Forty samples of raw milk cheese and 25 samples of raw milk itself were subjected to enrichment culture for Shiga-toxigenic Escherichia coli (STEC), and a single Stx2 positive strain was obtained from one of the cheese samples. Thus, aged cheeses in which the curd is subsequently heat treated (48°C) cannot be presumed to be STEC free. Infective Stx2 bacteriophages were induced from 3 STEC strains isolated elsewhere from raw milk and 1 STEC strain from aged cheese sourced in Italy. Data on E. coli host range, morphology, genome size, and genetic variation determined by restriction fragment length polymorphism and multi-locus genotyping are presented. Although all 4 bacteriophages were found to be short-tailed Podoviridae, they exhibited considerable variation in both genome size and content. This extended to the Stx2 genes themselves, whose sequences contained several point mutations, but these did not translate to amino acid substitutions.

Key words: Shiga-toxigenic Escherichia coli, milk and cheese, Shiga toxin bacteriophage, protected designation of origin dairy product

Short Communication

The production of Shiga toxins (Stx) is regarded as the main virulence factor of Shiga-toxigenic Escherichia coli (STEC) strains, which are responsible for severe human gastrointestinal diseases, typified by hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) as frequent complications (Cohen and Giannella, 1992). Most pathotypes, among them Escherichia coli O157, carry additional virulence markers such as intimin and enterohemolysin, encoded by eae and hly genes, respectively (LeBlanc, 2003). Shiga toxin genes are located in the genomes of bacteriophages, which are the natural vector for the spread of Shiga-toxigenic potential among E. coli and other Enterobacteriaceae through the phenomenon of lysogenic conversion (Herold et al., 2004; Gyles, 2007; Smith et al., 2007a; Strauch et al., 2008). Two Shiga toxin types exist: Stx1 and Stx2, which may or may not be present together in STEC strains (Nataro and Kaper, 1998) and although Stx1 is highly conserved, several variants of Stx2 exist for which evidence of enhanced pathogenicity exists (Friedrich et al., 2002). The ability of lysogens to give rise to infectious virions and to transduce indigenous E. coli strains within the host gastrointestinal tract of mice (Acheson, 1998; Gamage et al., 2003) and sheep (Cornick et al., 2006) has been demonstrated. Therefore, Stx phages are not only responsible for the origin of STEC, but also play a key role in the pathogenesis of HC and HUS with respect to the virulence and diffusion of pathology. In fact, the dissemination of these viral genes by transduction is considered the most likely mechanism for the dynamic emergence of new STEC serotypes (Schmidt et al., 1999; Schmidt, 2001; Bielaszewska et al., 2007).

Shiga toxin phages described thus far comprise double-stranded DNA with isomeric heads and long tails (Siphoviridae) or short tails (Podoviridae); they are members of the lambdoid family because they all exhibit the organization of genetic modules first described in bacteriophage lambda, even though their genomes have a mosaic structure (Allison, 2007). Usually for lambdoid phages, the ability of Stx phages to form multiple lysogens has been demonstrated (Fogg et al., 2007; Serra-Moreno et al., 2008) and, thus, the rapid evolution of STEC strains could have been driven by intracellular recombination events between multiple integrated prophages. Moreover, multiple copies of the Stx gene in a single E. coli genome may lead to enhancement of toxin production in vivo (Fogg et al., 2012).
Reports on the prevalence of STEC in different environments reveal that most of the strains recovered produce only 1 type of toxin; 2 to 54% of isolates possessed the Stx2 gene, 16 to 62% the Stx1 gene, and up to 38% carried both Stx1 and Stx2 genes (Muniesa et al., 2004; Dumke et al., 2006; Beutin et al., 2007; Slanec et al., 2009). Shiga toxin 2 and its variants are more important in foodborne infection outbreaks (Bertin et al., 2009). Evidence exists that Stx2 is more often associated with the progression of HC and HUS in pediatric patients and to identify the STEC strains associated with these syndromes (Tozzi et al., 2003). A recent episode of STEC foodborne infection involved 9 children in the Lombardy region, and in recognition of the risk associated with raw milk consumption, a ministerial ordinance requiring milk to be heated before consumption was issued (Ministero del Lavoro, della Salute e delle Politiche Sociali, 2009). To further establish and characterize the potential of raw milk and raw milk cheese to serve as a source of STEC, we describe, for the first time, Stx phages induced from E. coli isolated from Italian dairy products.

