Molecular and phylogenetic characterization of *Cryptosporidium* and *Giardia* from pigs and cattle in Denmark

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SUMMARY

The genetic diversity of *Cryptosporidium* spp. and *Giardia duodenalis* from dairy cattle and pigs in Denmark was determined in the present study. Faecal samples from 1237 pigs and 1150 cattle originating from 50 sow herds and 50 dairy herds, respectively, were analysed for the presence of the two parasites by immunofluorescence microscopy. A large proportion of the (oo)cyst containing samples were selected for molecular characterization. Sequencing and phylogenetic analysis of the 18S rDNA locus and/or the *HSP70* gene of 183 pig and 154 cattle isolates of *Cryptosporidium* revealed the presence of *C. suis*, pig genotype II, *C. parvum* (cattle genotype), *C. bovis*, *Cryptosporidium* deer-like genotype and a novel *C. suis*-like genotype. For both cattle and pigs, a host age-related change in distribution of species/genotypes was observed. The zoonotic *C. parvum* (cattle genotype) was most prevalent in young calves. For *Giardia*, 82 and 145 isolates from pigs and cattle, respectively, were analysed at the 18S rDNA locus and/or the *gdh* gene. *Giardia* isolates belonging to the zoonotic Assemblage A was found in both young and older calves, as well as in weaners and piglets, whereas cows seemed to be infected purely by isolates of the livestock group, Assemblage E.

Key words: *Cryptosporidium* spp., *Giardia duodenalis*, dairy cattle, pig, Denmark, genetic diversity, zoonotic genotypes.

INTRODUCTION

The protozoan parasites, *Cryptosporidium* and *Giardia* are ubiquitous and infect a wide range of vertebrates, including humans and domestic animals, often causing diarrhoea. *Cryptosporidium* and *Giardia* are shed in faeces as oocysts and cysts, respectively, and can be transmitted via the faecal/oral route for example by contaminated food or water. Whereas some species/genotypes are host-specific, others are potentially zoonotic (Cacciò *et al*. 2005). Oocysts and cysts have been found in run-off from agricultural areas, which thus constitute potential routes of transmission through contamination of drinking and recreational waters. While such effluents have been associated with outbreaks of cryptosporidiosis in humans (Barwick *et al*. 2003; Fayer, 2004; Thurston-Enriquez *et al*. 2005), this route of transmission of *Giardia* from animals to humans remains controversial (Thompson, 2004; Thompson and Monis, 2004).

At present, besides the 15 recognized *Cryptosporidium* species, a number of distinct genotypes have been described and several seem to be host specific (Cacciò *et al*. 2005; Fayer *et al*.). Most human infections are caused by the anthroponotic *C. hominis* and the zoonotic *C. parvum* (cattle genotype), but other species have also been reported to infect humans including, e.g. *C. suis* (Xiao *et al*. 2004). Previous reports have shown that pigs can be infected with the host-adapted *C. suis* and pig genotype II (Ryan *et al*. 2003, 2004) as well as *C. parvum* (cattle genotype) (Morgan *et al*. 1999). Besides *C. parvum* (cattle genotype) cattle can be infected with *C. andersoni, C. bovis* and *Cryptosporidium* deer-like genotype (Fayer *et al*. 2005; Olson *et al*. 2004; Santín *et al*. 2004).

The genus *Giardia* comprises 6 species. However, the only known species to cause human infections is *Giardia duodenalis* (synonym for *G. intestinalis*; *G. lamblia*). Isolates of *G. duodenalis*, which cannot be distinguished based on morphology, are commonly divided into different groups/genotypes (Assemblage A, B, C etc.) and subgroups/subgenotypes (A1, A2, BIII, BIV) based on genetic differences (Cacciò *et al*. 2005; Thompson and Monis, 2004). The host range of Assemblage A and B

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isolates include a wide variety of mammals, whereas isolates from the other groups are more host-specific, like the livestock genotype, Assemblage E. So far, only isolates from Assemblage A and B have been associated with human infections, and the zoonotic potential is still uncertain (Cacciò *et al*. 2005).

In Denmark, both parasites are known to occur in dairy cattle (Henriksen and Krogh, 1985; Iburg *et al*. 1996), and lately, *Cryptosporidium* has also been found in pigs (Enemark *et al*. 2003). Most recently, a large survey was undertaken, both to acquire local data on the prevalence within different age groups of production animals and, to acquire more insight into which herd management factors might be pivotal for the occurrence of these protozoan parasites (Maddox-Hyttel *et al*. 2006).

So far, few molecular studies of *Cryptosporidium* in Danish cattle and pigs have been made (Enemark *et al*. 2002*a*, *b*; Enemark *et al*. 2003) but none concerning *Giardia*. In order to assess the genetic diversity of *Cryptosporidium* spp. and *G. duodenalis* from Danish dairy cattle and pigs and thereby the zoonotic potential, an extensive study was made to determine the genotypes and subgenotypes of the two parasites obtained in the survey by Maddox-Hyttel *et al*. (2006), and most importantly, the distribution related to host age was examined. The distribution of the wide variety of novel and well-known species/genotypes of both parasites are described in the present study.

MATERIALS AND METHODS

Study design

The final study population comprised 50 dairy herds and 50 sow herds. Herds were selected from the Danish Central Husbandry Register using simple random sampling. Only herds with a minimum size of 50 reproducing female animals were selected. Each herd was visited once for the collection of faecal samples. On each farm, rectal faecal samples were collected from different age groups of animals, i.e. 5 lactating cows or sows, 10 calves less than 1 month ('young') or 10 suckling piglets (2 from each sow), and 10 calves 3–12 months ('older') or 10 pigs 8–30 kg ('weaners'). In order to only examine fresh samples in the laboratory, the farm visits were extended over a period of 1 year (August 2003–July 2004). At the laboratory, samples were kept at 5 °C until examination (for further details see Maddox-Hyttel *et al*. 2006). Samples were labelled according to animal species (cattle or pig), farm number (1–50) and animal number (1–25), e.g. cattle sample K3612 or pig sample S3021.

