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Outbreak of non-sorbitol-fermenting shiga toxin-producing *E. coli* O157:H7 infections among school children associated with raw milk consumption in Germany

Ausbruch bedingt durch nicht-sorbitol-fermentierende STEC O157:H7 unter Schulkindern in Verbindung mit dem Verzehr von Rohmilch in Deutschland

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Summary

After a day trip of an elementary school to a dairy farm in 2008 45 pupils aged 6–10 years became ill with symptoms of an acute gastroenteritis. Two children had to be hospitalized with haemolytic uremic syndrome. A retrospective cohort study was conducted to identify the source of infection. Non-sorbitol-fermenting shiga toxin-producing *E. coli* (STEC) O157:H7 could be detected in 26 participants (attack rate 24 % of all 109 participants). Statistical analysis with logistic regression revealed the strongest association of laboratory confirmed infection with the consumption of raw milk offered at the dairy farm (adjusted OR (aOR) = 10.04, p = 0.03). No significant statistical association was observed for any other food item or exposure. In addition STEC O157:H7 could be verified in faeces from cattle kept at the dairy farm. A comparison of isolates of STEC O157:H7 from human stool samples of confirmed cases with isolates from samples of cow faeces revealed identical patterns in the Pulsed Field Gel Electrophoresis (PFGE) and identical virulence factors. This investigation represents a very rare event where a strong association between the detection of STEC O157:H7 infections and the consumption of raw milk could be shown in an outbreak setting. A stronger awareness also about the risks of raw milk consumption is needed in the society to prevent future infections and outbreaks.

Keywords: non-sorbitol-fermenting STEC O157:H7, food-borne outbreak, raw milk, dairy farm

Zusammenfassung

Im Anschluss an einen Tagesausflug einer Grundschule auf einen Milchhof im Jahre 2008 erkrankten 45 Schüler im Alter zwischen 6 und 10 Jahren mit Symptomen einer akuten Gastroenteritis. Zwei Kinder mussten mit einem Hämolyisch-Urämischen Syndrom stationär behandelt werden. Eine retrospektive Kohortenstudie wurde daraufhin durchgeführt, um die Quelle der Infektionen aufzudecken. Bei 26 Teilnehmern (Attack rate 24 % von allen 109 Teilnehmern) konnten nicht-sorbitol-fermentierende, Shigatoxin-produzierende *E. coli* (STEC) O157:H7 isoliert werden. Die statistische Analyse mithilfe einer logistischen Regression zeigte den stärksten Zusammenhang von laborbestätigten Infektionen mit dem Verzehr von Rohmilch, die auf dem Milchhof angeboten wurde (adjustiertes OR (aOR) = 10,04; p = 0,03). Mit anderen Lebensmitteln oder anderen Expositionen auf dem Hof konnte kein statistisch signifikanter Zusammenhang gefunden werden. Darüber hinaus konnten nicht-sorbitol-fermentierende STEC O157:H7 in Kotproben der Rinder des Hofes nachgewiesen werden. Ein Vergleich von *E. coli* O157:H7 Isolaten aus Stuhlproben von bestätigten Fällen mit Isolaten aus Kotproben zeigte identische Muster in der Puls-Feld-Gel-Elektrophorese (PFGE) und identische Virulenzfaktoren. Diese Untersuchung zeigt ein seltenes Beispiel für einen höchstwahrscheinlich ursächlichen Zusammenhang zwischen nicht-sorbitol-fermentierenden STEC O157:H7-Infektionen und dem Verzehr von Rohmilch in einem Ausbruchsgeschehen. In der Bevölkerung muss ein stärkeres Bewusstsein auch für die Risiken, die mit dem Verzehr von Rohmilch verbunden sind, erreicht werden, um zukünftige Infektionen und Ausbrüche zu verhindern.

