Review of Prevalence and Diagnosis Methods of Cryptosporidium Spp

***Sabaa muhsen Farhan,**Ashraf Jamal Mahmoud Zangana, ***Mohammed J. Muhaidi, ****Akram Jassam Mohammed**

* University of Tikrit , collage of science, Tikrit, Iraq
** University of Tikrit , College of Education Women, Tikrit, Iraq
*** University of Fallujah · College of Applied Science, Al-Anbar, Iraq
****Ramadi Teaching Hospital For woman and children, Al-Anbar, Iraq
Corresponding Author:sabaamuhsen@gmail.com

Abstract

Worldwide, the apicomplexan parasite *Cryptosporidium* is responsible for significant diarrheal illness in both people and animals. The epidemiological, molecular, and clinical illness load of untreated *Cryptosporidium* infections in people in Al-Anbar province are all summarized in the current review. The disease is extremely common in both humans and animals, according to reports that have been retrieved about cryptosporidiosis in Iraq. However, the conclusions drawn from these reports are unclear and frequently used conventional techniques for detecting the infective stage of *Cryptosporidium*, the oocysts, in clinical samples. Numerous screening surveys are point prevalence studies that reported cryptosporidiosis as the cause of diarrhea in newborns and young children; other infections that cause diarrhea were not eliminated. Numerous investigations using a variety of environmental matrices revealed a high incidence of *Cryptosporidium* oocysts consuming tap water, which enables its spread to people and animals, in various regions of Iraq. There aren't many reports on the molecular characterisation of the various *Cryptosporidium* species found in Iraq, although both Cryptosporidium hominis and *Cryptosporidium parvum* have been found in human isolates, with the latter being more common in cattle, sheep, goats, and birds. To accurately determine the epidemiological condition of cryptosporidiosis, a nationwide investigation on sufficient numbers of samples from people of all ages in Al-Anbar province using cutting-edge diagnostic techniques is necessary. To further identify the species and subtypes of *Cryptosporidium* infecting people and animals, particularly during outbreaks, molecular genotyping research must be carried out in Iraq. Consequently, the parasite *Cryptosporidium* should be detected and monitored regularly.

Keywords: *Cryptosporidium* , *Cryptosporidium parvum*, Diarrhea, Human, diagnosis

Background

A harmful protozoan called *Cryptosporidium spp*. may be found in many hosts' gastrointestinal tracts. Ernest Edward Tyzzer originally identified this parasite in the gastrointestinal epithelium of mice in 1907. The first human infection was documented in 1976, occurring in both an adult and a kid during the same year. Due to outbreaks in daycare facilities, immunocompromised individuals, and instances of waterborne transmission, Public health worries have been raised by *Cryptosporidium spp* (Meireles 2010). *Cryptosporidium spp* is among the common waterborne parasites and a significant public health problem globally due to the resistance of its oocyst and the infectious form to most of the disinfectants used in water treatment plants. (Baldursson and Karanis 2011). The Coccidia Class was the original classification for this protozoan., but has recently undergone a gregarine classification in light of studies(Carreno, Matrin, and Barta 1999)(Karanis and Aldeyarbi 2011) that tracked the stages of evolution following the excision and sequencing of the 18S rRNA gene. In terms of morphology and phylogeny, *Cryptosporidium spp*. are more closely related to gregarine than to coccidia. The scientific community did not adequately address these findings until Clode *et al*.(Clode, Koh, and Thompson 2015) outlined them. Because of this, *Cryptosporidium spp.* is now classified in the subclass Cryptogregaria of the Class Gregarinomorphea, an epicellular class that includes organisms with an epimerite, a feeding organelle remarkably similar to that of gregarines but without apicoplast .It was also found through in vitro and in vivo research, that *Cryptosporidium spp*. may complete its development stages independently of a host cell(Gunasekera *et al*. 2020).

