESPT2 was started. Once the protocol was completed, 30 µl of the bacterial suspension obtained from the instrument were transferred to a Mac Conkey agar plate (Oxoid, Milan, ITA) for colonies isolation and molecular confirmation. Moreover, 100 µl of the bacterial suspension were subjected to a second incubation overnight in brain heart infusion (BHI) (Oxoid, Milan, ITA) to obtain a

greater amount of purified bacteria to use in subsequent analysis. Finally, 100 µl of the bacterial suspension (BSBM) were collected in a sterile tube and stored at -80°C for the subsequent molecular analysis.

2.3.2 Raw milk filters

To determine the sensitivity of the analytical protocol in filter samples, a filter cut in pieces of filter samples previously tested negative for *E coli*, were put in a specific plastic bag with the addition of 50 ml of peptone water and acriflavine at a concentration of 10 mg/l. Each piece was inoculated with low amounts (10^1 and 10^2 CFU/ml) of each of the *E. coli* serogroups O157, O26, O45, O103, O111, O121 and O145, and processed as described below.

The entire RMF, once defrosted, was cut using sterile scissors and put in specific plastic bags with the addition of 50 ml of buffered peptone water (Oxoid, Milan, ITA) and acriflavine (Sigma, Missouri, USA) at a concentration of 10 mg/l. The samples were homogenized in a BagMixer® 400 S (Interscience, Saint Nom, France) for four minutes and then incubated overnight at 37 °C, as recommended by the manufacturer. The samples were inoculated in the VIDAS® instrument following the same protocol described for BTM.

2.4 DNA extraction

Bacterial DNA was obtained from 100 µl of bacterial suspensions, isolated with VIDAS® instrument, and heat-shock treated following a protocol consisting of 2 cycles of boiling at 95°C for 10 minutes and freezing at -80°C for 10 minutes. After centrifugation of the boiled suspension, 1 µl of supernatant was used as template in PCR reactions.

2.5 *E.coli* serogroups identification by multiplex PCR

For the research and identification of O157 and the *E. coli* serogroups considered, a conventional multiplex PCR (mPCR) was used. The mPCRs were performed using 7 primer pairs published by Paddock et al. (2012). The mPCR reactions were performed on an iCycler Thermal Cycler instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA) and the mPCR conditions used were

the same as the ones reported by Paddock et al. (2012). The DNA bands were visualized and documented with a GelDoc 2000 Fluorescent Imaging System (Bio-Rad, Hercules, CA).

2.6 Single colony isolation from samples positive to mPCR

Once identified the BTM and RMF samples positive for the *E. coli* serogroup of our interest, DNA from a maximum of 50 different isolates/sample was extracted as described above. Then, each isolate was tested in mPCR to confirm the actual belonging to serogroup of interest and for HRM analysis, sequencing and virulence gene research.

2.7 qPCR HRM Analysis (qPCR HRMA)

To discriminate between VTEC and non-VTEC strains, isolates belonging to the non-O157 serogroups were subjected to the research of the processed for the identification of group-specific and VTEC-associated SNPs (Norman et al., 2012), using the qPCR-HRMA.

The primer design was manually performed through alignment, using the online version of the ClustalW programme (available at http://www.ebi.ac.uk/Tools/clustalw2/index.htmlref), of the sequences used by Norman et al. (2012), which include SNPs that are serogroup-specific and VTEC-associated. Each primer pair was chosen in order to discriminate the VTEC strains from non-VTEC strains within each serogroup of interest. In Supplementary File 1 are reported the primer pairs designed for each serogroup. The primer sequences were proved to be unique for *E. coli* species through a homology search using the Basic Local Alignment Search Tool program (available at http://blast.ncbi.nlm.nih.gov/). In order to verify the correct functionality, the primers were preliminary tested in a conventional PCR instrument (iCycler Thermal Cycler instrument (Bio-Rad Laboratories Inc., Hercules, CA, USA) on six strains of *E. coli* (non-O157) used as a reference, using the reagent quantity and the PCR condition of the real-time PCR described below.

