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Detection of zoonotic *Cryptosporidium* spp. in small wild rodents using amplicon-based next-generation sequencing

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ABSTRACT

Rodents may serve as reservoirs of zoonotic species of *Cryptosporidium*; however, data from molecular surveys in support of this hypothesis are still scarce. In this study, we screened faeces and rectal content from murid and cricetid rodents ($N = 58$) caught around three farms in Zealand, Denmark, for *Cryptosporidium* spp. by amplicon-based next-generation sequencing (NGS) of ribosomal genes. Selected samples were further examined using nested conventional PCR targeting SSU rRNA, gp60, and actin genes. *Cryptosporidium*-specific DNA was identified in 40/58 (69%) samples, and in 12 (30%) of the 40 positive animals, mixed cryptosporidial infections were observed. *Cryptosporidium ditrichi* was the species most commonly identified, found in 28 (48%) of the animals. *Cryptosporidium parvum* was identified in 4 (7%) of the animals, all of which were co-infected with *C. ditrichi*. The present study is the first to utilize NGS-based screening for *Cryptosporidium* species in wild rodents. Moreover, it is the first study to provide molecular data on *Cryptosporidium* in rodents sampled in Denmark and to detect DNA of *C. ditrichi* in *Mus musculus, Myodes glareolus,* and *Microtus agrestis*. The NGS approach was successfully applied to yield new knowledge, and the results showed that zoonotic species of *Cryptosporidium* are common in murid and cricetid rodents in Zealand, Denmark.

1. Introduction

More than 40 species and genotypes of *Cryptosporidium* have been identified in rodents (García-Livia et al., 2022). Both the murid

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and cricetid families include species that exhibit varying degrees of synanthropism (Baagøe and Jensen, 2007) and could be important reservoirs of human *Cryptosporidium* infections.

Over the past couple of years, Denmark has seen a rise in the number of detected *Cryptosporidium* infections in humans (Larsen et al., 2023; Johansen et al., 2023). This surge may primarily reflect a change in the testing of stool samples by the introduction of diagnostic PCR panels ('syndromic testing') featuring multiple intestinal pathogen targets, including *Cryptosporidium*, in some of the regional clinical microbiology departments. This means that *Cryptosporidium* is now part of the screening for pathogens involved in gastroenteritis and not only tested for upon special request. Molecular surveillance has led to the identification of several zoonotic species in human samples (Larsen et al., 2023), mainly *Cryptosporidium parvum*, but also *Cryptosporidium tyzzeri*, *Cryptosporidium ditrichi*, and *Cryptosporidium mortiferum* (also known as chipmunk genotype I (Tůmová et al., 2023)), which can all be hosted by rodents (Zhang et al., 2022). No molecular data has been available on *Cryptosporidium* spp. in wild rodents from Denmark, and so the potential role of rodents as a reservoir of human cryptosporidiosis in Denmark remains to be investigated.

DNA-based detection methods enable detection with high sensitivity as well as differentiation of species and genotypes of *Cryptosporidium*. Various genes are routinely used for differentiation; however, very few of the primers published to date are applicable across genetic variants of *Cryptosporidium*. Sequencing of rRNA genes is usually used for species and genotype differentiation, sometimes in combination with actin, heat shock protein 70, or other genes, whereas the glycoprotein (gp) 60 locus is used for subtyping (Lebbad et al., 2021). However, although the gp60 subtyping method published by Alves and colleagues (Alves et al., 2003) amplifies sequences from a variety of species, species-specific primers are needed to amplify the gp60 genes in species such as *Cryptosporidium meleagridis* (Stensvold et al., 2014), *Cryptosporidium viatorum* (Stensvold et al., 2015a), and *Cryptosporidium* chipmunk genotype I (Guo et al., 2015). What is more, for many species and genotypes infecting rodents (e.g., *C. ditrichi*), primers for molecular characterization of the gp60 gene are yet to be developed and published. Finally, any method depending on PCR followed by Sanger sequencing would be challenged in terms of detecting and differentiating *Cryptosporidium* infections of mixed species. Amplicon-based next-generation sequencing (NGS) of small subunit (SSU) ribosomal RNA (rRNA) genes, also known as metabarcoding (Chihi et al., 2022), has previously been used to detect and differentiate mixed *Cryptosporidium* infections in faecal samples from pigs (Stensvold et al., 2021), and could be a relevant method for exhaustive identification of *Cryptosporidium* species and genotypes found in rodents.

In the present study, DNA was extracted from faeces and rectal content of wild murid and cricetid rodents and screened for *Cryptosporidium* using amplicon-based NGS of nuclear ribosomal genes.

2. Materials and methods

2.1. Samples

In the context of rodent control, three farms in Northern Zealand, Denmark, agreed to participate in the current study. The animals housed at the farms included rabbits, cats, dogs, chickens, turkeys, ducks, pigs, sheep, horses, goats, ponies and donkeys.

Two types of traps, namely Ugglan Special trap No. 1 and Ugglan Special trap No. 2, were placed within a 500-m radius of the farms, instead of normal rodent traps used for pest control. The study area encompassing the three distinct farms covered an area of approximately 10 km^2.

Table 1
Number of host species positive for *Cryptosporidium* according to *Cryptosporidium* species (mixed infections were observed; see Table 2 for details).

