SVU-IJVS-8(3), 14-25 Print ISSN: 2535-1826 Online ISSN: 2535-1877

Research article Open Access

# Genetic Diversity of Cryptosporidium hominis in Humans and Sheep in Central Southern Iraq

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https://doi.org/ 10.21608/svu.2025.380836.1388
Submit Date: 02/05.2025; Revise Dat: 04/08/2025; Accept Date: 30/08/2025



## ABSTRACT

Cryptosporidium is a diarrhoeal obligate intracellular parasite. It is a common cause of waterborne outbreaks in infected animals and humans. To identify genetic variants linked to different hosts, DNA samples were analysed using genotyping platforms and sequencing technologies. This study included genotyping Cryptosporidium spp. from faecal samples from humans and sheep. Cryptosporidium spp. was performed molecularly based on the glycoprotein gene (GP60) by using a nested PCR reaction. Twenty-seven positive samples for Cryptosporidium spp. were amplified with the GP60 primer. The first cycle revealed 8 samples (5 human and 3 sheep) with C. hominis. were successfully sequenced. The nucleotide sequence demonstrated that the prevailing subtype of C. hominis was the IbA21G2 family.

**Keywords**: *Cryptosporidium hominis; genotyping; GP60; IbA21G2 family; mammals*

## INTRODUCTION

Cryptosporidiosis is a widespread parasitic disease among humans and animals worldwide. This parasite infects most vertebrates (Ryan *et al*.,2021). This disease is caused by *Cryptosporidium spp*., which is a member of the Coccidian family. This parasite is regarded as a Significant contributor to diarrhea in humans and other animals. The seriousness of cryptosporidiosis lies in the multiple ways of its transmission, as this disease is transmitted through direct contact with infected animals. It can be transmitted by contamination of water

and food sources with oocysts that play a role in the transmission (O'Leary *et al*., 2021).

Santin *et al*. (2020) found that Oocystozoites are present in 65-97% of surface water, such as rivers, lakes, and streams, and that water pollution is one of the major factors in the spread of parasite infestation. Due to the parasite's high prevalence in water, difficulty in getting rid of it, resilience to different sterilizers, and small size when it passes through filters, the parasite poses a "serious" hazard to water storage facilities. This parasite invades the digestive and respiratory systems' epithelial cells to finish its life cycle both sexually and asexually. One of the most crucial symptoms of parasite infection is watery diarrhea, which is produced by the parasite's growth inside the epithelial cells and the host's immunological reaction (Gerace *et al*., 2019).

As studies have shown, there are over 70 different subspecies of *Cryptosporidium spp*. the two most well-known and widespread species are *C. hominis* and *C. parvum* (Dong *et al*., 2020). Due to the specificity that *C. parvum* enjoys in that it infects most mammals, most studies have paid attention and researched to this type. Molecular studies have indicated differences in morphology and growth among parasites of this species that infect different hosts (Liu *et al*., 2020). Depending on the source of infection, these species' capacity to transmit between hosts and their capacity to spread differ. Parasites that have been identified in humans have not been found to infect mice or calves. On the other hand, parasites that were isolated from cows may infect people. Some parasites can infect wild animals or spread unintentionally from mother to fetus. Determining the type of parasite was necessary to account for these variations in the capacity and specificity of the host (Robertson *et al*., 2020).

With no vaccine or effective treatment, understanding the modes of transmission is critical to devising preventive measures. Since traditional methods of diagnosis are not capable enough to differentiating between distinct phenotypically species, genotyping methods are required (Cama *et al*., 2008). Therefore, accurate knowledge of the parasite prevalence and the most virulent species and genotypes is necessary to comprehend the dynamics of spread and transmission between various hosts (Sannella *et al*., 2019). Additionally, prior research suggested that various species or genotypes exhibit various clinical manifestations (Iqbal *et al*., 2011).

