Cryptosporidium in Cattle: From Observing to Understanding

R. Fayer, M. Santín and J.M. Trout

United States Department of Agriculture, Beltsville, MD, USA

Abstract

Cryptosporidium parvum is a zoonotic pathogen transmissible from a variety of animals to humans and is a considerable public health concern. Dairy cattle have been identified in numerous reports as a major source of environmental contamination with this pathogen. However, the vast majority of these reports have been based on microscopic examination of the organism in faeces from cattle. This chapter traces the progress of research on bovine cryptosporidiosis from the first observations of infection to the present understanding of susceptibility on the part of the bovine host and pathogenicity on the part of the parasites that constitute the taxa under the umbrella of the genus Cryptosporidium. It includes information based on molecular typing with the SSU rRNA gene and subtyping with the GP60 gene, which enables epidemiologists and others to trace the sources of Cryptosporidium-related outbreaks.

Introduction

Recent archaeological evidence from Egypt suggests that cattle were herded in prehistoric times, possibly from around 12,500 BC. It is not clear whether these cattle originally came from the 'Fertile Crescent'. Initially, it is presumed, cattle were killed for food, later they were used as draught animals, and for thousands of years cows' milk was consumed only by calves. As farming methods evolved, cattle became a major source of protein, milk and milk products, and leather. Over time, livestock production intensified to support growing human populations. Larger and larger herds became concentrated in increasingly smaller areas to maximize the efficiency of production. In industrialized countries, as well as less affluent areas of the world, the consequence of this evolution in animal husbandry was seen through the impact of enteric diseases on neonates, of which cryptosporidiosis is but one. Through the millennia of this close association between humans and cattle, enteric pathogens had billions upon billions of opportunities to adapt and to be readily transmitted between these hosts. Those that have are
of concern to public health and among them is the species *Cryptosporidium parvum*.

*Cryptosporidium* infections have been reported in cattle worldwide. Before the development and application of molecular methods to aid in species determination, numerous publications simply documented the presence of *Cryptosporidium* oocysts in cattle faeces. Subsequently, many other publications have identified *Cryptosporidium* species in cattle but with limited information on prevalence and herd management. Cryptosporidiosis, especially in calves, has been associated with a wide range of clinical signs from no apparent ill effects to severe morbidity, resulting in poor performance and production losses and in some instances mortality. From the perspective of human health, cattle have often been implicated as a source of zoonotic *Cryptosporidium* species. Risk of human infection has been based on physical contact with cattle, contamination of fresh fruits and vegetables with manure, and manure runoff from farms into drinking water supplies. With the goal of producing healthy cattle while protecting food and water supplies, studies have been undertaken to obtain a clear understanding of the species of *Cryptosporidium* that infect cattle, the prevalence of infection, and the relationship of these species to the age of the animals. There has been much progress in defining the *Cryptosporidium* species, genotypes, and some subtypes present in cattle. This chapter traces the progress of research on bovine cryptosporidiosis from the first observations of infection in cattle to our present understanding of susceptibility and pathogenicity. It includes those parasites that constitute the taxa under the umbrella of the genus *Cryptosporidium*. Application of this information can benefit both animal and human health.

**Early Reports of Cryptosporidiosis in Cattle**

The first report of bovine cryptosporidiosis appeared in 1971 and described stages of the parasite in tissue sections of the jejunum from an 8-month-old heifer with chronic diarrhoea (Panciera *et al.*, 1971). This observation was soon followed by others in which diarrhoeic dairy and beef calves, 2 weeks old and younger, had similar infections (Barker and Carbonell, 1974; Meuten *et al.*, 1974; Morin *et al.*, 1976). The association between the parasite and illness became stronger when it was reported that cryptosporidia were probably common enteropathogens of calves (Pohlenz *et al.*, 1978) and that *Cryptosporidium* was a pathogen in experimentally infected calves (Tzipori *et al.*, 1983). A survey of neonatal dairy calves in Maryland, USA, found *Cryptosporidium* in healthy as well as in diarrhoeic calves (Leek and Fayer, 1984). This finding led to a study of factors contributing to clinical illness in calves experimentally infected with *Cryptosporidium* obtained from pooled calves' faeces (Fayer *et al.*, 1985). In that study, clinical illness was not consistently found in neonatal dairy calves experimentally infected with 3.2–30 × 10⁶ oocysts, except when rotavirus and/or *Clostridium perfringens* was also present. It appeared that *Cryptosporidium* required the presence of other pathogens to produce illness. These findings gave rise to the following questions:

1. What was different in this study from reports in which cryptosporidiosis in calves was strongly associated with morbidity and mortality?
2. Were other isolates of the parasite more pathogenic or did other studies simply lack viral and bacterial assessments?
3. Why were some naturally infected calves healthy and others severely ill?

At that time there appeared to be a single species of Cryptosporidium that lacked host specificity, and answers to those questions would, in part, require the development of better taxonomic data.

In the next few years, cattle were recognized as hosts for two species, C. parvum and C. muris (Anderson, 1987), infecting the intestine and the abomasum, respectively, and represented by small and large oocysts (C. parvum, $\sim 4.5 \times 5.5 \mu m$ and C. muris, $\sim 5.5 \times 7.4 \mu m$). Molecular techniques and cross-species transmission studies eventually identified the abomasal form with the large oocysts as a new species, C. andersoni (Lindsay et al., 2000). Cryptosporidium parvum was then associated with diarrhoea in young calves and C. andersoni with asymptomatic adults.

As reports accumulated it became apparent that under farm and field conditions calves acquired infections shortly after birth. The highest prevalence of cryptosporidiosis in cattle was found at 1–3 weeks of age, with oocysts excreted for an average of 12 days and diarrhoea, when present, lasting an average of 8 days (reviewed by Santin and Trout, 2008).

What is the Immune Status of Calves and How Does it Affect Susceptibility to Infection?

Cell-mediated immunity had been shown to play an important role in control of C. parvum infections in mouse models, and by the early 1990s it was clear that HIV AIDS patients with low levels of T cells were extremely susceptible to cryptosporidiosis and other opportunistic pathogens, although little was known regarding the immune status of calves. To determine why young calves were so susceptible to infection, studies were designed to identify lymphoid cell populations at the site of infection in the ileum where lymphocytes could respond directly to the parasite (Pasquali et al., 1997; Canals et al., 1998). Intra-epithelial lymphocytes (IEL), and lamina propria lymphocytes (LPL) were collected from C. parvum-infected calves and uninfected control calves; the infected group was inoculated with oocysts at 1–2 days of age. Cells were collected from both groups at 7–9 days of age and analysed for phenotype and cytokine mRNA production. Significant increases in CD2$^+$, CD3$^+$, CD4$^+$ and CD8$^+$ T cells were observed in the IELs of infected versus uninfected calves. These findings were supported by reports of elevated IEL CD8$^+$ T cells in infected versus uninfected calves (Wyatt et al., 1997) and of elevated LPL CD4$^+$, CD8$^+$, and γ/δ T cells in infected versus uninfected calves (Abrahamsen et al., 1997). IELs and LPLs from uninfected calves contained much lower percentages of CD4$^+$ and CD8$^+$ T cells than found in adult cattle (Pasquali et al., 1997) which could explain the age-related susceptibility of neonatal calves while supporting early observations of others that T cell subsets protect against cryptosporidiosis in mice (Ungar et al., 1991; Chen et al., 1993; McDonald et al., 1994) and humans (Flanigan et al., 1992). Correlated with
the increases in CD4⁺ and CD8⁺ IELs was the finding of elevated IFN-γ and IL-12 mRNA in IELs and LPLs from the ileum of infected calves. However, no significant increases were detected in mRNA levels for IL-2, IL-4 or IL-10 (Canals et al., 1998). These cytokine and phenotypic cell differences in primary infected versus uninfected neonatal calves indicated that the susceptibility of these young calves to enteric infections had an immunological basis, the absence or extremely low levels of T cells.