Host species ('n' is the number of individuals tested)	*Cryptosporidium ditrichi*	*Cryptosporidium parvum*	*Cryptosporidium* sp. (JN172968 99% sim. or more)	*Cryptosporidium* sp. vole genotype II (99% sim. or more)	*Cryptosporidium* sp.	*Cryptosporidium* vole genotype or *Cryptosporidium microti*	Total number of animals infected
Apodemus flavicollis ($n = 18$)	11	1	2	1	0	0	13/18
Apodemus sp. ($n = 2$)	2	0	1	0	0	0	2/2
Apodemus sylvaticus ($n = 1$)	0	0	0	0	0	0	0/1
Micromys minutus ($n = 4$)	2	0	0	1	1	0	3/4
Microtus agrestis ($n = 5$)	1	0	0	0	1	1	2/5
Mus musculus ($n = 10$)	8	2	0	1	0	0	9/10
Myodes glareolus ($n = 18$)	4	1	4	6	2	0	11/18

Table 2
Overview of rodent rectal content and faecal samples included in the study, with host data (species, sex, body length, and body weight), tested for the presence of *Cryptosporidium* DNA using amplicon-based next-generation sequencing (NGS) of ribosomal genes. For some of the samples, PCR and Sanger sequencing of SSU rRNA, actin and/or gp60 genes were attempted, and *Cryptosporidium*-specific sequences were submitted to the NCBI nucleotide database.

Animal ID	Rodent species	Sex	Body length (cm)	Body weight (g)	*Cryptosporidium* NGS result	NGS result						Sanger sequence data available[a]
						C. ditrichi	*C. parvum*	*C.* sp. (JN172968 99% sim. or more)	*C.* sp. vole genotype II (99% sim. or more)	*C.* sp.	*C.* vole genotype or *C. microti*	
ab1	*Apodemus flavicollis*	M	7	14	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
ab2	*Myodes glareolus*	F	7.5	15	Pos	Neg	Neg	Pos	Neg	Neg	Neg	Actin: *Cryptosporidium* sp. Vole genotype IV [OR437325]
ab3	*Myodes glareolus*	NA	8.5	16	Pos	Neg	Neg	Neg	Pos	Pos	Neg	SSU rDNA: *Cryptosporidium* sp. (low quality) [OR428357]
ab4	*Apodemus flavicollis*	F	7	15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
ab5	*Myodes glareolus*	F	8	16	Pos	Pos	Neg	Neg	Pos	Neg	Neg	SSU rDNA: *Cryptosporidium* sp. [OR428358]
ab6	*Mus musculus*	F	6.5	13	Pos	Pos	Neg	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428359]
ab7	*Apodemus flavicollis*	M	8	18	Pos	Pos	Neg	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428360]
ab8	*Micromys minutus*	NA	5	9	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
ab9	*Mus musculus*	M	7.2	16	Pos	Pos	Neg	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428361]
ab10	*Mus musculus*	F	7.7	16	Pos	Pos	Pos	Neg	Neg	Neg	Neg	No
ab11	*Mus musculus*	M	8	18	Pos	Pos	Pos	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428362] and gp60: *C. parvum* IIdA22G1c [OR447415]
ab12	*Apodemus flavicollis*	F	8	20	Pos	Neg	Neg	Pos	Neg	Neg	Neg	No
bb1	*Myodes glareolus*	F	8.4	18	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
bb2	*Mus musculus*	M	7.5	15	Pos	Pos	Neg	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428363] and actin: *C. ditrichi* [OR437324]
bb3	*Apodemus flavicollis*	M	9	19	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
bb4	*Microtus agrestis*	F	9.5	20	Pos	Neg	Neg	Neg	Neg	Pos	Pos	SSU rDNA: *Cryptosporidium* sp. (low quality) [OR428364]
bb5	*Microtus agrestis*	F	10.5	32	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
bb6	*Microtus agrestis*	M	10	26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
bb7	*Mus musculus*	M	8.5	17	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
bb8	*Mus musculus*	F	8	15	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
bb9	*Mus musculus*	F	8	17	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Actin: *C. ditrichi* [OR437323]

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Table 2 (*continued*)

Animal ID	Rodent species	Sex	Body length (cm)	Body weight (g)	*Cryptosporidium* NGS result	NGS result						Sanger sequence data available[a]
						C. ditrichi	*C. parvum*	*C.* sp. (JN172968 99% sim. or more)	*C.* sp. vole genotype II (99% sim. or more)	*C.* sp.	*C.* vole genotype or *C. microti*	
cp1	*Micromys minutus*	F	6.5	9	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
cp2	*Apodemus flavicollis*	F	7.5	16	Pos	Pos	Neg	Neg	Neg	Neg	Neg	SSU rDNA: *C. ditrichi* [OR428365]
cp3	*Myodes glareolus*	M	8.5	17	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
cp4	*Myodes glareolus*	F	8.3	16	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
dp1	*Apodemus* sp.	F	7.56	15	Pos	Pos	Neg	Pos	Neg	Neg	Neg	Actin: *Cryptosporidium* sp. Vole genotype IV [OR437322]
dp2	*Myodes glareolus*	F	8	18	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
ej1	*Myodes glareolus*	F	8	16	Pos	Neg	Neg	Pos	Neg	Neg	Neg	No attempt
ej2	*Apodemus flavicollis*	M	11	40	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Actin: *C. ditrichi* [OR437318]
ej3	*Microtus agrestis*	F	9.5	30	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
ej4	*Apodemus flavicollis*	F	10.5	32	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No
fj1	*Myodes glareolus*	F	8.5	15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
fj2	*Micromys minutus*	M	5.5	7	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
fj3	*Mus musculus*	M	8.5	18	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
gp1	*Myodes glareolus*	F	8.2	18	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Actin: *Cryptosporidium* sp. Vole genotype IV [OR437321]
gp2	*Myodes glareolus*	F	8.5	19	Pos	Neg	Neg	Neg	Pos	Pos	Neg	No
gp3	*Myodes glareolus*	M	8.7	15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
gp4	*Myodes glareolus*	F	8	16	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
gp5	*Myodes glareolus*	M	8	16	Pos	Pos	Pos	Neg	Neg	Neg	Neg	SSU rDNA: *C. parvum* (low quality) [OR428366] and gp60: *C. parvum* IIaA16G3R1 [OR447416]
gp6	*Apodemus flavicollis*	F	7.5	15	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
gp7	*Apodemus flavicollis*	M	8.5	17	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Actin: *C. parvum* [OR437320] and gp60: *C. parvum* IIaA16G3R1 [OR447414]
hp1	*Apodemus flavicollis*	M	8	17	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
hp2	*Apodemus flavicollis*	F	9.5	25	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Actin: *C. ditrichi* [OR437319]

