THE PREVALENCE OF *CRYPTOSPORIDIUM SPP.* IN PRE- AND POST- WEANED CALVES

By

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Abstract

Cryptosporidium is a gastrointestinal parasite that affects multiple species of animals and humans. Its prevalence is of particular interest to the veterinary community as cattle are commonly infected, leading to lower production of milk and meat, causing monetary losses to cattle ranchers, and a subsequent increase in food prices. The three main species of *Cryptosporidium* that infect cattle are *C. parvum, C. andersoni,* and *C. bovis.* While related, each affects cattle very differently both in the age when infected and the symptomology. *C. parvum* is the most frequently found in newborn and pre-weaned calves, and triggers symptoms of diarrhea, fever, dehydration, and anorexia. In contrast, *C. andersoni* and *C. bovis* infect cattle post-weaning, with increased prevalence in age up to two years. While *C. bovis* is largely asymptomatic, C. *andersoni* elicits only subclinical symptoms of weight loss and reduced milk production. Currently there are no approved prevention/treatment regimens in place for *cryptosporidiosis*, with care being provided on a symptomatic basis only. qPCR was utilized to test calves from six cattle operations for the presence of *Cryptosporidium spp.,* and to determine the demographics of the cattle affected. The results from this research line up with prevalent knowledge of *Cryptosporidium spp.*

Introduction

Discovered in 1907, *Cryptosporidium* is a waterborne gastrointestinal parasite that affects most mammals, rodents, fish, and humans. In the family *Cryptosporiidae* and the phylum *Apicomplexa,* there are currently 19 recognized species (Ralston, 2010). Certain species of *Cryptosporidium* pose a significant challenge to the veterinary community, particularly in regards to calved up to 30 days of age. The infectious dose of *Cryptosporidium* is extremely small, leading to a high morbidity rate in calves (Sunnotel, 2006, Nasir, 2013). This is particularly an issue in feedlots and dairies, where one case can cause several hundred calves to become infected relatively

quickly. This increases the amount of time needed for labor and veterinary care to ensure all animals receive proper treatment and recover fully. *C. parvum, C. andersoni,* and *C. bovis* are three of the most prevalent species of crypto among cattle, and are explored in detail in this thesis.

Cryptosporidium parvum

Arguably the most common *Cryptosporidium* species found in cattle in the United States, *Cryptosporidium parvum* has an estimated 95% prevalence rate in US dairies among newborn calves less than 6 months old (Duranti, 2009). This high rate of infection couples with the highly symptomatic nature of *C. parvum,* which is unique to this *Cryptosporidium* species. When a calf is infected with *C. parvum*, the most common symptom is watery diarrhea, accompanied by fever, dehydration, anorexia and nausea, weight loss, and lethargy (Li, 2013, Fiuza, 2011). While *C. parvum* is not typically fatal, these symptoms require constant monitoring and may require medical intervention if they become too severe. Oral electrolytes are commonly provided to encourage rehydration, and subcutaneous fluids can be administered if necessary. If the fever spikes above 103.5 F^o an injection of a anti-inflammatory, such as banamine or prevail, can be used. Lowering the fever can often encourage an increase in appetite, which keeps the calf hydrated and reduces weight loss.

Morphologically, the *C. parvum* oocysts, which are the infectious stage shed in feces and found in the environment, are spherical and 4.5-5µm in diameter (Mirzai, 2014). The oocysts are transmitted through oral ingestion and can cause disease with as few as 10 oocysts (Ralston, 2010). Once in the digestive tract, oocysts excyst and release sporozoites which attach to and invade the epithelial cells of the terminal jejunum, ilum, cecum, and proximal colon and feed off of host cells to survive. The asexual life cycle of *C. parvum* is capable of auto-infection with the extracellular life stages, merozoites and sporozoites. The zoites replicate and infect other epithelial cells rapidly,

leading to the immense number of the sexual life stage of the oocysts in the digestive tract. Some of these oocysts are thin-walled and auto-infectious, and remain in the intestines, while the majority are thick-walled and leave the body in fecal matter, which is the primary route of transmission to other animals (Thompson, 2017). Diagnosis of *C. parvum* infection is performed via a fecal smear or floatation and microscopy, and can also be done using qPCR (Nasir, 2013).