Several genomic regions with moderate and low heterogeneity have been used to identify species and subspecies of the parasite, like *Cryptosporidium* oocytes wall protein (COWP), lectin gene, heat shock protein 70 kDa (HSP-70), rRNA small subunit gene (18ss rRNA), β-tubulin genes, the ITS-1, and ITS-2 intragenic regions (Guy et al., 2021). The GP 60 gene was chosen over other genes in studies aimed at identifying the evolutionary tree because it

contains conserved and other extremely heterogeneous sections, despite the fact that each gene has a special importance in diagnostic or taxonomic studies or to estimate the rate of spread (Guo *et al.,* 2022).

This study aimed to estimate the diversity of Cryptosporidium spp. genotypes and to assess the likelihood of transmission between various hosts.

## MATERIALS AND METHODS

### Collection of Samples

A study was conducted to collect one hundred faecal samples, consisting of 50 samples from humans and 50 from sheep, in rural and urban areas of central southern Iraq. The samples were gathered between September 2022 and April 2023. The human samples were sourced from children aged between 5 months and 6 years, with a distribution of 23 females and 27 males. These samples were obtained from various locations, including pediatric clinics, hospital auditors, and pet breeders. In parallel, the sheep samples, taken from grazing areas and animal cages, were from animals aged 2 months to 2 years, comprising 31 females and 19 males.

### Laboratory analysis

The analysis of the collected samples was conducted at the laboratory of Al-Iraqi company, utilizing microscopy techniques specifically for the examination of faecal smears. These smears were stained using the modified Ziehl-Neelsen method, as outlined by Angus (1987) and Anonymous (1991). To prepare the faecal smears, they were first placed on a microscope slide, air-dried, and then fixed with methanol for a duration of 5 minutes. Following fixation, the smears were stained with a dilute carbol fuchsin solution (1:10) for 3 to 5 minutes before being washed with tap water. The next step involved decolourizing the smears using acid alcohol, after which they were counter-stained with a 0.5% Methylene blue solution for 1 minute.

Once air-dried, these smear slides were examined under a microscope at 100x magnification. During the examination, *Cryptosporidium spp*. Oocysts were identified as pink to red, spherical to ovoid structures against a blue background. A sample was deemed positive for the presence of *Cryptosporidium spp*. If at least one morphologically distinct Oocyst was observed (Mergen *et al*., 2020).

DNA extraction, molecular detection and subtyping

**DNA extraction:**

Genomic DNA was isolated from 100 stool samples utilizing a DNA extraction kit provided by Bioneer, a company based in South Korea. The extraction process was carried out in accordance with the manufacturer's detailed instructions to ensure optimal results.

**Nested-PCR test:**

The initial PCR product (first cycle) was amplified using primers for the 18S rRNA gene (Table 1). The detection of sub-species, GP60 protein gene amplification for primary PCR products was done. PCR master mix was

prepared by adding PCR water (6.8µ), 10x buffer (2µ), dNTPs (2µ), MgCl2 (2µ), forward primer (1µ), reverse primer (1µ), Taq polymerase (0.2µ), template DNA (5µ), total volume (20µ). Then, n-PCR was performed with PCR water (10.8µ µL), 10x buffer (2µ), dNTPs (2µ), MgCl2 (2µ), forward primer (1µ), reverse primer (1µ), Taq polymerase (0.2µ), template DNA (1µ from the first round), total volume (20µ) (Abdou et al., 2022).

**Primers:**

The primers of the 18SrRNA gene and the differential gene (GP60 protein gene) 60-kDa glycoprotein were designed and prepared by Bioneer, South Korea (Table 1).

**Table 1:** Details of the primers used

| Primers | Sequences | Purpose | Ref. |
|---|---|---|---|
| 18SrRNAgen. PCR | F- AGACGGTAGGGTATTGGCCT | Detection *Cryptosporidium spp.* | (Al-Amery and Al-Amery, 2022) |
| | R- TCCTTGGCAAATGCTTTCGC | | |
| *18SrRNA*gen. Nested-PCR | F-AACGGGAATTAGGGTTCGA | | |
| | R-TGCTTTCGCATTAGTTTGTCTT | | |
| Gp60 gene (C. *hominis*) PCR | F- ATAGTCTCCGCTGTATTC | Detection *C. hominis* | (Shrivastava et al., 2020) |
| | R- GGAAGGAACGATGTATCT | | |
| Gp60 gene (C. *hominis*) Nested-PCR | F- TCCGCTGTATTCTCAGCC | | |
| | R- GCAGAGGAACCAGCATC | | |