Cryptosporidium spp. can develop biofilms in water or sewage treatment facilities, increasing the risk of outbreaks and thereby creating serious issues in the effective management of these resources. Additionally, microbial biofilms could develop in the human gut, exposing the host to infections both during and after

they have already occurred. Molecular biology methods have emerged as the basic tools for studying the epidemiology of numerous infectious agents, including *C. spp*. By using molecular techniques in taxonomy, Xiao *et al*. claim that one of the criteria that can be used to confirm the *Cryptosporidium* species evaluation is genetic data(Xiao and Feng 2017). There are currently 38 species of *Cryptosporidium* known, which include parasites that infest both mammals and amphibians(Fayer 2008).There are more than 40 genotypes that infect animals, but the two species most usually associated with human infections are *Cryptosporidium parvum* and *Cryptosporidium hominis*(Khan, Shaik, and Grigg 2018).

Molecular techniques usually rely on the 18S rRNA genes for the characterization of the *Cryptosporidium* genotype because they have 5 copies in each genome and exhibits low rates of variation due to a slower rate of evolution. Therefore, they are the preferred locus for animal samples that might harbor genotypes or species that have not yet been classified. The most popular technique for determining *C. hominis* subtypes in humans and *C. parvum* subtypes in ruminants and humans is the analysis of *GP60* glycoprotein in the DNA sequence(Xiao 2010) ; this gene is used for this purpose because of its tandem terminal repeats of the *TCA*, *TCG*, or *TCT* trinucleotides, thereby resembling a microsatellite sequence (also known as gp15/40). The classification of *C. ubiquitum*, *C. andersoni*, *C. parvum*, *C. hominis*, and *C. meleagridis* into families can also be based on the diverse subtypes, in addition to the differences in the levels of trinucleotide replications in sequences on regions without repeats because it is the marker in the genome of *C. spp* (the most observed polymorphism till date). In the human body, one of the primary targets of the human neutralizing antibody responses is the *GP60* protein; this protein is secreted by the *gp60* gene and detectable on the surface of the apical region of the invasive phases of the parasite(O'Connor *et al*. 2007).

The two *Cryptosporidium* species, *C. parvum* and *C. hominis*, are the subject of ongoing study into their families and subtypes, with some of the findings coming from other parts of the globe. The *Cryptosporidium* species has been divided into the following subtypes: *C. hominis* (Ia-Ik) has ten subtypes while *C. meleagridis* and *C. parvum* (IIa-IIt) have ten and nineteen subtypes, respectively(*Abe et al*. 2006) . Compared to *C. hominis* which is mostly found in Australia, England, and Portugal, families Ia, Id, and Ie are mostly seen in India, Peru, Kenya, Malawi, and the USA(Alves *et al*. 2006). If, Ib, and Id are the three families that are most frequently detected in South Africa; some samples from Portugal have also produced evidence for the If family(Leav *et al*. 2002) . Despite being thought of as a *Cryptosporidium* species unique to humans, *C. hominis* is being found in an increasing number of cases in animals. Dogs, rats, kangaroos, sheep, and goats have all been found to have *C. hominis*. The cosmopolitan subtype IbA10G2 is the most prevalent subtype in humans in these areas and is associated with animal infection(Zhang *et al*. 2020).

In addition to the *18S rRNA* and *gp60* gene targets, Other genes have also been utilized as molecular tools for the characterization of *C. species* and its sub-types following the availability of numerous molecular techniques (Table 2). When infected with *Cryptosporidium spp*., a person may display no symptoms, as only moderate clinical signs, like spontaneous diarrhea, or other more serious symptoms may occur. These people could develop a condition that lasts for months or even years. Cryptosporidiosis can be complicated in some cases and can cause the death of up to 50% of AIDS patients. In other cases, the lungs, pancreas, and hepato-biliary system may also be affected by cases of extra-intestinal cryptosporidiosis. It has been noted that the second-most prevalent symptom after intestinal infection is biliary cryptosporidiosis; this condition has been established in about 26% of AIDS patients.Specialized research is inadequate as a result of the rarity of respiratory tract infections and the possibility of underdiagnosis. The signs of this condition include chest pain, fever, coughing, dyspnea, and radiological abnormalities resembling those observed in other lung infections(Albuquerque et al. 2012).

