# Molecular epidemiology with subtype analysis of *Cryptosporidium* in calves in Belgium

T. GEURDEN[1]*, D. BERKVENS[2], C. MARTENS[3,4], S. CASAERT[1], J. VERCRUYSSE[1] *and* E. CLAEREBOUT[1]

[1] *Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*
[2] *Department of Veterinary Epidemiology, Prince Leopold Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium*
[3] *Department of Plant Systems Biology, VIB, Technologiepark 927, B-9052 Ghent, Belgium*
[4] *Bioinformatics and Evolutionary Genomics, Department of Molecular Genetics, Ghent University, Technologiepark 927, B-9052 Ghent, Belgium*

(*Received 18 April 2007; revised 18 June 2007; accepted 5 July 2007; first published online 31 August 2007*)

SUMMARY

The prevalence of *Cryptosporidium* in calves younger than 10 weeks was estimated in a cross-sectional epidemiological study on 100 dairy ($n=499$) and 50 beef ($n=333$) farms in East Flanders (Belgium), using a previously evaluated immunofluorescence assay (Merifluor®). The calf prevalence was 37% (95% Probability Interval (PI): 7–70%) in dairy calves and 12% (95% PI: 1–30%) in beef calves. To elucidate the genetic diversity, the *Cryptosporidium* 18S ribosomal DNA and the 70 kDa heat shock protein gene were targeted. In the majority of the samples *C. parvum* was present, although *C. bovis* was also identified, all but one in calves older than 1 month. The porcine-specific *C. suis* was identified in 1 beef calf. Subtyping of *C. parvum* positive isolates by sequence analysis of the 60 kDa glycoprotein gene indicated the presence of 4 allele IIa subtypes, along with 1 subtype IIdA22G1. The subtype IIaA15G2R1 was most prevalent, next to subtype IIaA13G2R1 and IIaA16G2R1, and a new subtype IIaA14G2R1. The results of the present study indicate a high prevalence of *Cryptosporidium* infections in calves in Belgium and confirm that these calves should be considered as a potential zoonotic reservoir for human infections.

Key words: *Cryptosporidium*, calf, molecular epidemiology, gp60 subtype, Belgium.

## INTRODUCTION

Worldwide *Cryptosporidium* is associated with diarrhoea in young calves (de Graaf *et al*. 1999). Parasite prevalence varies from 1% (Kvac *et al*. 2006) to 59% (Olson *et al*. 1997). Next to differences in breed (Geurden *et al*. 2006*b*; Kvac *et al*. 2006), the variation in prevalence is partially due to the choice of diagnostic technique, and several diagnostic assays have therefore been evaluated for use in epidemiological studies (Geurden *et al*. 2006*a*).

Since not all *Cryptosporidium* species in cattle have zoonotic potential, epidemiological research should include molecular identification to elucidate the relevance of cattle in the epidemiology of human cryptosporidiosis (Traub *et al*. 2005). Apart from the ubiquitous and zoonotic *C. parvum*, the ruminant-specific species *C. bovis* and *C. andersoni*, and the *Cryptosporidium* cervine genotype have been frequently reported in cattle (Caccio *et al*. 2005). Occasionally *C. suis* (Fayer *et al*. 2006), *C. felis* (Bornay-Llinares *et al*. 1999), *C. canis* (Fayer *et al*. 2001) and *C. hominis* (Smith *et al*. 2005) are reported in cattle. Human cryptosporidiosis can be due to infections with either the human-specific *C. hominis* or the zoonotic *C. parvum*, and in a more limited number of cases with *C. meleagridis*, which is also zoonotic (Pedraza-Diaz *et al*. 2001). In Europe *C. parvum* is the main cause of human cryptosporidiosis, while worldwide *C. hominis* is most prevalent (Caccio *et al*. 2005). At least part of the human *C. parvum* infections can be ascribed to contact with livestock (Hunter and Thompson, 2005), although the identification of *C. parvum* in stool samples does not conclusively implicate farm animals as the source of infection. Subtype analysis at the 60 kDa glycoprotein locus (Peng *et al*. 2001; Alves *et al*. 2006; Trotz-Williams *et al*. 2006), revealed both human specific and zoonotic subtypes within *C. parvum*. In most waterborne outbreaks, human sources of infection were identified (Peng *et al*. 1997). Since the majority of human cryptosporidial infections are, however, not outbreak related (Feltus *et al*. 2006), the importance of zoonotic transmission might be underestimated.

