Human Cryptosporidiosis: A Review and Staining Method

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HUMAN CRYPTOSPORIDIOSIS: A REVIEW AND STAINING METHOD

by

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ABSTRACT

Cryptosporidiosis is increasingly emerging as the most significant cause of infectious diarrhea in humans and livestock. In humans, studies show that Cryptosporidiosis disease is more prevalent in the immunocompromised and young ones. However, outbreaks from contaminated water sources have caused disease in healthy adults, too. Two main species of the protozoan causative agent, *Cryptosporidium parvum* and *Cryptosporidium hominis* are responsible for most human infections. Cattle, pets, mice, and other livestock can transmit the *Cryptosporidium parvum* variants to humans through fecal transmission. Other species of *Cryptosporidium* can infect domestic animals, but their host range is narrow; therefore, they are not of significant concern to human health. While most people can recover from the disease without medication, specific antidiarrhea drugs can treat the disease in people with a healthy immune system. But there are no approved drugs for immunocompromised adults or children. This review will cover the factors that support the survival of *Cryptosporidium* in humans, the current treatments, detection, prevention methods as well as an experiment to compare some staining methods used to detect *Cryptosporidium* in labs.
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INTRODUCTION

What is *Cryptosporidium*?

*Cryptosporidium* is a microscopic endoparasite known to survive in vertebrates' epithelial cells of the digestive and respiratory systems. Most of *Cryptosporidium*'s oocyst is spherical and measure between 4 and 6 µm (microns). The internal structures of the cell are unclear under a microscope, unlike some other types of protozoa, which makes it difficult to differentiate between the species (12). First discovered in 1895, *Cryptosporidium* was described as a free-living protozoan frequently found in the gastric glands of laboratory mice. Currently, this parasite is common in bovines as well as humans. Studies show that older humans and animals can carry these protozoa in their tissues, but it does not affect them in the same way that it affects and causes disease in younger and immunocompromised humans and animals (14,16,19,29).

Taxonomy and Parasite Structure

*Cryptosporidium* is a protozoan from the phylum Apicomplexa (12,16,19). This protozoan possesses unique organelles, such as microtubules and fibrils located at the apical end of the cell, as an adaptation for penetrating the host cell (figure 1,2,3). Initial research determined *Cryptosporidium*'s subtypes based on host selectivity, site of infection, and size; however, modern scientists recognized over 44 species found on a genetic basis through molecular techniques.
Figure 1. Shows *Cryptosporidium* oocysts. Yellow arrows point to yeast cells that stain red with methylene blue. Red arrows point to cells that did not stain with modified acid-fast stain (31).

Figure 2. Shows the *Cryptosporidium* cell structure and its parts. The apical takes part in penetrating the host’s cell. The storage organelles and the nucleus are in the central part of the cell. The mitochondria and the crystalloid body are located in the rear end (32).
Cell Life Cycle and Mechanisms of Infection

*Cryptosporidium* transmits through the fecal-oral route in its oocysts form (18,20). The oocyst is the only stage found outside a host and has four sporozoites surrounded by a thick multi-layered wall. *Cryptosporidium* reproduces sexually and asexually, and the shedding of the oocysts initiates three days after the infection. As a member of the Apicomplexa phylum, the parasite has a complex life cycle of eight days (18).

Temperature, pH, and bile salt concentration appear to be the three major environmental factors that promote the oocysts and parasites' survival in the host body. After ingesting the oocyst, the parasitic attack on the epithelial cells of the gastrointestinal tract begins. The four sporozoites excyst while serine proteases are activated. The cell surface glycoproteins such as P23 (15-kDa in size) initiates the sporozoites to travel along the gut's epithelium by gliding motility supported by the actinomoyosin motor (18).
The sporozoites attached themselves to the host's cell membrane with the help of apical complex proteins like thrombospondin-related adhesion proteins, which are 900 kDa (kilodaltons) in size, and 40 glycoproteins. The epicellular parasitophorous vascular membrane (PVM) of the host's cell surrounds the attached sporozoites. The sporozoites then mature into trophozoites. The trophozoites also develop in the PVM and produce type I meronts (schizonts) by asexual reproduction. Several cysteine proteases participate in sporozoites' engulfment, but the complete mechanism is not fully understood (18). Individual schizont contains 16 merozoites, and each of the merozoites affects a new healthy intestinal lining cell. Some merozoites reproduce new schizonts and repeat the cycle, while others form type II meronts containing four merozoites. These four merozoites infect new enterocytes and produce male and female gametes sexually. The female gametes, macrogametocytes, develop into macrogametes, and each male gametes, microgametocyte, holds 12 to 16 microgametes. These males and females’ gametes produce zygotes after fertilization, and the zygotes mature to form oocysts (18).
Figure 4. Illustration of Cryptosporidium parvum life cycle. Ingested (inhaled) is the parasite in its oocyst form, which enters and leaves the host body. a. sporozoite, b. sporozoite attached to the host cell. c. sporozoite matured into trophozoite and undergoes merogony (asexual reproduction) to form type I meront, d. Type I repeats the cycle. e. type I meront proceeds to form type II meront. f. type II meront reproduce sexually (gametogony) to form either g. female (macrogametocyte) or h. male (microgametocyte) gametes. Fertilization occurs between the macrogametocyte and the microgametocyte to produce i. zygote. The zygote formed is either j. matured and becomes k. thick-walled oocyst which leaves the host body, or the zygote is l. a thin-walled oocyst that remains in the host body and can auto-infect and repeat the cycle. (32).

