Cryptosporidium prevalence in calves and its effect on local water quality prior to abstraction and treatment

by Edwards, C., Katzer, F., Bartley, P.M., Roden, J. and Shaw, H.J.

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Cryptosporidium prevalence in calves and its effect on local water quality prior to abstraction and treatment

Ceri Edwards[a], Frank Katzer[b], Paul M. Bartley[b], Janet Roden[a], Hannah J. Shaw[a*]

[a] *Harper Adams University, Edgmond, Newport TF10 8NB, UK*

[b] *Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, EH26 0PZ, UK*

*Corresponding author.

E-mail address: hshaw@harper-adams.ac.uk

ABSTRACT

Cryptosporidium spp., particularly *Cryptosporidium parvum*, pose a significant threat to raw water quality and public health. Cryptosporidiosis, a gastrointestinal zoonotic disease, causes diarrhoea in dairy and beef production systems worldwide. Infected calves shed *Cryptosporidium* spp. oocysts in faeces, posing risks of contaminating surface water sources. Understanding the dynamics of *Cryptosporidium* contamination is crucial for effective water quality management. This study investigated *Cryptosporidium* spp. prevalence in neonatal calves and its potential impact on water quality before abstraction and treatment. The study analysed faecal samples from 1–3-week-old calves on two English dairy farms upstream of a water abstraction point. Initial screening used *C. parvum* immune chromatographic assays (ICT) on 47 faecal samples. This was followed by DNA extraction and species identification, with *gp60* subtyping. Raw and treated water quality data were analysed to determine *Cryptosporidium* oocyst counts. *Cryptosporidium parvum* was the predominant species in calves at both farms, with *gp60* subtype IIaA17G2R1 being the only subtype detected. Some calf samples revealed mixed infections with *C. parvum* and *C. ryanae*. Raw-water samples (n = 214) revealed a 50.00% positivity rate for *Cryptosporidium* oocysts, with 22.4% (24/107) containing *C. parvum/C. hominis* and the remainder 77.6% (83/107) *C. andersoni*. Water treatment significantly reduced oocyst counts ($P < 0.001$); however, due to the increase in popularity of bathing in rivers, oocysts present in raw river water may still prove a public health risk.

Keywords: *Cryptosporidium*; Calves; Water quality; Bathing Rivers; Public health

1. Introduction

The increasing demand for clean drinking and bathing water in both rural and urban populations has amplified concerns regarding the quality of surface and groundwater sources, particularly those located near agricultural operations. Species of *Cryptosporidium* are intracellular apicomplexan protozoan parasites with significant zoonotic potential. They pose a serious threat to water quality, particularly in regions where livestock farming and water abstraction sites coexist, as increasing water demand intensifies pressure on the availability of water sources (Thomson et al., 2017). Water treatment and public health authorities are increasingly concerned with how untreated surface water may act as a transmission vector for pathogens, including *Cryptosporidium* spp. oocysts, which are resilient to many conventional disinfection methods typically used on farms (Bushkin et al., 2013; Dixon, 2014). As such, understanding the environmental and biological dynamics of *Cryptosporidium* spp., particularly from livestock sources like calves, is crucial to developing robust strategies for safeguarding public water supplies and bathing rivers (Innes et al., 2020).

Cryptosporidium species, predominantly *C. parvum*, are well-known for their impact on both animal and human health (Chalmers and Katzer, 2013; Wells et al., 2015; Thomson et al., 2016; Xiao and Feng, 2017). The predominant species in cattle globally include *C. parvum*, *C. bovis*, *C. ryanae*, and *C. andersoni* (Chalmers and Katzer, 2013; Thomson et al., 2016). Over 90% of human *Cryptosporidium* infections are caused by *C. parvum* and/or *C. hominis* (Xiao and Feng, 2017; Feng et al., 2018). *Cryptosporidium parvum* is the predominant species among neonatal calves in Europe, detected as early as four days of age and persisting until approximately four weeks of age (Leitch and He, 2011; Shaw et al., 2021). Despite this, more localised studies are required to understand regional variations and the impact of different *Cryptosporidium* subtypes. An infected calf can shed billions of oocysts per day, creating a significant transmission risk to additional hosts (Parlange, 1999). The oocysts are shed in faeces and are immediately infectious, posing a significant risk of environmental contamination due to their rigid bilayer waxy coat of lipids, making them resilient to a wide range of environmental conditions (Uga et al., 2000). This durability enables the parasites to survive a variety of transport mechanisms, ultimately reaching surface watercourses *via* overland flow, particularly during periods of heavy rainfall, or through contaminated soil and grassland (Ong et al., 1996; Shaw, 2018).