* Corresponding author: Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium. Tel: +32 9 2647393. Fax: +32 9 2647496. E-mail: thomas.geurden@ugent.be

 
doi:10.1017/S0031182007003460 Printed in the United Kingdom

The objective of the present study was to obtain a reliable estimate of the parasite prevalence in dairy and beef calves using a previously evaluated diagnostic technique, and to identify the *Cryptosporidium* species in dairy, beef and clinically affected calves. By subgenotyping of the *C. parvum*-positive samples, the potential of calves as zoonotic reservoir for human infection was studied.

## MATERIALS AND METHODS

### *Study design*

A cross-sectional epidemiological study was conducted. In total, 100 dairy farms and 50 beef farms in the province of East-Flanders (Belgium) were randomly selected and visited on a single occasion between September 2001 and August 2005. All dairy calves were Holstein or local breed crossed with Holstein. All beef calves were Belgian White-Blue (BWB) or local breed crossed with BWB. Faecal specimens were collected from all calves below 10 weeks of age, present on the farm at the time of the visit. Faeces were scored either as normal, pasty or watery. The faeces were examined in the laboratory within 48 h after collection using a quantitative immunofluorescence assay (IFA; Merifluor *Cryptosporidium/Giardia* kit; Meridian Diagnostics Inc., Cincinnati, Ohio), using the same procedure as described by Geurden *et al.* (2006*a*). In short, 1 g of faeces was suspended in distilled water and strained through a layer of surgical gauze to withold large debris. After sedimentation for at least 1 h and centrifugation at 3000 ***g*** for 5 min, the sediment was resuspended in 1 ml of distilled water. After thorough vortexing, an aliquot of 10 $\mu$l was pipetted onto the IFA-slide. After staining, the entire slide was examined at a 400× magnification under a fluorescence microscope. A sample was considered positive if at least 1, clearly recognizable *Cryptosporidium* oocyst was identified. The number of *Cryptosporidium* oocysts per gram faeces was obtained by multiplying the total number of oocysts on the slide by 100. A subset of the dairy samples was used in a previous Bayesian analysis, in order to estimate both the sensitivity and the specificity of different diagnostic assays, including the IFA, in a bovine population (Geurden *et al.* 2006*a*).

### *Statistical analysis*

The results of the IFA were used to obtain the true calf prevalence, the farm prevalence, the intensity of cyst excretion and the prevalence in each age category. The true calf prevalence was estimated based on the number of negative and positive samples found in the present study, and on the 95% probability intervals of the sensitivity (19–34%) and specificity (88–99%) of the IFA (Geurden *et al.* 2006*a*). The farm prevalence was calculated as the number of farms with at least 1 positive calf, compared to the total number of farms in the study. The difference in age between dairy and beef calves, and between *C. parvum* and the *C. bovis*-positive calves was compared with a two-way Mann-Whitney U test, as was the difference in faecal consistency between infected and non-infected calves. Probability ($P$) values $<0{\cdot}05$ were considered to indicate a significant difference.

### *Molecular characterization*

In order to have a representative view of the different *Cryptosporidium* species and subtypes, *Cryptosporidium* positive isolates from both dairy and beef calves from the epidemiological study were selected for genotyping. Next to the samples from the epidemiological study, samples from calves younger than 6 weeks with clinical cryptosporidiosis, submitted to the diagnostic laboratory by practicing veterinarians, were selected.

DNA was extracted using the QIAamp® Stool Mini Kit (Qiagen) according to the manufacturer's instructions, incorporating an initial step of 3 freeze-thaw cycles (freezing in liquid nitrogen for 5 min and heating at 95 °C for 5 min) in the protocol to maximize oocyst lesions. The eluted DNA was dissolved in 15 $\mu$l of ultra-pure water.

