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A long-term study on the prevalence of shiga toxin-producing *Escherichia coli* (STEC) on four German cattle farms

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SUMMARY

The occurrence of Shiga toxin-producing *Escherichia coli* (STEC) was studied on four cattle farms. STEC were detected in 29–82% of the cattle. STEC with additional EHEC markers were detected on all farms. The occurrence of the complete virulence marker pattern (*stx1* and/or *stx2*, *eae*, $EHEC_{hlyA}$, *katP*, *espP*) was correlated with the presence of known STEC serotypes. STEC O26:H11 and O165:H25 with the complete pattern of virulence markers were the most prevalent. STEC O157 (H7/H-) STEC O103:H2 and STEC O145:H- were found sporadically. Five clonal subgroups of the STEC O26:H11 isolates were identified by pulsed-field gel electrophoresis. STEC O26:H11 were present in three groups of cattle. This serotype was detected in a single group over the entire fattening period. Most STEC O26:H11 with the complete pattern of potential virulence markers were found in clinically healthy cattle. These animals may represent a risk factor for farmers and consumers.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) represent important causative agents of diarrhoea, haemorrhagic colitis (HC), and the haemolytic-uraemic syndrome (HUS) [1–4]. STEC strains isolated from patients are also designated enterohaemorrhagic *Escherichia coli* (EHEC). Consequently, EHEC are considered a subgroup of STEC which can cause human disease. EHEC are an example of an 'emerging pathogen' [5].

The mechanisms by which EHEC strains cause disease are not completely understood. Virulence factors contributing to the pathogenesis include the production of two major phage-encoded toxins, Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2), that can be produced alone or in combination by individual strains. Stx1 and Stx2 are thought to cause the vascular endothelial damage observed in patients with HC and the HUS [6]. In addition to the expression of Stx, EHEC posses other virulence characteristics such as the ability to cause attaching-and-effacing lesions in the large intestine [7], and often contain a large (approximately 90 kb) plasmid that carries other potential virulence genes [8–11]. Cattle, cow milk, milk products (soft cheese, unpasteurized milk) or improperly cooked ground beef are believed to be principal sources of STEC and EHEC infections [12–14]. Cattle and other ruminants such as sheep and goats can harbour such strains in their faeces and are thus regarded as natural reservoirs of these pathogens

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[15]. EHEC can be transmitted to humans through direct or indirect contamination of food with faecal material. Moreover, humans can contract the infection by direct transmission of EHEC from infected animals or by secondary spread from person to person [16, 17].

Reported estimates of the prevalence of STEC on cattle farms in Europe vary widely. Blanco et al. [18, 19] reported a prevalence of 14% in calves in Galicia (north-western Spain) and prevalences between 35% and 37% in cows and calves on farms in the region of Lugo. An STEC-prevalence of 31% was found in cattle in Northern Ireland [20]. On Dutch dairy farms, the proportion of STEC-infected cattle varied between 0 and 61% [21]. In Germany, Richter et al. [22] isolated STEC from an estimated 48% of beef cattle. Weber et al. [23] reported an average prevalence of 35% ranging between 0 and 86% on different farms.

The aim of the present study was to analyse the epidemiological situation concerning STEC on German cattle farms by monitoring individual cattle from birth to slaughter. Entry, transmission and spread of the bacteria were investigated by molecular epidemiological methods.

MATERIALS AND METHODS

Study design

The study was conducted on three beef (farms A, B and D) and one dairy farm (C) located in Brandenburg and Saxony-Anhalt in northeastern Germany. Each farm was managed as a closed herd and all animals were kept indoors. On each farm groups of 25–30 cattle were monitored. Each animal amongst the beef groups was sampled at intervals of approximately 2–4 weeks from birth to slaughter. Each animal amongst the heifer group (farm C) was investigated from birth until May 1998, and then again from December 1998 until May 1999. Sampling was interrupted for the periods when the cattle were kept on pasture and were thus not accessible for regular examinations. The animals of two groups (farms B and D) were also sampled on the day of slaughter before transport to the abattoir, immediately upon arrival to the abattoir and just before they were slaughtered. Moreover, the carcasses of one group (farm D) were sampled immediately after the animals had been slaughtered.

Farm A. A total of 25 beef cattle were included in study group A (24 bulls, 1 heifer) which consisted of beef hybrid Hereford + Red Pied breed, Hereford + Black Pied breed and Red Pied breed + Red Pied breed. There were no calf losses in the rearing period. Two of 25 cattle were slaughtered in May 1997 for reasons deemed unrelated to this study.

Farm B. A total of 31 beef cattle were included in study group B (29 bulls, 2 heifers) which consisted of the Black Pied breed. During the first 5 months of the investigation, 9 of 31 calves died in the 6th–8th week of their lives. Calf losses rose temporarily to 50% of all new-born calves, and diarrhoea and pneumonia were recorded in many cattle. Rotavirus and coronavirus as well as *Cryptosporidium* spp. infections were detected in faecal samples. The bulls of the study group were sampled before and directly after slaughtering. The transport time to the abattoirs was 3–3·5 h.

Farm C. Study group C consisted of 25 heifers which belonged to the Black Pied breed. Eight of 25 heifers died in the first 3 months of the investigations. Calf losses were temporarily very high, and severe pneumonia was diagnosed in many calves.

Farm D. Two groups (D1 and D2) of cattle were studied. Group D1 comprised 23 bulls of the Black Pied breed. The group was managed as a closed herd. No calving losses were recorded in the rearing period. Before and immediately after slaughtering the bulls were sampled. The transport time between the farm and the abattoirs was 2–2·5 h. Group D2 consisted of 25 bulls of the Black Pied breed, managed as a closed herd. No calving losses were recorded in the rearing period. The investigation started after the cattle of group D1 had been slaughtered, so the investigation period was only 12 months (January–December 1999).

Sampling

On each visit, faecal samples (rectal swabs) were taken from each cattle. All animals were identified by ear tag numbers. The rectal swabs were transferred into sterile tubes and immediately transported to the laboratory, where the microbiological examination started within 3 h after sampling. Feed stuff samples ($n = 40$) and dust samples from cow-sheds ($n = 20$) were arbitrarily taken.

