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ORIGINAL PAPER

Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States

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Abstract *Cryptosporidium parvum* DNA from 175 neonatal calves on 16 farms in eight eastern states in the United States was subtyped by sequence analysis of the 60-kDa glycoprotein gene to determinate the parasite genetic diversity. Six subtypes of the IIa subtype family were found. Subtype IIaA15G2R1, which is the predominant *C. parvum* subtype in calves in many parts of the world, was identified in 77% of the *C. parvum* DNA from calves. Several farms had more than one *C. parvum* subtype and a few calves had infections with mixed subtypes. Distribution of subtypes differed geographically. Diversity of *C. parvum* in calves in eastern United States was lower than that previously seen in Michigan and southern Ontario. The high prevalence of one subtype in calves worldwide and frequent detection of this subtype in humans suggests that parasite fitness probably plays an important role in transmission of cryptosporidiosis among cattle and in zoonotic infections.

Introduction

Cattle are infected with at least four *Cryptosporidium* species: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and the *Cryptosporidium* deer-like genotype (Xiao et al. 2004; Fayer et al. 2006). A recent study showed that these *Cryptosporidium* spp. in cattle are age-related (Santin et al. 2004). *C. parvum*, the only prevalent zoonotic species in cattle, is responsible for about 85% of the *Cryptosporidium* infections in preweaned calves but only 1% of the *Cryptosporidium* infections in postweaned calves. Postweaned calves and older cattle are mostly infected with *C. bovis*, *C. andersoni*, and the *Cryptosporidium* deer-like genotype (Santin et al. 2004; Fayer et al. 2006). These findings clearly demonstrate that only neonatal calves are an important source of zoonotic cryptosporidiosis in humans. Neonatal calves are also the age group of cattle mostly affected by cryptosporidiosis in terms of prevalence of infection and the associated morbidity and mortality (Fayer et al. 1997).

Despite the clinical and public health importance of *C. parvum*, little is known about its transmission dynamics in cattle. Currently, the maintenance of the parasites on cattle farm and the role of herd-to-herd transmission in cryptosporidiosis epidemiology are not known. Recently, researchers have used highly discriminatory subtyping techniques to study the transmission of bovine *C. parvum* infections in a geographic area (Mallon et al. 2003a,b; Tanriverdi et al. 2006; Tanriverdi and Widmer 2006). These tools are very useful in tracking infection sources and examining transmission dynamics. The most commonly used subtyping tool is based on sequence analysis of the 60-kDa glycoprotein gene (GP60), which enables the identification of many subtype families of *C. parvum* and *Cryptosporidium hominis* and several subtypes within each subtype family (Strong et al. 2000; Peng et al. 2001, 2003a,b; Sulaiman et al. 2001, 2005; Glaberman et al. 2002; Leav et al. 2002; Alves et al. 2003, 2006; Sturbaum et al. 2003; Wu et al. 2003; Zhou et al. 2003; Chalmers et al. 2005; Abe et al. 2006; Trotz-Williams

Nucleotide sequence data reported in this paper are available in the GenBank database under the accession numbers DQ630514–DQ630519.

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et al. 2006). Of the two major *C. parvum* subtype families, the IIa subtype family is zoonotic, seen in both humans and calves, whereas the IIc subtype family is anthroponotic and is found only in humans (Alves et al. 2003; Xiao and Ryan 2004).

In the present study, *C. parvum* specimens from cattle farms in eight eastern states in the United States were subtyped and compared with results previously obtained from cattle farms in Michigan and neighboring Ontario, Canada.

Materials and methods

Specimens

All specimens used in this study were diagnosed as positive for *Cryptosporidium* by microscopy of immunofluorescence-stained fecal materials. They were further identified as *C. parvum* by a small subunit (SSU) rRNA-based nested PCR and DNA sequencing as previously described (Santin et al. 2004). A total of 189 *C. parvum*-positive specimens from neonatal calves less than 8 weeks old were used in the study. They were collected in 1996 and 1997 in four dairy farms in Ohio and in 2002 on two farms each in New York, Maryland, Virginia, North Carolina, and Florida and one farm each in Vermont and Pennsylvania (Table 1). At least 15 specimens were collected from each farm, but only those positive for *C. parvum* were used in the present study.

