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Soil and groundwater contamination with *Cryptosporidium spp*. oocysts: An environmental epidemiological study of pollution hotspots in rural areas of Diyala Province

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Abstract

This study aimed to assess the extent of soil and groundwater contamination by *Cryptosporidium spp.* egg cysts in rural areas of Diyala Governorate, and to identify environmental pollution hotspots and associated risk factors using microscopic examination combined with qualitative staining and morphometric analysis. Three hundred (300) environmental samples were collected, including 150 surface soil samples and 150 groundwater/well samples, from various agricultural and pastoral rural areas in the governorate between October 2025 and February 2026.

The soil samples were treated using the modified zinc sulfate flotation method, while the water samples were concentrated using (0.45 µm) and backwash centrifugation. All prepared smears were examined microscopically using modified acid-fast staining and modified saffron and methylene blue staining, with precise morphometric measurements of bag diameters performed using a graduated eyepiece. The results showed that the overall percentage of parasite contamination was %22.30 300/65)). Soil samples showed a higher contamination rate of 150/42) %26.55) compared to groundwater samples, which showed 150/23) %15.00). Analysis of contamination hotspots revealed that areas adjacent to dense livestock farms had the highest environmental contamination rate, accounting for 63% of all positive samples, followed by areas near cesspools and informal sewage treatment facilities, with rates ranging from 26.5% to 30%. This study confirms that relying on morphometric microscopy combined with double staining is a crucial, low-cost, and highly efficient epidemiological tool for monitoring environmental parasitic pollutants in rural Diyala, supporting One Health strategies.

Keywords: *Cryptosporidium spp*; Soil contamination Groundwater; Morphometric microscopy

1. Introduction

Parasitic diseases transmitted through environmental agents are considered one of the most significant challenges facing public health and environmental systems in rural and agricultural settings worldwide [1]. Among these pathogens, Cryptosporidium spp. stands out as a unique protozoan intestinal pathogen capable of causing acute gastroenteritis and severe watery diarrhea in humans and animals. This can progress to chronic and fatal conditions in individuals with acquired immunodeficiency or children under five years of age [2, 3]

The significant epidemiological and environmental success of this parasite is directly attributable to the characteristics of its infectious environmental stage, the oocysts. These cysts are characterized by a thick, multi-layered cell wall composed of carefully arranged protein, lipid, and sugar complexes, providing them with superior physical and mechanical protection against environmental inhibitors and temperature changes, as well as exceptional resistance to conventional chemical disinfection processes such as chlorination used in water treatment plants [4, 5].

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Cryptosporidiosis oocytes are transmitted via the oro-anal tract, with millions of cysts being shed in the feces of the infected host into the external environment [6]. In rural and agricultural settings, livestock—especially newborn calves and small ruminants— act as the primary zoonotic reservoir for the disease, shedding enormous quantities of infectious cysts into the soil surrounding barns and pastures; [7, 8]).

Rural communities in Diyala Governorate, eastern Iraq, rely heavily on agriculture and pastoralism due to their fertile geography and abundant irrigated land. However, these areas suffer from inadequate or nonexistent sewage systems, forcing residents to rely on unlined, rudimentary septic tanks for waste disposal. These tanks are designed to facilitate the spread of infectious diseases. Simultaneously, many farmers have resorted to digging shallow, unregulated wells as an alternative source of water for drinking, domestic use, and irrigating leafy crops [9]. The persistence of viable and infectious egg cysts in the soil for extended periods makes topsoil a continuous source and reservoir of contamination. Under the influence of various hydrological factors and intensive irrigation practices, including seasonal rainfall, these bags undergo vertical leaching and horizontal migration through soil pores and geological fissures. This facilitates their access to and contamination of nearby groundwater aquifers that feed these wells [10, 11]. Given the high economic cost of molecular techniques and their need for sophisticated infrastructure and equipment that may not always be available in the governorate's field and service laboratories, developing and implementing advanced microscopic protocols based on mechanical concentration and dual chemical staining methods, supported by morphometric analysis, represents the most sustainable and reliable academic option for large-scale epidemiological studies [12, 13].

