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Dynamics of verotoxin-producing *Escherichia coli* isolated from German beef cattle between birth and slaughter

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Abstract

A total of 85 cattle from three German beef farms were followed between birth and slaughter during a period of 2 years and monthly faecal samples were submitted for bacterial culture. Verotoxin-producing *Escherichia coli* (EC) were detected using a standard diagnostic cascade. Potentially pathogenic VTEC ($_p$VTEC) were defined as: positive for (1) verotoxin 1 (vt1) and eae, (2) positive for verotoxin 2 (vt2) and eae, (3) positive for both verotoxins 1 and 2 and eae, while verotoxinogenic EC ($EC_{vt1,2}$) were defined as: (1) positive for vt1, (2) positive for vt2 or (3) positive for both vt1 and vt2. There were 1587 observations (1462 valid) available for the statistical analysis including 6 (0.4%) samples from 6 (7.1%) different animals positive for VTEC O157, 78 (5.3%) pVTEC isolates and 389 (26.6%) $EC_{vt1,2}$ isolates. The median *day of the study* at first detection was 280 days for $EC_{vt1,2}$ and 315 days for pVTEC. The median *age at first detection* was: 121 days for $EC_{vt1,2}$ and 215 days for pVTEC. Time series analysis, survival analysis, and stochastic *SI* models were used to find differences in the population dynamics of $EC_{vt1,2}$ and pVTEC.

There was a strong farm and age effect for the first detection of $EC_{vt1,2}$ and for pVTEC while the seasonal effect was significant for the first $EC_{vt1,2}$ detections only.

With increasing age at first and all consecutive detections, $EC_{vt1,2}$ and pVTEC were detected less frequently. The serotype O157 was found more frequently together with detection of other serotypes of pVTEC in the same sample. The $EC_{vt1,2}$ were found more often together with pVTEC. The first

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$EC_{vt1,2}$ were on average found before the first pVTEC's and positive cross-correlations existed between $EC_{vt1,2}$ and pVTEC.

The critical duration for the shedding period above which the VTEC could propagate themselves on the farms by f.e. transmission between animals was found to be between 8 and 18 sampling intervals of 28 days (224–504 days) for $EC_{vt1,2}$, and between 5 and 6 sampling periods of 28 days each (140–168 days) for the pVTEC which is smaller than all critical shedding periods for $EC_{vt1,2}$. The reasons for $EC_{vt1,2}$ being isolated from faeces earlier than pVTEC are discussed.

Keywords: Verotoxin; VTEC; Transmission dynamics; Virulence factors; Cattle

1. Introduction

Verotoxinogenic *Escherichia coli* (VTEC) represent a threat to human health by causing outbreaks of (haemorrhagic) diarrhoea and colitis resulting in complications such as haemolytic uraemic syndrome (HUS), thrombocytic thrombocytopenic purpura (TTP) and death in very young children and elderly people (Karmali et al., 1985; Nataro and Kaper, 1998). The main reservoir for VTEC is believed to be the ruminant gastrointestinal tract (GI tract) (Rasmussen et al., 1999, Armstrong et al., 1996). Young heifers and calves until an age of 14 weeks are the age group with highest prevalence of VTEC found in faeces (Midgley et al., 1999). Weaned calves (2–6 months old) that were fed grain or molasses were found to be at higher risk for shedding VTEC O157 (Paiba et al., 2003; Rugbjerg et al., 2003; Synge et al., 2003). The shedding of VTEC declines with the increasing age of calves (Shaw et al., 2004). Contamination of the food chain, especially raw milk, cheese and undercooked meat, surface water, farm visits, high farm density, and person-to-person contact are considered to be the main risk factors for infection in humans (Dev et al., 1991; Griffin and Tauxe, 1991; Keene et al., 1994; Kistemann et al., 2004; Meng and Doyle, 1998; Nielsen et al., 2002; Reinders et al., 2001; Wilson et al., 1993).

The pathogenicity of VTEC is caused by several virulence factors that can be integrated into the bacterial genome (for example: LPS, gene coding for the serotype, intimin or eae), be located on plasmids among which the so-called ''large plasmid'' (haemolysin or Hly, katP, espP, colicin, etc.,) and be transmitted by bacteriophages (verotoxins 1 and 2 including their many subtypes) (Saunders et al., 1999). Verotoxin genes can be transferred between *E. coli* isolates by means of bacteriophages (Schmidt et al., 1999). According to literature, this re-combination takes place in the ruminant GI tract and may be the source of new potentially pathogenic VTEC (Saunders et al., 1999).

