Highlights

- Prevalence of *Cryptosporidium* ranged from 23.3% to 25%, across dairy cow farms from Belgium, France and the Netherlands
- *Cryptosporidium parvum* was associated with pre-weaned calves
- Subtyping of *gp60* gene revealed nine subtypes circulating among the farms
- We report for the first time a novel subtype: IIdA14G2R1
- Vertical transmission was not a major contributor to *Cryptosporidium* spread

Figure 1

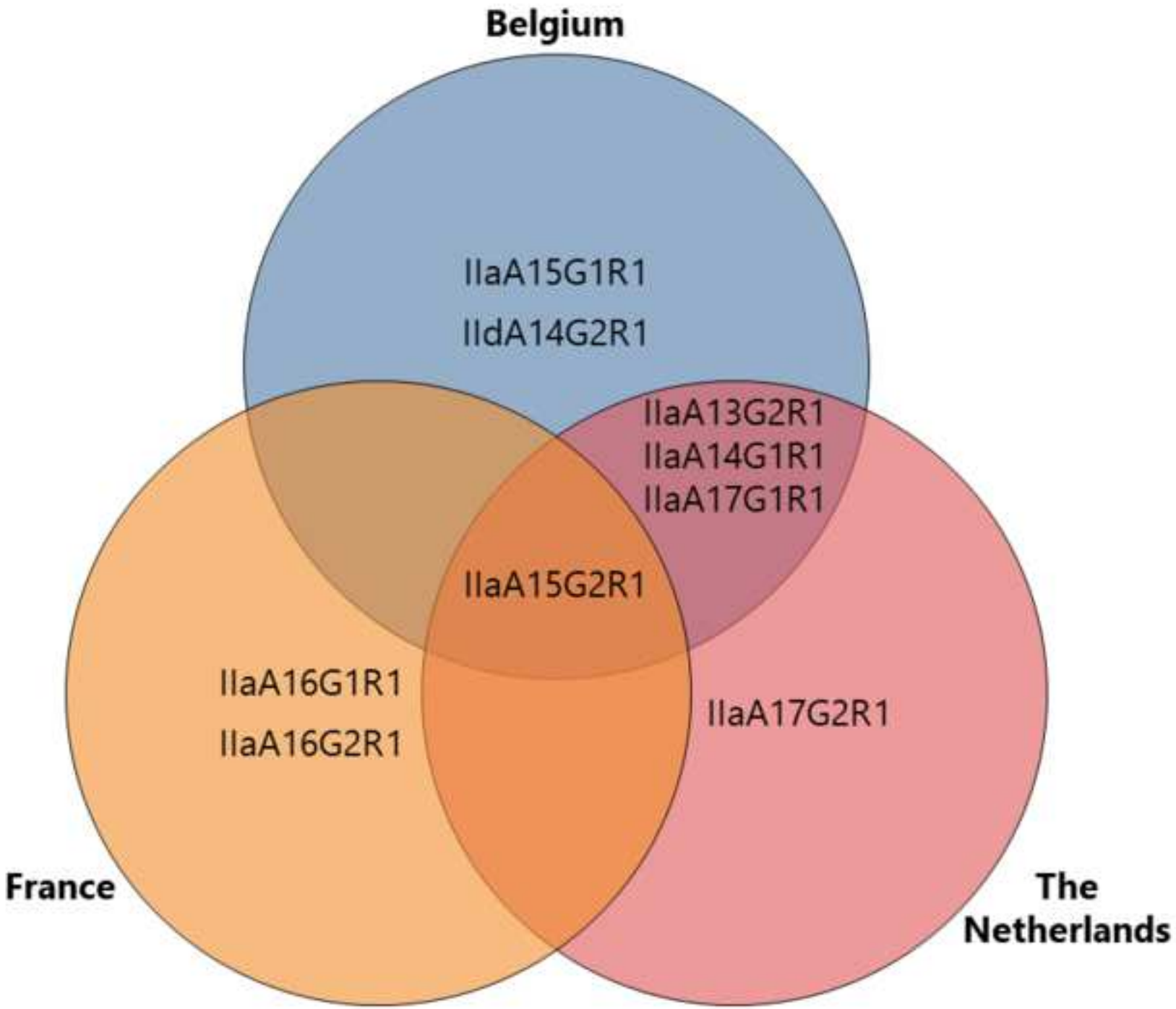

Figure 2

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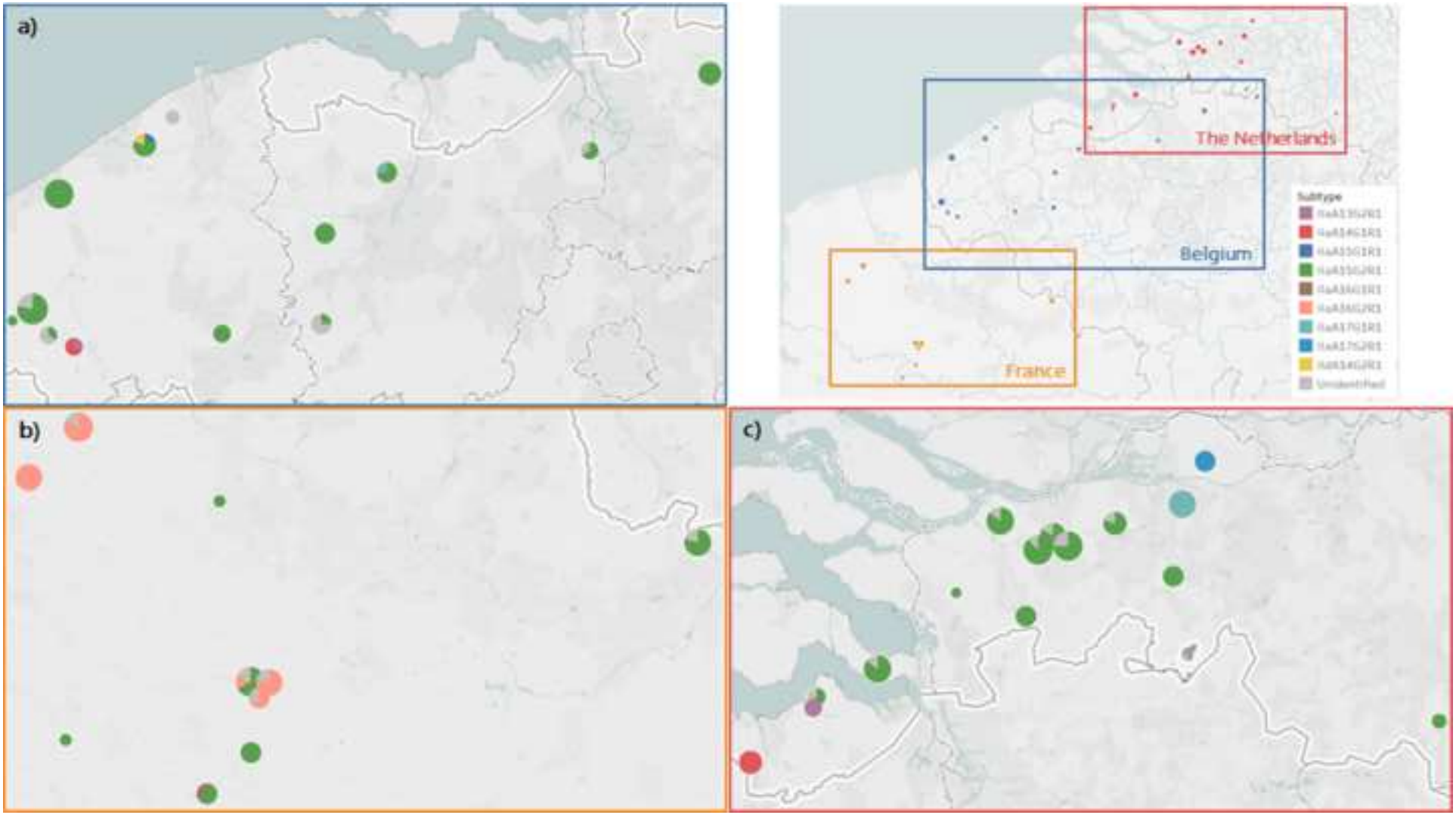

Figure 3

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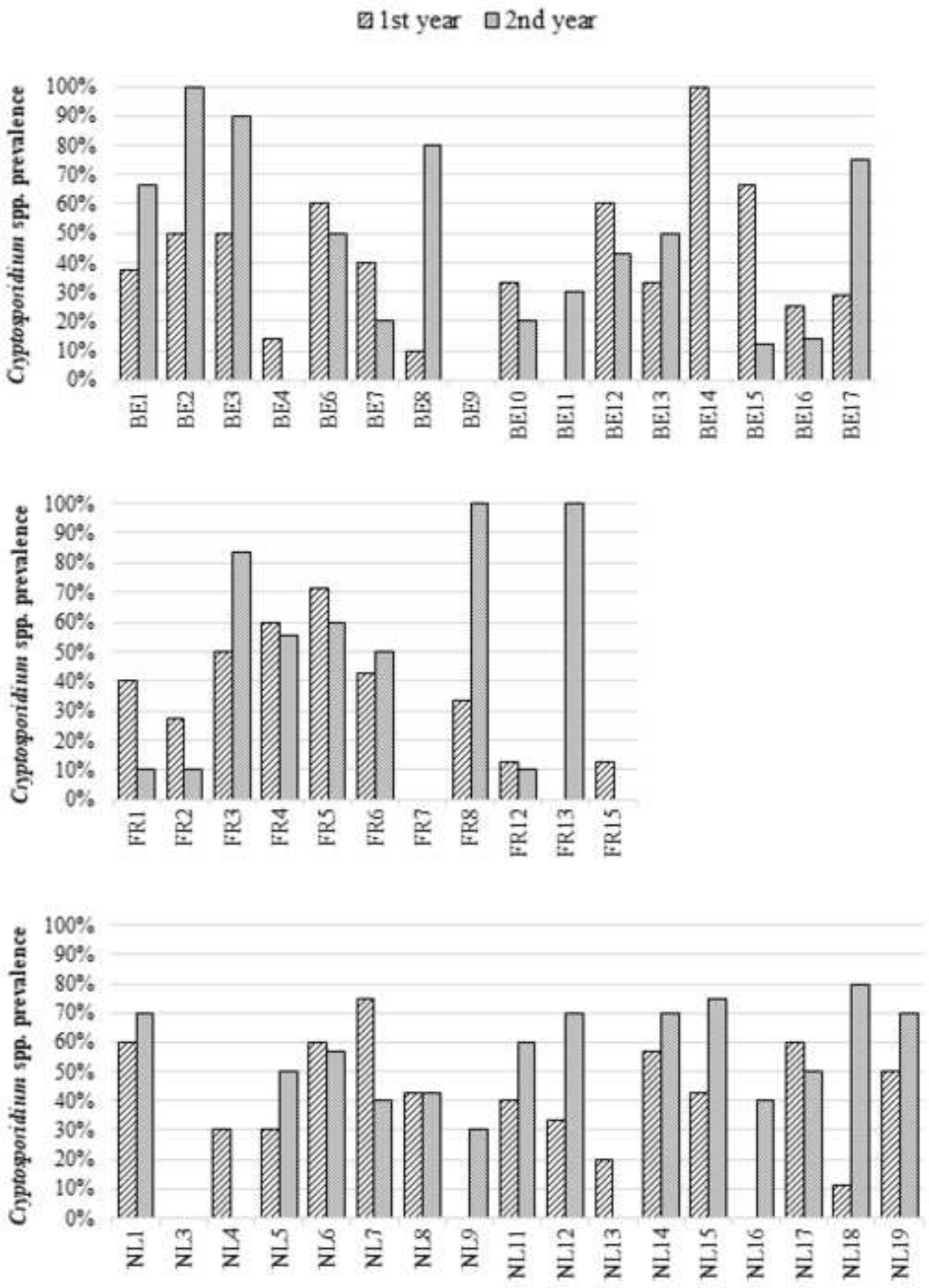

Table 1

Table 1. Occurrence of *Cryptosporidium* spp. and *C. parvum gp60* subtypes in dairy cattle from farms across Belgium, France and the Netherlands.