The real time PCR (qPCR) and HRM analysis (HRMA) were performed on a EcoTM® Real-Time PCR System (Illumina, Inc., San Diego, CA, USA) and all the reactions were carried out in 48 wells plates for qPCR sealed with the adhesive, and optically transparent, seals (Euroclone, Italy). The PCR reactions took place in a reaction mixture with a final volume of 15 µL, containing 2 µl of

template, 7.5 µl of SsoFast ™ EvaGreen® Supermix (Bio-Rad, Hercules, USA), 0,4 mM of each primers and ultrapure water for molecular biology (Invitrogen, Carlsbad, California, USA). The samples resulted positive in mPCR were tested in double in qPCR-HRMA in order to ensure the reproducibility of the test. As positive samples we added six strains of *E. coli* VTEC of reference, while the PCR reaction mix, devoid of DNA, was used as negative PCR control. The thermal protocols started with a denaturation-activation step at 95°C for 2 min, followed by a 35 cycles program of denaturation at 95°C for 15 s and annealing at different temperatures (depending by the primer pair, Supplementary File 1) for 30 sec. The following melting program comprised 3 steps: denaturation at 95°C for 15 s, renaturation at 55°C for 15 s, and finally melting with continuous fluorescence measurement (ramping 0.1°C/s) to 95°C, kept for 15 s. The HRM curve was analyzed using the Eco_v4.0 software as indicated in the paper of Albonico et al. (2014). Amplification products were run on 2% ethidium bromide agarose gel electrophoresis followed by UV-visualization to check for sensitivity and specificity of the assay.

2.8 Sequencing

To confirm the results of HRM analysis, the amplified samples were recovered, quantified using an agarose-gel quantification and the MassRuler^{TM} DNA ladder, Low Range ready to use (Fermentas, ThermoFisher Scientific, Massachusetts, USA) as molecular weight marker. The unpurified PCR products have been directly sequenced by an external company (Eurofins genomics, Regensburg, Germany) using a standard Sanger-sequencer. The obtained sequences were aligned to the expected target sequences using ClustalW program.

2.9 Virulence factors research using conventional PCR

In parallel, the single colonies isolated and belonging to the non-O157 serogroups (O26, O45, O103, O111, O121, and O145 serogroups) were tested by conventional PCR to identify the major virulence genes: *stx1* (Shiga toxin 1), *stx2* (Shiga toxin 2) and *eae* (intimin).

For the identification of *stx1* and *stx2* genes we used primers and PCR condition described by Cebula et al. (1995). For the identification of *eae* gene we used primers described by Noll et al.

(2015) and the PCR condition by Bai et al. (2012) with some minor modifications, as explained in Supplementary File 2.

All the amplified DNA was separated on 1,5% agarose gel and stained with 0.5 µg/ml of ethidium bromide. The DNA bands were visualized and documented with a GelDoc 2000 Fluorescent Imaging System (Bio-Rad, Hercules, CA).

3. Results

3.1 mPCR for the serogroup identification

The mPCR protocol and PCR reaction were first tested on the seven *E. coli* positive controls belonging to O157, O26, O45, O103, O111, O121, and O145 serogroups. The results confirmed the correct amplification of the primers in the adopted conditions and their ability to correctly discriminate the seven investigated serogroups.

3.2 VIDAS® UP ESPT protocol optimization

The results of molecular biology applied to the products of the isolation protocol performed as suggested by the manufacturer were not satisfactory when applied to experimentally contaminated samples. In fact, mPCR always gave negative results for every level (10^1 and 10^2) of experimental contamination of the milk samples. On the contrary, the conventional microbiological analysis of the products of the same isolation protocol, allowed the growth of the inoculated bacteria, though not in all tests. The mPCR results suggested that there was a deficiency of sensitivity in the procedure suggested by the manufacturer in the presence of relatively low concentrations of bacteria. To overcome this problem, we modified the protocol, including an overnight incubation in BHI of the immuno-concentrate solution obtained from VIDAS®. The results of this modification showed that the mPCR was able to confirm the microbiological data, if positive. Furthermore, mPCR was found to be a more sensitive technique than the conventional microbiology, identifying the O157 and the other non-O157 *E. coli* serogroups, even when conventional microbiological

results were negative. The lower limits of sensitivity achieved is 10^1 CFU/ml for each of the serogroups tested in both BTM and RMF samples.

