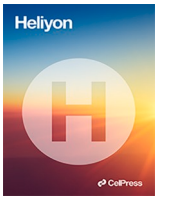
Heliyon 10 (2024) e38548

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Cryptosporidium spp. in German wildlife: Detection, regional occurrence and diversity in wild boar, roe, red and fallow deer

Claudia Jäckel [a,*], Iryna Hrushetska [a], Anne Mayer-Scholl [a], Jens A. Hammerl [a], Annette Johne [a], Carl Gremse [b], Denny Maaz [b,1], Karsten Nöckler [a], Martin Heinrich Richter [a]

[a] German Federal Institute for Risk Assessment (BfR), Department Biological Safety, Max-Dohrn Str. 8-10, 10589, Berlin, Germany
[b] German Federal Institute for Risk Assessment (BfR), Center for Land Use Related Evaluation Methods, One Health Approaches, Max-Dohrn Str. 8-10, 10589, Berlin, Germany

ARTICLE INFO

Keywords:
Sample preparation
Nested-PCR
Prevalence
Detection
Game
Cryptosporidium

ABSTRACT

Cryptosporidium is a cause of diarrheal infections responsible for a loss of human and animal welfare worldwide. The impact of the parasite is underestimated and the reported sources of infection are diverse, as it occurs in a wide variety of hosts. Wildlife has been reported as a notifiable source, but few studies are available on its occurrence in European wild boar and cervid species. To determine the occurrence of *Cryptosporidium* in game in Brandenburg, Germany, a molecular survey was conducted during the 2017 to 2020 hunting seasons. A total of 562 fecal samples from wild boar (*Sus scrofa*, $n = 262$), roe deer (*Capreolus capreolus*, $n = 174$), red deer (*Cervus elaphus*, $n = 62$), fallow deer (*Dama*, $n = 51$) and 13 samples of unspecified species were analyzed for both 18S ribosomal RNA (18S rRNA) and *Cryptosporidium* oocyst wall protein (COWP) gene sequence regions. PCR results showed that 21.2 % of the samples ($n = 119/562$) were positive for at least one target gene (18S rRNA: $n = 114$; COWP: $n = 14$), but differences in *Cryptosporidium* occurrence were observed within species and hunting seasons, with variations ranging from 1.8 % to 41.7 % (roe deer), respectively. Analysis of Sanger sequences of the 18S rRNA and COWP PCR products indicated that the *C.* sp. deer genotype was predominant in deer (roe deer: 86.7 %, red deer: 66.7 %, fallow deer: 58.8 %), while *C. suis* and *C. scrofarum* were mainly detected in wild boar (88.5 %). The human pathogenic species *C. parvum* was detected in only 1.2 % ($n = 7$) of the samples analyzed, but without a clear indication of a specific wild animal host. The highest *Cryptosporidium* diversity was found in wild boar and roe deer with five and four different species, respectively. Comparison of the 18S rRNA sequences with the designated reference revealed minor variations at several nucleotide positions in some isolates, possibly indicating evolutionary adaptations and the development of new subtypes. In conclusion, wildlife represents a reservoir for a diverse spectrum of *Cryptosporidium* species and may thus contribute to their environmental spread and the transmission to humans.

* Corresponding author. Unit Diagnostics, Pathogen Characterization, Parasites in Food, Department Biological Safety, German Federal Institute for Risk Assessment, Diedersdorfer Weg 1, 12277, Berlin, Germany.
E-mail address: claudia.jaeckel@bfr.bund.de (C. Jäckel).
[1] Current affiliation: Berlin-Brandenburg State Laboratory, Gerhard-Neumann-Straße 2, 15236 Frankfurt (Oder), Germany.

https://doi.org/10.1016/j.heliyon.2024.e38548
Received 29 December 2023; Received in revised form 24 September 2024; Accepted 25 September 2024
Available online 26 September 2024
2405-8440/© 2024 Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Cryptosporidiosis is a globally prevalent infection of the gastrointestinal tract of vertebrates and humans [1], with >8 million cases annually caused by different species of the protozoan parasite *Cryptosporidium* spp. (especially *C. hominis* and *C. parvum*) [2,3]. Clinical manifestations of the disease include prolonged watery diarrhea, vomiting, abdominal pain and fever [4,5] usually lasting for 2–3 weeks with the possibility of recurrence [6]. Infections are usually self-limiting, but can lead to severe or chronic manifestations, especially in children or immunocompromised individuals [5,7]. Hosts can be infected either directly through contact with contaminated humans and animals, or indirectly through ingestion of fecal-contaminated water and food, especially at low levels of processing. Infection is caused by the ingestion of a very small number (~10) of infective oocysts [8], which represent a stable and persistent environmental stage of its parasitic life cycle, in which they are able to survive harsh environmental conditions and standard disinfectants [9]. *Cryptosporidium* spp. have been identified as the etiologic agent of numerous small and large waterborne and foodborne outbreaks worldwide [10–13].

With 2407 confirmed cases in 2022, Germany notified the highest number of cryptosporidiosis cases since the beginning of reporting in 2001 [14]. However, the annual number of cases is expected to be higher [15] due to (i) a general lack of awareness of this pathogen, (ii) the challenging performance of the diagnostic methods (esp. microscopic detection), and (iii) the lack of harmonized molecular detection methods [16].

As game has been reported as a reservoir for *Cryptosporidium* spp. in Europe [17,18], the occurrence, regional distribution and annual variation of these parasites in Germany is of interest to support risk assessment along the food chain. Although *Cryptosporidium* spp. occur in the intestine of their hosts, infected animals can contaminate fields and recreational waters where low-grade foods (i.e., vegetables and fruits) are harvested. In addition, cross-contamination of meat can occur due to improper handling during the slaughtering process. In recent years, demand for game products is steadily increasing in Germany. While 24,742 tons of game (esp. wild boar, red and roe deer) were shot for consumption during the 2019/2020 hunting season, about 18,000 tons were imported from other European (Poland: 5732 tons, Spain: 2649 tons, Hungary: 2410 tons) and international countries (New Zealand: 2410 tons, Australia: 741 tons, USA: 130 tons) in 2019 [19]. To date, limited information is available on the prevalence and diversity of *Cryptosporidium* spp. in game in Europe. Existing datasets indicate substantial differences in detection rates of *Cryptosporidium* spp., ranging from 0.0 to 8.5 % in Poland [20] to 21.7 % in Spain [21]. To the best of our knowledge, there is no comprehensive data about the occurrence of this pathogen in German game population to support a risk assessment and consumer protection for game or derived products.

In this study, different preparation and extraction procedures were compared for reliable and sensitive detection of *C. parvum* oocysts by artificial spiking of fecal material and subsequent comparative molecular investigation. The most suitable procedure for sample preparation was further used to determine the occurrence of *Cryptosporidium* spp. in a regional molecular survey of 562 fecal samples from animals (esp. wild boar, roe deer, red deer, fallow deer) shot during the hunting seasons 2017 to 2020 in Brandenburg, Germany. Total DNA from fecal samples was subjected to nested PCR for the detection of the *Cryptosporidium*-specific target sequences 18S rRNA and COWP and epidemiological analysis. The amplified PCR products were further subjected to Sanger sequencing for comparative analysis on emerging sequence subtypes.

2. Materials & methods

2.1. Sample preparation for artificial spiking assays

For an initial evaluation of the efficacy of different methods for oocyst disruption and subsequent molecular detection, artificially spiked fecal samples were subjected to comparative analysis. Microscopically [22] and molecularly [23] confirmed *Cryptosporidium* spp.-free fecal samples from pigs (semi-solid fecal material) and sheep (solid fecal material) were provided by the experimental facility of the German Federal Institute for Risk Assessment (Berlin, Germany). For each assay, 200 mg of unprocessed feces were mixed with 100 µl of *C. parvum* oocysts (Lauenhain strain) from a 1:10 dilution series (10^6-10 oocysts/preparation) by vortexing. Each assay included a negative matrix control consisting of an unspiked fecal sample supplemented with 100 µl sterile water. All experiments were performed in triplicate. Following the different preparation procedures, samples were stored at 4 °C for a maximum of one week until analysis by nested PCR.

