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Prevalence of *Enterocytozoon bieneusi* in post-weaned dairy calves in the eastern United States

Abstract Fecal specimens were obtained from 3- to 8-month-old post-weaned dairy calves on farms in Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida. After removal of fecal debris by sieving and density gradient centrifugation, 59 of 452 calves (13%) from 11 farms in six states were found positive for *Enterocytozoon bieneusi* by PCR and DNA sequence analysis. Based on gene sequence data this genotype of *E. bieneusi* found in post-weaned calves was 100% identical to that found in pre-weaned calves in North America and differed by only two positions in 1,069 base pairs from specimens analyzed from humans. However, compared with previous reports, the prevalence of *E. bieneusi* was significantly higher in post-weaned than in pre-weaned calves from many of the same farms.

Introduction

Microsporidia, reported worldwide, are one of the most frequently identified pathogens in HIV-infected patients (Franzen and Müller 1999). *Enterocytozoon bieneusi* is the microsporidian species most frequently found in human infections, which are characterized by chronic diarrhea (van Gool et al. 1995; Kotler and Orenstein 1998). This species has also been found in other mammals, including pigs (Desplazes et al. 1996; Breitenmoser et al. 1999; Rinder et al. 2000; Dengjel et al. 2001), cattle (Rinder et al. 2000; Dengjel et al. 2001; Fayer et al. 2003a; Sulaiman et al. 2004), rhesus macaques (Mansfield et al. 1997), goats (Lores et al. 2002),

a llama (Dengjel et al. 2001), and cats and dogs (Mathis et al. 1999). It has also been found in wildlife including beavers, muskrats, otters, foxes, and raccoons (Sulaiman et al. 2003), and in chickens (Reetz et al. 2002). Spores of *E. bieneusi* have also been detected in surface waters (Dowd et al. 1998; Cotte et al. 1999). Despite the finding of *E. bieneusi* in a variety of domesticated and wild animals, neither the routes of transmission nor the age distribution of the hosts of this pathogen are known. In the most comprehensive prevalence study of *E. bieneusi* in domesticated animals, involving 413 pre-weaned dairy calves from six of 15 farms along the east coast of the United States, 13 calves were found positive (Fayer et al. 2003a). The present study was undertaken to examine post-weaned calves from the same farms to determine if the prevalence of infection is associated with age.

Materials and methods

Feces were collected from 452 3- to 8-month-old dairy calves. They were collected from calves on two farms each in Vermont, New York, Pennsylvania, Maryland, Virginia, North Carolina, and Florida (Table 1) directly from the rectum of each calf into plastic specimen cups that were immediately labeled, capped, and placed on ice in an insulated container. Feces were transported to the United States Department of Agriculture laboratory in Beltsville, Maryland, and processed within 1–3 days of collection.

Specimens were cleaned of fecal debris, concentrated, and DNA was extracted as previously described (Fayer et al. 2003a). PCR analysis of the small subunit rRNA gene (SSU-rRNA) of *E. bieneusi* was performed with specific primers that amplified ca. 1,100 bp, and all positive samples were sequenced as previously described (David et al. 1996; Fayer et al. 2003a). Sequence chromatograms from each strand were aligned and inspected using Lasergene software (DNASTAR, Madison, Wis.). SSU-rRNA coding sequences from microsporidia

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Table 1 The number of specimens positive for *Enterocytozoon bieneusi* by PCR from 3- to 8-month-old calves from farms examined in the present study (post-weaned calves) compared with 1- to 8-week-old calves from most of the same farms examined in a previous study (pre-weaned calves, Fayer et al. 2003a)

State	Farm	Pre-weaned calves		Post-weaned calves	
		No. of positive animals/no. of animals examined	Percentage of positives	No. of positive animals/ no. of animals examined	Percentage of positives
Vermont	VT-1	0/6	0	N/A	N/A
	VT-2	0/15	0	3/27	11.11
	VT-3	4/17	23.5	2/26	7.69
New York	NY-1	0/25	0	6/28	21.43
	NY-2	3/37	8.1	2/26	7.69
Pennsylvania	PA-1	1/24	4.2	17/49	34.7
	PA-2	0/25	0	N/A	N/A
	PA-3	N/A	N/A	0/29	0
Maryland	MD-1	1/39	2.6	1/35	2.86
	MD-2	2/27	7.4	3/26	11.54
Virginia	VA-1	0/22	0	0/21	0
	VA-2	0/17	0	0/40	0
North Carolina	NC-1	0/38	0	8/38	21.05
	NC-2	2/53	3.8	10/35	28.57
Florida	FL-1	0/40	0	5/43	11.63
	FL-2	0/28	0	2/29	6.89
Total		13/413	3.1	59/452	13.05

detected in the present study were compared with published sequences.

Results

Of the 452 specimens examined in the present study, 59 (13.05%) were found to be PCR positive (Table 1). Nucleotide sequences of the SSU-rRNA gene were obtained from all 59 isolates positive for *E. bieneusi* by PCR. When the sequences were compared to other SSU-rRNA sequences from GenBank they were found to be 100% identical in all PCR products with the corresponding published sequence of *E. bieneusi* isolates from cattle (Fayer et al. 2003a, GenBank accession no. AY257180).

