Molecular characterization of Danish *Cryptosporidium parvum* isolates

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SUMMARY

The genetic polymorphism among 271 Danish *Cryptosporidium* isolates of human and animal origin was studied by partial amplification and sequencing of the *Cryptosporidium* oocyst wall protein (COWP) gene, the 18S rDNA, and a microsatellite locus.† Furthermore, the microsatellite locus was studied directly using fragment analysis. A comparative analysis of DNA sequences showed the presence of 3 different subgenotypes (C1, C2 and C3) in *C. parvum* isolates from Danish cattle, with prevalences of 16·7, 17·2 and 73·1 % including 13 (7·0 %) mixed infections. Subgenotype C1 was significantly more prevalent ($P < 0{\cdot}001$) in the southern part of Denmark. In *Cryptosporidium* isolates of human origin the anthroponotic subgenotype H1 was identified, in addition to the zoonotic subgenotypes C1, C2, and C3. Of 44 human samples, 56·8 % were anthroponotic, whereas 40·9 % were zoonotic genotypes. One human isolate was characterized as *C. meleagridis*. The porcine *Cryptosporidium* isolates ($N = 4$) revealed a pattern which was genetically distinct from human and bovine isolates. *Cryptosporidium* in a hedgehog (*Erinaceus europaeus* L.) was identified for the first time. By microsatellite sequencing the hedgehog isolate showed a subgenotype distinct from the previously detected types. The assignment to subgenotype by microsatellite sequencing and fragment typing was 100 % identical in samples where results were achieved by both methods. In addition, the fragment analysis proved more sensitive, easier, faster, and less expensive compared to sequencing.

Key words: *Cryptosporidium*, genetic polymorphism, microsatellites, 18S rDNA, human genotypes, animal genotypes.

INTRODUCTION

The genus *Cryptosporidium* comprises a group of protozoan parasites that infect the gastrointestinal or respiratory tract of a broad variety of mammals, birds, reptiles and fish (Morgan *et al*. 1999*b*). *Cryptosporidium parvum* is the species most frequently documented as the cause of clinical cryptosporidiosis in man and domesticated animals (Morgan *et al*. 1999*b*), and increasingly recognized as a major cause of diarrhoea worldwide (Fayer, Speer & Dubey, 1997). However, other *Cryptosporidium* species have also been connected with human infections (Morgan *et al*. 2000*a*). The traditional approaches to identification based on host specificity, oocyst morphology, and site of infection do not provide a reliable method for speciation since several species are morphologically and developmentally indistinguishable (Xiao *et al*. 2000).

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† The nucleotide sequence data reported in this paper are available in the GenBank database under the accession number AF469174.

Molecular studies of *C. parvum* have demonstrated the existence of at least 2 genetically distinct subpopulations: one (genotype I, or the human type) is predominantly confined to humans as well as other primates, and maintained through an anthroponotic transmission cycle; and a second (genotype II, or the calf type) which is found in livestock in addition to humans, and implicated in zoonotic transmission (see Widmer, 1998 and Morgan *et al*. 1999*b* for reviews). Several outbreaks of cryptosporidiosis due to contamination of food, potable and recreational water have also been reported (Millard & Gensheimer, 1994; Fricker & Crabb, 1998), but the relative importance of the different routes of transmission has not yet been established.

Intragenotype variation has been identified in the 18S rRNA gene (Xiao *et al*. 1998), the thrombospondin-related adhesion protein (TRAP-C2) gene (Peng *et al*. 1997; Sulaiman *et al*. 1998), and the polythreonine [poly(T)] gene (Widmer *et al*. 1998*b*). Furthermore, analysis of the β-tubulin intron has revealed polymorphism within genotype I, and evidence of recombination between the two genotypes (Widmer *et al*. 1998*a*). However, other studies of the same region did not verify this variation within the genotypes, and no recombinant

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genotypes were found (Sulaiman *et al*. 1999; Rochelle *et al*. 1999).

A clonal population structure, as suggested by Morgan, Constantine & Thompson (1997), Gibbons *et al*. (1998), and Awad-El-Kariem (1999), has evident implications for understanding of the epidemiology of this parasite as well as for control strategies. The absence of recombinant genotypes found in several studies supports this theory (Spano *et al*. 1998; Morgan *et al*. 1999*b*). However, intragenotype variation has only been studied in a limited number of *Cryptosporidium* isolates so far; therefore molecular characterization of isolates from diverse hosts and different geographical origins is important in order to increase our knowledge of the population structure.

Studies of the *Cryptosporidium* genome have identified numerous simple sequence repeats (Liu *et al*. 1999; Strong, Gut & Nelson, 2000), and allowed for detailed analysis of the genetic polymorphism in the non-coding regions of the genome containing microsatellites (Aiello *et al*. 1999; Cacciò *et al*. 2000; Feng *et al*. 2000; Cacciò, Spano & Pozio, 2001). The aim of the present study was to analyse the extent of genetic diversity among *Cryptosporidium* isolates within a relatively restricted geographical area (Denmark). The secondary goal was to improve the typing system to facilitate both the identification of mixed infections, and the characterization of a large number of samples.