For the amplification of the *Cryptosporidium* 70 kDa heat shock protein (HSP-70) and the 18S ribosomal DNA gene (18S rDNA) gene, previously described PCR protocols were used (Morgan *et al.* 2001; Ryan *et al.* 2003). For the subgenotyping of *C. parvum* positive samples, the 60 kDa glycoprotein (gp60) was targeted (Peng *et al.* 2001). In all the above-mentioned PCR reactions bovine serum albumin (BSA) was added to a final concentration of $0{\cdot}1$ $\mu$g BSA/$\mu$l reaction mixture. Amplification products were visualized on 1·5% agarose gels with ethidium bromide. A positive (Plasmid DNA) and negative (PCR water) control sample were included in each PCR reaction. PCR products were purified using the Qiaquick® purification kit (Qiagen) and fully sequenced using the Big Dye Terminator V3.1 Cycle sequencing Kit (Applied Biosystems). Sequencing reactions were analysed on a 3100 Genetic Analyzer (Applied Biosystems) and assembled with Seqman II (DNASTAR, Madison WI, USA). Sequences were compared with known sequences by BLAST-analysis (Altschul *et al.* 1997) against the NCBI database. Furthermore, phylogenetic analysis was performed on more conserved positions of the nucleotide alignment created with MUSCLE (Edgar, 2004). The sequence alignments were manually improved using BioEdit (Hall, 1999). Distance matrices were calculated based on Poisson correction and trees were constructed with the neighbour-joining algorithm using the software

Table 1. The number ($n$) of animals included in the study, the true *Cryptosporidium* prevalence (in percentage), with the 95% Probability Interval, for different age categories in dairy and beef farms, and the farm prevalence

| | $n$ | <4weeks | 4–10weeks | <10weeks | Farm |
|---|---|---|---|---|---|
| Dairy | 499 | 68 (17–97) | 10 (1–27) | 37 (7–70) | 32 |
| Beef | 333 | 17 (1–44) | 11 (1–30) | 12 (1–30) | 24 |

package TREECON (Van de Peer and De Wachter, 1994). Bootstrap analysis with 500 replicates was performed to test the significance of the nodes.

## RESULTS

### *Epidemiological study*

A total of 499 dairy and 333 beef calves were examined with IFA for the presence of *Cryptosporidium* oocysts. There was an average number of 5 calves on these dairy farms, ranging from 1 to 19. The mean age of the dairy calves was $26{\cdot}43 \pm 0{\cdot}93$ (mean $\pm$ standard error) days ranging from 1 to 70 days, with 59% younger than 4 weeks and 40% younger than 2 weeks of age. Seventy-four percent of the dairy calves were female and 26% were male. On the beef farms there was an average number of 7 calves, ranging from 1 to 17. The mean age of the beef calves was $32{\cdot}7 \pm 1{\cdot}1$ days ranging from 1 to 70 days, with 47% younger than 4 weeks and only 22% younger than 2 weeks. The mean age of the calves on beef farms was significantly ($P<0{\cdot}001$) higher than on dairy farms. Fifty-two percent of the beef calves were female and 48% were male.

The true prevalence of *Cryptosporidium* is presented in Table 1. In the dairy calves a peak in prevalence was observed in calves between the ages of 2 and 4 weeks, whereas in beef calves no peak prevalence was observed. The number of excreted oocysts per gram of faeces (OPG) was determined using IFA. The geometric mean oocyst excretion in the dairy calves was 29425 OPG, ranging from 100 to $7 \times 10^7$ OPG. The highest individual OPG was observed in a 15-day-old calf. On the beef farms the geometric mean oocyst excretion in beef calves was 780 OPG, ranging from 50 to $3{\cdot}2 \times 10^4$ OPG. The highest individual OPG was observed in a 20-day-old calf. In some of the *Cryptosporidium* positive calves diarrhoea was observed, but both in the dairy and beef calves there was no correlation between faecal consistency and the presence of *Cryptosporidium* oocysts in the faeces ($P>0{\cdot}05$).

### *Molecular characterization*

The number of samples and the results of the molecular characterization are presented in Table 2. At least 1 isolate from each of the 32 *Cryptosporidium*-positive dairy farms was included. Isolates from 9 of the 12 positive beef farms were included, since no amplification product could be obtained from isolates of the 3 remaining farms. Both in beef and in dairy farms *C. parvum* was predominantly identified, next to the cattle-specific *C. bovis*. All but 1 of the calves infected with *C. bovis* were older than 4 weeks and the average age of the *C. bovis*-positive calves was higher ($P<0{\cdot}001$) than the age of the *C. parvum*-positive calves. The isolate of 1 beef calf was found to be closely related to the porcine-specific *C. suis*. In the samples from the calves with clinical cryptosporidiosis only *C. parvum* was identified. The identification on the 18S rDNA concurred with the identification on the HSP-70 gene in all but 2 samples, in which 1 of the 2 genes did not amplify. The phylogenetic analysis on the 18S rDNA is presented in Fig. 1. Four different subtypes of the *C. parvum* allele IIa were identified (IIaA13G2R1 ($n=1$), IIaA14G2R1 ($n=1$), IIaA15G2R1 ($n=84$), IIaA16G2R1 ($n=3$)). In 1 sample from a clinically affected calf, the subtype IIdA22G1 was identified. The presence of any specific *Cryptosporidium* species or *C. parvum* subtype was not correlated with clinical symptoms.