3.3. *E. coli* VTEC isolation with VIDAS® UP espt2 and serogroup identification by mPCR

Once the analytical protocol was optimized and verified, we analysed the 67 BTM samples, obtaining three positive samples: one sample was positive for both O157 and O145 serogroups, while two samples were positive for O45 serogroup.

Among the 67 RMF, we found a total of 20 positive samples, for at least one on the serogroups investigated. The results of the RMF analysis and the different serogroups identified, are listed in Table 1. Among these samples, four of them harboured a combination of two or more serotype. In detail, one sample was positive for O26 and O121, two samples were positive for O157 and O121, and one sample was positive for O157, O26, O103 and O121.

3.4 HRM-specific primers testing and analysis optimization

SNPs-specific primers that we have designed were first tested in conventional PCR to evaluate their target specificity and each primer has proved to be specific for the target of interest. All the SNPs-specific primers were then tested in HRMA using the positive controls VTEC strains to set up the reaction conditions and the thermal protocols.

3.5 HRM analysis on positive isolated colonies obtained from bulk milk and milking filter samples

HRM analyses were performed only on isolated colonies from samples positive belonging to non-O157 serogroups because, these latter ones are considered a hazard to human health, without any further investigation. Non-O157 serogroups were processed with HRMA to verify the informative SNPs presence that allows to discriminate the VTEC *E. coli* strains within each serogroup of our interest. In Table 2 are summarized the isolates obtained from each positive BTM sample and the result of their HRM analysis. In particular, the isolates obtained from the positive BTM sample for serogroup O145, showed the presence of the SNP in position 37 of the *wzy* gene (A/C) in its variant C, characterizing the VTEC O145 strains (with a melting curve identical to that of the O145 control

VTEC strain). Instead, the isolates obtained from the positive sample for serogroup O45, showed the presence of the SNP in position 721 of the *wbhQ* gene (C/A) in its variant A, characterizing the non-VTEC O45 strains (Figure 1), with a melting curve that differ from that of the O45 control VTEC strain.

The isolates obtained from the positive RMF samples were also tested by HRMA (Table 2). In particular, we observed that the isolates derived from the RMF samples positive for O45, O145 and O121 serogroup shown the presence of the SNP VTEC-correlated. Whereas, the isolates coming from the RMF samples positive for O103 and for O26 did not show the presence of the SNP VTEC-correlated.

The amplicon sequence confirmed the HRMA results, as shown in Table 2.

3.7 Virulence gene analysis

In parallel, all the isolated colonies obtained from the BTM and RMF samples result to be positive for one (or more) of the big six non-O157 serogroups in mPCR, and tested in HRMA, have been also screened for the presence/absence of the three major virulence gene: *stx1*, *stx2* and *eae*. The results are shown in Table 2. In particular, none of the samples tested was found to be positive for *stx1* and *stx2* gene, while some of them are positive for *eae* gene.

4. Discussion

In this work, conventional isolation and identification techniques were combined with some innovative methodologies in order to optimize the diagnostic protocol, improving both sensitivity and execution times. For the isolation and identification of the principal seven serogroups involved in VTEC outbreaks, we utilized the VIDAS® UP ESPT technology, coupled with a mPCR. The verification of the analytical sensitivity by applying our modified protocol confirms that the protocol developed is well suited for its application to BTM and RMF samples and it is also very effective in identifying low contamination (10^1 CFU/mcL) of VTEC associated *E. coli* serogroups considered.

Once optimized the methodology, we checked 67 BTM and the corresponding RMF of several Lombardy and Trentino Alto Adige's dairy herds to assess the O157 and the most important non-O157 *E. coli* serogroups presence. With this methodology, we identified three positive samples of the 67 BTM, and we found 20 positive samples among the 67 RMF samples tested. In literature, the prevalence of all the different investigated serogroups in raw milk and milk filters are not available. Giacometti et al. (2013), investigated the prevalence of O157 in raw milk sold by vending machines analyzing 60,907 samples from 1,239 milk vending machines, from 2008 to 2011, with a prevalence of 0 to 1.5% for *E. coli* O157:H7, and in our raw milk samples, the prevalence is comparable (1.49%). Giacometti et al. (2012), examined a total of 378 in-line milk filters for pathogen detection, with a O157 serogroup prevalence of 2.64%, 2.11% for O103 serogroup and 1.06% for O145 serogroup. In our study, we observed a higher prevalence for O157 (14.92%), O103 (4.48%) and O145 serogroup (2.98%).

