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Cryptosporidium Species of Dairy Cattle in the North Kazakhstan Region

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Abstract

Cryptosporidiosis is a zoonotic disease of farm livestock and humans which causes diarrhea and great economic disbenefit. This work aimed to identify *Cryptosporidium* species in the dairy cattle farm where the disease was noted in the North Kazakhstan Region. Freshly selected excrements specimens from 22 calves up to one month of age and 20 young animals up to one year old were examined by Heine (1982) for the presence of *Cryptosporidium* oocysts, as well as for detecting *C.parvum* with the Fassisi Fa Bodia immunochromatography test. In two samples from young cattle, in which oocysts were microscopically detected, but the presence of *C.parvum* was not confirmed, the Cryptosporidium species was determined using the molecular biological methods. DNA was extracted using the QIAamp Stool Kit. DNA quality was monitored by electrophoresis. DNA quantification analysis was performed on the NanoDrop 2000 spectrophotometer. The PCR program was performed on the Mastercycler Gradient amplifier. PCR products were purified using the Quick PCR Purification Kit. An analysis of nucleotide sequences was performed using the Finch TV v1.3.1. software and BLAST. As a result, oocysts revealed in 68% of calves, which gave a positive reaction to *C.parvum*. *Cryptosporidium* oocysts were found in 10% of young animals which samples showed a negative result for the presence of *C.parvum* antigens. However, in them the *C.andersoni* species DNA was identified. Thus, in the north of Kazakhstan, two zoonotic species of *Cryptosporidium* have been identified in cattle.

Keywords: *Cryptosporidium*; North Kazakhstan Region; cattle; molecular methods; *C.parvum*; *C.andersoni*.

Introduction

Cryptosporidiosis is a disease that affects many animal species and manifests itself in the form of abundant watery diarrhea, weight loss, appetite and sometimes causes death. At present, the disease is recognized as an important zoonosis (Chalmers, Katzer, 2013). *Cryptosporidium* is considered to be one of the significant causes of neonatal enteritis and calf diarrhoea worldwide (de Graaf, Vanopdenbosch, Ortega-Mora, Abbassi, Peeters, 1999; Fayer, Gasbarre, Pasquali, Canals, Almeria, Zarlenga, 1998; Thomson, 2016). 28 different species and an even greater number of *Cryptosporidium* genotypes have now been established (Chalmers, Katzer, 2013; Fayer, 2010). One of the most cosmopolitan zoonotic species is *C.parvum* (Chalmers, Katzer, 2013). Historically, the new species *Cryptosporidium* was named according to its

host, which was discovered for the first time on the assumption that cryptosporidia are host-specific. However, some species, such as *C.parvum* and *C.ubiquitum*, invade many different hosts.

Productive animals (cattle, small cattle, pigs) are infected by 8 *Cryptosporidium* species. In cattle there are usually four species: *C.parvum, C.bovis, C.ryanae* and *C.andersoni* (Thomson, 2016).

Differentiation of *Cryptosporidium* species found in humans and farm animals is important, as it makes it possible to identify the source of invasion in cases of outbreaks in the population and helps to choose the right control strategy (Chalmers, Elwin, Thomas, Guy, Mason, 2009; Gormley, Little, Chalmers, Rawal, Adak, 2011). During outbreaks of cryptosporidiosis in humans, the parasite is usually outranked to the species and even genotyped, whereas in veterinary practice only the parasite is most often confirmed. Although there are several detailed studies on molecular typing of *C.parvum* in calves at the regional level in many countries (Mammeri, Chevillot, Chenafi, Thomas, Julien, Vallee, Polack, Follet, Adjou, 2019), which have shown that the most significant reservoirs of *C.parvum* zoonotic subtypes (IIa, IId) are calves, which determine the risk of transmission of cryptosporidiosis from animals to humans (Thomson, 2016). Microscopic, immunoassay and polymerase chain reaction (PCR) methods are the most appropriate for the detection of *Cryptosporidium* (Chalmers, Katzer, 2013).

This article first describes the molecular genetic identification of *C.andersoni* in cattle in northern Kazakhstan.

