

Characterization of virulence factors in *Escherichia coli* isolated from diarrheic and healthy calves in Austria shedding various enteropathogenic agents

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ABSTRACT: Faecal samples from 230 diarrhoeic and healthy calves aged 0–6 weeks, from 100 farms in Austria, were examined between October 2004 and February 2005 for the presence of bacteria, especially Shiga toxin-producing *Escherichia coli* (STEC), viruses and parasites. *Escherichia coli* was detected in 17% of all the faecal samples and was more prevalent in healthy calves. However, *E. coli* F5 was identified only in one calf without diarrhoea. Overall, 35 out of the 230 (15.2%) samples analyzed carried the Shiga toxin gene: *stx1*, *stx2* or both *stx1* and *stx2* in their faeces, STEC. Nevertheless, out of 39 pathogenic *E. coli* positive samples observed, only two carried the Shiga toxin genes: *stx1*, in a diarrhoeic calf and both *stx1* and *stx2* in a healthy calf. *eaeA* and *Ehly* genes were detected more frequently in the strains from diarrhoeic calves 57.1% and 50.0%, respectively. *Clostridium perfringens* was detected in twenty-one samples, the most prevalent toxin type of *Clostridium perfringens* was found to be type A (76.2%). Other bacteria such as *Klebsiella* spp. and *Proteus* spp. were present in 1.3% and 0.4% of all samples. *Salmonella* spp. was not detected. The detection rates of other enteropathogens were 25.7% bovine coronavirus, 11.7% *Cryptosporidium* spp., 10.4% *Eimeria* spp., 9.1% group A rotavirus and *Giardia* spp. 6.1%. We demonstrated the presence of the STEC virulence genes in healthy and diarrhoeic Austrian calves but the importance of the virulence factors of STEC (*stx1*, *stx2*, *eae* and *Ehly*) in calf diarrhoea and systemic disease is not well defined. Therefore, further studies are necessary to identify reservoirs or potential sources of virulent STEC strains in order to establish control and prevention strategies for STEC associated diseases in animals and humans.

Keywords: diarrhoea; cattle; calves; enteropathogens; *Escherichia coli*; virulence genes; Shiga toxin

Infectious diseases, especially diarrhoea, are among the most important disorders in calves (Sivula et al., 1996; Bendali et al., 1999a; Svensson et al., 2003). Diarrhoea in neonatal calves is a syndrome of great aetiological complexity that causes economic losses directly through mortal-

ity and need for treatment, and indirectly from poor growth. In addition to the influence of various environmental, management, nutritional and physiological factors, the infectious agents capable of causing diarrhoea in the neonatal calf are numerous (Schumann et al., 1990; Bendali et al.,

1999b). Previous studies show that the most important infectious agents are group A rotavirus, bovine coronavirus, enterotoxigenic *Escherichia coli* (*E. coli*), *Salmonella* species and *Cryptosporidia* either singly or in combination (Moestl et al., 1984; Waltner-Toews et al., 1986; Steiner et al., 1997). However, additional agents can play a role in enteric diseases, such as bovine torovirus, parvovirus, pestivirus, calicivirus, astrovirus, adenovirus (Durham et al., 1989; Brown et al., 1990; Elschner, 1995; Deng et al., 2003), *Eimeria* spp., *Giardia*, *Clostridium perfringens*, *Campylobacter*, *Proteus* and *Klebsiella* (Snodgrass et al., 1986; Schulze, 1992). Bacterial infections are an important cause of morbidity and mortality in large animal neonates (Fecteau et al., 1997). Bacteraemia can rapidly lead to death or to infection in different organs such as in the meninges, joints or eyes. In addition to economic losses, diarrhoea in livestock is important because of the public health implications. Numerous infectious agents causing diarrhoea in animals are zoonotic and have been associated with food-borne diseases (Trevejo et al., 2005).

