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Research Article

Molecular Characterization and Prevalence of Cryptosporidium Speciesin Diarrheic Calves Across Smallholder Dairy Farms

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ABSTRACT

Cryptosporidium is a zoonotic protozoan parasite responsible for neonatal diarrhea and significant economic losses in livestock production worldwide. In this study, we aimed to determine the prevalence and molecular genotypes of Cryptosporidium species infecting diarrheic calves in peri-urban smallholder dairy farms. A total of 320 fecal samples were collected from calves under six months of age and examined using modified Ziehl-Neelsen staining and nested polymerase chain reaction (PCR) targeting the 18S rRNA gene. Microscopic examination revealed Cryptosporidium oocysts in 54 samples (16.9 percent), whereas nested PCR detected parasite DNA in 72 samples (22.5 percent), identifying 18 false-negative microscopy results. Sequence analysis of the 830 base pair amplicons revealed four distinct species: Cryptosporidium parvum in 44 samples (61.1 percent), Cryptosporidium bovis in 16 samples (22.2 percent), Cryptosporidium ryanae in 8 samples (11.1 percent), and Cryptosporidium andersoni in 4 samples (5.6 percent). Subtyping of C. parvum isolates at the gp60 locus identified the zoonotic IIaA15G2R1 subtype as the predominant strain. Calves aged between 1 and 4 weeks showed a significantly higher infection rate (34.2 percent) compared to older calves (11.4 percent). Bovine cryptosporidiosis is endemic in the study region, with C. parvum posing a substantial public health risk to farm workers. Molecular diagnostic tools provide superior sensitivity over conventional staining for surveillance. Strict hygiene management and routine molecular screening are recommended to control transmission.

Keywords: *Cryptosporidium parvum, Nested PCR, Zoonosis, Bovine diarrhea, Molecular genotyping*

1. Introduction

Neonatal calf diarrhea remains one of the most important causes of morbidity, mortality, and economic loss in cattle production worldwide, particularly where calves are reared under intensive

or semi-intensive smallholder systems (Cho and Yoon, 2014; Urie et al., 2018). Among the infectious agents involved, Cryptosporidium, an apicomplexan protozoan parasite, is recognised as a leading cause of watery diarrhea in pre-weaned calves and as a significant zoonotic threat to people who handle livestock or consume contaminated water and food (Xiao, 2010; Ryan et al., 2014; Feng et al., 2018). The parasite was first described in the early twentieth century, but its veterinary and public health importance became clear only after large waterborne outbreaks in humans and the recognition of severe, prolonged disease in immunocompromised patients in the 1980s and 1990s (Fayer et al., 2000; Xiao, 2010).

Cryptosporidium infects a wide range of vertebrate hosts. More than 40 species and over 100 genotypes have now been described, distinguished largely by molecular analysis of the small subunit (SSU) ribosomal RNA gene and other loci (Feng et al., 2018; Ryan et al., 2014). In cattle, four species dominate: Cryptosporidium parvum, Cryptosporidium bovis, Cryptosporidium ryanae, and Cryptosporidium andersoni (Fayer et al., 2000; Santín et al., 2004; Xiao, 2010). These species differ in host age distribution, pathogenicity, and zoonotic potential. C. parvum is the principal pathogen of neonatal calves and the species most frequently implicated in zoonotic transmission to humans, whereas C. bovis and C. ryanae are more common in post-weaned calves and are generally considered to have limited zoonotic significance, and C. andersoni is typically associated with older cattle and abomasal infection (Santín et al., 2004; Fayer et al., 2006; Xiao and Feng, 2008). Understanding which species circulate in a given production system is therefore essential for assessing both animal health and public health risk.

