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Use of Antibody Responses against Locus of Enterocyte Effacement (LEE)-Encoded Antigens To Monitor Enterohemorrhagic *Escherichia coli* Infections on Cattle Farms

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Enterohemorrhagic *Escherichia coli* (EHEC) is a significant zoonotic pathogen causing severe disease associated with watery and bloody diarrhea, hemorrhagic colitis, and the hemolytic-uremic syndrome (HUS) in humans. Infections are frequently associated with contact with EHEC-contaminated ruminant feces. Both natural and experimental infection of cattle induces serum antibodies against the LEE-encoded proteins intimin, EspA, EspB, and Tir and the Shiga toxins Stx1 and Stx2, although the latter are poorly immunogenic in cattle. We determined whether antibodies and/or the kinetics of antibody responses against intimin, Tir, EspA, and/or EspB can be used for monitoring EHEC infections in beef cattle herds in order to reduce carcass contamination at slaughter. We examined the presence of serum antibodies against recombinant O157:H7 *E. coli* intimin EspA, EspB, and Tir during a cross-sectional study on 12 cattle farms and during a longitudinal time course study on two EHEC-positive cattle farms. We searched for a possible correlation between intimin, Tir, EspA, and/or EspB antibodies and fecal excretion of EHEC O157, O145, O111, O103, or O26 seropathotypes. The results indicated that serum antibody responses to EspB and EspA might be useful for first-line screening at the herd level for EHEC O157, O26, and most likely also for EHEC O103 infections. However, antibody responses against EspB are of less use for monitoring individual animals, since some EHEC-shedding animals did not show antibody responses and since serum antibody responses against EspB could persist for several months even when shedding had ceased.

Enterohemorrhagic *Escherichia coli* (EHEC) causes bloody diarrhea and potentially sequelae like the hemolytic-uremic syndrome (HUS) in humans. Cattle are most frequently identified as the primary source of infection. EHEC generally colonizes the terminal rectum of cattle without causing disease. However, bacteria become shed in the feces. This shedding occurs typically intermittently over a long period in low numbers, as demonstrated in longitudinal studies of excretion by naturally infected cattle (1). However, a small proportion of cattle in a population positive for EHEC can, at any one time, shed high levels of EHEC, and as such be considered supershedders. Such animals are usually not a stable subset of the population, but they are considered to have a significant role (as yet unquantified) in the transmission and persistence of EHEC within the cattle population.

Following initial adherence of EHEC to the intestinal epithelium, a locus of enterocyte effacement (LEE)-encoded type III secreted protein translocation tube is formed, which connects the pathogen with its target cell (for reviews, see references 2 and 3). EspA is a major component of this tube, through which EspB, EspD, and Tir are delivered to the host cell. EspB and EspD form pores in the host cell membrane. EspB is also translocated into the host cell cytosol, where it triggers signal transduction events that mediate effacement of the microvilli and replacement with a pedestal-like structure. Tir becomes translocated to the host cell membrane, where it forms the receptor for the LEE *eaeA* gene-encoded intimin, expressed on the surface of the bacteria, resulting in intimate attachment to the host cell. A consequence of this interaction is a striking histopathological change known as attaching and effacing (A/E) lesion. Meanwhile, the bacteria produce toxins such as the Shiga toxins Stx1 and Stx2 (variants). However, unlike humans, ruminants lack vascular receptors for Stxs. Humans do have Gb3 on their intestinal crypt epithelial cells. Nevertheless, binding does not result in cytotoxicity due to exclusion of the toxin from the endoplasmic reticulum (reviewed in reference 4).

Both natural and experimental EHEC infections have shown that cattle develop serum antibodies against intimin, EspA, EspB, and Tir and the Shiga toxins Stx1 and Stx2 (5–7), although the latter are poorly immunogenic in cattle (8). Intimin, EspA, and EspB are more immunogenic in ruminants since oral infection of sheep with a Shiga toxin-negative O157:H7 *E. coli* strain induced antibody responses against intimin, EspA, and EspB (9). Responses against Tir were not examined in the latter study. Interestingly, antibody responses against these antigens decreased as EHEC shedding diminished. EHEC reinfection boosted the antibody responses against EspA and slightly less against EspB. Remarkably, antibody response against EspB remained high throughout the study even though shedding ceased (9). Nevertheless, these findings seemed to indicate that the presence of antibodies and/or the kinetics of antibody responses against

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the LEE-encoded proteins intimin, EspA, and/or EspB could be used for monitoring the EHEC infection status in cattle herds. Moreover, studying these antibody responses could help to elucidate (i) the interaction of different EHEC seropathotypes with the ruminant immune system and (ii) the possible correlation between intimin, EspA, and/or EspB antibodies and the prevalence of EHEC infections on the animal level (identification of supershedders and direct transmission between animals) and on the farm level.