Methods

Collection. *Per rectum* samples of excrements from 22 calves to one month's age and 20 young cattle aged up to one year - descendants of the first and second generation of high-born symmental cows imported from the Czech Republic - were taken at the dairy farm, which is unfavourable for cryptosporidiosis in the North Kazakhstan Region. We conducted a microscopic study on Heine (1982) samples for cryptosporidium oocysts as well as immunochromatographic analysis on *C.parvum* by Fassisi Fa Vosia commercial express test (Heine, 1982).

Molecular identification. In two samples from young cattle, in which oocysts were found microscopically, but the presence of *C.parvum* was not confirmed immunochromatographically, the species *Cryptosporidium* spp. was determined by molecular biological methods in the laboratory of the Agricultural Biotechnology Platform of S.Seifullin Kazakh Agro Technical University.

Extraction of oocysts. Oocysts of *Cryptosporidium* spp. were extracted from faeces using the saccharose flotation method. For this purpose 60 ml of faecal suspension was added to 250 ml flask with 90 ml of 45% sucrose solution and thoroughly stirred, then the suspension was centrifuged at

1000×g for 20 minutes. The supernatant was removed and the sediment was resuspended in 20 ml dH2O in a 50 ml tube, centrifuged for 5 minutes at 500×g, the supernatant was removed and the sediment resuspended in 20 ml dH2O. This stage was repeated until the supernatant became transparent (four times on average). After final washing, the sludge was resuspended in 10 ml of 10% sodium dodecylsulfate (SDS) and left at room temperature for 1 hour. The samples were then centrifuged at 500×g for 5 minutes, the supernatant was removed and the sludge resuspended in 10 ml dH2O. If any impurities still remained after this stage, 100 µl 2% sulfuric acid was added to promote flocculation. After the oocysts were extracted from the faeces, they were used to extract DNA.

Extraction of DNA. DNA extraction from the purified oocysteous suspension was performed using a modified method described by Karanis et al. followed by the QIAamp Stool Kit (Qiagen GmbH, Hilden, Germany) (Karanis, Plutzer, Halim, Igori, Ongerth, Nagasawa, 2007). In the first stage, the oocysts were destroyed using 10 freeze-thaw cycles in the presence of a lysis buffer in the thermoblock (TDB-120, Biosan, Latvia) and subjected to further treatment according to the instructions of the Qiagen manufacturer. DNA was eluted to 100 µl TE buffer and stored at -20°C.

Evaluation of quality and quantity of isolated DNA. The quality of genomic DNA was controlled by electrophoresis in 1.5% agarose gel. DNA quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Fisher). All measurements were carried out in a buffer solution (TE) at neutral pH. For preparation of working concentration of DNA (10 ng/mkl) TE was used.

PCR analysis. Amplification reactions were carried out in 25 µl volumes containing 10 ng DNA, 0.5Ed. Taq DNA Polymerase (Thermo Fisher), 0.2 mM each DNTF, 1 PCR buffer, 2.5 mM MgCl2, 10 pmols each primer. PCR program was executed on amplifier Mastercycler Gradient, (Eppendorf). The reaction parameters consisted of a hot start at 94°C for 1 min and then 35 cycles at 94°C for 10 s, 55°C for 30 s and 72°C for 1 min and final DNA completion at 72°C for 10 minutes.

DNA sequestering and data analysis. The resulting PCR products were cleaned from oligonucleotide residues with the Quick PCR Purification Kit (Invitrogen) following the manufacturer's instructions. The cleaned PCR products were then prepared for sequestering using the CEQ 8000 (Beckman Coulter). For this purpose, a standard set of reagents for cyclic sequestering using CEQ WellRED (Beckman Coulter) terminator dye was used. Thermocycling program was 30 cycles with temperature conditions: 96°C - 20 sec.; 50°C - 20 sec.; 60°C - 4 min.

The analysis of the obtained nucleotide sequences was performed using the Finch TV v1.3.1. software package and the international BLAST nucleotide sequence database.