Table 1 - *Cryptosporidium* species

Species	Main host	Site of infection
C. hominis	Human	Small intestine
C. parvum	Ruminants	Small intestine
C. meleagridis	Birds and human	Small intestine
C. felis	Cats	Small intestine
C. canis	Dogs	Small intestine
C. ubiquitum	Cattle, Ruminants, rodents,	Intestine

	primates	
C. viatorum	Human	Small intestine
C. cuniculus	European rabbits	Intestine
C. andersoni	Cattle	Abomasum
C. suis	Pigs	Small and large intestine
C. bovis	Cattle	Small intestine
C. erinacei	European hedgehog, horses	
C. scrofarum	Pig	Intestine
C. tyzzeri	Mouse, Rodents	Small intestine
C. xiaoi	Sheep and goats	
C. muris	House mouse, Rodents	Stomach

Table 2- lists the techniques for genotyping and subtyping *Cryptosporidium* using molecular epidemiology.

Target

- *SSU Rrna* - (*18S rRNA* gene)
- *60-kDa glycoprotein* gene - (*gp60*)
- *70-kDa heat shock protein* gene - (*hsp70*)
- *Oocyst wall protein* gene
- *Actin* gene
- *β-tubulin* gene
- *Thrombospondin-related adhesive protein* gene (*trap-C1 ad trap-C2)*
- *ITS1 of rRNA*
- *Dihydrofolate reductase* gene - *(dhfr)*

Molecular techniques

- Single-round and nested PCR
- PCR-RFLP
- Real-time PCR
- Single strand conformation polymorphism (SSCP) analysis
- Melting curve analysis
- Microarray
- DNA sequencing
- Multilocus fragment (MLFT) - sequence (MLST) typing
- New generation sequencing (NGS)

Laboratory diagnosis

If a patient has human cryptosporidiosis, it may be determined in the lab by looking for antibodies against *Cryptosporidium spp*., as well as antigens or genetic material. For a very long time, The diagnosis involves the flotation or centrifugation-sedimentation procedures used to concentrate the oocytes of the parasite during the parasitological investigation of stool samples (SPE). Monoclonal antibodies used in ELISA or immune-chromatographic testing have also been used for the detection of specific antigens of the parasite in fecal samples(McHardy *et al*. 2014). These monoclonal antibodies are also used to develop oocyst purification methods that dramatically improve the ability to identify parasites in environmental samples. These methods include the use of paramagnetic beads that have been adsorbed to anti-*Cryptosporidium* monoclonal antibodies for fluorescence-based detection. However, the speciation of *C. spp*. in samples cannot be achieved using these techniques. The molecular methods of parasite-specific DNA sequence identification have enabled not just a thorough evaluation of the taxonomy of species but also improved laboratory diagnosis by facilitating the classification of species and subtypes within these species (Khurana and Chaudhary 2018).

Stool parasitological examination

The aim of the microscopy detection methods based on stool sample concentration is to improve the chances of parasite detection in stool samples by comparing the densities of various fecal components, such as debris and lipids that might obscure parasite visibility. The two most often used techniques for concentrating oocysts are sedimentation utilizing gradients of ether- and formalin-based solvents, and flotation in saturated sucrose solutions (Khurana and Chaudhary 2018). For improved oocyst visualization, light field or phase contrast optical microscopy is indicated(Kar *et al*. 2011) . Oocysts look clear under a light field microscope, typically as slightly pinkish forms with granules within. Oocysts appear as sparkling bodies with black granules in phase contrast. . In 1923, Sheather's solution was introduced as a way of aiding the process of identifying different forms of parasites in animal fecal samples via a simple centrifugation-flotation method that requires a centrifugation-flotation process Sheather's solution (a hyper-saturated sucrose solution). This method causes parasitic structures to fluctuate so they can later be retrieved. This method requires the sedimentation of detritus in a high-density sucrose solution via centrifugation to promote the