Thus, we determined the presence of serum antibodies against recombinant O157:H7 *E. coli* intimin, EspA, EspB, and also Tir during a cross-sectional study on 12 beef cattle farms and during a longitudinal time course study on two beef cattle farms, We focused on a possible correlation between intimin, Tir, EspA, and/or EspB antibodies and the fecal excretion of EHEC O157, O145, O111, O103, or O26 seropathotypes.

MATERIALS AND METHODS

Experimental setup of the cross-sectional survey. Thirty-eight Flemish cattle farms delivering young EHEC O157 and/or non-O157 (EHEC O26, O103, O111, and O145)-excreting beef bulls for slaughter from December 2007 to June 2009 (10) were asked to cooperate in a cross-sectional EHEC survey. Twelve farmers (farms A to L) were willing to participate.

Samples were taken from August 2009 to October 2009. The random sample size was calculated based on a confidence level of 95% and a 10% accepted absolute error (11) (see Table 2). Within each farm, bulls were stratified in four different age groups (<8 months old, 8 to 12 months old, 12 to 18 months old, and 18 to 24 months old). The sample size was divided proportionally among the four groups. When the proportion of bulls was too small to satisfy sample size constraints, cows were added to the group. Every herd was examined once collecting paired fecal (rectal sampling) and blood (*v. jugularis*) samples of each individual animal. Fecal samples were transported on ice and immediately processed upon arrival in the laboratory. Blood was stored overnight at room temperature and subsequently centrifuged (380 × *g*, 4°C) to collect serum. Serum samples were stored at −20°C until tested.

Experimental setup of the longitudinal study. To study the natural time course of EHEC infections in cattle, 12-month-old clinically healthy bulls ($n = 20$) housed on two cattle farms (farms D and H) were monitored during the following 36 and 42 weeks, respectively, from September 2010 to August 2011, and the animals were then transported to the slaughterhouse. Farms were selected based on the following. Farms D and H were seronegative at the start of the survey but, 10 months earlier, farm D was diagnosed as positive for EHEC O157, whereas 11 months earlier farm H was diagnosed positive for EHEC O26 and O103 (M.-A. Joris et al., unpublished results). Paired fecal (rectal sampling) and blood (*v. jugularis*) samples were collected from each animal, starting at the age of 12 months and then every 6 weeks thereafter. Samples were transported and stored as during the cross-sectional survey.

Isolation and identification of EHEC O26, O103, O111, and O145. Isolation was performed as described by Possé et al. (12) using selective agars. Briefly, 25 g of each fecal sample was enriched during 24 h at 42°C in 225 ml of tryptone soy broth (TSB) supplemented with 8 mg of novobiocin liter^{-1}, 16 mg of vancomycin liter^{-1}, 2 mg of rifampin liter^{-1}, 1.5 g of bile salts liter^{-1}, and 1.0 mg of potassium tellurite liter^{-1}. After 6 h of incubation, 100 μl was plated onto O26, O111, O103, and O145 differential agar medium. After 24 h of enrichment, Shiga toxin-producing *E. coli* (STEC) was isolated by plating and by a serotype-specific immunomagnetic separation (IMS). For serotypes O26 and O103, Dynabeads (Invitrogen, Paisley, United Kingdom) were used, whereas for serotypes O111 and O145, Captivate beads (Lab M, Lancs, United Kingdom) were applied according to the manufacturer's instructions. The selective agar plates were incubated at 37°C for 24 h. Up to three suspected colonies were transferred to serogroup-specific confirmation media. Isolates with a suspected morphology on both media were characterized by molecular (PCR) serotyping using the O-unit flippase gene (*wzx*) (13). Primers and cycle conditions (thermal cycler; Gene Amp 9700; Applied Biosystems) used for the detection of O26, O103, O111, O145, and O157 serotypes are shown in Table 1. The amplified products were visualized by standard gel electrophoresis using 8 ml of the PCR product mixture on 1.5% agarose gels in 1× TAE buffer (0.1 M Tris, 0.1 M acetic acid. and 0.002 M sodium-EDTA). Gels were stained using ethidium bromide (1 mg/ml) and photographed under UV light.