Escherichia coli and their subtypes (O26, O111, O118 and O157) are firmly associated with emergent food-borne diseases, especially Shiga toxin-producing *Escherichia coli* (STEC). The pathogenicity of STEC O157:H7 is associated with a number of virulence factors, including Shiga toxin 1 (encoded by the *stx1* gene), Shiga toxin 2 (encoded by the *stx2* gene), intimin (encoded by the *eaeA* gene) and enterohaemolysin (encoded by the *Ehly* gene) (Dean-Nystrom et al., 1997; Kang et al., 2004). In humans, enterohaemorrhagic *E. coli* (EHEC), a subset of STEC, is associated with severe systemic disease as haemorrhagic colitis (HC), haemolytic-uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP), especially in infants, young children and in the elderly (Nataro and Kaper, 1998; Paton and Paton, 1998a). The production of Shiga toxin appears to be essential but not solely responsible for the pathogenic effect. However, Shiga toxins seem to play a major role in the pathogenesis of haemorrhagic colitis and haemolytic-uremic syndrome by inducing microvascular changes *in vivo* and are cytotoxic to selected cell lines *in vitro*. Intimin facilitates the adherence to intestinal villi producing attaching and effacing lesions, and although the role of enterohaemolysin in an intestinal disease is unclear, it has been suggested that enterohaemolysins may enhance the effects of Shiga toxins (Holland et al.,

1999; Caprioli et al., 2005). Sporadic cases or large STEC outbreaks in humans are associated with the consumption of raw or undercooked meat of food animals and other foods contaminated by animal faeces, and also by contact with STEC-positive animals or with their environment (Paton and Paton, 1998b). Numerous studies in several countries have shown that cattle are implicated as the principal reservoir of STEC in their gastrointestinal tract, the organism has also been reported in sheep, goats, water buffalos, and deer (Wieler et al., 1998; Osek et al., 2000). However, the organism did not appear to be pathogenic in older calves and adults (Kang et al., 2004; Irino et al., 2005).

This paper describes the variety of bacteria found in neonatal diarrhoeic calves, and determines the presence of most of the known virulence factors of Shiga toxin-producing *Escherichia coli* (STEC) from healthy and diarrhoeic calves that were examined in the course of a survey carried out in Lower Austria and Styria (Austria) using diagnostic techniques designed primarily to detect the principal enteropathogens (Haschek et al., 2006).

MATERIAL AND METHODS

Study design

The study was conducted between October 2004 and February 2005. A total of 230 diarrhoeic and healthy calves from 100 farms in Lower Austria and Styria were examined. Calves between 0 and 6 weeks of age were evaluated. On 50 of these farms (“diseased” farms), one or more calves were suffering from acute diarrhoea at the time of sampling. The other 50 farms (“healthy” farms) with a similar structure and geographical distribution had been free from calf diarrhoea for at least three weeks prior to the sampling procedure. On the “diseased” farms, sick calves and, if available, the same number of healthy neighbour calves were selected randomly. On the corresponding “healthy” farm, (if available) an equivalent number of calves were included in the study.

The health status of each calf was evaluated by clinical examination. Healthy calves had to be free from diarrhoea, whereas sick calves showed abnormal faecal consistency and/or signs of dehydration and weakness. Farm management characteristics were evaluated, including parameters like farm size, animal housing, cleaning and disinfection, feeding of the calves and vaccination of the cows.

Faecal samples

The faecal samples were obtained after digital stimulation of the rectal mucosa, then they were collected into sterile plastic tubes and submitted to the laboratory on ice packs in chests. Samples were processed within 24–48 h after reception.

Faeces examination

Bacteriological examination

Bacteriological analyses were carried out at the Austrian Agency for Health and Food Safety (AGES) in the Institute for Veterinary Disease Control, Moedling. Faecal samples were tested for the presence of *Clostridium perfringens* and Enterobacteriaceae like *E. coli*, *Salmonella*, *Proteus* and *Klebsiella*.

***E. coli*.** The faecal samples were cultivated on MacConkey agar and blood agar plates to assess the enterohaemolysin activity. After overnight incubation at 37°C, lactose fermenting colonies (LFC) with the typical appearance of *E. coli* and colonies surrounded by clear zones of haemolysis were chosen from each sample. *E. coli* strains were identified by biochemical assays, including oxidase and indole, in some suspicious colonies the confirmation was realized with hydrogen sulphide, citrate and urease. The presence of haemolytic or mucoid colonies was taken as evidence of *E. coli* pathogenicity. Four coliform colonies were subcultured for 24 h at 37°C on Minca-Isovitalex solid media before testing for F5 by the slide-agglutination method with specific antiserum (Vaccines Manufacturers Dessau-Tornau GmbH®) (Gershwin, 1990). The remaining bacteria obtained from the MacConkey agar plate were

suspended in PBS (phosphate buffered saline) in the presence of 10% glycerol and stored at –20°C until analyses.