What Accounts for the Variability in Severity of Infections?

Although pre-weaned calves are highly susceptible, infection with different isolates of C. parvum appears to result in variation in the severity of diarrhoea and number of oocysts produced. Such variation with different isolates has been demonstrated in human infectivity studies and in calves (Pozio et al., 1992; Okhuysen et al., 1999). Clinical cryptosporidiosis could not always be induced in experimental calf infections at inoculating doses of 3.2–30 × 10⁶ C. parvum oocysts per calf (Fayer et al., 1985). Subsequently, inoculation of calves with 1 × 10⁸ oocysts of a bovine C. parvum isolate from Alabama, USA, consistently induced severe clinical disease in experimentally infected calves (Fayer and Ungar, 1986). Another isolate of C. parvum from Auburn, Alabama (AUCP-1) was transmitted from one Cryptosporidium-naïve calf to another (26 calves) over a period of 3 years; these infections consistently resulted in oocyst production rates of 0.3–41.5 × 10⁶/g of faeces for 1 day or more during the patent period, accompanied by profuse diarrhoea (Fayer et al., 1997). Then, unexpectedly, over a period of the next six serial passages through calves, the severity of clinical signs and oocyst output steadily decreased until inoculation with 1.5 × 10⁶ oocysts resulted in no diarrhoea and the recovery of fewer than 1 × 10⁶ oocysts per calf. A similar decrease in productivity and pathogenicity with the same isolate occurred at Colorado State University. The same isolate was also serially transmitted, over a 3-year period, through 40 groups of C57BL/6 mice (four mice per group) immunosuppressed with dexamethasone. The mice showed no clinical signs but oocyst output remained consistently high. When oocysts from the mice were transmitted to a calf, the AUCP-1 isolate once again exhibited the earlier pathogenicity and oocyst production. It was not determined what factors led to the loss of pathogenicity in progressive serial passage through calves or why pathogenicity was unaffected by passage through mice; however, one might speculate that the original isolate contained multiple genetically distinct subpopulations that were selected for or against during passage through a particular host.

Selection

In an attempt to use selection to obtain a non-pathogenic precocious strain of C. parvum, an experiment was designed in much the same way as the two live commercial vaccines Paracox and Livacox were selected for in poultry (Fayer, 1994). Ten calves were serially infected with oocysts collected from the previous
calf on the first day of the patent period. Instead of the expected decrease in pathogenicity, as seen with coccidia, the duration and severity of diarrhoea steadily increased from calf 1 through to calf 9, although no pattern was seen in number of oocysts shed per calf. Perhaps it was the ability of C. parvum to auto-infect, unlike the Eimeria species in the coccidiosis vaccines, that invalidated the concept of a precocious non-pathogenic immunizing strain of C. parvum. These observations of different isolates and of serial passage of specific isolates of C. parvum demonstrate that not only do differences in pathogenicity exist among field isolates but also that both the pathogenicity and fecundity of an isolate can change over time.