Isolation of STEC

The following set of methods was used for isolation of STEC:

Pre-enrichment

The samples were shaken in the sampling tubes with 2 ml of phosphate buffer (5 min, room temperature),

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Table 1. *PCR primers and conditions for the detection of virulence marker genes*

Primers and sequences (5′–3′)		Target	PCR conditions			Product length (bp)	References
			Denaturation	Annealing	Extension		
MK1	ttt acg ata gac ttc tcg ac	*stxA*	94 °C, 60 s	44 °C, 60 s	72 °C, 90 s	230	[24]
MK2	cac ata taa att att tcg ctc			30 cycles			
Hly A1	ggt gca gca gaa aaa gtt gta g	$EHEC_{hlyA}$	94 °C, 30 s	57 °C, 60 s	72 °C, 90 s	1551	[11, 25]
Hly A4	tct cgc ctg ata gtg ttt ggt a			30 cycles			
Hyg5	ccc gaa ttc ggc aca agc ata agc	*eae*	94 °C, 30 s	52 °C, 60 s	72 °C, 60 s	800	[26–29]
Hyg6	ccc gga tcc gtc tcg cca gta ttc g			30 cycles			
wkat-B	ctt cct gtt ctg att ctt ctg g	*katP*	94 °C, 30 s	56 °C, 60 s	72 °C, 40 s	2125	[8]
wkat-F	aac tta ttt ctc gca tca tcc			30 cycles			
EspA	aaa cag cag gca ctt gaa cg	*espP*	94 °C, 30 s	56 °C, 60 s	72 °C, 150 s	1830	[9]
EspB	gga gtc gtc agt cag tag at			30 cycles			

then 50 μl of each sample were transferred into 3 ml of Luria Bertani broth (LBB). The pre-enrichment cultures were grown overnight at 37 °C on a rotary shaker (180 rpm).

Pre-screening by PCR

1 ml aliquots of the LBB cultures were harvested by centrifugation (12000 ***g***, 10 min), and washed three times in distilled water. Each pellet was resuspended in 200 μl distilled water, boiled at 95 °C for 20 min, and sonicated for 2 min in an ultrasonic water bath UM2 (Unitra Olsztyn, Poland). Shiga toxin genes (*stx*) 1 and 2 were detected by PCR using primer pair MK1/MK2 ([24]; Table 1).

Isolation of single colonies

Aliquots of the same pre-enrichment cultures of PCR-positive samples were diluted $1:10^5$ and $1:10^6$ in PBS, 50 μl was plated on McConkey agar and grown overnight at 37 °C. Between 5×10^2 and 2×10^3 colonies resulted from this cultivation. Colonies were pre-cooled for 30 min at 4 °C. A nylon membrane disk (Nylon Membranes for Colony and Plaque Hybridization, Roche Diagnostics GmbH, Mannheim, Germany) was placed onto the surface of the agar plate and the orientation marked. Subsequently, the membrane disk was removed and placed on filter paper soaked with denaturation solution (0·5 M NaOH, 1·5 M NaCl) for 15 min, then transferred to filter paper soaked with neutralization solution (1·5 M NaCl, 1·0 M Tris-HCl, pH 7·4) for 15 min and air-dried. The dry membrane was baked for 60 min at 80 °C, then equilibrated with 2× SSC (1× SSC is 0·15 M NaCl, 0·015 M sodium citrate [pH 7·0]) for 10 min and placed on filter paper soaked with dilute proteinase K (14–22 mg/ml, Roche) 1:10 in 2× SSC to remove cell debris.

DNA-probes were labelled with digoxigenin (DIG) with MK1/MK2 primers using the PCR DIG Probe Synthesis Kit (Roche) as recommended by the manufacturer. DIG Easy Hyb solution (Roche Diagnostics GmbH, Mannheim, Germany) was used for pre-hybridization and hybridization of nylon membranes. MK1/MK2-positive colonies were detected by the DIG Nucleic Acid Detection Kit (Roche) as recommended by the manufacturer. Up to 10 positive colonies per sample were selected for further characterization [30].

Characterization of STEC isolates

All isolated colonies were further analysed for the presence of additional virulence markers (*stx1*, *stx2*, *E. coli* attaching and effacing gene [*eae*], EHEC haemolysin gene [$EHEC_{hlyA}$], katalase-peroxidase gene [*katP*], and the serine protease gene [*espP*]) by colony hybridization. To this end, isolated colonies were cultivated on the required number of nitrocellulose filters (Millipore Corporation, Bedford, MA) and probed with labelled amplicons or oligonucleotides (Table 1).

The *stx1* and *stx2* oligonucleotides [31] were purchased (TIB MOLBIOL, Berlin, Germany), radiolabeled at their 5′ ends using T4 polynucleotide kinase (Roche) and [γ-^{32}P]ATP (Nycomed Amersham, Braunschweig, Germany), and purified by size exclusion chromatography using Sephadex G-50 columns (Roche). Pre-hybridization and hybrid-

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ization of nitrocellulose filters were carried out as recommended [31]. The visualization of signals was performed by autoradiography on Kodak films. All other DNA probes (*eae*, $EHEC_{hlyA}$, *katP*, and *espP*) were synthesized by PCR [32], radiolabelled by nick translation (Nick translation kit, Roche) in the presence of [α-^{32}P]dCTP (Nycomed Amersham) and purified by size exclusion chromatography in Sephadex G-50 columns (Roche). Pre-hybridization and hybridization of nitrocellulose filters were performed as described [33].

Serotyping

Serotyping of the O antigen was carried out with a limited set of 43 commercial O antisera (Denka Seiken, Japan, obtained from LD Labor Diagnostika GmbH, Heiden, Germany) according to the instructions of the manufacturer. All STEC with additional virulence markers were tested by serotyping.

Complete O- and H-serotypes were determined for potential EHEC isolates at the National Reference Centre for Enteric Pathogens, Hygiene Institut Hamburg, Germany. *E. coli* possessing at least *eae* and $EHEC_{hlyA}$ in combination with *stx1* and/or *stx2* genes were regarded as potential EHEC isolates.