The isolates were further examined for the presence of virulence genes using a multiplex PCR targeting the Shiga toxin type 1 (stx_1)-, the Shiga toxin type 2 (stx_2)-, intimin (*eaeA*)-, hemolysin (*hlyA*)-, and TTSS effector protein EspB (*espB*)-encoding genes (14–16). The primers and cycle conditions are shown in Table 1.

Isolation and identification of EHEC O157. A sample of feces (25 g) was added to 225 ml of modified TSB supplemented with 0.25 ml of novobiocin. After 6 h at 42°C, IMS using specific Dynabeads (Invitrogen) was performed according to the manufacturer's recommendations. Then, 100 μl was plated onto cefixime-tellurite sorbitol-MacConkey agar (Oxoid), followed by incubation overnight at 42°C. Up to three suspected colonies were transferred to tryptone soy agar (Oxoid), incubated for 24 h at 37°C, and serologically identified with an O157 latex agglutination test kit (Oxoid). Identification was further confirmed biochemically and by use of an EHEC O157-specific multiplex PCR targeting the *rfb* (O-antigen-encoding) and *fliC* (H-antigen-encoding) genes (17). EHEC O157 strains were further examined by (14–16). The primers and cycle conditions are shown in Table 1.

Recombinant intimin, Tir, EspA, and EspB. Plasmids pCVD468 and pCVD469 were used for the recombinant expression of EspA and EspB, respectively (18). Plasmids pTir and pMW103 were used to express Tir and the C-terminal 380 amino acids of intimin-γ (referred to as intimin), respectively (19). Briefly, transformed *E. coli* was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and recombinant His-tagged proteins were purified by nickel-affinity chromatography. Recombinant intimin, Tir, EspA, and EspB were subsequently used in antibody enzyme-linked immunosorbent assays (ELISAs).

Antibody responses to LEE-encoded antigens. Antibody responses to LEE-encoded antigens were measured by using a ELISA (9). Briefly, sera were heat inactivated (30 min at 56°C) and kaolin treated (20). Polysorp 96-well plates (Nunc, Roskilde, Denmark) were coated with 200 ng of recombinant intimin, Tir, EspA, or EspB/well in phosphate-buffered saline (PBS; pH 7.3), followed by incubation overnight at 4°C. Nonspecific binding sites were blocked during 1 h at 37°C by adding 250 μl of PBS supplemented with 1% bovine serum albumin per well. Plates were incubated for 1 h (37°C) with 2-fold serial dilutions of serum in PBS starting at a dilution of 1:10 till 1:160, followed by incubation for 1 h (37°C) with 1:5,000 horseradish peroxidase-conjugated anti-cow IgG(H+L) rabbit antibodies (DakoCytomation, Glostrup, Denmark). In between steps, the plates were washed three times with PBS containing 0.2% Tween 20. After the addition of ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid); Roche Diagnostics, Vilvoorde, Belgium], the optical density was measured at 405 nm (OD_{405}) by using a Tecan SpectraFluor. The results were positive if the absorbance exceeded the cutoff value being the mean OD_{405} values of the negative controls ± three times the standard deviation. Sera of experimentally infected (EHEC O157) calves and of a negative control group were used as positive and negative controls, respectively.

Statistical analysis. Statistical analyses were done using a nonparametric Mann-Whitney U test. The results were considered significantly different at $P < 0.05$.