***Salmonella*.** *Salmonella* species were isolated by enrichment of faeces in selective broths. The faecal swab was added to 10 ml of the peptone saline solution. After 24 h of incubation at 37°C, samples were plated on MSRV (modified semi-solid Rappaport-Vassiliadis) selective supplement and incubated for 48 h at 42°C. Suspect colonies with typical characteristics of *Salmonella* were subcultured on XLD (xylose lysine deoxycholate) agar and SMII (Salmonella Medium II) agar for 24 h at 37°C. Those having *Salmonella*-type reactions were tested for agglutination (Waltner-Toews et al., 1986).

***Clostridium perfringens*.** Samples were cultivated initially in blood agar plates (sheep-blood agar) at 37°C in anaerobic jars for 24–48 h. Colonies producing haemolytic reactions such as double zone of haemolysis were subcultured in a selective agar medium (Schaedler, Gentamicin and Neomycin agar) anaerobically at 37°C for 24 h. In the colonies with the evident double zone of haemolysis characteristic of *Clostridium perfringens* the CAMP test was performed for confirmation (Hansen and Elliott, 1980). Finally multiplex-polymerase chain reaction (multiplex-PCR) was carried out for the detection of the major *Clostridium perfringens* toxins as described by Yoo et al. (1997).

***Proteus* and *Klebsiella*.** The presences of other Enterobacteriaceae were tested by Enterotube test, according to the instructions of the manufacturers.

PCR amplification

Escherichia coli isolates were examined by means of a duplex-PCR using specific primers to deter-

Table 1. Primer sequences for virulence gene amplification

Virulence factor	Reference	Primer code	Primer sequence 5'–3'	Product size (bp)
<i>stx1</i>	Brian et al., 1992	Stx1-f	AAA TCG CCA TTC GTT GAC TAC TTC T	366
		Stx1-r	TGC CAT TCT GGC AAC TCG CGA TGC A	
<i>stx2</i>	Brian et al., 1992	Stx2-f	CAG TCG TCA CTC ACT GGT TTC ATC A	282
		Stx2-r	GGA TAT TCT CCC CAC TCT GAC ACC	
<i>eaeA</i>	Gallien, 2003	SK-1	CCC GAA TTC GGC ACA AGC ATA AGC	629
		SK-2	CCC GGA TCC GTC TCG CCA GTA TTC G	
<i>Ehly</i>	Gallien, 2003	hlyA-1	GGT GCA GCA GAA AAA GTT GTA G	1 551
		hlyA-2	TCT CGC CTG ATA GTG TTT GGT A	

mine the presence of *stx1* and *stx2* genes (Brian et al., 1992). Samples showing a positive PCR result for one or both *stx1* or *stx2* were tested for the presence of *eaeA* and *Ehly* genes (Gallien, 2003). Primer sequences for *stx1*, *stx2*, *eaeA* and *Ehly* are indicated in Table 1. Template DNA was prepared from a pure culture of isolates, cultivated in MacConkey broth for 18 h at 37°C. Briefly: 100 µl from this culture were centrifuged at 5 000 × g for 5 min and washed with PBS. The pellet was resuspended in 25 µl PBS, heated at 95°C for 15 min and this was the final sample for the PCR template. Duplex-PCR was performed in a 25 µl final reaction volume containing 0.2 µM primers (Invitrogen), 200 µM (2.5 mM) dNTPs (Invitrogen), 2.5 µl PCR buffer containing 1.5 mM MgCl₂ (Applied Biosystems Austria), 0.625 Units AmpliTaq[®] DNA Polymerase (Applied Biosystems Austria), 15.875 µl PCR-grade water and 2.5 µl of template DNA. The cycling condition in a Gene_Amp[®] 9700 thermocycler (Applied Biosystems Austria) had different characteristics for each virulence factor and was as follows: initial denaturation at 94°C for 5 min, denaturation at 95°C for 40 s (during 35 cycles), annealing 65°C for 20 s and extension 72°C for 45 s during 35 cycles and final elongation at 72°C for 5 min (Table 2). PCR amplicons were analysed by gel electrophoresis in a 1.5% agarose gel (Sigma), stained with ethidium bromide and visualised under UV illumination. A 100 bp DNA ladder (Sigma) was used as the molecular weight marker. For each PCR run an *E. coli*-positive and DNA-negative (distilled water only) control was used.