Antimicrobial susceptibility testing

Potential EHEC isolates were tested for antimicrobial susceptibility against the following compounds using agar diffusion tests (ISO-sensitest-agar, Oxoid GmbH, Wesel, Germany): tetracycline (TET, 30 μg), trimethoprim-sulphamethoxazole (SXT, 23·75 μg/1·25 μg), ampicillin (AMP, 10 μg), gentamicin (GEN, 10 μg), kanamycin (KAN, 30 μg), chloramphenicol (CHL, 30 μg), neomycin (NEO, 30 μg), streptomycin (STR, 25 μg), nitrofurantoin (NIT, 100 μg) and furazolidone (FR, 100 μg) (all substances on test sheets; Oxoid GmbH, Wesel, Germany). Distribution of the test sheets on ISO-sensitest-agar was performed with a dispenser (Oxoid). The results were analysed according to DIN 58 940, part 3 [34].

Plasmid profile analysis

Two colonies of *E. coli* were suspended in 200 μl of alkaline lysis solution (50 mM Tris-base, 3% SDS, 0·05 N NaOH) and incubated at 56 °C for 1 h in a water bath. 800 μl phenol/chloroform solution (1:1) was added and the sample mixed by vortexing. After centrifugation at 12000 ***g*** for 10 min at room temperature in a Biofuge15R® (Heraeus Instruments GmbH, Hanau, Germany), 25 μl of the supernatant was analysed by agarose gel electrophoresis (0·6% agarose gel cast in 1 × Tris-acetate/EDTA electrophoresis buffer containing 0·5 μg/ml ethidium bromide).

Genomic typing

Genomic typing of O26, O157, O165, O103 and rough forms of STEC isolates was performed by pulsed-field gel electrophoresis (PFGE). Genomic DNA was digested in agarose plugs with *Xba*I (20 U; New England Biolabs GmbH, Schwalbach, Germany) at 37 °C for 4 h. The resulting fragments were resolved by rotating field gel electrophoresis (ROFE) with a Rotaphor R22 apparatus (Biometra GmbH, Göttingen, Germany) at a constant voltage of 200 V, constant angel of 120° for 18 h at 14 °C and a linearly ramped pulse time of 5–50 sec. Interpretation of the PFGE patterns was performed by visual inspection and computer analysis (phoretix 1d electrophoresis image analysis, Phoretix International, Newcastle upon Tyne, UK). Isolates yielding patterns with less than 80% identity in the densitograms were classified as different clones and coded with different numbers. Patterns within a clone (one or more fragment difference) were coded with equal numbers and different small letters.

Data analysis and statistical procedures

A programme written in CA-Visual Objects, version 2.0 for Windows95 (Computer Associates International Inc., New York, USA), was used for the documentation and analysis of data. Statistica 5.1 for Windows (StatSoft Inc., Tulsa, OK, USA), Excel 7.0 and PowerPoint 7.0 (Microsoft Corp., Redmond, USA) were used for statistical analysis and graphical presentation. Non-parametric statistical comparisons between groups were performed using the χ^2-test or Fisher's exact test as appropriate.

RESULTS

Samples and isolates

A total of 2163 faecal samples, in which coliform bacteria were detected, were screened by PCR for *stx*.

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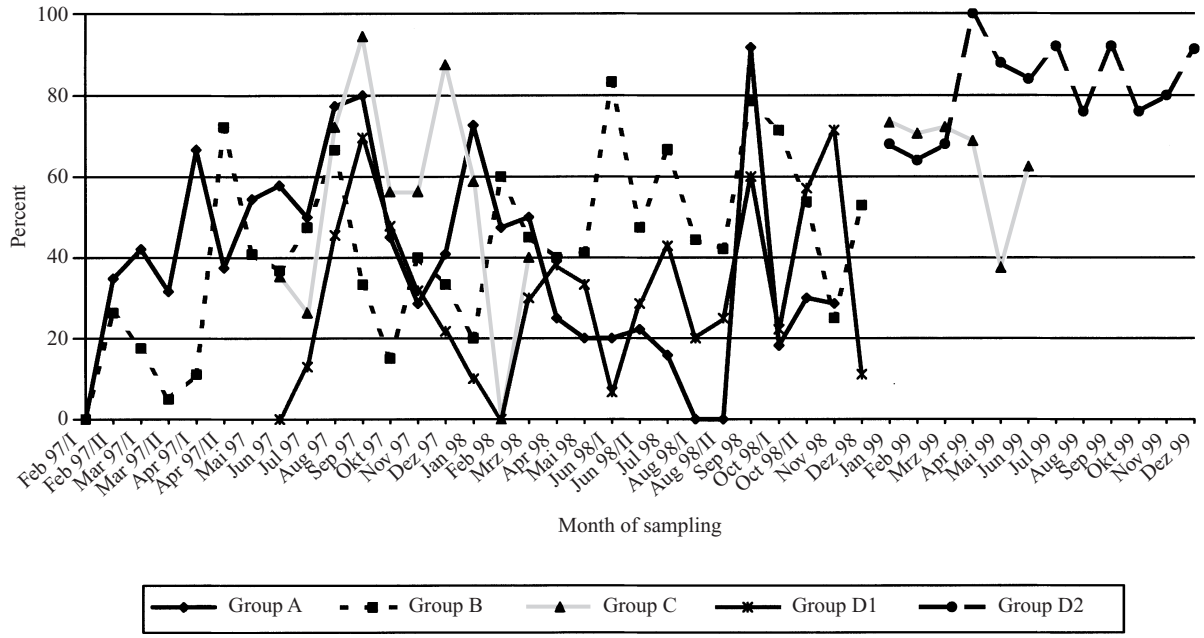

Fig. 1. Percentage of STEC-positive cattle on the individual days of sampling in all study groups on farms A–D.

Stx sequences were detected in 970 samples (44·8 %). The isolation of single STEC colonies by colony blot hybridization was accomplished for 810 samples.

