Whole-Genome Characterization and Strain Comparison of VT2f–Producing *Escherichia coli* Causing Hemolytic Uremic Syndrome

Laura Grande, Valeria Michelacci, Roslen Bondì, Federica Gigliucci, Eelco Franz, Mahdi Askari Badouei, Sabine Schlager, Fabio Minelli, Rosangela Tozzoli, Alfredo Caprioli, Stefano Morabito

Verotoxigenic *Escherichia coli* infections in humans cause disease ranging from uncomplicated intestinal illnesses to bloody diarrhea and systemic sequelae, such as hemolytic uremic syndrome (HUS). Previous research indicated that pigeons may be a reservoir for a population of verotoxigenic *E. coli* producing the VT2f variant. We used whole-genome sequencing to characterize a set of VT2f-producing *E. coli* strains from human patients with diarrhea or HUS and from healthy pigeons. We describe a phage conveying the *vtx2f* genes and provide evidence that the strains causing milder diarrheal disease may be transmitted to humans from pigeons. The strains causing HUS could derive from VT2f phage acquisition by *E. coli* strains with a virulence genes asset resembling that of typical HUS-associated verotoxigenic *E. coli*.

Verotoxigenic *Escherichia coli* (VTEC) infections in humans cause a wide spectrum of clinical manifestations ranging from uncomplicated forms of intestinal illnesses to bloody diarrhea and systemic sequelae, such as hemolytic uremic syndrome (HUS) (*1*). The most severe forms are caused by the damage inflicted by the verocytotoxins (VTs) to the target cells in the intestinal mucosa and the renal blood vessels (*1*). The genes encoding the verocytotoxins (*vtx*) are harbored by lambdoid bacteriophages, which can be transferred to multiple bacterial hosts, generating a great diversity in the bacterial types that produce such toxins (*2*).

The most well-known VTEC serogroup, O157, inhabits the gastrointestinal tract of ruminants, especially cattle. However, this and other VTEC serotypes have been isolated from the feces of several other animal species, including deer, pigs, horses, cats, dogs, and wild birds (*3*).

During a program aimed at the control of the pigeon population in Rome, Italy during 1998, G. Dell'Omo et al. observed that this animal species was a carrier of VTEC (*4*). In that study, VTEC of multiple serogroups were isolated from ≈10% of the animals tested. Of 16 VTEC, 15 carried the *eae* gene encoding the intimin and featured genetic determinants that produced a subtype of verocytotoxin type 2 not described before, later designated VT2f (*4–6*). The finding of such a high prevalence of VTEC in pigeons living in Rome led to further research into these bacteria in this and other bird species worldwide. Almost all these studies succeeded in isolating VTEC, with prevalence ranging 3% to >19% in different countries and bird species; most VTEC isolated from pigeon feces and cloacal swab samples harbored the genes encoding the VT2f subtype (*7–10*). These findings emphasize the existence of a strict association between VTEC carrying the *vtx2f* genes and pigeons, which represent a reservoir for such strains.

Data on human illness attributable to VT2f-producing *E. coli* has been scarce until recent reports from Germany and the Netherlands described the isolation of such strains from diarrheal stool specimens from humans (*11*,*12*). Furthermore, in the Netherlands, an HUS case was recently reported to be associated with the presence of a VT2f-producing O8:H19 strain (*13*). We aimed to characterize at the whole-genome level 3 *E. coli* strains that produced the VT2f isolated from HUS and to investigate their relationships with VT2f-producing *E. coli* isolated from human diarrheal cases and from the pigeon reservoir.

Author affiliations: Istituto Superiore di Sanità, Rome, Italy (L. Grande, V. Michelacci, R. Bondi, F. Gigliucci, F. Minelli, R. Tozzoli, A. Caprioli, S. Morabito); National Institute for Public Health and the Environment, Bilthoven, the Netherlands (E. Franz); Islamic Azad University Faculty of Veterinary Medicine, Garmsar Branch, Garmsar, Iran (M. Askari Badouei); Austrian Agency for Health and Food Safety, Graz, Austria (S. Schlager)

DOI: http://dx.doi.org/10.3201/eid2212.160017

Materials and Methods

Bacterial Strains

We investigated 22 Vt2f -producing *E. coli* strains. Eight previously described strains were isolated from pigeons in Italy (*4*); eleven strains were isolated in the Netherlands from fecal specimens from humans with diarrhea during

2008–2012 and are part of the collections held at the National Institute for Public Health and the Environment in the Netherlands (RIVM) (*12*). Of the 3 VT2f-producing *E. coli* from HUS patients, 1 was isolated in Austria in 2013 and 2 in Italy during 2013–2014. A total of 23 unrelated VTEC non-O157 strains that produced VT1 and/or VT2 subtypes other than VT2f have been used for the comparison of the profiles of virulence genes with those of the VT2f-producing isolates (Table 1).

Whole-Genome Sequencing of *E. coli* Strains

Sequencing of the strains isolated from fecal samples from humans with diarrhea and from pigeons was outsourced to the Central Veterinary Institute, Wageningen University (Lelystad, the Netherlands). Genome sequences were obtained by using a TruSeq protocol on an Illumina MiSeq PE300 platform (Illumina, San Diego, CA, USA). The genomes of the 3 VT2f-producing isolates from HUS patients were sequenced by using an Ion Torrent PGM (Thermo Fisher Scientific, Waltham, MA, USA) according to 400-bp protocols for library preparation through enzymatic shearing, Ion OneTouch2 emulsion PCR, enrichment, and Hi-Q sequencing kits (Thermo Fisher Scientific).

The whole-genome sequences (WGSs) of the 23 non-O157 VTEC strains are part of the European Molecular Biology Laboratory's European Nucleotide Archive Study (http://www.ebi.ac.uk/ena; accession no. PRJEB11886). The raw reads have been subjected to quality check through FastQC and trimmed with FASTQ positional and quality trimming tool to remove the adaptors and to accept 20 as the lowest Phred value (*14*).