Clostridium perfringens isolates were examined by multiplex-PCR; the confirmed colonies in bacteriological assays were harvested and transferred into 200 µl 1 × PBS. After boiling for 15 min, DNA extraction was realized with a commercial high pure PCR template preparation kit (Roche),

according to the manufacturer's instructions. For the detection of the major toxin the protocol of multiplex-PCR diagnostics was carried out (Meer and Songer, 1997; Baums et al., 2004) using primer sets designed to identify the presence of genes encoding *Clostridium perfringens* α toxin, β toxin, ε toxin or ι toxin.

Antimicrobial susceptibility testing

Susceptibility to different classes of antimicrobial agents was evaluated for *E. coli* and *Cl. perfringens* isolates, using standard disk-diffusion methods in Mueller Hinton agar and blood agar, respectively, and using the inhibition-zone patterns. The zone diameter interpretative criteria used to classify an isolate as susceptible, intermediate or resistant were interpreted according to the international standard guidelines AVID (Arbeitskreis fuer veterinärmedizinische Infektionsdiagnostik). The disks containing the following amount of antibiotics were used: ampicillin 10 µg, enrofloxacin 5 µg, gentamicin 10 µg, neomycin 30 µg, trimethoprim/sulphamethoxazole 1.25/23.75 µg, tetracycline 30 µg, kanamycin 30 µg, penicillin 10 µg and erythromycin 15 µg.

Virological examination. Faecal samples were tested for the presence of group A rotavirus, bovine coronavirus and bovine torovirus-specific nucleic acids by RT-PCR (Reverse Transcription-Polymerase Chain Reaction). Methods and results were described by Haschek et al. (2006).

Parasitological examination. Laboratory analysis of the faecal samples was conducted using a quantitative centrifugation concentration flotation technique. For each sample, 10 g of faeces were processed using a sugar solution as the flotation medium to recover *Cryptosporidium* spp., *Eimeria*

Table 2. PCR conditions for the detection of virulence genes in *E. coli* strains

Action	<i>stx1</i> and <i>stx2</i>			Intimin (<i>eaeA</i>)			Enterohemolysin <i>Ehly</i>		
	temperature (°C)	duration	cycles	temperature (°C)	duration	cycles	temperature (°C)	duration	cycles
Initial denaturation	94	5 min	1×	94	5 min	1×	94	5 min	1×
Denaturation	95	40 s	35×	94	30 s		94	30 s	
Annealing	65	20 s	35×	52	60 s	30×	57	60 s	30×
Extension	72	45 s	35×	72	60 s		72	90 s	
Elongation	72	5 min	1×	72	7 min	1×	72	7 min	1×
Cooling	4	infinite		4	infinite		4	infinite	

spp. oocysts and *Giardia* spp. cysts. Microscopic examination was realized using bright field and phase contrast microscopy. The sample was considered positive for the respective parasite when *Giardia* spp. cysts, *Cryptosporidium* spp. oocysts and *Eimeria* spp. oocysts were detected in the specimen with the correct morphology, optical properties, internal structure, size and shape. The intensity of the infection was estimated semi-quantitatively according to the average number of oocysts in different randomly selected fields (Uga et al., 2000).

Statistical analysis

The statistical analysis was used to verify the data and generate the descriptive statistics. The statistical evaluation was done using the SPSS for Windows package version 11.5.1. (SPSS Inc., Chicago, USA). First of all possible associations between the health status of the single animals and/or the health status of the farms and the data collected referring to management characteristics of the farms were investigated by χ^2 -test. If an association could be proved, the respective variable was included in the statistical model evaluating the difference in the distribution of enteropathogen positive/negative animals referring to the health status of the animal.

RESULTS

Faecal samples were obtained from 230 diarrhoeic and healthy calves on 100 farms. 140 of the evaluated calves were clinically healthy at the time of sampling. 35 of these (140) animals were healthy neighbour calves from diarrhoeic farms and 105 from healthy farms. 90 calves showed signs of diarrhoea at the time of sampling. 67.4% of calves included in the study were of dairy breeds, 11.7% beef breeds and 20.9% cross breeds. The animal population comprised 44.3% males and 55.7% females.