separation of coccidian oocysts and protozoa cysts, The density gradient and fluctuation of parasite evolutionary forms (such as cysts and Oocysts) are facilitated during the centrifugation step by introducing additional solutes, such as zinc sulfate into the centrifugation mixture. Staining procedures are always performed after the concentration steps to improve the visibility of the cysts. Studies on coccidia have utilized a variety of staining methods, such as Kinyoun, Ziehl-Neelsen, methylene blue, modified Koster staining, negative staining, safranin, auramine, etc. to improve the visibility of cysts (Mirhashemi *et al*. 2015).

The most widely used method for detecting *Cryptosporidium spp*. oocysts is still hot-resistant acid staining because of its cost, ease of use, and enhanced dye setting. The morphological analysis of oocysts cannot be used to identify *Cryptosporidium* species since they are so small (4–8 m), they may have imperceptible morphological differences, or they may be similar between different species(Fall *et al*. 2003).

The size of the oocysts may make them difficult to notice, hence it is critical to boost the sensitivity of these staining techniques. Additional factors decreasing the sensitivity of this technique for morphological assessment include the occasional removal of cysts from feces and the necessity of professional detection and identification of parasites. False-positive results can also result from misclassifying staining yeasts such *Cryptosporidium spp*. and fat bubbles. Other stains that can be used in coccidia research include Malachite Green, Giemsa, Auramine, Nigrosin, and Light Green. It is difficult to standardize the diagnosis process for these protozoa in diverse clinical laboratories since the sensitivity and specificity of these dyes are uncertain. The histological study using autopsy and biopsy materials may also be frequently employed to determine the parasite's identity and track its evolutionary forms(Chalmers and Giles 2010).

The frequently used method of hematoxylin-eosin staining is used to see *Cryptosporidium* in tissues. Basophilic spherical structures, measuring 2.0 to 7.5 m, can be detected on the surface of epithelial cells in histological sections. Two more techniques that can be used are stains composed of silver and Schiff periodic acid. Transmission electron microscopy can be used to examine the parasites' ultrastructural characteristics(Valigurová *et al.* 2008) . Although the basic approach for identifying *Cryptosporidium spp*. is fresh or formalin-preserved fecal analysis, the parasitological diagnosis has a number of shortcomings: It takes time, requires a skilled microscopist to detect the organisms, and may not be very sensitive. Another use for which this method fails is the examination of samples that have been subjected to adverse conditions, such as frozen feces or environmental samples, which may alter the morphology of the parasite. It's crucial to remember that employing microscopic methods makes it impossible to determine the species or genotypes that are causing the disease(Jex *et al*. 2008).