We subjected the sequences obtained with the Ion Torrent apparatus to de novo assembly by using the tool SPADES (*15*) and those from Illumina by using the A5 pipeline (*16*). The genomes have been assembled in several contigs ranging from 42 to 495 (mean 225), with N50 values (the length of the smallest contig among the set of the largest contigs that together cover at least 50% of the assembly) between 40,736 and 347,638 (mean 152,953). All the contigs were uploaded to the EMBL European Nucleotide Archive (accession no. PRJEB12203). We made annotations by using the Prokka tool (*17*). All the bioinformatics tools used are available on the Aries public Galaxy server (https://w3.iss.it/site/aries/).

Virulence Gene Profile Analysis and Serotyping

The presence of *vtx2f* and *eae* genes has been assessed by PCR by using primers and conditions described elsewhere (*5*,*18*). The activity of VT2f has been evaluated by Vero cell assay (VCA) as previously described (*19*).

We performed detection of the virulence genes *cif, efa1, espABCFIJP, etpD, iha, iss, katP, lpfa, nleABC, tccP, tir, toxB, ehxA*, and *espP* and the serotype determination in silico on the WGSs. We used blastn (available on the Aries public Galaxy server at https://w3.iss.it/site/aries/) to search databases containing the reference sequences of all the known virulence and serotype-associated genes of pathogenic *E. coli* (*20*). To perform the principal component analysis of the virulence gene profiles, we

Table 1. Characteristics of non-O157 verotoxigenic *Escherichia coli* strains used in a comparative analysis of the virulence profile of VT2f-producing strains from humans and the animal reservoir*

Strain	Serogroup	Source†	Year of isolation	Virulence gene profile
ED017	O26	HUS	1989	*eae vtx1*
ED075	O26	Diarrheal feces	1990	*eae vtx1*
ED180	O26	HUS	1994	*eae vtx2*
ED195	O26	HUS	1994	*eae vtx1*
ED392	O26	Diarrheal feces	1998	*eae vtx1*
ED411	O26	HUS	1999	*eae vtx2*
ED423	O26	Diarrheal feces	1999	*eae vtx1*
ED654	O26	HUS	2007	*eae vtx2*
ED669	O26	HUS	2008	*eae vtx1*
ED676	O26	HUS	2008	*eae vtx2*
ED729	O26	Diarrheal feces	2010	*eae vtx1*
ED766	O26	HUS	2010	*eae vtx2*
ED657	O145	HUS	2007	*eae vtx2*
ED603	O121	HUS	2004	*eae vtx2*
ED073	O111	Diarrheal feces	1990	*eae vtx1*
ED082	O111	HUS	1990	*eae vtx1*
ED142	O111	HUS	1992	*eae vtx1 vtx2*
ED178	O111	HUS	1994	*eae vtx1 vtx2*
ED608	O111	HUS	2005	*eae vtx1 vtx2*
ED664	O111	HUS	2007	*eae vtx2*
ED672	O111	HUS	2008	*eae vtx1 vtx2*
ED287	O103	Bovine	1998	*eae vtx1*
ED728	O103	Bloody diarrheal feces	2010	*vtx1*

*All samples are from humans except strain ED287. HUS, hemolytic uremic syndrome.
†HUS samples were isolated from feces.

used SAS/IML studio software version 3.4 (SAS Institute, Inc., Cary, NC, USA).

We investigated plasmid profiles by using PlasmidFinder (*21*; https://cge.cbs.dtu.dk//services/all.php). The intimin subtyping has been performed in silico through a BLAST search (*22*) of the *eae* gene sequences from the WGS against the National Center for Biotechnology Information nucleotide repository. The intimin types of the VT2f-producing strains isolated from pigeons have been published (*6*,*10*).

rpoB Sequencing and Analysis

Amplification and sequencing of the *rpoB* gene were conducted to discriminate between *E. coli* and *E. albertii* species, as previously described (*23*). The amplicons were purified with the SureClean Plus kit (Bioline, London, UK) and sequenced using the BigDye Terminator v1.1 kit on a Genetic Analyzer 3130 (Thermo Fisher Scientific). The obtained sequences were trimmed and aligned to the reference sequences as indicated (*23*), using the Clustal Omega free software (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Typing

We determined *E. coli* phylogenetic groups by using the method of Clermont et al. (*24*). We carried out multilocus sequence typing (MLST) of the VT2f isolates in silico according to the scheme proposed by Wirth et al. (*25*). We analyzed the assembled sequences by using blastn to search the MLST database downloaded from the Internet site of the MLST.UCC Mark Achtman database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/).

Single-Nucleotide Polymorphism (SNP) Analysis

We analyzed SNPs by using the tool kSNP3 (*26*) available on the Galaxy project instance Aries (https://w3.iss.it/site/aries/). We set a kmer value of 23.

Results

Characterization of the VT2f-Producing *E. coli* Strains

Serotyping

Of 11 VTEC strains isolated from humans with diarrhea, 5 belonged to the O63:H6 serotype. The remaining 6 isolates contained the $fliC_{H6}$ (3 strains), $fliC_{H7}$ (1 strain), and $fliC_{H34}$ (2 strains) genes (Table 2) and belonged to serogroups O96, O113, O132, O145, and O125. For 1 isolate, the O-antigen–associated genes could not be identified (Table 2) (*12*).

Molecular serotyping of the 8 VT2f-producing strains isolated from pigeons showed that all the isolates had the $fliC_{H2}$ and the O4, O45, O75, and O128 serogroup-associated genes. The O-antigen genes could not be identified for the isolate ED 366 (Table 2). The HUS-associated

Table 2. Characteristics of VT2f-producing *Escherichia coli* investigated in a comparative analysis of the virulence profile of strains isolated from humans with mild and severe disease and from the animal reservoir*