## DISCUSSION

The true *Cryptosporidium* prevalence was estimated taking the IFA test characteristics into account, which were previously estimated for use in epidemiological studies. The low sensitivity of IFA for the diagnosis of infection in calves older than 1 month is hereby taken into account, providing a more reliable estimate of the true prevalence (Geurden *et al.* 2006*a*). The peak prevalence of *Cryptosporidium* in dairy calves was observed in calves younger than 1 month, confirming previous studies (O'Handley *et al.* 1999; Nydam *et al.* 2001; Maddox-Hyttel *et al.* 2006). In the beef calves there was no obvious age-related infection pattern, possibly due to the overall low *Cryptosporidium* prevalence in beef calves. The low prevalence in beef calves can largely be attributed to the significantly higher age of the beef calves compared to dairy calves. Calf age is an important risk factor for infection with *Cryptosporidium* (O'Handley *et al.* 1999), with a maximum risk for infection between 9 and 12 days of age (Maldonado-Camargo *et al.* 1998; Castro-Hermida *et al.* 2002*a*, *b*). In the present study only 22% of the beef calves was younger than 2 weeks, compared to 40% of the dairy calves. In previous studies, housing and differences in management have also been advocated as a possible risk factor for infection (Kvac *et al.* 2006). Often, beef calves are raised in cow-calf herds, with less risk for cryptosporidial infection (Gow and Waldner, 2006) due to the more efficient transfer of colostrum and lower calf stocking

Table 2. Molecular characterization of *Cryptosporidium* in the dairy, beef and clinically affected calves, with the mean age and age range for each species

(*n*, Total number of samples; HSP-70, 70 kDa Heat Shock Protein; 18S, 18S ribosomal DNA; gp60, 60 kDa glycoprotein; NA, No Amplification; nd, not done.)

| | *n* | | HSP 70 | 18S | GP60 | Age (range) |
|---|---|---|---|---|---|---|
| Dairy | 73 | *C. parvum* | 67 | 67 | 52 IIa A15 G2 R1<br>1 IIa A14 G2 R1<br>2 IIa A16 G2 R1<br>1 IIa A13 G2 R1 | 24·8 (3–68) |
| | | *C. bovis* | 6 | 6 | nd | 53·8 (22–69) |
| | | NA | 0 | 0 | 11 | |
| Beef | 11 | *C. parvum* | 7 | 6 | 5 IIa A15 G2 R1 | 18·4 (3–46) |
| | | *C. bovis* | 2 | 3 | nd | 51·3 (36–70) |
| | | *C. suis* | 1 | 1 | nd | 27 |
| | | NA | 1 | 1 | 2 | |
| Clinical | 31 | *C. parvum* | 31 | 31 | 27 IIa A15 G2 R1<br>1 IIa A16 G2 R1<br>1 IId A22 G1 | <6 weeks |
| | | NA | 0 | 0 | 2 | |
| Overall | 115 | *C. parvum* | 105 | 104 | 84 IIa A15 G2 R1<br>1 IIa A13 G2 R1<br>1 IIa A14 G2 R1<br>3 IIa A16 G2 R1<br>1 IId A22 G1 | |
| | | *C. bovis* | 8 | 9 | nd | |
| | | *C. suis* | 1 | 1 | nd | |
| | | NA | 1 | 1 | 15 | |