The population transmission dynamics of verotoxinogenic *E. coli* are supposedly a mixture of the transmission dynamics of mobile virulence factors such as verotoxin genes within the animals and the transmission dynamics of verotoxinogenic *E. coli* isolates between animals (Saunders et al., 1999). Competition and dominance of certain strains of *E. coli* could be complementary explanations for the shedding of one predominant *E. coli* isolate over time (Midgley et al., 1999). The aim of this study was to compare the dynamics

of $EC_{vt1,2}$ and pVTEC in beef cattle between birth and slaughter pre-supposing transmission of *E. coli* between animals.

2. Materials and methods

2.1. Data set

A total of 85 cattle from three German beef farms (farm 1: 29 out of 100, farm 2: 33 out of 300, farm 3: 23 out of 46 animals) were followed during a period of about 2 years from 1997 to 1999 between birth and slaughter and monthly, individual faecal samples were submitted for bacterial culture (Geue et al., 2002). The farms 1–3 in the current analysis correspond to the cohorts A, B and D1 in Geue et al. (2002). The effective study period for the analysis was: farm 1: 751 days, farm 2: 665 days and farm 3: 661 days long. The sampling interval was 28 days. Verotoxin-producing *Escherichia coli* (EC) were detected using a standard diagnostic cascade. Four seasons were defined, season 1 from March to May, season 2 from June to August, season 3 from September to November and season 4 from December to February.

2.2. Diagnostic cascade

Briefly, after overnight enrichment, the sample was pre-screened for the presence of verotoxin genes using PCR. In case of a positive result, the sample was plated on McConckey agars and filter hybridisation with a 230 bp probe for verotoxin was used to detect verotoxin positive colonies of *Escherichia coli* (EC). Several virulence factors (verotoxin 1: vt1, verotoxin 2: vt2, and intimin: eae) were determined for a maximum of ten colonies of EC per faecal sample using multiple hybridisations. The test results were interpreted as the fraction positive or negative out of 10 colonies tested per sample and date. Potentially pathogenic VTEC ($_{p}$VTEC) were defined as: positive for (1) vt 1 and eae, (2) positive for vt2 and eae, (3) positive for both vt1 and vt2 and eae, while verotoxinogenic EC ($EC_{vt1,2}$) were defined as (1) positive for vt1, (2) positive for vt2 or (3) positive for both vt1 and vt2. The pVTEC were submitted for serotyping. Details of the laboratory procedures are described in Geue et al. (2002).

2.3. Statistical analysis

2.3.1. Descriptive statistics

Median and standard error of the median (S.E.M.) were calculated for day of the study and age at first and all detections of $EC_{vt1,2}$ and pVTEC stratified by farm. Detection of first and all isolates of $EC_{vt1,2}$ and pVTEC were stratified by season. The statistical analysis was done using SPSS 9.0 for Windows (SPSS Inc., 2005).

Time series analysis and ARIMA models were used to find the lags in samplings intervals that correlated with detection of $EC_{vt1,2}$ and pVTEC (Dohoo et al., 2003). Survival analysis including cumulative survival plots for the first and all detections of $EC_{vt1,2}$ and pVTEC were used to illustrate the distribution of isolates over farms and

seasons. The days of the study before the first sample are defined to be left-censored data and days after culling or slaughtering are right-censored data. Cox regression models were used to analyse the effect of farm, age at first and consecutive detections, season, co-isolation of VTEC O157 and three interaction terms (age × season, age × farm, season × farm) on the first or consecutive detections of $EC_{vt1,2}$ and pVTEC, respectively (Dohoo et al., 2003).