DNA extraction and PCR

Total DNA was extracted from *Cryptosporidium* oocysts, concentrated by cesium chloride or sucrose floatation, using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). A fragment of the GP60 gene of approximately 850 bp was amplified by nested PCR as previously described, using 1 or 2 μl of the extracted DNA in primary PCR (Alves et al. 2003). To reduce PCR inhibition, 400 ng/μl of nonacetylated bovine serum albumin (Sigma, St. Louis, MO, USA) was used in primary PCR. Each DNA was analyzed by PCR twice with the aim to generate at least one positive GP60 product for each *C. parvum* specimen.

DNA sequencing and phylogenetic analysis

PCR products were sequenced in both directions with the forward and reverse primers used in secondary PCR and an intermediary sequencing primer (5′-GAGATATATCTTG TTGCG-3′). In most cases, two PCR products from each specimen were sequenced. The nucleotide sequences obtained in this study were aligned with reference sequences retrieved from the GenBank using the program ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). The recently proposed nomenclature was used in naming *C. parvum* subtypes (Sulaiman et al. 2005).

Table 1 *C. parvum* GP60 subtypes in calves on dairy farms in eight eastern states in the United States

State	Farm	No. of GP60 PCR positive/no. of specimens	Subtype
Vermont	VT-2	7/7	IIaA15G2R1 (4) IIaA17G2R1 (1) IIaA11G2R1+ IIaA15G2R2 (1) IIaA11G2R1+ IIaA15G2R1 (1)
New York	NY-1	13/13	IIaA15G2R1 (12) IIaA11G2R1 (1)
	NY-2	7/9	IIaA15G2R1 (6) IIaA15G2R2 (1)
Pennsylvania	PA-1	11/13	IIaA15G2R1 (11)
Ohio	OH-1	12/12	IIaA15G2R1 (12)
	OH-2	14/15	IIaA15G2R1 (14)
	OH-3	12/12	IIaA15G2R1 (12)
	OH-4	7/7	IIaA17G2R1 (6) IIaA15G2R1 (1)
Maryland	MD-1	8/10	IIaA15G2R1 (8)
	MD-2	7/8	IIaA15G2R1 (7)
Virginia	VA-1	4/4	IIaA15G2R1 (4)
	VA-2	11/11	IIaA15G2R1 (11)
North Carolina	NC-1	8/8	IIaA11G2R1 (8)
	NC-2	32/35	IIaA15G2R1 (32)
Florida	FL-1	13/14	IIaA18G2R1 (9) IIaA19G2R1 (3) IIaA18G2R1+ IIaA19G2R1 (1)
	FL-2	9/11	IIaA15G2R2 (9)
Total	16 farms in 8 states	175/189	6 subtypes

Results

GP60 sequences

Of the 189 DNA extractions from neonatal calves previously diagnosed to be infected with *C. parvum*, 175 generated GP60 PCR products of the expected size in nested PCR. Those that failed in GP60 PCR mostly had inconsistent amplification in repeated SSU rRNA PCR analyses, indicative of low levels of infections. The secondary GP60 PCR products were all sequenced successfully with the forward and reverse primers used in the secondary PCR and the intermediary sequencing primer.

The alignment of sequences obtained with reference sequences downloaded from GenBank indicated that all sequences obtained from the study belonged to the *C. parvum* subtype family IIa. Almost all sequences were identical to each other in the nonrepeat region, except that some sequences had two copies of the sequence ACATCA immediately after the trinucleotide repeats instead of one copy. In the trinucleotide repeat region, all sequences obtained had two copies of the TCG repeat and 11–19 copies of the TCA repeat.