Of 14 farms in seven states, calves excreting spores of *E. bieneusi* were found on 11 farms in six states (Table 1). Positive calves were detected on farms in Vermont, New York, Pennsylvania, Maryland, North Carolina, and Florida but not in Virginia. The prevalence of infection by *E. bieneusi* ranged from 0% on farms designated as PA-3, VA-1 and VA-2 to 34.7% on PA-1 (Table 1).

Prevalence data in the present study for *E. bieneusi* infections in post-weaned dairy calves were compared with the data previously obtained for pre-weaned dairy calves (Fayer et al. 2003a). The prevalence was higher in post-weaned than in pre-weaned calves (13 vs 3%) (Table 1). This difference is highly significant (Fisher's test, $P < 0.0001$). In the previous study in pre-weaned calves, calves on six farms were positive versus 11 farms with positive post-weaned calves in the present study. All farms with positives for *E. bieneusi* in pre-weaned calves were positive in this study, and in four of them the prevalence had increased.

Discussion

E. bieneusi has been reported from cattle in Europe (Rinder et al. 2000; Dengjel et al. 2001) and in North America (Fayer et al. 2003a). The only large-scale prevalence study in cattle was age biased and limited to pre-weaned dairy calves (Fayer et al. 2003a). The present study, also age biased, targeted post-weaned dairy calves from most of the locations where pre-weaned calves were studied. Because specimen collection times were based on the availability of animals and personnel, seasonal and climate-biased data were not obtained.

Over a widely dispersed geographic area, from Vermont to Florida, calves on 11 of 14 farms were found positive for *E. bieneusi* by PCR. No regional differences in prevalence were detected. Gene sequence data supported the PCR findings. Of the 452 post-weaned calves examined in this study 59 (13%) were infected with *E. bieneusi*. These findings represent a marked increase from the 3% prevalence found in pre-weaned dairy calves from most of the same farms a year earlier (Fayer et al. 2003a). Of the 13 farms where calves were examined in successive years, the prevalence of infection with *E. bieneusi* increased with the age of the calves on 9 farms, remained the same on 2 farms, and decreased on 2 farms (Table 1). The underlying cause(s) for the increased prevalence noted with increased age has not been determined but several possibilities can be suggested. We speculate that prevalence could be related to diet (milk versus pelleted or grain-based feed), to physiological differences associated with single stomach digestion versus rumen function, to maturation of receptor sites on host cells, or possibly to the longer exposure time of older animals to parasites in the environment, perhaps facilitated by a change from

individual housing provided for younger animals to the group housing of older calves.

Studies conducted on pigs identified a similar age-related trend towards higher prevalence of infection in older animals. Breitenmoser et al. (1999) found a higher prevalence in weaning pigs than nursing pigs (70% vs 23%). Also, Buckholt et al. (2002) found a higher percentage of infection in pigs weighing more than 10 kg than in those weighing less than 10 kg (37% vs 16%). Those weighing more would be older animals, supporting the increase of prevalence related to age.

The organisms detected in the present study contained a SSU rRNA sequence identical to that found in pre-weaned calves in North America (Fayer et al. 2003a). This sequence differs from those reported in specimens from humans at only two positions within a total of 1,069 bp analyzed (Fayer et al. 2003a). Previous studies have utilized molecular techniques to identify genotypes of *E. bieneusi* from different hosts (Breitenmoser et al. 1999; Rinder et al. 2000; Dengjel et al. 2001; Buckholt et al. 2002; Sulaiman et al. 2003, 2004). Some of these studies revealed the lack of a transmission barrier by demonstrating that some *E. bieneusi* genotypes were shared by humans and animals, thereby suggesting that *E. bieneusi* could be a zoonotic pathogen (Dengjel et al. 2001). However, host-adapted genotypes also exist (Sulaiman et al. 2003, 2004). The analysis of the sequences of the ITS region of the rRNA gene disclosed five genotypes of *E. bieneusi* in cattle, four of which were host-adapted but one (BEB5) clustered with a group known to infect humans (Sulaiman et al. 2004).

The identification of *Enterocytozoon* in cattle feces is not definitive proof of an intestinal infection in calves, leaving the possibility of simple transit though the gut of spores originating from water or food. This possibility has been considered and cannot be discounted. Proof of intestinal infection would require that tissue specimens be acquired by biopsy or necropsy and examined for intracellular microsporidia, an economically prohibitive option. A previous study using generic primers detected none of ten calf fecal specimens spiked at a concentration of 100 spores/g of feces as positive and six of ten specimens spiked at 1,000 spores/g as positive, indicating that relatively large numbers of spores must be present for a specimen to be identified as positive (Fayer et al. 2003b). It appears unlikely that such large numbers would be routinely acquired by accidental ingestion in food and water by large numbers of calves on 11 farms in six states.

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