2.2. Oocyst disruption by preliminary treatment of oocysts

To optimize oocyst disruption, different treatment approaches were compared with the in-house gold standard method used. The following methods were compared: (i) heat treatment (20 min, 95 °C), (ii) thawing/freezing (five cycles of 5 min liquid nitrogen, 1 min 95 °C), (iii) proteinase K treatment (20 mg/ml, 3 h, 55 °C), (iv) ultrasound (6 min, 60 %, amplitude 0.5), (v) ultrasound combined with zirconia beads (1 mm, 1.5 g), (vi) homogenization by bead disruption via Fastprep24 (Lysing matrix E, 40 s, 6 m/s) and (vii) Fastprep24 performed for three times (Lysing matrix E, 3 × 40 s, 6 m/s). In general, the prepared samples were centrifuged (1 min, 13,000×*g*) and the supernatant was employed as a template for the PCR reaction. To evaluate the efficacy of the tested methods, untreated spiked samples were analyzed by nested PCR in parallel. Furthermore, Accustart II PCR Tough Mix (Quantabio, Beverly, MA, USA) was employed for oocyst lysis by heat instead of DreamTaq PCR reagents according to the manufacturer's recommendations, in order to minimize the impact of sample inhibitors.

2.3. Oocyst disruption using commercial extraction kit systems

The efficacy of several commercial DNA extraction kits for the detection of *Cryptosporidium*-specific DNA was evaluated. The PSP® Spin Stool DNA Kit (Stratec, Birkenfeld, Germany), the innuPREP Stool DNA Kit (Analytik Jena AG, Jena, Germany) and the GeneAll Exgene™ Stool DNA Kit (Cambio Ltd, Cambridge, UK) were employed in accordance with the manufacturer's instructions. The NucleoSpin DNA Stool Kit (Macherey-Nagel, Düren, Germany) was employed according to the manufacturer's recommendation, as well as according to the specification of the Consultant Laboratory for *Cryptosporidium* in Animals (Leipzig, Germany). This involved an additional step of ultrasonic treatment for 6 min (power 60 %, amplitude 0.5) (UP200St, Hielscher, Teltow, Germany). The manufacturer of the QIAamp Stool Kit (Qiagen, Hilden, Germany) recommends two different temperatures for lysis (70 °C and 95 °C). Both of these temperatures were compared in this study. Finally, the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, California, USA) was included in the study and compared with the other kits in two variations. In both cases, Lysing Matrix E was employed, with the following protocols: (i) homogenization once with a Fastprep24™ (40 s, 6 m/s) (MP Biomedicals, Santa Ana, California, USA) and (ii) homogenization for three times (3 × 40 s, 6 m/s). A summary of the systems tested and the specifications used is also presented in Table 1.

2.4. Sampling of game

Fecal samples of game were collected during driven hunts organized by the German Forest Service and the Frankenförder Forschungsgesellschaft mbH during the hunting seasons (September to January) of the years 2017/18, 2018/19 and 2019/20 [24]. The present study was conducted in 14 districts of seven counties of two Federal Forestry Enterprises of the Federal State of Brandenburg, Germany. Access to hunting grounds was provided by the German Institute for Federal Real Estate (BImA). All animals were legally hunted for human consumption and game management. The age of the animals was determined by experienced hunters on the basis of a number of factors, including tooth eruption, tooth replacement, tooth wear, physical appearance, and, where possible antler development. According to these criteria, they were divided into three age groups (juveniles: <1 year, yearlings: 1–2 years, adults: >2 years old).

Samples were collected by experienced hunters and employees of the German Federal Forestry Service directly from the rectum of the animals during evisceration. They were collected in sterile, sealable cups and transported within 12 h in a cool box to the German Federal Institute for Risk Assessment for storage at 4 °C and subsequent analysis.

For some samples ($n = 13$) specification about species, age and sex were not provided by the hunters. However, these samples were included into the molecular survey and subsequent typing of PCR-products, but could not be included in the epidemiological analysis.

2.5. DNA extraction of game samples

Unprocessed fecal samples were used for genomic DNA extraction using the NucleoSpin DNA Stool Kit (Macherey-Nagel, Düren, Germany) according to a modified procedure. In brief, 200–220 mg feces were dissolved in 800 µl of resuspension buffer. For an

Table 1
Results of the comparison of different disruption methods for *C. parvum* oocysts in feces.

Disruption of oocysts via	Disruption principle	18S rRNA (oocysts/ml)	COWP (oocysts/ml)
Without pretreatment		n. d.	10^7
Different pretreatments			
Heat (20 min, 95 °C)	Thermal	10^7	10^5–10^6
Heat (20 min, 95 °C) + AccuStart II PCR Tough Mix	Thermal	10^6	10^6
Thawing/Freezing (5 cycles à 5 min liquid nitrogen, 1 min 95 °C)	Mechanical	n.d.	10^7
Ultrasound (6 min, 60 %, amplitude 0.5)	Mechanical	n.d.	n.d.
Ultrasound + zirconia beads (1 mm; 1.5 g) (6 min, 60 %, amplitude 0.5)	Mechanical	n.d.	10^7
Proteinase K (20 mg/ml)	Enzymatic	n.d.	10^7
Fastprep24 (Lysing Matrix E) (40 s, 6 m/s)	Mechanical	10^7	n.d.
3x Fastprep24 (Lysing Matrix E) (3 × 40 s, 6 m/s)	Mechanical	10^7	n.d.
Kitsystem incl. pretreatment			
NucleoSpin DNA Stool	Mechanical (Beads)	10^2–10^3	10^2–10^3
NucleoSpin DNA Stool + Ultrasound (6 min, 60 %, amplitude 0.5)	Mechanical (Beads)	10^2	10^2–10^3
QIAamp Stool kit (Lysis at 70 °C)	Enzymatic (Proteinase K)	10^5	10^4–10^5
QIAamp Stool kit (Lysis at 95 °C)	Enzymatic (Proteinase K)	10^5	10^4
PSP® Spin Stool DNA kit	Mechanical (Beads)/enzymatic (Proteinase K)	10^2–10^3	10^2–10^3
innuPREP Stool DNA kit	Enzymatic (Proteinase K)	10^2	10^2–10^3
GeneAll Exgene™ Stool DNA kit		10^2	10^2
FastDNA® SPIN kit for Soil (40 s, 6 m/s)	Mechanical (Beads)	10^7	n.e.
FastDNA® SPIN kit for Soil (3 × 40 s, 6 m/s)	Mechanical (Beads)	10^7	10^7

All approaches were analyzed in triplicate.
Abbreviation: n.d., not detectable; n.e., not evaluable, because of too many byproducts.

efficient disruption of the oocyst wall an additional ultrasonic treatment (UP200St, Hielscher, Teltow, Germany) for 6 min (power 60 %, amplitude 0.5) was employed. Following sonication, DNA extraction was conducted in accordance with the standard protocol. Subsequently, the DNA was eluted in 100 μl of HPLC-grade water and stored at −20 °C until analysis by nested PCR.

2.6. Molecular detection

The presence of *Cryptosporidium* spp. in the extracted DNAs was molecularly determined by nested PCRs targeting the oocyst wall protein (COWP) gene [25] and the small subunit of ribosomal RNA (18S) gene of *Cryptosporidium* [26]. All PCR reactions were conducted using the components of the DreamTaq PCR amplification system (Thermo Fisher Scientific, Dreieich, Germany) in a final volume of 25 μl. The master mix of each reaction consisted of 9.375 μl or 13.375 μl RNase-free water, 2.5 μl of 10 x DreamTaq Buffer, 2.5 μl dNTPs (2 mM each), 0.625 μl DreamTaq Polymerase, 2.5 μl forward and reverse primers (0.2 μM each) and 5 μl or 1 μl template DNA for primary and secondary amplification, respectively. Primers used in this study are presented in Table S1.