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Table 2 (*continued*)

Animal ID	Rodent species	Sex	Body length (cm)	Body weight (g)	*Cryptosporidium* NGS result	NGS result						Sanger sequence data available[a]
						C. ditrichi	*C. parvum*	*C.* sp. (JN172968 99% sim. or more)	*C.* sp. vole genotype II (99% sim. or more)	*C.* sp.	*C.* vole genotype or *C. microti*	
hp3	*Apodemus* sp.	M	8	19	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
ij1	*Myodes glareolus*	M	8	15	Pos	Neg	Neg	Neg	Pos	Neg	Neg	No attempt
ij2	*Myodes glareolus*	F	9	17	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No
ij3	*Apodemus flavicollis*	M	9	25	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
ij4	*Apodemus flavicollis*	M	8	16	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
jj1	*Apodemus flavicollis*	M	8	17	Pos	Neg	Neg	Pos	Pos	Neg	Neg	No attempt
kb1	*Myodes glareolus*	F	12	15	Pos	Neg	Neg	Pos	Pos	Neg	Neg	No attempt
kb2	*Mus musculus*	M	10	22	Pos	Neg	Neg	Neg	Pos	Neg	Neg	No attempt
kb3	*Micromys minutus*	M	6	7	Pos	Neg	Neg	Neg	Pos	Pos	Neg	No attempt
kb4	*Apodemus flavicollis*	M	8	15	Pos	Pos	Neg	Neg	Neg	Neg	Neg	No attempt
kb5	*Apodemus flavicollis*	M	8.5	16	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
kb6	*Microtus agrestis*	F	10	23	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
lb1	*Apodemus flavicollis*	F	7.5	17	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
lb2	*Myodes glareolus*	M	8.5	16	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt
lb3	*Apodemus sylvaticus*	M	9	20	Neg	Neg	Neg	Neg	Neg	Neg	Neg	No attempt

[a] "no attempt" – the sample was not subject to PCR and Sanger sequencing; "no" – the sample was subject to PCR and Sanger sequencing (primarily the actin and SSU rRNA loci) but sequencing either failed or yielded a sequence that did not represent *Cryptosporidium*.

Table 3
Overview of *Cryptosporidium* species identified based on DNA evidence (data extracted from the NCBI Database on March 3, 2023), by host species represented in this study. Shotgun data has not been included. Consensus sequences obtained in the present study are highlighted in bold.

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
Apodemus flavicollis	*C. apodemi*	MH913031	NA	MH912926	MH913073	NA	NA
		MH913032		MH912928	MH913075		
		MH913038		MH912934	MH913081		
		MH913039		MH912935	MH913082		
		MH913040		MH912936	MH913083		
	C. ditrichi	MG266032	NA	MG266045	MH913067	NA	NA
		MH913015		MH912939	MH913068		
		MH913017		MH912941	MH913070		
		MH913018		MH912942	MH913071		
		MH913019		MH912943	MG266040		
		MH913020		MH912944	MH913051		
		MH913021		MH912945	MH913052		
		MH913022		MH912946	MH913053		
		MH913025		MH912947	MH913054		
		MH913026		MH912948	MH913055		
		MG266031		MH912949	MH913056		
		MH913027		MH912950	MH913057		
		MH913028		MH912951	MH913060		
		MH913029		MH912952	MH913061		
		MG266030			MG266039		
		MH913008			MH913065		
		MH913011			MH913062		
		MH913012			MH913063		
		MH913013			MH913064		
		MH913010					
		OK605344					
		OK605328					
		OK605329					
		OK605330					
		OK605331					
		OK605333					
		OK605335					
		OK605336					
		OK605337					
		OK605340					
		OK605341					
		OK605342					
		OK605343					
		OK605345					
		OK605346					
		OK605347					
		OK605348					
		OK605349					
		OK605327					
		OK605334					
		OK605338					

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Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
		ab7 **bb3** **cp2** **ej2** **ej4** **gp6** **gp7** **hp1** **hp2** **ij4** **kb4**					
	C. hominis	MG266034	MG266037	MG266047	MG266042	NA	NA
	C. microti	MH913047 MH913048	NA	MH912962 MH912963	MH913090 MH913091	NA	NA
	C. muris	MH913049 MH913050	NA	MH912968 MH912969	MH913109 MH913110	NA	NA
	C. parvum	MH913045 MH913046 **gp7**	MH912987 MH912988 KU311672	AJ489216 MH912966 MH912967	MH913088 MH913089	NA	NA
	Cryptosporidium sp. apodemus genotype I	MH912991 MH912992 MH912993 MH912994 MH912995 MH912996 MH912997 MH912998 OK605332	MH912981 MH912982 MH912985 MH912979 MH912980 MH912983 MH912984 MH912986	MH912954 MH912955 MH912956 MH912957 MH912958 MH912959 MH912960 MH912961	MH913092 MH913093 MH913094 MH913095 MH913096 MH913097 MH913098 MH913099	NA	NA
	Cryptosporidium sp. apodemus genotype II	MH912999 MH913000 MH913002 MH913005	MH912975 MH912976 MH912970 MH912971	NA	MH913100 MH913103 MH913105 MH913106	NA	NA
	Cryptosporidium sp. mouse185	JN172968	NA	NA	NA	NA	NA
		ab12 **jj1**					
	Cryptosporidium sp. vole genotype II	OK605339	NA	NA	NA	NA	NA
		jj1					
	C. suis	KU311671	NA	NA	NA	NA	NA
	C. tyzzeri	MH913043	MH912990	MH912965	MH913086	NA	NA
	C. ubiquitum	KC962124	NA	NA	NA	NA	NA
Apodemus sylvaticus	*C. andersoni*	ON306384	NA	NA	NA	NA	NA
	C. apodemi	MH913041 MH913042	NA	MH912937 MH912938	MH913084 MH913085	NA	NA