Double-stranded RNA Virus-like Particles

Double-stranded RNA (dsRNA) virus-like particles have been found in Babesia, Trichomonas, Giardia, Leishmania and Eimeria (Hotzel et al., 1995). Two sizes of extrachromosomal dsRNAs were found in the cytoplasm of sporozoites of C. parvum and C. hominis but not in seven other species of Cryptosporidium (Khramtsov et al., 1997; Khramtsov and Upton, 2000). Sequence analysis showed distinctly different dsRNA sequences in isolates (species) from calves versus those from humans (Khramtsov et al., 2000). Small dsRNA sequences of isolates from 23 calves and 38 humans (Xiao et al., 2001) showed that isolates from the same outbreak had identical sequences; eight distinct nucleotide sequences were from cattle (C. parvum) and ten from humans (C. hominis). If any dsRNAs in Cryptosporidium are associated with pathogenicity this has not been demonstrated, but a recent study with our colleagues (M. Jenkins and J. Higgins, USDA, personal communication) has shown an association with fecundity. Calves infected with C. parvum-Beltsville oocysts produced substantially more oocysts than calves infected with C. parvum-Iowa oocysts. Increased fecundity was correlated with levels of C. parvum virus (CPV) as measured by real-time RT-PCR using CPV RNA-specific primers. The CPV signal in C. parvum-Beltsville sporozoites relative to C. parvum-Iowa was 3–4 times greater as measured by RT-PCR. The greater fluorescence intensity of C. parvum-Beltsville sporozoites labelled with antibodies to CPV 40 kDa capsid protein supported this observation. These findings suggest that CPV affects fecundity, which might in turn affect the severity of infection. What other factors affect severity of infection is not known.

Prevalence of Severe Morbidity and Mortality

There is no formal reporting system for cryptosporidiosis in cattle. Reports of cases, surveys and outbreaks provide information, but not on the same basis as the public health reports of ProMed, FoodNet and MMWR. At the US Department of Agriculture in Beltsville, Maryland, telephone calls from farmers and veterinarians attributing high morbidity and mortality to cryptosporidiosis in dairy and beef calves were frequent, peaking in the late 1980s through to the
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early 1990s, but steadily decreased in number until in 2006 no such calls were received. Whether the actual number of severe outbreaks declined or whether the association of severe diarrhoea and death of calves with cryptosporidiosis was no longer a ‘new’ phenomenon, and therefore consultation was no longer sought, is not known. Nevertheless, this informal barometer of activity suggests a change in the frequency of severe and economically important outbreaks perhaps due to a time when an exceptionally pathogenic strain of \textit{C. parvum} was present.

Species Other than \textit{C. parvum}

\textit{Cryptosporidium andersoni}, which colonizes the gastric glands of the abomasum, has been reviewed by Santin and Trout (2008). Infections, primarily in calves older than 4 weeks of age, produce no diarrhoea or visible clinical signs. Infections are more chronic (sometimes lasting over a year) and oocyst production is less than that of \textit{C. parvum}. Elevated plasma pepsinogen and decreased milk production have been attributed to infection. For other species and genotypes infecting cattle (Table 2.1), there are no reports of clinical signs, histological data or subclinical pathology.

Molecular Identification of Species in Cattle

For decades, microscopy was the sole method for detecting oocysts, first by direct faecal smears routinely stained, and later stained by IFA techniques. Microscopy

<table>
<thead>
<tr>
<th>\textit{Cryptosporidium} species/genotypes</th>
<th>Prevalence</th>
<th>Geographical location</th>
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<tbody>
<tr>
<td>\textit{C. parvum}</td>
<td>Frequent</td>
<td>Worldwide</td>
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<tr>
<td>\textit{C. bovis}</td>
<td>Frequent</td>
<td>Worldwide</td>
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<tr>
<td>\textit{C. andersoni}</td>
<td>Frequent</td>
<td>Worldwide</td>
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<tr>
<td>\textit{C. ryanae}</td>
<td>Frequent</td>
<td>Worldwide</td>
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<tr>
<td>\textit{C. hominis}</td>
<td>Rare</td>
<td>Scotland, India, Korea</td>
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<tr>
<td>\textit{C. suis}</td>
<td>Rare</td>
<td>USA and Zambia</td>
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<tr>
<td>\textit{C. suis}-like</td>
<td>Rare</td>
<td>Denmark</td>
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<tr>
<td>Pig genotype II</td>
<td>Rare</td>
<td>Denmark</td>
</tr>
<tr>
<td>\textit{C. felis}</td>
<td>Rare</td>
<td>Poland</td>
</tr>
<tr>
<td>\textit{C. canis}</td>
<td>Experimental infection only</td>
<td>USA</td>
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was time-consuming and required highly trained personnel. Unless oocysts differed significantly in size, species identification was impossible. PCR was found to be more sensitive than IFA and, when combined with gene sequencing, could differentiate species and genotypes of *Cryptosporidium*. The most frequently used gene for identification has been the SSU rRNA (small subunit ribosomal RNA) gene (the 18S gene). Molecular analysis of *Cryptosporidium* from cattle has identified seven species and three genotypes: *C. parvum*, *C. andersoni*, *C. bovis*, *C. canis*, *C. felis*, *C. hominis*, *C. suis*, *C. suis-like genotype*, *C. ryanae*, and *Cryptosporidium* pig genotype II (Bornay-Llinares et al., 1999; Fayer et al., 2001, 2006; Santín et al., 2004; Smith et al., 2005; Geurden et al., 2006; Park et al., 2006; Starkey et al., 2006; Feng et al., 2007; Langkjær et al., 2007) (Table 2.1).