Parasitological techniques

Purification and quantification of *Giardia* cysts and *Cryptosporidium* oocysts were carried out as described by Maddox-Hyttel *et al*. (2006). For each sample, 1 g of faeces was subject to purification resulting in a 2–3 ml suspension, which was used for further analysis. Based on immunofluorescence microscopy, the total numbers of oocysts (*Cryptosporidium*) and cysts (*Giardia*) were determined according to morphology.

DNA isolation and species/genotype assignment

Following purification and enumeration, 1·0–1·5 ml of the purified suspension from selected samples (for details on selection of samples for genotyping see *Statistical analysis*) was centrifuged at 8000 ***g*** for 2 min and total DNA was isolated from the pellet using the QIAamp® DNA Stool Mini Kit (QIAGEN GmbH, Hilden, Germany). The manufacturer's directions were followed except that the lysis temperature was increased to 95 °C, and the DNA was eluted with only 100 μl of Buffer AE.

For molecular typing of *Cryptosporidium* (species/genotypes), partial nucleotide sequences were obtained by polymerase chain reaction (PCR) amplification and sequencing of the small subunit ribosomal RNA gene (18S rDNA locus) (approximately 925 bp) and the *HSP70* gene (325 bp) according to Enemark *et al*. (2002*a*) and Morgan *et al*. (2001) with slight modifications. *C. parvum* (cattle genotype) isolates were further analysed by PCR amplification and sequencing of the microsatellite, ML1, according to the methods of Cacciò *et al*. (2000).

Molecular typing of *G. duodenalis* was performed by PCR and partial sequencing of the 18S rDNA locus (Read *et al*. 2002) and the *gdh* gene (Read *et al*. 2004) with modifications (see below).

PCR amplification

For all 5 loci, the PCR mix contained 1× PCR Gold Buffer, 1·5–2·0 mM $MgCl_2$, 1–2 units of AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Branchburg, New Jersey, USA), 120–160 μM dNTP and 12·5 pmol of each primer (primer sequences are shown in Table 1) in a total volume of 25 μl. The PCR mix included 1–5 μl of purified DNA as template for primary steps and 1 μl of primary PCR product for secondary steps. In addition, both the primary and the secondary step of the nested PCR of *Giardia* 18S rDNA contained approximately 5% dimethyl sulfoxide (DMSO), whereas the primary step of the semi-nested PCR of *Giardia gdh* and the PCR mix for *Cryptosporidium* 18S rDNA both contained 0·16% (w/v) bovine serum albumin (BSA).

The PCR was carried out in a Biometra T3 Thermocycler (Biometra GmbH i. L., Göttingen, Germany) with an initial hot start (95 °C for 10 min) and a final extension (72 °C for 7 min). In the case of *Cryptosporidium*, the 18S rDNA locus was amplified by 45 PCR cycles (94 °C for 60 sec, 57 °C for 25 sec,

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Table 1. Primer sequences used for PCR and sequencing

Locus	Primer	Sequence (5′-3′)	Reference
Giardia 18S rDNA	RH11	CAT CCG GTC GAT CCT GCC	Hopkins *et al*. (1997)
	RH4LM	GTC GAA CCC TGA TTC TCC G	Carolyn Read (personal communication)
	GiarF	GAC GCT CTC CCC AAG GAC	Read *et al*. (2002)
	GiarR	CTG CGT CAC GCT GCT CG	Read *et al*. (2002)
Giardia gdh	GDHeF	TCA ACG TYA AYC GYG GYT TCC GT	Read *et al*. (2004)
	GDHiRm	GTT RTC CTT GCA CAT CTC	This study
	GDHiF	CAG TAC AAC TCY GCT CTC GG	Read *et al*. (2004)
Cryptosporidium 18S rDNA	Cp18S-105	AAC AGT TAT AGT TTA CTT GAT AAT C	This study
	Cp18S-1031	TGA AGG AGT AAG GAA CAA CC	Enemark *et al*. (2002*a*)
Cryptosporidium HSP70	HSPF4	GGT GGT GGT ACT TTT GAT GTA TC	Morgan *et al*. (2001)
	HSPR4	GCC TGA ACC TTT GGA ATA CG	Morgan *et al*. (2001)
	HSPF3m	GCT GGT GAY ACT CAC TTG GGW GG	This study
	HSPR3m	CTC TTR TCC ATA CCA GCA TCC	This study
ML1	Cp-GAG-F	CTA AAA ATG GTG GAG AAT ATT C	Cacciò *et al*. (2000)
	Cp-GAG-R	CAA CAA AAT CTA TAT CCT C	Cacciò *et al*. (2000)

72 °C for 50 sec), the nested PCR of the *HSP70* gene by 30 cycles (94 °C for 30 sec, 58 °C for 20 sec, 72 °C for 30 sec) in the primary step and 45 cycles (94 °C for 25 sec, 60 °C for 18 sec, 72 °C for 25 sec) in the secondary step, whereas the microsatellite, ML1 was amplified by 40 cycles (94 °C for 30 sec, 53 °C for 30 sec, 72 °C for 25 sec). The nested PCR of the *Giardia* 18S rDNA locus was carried out with 40 and 45 cycles (95 °C for 30 sec, 59 °C for 20 sec, 72 °C for 20 sec) in the primary and secondary step, respectively, and for *gdh* 40 cycles (94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 30 sec) was used in the primary step and 45 cycles (94 °C for 30 sec, 56 °C for 25 sec, 72 °C for 30 sec) for the secondary step. Positive and negative controls were included with every reaction.