RESULTS

Cross-sectional survey. (i) Isolation and identification of EHEC. Nine of twelve (75%) herds were EHEC positive based on at least one culture-positive animal (Table 2). The number of culture positives within the nine infected herds ranged from 1 of 52 sampled

TABLE 1 Primers and PCR cycling conditions used in detection of serotypes and virulence genes[a]

Gene	Primer	Sequence (5′–3′)	Cycling conditions	Amplicon size (bp)	Reference
wzx	wzxO26-F	CAGAATGGTTATGCTACTGT	30 cycles of 20 s at 95°C, 40 s at 60 to 54°C, and 30 s at 72°C	423	13
	wzxO26-R	CTTACATTTGTTTTCGGCATC			
	WzxO103-F	TTGGAGCGTTAACTGGACCT	30 cycles of 20 s at 95°C, 40 s at 57°C, and 30 s at 72°C	321	13
	wzxO103-R	GCTCCCGAGCACGTATAAG			
	wzxO111-F	TAGAGAAATTATCAAGTTAGTTCC	30 cycles of 20 s at 95°C, 40 s at 62°C, and 30 s at 72°C	406	13
	wzxO111-R	ATAGTTATGAACATCTTGTTTAGC			
	wzxO145-F	CCATCAACAGATTTAGGAGTG	30 cycles of 20 s at 95°C, 40 s at 59°C, and 30 s at 72°C	609	13
	wzxO145-R	TTTCTACCGCGAATCTATC			
	wzxO157-F	CGGACATAAATGTGATATGG	30 cycles of 20 s at 95°C, 40 s at 60°C, and 30 s at 72°C	259	13
	wzxO157-R	TTGCCTATGTACAGCTAATCC			
stx_1	stx1-F	ACACTGGATGATCTCAGTGG	35 cycles of 1 min at 95°C and 2 min at 65°C for the first 10 cycles, gradually decreasing to 60°C by cycle 15, and 1.5 min at 72°C	614	15
	stx1-R	CTGAATCCCCCTCCATTATG			
stx_2	stx2-F	GGCACTGTCTGAAACTGCTCC	35 cycles of 1 min at 95°C and 2 min at 65°C for the first 10 cycles, gradually decreasing to 60°C by cycle 15, and 1.5 min at 72°C	255	14
	stx2-R	TCGCCAGTTATCTGACATTCTG			
eaeA	eaeA-F	GTGGCGAATACTGGCGAGACT	35 cycles of 1 min at 95°C and 2 min at 65°C for the first 10 cycles, gradually decreasing to 60°C by cycle 15, and 1.5 min at 72°C	890	15
	eaeA-R	CCCCATTCTTTTTCACCGTCG			
hlyA	hlyA-F	ACGATGTGGTTTATTCTGGA	35 cycles of 1 min at 95°C and 2 min at 65°C for the first 10 cycles, gradually decreasing to 60°C by cycle 15, and 1.5 min at 72°C	165	15
	hlyA-R	CTTCACGTGACCATACATAT			
espB	epsB-F	CGGGATCCCGTGAGATGGTCAC	30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C	600	16
	espB-R	CGCTCGAGGGTTGGACTTGACA			
$espB_{O157}$	espB/O157-F	GATAATACTCAAGTAACGATGGTT	30 cycles of 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C	920	16
	espB/O157-R	CCCAGCTAAGCGACCCGATT			
rfb_{O157}	PF8	CGTGATGATGTTGAGTTG	30 cycles of 1 min at 94°C, 1 min 53°C, and 1 min 72°C	420	17
	PR8	AGATTGGTTGGCATTACTG			
fliC H7	1806	GCTGCAACGGTAAGTGAT	30 cycles of 1 min at 94°C, 1 min 53°C, and 1 min 72°	948	17
	1809	GGCAGCAAGCGGGTTGGT			

[a] Cycling conditions and references apply to both primers in each primer pair.

animals (farm K; 3.4%) to 9 of 49 sampled animals (farm F; 18.4%). Serotypes O157, O103, and O26 were detected on 4 of 9 (44.4%), 3 of 9 (33.3%), and 4 of 9 (44.4%) EHEC-positive farms, respectively. Mixed EHEC infections were found on 2 of 12 (16.6%) farms (farms F and H). Serotypes O111 and O145 were not detected. Three of twelve (25%) herds (farms C, E, and G) were EHEC negative based on culture results in all sampled animals.

TABLE 2 Culture results for the cross-sectional study in 12 cattle herds

Herd	Herd size (no. of animals)	No. of animals sampled	No. of EHEC positives (%)	Serogroup(s)
A	118	53	4 (7.5)	O157
B	41	25	2 (8.0)	O157
C	51	31	0 (0.0)	
D	270	70	7 (10.0)	O157
E	15	12	0 (0.0)	
F	101	49	9 (18.4)	O103/O157
G	18	15	0 (0.0)	
H	47	31	3 (9.7)	O26/O103
I	47	31	3 (10.0)	O103
J	152	59	2 (3.4)	O26
K	74	52	1 (1.9)	O26
L	58	36	4 (11.1)	O26

When regarding unmixed EHEC isolates ($n = 23$), 11 of 13 (84.6%) EHEC O157 isolates contained the stx_1 and stx_2 genes, while all non-O157 isolates had stx_1 but lacked stx_2. All O157 serotypes, as well as all non-O157 serotypes, harbored the *eaeA*, *hlyA*, and *espB* genes.