2.3.2. Stochastic SI model

A stochastic population dynamic model was assumed including the compartment "*S*" for the animals where $EC_{vt1,2}$ or pVTEC, respectively, were not isolated from faeces and a second compartment "*I*" for the animals where the EC under study were detected.[1] In this way, a so-called *SI* model could be formulated for the transitions of animals from the states *S* into *I* over time. The transition between the state *S* and *I* was postulated to be a rare event, governed by a Poisson process at rate $\beta((SI)/N)$, where *N* stands for the total number of animals in the study cohort and β is the so-called transmission parameter, i.e. a measure for the ease of EC transmission between animals. The logarithm of the mean number of transitions from *S* to *I* is:

$$\log[E(\text{cases})] = \log(\beta) + \log\left[\frac{SI}{N}\right]$$

which is a generalized linear regression model with intercept $\log(\beta)$ and offset variable $\log[(SI)/N]$ (McCullagh and Nelder, 1989).

The critical shedding period for $EC_{vt1,2}$ and pVTEC, respectively, is xcrit = $1/\beta$. The critical shedding period represents the number of sampling intervals of 28 days each for which the reproduction ratio R_0 is greater than 1. This ratio R_0 is defined as the mean number of animals positive for EC resulting from one EC positive animal (Anderson and Britton, 2000). A reproduction ratio R_0 of greater than 1 means that the EC infection is spreading in the population. In the context of this study, a reproduction ratio R_0 of greater than 1 implies that the EC can propagate themselves in the cohorts of beef calves if they are shed during intervals of time that are longer than xcrit. First detections of $EC_{vt1,2}$ and pVTEC were used only. The generalized linear models were fitted using the 6th edition of GenStat (GenStat Committee, 2000).

3. Results

3.1. Descriptive statistics

There were 1587 observations recorded of which 125 observations had missing values. Every animal was sampled between 1 and 27 times (median farms 1 and 2: 24, S.E.M.: 2, farm 3: 21, S.E.M.: 1 samples per animal), including 6 (0.4%) samples from 6 (7.1%) different animals positive for VTEC O157, 78 (5.3%) pVTEC and 389 (26.6%) $EC_{vt1,2}$ isolates. The median *day of the study* at first detection was 280 days (S.E.M.: 9.3) for

[1] "*S*" commonly stands for "susceptible" and "*I*" stands for "infectious".

Table 1
The number of animals, selected characteristics of the animals on the day of first detection of VETEC and the number of samples in the study

	Number of animals	Number of samples
Total observations	85 (100%)	1587 (valid: 1462, 92.1%)
serotype O157	6 (7.1%)	6 (0.4%)
Season 1 (March to May)	84 (98.8%)	438 (27.6%)
Season 2 (June to August)	79 (92.9%)	470 (29.6%)
Season 3 (September to November)	66 (77.7%)	386 (24.3%)
Season 4 (December to February)	66 (77.7%)	293 (18.5%)
First detection of $ECvt_{1,2}$	66 (77.7%)	66 (4.5%)
First detection of pVTEC	43 (50.6%)	43 (2.9%)
All detections of $ECvt_{1,2}$		389 (26.6%)
All detections of pVTEC		78 (5.3%)

	Median (S.E.M.)
First detection of $ECvt_{1,2}$ (days of study)	280 (9.3)
First detection of pVTEC (days of study)	315 (21.1)
Age at first detection of $ECvt_{1,2}$ (days)	121 (14.4)
Age at first detection of pVTEC (days)	215 (44.1)

$EC_{vt1,2}$ and 315 days (S.E.M.: 21.1) for pVTEC. Age ranged between 1 and 738 days. The median *age at first detection* was: 121 days (S.E.M.: 14.4) for $EC_{vt1,2}$ and 215 days (S.E.M.: 44.1) for pVTEC, observations recorded per *season* were: 438 samples from March to May, 470 samples from June to August, 386 samples from September to November, and 293 samples from December to February. Information about the calf cohort and the descriptive results are illustrated in Tables 1 and 2.

Table 2
Descriptive information about the calf cohort including farm number and median age at first and last sampling, date of first or last sampling and median day of the study for the first and last sample, and at culling

	Farm 1			Farm 2				Farm 3		
	Age (days)	Date	Day of study	Age (days)	Date	Day of study		Age (days)	Date	Day of study
First sample										
Median	23	10/02/97	1	15	11/02/97	1		45	12/05/97	1
S.E.M.	7		3	26		28		4		0
Last sample										
Median	643	14/9/98	581	632	14/9/98	637		579	08/10/98	605
S.E.M.	51		49	45		47		19		19
Culled or killed										
Median	26	24/02/97	14	50	24/02/97	35	calf 1	240	01/12/97	294
S.E.M.	14		11	86		90	calf 2	329	19/01/98	343

Average number of samples per calf: farms 1 and 2: 24 (S.E.M.: 2), farm 3: 21 (S.E.M.: 1); number of calves sampled on farm 1: 29 (8 culled), farm 2: 33 (11 culled), farm 3: 23 (2 culled); the duration of the sampling period was: farm 1: 751 days, farm 2: 665 days, farm 3: 661 days.