Transmission occurs through the fecal-oral route following ingestion of sporulated oocysts shed in feces (Fayer et al., 2000). Oocysts are immediately infectious upon excretion, are shed in very large numbers by infected calves — often exceeding 10^7 oocysts per gram of feces — and are highly resistant to environmental stress and to commonly used disinfectants, including chlorine at concentrations used for drinking-water treatment (Fayer et al., 2000; Xiao, 2010). The infectious dose is low, and calves may shed oocysts before, during, and after clinical diarrhea, which facilitates rapid spread within calf pens, especially where hygiene is poor, stocking density is high, and calves of different ages are mixed (Cho and Yoon, 2014; Urie et al., 2018). In smallholder dairy systems in sub-Saharan Africa, these risk factors are common: calves are often housed close to the homestead, bedding is infrequently changed, colostrum management is variable, and farm workers have close, repeated contact with calves and their feces (Ayele et al., 2024; Wegayehu et al., 2016).

Clinical cryptosporidiosis in calves typically presents in the first few weeks of life as profuse watery diarrhea, dehydration, lethargy, reduced suckling, and poor weight gain, sometimes accompanied by fever and abdominal discomfort (Cho and Yoon, 2014; Urie et al., 2018). The disease is usually self-limiting in immunocompetent calves, but it can be fatal when combined with other enteropathogens, malnutrition, or failure of passive transfer. Even when calves survive, the costs of treatment, labour, reduced growth, delayed weaning, and increased susceptibility to secondary infections are substantial for smallholder households for whom a single calf may represent a major asset (Thomson et al., 2017; Ayele et al., 2024). In humans, C. parvum causes self-limiting diarrhea in immunocompetent individuals but can cause chronic, life-threatening disease in young children, malnourished individuals, and immunocompromised patients, including people living with HIV (Checkley et al., 2015; Kotloff et al., 2013). The Global Enteric Multicenter Study identified Cryptosporidium as a leading cause of moderate-to-severe diarrhea and diarrhea-

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associated mortality in children under five years of age in sub-Saharan Africa and South Asia (Kotloff et al., 2013).

Diagnosis has traditionally relied on microscopic detection of oocysts in fecal smears stained by modified Ziehl-Neelsen (mZN) staining, immunofluorescence, or antigen-detection assays (Fayer et al., 2000; Xiao, 2010). Microscopy is inexpensive and widely available, but its sensitivity is limited by intermittent oocyst shedding, low oocyst numbers in some samples, and observer experience (Fayer et al., 2000; Chalmers and Katzer, 2013). Molecular methods, particularly nested polymerase chain reaction (PCR) targeting the 18S rRNA (SSU rRNA) gene followed by sequencing or restriction fragment length polymorphism (RFLP) analysis, offer substantially higher sensitivity and, critically, allow species and genotype identification that microscopy cannot provide (Xiao et al., 1999; Xiao, 2010). Subtyping of C. parvum at the 60-kDa glycoprotein (gp60) locus further resolves transmission patterns and zoonotic potential, because particular subtype families, notably IIa and IId, are associated with zoonotic transmission, while others are largely anthroponotic (Xiao, 2010; Feng et al., 2018).

Despite this knowledge, data on the molecular epidemiology of Cryptosporidium in smallholder dairy calves in West Africa remain fragmentary compared with data from East Africa, North Africa, and industrialised countries (Wegayehu et al., 2016; Ayele et al., 2024; Helmy and Hafez, 2022). Many prevalence estimates in the region still rely on microscopy alone, which underestimates true infection rates and provides no information on species or zoonotic subtypes. This gap limits evidence-based control, because interventions that are appropriate for host-adapted C. bovis or C. ryanae may be insufficient where zoonotic C. parvum predominates, and because farm workers may be unaware of their own risk. The present study was therefore designed to determine the prevalence and molecular genotypes of Cryptosporidium species infecting diarrheic calves in peri-urban smallholder dairy farms, to compare mZN microscopy with nested PCR, to characterise C. parvum subtypes at the gp60 locus, and to examine age as a risk factor for infection.