Purification and sequencing of PCR products

PCR products were purified either directly from the PCR reaction or from 1·5–2·0% agarose gels after electrophoretic separation using High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). The purified PCR products were sequenced in both directions by MWG-BIOTECH AG, Ebersberg, Germany. Sequences were analysed using BioEdit version 5.0.9 (Hall, 1999) and compared with already known *Cryptosporidium* spp. or *Giardia* spp. sequences obtained from GenBank using ClustalX version 1.8 (Thompson *et al*. 1997). For several samples, the sequencing chromatogram showed evidence of mixed infections and was further examined. In many instances, this resulted in assignment of 2 different species/genotypes, which subsequently each represented 1 isolate. When the involved species/genotypes could not be defined, the samples were excluded from the analysis. Nucleotide sequences obtained in this study, which were not identical to sequences already published in GenBank, have been deposited in GenBank under the Accession numbers DQ182597 to DQ182611. The designation of isolates, which were characterized as novel genotypes or sub-genotypes, was according to the sample labelling system.

Phylogenetic analyses

The phylogenetic analyses of *Cryptosporidium* 18S rDNA, *HSP70* and *Giardia gdh* were based on alignments obtained from ClustalX with lengths of 812, 287, and 411 nucleotides, respectively. The analyses were performed using TREECON for Windows version 1.3b (Van de Peer and De Wachter, 1994), and the distance estimations were carried out using different equations: Jukes and Cantor (1969), Tajima and Nei (1984) and Jin and Nei (1990) (Kimura two-parameter model with a = 1, 2 or 0·5). The phylogenetic trees were constructed using neighbour-joining (NJ) algorithm. For each calculation, branch reliability was assessed using bootstrap analysis (1000 replicates). Trees resolved with different equations were compared to assure consensus of topologies.

Statistical analyses

All data analyses were stratified by age groups, due to large variation in prevalence and genotype distribution among the groups. Collected data were analysed using the procedure SURVEYMEANS in the software SAS®, version 8 (Statistical Analysis

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System, 1999), utilizing methods for analysis of complex survey samples.

When estimating the prevalence of infected animals the complex sample design was taken into account. Because selected animals were clustered into selected herd (herd was the primary sampling unit and animal the second sample unit) the variance of the prevalence of infected animals was estimated from the variation among the herds.

To take the unequal sample probability of animals (a result of a fixed number of animals being selected, within each herd, irrespective of herd size) into account, each observation was assigned the weight of the inverse sample probability in the estimation, which was defined as (1/(1/herd size)).

In order to estimate the distribution of genotypes, a subsample of the isolates was analysed by sequencing. Isolates for genotyping were selected successively within each herd, until at least 2 isolates per age group within each herd were sequenced. Because isolates were obtained from animals clustered into herds, the variance of the distribution of genotypes was estimated from the variation among the herds. In order to take the unequal sample probability of isolates into account (a fixed number of isolates was genotyped within each herd, irrespective of number of positive animals), each observation was assigned the weight of the inverse sample probability of the isolate to be genotyped, which was defined as (1/(number of genotyped isolates within an age group within a herd/number of isolates within an age group within a herd)).

The method of successive sampling of isolates for genotyping might introduce a bias in the estimated distribution of genotypes because samples with a high concentration of (oo)cysts might have a higher probability of a successful sequencing compared to samples with lower concentration. Therefore, an association between genotype and concentration of (oo)cysts will result in an overestimation of the occurrence of genotypes, which give rise to high (oo)cyst concentrations. To investigate whether or not this bias was present, we estimated (1) the association between selection for genotyping and the concentration of (oo)cysts in the sample (logistic regression), (2) the association between successful genotyping and the concentration of (oo)cysts in the sample (logistic regression), and (3) the association between genotype and concentration of (oo)cysts in the sample (Wilcoxon rank test).

Regarding subgenotyping using ML1, the degree of longitude and latitude were ascertained for 70 *C. parvum* (cattle genotype) isolates corresponding to 32 dairy herds, and the localities plotted on a map of Denmark. A spatial cluster analysis, using SaTScan version 4.0 (Kulldorff, 2003), was performed to evaluate possible geographical clustering of any of the 3 subgenotypes.

RESULTS

Initially, 1237 faecal samples from pigs and 1150 faecal samples from dairy cattle were analysed for the presence of *Giardia* spp. and *Cryptosporidium* spp. (Maddox-Hyttel *et al*. 2006). The estimated prevalences of animals of different age groups infected by *Cryptosporidium* and *Giardia* are presented in Table 2 and Table 3, respectively, to provide relevant background information for the genotyping results. The prevalence of *Cryptosporidium* oocyst excreting animals varied considerably between the different age groups, both for pigs and cattle. Also, for pigs the prevalence of *Giardia* cyst excreting animals varied considerably between the different age groups, whereas for cattle the age dependency was less prominent with 20% of the cows and 44% of the young calves excreting cysts. For details of within-herd prevalence of animals infected with *Cryptosporidium* and *Giardia*, see Maddox-Hyttel *et al*. (2006).

In order to examine the genetic diversity of both *Cryptosporidium* spp. and *G. duodenalis* in cattle and pigs, a number of (oo)cyst containing samples from animals of different age groups were selected for genotyping. Statistical analyses revealed no significant association between samples selected for genotyping and the concentration of (oo)cysts in the sample neither for pigs nor for cattle (data not shown). In addition, there was no significant association between successful genotyping and the concentration of (oo)cysts in the sample (data not shown).

Genotyping of Cryptosporidium

In this study, 183 pig and 154 cattle isolates of *Cryptosporidium* were successfully analysed by sequencing of the 18S rDNA locus and/or of the *HSP70* gene. As a result, the previously described species/genotypes *C. suis*, pig genotype II, *C. parvum* (cattle genotype), *C. bovis* and *Cryptosporidium* deer-like genotype, as well as a novel genotype here referred to as *C. suis*-like genotype (i.e. *C.* sp. K4515 in GenBank) were identified. The age specific distributions of the *Cryptosporidium* species/genotypes are presented in Table 2. The number of isolates obtained from adult animals was low, and genotyping of isolates obtained from sows was unsuccessful. The three genotyped isolates from cows were all *C. parvum* (cattle genotype). Within each age group there was a dominating genotype, and this was not the same when comparing piglets to weaners or young to older calves. For example while pig genotype II was the most prevalent in weaners, the majority of piglets were infected with *C. suis*. Finally, the zoonotic *C. parvum* (cattle genotype) was most prevalent in young calves, while it was not detected in pigs of any age group.