Country	Farm	Prevalence % (positives/ total screened)	*Cryptosporidium* spp. present (no. isolates identified)						*C. parvum gp60* subtypes
			C. parvum	*C. bovis*	*C. ryanae*	*C. andersoni*	*C. parvum*/ *C. bovis* (co-infection)	*C. parvum*/ *C. ryanae* (co-infection)	
Belgium	BE1	25% (5/20)	5	-	-	-	-	-	IIaA15G2R1 (5)
	BE2	50% (5/10)	3	1	1	-	-	-	IIaA15G2R1 (3)
	BE3	45% (9/20)	9	-	-	-	-	-	IIaA15G2R1 (7)
	BE4	0% (0/19)	-	-	-	-	-	-	-
	BE5	38.9% (7/18)	3	3	1	-	-	-	IIaA15G2R1 (1)
	BE6	20% (4/20)	3	-	-	-	-	1	IIaA15G2R1 (3) IIaA17G1R1 (1)
	BE7	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (4)
	BE8	45% (9/20)	8	-	1	-	-	-	IIaA15G2R1 (8)
	BE9	20% (4/20)	2	1	1	-	-	-	-
	BE10	25% (5/20)	4	-	-	1	-	-	IIaA15G2R1 (1)
	BE11	20% (4/20)	3	-	-	1	-	-	IIaA15G2R1 (2)
	BE12	33.3% (7/21)	2	3	1	-	-	1	IIaA14G1R1 (1) IIaA13G2R1 (1)
	BE13	29.4% (5/17)	1	2	-	-	2	-	IIaA14G1R1 (2) IIaA13G2R1 (1)
	BE14	5% (1/20)	-	1	-	-	-	-	-
	BE15	20% (4/20)	1	3	-	-	-	-	IIaA15G2R1 (1)
	BE16	5.3% (1/19)	-	1	-	-	-	-	-
	BE17	35% (7/20)	4	2	-	-	1	-	IIaA15G2R1 (3) IIaA15G1R1 (1) IIdA14G2R1 (1)
France	FR1	10% (2/20)	1	-	-	1	-	-	IIa15G2R1 (1)
	FR2	10% (2/20)	-	1	1	-	-	-	-
	FR3	45.5% (5/11)	5	-	-	-	-	-	IIaA15G2R1 (3)
	FR4	29.4% (5/17)	5	-	-	-	-	-	IIaA16G2R1 (3)
	FR5	35% (7/20)	6	-	-	1	-	-	IIaA16G2R1 (5)
	FR6	27.3% (3/11)	3	-	-	-	-	-	IIaA15G2R1 (2) IIaA16G1R1 (1)
	FR7	25% (5/20)	2	1	1	-	1	-	IIaA16G2R1 (2)
	FR8	38.9% (7/18)	3	-	2	2	-	-	IIaA15G2R1 (3)
	FR9	38.9% (7/18)	6	-	1	-	-	-	IIaA15G2R1 (4) IIaA16G2R1 (1)
	FR10	0% (0/14)	-	-	-	-	-	-	-
	FR11	0% (0/14)	-	-	-	-	-	-	-
	FR12	10% (2/20)	-	2	-	-	-	-	-
	FR13	40% (8/20)	4	3	-	-	1	-	IIaA16G2R1 (4)
	FR14	0% (0/6)	-	-	-	-	-	-	-
	FR15	28.6% (2/7)	1	-	-	1	-	-	IIaA15G2R1 (1)
The Netherlands	NL1	35% (7/20)	7	-	-	-	-	-	IIaA15G2R1 (6)
	NL2	16.7% (3/18)	3	-	-	-	-	-	IIaA15G2R1 (2)
	NL3	15% (3/20)	-	1	1	1	-	-	-
	NL4	10% (2/20)	-	2	-	-	-	-	-
	NL5	25% (5/20)	5	-	-	-	-	-	IIaA14G1R1 (5)
	NL6	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (4)
	NL7	20% (4/20)	2	2	-	-	-	-	IIaA15G2R1 (2)
	NL8	25% (5/20)	-	5	-	-	-	-	-
	NL9	15% (3/20)	3	-	-	-	-	-	IIaA13G2R1 (3)
	NL10	25% (5/20)	1	4	-	-	-	-	IIaA15G2R1 (1)
	NL11	31.6% (6/19)	6	-	-	-	-	-	IIaA15G2R1 (5)
	NL12	40% (8/20)	7	1	-	-	-	-	IIaA15G2R1 (6)
	NL13	0% (0/20)	-	-	-	-	-	-	-
	NL14	40% (8/20)	8	-	-	-	-	-	IIaA15G2R1 (6)
	NL15	20% (4/20)	4	-	-	-	-	-	IIaA15G2R1 (4)
	NL16	20% (4/20)	4	-	-	-	-	-	IIaA17G2R1 (4)
	NL17	25% (5/20)	5	-	-	-	-	-	IIaA15G2R1 (4)
	NL18	42.1% (8/19)	8	-	-	-	-	-	IIaA15G2R1 (7)
	NL19	35% (7/20)	7	-	-	-	-	-	IIaA17G1R1 (7)

Table 2. Comparison of *Cryptosporidium* spp. prevalence and *gp60* subtypes present in neonatal calves across Belgium, France, and the Netherlands over 2 years.

Country	Cohort	% Prevalence	*Cryptosporidium* spp. present (% of positive samples)			*C. parvum gp60* subtypes
			C. parvum	*C. bovis*	*C. ryanae*	
Belgium	Year 1	35.6 (37/104)	89.2	10.8	-	IIaA13G2R1 IIaA15G2R1 IIaA16G3R1 IIaA17G2R1
	Year 2	39.8 (45/113)	84.4	13.3	2.22	IIaA13G2R1 IIaA15G2R1
France	Year 1	33.7 (26/77)	84.6	15.4	-	IIaA15G2R1 IIaA16G2R1
	Year 2	38.3 (28/73)	89.3	3.57	7.14	IIaA15G2R1 IIaA16G1R1 IIaA16G2R1
Netherlands	Year 1	35.9 (52/145)	96.2	1.92	1.92	IIaA13G2R1 IIaA14G1R1 IIaA15G2R1
	Year 2	46.7 (71/152)	95.8	4.23	-	IIaA13G2R1 IIaA14G1R1 IIaA15G2R1 IIaA17G1R1 IIaA17G2R1

Follow-up investigation into *Cryptosporidium* prevalence and transmission in Western European dairy farms

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ABSTRACT

Cryptosporidium parvum is an enteric parasite and a major contributor to acute enteritis in calves worldwide, causing an important~~significant~~ economic burden for farmers. This parasite poses a major public health threat through transmission between livestock and humans. Our previous pilot study in Western Europe revealed a high prevalence of *Cryptosporidium* in calves of dairy farms. In the sequel study herein, 936 faecal samples were collected from the same 51 dairy farms across Belgium, France, and the Netherlands. Following DNA extraction, *Cryptosporidium* screening was carried out using nested-PCR amplification targeting the *SSU* rRNA gene. All positive samples were sequenced, and phylogenetic analyses were used to identify the *Cryptosporidium* spp.~~species~~ present. The 60 kDa glycoprotein (*gp60*) gene was also sequenced to determine the *C. parvum* subtypes present. Prevalence of *Cryptosporidium* ranged from 23.3% to 25%, across the three countries surveyed. The parasite was found in most of the farms sampled, with 90.2% testing positive. *Cryptosporidium parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* were all identified, with the former being the most predominant, representing 71.4% of all infections. *Cryptosporidium parvum* was associated with pre-weaned calves, while other species were associated with older animals. Subtyping of *gp60* gene revealed nine subtypes, eight of which have ~~been~~ previously ~~been~~ reported to cause clinical disease in humans. Similarly to the first study, vertical transmission was not a major contributor to *Cryptosporidium* spread. Our study highlights the need for further investigation into cryptosporidiosis~~*Cryptosporidiosis*~~ transmission, and future studies will require~~likely requiring~~ a One Health approach to reduce the impact of this disease.

INTRODUCTION

Cryptosporidiosis is an enteric disease caused by the apicomplexan parasitesparasite *Cryptosporidium* (Shirley et al., 2012). Most cases of zoonotic cryptosporidiosis cases are associated with livestock. Cattle, in particular, have long been established as a key reservoir of infection with numerous global reports of human cryptosporidiosis cases linked to close contact with thosethe animals (Chalmers and Giles, 2010; Xiao and Feng, 2008). In the majority of these, *C. parvum* and *C. hominis* are the main species responsible species (Leitch and He, 2011; Xiao and Feng, 2008).

Bovine cryptosporidiosis also has a major financial impact for cattle farmers (Chalmers and Giles, 2010; Mosier and Oberst, 2000; Widmer et al., 2020). In this livestock, *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* are the main infectious species, with *C. parvum* being the most common (Cho and Yoon, 2014; De Graaf et al., 1999; Feng et al., 2018). *Cryptosporidium parvum* is a major cause of acute enteritis in cattle, causing neonatal diarrhoea. In most cases, the disease is self-limiting, though persistent diarrhoea can lead to emaciation, fatigue, and severe dehydration. While fathalitymortality is generally low, long-term adverse health consequences may arise (Cho and Yoon, 2014; Thomson et al., 2017). Significant differences in weight gain havehas been observed between calves who had severe cryptosporidiosis as neonates and calves that had not (Shaw et al., 2020). Thus, farmers may incur economic losses through dead livestock, treatment of sick animals and reduced production due to retarded animal growth (Bennett, 2003; Hawkins et al., 2019; Stott and Gunn, 1997). Additionally, cryptosporidiosis may exacerbate concurrent infections with other pathogens (Delling and Daugschies, 2022; García et al., 2000) and);, infected animals are more susceptible to developing severe cryptosporidiosis. It is difficult for farmers to know how to manage spread of infection, as there is insufficient data on risk factors for *Cryptosporidium* infection to cattle on dairy farms. A recent systematic review found no consistent risk or protective factors for preventing *C. parvum* infection (Brainard et al., 2020).