Interestingly, this is the first published report in Italy of positive recovery of O45 and O121 serogroups in milk and milk filters. Here, again, it justifies the special attention that non-O157 serogroups are beginning to have, even in Europe, and the need of screening methodology to quickly identify these serogroups. Finally, in some samples we detected the presence of more than one investigated serogroups at the same time. This evidence is of particular importance in the epidemiology of VTEC infections.

Once screened the seven serogroups of interest in our samples, we evaluated the presence of specific virulence factors of our positive, non-O157 samples through two parallel molecular biology methods: virulence gene research by PCR and HRMA and sequencing. Moreover, for the first time, we applied the HRMA to identify and discriminate the presence/absence of informative SNPs that Norman et al. (2012) with mass spectrometry found to be correlated to VTEC status. Successfully, the HRM-specific primers that we designed and the HRMA protocol that we optimized showed to be able to accurately identify the presence or absence of the mutation for each SNP analysed, as confirmed by the sequencing. Nevertheless, even if no one of our samples was positive for *stx1* and

stx2 genes (and just few *eae* genes), some of our samples showed the presence of the SNP correlated with VTEC status. If we compare the results of the two methods, it clearly appears that the presence of the SNP in the target serogroups genes is not correlated to the presence of either *stx1* or *stx2* genes. This apparent discrepancy with the results of Norman et al., (2012) should be further investigated in a larger comparative study.

This was a preliminary study aiming to develop and optimize the diagnostic protocol. The following step could be the application of this newly developed methodology to a bigger number of dairy herds and to individual cows to establish the serogroups prevalence among Italian herds, and to identify herd at risk.

5. Conclusions

In this paper, we applied, for the first time, the VIDAS® UP ESPT technology, coupled with mPCR, to quickly detect the seven serogroups more frequently associated with VTEC infections in BTM and RMF. This is a new approach to investigate the presence of the O157 and big six non-O157 serogroups at farm level and not to identify VTEC hazard only once the product is processed and/or is ready to be consumed.

The development of this new analytical protocol represents a genuine progress in the prevention of the spread of these pathogens identifying the hazard at the primary production level, and opens new horizons to expand the investigation on the VTEC distribution with potential significant positive impact in food safety.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Results of testing the RMF samples with VIDAS® UP ESPT2 protocol followed by mPCR, prevalence of the different serogroups.

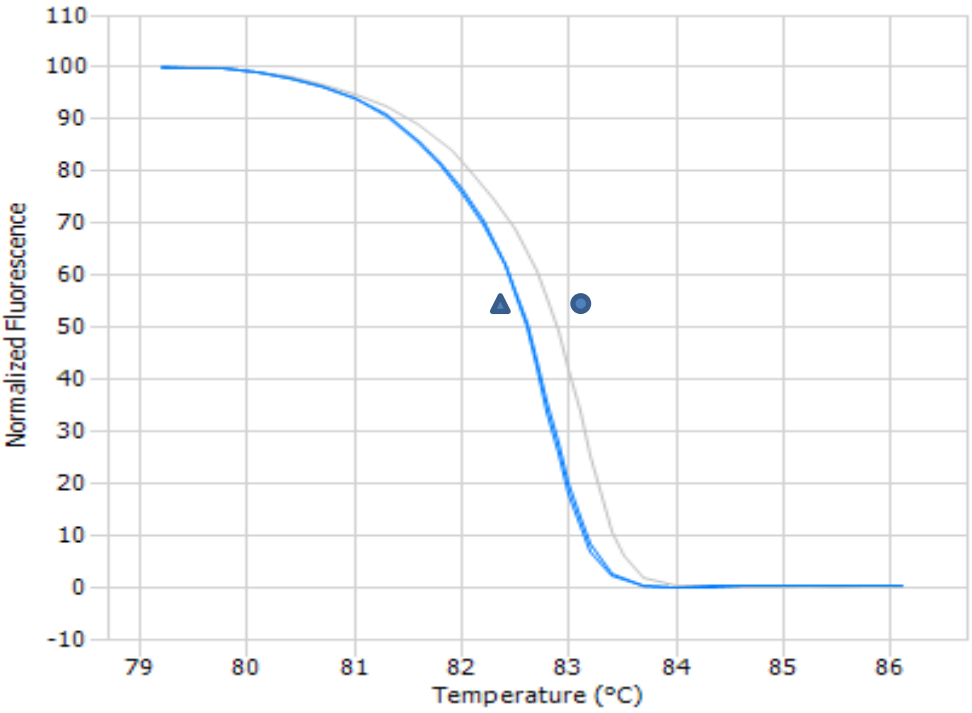
Serogroups	N° of positive sample	Percentage
O157	10	14,92
O26	5	7,46
O121	4	5,97
O103	3	4,48
O145	2	2,98
O45	2	2,98