Both parts of the COWP PCR consisted of 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s and elongation at 72 °C for 50 s. The initial denaturation and final elongation steps were performed at 94 °C for 3 min and 72 °C for 7 min, respectively.

The 18S rRNA PCR was conducted as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 95 °C for 45 s, 55 °C for 45 s and 72 °C for 1 min and a final elongation at 72 °C for 10 min [23]. The second part of this PCR was conducted in the same manner, with the exception of an annealing temperature of 58 °C. The presence of positive PCR products was confirmed by gel electrophoresis. The purification of the positive PCR products was conducted using the MSB® Spin PCRapace Kit (Stratec, Birkenfeld, Germany) in accordance with the manufacturer's instructions, followed by commercial Sanger sequencing by Eurofins Genomics (Ebersberg, Germany).

2.7. Species determination, phylogenetic relationship and statistical analysis

Species identification was performed either by restriction fragment length polymorphism (RFLP) of the second PCR product of 18S (endonucleases *Ssp*I & *Vsp*I; Thermo Fisher Scientific, Dreieich, Germany) or COWP PCR (*Rsa*I & *Alu*I; Thermo Fisher Scientific) as previously described [25,27] or by target gene sequence analysis. Sanger sequences of PCR products were aligned and adjusted using SeqMan Pro 17 (DNASTAR Lasergene v 17, Inc., MA, USA) against the available Refseq sequences in GenBank (NCBI, National Center for Biotechnology Information, BE, USA). Phylogenetic relationships were determined using MEGA version X [28] with the UPGMA method and the Kimura 2-parameter model [29]. A statistical analysis was conducted using Fisher's exact test in GraphPad Prism (version 8.2.0; GraphPad Software, La Jolla, CA, USA). Differences were considered as significant at $p \leq 0.05$.

3. Results

3.1. Evaluation of methods and extraction kits for reliable molecular detection of Cryptosporidium spp. in fecal samples

The efficacy of various methodologies for the reliable detection of *Cryptosporidium* spp. was evaluated by analyzing artificially spiked fecal samples exhibiting different concentrations of oocysts for the presence or absence of COWP and 18S rRNA target sequences (Table 1). The choice of the compared kits and pretreatments was guided by the following criteria: ease of handling, low costs, already published techniques and the broad diversity of employed extraction principles. We had further decided to investigate the performance of very simple procedures using physical (e.g., heat), mechanical (e.g., beads) or enzymatic (e.g., proteinase K) treatment principles as potential alternatives for easy and cheap sample prescreening.

As summarized in Table 1, many of the kit systems were demonstrated to be suitable for a reliable preparation of *Cryptosporidium* spp. oocysts from fecal samples. Nevertheless, some of the kits yielded only detection results in samples comprising high numbers of oocysts, rendering them unsuitable for the sensitive detection of these parasites. In contrast, the kits from Macherey-Nagel, Stratec, Analytic Jena and Cambio Ltd. were all found to be capable of detecting 10^2-10^3 oocysts/g of fecal sample. The combination of commercial extraction systems with mechanical, physical and/or enzymatic sample pretreatments did not result in relevant improvements in oocyst detection. The results of the study indicated that the use of simple pretreatment strategies without further extraction yielded the poorest detection results, as shown in Table 1. The detection limit varied between 10^5 and 10^6 oocysts/g of feces following heat treatment, and 10^7 oocysts/g of feces and no detection obtained following thawing/freezing, ultrasound, ultrasound combined with beads, proteinase K and homogenization with Fastprep24, respectively.

A subsequent investigation on the occurrence of *Cryptosporidium* spp. in game of specific regions of Brandenburg was carried out with the NucleoSpin DNA Stool kit with an additional step of ultrasonic treatment.

3.2. Cryptosporidium spp. oocysts are widely distributed in fecal samples of wildlife hunted in different regions of the German federal state Brandenburg

A total of 562 fecal samples from hunted animals were provided for experimental investigation during the hunting seasons of 2017/18, 2018/19, and 2019/20 (Table 2). Approximately two-thirds of the samples originated from female animals (60.1 %), whereas only 36.7 % and 3.2 % could be assigned to animals of male or unknown sex, respectively. The age distribution of hunted animals was found to be more similar, with 37.4 %, 31.4 % and 28.0 % belonging to age groups 0, 1 or 2, respectively. A total of 3.0 % of the examined samples could not be assigned to any of the described age groups. Of the 562 hunted animals, 262 were identified as wild boar (*Sus*

scrofa), 174 as roe deer (*Capreolus capreolus*), 62 as red deer (*Cervus elaphus*), 51 as fallow deer (*Dama*) and 13 for which no further information was available regarding their classification into the aforementioned game types. The samples were obtained from seven counties belonging to two Federal Forestry Enterprises as illustrated in Fig. 1. The results of molecular detection targeting 18S rRNA and COWP genes for the individual hunting seasons and the distribution of the samples are presented in Fig. 1(A and B).

The results of the molecular screening indicated a broad distribution of *Cryptosporidium* spp. oocysts across the different hunting grounds. None of the seven districts of Brandenburg were found to be free of *Cryptosporidium* spp. The distribution of the positive samples among the tested wildlife, according to animal species, sex and age group is presented in Table 2. A total of 119 out of 562 samples exhibited positive signals for at least one of the target genes examined. Nevertheless, 114 samples were positive for 18S rRNA, while only 14 samples yielded positive PCR signals for COWP. Of these, five samples were exclusively identified by COWP PCR. The data demonstrate that there were substantial variations in the number of samples with positive molecular results across the different hunting years, with differences observed both at the level of animal species and in terms of the total number of animals. This annual

Table 2
Occurrence of *Cryptosporidium* spp. in wild game differentiated by host species, sex, age group and hunting season: samples were considered positive for *Cryptosporidium* if at least one gene was detectable by PCR and could be determined as *Cryptosporidium* specific by Sanger sequencing or RFLP, respectively.

