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Investigation of human infections with verocytotoxin-producing strains of *Escherichia coli* (VTEC) belonging to serogroup O118 with evidence for zoonotic transmission

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SUMMARY

Twenty verocytotoxigenic *Escherichia coli* (VTEC) O118 strains isolated between 1996 and 1998 from human patients in Germany were analysed for their serotypes, their virulence markers and their epidemiological relatedness. Three strains were typed as O118:H12, these carried only the VT2d-Ount variant gene and were not associated with diarrhoea or haemolytic uraemic syndrome (HUS). Seventeen strains were serotyped as O118:H16 or O118:non-motile (NM). These carried all the genes for VT1, *eae* and EHEC-haemolysin. The O118:H16/NM strains were from diarrhoea (13 cases) and HUS (2 cases). Sixteen of the patients were young infants and most infections were associated with a rural environment. Evidence for zoonotic transmission from cattle to humans was found in two cases. The epidemiological relationship between the human and bovine O118:H16/NM isolates was indicated by homogeneous plasmid patterns and by very similar *Xba*I restriction patterns obtained by pulsed-field gel electrophoresis. VTEC O118:H16/NM are emerging pathogens in Germany and should be classified as new enterohaemorrhagic *E. coli* (EHEC) types.

INTRODUCTION

The production of verocytotoxins (VT) also called Shiga toxins (Stx) is associated with certain strains of *Escherichia coli* which occur frequently in faeces of domestic animals, particularly in ruminants [1]. Verocytotoxin producing *E. coli* strains (VTEC) have been shown to be heterogenous for their O-(lipopolysaccharide) and H-(flagellar) serotypes and certain VTEC types called enterohaemorrhagic *E. coli* (EHEC) are associated with severe diseases in humans such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Production of VT, the ability to cause attaching and effacing lesions in the intestinal mucosa and a plasmid-encoded haemolysin have been identified as virulence traits which are characteristic for typical EHEC [1–3].

Most studies on EHEC have been performed on *E. coli* O157:H7, which has caused numerous outbreaks and sporadic infections in humans worldwide [4]. This organism is resistant to a variety of physico-chemical stress factors and fewer than 100 viable bacteria are sufficient to cause an infection in humans. These properties facilitate the transmission of *E. coli* O157:H7 from various sources such as contaminated foodstuffs or water, the farm environment, or by direct contact with infected humans or animals [5, 6].

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Besides *E. coli* O157, strains belonging to serotypes O26:H11, O103:H2, O111:H$^-$ and O113:H21 are described as the most common EHEC types [3]. However, much less is known about the epidemiology, the possible sources of infection and modes of transmission of non-O157 EHEC strains [7–9].

We have recently investigated the role of non-O157 VTEC strains as pathogens of humans in Germany [10]. Strains showing EHEC virulence traits and caused HC and HUS in humans were associated with serogroups O26, O103, O111, O118 and O145. EHEC O118 was found as a new pathogen in humans in Germany and healthy as well as diseased cattle were identified as a reservoir for EHEC O118 strains [10–14]. In the current study, 20 cases of human infections with *E. coli* O118 in Germany were investigated. Most of these infections were associated with a rural environment and two cases had a direct link between cattle and humans. The results indicate that *E. coli* O118:H16 is an emerging EHEC type in human patients in Germany and that cattle are an important source of human infections with EHEC O118 strains.

MATERIAL AND METHODS

Isolation of VTEC from human and bovine faeces

VTEC were detected and isolated from human stool samples by the combined use of different detection methods, as recently described [10, 15]. Faecal samples from cattle were screened for VT by means of an enzyme-immunoassay (Premier EHEC, Meridian Diagnostics). VT-positive samples were inoculated on selective media (MacConkey sorbitol agar, Fluorocult *E. coli* O157:H7 agar) and on enterohaemolysin–agar plates which were incubated overnight at 37 °C [15]. Colonies showing an enterohaemolytic phenotype were investigated for VT1 and VT2 genes using primers MK1 and MK2 [16]. Alternatively, VT-positive colonies were identified by means of a colony-immunoblot [17].