Historically, *Cryptosporidium* detection has relied on microscopic examination of faecal smears for the presence of oocysts. As this method suffers from low sensitivity and specificity (Adeyemo et al., 2018), *Cryptosporidium* infection may not be detected in samples with low oocyst levels. Molecular-based diagnostic techniques have been used to increase sensitivity of *Cryptosporidium* detection along with ability to identity infecting species and subtypes. Subtype analysis can shed further light on potential zoonotic transmission by identifying the ones that occur in both humans and cattle. With increased molecular typing

studies, distinctions in the epidemiology of subtypes may also be discovered. This information could help ascertain how best to track and potentially tackle infection spread in certain areas.

A previous 2019–2020 study using samples from dairy cattle farms in Belgium, France and the Netherlands indicated a prevalence of 20.8–25.7% (Pinto et al., 2021), with numerous zoonotic *C. parvum* subtypes identified. Prior to this, prevalence investigations in these countries were infrequent and often did not utilise molecular detection. The study herein is a an~~longitudinal~~ investigation of *Cryptosporidium* across the same dairy farms one year later. We used the *SSU* rRNA and *gp60* genes for species and subtype level characterisation to investigate *C. parvum* diversity and transmission dynamics. As such, our study complements previous molecular work, provides temporal information on *Cryptosporidium* infection at the farm level and contributes to future investigations aiming to discern transmission networks.

MATERIALS AND METHODS

Sample collection

From September 2020 to July 2021, veterinarians collected a total of 936 faecal samples from 51~~fifty-one~~ dairy farms were – 17 in Belgium, 15 in France and 19 in the Netherlands. We aimed to collect samples from up to 10 calves (up to 3 months of age) and their mothers. In cases where farms had less than 10 calves, fewer samples were collected. Diarrheic and apparently health~~asymptomatic~~ animals were included in a random selection. Faeces were taken directly from the rectum, using a single pair of disposable gloves. This study was conducted as a cross-border collaboration under the Health for Dairy Cows (H4DC) project, funded by the Interreg-2-seas programme. This is a European Territorial Cooperation program covering the Flanders region of Belgium, the Hauts-de-France region in France and the western~~west~~ part of the Netherlands. These regions experience similar maritime temperate climates. The main objective of the project is to reduce the disease burden and economic impact of *Cryptosporidium* spp. on dairy farms.

DNA extraction

Immediately after defecation, faeces were collected into sterile tubes, and stored on ice. DNA extraction was carried out using PureLink™ Microbiome DNA Purification Kit (ThermoFisher) according to manufacturer's instructions, with slight modifications. ~~650 µL of~~ S1 Lysis Buffer (650 µL) and ~~100 µL of~~ S2 Lysis Enhancer (100 µL) was added to each sample. They were then incubated at 65°C and then vortexed on maximum speed for 13 min each. After addition of S3 Clean-up Buffer, samples were incubated at 4°C for 10 min. Following application of S6 Elution Buffer to the spin column, samples were incubated at room temperature for 3 min prior to centrifugation. Genomic DNA was stored at -20°C until 18S rRNA and *gp60* PCR reactions were carried out. Leftover DNA and fecal~~stool~~ samples were~~are~~ stored long-term in a -80 ºC freezer.

Cryptosporidium spp. screening and molecular genotyping

Nested-PCR amplification of a 631-bp region of the 18S rRNA gene was used to screen for *Cryptosporidium* spp. (Ziegler et al., 2007). The external primers used were 5´-GATTAAGCCATGCATGTCTAA-3´ (forward) and 5´-TTCCATGCTGGAGTATTCAAG3´ (reverse). The internal primers were 5´-CAGTTATAGTTTACTTGATAATC-3´ (forward)

and 5´-CCTGCTTTAAGCACTCTAATTTTC-3´ (reverse). Each PCR mixture contained 1 µL of DNA, 0.4 µM each of forward and reverse primers, 12.5 µL of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 9.5 µL nuclease-free water. External PCR cycling conditions were: denaturation for 2 min at 94°C, followed by 24 cycles at 94°C for 50 s, 53°C for 50 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. Internal PCR cycling conditions were: denaturation for 2 min at 94°C, followed by 30 cycles at 94°C for 50 s, 56°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. Positive (genomic DNA from a pure culture of *C. parvum* IOWA oocysts) and negative (using sterile water as a template) controls were included in both reactions. Following separation and excision from a 2% agarose gel, DNA was extracted using GeneJET Gel Extraction Kit (ThermoFisher Scientific). All positive samples were then bidirectionally sequenced (Eurofins Genomics) and chromatograms were manually assessed for quality, with ambiguous bases trimmed. For species level identification, BLAST searches were performed using obtained sequences as queries against the nucleotide database in GenBank. Following alignment with reference sequences, polymorphisms were identified.

gp60 subtyping of *Cryptosporidium parvum*

Nested-PCR of the *gp60* gene was carried out to determine the subtype of *Cryptosporidium* 18S rRNA PCR-positive samples (Alves et al., 2003). The external primers used were 5´-ATAGTCTCCGCTGTATTC-3´ (forward) and 5´-GGAAGGAACGATGTATCT-3´ (reverse). The internal primers were 5´-TCCGCTGTATTCTCAGCC-3´ (forward) and 5´- GCAGAGGAACCAGCATC-3´ (reverse). Each PCR mixture contained 2 µL of DNA, 0.2 µM each of forward and reverse primers, 15 µL of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 11.8 µL nuclease-free water. Cycling conditions for both internal and external PCR reactions were: a denaturation step for 3 min at 94°C, followed by 35 cycles at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Positive and negative controls were included in both reactions. Following separation and excision from a 2% agarose gel, DNA was extracted using GeneJET Gel Extraction Kit (ThermoFisher Scientific). All positive samples were then bidirectionally sequenced (Eurofins Genomics), and chromatograms were manually assessed for quality, with ambiguous bases trimmed. Subtypes were determined using established standard nomenclature (Sulaiman et al., 2005). BLAST searches were performed using newly generated sequences as queries against the nucleotide database in

GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Following alignment with reference sequences, polymorphisms were identified.

Phylogenetic analysis

The obtained sequences were aligned with each other as well as reference sequences from GenBank by MAFFT v.7 (https://mafft.cbrc.jp/alignment/server). Sequence alignment was manually inspected using BioEdit v7.0.5.3 (https://bioedit.software.informer.com). Best DNA/Protein phylogeny models were selected using the MEGA11 software (Tamura et al., 2021). The Tamura 3-parameter model (Tamura, 1992) was selected. Phylogenetic trees were inferred using maximum likelihood (ML), with the substitution model that best fit the alignment selected using the Bayesian information criterion. Bootstrap support for branching was based on 1,000 replications. An 18S sequence from *Monocystis agilis* (Accession number: AF457127) was used to root ML tree.

Comparison to preceding study and statistical analyses

Only neonatal calves aged under one month old were~~have been~~ included for comparison. Farms with less than three calves within this age range have been excluded from comparison. In the preceding study, *gp60* subtyping was only performed on samples confirmed as *C. parvum* through *SSU* rRNA gene sequencing. In this study, *gp60* screening was carried out on all samples that were PCR-positive for *SSU* rRNA, which revealed mixed infections in some animals. For comparison between the two time points these samples have been identified based on their *SSU* rRNA gene. Furthermore, the *SSU* rRNA gene of some samples could not be successfully sequenced, but the *gp60* gene could. These have been excluded in the prevalence count, as only *SSU* rRNA confirmed positive samples were reported in the previous study (Pinto et al., 2021). Comparison of prevalence between the two cohorts was performed by using the Mann-Whitney U test. Cochran Q tests were used to determine heterogeneity between farms within the same year and country. The mixed effects multilevel negative binomial modelling was done with STATA™ v17.0 with farm as the level. STATSDirect v. 3.3.5 was used to compare prevalence between countries within the same year and across the 2~~two~~ years.

RESULTS

Prevalence of *Cryptosporidium* in Belgian, French, and Dutch dairy farms

In Belgium, 324 fecal~~stool~~ samples across 17 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 25% with 81/324 specimens positive for *Cryptosporidium* spp. **(Table 1)**. Prevalence varied across farms from 0% to 50%. The majority of the positive samples were identified as *C. parvum* (64.2%, 52/81) with 45 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and four samples showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for three *SSU* rRNA PCR-positive samples. Nonetheless, *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 14/17 farms. The next most abundant species found was *C. bovis* (20.9%, 17/81). Additionally, three samples had co-infections of *C. bovis* and *C. parvum*. Nineteen samples had 100% nucleotide identity to the reference sequence AB777173.1 (from a calf in Egypt), while one sample had 100% nucleotide identity to the reference sequence MZ021459.1 (from a calf in Belgium in a previous study on these farms (Pinto et al., 2021)). The next most prevalent species was *C. ryanae* (6.2%, 5/81). Additionally, two samples showed mixed infection of *C. ryanae* and *C. parvum*. Five samples were 100% identical to the reference sequence FJ463193.1, while one sample had 100% nucleotide identity to the reference sequence KT922233.1 and another showed 99% identity to the same sequence. The least common species was *C. andersoni* (2.5%, 2/81). One sample showed 100% nucleotide identity to the reference sequence AB513856.1 (from cattle in Egypt), while the other was 100% identical to FJ463171.1 (from cattle in China).

In France, 236 fecal~~stool~~ samples across 15 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 23.3% with 55/236 specimens positive for *Cryptosporidium* spp. Prevalence varied across farms from 0% to 45.5%. Most of the positive samples were identified as *C. parvum* (65.5%, 36/55) with 33 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and one sample showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for two *SSU* rRNA PCR-positive samples, but *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 10/15 farms, with occurrence ranging from 5% to 45.5%. The next most common species was *C. bovis* (12.7%, 7/55). Additionally, two samples had co-infections of *C. bovis* and *C. parvum*. Eight samples showed 100% nucleotide identity to the reference sequence AB777173.1, while

one sample showed 100% nucleotide identity to the reference sequence MZ021459.1. Equal levels of *C. ryanae* and *C. andersoni* were detected at 9.1% (5/55) each. Two *C. ryanae* variants were found with three samples showing 100% identity to KT922233.1 and two samples showing 100% nucleotide identity to the reference sequence FJ463193.1. Two *C. andersoni* variants were found with three isolates showing 100% nucleotide identity to reference sequence FJ463171.1 and two showing 100% nucleotide identity to AB513856.1.

In the Netherlands, 376 fecal~~stool~~ samples across 19 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 24.2% with 91/376 specimens positive for *Cryptosporidium* spp. 18/19 farms contained at least one positive samples with prevalence ranging from 0% to 42.1% across farms. Most positive samples were identified as *C. parvum* (81.3%, 74/91) with all 74 isolates showing 100% nucleotide identity to the reference sequence AH006572.2. At the farm level, *C. parvum* was present in 8/19 farms. The next most prevalent species was *C. bovis* at (16.5%, 15/91), with 13 isolates showing 100% nucleotide identity to the reference sequence AB777173.1 and two isolates showing 100% nucleotide identity to MZ021459.1. One sample was positive for *C. ryanae* with 99% sequence identity to FJ463193.1, and one for *C. andersoni* with 100% nucleotide identity to AB513856.1. No mixed infections were found.

All *SSU* rRNA sequences have been submitted to GenBank under accession numbers OP975438 – OP975657.