Prevalence of STEC

The average prevalence of STEC-positive cattle on the farms varied between 28·8 % (farm D, group D1) and 81·5 % (farm D, group D2). An average prevalence of 40 % was found in groups A and B and a prevalence of more than 60 % was detected in group C. The differences in prevalence were significant between the groups A and C ($\chi^2 = 40{\cdot}10$, $P < 0{\cdot}001$), A and D (D1 $\chi^2 = 17{\cdot}46$, $P < 0{\cdot}001$; D2 $\chi^2 = 133{\cdot}53$, $P < 0{\cdot}001$), B and C ($\chi^2 = 37{\cdot}57$, $P < 0{\cdot}001$), B and D (D1 $\chi^2 = 19{\cdot}66$, $P < 0{\cdot}001$; D2 $\chi^2 = 129{\cdot}36$, $P < 0{\cdot}001$), C and D (D1 $\chi^2 = 93{\cdot}18$, $P < 0{\cdot}001$; D2 $\chi^2 = 25{\cdot}61$, $P < 0{\cdot}001$) and between both groups D1 and D2 on farm D ($\chi^2 = 210{\cdot}99$, $P = 0{\cdot}000$). No significant prevalence differences were found between groups A and B ($\chi^2 = 0{\cdot}07$, $P = 0{\cdot}795$).

The prevalence of STEC on the individual days of sampling in all groups of the cattle farms was analysed (Fig. 1). Considerable differences could be demonstrated between the groups and individual days of sampling. The differences ranged from failure to detect potential EHEC in individual groups (group A, 23rd and 24th days of sampling; group C, 16th day of sampling; group D, 7th and 15th days of sampling) to the detection of STEC in 70–90 % or even more in the same groups. The highest prevalence was observed on the 11th day of sampling in group C and on the 25th day of sampling in group A, when 94·4 % or 91·7 % of STEC positive cattle were found, respectively. By contrast to all other groups, the STEC prevalence was very high in group D2 on farm D throughout the study period. 70–90 % STEC-positive samples were detected on all days of sampling in group D2; on the 33rd day of sampling, all cattle were STEC-positive in this group.

STEC were detected at least once in a sample of each cattle in all study groups. In group D2, four animals tested positive for STEC on each sampling date. Based on the percentage of STEC-positive samples of each individual cattle in the study period, all cattle were grouped into five classes:

Class 1: $0\,\% \leqslant x < 10\,\%$
Class 2: $10\,\% \leqslant x < 25\,\%$
Class 3: $25\,\% \leqslant x < 50\,\%$
Class 4: $50\,\% \leqslant x < 75\,\%$
Class 5: $75\,\% \leqslant x \leqslant 100\,\%$

Most cattle of group A (18 cattle [75 %]) and B (15 cattle [62·5 %]) fell into class 3 (Fig. 2). A small number of cattle on both farms ranged between 10 % and 25 % (class 2 – group A: 3 cattle [12·5 %], group B: 4 cattle [16 %]) or 50 % and 75 % (class 4 – group A: 3 cattle [12·5 %], group B: 5 cattle [20 %]), respectively. The number of animals that had to be attributed to the chosen classes varied distinctly

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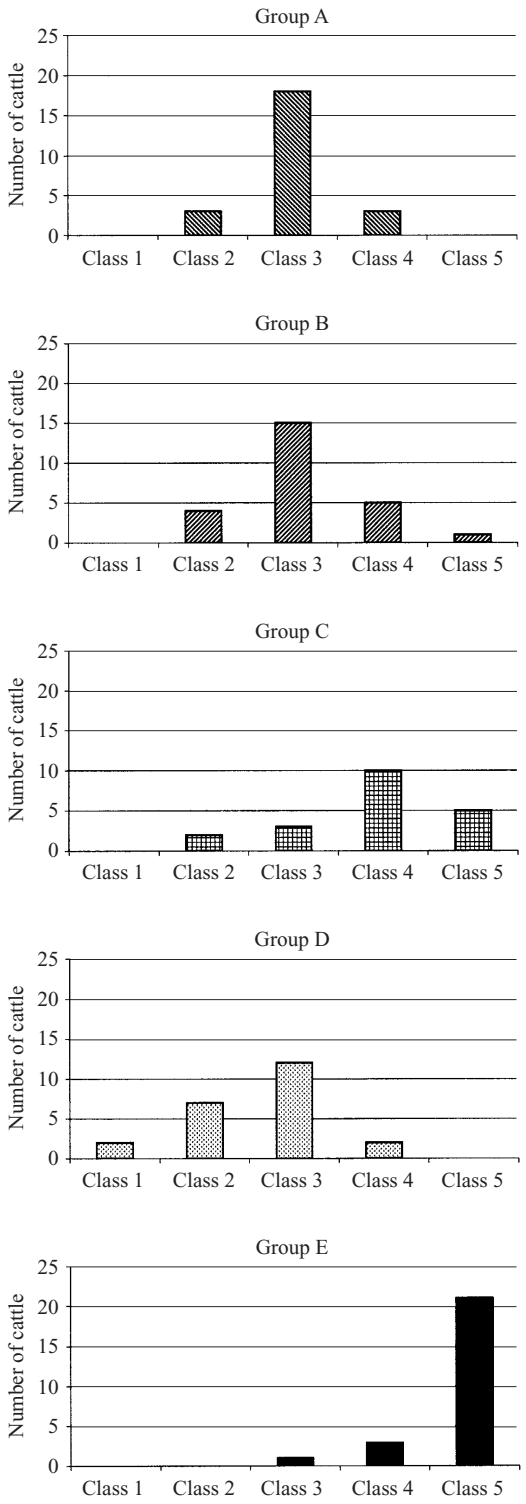

Fig. 2. Individual cattle classified according to the percentage of STEC-positive samples: class 1, $0\% \leqslant x < 10\%$; class 2, $10\% \leqslant x < 25\%$; class 3, $25\% \leqslant x < 50\%$; class 4, $50\% \leqslant x < 75\%$; class 5, $75\% \leqslant x \leqslant 100\%$.

between individual study groups. In group D1, 9 animals (39·1%) fell into classes 1 and 2 with a low percentage of STEC (class 1: 2 cattle [8·7%], class 2: 7 cattle [30·4%]). In contrast, the proportion of animals attributed to the classes with higher percentages was increased in groups C and D2. Twenty animals (75%) in group C and 24 cattle (96%) in group D2 fell in classes 4 and 5. More than 75% of STEC-positive samples (class 5) were detected in 21 of 25 (84%) cattle in group D2 during the entire study period. No STEC were found in samples of feedstuff or dust.