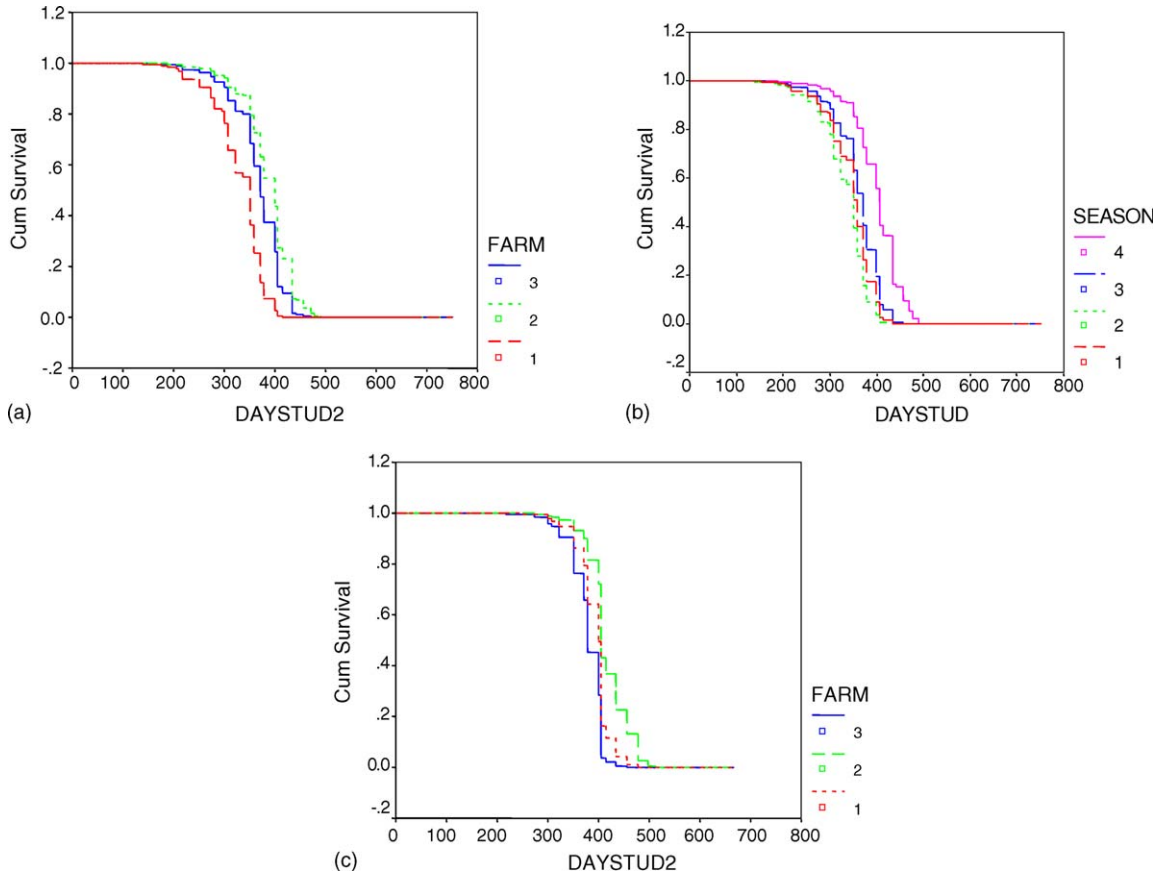

Fig. 1. Cumulative survival plots for the proportion of all verotoxin 1 and/or 2 positive samples (a) per farm, (b) per season; positive events: (389, 26.6%), and (c) for all potentially pathogenic VTEC per farm; positive events: 78 (5.3%), $n = 1462$, 125 missing values; DAYSTUD2: day of the study.