Cryptosporidium parvum subtyping through *gp60* analysis

Cryptosporidium parvum was detected in 71.3% (162/227) of *Cryptosporidium* positive samples and found in 39 farms. PCR amplification and sequencing of the *gp60* gene was used to identify the specific *C. parvum* subtypes present in these three countries. All *18S* rRNA PCR-positive samples were further screened using nested PCR of the *gp60* gene. Of these, the *gp60* gene was successfully amplified and sequenced in 145 samples. 24 *C. parvum* positive sequences were not successfully subtyped. Sequence analysis revealed the presence of nine subtypes, eight of which belong to the IIa subtype family and one belonging to the IId family **(Figure 1)**.

The most abundant subtype found overall was IIaA15G2R1, representing 61.7% of *C. parvum*-positive samples Ninety-eight isolates had 100% nucleotide identity to the reference sequence DQ630518.1 and two were 99% identical to the same sequence. This subtype was also the most widely distributed, occurring in 71.8% (28/39) of *C. parvum*-positive farms. This

subtype represented 73.1%, 41.7% and 63.5% of all *C. parvum* positive samples, in Belgium, France and the Netherlands, respectively. It was the most prevalent subtype in Belgium and the Netherlands. This subtype predominantly occurred in calves, with only two isolates occurring in a dam.

The second most common subtype in this study was IIaA16G2R1. Seventeen isolates were found with 100% nucleotide identity to reference sequence DQ192505.1, all of which were found in France. This subtype was found in five farms and represented 47.2% of total *C. parvum* infections in the French farms. This subtype also predominantly occurred in calves, with only one isolate occurring in a dam.

Eight isolates of IIaA14G1R1 were identified – six with 100% nucleotide identity to reference sequence AM937017.1 and two with 99% nucleotide identity to the same sequence. This subtype occurred in three farms, representing 5.8% and 6.8% of *C. parvum* infections in Belgium and the Netherlands, respectively.

Eight isolates of IIaA17G1R1 were identified – seven with 100% nucleotide identity to reference sequence GQ983359.1, which were all located in one Dutch farm. The remaining isolate occurred in a Belgian farm and had 99% nucleotide identity to the same sequence. This was the sole subtype present in two farms, representing 1.9% and 9.5% of *C. parvum* infections in Belgium and the Netherlands, respectively.

Five isolates of IIaA13G2R1 were identified – four with 100% nucleotide identity to reference sequence DQ192502.1 and one isolate with 99% homology to the same sequence. This was the sole subtype present in three farms, representing 3.8% and 4.1% of *C. parvum* infections in Belgium and the Netherlands, respectively.

IIaA17G2R1 was found exclusively in the Netherlands. Four isolates were identified with 100% nucleotide identity to DQ630516.1. This subtype occurred on one farm and represented 5.4% of *C. parvum* infections. IIaA15G1R1 (99% nucleotide identity to AB777872) and IIaA16G1R1 (100% nucleotide identity to DQ192504.1), each occurred once in Belgium and France, respectively. Lastly, a novel isolate was identified and marked as IIdA14G2R1. This is the first report of this subtype worldwide and is the only subtype belonging to the IId family. Subtypes IIaA17G1R1, IIaA13G2R1, IIaA17G2R1, IIaA15G1R1 and IIaA16G1R1 were all found exclusively in calves. Geographical distribution of subtypes is visualised in **Figure 2**.

All *gp60* sequences have been submitted to GenBank under accession numbers OP978513 – OP978657.

Comparison to previous sampling study

This work is a follow-up to a prevalence study carried out between 2019 to 2020 (Pinto et al., 2021). Methodology was slightly altered between the two studies. In the preceding study, *gp60* PCR and subtyping was only carried out on samples confirmed as *C. parvum* positive through *SSU* rRNA PCR and sequencing. Herein subtyping was performed in all *Cryptosporidium SSU* rRNA positive samples. As such, we were able to detect *Cryptosporidium* co-infections. For comparability between the cohorts, prevalence figures in the second cohort have been adjusted accordingly. Furthermore, only animals under 28 days old have been included for comparison.

While overall *Cryptosporidium* prevalence was higher in all three countries in the follow-up study **(Table 2)** this difference was not statistically significant (Mann-Whitney, U= U=813.5, p=0.197). In both cohorts, *C. parvum* is consistently the predominant species present in all countries, representing 91% of total infections. The difference in *Cryptosporidium* prevalence between the two studies was not uniform across all farms **(Figure 3).** In Belgium, seven farms showed an increase in prevalence amongst neonatal calves in the follow-up study, three of which with an increase two-fold or higher. While BE11 farm was initially *Cryptosporidium* negative, in the following year 30% of neonatal calves sampled tested positive. Incidence had decreased in eight farms, four of which had reduced by at least half. No infections were detected in BE14 farm, despite 100% of animals sampled the previous year testing positive. In France, prevalence decreased in six farms. Four farms showed an increase in prevalence amongst neonatal calves in the follow-up study, most dramatically seen in FR8 and FR13. *Cryptosporidium* incidence in the FR8 tripled in the subsequent study, while in FR13 100% of sampled calves testing positive despite initially testing negative in the previous year. In the Netherlands, ten farms displayed an increase in prevalence in the follow-up sampling, most markedly seen in NL18. Incidence in five farms decreased. In NL4 and NL13, no infections were found despite presence of the parasite in cohort 1. Two additional subtypes – IIaA17G1R1 and IIa17G2R1 were detected in the follow-up study.

Across countries, no neonatal infections were detected in farms BE9, FR7 and NL3 in either year. Prevalence in BE4, FR12 and FR15 remained at 10-14% in both years. There was no significant effect observed between countries, however an increase of prevalence from 1st to 2nd sampling year was noted (**Table S1**). Within the same country and same year Cochran Q was frequently significant suggesting that differences between farms reflect real differences rather than just random variation. This would indicate that~~. Nonetheless, variance~~

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~~remaining at >0 indicates remaining heterogeneity and so it does raise the issue of how some farms~~ may indeed be better at controlling *Cryptosporidium* infections than others ~~because of factors yet to be elucidated~~.

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DISCUSSION

Cryptosporidium species in cattle

Amplification and sequencing of *SSU* rRNA gene revealed high presence of *Cryptosporidium* at the farm level, with 90.2% of farms containing at least one positive sample across the three countries surveyed. Our results are in accordance with a previous cattle study in France, reporting prevalence of 88.4-100% at the farm level (Follet et al., 2011; Mammeri et al., 2019). Conversely, a previous study in Belgium reported just 32% of farms sampled as containing *Cryptosporidium*. This could be attributed to the less sensitive method of detection used (i.e. immunofluorescence assays of faecal smears). However, other molecular studies utilising *SSU* rRNA PCR amplification in Europe have also reported lower *Cryptosporidium* at the farm level, ranging from 44.5-66.0% (Díaz et al., 2021; Santoro et al., 2019). On the other hand, as the cows in this study were only sampled at one time point, the number of infections may be underestimated. Oocyst shedding can be intermittent (Shaw et al., 2021), and numerous longitudinal studies in farms have observed that almost all calves will shed oocysts over the course of the sampling period (Rieux et al., 2014, 2013a, 2013b).

Cryptosporidium infections were predominantly identified as *C. parvum*, comprising 64.2-81.32% of total infections across the three countries. These results are reflected in previous studies in Belgium (Geurden et al., 2007), France (Follet et al., 2011), and the Netherlands (Wielinga et al., 2008). Beyond these countries, the ubiquity of *C. parvum* in cattle has been reported extensively worldwide. However, *SSU* rRNA PCR amplification has been previously shown to selectively amplify the dominant species in a sample (Cama et al., 2006). As *C. parvum* infections have been suggested to result in higher oocyst shedding density, mixed infections with *C. bovis*, *C. ryanae* and *C. andersoni* are effectively concealed from detection.

High occurrence of zoonotic *C. parvum gp60* subtypes

To assess zoonotic risk of *Cryptosporidium* infection in cattle, *C. parvum* subtype identification was carried through amplification and sequencing of the *gp60* gene. Nine subtypes, eight of which belong to the IIa family, were identified across the three countries. The geographic distribution of these subtypes is broad (**Table S2**) and almost all are of known zoonotic risk, but each to a different extent. The most prevalent and widespread subtype in all three countries was IIaA15G2R1 in 66.7% of *C. parvum*-positive farms, closely matching the year one study (Pinto et al., 2021). This is also the most predominant subtype worldwide (Xiao,

2010) and has been described as "hyper-transmissible" (Feng et al., 2018) being responsible for the majority of acute clinical disease in humans (Feng et al., 2018; Xiao, 2010). The second most common subtype was IIaA16G2R1, which was found only in the French farms representing 44.7% of infections. It has been previously implicated in numerous individual human cryptosporidiosis cases globally (Azcona-Gutiérrez et al., 2017; Chalmers, 2012; Herges et al., 2012; Hijjawi et al., 2017; Nolan et al., 2009; Sharbatkhori et al., 2015). IIaA14G1R1 was found only in three farms across Belgium and the Netherlands. This subtype has been associated with zoonotic transmission, with human outbreaks noted in the UK, Norway and New Zealand (Garcia-R et al., 2020; Robertson et al., 2019; Smith et al., 2021). The highly zoonotic IIaA17G1R1 was found at low prevalence in Belgium and the Netherlands. Human cryptosporidiosis cases of this subtype have been reported worldwide (Chalmers et al., 2019, 2011; Hailu et al., 2021; Hatalova et al., 2022; Insulander et al., 2013; Ranjbar et al., 2016; Soba and Logar, 2008; Wielinga et al., 2008). Subtypes IIaA13G2R1, IIaA17G2R1, IIaA15G1R1 and IaA16G1R1 were found at very low prevalence matching previous results (Benhouda et al., n.d.; Geurden et al., 2007; Smith et al., 2014; Wielinga et al., 2008; Yasur-Landau et al., 2021). Their zoonotic potential appears low, as only sporadic cases in humans and outbreaks have been reported (Braima et al., 2019; Chalmers et al., 2019; Feltus et al., 2006; Guy et al., 2021; Herges et al., 2012, 2012; Hijjawi et al., 2017; Iqbal et al., 2012; Koehler et al., 2014; Ma et al., 2019; Nolan et al., 2009; O' Leary et al., 2020; Valenzuela et al., 2014; Waldron et al., 2011, 2009). In summary, the wide range and the persistent presence of subtypes detected across all counties, suggest a circulation and a high risk of zoonotic transmission of this parasite.

Age-associated differences in *Cryptosporidium* spp. infection

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Age-related variance of infection was observed (**Table S3**) with prevalence in calves being much higher than in dams across Belgium, France, and the Netherlands. The inverse correlation of parasite burden with age has been observed numerous times in cattle (Kváč et al., 2006; Maddox-Hyttel et al., 2006; Santín et al., 2004; Wielinga et al., 2008). However, while the methods used in this study are far more sensitive than conventional microscopic detection, they may not be sufficient when processing fecal~~stool~~ samples from adult cows. Efficient DNA extraction is vital for accurate detection of infection. Adult cows' faecal pats are much larger and contain more fibrous material (Wells et al., 2016). As such, oocysts can be "diluted" in these samples and DNA extracts may contain higher levels of PCR inhibitors

(Schrader et al., 2012). In this study, DNA extraction was carried out on just 0.2 g of each faecal sample so reported infection in adults may be underestimated. Indeed, when utilising a method to concentrate oocysts prior to PCR screening, 91% of sampled adult cows tested positive (Wells et al., 2015).

