SciForum
MOL2NET

Cryptosporidium spp. FREQUENCY IDENTIFIED THROUGH ZN STAIN IN LAMBS FROM MICHOACÁN, MÉXICO

María Uxúa Alonso-Fresán [1,*], Ana Dalia Ocaña-Soto [2], Soledad Díaz-Zarco [3], Alberto Barbabosa-Pliego [4], Juan Carlos Vázquez-Chagoyán [5], Benjamín Valladares-Carranza [6] and Valente Velázquez-Ordoñez [7]

[1] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km. 15.5 Carretera Toluca-Atlacomulco, CP. 50200;
[2] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, El Cerrillo Piedras Blancas, CP. 50090;
[3] Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, El Cerrillo Piedras Blancas, CP. 50090; sol_zarco@yahoo.com.mx;
[4] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km. 15.5 Carretera Toluca-Atlacomulco, CP. 50200; abarbabosa@yahoo.com.mx
[5] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km. 15.5 Carretera Toluca-Atlacomulco, CP. 50200; jcvch@yahoo.com
[6] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km. 15.5 Carretera Toluca-Atlacomulco, CP. 50200; benvac2004@yahoo.com.mx
[7] Centro de Investigación y Estudios Avanzados en Salud Animal, Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Km. 15.5 Carretera Toluca-Atlacomulco, CP. 50200; valentevelazquez@hotmail.com

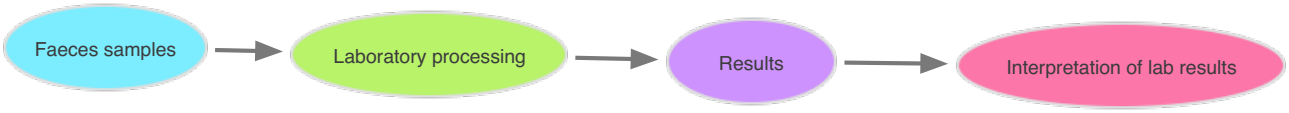
* Author to whom correspondence should be addressed; E-Mail: muaf@uaemex.mx; Tel.: +52-722-296-5555; +52-722-296-8980.

Received:28-11-2016 / Accepted: / Published:

Cryptosporidium spp. is a protozoan frequently observed in mammals, with worldwide distribution, that may cause disease in the respiratory and/or digestive tract, with economic loss. In order to find the frequency of *Cryptosporidium spp.* in lambs aging 0-60 days, four communities in Tlalpujahua, Michoacán, México, were sampled. One hundred and twenty two faeces samples were taken directly from the anus. Samples were processed and stained using ZN for parasite identification. Chi-square test was calculated according to location, age, sex, breed and farm's type of production. A frequency of 90-100% was obtained, with no statistical difference in variables. It is concluded that high frequency (90-100%) of *Cryptosporidium spp*. was found using ZN stain in lambs aging 0-60 days in Tlalpujahua, Michoacán, with no difference regarding location, age, sex, breed and production system.

Keywords: *Cryptosporidium spp.*; lambs; 0-60 days; ZN stain; location, age; sex; farm's type of production system

Graphical Abstract: *<<Insert a figure to summarize your work, mandatory>>*



.

Introduction:

In 1907 Tyzzer described a protozoan frequently observed in lab mice, which was named *Cryptosporidium* (hidden oocysts), establishing its characteristics in 1910 as well as its biological cycle. By 1984 it had already been identified in 20 different species (Tzipori, 1985). It can cause diarrhea in mammals such as beef, sheep, goats, horses and humans as primary agent provoking intestinal and respiratory infection with important economic loss (Robertson *et al.*, 2014). The first human case was reported in 1976, with few cases reported afterwards until it became important in HIV and immunosupressed patients. *Cryptosporidium* are small rounded or oval oocyts, with an average of 5 µm (Fayer and Ungar 1986). It is an opportunistic organism which infects the respiratory or digestive tract or both causing asymptomatic infections with anorexia to depression with or without diarrhea (Tzipori, 1985). Infection occurs even with a small number of oocysts. It can infect a large numbr of animals and can be found in several organs of the host, such as alveoli, bronchioles and trachea, suggesting variable and complex symptoms (Zu *et al.,* 1992). Transmission ocurrs through oral-faecal route. Oocysts excreted in faeces are sporulated, therefore they are immediately infectious. The average incubation period is four days (Merck, 2007). Some experiments have shown that only 10 oocysts may cause infection. In large intestine, esporozoites are released and infect the epithelium where they cause diffuse moderate atrophy in the villi and hyperplasia in the yeyunum (Zu *et al.,* 1992). Experimental infections in laboratory animals show that *Cryptosporidium spp.* is transmitted through oocyst ingestion that pass from the host to the environment (Ernest *et al.,* 1986). Infective forms are resistant to common disinfectants and can survive for several months. 50% Ammonia and 30% formaldehyde for 30 minutes are capable of destroying *Cryptosporidium* oocysts (Fayer and Ungar, 1986).

Four representative species were described: one isolated from mammals (*Cryptosporidium muris*), one from birds (*Cryptosporidium meleagridis*), one from reptiles (*Cryptosporidium croieli*) and one from fish (*Cryptosporidium nasorum*), when not enough information was available. *Cryptosporidium parvum* was included afterwards (Clark and Sears, 1996). It was thought that the parasite was species specific, until cross transmission infections were reported (Xiao, 2004). OIE Manual (2016) includes 26 valid *Cryptosporidium* species, from which C. *parvum*, C. *andersoni*, C. *baileyi*, C. *meleagridis* and C. *galli* have caused morbility and outbreaks in herds.

Cryptosporidium spp. causes a self-limiting enterocolitis worldwide. It is common in young ruminants specially in calves and lambs, causing weight loss and watery diarrhea (Merck, 2007). Diarrhea has been observed in animals from 5 to 15 days of age, in which other pathogens such as rotavirus, coronavirus, *Clostridium perfringens* or enterotoxicogenic *Escherichi coli* may be involved, and a high mortality rate has been observed in calves when extreme low environmental temperature occur. Intestinal malabsorption and fluid loss due to the infection, avoids the absorption of nutrients from diet (Fayer and Ungar, 1986). Natural or experimental infection show changes in the mucosa, with villi atrophy, crypt hyperplasia and cellular inflammation. Diarrhea is caused by impaired digestion provoked by membrane loss in the superior large intestine, reducing the ileon's absorption capacity. Some research studies mention that *Cryptosporidium* in pigs,

cats and dogs may cause subclinical infections, as well as in goats and sheep (Tzipori, 1985). Attention has been focused on pathogenesis studies to understand the severity of the disease (Zu *et al.,* 1992). Prepatent period lasts 3 to 5 days, where death is rarely present. Generally, the animals show spontaneous recovery (Fayer and Ungar, 1986). Lambs aging 1 to 3 weeks seem to be at highest risk. They show anorexia, weight loss, diarrhea and tenesmus. Nevertheless, cryptosporidiosis may be present without any clinical signs. Distal large intestine is the most affected (Merck, 2007).

Faecal oocyst elimination is correlated to the intensity of the infection, which progressively decreases up to the third week. This may indicate that animals might develop resistance (Pollok *et al.,* 2001).

Treatment

There is no effective treatment for cryptosporidiosis, but it is a self-limiting infection, where supportive treatment including oral and parenteral rehydration is suggested (Merck, 2007). There are several drugs which have been proven in human and animals with unfavorable results (Fayer and Ungar, 1986). Halofuginone and sulfamides are partially effective in neonates (Cordero *et al*., 2002).

Prevention and control

Cryptosporidium oocysts may remain viable for a year in aqueous solution, without direct sunlight and environmental temperature between 15 to 35 °C (Merck, 2007). It is also resistant to commercial disinfectants. These are the reasons why it is difficult to eliminate from the environment (Fayer and Ungar, 1986). Water consumption should be avoided in order to control its propagation when there is suspicion of water contamination (Whirmire and Harp, 1991). *Cryptosporidium* oocysts may be destroyed in the environment using 5% ammonia (Monis, King and Keegan, 2014).