RESULTS

In a microscopic study, oocysts were found in 15 of 22 calves (68%), all samples with parasitic elements in this group of animals during immunochromatographic testing gave a positive reaction to *C.parvum.*

In young cattle, Cryptosporidium oocysts were found in only two samples (10%), which gave a negative result on the presence of antigens of cryptosporidium of this species.

At the next stage, the work was carried out with samples of two samples of faeces with oocysts of the second sex and age group of animals, where the presence of *C.parvum* was not confirmed by immunological testing.

Genomic DNA was isolated from purified oocysts from two samples, the concentration of which is shown in Table 1.

Table 1. Quantity of isolated DNA

№	Number of sample	Type of sample	Concentration of DNA	The unit of measurement
1	2	DNA	875,23	ng/µl
2	36	DNA	1231,56	ng/µl

Qualitative indices of DNA isolated by electrophoresis method in 1.5% agarose gel are shown in Figure 1.

Electrophoresis revealed the presence of high quality genomic DNA and absence of impurities of various organic solvents, which allowed to use these DNA samples for PCR analysis.

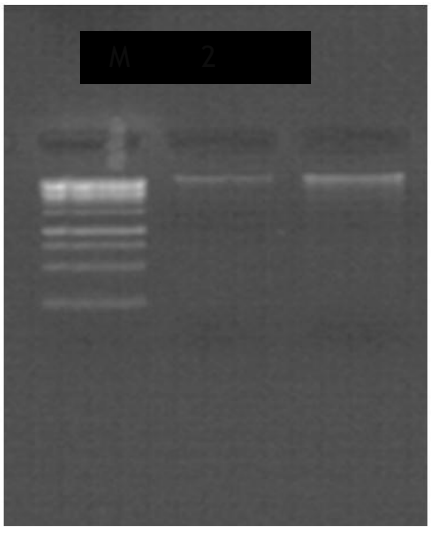

Figure 1. Electrophoregram of isolated DNA

Nota bene: 2nd and 36th samples of DNA; M-marker 1kb DNA Ladder (Sigma-Aldrich).

The Nested PCR method by Jiang et al. was used to carry out PCR analysis (Jiang, Alderisio, Xiao, 2005). Two sets of oligonucleotide primers (Table 2) were used to amplify a fragment of SSU (small-subunit) rRNA gene approximately 840 base pairs long.

DNA amplification was performed using the above method in two stages on the Mastercycler Gradient amplifier (Eppendorf).

Figure 2 shows that as a result of PCR in the studied samples was amplified fragment length of about 840 bps.

Table 2. Nucleotide sequence of primers

№	Name of primers	Sequence 5' → 3'	Name of gene
1	F1	TTCTAGAGCTAATACATGCG	SSU (smallsubunit) rRNA
2	R1	CCCATTTCCTTCGAAACAGGA	
3	F2	GGAAGGGTTGTATTTATTAGATAAAG	
4	R2	CTCATAAGGTGCTGAAGGAGTA	

The resulting PCR products were cleaned with the Quick PCR Purification Kit (Invitrogene) and sequenced on the CEQ 8000 (Beckman Coulter) using the standard CEQ WellRED (Beckman Coulter) cyclic sequestering and terminal dyeing chemicals. Thermocycling program was 30 cycles with the temperature regime: 96°C - 20 sec.; 50°C - 20 sec.; 60°C - 4 min.

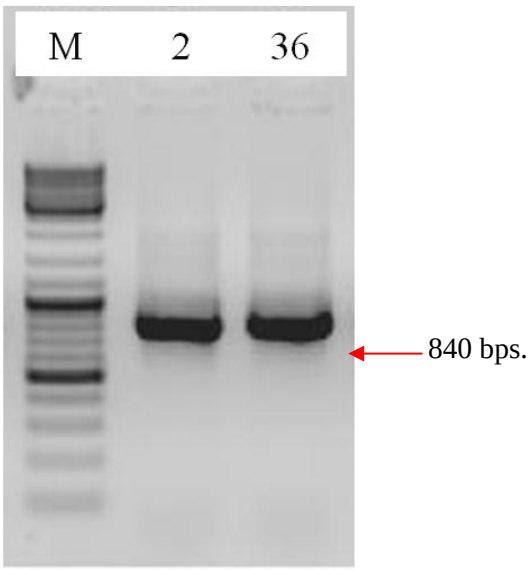

Figure 2. Electrophoregram of PCR products of amplification of SSU rRNA fragment of DNA gene

Nota bene: 2nd и 36th samples of DNA; M-marker 100 bp DNA Ladder (Thermo Fisher).