Source and strain	Year isolated	Serotype	Phylotype	MLST	LEE	*adfO*	*efa1*	*cif*	*nleA*	*nleB*	*nleC*	*Hly*	*katP*	*espP*	Intimin type
Human diarrhea															
M856	2008	ONT:H6	B2	ST583	+	+	–	+	–	+	+	–	–	–	α-2
M858	2008	O125:H6	B2	ST583	+	+	–	+	–	+	–	–	–	–	α-2
M859	2009	O113:H6	B2	ST121	+	+	–	+	–	–	–	–	–	–	α-2
M884	2011	O96:H7	B2	ST28	+	+	–	+	+	+	–	–	–	–	β-2
M885	2011	O132:H34	B2	ST582	+	+	–	–	–	+	+	–	–	–	β-2
M900	2012	O145:H34	B2	ST722	+	+	–	–	–	+	–	–	–	–	ι
BCW5711	2012	O63:H6	B2	ST583	+	+	–	+	+	–	+	–	–	–	α-2
BCW5746	2012	O63:H6	B2	ST583	+	+	–	+	–	–	+	–	–	–	α-2
BCW5743	2012	O63:H6	B2	ST583	+	+	–	+	–	–	+	–	–	–	α-2
BCW5739	2012	O63:H6	B2	ST583	+	+	–	+	–	–	+	–	–	–	α-2
BCW5717	2012	O63:H6	B2	ST583	+	+	–	+	–	–	+	–	–	–	α-2
Pigeon															
ED360	1997	O45:H2	B1	ST20	+	+	–	+	+	+	+	–	–	–	β
ED361	1997	O75:H2	B1	ST20	+	+	–	+	+	+	+	–	–	–	β
ED363	1997	O4:H2	B1	UNK	+	+	–	+	+	+	+	–	–	–	β
ED366	1997	ONT:H2	B1	ST2685	+	+	–	+	+	+	+	–	–	–	β
ED369	1997	O45:H2	B1	ST20	+	+	–	+	+	+	+	–	–	–	β
ED377	1997	O4:H2	B1	UNK	+	+	–	+	+	+	+	–	–	–	β
ED430	2000	O45:H2	B1	ST20	+	+	–	+	+	+	+	–	–	–	β
ED444	2000	O128:H2	B1	ST20	+	+	–	+	+	+	+	–	–	–	β
HUS															
EF453	2013	O80:H2	B1	ST301	+	+	+	–	+	+	+	+	–	+	ξ
EF467	2013	O26:H11	B1	ST21	+	+	+	+	+	+	+	+	+	+	β
EF476	2014	O55:H9	B1	ST301	+	+	+	–	+	+	+	+	–	+	ξ

*Human samples were diarrheal or fecal samples from HUS cases and pigeon samples were feces from asymptomatic birds. LEE, locus of enterocyte effacement; MLST, multilocus sequence type; UNK, unknown; +, positive; –, negative.

VT2f-producing *E. coli* strains EF453 and EF476 belonged to serotypes O80:H2 and O55:H9, respectively, while strain EF467 was O26:H11.

Virulence Gene Profiles

The *E. coli* strains carrying the *vtx2f* and isolated from pigeons have been previously reported to produce an active VT2f (*6*). As expected, culture supernatants from VT2f-producing strains isolated from human diarrhea and HUS induced a cytopathic effect on Vero cells morphologically compatible with that caused by verocytoxins.

All the VT2f-producing strains included in the study were positive for the *eae* gene (Table 2) and displayed the presence of the entire locus of enterocyte effacement (LEE) (data not shown). Most of the *E. coli* VT2f-producing strains isolated from diarrheal cases harbored the α-2 intimin type (8/11), followed by the β-2 (2/11) and ι (1/11) types. The VT2f-strains isolated from pigeons had been previously described to have the β-intimin (*6*) in most cases and, more rarely, the α-2 intimin type (*10*). Of 3 HUS-associated VT2f-producing strains, 2 (EF453 and EF476) carried the ξ intimin type and 1 (EF467) had the β intimin (Table 2).

All the pigeon and HUS isolates possessed the complete set of non–LEE-encoded effectors assayed (*nleA, nleB* and *nleC*) (*27*), whereas the isolates from human diarrhea cases displayed an unequal presence of these genes (Table 2). The *efa1* gene, hallmark of the OI-122 pathogenicity island (*28*), was not identified in the isolates from pigeons or from human diarrheal specimens; neither were the genes *ehxA*, *espP* and *katP*, usually present on the large virulence plasmid of VTEC O157 and other VTEC associated with severe human disease (Table 2). However, the gene *adfO*, present on the OI-57 (*29*), was detected in all the strains investigated (Table 2).

The HUS strains EF453, EF467, and EF476 had the entire *efa1* gene. Strain EF467 also had the *ehxA*, *espP*, and *katP* genes; the EF453 and EF476 strains had the *ehxA* and *espP* genes only (Table 2). The analysis of the plasmid profiles substantiated the finding that the 3 HUS-associated strains carried the large virulence plasmid of VTEC, revealing the presence of a sequence 100% homologous to the replicon sequence of the pO26-CRL plasmid from a VTEC O26:H- (GenBank accession no. GQ259888.1), which harbors the genes *ehxA*, *espP*, and *katP*.

On the basis of plasmid profiles analysis, 7 of 11 *E. coli* VT2f-producing strains isolated from human diarrheal feces seemed to have the replicon sequence of the plasmid pSFO (GenBank accession no. AF401292) encoding the enterohemolysin and a cluster of *pap*-like genes called *sfp* in a sorbitol-fermenting *E. coli* O157 (*30*). However, the analysis of the WGSs failed to identify the *ehxA* and the *pap*-like sequences, suggesting that the entire pSFO plasmid was not present.

Principal component analysis of the virulence genes profiles showed that the HUS isolates producing VT2f clustered with the set of non-O157 VTEC isolates used for comparison, rather than with the other VT2f-producing strains (Figure 1). Conversely, the VT2f-producing strains from diarrhea and from pigeons grouped together and apart from the HUS strains (Figure 1).

Phylogenetic Analyses

rpoB Analysis

All the VT2f-producing isolates had an *E. coli*-related *rpoB* sequence (*23*). This finding verified that all the strains investigated were *E. coli*.

Typing

All VT2f-producing *E. coli* isolates from pigeons and the strains isolated from HUS belonged to the B1 phylogenetic group. All the strains isolated from human diarrheal feces were of phylotype B2 (Table 2).

By MLST, most of the pigeon strains investigated (5/8) belonged to sequence type (ST) 20; 1 was ST2685, and 2 were of unknown ST (Table 2), mainly because of the absence of a recognizable *adk* gene sequence. The 5 O63:H6, the 1 O125:H6, and the 1 ONT:H6 VTEC strains from diarrheal fecal specimens belonged to ST583; of the remaining 4 strains, 1 each was of sequence types ST28, ST121, ST582, and ST722 (Table 2).