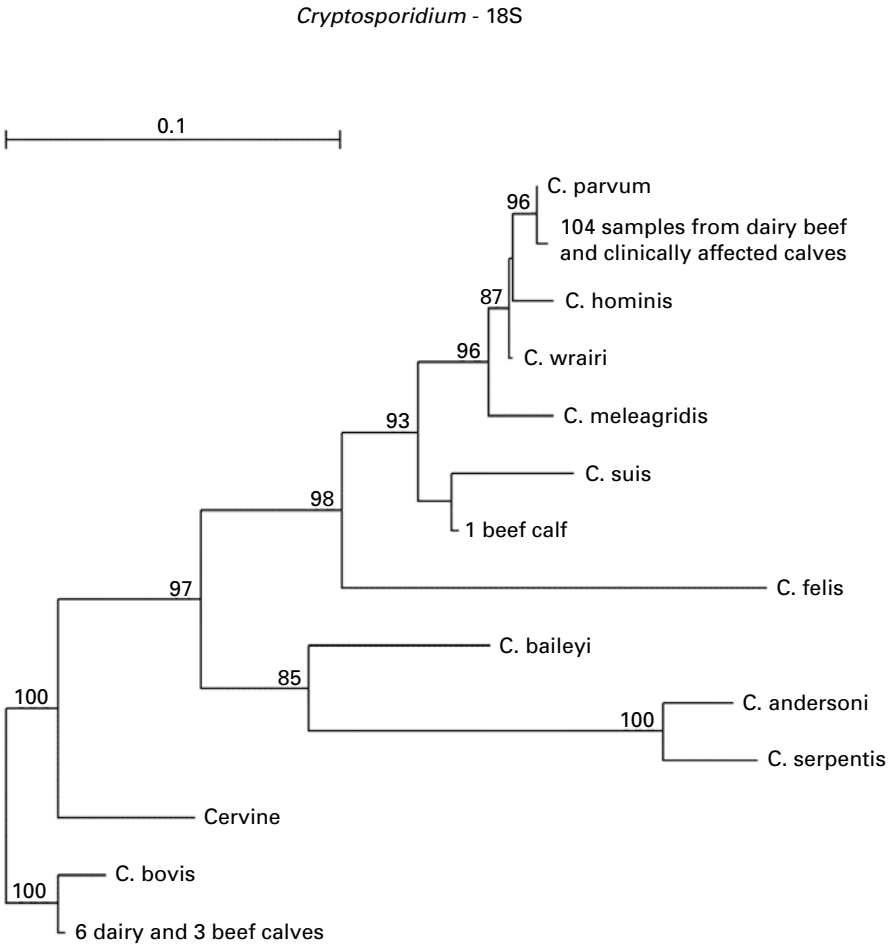


Fig. 1. Phylogenetic relationship of *Cryptosporidium* isolates (18S rDNA). Only bootstrap-values >70 are reported.

densities. Furthermore, cow-calf herds tend to stay on pasture, where the *Cryptosporidium* oocysts are more exposed to dessication and direct sunlight (Atwill *et al*. 1999; Geurden *et al*. 2006*b*). However, in the present study, both housing and management were similar on dairy and beef farms, with immediate separation of calves from the dam and rearing in either individual or group housing.

Since at least part of the human *C. parvum* infections can be ascribed to contact with livestock (Hunter and Thompson, 2005), it has been advocated to include tools for molecular identification in epidemiological studies (Traub *et al*. 2005). However, only a limited number of studies did so in the United States (Santin *et al*. 2004; Fayer *et al*. 2006) and in Australia (Becher *et al*. 2004). In Europe, a similar approach was used in Denmark and in Serbia-Montenegro (Langkjaer *et al*. 2007; Misic and Abe, 2007). In the present study, the guidelines for molecular identification of *Cryptosporidium* (Caccio *et al*. 2005) were taken into account, emphasizing that the genetic characterization of *Cryptosporidium* isolates should be based on 2 genetic loci, of which at least one is the conserved 18S rDNA gene. The genotyping results of the HSP-70 and 18S rDNA gene concurred in all but 2 samples, in which 1 of the 2 genes did not amplify. The majority of the *Cryptosporidium*-positive samples from the dairy and beef calves was identified as *C. parvum*. Since the calves included in the epidemiological study were all younger than 70 days, the current results are in accordance with previous findings that *C. parvum* is the most prevalent species in pre-weaned calves (Santin *et al*. 2004). The 70-days age limit might also account for the limited number of *C. bovis* infections, and for the absence of *C. andersoni* or the *Cryptosporidium* cervine genotype in the present study. All but 1 of the *C. bovis* infections were identified in calves older than 1 month, confirming that *C. bovis* is more prevalent in older calves compared to neonatal calves (Santin *et al*. 2004). In 1 beef calf *C. suis* was found, indicating a rather erratic parasitism of this *Cryptosporidium* species in cattle (Fayer *et al*. 2006). In the clinically affected calves solely *C. parvum* was identified, confirming the relevance of *C. parvum* in the aetiology of neonatal diarrhoea (de Graaf *et al*. 1999).