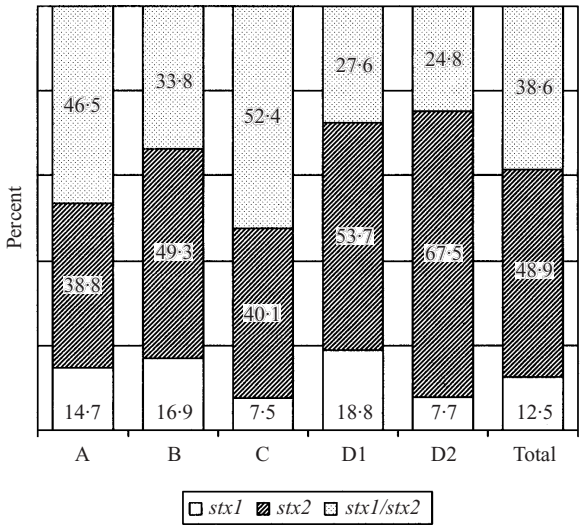

Fig. 3. Percentage of isolates with *stx1*-, *stx2*- and *stx1/stx2* genes.

Detection of additional virulence markers

Individual colonies that carried *stx1* or *stx2* could be isolated from 60·5% of the samples which had been positive for *stx* in the pre-screening tests. In most isolates *stx2* (48·9%) was found, followed by colonies carrying both *stx* genes (38·6%). Isolates harbouring only *stx1* were detected in 12·5% of all *stx* positive colonies. Isolates with both *stx* genes were predominantly found in groups A and C (Fig. 3) where 46·5% or 52·7% of *stx1/stx2* were detected, respectively. By contrast, isolates harbouring only the *stx2* prevailed in groups B and D where 49·3% (group B), 51·7% (group D1) and 68·6% (group D2) were found. Isolates carrying only *stx1* played a minor role in all groups.

STEC with additional EHEC virulence markers (*eae*, $EHEC_{hlyA}$, *katP*, *espP*) were detected on all four farms. $EHEC_{hlyA}$ and *espP* were found in a high proportion of isolated colonies. $EHEC_{hlyA}$ was found in 26·5% ($n = 1647$) of the isolates and *espP* in 28% ($n = 1740$) of the colonies. 20% ($n = 1242$) of the

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Table 2. *Distribution of potential EHEC samples according to serotype and farm*

	Farms			
Serotype	A	B	C	D
O20:H-NT	1	1	1	1
O20:H44	1			
O22:H16			1	
O26:H11	12		6	1
O26:H2*	1			
O26:H32	1			
O26:H-			1	
O74:H-		1		
O79:H-		1		
O79:H-NT		1		
O84:H-	1			
O103:H2	1			1
O110:H2			1	
O112:H2			1	
O119:H6		1		
O119:H40				1
O125:H47				1
O126:H20			1	
O128:H-NT				1
O145:H-		1		1
O149:H-NT	1			
O156:H-NT		2		1
O156:H1		1		
O156:H4		2		
O157:H7	3	1		
O157:H-	1			1
O159:H-NT	1			
O165:H25		9		
O172:H-		1		1
O-NT:H-NT	1	1		
O-NT:H-	2	2		2
O-NT:H4	1			
O-NT:H25	1	1		
O-NT:H32	1			
O-NT:H37	1			
ONT:H40	1			
O-RF:H-		5		
O-RF:H4				1
O-RF:H10	1			
O-RF:H27				1
O-RF:H32		1		

O-RF:O antigen rough and serotype not determined; O-NT or H-NT:O or H antigen did not agglutinate in any serotype specific antiserum.

* The *fliC* gene of strain O26:H2 had a nucleotide sequence identical with that of *E. coli* O26:H11 (H. Karch, unpublished data). Accordingly, the strain displayed a *fliC*-RFLP pattern that was identical with that of *E. coli* H11 reference strains.

isolates carried both genes. *katP* was found in 8% ($n = 495$) of the colonies. *Eae* was detected in 6·2% ($n = 387$) of all isolated colonies. 351 *eae*-positive isolates also carried an *stx* gene. In a total of 144 isolates (2·3%), all four additional virulence marker genes were detected by DNA-hybridization. A total of 128 of these isolates also carried one or two *stx* genes.

The 1647 $EHEC_{hlyA}$- and 1740 *espP*-positive isolates as well as the 1242 isolates carrying a combination of both genes was significantly higher than that of isolates positive for other virulence marker genes (including isolates with more than one virulence marker gene) on all farms. The combination of all four virulence marker genes was most frequently found in isolates also carrying *stx2*. The combination of all four virulence marker genes with both *stx* genes was detected on several sampling dates only in group A. In groups B and D1, the number of isolates harbouring a combination of all four virulence marker genes with *stx2* was significantly higher than the number of isolates with a combination of all four virulence marker genes with *stx1*, or both *stx1* and *stx2* (Fisher's exact test, $P < 0·01$). In group A, the number of isolates carrying a combination of all four virulence marker genes with both *stx* genes was significantly higher than the number of isolates with all other combinations of *stx* genes (Fisher's exact test, $P < 0·01$). A very low proportion of *eae*-positive isolates and isolates harbouring combinations with *eae* was observed in group C.

Serotyping

Out of 2320 isolates harbouring additional virulence markers the O-serogroup of 708 isolates could be determined by using commercial antisera. A wide range of serogroups was detected in the isolates. Many of the typed isolates were classified in serogroups O8 ($n = 142$), O26 ($n = 59$), O165 ($n = 54$) and O153 ($n = 286$). Smaller numbers of isolates were typed as O27 ($n = 8$); O114 ($n = 9$), O115 ($n = 9$), O125 ($n = 11$), O126 ($n = 11$), O128 ($n = 10$), O146 ($n = 10$), O157 ($n = 16$), O158 ($n = 16$), O159 ($n = 7$), O166 ($n = 9$), O168 ($n = 14$) and O169 ($n = 8$). One to three isolates were grouped in other serogroups (O1, O6, O15, O18, O28ac, O29, O44, O55, O78, O108, O111, O119, O124, O127a, O142, O164). Many isolates ($n = 1612$) could not be typed since the respective O antigen-specific antisera were not commercially available.