Materials and Methods: Sample recollection was performed from November 2014 to April 2015, by taking faeces directly from the anus of lambs aging 1 to 60 days in farms from four communities of Tlalpujahua, Michoacán, located at 2,337 metres over sea level, with warm climate and rainfall during summer. It has an anual rainfall of 1,003 mm and temperatures of 6.1 to 22.7 ºC (INEGI, 2011). Samples were identified and refrigerated until processed in CIESA-FMVZ-UAEM.

Positivity rate was established using modified Ziehl Neelsen stain (Henriksen and Polenz, 1981), where positive animals had more than three oocyts per field.

Sample processing

Distilled sterile water was added to faeces, filtered through gause and centrifuged at 1500 rpm for 5 minutes. Liquid was decanted and sediment preserved using 5% potassium dichromate until stained with Ziehl-Neelsen for microscopical identification (100X). A positive control was used per smear.

The number of samples was determined using finite population formula (Herrera, 2014), with a final calculation of 122 samples.

Statistical differences were calculated using chi-square test (Petrie and Watson, 2013).

Results and Discussion: In the majority of the farms which were sampled, there was no defined management programme. In all of them animals were mainained together: female and male with different ages, without a hygiene or reproductive control programme, representing a risk factor for *Cryptosporidium* spp. in the herds.

Ziehl-Neelsen stain is rapid, economical and differential, used for identification of Apicomplexans (intestinal coccidia) (Vohra, Shaura and Chaudhary, 2012).

Regarding *Cryptosporidium* spp. frequency, the highest was found in San Rafael (100%) (Table 1). In this municipality, the weather is warm with rainfall during summer. Anual rainfall is 1,003 mm with temperatures ranging from 6.1 to 22.7ºC (INEGI, 2011). According to Cordero (2002), it has a worldwide distribution infecting domestic and wild animals including mammals, birds, reptiles and fish. *Cryptosporidium* oocysts are infective as soon as they are excreted through faeces and are perfectly adapted for survival in the enfironment, resistant to variable conditions except for desecation and freezing. Xiao *et al.,* (1994) reported a prevalence of *Cryptosporidium parvum* del 100% in newborn diarrhaeic lambs and 78.3% in lambs 2 to 3 weeks of age in warm-cold weather with constant rainfall.

Table 1 shows a high positivity percentage to *Cryptosporidium* spp in lambs, ranging from 90

to 100 %. In Mexico García and Lara (2000) found a prevalence of 29.9% in lambs; Alonso (2007), 34.33%; and Medina and Medina (2007), 51.82%. Causapé (2002) found in Spain 59%.

There are no reports of *Cryptosporidium* spp in Tlalpujahua Michoacán municipality. In this study the frequency found was related to different conditions that could represent a risk factor in sheep herds such as: location, age, sex, breed and production system.

Regarding age, table 2 shows high *Cryptosporidium* spp. positivity rate, higher than 90% in all ages. All groups showed a frequency over 90%; nevertheless, the group with the highest frequency was the one aging 16-30 days with 97% (Table 2). These results are different from the ones reported by Alonso *et al.,* (2005) who found a general prevalence of 34.33% in asymptomatic lambs, same characteristic as the ones sampled in this study. Asymptomatic carriers represent an important risk factor because they can turn into an infection source for the herd. According to Cordero, (2002) the main clinical sign in sheep with cryptosporidiosis is diarrhea, its severity will depend in factors such as age, immune system and environmental conditions among others.

Table 3 shows *Cryptosporidium* spp. frequency according to sex, where there is no difference in gender (higher than 90%). Frequency in females was 98% and 92% in males (Table 3), which are considered high. Valenzuela *et al.,* (1991) reported a prevalence of 66.6% in lambs aging 7 days or more, without relation to sex in infected animals.

Table 4 shows *Cryptosporidium* spp. in lambs according to breed, where a positivity of more than 90% exists, similar to the other factors that were analysed. Predominant breeds in Tlalpujahua municipality were Suffolk and Criolla. They had a frequency of 93 and 96% respectively (Table 4). Romero *et al.* (2016) found a prevalence of 60-75% in different breeds.