We observed that the proportion of infecting *Cryptosporidium* spp. differed across age groups (**Table S3**). *Cryptosporidium parvum* was most commonly detected in young neonatal calves up to three weeks old, while *C. bovis* and *C. ryanae* became more prevalent in mothers. The age-related variation in infecting *Cryptosporidium* spp. has been observed extensively including within these same countries and elsewhere throughout Europe (Díaz et al., 2021; Follet et al., 2011; Geurden et al., 2007; Mammeri et al., 2019; Santoro et al., 2019; Soba and Logar, 2008). As *C. parvum* is the main 'cattle-infecting' species causing clinical disease, this result confirms that neonatal calves are particularly at risk for bovine cryptosporidiosis.

Co-infections were only observed in calves; however, this is likely attributable to the method in which mixed infections were identified. Co-infections were detected through a positive non-*C. parvum SSU* rRNA and specific *gp60* identities. As such, only mixed infections containing *C. parvum* were detected which would tend to discount older animals due to association of *C. parvum* with neonates. It is possible other mixed infections were present in the dams, but combinations of *C. bovis*, *C. ryanae* and *C. andersoni* could not be recognised. Since the focus of this study was *C. parvum*, future molecular work should explore techniques to better uncover mixed infections (Dettwiler et al., 2022).

Temporal *Cryptosporidium* infection

Cryptosporidium infection rates in Belgian, French, and Dutch calves over two years are similar. Nonetheless, large variation at the farm level is noted. Specifically, some farms demonstrated consistently low levels of infections, while others showed markedly reduced prevalence in the subsequent study. This is suggestive of variable effectiveness in infection control measures used in the different farms (Brainard et al., 2020). Further investigation into these farms may reveal best practices in reducing infections in neonatal calves.

Based on *gp60* analysis, two additional subtypes were detected in Dutch farms in this follow-up study (Pinto et al., 2021). While this could suggest parasite inflow into these farms, it is equally possible these subtypes were present in the initial study but underrepresented in the *C. parvum* population and therefore not identified. Given that both studies found vertical

transmission to be negligible, it is highly likely infection is originating from an environmental source. Being a known waterborne parasite, the organism has been found in environmental sources numerous times (Brankston et al., 2018; Rose, 1997). Analysis of *Cryptosporidium* in the surrounding farm environment could reveal potential sources of infection as well as routes of transmission.

Future avenues and concluding remarks

Further monitoring of *Cryptosporidium* is paramount to reducing disease burden in cattle. Development of a standardised molecular protocol, including next generation sequencing approaches (Wang et al., 2022) for species and subtype identification would allow for better comparability between studies as well as for tracking spread of different subtypes. While *gp60* is suitable for revealing subtype diversity within a species, additional loci are needed for intra-subtype analyses. This is particularly important regarding "hypertransmissable" subtypes, such as IIaA15G2R1. Despite this subtype being the most prevalent and widespread, the obtained isolates showed little *gp60* genetic variation, even across countries. Hence, transmission dynamics are impossible to discern as yet.

Age has been identified as a key risk factor for *Cryptosporidium* infection however other risk factors regarding animal husbandry should also be explored. Furthermore, as there was significant variation in prevalence between farms, investigations into how farm management practices differ may reveal effective infection control strategies. This would help ease the significant economic impact on farmers (Brainard et al., 2020; Brook et al., 2008; Shaw et al., 2020).

A One Health approach is essential to properly tackle disease burden and eliminate transmission routes of this parasite (Innes et al., 2020). Monitoring of the parasite in cattle moving across borders should be encouraged to prevent infection spread to uncontaminated areas (Pilarczyk et al., 2009). As outbreaks of *Cryptosporidium* are commonly associated with waterborne transmission, sampling of the surrounding water bodies, may reveal other reservoirs of disease as well as possible indirect routes of transmission through contaminated water runoff from farms. Reverse zoonoses may also be taking place, where humans are in fact responsible for infection spread to animals (Messenger et al., 2014). Farm professionals, veterinarians and other visitors may spread oocysts via contaminated clothing, shoes, and vehicles. It is possible all these modes of transmission play a role in cryptosporidiosis spread and cycling.

The high levels of *Cryptosporidium* detected in this and the previous study reinforce~~reinforces~~ the role of dairy farms as a key reservoir of this parasite. Our findings pinpoint to a high risk of zoonotic transmission with *C. parvum* as the predominant species found across all three countries and all *gp60* subtypes identified here having previously been detected in human cryptosporidiosis cases.

Figure Legends:

Figure 1. Venn diagram with all observed *Cryptosporidium*~~*C.*~~ *parvum gp60* subtypes across Belgium, France, and the Netherlands

Figure 2. Geographical distribution of *Cryptosporidium parvum gp60* subtypes in dairy farms across a) Belgium, b) France and c) the Netherlands.
IIaA13G12R1 (purple), IIaA14G1R1 (red), IIaA15G1R1 (dark blue), IIaA15G2R1 (green), IIaA16G1R1 (brown), IIaA16G2R1 (peach), IIaA17G1R1 (turquoise), IIaA17G2R1 (blue). *C. parvum* positive samples with unsuccessful *gp60* sequencing are indicated in grey. Pie charts are proportional to number of *C. parvum* positive samples identified per farm

Figure 3. Comparison of *Cryptosporidium* prevalence in neonatal calves across two sampling studies in a) Belgium, b) France, c) The Netherlands.

Supplementary Figure 1. A maximum likelihood (ML) tree based on the 18S rRNA gene sequences of *Cryptosporidium*~~*C.*~~ *bovis*, *C. ryanae* and *C. andersoni* obtained in Belgium, France, and the Netherlands. Bootstrap values for nodes with more than 50% support are shown. ML tree was rooted with an 18S rRNA sequence from *Monocystis agilis* (AF457127).

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Follow-up investigation into *Cryptosporidium* prevalence and transmission in Western European dairy farms

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ABSTRACT

Cryptosporidium parvum is an enteric parasite and a major contributor to acute enteritis in calves worldwide, causing an important economic burden for farmers. This parasite poses a major public health threat through transmission between livestock and humans. Our previous pilot study in Western Europe revealed a high prevalence of *Cryptosporidium* in calves of dairy farms. In the sequel study herein, 936 faecal samples were collected from the same 51 dairy farms across Belgium, France, and the Netherlands. Following DNA extraction, *Cryptosporidium* screening was carried out using nested-PCR amplification targeting the *SSU* rRNA gene. All positive samples were sequenced, and phylogenetic analyses were used to identify the *Cryptosporidium* spp. present. The 60 kDa glycoprotein (*gp60*) gene was also sequenced to determine the *C. parvum* subtypes present. Prevalence of *Cryptosporidium* ranged from 23.3% to 25%, across the three countries surveyed. The parasite was found in most of the farms sampled, with 90.2% testing positive. *Cryptosporidium parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* were all identified, with the former being the most predominant, representing 71.4% of all infections. *Cryptosporidium parvum* was associated with pre-weaned calves, while other species were associated with older animals. Subtyping of *gp60* gene revealed nine subtypes, eight of which have previously been reported to cause clinical disease in humans. Similarly to the first study, vertical transmission was not a major contributor to *Cryptosporidium* spread. Our study highlights the need for further investigation into cryptosporidiosis transmission, and future studies will require a One Health approach to reduce the impact of this disease.

INTRODUCTION

Cryptosporidiosis is an enteric disease caused by the apicomplexan parasites *Cryptosporidium* (Shirley et al., 2012). Most cases of zoonotic cryptosporidiosis are associated with livestock. Cattle, in particular, have long been established as a key reservoir of infection with numerous global reports of human cryptosporidiosis cases linked to close contact with those animals (Chalmers and Giles, 2010; Xiao and Feng, 2008). In the majority of these, *C. parvum* and *C. hominis* are the main responsible species (Leitch and He, 2011; Xiao and Feng, 2008).

Bovine cryptosporidiosis also has a major financial impact for cattle farmers (Chalmers and Giles, 2010; Mosier and Oberst, 2000; Widmer et al., 2020). In this livestock, *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* are the main infectious species, with *C. parvum* being the most common (Cho and Yoon, 2014; De Graaf et al., 1999; Feng et al., 2018). *Cryptosporidium parvum* is a major cause of acute enteritis in cattle, causing neonatal diarrhoea. In most cases, the disease is self-limiting, though persistent diarrhoea can lead to emaciation, fatigue, and severe dehydration. While fathality is generally low, long-term adverse health consequences may arise (Cho and Yoon, 2014; Thomson et al., 2017). Significant differences in weight gain have been observed between calves who had severe cryptosporidiosis as neonates and calves that had not (Shaw et al., 2020). Thus, farmers may incur economic losses through dead livestock, treatment of sick animals and reduced production due to retarded animal growth (Bennett, 2003; Hawkins et al., 2019; Stott and Gunn, 1997). Additionally, cryptosporidiosis may exacerbate concurrent infections with other pathogens (Delling and Daugschies, 2022; García et al., 2000) and infected animals are more susceptible to developing severe cryptosporidiosis. It is difficult for farmers to know how to manage spread of infection, as there is insufficient data on risk factors for *Cryptosporidium* infection to cattle on dairy farms. A recent systematic review found no consistent risk or protective factors for preventing *C. parvum* infection (Brainard et al., 2020).

Historically, *Cryptosporidium* detection has relied on microscopic examination of faecal smears for the presence of oocysts. As this method suffers from low sensitivity (Adeyemo et al., 2018), *Cryptosporidium* infection may not be detected in samples with low oocyst levels. Molecular-based diagnostic techniques have been used to increase sensitivity of *Cryptosporidium* detection along with ability to identity infecting species and subtypes. Subtype analysis can shed further light on potential zoonotic transmission by identifying the ones that occur in both humans and cattle. With increased molecular typing studies, distinctions

in the epidemiology of subtypes may also be discovered. This information could help ascertain how best to track and potentially tackle infection spread in certain areas.

A previous 2019–2020 study using samples from dairy cattle farms in Belgium, France and the Netherlands indicated a prevalence of 20.8–25.7% (Pinto et al., 2021), with numerous zoonotic *C. parvum* subtypes identified. Prior to this, prevalence investigations in these countries were infrequent and often did not utilise molecular detection. The study herein is a an investigation of *Cryptosporidium* across the same dairy farms one year later. We used the *SSU* rRNA and *gp60* genes for species and subtype level characterisation to investigate *C. parvum* diversity and transmission dynamics. As such, our study complements previous molecular work, provides temporal information on *Cryptosporidium* infection at the farm level and contributes to future investigations aiming to discern transmission networks.

MATERIALS AND METHODS

Sample collection

From September 2020 to July 2021, veterinarians collected a total of 936 faecal samples from 51 dairy farms were – 17 in Belgium, 15 in France and 19 in the Netherlands. We aimed to collect samples from up to 10 calves (up to 3 months of age) and their mothers. In cases where farms had less than 10 calves, fewer samples were collected. Diarrheic and apparently health animals were included in a random selection. Faeces were taken directly from the rectum, using a single pair of disposable gloves. This study was conducted as a cross-border collaboration under the Health for Dairy Cows (H4DC) project, funded by the Interreg-2-seas programme. This is a European Territorial Cooperation program covering the Flanders region of Belgium, the Hauts-de-France region in France and the western part of the Netherlands. These regions experience similar maritime temperate climates. The main objective of the project is to reduce the disease burden and economic impact of *Cryptosporidium* spp. on dairy farms.