			Sex		P value[a]	Age Group			P value[a]
	Total	Positive (%)	Male	Female		0	1	2	
2017/18									
Roe deer	60	25 (41.7 %)	12/25 (48.0 %)	13/35 (37.1 %)	0.4365	16/28 (57.1 %)	1/11 (9.1 %)	8/21 (38.1 %)	**0.0355***
Wild boar	117	27 (23.1 %)	13/38 (34.2 %)	14/79 (17.7 %)	0.0614	19/43 (44.2 %)	6/58 (10.3 %)	2/16 (12.5 %)	**<0.0001******
Red deer	40	4 (10.0 %)	3/13 (23.1 %)	1/26 (3.9 %)	0.0991	0/9 (0.0 %)	2/12 (16.7 %)	2/18 (11.1 %)	0.5558
Unknown origin	13	5 (38.5 %)	-	-		-	-	-	
Total	**230**	**61 (26.5 %)**	**28/76 (36.8 %)**	**28/140 (20.0 %)**		**25/80 (31.3 %)**	**9/81 (11.1 %)**	**12/55 (21.8 %)**	
2018/19									
Roe deer	55	1 (1.8 %)	1/15 (6.7 %)	0/40 (0.0 %)	0.2727	0/17 (0.0 %)	1/14 (7.1 %)	0/24 (0.0 %)	>0.9999
Wild boar	50	5 (10.0 %)	3/25 (12.0 %)	2/25 (8.0 %)	>0.9999	4/23 (17.4 %)	1/18 (5.6 %)	0/9 (0.0 %)	0.1668
Red deer	10	0 (0.0 %)	0/5 (0.0 %)	0/5 (0.0 %)	>0.9999	0/6 (0.0 %)	0/1 (0.0 %)	0/3 (0.0 %)	>0.9999
Total	**115**	**6 (5.2 %)**	**4/45 (8.9 %)**	**2/70 (2.9 %)**		**4/46 (8.7 %)**	**2/33 (6.1 %)**	**0/36 (0.0 %)**	
2019/20									
Roe deer	59	4 (6.8 %)	1/15 (6.7 %)	3/44 (6.8 %)	>0.9999	3/16 (18.8 %)	0/16 (0.0 %)	1/27 (3.7 %)	0.0569
Wild boar	95	29 (30.5 %)	12/46 (26.1 %)	16/45 (35.6 %)	0.3696	21/48 (43.8 %)	3/27 (11.1 %)	4/16 (25.0 %)	**0.0061****
Red deer	12	2 (16.7 %)	1/6 (16.7 %)	1/6 (16.7 %)	>0.9999	2/7 (28.6 %)	0/2 (0.0 %)	0/3 (0.0 %)	0.4697
Fallow deer	51	17 (33.3 %)	8/18 (44.4 %)	9/32 (28.1 %)	0.3518	5/13 (38.5 %)	6/17 (35.3 %)	6/20 (30.0 %)	0.7413
Total	**217**	**52 (24.0 %)**	**22/85 (25.9 %)**	**29/127 (22.8 %)**		**31/84 (36.9 %)**	**9/62 (14.5 %)**	**11/66 (16.7 %)**	
All years									
Roe deer	174	30 (17.2 %)	14/55 (25.5 %)	16/119 (13.5 %)	**0.0128***	19/61 (31.2 %)	2/41 (4.9 %)	9/72 (12.5 %)	**0.0006*****
Wild boar	262	61 (23.3 %)	28/109 (25.7 %)	32/149 (21.5 %)	0.4579	44/114 (38.6 %)	10/103 (9.7 %)	6/41 (14.6 %)	**<0.0001******
Red deer	62	6 (9.7 %)	4/24 (16.7 %)	2/37 (5.4 %)	0.2001	2/22 (9.1 %)	2/15 (13.3 %)	2/24 (8.3 %)	>0.9999
Fallow deer	51	17 (33.3 %)	8/18 (44.4 %)	9/32 (28.1 %)	0.3518	5/13 (38.5 %)	6/17 (35.3 %)	6/20 (30.0 %)	0.7413
Unknown origin	13	5 (38.5 %)	-	-		-	-	-	
Total	**562**	**119 (21.2 %)**	**54/206 (26.2 %)**	**59/337 (17.5 %)**		**70/210 (33.3 %)**	**20/176 (11.4 %)**	**23/157 (14.7 %)**	

[a] Statistical analysis was performed with GraphPad Prism 8. For identification of a statistical significance between male and female or age groups (age group 0 compared to age groups 1 and 2) Fisher`s exact test was employed. Significant results are marked in bolt and colored in dark grey. The strength of significance is ranked between * (weak) to **** (strong).

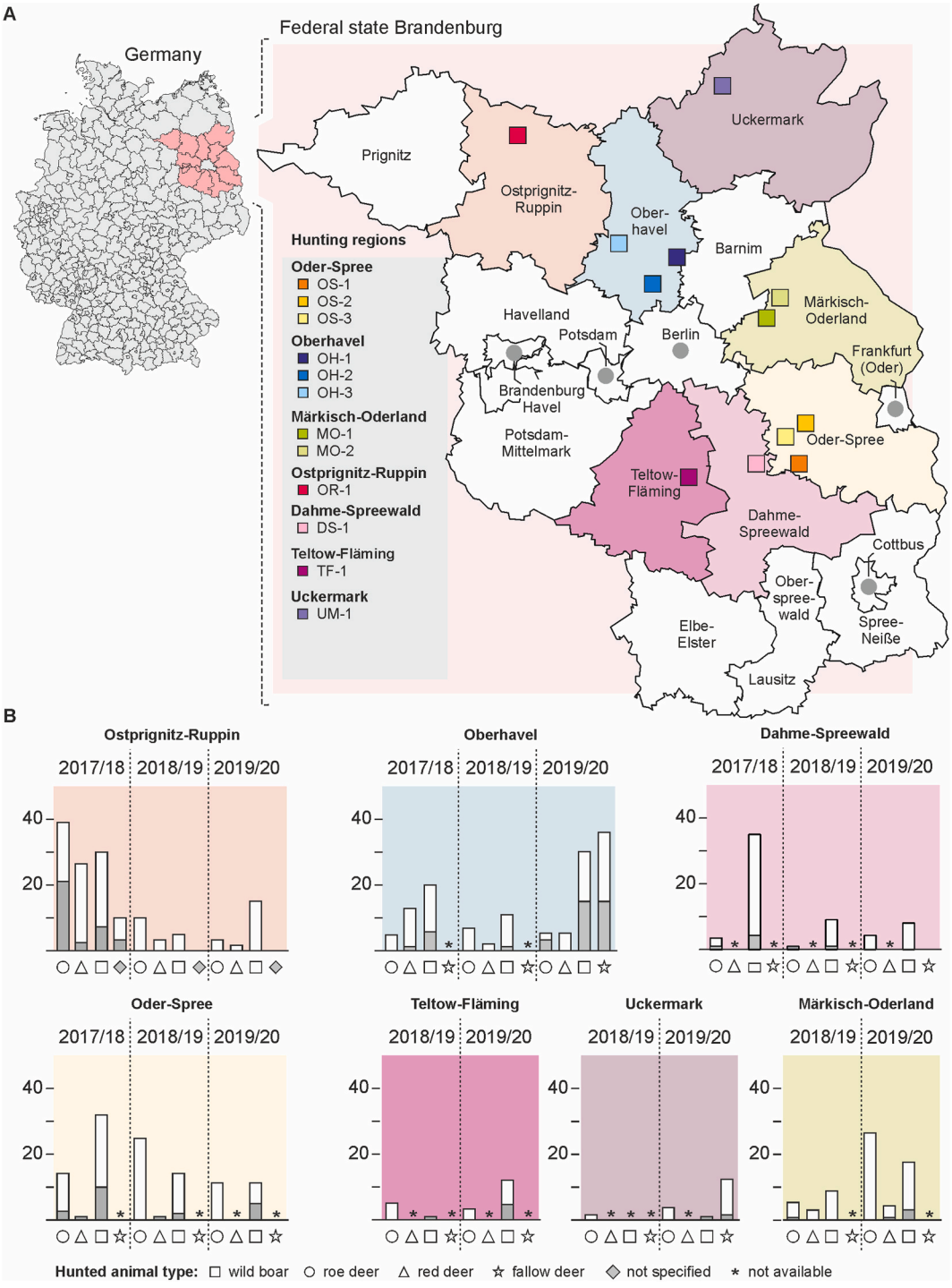


Fig. 1. Occurrence of *Cryptosporidium*-positive samples in the different hunting grounds and districts of the federal state Brandenburg. In the upper part (A), the location of the federal state Brandenburg and its districts is given. The colored squares indicate the location of the hunting grounds in the respective districts. In the lower part (B), regional information (district-specific) about hunted game species in the three different hunting seasons (2017/18, 2018/19 and 2019/20) is given. The respective symbols indicate the species as specified in the figure. White bars indicate the total number of hunted animals of the respective type, while the grey represent the total number of samples, which was assigned to be *Cryptosporidium*-positive. Asterisks in the diagrams indicate, that the respective animal type in the hunting season was not available for analysis.

fluctuation in prevalence is most evident in roe deer. In the 2017/18 season, 41.7 % of roe deer examined were found to be positive for *Cryptosporidium* spp. In contrast, in the 2018/19 and 2019/20 seasons, only 1.8 % and 6.8 % of the animals, respectively, tested positive for this pathogen.