Signs of diarrhoea in calves housed in large-group pens were significantly ($P = 0.043$) more severe than those in individually housed calves. The incidence of the different examined enteropathogens and their distribution among clinically healthy and diarrhoeic calves are shown in Table 3. (The final sum of percent in calves with diarrhoea and healthy calves is above 100% due to concurrent infections with different microorganisms at the same time in some samples).

As described by Haschek et al. (2006), the most frequently detected enteropathogen was bovine coronavirus (25.7% positive). With the second highest prevalence pathogenic *E. coli* could be found in 39 out of the 230 (17%) of all examined samples. However, *E. coli* with the F5 antigen was identified

Table 3. Number and percentage of samples tested positive for various enteropathogens in diarrhoeic and healthy calves

Enteropathogen	Calves with diarrhoea ($n = 90$)		Healthy calves ($n = 140$)		All samples ($n = 230$)	
	n	%	n	%	n	%
Virus						
Coronavirus	36	40.0	23	16.4	59	25.7
Rotavirus	19	21.1	2	1.4	21	9.1
Parasites						
<i>Cryptosporidia</i>	23	25.6	4	2.9	27	11.7
<i>Giardia</i> spp.	4	4.4	10	7.1	14	6.1
<i>Eimeria bovis</i>	7	7.8	2	1.4	9	3.9
<i>Eimeria</i> spp.	6	6.7	9	6.4	15	6.5
Bacteria						
<i>E. coli</i>	17	18.9	22	15.7	39	17.0
<i>E. coli</i> F5	0	0.0	1	0.7	1	0.4
<i>Salmonella</i> spp.	0	0.0	0	0.0	0	0.0
<i>Cl. perfringens</i>	9	10.0	12	8.6	21	9.1
<i>Proteus</i> spp.	1	1.1	0	0.0	1	0.4
<i>Klebsiella</i> spp.	3	3.3	0	0.0	3	1.3

only in one calf without diarrhoea. *E. coli* was detected as single enteropathogen in 15 calves (6.5%). Three of these animals showed signs of diarrhoea and 12 were healthy animals. The PCR assay correctly determined the presence or absence of *E. coli* virulence genes such as *stx1*, *stx2*, *eaeA* and *Ehly* as it is illustrated in Figure 1.

Overall, 39 out of the 230 (17%) calves studied were positive for pathogenic *E. coli*, out of these, only two (5.1%) calves carried the Shiga toxin genes STEC: *stx1* in a diarrhoeic calf and both *stx1* and *stx2* in a healthy calf. However, we have analysed all the samples including non-pathogenic *E. coli* for the presence of Shiga toxins and virulence genes. The distribution of *stx1*, *stx2*, *eaeA* and *Ehly* Shiga toxin genes among the healthy and diarrhoeic calves is shown in Table 4. Thirty-five out of the 230 (15.2%) samples observed carried the Shiga toxin gene: *stx1*, *stx2* or both in their faeces. The *stx1* gene was identified more frequently than the *stx2* gene, with 12.2% and 7.8%, respectively. The presence of virulence genes *eaeA* and *Ehly* was established only in samples

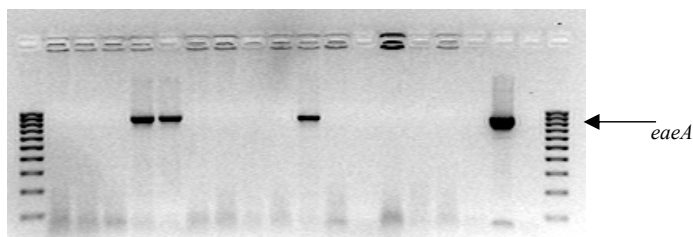
positive for Shiga toxin. 12 out of the 35 (34.3%) Shiga toxin gene positive samples were positive for the *eaeA* gene, and 16 (45.7%) were positive for the *Ehly* gene. Both virulence genes (*eaeA* and *Ehly*) in the same sample were observed in 25.7% of the Shiga toxin positive gene samples. In addition, *eaeA* and *Ehly* virulence genes were more evident in strains from diarrhoeic calves (57.1% and 50.0%, respectively) than in healthy calves (19.1% and 42.8%, resp.).

