



# *Cryptosporidium parvum* – zoonotic subtype IIdA15G1 in a Slovakian patient

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## Abstract

**Introduction and objectives.** The parasite *Cryptosporidium* spp. is an intracellular protozoa which has a broad range of hosts and zoonotic potential. It presents a serious health risk for agricultural workers and veterinarians. The aim of the study was to identify the species and subtypes of *Cryptosporidium* occurring in a veterinary student who came into contact with calves on a farm.

**Materials and method.** The Ziehl-Neelsen staining technique was employed to confirm the presence of *Cryptosporidium* oocysts. ELISA test was applied to detect coproantigen in faecal specimens. Nested PCR was used to amplify a small ribosomal subunit (SSU rRNA) and sequencing of the GP60 gene served to identify the zoonotic subtypes.

**Results.** The nested PCR allowed to confirm the *C. parvum* species; subsequently, the IIdA15G1 zoonotic subtype was identified.

**Conclusion.** This is the first confirmed case in Slovakia of human cryptosporidiosis caused by the unique subtype IIdA15G1.

## Key words

Slovakia, nested PCR, *Cryptosporidium* spp, zoonotic subtype, veterinary student

## INTRODUCTION

*Cryptosporidium* spp. are intracellular protozoa with a broad host range and zoonotic potential [1, 2]. Until now, 38 valid species of *Cryptosporidium* genus have been described; *Cryptosporidium hominis* and *Cryptosporidium parvum* are responsible for most human infections [3, 4]. The infection spreads via the faecal-oral transmission of oocysts from the environment, by a direct contact with infected persons and animals, or by ingestion of contaminated water and food [5, 6]. *Cryptosporidium* spp. show affinity to the epithelial cells of duodenal enterocytes. The disease is manifested by watery diarrhoea, abdominal pain, malabsorption, nausea, dehydration and body weight loss [7, 8]. The immune competence of the host, his congenital and acquired immunity significantly affect the course and severity of disease [7]. For the profoundly immunocompromised, such as people who are malnourished, very young infants or people who have a coexisting health problem (untreated HIV/AIDS infection) which leads to T-cell immunodeficiency, symptoms can be severe, prolonged and even life-threatening [9, 10]. Spontaneous recovery is rare in such patients, and moreover, the infection often disseminates into other organs (oesophagus, biliary tract) [9, 10, 11]. Cryptosporidiosis can also cause persistent symptoms in immunocompetent

subjects, present with profuse watery diarrhoea and abdominal pain which begins seven to fourteen days after being infected. The symptoms last for 8–20 days; later the patients recover spontaneously, but some of these symptoms may be indicative of post-infectious irritable bowel syndrome [12]. In Slovakia, records on the occurrence of cryptosporidiosis are scarce. The first record dates back to 1987 when seven cases caused by *Cryptosporidium* genus were detected in patients with HIV/AIDS [13]. The second documented case of the infection caused by the *C. hominis* species was described in two children in 2013. The DNA typing identified IbA10G2 subspecies of *C. hominis* [2]. Petrincová et al. (2015), in two kidney transplant recipients were detected species/genotypes *Cryptosporidium parvum* IIaA13G1T1R1 and *Cryptosporidium hominis* IaA11G2R8. In two immunocompetent patients were identified *Cryptosporidium muris* and *C. hominis* IbA10G2T1 and *C. hominis* IbA11G2 in one sample [14]. Hatalová et al. (2019) detected IIaA17G1R1 zoonotic subtype of *C. parvum* in immunosuppressed patients [15]. Nevertheless, *Cryptosporidium* spp. is underdiagnosed and underreported in most countries, despite being one of the communicable diseases for which surveillance is mandatory in the European Economic Area (EEA) and the European Union (EU) countries [16]. In our study, we present the case of human cryptosporidiosis in Slovakia induced by the zoonotic subtype IIdA15G1 in a veterinary student who came into contact with calves on a farm. This case report of *Cryptosporidium* infection in a human confirms the possible role of cattle in the zoonotic transmission of this pathogen, and indicates a potential danger of outbreaks of zoonotic

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cryptosporidiosis and the need for strict control and hygiene practices during handling of young animals, especially calves. The identification and genotyping of this pathogen in Slovakia, completes the epidemiological situation in Europe for *Cryptosporidium* zoonotic species.