**Prevalence of Species in Cattle**

In large-scale studies of cattle, *C. parvum*, *C. andersoni*, *C. bovis* and *C. ryanae* were found most frequently (Santín et al., 2004; Xiao et al., 2004; Fayer et al., 2006; Geurden et al., 2006; Feng et al., 2007; Langkjær et al., 2007). Other species and genotypes were found infrequently or rarely. *Cryptosporidium hominis* was found in a few cattle in Scotland, India and Korea (Smith et al., 2005; Park et al., 2006; Feng et al., 2007), *C. suis* was found in one calf in the USA and another in Zambia (Fayer et al., 2006; Geurden et al., 2006), *C. suis-like genotype* and *Cryptosporidium* pig genotype II were found in three cattle and one cow in Denmark, respectively (Langkjær et al., 2007), *C. felis* was found in a cow in Poland (Bornay-Llinares et al., 1999), and *C. canis* infected calves experimentally but natural infections have not been detected (Fayer et al., 2001) (Table 2.1).

**Large-scale Cross-sectional Study and Longitudinal Study of Species Prevalence Related to Age of Cattle**

A large-scale study involving 1615 cattle was conducted over a period of 4 consecutive years (Santín et al., 2004; Fayer et al., 2006, 2007). Each year 15 commercial dairy farms were included, two or three from each of seven states, ranging from Vermont to Florida along the east coast of the USA. Each year an attempt was made to collect faeces from 30 or more cattle from each farm. During the first 2 years, calves from 5 days to 11 months of age were examined. During the third year, dairy heifers 1–2 years of age were examined. During the fourth year, cows over 2 years of age were examined. Most of the farms visited in the first year were visited again in the following years, but in a few cases, because of management practices, either the required age or the required number of cattle were not available and substitute farms were introduced into the study. Based on SSU rRNA gene sequencing from PCR-positive specimens, virtually all infections in pre-weaned calves (<8 weeks of age) were *C. parvum*, with low levels of *C. bovis*, *C. ryanae* and *C. andersoni* in some calves. The number
of *C. parvum* infections decreased markedly in post-weaned calves (2–11 months of age), while the number of calves with *C. bovis*, *C. ryanae* and *C. andersoni* increased; *C. bovis* was the predominant species in this age group. In cattle older than 12 months, *C. andersoni* was the most prevalent species. In Denmark, a similar pattern was observed: *C. parvum* was the predominant species in calves younger than 1 month of age, and *C. bovis* became the predominant species in calves 3–12 months of age (Langkjær et al., 2007). Both *C. bovis* and *C. ryanae* have been found to be geographically widespread in cattle (Santin et al., 2004; Fayer et al., 2006, 2007; Starkey et al., 2006; Feng et al., 2007; Langkjær et al., 2007). *Cryptosporidium andersoni* constitutes the majority of infections in mature cattle in Denmark, the Czech Republic, Japan and the USA (Enemark et al., 2002; Kvac and Vitovec, 2003; Sakai et al., 2003; Fayer et al., 2006, 2007; Kvac et al., 2006) with the exception that Langkjær et al. (2007) in Denmark, did not detect this species in cattle of any age. The prevalence of *Cryptosporidium* in cattle declines significantly with age (Santin et al., 2004; Fayer et al., 2006, 2007; Kvac et al., 2006; Langkjær et al., 2007). Therefore, species and genotypes found in mature cattle are actually present in relatively few animals.