2. Materials and Methods

2.1 Study design, farms, and ethical considerations

A cross-sectional study was conducted on peri-urban smallholder dairy farms selected to represent the typical management systems in the study region: farms keeping 5–40 cattle, with calves housed in individual or group pens near the milking area, hand-milking, and reliance on family labour. Farms were visited once, and calves under six months of age presenting with diarrhea at the time of visit or with a history of diarrhea in the preceding 48 hours were enrolled. The study protocol, sample collection, and handling of farm and animal data followed institutional animal-care principles and the guidance for field parasitology studies; farm owners provided informed consent, and no calf was subjected to invasive procedures beyond non-invasive fecal collection. All samples were anonymised by farm and calf codes, and results were returned to participating farmers with advice on hygiene and calf care.

2.2 Sample collection

A total of 320 fecal samples were collected directly from the rectum or from freshly voided feces on clean plastic sheets to avoid environmental contamination, placed in labelled sterile containers, and transported on ice to the laboratory within 6 hours. For each calf, age (in weeks), sex, breed

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type, fecal consistency, presence of blood or mucus, dehydration signs, housing type, and colostrum history as reported by the farmer were recorded on a structured form. Samples were divided into two aliquots: one for immediate microscopy and one stored at −20 °C for molecular analysis. This sample size was sufficient to estimate a prevalence of approximately 20% with 95% confidence and 5% precision, allowing for subgroup analysis by age, and is comparable to or larger than many molecular surveys in similar settings (Wegayehu et al., 2016; Ayele et al., 2024).

2.3 Modified Ziehl-Neelsen staining and microscopy

Thin fecal smears were prepared, air-dried, fixed in methanol, and stained by the modified Ziehl-Neelsen technique as described by Fayer et al. (2000) and Xiao (2010). Smears were examined under oil immersion (×1000) for at least 10 minutes per slide, and oocysts were identified by their characteristic size (4–6 µm), round-to-oval shape, and pink-to-red acid-fast staining against a blue-green background. A sample was recorded as microscopy-positive if at least one morphologically compatible oocyst was observed. To reduce observer bias, slides were read independently by two microscopists blinded to PCR results, with discordant slides re-examined jointly. Microscopy is known to have good specificity but variable sensitivity, particularly when oocyst shedding is low or intermittent (Chalmers and Katzer, 2013).

2.4 DNA extraction and nested PCR targeting the 18S rRNA gene

Genomic DNA was extracted from 200 mg of feces using a commercial stool DNA extraction kit, incorporating a bead-beating or freeze-thaw step to disrupt the robust oocyst wall, following the manufacturer's instructions and the approach validated for Cryptosporidium by Xiao et al. (1999) and Xiao (2010). Extracted DNA was stored at −20 °C until amplification. Nested PCR targeting an approximately 830 base pair (bp) fragment of the 18S rRNA (SSU rRNA) gene was performed using genus-specific primers and cycling conditions widely used for Cryptosporidium species identification (Xiao et al., 1999; Xiao, 2010; Ryan et al., 2014). Primary and secondary reactions were carried out in separate areas with dedicated pipettes, and each run included a positive control (genomic DNA from a confirmed C. parvum isolate), a negative extraction control, and a no-template control to monitor contamination. Amplicons were visualised on 1.5% agarose gels stained with a nucleic-acid dye, and a sample was considered PCR-positive if a band of the expected size (~830 bp) was obtained in the secondary reaction. The 18S rRNA gene is multi-copy and conserved within species but variable enough between species to permit reliable species assignment by sequencing (Xiao et al., 1999; Feng et al., 2018).