The bacterial strains used in this work are listed in Table 1. Strains were routinely cultured in lysogeny broth (LB; Bertani, 2004; BD Difco, Franklin Lakes, NJ), with or without 1.5% (wt/vol) agar (Merck KGaA, Darmstadt, Germany). Phage buffer (PB; LB broth supplemented with 10 mM CaCl2; Sambrook and Russell, 2001) was used for phage propagation. Escherichia coli CNCTC 6896, a recA441 mutant of laboratory strain K12, was the indicator strain used for routine propagation of the bacteriophages.

In this study, 40 samples of raw milk cheese (fresh and aged) and 25 samples of raw milk (from goat and cow), collected from different Alpine regions in Northern Italy, were analyzed for STEC presence. Ten grams of each sample was added to 90 mL of LB, supplemented with 20 mg/L of novobiocin (Sigma, St. Louis, MO; Doyle and Schoeni, 1987) homogenized in a Stomacher 400C (Seward, Worthing, UK) for 3 min and incubated at 37°C for 24 h. Two microliters of this preenrichment culture were then subjected to multiplex PCR using

### Table 1. Bacterial strains and bacteriophages

<table>
<thead>
<tr>
<th>Strain/phage</th>
<th>Relevant characteristics</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D3.07</td>
<td>E. coli O157:H7, Stx2, eae, hlyA</td>
<td>Fontina cheese</td>
<td>This work</td>
</tr>
<tr>
<td>181181/2</td>
<td>E. coli O157:H7, Stx2, eae, hlyA</td>
<td>Cow raw milk</td>
<td>IZSLER4</td>
</tr>
<tr>
<td>208164/2</td>
<td>E. coli O157:H7, Stx2, eae, hlyA</td>
<td>Cow raw milk</td>
<td>IZSLER4</td>
</tr>
<tr>
<td>EC34</td>
<td>E. coli O157:H7, Stx2, eae, hlyA</td>
<td>Goat raw milk</td>
<td>Piceozzi et al. (2005)</td>
</tr>
<tr>
<td>SO14</td>
<td>E. coli O157:H7, Stx2, eae, hlyA</td>
<td>Cow raw milk</td>
<td>CEPI5</td>
</tr>
<tr>
<td>NCCTC 6896</td>
<td>E. coli Lambda-K12 indicator strain</td>
<td>CNCTC4</td>
<td>CNCTC4</td>
</tr>
<tr>
<td>NCCTC 6246</td>
<td>E. coli indicator strain</td>
<td>Muniesa et al. (2004)</td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>E. coli indicator strain</td>
<td>James et al. (2001)</td>
<td></td>
</tr>
<tr>
<td>DM1187</td>
<td>E. coli K12 derivative, constitutive RecA expression, indicator strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC1061</td>
<td>E. coli K12 derivative, indicator strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC 12079</td>
<td>E. coli O157:H7, Stx2, eae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WG5</td>
<td>E. coli C derivative, indicator strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦGV8</td>
<td>Stx phage induced from 1D3.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦGV2412</td>
<td>Stx phage induced from 181181/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦBR2</td>
<td>Stx phage induced from 208164/2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΦL34</td>
<td>Stx phage induced from EC34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Unypeable.
2Istituto Zooprofilattico Sperimentale per la Lombardia e l’Emilia Romagna (Brescia, Italy).
3Centro Enteropatogeni Italia Settentrionale (Milan, Italy).
4Czech National Collection of Type Cultures (Prague, Czech Republic).
5National Collection of Type Cultures (Salisbury, UK).
specific primers for amplification of Stx1, Stx2, eaeA, and hlyA genes (Fagan et al., 1999). Amplifications were performed using 1 U of Taq DNA polymerase (5 Prime GmbH, Hamburg, Germany) in a 25-μL reaction comprising 200 μM deoxyribonucleotide triphosphate (dNTP) mix (Fermentas, Vilnius, Lithuania), 3 mmol of MgCl2/L, and 200 nmol/L of each primer. Cycle conditions, carried out in a T-Gradient Biometra Thermocycler (Biometra, Göttingen, Germany) consisted of an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 40 s and extension at 72°C for 90s, followed by a final extension at 72°C for 5 min. Polymerase chain reaction products were separated on a 1.5% agarose gel stained with 0.3 μg/mL of ethidium bromide in Tris-acetate-EDTA (TAE) buffer (40 mmol of Tris-acetate/L and 1 mmol of EDTA/L, pH 8.0). Deoxyribonucleic acid fragments were visualized and photographed under UV illumination. A 100-bp DNA Ladder Plus (Fermentas) was included in each gel as molecular size marker.