**Sequencing:**

The PCR products were purified from a 1.5% agarose gel using the NucleoSpin® gel and PCR clean-up kit from Bioneer Korea. The 18S rRNA and GP60 gene amplicons were directly sequenced using the ABI 3100 Genetic Analyzer and the BigDye Terminator v3.1 Cycle Sequencing kit. All sequences were aligned using the MEGA 7.0 software. The homologous sequences of *Cryptosporidium spp*. were deposited in GenBank. Phylogenetic evolution and comparison trees were created using the MEGA 7.0 software.

**Ethical Approval:**

The current study was conducted following the recommendations of the International Guide for the Care and Use of Animals and the guidelines issued by the College of Veterinary Medicine at Al-Qadisiyah University, under announcement No. 576 in 2018.

**RESULTS**

**survey rate**

In a study involving 100 faecal samples, *Cryptosporidium spp*. was detected in 22 samples, resulting in an infection rate of 22%. After applying an acid-fast stain, the overall prevalence of infection was found to be (13/50)

26% in humans and (9/50) 16% in sheep (Figure 1). Molecular testing (first cycle) for the 18S rRNA gene revealed the presence of the parasite in 27 samples, which corresponds to a prevalence rate of 27%, as detailed in Table 2.

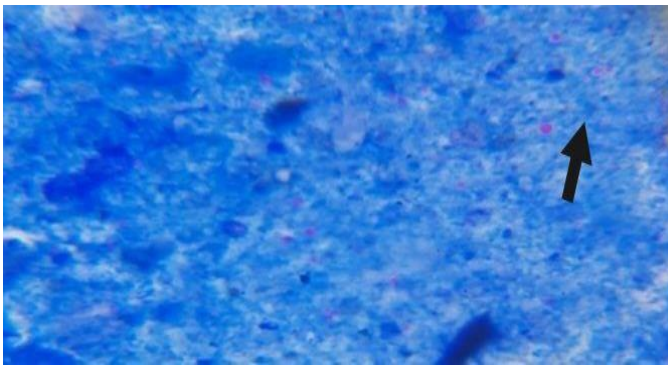

**Figure 1:** Oocysts of Cryptosporidium *spp* (100x).

**Nested PCR test**

DNA genome samples were subjected to molecular analyses by nested PCR using the 18S rRNA gene in order to identify the species of *Cryptosporidium spp*. 29 samples had a unique band of 835 bp following electrophoresis, confirming the presence of *Cryptosporidium spp* (Figure 2; Table 2

**Table 2:** Prevalence of *Cryptosporidium spp*. in study samples.

| Host | Total samples | Microscopic Test (%) | Molecular test | Nested PCR | Intensity of infection |
|---|---|---|---|---|---|
| **Human** | 50 | 13(26%) | 17(34%) | 11(22%) | 17(34%) |
| **Sheep** | 50 | 9(18%) | 10(20%) | 8(16%) | 10(20%) |
| **Total** | 100 | 22(22%) | 27(27%) | 29(29%) | 27(27%) |