Cryptosporidium andersoni

Another frequently seen species of *Cryptosporidium* is *C. andersoni*, which is the most common species isolate in cattle in China. While for the most part asymptomatic, *C. andersoni* has been known to cause subclinical symptoms of weight loss, gastritis, and reduced milk production in post-weaned and adult cattle (Guang-Hui, 2013, Fiuza, 2011). Infections are often chronic, lasting months to years with no clinical signs, with cattle excreting only small numbers of oocysts (Fiuza, 2011). *C. andersoni* is found within the abomasal gastric pits of cattle, and can cause mucosal epithelial atrophy. Te oocyst stage is spherical in shape and is the largest of the *Cryptosporidium* species at 6-8 micrometers in size (Masuno, 2006).

There are two known subtypes of *C. andersoni*, Type A and Type B, which have genetically distinct forms of 18S rRNA gene. Type A was determined to be the original *C. andersoni* species, while Type B has a single inserted nucleotide that makes it genetically distinct. Both types were able to infect SCID mice when presented, and no other differences have currently been noted (Matsubayashi, 2008).

Cryptosporidium bovis

While morphologically indistinguishable from *C. parvum*, *C. bovis* is a genetically distinct *Cryptosporidium* species that was previously known as *Cryptosporidium* Genotype Bovine B.

While also found in slightly older calves ranging from three to eleven months of age, *C. bovis* has also been known to be asymptomatic. *C. bovis* is adapted to cattle but can also infect sheep, yaks, and goats. It does not cause significant disease in animals and humans. (Feltus, 2008, Fayer, 2005, Navarro, 2007)

Methods

Many of the samples used for this research were collected by a former graduate student who previously worked in the Rigg's lab (Shelby Wendel). The current research reported here picked up where her research left off, in determining the demographics of cattle that tested positive for any of the three species of *Cryptosporidium.* There were 52 samples collected from five cattle operations in Arizona - two Beef operations and three Dairy operations. Each sample had been processed to isolate DNA from the Cryptosporidium oocysts using QIAamp Fast DNA Stool Isolation kit (Qiagen, Gaithersburg, MD). Total DNA in the samples was quantified by Nanodrop (Nanodrop Technologies, Wilmington, DE) and then stored at -20°C. In this research the samples were thawed and tested with qPCR to determine if the sample contained any of the three species of *Cryptosporidium – C. parvum, C. andersoni,* and *C. bovis*.

To run the qPCR, the *C. parvum* SSU rRNA (Genbank no. AF161856 for *C. parvum,* EF514234 for *C. bovis,* and AF093496 for *C. andersonii*) was chosen as the target for primers (ChvF18S 5'-CAATAGCGTATATTAAAGTTGTTGCAGTT-3'; ChvR18S 5'-CTGCTTTAAGCACTCTAATTTTCTCAAA-3') (Burnett et al. 2013). An approximate 107-bp sequence from conserved region of the SSU rRNA gene was selected. Taqman probes were designed to bind to the hypervariable region of the gene (for *C. parvum* Cp18S 5'-FAM/GTTAATAATTTATATAAAATATTTTGATG/NFQ-MGB-3', for *C. bovis* Cb18S 5'-

NED/AAAAGCTCGTAGTTAATCTTCTGTTA/NFQ-MGB-3', and for *C. andersonii* Ca18S 5'-FAM/CCAAGGTAATTATTATATTATC/NFQ-MGB-3'. Each sample was performed in triplicate to ensure that the run was accurate and the standard deviation was small. Each 25 ml reaction mixture contained 5µl of DNA from the sample or standard and 20µl of the master mix – a combination of qPCR Taqman Fast master mix (Thermofisher/Invitrogen, Grand Island, NY), forward and reverse primer (Thermofisher/Invitrogen), Taqman probe (Thermofisher/Invitrogen) for the species being tested for, and sterile HPLC water. For the No Template Control, 5 µl of sterile water was added to 20 µl of master mix (Burnett et al. 2013). The seven *C. parvum* standards used contained DNA from known *C. parvum* oocysts concentrations of 1E6, 5E5, 1E5, 5E4, 1E4, 5E3, and 1E3, respectively.

Each reaction of qPCR was set up for 10 minutes of initial heating at 95^0 C, then 50 cycles of 15 seconds at 95^0 C then 1 minute at 58^0 C (Burnett, 2013). The results were then analyzed using the StepOne software to determine the amount of *Cryptosporidium* and species found in each sample, if any. Additionally, the standard deviation between identical wells was assessed, and any samples that did not replicate were flagged and excluded.