DNA extraction

Immediately after defecation, faeces were collected into sterile tubes, and stored on ice. DNA extraction was carried out using PureLink™ Microbiome DNA Purification Kit (ThermoFisher) according to manufacturer's instructions, with slight modifications. S1 Lysis Buffer (650 µL) and S2 Lysis Enhancer (100 µL) was added to each sample. They were then incubated at 65°C and then vortexed on maximum speed for 13 min each. After addition of S3 Clean-up Buffer, samples were incubated at 4°C for 10 min. Following application of S6 Elution Buffer to the spin column, samples were incubated at room temperature for 3 min prior to centrifugation. Genomic DNA was stored at -20°C until 18S rRNA and *gp60* PCR reactions were carried out. Leftover DNA and fecal samples were stored long-term in a -80 °C freezer.

Cryptosporidium spp. screening and molecular genotyping

Nested-PCR amplification of a 631-bp region of the 18S rRNA gene was used to screen for *Cryptosporidium* spp. (Ziegler et al., 2007). The external primers used were 5´-GATTAAGCCATGCATGTCTAA-3´ (forward) and 5´-TTCCATGCTGGAGTATTCAAG3´ (reverse). The internal primers were 5´-CAGTTATAGTTTACTTGATAATC-3´ (forward) and 5´-CCTGCTTTAAGCACTCTAATTTTC-3´ (reverse). Each PCR mixture contained 1 µL

of DNA, 0.4 μM each of forward and reverse primers, 12.5 μL of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 9.5 μL nuclease-free water. External PCR cycling conditions were: denaturation for 2 min at 94°C, followed by 24 cycles at 94°C for 50 s, 53°C for 50 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. Internal PCR cycling conditions were: denaturation for 2 min at 94°C, followed by 30 cycles at 94°C for 50 s, 56°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 10 min. Positive (genomic DNA from a pure culture of *C. parvum* IOWA oocysts) and negative (using sterile water as a template) controls were included in both reactions. Following separation and excision from a 2% agarose gel, DNA was extracted using GeneJET Gel Extraction Kit (ThermoFisher Scientific). All positive samples were then bidirectionally sequenced (Eurofins Genomics) and chromatograms were manually assessed for quality, with ambiguous bases trimmed. For species level identification, BLAST searches were performed using obtained sequences as queries against the nucleotide database in GenBank. Following alignment with reference sequences, polymorphisms were identified.

gp60 subtyping of *Cryptosporidium parvum*

Nested-PCR of the *gp60* gene was carried out to determine the subtype of *Cryptosporidium* 18S rRNA PCR-positive samples (Alves et al., 2003). The external primers used were 5´-ATAGTCTCCGCTGTATTC-3´ (forward) and 5´-GGAAGGAACGATGTATCT-3´ (reverse). The internal primers were 5´-TCCGCTGTATTCTCAGCC-3´ (forward) and 5´- GCAGAGGAACCAGCATC-3´ (reverse). Each PCR mixture contained 2 μL of DNA, 0.2 μM each of forward and reverse primers, 15 μL of 2× PCRBIO Taq Mix Red (PCR Biosystems) and 11.8 μL nuclease-free water. Cycling conditions for both internal and external PCR reactions were: a denaturation step for 3 min at 94°C, followed by 35 cycles at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. Positive and negative controls were included in both reactions. Following separation and excision from a 2% agarose gel, DNA was extracted using GeneJET Gel Extraction Kit (ThermoFisher Scientific). All positive samples were then bidirectionally sequenced (Eurofins Genomics), and chromatograms were manually assessed for quality, with ambiguous bases trimmed. Subtypes were determined using established standard nomenclature (Sulaiman et al., 2005). BLAST searches were performed using newly generated sequences as queries against the nucleotide database in

GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Following alignment with reference sequences, polymorphisms were identified.

Phylogenetic analysis

The obtained sequences were aligned with each other as well as reference sequences from GenBank by MAFFT v.7 (https://mafft.cbrc.jp/alignment/server). Sequence alignment was manually inspected using BioEdit v7.0.5.3 (https://bioedit.software.informer.com). Best DNA/Protein phylogeny models were selected using the MEGA11 software (Tamura et al., 2021). The Tamura 3-parameter model (Tamura, 1992) was selected. Phylogenetic trees were inferred using maximum likelihood (ML), with the substitution model that best fit the alignment selected using the Bayesian information criterion. Bootstrap support for branching was based on 1,000 replications. An 18S sequence from *Monocystis agilis* (Accession number: AF457127) was used to root ML tree.

Comparison to preceding study and statistical analyses

Only neonatal calves aged under one month old were included for comparison. Farms with less than three calves within this age range have been excluded from comparison. In the preceding study, *gp60* subtyping was only performed on samples confirmed as *C. parvum* through *SSU* rRNA gene sequencing. In this study, *gp60* screening was carried out on all samples that were PCR-positive for *SSU* rRNA, which revealed mixed infections in some animals. For comparison between the two time points these samples have been identified based on their *SSU* rRNA gene. Furthermore, the *SSU* rRNA gene of some samples could not be successfully sequenced, but the *gp60* gene could. These have been excluded in the prevalence count, as only *SSU* rRNA confirmed positive samples were reported in the previous study (Pinto et al., 2021). Comparison of prevalence between the two cohorts was performed by using the Mann-Whitney U test. Cochran Q tests were used to determine heterogeneity between farms within the same year and country. The mixed effects multilevel negative binomial modelling was done with STATA™ v17.0 with farm as the level. STATSDirect v. 3.3.5 was used to compare prevalence between countries within the same year and across the 2 years.

RESULTS

Prevalence of *Cryptosporidium* in Belgian, French, and Dutch dairy farms

In Belgium, 324 fecal samples across 17 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 25% with 81/324 specimens positive for *Cryptosporidium* spp. **(Table 1)**. Prevalence varied across farms from 0% to 50%. The majority of the positive samples were identified as *C. parvum* (64.2%, 52/81) with 45 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and four samples showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for three *SSU* rRNA PCR-positive samples. Nonetheless, *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 14/17 farms. The next most abundant species found was *C. bovis* (20.9%, 17/81). Additionally, three samples had co-infections of *C. bovis* and *C. parvum*. Nineteen samples had 100% nucleotide identity to the reference sequence AB777173.1 (from a calf in Egypt), while one sample had 100% nucleotide identity to the reference sequence MZ021459.1 (from a calf in Belgium in a previous study on these farms (Pinto et al., 2021)). The next most prevalent species was *C. ryanae* (6.2%, 5/81). Additionally, two samples showed mixed infection of *C. ryanae* and *C. parvum*. Five samples were 100% identical to the reference sequence FJ463193.1, while one sample had 100% nucleotide identity to the reference sequence KT922233.1 and another showed 99% identity to the same sequence. The least common species was *C. andersoni* (2.5%, 2/81). One sample showed 100% nucleotide identity to the reference sequence AB513856.1 (from cattle in Egypt), while the other was 100% identical to FJ463171.1 (from cattle in China).

In France, 236 fecal samples across 15 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 23.3% with 55/236 specimens positive for *Cryptosporidium* spp. Prevalence varied across farms from 0% to 45.5%. Most of the positive samples were identified as *C. parvum* (65.5%, 36/55) with 33 of them showing 100% nucleotide identity to the reference sequence AH006572.2, and one sample showing 99% nucleotide identity to the same sequence. We were unable to obtain good quality sequences for two *SSU* rRNA PCR-positive samples, but *C. parvum* identity was confirmed through positive *gp60* PCR and subsequent sequencing. At the farm level, *C. parvum* was present in 10/15 farms, with occurrence ranging from 5% to 45.5%. The next most common species was *C. bovis* (12.7%, 7/55). Additionally, two samples had co-infections of *C. bovis* and *C. parvum*. Eight samples showed 100% nucleotide identity to the reference sequence AB777173.1, while

one sample showed 100% nucleotide identity to the reference sequence MZ021459.1. Equal levels of *C. ryanae* and *C. andersoni* were detected at 9.1% (5/55) each. Two *C. ryanae* variants were found with three samples showing 100% identity to KT922233.1 and two samples showing 100% nucleotide identity to the reference sequence FJ463193.1. Two *C. andersoni* variants were found with three isolates showing 100% nucleotide identity to reference sequence FJ463171.1 and two showing 100% nucleotide identity to AB513856.1.

In the Netherlands, 376 fecal samples across 19 farms were screened. Amplification of the *SSU* rRNA and *gp60* genes showed a prevalence of 24.2% with 91/376 specimens positive for *Cryptosporidium* spp. 18/19 farms contained at least one positive samples with prevalence ranging from 0% to 42.1% across farms. Most positive samples were identified as *C. parvum* (81.3%, 74/91) with all 74 isolates showing 100% nucleotide identity to the reference sequence AH006572.2. At the farm level, *C. parvum* was present in 8/19 farms. The next most prevalent species was *C. bovis* at (16.5%, 15/91), with 13 isolates showing 100% nucleotide identity to the reference sequence AB777173.1 and two isolates showing 100% nucleotide identity to MZ021459.1. One sample was positive for *C. ryanae* with 99% sequence identity to FJ463193.1, and one for *C. andersoni* with 100% nucleotide identity to AB513856.1. No mixed infections were found.

All *SSU* rRNA sequences have been submitted to GenBank under accession numbers OP975438 – OP975657.

Cryptosporidium parvum subtyping through *gp60* analysis

Cryptosporidium parvum was detected in 71.3% (162/227) of *Cryptosporidium* positive samples and found in 39 farms. PCR amplification and sequencing of the *gp60* gene was used to identify the specific *C. parvum* subtypes present in these three countries. All *18S* rRNA PCR-positive samples were further screened using nested PCR of the *gp60* gene. Of these, the *gp60* gene was successfully amplified and sequenced in 145 samples. 24 *C. parvum* positive sequences were not successfully subtyped. Sequence analysis revealed the presence of nine subtypes, eight of which belong to the IIa subtype family and one belonging to the IId family **(Figure 1)**.

The most abundant subtype found overall was IIaA15G2R1, representing 61.7% of *C. parvum*-positive samples Ninety-eight isolates had 100% nucleotide identity to the reference sequence DQ630518.1 and two were 99% identical to the same sequence. This subtype was also the most widely distributed, occurring in 71.8% (28/39) of *C. parvum*-positive farms. This

subtype represented 73.1%, 41.7% and 63.5% of all *C. parvum* positive samples, in Belgium, France and the Netherlands, respectively. It was the most prevalent subtype in Belgium and the Netherlands. This subtype predominantly occurred in calves, with only two isolates occurring in a dam.

The second most common subtype in this study was IIaA16G2R1. Seventeen isolates were found with 100% nucleotide identity to reference sequence DQ192505.1, all of which were found in France. This subtype was found in five farms and represented 47.2% of total *C. parvum* infections in the French farms. This subtype also predominantly occurred in calves, with only one isolate occurring in a dam.