For the isolation of presumptive STEC strains, Stx-positive samples were tested by colony hybridization with a DNA probe. For colony dot-blot hybridization, aliquots of the enrichment culture samples were plated onto Chromocult TBX agar (BD Difco). After incubation at 42°C for 24 h, the appropriate dilution with the highest number of separated colonies was selected. Colonies were transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Inc., Piscataway, NJ) according to a standard procedure (Sambrook and Russell, 2001) and hybridized at 65°C with a digoxigenin-labeled Stx probe. The Stx probes were prepared by labeling PCR amplicons from E. coli O157:H7 NCTC 12079 using a DIG High Prime kit (Roche Diagnostics GmbH, Mannheim, Germany) according to Kaufmann et al. (2006). Stringent hybridization was achieved with the DIG-DNA Labeling and Detection Kit (Roche Diagnostic Ltd., Lewes, UK) according to the manufacturer’s instructions.

Shiga-toxigenic Escherichia coli strains were cultured in PB (optical density at 600 nm of 0.45 to 0.55) and treated with norfloxacin (1 μg/mL; Matsushiro et al., 1999) at 37°C for 1 h to induce phages. The cultures were diluted 10-fold in PB and allowed to recover at 37°C for 2 h. One hundred microliters of appropriate dilutions was mixed with 100 μL of PB and 500 μL of an exponential-phase culture of E. coli strain CNCTC 6896 and examined through plaque assay using a double-layer agar method (Muniesa and Jofre, 2004). To detect the presence of the Stx genes in phages, the plaques were transferred to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Inc.) according to a standard procedure (Sambrook and Russell, 2001) and hybridized with digoxigenin-labeled Stx probe as described above for colony dot-blot hybridization.

After filtration through a 0.45-μm membrane, phage suspensions were subjected to purification in a CsCl gradient according to a protocol modified from Sambrook and Russell (2001). Phage stock solutions were plated at low dilutions to produce semiconfluent lysis. After overnight incubation (16 h) at 37°C, the plates were overlaid with 3 mL of SM buffer (100 mmol of NaCl/L, 8 mmol of MgSO4/L, and 50 mmol of Tris-HCl/L, pH 7.5) and stored overnight (16 h) at 4°C. The SM buffer was removed and the top agar was scraped into a sterile glass centrifuge tube, stirred with 10% (vol/vol) SM buffer, centrifuged at 10,000 × g for 10 min at room temperature, and the supernatant recovered. Chloroform (30 μL) was added to each 10-mL aliquot of phage suspension to kill any remaining cells. Deoxyribonuclease (5 μg/mL) and RNase (1 μg/mL; Sigma) were added to remove any bacterial nucleic acids, and the solution incubated for 1 h at 37°C. Bacteriophages were pelleted by ultracentrifugation at 100,000 × g for 30 min at room temperature, resuspended in SM buffer, and subsequently purified by CsCl density preformed gradient (d = 1.3–1.7 g/mL) ultracentrifugation at 100,000 × g for 1 h at room temperature. The visible band was collected and dialyzed with a 30-kDa cut-off membrane at 4°C overnight (16 h) in dialysis buffer (10 mmol of Tris-HCl/L, pH 7.4; 10 mmol of MgSO4/L; and 0.5 mol of NaCl/L) to remove CsCl.

To evaluate the ability of these Stx phages to infect other hosts in addition to E. coli CNCTC 6896, E. coli CNCTC 6246, DH5α, DM1187, MC1061, and WG5 strains were tested. A drop of phage suspension from the Stx phage stocks was spotted onto a monolayer plate containing each indicator strain and, after incubation at 37°C overnight (16 h), plates with a clear zone of lysis were scored as positive. The morphology of viral particles was observed by transmission electron microscopy (Philips 201, 80 kV; Philips International BV, Eindhoven, the Netherlands), after laying the suspensions on 300-mesh copper specimen grids coated with carbon film and negatively stained with 2% (wt/vol) uranyl acetate (pH 4.5).

Phage DNA, extracted with a standard protocol (Sambrook and Russell 2001) from purified stocks, was subjected to RFLP analysis by digestion with the EcoRI restriction enzyme (Fermentas). Deoxyribonucleic acid fragments were separated by agarose (1% wt/vol) gel electrophoresis in TAE buffer at 5 V/cm and visualized by UV light after staining with ethidium bromide. Deoxyribonucleic acid standard fragments of MassRuler DNA Ladder Mix ready-to-use (Fermentas) were used to calculate the size of the fragments. Genomic length
was estimated by the sum of molecular weights of fragments generated by digestion. Purified bacteriophage DNA was also characterized using the multi-loci typing scheme based on lambdoid phage genome organization, using the methods described by Smith et al. (2007b).