Despite the existing environmental and health threat, combined ecoepidemiological studies investigating soil and groundwater contamination without human clinical intervention in Iraq, and in Diyala Governorate in particular, remain scarce and do not adequately address the scale of the risk [14, 15]. Therefore, this study aimed to quantitatively and qualitatively assess soil and groundwater contamination by the parasitic egg cysts of Cryptosporidium spp. in rural areas of Diyala Governorate, identify geographical and environmental contamination hotspots, and verify epidemiological indicators using robust morphometric and staining microscopic methods to provide a comprehensive environmental database that supports sustainable environmental management strategies.

2. Materials and Methods

2.1. Study Design and Sampling Area

This field-based epidemiological study, based on environmental cross-sectional analysis, was conducted in several villages and rural and agricultural areas of Diyala Governorate (including the rural areas of Baquba District, Al-Muqdadiyah Center, and Al-Khalis District) from October 2025 to February 2026. The sampling strategy was designed to target areas with high pastoral and agricultural activity and heavy reliance on wells. A total of 300 environmental samples were collected, divided equally into 150 soil samples and 150 groundwater (well) samples. Collection sites were precisely determined based on graduated distances from potential pollution hotspots, including the perimeters of livestock pens, the perimeters of absorption pits, and open agricultural fields [16].

2.2. Soil Sample Collection and Processing

Soil samples (approximately 500 g each) were collected from the exposed surface layer at a depth of 5–0 cm using sterile metal shovels and stored in labeled plastic bags [13]. In the laboratory, the samples were air-dried at room temperature and sieved using fine sieves to remove gravel and large suspended particles. To efficiently isolate egg cysts, 10 g of the washed soil was mixed with 40 mL of potassium phosphate buffer (PBS) supplemented with 0.1% Tween 80 to break the physical bonding forces and release the cysts adhering to soil particles and clay colloids [17].

After mixing the samples for 30 minutes, the Modified Zinc Sulfate Flotation Technique (MSFT) was applied using a zinc sulfate solution with a control specific gravity of 1.20. The samples were centrifuged at 3000 rpm for 10 minutes, and the thin surface layer containing the floating egg sacs was carefully collected and transferred to clean tubes [18].

2.3. Groundwater Sample Collection and Concentration

Water samples were collected from active domestic and agricultural wells, 5 liters per sample, in clean, pre-sterilized polyethylene containers [19]. The samples were concentrated in the laboratory using membrane filtration by passing the entire volume of water through 0.45 μm microfiltration membrane filters (Millipore, USA) under a vacuum suction system [20]. After filtration, the membrane was carefully scraped with a sterile blade and washed with slightly warmed PBS to loosen any trapped cysts. Centrifugation was then performed at 3500 rpm for 12 minutes to obtain the final concentrated precipitate, which was used to prepare the microscopic smears [21].

2.4. Staining Techniques and Morphometric Microscopy

Thin, uniformly sized smears of soil sediment and concentrated water were prepared on clean glass slides, allowed to air dry, and then chemically fixed with pure methyl alcohol (methanol) for 5 minutes to stabilize the cyst morphology. Two separate staining protocols were applied to each sample to ensure double diagnostic confirmation and rule out accidental results:

Modified Zeil-Nielsen Method (Acid-Fast/Kinyoun Stain): The slides were stained with concentrated carbol fuchsin under gentle heating, differentiated and bleached with dilute sulfuric acid (1%) for 1 minute, and then stained with methylene blue as a contrast stain for 2 minutes (Garcia, 2020). Under this staining, egg cysts appear as spherical or oval shapes stained a bright crimson or deep pink against a blue background, exhibiting a thick, double wall [22].

