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SHORT COMMUNICATION

Occurrence of *Cryptosporidium parvum* and *Giardia duodenalis* in healthy adult domestic ruminants

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Abstract To determine the prevalence and intensity of infection of *Cryptosporidium* spp. and *Giardia duodenalis* in healthy adult domestic ruminants, faecal samples were collected from 379 cattle of between 3 and 13 years old, 446 sheep and 116 goats selected at random from 60 dairy farms and 38 and 20 herds, respectively, in Galicia (NW Spain). *Cryptosporidium* spp. oocysts were detected in 32 cows (8.4%), 24 sheep (5.3%) and in nine goats (7.7%) from, respectively, 48.3% of the farms and 34.2 and 30.0% of the herds. The intensity of infection in cows ranged between 25 and 5,924 oocysts per gram of faeces (OPG), whereas in sheep and goats, the number of oocysts shed ranged from 8–515 OPG and from 17–782 OPG, respectively. Parasitization by *Cryptosporidium* spp. was significantly higher ($P<0.05$) in cows than in sheep and goats. *G. duodenalis* cysts were identified in 101 cows (26.6%), 86 sheep (19.2%) and 23 goats (19.8%) from, respectively, 96.6% of the farms and 92.1 and 90% of the herds. The number of cysts shed by cows ranged between 15 and 3,042 cyst per gram of faeces (CPG), whereas the intensity of infection in sheep and goats ranged from 16–3010 CPG and from 15–1845 CPG, respectively, and was significantly lower ($P<0.05$) than in cows and sheep. The number of *Cryptosporidium* spp. oocysts isolated from sheep and goats was insufficient for successful polymerase chain reaction analysis. Nevertheless, gene sequence analysis of the hsp70 and 18SrRNA genes of *Cryptosporidium* revealed the presence of only *C. parvum* in faecal samples from cows. Genotyping studies of the β-giardin and glutamate dehydrogenase genes of *G. duodenalis* revealed mainly assemblage E of *Giardia* in cows, sheep and goat faecal samples. Assemblage B of *G. duodenalis* was also detected in one sheep sample. These animals should be considered as a possible source of cryptosporidiosis and giardiosis, thereby maintaining the infections on farms and in herds.

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Introduction

The genera *Cryptosporidium* and *Giardia* have been recognised as important enteropathogens in various species of domesticated animals and in humans, as well as the most common causes of waterborne gastroenteritis. Many studies have been carried out recently to investigate the prevalence of these protozoans in domestic ruminants (Castro-Hermida et al. 2005b, 2006; Fayer et al. 2006). Infection by *Cryptosporidium* and *Giardia* has been associated with economic losses, through the occurrence of diarrhoea in productive animals, and also death of the animals. The age of the animals is one of the most important risk factors associated with cryptosporidiosis and giardiosis, with young animals being more susceptible to infection than adults. Nevertheless, it has been shown that asymptomatic adult domestic ruminants, especially cows, sheep and goats, may act as healthy carriers and

may be a source of infection for younger animals (Fayer et al. 2000b; Bomfim et al. 2005). *Cryptosporidium* oocysts and *Giardia* cysts have been demonstrated in run-off from agricultural areas (Slifko et al. 2000; Fayer 2004), and some outbreaks of cryptosporidiosis and giardiosis in humans have been attributed to contamination of drinking and bathing water with such effluents (Rose 2007).

Recent studies in the USA have suggested that cattle can be infected by at least four species of *Cryptosporidium*: *C. parvum*, *C. andersoni*, *C. bovis* and the *Cryptosporidium* deer-like genotype (Lindsay et al. 2000; Fayer et al. 2005, 2006, 2007; Feng et al. 2007). The occurrence of these *Cryptosporidium* spp. in cattle was shown to be age-related (Santín et al. 2004; Fayer et al. 2006). *Cryptosporidium parvum* infects the small intestine—primarily in pre-weaned calves—although also in humans and other animals, often causing diarrhoeal disease (Castro-Hermida et al. 2002; Santín et al. 2004; Kváč et al. 2006). *Cryptosporidium andersoni* infects the abomasums of juvenile and mature cattle; infection has been identified as a cause of reduced milk production, but has not been associated with other signs of disease and is not known to infect animals other than cattle (Lindsay et al. 2000; Enemark et al. 2002). *Cryptosporidium bovis* has been found to be the predominant species infecting calves between 2 and 11 months old, but is not associated with overt disease (Santín et al. 2004). Kváč et al. (2004) failed in their attempt to infect lambs experimentally with *C. andersoni*, and only *C. parvum* has been detected in sheep and goats.