Schlüsselwörter: nicht-sorbitol-fermentierende STEC O157:H7; Lebensmittel-bedingter Ausbruch; Rohmilch; Milchhof

Introduction

Consumption of raw milk has often been confirmed or considered as the source of Shiga toxin (Stx)-producing *E. coli* (STEC) infections with to some extend serious progression (Allerberger et al., 2001; Crump et al., 2002; Goh et al., 2002; Gillespie et al., 2003; Payne et al., 2003; Werber et al., 2007). Therefore, the selling of raw milk from dairy farms directly to the consumer is only permitted with the additional advice "... boil before consumption" by the German Food Law (Anonymous, 2007). Although infections with STEC are often considered being associated with the consumption of not well cooked meat and meat products of ruminants, unpasteurized milk or products thereof, are also regarded as risky food. In addition, food-borne outbreaks were reported after consumption of unpasteurized apple juice and contaminated vegetables (Ackers et al., 1998; Itoh et al., 1998; Cody et al., 1999; Barak et al., 2002). Direct contact with cattle or other farm animals and spread from person to person have also been reported as mode of transmission even in outbreaks where many persons were affected as shown previously (Gage et al., 2001; Allerberger et al., 2003; Guh et al., 2010).

Beutin et al. (1993) have shown that cattle and other ruminants are a natural reservoir for STEC. STEC are a group of intestinal pathogenic *E. coli* (Nataro and Kaper, 1998) and are further categorized by many serogroups and serotypes. Among the most common serogroups associated with severe diseases, such as O26, O91, O103, O111, and O157, non-sorbitol-fermenting STEC O157:H7 is the most frequently isolated serotype of STEC worldwide and also in Germany, especially in outbreaks as shown by Gyles (2007), Paton and Paton (1998) and Dreesman et al. (2008). Despite its frequent occurrence the pathogen is rarely found outside human cases (Alpers et al., 2009; Werber et al., 2011). Typical clinical symptoms of STEC infections in humans are characterized by spasmodic abdominal pain, vomiting and/or bloody diarrhoea. Most frequently, the first symptoms appear one to eight days after the infection (average two to three days).

The pathogenicity of STEC is determined by different virulence factors, especially Stx. There are two major types, Stx1 and Stx2. STEC may produce one or more representatives of these toxin types as shown by Nataro and Kaper (1998). Severe complications like the haemolytic uremic syndrome (HUS) are predominantly associated with Stx2-producing strains (Friedrich et al., 2002). HUS is usually characterized by haemolytic anaemia, renal failure and low platelet count. Bell et al. (1994) and Tarr at al. (2005) showed that HUS is the most common cause of acute renal failure in children in industrialized countries. Up to 15 % of infected persons, especially children under the age of 10 may develop a HUS during the infection.

On May 5th 2008, 109 participants (101 pupils, 8 teachers) from an elementary school went for a day trip to a dairy farm. At the farm the pupils had the opportunity to get in contact with different kinds of animals like horses, cows and chicken. Raw milk was offered by the farmer and consumed by most of the pupils and some teachers. The day after the trip the first children became ill with diarrhoea. During the following days 45 participants of the daytrip (41.3 %) developed symptoms of an acute gastroenteritis with diarrhoea, vomiting and abdominal pain. Two children were diagnosed with HUS and had to be hospitalized.

Methods

In order to decide on infection control measures and identify the source of infection, several analyses were conducted. These included an retrospective cohort study in the cohort of school children to get epidemiological evidence for the source of infection, a laboratory analysis of human stool samples of all persons who were suspected being infected (291) as well as investigations of livestock at the farm to get microbiological evidence for the source of infection.

Retrospective cohort study

To identify the source of infections, a retrospective cohort study with 291 individuals was conducted by the local Public Health Department in Diepholz, Germany, in collaboration with the Governmental Institute of Public Health of Lower Saxony (NLGA). The total cohort included all participants of the day trip (109), family members of symptomatic participants (176) and employees of the dairy farm (6). All participants were provided with a standardized questionnaire on symptoms and exposures during the day trip.