The Stx2 gene of each phage was sequenced using the oligonucleotides S2Aup/S2Alp for Stx2 subunit A and GK3/GK4 for subunit B, as described by Muniesa et al. (2004) and using oligonucleotides VT1/VT2, as described by Kaufmann et al. (2006). Sequencing was provided by Primm Srl (Milan, Italy). Nucleotide sequence analysis, assembly of the sequences, protein translation, and BLAST analyses were performed with the GenDoc Multiple Sequence Alignment Editor (http://petang.cgut.edu.tw/Bioinfomatics/Softwares/SOFTWAREES/Soft-index.html), BioEdit Sequence Alignment Editor (http://www.ncbi.nlm.nih.gov/BioEdit/bioedit.html), and the ClustalW Multiple Sequence Alignment Program (http://www.ebi.ac.uk/clustalw/). Nucleotide sequences obtained in this work have been deposited in the GenBank database under accession numbers from JN193428 to JN193431.

Only 1 of the 40 cheese samples tested gave a positive result after the multiplex PCR assay on the preenrichment broth culture, showing a band for the Stx2 gene only at 779 bp. That same sample yielded a single STEC strain (named ID3.07), isolated and confirmed as Stx positive by colony-dot-blot hybridization. Amplification products for the eae and hlyA genes were not detected and the STEC strain could not be serotyped. The positive sample was an Italian protected designation of origin cheese produced from raw cow milk. None of the 25 samples of raw cow milk tested positive for the presence of the Stx gene by PCR amplification of extracted DNA. These results can be added to the limited data set that already exists for STEC occurrence in dairy products; Pradel et al. (2000) found that 60 out of 603 (10%) cheese samples examined were positive for Stx genes; Vernozy-Rozand et al. (2005) observed a STEC incidence of 13% in 1039 French raw milk cheeses; Rantsiou and Cocolin (2009) used quantitative PCR to report 18% Stx-positive samples in Piedmont traditional cheeses manufactured from raw milk; Stephan et al. (2008) and recently Zweifel et al. (2010) reported STEC prevalences of 4.9% in 796 samples and 5.7% in 1,502 samples, respectively, of soft, semi-hard, and hard cheeses made from Swiss cows, goats, and sheep raw milk collected at the producer level. The recovery here of an STEC strain (1D3.07), lysogenic for an inducible Stx phage (ΦGV8), from a sample of Fontina cheese that had undergone heating of the curd at 48°C and was ripened for no less than 3 mo at 12°C, supports a previous study (Zweifel et al., 2010) in which it was suggested that aged raw milk cheeses are a potential source of STEC.

Other STEC isolated from raw milk samples, obtained in previous studies or kindly supplied by Italian institutions, were also investigated (Table 1). Escherichia coli O157:H7 strains 181181/2, 208164/2, and EC34 yielded amplification products of the expected size in multiplex PCR for the Stx2, eae, and hlyA genes; SO14, an untypable E. coli O strain, was positive for Stx2 and hlyA genes, whereas our ID3.07 isolate, the other untypable strain, was positive for the Stx2 gene only. Induction of prophages by norfloxacin treatment was carried out on these 5 STEC strains, and infective bacteriophages were detected in 4 of the 5 strains (not SO14) by plaque assay. A single Stx2 phage was then purified from each one of the 4 viral lysates; the purified phages (Table 1) were again shown to be Stx positive by plaque hybridization assay. Detection by plaque assay is dependent on the susceptibility of host indicator strains, as evidenced here by the variation in host range found among only 4 purified Stx phages (Table 2). More meaningful data on the prevalence of Stx phages requires the application of direct viral DNA recovery protocols in combination with quantitative PCR-based quantification (Rooks et al., 2010).

All 4 phages had a similar morphology as determined by transmission electron microscopy (Figure 1). The viral particles were identified as members of the Podoviridae family, characterized by a short tail and icosahedral head; base plate-like structures were not observed. The heads had a diameter of 45 to 55 nm.