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Table 2. Estimated age specific prevalence of *Cryptosporidium* oocyst excreting animals and age specific distribution of *Cryptosporidium* species/genotypes within shedding animals

Host and age group		Animals/ genotyped isolates (herds)*	Distribution of *Cryptosporidium* genotypes					
Pigs	Age specific prevalence		*C. suis*	Pig genotype II				
Sows†	0·02 (0·00–0·04)§	10/0 (–)	—	—				
Weaners‡	0·74 (0·69–0·79)	356/170 (48)	24% (18–31)	76% (69–82)				
Piglets†	0·04 (0·02–0·07)	29/13 (8)	71% (37–100)	29% (0–63)				
Cattle			*C. parvum* (cattle genotype)	Pig genotype II	*C. bovis*	*C.* deer-like genotype	*C. suis*-like genotype	Atypical isolate
Cows	0·03 (0·01–0·05)	8/3 (2)	100% (–)	—	—	—	—	—
Older calves	0·25 (0·19–0·31)	129/61 (38)	4% (0–9)	—	73% (59–87)	14% (5–25)	4% (0–11)	4% (0–11)
Young calves	0·62 (0·55–0·70)	229/90 (44)	82% (71–92)	0% (0–1)	14% (4–24)	3% (0–6)	—	1% (0–4)

* The total number of oocyst excreting animals followed by the number of genotyped isolates. In parentheses, the number of herds from which results of genotyping were obtained.
† Based on 49 herds because one herd was without weaners.
‡ Based on 49 herds because one herd was without sows and piglets.
§ 95% confidence interval.

Table 3. Estimated age specific prevalence of *Giardia* cyst excreting animals and age specific distribution of genotypes within shedding animals

Host and age group		Animals/ genotyped isolates (herds)*	Distribution of *Giardia* genotypes				
Pigs	Age specific prevalence		A	D	K2521	E (rDNA)**	E
Sows†	0·04 (0·01–0·07)§	10/4 (4)	—	—	—	—	100% (–)
Weaners‡	0·39 (0·29–0·49)	190/77 (32)	12% (1–23)	1% (0–2)	1% (0–3)	25% (13–36)	62% (47–76)
Piglets†	0·03 (0·01–0·06)	15/1 (1)	100% (–)	—	—	—	—
Cattle			A	K4016	K2521	E (rDNA)	E
Cows	0·20 (0·14–0·26)	51/9 (7)	—	—	—	50% (0–100)	50% (0–100)
Older calves	0·44 (0·39–0·50)	221/66 (42)	6% (0–12)	—	0% (0–2)	21% (8–5)	72% (58–86)
Young calves	0·34 (0·27–0·40)	129/70 (39)	6% (0–14)	1% (0–3)	4% (0–10)	15% (4–6)	73% (61–86)

* The total number of cyst-excreting animals followed by the number of genotyped isolates. In parentheses, the number of herds from which results of genotyping were obtained.
** The isolates, which were genotyped as Assemblage E only at the 18S rDNA locus, but not sequenced successfully at the *gdh* gene.
† Based on 49 herds because one herd was without weaners.
‡ Based on 49 herds because one herd was without sows and piglets.
§ 95% confidence interval.

Several pigs seemed to be infected with both *C. suis* and pig genotype II, whereas very few cattle samples exhibited a mix of infections. For 3 cattle samples, the rDNA locus was identical to that of *C. bovis*, whereas *HSP70* was identical to that of *C. parvum* (cattle genotype). Based on the sequencing chromatograms, none of these samples seemed to contain a mix of infections and are therefore referred to as 'Atypical isolate' in Table 2.

A novel *C. suis*-like genotype was identified in 3 calves originating from 2 different herds. It showed 99% and 98% nucleotide identity to *C. suis* at the 18S rDNA and *HSP70* loci, respectively. Besides, 1 young calf was infected with pig genotype II.

Both in piglets and weaners there was a significant association between species/genotype and concentration of oocysts in the sample. Thus, the concentration was significantly ($P<0·05$) higher in

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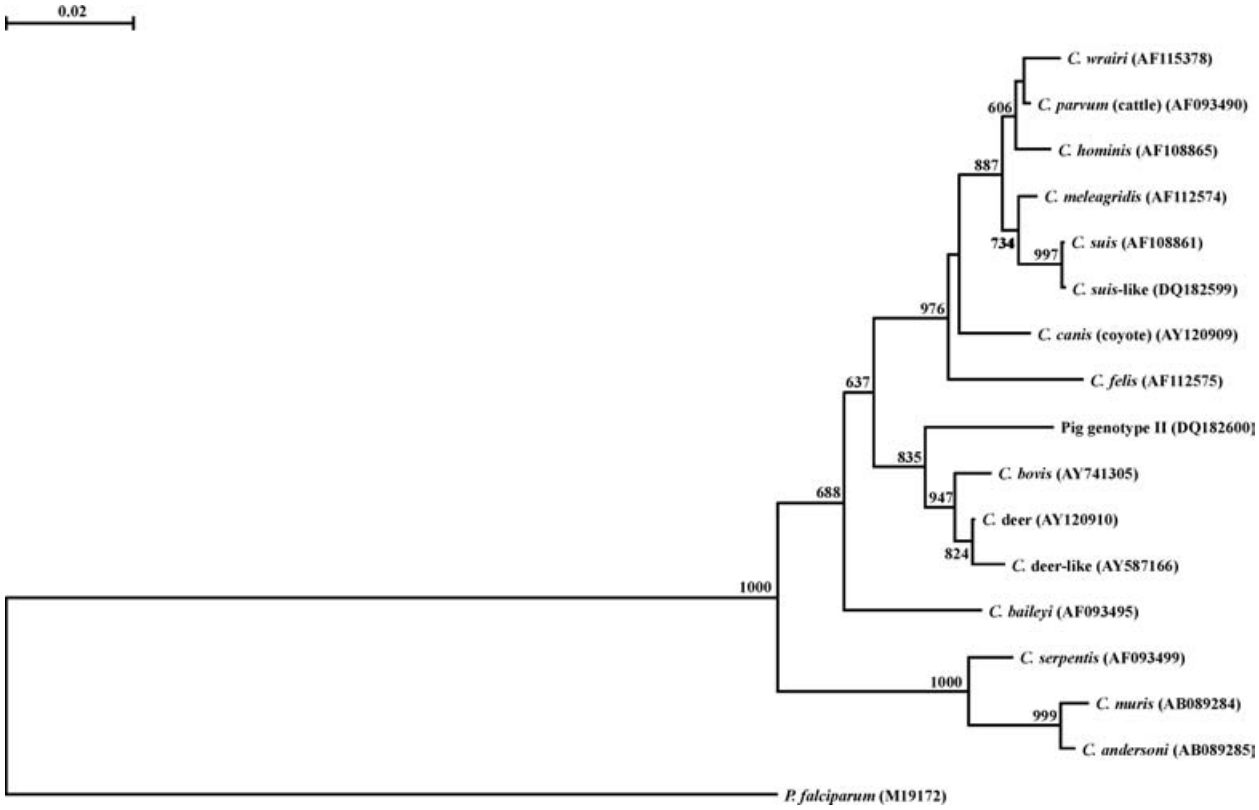

