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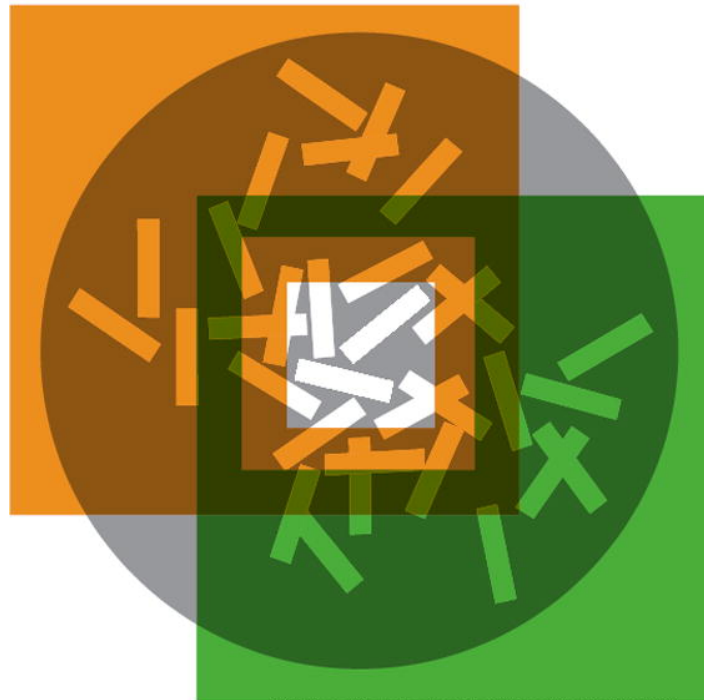


Volume 151, issues 2-4, 14 February 2008

ISSN 0304-4017

veterinary parasitology

An International Scientific Journal



Official Organ of the American Association of Veterinary Parasitologists (A.A.V.P.),
the European Veterinary Parasitology College (E.V.P.C.) and the World
Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)

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Veterinary Parasitology 151 (2008) 191–195

**veterinary
parasitology**

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High prevalence of *Cryptosporidium bovis* and the deer-like genotype in calves compared to mature cows in beef cow-calf operations

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Received 19 June 2007; received in revised form 17 October 2007; accepted 18 October 2007

Abstract

Recent studies have identified the novel, host adapted *Cryptosporidium bovis* and the deer-like genotype in dairy cattle from farms in the United States, China, India and Europe. This novel species and genotype appear to be more prevalent in older, post-weaned dairy cattle than previously thought. However, little information is available on their prevalence in beef cow-calf operations. In the present study, we determined the prevalence of *Cryptosporidium* species in 98 calves (6–8 months old) and 114 cows (>2 years old) in seven beef cow-calf herds in western North Dakota. DNA was extracted from fecal samples and *Cryptosporidium* spp. were identified by amplification of the 18S rRNA gene followed by sequencing or RFLP analysis. All seven herds tested positive for *Cryptosporidium*. Overall, 43/212 (20.3%) animals were positive. Only five of these positives were from cows. *C. bovis*, the deer-like genotype and *C. andersoni* were identified in 9.4, 6.6 and 1.4% of animals sampled, respectively. *C. parvum* was not identified in any of the positive samples. *C. bovis*, the deer-like genotype and *C. andersoni* were detected in 6/7, 5/7 and 2/7 herds, respectively. *C. bovis* and the deer-like genotype were primarily detected in calves, while *C. andersoni* was only detected in cows. Six isolates could not be typed. These results show a relatively high prevalence of *C. bovis* and the deer-like genotype in 6–8-month-old beef calves compared to cows older than 2 years in the seven herds studied.