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Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
	C. ditrichi	MH913016 MH913024 MH913009 ON306386	NA	MH912940	MH913069 MH913059 MH913066	NA	NA
	C. muris	ON306387	NA	NA	NA	NA	NA
	C. parvum	ON306388 AY282717	NA	NA	NA	NA	NA
	Cryptosporidium sp. CrCZ-27	NA	NA	AY282699	NA	NA	NA
	Cryptosporidium sp. HGC-2014	KM065510	NA	NA	NA	NA	NA
	C. tyzzeri	MH913044	MH912989	MH912964	MH913087	NA	NA
Myodes glareolus	*C. baileyi*	OK605409	NA	NA	NA	NA	NA
	C. parvum	NA	KU311670	AJ489215	NA	NA	NA
		gp5					
	Cryptosporidium sp.	KY644690 KY644691 KY644634 KY644635 KY644636 KY644637 KY644693 KY644692 KY644594 KY644595 OK605438 OK605434 OK605497 OK605458 KU311675 KU311677	NA	NA	KY657316 KY657317 KY657327 KY657318 KY657326 KY657351 KY657350	NA	NA
		ab3					
	Cryptosporidium sp. shrew genotype II	OK605459	NA	NA	NA	NA	NA
	Cryptosporidium sp. vole genotype II	OK605398 OK605399 OK605402 OK605403 OK605404 OK605455 OK605457 OK605476 OK605481 OK605484 OK605405 OK605406 OK605407 OK605408 OK605410	NA	NA	NA	NA	NA

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Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
		OK605411 OK605412 OK605416 OK605417 OK605419 OK605421 OK605422 OK605424 OK605425 OK605426 OK605428 OK605429 OK605430 OK605433 OK605435 OK605436 OK605439 OK605443 OK605444 OK605445 OK605446 OK605447 OK605448 OK605451 OK605452 OK605461 OK605472 OK605474 OK605479 OK605480 OK605485 OK605486 OK605489 OK605491 OK605496 OK605418 **ab3** **ab5** **ij1** **kb1**					
	Cryptosporidium sp. vole genotype III	OK605400 OK605466 OK605470 OK605414 OK605437 OK605440 OK605442	NA	NA	NA	NA	NA

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Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
		OK605449					
		OK605450					
		OK605453					
		OK605454					
		OK605456					
		OK605462					
		OK605463					
		OK605467					
		OK605468					
		OK605469					
		OK605473					
		OK605475					
		OK605488					
		OK605490					
		OK605493					
		OK605495					
		OK605498					
	Cryptosporidium sp. vole genotype IV	OK605477	NA	NA	NA	NA	NA
		OK605401					
		OK605413					
		OK605482					
		OK605494					
		OK605415					
		OK605420					
		OK605423					
		OK605427					
		OK605431					
		OK605432					
		OK605441					
		OK605471					
		OK605478					
		OK605483					
		OK605487					
		OK605492					
	Cryptosporidium sp. vole genotype IX	OK605465	NA	NA	NA	NA	NA
	Cryptosporidium sp. vole genotype VII	OK605397	NA	NA	NA	NA	NA
		OK605460					
		OK605464					
	Cryptosporidium sp. vole genotype VIII/ *C. microti*	**gp2**	NA	NA	NA	NA	NA
	C. ditrichi	**ab5**	NA	NA	NA	NA	NA
		cp4					
		dp2					
		gp5					
	Cryptosporidium sp. mouse185	**ab2**					NA
		dp2					
		ej1					
		gp1					

(*continued on next page*)

Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
		gp2					
		kb1					
Microtus agrestis	*C. microti*	OK605354	NA	NA	NA	NA	NA
		OK605362					
		OK605365					
		OK605371					
		OK605381					
		OK605361					
		OK605391					
		OK605387					
		OK605396					
		OK605380					
		OK605374					
	Cryptosporidium sp.	OK605355	NA	NA	NA	NA	NA
		OK605358					
		OK605370					
		OK605384					
		OK605368					
	Cryptosporidium sp. vole genotype II	OK605383	NA	NA	NA	NA	NA
	Cryptosporidium sp. vole genotype IX	OK605377	NA	NA	NA	NA	NA
		OK605359					
		OK605388					
		OK605376					
		OK605390					
		OK605392					
		OK605357					
		OK605373					
		OK605385					
		OK605389					
		OK605363					
	Cryptosporidium sp. vole genotype V	OK605356	NA	NA	NA	NA	NA
		OK605364					
		OK605382					
	Cryptosporidium sp. vole genotype VIII	OK605379	NA	NA	NA	NA	NA
		OK605378					
		OK605394					
		OK605393					
		OK605353					
		OK605360					
		OK605366					
		OK605367					
		OK605369					
		OK605372					
		OK605375					
		OK605386					
		OK605395					
	C. ditrichi	**ej3**	NA	NA	NA	NA	NA
	Cryptosporidium sp. UK E8	**bb4**	NA	NA	NA	NA	NA

(*continued on next page*)

Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
	Cryptosporidium sp. UK E4/*Cryptosporidium* sp. Vole genotype (V, VIII or IX)/ *C. microti*	**bb4**	NA	NA	NA	NA	NA
Mus musculus	*C. hominis*	EF570921 EF570921 EF570922 EF570922	NA	NA	NA	NA	NA
	C. muris	MN599008 KR090624 JQ073556 MN783640 AF248761	NA	KR090625	KR090628	KR090627	KR090626 HM358026 KR090632 KR090631 KR090629 KR090627 KR090630
	C. occultus	MG699178	NA	NA	MG699170	MG699174	NA
	C. parvum	AF093010 **ab10** **ab11**	JF727767 JF727768 JF727764 JF727766 JF727765 JF727769 JF727763	NA	NA	NA	NA
	Cryptosporidium sp.	MN599025 MN599022 MN599023 MN599024 MN783642 MH375331	NA	NA	NA	NA	NA
	Cryptosporidium sp. chipmunk genotype I	NA	NA	NA	NA	NA	MZ772043
	Cryptosporidium sp. CrMouBr23	KF176348	NA	NA	KF176346	NA	
	C. tyzzeri	GU951714 JQ073495 JQ073498 JQ073504 JQ073505 JQ073508 JQ073509 JQ073510 JQ073511 JQ073516 JQ073521 JQ073496 JQ073523 JQ073497 JQ073501 JQ073519 JQ073520 JQ073518 JQ073499	JQ073448 JQ073451 JQ073454 JQ073452 JQ073447 JQ073450 JQ073453 JQ073458 JQ073455 JQ073461 JQ073457 JQ073449 JQ073480 JQ073473 JQ073482 JX575577 JX575578 JQ073468 JQ073470	JQ073415 JQ073416 JQ073417 JQ073418 JQ073419 JQ073420 JQ073421 JQ073422 JQ073423 JQ073424 JQ073425 JQ073426 JQ073427 JQ073428 JQ073429 JQ073430 JQ073431 JQ073432 JQ073433	JQ073396 JQ073388 JQ073389 JQ073390 JQ073391 JQ073392 JQ073393 JQ073394 JQ073397 JQ073398 JQ073399 JQ073400 JQ073401 JQ073402 JQ073403 JQ073404 JQ073405 JQ073406 JQ073407		JQ073524 JQ073525 JQ073526 JQ073527 JQ073528 JQ073529 JQ073530 JQ073531 JQ073532 JQ073533 JQ073534 JQ073535 JQ073536 JQ073537 JQ073538 JQ073539 JQ073540 JQ073541 JQ073542

(*continued on next page*)

Table 3 (*continued*)

Host species	*Cryptosporidium* species	GenBank accession number (SSU rRNA)/**study ID**	GenBank accession number (gp60)	GenBank accession number (COWP)	GenBank accession number (actin)	GenBank accession number (hsp70)	GenBank accession number (other loci)
		JQ073506	JQ073474	JQ073434	JQ073408		JQ073543
		JQ073514	JQ073475	JQ073435	JQ073409		JQ073544
		JQ073502	JQ073476	JQ073436	JQ073410		JQ073545
		JQ073522	JQ073477	JQ073437	JQ073411		JQ073546
		JQ073515	JQ073456	JQ073438	JQ073413		JQ073547
		JQ073517	JQ073481	JQ073439	JQ073414		JQ073548
		JQ073494	JX575574	JQ073440	JQ073412		JQ073549
		JQ073503	JX575575	JQ073441	JQ073395		JQ073550
		JQ073507	JX575576	JQ073442			JQ073551
		JQ073500	JQ073463	JQ073443			JQ073552
		JQ073513	JQ073465	JQ073444			JQ073553
		JQ073512	JX575579	JQ073445			JQ073554
		JQ073483	JX575580	JQ073446			JQ073555
		JQ073484	JX575581				
		JQ073485	JQ073464				
		JQ073486	JQ073460				
		JQ073487	JQ073466				
		JQ073488	JQ073467				
		JQ073489	JQ073471				
		JQ073490	JQ073472				
		JQ073491	JQ073478				
		JQ073492	JQ073479				
		JQ073493	JQ073469				
		KJ569799	JQ073462				
			JQ073459				
			GU951713				
	C. ditrichi	**ab6**	NA	NA	NA	NA	NA
		ab9					
		ab10					
		ab11					
		bb2					
		bb8					
		bb9					
		fj3					
	Cryptosporidium sp. vole genotype II	**kb2**	NA	NA	NA	NA	NA
Micromys minutus	*Cryptosporidium* sp. apodemus genotype II isolate Mimi- FIN1	OK605352	NA	NA	NA	NA	NA
	C. ditrichi	**ab8**	NA	NA	NA	NA	NA
		cp1					
	Cryptosporidium sp. vole genotype II	**kb3**	NA	NA	NA	NA	NA
	Cryptosporidium sp.	**kb3**					NA

Abbreviation: NA – Not Available.

Sampling was carried out from mid-October until December 1st, 2019. Before initiating the sampling, the traps underwent a thorough cleaning with high-pressure water, without soap. Gloves were worn while handling the traps to minimize any human scent interference. Additionally, the traps were set opened at the sampling site 24 h prior to the first trappings. To attract rodents, the trapping compartment of each trap was baited with a piece of wild apple and a small quantity of oats. The bait was replaced with fresh supplies whenever the traps were collected or reused. Traps were strategically placed in the hedgerows surrounding the farms.

The traps were placed before sunset and retrieved in the morning, the period ranging from 16 to 18 h according to daylight changes. Captured rodents were transported to the laboratory inside the traps, in order to minimize stress. At the laboratory, the rodents were anesthetized and euthanized with carbon dioxide (CO_2) by submerging the trap into a box prefilled with CO_2.

Measurements of the weight, body length, and tail length of each animal were obtained post mortem. Host species were determined and noted using the identification key developed by Secher Jensen (1993). The gastrointestinal tract of each animal was extracted. Faeces and rectal content were collected and immediately frozen at −21 °C until DNA extraction.

2.2. Ethical considerations

The collection of wild rodents was conducted as part of pest control activities on the participating farms. All handling and the euthanasia of the rodents were carried out in a way to minimize stress to the animals.