A suspected case was defined as a participant of the day trip on May 5th 2008 with an onset of diarrhoea (more then three liquid stools per day) or vomiting until May 13th 2008 or laboratory confirmation for a STEC infection by PCR (including all participants with serotypes different to STEC O157:H7 and participants without a serotype result). Based on the average incubation period for STEC infections of one to eight days, only participants with an onset of symptoms between May 6th and May 13th were assessed as suspected cases. A confirmed case was defined as a participant of the day trip on May 5th 2008 with a verified STEC infection with serotype STEC O157:H7. Non-cases were defined as participants with no diagnosed STEC infection and without declared onset of diarrhoea or vomiting until May 13th 2008. The epidemic curve shown in figure 1 represents the cases that provided information on the day of onset. The onset of the first symptoms (diarrhoea and/or vomiting) was considered as onset of disease.

Epidemiological analyses aimed to assess the association between food items and exposures from the questionnaire and the infection status. For this analysis, only confirmed cases in relation to non-cases (neither confirmed nor suspected) were considered, whereas suspected but non-confirmed cases were excluded.

The association was assessed by means of the relative risk (RR) and the chi^2-test. To adjust for correlations among exposures, multivariable logistic regression was conducted. Only variables that showed a significant association with the STEC O157:H7 infection in the univariable analysis plus age and sex were selected as variables in the multivariable model. The results were presented by means of adjusted odds ratio (aOR) with its 95 % confidence interval (CI). P values less than 0.05 were considered statistically significant. In case of sparse data, association was tested by means of Fisher's exact test. Quantitative properties of cases and non-cases, like age and amount of raw milk consumed were compared using a t-test. Data entry was performed with Epi Info™ version 3.3.2 (CDC, Atlanta, GA, USA). STATA/IC version 9.2 was used for statistical analysis.

Laboratory investigation

Stool samples of all participants (109), all family members of symptomatic participants (176) and of the employees (6)

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of the dairy farm were taken for microbiological investigation. Stool samples were analysed for STEC and other pathogens as possible agents of acute gastroenteritis at the NLGA. An ELISA (r-Biopharm AG, Darmstadt, Germany) was used as a screening test for Stx 1/2. Detection of Stx1 or Stx2 by ELISA was confirmed by PCR (Primer: STEC-1, STEC-2 for light cycler PCR, TIB Molbiol GmbH, Berlin, Germany). In case of a positive PCR result for any *stx*, the pathogen was cultured and isolated for further serotyping (Sifin Shiga Toxin Colony Immunoblot, Sifin, Berlin, Germany). Other virulence factors (*eae*, E-*hlyA*) were detected by PCR (Primer: eaeAF, eaeAR, hlyAF, hlyAR for light cycler PCR TIB Molbiol GmbH, Germany) from isolated samples. A STEC infection was only confirmed for samples with a positive PCR result for *stx*. Sorbitol fermentation was detected using Sorbit-MacConkey Agar (Heipha Diagnostika GmbH, Eppelheim, Germany).

In the National Reference Laboratory for *Escherichia coli* in Berlin, Germany, PCR positive samples were serotyped and characterized by molecular methods as shown by Beutin et al. (2007). Pulsed field gel electrophoresis (PFGE) was performed in the National Reference Laboratory for *Escherichia coli* (Berlin) as well as in the National Reference Center for Salmonella and Other Enteric Pathogens (Wernigerode) using a standardized PulseNet protocol (Gerner-Schmidt and Scheutz, 2006).

Environmental investigation

Approximately two weeks after the outbreak, the dairy farm was inspected by the LAVES, Veterinary Institute Hannover in regard to hygienic handling of the milking process and the storage of the milk. To identify a potential reservoir host of STEC within the herd cattle faeces and milk from the dairy farm were tested for STEC and other pathogens. In total, 20 raw milk samples were collected on four different days between May 16th and May 28th 2008 (one sample of 200 ml) from one bulk tank. Furthermore 443 faecal samples were collected from 443 animals on three different days between May 20th and May 27th 2008. These samples were pooled and analysed in 88 collective samples. A RIDASCREEN® Verotoxin ELISA (r-Biopharm AG, Darmstadt, Germany) was used as a screening test for Stx 1/2 according to the manufacturers' instructions. Therefore, an aliquot of the pre-enrichment of these samples in tryptic soy broth, supplemented with Novobiocin (Merck KGaA, Darmstadt, Germany) was inoculated into a tryptic soy bouillon broth supplemented with Mitomycin C (r-Biopharm AG, Darmstadt, Germany).