With the rising popularity of wild swimming and recreational water activities across the UK, there has been a surge in public campaigns advocating for new official bathing water designations. These designations aim to improve water quality monitoring and protect public

health, especially in frequently used natural swimming spots (Burnett and Sutherland, 2025). Ingesting *Cryptosporidium* oocysts in contaminated water poses a public health risk: *Cryptosporidium* spp. are of key interest because of the parasites' extremely low infective dose (Dillingham et al., 2002). Surface water, especially rivers, streams and reservoirs near agriculturally dense areas are particularly vulnerable to microbial contamination (EPA, 2015). Studies have identified a direct correlation between livestock density in catchments and elevated *Cryptosporidium* spp. oocyst concentrations in adjacent water bodies (Robinson et al., 2011). Raw water intakes in proximity to grazing land found significant levels of *C. parvum*, closely matching the predominant species found in local herds (McDonald et al., 2010; Wells et al., 2015). Between 2017–2022, 416 global waterborne protozoan outbreaks occurred, with *Cryptosporidium* spp. accounting for 77.4% of cases (Bourli et al., 2023). These outbreaks are not only limited to rural or developing regions; developed regions, including the UK, have recorded human *Cryptosporidium* spp. outbreaks linked to surface water contamination after treated water was in supply. A notable example is the 2024 incident in South Devon, where 57 cases of cryptosporidiosis were confirmed and more than 16,000 residents were issued a "boil water" notice after a faulty air valve compromised the water supply (UKHSA, 2024).

Cryptosporidium spp. are difficult to eliminate from the catchment using chemical means because of their resilient structure and protective outer shell (Lorenzo et al., 2018). Therefore, it is essential that water treatment plants are designed with catchment risk in mind. To address this, a multi-barrier approach is often employed, typically including clarification, various filtration methods such as rapid gravity filtration and/or slow sand filters, and if necessary, membrane filtration, UV or ozone in combination to provide an appropriate level of *Cryptosporidium* treatment (DWI, 2024). These treatment methods are expected to achieve a 99.9% (3 log) removal (UKWIR, 2000). End-point testing is a regulatory requirement in England and Wales (DWI, 2024). Surface works treatment is designed to cope with a higher loading of *Cryptosporidium* oocysts than pristine groundwaters; therefore, low level numbers of oocysts in the raw water would not be a cause for concern. The raw water *Cryptosporidium* trigger levels for concern depend on the capability of the processes on site, only causing concern if the challenge indicated is near to the design capability of the water treatment works.

The Drinking Water Inspectorate (DWI) requires water companies to obtain information on the microbiological quality of water, the effectiveness of drinking water treatment, and to ensure that water quality meets specifications for indicator parameters. In the rare event of a *Cryptosporidium* breakthrough, the DWI expects water companies to act swiftly by implementing effective mitigation measures immediately, such as optimising treatment and issuing a "boil water" notice to protect the public health (DWI, 2024). Monitoring includes

sampling raw surface water upstream of treatment plants to identify challenges to the water treatment processes and assessing the catchment for any changes. Treated water is tested continuously in large volumes (> 1000 litres) on rigs at the point where treated water enters the distribution system to ensure the recovery of low levels of oocysts, should they be present. (DWI, 2024).

Whilst existing literature has explored *Cryptosporidium* spp. prevalence in livestock, the impact on water quality at catchment sites remains significantly understudied. This study aims to address this gap by focusing on calves as potential reservoirs for *Cryptosporidium* spp. oocysts and assessing raw water quality near an abstraction point in a specific catchment area. This research provides valuable insights into the contamination risks in local water sources. Further research is needed to ascertain whether other livestock or wildlife species in this catchment serve as a reservoir for the parasites.

The aims of this study were: (i) to determine *Cryptosporidium* spp. presence on farms situated near water catchments; (ii) evaluate the potential zoonotic risk to identify contamination sources; (iii) determine total oocyst counts between raw and treated water to assess treatment efficacy; and (iv) to determine annual oocyst count variability to understand the environmental impact on *Cryptosporidium* prevalence.

2. Materials and methods

2.1. Farm and animal selection

The study was conducted on two dairy farms in England known to have a history of cryptosporidiosis in Holstein-Friesian calves, as well as nearby human outbreaks (Ball, 2024). Located just 20–25 miles (40 km) upstream from a water abstraction point, both farms are in an area where *Cryptosporidium* spp. have previously been identified in livestock and environmental raw water samples. Both farms operated as all-year-round calving herds. The study included all female calves aged 1–3 weeks that were scouring at the time of sampling. These calves were housed in outdoor group pens (up to five per hutch) with straw bedding, fed milk twice daily, and given constant access to clean water. Sample details are provided in Supplementary file 1: Table S1.

2.2. Sample collection

2.2.1. Calf samples

Faecal samples were collected from 47 Holstein-Friesian calves across both farms in September 2023. On 11-Sep-2023, Twenty samples were collected from Farm 1 and three from Farm 2. On 25-Sept-2023, 21 samples were collected from Farm 1 and three from Farm 2. Fresh faecal material was collected directly from the ground of the calves' housing pens. Individual calf identification was ensured through observation during defecation. Samples were stored at 4 °C.

2.2.2. ICT BIO K 387 Dipfit *Cryptosporidium parvum* test strips

During faecal sampling, lateral flow ICT BIO K 387 DipFit (Bio-X Diagnostics, Rochefort, Belgium) *C. parvum* test strips were employed for initial detection on Farms 1 and 2, providing qualitative results. A small portion of the faecal sample was collected, diluted and homogenised to prevent foam formation. The ICT device was promptly inserted into the sample pot, due to its short stability. After a 10-min incubation period, readings were recorded. A positive result was indicated by two horizontal lines, while a negative result displayed only a single line (Supplementary file 2: Figure S1). The kits included an internal control, evidenced by a complete line at the base of the device, eliminating the need for separate controls (Atwal et al., 2022). All samples were retained for DNA extraction (*Section 2.3*).

