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Short Communication

Cryptosporidium parvum infection in Calves from an animal farm in Slovakia

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Abstract

Cryptosporidium spp. is opportunistic pathogen that infect a wide range of animals, including mammals and birds. In the present paper we focused our attention on faecal samples of calves younger than 35 days bred on a cattle farm in Slovakia. The total of collected faecal was 54 and applying the Ziehl-Neelsenacid resistant staining technique, we confirmed the presence of oocysts in 31.5% of smears. And for confirmationwas used molecular methods with identificationthe *Cryptosporidiumparvum* species and on the basis of the gp60 gene we confirmed two zoonotic subtypes, IIdA17G1 and IIaA17G1R1.

INTRODUCTION

Cryptosporidium are intracellular protozoa with a broad host range, cosmopolitan prevalence and zoonotic potential [1]. Until now, 38 valid species of *Cryptosporidium* genus have been described; out of them, more than 20 were reported in humans while *Cryptosporidiumhominis* and *Cryptosporidiumparvum* are responsible for most human infections [2,3,4]. *Cryptosporidium*spp, represent a significant cause of diarrhoea in farm animals all over the world. The main reservoirs of pathogenic species *Cryptosporidium* spp. include farm animals, especially beef cattle [5]. The impact of a parasite is the strongest in young cattle animals with insufficient colostral immunity. Getting into contact with infected animals represents a risk also for farm staff, veterinary doctors, immune deficient and immune suppressed individuals [6,7,8]. Oocysts are most frequently transmitted directly via the faecal-oral route [9] but they can also be transmitted through contaminated water, feeds, or tools [10,11]. Calves very often become infected within the first week of their lives [age ≤ 30 days] [12]. Typical findings include watery diarrhoea, sometimes accompanied with lethargy, fever, dehydration, and overall poor condition of an animal. Infection may disappear spontaneously within 1-2 weeks; in some cases, it may have a fatal end [13,14]. The species that commonly occur in beef cattle include *Cryptosporidiumparvum*, *Cryptosporidiumbovis*, *Cryptosporidiumryanae* and *Cryptosporidiumandersoni* [15]. The distribution of such species depends on their age: *C. parvum* prevails in pre-weaned calves [up to 2 months of age] [16,17]; *C. bovis* and *C. Ryanae* are detected in young cattle animals [3-12 months] [18,19,20] and *C. andersoni* mostly occurs in adult beef cattle older than 12 months [21]. Pre-weaned calves are therefore regarded as important reservoirs of *C. parvum* oocysts that are infectious for humans [22]. From the epidemiology point of view, in *Cryptosporidium* species itis very important to perform a molecular analysis. Due the highly polymorphic GP60 gene, numerous subtype families of *C. parvum*have been described. The subtypisation is based on the sequence analysis of 60-kDa glycoprotein gene [GP60] containing highly polymorphic microsatellite sequences with variable number of TCA and TCG repeats. The analysis of GP60 gene proved that approximately 98% of *Cryptosporidium* isolates from calves belong to the IIa subtype family that is of the zoonotic nature, and it may also be identified in humans [23]. IIa and IId subtype families prevail in animals and humans all over the world [24]. These families have been detected in Europe [Hungary, Germany, Portugal, Sweden, Ireland, Spain, Belgium, Romania, United Kingdom, Netherlands, Serbia and Montenegro] [25,26,27], Asia [Kuwait, Iran, Jordan, India, Malaysia and China] [28,29], Egypt, and Australia [30].

Slovakia now belongs to the countries with confirmed presence of IIaA17G1R1 subtype in beef cattle [31] and in oncological patients [32]. Our objective was to identify subtypes of *Cryptosporidium* spp. in calves younger than 35 days, as currently there are very little records on the prevalence of this species in calves in Slovakia. There is only one paper published in the English language, i.e. by Danišová et al., 2016 [5], which confirmed the 70 % prevalence of *C. parvum* in calves younger than 1 month.

The purpose of the present paper was to identify the species and subtypes occurring in calves in Slovakia.

MATERIALS AND METHODS

On a farm in ZemplínskaTeplica village, Trebišov District, we collected 54 samples of faeces from calves aged less than 35 days; the samples were collected in the period from October 2017 to November 2018. Faecal smears were stained using Ziehl-Neelsen stain method [33]. The stained oocysts appeared to be spherical formations of pink colour on the blue-green background. The amplification of the SSU rRNA segment sized 820bp was carried out using the nested PCR [34] which confirmed the presence of *C. parvum* species. Subsequently, the subtypisation of 5 selected products was carried out. It was based on the sequence analysis of the GP60 locus according to Alves et al. [2003] [35]. DNA extraction was carried out using the commercial ZR Fecal DNA MiniPrep™ kit [Zymo Research, USA] according to the manual recommended by the manufacturer. The reaction mixture for a PCR reaction contained 50 µl of the mixture [Master Mix "OneTaq 2x MM" [New England Biolabs]], primers [forward and reverse] in the concentration of 33µM, H_2O,and DNA. The amplification of the segment sized SSU rRNA [~ 820 – 850 bp] was carried out using the nested PCR.The content of the second PCR reaction [nest 2] was similar to the first one, except for the use of the primary product as a template for the second PCR reaction [nest 2]. The PCR reactions included the positive and negative controls. A microscopically confirmed positive sample was used as a positive control. The program 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.GP60 gene [850 bp] was also amplified using the nested PCR. Both PCR resctions [nest1, nest2] included 40 cycles consisting of thermal steps of exposure to 95 °C for the period of 45 s, exposure to 52 °C for the period of 45 s, and exposure to 72 °C for 1 minute, with the initial step of denaturation at 95 °C for 3 minutes and the final amplification at 72°C for the period of 10 minutes. Positive 820 – 850 bpPCR products were visualized on at 1.5 % agarose gel, using the Gel red [BiotiumInc CA Hayward USA] and visualized under the UV light.