Adult ruminants are generally considered refractory to heavy infections by *Cryptosporidium* spp. and *G. duodenalis* and associated clinical diseases because of the strong immune response that they produce. Nevertheless, these animals can act as a source of infection for younger animals, especially during the periparturient period (Fayer et al. 2000a; Ralston et al. 2003; Castro-Hermida et al. 2005a). The most commonly detected genotypes of *Giardia duodenalis*, i.e. assemblages A and B, have the widest host ranges, including humans and a variety of other animals such as cattle. Assemblages C and D have been reported only in dogs, and assemblages E, F and G have been reported only in livestock, cats and rats, respectively (Monis et al. 2003; Trout et al. 2005). There is currently scant information available on the prevalence of *Cryptosporidium* spp. and *G. duodenalis* and the genotypes present in adult domestic ruminants (cows, sheep and goats), especially healthy specimens, in Europe. The purpose of the present study was to document the prevalence, intensity of infection and molecular characterisation of *Cryptosporidium* spp. and *G. duodenalis* isolates from healthy adult domestic ruminants in Galicia (NW Spain).

Materials and methods

Samples collection and examination

The study was carried out in Galicia, in the northwest of Spain, a region characterised by a high density of domestic ruminants, especially cattle. Faecal samples were collected from healthy and asymptomatic cattle (379) of between 3 and 13 years old, selected at random from 60 dairy farms, and from healthy, asymptomatic sheep (446) and goats (116), selected at random from 38 and 20 herds, respectively. The samples were taken directly from the rectum with sterile plastic gloves. For each animal, the sampling date, origin, age and identification number were recorded. The samples were transported to the laboratory in a cool box and then stored for a maximum of 24 h before analysis. Between 2 and 4 g of faeces were weighed, and *Cryptosporidium* spp. oocysts and *G. duodenalis* cysts were detected by a direct immunofluorescence technique with monoclonal antibodies (IFAT; Aqua-Glo G/C Direct, FL, comprehensive kit, Waterborne, New Orleans, LA, USA) according to the manufacturer's instructions. The samples were observed by fluorescence microscopy at 400× magnification. The number of oocysts per gram of faeces (OPG) and the number of cysts per gram of faeces (CPG) was calculated by {number of oocysts or cysts identified/[volume of sample examined (ml) × weight of faeces (g)]}. Positive samples, identified by microscopy, were processed for parasite isolation before DNA extraction. The samples were cleaned by CsCl gradient centrifugation, and *Cryptosporidium* spp. oocysts and *Giardia* cysts were purified by immunomagnetic separation with magnetic beads coated with a monoclonal antibody (GC-Combo; Dynal, Invitrogen, Oslo, Norway).

DNA extraction

Total DNA was extracted from each CsCl-cleaned faecal sample and concentrated by immunomagnetic separation by the use of a QIAmp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions with slight modifications. The whole volume of processed faeces was used for DNA extraction, and this sample was incubated overnight at 55°C with the lysis ATL buffer and proteinase K (20 mg/ml) provided by the manufacturer. The nucleic acid was also concentrated by elution in 100 µl of AE buffer.