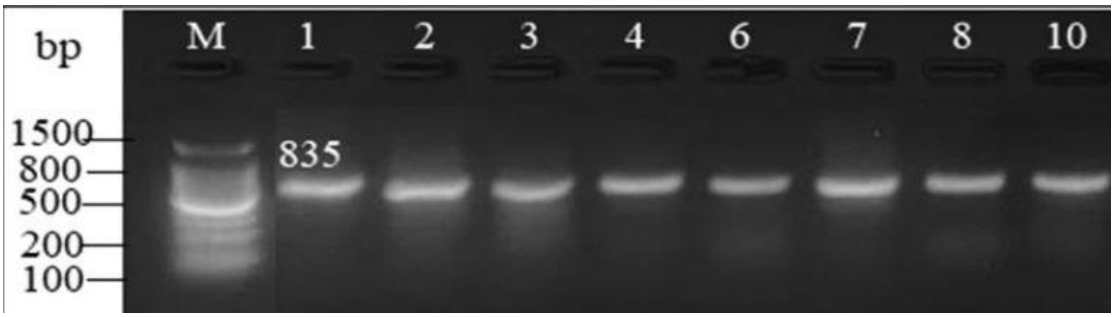


**Figure 2:** Amplification of the 18S rRNA gene for positive *Cryptosporidium spp.* samples (835 bp) were shown by Agarose gel electrophoresis

**Nested PCR with gp-60 gene**

In a recent study, positive samples for *Cryptosporidium spp*. were amplified using the GP 60 primer. The initial cycle identified eight samples – five from human sources and three from sheep – confirmed to contain *C. hominis*. Out of these, seven samples were successfully sequenced, revealing that the dominant subtype of *C. hominis* belonged to the IbA21G2 family. The nucleotide sequences obtained were compared with existing data in the GenBank database at the National Centre for Biotechnology Information (NCBI) through the NCBI BLAST tool. This analysis uncovered the

presence of new and distinct isolates from Iraq. Furthermore, the newly generated sequences have been deposited in GenBank under private accession numbers, as detailed in Table 3 and Figure 3.

**Table 3:** The Homology Sequence Identity (%) between the submitted genotype isolates from NCBI-BLAST and the local *C. hominis*

| Host | Accession number | NCBI-BLAST Homology Sequence identity (%) | | | |
|---|---|---|---|---|---|
| | | Identical genotypes | Accession number | Country | Identity (%) |
| **Human** | OR360262 | IbA9G3 | MT952955 | Australia | 100 |
| | OR360263 | IbA21G2 | FJ153239 | China | 100 |
| | OR360264 | IbA21G2 | FJ153239 | China | 100 |
| | OR360265 | IbA21G2 | MW984369 | Iraq | 100 |
| **Sheep** | OR360267 | IbA21G2 | MZ787830 | Iraq | 100 |
| | OR360268 | IbA21G2 | FJ153239 | China | 100 |
| | OR360269 | IbA21G2 | MW984369 | Iraq | 100 |

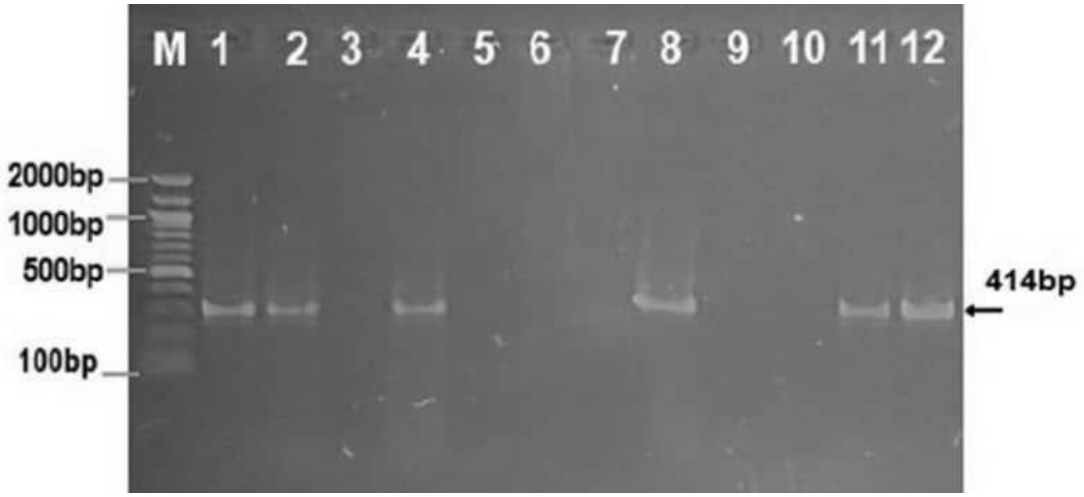


**Figure 3:** Amplification of gp60 (414 bp) for positive *C. hominis* samples was shown by Agarose gel electrophoresis.

**The phylogenetic analysis**

The phylogenetic analysis was performed using. The MEGA 11 software was used to generate a neighbor-joining phylogram (Figure 4.

