

Detection and identification of six *Cryptosporidium* species in livestock in Slovakia by amplification of SSU and GP60 genes with the use of PCR analysis

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Abstract

Introduction. In this study we examined 200 faecal samples from pigs and calves with suspected cryptosporidiosis were examined by the PCR methods: nested PCR for amplification of SSU region; nested PCR for amplification of GP60 region; and with restriction analysis of DNA (PCR-RFLP). The sequencing identified the following species: *Cryptosporidium muris* (2), *Cryptosporidium andersoni* (1), *Cryptosporidium bovis* (4), *Cryptosporidium suis* (2), *Cryptosporidium scrofarum* (10), mixed infection caused by *C. scrofarum* and *C. muris* (1), and *Cryptosporidium parvum* (10) genotype A subtype IIaA17G2R1.

Results and conclusions. The findings suggest that livestock can be an important source of zoonotic species or genotypes of *Cryptosporidium*, which may adversely affect the public health of human populations. This is the first time in our country that the *Cryptosporidium* species has been identified in livestock in Slovakia. The identification and genotyping of this pathogen in Slovakia, completes the epidemiological situation in Europe for *Cryptosporidium* species.

Keywords

Cryptosporidium spp., cattle, pig, zoonotic potential, gene SSU, gene GP60

INTRODUCTION

The diarrheal syndrome is a common and economically significant disease of livestock. In breeding conditions with low hygienic standards, this syndrome is known to cause high morbidity and mortality. *Cryptosporidium* spp. are one of several possible etiologic agents that may be responsible for significant neonatal morbidity [1]. The main reservoir of pathogenic *Cryptosporidium* spp. is among livestock, especially calves, lambs, kids and weaners. Cases of *Cryptosporidium* infection have been reported in Slovakia only rarely. Therefore, information about the occurrence of individual *Cryptosporidium* species and the prevalence of cryptosporidiosis is sparse.

Cryptosporidium infections in weaners and pigs are mostly asymptomatic or rarely exhibit overt clinical symptoms. Cryptosporidiosis in pigs is usually caused by three different intestinal species of *Cryptosporidium*: *Cryptosporidium parvum*, *Cryptosporidium suis*, and *Cryptosporidium scrofarum* (*Cryptosporidium* pig genotype II), which has been described as 'natural infections' in pigs and may be caused by *Cryptosporidium* specific for other hosts. The gastric *Cryptosporidium muris* has been detected in pig manure collected from Irish farms [2]. In eastern China, a *C. parvum* mouse genotype was isolated from porcine faeces [3], whose typical natural hosts are rodents [4]. Naturally occurring *Cryptosporidium* infection in pigs are most commonly found in weaners more than one month old, but younger than six months of age. Infections of piglets younger than one month and in adult pigs are described less frequently.

The course of *Cryptosporidium* infections in pigs is different from that in other livestock or in humans. Diarrhoea in pigs is usually a multifactorial problem and can be caused by a wide variety of pathogens (coccidia, *Strongyloides* sp., *Salmonella* spp., *Clostridium perfringens*, *Escherichia coli*, adenoviruses, rotaviruses, and circoviruses [5, 6, 7]), either separately or in combination with other opportunistic pathogens (*Encephalitozoon intestinalis*, *Enterocytozoon bieneusi*, and *Cryptosporidium* spp.). Experimental infections have demonstrated that pigs are susceptible to other gastric *Cryptosporidium*, i.e. *C. meleagridis* and *C. hominis* [8].

Unlike in pigs, the prevalence of cryptosporidiosis in calves ranges from 2.4% – 100% worldwide [9]. Cattle are mainly infected by four *Cryptosporidium* species: *Cryptosporidium parvum*, *Cryptosporidium bovis*, *Cryptosporidium andersoni*, and *Cryptosporidium deer-like* genotype [10]. The zoonotic potential has been confirmed in only one species, namely, *C. parvum*. Other species and genotypes of *Cryptosporidium* reported in cattle are; *Cryptosporidium ubiquitum*, *Cryptosporidium xiaoi* and *Cryptosporidium muris*. The latter has a wide host range which includes mice and other rodents, some ruminants, primates, dogs, cats, rabbits and humans [11]. There has also been described a mixed infection caused by *C. bovis* and *C. ryanae* and a new genotype in the yak (*Bos grunniens*; 12). Studies have shown that *Cryptosporidium* infections in cattle are not limited by age, but by species (genotype) of *Cryptosporidium*, which causes these protozoan infection.