Secondary PCR products were sent for purification and sequencing to the Microsynth Laboratory in Wien. The final sequences were compared to the known sequences using the BLAST–analysis in the NCBI database [http://blast.ncbi.nlm.nih.gov].

RESULTS AND DISCUSSION

Cryptosporidiosis is a globally prevalent parasitic disease affecting animals and humans [36]. Gastrointestinal tract diseases induced by species of the *Cryptosporidium* genus facilitate transmission among individuals and environmental contamination. A hygiene level significantly affects the ability of excreted oocysts to survive in the external environment where they are infectious for as long as one year [6]. Cryptosporidiosis may develop as an opportunistic infection or a secondary disease. Over the last few years, numerous reports on infections caused by *Cryptosporidium* spp. have been published [2,3,4,5]; this proves that this parasite has spread globally. Cattle may become infected with four species of *Cryptosporidium* spp.: *C. parvum*, *C. bovis, C. andersoni*and *C. Ryanae* [15]. *C. Parvum* is the most important zoonotic species [4]; most frequently occurs in calves aged less than 2 months and is responsible for 80% of *Cryptosporidium* infections in these animals [37]. *C. parvum* belongs to the group of parasites affecting a host's intestine; in beef cattle, it mainly infects jejunum and ileum. Clinical symptoms persist for the period of 4-7 days in form of watery diarrhoea, depression, malnutrition and abdominal pain [38,39]. The prevalence relates to the method and extent of breeding and the density of farm animals population [6].

In Netherlands, the prevalence of *Cryptosporidium parvum* in beef cattle animals younger than three moths represents approximately 64% [40]. In the Czech Republic, the prevalence of infected cattle animals younger than 2 months was 21.5% [in 2011]. According to Kaupke, the prevalence of cryptosporidiosis in Poland reached the value of 22.5% in 2015 [41]. In Columbia, the prevalence of infected calves younger than 35 days represented as much as 26.6% [42]. In Slovakia, the infection induced by *C. parvum* was detected in the 27-30% prevalence, often with the 60% mortality [43 – available only in the Slovak language]. Danišová et al., 2016 confirmed the 70 % prevalence of *C. parvum* in calves younger than 1 month [5].

In calves from the farm in Zemplínska Teplica we diagnosed *Cryptosporidium* spp. oocysts in 31.5% of faecal smears [17/54] using the Ziehl Neelsen stain method. Applying the PCR method, we confirmed the zoonotic species *C. parvum*. Five positive faecal samples were used for genotyping. The subtypisation of products was carried out on the GP60 locus. The comparison of the obtained sequences and the reference sequences acquired from the GenBank showed that the isolates belonged to IId and IIa subtypes. The IIdA17G1 subtype [GenBank KY499053.1] was identified in four isolates, and the IIaA17G1R1 subtype [GenBank JX258865.1] in one isolate. The IIdA17G1 subtype of *C. parvum* in beef cattle belongs to zoonotic subtypes and in Europe it was also confirmed in humans [44,32], in samples of treated water [44], as well as in small ruminants, for example in Spain [46]. In 2017, Hatalová confirmed the presence of the IIaA17G1R1 subtype in Slovakia in beef cattle [31]. Particularly this subtype that we have identified is associated with zoonotic transmission confirmed in humans e.g. in patient from Great Britain [44]; in 12 patients from Slovakia with oncological diseases [32].

CONCLUSION

Domestic ruminants [especially calves] represent typical hosts for *Cryptosporidium* spp. Within the present study we examined faecal samples collected from calves younger than 35 days. We confirmed the presence of *C. parvum* species and*C. Parvum* zoonotic subtype families IId and IIa. Calves come into close contact with humans [especially with farm workers, veterinary doctors, students of veterinary school…etc] and thus represent a significant risk in terms of cryptosporidiosis spreading. The contaminated water and food by oocysts of *Cryptosporidium* also represents an important pathway of infection.

There is currently no efficient therapy of cryptosporidiosis. It is recommended to initiate symptomatic treatment of diarrhoea and dehydration; in more severe cases, halofuginoneis

recommended for affected calves [37]. The most efficient measures include improvement of breeding conditions [clean boxes for calving and calves and separation of infected calves] and regular sanitation of the environment. It is also very important to provide veterinary care and perform regular monitoring of cryptosporidiosis occurrence.

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