Characterization of VTEC isolates

VT-positive colonies were examined for their O:H serotypes as described [18]. The presence of the *eae* gene was determined by PCR using two different primer pairs [10, 19]. Genotyping of VT genes was performed by PCR using primers KS7 and KS8 for VT1 [19] and primers LP43 and LP44 for VT2 [20]. Subtyping of genetic variants of VT2 was performed as described by Piérard and colleagues [21]. The enterohaemolytic phenotype was investigated on enterohaemolysin–agar as described [15]. The presence of EHEC–haemolysin gene sequences was determined by PCR using primers EHA1 and EHA2 [22].

Plasmid and pulsed-field gel electrophoresis (PFGE) profiles of VTEC O118 strains

Plasmid DNA from wild-type *E. coli* strains was prepared from 5 ml overnight cultures of bacteria as described [23]. Plasmid DNA was separated on 0·7% agarose gels and stained with ethidium bromide. Preparation of total genomic DNA, *Xba*I digestion, and pulsed-field gel electrophoresis were performed as described [24].

Southern hybridizations for detection of EHEC-*hly*A sequences

The 1551 bp fragment of EHEC-*hly*A was generated from VTEC O157:H7 strain EDL933 by PCR using primers EHA1 and EHA2 as described [22]. DNA was labelled with digoxigenin-11-dUTP with the Boehringer DIG-DNA labelling and detection system (Boehringer–Mannheim, Mannheim, Germany) according to the instructions of the supplier. Hybridization reactions and subsequent washing steps were performed at conditions of high stringency as described [25].

RESULTS

Detection of Shiga-toxin producing O118 strains in human patients

Between July 1996 and September 1998 20 patients with a faecal VTEC O118 isolate were identified in our laboratory (Table 1). Sixteen of the patients were living in rural areas in different parts of Germany (Bavaria, North Rhine-Westphalia and Lower Saxony). Four patients were from urban areas. All cases were reported as sporadic and were not linked. Eighteen patients were children and 16 of these were under 5 years of age. Fifteen patients were male and 5 were female. Clinical data were obtained for 18 patients. Two of these showed no symptoms of enteric disease, 1 patient showed abdominal pain only, 11 had non-bloody diarrhoea, 2 patients showed bloody

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Table 1. *VTEC O118 infections in human patients in Germany (1996–8)*