MATERIALS AND METHODS

Sources of parasite isolates and clinical information

Cryptosporidium oocysts were purified from a total of 199 faecal samples from Danish animals (193 cattle, 1 lamb, 4 piglets and 1 isolate from a hedgehog (*Erinaceus europaeus* L.)). The isolates were randomly selected from *Cryptosporidium*-positive faecal samples submitted to the Danish Veterinary Institute (DVI) during a period from October 1997 to August 2001. The specimens were collected by veterinarians from individual animals with watery diarrhoea and/or herds with high mortality in the young animals. Included in the study was also one reference isolate (the Copenhagen calf laboratory strain, CPB 0) propagated at DVI since 1990. Furthermore, 72 human specimens representing all *Cryptosporidium*-positive samples, which were submitted to Statens Serum Institut, DK during the same period, were studied. These samples were from patients with protracted diarrhoea diagnosed as having sporadic cryptosporidiosis. The sample size of the human specimens was small, less than 1 g, and several samples were dried before DNA extraction.

Limited epidemiological information (details of outbreaks, duration of clinical symptoms, age, sex, recent foreign travel, geographical region, date of collection, etc.) was collected for all samples from the original request form submitted with the sample. No information as regards immunocompromising conditions was given.

Cryptosporidium-positive samples were identified by the modified Ziehl Neelsen technique (Henriksen & Pohlenz, 1981), frozen without preservatives at −18 °C or suspended in cold 2·5% (w/v) aqueous potassium dichromate solution, and stored at 4 °C until concentration with diethyl ether, as described by Peeters & Villacorta (1995). Sources of parasites are shown in Table 1.

Oocyst disruption and DNA extraction

Oocyst disruption and DNA purification was done essentially as described by McLauchlin *et al*. (1999). Approximately 200 μl of oocyst suspension was added to 900 μl of 10 M guanidinium thiocyanate in 0·1 M Tris–HCl (pH 6·4) plus 0·2 M EDTA (pH 8·0), and 2% (w/w) Triton X-100, together with 0·3 g of 0·5 mm-diameter zirconia beads (Stratech Scientific, Luton, UK) plus 60 μl of isoamyl alcohol. The tube was shaken in a cell disruptor (FP 120 FastPrep, Savant Instruments, Inc., USA) for 30 sec at maximum speed, left at room temperature for 5 min, and centrifuged. Coarse activated silica suspension (100 μl; Sigma Chemical Co., USA) was added to the supernatant, and this was incubated at room temperature for 30 min with gentle agitation. The supernatant was discarded, and the pellet was washed: twice with 200 μl of 10 M guanidinium thiocyanate in 0·1 M Tris–HCl (pH 6·4), twice with 200 μl of ice-cold 80% ethanol and once with 200 μl of ice-cold acetone. The pellet was then dried at 55 °C for 10 min, and the DNA was eluted into 150 μl of water, vortex mixed and incubated at 55 °C for 10 min. The supernatant was recovered by centrifugation and used directly for PCR amplification.

Polymerase chain reaction (PCR) amplification

For genotype assignment (i.e. I or II), a PCR method targeting the *Cryptosporidium* oocyst wall protein (COWP) gene was used, combined with either restriction fragment length polymorphism (RFLP) or sequencing (Spano *et al*. 1997). Amplification was performed on 5–10 μl of extracted DNA in a total volume of 50 μl using the primer pair cry-9 and cry-15. Positive (previously tested samples of known genotype) and negative (buffer only) controls were included in each batch of tests. For human samples, 5 μl of the primary PCR product was purified with the High Pure PCR Product Purification kit from Roche Diagnostics. Purified PCR product was subjected to a nested PCR using internal primer cry-9a and cry-15a. Primer sequences are shown in Table 2.

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Table 1. Sources of parasites and methods used to characterize 271 Danish *Cryptosporidium* isolates

		Methods*			
Number of isolates	Host	COWP	18S rDNA	GAG-seq.	F-type
72	Human	29	62	62	72
193	Bovine	193	18	114	193
1	Ovine	1	1	1	1
4	Porcine	4	4	4	4
1	Hedgehog	N.T.	1	1	1

* COWP: PCR analysis of the *Cryptosporidium* oocyst wall protein gene; 18S rDNA: PCR amplification of a fragment of the 18S rDNA region and sequencing; GAG-seq.: PCR amplification of a GAG microsatellite and sequencing; F-type: direct typing of GAG microsatellites (fragment typing).
N.T., Not tested.