Of the *Cryptosporidium* species commonly found in calves, *C. parvum* is known to infect human patients. The molecular analysis on the gp60 gene identified all but 1 of the *C. parvum* positive calf isolates as the zoonotic IIa allele. All *C. parvum* IIa subtypes identified in the present study, except the IIaA14G2R1 subtype, have previously been described in calves. The IIaA15G2R1 subtype was predominantly identified, as in previous studies in Portugal (Alves *et al*. 2006), in the UK (Chalmers *et al*. 2005), and in Northern America (Peng *et al*. 2003; Trotz-Williams *et al*. 2006; Xiao *et al*. 2007). Although there are no data on the distribution of *C. parvum* subtypes in human patients in Belgium, in other countries the IIaA15G2R1 subtype is known to frequently infect human patients (Chalmers *et al*. 2005; Feltus *et al*. 2006). The occurrence of subtype IId in calves is rare, but has previously been reported (Alves *et al*. 2003, 2006). Contrary to a previous report (Xiao *et al*. 2007), the distribution of the *C. parvum* subtypes did not differ geographically in the present study, and the occurence of a subtype other than the predominant IIaA15G2R1 was not linked to a particular region. As expected, the anthroponotic *C. parvum* allele IIc was not found in any of the calves.

The present study demonstrated a high prevalence of *C. parvum* infections in Belgium, especially in dairy calves and to a lesser extent in beef calves. The results of the subtyping confirm that young calves should be considered as potential zoonotic reservoirs for human infections, although further studies are needed to elucidate the distribution of *C. parvum* subtypes in human patients in Belgium.

## REFERENCES

**Atwill, E. R., Johnson, E., Klingborg, D. J., Veserat, G. M., Markegard, G., Jensen, W. A., Pratt, D. W., Delmas, R. E., George, H. A., Forero, L. C., Philips, R. L., Barry, S. J., McDougald, N. K., Gildersleeve, R. R. and Frost, W. E.** (1999). Age, geographic, and temporal distribution of fecal shedding of *Cryptosporidium parvum* oocysts in cow-calf herds. *American Journal of Veterinary Research* **60**, 420–425.

**Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J.** (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.

**Alves, M., Xiao, L., Antunes, F. and Matos, O.** (2006). Distribution of *Cryptosporidium* subtypes in humans and domestic and wild ruminants in Portugal. *Parasitology Research* **99**, 287–292.

**Becher, K. A., Robertson, I. D., Fraser, D. M., Palmer, D. G. and Thompson, R. C.** (2004). Molecular epidemiology of *Giardia* and *Cryptosporidium* infections in dairy calves originating from three sources in Western Australia. *Veterinary Parasitology* **123**, 1–9.

**Bornay-Llinares, F. J., da Silva, A. J., Moura, I. N., Myjak, P., Pietkiewicz, H., Kruminis-Lozowska, W., Graczyk, T. K. and Pieniazek, N. J.** (1999). Identification of *Cryptosporidium felis* in a cow by morphologic and molecular methods. *Applied and Environmental Microbiology* **65**, 1455–1458.

**Caccio, S. M., Thompson, R. C., McLauchlin, J. and Smith, H. V.** (2005). Unravelling *Cryptosporidium* and *Giardia* epidemiology. *Trends in Parasitology* **21**, 430–437.

**Castro-Hermida, J. A., Gonzalez-Losada, Y. A. and Ares-Mazas, E.** (2002*a*). Prevalence of and risk factors involved in the spread of neonatal bovine

cryptosporidiosis in Galicia (NW Spain). *Veterinary Parasitology* **106**, 1–10.

**Castro-Hermida, J. A., Gonzalez-Losada, Y. A., Mezo-Menendez, M., Ares-Mazas, E.** (2002*b*). A study of cryptosporidiosis in a cohort of neonatal calves. *Veterinary Parasitology* **106**, 11–17.