Clostridium perfringens was isolated in 10% of the diarrhoeic and 8.6% of the healthy calves. *Cl. perfringens* was the only detectable enteropathogen in six calves (2.6%), two diarrhoeic and four healthy animals. Multiplex-PCR assay was used to type 21 field isolates of *Clostridium perfringens* identified by conventional tests. Out of these 21 samples 16 (76.2%) were type A. Other bacteria such as *Klebsiella* spp. and *Proteus* spp. were present in 1.3% and 0.4% of all samples.

21.1% of diarrhoeic calves and 1.4% of healthy calves were positive for group A rotavirus and 11% of sick calves and 0.7% of healthy calves were positive for bo-

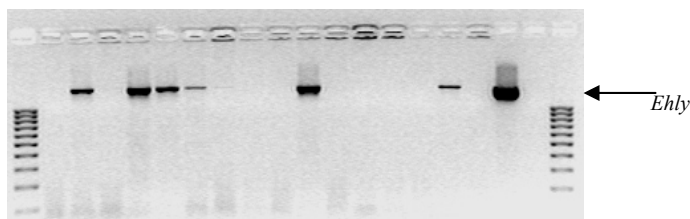
eaeA

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 K+K- M



Ehly

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 K+K- M



stx1, *stx2*

M 1 2 3 4 5 6 7 8 9 10 K+K- M

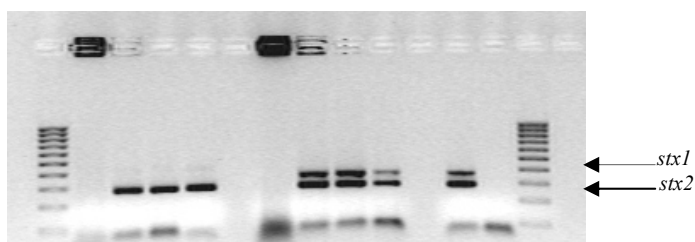


Figure 1. Representative gels for duplex-PCR amplification of DNA extracted from selected *E. coli* isolates from calves showing the presence of diverse virulence genes. *eaeA* (863 bp) and *Ehly* (1551 bp) genes in the top picture, and *stx1* (366 bp) and *stx2* (282 bp) genes in the bottom picture. Lane M (100 bp) = DNA size marker; lane 1–16 = calf origin *E. coli*; lane K+ and K- = positive and negative controls, respectively. The relative positions in the gel of predicted size of PCR products are indicated by arrowheads on the right sides

Table 4. Distribution of virulence factors of *Escherichia coli* strains isolated from healthy and diarrhoeic calves

Virulence factor	Calves with diarrhoea		Healthy calves		All samples	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
<i>stx1</i>	12/90	13.3	16/140	11.4	28/230	12.2
<i>stx2</i>	8/90	8.9	10/140	7.1	18/230	7.8
<i>stx1, stx2</i>	6/90	6.7	5/140	3.6	11/230	4.8
<i>eaeA</i> *	8/14	57.1	4/21	19.0	12/35	34.3
<i>Ehly</i> *	7/14	50.0	9/21	42.9	16/35	45.7
<i>eaeA, Ehly</i>	5/14	35.7	4/21	19.0	9/35	25.7

*The detection of these virulence factors was carried out only in samples positive for *stx1*, *stx2* or both *stx1* and *stx2*

vine torovirus. *Eimeria bovis* and *zurnii* oocysts were detected in both groups of calves with a prevalence of 7.8% in diarrhoeic and 1.4% in healthy calves. A total of 6.1% of the samples were positive for *Giardia* spp. cysts and 11.7% for *Cryptosporidium* oocysts. No *Salmonella* species were detected in any of the analysed samples (Haschek et al., 2006).

Considering the prevalence in diarrhoeic calves in this study bovine coronavirus was the most common pathogen (40%), followed by *Cryptosporidia* (25.6%), group A rotavirus (21.1%), *E. coli* (18.9%) and *Clostridium perfringens* (10%).