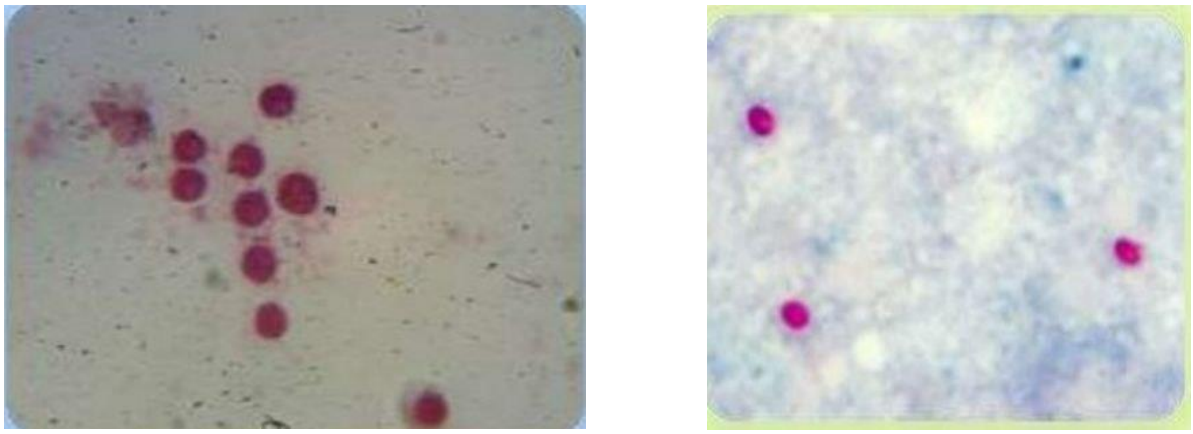

Figure 1*: Cryptosporidum spp*. under microscope(100x) with Ziehl-Neelsen

Immunological methods

Immunological methods are available for the rapid, accurate, and straightforward detection of antigens. For species differentiation in immunological research, Antigens can also be easily, quickly, and accurately detected using immunological techniques. Monoclonal antibodies produced against specific targets are needed for species differentiation in immunological studies. Even though these monoclonal antibodies are more expensive, they are more sensitive than the conventional staining methods. The diagnosis of diseases caused by *Giardia lamblia*, *Entamoeba histolytica/Entamoeba dispar*, or *Cryptosporidium spp*., based on the identification of antigens

(coproantigens) has been considered helpful because, in these disease states, there is a high rate of fecal clearance of the causative agents. The identification of *Cryptosporidium spp*. in water samples can also be done using immunological methods, such as indirect immunofluorescence techniques(Neto *et al*. 2010).

Analysis using the filtration-concentration methods requires different volumes of water depending on the type of water samples analyzed; this increases the sensitivity of the fluorescence-labeled monoclonal antibodies and *DAP* (4',6'-diamidino-2-phenylindole) in the detection of oocysts (Neto *et al*. 2010). The serum of infected persons can also be used for the detection of circulating antibodies, but the problem with this method is that it cannot be used to distinguish between the stages of infection (present or past); this has a negative consequence on the test value, especially when analyzing people from endemic regions with high parasite load. Another problem of this serum antigen-based method is that since several species under the genus *Cryptosporidium* share antigens, it is difficult to distinguish between species using the development of antibodies against oocyst wall antigens. To address this problem, scholars have recommended the development of specific, accurate, and sensitive molecular techniques for the detection of DNA fragments of parasites during active infections(Navarro-i-Martinez, del Águila, and Bornay-Llinares 2011) .

ACKNOWLEDGMENTS

This work was supported by department of biology, collage of science , Tikrit university.