Fig. 1. Phylogenetic relationship of *Cryptosporidium* isolates using partial 18S rDNA sequences. The evolutionary distances were calculated with the Jukes and Cantor model and used to construct the neighbour-joining tree. Bootstrap values (>500) out of 1000 are indicated at each node. Accession numbers for sequences obtained from GenBank are given in parentheses. The sequence of *Plasmodium falciparum* was used as an outgroup. The distance scale indicates 0·02 substitutions/site.

samples genotyped as *C. suis* (range for piglets was 3600 to 5 920 000 oocysts per gramme faeces (OPG) and for weaners 200 to 1 480 000 OPG) than in samples genotyped as pig genotype II (range for piglets was 400 to 28 600 OPG and for weaners 200 to 228 800 OPG). In cattle there was no association between genotype and concentration of oocysts in any of the age groups.

For most *C. parvum* (cattle genotype) isolates the sequencing of the 18S rDNA locus revealed the presence of the 2 different alleles, Type A and Type B (99% nucleotide identity). For the phylogenetic analyses, the sequence of the allele Type A was used (Fig. 1). In general, the predominant allele was Type A; however, the sequencing chromatogram from 4 isolates revealed purely Type B. In addition, the 18S rDNA sequence of at least 4 of the 15 isolates of the *Cryptosporidium* deer-like genotype showed evidence of multiple alleles. For this genotype, the amplification at the *HSP70* gene was mostly unsuccessful, presumably due to sequence differences at the priming sites.

C. parvum (cattle genotype) were obtained from 33 different dairy herds. In total, 70 isolates from 32 herds were further subgenotyped using the microsatellite, ML1 and revealed the presence of the alleles ML1-238, ML1-226 and ML1-223 previously named C1, C2 and C3, respectively. Only 1 allele was detected in any single herd. Regarding the geographical distribution, the spatial cluster analysis revealed no significant geographical clustering of any of the subgenotypes (data not shown).

Phylogenetic relationship of Cryptosporidium

The phylogenetic analyses of the 18S rDNA and the *HSP70* gene are largely consistent, but there are some differences between the obtained trees (Figs 1 and 2). The location varies for both *C. baileyi* and *C. meleagridis* in addition to the *C. canis*/*C. felis* group, which most likely can be explained by low branch support. In both the 18S rDNA and the *HSP70* tree, one of the conserved groups is made up of pig genotype II, *C. bovis* and *Cryptosporidium* deer-like genotype, whereas within the group that includes *C. suis* and *C. parvum* (cattle genotype) the relationship is more uncertain. As expected, the novel *C. suis*-like genotype groups relates most closely with *C. suis* in both phylogenetic trees.

Genotyping of Giardia

In total, 227 *Giardia* isolates were analysed at the 18S rDNA locus and/or the *gdh* gene. It was possible to

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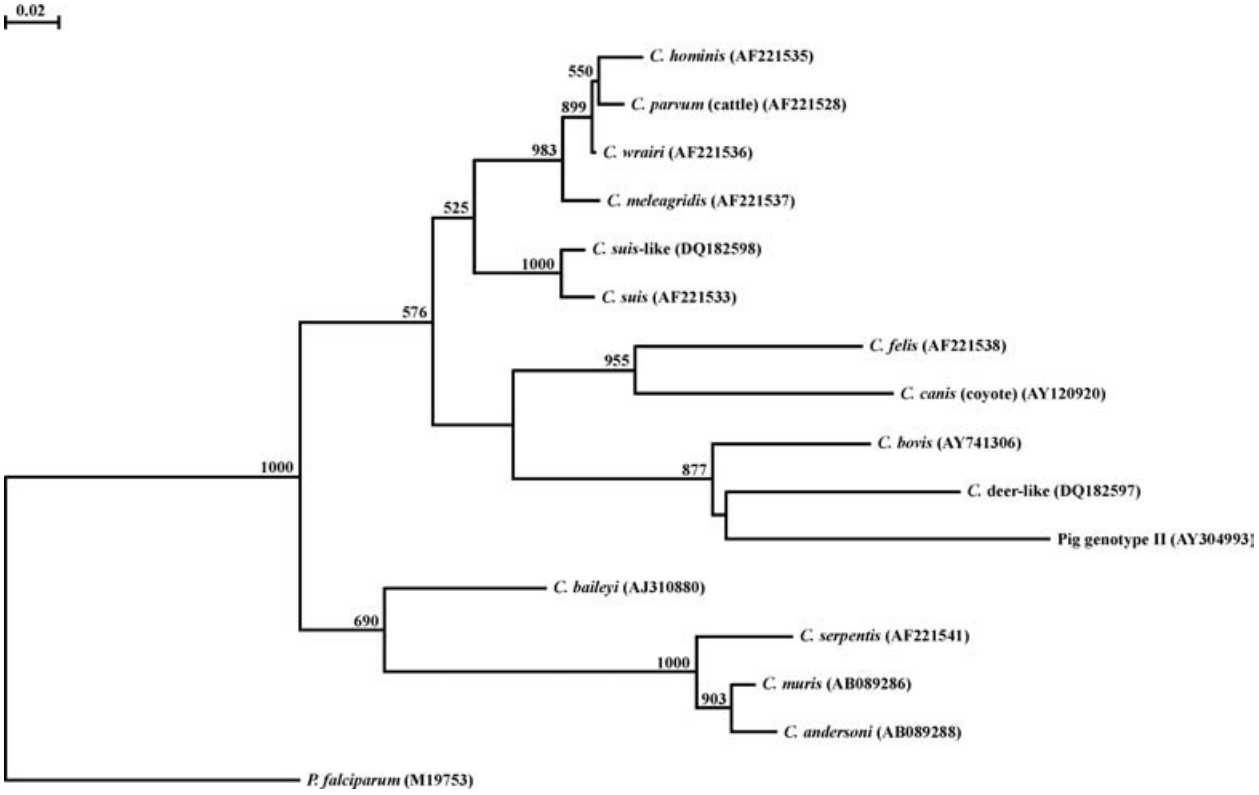