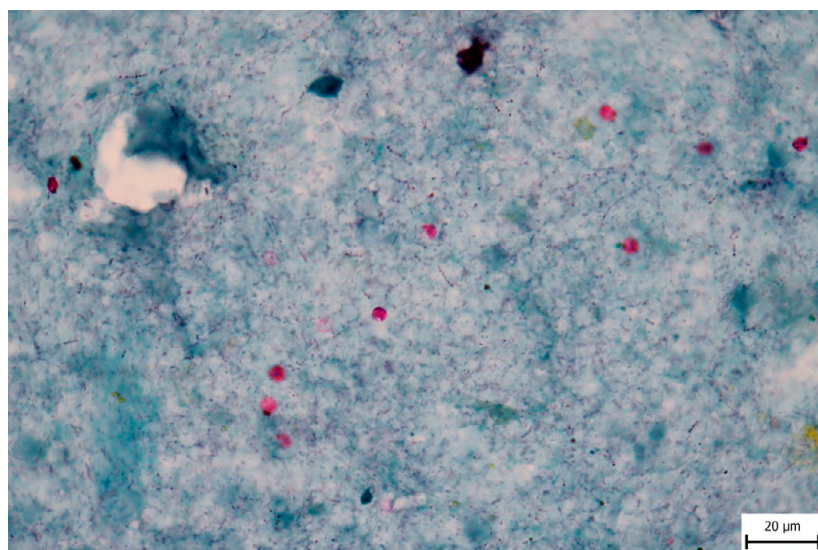
## CASE REPORT

**Case presentations.** In September 2018, a 23-year-old veterinary student was admitted to the University Hospital in Košice with gastrointestinal problems. He denied any fever or blood or mucus in his faeces. Stool cultures were negative for *Shigella*, *Salmonella*, *E. coli*, *Campylobacter*, *Yersinia* and *Bacillus cereus*. The infection was severe and presented as high frequency diarrhoea (10 episodes per day), weight loss (8 kg in 10 days), depression, flatulence, abdominal pain and dehydration. The gastrointestinal symptoms lasted for seven days after which the patient's condition gradually improved. During hospitalisation, the patient received supportive therapy (diet, infusions) and faecal specimens were collected on three subsequent days for coprological examinations. The samples were examined at the Institute of Parasitology in University of Veterinary Medicine and Pharmacy in Košice. In order to confirm the suspected cryptosporidiosis, the ELISA test was used for proof of the coproantigen, with a positive result. The coproantigen was detected by means of a commercially available kit, the CRYPTOSPORIDIUM (FAECAL) using Diagnostic Automation, INC, Calabasas, USA, according to the manufacturer's instructions. A faecal smear was prepared to investigate the presence of *Cryptosporidium* oocysts. The samples were stained with a special acid-resistant stain according to Ziehl–Neelsen [17]. Microscopic examination allowed identification of the *Cryptosporidium* oocysts which appeared as pink-purple subspherical formations against a blue-green background (Fig. 1). The samples did not show the presence of eggs/oocysts of other parasites. Total DNA was extracted from 200–300 mg stool by using the ZR Fecal DNA MiniPrep™ kit (Zymo Research, USA) for DNA, following the manufacturer's instructions. DNA samples were stored at -20 °C until further use.

**Molecular analysis.** To identify *Cryptosporidium* spp. in the stool samples, a fragment covering 18S rRNA and GP60 gene were amplified by nested PCR. The amplification of the SSU rRNA segment sized 820 bp confirmed the presence of *C. parvum* species. The programme of amplification was as follows: initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of 94 °C/45 sec, 55 °C/45 sec and 72 °C/60 sec, followed by a final extension of 72 °C/7 min. The secondary PCR reaction components and conditions were identical to those applied in the first PCR [18]. Subsequently, the subtyping of selected products was carried out based on the sequence analysis of the GP60 locus, according to Alves et al. (2006). The GP60 gene (~ 820–864 bp), the programme of amplification was as follows: initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of 95 °C/45 sec, 52 °C/45 sec and 72 °C/60 sec, followed by a final extension of 72 °C/10 min. The secondary PCR reaction components and conditions were identical to those applied in the first PCR (Tab. 1) [19]. The positive PCR products were visualised on 1.5% agarose gel under UV light, using Gel red (Biotinum Inc CA Hayward USA). Subsequently, all positive PCR products were sent for purification and sequencing to the Microsynth Laboratory (Vienna, Austria), and the sequences were compared with the known sequences using BLAST-tool

**Table 1.** Primers used in the study

Gene	PCR	Primers 5'- 3'	Product size [bp]
SSU rRNA [19]	First amplification	Forward – TTCTAGAGCTAATACATGCG	~1,250
		Reverse – CCCTAATCCTTCGAAACAGGA	
	Nested amplification	Forward – GGAAGGGTTGTATTATTAGATAAAG	820
		Reverse – AAGGAGTAAGGAACAACCTCCA	
GP60 [20]	First amplification	Forward – ATAGTCTCCGCTGTATTC	~1,250
		Reverse – GGAAGGAACGATGTATCT	
	Nested amplification	Forward – TCCGCTGTATTCTCAGCC	~820–850
		Reverse – GCAGAGGAACCGATC	



**Figure 1.** Ziehl-Nielsen acid-resistant stain – identification of *Cryptosporidium* oocysts ×1,000

(NCBI, USA). Genotyping of the GP60 was performed by means of Geneious software (Biomatters Ltd., New Zealand) and published in the GenBank database under Accession No. MK948612.