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Keywords: *Cryptosporidium*; *Cryptosporidium bovis*; *Cryptosporidium* deer-like genotype; *Cryptosporidium andersoni*; Beef; Cows; Calves

1. Introduction

Cryptosporidium, a protozoan intestinal parasite, commonly infects dairy and beef cattle (Santin et al., 2004; Fayer et al., 2006; Trotz-Williams et al., 2006; Kvac et al., 2006; Plutzer and Karanis, 2007; Thompson et al., 2007). Among the species and genotypes found in

cattle, *C. parvum* has zoonotic potential and is a frequent cause of human cryptosporidiosis (Morgan et al., 1999; Fayer et al., 2000; Xiao et al., 2002). Other species and genotypes, including *C. andersoni*, *C. bovis*, and the deer-like genotype are considered as cattle adapted (Lindsay et al., 2000; Fayer et al., 2005; Feng et al., 2007) and are not significant causes of human disease. Identifying factors that contribute to the occurrence of different species and genotypes in cattle is critical to understanding the role that cattle may play in the transmission of human pathogenic *Cryptosporidium*.

Recent studies have identified a complex, age-related distribution of *Cryptosporidium* species and genotypes

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in dairy cattle (Santin et al., 2004; Fayer et al., 2006, 2007). *Cryptosporidium parvum* is reported to primarily infect pre-weaned (5 days to 2 months) dairy calves, causing a diarrheal disease (Santin et al., 2004). *C. bovis* and the deer-like genotype are found to predominate in 3 months to 2 years old dairy cattle (Fayer et al., 2006). However, the prevalence of *C. bovis* and the deer-like genotype are reported to decrease in mature dairy cattle (>2 years old), and this decrease is accompanied by a corresponding increase in the prevalence of *C. andersoni* (Fayer et al., 2007).

It is not yet known whether a similar pattern of *Cryptosporidium* infection occurs in beef cattle. *C. bovis* (previously Bovine B genotype) was identified in a single beef animal in Maryland (Xiao et al., 2002); while the deer-like genotype has yet to be identified in beef cattle. We undertook a study to determine the extent to which the age-related distribution of *Cryptosporidium* species and genotypes in beef cattle was similar to that previously reported in dairy cattle. This information will help determine the public health risk associated with *Cryptosporidium* in beef cattle.

2. Materials and methods

2.1. Sources and collection of isolates

Fecal samples were collected from 212 beef cattle (98 calves and 114 cows) originating from seven cow-calf breeding farms in the counties of Billings, Dunn, Mercer and Stark in western North Dakota (Fig. 1).

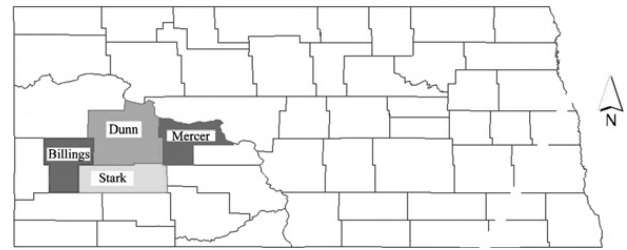


Fig. 1. Map of North Dakota showing the counties where beef cow-calf herds were sampled.

Table 1 shows the number of calves and cows sampled by herd and county. A random sample of at least 30 cattle (calves and cows) was selected from each of the seven herds that participated in the study. Only calves were sampled in one herd, while in two herds samples were collected from cows only. Calves were born indoors in March or April, pastured in May and weaned in the months of October to November. Fecal samples were collected beginning in September through November. All calves in this study were sampled before weaning (6–8 months old). All cows sampled were >2 years old. The exact age of the cows was undetermined but estimated to range from 2 to 7 years.