Infection of Cryptosporidium in individuals with immune disorders is related to another type of asexual reproduction of the trophozoites called sporogony. Two types of oocysts, thin and thick-walled, are the product of this asexual reproduction. The thick-walled oocysts leave the host body through feces. The thin-walled oocysts remain in the gastrointestinal tract, as they can initiate autoinfection by infecting the microvilli, increasing the infection's severity (18).
Figure 5. Showing several stages of parasite development in culture. Green arrows show final result of asexual reproduction of type I meronts. White arrows show new parasitic attack. Pink arrows points to macrogametocyte while blue arrows are microgametocyte (34)

The Disease

Cryptosporidiosis is therefore a gastrointestinal disease caused by the protozoa of Cryptosporidium. Symptoms include nausea/vomiting, loss of weight and appetite, stomach/abdominal aches, fever, watery diarrhea, and dehydration that can lead to more complicated issues, including death in the younger ones. Some people display no symptoms at all (14,18).
Modes of Transmission

Figure 6. Cryptosporidium Modes of transmission. (A) oocysts transmissions: waterborne, zoonotic, foodborne, and airborne. (B) person to person transmission. 1A. ingestion of the oocysts through fecal-oral-route. B. inhalation of the oocyst (uncommon). 2. Parasitic attack in the small intestine. 3. Thick-walled oocysts (31).

Waterborne Transmission

Cryptosporidiosis is the leading waterborne-related disease in the United States. It is among the most significant waterborne disorders globally. Cryptosporidium resists most water purification methods; therefore, it can infect a large group of people within a short period and cause an outbreak. This transmission mode is commonly known to be the most critical (26).

The oocytes of Cryptosporidium usually reside on the surfaces of water. In the late 1990s, studies tracked the oocysts in 17 to 27% of treated water and 9.5 to 22% of groundwater (22). Cryptosporidium oocysts escaping many waters purification is still a concern in the United States. Recreational waters are the second largest endemic sources for contracting the disease after drinking water. Bodily contaminated and incontinent
persons can dispense the oocysts at these locations. This increases the potential, since the oocysts can withstand treatment methods, including chlorination (26).

Foodborne Transmission

Contaminated raw food like milk, fruits, vegetables, and seafood have been linked to foodborne outbreaks globally. The first foodborne case in the United States was noted in 1993 and was associated with apple cider. Between 2009 and 2017, the Centers for Disease Control and Prevention reported 13 outbreaks of Cryptosporidium related to pasteurized milk or apple cider only. In fall 1993, a wave of cryptosporidiosis occurred in central Maine after drinking fresh pressed apple juice from an agricultural fair. The fair was held near one of the cattle grazing lands, and 160 cases of cryptosporidiosis were among the fair attendees (12).

Person to Person Transmission

While humans are likely to increase the potential of this waterborne and foodborne disease, they can transmit the disease in many other ways, including direct contact. An infected individual can dispatch the oocysts through handshakes, other bodily contacts, or indirect contacts such as leaving the oocysts on fomites. Few studies propose the possibility of acquiring the disease sexually, especially after their data show the higher prevalence of cryptosporidiosis among homosexual men. Other behaviors, including inadequate hygiene practices, can easily exacerbate the transmission process.


Animal to Person Transmission

This type of transmission is not limited to bovines, as many studies showed that many animals could disperse the oocysts across large areas. In a study, birds carrying the oocysts infected mice in a place they rested while traveling along their migration route. Another study demonstrated how cockroaches carrying the oocysts in their intestinal tracts could have transmitted the disease to a child diagnosed with cryptosporidiosis in a house (12). In an experiment with beetles, their feces carried lower numbers of oocysts than digested; however, their external body surfaces transported large quantities of the oocysts. In addition, houseflies, rotifers, and other domestic animals are possible carriers, even though the disease does not necessarily affect them (12). A 2020 study in Canada focused on oysters produced in one of the large bays suggests the high risk of oysters being a source of Cryptosporidium infection (21).

Airborne Transmission

Several research reported cases of Cryptosporidium affecting the respiratory system of birds more frequently than in mammals. Cases with pulmonary cryptosporidiosis are rare, which makes this transmission mode the least source. However, there are data relating high cough rates and other pulmonary diseases in people with immune-related infections and in children (12).

Significance

Cryptosporidiosis is globally known to infect both immunocompromised and healthy individuals. Age and health conditions determine its severity. The disease usually
persists in children and individuals with immunity complications such as HIV, malnutrition, and post-transplantation, and it can lead to death. Researchers believe that Cryptosporidium infection is underdiagnosed globally and in the United States. Locally, about 30% of healthy children and adult tested to carry the antibodies to Cryptosporidium (Brett et al, 2003).