(ii) Serum antibodies against LEE-encoded antigens. Serum samples were tested for the presence of antibodies against intimin, Tir, EspA, and EspB (Fig. 1). For intimin and Tir, no significant differences could be observed between mean OD values for EHEC-positive and EHEC-negative cattle farms (C, E, and G). The mean OD values for serum antibodies against EspB, and especially against EspA, differed considerably (albeit not significantly) between EHEC-positive and EHEC-negative farms. EspA and EspB ELISAs for all animals of negative farms revealed OD values below the cutoff value, whereas 31.6 and 49.9% of the examined animals of all EHEC-positive farms revealed OD values above the cutoff value for the EspA and EspB ELISAs, respectively. Total of 13.5 and 11.5% of the animals on the farm with the lowest percentages of EHEC-excreting animals had serum antibodies against EspA and EspB, respectively. Totals of 18.4 and 34.7% of the animals on the farm with the highest percentage of EHEC

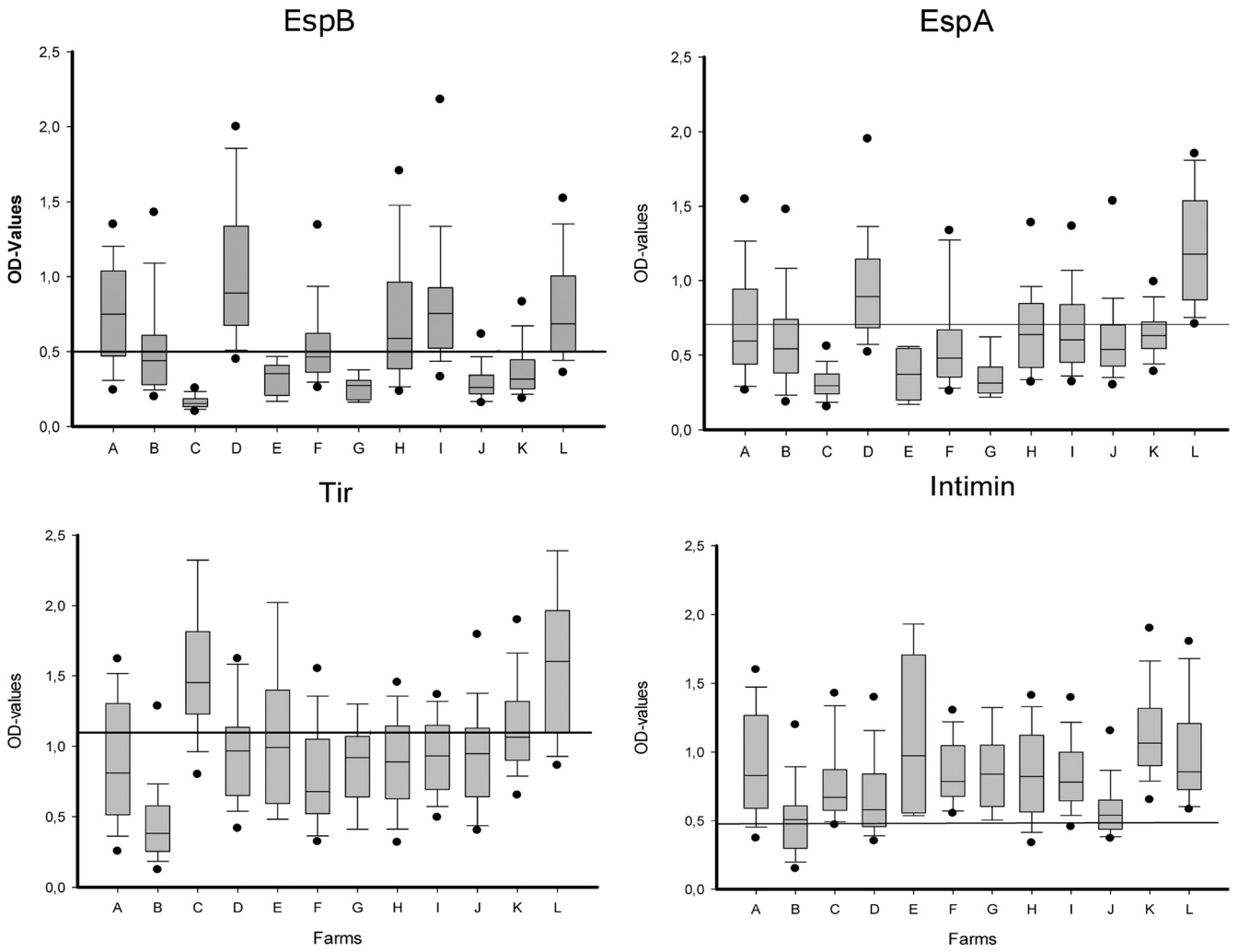

FIG 1 Boxplots comparing serum antibody responses to intimin, Tir, EspA, and EspB on 12 cattle farms. Horizontal lines within the boxes mark the median. Upper and lower horizontal box lines represent the 25th and 75th percentiles. Whiskers above and below the box indicate the 95th and 5th percentiles. Outliers are marked as dots. The horizontal line within the figure represents the cutoff value.

excreting animals had serum antibodies against EspA and EspB, respectively.

Longitudinal study. (i) Isolation and identification of EHEC. The longitudinal study was performed on herds D and H (Table 3). For farm D, EHEC O157 and O26 were isolated 18 times and once, respectively. For farm H, EHEC O27 and O111 were isolated 10 times and twice, respectively. EHEC O157 isolates contained the stx_1 and stx_2 genes, whereas all non-O157 isolates had stx_1 but lacked stx_2. All O157 serotypes, as well as all non-O157 serotypes, harbored the *eaeA*, *hlyA*, and *espB* genes.

At the beginning, 4 of 10 (40%) animals of farm D were EHEC O157 culture positive. Subsequently, 8 of 10 (80%) animals excreted EHEC O157 on at least one sampling time point. Thus, EHEC was shed intermittently. Moreover, one of the EHEC O157 animals also became culture positive for EHEC O26, albeit only at one sampling time point. Thus, simultaneous infections with different serotypes were rare. Animals were apparently still excreting EHEC O157 while being slaughtered, since 3 of 10 (30%) animals were culture positive at the final sampling time point.