Table 5 shows *Cryptosporidium* spp percentage according to production system, with similar results in extensive and semi-intensive systems.

We found a frequency of 90% of *Cryptosporidium* spp in semi-intensive productive systems and 96% in the extensive (Table 5). This frequency was probably influenced by inadequate hygiene conditions in the farms. In both systems lambs are in direct contact with ewes, which might act as reservoir. Alonso *et al.*, (2005) found a prevalence of 34% in ewes from family production farms in the State of Mexico.

Almost all the farms in Tlalpujahua, Michoacán municipality are classified as extensive. Partida *et al*., (2013) describes their characteristics: grazing in native vegetation, with almost no food supplements and parturitions during autumn and winter, rustic facilities of wood mainly and soil floor with almost no hygiene programmes, where faeces remain as bed material. Delafosse *et al.,* (2006) found a high prevalence (44%) in farms with soil floor.

Alonso, (2007) concluded that risk factors are herd size and grazing place (≤100 and >100 animals) (OR=2.6, $IC_{95\%}$ 1.8-3.6) and (OR = 1.467, $CI_{95\%}$ 1.102-1.951), respectively.

It is important to denote that no statistical differences were found between the different risks analysed.

.

.

.

. **Table 1.** *Cryptosporidium* spp. frequency in lambs aging 15 to 90 days in four communities in Tlalpujahua, Michoacán.

COMMUNITY	No. OF SAMPLES	FREQUENCY	
		POSITIVE	PERCENTAGE
EL CAPULIN	46	44	95[a]
CERRO PRIETO	30	27	90[a]
SAN RAFAEL	22	22	100[a]
LAS CANTERAS	25	24	96[a]
TOTAL	123	117	

Source: original data; p>0.05

Table 2. *Cryptosporidium* spp. frequency in lambs according to age in Tlalpujahua, Michoacán.

AGE /DAYS	No. OF SAMPLES	FREQUENCY	
		POSITIVE	PERCENTAGE
1 – 15	45	43	95[a]
16 – 30	47	46	97[a]
31 – 45	21	19	90[a]
46 – 60	10	9	90[a]
TOTAL	123	117	

Source: original data; p>0.05

Table 3. *Cryptosporidium spp.* frequency according to sex in lambs aging 15 to 60 days in Tlalpujahua, Michoacán.

SEX	No. OF SAMPLES	FREQUENCY	
		POSITIVE	PERCENTAGE
FEMALE	57	56	98[a]
MALE	66	61	92[a]
TOTAL	123	117	

Source: original data; p>0.05

Table 4. *Cryptosporidium* spp. frequency according to breed in lambs aging 15 to 60 days in Tlalpujahua, Michoacán.

BREED	No. OF SAMPLES	FREQUENCY	
		POSITIVE	PERCENTAGE
SUFFOLK	45	42	93[a]
CRIOLLO	78	75	96[a]
TOTAL	123	117	

Source: original data; p>0.05

Table 5. *Cryptosporidium* spp frequency according to production system in lambs aging 15 to 60 days in Tlalpujahua, Michoacán

PRODUCTION SYSTEM	NO. OF SAMPLES	FREQUENCY	
		POSITIVE	PERCENTAGE
SEMI-INTENSIVE	30	27	90[a]
EXTENSIVE	93	90	96[a]
TOTAL	123	117	

Source: original data; p>0.05

.**Conclusions:** A high frequency (90-100%) of Cryptosporidium spp. identified using ZN stain was found in lambs aging 0-60 days in Tlalpujahua, Michoacán, where no difference was found regarding location, age, sex, breed and production system.