The risk of outbreaks increases as the global market increases, and as people prefer buying food instead of preparing it themselves. Prevention of such a disease requires good hygiene practices, and one cannot control it without careful attention to what one eats. Unfortunately, food handlers can easily cause a rise in contaminated food with the slightest negligence. As the detection method is not as recurrent as needed to prevent the spread of the disease, controlling the condition can also be challenging.

Some Biological Methods for Tracking/Detecting

While many successful methods enable the detection of Cryptosporidium, most methods are time- and funds-consuming. It is necessary to conduct more research to improve the currently used methods and facilitate faster and more accurate detection. Table 1 presents the current methods used and how effective each method is.
Table 1. List of some of the methods used in Cryptosporidium’s detection.

<table>
<thead>
<tr>
<th>Method</th>
<th>Examples</th>
<th>Effectivity</th>
<th>Affordability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>Staining: e.g., acid fast (AF)-modified Ziehl-Nelsen (9,24)</td>
<td>Lacks sensitivity and specificity</td>
<td>- Simple and cheap material - Labor intensive</td>
</tr>
<tr>
<td></td>
<td>Phase-contrast microscopy (PCM) (24)</td>
<td>Specific but not sensitive</td>
<td>inexpensive</td>
</tr>
<tr>
<td></td>
<td>Hematoxylin and eosin (HE) (40)</td>
<td>97.67% Sensitive, 85.08% Specific</td>
<td>Practical and cost effective</td>
</tr>
<tr>
<td>Immunological based (Antigen testing)</td>
<td>Fluorescent antibodies (IFA) (12,24)</td>
<td>Better sensitivity (22% more)</td>
<td>- More expensive - Requires fluorescence microscope - Labor intensive</td>
</tr>
<tr>
<td></td>
<td>Reverse Passive Haemagglutination (12)</td>
<td>Non-specific due to cross reactivity</td>
<td>Requires more tools for further verification</td>
</tr>
<tr>
<td></td>
<td>Enzyme-linked immunosorbent assays (ELISAs) and the likes; EIAs, dipstick assays (9,12,24)</td>
<td>Sensitivity varies from 70 to 100%</td>
<td>Save time and manageable</td>
</tr>
<tr>
<td></td>
<td>Rapid test; biosensor (24)</td>
<td>Lower sensitivity and specificity for other species</td>
<td>Requires confirmation</td>
</tr>
<tr>
<td></td>
<td>Solid phase immunochromatographic assays (12,24)</td>
<td>Non-specific but sensitive</td>
<td>Save time and manageable</td>
</tr>
<tr>
<td>Molecular</td>
<td>DNA sequencing (27, 28, 29)</td>
<td>100% Specific and sensitive</td>
<td>Expensive</td>
</tr>
<tr>
<td></td>
<td>Polymerase chain reaction (PCR) (12,13,15,20,24)</td>
<td>100% Specific and sensitive</td>
<td>Expensive and time consuming</td>
</tr>
</tbody>
</table>

Images of Some of the Three Detecting Methodologies Discussed Above

Figure 7. This shows both Hematoxylin & eosin (HE) Staining and Fluorescent antibodies (IFA). In the H&E staining, the white arrows show the reduction of the villi’s height of the mouse ileum after 48 hours of C. parvum infection. Secondary immunofluorescent (IFA) staining authenticates the infection in green after using DAPI to stain the cell nuclei blue (37).
Figure 8. *Cryptosporidium parvum* gene expression in different stages of the oocysts’ life cycle. **A.** Shows comparisons based on the DESeq2 normalized gene expression of all the oocysts stages. **B.** Exclude the Sporozoite stages. **C and D** show the gene-wise z-score expression. **E.** Shows up and down-regulation of sporozoites, in vivo/ in vitro stages, and the stages in between (36).
Curriculum Knowledge and New Findings

*Cryptosporidium* Current Reports

*Cryptosporidium* is still a significant health concern globally. Studies identified 44 species and 120 genotypes approximately, but cases are still underreported and underdiagnosed. *Cryptosporidium hominis* and *Cryptosporidium parvum* are the two species that commonly affect humans (1,2,11,13,30). These two species, *Cryptosporidium hominis* and *Cryptosporidium parvum*, account for 66% and 22%, respectively, of reported cases (11). Multiple research projects have established *Cryptosporidium hominis* to be of the human base. Therefore, *Cryptosporidium hominis* is an anthropoontic transmission. On the other hand, *Cryptosporidium parvum* is a zoonotic transmission, as multiple animals, including domestic ones, account for their spread (2,4,5,6,7,8,10,11,12,13,14,30). Table 2 shows the list of the species known as of April 2022 (31).