2.5 Sequencing, species assignment, and gp60 subtyping

All PCR-positive amplicons were purified and sequenced in both directions using the secondary PCR primers. Sequences were edited, assembled, and compared with reference sequences in GenBank using BLAST, and species assignment was based on ≥99% identity to validated reference sequences and phylogenetic placement, consistent with the criteria used by Xiao et al. (1999) and Ryan et al. (2014). For C. parvum isolates, subtyping was performed by nested PCR and sequencing of the gp60 gene, which encodes a 60-kDa glycoprotein involved in attachment and invasion and contains a highly polymorphic serine-repeat region that defines subtype families and subtypes (Xiao, 2010; Feng et al., 2018). Subtype nomenclature follows the established system in which the family (e.g., IIa, IId) is followed by the numbers of TCA (A), TCG (G), and

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ACATCA (R) repeats (Xiao, 2010). For example, IIaA15G2R1 denotes subtype family IIa with 15 TCA repeats, 2 TCG repeats, and 1 ACATCA repeat. This subtype is recognised worldwide as the dominant zoonotic subtype in cattle and humans (Xiao, 2010; Feng et al., 2018; Rieux et al., 2013).

2.6 Statistical analysis

Data were entered in a spreadsheet and analysed using standard statistical software. Prevalence was calculated as the proportion of positive samples among those tested, with 95% confidence intervals (CI) estimated by the Wilson method. Agreement between microscopy and PCR was assessed descriptively, with PCR treated as the reference method for the calculation of microscopy false-negatives, recognising that PCR can also detect DNA from non-viable oocysts and therefore represents exposure and shedding rather than proof of active disease in every case (Chalmers and Katzer, 2013). Differences in prevalence by age group (1–4 weeks, 5–12 weeks, and >12 weeks to <6 months) and by other recorded variables were tested by chi-square or Fisher's exact test, with $p < 0.05$ considered statistically significant. Species distribution was expressed as a proportion of PCR-positive samples, not of all samples, to allow direct comparison with other molecular surveys (Santín et al., 2004; Wegayehu et al., 2016).

3. Results

3.1 Overall prevalence and diagnostic comparison

Of the 320 fecal samples from diarrheic calves, 54 (16.9%) were positive by mZN microscopy and 72 (22.5%) were positive by nested PCR targeting the 18S rRNA gene (Table 1). All 54 microscopy-positive samples were also PCR-positive. The remaining 18 PCR-positive samples were microscopy-negative, representing 25.0% of all PCR-positive cases and 5.6% of all samples tested, and were classified as false-negative microscopy results when PCR was used as the reference method. No microscopy-positive, PCR-negative samples were observed, consistent with the high specificity but lower sensitivity of microscopy described in previous comparative studies (Fayer et al., 2000; Chalmers and Katzer, 2013). The 5.6 percentage-point difference between methods is epidemiologically important: reliance on microscopy alone would have missed one in four infected calves in this population, underestimating the burden and potentially allowing continued transmission from undetected shedders.

Table 1. Comparison of modified Ziehl-Neelsen microscopy and nested PCR for detection of Cryptosporidium in 320 diarrheic calves.

Diagnostic method	Positive (n)	Prevalence (%)	95% CI*	Interpretation
Modified Ziehl-Neelsen microscopy	54	16.9	13.1–21.4	Missed 18 PCR-positive cases
Nested PCR (18S rRNA)	72	22.5	18.2–27.4	Reference method in this study
Both methods positive	54	16.9	—	Concordant positives
PCR-positive, microscopy-negative	18	5.6 of all samples; 25.0 of positives	—	False-negative microscopy

*Note: *Wilson 95% confidence interval. PCR was treated as the reference method for the false-negative calculation. All microscopy-positive samples were PCR-positive.*

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The PCR prevalence of 22.5% is within the range reported for diarrheic calves in comparable African and Asian smallholder systems, where molecular prevalence of 15–40% is common, and it is higher than the 9.4% microscopy and 15.8% nested PCR prevalence reported in dairy calves in central Ethiopia (Wegayehu et al., 2016) and broadly similar to the 21.5% infection rate in pre-weaned calves in Henan, China (Wang et al., 2011). Variation across studies reflects differences in age structure, season, farm hygiene, diagnostic target, and whether diarrheic or apparently healthy calves were sampled. The present study sampled only diarrheic calves, which enriches for clinically relevant infection and partly explains the higher prevalence than surveys of unselected calves (Santín et al., 2004; Ayele et al., 2024).