Fig. 2. Phylogenetic relationship of *Cryptosporidium* isolates using partial nucleotide sequences of the *HSP70* gene. The evolutionary distances were calculated by Jukes and Cantor model and used to construct the neighbour-joining tree. Bootstrap values (>500) out of 1000 are indicated at each node. Accession numbers for sequences obtained from GenBank are given in parentheses. The sequence of *Plasmodium falciparum* was used as an outgroup. The distance scale indicates 0·02 substitutions/site.

obtain the *gdh* sequence for 168 isolates. The *gdh* gene, which is less conserved than the 18S rDNA locus, can be used for subgenotyping. Therefore, the final assignment of genotype/subgenotype was preferentially based on the *gdh* sequence. Otherwise the genotype was defined by the 18S rDNA. The age specific distributions of genotypes are presented in Table 3, whereas details on subgenotypes are described below. Mostly, there was a correlation between the sequencing results of the 18S rDNA locus and the *gdh* gene, but for some isolates this was not the case.

In this study, 2 novel genotypes, K2521 and K4016, were found. When isolates, which were characterized as K2521 based on the *gdh* gene, were analysed at the 18S rDNA locus they seemed to belong to Assemblage E. According to the phylogenetic relationship of the *gdh* sequences these isolates did not belong to Assemblage E (Fig. 3). The contradiction between the results from the 18S rDNA and *gdh* sequences was most likely caused by the lack of variation at the 18S rDNA locus. Isolates that were only analysed at the 18S rDNA locus and found to belong to Assemblage E could in principle either truly belong to Assemblage E or be identical to K2521. Therefore, these isolates were referred to as Assemblage E (rDNA) (Table 3).

All 4 genotyped isolates from sows belonged to the Assemblage E (Table 3). This was also the most frequent among weaners, which additionally had 12% of the isolates belonging to the Assemblage A. In all age groups of cattle, the Assemblage E was the predominant. The pattern of distribution of *Giardia* genotypes among young and older calves was similar to that of weaners, including 6% of the isolates from young and older calves belonging to Assemblage A. Similar to sows, no potential zoonotic genotypes were found in cows.

In pigs there was no association between genotype and concentration of cysts in any of the age groups (data not shown). In cows and young calves there was a significant association between genotype and concentration of cysts in the sample ($P<0{\cdot}05$), whereas the same association was only marginally significant in older calves ($P=0{\cdot}06$). In particular, the concentration was much higher in the Assemblage E group (range for cows was 600 to 1200 cysts per gramme faeces (CPG); range for young calves was 400 to 6 000 000 CPG; range for old calves 200 to 2 100 000 CPG) and lower in the Assemblage E (rDNA) group (range for cows was 200 to 400 CPG; for young calves 1000 to 320 000 CPG; and for old calves 200 to 179 200 CPG) than expected when assuming no association.

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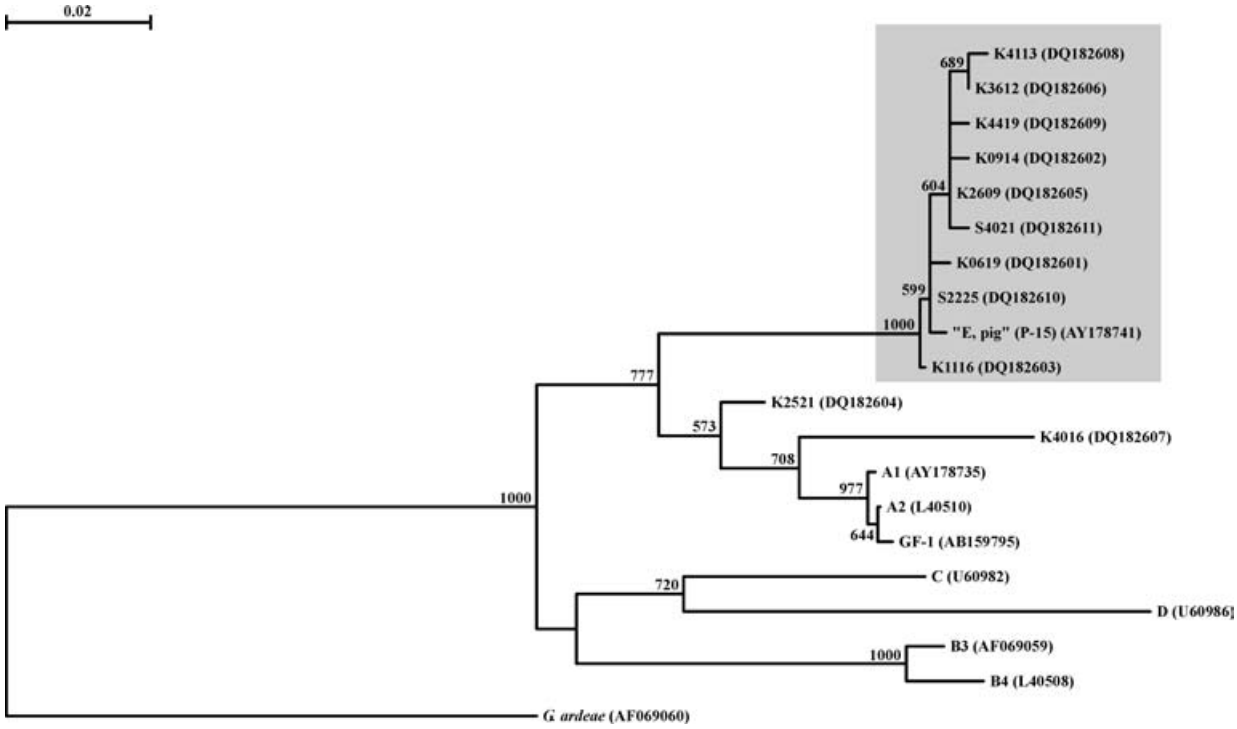