GP60 IIa subtypes

Altogether, six *C. parvum* IIa subtypes were found in the neonatal calves. They were named based on the number of TCA and TCG repeats in the trinucleotide repeat region and the ACATCA sequence, using the recently proposed nomenclature system (Sulaiman et al. 2005). The most common subtype was IIaA15G2R1 subtype, which had 15 copies of the TCA repeat (represented by the letter A), two copies of the TCG repeat (represented by the letter G), and one copy of the ACATCA sequence (represented by the letter R). This subtype was seen in 135 of the 175 GP60-positive animals. A similar subtype but with two copies of the ACATCA sequence, IIaA15G2R2, was seen in 11 of the 175 positive animals. The other four subtypes were IIaA11G2R1, IIaA17G2R1, IIaA18G2R1, and IIaA19G2R1, seen in 11, 10, 7, and 4 animals, respectively (Table 1).

Frequency of GP60 subtypes on farms

The subtype IIaA15G2R1 was also the most widely distributed *C. parvum*, seen on 14 of the 16 study farms and in all states except Florida (Table 1). Other subtypes were much more geographically restricted, with IIaA15G2R2 and IIaA11G2R1 each seen on three farms in two states and IIaA17G2R1 on two farms in two states. In contrast, IIaA18G2R1 and IIaA19G2R1 were seen on only one farm in Florida. Over half of the farms had only one *C. parvum* subtype in calves. However, one farm each in Florida and Ohio and two farms in New York each had two *C. parvum* subtypes, and the farm in Vermont had four subtypes in calves. Two calves in Vermont and one calf in Florida were concurrently infected with two *C. parvum* subtypes (Table 1).

Discussion

Multiple *C. parvum* subtypes were identified in DNA extracts from feces collected from neonatal calves. Six IIa subtypes were identified from calves on 16 farms in eastern United States. Previously, the transmission of *C. parvum* in calves was examined by GP60 sequence typing with modest numbers of specimens only in Michigan, southern Ontario, and Portugal (Alves et al. 2003, 2006; Peng et al. 2003b; Trotz-Williams et al. 2006). Seven, six, and two IIa subtypes were found in 36, 34, and 72 bovine specimens subtyped, respectively (Table 2). Considering previous results from Michigan and Ontario and the much larger sample size in the present study, the finding of only six IIa subtypes in calves on diverse farms in eastern United States is surprising. It is possible that *C. parvum* genetic diversity is greater in northern states and provinces in North America, as the number of GP60 IIa subtypes seen in Michigan and Ontario in previous studies and Vermont in the present study is higher than the number of subtypes found in other states. As in Michigan (Peng et al. 2003b), several farms in the present study had more than one *C. parvum* subtypes in calves at the time of sampling. In addition, two calves in Vermont and one calf in Florida had concurrent infection of mixed IIa subtypes (Table 1).

The subtype IIaA15G2R1 was responsible for 77% of *C. parvum* infections in calves in this study, but for a much lower percentage in Michigan and Ontario (Table 2). In contrast, 85% of *C. parvum* infections in calves and other ruminants in Portugal had this subtype. On most farms where IIaA15G2R1 subtype was present few other subtypes were found, whereas other subtypes were found mostly on farms where IIaA15G2R1 did not predominate (Table 1). These findings parallel the low subtype diversity found in calves in Portugal where IIaA15G2R1 is the major subtype and the high subtype diversity in Michigan and Ontario where IIaA15G2R1 is less frequent (Table 2).

The high prevalence of IIaA15G2R1 in calves in the United States and Canada may be due to the fitness of the parasite, as it is also the predominant IIa subtype in calves and humans all over the world (Table 2). So far, it has been seen with high frequency in the United States, Canada, Portugal, Slovenia, Australia, Kuwait, and Japan (Table 2). The only possible exception is the United Kingdom, where it was seen less frequently in humans who were infected with many other IIa subtypes (Table 2) (Glaberman et al. 2002; Chalmers et al. 2005). Many widely used laboratory isolates of *C. parvum* belong to this subtype, such as the Iowa, UCP, Tamu, KSU1, Texas, Maine Apple Cider, and Glasgow isolates (Table 2). It is interesting to note that subisolates of the Iowa isolate, passaged in different laboratories over many years, differ from each other by one nucleotide in the GP60 gene; the parasite from Pleasant Hill Farm has a GP60 sequence identical to IIaA15G2R1 seen worldwide, whereas the parasite from the University of Arizona has a “G” to “T” nucleotide change after the trinucleotide repeats, which results in an amino acid change and is not seen in any other IIa subtypes (Sturbaum et al. 2003).