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Table 3. *Temporal distribution of potential EHEC clones isolated from group A*

Year/month	Ear tag no. of cattle	*stx1*	*stx2*	*eae*	hly_{EHEC}	*katP*	*espP*	Serotype	Clone
1997–02	104	+	+	+	+	+	+	O26:H11	26-1a
	109	+	+	+	+	+	+	O26:H11	26-1a
	141	+	+	+	+	+	+	O26:H11	26-1a
1997–03	788	+	+	+	+	+	+	O26:H11	26-1b
	141	+	+	+	+	+	+	O26:H11	26-2a
1997–04	147	+		+	+	+	+	O26:H11	26-2a
1997–05	144	+		+	+	+	+	O26:H11	26-2b
1997–06	112	+	+	+	+	+	+	O103:H2	103-1
	116		+	+	+	+	+	O157:H7	157-1
1997–09	144	+	+	+	+	+	+	O26:H2*	26-1c
1997–12	147	+		+	+	+	+	O26:H11	26-1c
1998–12	110	+			+	+	+	O26:H32	26-3
	126	+	+	+	+	+	+	O157:H-	157-2
	144	+	+	+	+	+	+	O26:H11	26-1c
1998–03	147	+	+	+	+	+	+	O26:H11	26-1c
1998–06	144	+	+	+	+	+	+	O26:H11	26-1d
1998–08	141	+	+	+	+	+	+	O157:H7	157-2

* The *fliC* gene of strain O26:H2 had a nucleotide sequence identical with that of *E. coli* O26:H11 (H. Karch, unpublished data). Accordingly, the strain displayed a *fliC*-RFLP pattern that was identical with that of *E. coli* H11 reference strains.

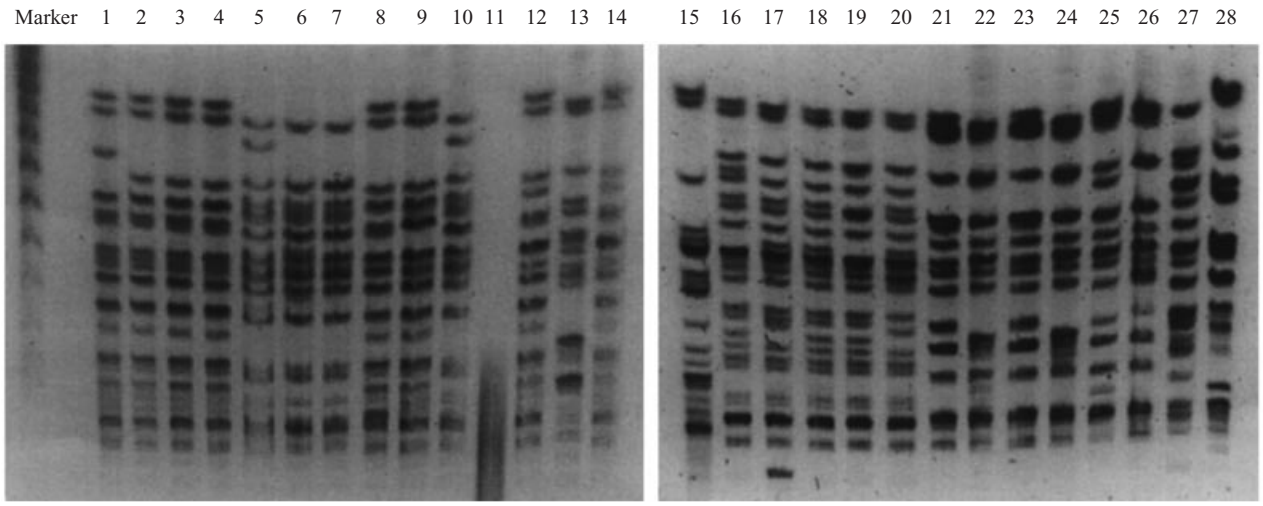

Fig. 4. Pulsed-field gel electrophoresis of STEC-O26-, STEC-O157- and STEC-O103-isolates digested with *Xba*I. Lanes 1–14, STEC-O26 from farm A: lanes 2–4, clone 26-1a; lane 1, clone 26-1b; lanes 8, 11, 12, 14, clone 26-1c (lane 11 – autodigested); lane 9, clone 26-1d; lanes 5, 10, clone 26-2a; lanes 6, 7, clone 26-2b; lane 13, clone 26-3. Lane 15, STEC-O26 from farm D: lane 15, clone 26-4. Lanes 16–20, STEC-O26 from farm C: lane 16, clone 26-5a; lanes 17, 20, clone 26-5b; lane 18, clone 26-5c; lane 19, clone 26-5d. Lanes 21–24, STEC-O157 from farm A: lanes 21, 23, clone 157-1a; lanes 22, 24, clone 157-2a. Lane 25, STEC-O157 from farm B. lane 25, clone 157-1b. Lane 26, STEC-O157 from farm D: lane 26, clone 157-2b. Lane 27, STEC-O103 from farm A: lane 27, clone 103-1. Lane 28, STEC-O103 from farm D: lane 28, clone 103-2.

Complete O- and H-serotype patterns were determined for all 93 potential EHEC isolates (presence of at least *eae* and $EHEC_{hlyA}$ in combination with *stx1* and/or *stx2*; single isolates of up to 10 positive isolates per sample were serotyped). The potential EHEC isolates belonged to a small number of serogroups (Table 2). 23·7% of the samples belonged to serogroup O26, and isolates of this serogroup were

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found in 3 of the 4 farms investigated. Isolates of serogroup O165 were detected in 9 samples exclusively in group B, while O26 isolates were not found in this group. Rarer serotypes were O157 (6 samples), O156 (6 samples), O20 (5 samples), O103 (2 samples), O119 (2 samples), O145 (2 samples) and O22 (1 sample).