Application of the PCR method and its modifications (PCR-RFLP) with the use of appropriate primers and restriction enzymes, significantly improved diagnostics, mainly in terms of detection, identification of species and subtyping of *Cryptosporidium* spp. [13], because this pathogen has zoonotic potential. Cryptosporidiosis can have an effect on the public health of humans. Identified species

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of *Cryptosporidium* completes the epidemiological situation in neighbouring countries and indicates the necessity for especially monitoring the zoonotic species.

MATERIALS AND METHOD

Study population – samples. Samples of faeces were collected from 100 pigs and 100 calves from two farms (Strážske, Zemplínska Teplica) in Eastern Slovakia in September 2013 and 2014, and divided into groups according to age (Tab. 1). Faecal samples were collected only from animals with clinical signs (anorexia, diarrhoea, abdominal pain, and weight loss to cachexia) indicating suspected cryptosporidiosis.

Molecular analysis – DNA isolation. Genomic DNA was extracted from the 100 mg stool samples using the DNA-Sorb-B Nucleic acid Extraction kit (AmpliSence), according to the manufacturer's instructions. Before extraction, the stools we homogenized to disrupt the oocysts at 6,500 rpm for 90 seconds, with the addition of 0.5-mm-glass beads, 1.0-mm-zircon beads and 300 µl lysis solution in a homogenizer, Precellys 24 (Bertin Technologies, France). After purification, the DNA was stored at 20° C until use in the nested PCR.

Nested PCR, PCR-RFLP – Electrophoresis, Sequencing. For the nested PCR, a modified protocol [13, 14] was used with genus-specific primers Xiao F2/ Xiao R2 (819–825 bp) and VKSS F1/VKSS R2 (345–355 bp), and a protocol described by Leetz et al. [14] using the outer primers VKSS F1/VKSS R1 (658–662 bp) and the inner primers VKSS F2 / VKSS R2 (245–250 bp) for amplification of the SSU region of the *Cryptosporidium* species DNA. Secondary PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized by UV light with a wavelength of 312 nm [15].

Positive samples were repeatedly analyzed by PCR-RFLP, where for restriction of the primary PCR product with the length of 819–825 bp (primers: Xiao F2/Xiao R2), two

restriction enzymes were used: *Ssp I* (identification of species) and *PshB I* (*Vsp I*, identification of genotypes; Takara BIO INC., Japan) with an incubation at 37 °C/12 hours [13]. The products of restriction were separated on 2% agarose gel and visualized after staining with RedGel dye by UV light.

For confirmation of the *Cryptosporidium* spp. after PCR-RFLP and nested PCR (VKSS primers), all positive samples were sent for sequencing. The sequences were compared with known sequences with BLAST in the NCBI database.

Samples positive for *Cryptosporidium parvum* were analyzed once again with nested PCR with species-specific primers gp15 F1/ gp15 R1 (980–1,000 bp) and gp15 F2/ gp15 R2 (450 bp) which are used for amplification of the GP60 region and for identification of the genotype and subtype of *C. parvum*. The PCR products were again sent for sequencing and the sequences genotyped.

PCR reaction mix. The volumes of the PCR reaction mixtures in both cases were 50 µl, from which the DNA sample was 5 µl. In these reactions, primers with a concentration of 0.2 µM and 5 U Taq DNA polymerases (FIREPol) were used.

The PCRs were run in a thermocycler (XP Thermal Cycler Blocks) with an initial denaturation of 95 °C for 5 min., followed by 35 cycles of 95 °C for 1 min., 60/ 61/69 °C for 1 min., and 72 °C for 2 min. A final elongation step of 72 °C for 7 minutes was included to ensure the complete extension of the amplified products.

Annealing temperature for the used primers Xiao F2/Xiao R2–60 °C, VKSS F1/VKSS R2 and gp15 F1/gp 15 R1–61 °C, gp15 F2/ gp 15 R2–69 °C.