Modified Safranin-Methylene Blue Stain: This method was used as a confirmatory technique to enhance color contrast. Smears were treated with a hot safranin solution, acid- treated, and then stained with methylene blue. This resulted in *Cryptosporium* cysts appearing as a distinctive orange-red color and transparent, easily distinguishable from environmental contaminants [23].

All slides were blindly examined using high-power oil-based lenses (100x). To ensure accurate diagnosis and avoid confusion with fungal spores or acid-resistant yeasts, standard morphometric verification of the diameters and dimensions of the diagnosed cysts was performed using a graduated ocular micrometer, pre-calibrated with a stage micrometer. This confirmed that the diameters fell precisely within the standard morphological range for adult *Cryptosporum*4.2) to 5.5 µm), while also observing the internal sporozoites and the residual body [24].

2.5. Statistical Analysis

The collected data were entered and statistically analyzed using the Statistical Package for the Social Sciences (SPSS version 26). The chi-square test was used to compare pollution levels between soil and water samples and to determine the statistical significance of differences between different pollution hotspots. Differences were considered statistically significant at a p < .0.05

3. Results and Discussion

The results of double morphometric microscopy of environmental samples collected from rural areas of Diyala Governorate revealed clear and alarming levels of parasitic contamination. Of the 300 environmental samples examined, 65 showed confirmed contamination with standard morphological characteristics of *Cryptosporidium spp*. egg sacs, representing an overall contamination rate of .%22.30. Data analysis based on the type of environmental medium showed a statistically significant difference (P < 0.05) between soil and water contamination. Surface soil samples showed a significantly higher contamination rate of 42) %26.55 positive samples out of 150 tested), while groundwater samples showed a contamination rate of 23) %15.00 positive samples out of 150 tested), as detailed in Table .1

Table 1 Contamination rate of soil and groundwater samples with *Cryptosporum* parasite in rural Diyala

Probability Value	Percentage of Contamination	Number of Microscopically Positive Samples	Total Number of Samples	Environmental Sample Type
P<0.031	26.55%	42	150	Surface soil samples
P<0.040	15.00%	23	150	Groundwater (well) samples
--------	22.30%	65	300	Total

Regarding the distribution of infectious disease based on proximity to environmental pollution hotspots, laboratory studies showed that livestock pens and animal housing areas represent the greatest source and risk for the dispersal of egg sacs in the environment. These hotspots resulted in the highest positive rates, with 26 soil samples testing positive (63.00% of the total positive soil samples) and 14 groundwater samples testing positive (62.00% of the total positive groundwater samples). Hotspots adjacent to cesspools and primitive human waste disposal sites ranked second in terms of risk, registering 26.5% of soil samples and 30% of well water samples. These rates decreased significantly in open agricultural fields far from direct animal and human activities, registering 10.0% and 8.2%, respectively (Table 2)

Table 2 Pollution record and percentages in general weather conditions according to environmental conditions

Percentage %	**Number of positive water samples**	**Percentage %**	**Number of positive soil samples**	**Nature of the surrounding environmental pollution hotspot**
62.00%	14	63.00%	26	Area of livestock and animal farms
30%	8	26.5%	10	Area of absorption pits and informal drainage systems
8.2%	2	10%	4	Open and relatively clean agricultural fields
100%	24	100%	40	Total

The results obtained in this field epidemiological study confirm that the rural environments of Diyala Governorate are subject to clear and high levels of pollution by the parasitic oocysts of *Cryptosporidium spp.* This finding directly reflects the health and environmental conditions of agricultural areas that lack centralized waste and water treatment systems [9]. The overall pollution rate of 21.33% is very close to and similar to what researchers recorded in parallel environmental studies in the central governorates of Iraq. It also supports the international reports issued by [13] and [20] which confirmed that intestinal parasites represent a constant and sustainable environmental pollutant in the rural areas of developing countries.