In Developing Countries

*Cryptosporidium* is a recognized cause of mild to profuse diarrhea in developing countries. It accounts for about 48,000 mortalities in children under five years and approximately 4.2 million modalities. Globally, the death rates in children have significantly decreased by 60%, and its medical consequences have been reduced by 13% from 2000 to 2016. However, the variants are unresponsive to treatments, making the disease still of concern. *Cryptosporidium* is also a causative agent of children's intrauterine growth restriction (IUGR). Many studies have recorded body mass, weight, and height deficiency cases worldwide, linking them to cryptosporidiosis as a basal cause (25, 30).
Incidents of *cryptosporidiosis* are prevalent in individuals with impaired immunity in developing countries. Individuals with HIV/AIDS primarily show multiple episodes of the disease. Figure 7 shows the percentage of immunodeficient persons that developed *cryptosporidiosis in Africa, Asia, Europe, and South America, as collected from epidemiological research in a review (30).*
Table 2. List of the current discovered species of Cryptosporidium and their hosts (10, 15, 25, 28, 30, 31)

Factors contributing to the increasing cases in developing countries include overcrowding, poor drinking water, young age, household diarrhea, and immunity.
Although very few waterborne links have been detected, inadequate drinking water treatments promote the potential of infection. The severity of the illnesses appears to be milder in underdeveloped countries. Diarrhea occurs in about a third of cases and lasts one to two weeks. Furthermore, the shedding of oocytes can continue to occur up to 39.5 days after infection.

Some rare positive factors that lessen the frequency include breastfeeding in Cameroon and Botswana and animal contact in Mozambique. However, in a study in Malaysia for children over two years of age, prolonged lactation was a negative factor. Again, in immunocompromised HIV/AIDS persons, male gender, and sharing water sources are risk factors. Most of the studies in underdeveloped areas show that populations are highly immune. Cryptosporidiosis tends to be anthroponotic more than it is zoonotic in these countries. However, the incidents are still underreported and underdiagnosed. Most of these populations are asymptomatic, possibly because higher rates of exposure increase the amount of immunoglobin-Es people have against the protozoa which might help reduce infection severity.

Figure 9. This shows the percentage of immunocompromised individuals with Cryptosporidium (30).
Developed Countries

Developed Countries show even distribution of cases among adults and children, immunocompetent and immunosuppressed individuals, likely because less contact with animals and the environment has reduced transmission and means most people lack immunoglobin-Es against the parasite. In contrast to the developing countries, developed countries' risk factors include recreational waters, foodborne, traveling, waterborne, and animal contacts. In industrialized areas, infections with both *C. hominis* and *C. parvum* affect people equally. Records show that cases experience mild to severe diarrhea, which makes diarrhea the most common symptom. Diarrhea lasts about two weeks in the immunocompetent, and the immune system usually takes care of it without special treatment (3,4,6,7,11,13).

Children, the elderly, individuals with malnutrition, and various conditions of infectious, immune individuals such as HIV/AIDS, are at high risk of being hospitalized from cryptosporidiosis. Abdominal pain and gastroenteritis are also prominent signs of the infection. Immunosuppressed patients have also complained of non-gastrointestinal symptoms like hepatitis, pulmonary diseases, and cholecystitis (3,7).

Surprisingly, industrialized locations have proven to have more outbreaks than expected in comparison to unindustrialized places. Many factors may account for this difference, and they can involve reduced immunity due to better sanitation and hygiene, which minimizes the encounter of diseases (30). New Zealand, for instance, has a history of outbreaks despite the developed health and living conditions. In 2001 and 2002, 32.3 and 26.1 out of 100,000 in a population were diagnosed with *cryptosporidiosis in New Zealand*. The situation still persists as the data in 2013 reported 30.3 per 100,000 area and
12.9 per 100,000 area in 2014. New *cryptosporidium* species—*C. meleagridis* and *C. cuniculus*—have also appeared in patients, raising more concerns in New Zealand (13).

A study in England documented 3,500 cases of cryptosporidiosis in 2013 and 5,500 in 2015. Common symptoms patients reported include onset of profuse diarrhea followed by vomiting, weight loss, and abdominal pains. More advanced cases of diarrhea caused fever, fatigue, muscle spasms, and weakness. Durations of these signs were between 3 weeks to 12.7 days (7).

In southwest Ontario, Canada, about 3 cases of cryptosporidiosis per 100,000 population is the current projection of the infection. Transmission sources are more zoonotic and associated with livestock, municipal, and surface waters (4). Other studies have related the sources to agricultural practices because many cases are caused by foodborne including some kinds of seafood in higher quantity (21). A study showed almost all dairy calves in southern Ontario carry *C. parvum*, and the human cases are at their peak during the spring. *C. hominis*, on the other hand, rises during summer through mid-fall (4).

A 2018 study focused on the immunocompromised person in France demonstrates the high-risk organ transplant patients face due to cryptosporidiosis. Usually, the infection manifests within the first six months of transplantation and accounts for 49% of the cases. HIV/AIDS patients followed, marking 30% of the cases; in 2010, the majority of infection was in HIV/AIDS patients. Although the persons experienced the same symptoms, their conditions shorten the duration and are highly life-threatening. Most of the exposure in France is drinking water, including bottled water (11).