PCR products were directly sequenced in both directions. Sequences were aligned and completed using ChromasPro, Bioedit and Clustal X, and compared with known sequences in the National Centre for Biotechnology Information GenBank database. The sequences generated in the presented study have been deposited in the GenBank database under accession numbers (described in the Results). The sequences

Table 1. Detected species of *Cryptosporidium*

group	Age/ category	n	Intestinal species				Stomach species		Mixed infection
			<i>C. parvum</i>	<i>C. bovis</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. muris</i>	<i>C. andersoni</i>	<i>C. scrofarum</i> , <i>C. muris</i>
pig	Strážske 2013	piglets	5			2	2		1
		weaners	4		2	2			
		sows	6			1		1	
	Zempl. Teplica 2013	piglets	7						
		weaners	27						
	Strážske 2014	piglets	5						
		weaners	12			1			
	Zempl. Teplica 2014	piglets	8						
		weaners	26				4		
	Σ pigs		100		2	10	2	1	1
calf	Strážske 2013	< 1 month	5	2					
	Zempl. Teplica 2013	< 1 month	10	7					
		> 1 month	10	1	2				
	Strážske 2014	< 1 month	9						
	Zempl. Teplica 2014	> 1 month	66	2					
	Σ calves		100	10	4				
Σ animals		200	10	4	2	10	2	1	1

identified as *Cryptosporidium* spp are used for phylogenetic analysis and phylogenetic tree formation. The sequences were controlled by the GeneEdit programme and modified in the Bioedit program programme. In the phylogenetic process and evaluation of the monitored sequence, the RAxML programme was used [16]. The resulting phylogenetic tree is shown using Figtree and edited in Adobe Illustrator CC, 2014.

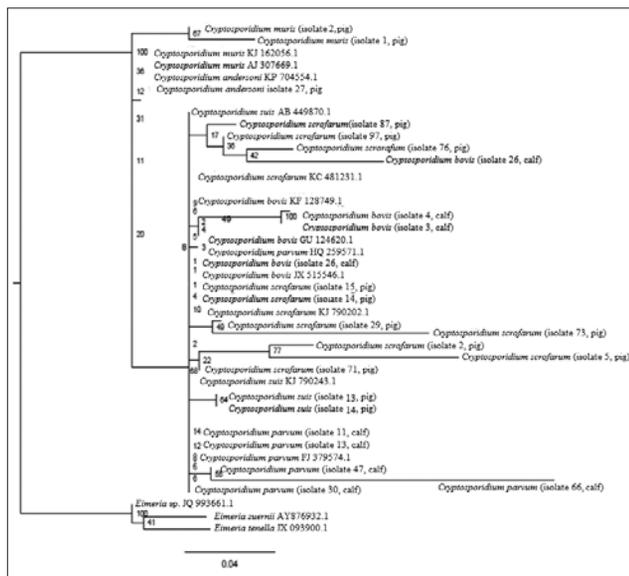


Figure 1. Evolutionary relationships among *C. muris*, *C. andersoni*, *C. suis*, *C. scrofarum*, *C. parvum*, *C. bovis* inferred from a partial fragment of the 18S rRNA gene

RESULTS

In the faecal samples of animals with suspected clinical cryptosporidiosis, the presence of the following species were confirmed in pigs: *C. andersoni* (1; KP704554), *C. suis* (2; KP704556), and *C. scrofarum* (10; KP704557). By repeated analysis of nested PCR with primers VKSS, the species *C. muris* (KP704555) were also identified in two weaners, and a mixed infection with *C. scrofarum* and *C. muris* confirmed in one weaner. In calves, infections were found with the species *C. bovis* (4; KP704558) and *C. parvum* (10; KP704559) genotype A subtype IIaA17G2R1 (KP704560). Prevalence of cryptosporidiosis in calves was 15% and in pigs – 14%.

The analyzed samples of the reference sequence (NCBI GenBank), and the 'outgroup' group consisted of the sequence *Eimeria* spp. A phylogenetic tree was constructed and the phylogenetic analysis highlighted not only the affinities and evolutionary branching between sequences of different types of *Cryptosporidium* spp. analysed, but also within the same species. The sequences obtained for *C. parvum* lie on a branch with a reference sequence of *C. parvum* and *C. bovis* analyzed sequences lie along the tree to the reference sequence of *C. bovis*, as well as *C. suis* and *C. scrofarum*, indicating also the close familial relationships within the intestinal species of *Cryptosporidium* spp. analyzed sequences. The phylogenetic tree also shows the differences in the types of gastric and intestinal species of *Cryptosporidium*.