3.3. The occurring species of Cryptosporidium are predominantly dependent on the respective host

The results of Sanger sequencing of the 18S rRNA and COWP PCR products and sequence comparison to reference sequences (similarity analysis) in public databases permit the assignment of the *Cryptosporidium* species in relation to the species of the sampled animals (Table 3). The majority of the species identified could be assigned to the eponymous host. The *C.* sp. deer genotype was the predominant species in roe (86.7 %), red (66.7 %) and fallow deer (58.8 %), while the porcine species *C. suis* and *C. scrofarum* were mostly identified in wild boar (88.5 %). The greatest diversity of determined Cryptosporidia was observed in wild boar, with five different species identified. This was followed by four species in roe deer and two different species in red and fallow deer. The number of species identified is consistent with the number of samples analyzed for the respective type of animal. The greater the number of animals of a given species sampled, the greater the diversity of the detected Cryptosporidia.

The amplified sequence of the 18S rRNA gene exhibited variations that were subjected to phylogenetic analysis, as illustrated in Fig. 2. The phylogenetic analysis revealed that the individual sequences exhibited a high degree of conservation. The majority of the isolates from this study exhibited identical characteristics to those of the respective reference. Nevertheless, for some isolates, minor variations were identified at several nucleotide positions, which may suggest the emergence of developing subtypes (Fig. 2). The occurrence of variations is not confined to a specific geographical region.

4. Discussion

4.1. Evaluation of sample processing methods on molecular oocyst detection

As previously reported, the reliability of *Cryptosporidium* prevalence data is contingent upon the quality of sample preparation, DNA extraction procedures, and target sequence selection [30]. To ascertain the efficacy of disparate pretreatment procedures or commercial extraction systems for the detection of the *Cryptosporidium* targets COWP and 18S (SSU) rRNA, a comprehensive evaluation of artificially spiked fecal samples (*C. parvum*) was conducted for the first time. The results demonstrate that the majority of commercial extraction systems exhibit superior oocyst detection rates (10^2–10^5 oocysts/ml), whereas individual pretreatment procedures are less effective (10^5–10^7 oocysts/ml). With regard to the extraction principle for oocyst disruption, no beneficial effects were observed with either enzymatic or mechanical treatment. However, due to the lack of information regarding the operating procedures and the composition of the buffers of extraction kits a reliable interpretation of influencing factors is impossible. The efficacy of the DNA extraction process is contingent upon the integration of an exhaustive oocyst disruption methodology with an efficient DNA preparation procedure. This integration should be specifically tailored to the complexity of the samples studied. The advantageous effects of kit systems for enhanced oocyst DNA detection have been demonstrated on previous occasions [30–33]. For reliable determination of the *Cryptosporidium* occurrence in fecal samples of hunted game, the NucleoSpin DNA Stool kit with an additional step of ultrasonic treatment was used.

Table 3
Results for *Cryptosporidium* species found in sampled game.

Sampled animal species	Total number of samples (*n*)	Number of positive samples (%)	Determined species	Number of determined species (%)
Roe deer	174	30 (17.2 %)	*C.* sp. deer genotype	26 (86.7 %)
			C. parvum	2 (6.7 %)
			C. sp. vole genotype III	1 (3.3 %)
			C. scrofarum	1 (3.3 %)
Red deer	62	6 (9.7 %)	*C.* sp. deer genotype/*C.ryanae*	5 (66.7 %)
			C. parvum	1 (16.7 %)
Fallow deer	51	17 (33.3 %)	*C. ryanae*/*C.* sp. deer genotype	10 (58.8 %)
			C. ubiquitum	7 (41.2 %)
Wild boar	262	61 (23.3 %)	*C. scrofarum*/*C. sp. pig genotype II*	46 (75.4 %)
			C. suis	8 (13.1 %)
			C. parvum	3 (4.9 %)
			C. ubiquitum	3 (4.9 %)
			C. ryanae/*C.* sp. deer genotype	2 (3.3 %)
Unknown game origin	13	5 (38.5 %)	*C.* sp. deer genotype	3 (60.0 %)
			C. scrofarum/*C.* sp. pig genotype II	1 (20.0 %)
			C. parvum	1 (20.0 %)

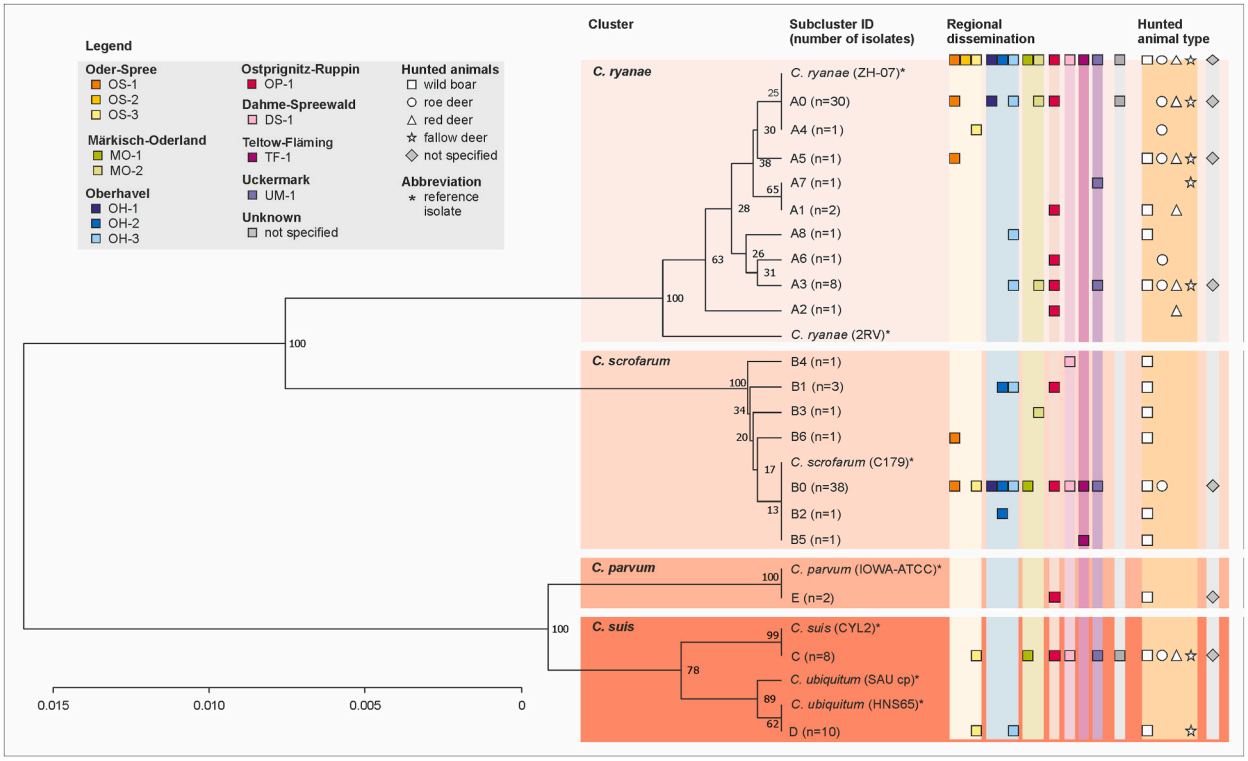


Fig. 2. Phylogenetic clustering of *Cryptosporidium*-positive samples based on the target sequence of 18S rRNA gene. Sequence comparison was conducted using MEGA (version X) and reference sequences (indicated by asterisks) of selected target species were added. The accession numbers of the reference sequences are the following: MK982509.1 (*C. ryanae* ZH-07), JN400880.1 (*C. ryanae* 2RV), MN243617.1 (*C. scrofarum* C179); CP044422.1 (*C. parvum* IOWA-ATCC), KJ790240.1 (*C. suis* CYL2), GQ337959.1 (*C. ubiquitum* SAU cp) and EU827425.2 (*C. ubiquitum* HNS65). The resulting clusters were colored and assigned to specific species types for better visualization. Beside the clusters, the ID of the subclusters is given including information about the number of determined samples in brackets. Each subcluster is defined by the individual exchange of at least one nucleotide. On the right, information about the regional dissemination and the respective animal type is given. Colored squares indicate the occurrence of the subcluster ID in the respective region. The symbols indicate the association of the subcluster ID to the respective types of hunted animals. Further information on the coding of the symbols and colors is given in the legend of the figure. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4.2. *Cryptosporidium* detection in fecal samples from game