2.2.3. Water

Ten litres of raw water were sampled at a surface water abstraction site, with investigational samples collected January to October 2023. This coincided with faecal sampling on 11-Sep-2023 and 25-Sep-2023. Samples were analysed by the Severn Trent Water Laboratory, Coventry, UK in accordance with the standard protocols outlined in Section 14 of *The Microbiology of Drinking Water* (Environment Agency, 2010). *Cryptosporidium* oocysts were isolated from other particulate matter in the sample using immunomagnetic separation (IMS). Following separation, the oocysts were stained with fluorescein isothiocyanate (FITC) - labelled anti-*Cryptosporidium* monoclonal antibodies to stain the cell wall (Supplementary file 2: Figure S2) and the DNA stain 4′,6-diamidino-2-phenylindole (DAPI) to visualise internal structures such as sporozoites (Supplementary file 2: Figure S3). The identification of the cell as *Cryptosporidium* sp. was then confirmed using differential interference contrast (DIC) microscopy by identifying characteristic oocyst features, and the cell was measured (Supplementary file 2: Figure S4). The authors were provided with data including date of sampling, oocyst size and total number observed. Oocysts sized 4–6 μm indicated a potentially zoonotic species such as *C. parvum* or *C. hominis* (for the remainder of this paper, these will be referred to as *C.parvum/C. hominis*). Larger oocysts (6–8 μm) were considered likely to be *C.*

andersoni (Pakes and Gerrity, 1994; Masuno et al., 2006). Rainfall data from January to October 2023 was obtained from the Met Office to enable comparisons with oocyst count.

2.3. DNA extraction

Before DNA extraction, 200 µl of watery faecal samples or an inoculation loop of solid faecal sample was added to 1 ml TE buffer (10 mM Tris-HCl, 0.5 mM EDTA) where they were stored at 4 °C until they were ready for further processing. The DNA extraction kit used was Macherey-Nagel, Nucleospin Tissue kit (Düren, Germany). The method followed Wells et al. (2016) with no required changes. Samples underwent centrifugation 4000× *g* for 15 min, supernatant removal and resuspension in 200 µl Buffer T1. After 10 freeze-thaw cycles in liquid nitrogen and a 56 °C water bath, 25 µl Proteinase K was added and the sample incubated overnight at 56 °C. Then, 200 µl Buffer B3 was added, followed by a 70 °C incubation for 10 min. The samples were centrifuged at 11,000× *g* for 5 min, and the supernatant was combined with 210 µl 100% ethanol after centrifugation. This was centrifuged at 11,000× *g* for 1 min. The process included washing with 500 µl Buffer BW and 600 µl Buffer B5, each followed by centrifugation, with the resulting flow-through discarded after each step. Finally, DNA was eluted with 100 µl dH_2O and centrifuged at 11,000× *g* for 1 min before storage at -20 °C.

2.4. 18S PCR

Cryptosporidium in calves was detected using a nested species-specific PCR for the 18S rRNA gene region as previously described (Brook et al., 2009; Thomson et al., 2016; Wells et al., 2016). Samples were run in duplicate, alongside a DNA extraction control and a negative control consisting of distilled PCR water. A sample was deemed positive if at least one replicate tested positive. The primary PCR followed Xiao et al. (2000), with a second-round reaction using species-specific forward primers and a reverse 18S rRNA primer (Thomson et al., 2016) (Table 1) alongside GoTaq Green mastermix (Promega Corporation, Wisconsin, USA). Products from the first PCR round were diluted with 50 µl dH_2O and 1µl of the product was utilised as a template for the second PCR round, with reactions brought to 20 µl using dH_2O.

PCR conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles (94 °C for 45 s, 55 °C for 45 s, 72 °C for 60 s), and a final extension step at 72 °C for 7 min. PCR products were run on a 2% agarose gel, stained with GelRedTM (Biotium, California, US), and visualised under UV light using an inGenus 3 gel documentation system (Syngene, Cambridge, UK). Band sizes were estimated relative to a 25 bp HyperLadder (Bioline, London, UK). Species confirmation (Table 1) involved measuring band distance relative to the ladder.

2.5. *gp60* PCR amplification

Amplification of the *gp60* gene by nPCR was performed on 41 *C. parvum*-positive samples identified using the 18S gene amplification. Positive products were amplified using a modified nested PCR method by Brook et al. (2009), incorporating forward and reverse primers (Table 2), positive and negative controls and triplicate testing. PCR products were electrophoresed on a 1.5% agarose gel, alongside a 100 bp Promega ladder (Promega Corporation, Wisconsin, USA), stained with GelRedTM (Biotium, California, USA), and run for 90 min at 120 V. The gel was then examined under UV light using an AlphaImager 2000 (Alpha Innotech Corporation, California, US) before sequencing.

2.6. PCR purification and sequencing

In accordance with manufacturer instructions, PCR amplicons were purified utilising the Promega Wizard SV gel and PCR clean-up equipment (Promega, Southampton, UK). DNA concentration was determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), purified amplicons were then stored at 4 °C and preserved at -20 °C for extended durations.