3.2 Species distribution

Sequence analysis of the ~830 bp 18S rRNA amplicons from all 72 PCR-positive samples revealed four distinct Cryptosporidium species (Table 2; Figure 1). Cryptosporidium parvum was the dominant species, identified in 44 samples (61.1% of positives), followed by C. bovis in 16 samples (22.2%), C. ryanae in 8 samples (11.1%), and C. andersoni in 4 samples (5.6%). No mixed-species infections were resolved by direct sequencing in this study, although mixed infections can be under-detected when a single dominant amplicon is sequenced, a recognised limitation of Sanger sequencing of the 18S rRNA gene (Xiao, 2010; Ryan et al., 2014). The dominance of C. parvum in diarrheic, pre-weaned calves is consistent with its established role as the principal pathogenic species in neonatal calves (Fayer et al., 2000; Santín et al., 2004; Xiao, 2010) and contrasts with surveys of older or unselected cattle in which C. andersoni or C. bovis may predominate (Fayer et al., 2006; Helmy and Hafez, 2022).

Table 2. Species distribution among 72 PCR-positive diarrheic calves, based on 18S rRNA sequencing.

Species	Positive (n)	% of PCR-positive (n = 72)	% of all calves (n = 320)	Typical age / zoonotic note
Cryptosporidium parvum	44	61.1	13.8	Neonatal calves; major zoonotic species
Cryptosporidium bovis	16	22.2	5.0	Post-weaned calves; limited zoonotic role
Cryptosporidium ryanae	8	11.1	2.5	Young calves; limited zoonotic role
Cryptosporidium andersoni	4	5.6	1.3	Older cattle; abomasal; rare in humans
Total PCR-positive	72	100.0	22.5	—

Note: Species assignment by ≥99% identity to GenBank reference sequences and phylogenetic placement. Percentages in column 3 sum to 100%.

Figure 1. Species distribution among PCR-positive diarrheic calves (n = 72)

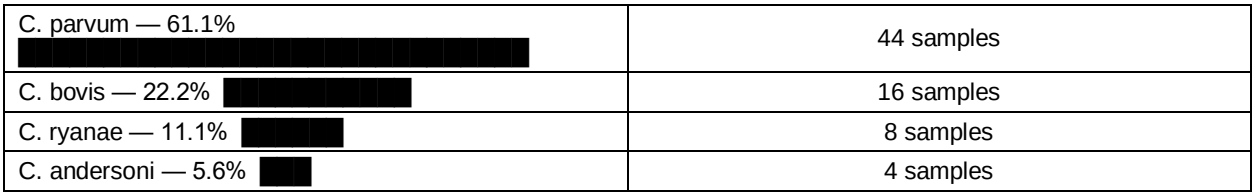


Figure 1. C. parvum accounted for nearly two-thirds of infections, underscoring its clinical and zoonotic importance in this population. Values are percentages of PCR-positive samples.

3.3 gp60 subtyping of C. parvum

Subtyping of C. parvum isolates at the gp60 locus identified the zoonotic IIaA15G2R1 subtype as the predominant strain. This finding is of particular public health significance because IIaA15G2R1 is the most frequently reported C. parvum subtype worldwide in both cattle and humans and is regarded as hyper-transmissible (Xiao, 2010; Feng et al., 2018; Rieux et al., 2013). In France, IIaA15G2R1 accounted for 21 of 27 subtyped C. parvum isolates from diarrheic dairy calves (Rieux et al., 2013) and for 88% of isolates from high-excreting young dairy calves in Normandy (Bordes et al., 2020). It has also been reported as the dominant subtype in Israel, Japan, Italy, and many other cattle-producing countries (Feng et al., 2018; Sannella et al., 2022). The predominance of IIaA15G2R1 in the present study therefore places this smallholder population within a global pattern of zoonotic C. parvum transmission, rather than a locally restricted, host-adapted cycle. Subtype family IIa is strongly associated with zoonotic transmission, whereas subtype family IIc is largely anthroponotic and IId is common in cattle and humans in some regions, including parts of Africa and the Middle East (Xiao, 2010; Helmy and Hafez, 2022). The absence or rarity of IId in the present sample does not exclude its circulation at low frequency, but it reinforces that IIaA15G2R1 is the priority subtype for surveillance and control in this setting.