Gene amplification by PCR and DNA sequence analysis

Polymerase chain reaction (PCR) was performed to amplify fragments of the 18SrRNA gene (1,325 bp in the primary reaction and 826 or 824 bp in the second reaction

Table 1 Prevalence and intensity of infection by *C. parvum* and *G. duodenalis* in asymptomatic adult domestic ruminants

	C. parvum			*G. duodenalis*		
Animals	Prevalence (%)	Min–Max (OPG)	Mean (OPG)	Prevalence (%)	Min–Max (CPG)	Mean (CPG)
Cows	8.4	25–5,924	920[a]	26.6	15–3,042	173
Sheep	5.3	8–515	53	19.2	16–3,010	324
Goats	7.7	17–782	184	19.8	15–1,845	113[b]

OPG Number of oocysts per gram of faeces, *CPG* number of cysts per gram of faeces
[a] The mean intensity of infection was significantly higher ($P<0.05$) than in sheep and goats.
[b] The mean intensity of infection was significantly lower ($P<0.05$) than in cows and sheep.

depending on isolates) and the hsp70 gene (346 bp) of *Cryptosporidium*, as previously described by Xiao et al. (1999) and LeChevallier et al. (2003). The procedures followed for the *Giardia* β-giardin gene (753 bp in the primary reaction, and 384 bp in the second reaction) and GDH (glutamate dehydrogenase, ≈432 bp) were as described by Cacciò et al. (2002) and Read et al. (2004). Sequence chromatograms were aligned with ProSeq® 2.0 software and inspected with ChromasPro® software. The sequences obtained were compared against a database (GenBank at www.ncbi.nlm.nih.gov/BLAST) obtained with BLAST software to determine the species and genotype of *Giardia* and *Cryptosporidium*.

Statistical analysis

The prevalence and intensity of infection were compared by a test of comparison of proportions and the Kruskal–Wallis test (non-parametric analysis of variance), respectively, with GraphPad InStat® (version 3.05). Differences were considered statistically significant at $P<0.05$.

Results

Cryptosporidium spp. oocysts were detected in 32 cows (8.4%), 24 sheep (5.3%) and in nine goats (7.7%) from 29 dairy farms (48.3%), 13 herds (34.2%) and six herds (30.0%), respectively. The intensity of infection in cows ranged between 25 and 5,924 OPG (mean 920 OPG), whereas in sheep and goats, the number of oocysts shed ranged from 8–515 OPG (mean 53 OPG) and from 17–782 OPG (mean 184 OPG), respectively. Moreover, parasitization by *Cryptosporidium* spp. was significantly higher ($P<0.05$) in cows than in sheep and goats. *G. duodenalis* cysts were identified in 101 cows (26.6%), 86 sheep (19.2%) and 23 goats (19.8%) from, respectively, 96.6% of the farms and 92.1 and 90% of the herds. The number of cysts shed by cows ranged between 15 and 3,042 CPG (mean 173 CPG), whereas the intensity of infection in sheep and goats ranged from 16–3,010 CPG (mean 324 CPG) and from 15–1,845 CPG (mean 113 CPG), respectively, and was significantly lower ($P<0.05$) than in cows and sheep (Table 1). The prevalence of *G. duodenalis* in dairy farms and herds of sheep and goats was significantly higher ($P<0.05$) than the prevalence of *Cryptosporidium* spp. Moreover, the percentage of asymptomatic adult domestic ruminants infected by *G. duodenalis* was significantly higher ($P<0.05$) than the percentage of the animals infected by *Cryptosporidium* spp.

Genotyping studies were only carried out with faecal samples from animals with a high intensity of infection because PCR amplification of the DNA of *Cryptosporidium* spp. and *Giardia* isolated from samples containing low numbers of parasites (<800 OPG or CPG) was not possible. Even when an insufficient quantity of faeces was available for molecular analysis, each positive slide (as evaluated by the immunofluorescence antibody technique, IFAT) was rinsed with dH_2O with the aim of recovering oocysts and cysts. Nevertheless, when the quantity was low (less than one oocyst or cyst per field of view), few parasites were recovered, and PCR was unsuccessful. Finally, only *C. parvum* was identified in ten positive PCR from cattle samples. Unfortunately, we were not able to detect any positive PCR for *Cryptosporidium* spp. in any of the faecal samples from sheep or goat because of the small amount of faecal material available and low parasite loading. Two genotypes of *G. duodenalis*—assemblages E and B—were identified in asymptomatic adult domestic ruminants. Assemblage E was detected in four faecal samples from cows, in 11 faecal samples from sheep and in one goat faecal sample. Assemblage B was identified only in one sheep faecal sample.