Of 3 HUS-associated VT2f-producing *E. coli*, 2 (EF453 and EF476) belonged to ST301; strain EF467 was of ST21 (Table 2). All of the STs belonged to different clonal complexes or to any clonal complex, indicating that they were not related each other (data not shown).

SNP Analysis

A parsimony tree representing the core-genome SNPs analysis (Figure 2) shows that VT2f-producing strains from pigeons, human diarrheal feces, and HUS cases cluster apart from each other and from other VTEC strains used for comparison. The HUS-associated EF467 strain clusters together with the group of VTEC non-O157 from human disease, in agreement with the principal component analysis (Figures 1, 2).

Identification of a Bacteriophage Containing the *vtx2f* Genes

The contigs containing the *vtx2f* genes in the different strains ranged 2,500–68,480 bp in size. Upon annotation, they showed the presence of phage-associated genes in the proximity of *vtx2f*, including those encoding the

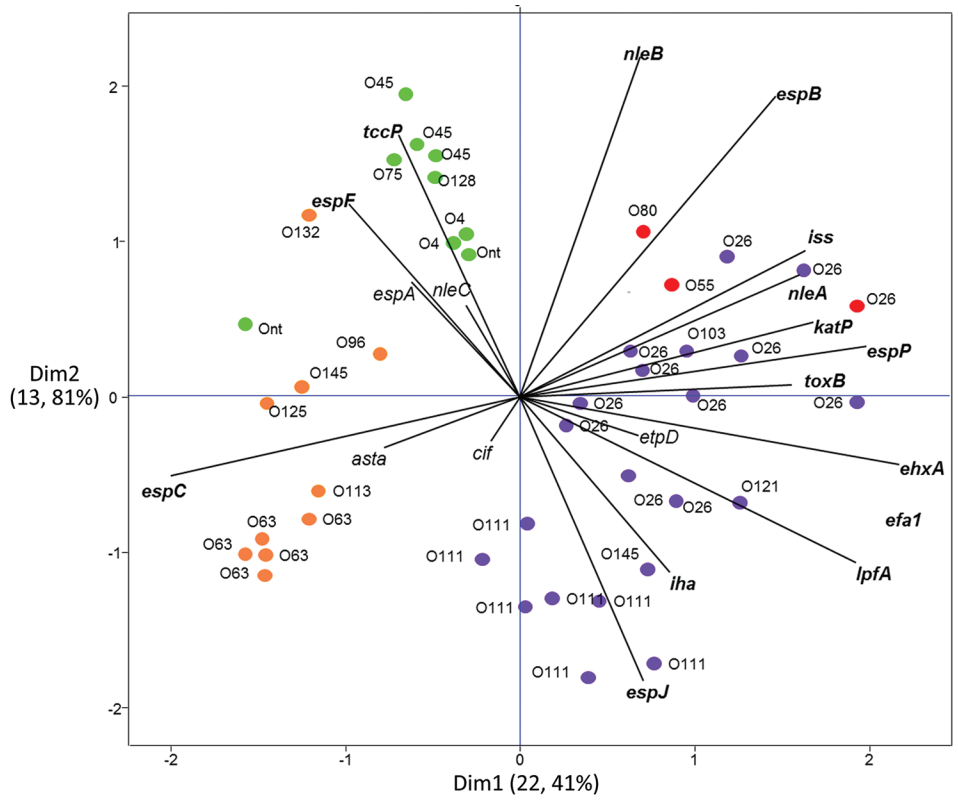

Figure 1. Principal component analysis of the virulence profiles of VT2f-producing verotoxigenic *Escherichia coli* (VTEC) strains isolated from fecal samples from human uncomplicated diarrheal case-patients (orange), human hemolytic uremic syndrome patients (red), and pigeon feces (green). Non-O157 VTEC that do not produce VT2f are indicated in purple. This analysis clusters isolates on the basis of similarities in virulence gene content. Isolates clustering together have similar virulence profiles. Lines represent individual virulence genes. The projected length of the lines on the horizontal and vertical axes indicates the strength of the contribution of a specific virulence marker to the separation of clusters. Dim1 and Dim2 represent the first 2 dimensions of the analysis and indicate the fraction of variation in the dataset that can be explained by the included variables (i.e., virulence genes).

antitermination protein Q, the lysis protein S, a phage terminase, an integrase, and tail-assembly proteins.

We used WGS of HUS strain EF467 to assemble a partial VT2f phage sequence of 38,594 bp (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/12/16-0017-Techapp1.pdf). Analysis of the construct highlighted the absence of most of the genes normally involved in the regulation of the switch between the lysogenic state and the lytic cycle of lambdoid phages, such as *cro*, *cI*, *cII*, *cIII*, and N. We confirmed these findings by mapping the raw reads of the WGS of the strain EF467 against the VT2 reference bacteriophage BP933W (GenBank accession no. AF125520) (not shown).

We confirmed the genomic structure of VT2f phage obtained in silico by 4 PCRs performed on the total DNA extracted from strain EF467 by using primer pairs designed on the construct's map and by using a restriction fragment length polymorphism analysis on the obtained PCR fragments (Table 3; online Technical Appendix). The partial VT2f phage sequence was deposited into the EMBL database (accession no. LN997803).

The portion of the VT2f phage spanning the *xerC* gene and the tRNA-Gly and tRNA-Thr loci (online Technical Appendix) was found in the draft genomes of all the VT2f-producing strains. The region downstream to the tRNA loci was also detected, but with different degrees of variation among the WGSs; for example, the presence of an additional DNA stretch of ≈24 kb in the strain BCW5746 (online Technical Appendix).

The presence of a similar phage structure was confirmed by using a long PCR approach in the other 2 VT2f-producing *E. coli* from HUS cases and in 2 strains from pigeons (strains ED377 and ED363). All the strains showed the expected amplicons with PCR 2 and 3 together with the expected product of PCR 1 in 2 pigeon isolates; all the isolates tested did not yield any amplicon with PCR 4 (Figure 3). A comparative analysis of the *xerC* sequences from all the VT2f phage constructs returned a high degree of variation in its sequence, explaining the observed absence of the PCR4-specific amplicon (data not shown). Finally, a BLAST search by using this VT2f phage construct from strain EF467 returned only partial similarity with phages identified in different *Enterobacteriaceae* but did not retrieve similar structures.