2.2. DNA extraction

DNA was extracted from 0.2 g fecal samples by alkaline digestion and phenol–chloroform extraction, and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) as previously described (Peng

Table 1

Overall prevalence of *Cryptosporidium* and prevalence of individual species/genotypes in 6–8-month calves and >2 years old cows by county and herd

County	Herd	Age	n	<i>Cryptosporidium</i>		<i>C. bovis</i>		Deer-like genotype		<i>C. andersoni</i>	
				Positive	Prevalence (%)	Positive	Prevalence (%)	Positive	Prevalence (%)	Positive	Prevalence (%)
Billings	B1	6–8 months	12	4	33.3	1	8.3	3	25	0	0
		>2 years	20	0	0	0	0	0	0	0	0
	B2	6–8 months	26	6	23.1	3	11.5	3	11.5	0	0
		>2 years	4	2	50	0	0	0	0	2	50
Dunn	D1	6–8 months	15	10	66.7	7	46.7	2	13.3	0	0
		>2 years	15	0	0	0	0	0	0	0	0
	D2	>2 years	30	1	3.3	0	0	1	3.3	0	0
Mercer	M	6–8 months	15	9	60	3	20	5	33.3	0	0
		>2 years	15	0	0	0	0	0	0	0	0
Stark	S1	>2 years	30	2	6.7	1	3.3	0	0	1	3.3
		S2	6–8 months	30	9	30	5	16.7	0	0	0
Total			212	43 ^a		20		14		3	

^a Six of the 43 isolates recovered during this study could not be typed to the species or genotype level.

et al., 2003). Briefly, 66.6 μ l of 1 M KOH and 18.6 μ l of 1 M DTT (Dithiothreitol) was added to a 1.5 ml centrifuge tube containing 100–200 μ l of stool. The samples were incubated at 65 °C for 15 min, neutralized with 8.6 μ l of 25% HCl and buffered with 160 μ l of 2 M Tris–HCl (pH 8.3). DNA was extracted with 250 μ l of phenol:chloroform:isoamyl alcohol (Invitrogen, Carlsbad, CA), mixed and centrifuged at 3330 \times *g* for 5 min (IEC Micromax centrifuge). The supernatant was removed to a 2.0 ml Eppendorf tube containing 1.0 ml of buffer ASL from the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA). The DNA was further purified in accordance with manufacturer's instructions and stored at –20 °C until required.

2.3. Species/genotype identification

Cryptosporidium species and genotypes were identified by sequencing or RFLP analysis of an amplified fragment of the 18S rRNA gene. RFLP analysis was used for PCR products that were refractory to sequencing. A fragment of the 18S rRNA gene was amplified using a nested PCR as previously described (Santin et al., 2004). The primary PCR reaction amplified a 1300-bp fragment using primers 18SFor1 5'-TTCTAGAGCTAATACATGCG-3' and 18SRev1 5'-CCCATTTTCCTTCGAAACAGGA-3'. Primary PCR reactions contained 1 μ l of template DNA, 1 \times PCR buffer (Promega, Madison, WI), 3 mM MgCl₂, 0.2 mM of each dNTP, 1 μ M of each primer, 2.5 U of *Taq* DNA polymerase, and 2 μ l of non-acetylated BSA (10 mg/ml) (New England Biolabs, Beverly, MA) in a 50 μ l reaction volume. Primary PCR cycling conditions consisted of an initial denaturation of 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 60 s, with a final extension of 72 °C for 7 min. The secondary PCR reaction amplified an 830-bp fragment within the primary PCR product using primers 18SFor2 5'-GGAAGGGTTGTATTTAT-TAGATAAAG-3' and 18SRev2 5'-AAGGAGTAAG-GAACAACCTCCA-3'. Reaction conditions were similar to those described for primary PCR with the exception that 1 μ l of primary PCR product was used as template and the MgCl₂ concentration was 1.5 mM. Cycling conditions for the secondary PCR consisted of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 2 min. Secondary products of expected size were digested using *Vsp*I, *Ssp*I (Xiao et al., 1999) and *Mbo*II (Feng et al., 2007) (New England Biolabs, Beverly, MA) at 37 °C. Digested products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and

visualized under UV transillumination. Species/genotype assignment was made by comparing RFLP profiles to known profiles reported in the literature (Feng et al., 2007). Secondary PCR products were purified using the Wizard SV gel and PCR product clean-up system, in accordance with manufacturer's instructions (Promega, Madison, WI), and sequenced in both directions using 18SFor2 and 18SRev2 primers. Sequencing was performed on an ABI 3730 DNA Analyzer (Iowa State University, Ames, Iowa). Sequences were assembled and edited using SeqMan (DnaStar, Madison, WI), and compared to non-redundant sequences in GenBank™ using the BLAST algorithm.

