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Hemolytic–uremic syndrome associated with enterohemorrhagic *Escherichia coli* O26:H infection and consumption of unpasteurized cow's milk

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Background: Enterohemorrhagic *Escherichia coli* (EHEC) O26 has emerged as a significant cause of hemolytic-uremic syndrome (HUS). The source and the vehicle of contamination with EHEC O26 are not often identified. We report two Austrian cases of HUS due to *E. coli* O26:H$^-$ affecting an 11-month-old boy and a 28-month-old girl in which transmission through unpasteurized cow's milk was positively identified.

Methods and Results: Using automated ribotyping and pulsed-field gel electrophoresis (PFGE), the isolates (which yielded the virulence genes *stx2*, *eae*, and *hly*) were indistinguishable from each other. An epidemiologic investigation revealed that the children had stayed in the same hotel. Both patients had consumed unpasteurized cow's milk from the breakfast buffet. Fecal samples were taken from the cows of the farm producing the incriminating milk, and one of three cattle EHEC O26:H$^-$ isolates had a PFGE pattern indistinguishable from that of the patients' strains.

Conclusions: These two cases of *E. coli* O26 infection illustrate the hazards associated with the consumption of raw milk, and underline the importance of microbiological diagnostic approaches able to detect sorbitol-fermenting, non-O157 EHEC.

INTRODUCTION

Hemolytic–uremic syndrome (HUS) is often associated with enterohemorrhagic *Escherichia coli* (EHEC) infections, particularly those due to EHEC O157.[1] Other EHEC serogroups, especially O26, have recently emerged as significant causes of HUS.[1-4] While the epidemiology of EHEC O157 infection has been thoroughly investigated, little is known about sources and the vehicles of contamination with EHEC O26. We describe here two EHEC O26-associated HUS cases in which transmission through unpasteurized cow's milk was identified.

MATERIALS AND METHODS

Microorganisms

Stools were investigated for EHEC using methods described previously.[5,6] Briefly, stools were enriched in GN Broth Hajna (Difco Laboratories, Detroit, MI, USA), and 1 mL of the enrichment culture was investigated for *E. coli* O157 using an immunomagnetic separation procedure and subsequent culture of magnetic beads with attached O157 bacteria on sorbitol MacConkey (SMAC) (Difco Laboratories) agar and cefixime-tellurite–SMAC agar.[7] To detect non-O157 ETEC, 100 µL of enriched culture was inoculated on SMAC agar and on enterohemolysin agar (Sifin GmbH, Berlin, Germany). Overnight bacterial growth from the plates was harvested in 1 mL of saline and screened for the presence of genes encoding for Shiga toxin production (*stx1* and *stx2*) by PCR with primers KS7–KS8[5] and LP43–LP44,[8] respectively, and conditions described previously.[5,6] The identification of EHEC in PCR-positive cultures was performed from enterohemolysin agar by analysis of colonies which displayed an enterohemolytic phenotype for *stx1* and *stx2* using primers KS7–KS8 (*stx1B*) and GK3–GK4 (*stx2B*, *stx2cB*), as described

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previously.[5,6] stx_2 and stx_{2c} were differentiated by restriction analysis of GK3/GK4 amplification products using *Hae*III or *Fok*I, as described by Rüssmann et al.[9] The isolates were investigated for *eae* encoding intimin and for the enterohemolysin-encoding gene (EHEC-*hlyA*) by PCR with primers SK1–SK25 and Hly1–HlyA4.[10] Isolated EHEC strains were serotyped and investigated for sorbitol fermentation on SMAC agar. Production of enterohemolysin was investigated on blood agar containing washed human erythrocytes.[10]

Automated ribotyping

EHEC O26 isolates were ribotyped by use of an automated microbial characterization system (RiboPrinter, Qualicon, Wilmington, DE, USA), as described previously.[11] In brief, isolated colonies were plated onto brain heart infusion agar (BBL, Cockeysville, MD, USA) plates. After overnight growth, each sample was suspended in lysis buffer and heated at 80°C for 10 min. The samples were then processed by use of the aforementioned automated microbial characterization system. In the first step, samples were treated with a lysing agent to release the DNA. The DNA was then digested to completion with the restriction enzyme *Pvu*II. The resulting DNA restriction fragments were transferred to an agarose gel cassette. Using a process known as direct blot electrophoresis, the DNA fragments were size separated and transferred to a moving nylon membrane. After denaturation, each membrane was hybridized with a chemically labeled rRNA operon from *E. coli*. The membrane was washed and treated with blocking buffer and an antisulfonated DNA antibody–alkaline phosphate conjugate. Unbound conjugate was removed, and a chemiluminescent substrate applied. This step makes each electrophoresis band containing genetic information from the rRNA genes visible to a custom CCD camera in the RiboPrinter system. Through the customized software of this system, each sample was characterized into a pattern (ribotype).