Table 2 *C. parvum* GP60 IIa subtypes in humans and ruminants in published studies

Parasite source	Host	Subtype	Reference
US and others	Human and calf isolates kept in calves	IIaA15G2R1 (1 Iowa, 2 Maine, and Peru, UCP, Tamu, and KSU1 isolates kept in Arizona) IIaA16G3R1 (2 calves in Ontario) IIaA15G2R2 (Zaire isolate kept in Maine) IIaA17G2R1 (Brazil isolate kept in Arizona) IIaA18G5R1 (1 calf in Idaho)	(Strong et al. 2000)
US and UK	Lab isolates kept in calves	IIaA15G2R1 (Iowa[a], Texas, Maine, and Glasgow isolates) IIaA17G1R1 (Moredun isolate)	(Sturbaum et al. 2003)
Michigan, US	Calves, humans, and water	IIaA15G2R2 (10 calves, 1 human, 1 water) IIaA15G2R1 (8 calves, 1 human) IIaA16G2R1 (8 calves) IIaA16G1R1 (3 calves) IIaA16G3R2 (2 calves) IIaA16G3R1 (2 calves) IIaA16G2R2 (1 human)	(Peng et al. 2003b)
Ohio	Human and apple cider in one apple cider-associated outbreak	IIaA17G2R1 (4 humans, 1 apple cider) IIaA15G2R1 (3 humans) IIaA15G2R1+IIaA17G2R1 (1 human)	(Blackburn et al. 2006)
Ontario, Canada	Calves and humans	IIaA15G2R1 (10 calves) IIaA16G2R1 (9 calves, 1 human) IIaA16G3R1 (8 calves) IIaA16G1R1 (4 calves) IIaA13G2R1 (2 calves) IIaA17G2R1 (2 calves, 2 humans) IIaA15G2R2 (1 human) IIaA18G3R1 (1 calf)	(Trotz-Williams et al. 2006)
N. Ireland	Humans	IIaA18G3R1 (32) (24 from outbreak A, 8 from outbreak C) IIaA20G3R1 (7) (5 from outbreak A) IIaA15G2R1 (2) IIaA17G3R1 (2) IIaA17G2R1 (1) IIaA19G2R1 (1) IIaA19G3R1 (1) IIaA20G4R1 (1) IIaA21G6R1 (1)	(Glaberman et al. 2002)
UK	Humans and animals	IIaA17G1R1 (9 humans in 3 outbreaks, 1 water) IIaA20G3R1 (1 human, 2 calves in an outbreak)	(Chalmers et al. 2005)
Slovenia	Humans and calves	IIaA17G1R1 (4 calves)[b] IIaA15G2R1 (3 humans) IIaA16G2R1 (1 human)	(Stantic-Pavlinic et al. 2003)
Portugal	Humans and ruminants	IIaA15G2R1 (9 humans, 61 calves, 1 lamb, 1 deer, 9 zoo ruminants) IIaA16G2R1 (7 calves)	(Alves et al. 2003, 2006)
Australia	Humans	IIaA15G2R1 (10) IIaA20G3R1 (4) IIaA18G3R1 (2)	(Chalmers et al. 2005)
Japan and Italy	Human and calves	IIaA15G2R1 (1 human, 1 calf in Japan) IIaA13G2R1 (1 calf in Italy)	(Wu et al. 2003)
Japan	Human and calves	IIaA15G2R1 (1 human, 3 calves)	(Abe et al. 2006)
Kuwait	Humans	IIaA15G2R1 (25) IIaA15G1R1 (2)[b]	(Sulaiman et al. 2005)

[a] With a change of "G" to "T" after the trinucleotide repeats
[b] With a change of "A" to "G" after the trinucleotide repeats