Molecular amplification of the COWP and 18S (SSU) rRNA target sequences revealed an overall occurrence of *Cryptosporidium* spp. in fecal samples from game hunted between 2017 and 2020 in Brandenburg, Germany, with a total of 21.2 % of samples testing positive (n = 119/562). As this study is based on the detection of both target sequences, the individual interpretation of the loci provides substantially different detection rates (COWP: n = 14/562 vs. 18S rRNA: n = 114/562). However, no single target gene could be identified as the preferred option, as the detection rate of the species *C. parvum* in this study was higher with COWP, while 18S rRNA amplification also covers other species, but is partially lacking in its detection of *C. parvum*. As the specificity of detection for both loci has yet to be described in detail, the outcome of surveillance studies that have employed these target genes must be considered with caution. The use of the 18S rRNA locus may provide a comprehensive overview of the occurrence of a wide range of *Cryptosporidium* species, partially including *C. parvum*-positive samples. In contrast, the COWP locus is more specific, primarily detecting a broader spectrum of the human pathogenic species *C. parvum*. In this study, the detection rates of individual target sequences were aggregated to achieve comprehensive coverage of *Cryptosporidium*-positive samples. The overall occurrence of *Cryptosporidium* is slightly higher, but in accordance with data sets (roe deer: 1.3–9 % [34,35] vs. 17.2 %; red deer: 0.3–4.6 % [36,37] vs. 9.7 %; wild boar: 0.8–21.7 % [21,38] vs. 23.3 %) of surveillance studies previously published. As this study encompasses the initial comprehensive surveillance and detailed analysis of three successive hunting seasons, extensive variations in the detection rates from the individual animal types could be demonstrated. These variations are markedly disparate between the seasons, reflecting considerable geographic and climatic differences in the occurrence of *Cryptosporidium* spp., which varied considerably among the analyzed animal types, regions, and hunting periods. A comparison of the results with those of other studies is challenging due to the influence of various biotic (e.g., animal type, population density) and abiotic factors (e.g., weather, climate).

Furthermore, methodological issues, such as low recovery rates, weak oocyst disruption, and improper detection assays, can significantly impact the results of prevalence studies, leading to misinterpretation. Therefore, it is essential to maintain a gold-standard

method in cross-national studies that provides reliable and comparable results, which can be interpreted in conjunction with the biotic and abiotic conditions of individual geographic regions.

4.3. Occurrence of Cryptosporidium in different animal types

The occurrence of *Cryptosporidium* in wild boar in this study (23.3 %) is slightly higher than that observed in other European studies, which ranged from 0.0 to 21.7 % [20,21,35]. Nevertheless, it also corroborates the existence of considerable regional or animal-specific variation in published datasets. In addition to the mean value, local detection rates of up to 54.6 % were observed for a hunting ground situated in Ostprignitz-Ruppin, while detection in wild boar exhibited fluctuations between 10.0 % (2018/19) and 30.5 % (2019/20) across different hunting periods. To ascertain the influence of various factors, it is necessary to align annual data from long-term studies with climate and animal population data. In contrast to the data presented here, the majority of existing studies have not provided substantial insights into the long-term dynamics of the *Cryptosporidium* population in specific geographical regions. The findings of our three-year study did not identify any statistically significant differences in the occurrence of *Cryptosporidium* spp. based on the sex of the animals, contrary to reports that *Cryptosporidium* spp. is more prevalent in younger animals, particularly in pigs and wild boar [20,36,39–41]. These findings are in accordance with the detection rate ($p < 0.0001$; 72.1 % of positive wild boars in the age group 0) observed in our study (Table 2).

As with wild boar, the overall occurrence of *Cryptosporidium* spp. among cervids was 18.9 %, exhibiting considerable variations between different animal species. The lack of comprehensive datasets, particularly for the animal species under investigation, precludes meaningful comparison and interpretation. These datasets are not in accordance with the prevalence data for *Cryptosporidium* spp. in other cervids, such as sika deer [42], white tailed deer, reindeer or moose [43]. Nevertheless, the detection rates in roe deer (17.2 %) and red deer (9.7 %) were considerably higher than the ranges previously described for different countries [35,44–46]. European prevalence data in fallow deer range from 6 % (Great Britain) [47], to 33.3 % (this study) and 100 % (Portugal) [48]. The reasons for differences in prevalence between species are typically not presented in a convincing manner. However, they may include the probability of an exposure of the parasite to its host based on the regional distribution, diverging environmental conditions, or distinct behaviors of the animal species (e.g., consumption habits, agility). It is also conceivable that the observed differences may be attributed to variations in host susceptibility or pathogen virulence. Similar fluctuations in oocyst detection were identified between the hunting years for roe deer, with rates of 41.7 % in 2017/18, 1.8 % in 2018/19, and 6.8 % in 2019/20. The reasons for this may be associated with the availability and abundance of water between the individual years, for example, geographic differences in the hunting grounds and the amount of rainfall. It is also noteworthy that a sex-specific distribution was observed in roe deer, with males being more frequently affected by *Cryptosporidium* infections than females ($p = 0.0128$). This tendency has also been observed in the other cervids analyzed in this study, although the results are not statistically significant. Conversely, two studies have considered their data regarding a possible sex dependency, identifying a greater occurrence of infection in females than males, although also without statistical significance [42,43].

4.4. Dissection of Cryptosporidium species

The most prevalent *Cryptosporidium* species identified in this study was *C. scrofarum* ($n = 48$), the deer specific genotype ($n = 46$), *C. ubiquitum* ($n = 10$), and less frequently, the species *C. suis* ($n = 8$), *C. parvum* ($n = 7$), and the vole genotype ($n = 1$). These findings were determined through species determination on the basis of the 18S rRNA sequence. The majority of these species have previously been linked to human infections, thereby representing a potential threat to human health. For instance, *C. parvum* and *C. hominis* are responsible for approximately 95 % of human infections [49,50]. *Cryptosporidium parvum* and *C. ubiquitum* have previously been linked to water-associated outbreaks [51–53]. While *C. ubiquitum* was only detected in wild boar and fallow deer in this study, *C. parvum* could be detected in almost all examined animal species (except fallow deer). It was previously hypothesized that deer could act as a source of contamination of environmental water with *Cryptosporidium* [43]. Furthermore, wild animals represent a risk factor for the contamination of other wild or domestic animals, thereby acting as a potential source for the transmission in the environment [42], and consequently also for humans.

Fig. 2 depicts the phylogenetic relationship between the identified *Cryptosporidium* species. All samples of the determined *C. parvum*, *C. suis*, and *C. ubiquitum* (also known as the cervine genotype) are identical to the respective reference sequence. However, the species *C. scrofarum* and the deer-specific genotype exhibit a greater degree of sequence variation. While the majority of sequences are identical to the reference sequences (*C. scrofarum*: 82.6 %; *C.* deer genotype: 65.2 %), six and eight additional subclusters can be distinguished for *C. scrofarum* and *C.* deer genotype, respectively. The definition of each subcluster is contingent upon the exchange of at least one nucleotide. A limited number of authors have conducted sequence analysis in their studies on the prevalence of *Cryptosporidium* spp. in wild animals [21,42,43,46,54]. Only a few variations were identified in the respective reference sequences for the deer genotype [46], *C. scrofarum* and *C. suis* [21].