The second part of this research involved collecting fecal samples from 23 five-month old calves from the University of Arizona Feedlot. These samples were collected directly from the rectum of the calves, and then processed to isolate the DNA. The procedure used to isolate DNA via the QIAGEN Fast DNA Mini STOOL KIT is as follows: Samples were measured out to ~200mg feces, and then combined with 1ml of InhibitEX buffer, vortexed thoroughly, and incubated for five minutes in a 95 C water bath. The samples were then run through five freeze-thaw cycles, then vortexed and centrifuged at 16,363 rcf for two minutes. 200µl of supernatant were added to a new microfuge tube along with 15µl proteinase K, vortexed, and then 4µl of Rnase

A, Dnase-free was added and vortexed again to mix. Next, 200µl of Buffer AL was added to the solution, mixed thoroughly, and incubated at 70^{o} C in a dry-bath for 10 minutes. The samples were then centrifuged for 30 seconds at 16,363 rcf to remove any droplets of water from the lids and walls of the tubes, and then 200µl of ethanol was added to bring the DNA out of solution. Samples were vortexed again, and centrifuged for 30 seconds. The samples were then transferred into QIAmp spin columns with 2ml collecting tubes, and centrifuged at 16,363 rcf for two minutes to remove buffer. The filtrate was discarded, and the spin column placed into a new collecting tube. 500µl of Buffer AW1 with ethanol was added, and the samples were again centrifuged at 16,363 rcf for two minutes. The filtrate was discarded and a new collection tube was added. Then 500µl of Buffer AW2 with ethanol was added to the sample, and was centrifuged at 16,363 rcf for 8 minutes. The filtrate was discarded and the spim column was placed in a 1.5ml centrifuge tube. 200µl of Buffer ATE was added to bring the DNA back into solution. The sample was incubated at room temperature for one minute, then centrifuged again at 16,363 rcf for two minutes. Another 200µl of Buffer ATE was added, and the sample was centrifuged again. The spin column was discarded, and the filtrate, which contained the purified DNA, saved and frozen. The purified DNA from these samples was then tested via the qPCR described above to identify the presence of *Cryptosporidium spp.*

Results

The original research and PCR performed by the previous graduate student found that *C. parvum* and *C. bovis* were found to have a much higher prevalence in pre-weaned calves two weeks old. *C. andersoni* was found exclusively in older, post-weaned calves of 3.5-4 months old. The qPCR run on these same samples later found similar results. Additionally, it should be noted

that it is possible for calves to be infected with more than one species of *Cryptosporidium* at a time.

Cattle Operations	Calves Sampled	# of positive calves	*C. parvum*	*C. bovis*	*C. andersoni*
Beef Operation 1: 9-15 days old	20	6	5	1	0
Beef Operation 1: 3.5-4 months old	20	6	1	2	4
Beef Operation 2: 9-15 days old	31	17	15	7	0
Beef Operation 2: 3.5-4 months old	28	7	1	5	2
Dairy Operation 1: 9-15 days old	27	17	17	5	0
Dairy Operation 1: 3.5-4 months old	16	4	0	4	0
Dairy Operation 2: 9-15 days old	27	8	7	3	0
Dairy Operation 2: 3.5-4 months old	21	7	0	4	5
Dairy Operation 3: 9-15 days old	28	11	11	4	0
Dairy Operation 3: 3.5-4 months old	28	10	0	10	5
UA Feedlot: 4.5 months old	23	7	7	0	0

In line with recent studies, it was found that *C. parvum* is found almost exclusively in pre-weaned calves, while *C. andersoni* infected post-weaned calves up to five months old. *C. bovis* appeared to fall in the middle, causing infections in both pre- and post-weaned calves. The previous data used by the former graduate student supports this idea, however the calves from the UA feedlot had a very low prevalence of any *Cryptosporidium* infection. Only seven of the 23 calves sampled were positive for *C. parvum*, and at very low levels, which indicates they could have had a previous infection when younger which has never fully resolved. However, the lack of *C. bovis* and *C. andersoni* is contrary to what was expected, given the previous data from the other five cattle operations and what is known from prior research.

Discussion

This research yielded both unexpected and contradictory results. While the original samples showed *Cryptosporidium* infection prevalence in line with what is commonly accepted, the new samples from the UA Feedlot showed a very low prevalence of infection, and the infection that was present was entirely *C. parvum.* A possible explanation for this is excellent herd management and disease prevention on the part of the UA Feedlot. If *Cryptosporidium* can be controlled and eliminated, it cannot spread and will remain eradicated until a new exposure brings it back to prevalence. If the UA Feedlot is careful with new calves and adult cattle brought into the herd and maintain rigorous disease prevention plans, they may never experience significant problems with *Cryptosporidium* infection.