2.3. DNA extraction and molecular analyses

To initiate the DNA extraction of the faecal pellets and rectal content samples, the following procedure was employed. First, 500 μL of lysis buffer (NUCLISENS ® easyMAG ® Lysis Buffer, bioMérieux SA, Marcy-l'Etoile, France) and 100 zirconium beads (1.4 mm Zirconium Beads, OPS DIAGNOSTICS, Lebanon, New Jersey, USA), were added to the samples (0.2 g material per sample). These samples were subjected to mechanical lysis using the TissueLyser II (Schwingmühle TissueLyser 2, QIAGEN GmbH, Hilden, Germany) for a duration of two minutes, operating at an oscillation frequency of 30.0 Hz. This initial step involved a combination of mechanical and chemical lysing methods, which is essential for effective faecal DNA collection and aligns with an established protocol recommended for human faeces (Eeckhout and Wullaert, 2018).

Next, the samples were centrifuged for five minutes at 20,238 ×*g*. Following centrifugation, 100 μL of the supernatant from each sample were processed using the NucliSENS eMAG automated platform (bioMérieux SA, Marcy-l'Etoile, France). The nucleic acid extraction process utilized magnetic silica particles to separate the DNA from each sample. Once the eMAG extraction was complete, the DNA was stored at −21 °C until molecular analyses.

The DNA samples were analysed by the metabarcoding assay as previously described (Stensvold et al., 2021; Stensvold et al., 2020). Briefly, the method employs PCR-based amplification, which utilizes a set of primers targeting 16S ribosomal genes and three sets of primers targeting 18S ribosomal genes. The G3 and G6 primers target the hyper-variable regions V3-V4 of the 18S gene, while the G4 primers target V3-V5 (PCR 1). After PCR 1, an adaptor PCR (PCR 2) is performed, and the DNA concentration is quantified using the Quant-iT high sensitivity dsDNA Assay Kit (Thermo Fisher Scientific, Eugene, Oregon, USA). The PCR 2 products are then pooled in equimolar amounts across samples. Undesirable DNA amplicons are removed from the pooled amplicon library (PAL) through purification using Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Indianapolis, USA). The resulting purified PAL was diluted to a final concentration of 11.5 pM DNA in 0.001 N NaOH and used for sequencing on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA). The library is sequenced using the 500-cycle MiSeq Reagent Kit V2 in a 2 × 250 nucleotide setup. For data analysis, raw reads are processed using the "BION" package. This involves quality trimming, read pairing, and chimera filtering before taxonomic classification of the sequences. The sequences derived from the three 18S rRNA targets are compared against the SILVA database (https://www.arb-silva.de/) in combination with an in-house database for taxonomic classification. No further investigations regarding 16S sequences were made within the current study.

From the BION server, fasta files containing DNA sequences were downloaded for *Cryptosporidium* and organized into sample-specific files. To produce consensus sequences, multiple sequence alignment was used, assisted by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/; last accessed 24 August 2023). Each alignment was visually inspected to identify sequence variation that could indicate genetic diversity, distinguishing these from potential errors in PCR or sequencing. Consensus sequences were generated for major clusters of nearly identical sequences (noting sporadic single nucleotide polymorphisms [SNPs] that were likely introduced by PCR or sequencing errors). These consensus sequences were recorded and queried against the NCBI Database (https://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed 24 August 2023). The sequences are available on Mendeley Data (https://data.mendeley.com/datasets/mc8gt35xb9/1).

Since the distribution across the entire dataset of the number of *Cryptosporidium*-specific reads per sample was skewed with most samples having reads <1000, and some even with <100 reads, ten DNA samples were randomly chosen for real-time PCR analysis for *Cryptosporidium*-specific small subunit ribosomal DNA (Berg et al., 2021) to establish whether the samples were indeed positive or whether the few reads observed for some of the samples might reflect sequence tag carryover from strongly positive samples (i.e., mechanical contamination).

Moreover, a random selection of DNA samples was subject to nested conventional PCR targeting *Cryptosporidium*-specific SSU rRNA genes (Xiao et al., 2001; Xiao et al., 1999), gp60 genes (Alves et al., 2003), and actin genes Sulaiman et al., 2002, with sequencing of those PCR products that could be considered specific for *Cryptosporidium* based on evaluation of PCR product size.

Sequences representing the SSU rRNA, actin and gp60 genes were submitted to the NCBI Database with the following accession numbers; OR428357–OR428366, OR437318–OR437325, and OR447414–OR447416, respectively.

2.4. Data extraction from GenBank

To obtain an overview of species of *Cryptosporidium* detected by others in the host species sampled in the present study, the NCBI Database was queried in March 2023. Briefly, genus names were entered in the search field along with Boolean operators as applicable (e.g., "*Cryptosporidium*" AND "*Apodemus*"). The resulting list of hits was then subject to filtering using the filters and other features available at the site where relevant to ensure the inclusion of 18S sequences only and exported (Table 3).

3. Results

A total of 58 individual rodents were sampled (Table 1), including 21 belonging to the genus *Apodemus*, of which 18 were *Apodemus flavicollis* and one was *Apodemus sylvaticus*. Other species sampled included *Myodes glareolus* ($n = 18$), *Mus musculus* ($n = 10$), *Microtus agrestis* ($n = 5$), and *Micromys minutus* ($n = 4$).

A total of 40 animals (69%) were found positive for *Cryptosporidium* sp. The species most commonly detected was *C. ditrichi,* found in 28 animals (70% of the positive animals), comprising species of *Apodemus, Micromys, Microtus, Mus,* and *Myodes*. *Cryptosporidium parvum* was identified in four animals (10% of the positive animals), whereas 12 *Cryptosporidium*-positive animals could only be typed to the genus *Cryptosporidium* (99%–100% similarity with reference sequences in the NCBI Nucleotide Database) (Table 2). Mixed cryptosporidial infections were observed in 12 animals (21% of the positive animals). All the four animals (*M. musculus* ($n = 2$), *M. glareolus* ($n = 1$), and *A. flavicollis* ($n = 1$)) that harboured *C. parvum* were also infected with *C. ditrichi*.