Despite the high prevalence of IIaA15G2R1 worldwide, there is geographic segregation in some *C. parvum* subtypes. The distribution of IIa subtypes in calves and humans in Michigan and neighboring southern Ontario is very similar, and most of the IIa subtypes there have 16 copies of the TCA repeat. Several subtypes common in Michigan and Ontario, such as IIaA16G2R1 and IIaA16G3R1, were not seen in this study (Table 2). Likewise, IIaA18G2R1 and IIaA19G1 were seen in calves on only one farm in Florida in this study, and many of the IIa subtypes seen in humans in Northern Ireland were not seen in other areas (Table 2). Even though an earlier multilocus typing study in Scotland showed no geographic isolation of *C. parvum* subtypes (Mallon et al. 2003b), results of a recent similar study in Turkey and Israel demonstrated the occurrence of geographically unique *C. parvum* subtypes in calves (Tanriverdi et al. 2006).

The *C. parvum* subtype IIaA15G2R1 is also the most commonly identified zoonotic *C. parvum* infection in humans. It was found in humans in the United States, United Kingdom, Portugal, Slovenia, Australia, Japan, and Kuwait (Table 2). It is likely the major subtype responsible for zoonotic cryptosporidiosis in areas with intensive animal husbandry, even though an endemicity of IIaA15G2R1 was found in children in Kuwait City (Sulaiman et al. 2005). Three other subtypes seen in calves in this study, IIaA15G2R2, IIaA17G2R2, and IIaA19G2R1, were also previously reported in humans (Table 2), indicating that all IIa subtypes have the potential to infect humans. The anthroponotic *C. parvum* IIc subtype family, which is the most common type of *C. parvum* in humans in most countries (Alves et al. 2003; Xiao et al. 2003; Xiao and Ryan 2004), were expectedly not seen in calves in this and any previous studies. The *C. parvum* IId subtype family previously seen in a few calves and humans in Portugal was not detected in US calves (Alves et al. 2006), demonstrating that this group of *C. parvum* is also not a major zoonotic pathogen in North America.

In addition to the studies of transmission of *C. parvum* in calves, GP60 subtyping was also used in tracking the source of contamination in waterborne and foodborne outbreaks of cryptosporidiosis (Glaberman et al. 2002; Xiao et al. 2003; Chalmers et al. 2005; Blackburn et al. 2006; Wheeler et al. 2006). Even though in several occasions a direct linkage at the subtype level between outbreak cases and the implicated food or water has been made (Glaberman et al. 2002; Blackburn et al. 2006), the power of the approach was compromised by the lack of baseline data on the distribution of *C. parvum* subtypes in the same geographic area. A noticeable exception is the investigation of an outbreak of cryptosporidiosis in Ohio in 2003 associated with consumption of ozonated apple cider in Blackburn et al. (2006). In this outbreak, two *C. parvum* subtypes, IIaA15G2R1 and IIaA17G2R1, were found in eight outbreak cases, and the IIaA17G2R1 subtype was further found in epidemiologically implicated apple cider. Existing data collected from dairy farms several years ago, reported now in the present study, have already demonstrated the occurrence of these two *C. parvum* subtypes in calves in Ohio, supporting the conclusion of environmental investigation that cattle might be a source of contamination of apples used in making the cider.

In conclusion, results of the subtyping study showed the uniqueness of *C. parvum* transmission on farms in eastern United States, with one predominant subtype in neonatal calves. The widespread occurrence of the subtype in calves in many parts of the world demonstrates the role of genetic fitness of the parasite in the transmission of cryptosporidiosis, and the report of this subtype in humans in many areas raises some concerns about the selection of highly infectious strains by intensive animal husbandry practices. The finding of apparent differences in the distribution of *C. parvum* subtypes in northern and southern regions in North America is also intriguing. Similar studies in other geographic areas and other agricultural settings would serve to increase our understanding of the transmission dynamics of cryptosporidiosis in cattle and the zoonotic potential of *C. parvum*.

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