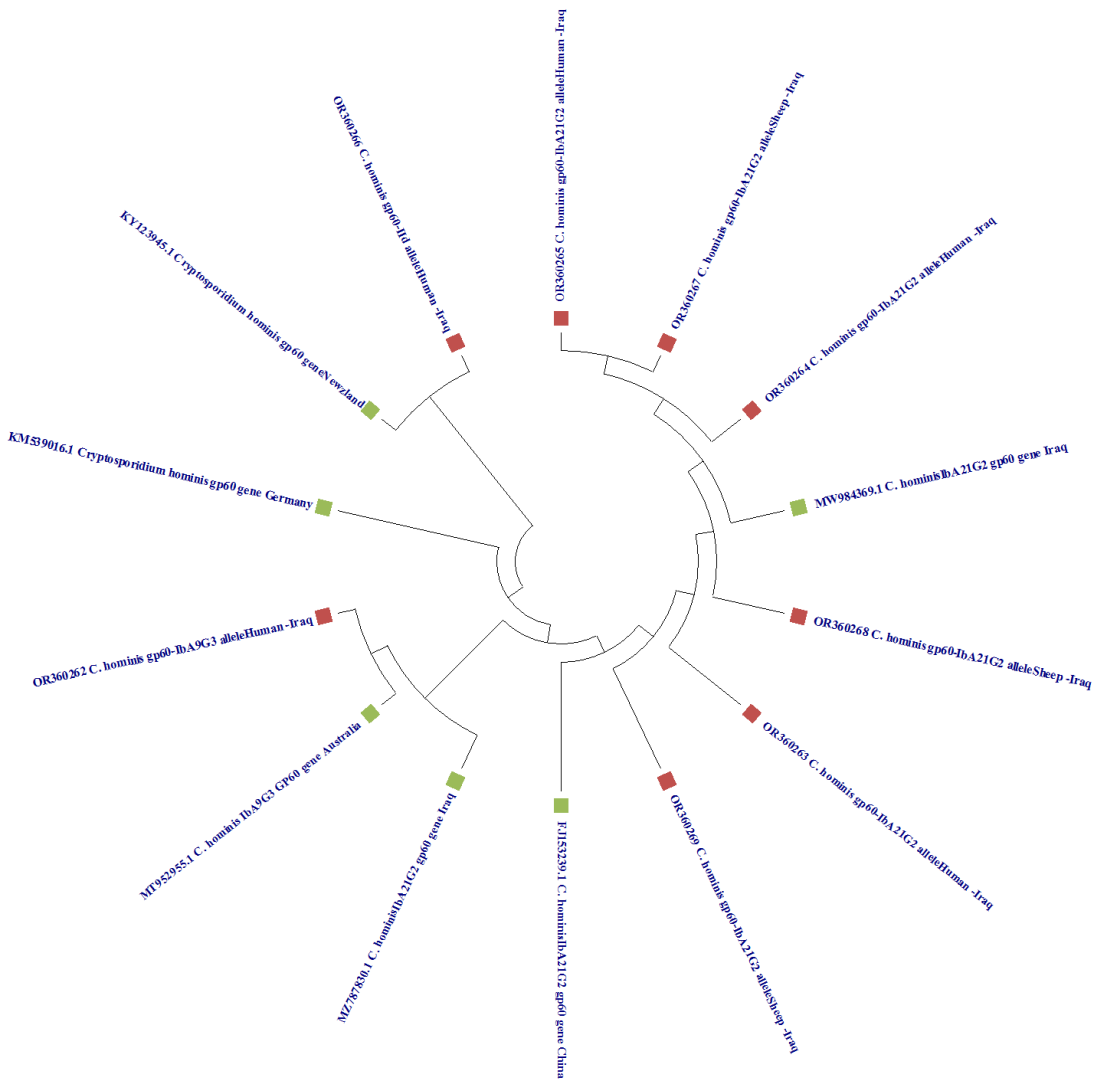


**Figure 4:** A partial SSU rRNA gene sequence from *C. hominis* used for phylogenetic tree analysis. MEGA 11.0 was used to create the phylogenetic tree using the maximum likelihood method (brown: current result; green: previous studies).