Table 2. Three molecolar methods comparison: HRM analysis for the identification of the informative SNPs VTEC-associated, sequencing results and virulence gene (*stx1*, *stx2*, *eae*) amplification by PCR.

Sample ID	Serogroup	N° of isolates	Gene target SNP position	Informative SNP/ Nucleotide mutation	Nucleotide identification by sequencing	VTEC prediction by HRM	*stx1*	*stx2*	*eae*
L64	O45	1	*wbhQ* 721	A → C	A	no	neg	neg	neg
L34	O145	1	*wzy* 37	A → C	C	yes	neg	neg	neg
F31	O26	6	*fnl1* 88	G → A	G	no	neg	neg	pos
			wzx 953	T → G	T	no	neg	neg	
F38	O26	9	*fnl1* 88	G → A	G	no	neg	neg	pos
			wzx 953	T → G	T	no	neg	neg	
F45	O26	1	*fnl1* 88	G → A	G	no	neg	neg	neg
			wzx 953	T → G	T	no	neg	neg	
F46B	O26	7	*fnl1* 88	G → A	G	no	neg	neg	neg
			wzx 953	T → G	T	no	neg	neg	
F46C	O26	8	*fnl1* 88	G → A	G	no	neg	neg	neg
			wzx 953	T → G	T	no	neg	neg	
F51	O45	4	*wbhQ* 721	A → C	C	yes	neg	neg	neg
F36	O103	1	*wbtD* 935	C → T	C	no	neg	neg	neg
F45	O103	5	*wbtD* 935	C → T	C	no	neg	neg	neg
F31	O121	2	*vioA* 313	C → T	T	yes	neg	neg	neg
F45	O121	1	*vioA* 313	C → T	T	yes	neg	neg	neg

F46A	O121	1	*vioA* 313	C → T	T	yes	neg	neg	neg
F65	O121	1	*vioA* 313	C → T	T	yes	neg	neg	neg
F49	O145	8	*wzy* 37	A → C	C	yes	neg	neg	pos

Figure 1. Example of results obtained with the HRM analysis on isolate belonging to serogroup O45 for the SNP (C/A) research in position 721 of the *wbhQ* gene.



In this case, the melting curve obtained from the samples (circle) is different from the melting curve of the VTEC reference for O45 (triangle), in particular it is shifted to the right, thus indicating the presence of the nucleotide base C in our samples, which therefore requires a higher thermal strength to break the three hydrogen bridges with respect to that necessary for the nucleotide base A, present on the filament of the mutate reference VTEC. The amplicon melting temperature of our samples is greater than that the one of the amplicons of the reference VTEC strains.

Highlights:

- New approach for serogroups associated with VTEC status identification
- Combination of VIDAS® UP ESPT technology and multiplex PCR to detect *E.coli* VTEC
- Once optimized, the protocol was used for the analysis of raw milk and milk filters
- First isolation of O45 and O121 serogroups in raw milk and milk filters in Italy