Detection of Stx by ELISA was confirmed by PCR. Stx producing colonies were identified by a colony immunoblot as shown by Klie et al. (1997), sub-cultivated, isolated and finally sent to the National Reference Laboratory for *E. coli*, BfR, Berlin, for further characterisation.

PFGE Cluster analysis

In order to determine the degree of genetic relatedness between the strains, the STEC O157:H7 isolates from stool samples of confirmed cases and from cattle faeces were compared for their *XbaI* macrorestriction patterns by PFGE. Images were analysed using BioNumerics software (version 5.10; Applied Maths, Ghent, Belgium). Percentages of similarity between fingerprints were determined using the band-based Dice coefficient and a 1.50 % band position tolerance. The unweighted pair group method using average linkages was used for generating a dendrogram.

Results

Retrospective cohort study

The questionnaires of all 109 participants of the day trip (101 pupils, 8 teachers) were available for further epidemiological analysis. The median age of this cohort population was nine years (range 6 to 57 years); 58 (53.2 %) were female.

Twenty-nine participants (27 %) fulfilled the case definition as suspected cases (Median age 12 years; range 7 to 46 years, 38 % male) and 26 participants (24 %) fulfilled the case definition as confirmed cases. The median age of the confirmed cases was eight years (range 7 to 12 years), 62 % were male.

The progression of the outbreak is shown by the epidemic curve in figure 1 according to the case definition. Only confirmed and suspected cases that provided information on the day of onset (27 of 55) are shown. The median duration of the symptoms was three days (range 1 to 28 days). Four cases still had clinical manifestations at the time of the interview.

The consumption of raw milk was the only risk factor significantly associated with the STEC O157:H7 infection in univariable analysis (RR=6.45; p=0.01). Only one case declared not to have drunken any milk at the dairy farm. No other risk factor showed significant association with this STEC infection (Tab. 1).

After adjustment for age and sex the consumption of raw milk still showed a significant association with the disease status (aOR = 10.04; 95 %-CI = 1.21–83.09; p = 0.03).

On average, confirmed cases consumed higher amounts of raw milk than non-infected persons (4.3 vs. 2.6 glasses) (t-test: p = 0.02). One additional glass of milk elevated the odds of becoming a case by 28 % (OR = 1.28; 95 %-CI = 1.03–1.59; p = 0.03).