3.2. Time series analysis

Sixty-six of the total of 85 animals (77.7%) had time series with more than eight observations and were included in the time series analysis. Of the 85 animals, 47 (71.2%) for $EC_{vt1,2}$ and 35 (53.0%) for pVTEC could be modelled using ARIMA (2 1) or (1 1) models including statistically significant negative coefficients for the autoregression with lags 2 or 1 after correcting for positive linear trends. This means that frequent detections of $EC_{vt1,2}$ and pVTEC 1 or 2 sampling intervals earlier autocorrelated with less frequent detections of $EC_{vt1,2}$ and pVTEC on the current day of the study and that the overall trend for frequency of the detections was increasing. A second group of 1 (1.5%) animal for pVTEC and 5 (7.6%) animals for $EC_{vt1,2}$ had higher lags of correlations (1 time lag 4 and 5 times lag 3) and negative autocorrelation coefficients as well. Cross-correlations between $EC_{vt1,2}$ and pVTEC O157 or $EC_{vt1,2}$ and pVTEC were positive and significant on the same day only. This implied that the more $EC_{vt1,2}$ were detected in the current faecal sample the more pVTEC were found amongst the other colonies of EC examined from the same sample.

3.3. Survival analysis and Cox regression model

Cumulative survival plots for the detection of all $EC_{vt1,2}$ and pVTEC during the study are shown in Fig. 1. In Fig. 1a and c, the plots for the detection of all $EC_{vt1,2}$ and pVTEC are stratified per farm and Fig. 1b shows the detection of all $EC_{vt1,2}$ stratified per season. There was a strong farm effect for $EC_{vt1,2}$ and pVTEC, but the seasonal effect was found for $EC_{vt1,2}$ only. Increasing numbers of first $EC_{vt1,2}$ detections were found between March and November, while the smallest number of $EC_{vt1,2}$ was found between December and February. When subtracting the time of first detection for the $EC_{vt1,2}$ (median: 95 days, 95% CI: 70–119) from the first detection of pVTEC (median: 235 days, 95% CI: 185–284) the differences were all positive with a median of 140 days and a 95% CI of 109–170 days. This means that the first $EC_{vt1,2}$ were detected earlier than the first pVTEC.

The initial coefficients, interaction terms and results of four Cox regression models are shown in Table 3. The two left columns of results show the odds ratios (OR) and 95% confidence intervals for the regression coefficients at the detection of first or all $EC_{vt1,2}$ while the two right columns show the OR's for coefficients at the detection of first or all pVTEC. It can be seen that farms 1 and 2 have relatively less first and all detections of $EC_{vt1,2}$ and pVTEC compared to the reference farm 3 except for all detections of $EC_{vt1,2}$ where farm 1 has more events recorded compared to farm 3. Increasing age of detection correlates less frequently with first and all detections of $EC_{vt1,2}$ and pVTEC and serotype O157 was found more frequently in the presence of $EC_{vt1,2}$. A very strong seasonal effect was found for the first and all detections of $EC_{vt1,2}$ only, where the warmer months of the year (March–October) correlated increasingly more frequently with first and all detections of $EC_{vt1,2}$. During the cold months of the year (December–February), $EC_{vt1,2}$ were found less frequently. When excluding all observations of detections of verotoxin genes in pVTEC in one sample then the remaining $EC_{vt1,2}$ were found significantly more frequently together with pVTEC in the same sample.

Table 3
Cox regression models including OR's and 95% confidence intervals for the first and all detections of EC with verotoxin 1 and/or 2 and potentially pathogenic VTEC detected on a given day of the study period; all OR were found to have a $P < 0.05$ or as indicated by a *: $P < 0.1$, n.s.: not significant, n.a.: not analysed; observations included: 1462; positive events: 389 (26.6%) for vt1 and/or 2, 66 (4.5%) for first vt1 and/or 2, 78 (5.3%) for vt 1 and/or 2 and eae, and 43 (2.9%) for first vt1 and/or 2 and eae