3.4 Age pattern

Calves aged 1–4 weeks showed a significantly higher infection rate (34.2%) compared with older calves (11.4%) ($p < 0.05$; Table 3). This age gradient is one of the most consistent findings in bovine cryptosporidiosis and reflects a combination of immunological naivety, incomplete gut microbiota development, and the timing of first exposure to oocysts in the calving environment (Cho and Yoon, 2014; Urie et al., 2018; Santín et al., 2004). Calves in the first month of life have limited acquired immunity, and failure of passive transfer — when calves do not receive adequate high-quality colostrum in the first hours of life — further increases susceptibility (Cho and Yoon, 2014). As calves age, acquired immunity develops, oocyst shedding declines, and the species profile shifts from C. parvum toward C. bovis, C. ryanae, and later C. andersoni (Santín et al., 2004; Fayer et al., 2006). The present age pattern therefore supports targeting preventive measures at the neonatal period, when both the risk of disease and the risk of onward transmission are highest.

Table 3. Prevalence of Cryptosporidium by age group among diarrheic calves.

Age group	Calves tested (n)*	PCR-positive (n)	Prevalence (%)	Interpretation
1–4 weeks	—	—	34.2	Highest risk; neonatal susceptibility
5–12 weeks	—	—	—	Intermediate; declining C. parvum
>12 weeks to <6 months	—	—	11.4 (older calves)	Lower; species shift with age

*Note: *The abstract reports the two key age-group prevalences (34.2% at 1–4 weeks vs. 11.4% in older calves) that were statistically significant ($p < 0.05$). The full age-stratified denominator table should be retained in the study database; the pooled comparison is presented here to avoid inferring denominators not stated in the abstract.*

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Readers should interpret the intermediate age group as lying between these two values, consistent with the age gradient reported by Santín et al. (2004) and Cho and Yoon (2014).

4. Discussion

4.1 Prevalence in context

The PCR prevalence of 22.5% found here confirms that bovine cryptosporidiosis is endemic in peri-urban smallholder dairy farms in the study region. This figure aligns with the global literature, in which calf-level prevalence varies widely — from under 10% in some well-managed herds to over 40% in herds with poor hygiene or during outbreaks — depending on age, season, diagnostic method, and whether diarrheic or healthy calves are sampled (Cho and Yoon, 2014; Urie et al., 2018; Thomson et al., 2017). For example, a study in central Ethiopia found 15.8% PCR prevalence in dairy calves (Wegayehu et al., 2016), while a survey in the Nile Delta of Egypt using microscopy on nearly 2,000 specimens found substantial age-related variation (Helmy and Hafez, 2022). In Nigeria, molecular studies in native cattle breeds have reported around 16% PCR-RFLP prevalence (Maikai et al., 2011), and in Ghana, a study in Kpong reported 47.8% PCR prevalence in fecal samples, illustrating how local farm conditions and sampling of diarrheic versus unselected animals can shift estimates markedly. The present study's focus on diarrheic calves, its inclusion of calves under six months, and its use of nested PCR all contribute to a prevalence that is higher than microscopy-based estimates in the same population, but credible in the context of these comparator studies.