Acknowledgments

The authors would like to thank producers from Tlalpujahua, Michoacán for kindly permitting taking the samples from the lambs. Also, CIESA FMVZ UAEM for allowing the use of its laboratory facilities.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Alonso-Fresán, MU; García-Alvarez, A.; Salazar-García, F.; Vázquez-Chagoyán, JC; Pescador-Salas, N. and Saltijeral-Oaxaca, J. Prevalence of Cryptosporidium spp. in asymptomatic sheep in family flocks from Mexico State. *J. Vet. Med. B.*, 2005, 52, (482–483), DOI 10.1111/j.1439-0450.2005.00889.x.
2. Alonso-Fresán, MU.; Vázquez-Chagoyán, JC.; Velázquez-Ordoñez, V.; Pescador-Salas, N. and Saltijeral-Oaxaca, J. Sheep management and cryptosporidiosis in central Mexico. *Trop. Anim. Health Prod.*, 2009, 41, (431-436), DOI 10.1007/s11250-008-9208-4.
3. Causapé, AC.; Quílez, J.; Sánchez-Acedo, C.; del Cacho, E.; López-Bernad, F. Prevalence and analysis of potential risk factors for Cryptosporidium parvum infection in lambs in Zaragoza (northeastern Spain). *Vet Parasitol.,* 2002, 104, (287-298) DOI 10.1016/S0304-4017(01)00639-2.
4. Clark, DP. and Sears, CL. The Pathogenesis of Cryptosporidiosis. *Parasitol Today*, 1996, 132, (221-225), DOI 10.1016/0169-4758(96)10018-1.
5. Cordero del C, M.; Rojo, VFA.; Martínez, FAR.; Sánchez, AC.; Hernández, RS.; Navarrete, LCI.; Díez, BP.; Quiroz, RH. and Carvalho, VM. *Parasitología Veterinaria*; Mc Graw Hill Interamericana, España, 2002; pp. 195-259.
6. Delafosse, A.; Castro-Hermida, JA.; Baudry, C.; Ares-Mazás, E. and Chartier, C. Herd-level risk factors for Cryptosporidium infection in dairy-goat kids in western France, *Prev. Vet. Med.,* 2006, 77, (109-121). DOI 10.1016/j.prevetmed.2006.07.001.
7. Ernest JA, Blagburn BL, Linsday DS. Infection Dynamics of *Cryptosporidium parvum* (*Apicomplexa, Criptosporidae*) in neonaltal mice (*Mus musculus*), *J. Parasitol.,* 1986, 72, (796-798), DOI 10.2307/3281485.
8. Fayer, R.; and Ungar, BL. *Cryptosporidium spp*. and Cryptosporidiosis. *Microbiol Rev*, 1986, 50, (458-483), PMCID: PMC373083.
9. Garcia, PC. and Lara, GF. Prevalencia de *Cryptosporidium spp.* en ovinos de cuatro municipios del Estado de México. Tesis de Licenciatura. Facultad de Medicina Veterinaria y Zootencia, Universidad Autónoma del Estado de México, Toluca, México, 2000.
10. Henriksen, SA. and Pohlenz. Staining of cryptosporidia by a modified Ziehl Neelsen technique. *Acta Vet. Scand*., 1981, 22, (594-596), PMID: 6178277.
11. Herrera, CM. 2016. Fórmula para el cálculo de la muestra para poblaciones finitas. http://investigacionpediahr.files.wordpress.com/2011/01/formula-para-cc3a1lculo-de-la-muestra-poblaciones-finitas-var-categorica.pdf. Available online: (26/11/2016).
12. INEGI. 2011. Perspectiva estadística, Michoacán de Ocampo. Available online:
13. http://www.inegi.gob.mx/est/contenidos/espanol/sistemas/perspectivas/perspectiva-mic.pdf (21/10/14).
14. Medina, LS. and Medina, LE. Determinación de la prevalencia de Cryptosporidium spp. en corderos lactantes en tres unidades de producción ovina en el Estado de México utilizando dos técnicas de tinción. Tesis de Licenciatura. Facultad de Medicina Veterinaria y Zootencia, Universidad Autónoma del Estado de México, Toluca, México, 2007.