All examined animals of farm H were culture negative at the first sampling time point (Table 3). Twelve weeks later, 3 of 10 (30%) animals were excreting EHEC O26. Thereafter, 7 of 10 (70%) animals excreted EHEC O26 on at least one sampling time point, and 1 of them (animal 2) excreted EHEC O111 at the end of the study. A second animal (animal 1) excreted only EHEC O111 at the final sampling time point. The remaining two animals remained negative throughout the study. Animals of farm H could not be tested just before slaughter (sampling time point 8) since the farmer forgot to inform us regarding the slaughter data. Thus, as for farm D, EHEC was shed intermittently, and simultaneous infections with different serotypes were rare.

(ii) Antibody responses against LEE-encoded antigens. Regarding the whole sampling period, all animals (100%) tested at least once positive for Tir antibodies, whereas 16 of 20 (80%), 15 of 20 (75%), and 11 of 20 (55%) tested positive at least once for intimin, EspB, or EspA antibodies, respectively. However, fecal excretion was not always correlated with serum antibody responses to EspA, intimin, and Tir, but when these responses occurred, their time course was similar, having a maximum duration of 2 months.

TABLE 3 Longitudinal survey of EHEC shedding and EspB serology in farms D and H

Herd and animal[a]	EspB-seropositive and culture-positive cattle at sample point[b]: 1	2	3	4	5	6	7	8
Herd D								
1			O157		O157	O26		O157
2								
3				O157				
4								
5				O157	O157	O157		
6	O157			O157	O157			O157
7			O157					O157
8	O157		O157					
9	O157		O157					
10	O157							
Herd H								
1							O111	NS
2					O26		O111	NS
3								NS
4			O26			O26		NS
5					O26			NS
6			O26		O26			NS
7					O26			NS
8								NS
9			O26			O26		NS
10					O26			NS

[a] For herd D, animals were sampled at 8 (6-weekly) sampling time points; for herd H, animals were sampled at 7 (6-weekly) sampling time points.
[b] NS, not sampled. Shading indicates that an animal is seropositive for EspB.