A longitudinal study involving 30 Holstein cattle on a single dairy farm was conducted over a 2-year period (M. Santin et al., 2008). Faeces were examined consecutively at weekly, two-weekly and then at monthly intervals from 1 week to 24 months of age for the presence of *Cryptosporidium* oocysts using a two-step nested PCR protocol to amplify the SSU rRNA gene. Every PCR-positive product was purified and sequenced. *Cryptosporidium parvum*, *C. bovis*, *C. andersoni* and *C. ryanae* were detected in every animal and their presence was strongly associated with the age of the animal. This longitudinal study strongly supports the findings of the cross-sectional studies. In the longitudinal study all pre-weaned calves were infected with *C. parvum*, followed in the post-weaned cattle by *C. bovis* and *C. ryanae* infections, and then in the older cattle *C. andersoni* infections.

**Subtyping of *C. parvum* and *C. hominis***

Because *C. parvum* is the zoonotic species most commonly identified in cattle, it became the focus of subtyping to better determine its genetic diversity and to provide a more accurate tool for source tracking. Most subtyping protocols have been based on the 60 kDa glycoprotein gene (GP60). Sequence analysis of GP60 has revealed several subtype families (alleles) and subtypes within those families for *C. parvum* and *C. hominis* (Cacciò et al., 2000; Strong et al., 2000; Feng et al., 2001, 2003; Alves et al., 2003; Tanriverdi et al., 2003; Wu et al., 2003; Xiao and Ryan, 2004).

Most cattle isolates belong to the *C. parvum* subtype family Ila, which is the zoonotic subtype family. Within this family the most common subtype found worldwide was IIA15G2R1. For example, in Portugal 61 of 72 isolates were positive for this subtype (Alves et al., 2003, 2006). Likewise, in India 5 of 9 were positive (Feng et al., 2007), and in the USA 135 of 175 were positive for this subtype (Xiao et al., 2007). Greater diversity was found within allele Ila in
Northern Ireland (Thompson et al., 2007). Subtypes IIA18G3R1 and IIA15G2R1 were found in 120 and 28 of 214 isolates, respectively.

The identity of IIA15G2R1 as the subtype most prevalent in calves and frequently detected in humans worldwide (Strong et al., 2000; Glaberman et al., 2002; Alves et al., 2003, 2006; Peng et al., 2003; Stantic-Pavlinic et al., 2003; Wu et al., 2003; Chalmers et al., 2005; Sulaiman et al., 2005; Abe et al., 2006; Trotz-Williams et al., 2006), as well as other subtypes identified by analysis of the GP60 gene, provides an important tool to accurately identify and track Cryptosporidium of veterinary and public health concern. The recent finding that C. parvum subtypes IIA15G2R1 and IIA17G2R1 were detected in stools from 12 laboratory-confirmed case patients who drank apple cider at a school outing in Ohio, and that subtype IIA17G2R1 was also detected in the remains of some of the cider (Blackburn et al., 2006) illustrates the usefulness of subtyping. It is interesting to note that the same two subtypes were detected in a high percentage of faeces from cattle in Ohio (Xiao et al., 2007).

Although these initial findings based on subtyping with GP60 have already served to provide a greater understanding of the transmission dynamics of C. parvum and C. hominis, the number of isolates examined is relatively few, so much more can still be learned. For other species and genotypes of Cryptosporidium affecting cattle and humans, additional typing tools are needed to reach a similar level of identification and understanding.

References


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polymorphism (RFLP) and RFLP-single-strand conformational polymorphism analyses. Applied and Environmental Microbiology 69, 4720–4726.