## DISCUSSION

Cryptosporidiosis is a zoonotic disease that poses a significant risk for the transmission of pathogens between humans and animals, as well as for outbreaks related to water and food sources. This is partly due to the resilience of its oocysts to common disinfectants, including chlorination used in drinking and recreational water (Ryan *et al*., 2021). The disease is especially concerning in low-income areas and among both immunocompetent and immunocompromised individuals (Ryan *et al*., 2014) .

One of the key factors contributing to the effective spread of *Cryptosporidium* as a parasite is that an infected person can produce a large number of oocysts. These oocysts can withstand moderate environmental conditions and persist in humid environments for extended periods, which can lead to infections. Experimental

studies involving humans have shown that a dose of as few as 10 to 100 *C. hominis* oocysts is capable of causing infection (Chappell *et al*., 2006).

*Cryptosporidium hominis* ranks first in humans and sheep (Ayres Hutter *et al*., 2020). The accidental transmission of infection between humans and different animals results from sharing the same water sources (especially in rural areas) and the direct intervention of humans in raising and caring for animals (Alali *et al*., 2021). Factors such as living conditions, nutritional status, personal hygiene, immune status, socio-economic criteria, and variations like the region and the type of test used for diagnosis affect the prevalence rates and transmission between hosts (Yang *et al*.,2021).

The use of standard correlation indicators such as 18s rRNA and GP60 and their measurement between alleles is necessary to identify these genotypes and estimate the time of their appearance or the degree of kinship between them. It is zero in single-origin populations and positive in divergent populations (Cacciò *et al*.,2015).

The glycoprotein (60 kD) gene is widely used in molecular and diagnostic research due to its highly variable and conserved sequences. These characteristics make it easier to design species-specific primers. There are small sequence variations within isolates among different copies in numerous *Cryptosporidium* species and genotypes. The gp60 gene, which can be used with all isolates from hosts, is more accurate than other genes for diagnosing and differentiating between species in different hosts (Abeywardena *et al*., 2014).

Most of the molecular heterogeneity in the GP60 gene is variation in the number of triplet coding repeats (TCA, TCG, or TCT) at the 5′ ends (gp40) of the coding region. These variations in the frequencies of the triplet code produce various sub-generic phenotypes, which have been given specific names to distinguish them. The roman numeral II means that the strain belongs to *C. parvum*, and internal changes in the structure and arrangement of the three gene codons are expressed in lowercase letters; for example, IIa denotes heterogeneity in the codons TCA, and TCG (Liu *et al*., 2014).

## CONCLUSION

Our findings indicate that the occurrence of cryptosporidiosis in the studied region is relatively low. To effectively manage and minimize the transmission of this disease between animals and humans, it is crucial to enhance awareness regarding the risk factors, sources of infection, and modes of transmission. Furthermore, we recommend conducting additional molecular research in various areas of the country to better understand species distribution and assess the overall impact of the disease on a national scale.

## DECLARATIONS


### FUNDING

The authors affirm that they have not received any grants, funds, or other assistance for this manuscript.


### AUTHORS' CONTRIBUTION

The authors attested to having taken part in accepting public accountability for the content, which encompasses the idea, research, composition, and editing of the manuscript.