Fig. 3. Phylogenetic relationship of *Giardia duodenalis* isolates using partial nucleotide sequences of the *gdh* gene. The evolutionary distances were calculated by the Jukes and Cantor model and used to construct the neighbour-joining tree. Bootstrap values (>500) out of 1000 are indicated at each node. Accession numbers for sequences obtained from GenBank are given in parentheses. The sequence of *Giardia ardeae* was used as an outgroup. The distance scale indicates 0·02 substitutions/site. The shaded box indicates the Assemblage E group.

Phylogenetic relationship and subgenotypes of Giardia

The *gdh* sequences of the different subgenotypes found in this study were compared to already known sequences obtained from GenBank (Fig. 3). The large Assemblage E group is comprised of subgenotypes that only differ by few nucleotides. For cattle more than 90% of the isolates belonging to this group were either K3612 or K2609, whereas for pig isolates roughly 60% were 'E, pig' and more than 20% were S2225. Interestingly, only 1 isolate identical to S2225 was found in cattle. Likewise, K3612 was only found in 4 different pig herds and no pig isolates were identical to K2609.

The 2 novel genotypes, K2521 and K4016, were closely related to Assemblage A (Fig. 3); K2521 was found in 3 different cattle herds and a single pig herd, whereas only 1 cattle isolate was characterized as K4016. *Giardia* isolates belonging to Assemblage A were found in both cattle and pig herds. While all subgenotyped pig isolates were A1, all subgenotyped cattle isolates were identical to the previously identified ferret isolate, GF-1 (Fig. 3). Two pig isolates from the same herd were genotyped at the rDNA locus as Assemblage D (Table 3), but confirmation at the *gdh* gene was unsuccessful. Both pigs had very low excretion of cysts, and the possibility of passive transfer as a result of coprophagy cannot be excluded. In this study, no isolates belonging to Assemblage B or C were found.

DISCUSSION

Hitherto, little was known about the genetic diversity of *Cryptosporidium* spp. and especially *G. duodenalis* in Danish dairy cattle and pigs. This is a substantial study, and besides the widespread occurrence of the 2 parasites, it reports the distribution of the genetic variations in a large number of animals from many different herds.

In the case of *Cryptosporidium*, our study has shown that there is an age-related change in the species/genotypes infecting animals, thereby supporting observations from similar studies (Santín *et al*. 2004; Fayer *et al*. 2006). For pigs, we observed higher oocyst concentration in samples genotyped as *C. suis* than in samples genotyped as pig genotype II. In addition, we found that pig genotype II was the most prevalent in weaners whereas the majority of piglets were infected with *C. suis*. This is in contradiction to a previous Australian study in which 28 *Cryptosporidium* isolates from pigs were genotyped revealing equal numbers of *C. suis* and pig genotype II. No correlation between genotype and host age was found, but some pigs infected with pig genotype II seemed to have high excretion of oocysts (Ryan *et al*. 2003).

In this study, pigs were commonly infected with more than 1 species/genotype. In contrast, mixed infections seemed less common in cattle similar to findings by Santín *et al*. (2004) and Fayer

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et al. (2006), who observed a single case of mixed infection and none, respectively. Nevertheless, previous Danish findings revealed 7% (13 out of 193) mixed infections in *Cryptosporidium* isolates from cattle (Enemark *et al*. 2002*a*). Likewise, Scottish results have demonstrated the presence of 8–26% infections containing more than 1 genotype in ruminants (Mallon *et al*. 2003). In both of these studies results were obtained by separation of microsatellites using an ABI sequencer and GENESCAN software, indicating that the technique is of significance for the identification of mixed parasite populations.