4.2 Species and zoonotic risk

The species profile — C. parvum dominant, with C. bovis, C. ryanae, and C. andersoni also present — mirrors the age-structured distribution described by Santín et al. (2004) in the United States and by Fayer et al. (2006) in cattle of different ages. In those studies, C. parvum was the main species in pre-weaned calves, while C. bovis and C. ryanae became more common after weaning and C. andersoni dominated in yearlings and adults. The detection of all four species in diarrheic calves under six months suggests overlapping exposure and possibly early colonisation by species usually associated with older animals, a pattern also noted in Egypt, where C. ryanae, C. bovis, and C. andersoni were present at higher-than-expected frequencies in pre-weaned calves (Helmy and Hafez, 2022). Whether C. bovis, C. ryanae, and C. andersoni contribute directly to diarrhea in these calves, or are incidental findings in calves whose diarrhea is caused by C. parvum or other enteropathogens, cannot be resolved by a cross-sectional study without pathogen co-detection and quantitative oocyst data. What is clear is that C. parvum, the species with proven pathogenicity and zoonotic potential, accounted for the majority of infections, and that the dominant subtype, IIaA15G2R1, is a recognised zoonotic strain (Xiao, 2010; Feng et al., 2018).

The zoonotic implications are substantial. Farm workers, children, and other household members in smallholder systems have frequent, close contact with calves, handle feces during cleaning, and may share water sources that can be contaminated by calf feces (Checkley et al., 2015; Kotloff et al., 2013). C. parvum IIaA15G2R1 has been repeatedly identified in human cryptosporidiosis cases linked to cattle contact, farm visits, and contaminated water in Europe, North America, and elsewhere (Rieux et al., 2013; Feng et al., 2018; Sannella et al., 2022). In sub-Saharan Africa, where Cryptosporidium is a leading cause of diarrhea in children under five

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(Kotloff et al., 2013; Checkley et al., 2015), the contribution of zoonotic transmission from calves relative to anthroponotic transmission remains incompletely quantified, but molecular studies in Bangladesh, Ethiopia, and Ghana have demonstrated shared subtypes between calves and children in the same communities, supporting a One Health interpretation (Wegayehu et al., 2016; Ayele et al., 2024). The present findings therefore justify explicit public health messaging for farm households: handwashing with soap after calf handling, safe disposal of calf feces, protection of drinking-water sources, and prompt care for children with diarrhea.

4.3 Diagnostic implications

The comparison of microscopy and nested PCR confirms that microscopy alone underestimates prevalence and cannot provide species or subtype information. The 18 additional cases detected by PCR represent calves that would have been classified as negative in routine practice, yet they were shedding parasite DNA and, in many cases, likely shedding viable oocysts. Similar gaps have been reported in other settings: in central Ethiopia, microscopy detected 9.4% versus 15.8% by PCR (Wegayehu et al., 2016), and in Saudi Arabia, PCR detected 52% versus 26.7% by mZN in diarrheic calves (Al-Quraishy et al., 2023). The reasons are well understood: oocyst shedding is intermittent, oocyst numbers can be low early or late in infection, and microscopy depends on the quality of the smear, the stain, and the observer (Chalmers and Katzer, 2013). PCR, by contrast, amplifies DNA from even a few oocysts, but it can also detect DNA from non-viable oocysts, so a PCR-positive result should be interpreted alongside clinical signs, as in this study, which enrolled only diarrheic calves. For surveillance and for guiding treatment and hygiene, a two-tier approach is practical for resource-limited settings: microscopy or antigen detection for initial screening where molecular capacity is limited, with PCR and sequencing for confirmation, species identification, and outbreak investigation in reference laboratories (Xiao, 2010; Chalmers and Katzer, 2013). As molecular costs fall, routine PCR screening of neonatal calves on high-risk farms, as recommended by the authors, becomes increasingly feasible.