Following purification, 20 positive *gp60* PCR amplicons (approximately 100 ng DNA and 2 µl of 10 mM primer, in a final volume of 17 µl) were forwarded for Sanger sequencing (MWG Eurofins, Bavaria, Germany). Overlapping forward and reverse sequences were used to create consensus sequences for every sample by using SeqManPro and Mega Sequence Alignment Software (DNASTAR, Wisconsin, USA). Identification of *gp60* genotypes were determined by physically counting the amount of trinucleotide repeats found in particular conserved sequence regions (Chalmers et al., 2019). A *C. parvum* infection with mixed *gp60* genotypes was considered present if multiple peaks were observed in the *gp60* sequence, with a secondary peak exceeding 0.25 (Robinson et al., 2022).

2.7. Statistical analysis

Statistical analysis was performed using GenStat 23rd edition (VSNi, Cambridge, UK) employing Kruskal-Wallis and Mann-Whitney tests. If $P < 0.05$, the results were deemed significant. Regression analysis explored the impact of monthly rainfall on *Cryptosporidium* counts in raw water.

3. Results

3.1. Oocyst concentration in treated and raw water

Statistical analysis revealed a contrast between oocyst counts in treated and raw water samples. Treated water has a median of 0.00 oocysts/10 l, with both the 25th and 75th percentiles at 0.00, and a mean of 0.02 oocysts/10 l, indicating that *Cryptosporidium* oocysts are very rarely detected in these samples. In contrast, raw water has a median of 7.00 oocysts/10 l, a mean of 13.95 oocysts/10 l, with the 25th percentile at 2.00 and the 75th percentile at 18.00 oocysts/10 l, indicating significant contamination and variability.

3.2. Annual variability in oocyst counts

Oocyst counts varied significantly across different months (Kruskal Wallace one-way analysis of variance, H (5) = 75.57, $P < 0.001$), indicating that environmental factors significantly influence *Cryptosporidium* spp. prevalence in the raw water. Oocyst counts in raw water from January to October 2023 (Table 3, Fig. 1) showed the highest median and greatest variability in January, reflecting significantly elevated *Cryptosporidium* spp. levels in some samples. February, March, April and October exhibited moderate to low median counts with variable levels of dispersion. From May through to September, oocyst counts remained consistently low, with median values near zero, indicating minimal contamination during this period.

Rainfall varied throughout the year (Met Office, 2024) (Fig. 2), peaking in January, March, July and October, with the highest levels in October (147.2 mm) and March (119.2 mm). Regression analysis of monthly *Cryptosporidium* spp. total counts and rainfall showed a weak positive trend, with wide confidence intervals, especially at extreme and low rainfall values. The regression analysis was not statistically significant, $F(1, 9) = 0.06$, $p = 0.813$, indicating that rainfall did not significantly explain variation in *Cryptosporidium* spp. counts. *Cryptosporidium* spp. total counts varied, peaking in January at 1728 oocysts/10 l, correlating with high rainfall in January, followed by a secondary peak in March also mirrored with a high rainfall total. However, despite high rainfall in July and October, the *Cryptosporidium* spp. total counts in the raw water samples were 0 and 58 oocysts/10 l, respectively. *Cryptosporidium* spp.oocyst counts, (reaching 58 oocysts in total) in October, corresponded with the highest recorded monthly rainfall of 147.2 mm.

3.3. On-farm *Cryptosporidium* prevalence

Faecal sampling on the initial day (11-Sep-2023) showed no *Cryptosporidium* spp. oocysts in the raw water. However, faecal samples taken on the second day of sampling (25-Sep-2023) coincided with the detection of *Cryptosporidium* in water samples shortly after (Fig. 3). Raw water samples showed high variability in oocyst counts, peaking at 7 oocysts/10 l per recorded day in October.

3.4. *Cryptosporidium* species in calves

DipFit ICT screening revealed 100% positivity (41/41) for *Cryptosporidium* from Farm 1, while all samples from Farm 2 were negative (0/6). Molecular identification confirmed *C. parvum* as the dominant species in Farm 1, detected in 100% of samples; in 9.8% of these (4/41), mixed species infection with *C. parvum* + *C. ryanae* was detected. *Cryptosporidium parvum* was detected in 50% of samples from Farm 2 (3 out of 6), while the remaining samples tested negative for the four *Cryptosporidium* species commonly found in cattle (Thomson et al., 2017) (Fig. 4). The *gp60* subtyping was conducted on 20 samples with high DNA concentration, which included 17 from Farm 1 and 3 from Farm 2 to ensure research validity (Wells et al., 2016). Among these, 70.6% (12/17) of Farm 1 samples had genotype IIaA17G2R1, and 29.4% (5/17) showed mixed infections with the same genotype (mixed genotype infection), suggesting concurrent infections or sample contamination. All samples from Farm 2 (100%, 3/3) exhibited genotype IIaA17G2R1 (Supplementary file 1: Table S1). No amplification failures occurred, indicating the predominant genotype on both farms is IIaA17G2R1.