The sequencing product chromatogram (Figure 3) shows distinct peaks with high levels of fluorescence over the entire sequence, allowing for an unambiguous interpretation of the results that

were used to identify the species using BLAST.

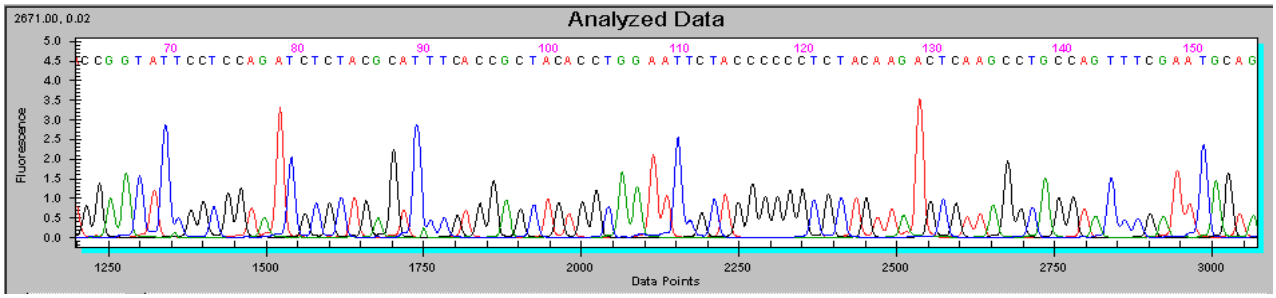

Figure 3. Chromatogram of sequestering

When identifying DNA, account was taken of three species that have the maximum identity with the sequences being analyzed. As a result, it was established that the SSU rRNA nucleotide sequence of the gene from two investigated samples has the maximum identity (99%) with the simplest species *Cryptosporidium* andersoni. Thus, DNA of *C.andersoni* species was identified by molecular genetic methods in both samples.

Discussion

The present studies have shown that of the eight *Cryptosporidium* species described in cattle, small cattle and pigs, *C.parvum* and *C.iersoni* species are common in cattle breeding enterprises in Kazakhstan. The data obtained generally agree with the information that *C.andersoni* is the most common species in older cattle (Fayer, Santin, Dargatz, 2010; Feng, Alderisio, Yang, Blancero, Kuhne, Nadareski, Reid, Xiao, 2007; Santin, Trout, Fayer, 2008; Xiao, 2010). This species is rarely found in weaning calves and this indicates that adult cattle are not at risk as a source of infection for newborn calves (Amer, Harfoush, He, 2010; Kvac, Hromadova, Kvetonova, Rost, Sak, 2011; Wang, Wang, Sun, Zhang, Jian, Qi, Ning, Xiao, 2011).

C.iersoni is known to occur mainly in the rennet of cattle and small cattle, does not cause diarrhea and is characterized by much lower oocystic excretion compared with *C.parvum* (Kvac, et al., 2011). However, some data suggest that *C.iersoni* infection causes moderate clinical severity and affects weight and dairy productivity (Ralston, Thompson, Pethick, McAllister, Olson, 2010). Compared to the other three *Cryptosporidium* species of cattle, *C. andersoni* oocysts are relatively larger in size and morphologically more elongated, making it difficult to diagnose and differentiate species through microscopy (Fayer, 2010). More advanced species identification methods are sequencing analysis of gene fragments or PCR-RFLP, which are time and cost intensive (Thomson, 2016).