Within the small sample size that this research reviewed, there were no significant differences in the prevalence of *Cryptosporidium spp.* between dairy and beef cattle. Both the beef and the dairy operations had infection rates of 25-60% for *C. parvum*, 5-30% for *C. bovis*, and 0-25% for *C. andersoni.* This indicates that both industries have an equal likelihood of being affected by *cryptosporidiosis* and should take similar preventative measures to lessen the chance of infection. Moreover, similar infection rates mean that food prices are equally at risk of being increased due to *Cryptosporidium spp.*

The original intention of this thesis was to go further and analyze the DNA of the *Cryptosporidium spp.* found in order to look for genetic differences that may be causing the variance in age of cattle affected and symptoms elicited. However, due to unforeseen time constraints that became present in the lab, this research was not able to be completed. If done, this further research could have analyzed in more depth the specific disease causing mechanisms of each species of *Cryptosporidium*, and how that could be manipulated or disabled when testing

possible treatments for infection. Currently, there is very little known about the genetic differences in these three species, and additional research into the specific genomics could reveal a wealth of information that could be used to improve upon treatments and therapies.

Citations

Burnet, J.B, L. Ogorzaly, A. Tissier, C. Penny, H.M. Cauchie (2013), Novel quantitative TaqMan real-time PCR assays for detection of Cryptosporidium at the genus level and genotyping of major human and cattle-infecting species. *Journal of Applied Microbiology*, 114: 1211-1222. doi:10.1111/jam.12103

Duranti, A., Cacciò, S. M., Pozio, E., Di Egidio, A., De Curtis, M., Battisti, A. and Scaramozzino, P. (2009), Risk Factors Associated with *Cryptosporidium parvum* Infection in Cattle. *Zoonoses and Public Health*, 56: 176–182. doi:10.1111/j.1863-2378.2008.01173.x URL: http://zp9vv3zm2k.search.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Risk+Factors+Associated+with+Cryptosporidium+parvum+Infection+in+Cattle&rft.jtitle=Zoonoses+and+Public+Health&rft.au=A+Duranti&rft.au=S+M+Caccio&rft.au=E+Pozio&rft.au=A+Di+Egidio&rft.date=2009-05-01&rft.pub=Blackwell+Publishing+Ltd&rft.issn=1863-1959&rft.eissn=1863-2378&rft.volume=56&rft.issue=4&rft.spage=176&rft_id=info:doi/10.1111%2Fj.1863-2378.2008.01173.x&rft.externalDocID=1674075731&paramdict=en-US

Fayer, R., Santin, M., & Xiao, L. (2005). Cryptosporidium bovis N. Sp. (Apicomplexa: Cryptosporidiidae) in Cattle (Bos taurus). *The Journal of Parasitology, 91*(3), 624-629. Retrieved from http://www.jstor.org.ezproxy2.library.arizona.edu/stable/20059725

Feltus, Dawn, Catherine W.Giddings, Margaret L.Khaitsa, John M.McEvoy (2008), High prevalence of *Cryptosporidium bovis* and the deer-like genotype in calves compared to mature cows in beef cow-calf operations, *Veterinary Parasitology***,** Volume 151, Issues 2–4, 14 February 2008, Pages 191-195 https://doi.org/10.1016/j.vetpar.2007.10.012

Fiuza, V., Almeida, A., Frazão-Teixeira, E., Santín, M., Fayer, R., & Oliveira, F. (2011). Occurrence of Cryptosporidium andersoni in Brazilian Cattle. *The Journal of Parasitology, 97*(5), 952-953. Retrieved from http://www.jstor.org.ezproxy2.library.arizona.edu/stable/23019159

Guang-Hui Zhao, Wan-Xin, R., Gao, M., Qing-Qing Bian, Hu, B., Mei-Mei Cong, . . . Long-Xian, Z. (2013). Genotyping cryptosporidium andersoni in cattle in shaanxi province, northwestern china. PLoS One, Volume 8, Issue 4 URL: https://search-proquest-com.ezproxy4.library.arizona.edu/docview/1330900695?pq-origsite=summon#

Li, S., W. Li, , Z. Yang, S. Song, J. Yang, P. Gong, W. Zhang, K. Liu, J. Li, G. Zhang, X. Zhang (2013) Infection of Cattle With Cryptosporidium parvum: Mast Cell Accumulation in Small Intestine Mucosa *Veterinary Pathology*, Vol 50, Issue 5, pp. 842 - 848 https://doi-org.ezproxy4.library.arizona.edu/10.1177/0300985813476055