Based on the individual calf records, 40 (44.4%) of the diarrhoeic calves received antimicrobial therapy. Gentamicin, sulphonamides, ceftiofur sodium, fluorquinolone, and procaine penicillin were the most frequently used antimicrobials. The percentages of resistance in the 39 *E. coli* positive isolates in ascending order were: gentamicin 0%, enrofloxacin 11 (28.2%), neomycin 13 (33.3%), sulphonamides 17 (43.5%), ampicillin 25 (64.1%) and tetracycline 26 (66.6%). The *E. coli* strains were very susceptible to enrofloxacin 28 (71.8%) and highly susceptible to gentamicin (100%). Thus, most of the strains showed multi-drug resistance: 69.2% of the isolates were resistant to two antibiotics at least, 53.8% to three antibiotics at least and 38.4% were resistant to four or more antibiotics.

DISCUSSION

Diarrhoea due to the enterotoxigenic *Escherichia coli* (ETEC) is one of the most frequent bacterial diseases in neonatal calves and the predominant pathogen cultured from calves with septicaemia (Lofstedt et al., 1999). *E. coli* was the bacterial agent cultured with the highest frequency from diarrhoeic and healthy calves in our study,

a finding that agrees with results of Bendali et al. (1999a) and Garcia et al. (2000). This study is the first to estimate the presence of virulence factors of Shiga toxin-producing *Escherichia coli* (STEC) from healthy and diarrhoeic calves and their relation with other enteropathogens in Austria. 15.2% of the analysed samples in the present study were positive to *stx1*, *stx2* or both. Our results contrast with other studies from Austria of Much (1996) where 18.5% of the calves sampled were positive to *stx* genes and of Awad-Masalmeh (2004) where *stx1* and *stx2* were identified in 10.1% and 17.8%, respectively. The data in this study are consistent with that of others who demonstrated the prevalence of Shiga toxin (virulence factors) producing *E. coli* in the faeces of young calves in Germany (Wieler et al., 1998), USA (Holland et al., 1999) and Brazil (Leomil et al., 2003) and confirmed the importance of calves as a reservoir of STEC, but comparably higher than that of Osek et al. (2000) with 7.6% in Poland.

Stx production is essential but not sufficient for STEC virulence, however, epidemiological studies have revealed that *stx2* is more associated with severe human disease (HC and HUS) than *stx1* (Beutin et al., 2004; Caprioli et al., 2005). The prevalence of *stx2* genes among the isolates was 7.8% indicating a potential source of infection induced by STEC.

In the diarrhoeic calves, eight (57.1%) of the isolated STEC strains were *eaeA*-positive. The distribution of the *eaeA* genes was equal among *stx1* positive and *stx2* positive *E. coli*. It has been reported that in calves the *eaeA* gene and intimin have a defined role in causing the attaching and effacing (AE) lesions (Dean-Nystrom et al., 1998). Moreover, bovine STEC with the *eaeA* genetic virulence markers may also be able to induce AE lesions in humans; therefore, they may be a poten-

tial risk for severe human infections (Boerlin et al., 1999). Although the influence or importance of how enterohaemolysin might contribute to the pathogenesis of STEC infection in calves has not been defined yet, it has been suggested that enterohaemolysins may complement the effects of shiga toxins enhancing their virulence (Nataro and Kaper, 1998). In our study the distribution of *Ehly* genes was 50% in the diarrhoeic STEC positives and 42.9% in the healthy STEC positives, in accordance with the results of Osek et al. (2000). The demonstration of the virulence factors in diarrhoeic and healthy calves confirms the role of cattle as a reservoir and their faeces as a source of contamination for food, environment and humans.

The prevalence of *E. coli* F5 as an enteropathogen producing diarrhoea in neonatal calves has been documented (Waltner-Toews et al., 1986; Mahdi-Saeed et al., 1993). However, Ganaba et al. (1995) reported *E. coli* F5 in 4–12 weeks old clinically healthy calves not finding an association between this enteropathogen and the occurrence of diarrhoea. In the present study *E. coli* F5 was detected only in one sample of a healthy calf suggesting the low relevance of this enteropathogen in this syndrome, in agreement with De Verdier Klingenberg and Svensson (1998).