Case no.	Sex	Age (year, months)	Disease	Date of isolation (month/year)	Geographic region	Serotype	*eae**	Ehly†	VT-type
5482	M	36 y	HIV, diarrhoea, HUS	1/96	Aachen, NRW‡	O118:H16	+	+	VT1
6175	M	1 y	Diarrhoea	7/96	Marktredwitz, Bavaria	O118:H16	+	+	VT1
7109			Bovine isolate to case 6175	7/96	Marktredwitz, Bavaria	O118:H16	+	+	VT1
6236	F	3 y, 5 m	Bloody diarrhoea	8/96	Winterberg, NRW	O118:H16	+	+	VT1
6365	M	1 y, 5 m	Severe diarrhoea	9/96	Aurich, LSA§	O118:H16	+	+	VT1
6525	M	2 y	Diarrhoea	11/96	Aachen, NRW	O118:H16	+	+	VT1
6585	M	10 m	Severe diarrhoea	11/96	Augsburg, Bavaria	O118:H16	+	+	VT1
6586	F	2 y, 7 m	Diarrhoea	12/96	Augsburg, Bavaria	O118:NM	+	+	VT1
6981			Bovine isolate to case 6586	12/96	Augsburg, Bavaria	O118:NM	+	+	VT1
6888	M	3 y, 8 m	Diarrhoea	5/97	Aachen, NRW	O118:H16	+	+	VT1
6905	M	1 y, 8 m	No data	5/97	Weiden, Bavaria	O118:H16	+	+	VT1
7000	M	1 y, 1 m	Diarrhoea	6/97	Munich, Bavaria	O118:H16	+	+	VT1
7014	F	1 y, 1 m	Diarrhoea	6/97	Lam, Bavaria	O118: H16	+	+	VT1
7035	M	1 y, 2 m	No data	7/97	Augsburg, Bavaria	O118:H16	+	+	VT1
7099	M	1 y, 4 m	Diarrhoea	8/97	Burglengenfeld, Bavaria	O118:H16	+	+	VT1
7451	M	4 y	Diarrhoea	1/98	Schwarzenfeld, Bavaria	O118:H16	+	+	VT1
7727	M	1 y, 10 m	Diarrhoea	7/98	Weiden, Bavaria	O118:NM	+	+	VT1
7786	M	2 y, 8 m	Bloody diarrhoea	8/98	Moers, NRW	O118:H16	+	+	VT1
7834	M	1 y, 6 m	Diarrhoea, HUS	9/98	Rastede, LSA	O118:NM	+	+	VT1
6069	F	13 y	Abdominal pain	7/96	Kürten, NRW	O118:H12	−	−	VT2d-Ount
6591	F	8 y	Asymptomatic	12/96	Cologne, NRW	O118:H12	−	−	VT2d-Ount
7164	M	59 y	Asymptomatic	8/97	Oberhaching, Bavaria	O118:H12	−	−	VT2d-Ount

* Investigated by PCR, † Ehly, EHEC-haemolysin, investigated by phenotype and by DNA-hybridization, ‡ NRW, North Rhine Westphalia, § LSA, Lower Saxony.