### COMPETING INTERESTS

No competing interests are disclosed by the authors

### CONFLICT OF INTEREST

The authors declare no competing

## REFERENCES


Abdou, N. E. M., AlAzemi, M. S., Al-Sayegh, M. T., Majeed, Q. A. (2022). Performance of diagnostic assays used to detect *Cryptosporidium* oocysts in faecal samples of cattle in Kuwait and genotyping of *Cryptosporidium* species. BMC Veterinary Research, 18(1): 1-11. https://doi.org/10.1186/s12917-022-03435-w

Abeywardena, H., Jex, A. R., Koehler, A. V., Rajapakse, R. J., Udayawarna, K., Haydon, S. R., Stevens, M. A. Gasser, R. B. (2014). First molecular characterization of *Cryptosporidium* and *Giardia* from bovines (*Bos taurus* and *Bubalus bubalis*) in Sri Lanka: unexpected absence of *C. parvum* from pre-weaned calves. Parasites and Vectors, 7: 75. http://www.parasitesandvectors.com/content/7/1/75

Alali, F., Abbas, I., Jawad, M., Hijjawi, N. (2021). *Cryptosporidium* infection in humans and animals from Iraq: A review. Acta Tropica, 220: 105946. https://doi.org/10.1016/j.actatropica.2021.105946

Al-Amery A. R., Al-Amery A. M. (2022). Molecular diagnosis of *Cryptosporidium* ssp. in water buffaloes at Babylon province, Iraq. Iraqi Journal of Agricultural Sciences,53(1):147-156. https://doi.org/10.36103/ijas.v53i1.1519

Ayres Hutter, J., Dion R., Irace-Cima A. (2020). *Cryptosporidium* spp.: Human incidence, molecular characterization and associated exposures in québec, Canada (2016-2017). PLoS One, 15: e0228986. https://doi.org/10.1371/journal.pone.0228986

Cacciò, S.M., de Waele, V., Widmer, G. (2015). Geographical segregation of *Cryptosporidium parvum* multilocus genotypes in Europe. Infect Genet Evol. 31(April):245–9. https://doi.org/10.1016/j.meegid.2015.02.008

Cama, V. A., Bern, C., Roberts, J., Cabrera, L., Sterling, C. R., Ortega, Y., Xiao, L. (2008). *Cryptosporidium* species and subtypes and clinical manifestations in children, Peru.

Emerging infectious diseases, 14(10): 1567. https://doi.org/10.3201/eid1410.071273

Chappell, C. L., Okhuysen, P. C., Langer-Curry, R., Widmer, G., Akiyoshi, D. E., Tanriverdi, S., Tzipori, S. (2006). *Cryptosporidium hominis*: experimental challenge of healthy adults. American Journal of Tropical Medicine and Hygiene, 75: 851–857. https://doi.org/10.4269/ajtmh.2006.75.851

Dong, S., Yang, Y., Wang, Y., Yang, D., Yang, Y., Shi, Y., Li, C., Li, L., Chen, Y., Jiang, Q., Zhou Y. (2020). Prevalence of *Cryptosporidium* infection in the global population: A systematic review and meta-analysis Acta Parasitol, 65 (4):882-889. https://doi.org/10.2478/s11686-020-00230-1

Gerace, E., Presti, V. D. M. L., Biondo, C. (2019). *Cryptosporidium* infection: epidemiology, pathogenesis, and differential diagnosis. European Journal of Microbiology and Immunology, 9(4): 119-123. https://doi.org/10.1556/1886.2019.00019

Guo, Y., Ryan, U., Feng, Y., Xiao, L. (2022). Emergence of zoonotic *Cryptosporidium parvum* in China. Trends in Parasitology, 38(4): 335-343. https://doi.org/10.1016/j.pt.2021.12.002

Guy, R. A., Yanta, C. A., Muchaal, P. K., Rankin, M. A., Thivierge, K., Lau, R., Boggild, A. K. (2021). Molecular characterization of *Cryptosporidium* isolates from humans in Ontario, Canada. Parasites & Vectors, 14(1): 1-14. https://doi.org/10.1186/s13071-020-04546-9

Iqbal, J., Khalid, N., Hira, P. R. (2011). Cryptosporidiosis in Kuwaiti children: association of clinical characteristics with *Cryptosporidium* species and subtypes. Journal of Medical Microbiology, 60(5): 647-652. https://doi.org/10.1099/jmm.0.028001-0