In contrast, 14 of 16 (87.5%) EHEC-excreting animals showed serum antibodies against EspB at the same time as the feces was positive or at the next sampling time point (Table 3). Once an animal became seropositive to EspB, it remained seropositive for the entire study period. Two of sixteen (13.3%) cattle were shedding EHEC at the beginning of the study (animals 9 and 10, herd D). However, they were seronegative for EspB, as well as for all other EHEC antigens tested and they remained seronegative during the whole sampling period. Three of 20 (15%) animals (animals 2 and 4, herd D; animal 8, herd H) shed no EHEC during our study and remained seronegative for all antigens tested, including EspB. The feces of one animal (animal 3, herd H) tested negative throughout the study, but nevertheless, antibodies against EspB were discovered from the forth-sampling point onwards. One animal (animal 1, herd H) became seropositive for EspB before excretion (O111) could be demonstrated.

DISCUSSION

Diarrheagenic *E. coli* strains are broadly categorized into 6 classes based on virulence mechanisms. One of these classes, enterotoxigenic *E. coli*, is the most common cause of diarrhea in beef and dairy calves in the first days of life. Two other classes, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) (reviewed in reference 3), are less well substantiated causes of diarrhea in calves. However, they are both important zoonotic pathogens and cattle are considered to be the main reservoir. Transmission occurs by fecally contaminated foodstuffs and contact with infected animals or a contaminated environment. EPEC infections are the leading cause of diarrhea in infants <2 years of age, while EHEC is responsible for a variety of clinical outcomes of disease in children and the elderly, ranging from diarrhea to hemorrhagic colitis and the life-threatening complications hemolytic-uremic syndrome (HUS) and trombotic thrombocytopenic purpura. EHEC is distinguished from EPEC by the production of Shiga toxins that can cause severe kidney damage, leading to HUS.

The morbidity and mortality associated with recent large EHEC outbreaks have highlighted the threat EHEC poses to public health. EHEC vaccines for ruminants are only partially effective. It is important to understand EHEC acquisition, how EHEC circulates in the herd, and how beef and milk may substantially become contaminated. This is especially true because of the phenomenon of a small proportion of individuals generating large numbers of infections due to supershedding of EHEC. The infectiousness of supershedders is substantially greater due to periods of high-density ($\geq 10^3$ CFU/g of feces) shedding (21). Consequently, there is an increasing demand for improved EHEC diagnostics in cattle and for measures for preventing EHEC contamination of beef and milk (22).

EHEC O157:H7 are comparatively easy to isolate because of unique biochemical properties. However, the other serotypes cannot easily be differentiated from nonpathogenic *E. coli* strains. Different PCRs and a DNA microarray for typing genetic variants of the LEE of EHEC have been designed for examining food, environmental samples, human feces, or cattle feces (14–17, 23). Verstraete et al. (24) recently demonstrated the benefit (higher sensitivity) of combining isolation and PCR for EHEC detection in food. We therefore used the same strategy for examining cattle feces. We used selective agars for Shiga toxin-producing EHEC serotypes (12) in combination with various multiplex PCRs for molecular serotyping and for the detection of multiple virulence genes. We also performed serology, focusing on antibodies against the LEE-encoded antigens intimin, Tir, EspA, and EspB in order to find a possible correlation between serum antibody responses and EHEC shedding.

The cross-sectional study revealed no correlation between antibodies against intimin or Tir and EHEC shedding, since culture negative herds had antibodies against intimin and/or Tir. However, the mean OD values for serum antibodies against EspB, and especially against EspA, differed considerably (albeit not significantly) between EHEC O157, O103, or O26 culture-positive and EHEC culture-negative farms. This could indicate that antibodies to EspA and EspB might be used as indicators for EHEC infection and that antibodies to intimin and Tir are the result of cross-reaction and/or of long-lasting responses against both antigens.