3.5. *Cryptosporidium* species in raw water

Microscopic analysis of water samples revealed two species, i.e. *C. parvum/C. hominis* (oocyst diameter of 4–6 μm) and *C. andersoni* (oocyst diameter of 6–8 μm), present at varying concentrations. While both species were detected intermittently, *C. andersoni* was significantly more prevalent, as supported by Kruskal-Wallis analysis ($H = 10.27$, $P = 0.001$). Overall, 50.00% of raw water samples ($n = 214$) were positive for *Cryptosporidium* spp., with *C. parvum/C. hominis* detected in 22.4% ($n = 24$) of the positive cases. The remainder 77.6% (83/107) was positive for *C. andersoni*.

3.6. Sensitivity of DipFit ICT and PCR

Comparative analysis of ICT and PCR testing demonstrated that PCR is a more reliable and sensitive method. While ICT identified 93.2% of samples as positive, PCR detected 100%, confirming that all samples were indeed positive. This highlights the superior ability of PCR to accurately detect infections that ICT testing missed. Both methods showed full agreement in negative control samples, supporting their reliability. However, PCR not only provided higher sensitivity and specificity but also enabled the detection of mixed-species infections with *C. ryanae* and the identification of *C. parvum* subtypes, such as IIaA17G2R1, which ICT does not offer, even though it does offer rapid, on-site test results.

4. Discussion

This study revealed significant annual variation in *Cryptosporidium* oocyst counts in raw water ($P < 0.001$), with the lowest levels recorded from May through to September and a pronounced peak in January. The small increase in *Cryptosporidium* spp. counts (58 oocysts/10 l) in October, coincided with the highest monthly rainfall (147.2 mm), supporting previous research that links rainfall and surface runoff to increased oocyst transport into water bodies (Keeley and Faulkner, 2008; Jagai et al., 2009; Wells et al., 2015; Young et al., 2015).

When testing water samples, multiplex PCR is a useful method for identifying the species and genotype of the *Cryptosporidium* spp. present but, unless real-time PCR is used, it does not afford enumeration. The water company achieves this through microscopy and a total oocyst count. In the water industry, filtration is used to collect and concentrate any oocysts in the water sample before microscopy is used to determine whether defined characteristic cellular features are visible that identify the cell as an oocyst. This method also allows for quantification. The efficiency of both PCR and microscopy in identifying *Cryptosporidium* spp. oocysts in water depends on the quantity and quality of samples examined. Xiao et al. (2006) found limited sensitivity in detecting *Cryptosporidium* spp. in water using both microscopy and PCR. These authors found inconsistent results (positive and negative) between duplicate or quadruplicate samples in 6 out of 14 rain occurrences, suggesting that, to be assured of accuracy, multiple subsamples would have to be analysed. The turbidity of tested water samples may influence the number of oocysts seen and subsequently detected by PCR. A study investigating *Toxoplasma gondii* oocysts found that experiments involving spiked water samples can give negative results (Wells et al., 2015). Although the study of Wells et al. (2015) focused on *T. gondii*, it suggests that poor water quality can similarly reduce the sensitivity of oocyst detection methods within a

research laboratory. In the UK, all water companies operate under the same regulatory analysis framework, Section 14 of *The Microbiology of Drinking Water* (Environment Agency, 2010). This guidance provides several methodologies deemed appropriate for the concentration of oocysts from a sample and uses microscopy for identification and enumeration of oocysts, which the DWI consider the most robust technique for environmental samples. Standardisation in analytical method ensures that results are reliable and comparable across different samples and between companies. Any changes to *The Microbiology of Drinking Water* must be approved by the DWI, ensuring that updates are strictly regulated and controlled.

The findings within this study reinforce the role of environmental factors (particularly rainfall and agricultural practices) in seasonal contamination patterns of *Cryptosporidium* and the associated risk of human infection, as highlighted in earlier studies (Bridgman et al., 1995; ; Wilkes et al., 2011). Bathing waters in England and Wales are monitored by the Environment Agency (EA) (Burnett and Sutherland, 2025). The Bathing Waters Regulations (2013) require monitoring of intestinal enterococci and *E.coli*; however, the document notably omits any mention of *Cryptosporidium*, a concerning oversight given the parasites' ability to travel long distances in surface water, particularly from upstream agricultural sources (The Bathing Waters Regulations., 2013; Wells et al., 2015). This omission highlights the requirement to raise public awareness about the environmental transmission pathways of *Cryptosporidium* spp. and the associated risks to human health. The presence of *C. parvum* in both the water and livestock in the surrounding catchment may suggest a potential link between the cattle contaminating the watercourses, aligning with findings that emphasise the role of farming practices in pathogen transmission and their impact on water quality (Robertson et al., 2013; Golomazou et al., 2024). To mitigate this, water companies may collaborate with local farmers to reduce oocyst burden by implementing buffer strips, providing funding towards water course fencing and educating farmers on animal health and waste management (Widmer and Lee, 2010; Cunningham, 2022).