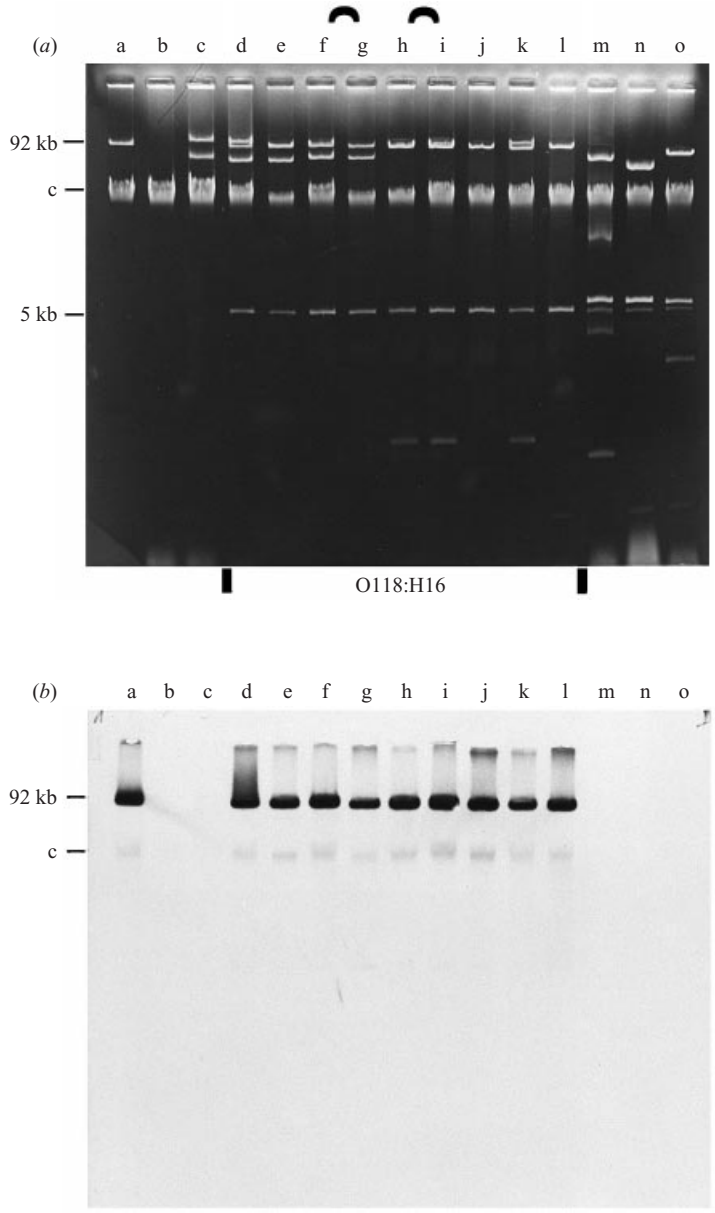

Fig. 1. (*a*) Agarose gel (0·7%) electrophoresis of plasmids of representative VTEC O118 and control strains. Lanes a–c control strains. a, EDL933 (O157:H7) carries 92 kb plasmid pO157 [22]; b, JC3272 (*E. coli* K-12, plasmid-free); c, TPE1597 (*E. coli* K-12 carrying plasmids of > 92 kb and < 92 kb); lanes d–o are VTEC O118 strains from Table 1; lane d, 6525; e, 5482; f, 6175; g, 7109; h, 6586; i, 6981; j, 6585; k, 6888; l, 6905; m, 6991; n, 6069; o, EH250 (O118:H12), reference strain for VT2d-Ount, kindly donated by D. Piérard, Brussels, Belgium [21]. Lanes with epidemiologically related strains from human patients and cattle are labelled with an arc. The left side of the gel shows the position of the 92 kb plasmid pO157 (92 kb) and of the chromosomal band (c). (*b*) Southern hybridization of plasmids separated on (A) with the EHEC-*hly*A probe.

diarrhoea and 2 others developed haemolytic uraemic syndrome (HUS) with a diarrhoeal prodrome (Table 1).

Characterization of VTEC O118 strains

E. coli O118:H16 or O118:non-motile (NM) were isolated from 17 cases (Table 1). The *E. coli* O118:H16 and O118:NM isolates were similar for their virulence traits, all were positive for VT1, the *eae* gene, and for the enterohaemolytic phenotype. All strains carried a plasmid of approx. 92 kb which hybridized with the EHEC-*hly*A specific gene probe (Fig. 1, data not shown). These strains were classified as typical EHEC according to the terminology of Nataro and Kaper [3]. The remaining three isolates all belonged to

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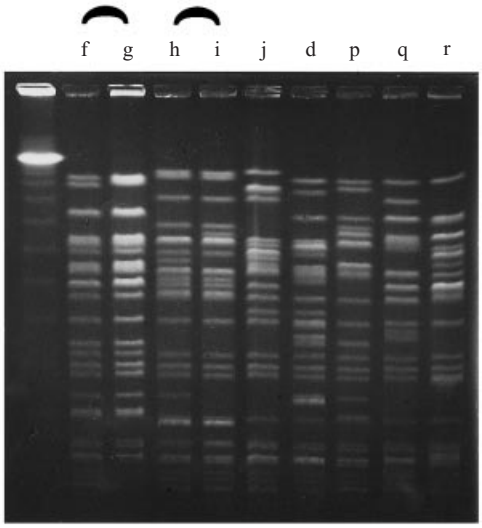

Fig. 2. (D) *Xba*I digested total DNA of nine representative VTEC O118 strains separated by PFGE. Lanes are labelled with letters corresponding to Figure 1. First lane (unlabelled) molecular weight standard (lambda concatemers, Bio-Rad Laboratories, Richmond, CA, USA). Lanes: f, 6175; g, 7109; h, 6586; i, 6981; j, 6585; d, 6525; p, 7019; q, 7035; r, 6365. Lanes with epidemiologically related strains from human patients and cattle are labelled with an arc.

serotype O118:H12 and originated from two asymptomatic patients and from a patient suffering from abdominal pain (Table 1). The non-haemolytic O118:H12 strains were all positive for a recently described VT2 variant, called VT2d-Ount [21] but were negative for other VT-types and for *eae*- and EHEC-haemolysin specific gene sequences (Table 1, Fig. 1). Accordingly, the VTEC O118:H12 strains were thus classified as atypical EHEC [3].