Liu, A., Gong, B., Liu, X., Shen, Y., Wu, Y., Zhang, W., Cao, J. (2020). A retrospective epidemiological analysis of human *Cryptosporidium* infection in China during the past three decades (1987-2018). PLoS neglected tropical diseases, 14(3): e0008146. https://doi.org/10.1371/journal.pntd.0008146

Liu, H., Shen, Y., Yin, J., Yuan, Z., Jiang, Y., Xu, Y., Pan, W., Hu, Y., Cao, J. (2014). Prevalence and genetic characterization of *Cryptosporidium*, *Enterocytozoon*, *Giardia* and *Cyclospora* in diarrheal outpatients in China. BMC Infectious Diseases, 14: 25. https://doi.org/10.1186/1471-2334-14-25

Mergen, K., Espina, N., Teal, A., Madison-Antenucci, S. (2020). Detecting *Cryptosporidium* in stool samples submitted to a reference laboratory. The American

Journal of Tropical Medicine and Hygiene, 103(1): 421. https://doi.org/10.4269/ajtmh.19-0792

O'Leary, J. K., Sleator, R. D., Lucey, B. (2021). *Cryptosporidium* spp. diagnosis and research in the 21st century. Food and waterborne parasitology, 24: e00131. https://doi.org/10.1016/j.fawpar.2021.e00131

Robertson, L. J., Johansen, O. H., Kifleyohannes, T., Efunshile, A. M., Terefe, G. (2020). *Cryptosporidium* infections in Africa—how important is zoonotic transmission? a review of the evidence. Frontiers in veterinary science, 7: 575881. https://doi.org/10.3389/fvets.2020.575881

Ryan, U. M., Feng, Y., Fayer, R., Xiao, L. (2021). Taxonomy and molecular epidemiology of *Cryptosporidium* and *Giardia* - a 50-year perspective (1971-2021). International journal for parasitology, 51(13-14): 1099–1119. https://doi.org/10.1016/j.ijpara.2021.08.007

Ryan, U., Fayer, R., Xiao, L. (2014). *Cryptosporidium* species in humans and animals: current understanding and research needs. Parasitology, 141(13): 1667–1685. https://doi.org/10.1017/S0031182014001085

Rzeżutka, A., Kaupke, A. (2013). Occurrence and molecular identification of *Cryptosporidium* species isolated from cattle in Poland. Veterinary parasitology, 196(3-4): 301–306. https://doi.org/10.1016/j.vetpar.2013.03.009

Sannella, A. R., Suputtamongkol, Y., Wongsawat, E., Cacciò, S. M. (2019). A retrospective molecular study of *Cryptosporidium* species and genotypes in HIV-infected patients from Thailand. Parasites & vectors, 12(1): 91. https://doi.org/10.1186/s13071-019-3348-4

Santin M. (2020). *Cryptosporidium* and *Giardia* in Ruminants. The Veterinary clinics of North America. Food animal practice, 36(1): 223–238. https://doi.org/10.1016/j.cvfa.2019.11.005

Shrivastava, A. K., Panda, S., Kumar, S., & Sahu, P. S. (2020). Two novel genomic DNA sequences as common diagnostic targets to detect Cryptosporidium hominis and Cryptosporidium parvum: Development of quantitative polymerase chain reaction assays, and clinical evaluation. Indian journal of medical microbiology, 38(3-4): 430-440. https://doi.org/10.4103/ijmm.IJMM_20_114

Valenzuela, C. A., Martínez, A. A. (2017). Caracterización molecular de los subtipos de la GP60 de *Cryptosporidium parvum* y *Cryptosporidium hominis* alrededor del mundo. Revista MVZ Córdoba, 22(3):

6339-6354. https://doi.org/10.21897/rmvz.1138

Yang, X., Guo, Y., Xiao, L., Feng, Y. (2021). Molecular Epidemiology of Human Cryptosporidiosis in Low- and Middle-Income Countries. Clinical microbiology reviews, 34(2): e00087-19. https://doi.org/10.1128/CMR.00087-19.