The predominance of zoonotic genotype II in the calves may lead to human infections through direct contact or contaminated bathing rivers, as this genotype has been reported in humans before (Nichols and McLauchlin, 2003). Lower oocyst levels from February to April compared to the January peak, likely reflect the decline in neonatal shedding post-calving, consistent with earlier reports (Lake et al., 2006; Sterk et al., 2016). While the high variability in January suggests contamination driven by environmental or seasonal factors such as rainfall, land-use changes and temperature fluctuations may also contribute, potentially explaining the slight positive trend observed in the regression analysis. Our study found no consistent correlation between rainfall and oocyst levels, aligning with findings that precipitation may dilute concentrations (Carmena, 2010; Li et al., 2019; Helmy and Hafez, 2022). The absence of

detections during high-rainfall months (July and November) further highlights the complexity of environmental influences on oocyst transport. It is worth noting that raw water grab samples were not continuously monitored for *Cryptosporidium* spp., so the data collected may have missed river flushes. The frequency of sampling is determined by the site risk as identified in the Drinking Water Safety Plan (DWI, 2024). Implementing vegetative buffers and improved catchment management could help reduce contamination risks at water abstraction points, particularly with climate change projected to increase precipitation rates in some areas across the world (Chhetri et al., 2017).

Cryptosporidium parvum was the most prevalent species detected in calf faecal samples, present in 100% (n = 41) of samples from Farm 1 and in 50% (n = 6) of samples from Farm 2, consistent with earlier studies identifying *C. parvum* as the most dominant species found in neonatal calves (Brook et al., 2009; Rieux et al., 2013; Wells et al., 2015). We used a multiplex PCR method capable of detecting mixed infections (Thomson et al., 2019). This enhanced detection method is critical for assessing public health risk from calves, as less pathogenic species such as *C. bovis* or *C. ryanae* can mask low-level infections with the zoonotic *C. parvum* (Thomson et al., 2016). Reports have shown up to 75% of farm workers contract the parasite from infected livestock, highlighting the urgent need for effective biosecurity protocols (Klous et al., 2016; Golomazou et al., 2024). This is further supported by Khalil et al. (2018), who estimated a global burden of 4.2 million disability-adjusted life years (DALYs) lost due to the disease. Mixed infections with *C. parvum* and *C. ryanae* were also detected, demonstrating that calves aged 1–3 weeks can simultaneously harbour multiple *Cryptosporidium* species, highlighting the occurrence of co-infections at an early age (Rieux et al., 2013), though the early presence of *C. ryanae* contrasts with studies that associate it with post-weaned calves (Xiao, 2010; Santin, 2020). This variation may reflect differences in sampling design, as spot sampling (as used here) may capture different shedding dynamics than longitudinal studies, which better illustrate disease patterns (Shaw et al., 2021).

Cryptosporidium parvum IIa is a globally prevalent zoonotic subtype, most commonly found across all continents except Africa and Asia (Chen et al., 2023; Buchanan et al., 2024). The predominant *gp60* subtype identified in this study was *C. parvum* IIaA17G2R1, detected on both farms. Notably, 29.4% (5/17) of the samples showed mixed infections with different *gp60* genotypes, suggesting concurrent *C. parvum* infections. Although IIaA15G2R1 is typically the most common subtype in UK cattle (Wells et al., 2015), the presence of IIaA17G2R1 aligns with reports from other countries and UK cases, where it accounted for 4.3% (n = 129/2911) of the eight most commonly reported genotypes in calves (Thompson et al., 2007; Toledo et al., 2017; Buchanan et al., 2024). This suggests that while IIaA15G2R1 remains dominant in the UK,

IIaA17G2R1 is also a notable and widespread subtype, reflecting its broader geographical distribution and potential for zoonotic transmission. Previous studies suggest that closed herds often harbour a single dominant genotype (Brook et al., 2009; Silverlas and Blanco-Penedo, 2013), with calf-to-calf transmission likely, as observed in this study (Bartley et al., 2024). This is exacerbated by the low infectious dose of *C. parvum*, allowing rapid spread (Costa et al., 2021). Although IIaA17G2R1 is less frequently reported in the UK, its dominance here may reflect localised transmission dynamics or environmental persistence. All Farm 2 samples tested negative on initial screening using DipFit ICT testing but tested positive using PCR techniques, highlighting the sensitivity of PCR over pen-side DipFit tests (Thomson et al., 2019).

The detection of identical *gp60* genotypes on both farms shows a likely consistent genotype within the catchment. Further resolution would require multilocus-based genotyping to trace transmission pathways more precisely (Risby et al., 2023; Bartley et al., 2024). To better understand the impact of *C. parvum* on water quality, multilocus-based genotyping could distinguish between genotypes on farms (Morris et al., 2019; Bartley et al., 2024). Typing more samples from various hosts, including livestock and wildlife, is essential to confirm if the *Cryptosporidium* spp. found in calves are linked to the watercourse or another source (Chalmers, 2012; Wells et al., 2015). Periodic farm re-testing could identify emerging genotypes, providing ongoing insights into *Cryptosporidium* infection dynamics (Thomson et al., 2016; Bartley et al., 2024). Given limited studies on *Cryptosporidium* spp. in calves and its impact on raw water quality, other UK regions should be explored.