Discussion

VTEC producing the VT2f subtype have long been considered a minor public health problem because of their rare association with human infections (*31–34*). Recently, however, an increasing number of reports of human diseases caused by infection with these *E. coli* strains have

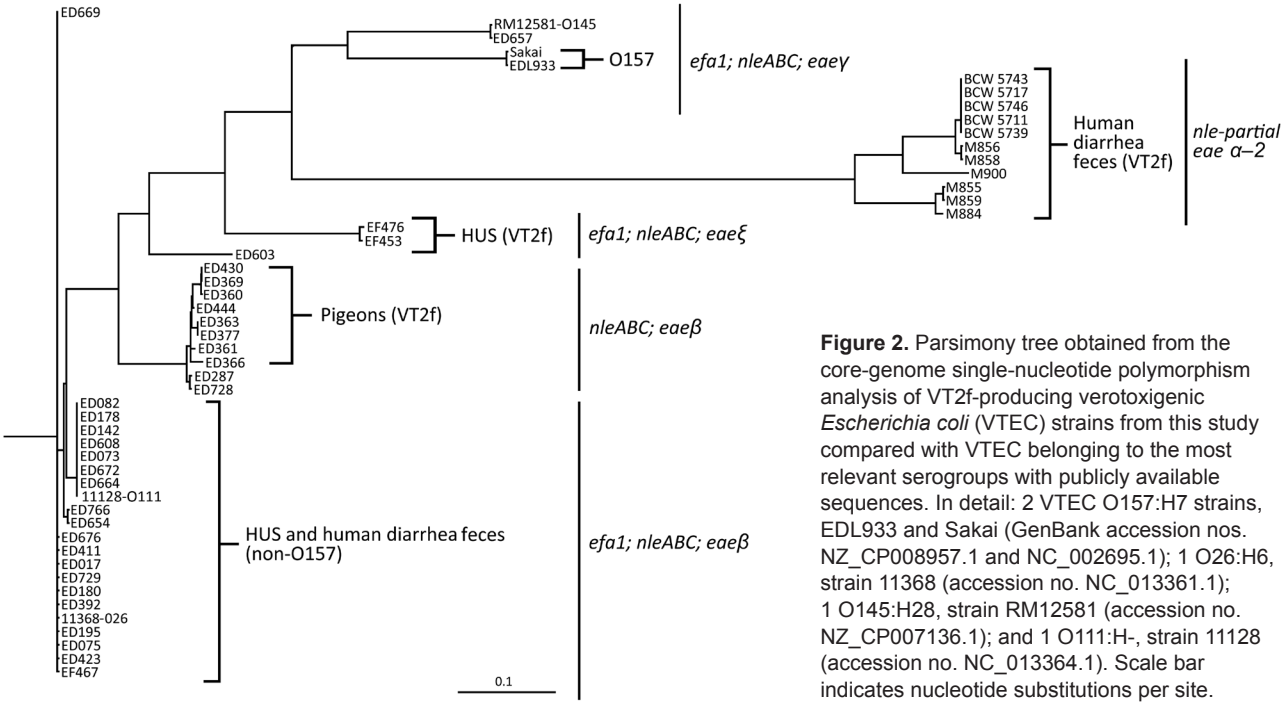

Figure 2. Parsimony tree obtained from the core-genome single-nucleotide polymorphism analysis of VT2f-producing verotoxigenic *Escherichia coli* (VTEC) strains from this study compared with VTEC belonging to the most relevant serogroups with publicly available sequences. In detail: 2 VTEC O157:H7 strains, EDL933 and Sakai (GenBank accession nos. NZ_CP008957.1 and NC_002695.1); 1 O26:H6, strain 11368 (accession no. NC_013361.1); 1 O145:H28, strain RM12581 (accession no. NZ_CP007136.1); and 1 O111:H-, strain 11128 (accession no. NC_013364.1). Scale bar indicates nucleotide substitutions per site.

populated the literature (*11*,*12*,*32*,*35*). The diversity of *vtx2f* gene sequences compared with other *vtx2* subtype genes may have played a role in underestimating the global burden of such infections. PCR primers mostly used for the detection of *vtx2* genes in clinical specimens and the vehicles of infection have been proven to be unable to amplify the *vtx2f* gene (*36*). In addition, the recent description of another *eae*-positive *Escherichia* species often isolated from birds, and sometimes carrying *vtx2f* genes, *E. albertii*, added a further element of confusion. *E. albertii* has been associated both with gastroenteritis in humans and with healthy and diseased birds (*37*,*38*), but this species is difficult to distinguish from *E. coli* when using the usual biochemical or molecular assays.

Most human infections with VTEC producing VT2f have been reported as uncomplicated diarrheal cases (*11*,*12*), which may also have accounted for the underestimation of these infections. Because such cases are not actively surveyed in many countries, these infections may have been overlooked. The recent description of an HUS case associated with a VT2f-producing *E. coli* (*13*) changed the perspective on VT2f-producing *E. coli* and the associated disease, making it necessary to update the current paradigm of HUS-associated VTEC.

Table 3. PCR and restriction fragment length polymorphism analysis conditions used to verify VT2f phage structure in a comparative analysis of the virulence profile of human and zoonotic VT2f-producing *Escherichia coli* strains*

Analysis	Primer name	Sequence, 5′→3′	Position	Thermal profile	Amplicon size, bp	Restriction enzyme (obtained fragments, bp + bp)
PCR1	φ-*vtx2f*_1FW	caccatatcccagcaactgc	1,985–2,005	95°C for 2 min, 30× (94°C for 30 s, 53°C for 30 s, 70°C for 9 min); 72°C for 10 min	6,331	*Pvu*I (1,773 + 4,558)
	φ -*vtx2f*_1RV	gttggcggttccgactacaa	8,315–8,296			
PCR2	φ -*vtx2f*_2FW	gcgcatcaccacttcatctt	8,337–8,357	95°C for 2 min, 30× (94°C for 30 s, 53°C for 30 s, 70°C for 9 min), 72°C for 10 min	8,166	*Hind*III (1,855 + 6,311)
	128–1	agattgggcgtcattcactggttg	16,502–16,479			
PCR3	φ -*vtx2f*_3FW	ggagtggatattgccgacct	16,808–16,827	95°C for 2 min, 30× (94°C for 30 s, 53°C for 30s, 70°C for 9 min), 72°C for 10 min	3,927	*Bgl*II (1,310 + 2,617)
	φ -*vtx2f*_3RV	gtcttcctgctgaggcgatc	20,734–20,715			
PCR4	φ -*vtx2f*_4FW	taatcgcggccgtactcaag	22,172–22,191	95°C for 2 min, 30× (94°C for 30 s, 53°C for 30 s, 70°C for 9 min), 72°C for 10 min	8,808	*Nco*I (5,029 + 3,779)
	φ -*vtx2f*_4RV	tgttcagctccaccttacgg	30,979–30,960			