Clonal relationships

A combination of the data on serotype, virulence pattern, plasmid profile and PFGE pattern were used to assess the clonal relationships of STEC O26, O157, O165, O103 and of rough form isolates.

Among the STEC O26 isolates five different clones could be distinguished. Three of them were present in group A (clones 26-1, 26-2, 26-3), one on farm D (clone 26-4) and one in group C (clone 26-5). Small variations within individual clones became evident during the course of the investigation in group A (Table 3, Fig. 4, lanes 2–4 [clone 26-1a], lane 1 [clone 26-1b], lanes 8, 11, 12, 14 [clone 26-1c], lane 9 [clone 26-1d] and lanes 5, 10 [clone 26-2a], lanes 6, 7 [clone 26-2b]. Two clones were also detected in each of STEC O157 and STEC O103 serogroups.

A plasmid profile analysis revealed no differences between the STEC O165 isolates (Fig. 5*a*). This result was confirmed by the PFGE patterns, although small variations within presumed clones were observed (Fig. 5*b*, lane 1 [clone 165-1a], lanes 2–4 [clone 165-1b], lanes 5–7, 9 [clone 165-1c]). Several differences could be demonstrated among the rough form STEC isolates. There was no indication for a clonal relationship of these (O-RF : H-) with the STEC O165 isolates (Fig. 5*b*, lanes 10–14).

Antimicrobial susceptibility testing

All potential EHEC isolates ($n = 93$) were examined for potential antimicrobial resistances. Seventy-three isolates (78·5%) were resistant to GEN, 54 (58·1%) to CHL, 54 (58·1%) to STR and 72 (77·4%) to FR. The difference in the number of GEN- and FR- resistant isolates was not statistically significant between the study groups. By contrast, the number of isolates resistant to CHL was significantly higher in group B as compared with all other study groups, whereas the number of isolates with resistance to STR was significantly lower in the group B ($P < 0{\cdot}01$). All 14 potential EHEC isolates on farm D were resistant to STR.

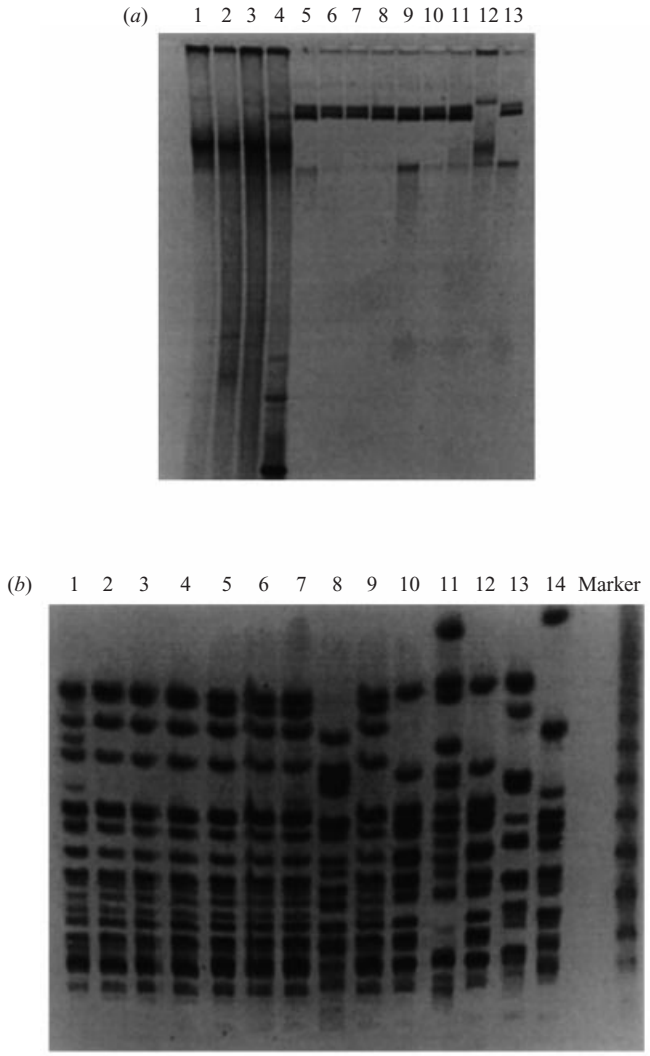

Fig. 5. (*a*) Plasmid profiles of STEC-O165 from farm B. Lanes 1–4, markers (PIP40a, R64, RSF, V517), lanes 5–13, STEC-O165. (*b*) Pulsed-field gel electrophoresis of STEC-O165 and STEC-O-RF-isolates digested with *Xba*I from farm B: lanes 1, clone165-1a; lanes 2–4, clone165-1b; lanes 5–7, 9, clone 165-1c; lanes 10–14, STEC-O-RF. Re-testing of isolate 8 [lane 12 in Fig. 5*a* and lane 8 in Fig. 5*b*, same isolate] showed that it is not an STEC-O165 isolate.

Four isolates from farm A displayed multiple resistances. Resistance against STR, CHL, GEN, TET and AMP was found in three isolates, and additional resistance against SXT was detected in the remaining isolate.

Influence of transport

A potential influence of the duration of transport of cattle to the abattoirs was investigated by analysing samples taken before and after transportation (Table

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Table 4. *STEC findings of samples of cattle in farms B and D on the day of slaughter*

Farm B			Farm D			
	Sample type			Sample type		
Ear tag no. of cattle	Shedding	Abattoir	Ear tag no. of cattle	Shedding	Abattoir	Surface
460	+	+	5063	+	+	—
462	—	—	5064	—	—	—
463	+	—	5066	—	—	—
465	+	—	5067	—	—	—
468	—	—	5069	+	—	—
471	+	—	5070	—	—	—
472	—	—	5071	—	—	—
473	—	—	5072	—	+	—
474	—	+	5074	—	+	—
476	—	—	5075	—	—	—
478	—	—	5076	—	—	—
481	+	+	5077	+	—	—
			5078	—	—	—
			5079	—	—	—
			5080	—	—	—
			5081	—	+	n.i.*
			5082	+	—	—
			5085	—	—	—

* n.i., not investigated.

4). Neither a statistically significant difference nor a trend became evident.