References

1. Abe, Niichiro, Makoto Matsubayashi, Isao Kimata, and Motohiro Iseki. 2006. "Subgenotype Analysis of Cryptosporidium Parvum Isolates from Humans and Animals in Japan Using the 60-KDa Glycoprotein Gene Sequences." *Parasitology Research* 99 (3): 303–5. DOI: 10.1007/s00436-006-0140-0
2. Albuquerque, Yvana Maria Maia de, Márcia Cristina Fraga Silva, Ana Luiza Magalhães de Andrade Lima, and Vera Magalhães. 2012. "Pulmonary Cryptosporidiosis in AIDS Patients, an Underdiagnosed Disease." *Jornal Brasileiro de Pneumologia* 38: 530–32.
3. Alves, Margarida, Lihua Xiao, Francisco Antunes, and Olga Matos. 2006. "Distribution of Cryptosporidium Subtypes in Humans and Domestic and Wild Ruminants in Portugal." *Parasitology Research* 99 (3): 287–92. DOI: 10.1007/s00436-006-0164-5
4. Baldursson, Selma, and Panagiotis Karanis. 2011. "Waterborne Transmission of Protozoan Parasites: Review of Worldwide Outbreaks–an Update 2004–2010." *Water Research* 45 (20): 6603–14. DOI: 10.1016/j.watres.2011.10.013
5. Carreno, Ramon A, Donald S Matrin, and John R Barta. 1999. "Cryptosporidium Is More Closely Related to the Gregarines than to Coccidia as Shown by Phylogenetic Analysis of Apicomplexan Parasites Inferred Using Small-Subunit Ribosomal RNA Gene Sequences." *Parasitology Research* 85 (11): 899–904. DOI:10.1007/s004360050655
6. Chalmers, R M, and M Giles. 2010. "Zoonotic Cryptosporidiosis in the UK–Challenges for Control." *Journal of Applied Microbiology* 109 (5): 1487–97. DOI: 10.1111/j.1365-2672.2010.04764.x
7. Clode, Peta L, Wan H Koh, and R C Andrew Thompson. 2015. "Life without a Host Cell: What Is Cryptosporidium?" *Trends in Parasitology* 31 (12): 614–24. DOI: 10.1016/j.pt.2015.08.005
8. Fall, Abbie, R C Andrew Thompson, Russell P Hobbs, and Una Morgan-Ryan. 2003. "Morphology Is Not a Reliable Tool for Delineating Species within Cryptosporidium." *Journal of Parasitology* 89 (2): 399–402. DOI: 10.1645/0022-3395(2003)089[0399
9. Fayer, R. 2008. "General Biology in: Fayer, R. and Xiao, L. Editors. Cryptosporidium and Cryptosporidiosis. Edition. Taylors and Francis Groups. USA. Pp: 1-42. 28-Mohammed N. Al-Shahery and Manal H. Hassan.(2007). Immunological and Experimental Study for Cryptosporidiosis." *J. Iraq. Vet. Sci* 21 (1): 129–40. DOI:10.1186/1756-3305-1-47
10. Gunasekera, Samantha, Alireza Zahedi, Mark O'Dea, Brendon King, Paul Monis, Benjamin Thierry, Jillian M. Carr, and Una Ryan. 2020. "Organoids and Bioengineered Intestinal Models: Potential Solutions to the Cryptosporidium Culturing Dilemma." *Microorganisms* 8 (5): 715. DOI: 10.3390/microorganisms8050715
11. Jex, A R, H V Smith, P T Monis, B E Campbell, and R B Gasser. 2008. "Cryptosporidium—Biotechnological Advances in the Detection, Diagnosis and Analysis of Genetic Variation." *Biotechnology Advances* 26 (4): 304–17. doi:10.1016/j.biotechadv.2008.02.003
12. Kar, Sirri, Sandra Gawlowska, Arwid Daugschies, and Berit Bangoura. 2011. "Quantitative Comparison of Different Purification and Detection Methods for Cryptosporidium Parvum Oocysts." *Veterinary Parasitology* 177 (3–4): 366–70. DOI: 10.1016/j.vetpar.2010.12.005