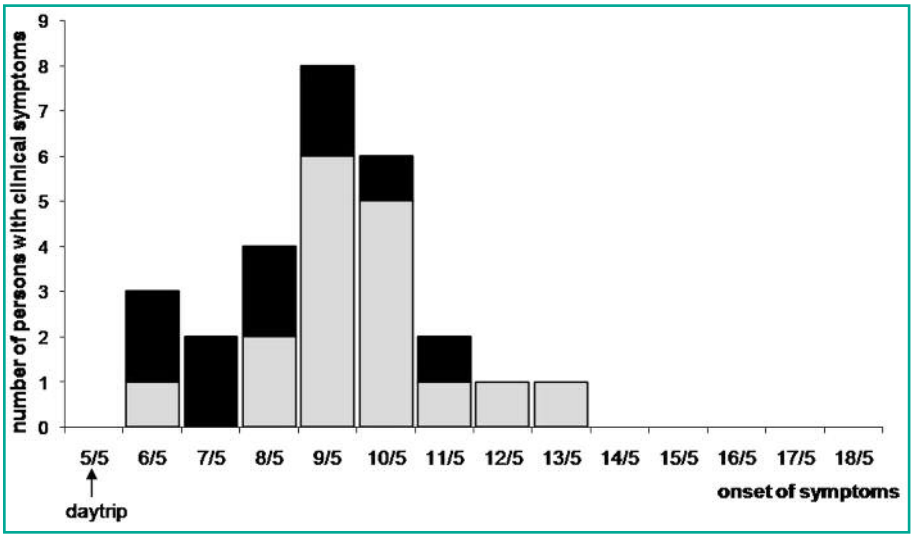

FIGURE 1: *Epidemic curve of STEC infections for participants with information about the day of onset (n = 27). Black, confirmed cases; grey, suspected cases.*

TABLE 1: *Results of univariable analysis of selected risks of exposure for confirmed cases in relation to non cases.*

Exposure	Exposed			Unexposed			RR	CI	p-value
	Total	CC	AR%	Total	CC	AR%			
Consumption of raw milk	62	25	40.32	16	1	4.55	6.45	[0.94–44.08]	0.01
Contact to poultry	29	11	37.93	51	15	29.41	1.29	[0.69– 2.42]	0.43
Contact to horses	46	16	34.78	34	10	29.41	1.18	[0.61– 2.27]	0.61
Contact to calves	68	14	35.29	12	2	16.67	2.12	[0.57– 7.81]	0.20
Contact to cows	48	15	31.28	32	11	34.38	0.91	[0.48– 1.72]	0.77
Gender	40	16	40.00	40	10	25.00	1.60	[0.83– 3.09]	0.15

CC: confirmed cases; AR: Attack Rate; RR: Relative Risk; CI: Confidence interval

Laboratory investigation

Stool samples from 291 persons were investigated. In total, 49 persons (16.8 %) were tested positive (PCR for *stx1, stx2*) for STEC (Tab. 2). Of these, 42 were participants of the day trip (38.5 %) and five were members of the dairy farmers' family or their employees. The two remaining were family members of participants with STEC infections. Two children with HUS had to be hospitalized. Neither the members of the dairy farmers' family, their employees or the two family members of STEC positive participants reported any clinical symptoms in regard to the case definition.

Twenty seven (55 %) of the 49 persons positive for STEC by PCR carried a STEC O157:H7 (tab. 1). With the exception of a brother of one participant all other persons with this serotype attended the day trip to the dairy farm. Three participants carried STEC of the serotypes (Ont:H2, O76:H19, O8:Hnt). One mother of a participant carried STEC with the serotype Ont:H7. STEC with two different serotypes (Ont:H-, O153:Hnt) were detected within the dairy farmers' family and employees. In 15 isolates form symptomatic persons no STEC could be detected. STEC could not be detected among the attending teachers.

Environmental investigation

The investigation on the dairy farm revealed no noticeable hygienic problems in regard to the milking process or the storage of the milk in bulk tanks. Nine different STEC serotypes were isolated from 25 of 443 faecal samples from cattle of the dairy farm. Similar to the human stool samples, STEC O157:H7 was the predominant serotype in the cow faeces (tab. 3). One isolate with serotype STEC O2:H27 with toxin type Stx2 was obtained by investigation of 20 raw milk samples of the dairy farm.

Cluster analysis

All selected STEC O157:H7 isolates from stool samples of confirmed cases and from cattle faeces revealed identical or nearly identical PFGE patterns. By cluster analysis, all of these isolates were assigned to a common cluster with a high similarity of 98–100 %. Reference isolates unrelated to the outbreak only showed a low degree of similarity (82–84 %) and were arranged outside the outbreak cluster (fig. 2).