*Analysis for PCR2, primer 128–1 from (*5*); all other data were compiled for this study. All the long PCR described were performed with the GoTaq Long PCR Master Mix (Promega, Madison, WI, USA) according to manufacturer's instructions. Primer positions refer to the phage sequence deposited into the EMBL database (accession no. LN997803).

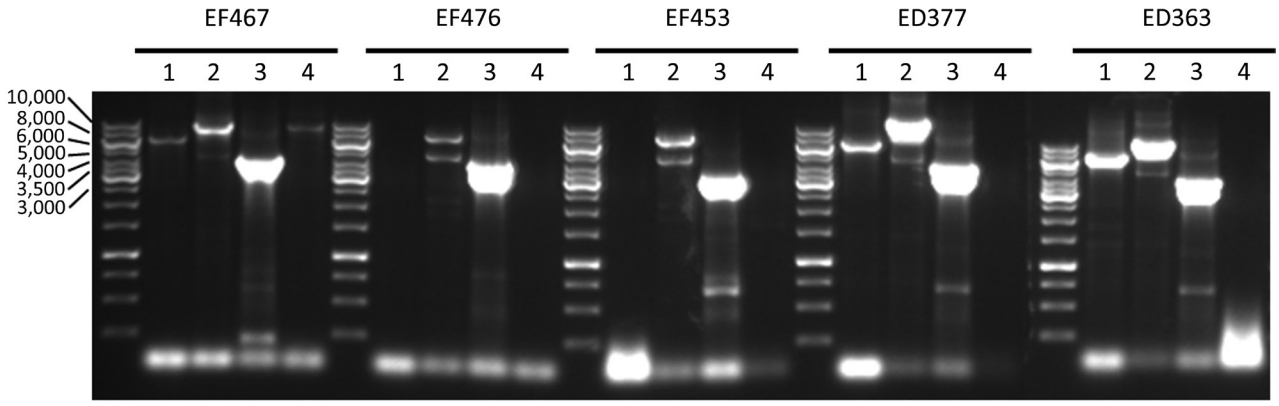

Figure 3. Long PCR analysis of the VT2f phage of verotoxigenic *Escherichia coli* (VTEC) strains isolated from fecal samples from humans with hemolytic uremic syndrome (EF467, EF476, EF453) and from pigeon feces (ED363, ED377). Numbers at left indicate bps; lane numbers indicate PCR 1 to PCR 4. The expected size of the amplicons were 6,331 bp (PCR 1), 8,166 bp (PCR 2), 3,927 bp (PCR 3), and 8,808 bp (PCR 4).

We provide evidence that the VT2f-producing *E. coli* isolated from HUS cases display the complete set of virulence genes described in the typical HUS-associated VTEC (Table 2; Figure 1) (*28*). All VT2f strains from HUS that we examined were positive for pathogenicity island OI-122 (*28*) and the large virulence plasmid first described in VTEC O157 (Table 2) (*39*); the strains from pigeons or from humans with uncomplicated diarrhea did not have these virulence-associated mobile genetic elements (Table 2) (*10–12*,*32*,*40*).

Our study also showed that the LEE was complete in all the genomes investigated, but a complete set of *nleABC* genes was found only in strains from pigeons and from humans with HUS (Table 2), indicating that the VT2f-producing isolates investigated belonged to 3 distinct main virulotypes or subpopulations (Table 2). The intimin subtyping supported this observation. Of 11 diarrheal isolates, 8 had the α-2 gene; all the pigeon isolates had a β intimin coding gene. Finally, 2 of the 3 strains from HUS showed the presence of a gene encoding the ξ intimin (Table 2). Furthermore, the analysis of core genome SNPs confirmed the existence of different subpopulations of VT2f-producing *E. coli* (Figure 2). The analysis of the virulence genes suggests that different populations of VT2f-producing *E. coli* exist and have different potential to cause human disease on the basis of the virulotype to which they belong.

VT2f-producing *E. coli* strains isolated from uncomplicated human cases of diarrhea have been reported in the literature as being ST20 (*11*), which is the same sequence type we identified in most pigeon isolates; this ST was also described in VT2f-producing *E. coli* isolated from pigeons in Japan (*40*). The same study also described an animal isolate of ST722, which was found in 1 strain isolated from human diarrheal feces in our study (Table 2). Similarly, the serotypes in some cases appeared to overlap isolates from pigeons and human cases of diarrhea, such as the serotype O128:H2 that we found in 1 pigeon isolate that was also reported in isolates from human cases of diarrhea in Germany (*11*).

Altogether, these observations indicate that the VT2f-producing *E. coli* causing diarrhea in humans could be a subpopulation of those inhabiting the pigeon reservoir. Alternately, information on the serotypes, ST, and principal component analysis of virulence genes profiles supports the hypothesis that the HUS VT2f-producing strains are more similar to the non-O157 VTEC often isolated from samples from humans with severe disease (Figure 1) than to the other VT2f-producing *E. coli* from humans with diarrhea or from asymptomatic pigeons. This hypothesis suggests that the HUS VT2f-producing strains represent a distinct population of VTEC; whether they are part of the pigeon intestinal flora or arise from an acquisition of the *vtx2*-phage is difficult to ascertain.

The phylogeny of VTEC of different serogroups, investigated by core SNP analysis, showed that the different VT2f-producing *E. coli* cluster into different subpopulations that include strain EF467 grouping together with non-O157 VTEC strains from humans with disease (Figure 2). However, the results from SNP analysis for VTEC of multiple serogroups should be carefully evaluated; the population structure of VTEC belonging to serogroups other than O157 and O26 has not been completely investigated yet.