Earlier findings by Xiao *et al*. (2002*b*) indicated that *C. bovis* was not prevalent in cattle. Our study and the studies by Santín *et al*. (2004) and Fayer *et al*. (2006) have proven otherwise. As mentioned by Santín *et al*. (2004) this is most likely due to the fact that this species is found in animals older than those most often examined. This clearly shows the importance of broader studies. In our study, *C. bovis* caused 14% and 73% of the infections in young and older calves, respectively. In comparison, Santín *et al*. (2004) reported 9% of the positive specimens from pre-weaned calves to be infected with *C. bovis*, which was also the case in 55% of positive specimens from post-weaned calves. In the most recent study by Fayer *et al*. (2006), *C. bovis* caused 35% of the *Cryptosporidium* infections in 1 to 2-year-old calves. Equal to our observations for the older calves, the latter study reported that the *Cryptosporidium* deer-like genotype accounted for another 15% of the infections. *C. andersoni*, which was found previously in Danish cattle (Enemark *et al*. 2002*b*), was not detected in the present study. This is probably explained by the fact that *C. andersoni* is primarily found in older animals (Olson *et al*. 2004), and that only 3 isolates from cows could be successfully genotyped in the present study. Interestingly, 1 young calf was infected with pig genotype II, which until now has appeared to be pig-specific. As seen by the phylogenetic analysis, pig genotype II most likely represents an individual species yet to be described. Furthermore, the *C. suis*-like genotype was isolated from 3 older calves. In comparison, 1 calf was infected with *C. suis* in the study by Fayer *et al*. (2006). The presence of the pig genotype II and the *C. suis*-like genotype in cattle cannot be explained by proximity between pigs and cattle, since pigs were not present on any of the 3 involved cattle farms.

In the case of *C. parvum* (cattle genotype), no significant clustering of the subgenotypes of ML1 was observed in this study, even though clustering of ML1-238 has previously been detected in Denmark (Enemark *et al*. 2002*a*).

The *HSP70* gene could only be amplified for 2 out of 15 isolates of the *Cryptosporidium* deer-like genotype, corresponding to previous descriptions of a lack of amplification at this locus (Ryan *et al*. 2005). Even though the degenerate primers were further modified in our study, extensive interspecies variation in the primer regions could still cause insufficient annealing. In addition, the heterogeneity in the primer regions can cause differences in amplification efficiency, which in the case of mixed infections can lead to underestimation of some species/genotypes.

The outcome of the phylogenetic analyses was in agreement with results from other studies, including differences between the 18S rDNA and the *HSP70* loci like for *C. baileyi* (Ryan *et al*. 2005; Fayer *et al*. 2005).

One purpose of this study was to examine the distribution of potentially zoonotic *Cryptosporidium* isolates. Presently, *C. parvum* (cattle genotype) is recognized as zoonotic, but for most of the other isolates found in this study the information on zoonotic potential remains limited. However, the fact that *C. suis* has been identified in immunocompetent humans (Xiao *et al*. 2002*a*) and the recent detection of pig genotype II in secondary sewage (Ryan *et al*. 2005), along with the widespread occurrence of *C. parvum* (cattle genotype) infection all suggest that pig and/or cattle faeces, if used as untreated manure on open fields, could be a concern in the transmission of potentially zoonotic *Cryptosporidium* to the environment and, thus, recreational waters.

The high prevalence of *Giardia* in both cattle and pigs in Denmark allowed genotyping of a couple of hundred isolates. Thus, this is the largest study of *Giardia* in pigs, and comparable to the extensive studies of cattle by Trout *et al*. (2004, 2005).

Because *Giardia* belonging to Assemblage A was observed in Danish cattle, they represent the potential for zoonotic transmission. From all over the world, similar observations have been reported in several other studies (O'Handley *et al*. 2000; van Keulen *et al*. 2002; Huetink *et al*. 2001; Appelbee *et al*. 2003; Trout *et al*. 2004, 2005; Itagaki *et al*. 2005; Lalle *et al*. 2005), with the exception of Becher *et al*. (2004), who only recovered cattle isolates belonging to Assemblage E. As in the studies by Trout *et al*. (2004, 2005), we found that the level of Assemblage A was relatively constant (6%) in young and older calves. Thus, we did not see a host age related change in distribution of zoonotic genotypes as was the case of *Cryptosporidium*.

In all age groups of cattle there was a significant association between genotype and concentration of cysts in the sample. In particular, the concentration was much higher in the Assemblage E group and lower in the Assemblage E (rDNA) group (those genotyped only at the 18S rDNA locus) than expected when assuming no association. The lack of amplification at the *gdh* gene might be related to low concentration of cysts and thereby could explain the above association. A correlation between specific subgroups and host origin was observed within Assemblage E. This indication of some subgenotypes

being more or less host adapted is similar to the report by Monis *et al*. (2003), who observed host-specific substructuring within Assemblage E, when analysing isolates from sheep, calves and pigs by allozymic analysis.

In the case of the isolate K2521, discrepancy was observed between the results obtained at the 18S rDNA locus and the *gdh* gene. Similarly, Read *et al*. (2004) found that several of their *Giardia* isolates, which grouped with the non-zoonotic genotypes at the 18S rDNA locus, conversely grouped with the potentially zoonotic isolates at the *gdh* gene. Because the *gdh* gene is less conserved and the sequence is longer than the 18S rDNA, the *gdh* allows the discrimination between the various subgenotypes of *Giardia*. These observations emphasize the importance of more consistent methods for *Giardia* genotyping in the future. For example, in the attempt to elucidate the heterogeneity of *G. duodenalis* it is important that different laboratories use the same set of molecular markers in order to compare the results. Thereby, identical subgenotypes could be named according to a common nomenclature.

While isolates of the *G. duodenalis* A1 group are potentially zoonotic, most isolates of the A2 group have been found to infect only humans; however more recently, also isolates from cattle, dogs and horses have been placed in this group (Traub *et al*. 2004, 2005; Lalle *et al*. 2005). For cattle, the GF-1 (Abe *et al*. 2005) groups with the A2 subgenotype in the phylogenetic analysis. The GF-1 isolate has previously been placed in the A1 group (Abe *et al*. 2005) and a likely explanation for the inconsistency of the results is low branch support in the phylogenetic analyses. Regardless of subgenotype, the Danish cattle are infected with *Giardia* belonging to Assemblage A and, furthermore, the Danish pigs with *Giardia* of the A1 subgenotype, thus also with regard to *Giardia* there may be reason for public health concern when spreading manure on the open fields.

At present, a project on genotyping human isolates of *Giardia* and *Cryptosporidium* is being conducted in our laboratory and will provide the opportunity to compare results from both the human and the livestock population. If the genotypes found are identical, it may shed light on potential zoonotic species/genotypes and possible routes of transmission.

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