The history of *Cryptosporidiosis* outbreaks in the United States shows an increase of about 13% per year. Public health agents documented 444 waves
of *Cryptosporidiosis* epidemic in forty states and Puerto Rico from 2009 to 2017. Exposures include bovine contact causes 14.6% of these outbreaks, childcare institutions account for 12.6%, and about 35.1% of the waves are related to recreational waters. These 444 outbreaks resulted in 7465 cases, of which 287 involved hospitalization and one patient died. According to the CDC, 1020 laboratories reported cases of *cryptosporidiosis* by July of 2021. Between July and September of 2021, the laboratories reported an increase in 402 *cryptosporidiosis* patients.

In the industrialized countries, *C. parvum* is a potential cause of irritable bowel syndrome (IBS) if infection symptoms are prolonged (6,7,24, 25,30). These IBS symptoms can extend to a year after infection (7). Studies show that the outbreaks are seasonal, depending on temperature and moisture in the air. Seasonal waves are associated with specific species; *C. parvum* prevails in spring, and *C. hominis* proceed when the weather is warmer through fall (4,13).

<table>
<thead>
<tr>
<th>Reasons</th>
<th>Developing Countries</th>
<th>Developed Countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drinking Water</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Recreational Water</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Crowd areas</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Transplantation</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Zoonotic rather than anthroponic</td>
<td>Low</td>
<td>leveled</td>
</tr>
<tr>
<td>Anthroponotic rather than Zoonotic</td>
<td>High</td>
<td>leveled</td>
</tr>
</tbody>
</table>

Table 3. Summary of the difference between industrialized and non-industrialized countries in *Cryptosporidium* transmission methods (30).

**Immunity Against Cryptosporidium**

Human immunity to *cryptosporidiosis* is still unclear, and the studies about this process are inadequate to explain the whole mechanism incorporated. Several studies show
that in the case of *C. parvum* attack on the host's epithelial cell, the parasite transports its RNA transcripts into the host's cell, which reduces gene transcription of the host's cell, and facilitates the parasite's survival. A recent study in 2017 revealed that, through epigenetic histone methylation, *Cryptosporidium* transports Cdg7_Flc_0990 into the host's epithelial cells. This transport of the parasite's proteins causes the suppression of the transcription of interleukin 33 (IL33) genes, LRP5, and SLC7A8 in the host's cell (DOP).

**Prevention Methods**

Waterborne transmission is by far the most source of *Cryptosporidium* worldwide. Poor drinking water is still an issue in developed countries, as it is in developing countries for different reasons. Oocysts can escape purification methods and enter municipal water in developed areas with cleaner drinking water. In a few cases, oocysts got into bottled water. Recreational water is another significant water source related to these places. Incontinence individuals, including diapers-wearing toddlers and children, is a risk factor. Sharing water sources is the second major factor. In contrast to non-industrialized regions, water and water treatment techniques are generally inadequate.

People can preclude the potential of contracting cryptosporidiosis by practicing sound sanitation and good personal hygiene. Based on the global analysis of the cases of cryptosporidiosis, humans transmit the disease to one another easily and quickly. With waterborne, the best prevention methods involve personal hygiene and avoiding sharing public water sources if possible. Studies show that the oocyst cannot resist too low and too high temperatures, so using this procedure may reduce the contacts.
Hygienic food handlers can reduce exposure to foodborne tremendously. Over 30 articles demonstrate that food handlers can prevent incidents of foodborne exposure to Cryptosporidium. Correct personal hygiene and sanitation of people in agriculture, such as farmers, food transporters, dairy producers, and commercial food workers such as retailers, caterers, and waiters, is crucial. Sanitation of places where food is handled is equally a critical precaution measure. Individuals serving food at home should carefully wash food and vegetables before consuming them. Proper cooking of food is also a critical factor in reducing infections. Especially seafood, well cooking eliminates its risk of Cryptosporidium (22).

Airborne Transmission is a sporadic case, and very little research mentioned it. Immunocompromised and people with pulmonary diseases are at higher risk of being victims. Standard airborne precautions like wearing a mask are of importance.

Person to Person & Animal to Person Transmission: This contact method, as the passed methods, requires personal hygiene and quality sanitation for obviation. As fomites and surfaces are at high risk of contamination, proper handwashing techniques and regular sanitation of such faces are essential. Farmers and pet owners need to monitor and isolate animals until cured. Teachers, daycare personnel, industry, and institutional workers must also pay attention to prevent exposure (18).