Eight isolates of IIaA14G1R1 were identified – six with 100% nucleotide identity to reference sequence AM937017.1 and two with 99% nucleotide identity to the same sequence. This subtype occurred in three farms, representing 5.8% and 6.8% of *C. parvum* infections in Belgium and the Netherlands, respectively.

Eight isolates of IIaA17G1R1 were identified – seven with 100% nucleotide identity to reference sequence GQ983359.1, which were all located in one Dutch farm. The remaining isolate occurred in a Belgian farm and had 99% nucleotide identity to the same sequence. This was the sole subtype present in two farms, representing 1.9% and 9.5% of *C. parvum* infections in Belgium and the Netherlands, respectively.

Five isolates of IIaA13G2R1 were identified – four with 100% nucleotide identity to reference sequence DQ192502.1 and one isolate with 99% homology to the same sequence. This was the sole subtype present in three farms, representing 3.8% and 4.1% of *C. parvum* infections in Belgium and the Netherlands, respectively.

IIaA17G2R1 was found exclusively in the Netherlands. Four isolates were identified with 100% nucleotide identity to DQ630516.1. This subtype occurred on one farm and represented 5.4% of *C. parvum* infections. IIaA15G1R1 (99% nucleotide identity to AB777872) and IIaA16G1R1 (100% nucleotide identity to DQ192504.1), each occurred once in Belgium and France, respectively. Lastly, a novel isolate was identified and marked as IIdA14G2R1. This is the first report of this subtype worldwide and is the only subtype belonging to the IId family. Subtypes IIaA17G1R1, IIaA13G2R1, IIaA17G2R1, IIaA15G1R1 and IIaA16G1R1 were all found exclusively in calves. Geographical distribution of subtypes is visualised in **Figure 2**.

All *gp60* sequences have been submitted to GenBank under accession numbers OP978513 – OP978657.

Comparison to previous sampling study

This work is a follow-up to a prevalence study carried out between 2019 to 2020 (Pinto et al., 2021). Methodology was slightly altered between the two studies. In the preceding study, *gp60* PCR and subtyping was only carried out on samples confirmed as *C. parvum* positive through *SSU* rRNA PCR and sequencing. Herein subtyping was performed in all *Cryptosporidium SSU* rRNA positive samples. As such, we were able to detect *Cryptosporidium* co-infections. For comparability between the cohorts, prevalence figures in the second cohort have been adjusted accordingly. Furthermore, only animals under 28 days old have been included for comparison.

While overall *Cryptosporidium* prevalence was higher in all three countries in the follow-up study **(Table 2)** this difference was not statistically significant (Mann-Whitney, U= U=813.5, p=0.197). In both cohorts, *C. parvum* is consistently the predominant species present in all countries, representing 91% of total infections. The difference in *Cryptosporidium* prevalence between the two studies was not uniform across all farms **(Figure 3).** In Belgium, seven farms showed an increase in prevalence amongst neonatal calves in the follow-up study, three of which with an increase two-fold or higher. While BE11 farm was initially *Cryptosporidium* negative, in the following year 30% of neonatal calves sampled tested positive. Incidence had decreased in eight farms, four of which had reduced by at least half. No infections were detected in BE14 farm, despite 100% of animals sampled the previous year testing positive. In France, prevalence decreased in six farms. Four farms showed an increase in prevalence amongst neonatal calves in the follow-up study, most dramatically seen in FR8 and FR13. *Cryptosporidium* incidence in the FR8 tripled in the subsequent study, while in FR13 100% of sampled calves testing positive despite initially testing negative in the previous year. In the Netherlands, ten farms displayed an increase in prevalence in the follow-up sampling, most markedly seen in NL18. Incidence in five farms decreased. In NL4 and NL13, no infections were found despite presence of the parasite in cohort 1. Two additional subtypes – IIaA17G1R1 and IIa17G2R1 were detected in the follow-up study.

Across countries, no neonatal infections were detected in farms BE9, FR7 and NL3 in either year. Prevalence in BE4, FR12 and FR15 remained at 10-14% in both years. There was no significant effect observed between countries, however an increase of prevalence from 1st to 2nd sampling year was noted (**Table S1**). Within the same country and same year Cochran Q was frequently significant suggesting that differences between farms reflect real

differences rather than just random variation. This would indicate that may indeed be better at controlling *Cryptosporidium* infections than others.

DISCUSSION

Cryptosporidium species in cattle

Amplification and sequencing of *SSU* rRNA gene revealed high presence of *Cryptosporidium* at the farm level, with 90.2% of farms containing at least one positive sample across the three countries surveyed. Our results are in accordance with a previous cattle study in France, reporting prevalence of 88.4-100% at the farm level (Follet et al., 2011; Mammeri et al., 2019). Conversely, a previous study in Belgium reported just 32% of farms sampled as containing *Cryptosporidium*. This could be attributed to the less sensitive method of detection used (i.e. immunofluorescence assays of faecal smears). However, other molecular studies utilising *SSU* rRNA PCR amplification in Europe have also reported lower *Cryptosporidium* at the farm level, ranging from 44.5-66.0% (Díaz et al., 2021; Santoro et al., 2019). On the other hand, as the cows in this study were only sampled at one time point, the number of infections may be underestimated. Oocyst shedding can be intermittent (Shaw et al., 2021), and numerous longitudinal studies in farms have observed that almost all calves will shed oocysts over the course of the sampling period (Rieux et al., 2014, 2013a, 2013b).

Cryptosporidium infections were predominantly identified as *C. parvum*, comprising 64.2-81.32% of total infections across the three countries. These results are reflected in previous studies in Belgium (Geurden et al., 2007), France (Follet et al., 2011), and the Netherlands (Wielinga et al., 2008). Beyond these countries, the ubiquity of *C. parvum* in cattle has been reported extensively worldwide. However, *SSU* rRNA PCR amplification has been previously shown to selectively amplify the dominant species in a sample (Cama et al., 2006). As *C. parvum* infections have been suggested to result in higher oocyst shedding density, mixed infections with *C. bovis, C. ryanae* and *C. andersoni* are effectively concealed from detection.

High occurrence of zoonotic *C. parvum gp60* subtypes

To assess zoonotic risk of *Cryptosporidium* infection in cattle, *C. parvum* subtype identification was carried through amplification and sequencing of the *gp60* gene. Nine subtypes, eight of which belong to the IIa family, were identified across the three countries. The geographic distribution of these subtypes is broad (**Table S2**) and almost all are of known zoonotic risk, but each to a different extent. The most prevalent and widespread subtype in all three countries was IIaA15G2R1 in 66.7% of *C. parvum*-positive farms, closely matching the year one study (Pinto et al., 2021). This is also the most predominant subtype worldwide (Xiao,

2010) and has been described as “hyper-transmissible” (Feng et al., 2018) being responsible for the majority of acute clinical disease in humans (Feng et al., 2018; Xiao, 2010). The second most common subtype was IIaA16G2R1, which was found only in the French farms representing 44.7% of infections. It has been previously implicated in numerous individual human cryptosporidiosis cases globally (Azcona-Gutiérrez et al., 2017; Chalmers, 2012; Herges et al., 2012; Hijjawi et al., 2017; Nolan et al., 2009; Sharbatkhori et al., 2015). IIaA14G1R1 was found only in three farms across Belgium and the Netherlands. This subtype has been associated with zoonotic transmission, with human outbreaks noted in the UK, Norway and New Zealand (Garcia-R et al., 2020; Robertson et al., 2019; Smith et al., 2021). The highly zoonotic IIaA17G1R1 was found at low prevalence in Belgium and the Netherlands. Human cryptosporidiosis cases of this subtype have been reported worldwide (Chalmers et al., 2019, 2011; Hailu et al., 2021; Hatalova et al., 2022; Insulander et al., 2013; Ranjbar et al., 2016; Soba and Logar, 2008; Wielinga et al., 2008). Subtypes IIaA13G2R1, IIaA17G2R1, IIaA15G1R1 and IaA16G1R1 were found at very low prevalence matching previous results (Benhouda et al., n.d.; Geurden et al., 2007; Smith et al., 2014; Wielinga et al., 2008; Yasur-Landau et al., 2021). Their zoonotic potential appears low, as only sporadic cases in humans and outbreaks have been reported (Braima et al., 2019; Chalmers et al., 2019; Feltus et al., 2006; Guy et al., 2021; Herges et al., 2012, 2012; Hijjawi et al., 2017; Iqbal et al., 2012; Koehler et al., 2014; Ma et al., 2019; Nolan et al., 2009; O’ Leary et al., 2020; Valenzuela et al., 2014; Waldron et al., 2011, 2009). In summary, the wide range and the persistent presence of subtypes detected across all counties, suggest a circulation and a high risk of zoonotic transmission of this parasite.

Age-associated differences in *Cryptosporidium* spp. infection

Age-related variance of infection was observed (**Table S3**) with prevalence in calves being much higher than in dams across Belgium, France, and the Netherlands. The inverse correlation of parasite burden with age has been observed numerous times in cattle (Kváč et al., 2006; Maddox-Hyttel et al., 2006; Santín et al., 2004; Wielinga et al., 2008). However, while the methods used in this study are far more sensitive than conventional microscopic detection, they may not be sufficient when processing fecal samples from adult cows. Efficient DNA extraction is vital for accurate detection of infection. Adult cows’ faecal pats are much larger and contain more fibrous material (Wells et al., 2016). As such, oocysts can be “diluted” in these samples and DNA extracts may contain higher levels of PCR inhibitors (Schrader et

al., 2012). In this study, DNA extraction was carried out on just 0.2 g of each faecal sample so reported infection in adults may be underestimated. Indeed, when utilising a method to concentrate oocysts prior to PCR screening, 91% of sampled adult cows tested positive (Wells et al., 2015).

We observed that the proportion of infecting *Cryptosporidium* spp. differed across age groups (**Table S3**). *Cryptosporidium parvum* was most commonly detected in young neonatal calves up to three weeks old, while *C. bovis* and *C. ryanae* became more prevalent in mothers. The age-related variation in infecting *Cryptosporidium* spp. has been observed extensively including within these same countries and elsewhere throughout Europe (Díaz et al., 2021; Follet et al., 2011; Geurden et al., 2007; Mammeri et al., 2019; Santoro et al., 2019; Soba and Logar, 2008). As *C. parvum* is the main 'cattle-infecting' species causing clinical disease, this result confirms that neonatal calves are particularly at risk for bovine cryptosporidiosis.

Co-infections were only observed in calves; however, this is likely attributable to the method in which mixed infections were identified. Co-infections were detected through a positive non-*C. parvum SSU* rRNA and specific *gp60* identities. As such, only mixed infections containing *C. parvum* were detected which would tend to discount older animals due to association of *C. parvum* with neonates. It is possible other mixed infections were present in the dams, but combinations of *C. bovis*, *C. ryanae* and *C. andersoni* could not be recognised. Since the focus of this study was *C. parvum*, future molecular work should explore techniques to better uncover mixed infections (Dettwiler et al., 2022).