Environmental contamination, including unchanged bedding on the farm, likely contributed to infection persistence, consistent with findings on the resilience of *Cryptosporidium* and reinfection potential due to its ubiquitous nature (Goater et al., 2014; Wells et al., 2015). In this study, early infection in calves (as young as 7 days) supports rapid post-natal transmission, in line with the known prepatent period (Tzipori et al., 1983). Since only environmental samples were collected, it is unclear how many neonatal calves were actively shedding oocysts (Shaw, 2018). The absence of other species, such as *C. bovis*, typically found in pre-weaned calves, may be attributed to the fact that the animals were housed exclusively with others of a similar age, limiting exposure to a broader range of *Cryptosporidium* species (Silverlas et al., 2010; Wang et al., 2011). The techniques employed in this study may have lacked the sensitivity to detect low-level subtypes, potentially underestimating species diversity. This is supported by Thomson et al. (2016), who found that while calves can be co-infected with multiple subtypes, preferential amplification within the gut may result in the dominance of a single detectable genotype.

This study found significantly higher oocyst counts in raw water compared to treated water ($P < 0.001$), with median values of 7 and 0 oocysts/10 l, respectively. These findings are consistent with previous studies demonstrating the effectiveness of treatment processes in reducing *Cryptosporidium* spp. loads (Wallis et al., 1996; Skerrett and Holland, 2000), underscoring the importance of robust water treatment in protecting public health (Chamlers, 2012; Moussa et al., 2023). These results clearly show the effectiveness of treatment and ensure the safety of the final water. Oocyst detection in final water was negligible in this study. Given the potential of a very low infectious dose, especially in vulnerable populations (Pouillot et al., 2004), maintaining treatment efficacy is critical, particularly in areas with a history of contamination (Johnson et al., 2012). Effective operation and regular maintenance of treatment systems are critical to ensuring consistent water safety. Even with a well-designed infrastructure, lapses in maintenance can compromise performance, especially during extreme weather events. Despite advancements in filtration technology, studies have shown that even high-performing systems may fail to remove oocysts during periods of heavy contamination (Goh et al., 2005; Pollock et al., 2008). Continual monitoring to assess catchment risk and employ appropriate treatment is vital as dictated by the DWI regulations (DWI, 2024). Drinking water-associated outbreaks in the UK are now exceptionally rare, and the detection of *Cryptosporidium* spp. oocysts in treated water does not necessarily indicate a failure in water treatment processes. For instance, a recent outbreak in Devon was attributed to a malfunctioning air valve in the distribution network rather than treatment inefficiency (Harris, 2024). Supporting this, Chalmers et al. (2019) reported that among 178 outbreaks investigated in the UK between 2009 and 2017, only two (approximately 1%) were linked to public drinking water supplies. In contrast, the majority were associated with animal contact (42%) or exposure to recreational water sources (46%) such as bathing rivers or swimming pools. This further highlights the public health risk posed by *Cryptosporidium*, which can cause serious gastrointestinal illness. Despite its risk and prevalence in recreational waterborne outbreaks, it is not included in routine bathing water monitoring, which focuses only on bacterial indicators (The Bathing Water Regulations, 2013). With the rise in wild swimming, there is a growing need to reconsider the adequacy of existing monitoring frameworks in bathing water to ensure they reflect the full spectrum of microbial risks. The results of this study, based within England, indicate the success of the treatment used for drinking water. However, consuming river water may pose a risk. The difficulty in dealing with a poor oocyst recovery of < 50% (Thompson and Chalmers, 2002), poor quality and highly turbid water samples also emphasise the necessity for further research into detection methods (Wells et al., 2015).

Environmental contamination was evident in the study, with 50% of raw water samples testing positive for *C. andersoni* or *C. parvum*/*C. hominis.* A significant difference in oocyst size was observed ($P < 0.001$), with *C. andersoni*, the more prevalent species in this study, typically associated with adult cattle and considered non-zoonotic (Wang et al., 2011). This contrasts with Wells et al. (2015), who reported alignment between livestock and waterborne species. Tang et al. (2011) found that the timing of spreading manure significantly impacts environmental contamination, possibly explaining the presence of *C. andersoni* in water contaminated by slurry from adult cattle during high rainfall. The detection of *C. parvum/C. hominis*, a zoonotic pathogen found in 22.4% of positive raw water samples, indicates potential human health risks and links to livestock sources, consistent with Robinson et al. (2011) and McDonald et al. (2010).

Although calves in this study were not grazing, their presence on non-hard surfaces likely increased runoff and oocyst transfer during rainfall, reinforcing previous findings on environmental transmission between *Cryptosporidium* species in surface waters and those expelled by cattle and wildlife (Robinson et al., 2011; Wells et al., 2015). Wildlife activity in the catchment may also contribute to contamination, though this was not explored in our study (Mizuki et al., 2020). Although PCR and genotype-level confirmation of *C. parvum* in raw water samples was not feasible, due to low oocyst concentrations and resource constraints, its detection in livestock suggests the potential for zoonotic transmission into local water sources. Currently, water company protocols rely primarily on oocyst size for species identification, a method with limited sensitivity that risks misclassification (Xiao and Fayer, 2008). To mitigate this, water companies will act on *Cryptosporidium* oocyst detection regardless of size/classification (DWI, 2024). Upon finding the presence of *Cryptosporidium* oocysts, in some circumstances, water companies will send the slides for genotyping to support further investigation. However, when there is a known or suspected risk of *Cryptosporidium* spp. in the catchment, companies are more likely to respond by enhancing and increasing vigilence of the monitoring of water treatment processes rather than conducting additional sampling. Incorporating detailed genotyping techniques such as multilocus-based genotyping is highly beneficial for epidemiological purposes (Robinson et al., 2022). However, there are difficulties in using multilocus-based methods when there are limited number of oocysts in the samples.