At the first characterization of the *vtx2f* genes, it was proposed that they were, similar to other VT-coding genes, located on bacteriophages (*5*). Our study confirms this hypothesis and shows that such a phage apparently does not have similar counterparts in the VT-phage genomes reported in the National Center for Biotechnology Information nucleotide repository (http://www.ncbi.nlm.nih.gov/). In addition, we observed that VT2f phage was very similar

in all the VT2f-producing *E. coli* investigated (Figure 3; online Technical Appendix), suggesting that the *vtx2f* genes are present in phages sharing a common ancestor that is different from other phages with the other *vtx1*/*vtx2* subtypes.

In conclusion, we provide evidence that human infections with VT2f-producing *E. coli* are zoonotic diseases transmitted from pigeons. Such an animal reservoir may either directly disseminate VTEC strains causing diarrhea or indirectly release VT2f phages in the environment, which can in turn lysogenize *E. coli* strains that contain accessory virulence determinants and confer them the ability to cause HUS. The isolation of VT2f-producing *E. coli* with a virulence gene profile related to the other HUS-associated VTEC suggests that the severity of the symptoms induced by infection may depend more on the ability to achieve a proficient colonization of the host gut mucosa rather than on the subtype of the produced toxin.

Dr. Grande is a researcher in the field of molecular microbiology. Most of her research activities have been at the European Union Reference Laboratory for *E. coli,* in the unit of Foodborne Zoonoses of the Italian National Institute of Health in Rome, Italy. Her research interests include the investigation and characterization of mobile genetic elements encoding virulence determinants in pathogenic *E. coli*.

References

1. Tozzoli R, Scheutz F. Diarrheagenic *Escherichia coli* infections in umans. In: Morabito S, editor. Pathogenic *Escherichia coli*: molecular and cellular microbiology. Poole (UK): Caister Academic Press; 2014. p. 1–18.
2. Tozzoli R, Grande L, Michelacci V, Ranieri P, Maugliani A, Caprioli A, et al. Shiga toxin–converting phages and the emergence of new pathogenic *Escherichia coli*: a world in motion. Front Cell Infect Microbiol. 2014;4:80. http://dx.doi.org/10.3389/fcimb.2014.00080
3. Caprioli A, Morabito S, Brugere H, Oswald E. Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet Res. 2005;36:289–311. http://dx.doi.org/10.1051/vetres:2005002
4. Dell'Omo G, Morabito S, Quondam R, Agrimi U, Ciuchini F, Macrì A, et al. Feral pigeons as a source of verocytotoxin-producing *Escherichia coli*. Vet Rec. 1998;142:309–10. http://dx.doi.org/10.1136/vr.142.12.309
5. Schmidt H, Scheef J, Morabito S, Caprioli A, Wieler LH, Karch H. A new Shiga toxin 2 variant (Stx2f) from *Escherichia coli* isolated from pigeons. Appl Environ Microbiol. 2000;66:1205–8. http://dx.doi.org/10.1128/AEM.66.3.1205-1208.2000
6. Morabito S, Dell'Omo G, Agrimi U, Schmidt H, Karch H, Cheasty T, et al. Detection and characterization of Shiga toxin-producing *Escherichia coli* in feral pigeons. Vet Microbiol. 2001;82:275–83. http://dx.doi.org/10.1016/S0378-1135(01)00393-5
7. Nielsen EM, Skov MN, Madsen JJ, Lodal J, Jespersen JB, Baggesen DL. Verocytotoxin-producing *Escherichia coli* in wild birds and rodents in close proximity to farms. Appl Environ Microbiol. 2004;70:6944–7. http://dx.doi.org/10.1128/AEM.70.11.6944-6947.2004
8. Farooq S, Hussain I, Mir MA, Bhat MA, Wani SA. Isolation of atypical enteropathogenic *Escherichia coli* and Shiga toxin 1 and 2f-producing *Escherichia coli* from avian species in India. Lett Appl Microbiol. 2009;48:692–7.
9. Kobayashi H, Kanazaki M, Hata E, Kubo M. Prevalence and characteristics of *eae*- and *stx*-positive strains of *Escherichia coli* from wild birds in the immediate environment of Tokyo Bay. Appl Environ Microbiol. 2009;75:292–5. http://dx.doi.org/10.1128/AEM.01534-08
10. Askari Badouei M, Zahraei Salehi T, Koochakzadeh A, Kalantari A, Tabatabaei S. Molecular characterization, genetic diversity and antibacterial susceptibility of *Escherichia coli* encoding Shiga toxin 2f in domestic pigeons. Lett Appl Microbiol. 2014;59:370–6. http://dx.doi.org/10.1111/lam.12288
11. Prager R, Fruth A, Siewert U, Strutz U, Tschäpe H. *Escherichia coli* encoding Shiga toxin 2f as an emerging human pathogen. Int J Med Microbiol. 2009;299:343–53. http://dx.doi.org/10.1016/j.ijmm.2008.10.008
12. Friesema I, van der Zwaluw K, Schuurman T, Kooistra-Smid M, Franz E, van Duynhoven Y, et al. Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011. Euro Surveill. 2014;19:26–32. http://dx.doi.org/10.2807/1560-7917.ES2014.19.17.20787
13. Friesema IH, Keijzer-Veen MG, Koppejan M, Schipper HS, van Griethuysen AJ, Heck ME, et al. Hemolytic uremic syndrome associated with *Escherichia coli* O8:H19 and Shiga toxin 2f gene. Emerg Infect Dis. 2015;21:168–9. http://dx.doi.org/10.3201/eid2101.140515
14. Cuccuru G, Orsini M, Pinna A, Sbardellati A, Soranzo N, Travaglione A, et al. Orione, a web-based framework for NGS analysis in microbiology. Bioinformatics. 2014;30:1928–9. http://dx.doi.org/10.1093/bioinformatics/btu135
15. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77. http://dx.doi.org/10.1089/cmb.2012.0021
16. Tritt A, Eisen JA, Facciotti MT, Darling AE. An integrated pipeline for de novo assembly of microbial genomes. PLoS ONE. 2012;7:e42304. http://dx.doi.org/10.1371/journal.pone.0042304
17. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30:2068–9. http://dx.doi.org/10.1093/bioinformatics/btu153
18. Paton AW, Paton JC. Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J Clin Microbiol. 1998;36:598–602.
19. Caprioli A, Nigrelli A, Gatti R, Zavanella M. Isolation of verotoxin-producing *Escherichia coli* from slaughtered pigs. Eur J Clin Microbiol Infect Dis. 1993;12:227–8. http://dx.doi.org/10.1007/BF01967122
20. Joensen KG, Tetzschner AM, Iguchi A, Aarestrup FM, Scheutz F. Rapid and easy in silico serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. J Clin Microbiol. 2015;53:2410–26. http://dx.doi.org/10.1128/JCM.00008-15
21. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014;58:3895–903. http://dx.doi.org/10.1128/AAC.02412-14
22. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990;215:403–10. http://dx.doi.org/10.1016/S0022-2836(05)80360-2
23. Lindsey RL, Fedorka-Cray PJ, Abley M, Turpin JB, Meinersmann RJ. Evaluating the occurrence of *Escherichia albertii* in chicken carcass rinses by PCR, Vitek analysis, and sequencing of the *rpo*B gene. Appl Environ Microbiol. 2015;81:1727–34. http://dx.doi.org/10.1128/AEM.03681-14

24. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013;5:58–65. http://dx.doi.org/10.1111/1758-2229.12019
25. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in *Escherichia coli*: an evolutionary perspective. Mol Microbiol. 2006;60:1136–51. http://dx.doi.org/10.1111/j.1365-2958.2006.05172.x
26. Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics. 2015;31:2877–8. http://dx.doi.org/10.1093/bioinformatics/btv271
27. Dean P, Kenny B. The effector repertoire of enteropathogenic *E. coli*: ganging up on the host cell. Curr Opin Microbiol. 2009;12:101–9. http://dx.doi.org/10.1016/j.mib.2008.11.006
28. Karmali MA, Mascarenhas M, Shen S, Ziebell K, Johnson S, Reid-Smith R, et al. Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichia coli* seropathotypes that are linked to epidemic and/or serious disease. J Clin Microbiol. 2003;41:4930–40. http://dx.doi.org/10.1128/JCM.41.11.4930-4940.2003
29. Imamovic L, Tozzoli R, Michelacci V, Minelli F, Marziano ML, Caprioli A, et al. OI-57, a genomic island of *Escherichia coli* O157, is present in other seropathotypes of Shiga toxin–producing *E. coli* associated with severe human disease. Infect Immun. 2010;78:4697–704. http://dx.doi.org/10.1128/IAI.00512-10
30. Brunder W, Karch H, Schmidt H. Complete sequence of the large virulence plasmid pSFO157 of the sorbitol-fermenting enterohemorrhagic *Escherichia coli* O157:H- strain 3072/96. Int J Med Microbiol. 2006;296:467–74. http://dx.doi.org/10.1016/j.ijmm.2006.05.005
31. Friedrich AW, Bielaszewska M, Zhang WL, Pulz M, Kuczius T, Ammon A, et al. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J Infect Dis. 2002;185:74–84. http://dx.doi.org/10.1086/338115
32. Jenkins C, Willshaw GA, Evans J, Cheasty T, Chart H, Shaw DJ, et al. Subtyping of virulence genes in verocytotoxin-producing *Escherichia coli* (VTEC) other than serogroup O157 associated with disease in the United Kingdom. J Med Microbiol. 2003; 52:941–7. http://dx.doi.org/10.1099/jmm.0.05160-0
33. Seto K, Taguchi M, Kobayashi K, Kozaki S. Biochemical and molecular characterization of minor serogroups of Shiga toxin–producing *Escherichia coli* isolated from humans in Osaka prefecture. J Vet Med Sci. 2007;69:1215–22. http://dx.doi.org/10.1292/jvms.69.1215
34. van Duynhoven YT, Friesema IH, Schuurman T, Roovers A, van Zwet AA, Sabbe LJ, et al. Prevalence, characterisation and clinical profiles of Shiga toxin–producing *Escherichia coli* in the Netherlands. Clin Microbiol Infect. 2008;14:437–45. http://dx.doi.org/10.1111/j.1469-0691.2008.01963.x
35. Buvens G, De Rauw K, Roisin S, Vanfraechem G, Denis O, Jacobs F, et al. Verocytotoxin-producing *Escherichia coli* O128ab:H2 bacteremia in a 27-year-old male with hemolytic-uremic syndrome. J Clin Microbiol. 2013;51:1633–5. http://dx.doi.org/10.1128/JCM.03025-12
36. Feng PC, Jinneman K, Scheutz F, Monday SR. Specificity of PCR and serological assays in the detection of *Escherichia coli* Shiga toxin subtypes. Appl Environ Microbiol. 2011;77:6699–702. http://dx.doi.org/10.1128/AEM.00370-11
37. Oh JY, Kang MS, Hwang HT, An BK, Kwon JH, Kwon YK. Epidemiological investigation of *eae*A-positive *Escherichia coli* and *Escherichia albertii* strains isolated from healthy wild birds. J Microbiol. 2011;49:747–52. http://dx.doi.org/10.1007/s12275-011-1133-y
38. Ooka T, Tokuoka E, Furukawa M, Nagamura T, Ogura Y, Arisawa K, et al. Human gastroenteritis outbreak associated with *Escherichia albertii*, Japan. Emerg Infect Dis. 2013;19:144–6. http://dx.doi.org/10.3201/eid1901.120646
39. Karch H, Heesemann J, Laufs R, O'Brien AD, Tacket CO, Levine MM. A plasmid of enterohemorrhagic *Escherichia coli* O157:H7 is required for expression of a new fimbrial antigen and for adhesion to epithelial cells. Infect Immun. 1987;55:455–61.
40. Murakami K, Etoh Y, Ichihara S, Maeda E, Takenaka S, Horikawa K, et al. Isolation and characteristics of Shiga toxin 2f-producing *Escherichia coli* among pigeons in Kyushu, Japan. PLoS ONE. 2014;9:e86076. http://dx.doi.org/10.1371/journal.pone.0086076

Address for correspondence: Laura Grande, European Union Reference Laboratory for *E. coli*, Veterinary Public Health and Food Safety Department, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy; email: laur.grande@gmail.com