	vt1 and/or 2 OR (95% CI)	first vt1 and/or 2 OR (95% CI)	vt1,2 and eae OR (95% CI)	first vt1, 2 and eae OR (95% CI)
Farm 1	2.65 (0.99–7.13)	0.02 (0.004–0.10)	0.55 (0.26–1.19)	0.59 (0.24–1.47)
Farm 2	0.61 (0.34–1.10)*	0.02 (0.01–0.08)	0.26 (0.12–0.57)	0.31 (0.12–0.79)
Farm 3	Ref.	Ref.	Ref.	Ref.
Age at detection (days)	0.97 (0.97–0.974)	0.98 (0.97–0.99)	0.96 (0.95–0.98)	0.97 (0.96–0.98)
O157 (yes/no)	n.s.	n.s.	1.40 (1.18–1.65)	n.s.
Season 1	4.17 (1.47–11.78)	0.81 (0.09–7.07)	n.s.	n.s.
Season 2	5.77 (2.45–13.58)	21.61 (6.26–74.57)	n.s.	n.s.
Season 3	2.82 (1.63–4.89)	4.02 (1.54–10.52)	n.s.	n.s.
Season 4	Ref.	Ref.	Ref.	Ref.
vt1 and/or 2 excl. pVTEC	n.a.	n.a.	1.11 (1.04–1.79)	n.a.
Age × season	n.s.	n.s.	n.s.	n.s.
Age × farm	n.s.	n.s.	n.s.	n.s.
Season × farm	1.33 (1.12–1.58)	n.s.	n.s.	n.s.

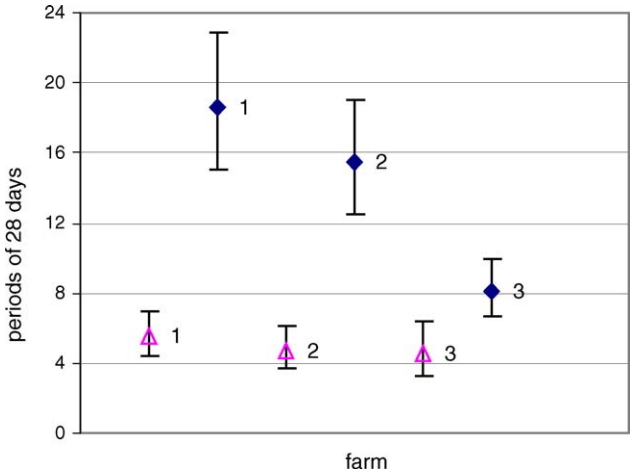

Fig. 2. Critical shedding period (xcrit = 1/β in periods of 28 days, 95% CI) above which the transmission parameter R_0 was found to be greater than 1 (indicating propagation of disease) for potentially pathogenic VTEC (triangles) and EC with verotoxin 1 and/or 2 genes (diamonds) on three farms (29, 33, and 23 calves on farms 1, 2, and 3) sampled at 28 days interval between birth and slaughter.

3.4. Stochastic SI model

The critical shedding period above which the reproduction ratio R_0 is greater than 1 was found to be between 8 and 18 sampling intervals of 28 days (224–504 days) for $EC_{vt1,2}$, and between 5 and 6 sampling periods of 28 days each (140–168 days) for the pVTEC which is smaller than all critical shedding periods for $EC_{vt1,2}$. The critical shedding periods for $EC_{vt1,2}$ were significantly larger and different between farms 1 and 2 when compared to 3. This was in contrast to the critical shedding periods for pVTEC that were statistically non-different between farms. The critical shedding periods for $EC_{vt1,2}$ and pVTEC per farm are shown in Fig. 2.

4. Discussion

The dynamics of $EC_{vt1,2}$ and pVTEC were different as found during survival analysis and the stochastic *SI* transmission modelling. Median age at first detection of $EC_{vt1,2}$ and pVTEC were 121 days (S.E.M.: 14.4) for $EC_{vt1,2}$ and 215 days (S.E.M.: 44.1) for pVTEC, respectively, and when considering the detection of first and all consecutive detections of $EC_{vt1,2}$ and pVTEC the older calves seemed to shed $EC_{vt1,2}$ and pVTEC less frequently as found in the Cox regression model. This finding matches peak incidences of VTEC shedding reported for calves around 14 weeks of age (Midgley et al., 1999), very young calves shedding VTEC O26 (Shaw et al., 2004) or 2–6 months of age (Paiba et al., 2003). A strong seasonal effect was found for $EC_{vt1,2}$ only and not for the pVTEC. The seasonal shedding patterns for VTEC are controversial. Some authors found a seasonal shedding

pattern for VTEC O157 and others did not (Meyer-Broseta et al., 2001; Nielsen et al., 2002). The reason why pVTEC were not found to have a seasonal pattern in this study may be a consequence of the many non-O157 serotypes found and analysed in this study, where some do have a seasonal patterns and others may not. Most studies are focussed on pVTEC O157 though and non-O157 serotypes are getting more and more attention (Caprioli et al., 1997; Pearce et al., 2004; Potter et al., 2004; Shaw et al., 2004).