Masuno, K., T. Yanai, A. Hirata, K. Yonemaru, H. Sakai, M. Satoh, T. Masegi, Y. Nakai (2006), Morphological and Immunohistochemical Features of *Cryptosporidium andersoni* in Cattle, *Veterinary Pathology*, Volume: 43 issue: 2, page(s): 202-207
URL: http://journals.sagepub.com.ezproxy4.library.arizona.edu/doi/full/10.1354/vp.43-2-202

Matsubayashi, Makoto, Saki Nagano, Toshimasa Kita, Tsuguto Narushima, Isao Kimata, Motohiro Iseki, Tomoya Hajiri, Hiroyuki Tani, Kazumi Sasai, Eiichiroh Baba (2008). Genetical survey of novel type of *Cryptosporidium andersoni* in cattle in Japan, *Veterinary Parasitology* Volume 158, Issues 1–2, 25 November 2008, Pages 44-50, https://doi.org/10.1016/j.vetpar.2008.08.021

Mirzai, Y., Yakhchali, M., & Mardani, K. (2014). *Cryptosporidium parvum* and *Cryptosporidium andersoni* infection in naturally infected cattle of northwest Iran. *Veterinary Research Forum : An International Quarterly Journal*, *5*(1), 55–60.
URL: http://zp9vv3zm2k.search.serialssolutions.com/?ctx_ver=Z39.88-2004&ctx_enc=info%3Aofi%2Fenc%3AUTF-8&rfr_id=info%3Asid%2Fsummon.serialssolutions.com&rft_val_fmt=info%3Aofi%2Ffmt%3Akev%3Amtx%3Ajournal&rft.genre=article&rft.atitle=Cryptosporidium+parvum+and+Cryptosporidium+andersoni+infection+in+naturally+infected+cattle+of+northwest+Iran&rft.jtitle=Veterinary+Research+Forum&rft.au=Yousef+Mirzai&rft.au=Mohammad+Yakhchali&rft.au=Karim+Mardani&rft.date=2014-03-01&rft.pub=Veterinary+Research+Forum&rft.issn=2008-8140&rft.eissn=2322-3618&rft.volume=5&rft.issue=1&rft.spage=55&rft.externalDocID=3284954261&paramdict=en-US

Nasir, A., Avais, M., Khan, M., Khan, J., Hameed, S., & Reichel, M. (2013). Treating Cryptosporidium parvum Infection in Calves. *The Journal of Parasitology*, *99*(4), 715-717. Retrieved from http://www.jstor.org.ezproxy4.library.arizona.edu/stable/41982078

Navarro-I-Martinez, L., Da Silva, A., Bornay-Llinares, F., Moura, L., Del Aguila, C., Oleaga, A., & Pieniazek, N. (2007). Detection and Molecular Characterization of Cryptosporidium bovis-like Isolate from a Newborn Lamb in Spain. *The Journal of Parasitology*, *93*(6), 1536-1538. Retrieved from http://www.jstor.org.ezproxy2.library.arizona.edu/stable/40058960

Ralston, B. , Thompson, R. , Pethick, D. , McAllister, T. and Olson, M. (2010), *Cryptosporidium andersoni* in Western Australian feedlot cattle. *Australian Veterinary Journal*, 88: 458-460. doi:10.1111/j.1751-0813.2010.00631.x

Sunnotel, O., Lowery, C.J., Moore, J.E., Dooley, J.S.G., Xiao, L., Millar, B.C., Rooney, P.J. and Snelling, W.J. (2006), *Cryptosporidium*. *Letters in Applied Microbiology*, 43: 7–16. doi:10.1111/j.1472-765X.2006.01936.x
URL: http://onlinelibrary.wiley.com/doi/10.1111/j.1472-765X.2006.01936.x/full

Thomson, Sarah, Carly A. Hamilton, Jayne C. Hope, Frank Katzer, Neil A. Mabbott, et al.. (2017) Bovine cryptosporidiosis: impact, host-parasite interaction and control strategies. *Veterinary Research*, BioMed Central, 48 (1), pp.42. <10.1186/s13567-017-0447-0>. <hal-01574200>
URL: https://hal.archives-ouvertes.fr/hal-01574200/document

Wyatt, C.R., M.W. Riggs, and R. Fayer. (2010) Cryptosporidiosis in Neonatal Calves, *The Veterinary Clinics of North America, Food Animal Practice* – Emerging, Reemerging and Persistent Infectious Diseases in Cattle. S. Kapil, D.S McVey Eds. 26 (1): 89-103.