Table 2. Polymorphic loci analysed

Locus	Primer (sequence)	Method	Reference
COWP	Cry15 (5′-GTAGATAATGGAAGAGATTGTG-3′) Cry9 (5′-GGACTGAAATACAGGCATTATCTTG-3′)	PCR + sequencing	Spano *et al.* (1997)
	Cry15a (5′-GATTGTGTTGCATTCACTATG-3′) Cry9a (5′-TAATACTGTACCTGGAGGGCA-3′)	Nested PCR + sequencing	This study
18S rDNA	Cp18S-108 (5′-GTTATAGTTTACTTGATAATCTT-3′) Cp18S-1031 (5′-TGAAGGAGTAAGGAACAACC-3′)	PCR + sequencing	This study
G 35348	(5′-CTAAAAATGGTGGAGAATATTC-3′) (5′-CAACAAAATCTATATCCTC-3′)	PCR + sequencing	Cacciò *et al.* (2000)
G 35348	Cp-GAG-F-*Bam* (5′-CGGGATCCATGAGCTAAAAATGGTGG-3′) Cp-GAG-R-FAM (5′-CAACAAAATCTATATCCTC-3′)	PCR + fragment typing	This study
G 35348	Cp-GAG-F-*Bam* (5′-CGGGATCCATGAGCTAAAAATGGTGG-3′) Cp-GAG-R-*Eco* (5′-GGAATTCAACAAAATCTATATCCT-3′)	Cloning	This study

Analysis of the 18S rDNA was applied to identify *Cryptosporidium* at the species level in addition to differentiation between the bovine (Cpb), the porcine (Cpp), and the human (Cph) genotype of *C. parvum* (Morgan *et al.* 1998). The primers used to amplify an approximately 925 bp product from the 18S rDNA were Cp18S-108, forward and Cp18S-1031, reverse (Table 2). The PCR mix consisted of 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 2·5 mM $MgCl_2$, each of the 4 deoxynucleotides at a concentration of 200 μM, 0·5 units *Taq* polymerase, 130 ng of each primer, and 2 μl of extracted DNA in a final volume of 50 μl. Reactions were performed on an automatic thermocycler. The templates were subjected to 35 amplification cycles (94 °C for 60 s, 55 °C for 60 s, 72 °C for 90 s) followed by one 5 min extension period at 72 °C.

For subgenotyping of *C. parvum* amplification of a locus containing a GAG microsatellite (GenBank accession number G35348) was carried out basically as described by Cacciò *et al.* (2000).

Moreover, the primers Cp-GAG-F-Bam, and Cp-GAG-R-FAM (labelled with 6 carboxyfluorescin, FAM) were designed for direct amplification of the GAG trinucleotide repeats (fragment typing), and the templates subjected to 27 amplification cycles (94 °C for 60 s, 50 °C for 60 s, 72 °C for 60 s) followed by 5 min extension at 72 °C. With the above-mentioned primers the predicted fragment sizes of the different GAG types described by Cacciò *et al.* (2000) were the following: H1: 243 bp; H2: 249 bp; C1: 252 bp; C2: 240 bp; C3: 237 bp; C4: 234 bp. For cloning the primers Cp-GAG-F-*Bam*, and Cp-GAG-R-*Eco* were used.

Genotype assignment by amplification of the COWP gene fragment was completed in all samples from animal hosts, whereas limited accessibility of *Cryptosporidium* DNA from human hosts led to genotyping of only a fraction of these samples. Likewise, studies of the 18S rDNA from the bovine specimens were restricted to a small part of these samples because the method revealed no variation in the bovine isolates. Finally, after the analysis of a total of 176 human and bovine samples without finding any disagreement between the methods, the fragment typing technique was chosen for charac-

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Table 3. Geno- and subgenotyping results from 192 bovine *Cryptosporidium parvum* isolates analysed by PCR amplification of the COWP gene, the 18S rDNA, microsatellite analysis (GAG-seq.), and/or direct typing of GAG microsatellites (fragment typing/F-type)

Number of isolates		COWP	18S rDNA	GAG-seq.	F-type
Total, examined:		192	17	113	192
C1	2	II	Cpb*	C1	C1
	1	II	Cpb		C1
	7	II		C1	C1
	6	II			C1
	3			C1	C1
	5				C1
Subtotal	24	16	3	12	24
C2	3	II	Cpb	C2	C2
	3	II		C2	C2
	6	II			C2
	4			C2	C2
	9				C2
Subtotal	25	12	3	10	25
C3	10	II	Cpb	C3	C3
	1	II	Cpb		C3
	30	II		C3	C3
	33	II			C3
	21			C3	C3
	29				C3
Subtotal	124	74	11	61	124
Mixed	1	II			C1 + C2
	2	II		C3	C1 + C3
	3			C3	C1 + C3
	1				C1 + C3
	1			C2	C2 + C3
	2	II		C3	C2 + C3
	1			C3	C2 + C3
	1	II			C2 + C3
	1				C2 + C3
Subtotal	13	6		8(1)	13
Total	186	108	17	92	186

* Cpb, *Cryptosporidium parvum*, bovine genotype.

terization of the remaining isolates. The numbers of samples analysed by the different techniques are summarized in Table 1.

DNA sequencing and fragment analysis

The PCR products were purified and sequenced according to the manufacture's instructions using the PRISM dRhodamine Dye Terminator Cycle sequencing kit (PerkinElmer/Applied Biosystems, Foster City, CA, USA). Aliquots (5 μl) of the amplicons were sequenced in both directions on ABI 373A/377 automatic sequencers and compared with the sequences of *C. parvum* from the human genotype (AF093492), the bovine genotype (AF093490), the porcine genotype (AF108861) and *C. meleagridis* (AF112574) available in GenBank.