The observed discrepancies in variability between the various species may be attributed to the disparate number of samples available for each species. In species with a low number of detections, no variations from the reference genome are observed. In contrast, species that were detected in higher quantities exhibited variations in their genomes. As has been previously demonstrated, the majority of identified *Cryptosporidium* species can be attributed to the host from which the species was initially described. However, some species, such as *C. parvum* and *C. ubiquitum*, appear to demonstrate the capacity to infect hosts other than those typically associated with them.

It is also essential to recognize the constraints of this study. The availability of the animals for sampling is dependent on the success

of the hunting expeditions and the guidelines established by the hunting committee. Hunters are engaged to shoot young animals or animals with a decreased health status, which may include animals with physical problems. In some cases, the sample size or available data for hunted animals was insufficient, resulting in their exclusion from the study. As a result, the hunters were unable to guarantee a uniform distribution of the animals according to their respective categories, age groups, or health status. In certain instances, additional constraints were imposed by the owners of the hunting grounds or the hunting committee, for example, the protection of specific animal types. Furthermore, the lack of data for specific hunting regions was due to logistical constraints that prevented access to these regions. As a result, a comparative analysis of the various target regions and animals could only be partially conducted.

In conclusion, the study provides an interesting insight into the variations in the occurrence and diversity of *Cryptosporidium* in samples of game hunted between 2017 and 2020 in different regions of the federal state of Brandenburg, Germany. However, it also highlights the need for comprehensive and cross-national 'One Health' surveillance studies to reliably determine the influencing biotic (i.e., animal population density, cross-animal interactions, persistence of parasites in the environment) and abiotic (i.e., climatic conditions, seasonality, geographic differences) factors influencing regional variation. In addition, the suitability of different methods of sample preparation, oocyst DNA extraction and detection was investigated, which revealed substantial differences in the detection rate of *Cryptosporidium* that need to be taken into account when evaluating and comparing the results of previous studies. The observed differences highlight the need for the utilization of a gold standard method to ensure the reliability and comparability of results across disparate studies. Overall, the detected occurrence of *Cryptosporidium* in wild game fit in the range of previously published data sets, indicating a potential for exposure to human pathogenic species (i.e., *C. parvum*) found in different animal species for people who come in contact with the animals (i.e., hunters) or prepare or consume wild game meat.

Data availability

Sequence data from Sanger sequences of the 18S target gene were deposited into the GenBank database and are available under GenBank accession numbers PQ471495-PQ471513. All the data analysis results obtained during this study are included in the manuscript and its Supplementary Information.

CRediT authorship contribution statement

Claudia Jäckel: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Iryna Hrushetska:** Writing – review & editing, Investigation. **Anne Mayer-Scholl:** Writing – review & editing, Funding acquisition. **Jens A. Hammerl:** Writing – review & editing, Visualization, Supervision, Software, Investigation, Data curation. **Annette Johne:** Writing – review & editing, Funding acquisition. **Carl Gremse:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Denny Maaz:** Writing – review & editing, Methodology, Investigation. **Karsten Nöckler:** Writing – review & editing, Project administration, Funding acquisition. **Martin Heinrich Richter:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors thank Zaida Melina Renteria Solis (Consultant laboratory for *Cryptosporidium* in animals, University of Leipzig, Germany) for providing *C. parvum* oocysts for artificial spiking experiments and general support. Luis Eduardo Flores Landaverde, Kaya Stollberg, Smita Sutrave and Carolyn Kästner are greatly acknowledged for their support during the driven hunts and initial laboratory work. We thank the German Institute for Federal Real Estate (BImA), the Frankenförder Forschungsgesellschaft mbH, the German Forest Service and the German Federal Institute for Risk Assessment (BfR)-Center for Land Use Related Evaluation Methods, One Health Approaches for providing assistance and support for this study. This work was financially supported by a grant of the German Federal Institute for Risk Assessment (1322-689).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38548.

References

[1] Gómez-Romano, M.A.-O., M.A.-O. Rodríguez-Iglesias, and F.A.-O. Galán-Sánchez, Molecular Detection of Cryptosporidium spp. and Microsporidia in Human and Animal Stool Samples. LID - *10.3390/microorganisms12050918* [doi] *LID - 918.* (2076-2607 (Print)).