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) of *Xba*I-digested total DNA was performed as described previously.[3,11] Bacteria were grown at 37°C overnight from single colonies in 10 mL of 1% Bacto Tryptone (Difco Laboratories). Grown cultures were harvested by centrifugation and resuspended in the same volume of SE buffer (10 mM Tris-Cl, 25 mM EDTA, and 75 mM NaCl (pH 7.5)). After a second centrifugation, the bacterial pellet was resuspended in 1 mL of SE buffer. A 300-µL aliquot of concentrated bacteria was mixed rapidly in the same amount of molten 2% Rapid-Agarose (Gibco-BRL, Eggenstein, Germany), and this was followed immediately by placing the mixture in sample inserts in a Bio-Rad sample mold (Bio-Rad Laboratories, Richmond, CA, USA), as instructed by the supplier.

Lysis of bacteria embedded in agarose was performed by 15 h of incubation at 56°C in lysis buffer (10 mM Tris-Cl, 1 mM EDTA, 1% *N*-laurylsarcosine sodium salt (pH 9.5)) containing 0.5 mg of proteinase K (Boehringer Mannheim, Mannheim, Germany) per milliliter. After lysis, agarose plugs were washed five times in sterile TE buffer (10 mM Tris-Cl, 1 mM EDTA (pH 8.0)), and this was followed by a 1-h incubation at 37°C in the TE buffer containing RNase (final concentration, 20 µg/mL). For *Xba*I restriction endonuclease cleavage of genomic DNA, the agarose plugs were equilibrated for 1 h at 4°C in *Xba*I restriction enzyme buffer (Reac 2; Gibco-BRL), and this was followed by an overnight incubation in Reac2 containing 50 U of *Xba*I (Gibco-BRL) at 37°C. After enzymatic digestion, the agarose plugs were washed in 10 mM Tris-Cl/50 mM EDTA (pH 8.0) and kept at 4°C in the same type of buffer for long-term storage. PFGE was performed with the clamped homogeneous electric field (CHEF-DR II) system from Bio-Rad Laboratories, as instructed by the supplier. A slice of agarose plug was sealed into a well of 1% horizontal agarose gel as described in the Bio-Rad instruction manual. Gels were run for 22 h at a voltage of 6 V/cm at 14°C, with a pulse time increasing from 2.2 to 54.2 s and a field angle of 120°.

CASES AND EPIDEMIOLOGIC REVIEW

Patient A

On 12 July 2001, a 28-month-old girl was admitted to the University Childrens' Hospital in Graz, 11 days after returning from a 1-week holiday (23 June to 1 July). Prodromal mucoid diarrhea had started on 27 June, and improved after 3 days. Thereafter, increasing pallor—first noticed on 6 July—had been the predominant feature. On admission, laboratory investigation indicated non-oliguric renal failure (plasma creatinine 1.5 mg/dL; normal range for this age 0.3–0.6 mg/dL) and hemolytic anemia (hemoglobin 5.9 g/dL; normal range for this age 11.5–14.0 mg/dL), with 15% fragmentocytes. The platelet count was normal (185 000/mm³; normal range for this age 140 000–440 000/mm³). Lactic dehydrogenase was highly elevated (923 U/L; normal range for this age 120–340 U/L). Supportive therapy was administered, and renal function normalized within 7 days. A stool specimen taken on 14 July yielded EHEC O26:H⁻. A serum specimen, taken on the same day, showed the presence of antibodies to the lipopolysaccharide (LPS) of *E. coli* O26 when tested by ELISA as previously described,[12] but yielded negative results for the LPS from the other EHEC-related serogroups O157, O111, O103, and O145. The girl was discharged after 12 days of hospitalization.

Patient B

On 27 July 2001, an 11-month-old boy was admitted to the University Childrens' Hospital in Vienna, 13 days

after returning from a 1-week holiday (7 July to 14 July). Bloody diarrhea had started on 20 July; the first signs of kidney involvement (hematuria and proteinuria) were noticed on 27 July. On admission, laboratory investigation revealed mild renal failure (serum creatinine 0.66 mg/dL; normal range for this age 0.2–0.4 mg/dL), hemolytic anemia (hemoglobin 7.0 g/dL; normal range for this age 11.5–15.5 g/dL) with 5% fragmentocytes, and thrombocytopenia (platelets 66 000/mm^3; normal range for this age 150 000–400 000/mm^3). Lactic dehydrogenase was highly elevated (1450 U/L; normal range for this age 170–580 U/L). A stool specimen taken on 31 July yielded EHEC O26:H−. A serum specimen, taken on the same day, yielded antibodies against the *E. coli* O26 LPS, but gave negative results for the other EHEC-related serogroups. The patient recovered without dialysis, and was discharged after 17 days of hospitalization.