DISCUSSION

EHEC represent a major risk for human health since they can cause severe diseases such as HUS and HC which can be fatal [2–4, 17]. Although other modes of transmission exist, humans often contract the infection via contaminated food such as milk or ground beef [12–14]. It is therefore necessary to study the occurrence of STEC and EHEC in dairy and beef herds. In the present study, the occurrence of such strains was monitored in 5 groups on 4 farms over a period of 3 years.

A uniform cascade of methods was established, optimized and standardized for the isolation and characterization of STEC found in faecal samples of cattle. Consequently, comparable results could be obtained in all groups involved during a period of 3 years. The cascade of methods was equally suitable for the isolation of O157 and potential other EHEC serogroups. The results of these investigations should thus reflect the relative importance of individual serogroups during the study period.

Stx1- or *stx2*-positive colonies could only be isolated from 60·5% of *stx*-positive pre-screening cultures. The loss of phage encoding *stx* genes during the first cultivation step may be one reason for this phenomenon. Picking wrong colonies (5×10^2 to 2×10^3 grown colonies) and limitations in the sensitivity of the DNA-hybridization with oligonucleotide probes as compared to the MK1/MK2 PCR could also explain why in many cases no *stx*-positive colonies were recovered from *stx*-positive pre-screening cultures.

The prevalence of STEC varied widely between the study groups and farms. These results are in accord with previous studies performed by several investigators in the USA and Europe [18–23, 35–37]. It is of interest that large prevalence differences became evident even between two study groups (D1 and D2) kept successively on the same farm. While the average prevalence was relatively low during the fattening period of group D1, the STEC prevalence was very high in the following group D2 even though there were no apparent variations in farm management or any other obvious extrinsic factor that may have influenced the STEC prevalence. The finding that the proportion of STEC-positive samples varied between the individual days of sampling in the same group may be explained by alternating faecal shedding of

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STEC and from a circulation of these bacteria in the cattle herds with relatively frequent infections and re-infections of individual cattle.

An association between a high prevalence of STEC positive cattle and diarrhoea could not be shown in this or previous field studies [20, 35, 36, 38], although a causal relationship between STEC and diarrhoea has been observed in experimental infections [39–41]. Although the STEC prevalence was significantly higher in group C which also encountered severe calf losses and pneumonia, the number of potential EHEC isolates was low in this group. The highest percentage of STEC was detected in group D2 which had an excellent health status on all sampling dates throughout the study period, while the STEC prevalence was significantly higher in this group than in all other groups. In group B, the detection of coronavirus and rotavirus as well as *Cryptosporidium* spp. coincided with diarrhoea. It is thus possible that these agents caused diarrhoea alone. It is also possible, however, that STEC contributed to the losses in group B since it has been suggested that STEC can cause acute diarrhoea in conjunction with other infections and environmental influences (management, temperature, humidity, draught) which may particularly affect calves. Furthermore, Wieler [42] argued that Stx and other virulence factors of STEC may not only induce an acute toxic effect. The results of several investigations [43–46] showed a chronic or subclinical picture which may have been caused by toxins associated with STEC.

STEC with additional EHEC virulence marker genes were detected on all four farms. A high prevalence of STEC was not necessarily associated with a high percentage of potential EHEC. By contrast, a significantly lower percentage of potential EHEC was detected in the groups with the highest STEC prevalences (groups C and D2). The largest number of potential EHEC could be serotyped as O26:H11. However, the distribution of STEC O26:H11 in the study groups was very different. While O26:H11 was the dominant isolate in groups A and C, only a single sample with O26:H11 was found on farm D and none at all in group B.

The clonal analysis of O26:H11 isolates showed considerable differences in this particular serogroup. The clones varied considerably between individual groups, while individual clones displayed only minor differences over the course of the study. Interestingly, three different clones were detected in group A. Clones 1 and 2 were found throughout the study period in group A and may have persisted in the herd and/or individual cattle during a period of several months. The same was true for STEC O165:H25 which was detected exclusively in group B as a single clone. The minor variations observed within the clones of O26:H11 and O165:H25 could be due to interactions with the host or with other intestinal bacteria.

STEC O157 (H7/H-) and STEC O103:H2 were detected only sporadically in cattle investigated in this study as compared with O26:H11 or O165:H25. In the case of O157, this could be due to the fact that the immunomagnetic separation technique (IMS), which has been shown to be significantly more sensitive for isolation of *E. coli* O157 from bovine faeces than other detection techniques [47], was not used in this study. However, selective enrichment of O157 by use of IMS may lead to an over-estimation of this serogroup in relation to all other serogroups. Since the purpose of this study was an overview of the relevance of all serogroups potentially involved, it was necessary to avoid an over-estimation of any particular serogroup. Wieler and coworkers [48] frequently found STEC O118 in German cattle. However, we failed to isolate this EHEC in our study.

The serogroups of most potential EHEC isolates from cattle were identical with the serogroups isolated from human cases of HUS or HC. It has already been shown that some bovine STEC strains possess all features of human STEC strains that had caused HUS or HC [42]. Thus it is currently not possible to distinguish bovine STEC from human STEC. The results of our study confirm this view, at least as far as the detection of known virulence markers of STEC is concerned.

The results of antimicrobial resistance testing illustrate a general problem in animal production that has resulted from an inconsistent use of antibiotics. The statistically significant differences in the antimicrobial resistance against CHL and STR between strains on different farms could be related to the preferred use of these antibiotics on the respective farms.

The results of this study show that STEC can be regularly detected in German cattle herds over long periods, mainly in clinically healthy animals. The presence of STEC may represent a potential health risk for the farmers, for other people who have contact to the animals or their faeces and for consumers. In Germany, STEC O26 has been the most frequently isolated EHEC serogroup from cases

of human diseases during recent years [12, 49]. In our study, we also found isolates of these serogroups in cattle, and detected them even over longer periods. Thus, a natural elimination of these potential EHEC clones from the intestine of an infected animal cannot be expected. Future studies are needed to estimate the risk of infection for farmers and consumers and to develop control strategies for cattle herds. In case of STEC 026:H11, it is necessary to conduct a direct comparison of clonal relationships between cattle isolates from our study and those from cases of human disease [50].

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