C. andersoni is widespread in cattle and the environment. For example, it has been found that cattle are 23% infected with this species in selected dairy herds in Scotland, 17.3% in Brazil and 1.4% in

12 US states according to a fairly large sample (95,875 excrements samples) (Lindsay, Upton, Owens, Morgan, Mead, Blagburn, 2000). In a sample study in the Czech Republic 4% of cows educed oocysts of this species. Oocysts of *C. andersoni* were observed in feces in 4.5-7.9% of Holstein-Friesian cattle imported to this country from France and Germany (Pavla´sek, 1995). In our own study, we have carried out research on the second and third generation young cattle of the Simmental breed imported from the Czech Republic, which suggests that the detected *cryptosporidium* species are imported from central Europe.

Since *C.andersoni* was found mainly in domesticated cattle, it was referred to as a species that had a narrow specificity to its owners (Feng, Yang, Ryan, Zhang, Kvac, Koudela, Modry, Li, Fayer, Xiao, 2011). However, isolated cases of human *C.andersoni* infection have been described in different countries of the world. In addition, recent studies in China have shown that *C.andersoni* has caused outbreaks of diarrhoeal infection involving more than 90% of the people infected (Jiang, Ren, Yuan, Liu, Zhao, Liu, Chu, Pan, Cao, Lin, Shen, 2014). This has suggested that *C. andersoni* is becoming the new main species of *Cryptosporidium*, which infects humans, and its zoonotic transmission can come from animals to humans both directly and indirectly.

C. andersoni is known to frequently contaminate water sources and was found in rivers such as the Potomac (USA), Huangpu (China) and in aqueous effluent (Harbin, China). Oocysts from the parasite are supposed to release *C.iersoni*-contaminated people into urban aqueous effluent, and this contributes to further contamination of water sources, which may lead to anthropogenic transmission of *C.iersoni* (Feng, Zhao, Chen, Jin, Zhou, Li, Wang, Xiao, 2011).

The forgoing gives reason to believe that the organization of further molecular researches of Cryptosporidium animals, and also studying of dynamics of transfer of this sort between dairy calves, people and water sources is important for better understanding of epidemiology of cryptosporidiosis in various geographical regions of the country.

Conclusion

Molecular biology studies have shown that in the north of the Republic of Kazakhstan the species *Cryptosporidium parvum* and *C. andersoni*, which are zoonotic infection agents, are common in cattle.

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References

Amer, S., Harfoush, M., He, H. (2010). Molecular and phylogenetic analyses of *Cryptosporidium* spp. from dairy cattle in Egypt. *Journal Of The Egyptian Society Of Parasitology*, 40, 349-366.

Chalmers, R.M., Elwin, K., Thomas, A.L., Guy, E.C., Mason, B. (2009). Long-term Cryptosporidium typing reveals the aetiology and species-specific epidemiology of human cryptosporidiosis in England and Wales, 2000 to 2003. *Eurosurveillance*, 14.

Chalmers, R.M., Katzer, F. (2013). Looking for *Cryptosporidium*: the application of advances in detection and diagnosis. *Trends in Parasitology*, 29, 237-251.

de Graaf, D.C., Vanopdenbosch, E., Ortega-Mora, L.M., Abbassi, H., Peeters, J.E. (1999). A review of the importance of cryptosporidiosis in farm animals. *International Journal for Parasitology*, 29, 1269-1287.

Fayer, R. (2010). Taxonomy and species delimitation in Cryptosporidium. *Experimental Parasitology*, 124, 90-97.

Fayer, R., Gasbarre, L., Pasquali, P., Canals, A., Almeria, S., Zarlenga, D. (1998). Cryptosporidium parvum infection in bovine neonates: dynamic clinical, parasitic and immunologic patterns. *International Journal for Parasitology*, 28, 49-56.

Fayer, R., Santin, M., Dargatz, D. (2010). Species of *Cryptosporidium* detected in weaned cattle on cow-calf operations in the United States. *Veterinary Parasitology*, 170, 187-192.

Feng, Y., Alderisio, K.A., Yang, W., Blancero, L.A., Kuhne, W.G., Nadareski, C.A., Reid, M., Xiao, L. (2007). *Cryptosporidium* genotypes in wildlife from a new york watershed. *Applied and Environmental Microbiology*, 73, 6475-6483.