[2] O. World Health, WHO Estimates of the Global Burden of Foodborne Diseases: Foodborne Disease Burden Epidemiology Reference Group 2007-2015, World Health Organization, Geneva, 2015.
[3] U.M. Ryan, et al., Taxonomy and molecular epidemiology of Cryptosporidium and Giardia - a 50 year perspective (1971-2021), Int. J. Parasitol. 51 (13–14) (2021) 1099–1119.
[4] K. Sharma, S. Srivastava, V. Tak, Cryptosporidiosis in India and the world: a review, Infect. Disord. - Drug Targets 23 (2023).
[5] M.J. Hossain, et al., Clinical and epidemiologic features of cryptosporidium-associated diarrheal disease among young children living in sub-saharan africa: the vaccine impact on diarrhea in africa (VIDA) study, Clin. Infect. Dis. 76 (76 Suppl1) (2023) S97–S105.
[6] M. Rehn, et al., Post-infection symptoms following two large waterborne outbreaks of Cryptosporidium hominis in Northern Sweden, 2010-2011, BMC Publ. Health 15 (2015) 529.
[7] M.A. Mohamed, et al., Cryptosporidium species in HIV patients in Alexandria, Egypt: distribution and associated clinical findings, Trop. Biomed. 39 (1) (2022) 108–116.
[8] M.C. Hlavsa, et al., Outbreaks of illness associated with recreational water–United States, 2011-2012, MMWR Morb. Mortal. Wkly. Rep. 64 (24) (2015) 668–672.
[9] T.K. Graczyk, B. Fried, Human waterborne trematode and protozoan infections, Adv. Parasitol. 64 (2007) 111–160.
[10] A. Efstratiou, J.E. Ongerth, P. Karanis, Waterborne transmission of protozoan parasites: review of worldwide outbreaks - an update 2011-2016, Water Res. 114 (2017) 14–22.
[11] U. Ryan, N. Hijjawi, L. Xiao, Foodborne cryptosporidiosis, Int. J. Parasitol. 48 (1) (2018) 1–12.
[12] Franceschelli, A., et al., An Outbreak of Cryptosporidiosis Associated with Drinking Water in North-Eastern Italy, August 2019: Microbiological and Environmental Investigations. LID - *10.2807/1560-7917.ES.2022.27.35.2200038* [doi] *LID - 2200038.* (1560-7917 (Electronic)).
[13] Zahedi, A. and U. Ryan, Cryptosporidium - an Update with an Emphasis on Foodborne and Waterborne Transmission. (1532-2661 (Electronic)).
[14] Koch-institut, R., survstat@RKI 2.0. https://survstat.rki.de, 2023.
[15] G. Deksne, et al., Occurrence of Cryptosporidium spp. and giardia spp. infection in humans in Latvia: evidence of underdiagnosed and underreported cases, Medicina (Kaunas) 58 (4) (2022).
[16] R.C.A. Thompson, A. Ash, Molecular epidemiology of Giardia and Cryptosporidium infections - what's new? Infect. Genet. Evol. 75 (2019) 103951.
[17] E.S. El-Alfy, Y. Nishikawa, Cryptosporidium species and cryptosporidiosis in Japan: a literature review and insights into the role played by animals in its transmission, J. Vet. Med. Sci. 82 (8) (2020) 1051–1067.
[18] I. García-Presedo, et al., *Presence of Cryptosporidium scrofarum*, C. suis and *C.* parvum subtypes IIaA16G2R1 and IIaA13G1R1 in Eurasian wild boars (Sus scrofa), Vet. Parasitol. 196 (3–4) (2013) 497–502.
[19] D. Jagdverband, Wildbret(-fleisch)-einfuhren nach Deutschland. [Webpage], 2023-09-07]; Available from: https://www.jagdverband.de/sites/default/files/2023-03/2023-02_Infografik_Wildbreteinfuhren_Deutschland_2021_2022%20.jpg, 2023.
[20] K. Němejc, et al., Cryptosporidium suis and Cryptosporidium scrofarum in eurasian wild boars (Sus scrofa) in central Europe, Vet. Parasitol. 197 (3–4) (2013) 504–508.
[21] Martí-Marco, A.A.-O., et al., Molecular Detection and Epidemiology of Potentially Zoonotic Cryptosporidium Spp. And Giardia Duodenalis in Wild Boar (Sus scrofa) from Eastern Spain. *LID - 10.3390/ani13152501* [doi] *LID - 2501.* (2076-2615 (Print)).
[22] J. Heine, Eine einfache Nachweismethode für Kryptosporidien im Kot, Zentralblatt für Veterinarmed. - Reihe B 29 (4) (1982) 324–327.
[23] S. Taha, et al., Molecular characterization of bovine Cryptosporidium isolated from diarrheic calves in the Sudan, Parasitol. Res. 116 (11) (2017) 2971–2979.
[24] D. Maaz, et al., Standardised sampling approach for investigating pathogens or environmental chemicals in wild game at community hunts, Animals (Basel) 12 (7) (2022).
[25] F. Spano, et al., PCR-RFLP analysis of the Cryptosporidium oocyst wall protein (COWP) gene discriminates between C. wrairi and C. parvum, and between C. parvum isolates of human and animal origin, FEMS Microbiol. Lett. 150 (2) (1997) 209–217.
[26] L. Xiao, et al., Identification of species and sources of Cryptosporidium oocysts in storm waters with a small-subunit rRNA-based diagnostic and genotyping tool, Appl. Environ. Microbiol. 66 (12) (2000) 5492–5498.
[27] D.M. Roellig, L. Xiao, Cryptosporidium genotyping for epidemiology tracking, in: J.R. Mead, M.J. Arrowood (Eds.), Cryptosporidium: Methods and Protocols, Springer New York, New York, NY, 2020, pp. 103–116.
[28] S. Kumar, , et al.X. Mega, Molecular evolutionary genetics analysis across computing platforms, Mol. Biol. Evol. 35 (6) (2018) 1547–1549.
[29] M. Kimura, A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences, J. Mol. Evol. 16 (2) (1980) 111–120.
[30] O. Hachimi, et al., Evaluation of molecular-based methods for the detection and quantification of Cryptosporidium spp. in wastewater, Sci. Total Environ. 947 (2024) 174219.
[31] Y. Hawash, DNA extraction from protozoan oocysts/cysts in feces for diagnostic PCR, Kor. J. Parasitol. 52 (3) (2014) 263–271.
[32] J.C. Arona, et al., Comparison of four commercial immunomagnetic separation kits for the detection of Cryptosporidium, J. Water Health 21 (10) (2023) 1580–1590.
[33] G. Lindergard, et al., The sensitivity of PCR detection of Cryptosporidium oocysts in fecal samples using two DNA extraction methods, Mol. Diagn. 7 (3–4) (2003) 147–153.
[34] J.A. Castro-Hermida, et al., Prevalence of Cryptosporidium and Giardia in roe deer (Capreolus capreolus) and wild boars (Sus scrofa) in Galicia (NW, Spain), Vet. Parasitol. 179 (1–3) (2011) 216–219.
[35] A. Paziewska, et al., Distribution of Cryptosporidium and Giardia spp. in selected species of protected and game mammals from North-Eastern Poland, Ann. Agric. Environ. Med. 14 (2) (2007) 265–270.
[36] I.S. Hamnes, et al., Occurrence of Cryptosporidium and giardia in suckling piglets in Norway, Vet. Parasitol. 144 (3–4) (2007) 222–233.
[37] W. Zhao, et al., Prevalence and characterization of Cryptosporidium species and genotypes in four farmed deer species in the northeast of China, Front. Vet. Sci. 7 (2020) 430.
[38] W. Li, et al., Presence of zoonotic Cryptosporidium scrofarum, Giardia duodenalis assemblage A and Enterocytozoon bieneusi genotypes in captive Eurasian wild boars (Sus scrofa) in China: potential for zoonotic transmission, Parasites Vectors 10 (1) (2017) 10.
[39] S.E. Sanford, Enteric cryptosporidial infection in pigs: 184 cases (1981-1985), J. Am. Vet. Med. Assoc. 190 (6) (1987) 695–698.
[40] J. Quílez, et al., Prevalence of Cryptosporidium infections in pigs in Aragón (northeastern Spain), Vet. Parasitol. 67 (1) (1996) 83–88.
[41] M. Jeníková, et al., New view on the age-specificity of pig Cryptosporidium by species-specific primers for distinguishing Cryptosporidium suis and Cryptosporidium pig genotype II, Vet. Parasitol. 176 (2–3) (2011) 120–125.
[42] S. Kato, et al., Molecular identification of the Cryptosporidium deer genotype in the Hokkaido sika deer (Cervus nippon yesoensis) in Hokkaido, Japan, Parasitol. Res. 115 (4) (2016) 1463–1471.
[43] M. Santin, R. Fayer, Enterocytozoon bieneusi, giardia, and Cryptosporidium infecting white-tailed deer, J. Eukaryot. Microbiol. 62 (1) (2015) 34–43.
[44] S.A. Henriksen, H. Korsholm, Parasitological examination of faecal samples-the construction and use of a simple counting chamber [Parasitologisk undersøgelse af fæcesprøver-konstruksjon og anvendelse af et enkelt opbygget tællekammer], Dan. Veterinaertidsskr. 67 (1984) 1193–1196.
[45] Y. Feng, Cryptosporidium in wild placental mammals, Exp. Parasitol. 124 (1) (2010) 128–137.
[46] A. Dashti, et al., Occurrence and limited zoonotic potential of Cryptosporidium spp., Giardia duodenalis, and Balantioides coli infections in free-ranging and farmed wild ungulates in Spain, Res. Vet. Sci. 159 (2023) 189–197.
[47] A.P. Sturdee, R.M. Chalmers, S.A. Bull, Detection of Cryptosporidium oocysts in wild mammals of mainland Britain, Vet. Parasitol. 80 (4) (1999) 273–280.
[48] A. Lourenço, et al., Estudo Preliminar da Criptosporidiose em Gamos (Dama dama L.) da Tapada Nacional de Mafra (Portugal), Acta. Parasitol. 7 (2000) 29–31.
[49] X. Yang, et al., Molecular epidemiology of human cryptosporidiosis in low- and middle-income countries, Clin. Microbiol. Rev. 34 (2) (2021).

[50] R.M. Chalmers, et al., Analysis of the Cryptosporidium spp. and gp60 subtypes linked to human outbreaks of cryptosporidiosis in England and Wales, 2009 to 2017, Parasites Vectors 12 (1) (2019) 95.
[51] M. Gertler, et al., Outbreak of Cryptosporidium hominis following river flooding in the city of Halle (Saale), Germany, August 2013, BMC Infect. Dis. 15 (2015) 88.
[52] N. Li, et al., Subtyping Cryptosporidium ubiquitum,a zoonotic pathogen emerging in humans, Emerg. Infect. Dis. 20 (2) (2014) 217–224.
[53] S. Kato, J.C. Kissinger, Cryptosporidium: comparative genomics and pathogenesis, in: Evolution of Virulence in Eukaryotic Microbes, 2012, pp. 518–544.
[54] I. Garcia-Presedo, et al., *Presence of Cryptosporidium scrofarum*, C. suis and *C.* parvum subtypes IIaA16G2R1 and IIaA13G1R1 in Eurasian wild boars (Sus scrofa), Vet. Parasitol. 196 (3–4) (2013) 497–502.