For direct analysis of microsatellites by sizing amplicons and internal-lane standard (GeneScan-500 ROX, Perkin-Elmer Applied Biosystems, Warrington, UK) were detected on an ABI 377 automatic sequencer using the GeneScan 3.1 software.

Identification of simultaneous infection with two subgenotypes

During the fragment analysis-based typing of GAG repeats a few samples showed more than one peak. From 1 of these samples (CPB 110) the 2 amplicons were digested with *Bam*HI and *Eco*RI, cloned into the plasmid vector pUC19 (Yanisch-Perron, Vierira & Mesing, 1985) and transformants reamplified. The cloned fragments were sequenced as described above. At least 3 clones of each PCR product were sequenced in both directions.

To estimate the ability of microsatellite sequencing and fragment typing to identify mixed infections, a subgenotype C2 and a subgenotype C3 sample were mixed in various ratios spanning from 300:1 to 1:300, and subsequently analysed by sequencing and fragment analysis as described above.

Statistics

Degrees of longitude and latitude were ascertained for the 186 isolates corresponding to 171 cattle herds from which *C. parvum* was characterized, and the localities plotted on a map of Denmark. A spatial clusteranalysis, using the SaTScan software (Kulldorff *et al.* 1998), was performed to evaluate possible clustering of any of the 3 subgenotypes. The probability of finding subgenotype 1 inside and outside the marked area was calculated by dividing the observed findings with the total number of *Cryptosporidium* cases. Estimation of 95 % (2-sided) confidence intervals for the estimated prevalences was done according to Armitage & Berrry (1994). All contingency tables were analysed using Fisher's exact test or the Chi-square test as described in SAS© System for Elementary Statistical Analysis (SAS Institute Inc., USA). Both tests were used to test the null hypothesis of independence between the row and column variables of the table or the homogeneity of proportions in the table.

RESULTS

Heterogeneity among Cryptosporidium *isolates from cattle*

All of the 192 bovine field strains of *Cryptosporidium* analysed in the present study could be assigned to a specific geno- or subgenotype by one or more of the applied techniques (Table 3).

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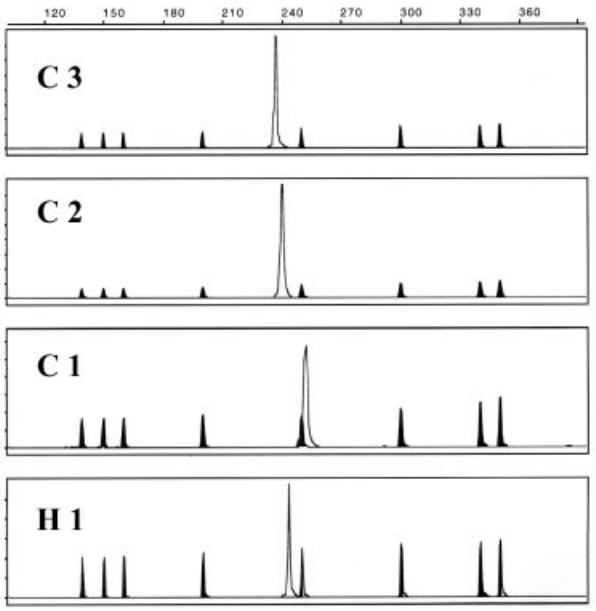

Fig. 1. Fragment typing of GAG microsatellites from Danish *Cryptosporidium parvum* isolates. GeneScan image demonstrating the different subgenotypes. Open peaks, *Cryptosporidium* fragments. Closed peaks, markers. C3: Cpb 37, 237 bp; C2: Cpb 54, 240 bp; C1: Cpb 65, 243 bp; H1: Cph 29, 243 bp.

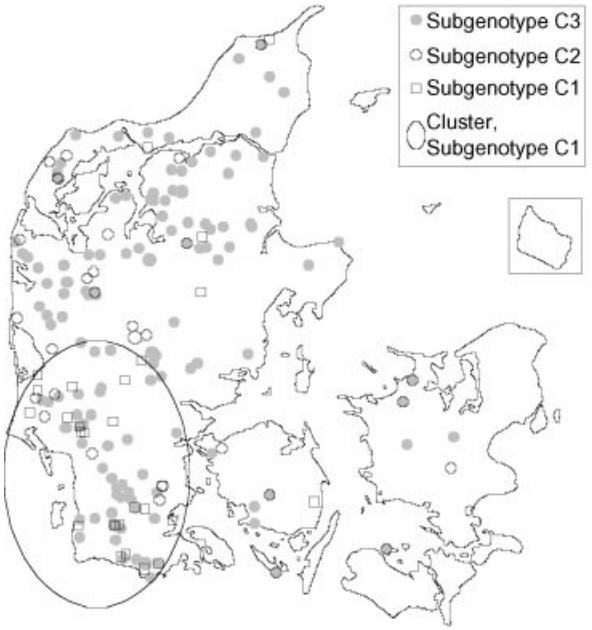

Fig. 2. Map of Denmark showing the geographical distribution of 186 bovine *Cryptosporidium parvum* isolates characterized as subgenotype C1, C2 and C3 or a mixture of these subgenotypes. The encircled area in the southern part of Jutland surrounds the farms identified in the spatial cluster analysis in which the prevalence of subgenotype C1 was significantly higher than in the rest of the country.