Analysis of PFGE of *Xba*I cleaved total DNA of the 20 *E. coli* O118 strains revealed individual patterns which differed for seven bands or more between the different isolates (Fig. 2, data not shown), indicating that the strains were epidemiologically unrelated to each other [26].

Evidence for transmission of VTEC O118 from cattle to humans

Two cases (nos. 6175 and 6586 in Table 1) of VTEC O118 infections in children living on private farms were identified. In both cases, children had contact with cattle, developed diarrhoeal disease and excreted VTEC O118:H16 or O118:NM, respectively. Because it is already well known that cattle are a reservoir of VTEC, epidemiological investigations on the bovine lifestock at these farms were performed.

Case no. 6175 was a 1-year-old boy living with his family on a farm in the region of Marktredwitz (Bavaria). In July 1996, he developed severe watery diarrhoea for a period of 15 days but members of his family had no symptoms of disease. Culture of a stool sample from the child isolated VTEC O118:H16. Two weeks later, samples from milk, milk filters and faecal specimens of the 45 adult cattle and 10 cattle calves living on the farm were investigated for VTEC. VTEC was isolated from faecal samples of 5 adult cattle (3 O157:H^-; 1 O82:H8 and 1 O-untypable strain) and from 2 calves (1 O118:H16 and 1 O-untypable strain). No VTEC were isolated from milk samples and milk filters. The boy did not consume raw milk but was known to have contact with the calves. The VTEC O118:H16 isolate from the boy (no. 6175) and from one calf (no. 7109) were indistinguishable by their plasmid profiles and differed only in one band in their PFGE-patterns of *Xba*I digested total DNA (Fig. 1, Fig. 2, lanes f and g). Both isolates were positive for VT1, *eae* and for EHEC-*hly*A (Table 1). A second survey on the VTEC-positive animals was performed 10 weeks later but VTEC O157 and VTEC O118 were no longer detectable.

Case no. 6586 was a girl aged 2 years 7 months who developed diarrhoea in November 1996. She lived with her family on a farm in the region of Augsburg (Bavaria). She did not consume raw milk but had contact with cattle on the farm. Examination of her stool samples yielded VTEC O118:NM. Faecal samples from 46 cows, 13 calves and 24 bulls from the farm were examined for VTEC 20 days after the incident became known and 4 animals were identified as VTEC excretors. VTEC O118:NM was isolated from one calf. The VTEC O118:NM from the girl (no. 6586) and the calf (no. 6981) had identical plasmid profiles and differed only in three bands in their PFGE patterns of *Xba*I digested total DNA (Fig. 1, Fig. 2, lanes h and i). The human and the bovine isolates were positive for VT1, *eae* and EHEC-*hly*A (Table 1).

DISCUSSION

VTEC O118 are known as pathogens in cattle and have been isolated from faeces of healthy and diseased cattle in Belgium, Canada, Germany and Sri Lanka [11–13, 27]. In Belgium and Germany, VTEC O118

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(O118:H16 and O118:NM) were the most frequent type found among *eae*-positive VTEC isolates which were isolated from cattle between 1989–96 [12, 27]. Before 1996, VTEC O118 were not detected in human patients in Germany but were sporadically isolated from humans in Belgium, Canada and the UK [28–30]. In contrast, 20 cases of patients infected with VTEC O118 in Germany were identified in our laboratory between July 1996 and September 1998 [10; this work]. The majority of human infections with VTEC O118:H16 and O118:NM occurred in a rural environment and cattle seem to be an important source of human infections [27, this work]. The VTEC O118:H16 and O118:NM strains isolated from humans were similar in their serotypes and in their virulence attributes to the bovine isolates from Germany and Belgium [14, 27].