Significant negative autocorrelations on the animal level were found for $EC_{vt1,2}$ only. Intraspecies interactions between the $EC_{vt1,2}$ strains in the GI tract, effects of immunity, differences in niches and shedding levels as well as differences in transmission rates could be the reason for this finding. Cross-correlations between $EC_{vt1,2}$ compared to pVTEC O157 and pVTEC were positive and significant on the same day only. This is in agreement with the positive correlations between the occurrence of verotoxin genes and eae genes reported by Shaw et al., 2004. The description of the bacterial interactions is a prerequisite for the modelling of the dynamics of VTEC in the ruminant GI tract in the future. The results of the analysis imply a difference in dynamics between $EC_{vt1,2}$ and pVTEC. This difference could be explained by differences in intestinal immunity, different levels of shedding, intermittent shedding of VTEC's, detection limits, age, seasonal and strain-related differences, VTEC re-infection of calves from the environment and differences in the transmission rates of the VTEC's on the farms.

Intestinal immunity changes and matures with age, which could be an explanation for the differences in dynamics. Given the large number of EC isolates in the environment, it is unlikely that $EC_{vt1,2}$ would systematically infect the calves earlier than the pVTEC if their exclusive source of infection were the environment. An environmental reservoir could prolong the presence of $EC_{vt1,2}$ and pVTEC in time and favour the propagation of $EC_{vt1,2}$ and pVTEC infections on the farms. Environmental reservoirs should therefore be studied for their exact impact on the between animal transmission dynamics in the future. Furthermore, pVTEC could be more effective during the between animal transmission as reflected by the finding that their critical shedding time for propagation of the infection in the population is significantly shorter compared to $EC_{vt1,2}$. The presence of intimin genes is believed to result in enhanced adhesion of these EC to the GI epithelia facilitating more prolonged infections. This is why vaccines against VTEC infections have been directed against intimins of VTEC (Potter et al., 2004). There are other virulence factors that may influence the competitive growth of selected EC strains in the ruminant GI tract. When shed extracellularly, verotoxins, katalase P and serinesterase P (the latter two are often associated with pVTEC) could inhibit the growth of the GI $EC_{vt1,2}$ and pVTEC (Brunder et al., 1999, 1996). Finally, the temporal pattern in the dynamics of $EC_{vt1,2}$ in relation to pVTEC could be explained by the re-combination of virulence factors in the GI tract of the calves as described in literature (Saunders et al., 1999), but the current study cannot prove this hypothesis.

The stochastic *SI* model used in this analysis is one of the most simple and yet meaningful transmission models. The model is independent of herd size which is why the number of animals followed were allowed to be a fraction of the total herd size while results can be extrapolated to the herd level and should still be comparable between farms. An alternative model could be an SIS model, where the animals return to a susceptible state after the infectious period and exact R_0 values could be estimated. The SIS and an SIR

(including a resistant compartment after the infectious state in time) modelling approach would require data about the more exact duration of shedding and therefore smaller sampling intervals than the data in the current study (Britton, 2003). Given the difficulties in detecting VTEC in faecal samples due to intermittent shedding of VTEC and shedding of small amounts of VTEC below the detection limit, the alternative SIS and SIR modelling approaches would depend more sensibly on the quality of the diagnostic test used for the VTEC detection. The combination of a diagnostic test evaluation as prior knowledge for the definition of the states in the SI, SIS and SIR models during a Bayesian approach to transmission dynamic modelling could be desirable. The diagnostic test evaluation for the detection of VTEC in this study has been reported (Döpfer et al., 2003). Diagnostic test evaluation, optimised sampling frequency given the intermittent and low shedding of VTEC in faeces and the sample size calculation for examining multiple isolates from one faecal sample (Singer et al., 2000; Altekruse et al., 2003) should all be combined into an integrated stochastic modelling approach to longitudinal field data in the future.

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