Analysis of the COWP gene fragment. Of the 192 bovine samples analysed by PCR amplification of the COWP gene, results were achieved from 108 (56·3 %), all identified as *C. parvum* genotype II.

Analysis of the 18S rDNA fragment. The 17 bovine specimens, studied by partial amplification of the 18S rDNA gene, were all identified as the 'bovine genotype'.

Sequencing of microsatellites. Of 113 bovine samples examined by amplification and sequencing of locus G35348, the general sequence quality was poor in several cases, leading to inconclusive typing in 21 (18·5 %) of the specimens. The remaining 92 isolates were characterized as subgenotype C1, C2, and C3.

Fragment typing of microsatellites. Using the fragment typing technique all but 6 (3·1 %) could be assigned to a specific subgenotype or a mixture of subgenotypes. Furthermore, it was possible with this technique, to obtain typing results directly from faeces without any previous concentration or purification of oocysts in 18 of 18 samples (data not shown).

Representative examples of typing results obtained by the fragment typing technique are presented in Fig. 1. The assignment to subgenotype by microsatellite sequencing and fragment typing was 100 % identical in samples where results were achieved by both methods.

Sequencing of the cloned PCR products showed distinct amplicons, either subgenotype C1 or C3, suggesting that the 2 original peaks truly reflect a mixed infection. The results suggested that sequence analysis could identify a mixed infection when the rare type was 1/3 of the frequent type. At a concentration of 1:10 or less the rare type would not be identified. Similar data for fragment analysis indicated a detection limit of the rare type between 1:30 and 1:100, thus suggesting fragment analysis as a superior technique for identification of mixed infections as compared to sequencing.

A total of 13 (7·0 %) mixed infections was identified by fragment typing (Table 3). Subgenotype C1 was found in 24 (12·9 %) of the samples; C2 was demonstrated in 25 (13·4 %), and C3 was prevalent in 124 (66·7 %) of the Danish cattle specimens analysed in this study. Including the mixed infections, the prevalences for C1, C2, and C3 were 16·7 % [11·6–22·8 %], 17·2 % [12·1–23·4 %], and 73·1 % [66·1–79·3 %].

At all times bovine isolates identified as genotype II by analysis of the COWP gene were characterized as one of the 'C types' by microsatellite analysis, and the 'bovine genotype' by analysis of the 18S rDNA gene. Estimated probability and exact binomial confidence limits for a bovine isolate with 18S rDNA Cpb being COWP I or equal a bovine isolate, COWP II being a Cph: $P = 0{\cdot}0\,\%$ [0·0 %; 20 %]. Likewise, the estimated probability for a bovine isolate identified as a 'C type' being COWP I or H1 was $P = 0{\cdot}0\,\%$ [0·0 %; 3·4 %].

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Table 4. Code, host, year, place of origin, geno- and subgenotyping results from 7 Danish *Cryptosporidium* isolates analysed by PCR amplification of the COWP gene, the 18S rDNA, microsatellite analysis (GAG-seq.), and direct typing of GAG microsatellites (fragment typing/F-type)

Code	Host	Year	Place of origin	COWP	18S rDNA†	GAG-seq.	F-type
CPB 0*	Calf	1990	Denmark	II	Cpb	C2	C2
CPO 12	Lamb	2000	Tappernoeje	II	Cpb	C2	C2
CPP 13	Piglet	2000	Viby	—	Cpp	—	—
CPP 17	Piglet	2000	Roedekro	—	Cpp	—	—
CPP 18	Piglet	2000	Agerskov	—	Cpp	—	—
CPP 19	Piglet	2000	Als	—	Cpp	—	—
CPA 16	Hedgehog	2001	Svendborg	N.T.	Cpb	‡	H1

* 'The Copenhagen calf laboratory strain', propagated at the Danish Veterinary Institute since 1990.
† Cpb, *Cryptosporidium parvum*, bovine genotype; Cpp, *Cryptosporidium parvum*, porcine genotype.
‡ Genetically distinct subgenotype (AF469174).
—, No PCR amplification.
N.T., Not tested.

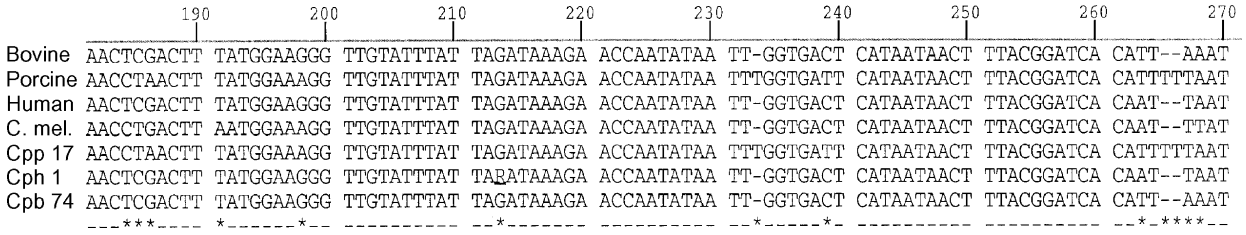

Fig. 3. Partial sequence alignment (approximate position: 533–590) of the 18S rDNA illustrating some of the sequence differences used to distinguish *Cryptosporidium* genotypes/species. Bovine: *C. parvum*, the bovine genotype (AF093490); Porcine: *C. parvum*, the porcine genotype (AF108861); Human: *C. parvum*, the human genotype (AF093492); C. mel.: *C. meleagridis* (AF112574), and representative porcine (Cpp 17), human, (Cph 1) and bovine (Cpb 74) *C. parvum* isolates.