2.4. Statistical analysis

For purposes of analysis, the cattle were divided into two age groups; calves (6–8 months old) and cows (>2 years old). Data were analyzed using Epi Info Version 3.3. The relative percentages of positive animals out of the total number of animals tested were computed and compared using Chi square tests of independence with the level of significance set at $P \leq 0.05$. In addition, comparisons of *Cryptosporidium* infection by age group and *Cryptosporidium* species/genotype were made using Fisher's Exact Test with significance set at $P \leq 0.05$.

2.5. GenBank™ accession numbers

Representative sequences from isolates in this study have been published in GenBank™ under the accession numbers EU203216, EU203217, EU220742 and EU220743.

3. Results

Positive *Cryptosporidium* samples for each county and herd for 6–8-month calves and >2 years old cows are shown in Table 1. All seven herds tested positive for *Cryptosporidium* with an overall animal prevalence of 20.3% (43/212). A significantly higher prevalence was observed in calves (38.8%) than in cows (4.4%) ($P < 0.001$). Results of Chi-squared analysis indicated that calves were at 14 times higher odds of being infected with *Cryptosporidium* than cows (95% CI, 5–48). *C. bovis* was identified in 9.4% of animals and was more prevalent in calves (19.2%) than cows (0.8%) ($P < 0.001$). Calves were at 27 times higher odds of being infected with *C. bovis* compared to cows (95% CI, 4–207). *C. bovis* was present in all herds where calves were sampled, with a prevalence ranging from 8.3 to

46.7% within the calf population of a herd. The deer-like genotype was identified in 6.6% of animals and, similar to *C. bovis*, was more prevalent in calves (13.2%) than cows (0.8%) ($P < 0.001$). Calves were at 17 times higher odds of being infected with the deer-like genotype when compared to cows (95% CI, 2–134). Both *C. bovis* and the deer-like genotype were recovered from different calves within the same herd in 4/5 herds where calves were sampled. Mixed infections with *C. bovis* and the deer-like genotype were not identified. One cow tested positive for *C. bovis* (Herd S1) and one cow tested positive for the deer-like genotype (Herd D2). *C. andersoni* was identified in 1.4% of animals and was only recovered from cows. *C. parvum* was not identified in any of the animals from this study. Isolates from six animals could not be typed by sequencing or RFLP analysis.

4. Discussion

Advances in *Cryptosporidium* typing tools have led to a reevaluation of the epidemiology of this parasite in cattle. The traditional view of cattle being primarily infected with *C. parvum* as neonates and *C. andersoni* as adults has been expanded following recent reports from dairy cattle in the eastern US and elsewhere (Santin et al., 2004; Fayer et al., 2006, 2007; Feng et al., 2007). A more complex, age-related distribution of *Cryptosporidium* species and genotypes is suggested, with *C. parvum* predominating in neonates, *C. bovis* and the deer-like genotype predominating at 3–11 months and *C. andersoni* being more prevalent in animals older than 2 years (Santin et al., 2004; Fayer et al., 2006, 2007). In addition, the overall prevalence of *Cryptosporidium* in dairy cows is reported to decrease with increasing age (Fayer et al., 2007). In the present study of beef cow-calf herds in North Dakota we found *Cryptosporidium* to be significantly more prevalent in 6–8 months calves than in cows older than 2 years. The calves were exclusively infected with *C. bovis* and the deer-like genotype, while *C. andersoni* was found only in cows. These findings suggest that the age-related distribution of *Cryptosporidium* species/genotypes in beef cow-calf herds is similar to that previously reported in dairy cattle.