A significant difference was observed in terms of the host spectrum of *C. ditrichi*, as 23/35 (66%) of animals that could be categorized as 'mouse' (*A. flavicollis*, *A. sylvaticus*, *M. musculus,* and *M. minutus*) were positive, whereas 5/23 (22%) animals categorized as 'vole' (*M. glareolus* and *M. agrestis*) were positive ($p = 0.0013$, Fisher's Exact Test). The overall *Cryptosporidium*-positivity rate was 27/35 (77%) among the mice and 13/23 (56%) among the voles. *Cryptosporidium*-positive animals had the same median weight as *Cryptosporidium*-negative animals (18 g; range, 7–40 g).

Ten of the samples with few reads from Illumina-based sequencing (range, 21–605 reads; mean, 184.5; median, 145.5) were randomly chosen for confirmation by real-time PCR. All were deemed positive, with cycle threshold values ranging from 28.79 to 36.90 (mean, 31.91; median, 32.07).

For a subset of the samples (Table 2), attempts were made to produce SSU ribosomal DNA (rDNA) and actin DNA sequences by PCR and Sanger sequencing to further confirm the species identified using the NGS data. Readable *Cryptosporidium*-specific SSU rDNA sequences were obtained from 10 samples and actin sequences were obtained from eight samples. In all cases, there was agreement between the NGS data and the Sanger sequencing data. In eight other *Cryptosporidium*-positive samples, sequencing of PCR products obtained by primers targeting the actin gene resulted in the generation of DNA sequences of trichomonad actin genes.

Three of the *C. ditrichi* NGS consensus sequences differed by multiple consistent SNPs from the remaining *C. ditrichi* sequences, but at least 3% genetic difference among *C. ditrichi* sequences can be acknowledged by analysis of GenBank data. No SSU rDNA sequences were obtained by the Sanger method for any of these three samples. However, actin DNA sequences were obtained for two of the samples; these were identical and differed by two consistent SNPs from *C. ditrichi* actin DNA sequences from two other samples.

Gp60-based subtyping of *C. parvum* yielded IIaA16G3R1 from two samples (*M. glareolus* and *A. flavicollis*), and IIdA22G1c from one sample (*M. musculus*) (Table 2). We were not able to produce a gp60 sequence from one selected sample.

Among *Cryptosporidium* not belonging to *C. ditrichi* and *C. parvum*, four samples from voles and three samples from mice produced NGS-based consensus sequences that shared at least 99% similarity to *Cryptosporidium* sp. vole genotype II (Table 2). Moreover, four samples from voles and two samples from mice produced NGS-based consensus sequences that shared at least 99% similarity to GenBank acc. no. JN172968, a sequence referred to as '*Cryptosporidium* sp. mouse185', found in *A. flavicollis* in Sweden (Backhans et al., 2013). Five other *Cryptosporidium* sequences were also found; these could not be assigned unambiguously to species or genotype.

Based on data from the present study and data available in the NCBI Database, an overview of the species and genotypes of *Cryptosporidium* by host species included in the present study is provided in Table 3.

4. Discussion

This study is the first to detect and differentiate *Cryptosporidium* in wild rodents sampled in Denmark using DNA-based methods and the first to survey *Cryptosporidium* infections in wild rodents using NGS-based methods. Species of *Cryptosporidium* known to be zoonotic were found in a substantial proportion of the studied animals; especially *C. ditrichi* (48%), but also *C. parvum* (7%).

The use of amplicon-based NGS for identification of different *Cryptosporidium* species in the samples allowed for detection of mixed cryptosporidial infections, which was seen in 30% of the infected animals. If detection and differentiation had relied solely on PCR and Sanger sequencing, issues with mixed chromatogrammes would probably have precluded differentiation of the species in mixed infections. This could also be the case, even if steps had been taken to isolate oocysts or in other ways enhance template to increase target DNA and reduce the amount of non-target DNA. The process of purifying/isolating oocysts is demanding (Ebrahimzade et al., 2014; Gharieb et al., 2019; Kar et al., 2010), and such methods might not readily be incorporated into standard laboratory procedures. Therefore, even though oocyst isolation might aid in the detection and differentiation of species in mixed infections and cloning of PCR products could have been an option as well, the amplicon-based NGS appears to be a convenient an effective method for investigating the diversity of *Cryptosporidium* in rodents.

Research applying NGS-based methods to identify intestinal pathogens in wild rodents is scarce. Almost all studies published to date focus solely on the detection of bacterial species targeting 16S rRNA (Gurbanov et al., 2022; Jahan et al., 2021; Koskela et al.,

2017). One study included data on 18S rRNA gene sequencing, but the authors obtained only a relatively low number of reads, which were not referred to at a more specific taxonomic level (Tanaka et al., 2014). Based on the literature searches, the present study appears to be the first to utilize NGS for detection and differentiation of protozoan parasites in samples from wild rodents.

The occurrence of *Cryptosporidium* in the current study is relatively high compared with results of previous studies (Zhang et al., 2022). One could think of several potential reasons for this. A high occurrence in the sampled rodents, compared with previous studies, cannot be ruled out. Data presented here could also add to the evidence that the NGS platform used might be more sensitive than the amplicon-based methods utilized previously and less challenged than PCR and Sanger sequencing when it comes to detecting mixed infections. All rodents included in this study were collected during the late autumn of 2019. Possible seasonal differences could be of significance due to the relatively low freeze/thaw tolerance of *Cryptosporidium* oocysts (Robertson and Gjerde, 2004).

In this study, an effort was made to produce actin and gp60 DNA sequences as well as longer SSU rDNA sequences using PCR and Sanger sequencing for a subset of the samples. As anticipated, the tests only succeeded for some samples, and typically for samples with relatively high numbers of reads (data not shown). The sequences resulting from Sanger sequencing of SSU rDNA differed in terms of sequence quality, and some of the samples positive for *Cryptosporidium* SSU rDNA by NGS produced trichomonad-specific DNA sequences when subjected to actin gene amplification using conventional PCR.