PCR detected a higher proportion of positive *Cryptosporidium* cases in calves (100%) compared to ICT (93.2%), highlighting its superior sensitivity. This finding aligns with previous research identifying PCR as the gold standard for low-level pathogen detection (Morgan et al., 1998; Ghallab et al., 2016). Although the present study did not replicate the full sensitivity and specificity reported in earlier work, minor discrepancies may reflect PCR inhibition, low oocyst

concentrations, or human error (Weber et al., 1991). However, due to the relatively small difference between ICT and PCR it is likely that a high level of oocysts were being shed by the calves. Further work should include quantitative analysis using real-time PCR.

Importantly, PCR detected both *C. parvum* and *C. ryanae*, whereas the DipFit ICT test identified only *C. parvum*, highlighting the broader diagnostic capacity of PCR, a key advantage for accurate epidemiological surveillance and outbreak source tracing (O'Leary et al., 2021). Although the DipFit ICT packaging specifies detection of *C. parvum*, cross-reactivity studies are necessary to assess whether it may also detect other *Cryptosporidium* species. Negative controls confirmed the reliability of both methods in accurately detecting the presence or absence of *Cryptosporidium* spp.

PCR is a highly sensitive method but requires specialised expertise, is time-consuming, and depends on costly, specialised equipment. It is therefore more commonly used in research laboratories. In contrast, ICT provide rapid, affordable, and field-friendly qualitative diagnostics suitable for use on farm. However, ICT has lower specificity (~75%) compared to PCR and is more susceptible to false-positive and false-negative results (Danisova et al., 2018; Manouana et al., 2020). Nonetheless, its simplicity and ease of use make it a valuable tool for preliminary screening in field-based settings (Cho and Yoon, 2014) and combining PCR and ICT enhances diagnostic accuracy. ICT is particularly helpful for guiding on-farm decisions by veterinarians and farmers, especially when treatment with paromomycin requires confirmed *Cryptosporidium* infections and may guide the farmer towards other treatment or preventative options, such as Halofuginone and the Bovilis Cryptium vaccine (Reijnders et al., 2025).

5. Conclusions

The findings of the present study prove relevant to various stakeholders, including farmers, water companies, advisors, scientists and public health officials. The study reveals a significant prevalence of *Cryptosporidium* spp., predominantly *C. parvum*, on the two farms examined. Historical evidence of *Cryptosporidium* spp. in the raw water at the point it is abstracted indicates recurrent transmission through the catchment area. The high prevalence of *C. parvum/C. hominis* in the raw water could pose a threat to public health due to zoonotic waterborne transmission *via* bathing rivers. In the samples analysed in this study, treatment was seen to be highly effective in reducing the presence of *Cryptosporidium* spp. within treated water. The same genotype of *C. parvum* was found on both farms illustrating a stable genotype in the catchment; however, multilocus genotyping would be required to confirm this. This

underscores the requirement for effective on-farm management, effective risk assessment of the catchment and continuous water monitoring to protect public health and ensure water safety.

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Ethical approval

The research protocol, including ethics and consent procedures, received approval from Harper Adams University Ethics Committee (Reference number: 0455-202307). Farmers consented to non-invasive faecal sampling of their animals and subsequent analysis, with no treatments administered, mitigating harm to livestock. Personal protective equipment (PPE) was used to minimise zoonotic transmission risk. Secondary water quality data were obtained from the regional water utility provider. Results of *Cryptosporidium* identification were shared with participating farmers to support improvements in livestock management and contribute to mitigating *Cryptosporidium*-related water quality risks. No amendments to the original research plan were necessary.

CRediT authorship contribution statement

Ceri Edwards: Conceptualisation, Methodology, Data collection, Investigation, Formal analysis, Visualisation. **Hannah J Shaw:** Conceptualisation, Methodology, Formal analysis, Investigation, Writing – review & editing, Visualisation, Supervision, Funding acquisition. **Frank Katzer:** Methodology, Writing – review & editing, Funding acquisition. **Paul M. Bartley:** Formal analysis, Writing – review & editing. **Janet Roden:** Formal analysis, Statistics, Writing & review and editing.

Declaration of competing interests

The authors declare that they have no known competing interests that could have influenced the work reported in this paper. Given their role as Co-Editor, Frank Katzer had no

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Figure legends

Fig. 1. Annual variability of *Cryptosporidium* oocyst counts in raw water quality results during 2023. The top of the grey box indicates the median value, the black dot indicates the 25th percentile, and the blue dot indicates the 75th percentile.

Fig. 2. Monthly rainfall and total *Cryptosporidium* spp. counts per 10 l of raw water. The blue bar represents the total rainfall (in mm), while the orange bar indicates the total number of *Cryptosporidium* spp. oocysts per 10 l of water.

Fig. 3. Detection of *Cryptosporidium* spp. (total oocyst count) in raw water samples corresponding to the timing of faecal sampling (grey dots indicate the water samples, and the red triangles indicate the timing of faecal sampling).

Fig. 4. *Cryptosporidium* species detected in faecal samples from calves of the two dairy farms selected.