The widespread occurrence of both *C. bovis* and the deer-like genotype in cattle reported in this and other studies (Fayer et al., 2006; Feng et al., 2007) is indicative of the host adapted nature of this species and genotype. While *C. parvum* is generally limited to neonatal calves with an immature immune system, *C. bovis* and the deer-like genotype are likely to possess

attributes that enable their survival and persistence in the presence of a mature immune response. Recently, Feng et al. (2007) reported finding *C. bovis* and the deer-like genotype in pre-weaned dairy calves suggesting that infection can occur at an early age and may be masked by high levels of *C. parvum* in neonatal calves. This would indicate that *C. bovis* and deer-like genotype infections persist in cattle from the first weeks to the first year of life. Our finding that infection was rare in cows older than 2 years, even when calves in the herd were infected, suggests that immunity does develop in older animals. This is in agreement with the results reported for dairy cattle (Fayer et al., 2007).

Comparing our findings with earlier studies of beef cattle is not possible in many cases because those studies did not identify *Cryptosporidium* isolates to the species or genotype level. For example, methods based on oocyst morphology may have misidentified *C. bovis* as *C. parvum* due to the similar size of the oocysts (Fayer et al., 2005). However, the ability to unambiguously identify *C. andersoni* by microscopy (the oocysts are considerably larger than *C. parvum* and *C. bovis*) (Lindsay et al., 2000) makes some comparisons possible. We found *C. andersoni* to occur infrequently with infections limited to older cows; generally supporting previous findings from beef cattle (Ralston et al., 2003; McAllister et al., 2005; Kvac et al., 2006). Kvac et al. (2006) reported that beef cows on pasture had a much lower prevalence of *C. andersoni* than those housed indoors, which may explain the relatively low prevalence of this species in our study. McAllister et al. (2005) reported a higher prevalence (10.6%) of *C. andersoni* (referred to as *C. muris* in their publication) in 2–14-year-old beef cows in Ontario than was observed in our study. Interestingly, McAllister et al. (2005) found *C. parvum* (identified by oocyst morphology) to be more prevalent (18.4% versus 10.6%) than *C. andersoni* in 2–14-year-old beef cattle. In light of what we now know, it is possible that those authors were actually isolating *C. bovis* and/or the deer-like genotype. Whether or not this was the case, the data does indicate that *C. andersoni* is not always the most prevalent species in older cattle.

We did not find *C. parvum* in 6–8-month-old calves or cattle older than 2 years in any of the seven herds tested. *C. parvum* is a cause of cryptosporidiosis in humans and is therefore a concern when assessing the risk to human health posed by cattle waste (Morgan et al., 1999; Fayer et al., 2000). *C. bovis*, the deer-like genotype, and *C. andersoni* are not significant causes of human disease. Therefore the 6–8-month-old beef calves and >2 years old beef cows in our study are not likely to be a public

health concern. As stated previously (Atwill et al., 1999), it is likely that the contribution of cattle (both dairy and beef) to human cryptosporidiosis is limited to calves less than 2 months of age.

MboII was useful in differentiating *C. bovis*, the deer-like genotype, and *C. parvum* in cases where isolates could not be sequenced. PCR amplification of the 18S rRNA gene followed by *VspI* and *SspI* digestion fails to differentiate these species and genotype due to the similar digestion patterns obtained. The use of *MboII*, which was first reported by Feng et al. (2007), is an economical alternative to sequencing for species identification. Furthermore, this method may be especially useful in identifying species in mixed infections, where direct sequencing of PCR products is likely to be unsuccessful.

5. Conclusion

The overall prevalence of *Cryptosporidium* and specifically the prevalence of *C. bovis* and the deer-like genotype are significantly higher in 6–8-month-old beef calves than cows older than 2 years in the seven herds studied. Our failure to find *C. parvum* in these age groups indicates that they are not likely to contribute to zoonotic cryptosporidiosis.

Acknowledgments

The authors are grateful to North Dakota EPSCoR, USDA special grant, Agro Security: Disease Surveillance and Public Health and USDA National Research Initiative (Grant Number: 2006-35102-17237) for funding.

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