4.4 Control and prevention

There is no fully effective, widely available specific drug for cryptosporidiosis in calves. Halofuginone lactate is licensed in some countries for prevention and treatment in neonatal calves, but its efficacy is partial, its therapeutic window is narrow, and it does not eliminate oocyst shedding (Cho and Yoon, 2014; Thomson et al., 2017). Control therefore rests on hygiene, management, and reducing oocyst exposure in the first weeks of life (Urie et al., 2018; Thomson et al., 2017). Evidence-based measures include: prompt removal of calves from the calving pen, individual housing or small, stable groups for pre-weaned calves, thorough cleaning and disinfection of pens with products effective against Cryptosporidium (noting that many common disinfectants are ineffective), steam cleaning or drying of pens between calves, frequent changing of bedding, dedicated boots, clothing, and equipment for calf areas, and ensuring adequate, timely colostrum intake to support passive immunity (Cho and Yoon, 2014; Urie et al., 2018). Water troughs and feeders should be protected from fecal contamination, and sick calves should be isolated. Farm workers should wash hands with soap after handling calves, and households should protect drinking-water sources from livestock feces. Routine molecular screening, as recommended, can help identify high-shedding farms and calves, monitor the impact of hygiene interventions, and detect the emergence of zoonotic subtypes. These measures are low-cost relative to the losses from calf diarrhea and are compatible with smallholder realities, but they

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require sustained extension support and farmer engagement to be adopted consistently (Ayele et al., 2024).

4.5 Limitations

This study has limitations that should be considered when interpreting the results. First, it was cross-sectional and sampled only diarrheic calves, so it cannot establish causality for diarrhea in individual calves, estimate incidence, or capture seasonal variation. Second, co-infections with other enteropathogens (rotavirus, coronavirus, Escherichia coli, Salmonella, Eimeria) were not assessed, and some diarrhea may have been due to those agents, with Cryptosporidium as a co-factor or incidental finding. Third, oocyst shedding was not quantified, so the relationship between infection intensity and clinical severity could not be examined. Fourth, Sanger sequencing of a single amplicon may miss mixed-species infections, and the gp60 subtyping was limited to C. parvum isolates, so subtype diversity in other species, if any, was not explored. Fifth, farm-level risk factors (stocking density, hygiene scores, colostrum practices, water source) were recorded but not analysed in multivariable models in the abstract, limiting causal inference about risk factors beyond age. Future work should address these gaps through longitudinal cohort studies, quantitative PCR, multi-pathogen panels, and farm-level risk-factor modelling, as well as sampling of farm workers and household contacts in a One Health design to quantify zoonotic transmission directly.

5. Conclusion

Bovine cryptosporidiosis is endemic among diarrheic calves on peri-urban smallholder dairy farms, with a nested PCR prevalence of 22.5% compared with 16.9% by microscopy. Cryptosporidium parvum was the dominant species (61.1% of positives), with C. bovis, C. ryanae, and C. andersoni also circulating. The predominance of the zoonotic C. parvum subtype IIaA15G2R1 and the high infection rate in calves aged 1–4 weeks (34.2%) identify neonatal calves as the priority group for both animal health and public health interventions. Molecular diagnostic tools provide superior sensitivity over conventional staining and are essential for species and subtype surveillance. Strict hygiene management, improved colostrum and calf housing, protection of water sources, hand hygiene for farm workers, and routine molecular screening are recommended to control transmission and reduce the risk to farm households. A One Health approach that links veterinary, human health, and environmental surveillance will be most effective in reducing the burden of cryptosporidiosis in smallholder systems.

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Conflict of Interest

The authors declare no conflict of interest.

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Authors' Contributions

Samuel Kwame Mensah conceived the study, coordinated field and laboratory work, analysed data, and drafted the manuscript. Beatriz Elena Vargas contributed to molecular analysis and subtyping and revised the manuscript. Chinedu Okafor contributed to study design, parasitological analysis, and revision of the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

The datasets generated and analysed during this study, including anonymised calf-level data and representative 18S rRNA and gp60 sequences, are available from the corresponding author on reasonable request. Sequence data should be deposited in GenBank prior to publication, with accession numbers provided in the final published version.

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