Temporal *Cryptosporidium* infection

Cryptosporidium infection rates in Belgian, French, and Dutch calves over two years are similar. Nonetheless, large variation at the farm level is noted. Specifically, some farms demonstrated consistently low levels of infections, while others showed markedly reduced prevalence in the subsequent study. This is suggestive of variable effectiveness in infection control measures used in the different farms (Brainard et al., 2020). Further investigation into these farms may reveal best practices in reducing infections in neonatal calves.

Based on *gp60* analysis, two additional subtypes were detected in Dutch farms in this follow-up study (Pinto et al., 2021). While this could suggest parasite inflow into these farms, it is equally possible these subtypes were present in the initial study but underrepresented in the *C. parvum* population and therefore not identified. Given that both studies found vertical

transmission to be negligible, it is highly likely infection is originating from an environmental source. Being a known waterborne parasite, the organism has been found in environmental sources numerous times (Brankston et al., 2018; Rose, 1997). Analysis of *Cryptosporidium* in the surrounding farm environment could reveal potential sources of infection as well as routes of transmission.

Future avenues and concluding remarks

Further monitoring of *Cryptosporidium* is paramount to reducing disease burden in cattle. Development of a standardised molecular protocol, including next generation sequencing approaches (Wang et al., 2022) for species and subtype identification would allow for better comparability between studies as well as for tracking spread of different subtypes. While *gp60* is suitable for revealing subtype diversity within a species, additional loci are needed for intra-subtype analyses. This is particularly important regarding "hypertransmissable" subtypes, such as IIaA15G2R1. Despite this subtype being the most prevalent and widespread, the obtained isolates showed little *gp60* genetic variation, even across countries. Hence, transmission dynamics are impossible to discern as yet.

Age has been identified as a key risk factor for *Cryptosporidium* infection however other risk factors regarding animal husbandry should also be explored. Furthermore, as there was significant variation in prevalence between farms, investigations into how farm management practices differ may reveal effective infection control strategies. This would help ease the significant economic impact on farmers (Brainard et al., 2020; Brook et al., 2008; Shaw et al., 2020).

A One Health approach is essential to properly tackle disease burden and eliminate transmission routes of this parasite (Innes et al., 2020). Monitoring of the parasite in cattle moving across borders should be encouraged to prevent infection spread to uncontaminated areas (Pilarczyk et al., 2009). As outbreaks of *Cryptosporidium* are commonly associated with waterborne transmission, sampling of the surrounding water bodies, may reveal other reservoirs of disease as well as possible indirect routes of transmission through contaminated water runoff from farms. Reverse zoonoses may also be taking place, where humans are in fact responsible for infection spread to animals (Messenger et al., 2014). Farm professionals, veterinarians and other visitors may spread oocysts via contaminated clothing, shoes, and vehicles. It is possible all these modes of transmission play a role in cryptosporidiosis spread and cycling.

The high levels of *Cryptosporidium* detected in this and the previous study reinforce the role of dairy farms as a key reservoir of this parasite. Our findings pinpoint to a high risk of zoonotic transmission with *C. parvum* as the predominant species found across all three countries and all *gp60* subtypes identified here having previously been detected in human cryptosporidiosis cases.

Figure Legends:

Figure 1. Venn diagram with all observed *Cryptosporidium parvum gp60* subtypes across Belgium, France, and the Netherlands

Figure 2. Geographical distribution of *Cryptosporidium parvum gp60* subtypes in dairy farms across a) Belgium, b) France and c) the Netherlands.
IIaA13G12R1 (purple), IIaA14G1R1 (red), IIaA15G1R1 (dark blue), IIaA15G2R1 (green), IIaA16G1R1 (brown), IIaA16G2R1 (peach), IIaA17G1R1 (turquoise), IIaA17G2R1 (blue). *C. parvum* positive samples with unsuccessful *gp60* sequencing are indicated in grey. Pie charts are proportional to number of *C. parvum* positive samples identified per farm

Figure 3. Comparison of *Cryptosporidium* prevalence in neonatal calves across two sampling studies in a) Belgium, b) France, c) The Netherlands.

Supplementary Figure 1. A maximum likelihood (ML) tree based on the 18S rRNA gene sequences of *Cryptosporidium bovis*, *C. ryanae* and *C. andersoni* obtained in Belgium, France, and the Netherlands. Bootstrap values for nodes with more than 50% support are shown. ML tree was rooted with an 18S rRNA sequence from *Monocystis agilis* (AF457127).

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Dear Editor,

I hereby submit the revised version of our manuscript Vetpar-D-22-577R1, entitled “Follow-up investigation into *Cryptosporidium* prevalence and transmission in Western European dairy farms”, on behalf of myself and all co-authors. We have addressed all of the reviewers’ suggestions, as detailed below (in red and italics). I hope that the improved manuscript is now suitable for publication

Reviewer Comments:

Reviewer #2:

The multi-authored manuscript describes an epidemiological survey on bovine Cryptosporidium in three western European countries, including the occurrence of different C. parvum subtypes and the analysis of possible risk factors for transmission. This work presents a follow-up study on this topic and extends to detailed genotyping with a very detailed analysis, and supports the previous findings that C. parvum is the prevailing and probably also the most important species in calves, due to its pathogenic potential in suckling calves and its zoonotic nature. The follow-up study not only enabled comparison of prevalence rates over several years, but also developed further in methodology as it changed the decision on C. parvum genotyping which enabled the detection of coinfections in the present study. The authors do not embark on a discussion whether this may have lead to a higher C. parvum prevalence on some of the farms that were re-sampled, but focus on the genotyping details instead and do not discuss possible reasons for prevalence differences, only mention this briefly in the Results and Discussion sections (maybe this is yet to come in another manuscript).
The manuscript is well written, the methods are outlined in a way that makes them easy to follow, the results are clearly presented and support the conclusions.

Comment: ***We would like to thank the reviewer for their positive comments on the manuscript.***

A few points should be addressed in a minor revision, listed according to their appearance in the manuscript:
Table 1: in the header "(co-infection)" should not be in italics; the table should be formatted in a way that enables portrait format on a single page if possible.

Comment: ***Both changes have been made. We still think that the font size is very small to have all the information in one page, but we have made the modification according to the reviewer’s suggestion.***

Abstract, last sentence: while zoonotic diseases usually require a One Health approach, it is not clear wat the impact of cryptosporidiosis is on humans in the countries that were enrolled in this study. Have clinical cases in humans with the reported genotypes been described from this area or is this a more general statement? This should be described more precisely in the Abstract. The statement that vertical transmission does not seem to play a role comes a bit as a surprise in the Abstract, since, unlike in the full text, sampling of calves and their mothers is not mentioned as such, this should also be clarified.

Comment: ***In regard to the One Health sentence, this is a general statement on future studies. We have modified the sentence to highlight this: “Our study highlights the need for further investigation into cryptosporidiosis transmission, and future studies will require a One Health approach to reduce the impact of this disease.”.***

We are a bit confused about the comment on the vertical transmission. We have stated several times within the text that we have sampled calves and their mothers: e.g. “We aimed to collect samples from up to 10 calves (up to 3 months of age) and their mothers.” in methods; “Cryptosporidium

parvum was most commonly detected in young neonatal calves up to three weeks old, while C. bovis and C. ryanae became more prevalent in mothers." in results.

Results: the term "stool" is used for human samples, while "fecal samples" is used for animals. It is suggested to use "fecal samples" (this is not mandatory, just a suggestion).

Comment: ***We have replaced eh word "stool" to the word "fecal" throughout the manuscript.***

Discussion: There is a strong focus on discussion of the detected genotypes (maybe this impression is not quite correct since a lot of the text in the pdf was erased but is still visible). The authors give hints on possible mechanisms of spread and transmission between cattle and from cattle to humans (and vice versa) and recommend NGS for more detailed genotyping. The "high risk of zoonotic transmission" is, however, not fully supported by the Discussion that does not bring forward data on human cryptosporidiosis in the three countries the samples in this study originated from. It would provide more stringency in the discussion to only include Europe in the discussion of the genotypes considered as zoonotic; after all, the data deal with cattle from 3 western European countries and not with a review on zoonotic genotypes worldwide. To not let the authors' work on compiling such data go unnoticed, it is suggested to collectively describe non-European findings in the format of a Table (supplementary data) instead of putting them into the text, and to just sum them up briefly in the text to put more focus on the geographic correlation between the findings in cattle and humans (to pinpoint the reviewer's concerns, cattle are certainly moved over long distances, however, for an outbreak in New Zealand oocysts shed by a calf on a European farm are not relevant). On the other hand. The now deleted data on the distribution of genotypes in cattle could also go into a supplementary table instead of being deleted (this is just a suggestion, if the authors do not want to follow this, the reviewer will not insist).

Comment: ***We would like to thank the reviewer for this comment. We have actually done this, based on previous comments on the previous revision of our manuscript. Supplementary Table S2 summarises all the information and in the discussion we just provide a small overview of the impact of the various subtypes.***

Reviewer #3:

Table 1 - replace "Incidence" with "Occurrence"

Comment: ***Done***

Keywords - display alphabetically

Comment: ***Done***

Abstract

… causing an important economic burden

Comment: ***Done***

… identify the Cryptosporidium spp. present

Comment: ***Done***

… have previously been reported

Comment: ***Done***

Replace Cryptosporidiosis [italics] with Cryptosporidium [roman]
Comment: ***Done***

Introduction

Cryptosporidiosis is an enteric disease caused by apicomplexan parasites of the genus Cryptosporidium…
Comment: ***Done***

Most cases of zoonotic cryptosporidiosis are associated…
Comment: ***Done***

… close contact with THOSE animals
Comment: ***Done***

are the main responsible species
Comment: ***Done***

Remark 1 - display references by year of publication
Comment: ***the format of the references are according to the journal's guidelines, which is an alphabetical order***

While FATHALITY is generally low…
Comment: ***Done***

Significant differences in weight gain HAVE been observed between calves WHICH had severe
Comment: ***Done***

García et al., 2000) AND infected animals are more susceptible to developing severe
Comment: ***Done***

Delete specificity: As this method suffers from low sensitivity (Adeyemo et al., 2018),
Comment: ***Done***

Delete longitudinal: The study herein is an investigation of Cryptosporidium across the same dairy farms one year later
Comment: ***Done***

Materials and methods (instead of Methods)
Comment: ***Done***

… from 51 dairy farms were
Comment: ***Done***

Remark 2 - replace asymptomatic with apparently healthy
Comment: ***Done***

and the WESTERN part of the Netherlands
Comment: ***Done***

650 µL of
S1 Lysis Buffer (650 µL) and S2 Lysis Enhancer (100 µL) WERE added to each sample.
Comment: ***Done***

Leftover DNA and stool samples WERE stored long-term in a -80 oC freezer
Comment: ***Done***

Only neonatal calves aged under 1 month old WERE included for comparison
Comment: ***Done***

Farms with less than three calves within this age range have been excluded from comparison.
Comment: ***Done***

… and across the 2 years
Comment: ***Done***

Write spp. in non-italic style
Comment: ***Done***

The high levels of Cryptosporidium detected in this and the previous study REINFORCE
Comment: ***Done***

Figure legends - write out Cryptosporidium at least once per legend
Comment: ***Done***

References - please standardize, including lowercase for titles (as much as possible)

Comment: ***Done***

Kind regards,

Anastasios Tsaousis (on behalf of all the co-authors)

Supplementary Figure 1