15. Merck & Co. Inc. Sistema Digestivo in Manual Merck de Veterinaria, 6th ed.; Kahn CM. and Line, S. Eds.; Editorial Oceano: Barcelona, España, 2007; Vol. I, pp. 111-381.
16. Monis, P.; King, B. and Keegan, A. Removal and inactivation of *Cryptosporidium* from water. In Cryptosporidium: parasite and disease. Cacciò, SM. and Widmer, G. Eds. Springer-Verlag. Austria, 2014, pp. 515-552.
17. OIE. Terrestrial Manual 2016, Chapter 2.9.4. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.09.04_CRYPTO.pdf. Available online: (26/11/2016).
18. Partida de la PJA.; Braña, VD.; Jiménez, SH.; Ríos RFG. and Buendía, RG. Producción de carne ovina. Libro Técnico No. 5, SAGARPA-INIFAP, Querétaro, México, 2013; pp. 6-20.
19. Petrie, A. and Watson, P. Statistics for Veterinary and Animal Science; Wiley-Blackwell, UK, 2013; pp. 112-125.
20. Pollok, RC.; Farthing, MJ.; Bajaj-Elliott, M.; Sanderson, JR. and McDonald, V. Interferon gamma induces enterocyte resistance against infection by the intracellular pathogen *Cryptosporidium parvum*. *Gastroenterology*, 2001, 120, (99-107), PMID: 11208718.
21. Robertson, LJ.; Bjöekman, C.; Axén, C. and Fayer, R. Cryptosporidiosis in farmed animals. In Cryptosporidium: parasite and disease. Cacciò, SM. and Widmer, G. Eds. Springer-Verlag, Austria, 2014; pp. 149-235.
22. Romero-Salas, D.; Alvarado-Esquivel, C.; Cruz-Romero, A.; Aguilar-Domínguez, A.; Ibarra-Priego, N.; Merino-Charrez, JO.; Pérez de León, AA. and Hernández-Tinoco, J. Prevalence of Cryptosporidium in small ruminants from Veracruz, Mexico. *BMC Vet Res.*, 2016, 12, (1-6), DOI 10.1186/s12917-016-0638-3.
23. Tzipori S. *Cryptosporidium*: notes en epidemiology and pathogenesis. *Parasitol. Today*, 1985, 1, (159-165), PMID: 15275573.
24. Vohra, P.; Sharma, M. and Chaudhary, U. A comprehensive review of diagnostic techniques for detection of *Cryptosporidium parvum* in stool samples. *IOSRPHR*, 2012, 2, (15-26), DOI 10.9790/3013-25701526.
25. Valenzuela, G.; Sci, MV.; Grandón, W.; Quintana, I. and Tadich TM. Prevalencia de Cryptosporidium spp. en corderos muertos en la provincia de Valdivia, Chile. *Arch. Med. Vet.*, 1991, XXIII, (81-83). Available online: https://books.google.com.mx/books?id=1bisKawjnqgC&pg=PA2&lpg=PA2&dq=valenzuela+1991+cryptosporidium&source=bl&ots=Em85z8v90W&sig=C069xu1l_dBc5jAMyUAcy5aEK_w&hl=es&sa=X&ved=0ahUKEwjoj5LipszQAhUQ12MKHcxsCWQQ6AEIUDAJ#v=onepage&q=valenzuela%201991%20cryptosporidium&f=false
26. Whirmire, WM. and Harp, JA. Characterization of bovine cellular and serum antibody responses during infection of *Cryptosporidium parvum*. *Infect. Immun.*, 1991, 58, (84-93), PMCID: PMC258357.
27. Xiao, L.; Herd, RP. and McClure, KE. Periparturient rise in the excretion of Giardia spp. cysts and Cryptosporidium parvum oocysts as a source of infection for lambs. *J. Parasitol.*, 1994, 80, (55-59), PMID: 8308659.
28. Xiao, L.; Fayer, R.; Ryan, U. and Upton, SJ. Cryptosporidium taxonomy: recent advances and implications for public health. *Clin. Microbiol. Rev.*, 2004, 17, (72-97), DOI

10.1128/CMR.17.1.72–97.2004.

29. Zu, SX.; Fang, GD.; Fayer, R. and Guerrant, RL. Cryptosporidiosis: Pathogenesis and immunology. *Parasitol. Today*, 1992, 8, (24-27), PMID: 15463522.