13. Karanis, P, and H M Aldeyarbi. 2011. "Evolution of Cryptosporidium in Vitro Culture." *International Journal for Parasitology* 41 (12): 1231–42. DOI: 10.1016/j.ijpara.2011.08.001
14. Khan, Asis, Jahangheer S Shaik, and Michael E Grigg. 2018. "Genomics and Molecular Epidemiology of Cryptosporidium Species." *Acta Tropica* 184: 1–14. DOI: 10.1016/j.actatropica.2017.10.023
15. Khurana, Sumeeta, and Preeti Chaudhary. 2018. "Laboratory Diagnosis of Cryptosporidiosis." *Tropical Parasitology* 8 (1):2 doi: 10.4103/tp.TP_34_17
16. Leav, Brett A, Malanie R Mackay, Akudo Anyanwu, Roberta M O'Connor, Ana Maria Cevallos, Gurpreet Kindra, Nigel C Rollins, Michael L Bennish, Richard G Nelson, and Honorine D Ward. 2002. "Analysis of Sequence Diversity at the Highly Polymorphic Cpgp40/15 Locus among Cryptosporidium Isolates from Human Immunodeficiency Virus-Infected Children in South Africa." *Infection and Immunity* 70 (7): 3881–90. DOI: 10.1128/IAI.70.7.3881-3890.2002
17. McHardy, Ian H, Max Wu, Robyn Shimizu-Cohen, Marc Roger Couturier, and Romney M Humphries. 2014. "Detection of Intestinal Protozoa in the Clinical Laboratory." *Journal of Clinical Microbiology* 52 (3): 712–20. doi: 10.1128/JCM.02877-13
18. Meireles, Marcelo Vasconcelos. 2010. "Cryptosporidium Infection in Brazil: Implications for Veterinary Medicine and Public Health." *Revista Brasileira de Parasitologia Veterinária* 19: 197–204. https://doi.org/10.1590/S1984-29612010000400002
19. Mirhashemi, Marzieh Ezzaty, Annetta Zintl, Tim Grant, Frances E Lucy, Grace Mulcahy, and Theo De Waal. 2015. "Comparison of Diagnostic Techniques for the Detection of Cryptosporidium Oocysts in Animal Samples." *Experimental Parasitology* 151: 14–20. DOI:10.1016/j.exppara.2015.01.018
20. Navarro-i-Martinez, Luis, Carmen del Águila, and Fernando J Bornay-Llinares. 2011. "Cryptosporidium: Un Género En Revisión. Situación En España." *Enfermedades Infecciosas y Microbiología Clínica* 29 (2): 135–43. DOI: 10.1016/j.eimc.2010.12.002
21. Neto, Romeu Cantusio, Luciana Urbano dos Santos, Maria Ines Zanoli Sato, and Regina Maura Bueno Franco. 2010. "Cryptosporidium Spp. and Giardia Spp. in Surface Water Supply of Campinas, Southeast Brazil." *Water Science and Technology* 62 (1): 217–22. https://doi.org/10.2166/wst.2010.312
22. O'Connor, Roberta M, Jane W Wanyiri, Ana Maria Cevallos, Jeffrey W Priest, and Honorine D Ward. 2007. "Cryptosporidium Parvum Glycoprotein Gp40 Localizes to the Sporozoite Surface by Association with Gp15." *Molecular and Biochemical Parasitology* 156 (1): 80–83. doi: 10.1016/j.molbiopara.2007.07.010.
23. Valigurová, Andrea, Miloslav Jirků, Břetislav Koudela, Milan Gelnar, David Modrý, and Jan Šlapeta. 2008. "Cryptosporidia: Epicellular Parasites Embraced by the Host Cell Membrane." *International Journal for Parasitology* 38 (8–9): 913–22. DOI: 10.1016/j.ijpara.2007.11.003
24. Xiao, Lihua. 2010. "Molecular Epidemiology of Cryptosporidiosis: An Update." *Experimental Parasitology* 124 (1): 80–89. DOI: 10.1016/j.exppara.2009.03.018
25. Xiao, Lihua, and Yaoyu Feng. 2017. "Molecular Epidemiologic Tools for Waterborne Pathogens Cryptosporidium Spp. and Giardia Duodenalis." *Food and Waterborne Parasitology* 8: 14–32. DOI:10.1016/j.fawpar.2017.09.002
26. Zhang, Zhenjie, Suhui Hu, Wentao Zhao, Yaqiong Guo, Na Li, Zezhong Zheng, Longxian Zhang, Martin Kváč, Lihua Xiao, and Yaoyu Feng. 2020. "Population Structure and Geographical Segregation of Cryptosporidium Parvum IId Subtypes in Cattle in China." *Parasites & Vectors* 13 (1): 1–10. doi: 10.1186/s13071-020-04303-y