The most abundant *Cryptosporidium* species in this study, *C. ditrichi,* was first described five years ago (Čondlová et al., 2018). Since then, *C. ditrichi* has been observed in wild rodents in Europe (Zhang et al., 2022); literature searches did not identify records of this species outside of Europe. Human infections with *C. ditrichi* have been confirmed in three individuals in Sweden, sampled at different times (2013, 2015 and 2018) and in different areas of the country (Beser et al., 2020). In 2022, the species was also identified in a human patient in Denmark (Larsen et al., 2023).

DNA-based evidence of *C. ditrichi* has predominantly been demonstrated in *Apodemus* sp. (Čondlová et al., 2019; Čondlová et al., 2018; Kivistö et al., 2021; Vioque et al., 2022; Zhang et al., 2022). The NCBI database also holds one sequence obtained from *Mus spretus* (acc. No. ON306385) (Vioque et al., 2022), one from *Martes foina (*acc. No. MN237649) (Perec-Matysiak et al., 2023), and ten sequences from three humans (Beser et al., 2020). The present study provides the first record of *C. ditrichi*-specific DNA in the genera *Micromys, Microtus, Mus,* and *Myodes.* According to Zhang and colleagues, the main host of *C. ditrichi* is *A. flavicollis* (Zhang et al., 2022), and based on phylogenetic analysis, Kivistö and colleagues found *C. ditrichi* in this mouse species to be of high zoonotic risk (Kivistö et al., 2021). The finding of *C. ditrichi* in two different marten species (*Martes martes and Martes foina*) in Poland (Perec-Matysiak et al., 2023) suggests an even broader host range. A gp60-based subtyping scheme for *C. ditrichi* that could be used to look for cryptic host specificity of the species remains to be established (Ryan et al., 2021). Efforts should also continue to map intraspecific diversity and to identify any cryptic host specificity or geographic distribution component within this species.

In the present study, there was a significantly higher occurrence of *C. ditrichi* in mice compared with voles. It is important to note, however, that the study was not designed to evaluate differences between the rodent families. A large study from Finland (Kivistö et al., 2021) carried out in 2010–2015 identified a relatively high occurrence of *Cryptosporidium* in both host families, with 36.8% of murids and 53.9% of cricetids infected. One of the most prevalent *Cryptosporidium* species in that study was *C. ditrichi*, which was detected in 21/66 *A. flavicollis*. Meanwhile, out of the 450 mice, voles and shrews collected all over Finland, none were positive for *C. parvum*.

Cryptosporidium parvum, the other zoonotic species observed in the present study, has not previously been described in rodents in Scandinavia, but it has been suggested to be the most dominant species among rodents in Europe and even worldwide (Zhang et al., 2022). With regards to *C. parvum*, rodent-specific *C. parvum* may exist; however, several different subtype families have been described in rodents, and at least some of these have also been observed in humans (Zhang et al., 2022), suggesting zoonotic potential.

Subtyping of *C. parvum* is of key relevance in efforts to map the transmission of *C. parvum*. Indeed, gp60 data available in the NCBI database indicate that rodents may very well be hosts of strains of *C. parvum* that can infect cattle and humans; e.g., IIaA16G1R1b (MH912987 and MH912988) and IIaA15G2R1 (JF727763–JF727769) (Table 3). In the present study, *C. parvum* could be subtyped in three out of four samples. Evidence of IIaA16G3R1 was obtained from *A. flavicollis* (OR447414) and *M. glareolus* (OR447416). This subtype is one of the most commonly detected ones in Scandinavia, observed in both humans and cattle in Denmark (Stensvold et al., 2015b) and it also appears common in cattle in other European countries such as Germany (Holzhausen et al., 2019) and Spain (Couso-Pérez et al., 2020). To date, only one study has observed this subtype in a rodent species, more specifically an urban rat (*Rattus* sp.) from Malaysia (Tan et al., 2019); however, the sequence (KY696270) differs from other IIaA16G3R1 sequences in the NCBI Database in that the last of the three 'TCG' repeats is located more towards the 3′-end of the repeat section than what is observed in other IIaA16G3R1 sequences.

Results from one sample (*M. musculus*) revealed *C. parvum* subtype IIdA22G1c (OR447415). IIdA22G1 has previously been identified in ovine, bovine and human hosts in several European countries (Vieira et al., 2015). Recently, in 2021, it was observed as the second most frequent subtype causing human infections in Sweden (37/296 successful subtypings) with most of the infections acquired within the country (Lebbad et al., 2021). The present study provides the first report of a rodent hosting this subtype. In fact, IIdA22G1 has only been reported once in a host that was not human, sheep or cattle, namely from a horse (KP272151); however, the sequence was only partial at the 5′-end, and so the total number of TCA and TCG triplets, and therefore the subtype, remains unclear.

In the present study, we found no evidence of *C. tyzzeri*, which has been identified on multiple occasions, especially in *M. musculus* (Table 3; (Hancke and Suárez, 2022)). More studies on higher numbers of rodents sampled in different parts of Denmark are warranted, especially considering that a human case of cryptosporidiosis due to *C. tyzzeri* was identified in Denmark recently (unpublished data).

In summary, a substantial proportion of the wild rodents sampled in this study tested positive for *Cryptosporidium*, often exhibiting mixed species/genotype infections. Rodents in the area might constitute a reservoir for cryptosporidiosis due to *C. parvum* and *C. ditrichi* in other hosts, including humans.

Abbreviations used: sim. – similarity; F – female; M – male; pos – positive; neg – negative; gp - glycoprotein; NGS – next-generation

sequencing; SSU - small subunit; rRNA - ribosomal RNA; PAL - pooled amplicon library; SNPs - single nucleotide polymorphisms.

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Disclosure statement

During the preparation of this work the authors used ChatGPT (Free Research Review, OpenAI) in order to rewrite or translate short sections or sentences. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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