As regards geographical distribution the spatial cluster analysis revealed a clustering of subgenotype C1 in the southern part of Jutland. The cluster area contains 66 farms from which 25 out of 78 samples were subgenotype C1 as illustrated in Fig. 2. The expected number of subgenotype C1 in the area was 12 resulting in a relative risk of 2·08, a Monte-Carlo ranking of 42/250.000 and thereby a P value = 0·00017. The proportion of subgenotype C1 outside and inside the marked area was 4·3 % [1·4–9·7 %], and 32·1 % [21·9–43·6 %] respectively. Based on the present findings, the chances of a C1 isolate being from Southern Jutland is 80·0 % [59·3–93·2 %]. The most likely cluster for subgenotype C2 and C3 had P values (based on 250.000 replications) of 0·37 and 0·11 respectively, thus no clustering was detected.

Heterogeneity among other Cryptosporidium *isolates*

Typing results from 'other' animals, including the 'Copenhagen calf laboratory strain' (CPB 0), are shown in Table 4. The CPB 0 strain, in addition to 1 isolate from a lamb, achieved identical typing results, as did 4 porcine *Cryptosporidium* isolates. The latter were not amplified with the primers used to analyse the COWP gene or the GAG microsatellites, whereas sequence analysis of an approximately 925 bp product of the 18S rDNA gene revealed a distinct genotype (Fig. 3) identical to a pig sequence from GenBank (AF108861). Furthermore, a *Cryptosporidium* isolate from a hedgehog (*E. europaeus* L.) characterized as the 'bovine genotype' by partial sequencing of the 18S rDNA, revealed a DNA sequence the size of subgenotype H1 by fragment typing, whereas sequencing of the locus containing GAG microsatellites showed a new, genetically distinct genotype (AF469174).

Heterogeneity among human Cryptosporidium *isolates*

Of the 72 human *Cryptosporidium* isolates examined during the present study, 44 could be typed by one or more of the applied techniques (Table 5). Typing results were obtained from 51·7, 43·5, and 44·4 % of the specimens analysed by PCR of the COWP gene, microsatellite sequencing, and fragment typing respectively, whereas results from the partial amplification of the 18S rDNA were only achieved from 19·4 % of the samples. Subgenotype H1 was found in

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Table 5. Geno- and subgenotyping results from 72 human *Cryptosporidium parvum* isolates analysed by PCR amplification of the COWP gene, the 18S rDNA, microsatellite analysis (GAG-seq.), and direct typing of GAG microsatellites (fragment typing/F-type)

Code	Year	Travel*	COWP	18S rDNA†	GAG-seq.	F-type
CPH 29	2000	The Canary Islands	I	Cph	H1	H1
CPH 52	2000	Central America	I	Cph	H1	H1
CPH 1	1997		—	Cph	H1	—
CPH 45	2000		I	Cph	—	H1
CPH 43	2000		—	Cph	—	H1
CPH 42	2000		—	Cph	—	—
CPH 44	2000		I	—	H1	H1
CPH 58	2000		I	—	H1	H1
CPH 21	1999	'Yes'	—	—	H1	H1
CPH 13	1999		I	—	H1	H1
CPH 50	2000		I		H1	H1
CPH 20	1999	Italy	I	—	H1	H1
CPH 22	1999		—	—	H1	H1
CPH 26	2000	Italy	—	—	H1	—
CPH 39	2000	Egypt	—	—	H1	—
CPH 63	2000		N.T.	—	H1	—
CPH 14	1999		N.T.	—	H1	—
CPH 64	2000	Greece	—	—	—	H1
CPH 23	1999		N.T.	—	—	H1
CPH 75	2001	Somalia	N.T.	—	—	H1
CPH 76	2001	Egypt	N.T.	—	—	H1
CPH 72	2001	[a]	N.T.	N.T.	N.T.	H1
CPH 73	2001	Asia	N.T.	N.T.	N.T.	H1
CPH 79	2001	Somalia	N.T.	N.T.	N.T.	H1
CPH 68	2001	Nepal	N.T.	N.T.	N.T.	H1
CPH 31	2000		—	—	C1	—
CHP 25	2000	[b]	II	Cpb	C2	C2
CPH 55	2000		II	Cpb	C2	C2
CPH 7	1998	Bolivia	II	Cpb	C2	—
CPH 17	1999		—	—	C2	C2
CPH 65	2000	Ethiopia	—	—	C2	C2
CPH 56	2000	[c]	N.T.	—	C2	C2
CPH 35	2000		—	—	C2	—
CPH 8	1998		N.T.	N.T.	N.T.	C2
CPH 57	2000		N.T.	N.T.	N.T.	C2
CPH 47	2000		II	Cpb	—	C3
CPH 48	2000		II	Cpb	C3	—
CPH 54	2000	Turkey	II	—	C3	C3
CPH 80	2001		N.T.	—	C3	C3
CPH 49	2000		N.T.	—	C3	C3
CPH 53	2000	Portugal	II	—	C3	—
CPH 81	2001		N.T.	—	—	C3
CPH 51	2000	Spain	—	—	—	C3
CPH 67	2000	Nepal	—	*C. mel.*	—	—
Total, examined			29	62	62	72
Positive typing results			15	12	27	32