Discussion

Although infections by *Cryptosporidium* spp. and *G. duodenalis* have been reported for cattle, sheep and goats in many parts of the world, the prevalence data have often varied markedly. Studies have commonly been carried out

in neonates and young animals, but less is known about the prevalence of infection in adult domestic ruminants, especially healthy animals. Galicia is a region characterised by a high density of domestic ruminants. The large number of farms and herds, the large proportion of adult animals (around 90%) and the large volume of faeces that they produce demonstrate the importance of adult animals as a potential source of environment contamination and as possible reservoirs of infectious parasites (Fayer et al. 2000b; Castro-Hermida et al. 2006).

In the present study, the prevalence and intensity of infection by both *C. parvum* and *G. duodenalis* in cows, sheep and goats samples were relatively low, but widespread. The results obtained reflect a serious situation, taking into account that the study was carried out on healthy adult animals and that the farms were selected at random and the only possible restriction was whether the farmers consented to the study. Moreover, as only one faecal sample was collected per animal, the prevalence data probably underestimates the actual number of infected animals.

Both prevalence and intensity of infection by *G. duodenalis* were significantly higher than for *C. parvum*, as has also been observed in other studies (Fayer et al. 2000b; Castro-Hermida et al. 2005b; Maddox-Hyttel et al. 2006). One possible reason for this is that *G. duodenalis* infections usually last longer than *C. parvum* infections, often becoming chronic, whereas *C. parvum* infections may be acute, but usually with spontaneous recovery over a relatively short time period. Therefore, *G. duodenalis* infections are more likely to be identified in single samples.

The use of the IFAT is a suitable method for determining the prevalence and intensity of infection by the protozoan parasites *Cryptosporidium* spp. and *G. duodenalis* isolated from faecal samples. This technique presents good affinity, intensity and specificity. The manipulation of samples is minimal, and the loss of parasites is low. Moreover, the need to observe the samples in the microscope guarantees the likelihood of correct identification. Most recently published prevalence studies of these parasites have involved microscopic methods (e.g. Fayer et al. 2000b; Ralston et al. 2003; Bomfim et al. 2005; Castro-Hermida et al. 2006; Maddox-Hyttel et al. 2006). Nevertheless, microscopic methods alone are not sufficient for identifying the species and genotypes infecting domestic ruminants. Therefore, when discussing animal cryptosporidiosis and giardiosis in a public health context, it is necessary to identify clearly the species or genotypes involved. Molecular characterisation of *Cryptosporidium* spp. and *G. duodenalis* has helped to clarify the confusion in the taxonomy, especially for *Cryptosporidium* spp., and to validate the existence of multiple species, genotypes or assemblages, knowledge of which is required for understanding the zoonotic potential and also the transmission routes of these parasites (Lalle et al. 2005; Boulter-Bitzer et al. 2007; Feng et al. 2007).

PCR-based approaches are also very sensitive, although they must be carried out with very clean purified samples. In the present study, IFAT was more sensitive than PCR for detecting *Cryptosporidium* oocysts and *G. duodenalis* cysts in faecal samples from cows, sheep and goats. Thus, after extraction of DNA from samples containing low numbers of parasites (<800 OPG or CPG), we were not able to amplify *Cryptosporidium* spp. and *Giardia* DNA by PCR. This may have been due to inhibitors present in the faeces, the small volumes used or to loss of parasites during concentration and purification before carrying out the PCR. Although PCR is a highly sensitive and useful technique, so far, we have only been able to apply it successfully to samples with a high parasite load, which makes purification of the oocysts/cysts easier. The PCR technique is therefore of rather limited use or faecal samples with low parasite loading. The present data provide no further information on the species or genotypes of *Cryptosporidium* that infect sheep and goats because of the poor success with the PCR technique. The protocol must be modified so that DNA of parasites can be amplified even when faecal samples contain only low numbers oocysts/cysts. In a study of *Cryptosporidium* spp. and *Giardia* in red foxes (*Vulpes vulpes*), Hamnes et al. (2007) did not obtain positive results with PCR detection of the *Cryptosporidium* SSU-rRNA gene and suggested that this was probably due to the low numbers of nucleated oocysts in the positive samples examined.