Clostridium perfringens is considered to be one of the common microorganisms that cause human and animal diseases. In addition, it is a part of their ecological community of the intestinal tract. Some type A strains produce an enterotoxin that causes diarrhoea in humans and most likely also in various domestic animals. *Cl. perfringens* type A strains have been associated with intestinal disorders in horses, piglets, dogs and calves. Thus the detection of *Cl. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *Cl. perfringens* infections and may be helpful in the development of effective preventive measures (Baums et al., 2004). The virulence of *Cl. perfringens* is determined by its prolific toxin-producing ability, including enterotoxins. *Cl. perfringens* strains are divided into five toxin types (A, B, C, D and E) on the basis of the production of four major lethal toxins: α , β , ϵ and ι (Al-Khaldi et al., 2004). The results of the present study indicate that *Cl. perfringens* type A is the serotype most frequently isolated from calves in Austria with a prevalence of 9.1%. However, only in two diarrhoeic calves was *Cl. perfringens* identified as single pathogenic agent. This fact could indicate that this enteropathogen does not have a significant clinical

relevance. The main toxin produced by the virulent strains is α toxin, which interferes with glucose uptake and energy production, furthermore, triggers the arachidonic acid metabolism and activates the secretion of enterocytes. Moreover, the pathogenic effect of *Cl. perfringens* is caused by the overgrowth of toxinogenic strains over normal intestinal flora, most commonly causing diarrhoea in calves (Songer, 1996). Compared with the standard *in vivo* typing technique the PCR method has been shown to be much more rapid, with results obtained within a few hours, and it is much more reliable showing an excellent specificity and sensitivity superior to those of the classical methods (Kadra et al., 1999).

Rotaviruses and bovine coronaviruses are the most commonly identified viral causes of diarrhoea of neonatal food animals (Holland, 1990; Athanassious et al., 1994). In our study the coronavirus was detected in 40% of the diarrhoeic calves, which is a higher percentage in comparison with other studies (Bendali et al., 1999a; Garcia et al., 2000). Group A rotavirus identification in this survey (21.1%) is in agreement with Alfieri et al. (2006), suggesting that the rotavirus is one of the more important causative agents in neonatal calf diarrhoea.

Cryptosporidium spp. oocysts were detected in 25.6% of the calves showing signs of diarrhoea and only in 2.9% of the healthy calves. This result is comparable to the findings reported in previous studies from other countries (Naciri et al., 1999; Kvac et al., 2006). Lower prevalence of *Cryptosporidium* spp. has been reported in western Canada (Gow and Waldner, 2006). The analysis of the relationship between diarrhoea and *Cryptosporidium* spp. infection revealed a *P*-value of < 0.0001 , indicating a strong and highly significant association between the infection with *Cryptosporidium* spp. and the occurrence of diarrhoea in calves. The results from this study suggest that *Cryptosporidium* spp. is one of the major aetiological agents of neonatal diarrhoea in calves, maybe due to several factors like early contamination soon after birth by contact with their dams, contaminated litters, asymptomatic carriers and contaminated environment (Castro-Hermida et al., 2002). However, this is also valid for other microorganisms.

The indiscriminate use of different kinds of antibiotics creates a potential health risk to animals and humans in terms of drug residues and the development of resistant bacterial strains. Recently published studies described an increased incidence of

resistance for different antimicrobials (Orden et al., 2000). The susceptibility pattern of *E. coli* observed in the present study indicates that the resistance patterns were more evident to antibiotics frequently used like ampicillin and tetracycline in accordance with Hariharan et al. (2004). In contrast to studies of Holland et al. (1999), all *E. coli* strains were highly sensitive to gentamicin. These results put the use of antibiotics in cases of diarrhoea into question. Constable (2004) recommended that in calves with diarrhoea without signs of systemic disease (normal appetite for milk, no fever), oral as well as parenteral antimicrobials should not be administered.

In conclusion, we demonstrated the presence of the STEC virulence genes in healthy and diarrhoeic Austrian calves but the importance of the virulence factors of STEC (*stx1*, *stx2*, *eae* and *Ehly*) in calf diarrhoea and systemic disease is not well defined. Therefore, further studies are necessary to identify reservoirs or potential sources of virulent STEC strains in order to establish control and prevention strategies for STEC associated diseases in animals and humans. Early recognition of the problem, as well as appropriate treatment, improvement of hygiene, housing and management may be beneficial in increasing survival rates. Because of the large number of aetiological agents, the prevention of neonatal diarrhoea in calves is difficult but should be focused on management factors.

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