The higher contamination rate recorded in soil samples (26.66%) compared to well water samples (16.00%) has a logistical explanation in the fact that topsoil is the "first and direct recipient and biological reservoir" for all forms of organic and fecal excretion from humans and animals [8, 25]. Cryptosporum egg sacs possess unique physical properties that allow them to adhere strongly to soil particles and fine clay colloids. This is further supported by their electrostatic surface charge and hydrophobic structure, preventing rapid erosion and allowing them to remain viable and infectious for extended periods, sometimes several months, benefiting from the high humidity and shade available under vegetation in rural Diyala [5, 11]. By reading and analyzing the data from the infection hotspots (Table 2), the crucial and dangerous role of livestock farms and breeding areas becomes clear, accounting for the lion's share of positive samples at 62.5%. This phenomenon is epidemiologically attributed to the zoonotic nature of cryptosporidiosis; the cattle, sheep, and goats prevalent in the villages of Diyala are classified as massive natural reservoirs and secretors of the parasite, especially young and newborn animals that excrete unimaginably large microscopic quantities, reaching millions of cysts per gram of feces [2, 6]. The lack of health awareness leads farmers to haphazardly wash their barns and collect animal manure in open areas adjacent to agricultural lands and water sources without heat treatment, thus reinforcing the epidemiological contamination ([7].

The most serious and critical aspect for public health is the proven contamination of groundwater wells at a rate of 16.00%. Most of the wells drilled in the rural areas of Diyala are rudimentary shallow wells (shallow wells) lacking proper engineering protection or impermeable cement lining. This contamination confirms the occurrence of vertical leaching and infiltration mechanisms. When heavy rains fall or crops are over- irrigated, water flows, carrying parasitic cysts from the contaminated soil. Due to the very small size of the cysts (6-4 micrometers), they can penetrate and pass through the interstitial spaces and pores of the soil to reach the groundwater, taking advantage of the close horizontal distances between the drilled wells and the absorption pits of farmers' houses, which are much less than the safe engineering and protective limits [10, 21]. This complex mechanism of biological contaminant translocation has been observed and validated globally in rigorous hydrological studies [3, 4] . The adoption of a methodology in this study that combined dual staining techniques with micro-morphometric measurements using a graduated lens effectively contributed to providing the microscopic results with high academic accuracy and reliability, overcoming the limitations of conventional microscopy. Specimens and fungal spores outside the standard range of parasite diameters (5.5–4.2 μm) were isolated and excluded, demonstrating that this methodology represents a crucial and cost-effective epidemiological alternative for contaminant monitoring [26]. Therefore, the continued use of unboiled or unfiltered well water by farmers and their families in rural Diyala explains the persistent cases of diarrhea and mysterious gastroenteritis in these areas (Xiao et al., 2024; [14].

4. Conclusion

The rural and agricultural environment of Diyala Governorate suffers from significant and widespread contamination with *Cryptosporidium spp.*, posing a serious epidemic risk. This contamination includes both soil and groundwater.

Surface soil appears to be a larger reservoir for parasitic cysts than groundwater due to direct contact with waste.

Livestock pens and makeshift cesspools (absorption pits) are the primary hotspots responsible for the ongoing environmental pollution in the region.

The mechanical concentration method followed by double staining and morphometric analysis has proven to be a highly reliable and cost-effective epidemiological tool, making it a viable alternative in the absence of molecular techniques.

Recommendations

The "One Health Approach" strategy must be adopted through regular and coordinated efforts between the Diyala Environment Directorate, veterinary hospitals, and the Health Department to monitor pollution hotspots.

Livestock breeders must be required to construct properly equipped, isolated enclosures, and the use of untreated organic animal manure in fertilizing agricultural land must be prohibited to ensure the elimination of egg sacs.

Municipal authorities must implement strict engineering regulations for the drilling of domestic water wells, ensuring a minimum distance of 30 to 50 meters between the well and the nearest cesspool as a preventative measure against vertical seepage.

Health awareness and guidance campaigns should be launched targeting residents of villages and rural areas in Diyala to highlight the dangers of untreated well water and encourage them to boil water or use high-efficiency home filtration systems before consumption.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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