## RESULTS AND DISCUSSION

This study presents the first case of human cryptosporidiosis caused by the *C. parvum* species, the IIdA15G1 subtype, in 23-year-old veterinary student who worked on a calves farm in Eastern part of Slovakia. The infectious oocysts were probably transmitted through contact with infected calves and subsequent poor hand hygiene. The presence of *Cryptosporidium* oocysts was detected in the stool samples examined by a specific staining technique (Ziehl-Neelsen). In order to prove the presence of coproantigen in the patient's faeces, the ELISA test was applied, with a positive result. Selected primers and the nested PCR confirmed the presence of a zoonotic species of *C. parvum*, the IIdA15G1 subtype. The GP60 sequence of the tested sample was submitted to the GenBank and accepted for publishing in GenBank databases under Accession No. MK948612. Genotyping analysis of the GP60 gene from the patient revealed homology with the IIdA15G1 subtype. *Cryptosporidium* spp. are protozoan parasites the importance of which has significantly increased over the last 2 decades. On the basis of the published evidence about the transmission of *Cryptosporidium* from animals to humans, cryptosporidiosis has been definitely classified as a zoonotic disease [1]. In humans, the infection is often caused by the zoonotic species *Cryptosporidium parvum* which is spread among young farm animals. Contact with infected calves is the main cause of the frequent occurrence of cryptosporidiosis for farmers, animal handlers or veterinary students all over the world [20]. There is an increased risk of *Cryptosporidium* spp. oocysts transmission by direct contact with calves clinically affected with diarrhoea; however, asymptomatic animals can also be the source of infection. The patient infected with *C. parvum* did not suffer from immunodeficiency, he was healthy individuals with an episodic occurrence of cryptosporidia. The patient was not treated with any drugs (except infusions). In Slovakia, this is the first case of human cryptosporidiosis induced by the subtype IIdA15G1 in a man who came into contact with calves on a farm. In Asia and Europe rare cases were identified only in calves with mild or moderate diarrhoea [21].

In October 2018, the positive case described above prompted us to examine faecal specimens from calves bred on the relevant calves farm. We diagnosed *Cryptosporidium* coproantigen in calves in 31.5% of samples (17/54) using the ELISA test. Applying the PCR method confirmed the zoonotic species *C. parvum* in 5 positive isolates. The harmonisation of the obtained sequences and the reference sequences acquired from the GenBank showed that the IIdA17G1 subtype (GenBank KY499053.1) was identified in 4 isolates, and the IIdA17G1R1 subtype (GenBank JX258865.1) in one isolate [22]. The positive isolates belonged to the subtypes of IIdA and IId families which are of a zoonotic nature, and it may also be identified in humans [7, 21]. In European countries, other zoonotic subtypes in veterinary students and farm visitors were rarely identified. In March 2012, outbreaks of cryptosporidiosis spread among schoolchildren who had come into contact with lambs and goat kids at a

holiday farm in Norway. Molecular methods identified the IIdA19G1R1 subtype of *C. parvum* [23]. In March 2013, 64 students in the fourth year of veterinary medicine studies in Sweden were subjected to parasitological screening. Oocysts of *Cryptosporidium* were detected in 6 cases, in 4 specimens were confirmed the IIdA16G1R1b subtype of *C. parvum*, 2 specimens were positive for the *C. parvum* IIdA24G1 subtype [24]. The above-mentioned findings confirmed that lambs, calves and kids serve as reservoirs of zoonotic subtypes of *C. parvum* which also affect humans. Preventive strategies should include increased hygiene measures on farms, personal hygiene measures, use of personal protective equipment (disposable gloves), disinfection of instrument, and the education of all people who come into contact with calves about cryptosporidiosis transmission [25, 26]. At present, the GP60 gene is the most appropriate genetic marker for the identification of subtypes of the *Cryptosporidium* species [27, 28]. Understanding the circulation of subgenotypes of *C. parvum* and *C. hominis* will facilitate implementation of efficient epidemiological measures aimed at prevention of the spread of cryptosporidiosis to humans.

## CONCLUSION

This is the first report of human cryptosporidiosis caused by *C. parvum* IIdA15G1 subtype determined by genotyping and subtyping. In the Slovak Republic, only several cases of an infection of humans caused by *Cryptosporidium* have been reported. The IIdA15G1 subtype, confirmed in Slovakia and only rarely occurring in other countries, induces mild or moderate diarrhoea [29]. The presented case report indicates a potential danger of outbreaks of zoonotic cryptosporidiosis and the need for strict control and hygiene practices during the handling of young animals, especially calves [30, 31].

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