* [a] Swimming pool-associated infection; [b] laboratory infection; [c] transmission from mother to child.
† Cph, *Cryptosporidium parvum*, human genotype; Cpb, *Cryptosporidium parvum*, bovine genotype; *C. mel.*, *Cryptosporidium meleagridis*.
—, No PCR amplification.
N.T., Not tested.

24 human isolates. One further isolate was identified as the 'human genotype' by analysis of the 18S rDNA only, giving a total of 25 (56·8%) [41·0–71·7%] of the human samples characterized as anthroponotic. The zoonotic subgenotypes C1, C2, and C3 were detected in 18 (40·9%) [26·3–56·8%] of

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the samples. Of the anthroponotic types, 12 isolates were recovered from patients with a history of recent travel whereas 5 of the zoonotic types were from patients with a record of travel. No significant differences were revealed between genotypes as regards the relative number of patients with a recent history of travel ($P = 0{\cdot}22$). One human isolate was identified as *C. meleagridis*.

Human isolates identified as genotype I by analysis of the COWP gene were characterized as H1 by sequencing or fragment typing of microsatellites, and the 'human genotype' by analysis of the 18S region. Genotype II isolates were identified as 'C types' by microsatellite analysis, and the 'bovine genotype' by analysis of the 18S rDNA. There was a significant coherence between COWP and 18S rDNA ($P = 0{\cdot}0179$), and COWP and microsatellite analysis ($P = 0{\cdot}0002$), respectively.

DISCUSSION

In the present study a heterogeneous collection of *Cryptosporidium* samples from 271 human and animal patients were characterized by a multilocus approach including sequence analysis of microsatellites. By this technique a total of 4 different subgenotypes were identified in the Danish isolates. As in the studies by Cacciò *et al*. (2000, 2001) the subgenotypes segregated into 2 large groups corresponding to genotype I and II, identified by analysis of the COWP gene and the 18S rDNA. Similar to the findings reported by Cacciò *et al*. (2000), who found subgenotype H1 in human isolates from Europe and America, H1 was also identified in the Danish samples. But whereas Cacciò *et al*. (2000) discovered a second subgenotype (H2) in 5 human isolates from Japan, no heterogeneity was seen among H types in the present study.

Three zoonotic subgenotypes (C1, C2, and C3) were detected in human as well as in animal isolates analogous to the findings of Cacciò *et al*. (2000). However, whereas Cacciò found C1 to account for the majority (16/26) of human infections caused by the zoonotic subgenotypes, we only identified one (1/18) C1 isolate in the human material. Subgenotype C4 that was earlier found in human and animal isolates from central and southern Italy (Cacciò *et al*. 2000, 2001) was not observed in the Danish samples.

The anthroponotic genotype was more prevalent than the zoonotic genotype in the human samples (56·8 versus 40·9 %); however, because of the limited data so far no conclusion regarding the relative distribution of genotypes in the human Danish population can be made. In a recent study from the United Kingdom, genotype I was detected in 37·8 % of 1705 *Cryptosporidium*-positive faecal samples from humans, whereas 61·5 % were attributed to genotype II (McLauchlin *et al*. 2000). These data were in agreement with previous European studies (Bonnin *et al*. 1996; Awad-El-Kariem *et al*. 1998; McLauchlin *et al*. 1999; Cacciò *et al*. 2000), but differed markedly from an American study of 50 cases of human cryptosporidiosis, showing that 82 % were due to genotype I, while 18 % were caused by genotype II (Sulaiman *et al*. 1999). Similarly, a study of 85 cases of cryptosporidiosis in Peruvian children demonstrated that 78·8 % were due to genotype I, whereas 9·4 % were identified as genotype II (Xiao *et al*. 2001). Variation might be present between different geographical areas with respect to the prevalence and the relative importance of the genotypes, but comparison of studies is difficult, and certain conclusions concerning this issue should await true prevalence studies, and the analysis of a much larger number of isolates from several different sources.

Within the series of cryptosporidiosis cases presented here, all bovine samples yielded genotype II, which is consistent with results from others (Awad-El-Kariem *et al*. 1995; Morgan *et al*. 1999*b*; McLauchlin *et al*. 2000).