Table 2. Characterization of purified Shiga toxin (Stx) phages

<table>
<thead>
<tr>
<th>Isolated phage</th>
<th>Capsid diameter (nm)</th>
<th>Tail length (nm)</th>
<th>Genome size (kbp)</th>
<th>Host range&lt;sup&gt;3&lt;/sup&gt;</th>
<th>% Identity to closest match sequences of Stx2 subunit A and B genes (accession number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΦGV8</td>
<td>52</td>
<td>8</td>
<td>61</td>
<td>CNCTC 6246</td>
<td>++</td>
</tr>
<tr>
<td>ΦGV2412</td>
<td>45</td>
<td>9</td>
<td>65</td>
<td>CNCTC 6896</td>
<td>++</td>
</tr>
<tr>
<td>ΦBR2</td>
<td>55</td>
<td>9</td>
<td>53</td>
<td>DH5α</td>
<td>++</td>
</tr>
<tr>
<td>ΦL34</td>
<td>52</td>
<td>10</td>
<td>58</td>
<td>DM1187</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MC1061</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WG5</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>3</sup>+++ = strong lysis; ++ = clear lysis; + = weak lysis; − = no lysis in the spot area.

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nm with short tails up to 10 nm in length (Table 2). Mean values were obtained by measuring the dimensions of 10 individual viral particles for each of the 4 phage preparations on at least 2 different microscopic preparations. RFLP analysis of phage DNA extracts generated a unique banding pattern for each (Figure 2) and the genome sizes estimated on the basis of the total sum of the restriction fragments were also distinct and in the range of approximately 53 to 61 kbp (Table 2). The ability of Stx phages to infect different host strains was evaluated by spotting the phage preparations on a lawn of each E. coli strain and scoring any lysis observed (Table 2). All phages were able to infect and lyse the phage propagation host E. coli CNCTC 6896; E. coli DM1187, which is a recA441 mutant that produces RecA protease constitutively and, therefore, cannot be lysogenized; and E. coli WG5, which possesses an attenuated host restriction modification system, supporting increased phage sensitivity. In contrast, E. coli CNCT 6246 and DH5α were not infected by any of the 4 phages, whereas E. coli MC1061 was sensitive to ϕGV8 and ϕL34 phages only. The ϕGV2412 and ϕBR2 phages, induced from 2 strains of E. coli O157:H7, had a similar host range to one another (Table 2).

The nucleotide sequences of the toxin genes confirmed that all of the 4 phages carried the Stx2 gene. This is in agreement with the suggested predominance of short-tailed Podoviridae carrying the Stx2 form of the Stx gene among inducible Stx phages (Smith et al., 2007; McDonald et al., 2010). The Stx2 subunit A and B gene sequences exhibited close homology to various previously described phage-borne Stx genes (Table 2; Supplementary Figure 1, available online at http://www.journalofdairyscience.org/): the Stx2 sequence of ϕBR2 was absolutely identical to that of the VTB60 phage induced from an O136:H E. coli strain (Muniesa et al., 2004); the ϕL34 Stx2 subunit A and B genes were sufficiently similar to the Shiga-like toxin from phage VTB324 induced from an O177:H25 E. coli strain (Muniesa et al., 2004) to enable its identification as an Stx2c variant (Meyer et al., 1992).

The genotypic variation detected in the 4 purified Stx phages is illustrated in Figure 3. As predicted by the data of Smith et al. (2007b), confirming the inherent genetic mosaicism of Stx phages, each purified bacteriophage had a unique profile based on the distribution of variants of specific loci. This was confirmed by the construction of a Jaccard single linkage dendrogram (Figure 3). The sequenced Stx phages ϕ24b, 933W, and P27, and bacteriophage lambda were also included in the analysis. All of the short-tailed phages (Podoviridae) share the tail fiber gene type VTTF (Figure 3); otherwise, the genetic heterogeneity indicated by the RFLP analysis (Figure 2) is reflected by the PCR based multi-loci typing.

In spite of abundant literature related to the characterization of Stx phages isolated from the environment, fecal material, sewage, and water (Gamage et al., 2004; Muniesa et al., 2004; Muniesa and Jofre, 2004; García-Aljaro et al., 2009), no data exist on phages induced from STEC isolated from milk or cheese. Here, 4 Stx...
phages were induced from STEC strains isolated from different sources and dairy products, yet all had similar morphologies and carried the *Stx* gene. Both the RFLP and multi-locus characterization data sets (Figures 2 and 3) confirm that Stx phages are genetically heterogeneous (Muniesa et al., 2004; Allison, 2007). This genetic variation also extended partly to the *Stx* gene itself, even in this small subset of induced Stx phages, further emphasizing the dynamic state of lambdoid phage evolution and its potential contribution to the emergence of new foodborne STEC strains.

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