The intensity of infection by *Cryptosporidium* spp. in cows ranged between 25 and 5,924 OPG, whereas in sheep and goats, the number of oocysts shed ranged from 8–515 OPG and from 17–782 OPG, respectively. The number *G. duodenalis* cysts shed by cows ranged between 15 and 3,042 CPG, whereas the intensity of infection in sheep and goats ranged from 16–3,010 CPG and from 15–1,845 CPG, respectively, figures that are similar to those of previous studies of apparently healthy animals (Ralston et al. 2003; Castro-Hermida et al. 2005a; Maddox-Hyttel et al. 2006).

The occurrence of *Cryptosporidium* spp. in cattle was shown to be age-related. Thus, recent studies reported that 85% of pre-weaned dairy calves were infected by zoonotic *C. parvum*, whereas only 0.4–2% of post-weaned calves, 1- to 2-year-old heifers and mature dairy cattle were infected by this species (Santín et al. 2004; Fayer et al. 2006, 2007; Feng et al. 2007). Moreover, in a study of *Cryptosporidium* infection in calves from dairy and beef herds in south Bohemia (Czech Republic), in relation to age and housing, Kváč et al. (2006) did not detect any *C.*

parvum infections in post-weaned calves. There are very few reports about *Cryptosporidium* spp. in cows of more than 3 years old, nevertheless, in the present study, only *C. parvum* was identified in ten PCR-positive cows, which indicates that these animals are also sources of zoonotic cryptosporidiosis for humans.

Genetic analysis for *G. duodenalis* showed that assemblage E was the most prevalent genotype detected in cows, sheep and goats. Thus, assemblage E was detected in four faecal samples from cows, in 11 faecal samples from sheep and in one goat faecal sample. This genotype is common in domestic ruminants, and there are no epidemiological or genetic data that support the zoonotic potential of this assemblage (Becher et al. 2004; Fayer et al. 2004; Hunter and Thompson, 2005; Trout et al. 2005). The present results are consistent with those of Ryan et al. (2005), who suggested that sheep may not be an important zoonotic reservoir for *G. duodenalis*, and those of Langkjaer et al. (2007) who concluded that cows are only infected by isolates of the livestock group, assemblage E. Other studies of cattle in Australia and North America have also indicated a minimal risk to public health related to the prevalence of *G. duodenalis* in livestock (O'Handley et al. 2000; Hoar et al. 2001).

However, it is reported that domestic ruminants are susceptible to infection by zoonotic genotypes of *G. duodenalis* and that assemblage A is the most commonly reported zoonotic genotype (Thompson et al. 2000). Moreover, it has been suggested that the patent periods may differ for genotype A (zoonotic) and genotype E (non-zoonotic; Trout et al. 2005). Interestingly, in the present study, only one isolate of *G. duodenalis* assemblage B (zoonotic genotype) was detected in a sheep faecal sample. Because of the small number of animals sampled, it is not clear whether the results represent differences in the geographical distribution of assemblage A, and further studies are required to confirm this hypothesis. Similar studies have been carried out in northern Portugal, which borders with Galicia (the combined area represents a large area of animal production and is denominated the "Galicia–Northern Portugal Euroregion") and have shown that the prevalent *G. duodenalis* genotype in livestock in the area were assemblages E, A and B (Mendonça, unpublished data). The results of the present study appear to suggest that healthy adult domestic ruminants may not be an important zoonotic reservoir of *G. duodenalis*, although these animals may harbour some *G. duodenalis* assemblages that are infective to humans. Nevertheless, the variation in the gene assemblages inhibits our ability to estimate the risk of these animals as a reservoir of *G. duodenalis* with the potential to infect humans.

The results of the current study show a low but widespread prevalence of *C. parvum* and *G. duodenalis* in healthy adult domestic ruminants. The genotyping information suggests that cows are a potential source of environmental contamination by *C. parvum*. The *G. duodenalis* harboured by these animals principally infect other domestic ruminants and wild animals, although humans can also become infected by exposure to infected sheep.

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