Control/Treatment

Individuals infected with cryptosporidiosis are treated based on each case's unique symptoms. While treatment methods are still under various studies, the US Food and Drug
Administration (FDA) approved only one medication, nitazoxanide (NTZ), to treat diarrhea related to Cryptosporidium (17,23,24). Clofazimine (CFZ) is a new potential medication as studies show its effectiveness in treating Cryptosporidium in vitro. CFZ is currently used to treat leprosy and is part of resistant tuberculosis (TB) multidrug treatment. CFZ is effective for TB and leprosy, but the available research results do not suggest using it as a Cryptosporidium drug (17).
Due to the intractable nature of Cryptosporidium, identifying it among other protozoans is challenging, especially with a low-cost budget. Previous studies show several images of Cryptosporidium; however, there is still uncertainty about its physical appearance that can differentiate it from other protozoa. Different staining methods are difficult to achieve for some types of samples that contain lots of organic material, animal cells, and other microbes. In this lab, we use three approaches to determine the best technique that enhances the viewing of Cryptosporidium under the microscope. We use samples of water, cow feces, and soil/bedding from farms, assuming that the University of Maine J. Witter Research and Teaching Farm would have some protozoa present. We also collected samples of water from puddles on the farm, the Penobscot river, and a small stream in Orono to use for comparison.

**Research Question & Goal**

What staining method allows the visualization of Cryptosporidium under the microscope and what other microbes in the samples do they stain? In addition, this research aims to train undergraduates on effective lab techniques and several staining methods.

**Methodology**

Sample fetching: We collected three different forms of samples from the University's farm: water, soil, and feces. We collected the sample in accordance to reduce contamination by first using hand sanitizer, wearing latex gloves, and collecting ‘cleaner’
samples first. Starting with the water sample, we fetched from each calf's drinking water in a labeled 50 ml plastic centrifuge tube. In the same way, we used a 50-ml labeled tube to collect the soil, and another to collect the feces samples, changing gloves in between. We collected samples from two dairy calves (1- to 3-week-old calves) kept in separate but adjacent pens in the calf barn at the Witter Farm. We collected samples from multiple days over the span of a week in August 2022.

Slides Preparation: The slides preparation is either by directly putting/smearing the samples on the glass slides or using the **floatation method**. In this method, we used a flotation cup to mix the specimen with a dense liquid and allow it to sit for at least 20 minutes to let the oocyst float and settle on the slides. Appendix 1 contains a detailed explanation of the steps.

We then allowed the slides to dry at room temperature or incubate them in the chamber base on our protocol and precede staining them. We used three staining procedures:

1. Putting a drop of iodine on the slides and then sealing the slide for viewing under the microscope (Appendix I).
2. Staining the slides based on the Modified Ziehl-Nelson protocol (Appendix II).
3. Using the crypt-a-glo kit (Appendix III).

Microscopy: We used three forms of the microscope to view the slides. 1. The simple light microscope to view both slides with iodine and the modified Ziehl-Nelson stain, 2. The Inverted compound microscope also views both slides with iodine and the modified
Ziehl-Nelson stain, and 3. the fluorescent microscope views the slides with crypt-a-glo stain.

We assessed results by visual inspection to determine the size of the microbe in question as Cryptosporidium is 4 - 6 µm in size and can only be seen easily at 100X. We also assessed results by whether the stain highlighted the cells as expected based on previous literature and examples on the CDC website.

**Results**

Out of over 100 images we took in this research, these three pictures below show a synopsis of them. A couple more images from this study are in appendix 4.

![Images](image1.png)

Figure 10. Staining with Modified Ziehl-Nelsen, at 100x from a puddle at the University's farm. We concluded that cysts stained green. Therefore, white arrows are possible cysts formed in the cell wall of the two large protozoa (uncertainty due to lack of adequate information on how the stain works on protozoa). The black arrows point to bacterial endospores. **B.** Iodine stain under Inverted light Microscope. The black arrow points at Cryptosporidium at 100x from a positive control sample. **C.** DAPI stain under the fluorescent microscope at 10x from a puddle at the University's farm.

**Discussion**

All the staining methods effectively stain protozoans of some kinds. However, it was challenging to confirm if they are Cryptosporidium or not. Ruminant animals, like cows, routinely carry certain types of protozoa in their digestive tract which aid in food
digestion and are beneficial. Livestock and other animals living outside commonly pick up protozoa from their environment. Previous studies of the parasite show several images of it with different shapes and sizes, making it difficult to verify that we viewed Cryptosporidium. However, comparing our images with previous studies, we identified some likely protozoans, including Coccidia, Giardia, Amoeba, and possible Cryptosporidium.

Although there are so many discrepancies in the color and what each staining method is supposed to stain, considering other characteristics of the protozoans’ cells facilitates recognizing them. The modified Ziehl-Nelson staining method enables the best projection of the protozoans, followed by Iodine staining, and the crypt-a-glo stains the least with the current limitations we faced. The likely major setback of the crypt-a-glo staining is the camera, as it is not the correct camera for viewing fluorescent stains. The camera continuously lost focus and needed to be readjusted. A new camera and updated video-imaging software would resolve this issue.

Generally, Cryptosporidium studies are insufficient to support observing and detecting it through microscopy, and many studies now rely on genomic identification which can be expensive. Due to the difficulty in confirming the microscopic visualization of the Cryptosporidium in animal feces and soil samples, the samples need at least confirmation of the parasites' presence. Also, obtaining larger samples from many hosts can help confirm the results.