Feng, Y., Yang, W., Ryan, U., Zhang, L., Kvac, M., Koudela, B., Modry, D., Li, N., Fayer, R., Xiao, L. (2011). Development of a multilocus sequence tool for typing *Cryptosporidium muris* and *Cryptosporidium andersoni*. *Journal of Clinical Microbiology*, 49(1), 34-41.

Feng, Y., Zhao, X., Chen, J., Jin, W., Zhou, X., Li, N., Wang, L., Xiao, L. (2011). Occurrence, source, and human infection potential of *Cryptosporidium* and *Giardia* spp. in source and tap water in Shanghai, China. *Applied and Environmental Microbiology*, 77(11), 3609-3616.

Gormley, F.J., Little, C.L., Chalmers, R.M., Rawal, N., Adak, G.K. (2011). Zoonotic

cryptosporidiosis from petting farms, England and Wales, 1992-2009. *Emerging Infectious Diseases*, 17, 151-152.

Heine, J. (1982). Eine einfache Nachweismethode für Kryptosporidien im Kot. *Zentralbl Veterinaermed*, 29, 324-327.

Jiang, J, Alderisio, K.A, Xiao, L. (2005). Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. *Applied and Environmental Microbiology*, 71, 4446-4454.

Jiang, Y., Ren, J., Yuan, Zh., Liu, A., Zhao, H., Liu, H., Chu, L., Pan, W., Cao, J., Lin, Y., Shen, Y. (2014). *Cryptosporidium andersoni* as a novel predominant *Cryptosporidium* species in outpatients with diarrhea in Jiangsu Province, China. *BMC Infectious Diseases*, 14, 555.

Karanis, P., Plutzer, J., Halim, N.A., Igori, Kh., Ongerth, H.J., Nagasawa, M.L. (2007). Molecular characterization of *Cryptosporidium* from animal sources in Qinghai province of China. *Parasitology Research*, 101, 1575.

Kvac, M., Hromadova, N., Kvetonova, D., Rost, M., Sak, B. (2011). Molecular characterization of *Cryptosporidium* spp. in pre-weaned dairy calves in the Czech Republic: absence of *C. ryanae* and management-associated distribution of *C. andersoni*, *C. bovis* and C. *parvum* subtypes. *Veterinary Parasitology*, 177, 378-382.

Lindsay, D.S., Upton, S.J., Owens, D.S., Morgan, U.M., Mead, J.R., Blagburn, B.L. (2000). C*ryptosporidium andersoni* n. sp. (Apicomplexa: Cryptosporiidae) from cattle, *Bos Taurus*. *Journal of Eukaryotic Microbiology*, 47(1), 91-95.

Mammeri, M., Chevillot, A., Chenafi, I., Thomas, M., Julien, Ch., Vallee, I., Polack, B., Follet, J., Adjou, K.T. (2019). Molecular characterization of *Cryptosporidium* isolates from diarrheal dairy calves in France. Veterinary Parasitology: Regional Studies and Reports, 18, 100323.

Pavla´sek, I. (1995). Findings of cryptosporidia and of other endoparasites in heifers imported into the Czech Republic. *Veterinarni Medicina*, 40, 333–336.

Ralston, B., Thompson, R.C., Pethick, D., McAllister, T.A., Olson, M.E. (2010). *Cryptosporidium andersoni* in Western Australian feedlot cattle. *Australian Veterinary Journal*, 88, 458-460.

Santin, M., Trout, J.M., Fayer, R. (2008). A longitudinal study of cryptosporidiosis in dairy cattle from birth to 2 years of age. *Veterinary Parasitology*, 155, 15-23.

Thomson, S. (2016). Cryptosporidiosis in farm livestock. Institute of Biodiversity Animal Health and Comparative Medicine. University of Glasgow, PhD thesis.

Wang, R., Wang, H., Sun, Y., Zhang, L., Jian, F., Qi, M., Ning, C., Xiao, L. (2011). Characteristics of *Cryptosporidium* transmission in preweaned dairy cattle in Henan, China. *Journal of Clinical Microbiology*, 49, 1077-1082.

Xiao, L. (2010). Molecular epidemiology of cryptosporidiosis: an update. *Experimental Parasitology*, 124, 80-89.