All patients infected with VTEC O118:H16 or O118:NM were children under the age of 5 years. The only exception was an adult patient suffering from AIDS who developed HUS after a diarrhoeal prodrome (case no. 5482 in Table 1). By analysis of 89 cases of non-O157 VTEC infections in humans, an association between a young age, severe diarrhoeal disease or HUS and infections with *eae*- and enterohaemolysin-positive VTEC strains was found [10]. Such VTEC types resemble EHEC O157 in their virulence markers and for the illness they cause in the patients. Among the non-O157 VTEC strains, those belonging to serogroups O26, O103, O111 and O145 are regarded as EHEC because they can cause bloody diarrhoea and HUS in humans and carry all virulence markers which are found in EHEC O157 strains [3, 9]. Our findings indicate that VTEC O118:H16 and O118:NM strains belong to the EHEC group. VTEC O118 strains were isolated from patients living in different parts of Germany and the possible spread of this new emerging EHEC type to other countries than Germany needs to be investigated.

EHEC O157 strains are known to be transmitted to humans in different ways, such as by consumption of contaminated food, by human to human contact, by direct or indirect contact with farm animals and a contaminated environment [4]. Direct and indirect contact with farm animals has been shown to be an important source of human infections with EHEC O157 on private and public farms [31–33]. Zoonotic transmission of EHEC O157 has been significantly associated with contact with farm animals [34]. In contrast, little is known about the importance of zoonotic and other modes of transmission of non-O157 VTEC strains [9]. The current findings indicate that cattle are an important source of zoonotic transmission of VTEC O118:H16 and VTEC O118:NM strains to humans. In these cases it is not known if the children were infected by direct or indirect contact with the animals. The findings indicate that human infections with non-O157 EHEC follow similar modes of transmission as have been described for EHEC O157 [4]. Further investigations are necessary to determine the importance of zoonotic transmission of other non-O157 EHEC types.

PFGE typing has proven to be a highly discriminative and reproducible method for investigation of outbreaks with EHEC O157 strains and epidemiologically related EHEC O157 strains have been identified by homogeneous PFGE patterns [35, 36]. In the current study, differences of seven-DNA bands or more were found between VTEC O118 strains which had no known epidemiological link to each other. In contrast, VTEC O118 strains from the human case nos. 6175 and 6586 showed minor differences in only 1 and 3 bands, respectively to their corresponding bovine isolates (nos. 7109 and 6981) (Fig. 2). According to the criteria used for bacterial strain typing the human and the bovine isolates are closely related [26]. The minor differences found between the bovine and the corresponding human strains might be explained by the fact that the bovine samples were taken 2–3 weeks after the human isolate was obtained. It has been reported that a longer time interval between sampling of strains in outbreaks increases the chance for random changes in the PFGE profiles of epidemiologically related strains [37].

Three of the VTEC O118 strains described in this study were serotyped as O118:H12. VTEC O118:H12 were different from O118:H16 and O118:NM VTEC in regard to their virulence factors, their plasmids and their PFGE patterns. Interestingly, VTEC O118:H12 were only positive for the VT2d-Ount variant but were negative for EHEC-associated virulence markers such as *eae*, and EHEC-haemolysin. It has been reported that the VT2d-Ount variant is not found in typical EHEC strains belonging to serogroups O157, O26, O103, O111 and O145 but is more frequent in those VTEC strains which lack EHEC-associated virulence markers and appear to be less virulent for humans [21]. Correspondingly, the VTEC O118:H12 strains from this study originated only from patients showing no or minor symptoms of gastrointestinal disease. In contrast to the VTEC O118:H16 and O118:NM strains, VTEC O118:H12 was not found

among *E. coli* from cattle or other animal species, indicating that both groups of VTEC O118 strains differ for their natural reservoirs [25, 27].

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