Epidemiologic review

Both *E. coli* O26:H− isolates yielded virulence genes *stx2*, *eae*, and *hly*. With the use of automated ribotyping (Ribotyper, Qualicon), both strains were indistinguishable from each other but clearly different from three other Austrian O26:H− strains isolated between 1998 and 2001 (Figure 1). An epidemiologic investigation revealed that the children were living in different cities in the provinces of Vienna and Styria, but had stayed at the same hotel (a 37-room spa, catering to parents with babies and young children) in the province of Carinthia. Both patients had consumed unpasteurized cow's milk from the breakfast buffet; the parents were not aware that this milk was untreated. The farm producing the incriminated milk was traced, and, on 14 August, fecal samples were collected from 18 cows held on the farm. Three of the 18 fecal specimens collected yielded EHEC O26:H−. Subtyping by PFGE revealed that one of these three bovine isolates was indistinguishable from the patients' strains.

DISCUSSION

EHEC O26:H11 and O26:H− have recently emerged as important causative agents of diarrhea and HUS all over Europe.[4,13] Zhang et al showed that the extensive genetic diversity of EHEC O26 provides a basis for molecular subtyping of this important non-O157 serogroup.[3] In the year 2001, four EHEC O26 infections were documented in Austria (total population: approximately 8 million). In addition to the two cases described in this report, a 65-year-old male patient acquired bloody diarrhea during a vacation in Bulgaria (stool cultures yielded EHEC O26:H−), and a 2-year-old girl presented with HUS (negative stool cultures after previous antibiotic treatment, and EHEC O26 infection proven by serology only). In Austria, the two HUS cases described in this report are the first EHEC O26 infections where the source of infection could be elucidated. Raw cow's milk as the source of infection is not surprising, since the importance of cattle as a reservoir of Shiga toxin-producing EHEC O26 is well documented.[14]

Austrian law does not allow the delivery of raw cow's milk to hotels; for the few exceptions, labeling with 'raw milk, boil before consumption' is mandatory.[15] The two cases of EHEC O26 infection reported here illustrate the hazards associated with the consumption of raw milk, a fact not yet recognized by the general public. Transmission of bacterial pathogens other than EHEC O26 with raw milk has been shown in Austria repeatedly, including cases from elementary school-related activities.[16–19] The manager of the hotel catering to babies and young children justified the use of untreated milk by a perceived 'general trend towards biological products'.

The two cases of EHEC O26 infection reported here also underline the importance of microbiological diagnostic approaches able to detect sorbitol-fermenting, non-O157 EHEC. Sorbitol-fermenting EHEC, like EHEC O26, would be missed by diagnostic methods
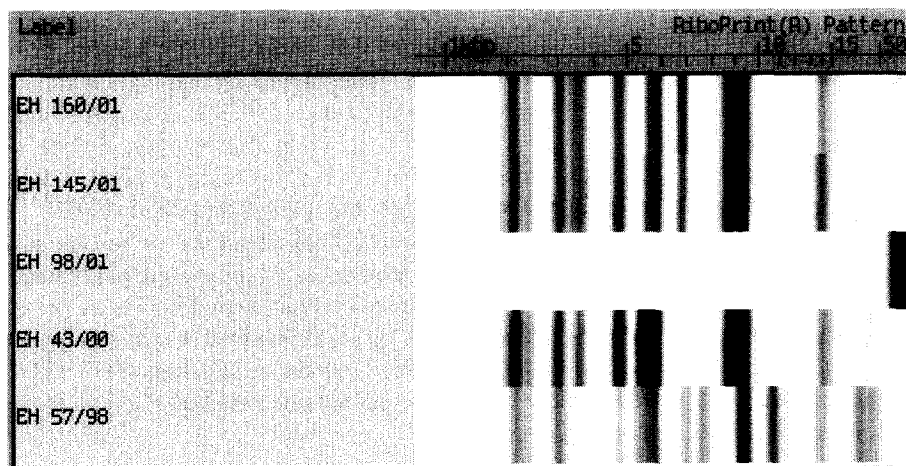

Figure 1. Ribogroups of *Escherichia coli* O26:H− isolates analyzed by the RiboPrinter system using PvuII as restriction enzyme (source of strains: EH160/01 from patient B; EH145/01 from patient A; EH98/01 isolated from a 65-year-old in 2001 after a vacation in Bulgaria; EH43/00 isolated from a 14-month-old girl in 2000 in Carinthia; EH57/98 isolated from an 11-month-old girl in 1998 in Styria).

purely relying on SMAC plates.[20] Therefore, *E. coli* strains from patients with HUS should be tested for the presence of Shiga toxin genes or the ability to produce the toxin.

The detection of antibodies to the LPS of *E. coli* O26 in patients' sera confirmed the etiologic role of EHEC O26 in both HUS cases. Several studies have shown that LPS serology, especially if performed with a panel of different O antigens, is a reliable and effective method of diagnosing EHEC infection in HUS patients.[12,21–24] Moreover, since the antibodies are serogroup specific, LPS serology provides useful epidemiologic information on the serogroup of the infecting strain, even if this cannot be isolated from the patient's stool due to late collection of the specimen or previous antimicrobial therapy.

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