This research has successfully enabled me to review and practice my laboratory procedures. I learned productive ways to collect samples from multiple hosts. By preparing over 50 slides, I am now more comfortable with the process. In addition, focusing on the
various staining methods has helped me understand and project some gaps between the studies. Although it was challenging to recognize the *Cryptosporidium* protozoa from the others, the research provides useful information that could help future studies.
REFERENCES


35. Eltahan R, Guo F, Zhang H, Xiang L, Zhu G. Discovery of ebselen as an inhibitor of Cryptosporidium parvum glucose-6-phosphate isomerase (CpGPI) by high-throughput


APPENDICES
APPENDIX I:

About: The floatation method uses dense liquids which have a higher specific gravity than parasite eggs or cysts, causing them to float to the top of the solution.
- Specific gravity is the weight of an object compared to the equal volume of water
- Specific gravity of water is 1.0, and most parasite eggs are 1.05 - 1.24 (heavier than water)
- Floatation solutions should be >1.24 to be heavier than parasite eggs

Choose a floatation solution which is heavier than the parasite you are detecting. Our preferred solution is:

1. **Table sugar/sucrose: 454 g dissolved in 355 ml water = 1.27 sp.g.**
   a. You must heat the water to get the sugar to dissolve
   b. After the sugar dissolves, you must add 2 ml of 37% formaldehyde (10% formalin) to preserve the sugar solution

Watch a video of the flotation prep here: [https://youtu.be/GbO5NW0w99Y](https://youtu.be/GbO5NW0w99Y)

To create the floatation:

1. Remove the green/blue center from the floatation cup, and use the bottom to jab into a fecal sample. Try to get 1 gram or about ½ of feces in the bottom of the tube.
2. Place the green/blue center back in the cup, and fill only halfway with fecal floatation solution.
3. Use the green/blue center to mix the sample up and down and get it to mix with the solution. You need to dislodge any parasites that are in the feces.
4. When you are done mixing, push the blue/green center down in the cup so that there is no gap between them on the rim (otherwise it will leak when you add more solution.
5. Fill the vial with enough flotation solution so that the meniscus is just level with the top of the tube.
6. Place a glass coverslip on top of the tube and allow it to stand for at least 20 minutes.
7. Liftoff coverslip and place on top of a glass slide, examine under 10X and 40X magnification, condenser in the down position, with low light. A drop of Lugol’s
iodine can be placed on the slide before placing the coverslip to enhance the identification of Giardia cysts.

8. Read the slide by scanning across side to side, from top to bottom. Be consistent in how you scan the slides, to make sure you’ve looked at all of it without repeating the same areas.
APPENDIX II: FECES SAMPLES

Collection:
You may not collect feces unless you have completed the IACUC training and have been included on our approved application. [IACUC application and related documents](mailto:sue.ishaq@maine.edu) for details.

Collect at least 5mL of feces into a 15ml or 50mL centrifuge tube with 90% ethanol to cover the sample, labeled with date, cow name/ID, and the pen that cow is in. Label as sample type (F = feces, S = soil, W = water).

Storage between collection and processing
Store in Rogers 110 fridge (4°C) until ready for processing, can be stored up to 1 week but number of parasite cysts will decay over time. Try to process them within 48 hours if possible.

Add information to the Sample tracking sheet
Microscopy to look for cysts or other parasite eggs:
For making fecal smear (CDC - DPDx - Diagnostic Procedures - Stool Specimens)
Place a small amount (about the size of your pinky finger nail) of stool on the slide and smear it so that it creates a thin area (thin enough for light to shine through, if too thick you will not be able to see anything in the microscope) to look at. This are does not have to be very large
If the sample is too solid, add a few drops of ultra-pure water and mix/spread out the sample so that it is not a clump and can be seen under a microscope.
Add a drop of ultra-pure water on top of the smear to act as mounting medium, and place coverslip on the slide.
View under microscope, a reference slide can be found here
https://www.merckvetmanual.com/multimedia/image/cryptosporidium-parvum-oocysts-fecal-smear (this uses Ziehl-Nelson method but does not have magnification labeled, the image is from a fecal smear)
https://www.researchgate.net/figure/Smear-shows-numerous-round-to-oval-oocysts-of-cryptosporidium-in-a-direct-smear-prepared_fig1_267932685 (modified ziehl-nelson stain used, 1000x magnification which is what our oil immersion is)
Cryptosporidium spp. oocysts are rounded and measure 4.2 to 5.4 µm in diameter
Start at 4x and focus. Then continue to the higher lenses and focus each time. The highest lens is oil immersion, place a drop of the immersion oil on the slide where the light comes through, DO NOT TURN TO THE OIL IMMERSION LENSE WITHOUT OIL ON THE SLIDE.
When you are done using the oil immersion lens dab the lens with lens paper. There is also oil immersion cleaner in the drawer. To use this put a drop of the cleaner on the lens paper or kimwipe if you are cleaning the lens or slide.
Record all findings here Sample tracking sheet

Soil Samples
Collection:
Collect the top 1 inch of soil into a collection container (50mL tube) fill with 90% ethanol, labeled with date, location/which pen, and location within pen if relevant (e.g. next to water trough). Label as sample type (F = feces, S = soil, W = water).