We examined this further during a longitudinal study on two farms. The results showed that antibodies to intimin, Tir, and EspA were short-lived, suggesting that antibodies to intimin and Tir in the culture-negative herds of the cross-sectional survey were indeed due to cross-reaction with other LEE-containing *E. coli*, such as EPEC. Antibodies to EspB, and to a lesser extent to EspA (data not shown), appeared in most animals following EHEC O157 and O26 infection or concomitant with an EHEC O157 and O26 infection and then remained present till the end of the experiment, even when shedding had ceased. Brettschneider et al. (7) also noticed a long persistence of EspB antibodies in experimentally infected cattle. The serum antibody response against EspA was short-lived, whereas the one against EspB was sustained for months. Thus, a combined EspA and EspB antibody detection method could be used to distinguish recent from former infections with EHEC O157 and O26. A correlate between EspB and EspA antibody responses and shedding of EHEC O111, or EHEC O145 could not be evaluated since EHEC O111 shedding was only

detected once in two animals on farm H at the very end of the sampling period and EHEC O145 shedders were absent.

Two of ten sampled animals of herd D remained bacteriologically and serologically negative, although housed in the same pen as eight shedders. This, according to Cobbold et al. (25), is not unusual, at least when supershedders are absent in the pen. However, one of ten sampled animals of herd D was an O157 supershedder. Thus, we cannot explain this finding. Probably, as stated by Vallance et al. (26), host immunogenetics influences the susceptibility to infection by A/E pathogens such as EHEC.

The most important conclusion is that serum antibody responses to EspB might be useful for a sensitive and cost-effective screening at herd level for EHEC O157, O26, and based on the cross-sectional survey, probably also for EHEC O103 infections. However, antibody responses to EspB are of less use for monitoring individual animals, since some EHEC-shedding animals did not show antibody responses and since serum antibody responses to EspB could persist for several months even when shedding had ceased.

Nevertheless, as stated before, we must be aware of possible serological cross-reactions with other LEE-containing *E. coli* present in cattle, such as EPEC. Although the pathogenesis of EPEC and EHEC is distinct (for reviews, see references 3 and 27), both carry the LEE, which is organized into five major operons encoding, among other proteins, intimin, Tir, EspA, and EspB. LEE5 contains the *tir-cesT-eae* operon, which encodes Tir, the Tir chaperon, and intimin, while LEE4 encodes the translocators EspA, EspB, and EspD, a chaperone for EspD (CesD2), the effector protein EspF, a chaperone for EspA (L0017 or Orf29), and a component of the T3SS (EscF) (reviewed in reference 2). The genetic variability of the LEE4 and LEE5 regions of EHEC and EPEC has been described (28). Major variants of Tir and intimin are related, to some extent, to the serogroups of the EHEC and EPEC strains, whereas minor variants can exist within a serogroup for the same major variant (29, 30). However, *eae* and *tir* genes of EHEC and EPEC strains are in general well conserved (31), and therefore our ELISA based on recombinant EHEC intimin or Tir certainly does not specifically detect antibodies to EHEC. The same is true for the EspA ELISA, since EspA presents a high level of amino acid sequence identify between different EHEC and EPEC serotypes (16). EspB, on the other hand, is more diverse (16), but serological cross-reaction with EPEC cannot be ruled out.

This is to our knowledge the first report on a longitudinal time course survey of serological responses against intimin, EspA, EspB, and Tir in naturally infected cattle. The observed immune responses were not protective as intermittent shedding occurred in seropositive animals. To our surprise, the antibody responses in cattle, which become mildly infected with EHEC, were in accordance with the ones observed in humans, where the infection causes severe intestinal lesions and disease, resulting in intense contacts with the immune system (32). Karpman et al. (32) also suggested that measurement of the antibody response to EspB could be used for the diagnosis of EHEC infections in humans. Interestingly, these researchers found the specificity and sensitivity of immunoblotting to be higher when recombinant EHEC EspB was used instead of recombinant EPEC EspB.

In conclusion, larger-scale studies on cattle farms, where EHEC circulate are surely needed to confirm our findings for EHEC O26, O103, and O157 and to extrapolate our results to additional EHEC serotypes, which were not present on the currently examined farms. In the future, the EspB ELISA might be used as a sensitive, cost-effective first-line EHEC screening test for cattle on a farm level allowing to differentiate highly infected farms from low-infected or EHEC-negative farms, followed by culture, preferably in combination with PCR for differentiation of EHEC or EPEC infections, if needed. However, nowadays, EPEC is considered of limited importance in developed countries (27).

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