**Chalmers, R. M., Ferguson, C., Caccio, S., Gasser, R. B., Abs, E. L. O. Y.G., Heijnen, L., Xiao, L., Elwin, K., Hadfield, S., Sinclair, M. and Stevens, M.** (2005). Direct comparison of selected methods for genetic categorisation of *Cryptosporidium parvum* and *Cryptosporidium hominis* species. *International Journal for Parasitology* **35**, 397–410.

**de Graaf, D. C., Vanopdenbosch, E., Ortega-Mora, L. M., Abbassi, H. and Peeters, J. E.** (1999). A review of the importance of cryptosporidiosis in farm animals. *International Journal for Parasitology* **29**, 1269–1287.

**Edgar, R. C.** (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.

**Fayer, R., Santin, M., Trout, J. M. and Greiner, E.** (2006). Prevalence of species and genotypes of *Cryptosporidium* found in 1–2-year-old dairy cattle in the eastern United States. *Veterinary Parasitology* **135**, 105–112.

**Fayer, R., Trout, J. M., Xiao, L., Morgan, U. M., Lai, A. A. and Dubey, J. P.** (2001). *Cryptosporidium canis* n. sp. from domestic dogs. *Journal of Parasitology* **87**, 1415–1422.

**Feltus, D. C., Giddings, C. W., Schneck, B. L., Monson, T., Warshauer, D. and McEvoy, J. M.** (2006). Evidence supporting zoonotic transmission of *Cryptosporidium* spp. in Wisconsin. *Journal of Clinical Microbiology* **44**, 4303–4308.

**Geurden, T., Berkvens, D., Geldhof, P., Vercruysse, J. and Claerebout, E.** (2006*a*). A Bayesian approach for the evaluation of six diagnostic assays and the estimation of *Cryptosporidium* prevalence in dairy calves. *Veterinary Research* **37**, 671–682.

**Geurden, T., Goma, F. Y., Siwila, J., Phiri, I. G., Mwanza, A. M., Gabriel, S., Claerebout, E. and Vercruysse, J.** (2006*b*). Prevalence and genotyping of *Cryptosporidium* in three cattle husbandry systems in Zambia. *Veterinary Parasitology* **138**, 217–222.

**Gow, S. and Waldner, C.** (2006). An examination of the prevalence of and risk factors for shedding of *Cryptosporidium* spp. and *Giardia* spp. in cows and calves from western Canadian cow-calf herds. *Veterinary Parasitology* **137**, 50–61.

**Hall, T. A.** (1999). BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

**Hunter, P. R. and Thompson, R. C.** (2005). The zoonotic transmission of *Giardia* and *Cryptosporidium*. *International Journal for Parasitology* **35**, 1181–1190.

**Kvac, M., Kouba, M. and Vitovec, J.** (2006). Age-related and housing-dependence of *Cryptosporidium* infection of calves from dairy and beef herds in South Bohemia, Czech Republic. *Veterinary Parasitology* **137**, 202–209.

**Langkjaer, R. B., Vigre, H., Enemark, H. L. and Maddox-Hyttel, C.** (2007). Molecular and phylogenetic characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark. *Parasitology* **134**, 339–350.

**Maddox-Hyttel, C., Langkjaer, R. B., Enemark, H. L. and Vigre, H.** (2006). *Cryptosporidium* and *Giardia* in different age groups of Danish cattle and pigs–occurrence and management associated risk factors. *Veterinary Parasitology* **141**, 48–59.

**Maldonado-Camargo, S., Atwill, E. R., Saltijeral-Oaxaca, J. A. and Herrera-Alonso, L. C.** (1998). Prevalence of and risk factors for shedding of *Cryptosporidium parvum* in Holstein Freisian dairy calves in central Mexico. *Preventive Veterinary Medicine* **36**, 95–107.

**Misic, Z. and Abe, N.** (2007). Subtype analysis of *Cryptosporidium parvum* isolates from calves on farms around Belgrade, Serbia and Montenegro, using the 60 kDa glycoprotein gene sequences. *Parasitology* **134**, 351–358.

**Morgan, U. M., Monis, P. T., Xiao, L., Limor, J., Sulaiman, I., Raidal, S., O'Donoghue, P., Gasser, R., Murray, A., Fayer, R., Blagburn, B. L., Lal, A. A. and Thompson, R. C.** (2001). Molecular and phylogenetic characterisation of *Cryptosporidium* from birds. *International Journal for Parasitology* **31**, 289–296.