Discussion

The epidemic curve of the outbreak with a mode (value of highest frequency of disease onset) four days after the day trip indicated a common point source of infection. STEC O157:H7 was detected as the causative agent of this outbreak. Evaluation of the epidemiological data revealed consumption of raw milk as the most plausible source of the outbreak. In the retrospective cohort study, the consumption of raw milk showed the strongest association with the STEC O157:H7 infection. It was the only factor with a significantly increased risk for the infection in the multivariable analysis in respect to possible confounding. No other explaining risk exposure outside the dairy farm could be found in the investigation, e. g. school canteen. Five people with STEC infection belonged to the farmers' family or their employees. Although, all of them regularly consume raw milk from the farm, they reported no diarrheal disease during the outbreak period. This finding is in accordance to other studies shown by Crump et al. (2002) and Wilson et al. (1996) showing that previous infection and frequent re-exposure to STEC O157:H7 may confer some protection against symptomatic infection.

Besides the two asymptomatic family members of STEC positive participants and the also asymptomatic farmers' family and their employees no other STEC infections were reported in the district during the observed time period.

TABLE 2: *Characteristics of STEC strains isolated from human stool samples.*

Number of isolates	*E. coli* serotype	Shiga toxin gene
27	O157:H7	*stx2*
1	Ont:H7	*stx2*
1	Ont:H-	*stx1, stx2*
1	Ont:H2	*stx1, stx2*
1	O76:H19	*stx1*
1	O8:Hnt	*stx2*
1	O153:Hnt	*stx1*
1	nt	*stx1*
15	nt	*stx2*

nt: nontypeable

TABLE 3: *Characteristics of STEC isolates from raw milk and cow faeces from the dairy farm.*

	number of isolates	*E. coli* serotype	Shiga toxin gene
raw milk	1	O2:H27	*stx2*
cow faeces	4	O2:H27	*stx2*
	1	O2:Hnt	*stx2*
	2	O150:H$^-$	*stx1, stx2*
	2	O156:H$^-$	*stx2*
	7	O157:H7	*stx2*
	1	O177:H$^-$	*stx2*
	4	O184:H27	*stx2*
	3	Ont:H$^-$	*stx2*
	1	O185:Hnt	–

nt: nontypeable

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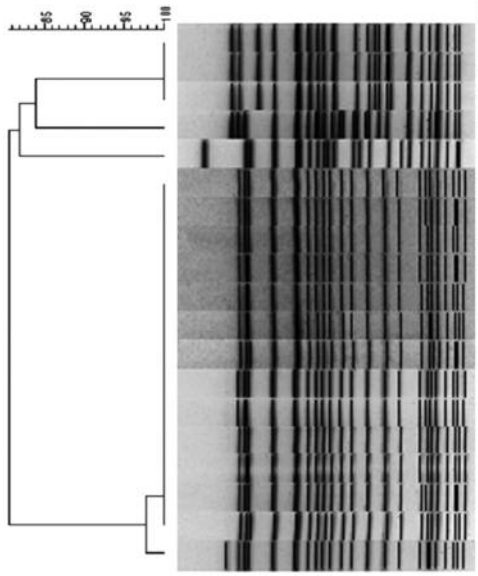

Lane	Source	Serogroup	H-type	IsolatDate	stx	E-hlyA	eae
1	gourmet salad1	O157	H-	2004	stx2	+	+
2	gourmet salad2	O157	H-	2004	stx2	+	+
3	n.a.	O157	H-	2004	stx2	+	+
4	cattle, meat	O157	H7	2002	stx2	+	+
5	hamburger	O157	H7	1982	stx1, stx2	+	+
6	human	O157	H7	2008	stx2	+	+
7	human	O157	H7	2008	stx2	+	+
8	human	O157	H7	2008	stx2	+	+
9	human	O157	H7	2008	stx2	+	+
10	human	O157	H7	2008	stx2	+	+
11	human	O157	H7	2008	stx2	+	+
12	human	O157	H7	2008	stx2	+	+
13	cattle, faeces	O157	H7	2008	stx2	+	+
14	cattle, faeces	O157	H7	2008	stx2	+	+
15	cattle, faeces	O157	H7	2008	stx2	+	+
16	cattle, faeces	O157	H7	2008	stx2	+	+
17	cattle, faeces	O157	H7	2008	stx2	+	+
18	cattle, faeces	O157	H7	2008	stx2	+	+
19	cattle, faeces	O157	H7	2008	stx2	+	+