Subgenotype C3 was found in 73·1 % of faecal samples from Danish cattle herds analysed in the present study, but the prevalence might have been biased by the fact that only samples from clinical cases were included in the study. Apart from our findings, C3 isolates were identified in 4 livestock samples from The Netherlands (Cacciò *et al*. 2000) as well as in several samples from one particular Dutch cattle herd (Huetink *et al*. 2001). As in the case of subgenotype C4, found only in central and southern Italy (Cacciò *et al*. 2000, 2001), the frequent finding of subgenotype C3 in Danish *Cryptosporidium* samples might imply a heterogeneous geographical distribution of subgenotypes which, among other things, could be correlated to patterns of farm animal trade as suggested by Cacciò *et al*. (2001).

Our study showed that, whereas subgenotype C3 was randomly distributed in Danish cattle herds, C1 was significantly more prevalent in the southern part of the Danish mainland. This is surprising because there are no obvious natural boundaries, and other studies have demonstrated C1 in calf, lamb, kid and humans from all over Italy in addition to calf isolates from the United States, Japan and other European countries (Cacciò *et al*. 2000, 2001; Huetink *et al*. 2001). The reasons for the differences in the geographical distribution of subgenotypes, whether correlated to i.e. farm-animal trade, management or herd size was not further investigated in the present study.

It is noteworthy that the 'Copenhagen calf laboratory strain', which has been propagated in a laboratory environment for more than 10 years, was genetically identical at the 3 distinct loci to the 'wild type' C2 strains analysed in our study. This indicates

that the Danish *C. parvum* population has been stable over time. Furthermore, a linkage disequilibrium between alleles at the independent loci examined in our study, was observed. According to Tibayrenc & Ayala (1991) these statements support the existence of a clonal population structure, and demonstrate the necessity of discriminative methods with a multilocus approach to be used in future studies for a critical evaluation of the population structure.

Of the 44 human *Cryptosporidium* samples typed in the present study, 1 was characterized as *C. meleagridis*, which normally infects turkeys (Fayer *et al*. 1997). However, there is increasing evidence of a much wider host range of this parasite than previously thought (Morgan *et al*. 2000*a*, *b*). So far, the findings of *C. meleagridis* in humans include 1 isolate from Switzerland and 1 from Kenya (Morgan *et al*. 2000*a*), 5 of 1705 cases of cryptosporidiosis in the United Kingdom (McLauchlin *et al*. 2000), and 7 of 85 infections in Peruvian children (Xiao *et al*. 2001).

Many studies worldwide indicate that *C. parvum* is not a single uniform species, but consists of several distinct genotypes or cryptic species, among those the 'pig genotype' (Morgan *et al*. 1999*a*, *b*). In accordance with these studies, our results from amplification of a fragment of the 18S rDNA region confirm that the porcine isolates are different from the 'bovine' and 'human genotype' of *C. parvum*. Furthermore, no products were obtained from these isolates with the primers used to amplify a fragment of the COWP gene or the GAG microsatellites (G35348 locus) of *C. parvum*. Our typing results also verify that the 'pig genotype' is conserved genetically across geographical areas, as the Danish isolates were identical to Swiss and Australian pig isolates analysed by Morgan *et al*. (1998). While strong molecular evidence of a pig specific *Cryptosporidium* species exists (Xiao *et al*. 1999; Morgan *et al*. 1999*a*) including the present results, there is still a need for further studies particularly of biological characteristics, before a final decision regarding the classification of the 'pig genotype' can be made.

The finding of a distinct subgenotype in a *Cryptosporidium* isolate from a hedgehog (*E. europaeus* L.) support the assumption that a series of host-adapted genotypes/strains/species of the parasite exist (Morgan *et al*. 1999*b*). In addition, this is, to our knowledge, the first reported finding of *Cryptosporidium* in hedgehogs (*E. europaeus* L.).

In several cases, sequencing of samples containing more than 1 subgenotype led to inclusive typing, which was one reason for the increased sensitivity obtained with the fragment typing. In addition, the GeneScan technology (fragment typing) made analysis of microsatellites easier, faster, and less expensive compared to sequencing. However, one must be aware that the results from the fragment typing simply reflect the size of the amplified fragment, which might entail misleading results, as in the case of the hedgehog isolate which was typed as H1, whereas sequencing revealed a distinct subgenotype.

In conclusion, we have characterized a collection of *Cryptosporidium* isolates from a relatively restricted geographical area. By the use of molecular techniques, we were able to recognize genotypes I and II, and further identify 4 subgenotypes of *C. parvum* in Denmark. Moreover, the existence of a specific 'pig genotype' was verified, and *Cryptosporidium* in a hedgehog was identified for the first time.

The results presented here also confirm that microsatellites are informative markers for the molecular identification of *C. parvum*. Furthermore, we have shown that the application of fragment typing to differentiate between the different subgenotypes is a cheap, reliable, and relatively fast alternative to sequencing of microsatellites. Nevertheless, it is important that the subtyping technique is only used in combination with a reliable method for species identification, and it is essential, that future molecular studies are conducted in conjunction with conventional biological studies to assess the correlation between genetic variation and clinically important characteristics.

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