**Nydam, D. V., Wade, S. E., Schaaf, S. L. and Mohammed, H. O.** (2001). Number of *Cryptosporidium parvum* oocysts or *Giardia* spp. cysts shed by dairy calves after natural infection. *American Journal of Veterinary Research* **62**, 1612–1615.

**O'Handley, R. M., Cockwill, C., McAllister, T. A., Jelinski, M., Morck, D. W. and Olson, M. E.** (1999). Duration of naturally acquired giardiosis and cryptosporidiosis in dairy calves and their association with diarrhea. *Journal of the American Veterinary Medical Association* **214**, 391–396.

**Olson, M. E., Guselle, N. J., O'Handley, R. M., Swift, M. L., McAllister, T. A., Jelinski, M. D. and Morck, D. W.** (1997). *Giardia* and *Cryptosporidium* in dairy calves in British Columbia. *Canadian Veterinary Journal* **38**, 703–706.

**Pedraza-Diaz, S., Amar, C. F., McLauchlin, J., Nichols, G. L., Cotton, K. M., Godwin, P., Iversen, A. M., Milne, L., Mulla, J. R., Nye, K., Panigrahl, H., Venn, S. R., Wiggins, R., Williams, M. and Youngs, E. R.** (2001). *Cryptosporidium meleagridis* from humans: molecular analysis and description of affected patients. *Journal of Infection* **42**, 243–250.

**Peng, M. M., Matos, O., Gatei, W., Das, P., Stantic-Pavlinic, M., Bern, C., Sulaiman, I. M., Glaberman, S., Lal, A. A. and Xiao, L.** (2001). A comparison of *Cryptosporidium* subgenotypes from several geographic regions. *Journal of Eukaryotic Microbiology* (Suppl.) 28S–31S.

**Peng, M. M., Wilson, M. L., Holland, R. E., Meshnick, S. R., Lal, A. A. and Xiao, L.** (2003). Genetic diversity of *Cryptosporidium* spp. in cattle in Michigan: implications for understanding the transmission dynamics. *Parasitology Research* **90**, 175–180.

**Peng, M. M., Xiao, L., Freeman, A. R., Arrowood, M. J., Escalante, A. A., Weltman, A. C., Ong, C. S., Mac Kenzie, W. R., Lal, A. A. and Beard, C. B.** (1997). Genetic polymorphism among *Cryptosporidium parvum* isolates: evidence of two distinct human

transmission cycles. *Emerging Infectious Diseases* **3**, 567–573.

**Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A. A. and Pavlasek, I.** (2003). Identification of novel *Cryptosporidium* genotypes from the Czech Republic. *Applied Environmental Microbiology* **69**, 4302–4307.

**Santin, M., Trout, J. M., Xiao, L., Zhou, L., Greiner, E. and Fayer, R.** (2004). Prevalence and age-related variation of *Cryptosporidium* species and genotypes in dairy calves. *Veterinary Parasitology* **122**, 103–117.

**Smith, H. V., Nichols, R. A., Mallon, M., Macleod, A., Tait, A., Reilly, W. J., Browning, L. M., Gray, D., Reid, S. W. and Wastling, J. M.** (2005). Natural *Cryptosporidium hominis* infections in Scottish cattle. *Veterinary Record* **156**, 710–711.

**Traub, R. J., Monis, P. T. and Robertson, I. D.** (2005). Molecular epidemiology: a multidisciplinary approach to understanding parasitic zoonoses. *International Journal for Parasitology* **35**, 1295–1307.

**Trotz-Williams, L. A., Martin, D. S., Gatei, W., Cama, V., Peregrine, A. S., Martin, S. W., Nydam, D. V., Jamieson, F. and Xiao, L.** (2006). Genotype and subtype analyses of *Cryptosporidium* isolates from dairy calves and humans in Ontario. *Parasitology Research* **99**, 346–352.

**Van de Peer, Y. and De Wachter, R.** (1994). TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Computional and Applied Bioscience* **10**, 569–570.

**Xiao, L., Zhou, L., Santin, M., Yang, W. and Fayer, R.** (2007). Distribution of *Cryptosporidium parvum* subtypes in calves in eastern United States. *Parasitology Research* **100**, 701–706.