FIGURE 2: *Dendrogram showing the relatedness of PFGE patterns for the NSF STEC O157:H7 isolates. (Lane 1–5, reference isolates unrelated to the outbreak; Lane 6–19, isolates of the outbreak: Lane 6–12, human stool samples; 13–19 samples of cattle faeces). The degree of similarity (%) is shown on the scale at the top left of the figure. stx, Shiga toxin gene(s).*

Only one confirmed case stated not to have drunken any raw milk during the day trip. The consumption of raw milk was a dose dependent risk factor. Data analysis revealed that the more volume of raw milk the participants had drunken, the higher was their chance of infection. In our analysis there was no significant association of contact to farm animals with STEC O157:H7 infection.

To reduce information bias a standardized questionnaire was used, nevertheless a possible recall bias can not totally be excluded. All participants of the day trip participated in the retrospective cohort study (100 % response). Thus there is no risk of selection bias.

The microbiological test results and comparisons of STEC isolates from stool samples and cattle faeces support the results of the epidemiological investigation. Both the virulence factors (*stx2*, E-*hlyA*, *eae*-γ) and other properties (sorbitol-fermenting- and beta-glucuronidase-negative) as well as the PFGE patterns of the isolates of STEC O157:H7 from cattle faeces and human stool samples were concordant. Whereas even two weeks after the daytrip the outbreak strain could be detected in faecal samples of the dairy cows, all attempts failed to isolate STEC O157:H7 in cumulated raw milk samples.

Raw milk as a possible source of STEC infections has often been described in the literature. Studies performed in the United States by Karns et al. (2007) and in Germany by Mäde and Stark (1996) showed that 11–23 % of samples obtained from bulk tank milk were contaminated with strains that harbour virulence factors of STEC. Asymptomatic cows can harbour different STEC types and shed them with their faeces. Raw milk can be contaminated with STEC during the milking process or the handling of the milk, thus untreated milk or products thereof may cause human STEC infections as shown by Beutin et al. (1993), Wilson et al. (1996) and Mäde and Stark (1996). Because cattle shed STEC intermittently, it can be difficult to obtain reproducible evidence for ongoing STEC carrier status in cattle (Midgley and Desmarchelier, 2001 and Schneider et al., 2008). During this investigation raw milk samples were taken from different milk tank batches. The original milk batch from which the participants drank during the day trip could not be investigated anymore.

In this investigation it was possible to show a strong association between the detection of non-sorbitol-fermenting STEC O157 infections and the consumption of raw milk in an outbreak setting. In the last years there have been quite a few outbreaks in Germany caused by STEC O157 as shown by Ammon et al. (1999), Dreesman et al. (2008), Alpers et al. (2009) and Werber et al. (2011). But most of these were primarily recognized by the appearance of HUS in patients whereas the source of infections could only be clarified in some of these outbreaks. In addition, a detection of an outbreak strain in the environment was rarely found.

Conclusion

The consumption of raw milk contaminated with a non-sorbitol-fermenting STEC O157:H7 is considered to be the source of this outbreak in school children. These STEC infections could have been prevented by heating the milk sufficiently or by delivery of pasteurized milk. There are legal regulations in Germany to prevent zoonotic infections due to consumption of raw milk. However, these must be followed by an effective awareness among dairy farmers and consumers. Following the outbreak, the farm owner was convicted not to distribute raw milk from this farm to groups any more. The image of raw milk as a healthy food product has to be reconsidered. The hygiene production of raw milk cannot completely eliminate the risk for contamination with pathogens. Only heat treatment like pasteurization can substantially inactivate pathogens in milk and effectively prevent disease transmission (LeJeune and Rajala-Schultz, 2009). Specific information is required especially for supervisors in schools, kindergartens and nurseries as well as youth groups and parents.

Acknowledgment

In this outbreak the intense cooperation of all participants contributed decisively to the investigation of the source of the outbreak.

Declaration of interests

None.

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