Storage between collection and processing:
Store in Rogers 110 fridge (4°C) until ready for processing, can be stored up to 1 week but number of parasite cysts will decay over time.

Add information to the Sample tracking sheet

Microscopy to look for cysts or other parasite eggs:
Protocol is modified from Hong et al. 2014: Detection of Cryptosporidium parvum in Environmental Soil and Vegetables

Filter soil through cheesecloth by putting cheesecloth over a 50mL centrifuge tube or 200mL beaker (make sure it is clean), putting the soil in the cheesecloth and squeezing it, run DI water through cheesecloth if solution is too thick/you don’t think you would be able to see through it on a microscope

Alternate method still being developed

Put soil in drawstring teabag
Stuff tea bag into 50mL centrifuge tube, fill with saturated NaCl solution all the way to make it convex, place slide on top of tube and wait 30 min

Place coverslip on microscope and view under microscope

Use the supernatant to make a slide for staining. Use a plastic pipette dropper so place one or two drops of solution onto slide

Allow slide to dry (if no water was added and it is just the ethanol from the soil samples, it will dry quickly, if not, placing them under the biosafety hood in 111 with the fan on will make them dry faster (only if you are trained to use the hood))

Stain according to Modified Ziehl-Nelson procedure found later in the document

View under microscope, a reference slide can be found here

https://www.merckvetmanual.com/multimedia/image/cryptosporidium-parvum-oocysts-fecal-smear (this uses ziehl-nelson method but does not have magnification labeled, the image is from a fecal smear)

https://www.researchgate.net/figure/Smear-shows-numerous-round-to-oval-oocysts-of-cryptosporidium-in-a-direct-smear-prepared_fig1_267932685 (modified ziehl-nelson stain used, 1000x magnification which is what our oil immersion is)

Cryptosporidium spp. oocysts are rounded and measure 4.2 to 5.4 μm in diameter

Start at 4x and focus. Then continue to the higher lenses and focus each time. The highest lens is oil immersion, place a drop of the immersion oil on the slide where the light comes through. DO NOT TURN TO THE OIL IMMERSION LENSE WITHOUT OIL ON THE SLIDE.

When you are done using the oil immersion lens dab the lens with lens paper. The slide can be cleaned with kimwipes. There is also oil immersion cleaner in the drawer. To use this put a drop of the cleaner on the lens paper or kimwipe if you are cleaning the lens or slide.

Record all findings here Sample tracking sheet

Water Sample
Collection

Water Sample Collection
Collect water from animal pen troughs or standing water in puddles in the pen, in a 50mL centrifuge tube. Label with date collected, and which pen collected from. Label as sample type (F = feces, S = soil, W = water).

Storage between collection and processing:
Store in Rogers 110 fridge (4°C) until ready for processing, can be stored up to 1 week but number of parasite cysts will decay over time.
Add information to the Sample tracking sheet

Microscopy to look for cysts or other parasite eggs:
Protocol from: Assessment of Giardia and Cryptosporidium Assemblages/Species and Their Viability in Potable Tap Water in Beni-Suef, Egypt Using Nested PCR/RFLP and Staining - PMC

Place a folded Whatmans filter (so that it looks like a funnel) on top of a new 50mL centrifuge tube, use a 1 um pore size, whatman filter size 5, 6 or 597
Pour the water sample through the paper membrane filter slowly, and try to concentrate it on a small surface area
Take the wet portion of the filter paper and place in a small tube, cover with PBS
Allow that to sit for at least 15 minutes, then centrifuge for 10 minutes at room temperature or cooler, at 6000 x G (RCF)
Remove the supernatant and use a plastic dropper pipette to place one or two drops from the very bottom of the tube onto a slide
Allow slides to air dry
Stain samples using Modified Ziehl-Nelson protocol below
View under microscope, a reference slide can be found here
https://www.merckvetmanual.com/multimedia/image/cryptosporidium-parvum-oocysts-fecal-smear (this uses ziehl-nelson method but does not have magnification labeled, the image is from a fecal smear)
https://www.researchgate.net/figure/Smear-shows-numerous-round-to-oval-oocysts-of-cryptosporidium-in-a-direct-smear-prepared_fig1_267932685 (modified ziehl-nelson stain used, 1000x magnification which is what our oil immersion is)
Cryptosporidium spp. oocysts are rounded and measure 4.2 to 5.4 µm in diameter
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When you are done using the oil immersion lens, dab the lens with lens paper. The slide can be cleaned with kimwipes. There is also an oil immersion cleaner in the drawer. To use this, put a drop of the cleaner on the lens paper for cleaning lens or kimwipe for cleaning slides.
Record all findings here Sample tracking sheet
APPENDIX III

AUTHOR BIOGRAPHY

Amatullah Ahmad is an Honors and Biology student at the University of Maine.