DISCUSSION

By restriction analysis RFLP it is possible to diagnose and identify *Cryptosporidium* spp. in various hosts in a relatively short time. With RFLP analysis, 4 species of *Cryptosporidium* were identified, with a prevalence of 15.0% in pigs. The prevalence of *Cryptosporidium* infections in pigs has been reported to be 30–34%, with various representations in all age categories. *Cryptosporidium* infections have been reported worldwide, mainly in pigs before and after weaning which were in the age category of 1.5–3 months [5, 17]. In older animals with a prevalence of infection of 5–12%, cryptosporidiosis, it has been found to be less common in the age category of 5.5–6 months [18]. The presence of *Cryptosporidium* in older pigs varies. Xiao et al. [19], Atwill et al. [20] and Maddox-Hyttel et al. [17] reported the total absence of disease in this age category. On the contrary, in 2007–2008, Kváč et al. (unpublished results) detected in the Czech Republic a prevalence higher than 21%, and Quilez et al. [21] reported prevalences of 30–34% in 2–6 months old pigs.

The infection in pigs is primarily localized in the small and large intestines. Vítovec et al. [22] identified in their study a similarity of gastric *Cryptosporidium*: *C. muris* and *C. andersoni*, with *C. suis*, oocysts, which were localized in the glands of the lymphogranular complex in the submucosa of the colon and the rectum. *C. suis* frequently becomes disseminated to other parts of the gastrointestinal tract. In addition to the gastrointestinal tract, *Cryptosporidium* of pigs can infect surrounding organs. *C. muris* has a wide host range that includes mainly mice and other rodents that are possible reservoir of *Cryptosporidium* infections and have zoonotic potential. *C. muris* has been identified in pig faeces in the Czech Republic [23], in China [3], in pig manure (USA; 2) and in swine waste lagoons and sprayed fields (USA; 24). In their study, Kváč et al. [25] indicated that infections caused by *C. muris* in pigs after weaning, is not active, and therefore this group is not susceptible to infection by this species, but *C. suis* and *C. scrofarum* are highly pathogenic for pigs and the presence of these species in pigs after weaning was also found in the presented study. The infection of piglets by *C. muris* and mixed infection with *C. muris* and *C. scrofarum*, may indicate an active cryptosporidiosis in this age group due to the lack of passive immunity which may cause a high susceptibility to the pathogens, including parasitic infections.

The important aspect of the current work is the detection of *C. muris*, confirmed only with the pair of primers VKSS that detects *C. muris* and also *C. parvum*, *C. hominis*, *C. andersoni*, *C. meleagridis*, *C. baileyi*, *C. serpentis* and *C. wairi* [14, 26]. Based on these results, it is suspected that the primer VKSS is probably more sensitive for small fragments of DNA and specific for *C. muris* in the PCR analysis.

The fact that *C. scrofarum* was identified not only in weaners, but also in one sow, correlates with the results of studies by Kváč et al. [27] and Quilez et al. [21]. It is also important to pay attention to the fact that this species was diagnosed in an immunocompromised human [28], which confirms the zoonotic potential of *C. scrofarum*. From an epidemiological point of view, it would therefore be desirable to monitor the occurrence of this species in the human population.

Other *Cryptosporidium* species with zoonotic potential in the presented study group of animals were identified

in calves. The prevalence of *Cryptosporidium* infections in calves on the farms in this study was 14%. However, Santín et al. [29], Rieux et al. [30] detected 92–100% prevalence of *Cryptosporidium* infections in calves. The difference in the prevalence of individual *Cryptosporidium* species is closely linked not only to the geographic locality and the climatic conditions, but also with selection of the study group, the type of breeding and the diagnostic methods used. Numerous studies have reported infections caused by *C. bovis* (43.08%), *C. parvum* (36.92%), *C. ryanae* (7.69%) as the most common in calves younger than one month of age [30, 31, 32, 33], which was also shown by the presented study with a prevalence of infection by *C. parvum* of 35%. Kváč et al. [34], Fayer et al. [34], and Santín et al. [32] described in their studies that 80–90% of the calves under one month of age with clinical cryptosporidiosis were infected by *C. parvum*. In the current study, not only *C. parvum* were identified we identified, but also infections caused by *C. bovis* in both groups of calves.

The most widespread subtypes of *Cryptosporidium parvum* in Europe are IIAA18G2R1, which is common in calves in the surrounding countries of Hungary [36], Sweden [37] and France [30], or the subtype IIAA15G2R1 reported not only in the cattle, but also in humans in Portugal [38], Slovenia [39], Netherlands [40] and France [41, 42] which indicates a possible zoonotic potential.

Genotyping allowed identification of subtype IIAA17G2R1, detected only in calves in Europe, in Northern Ireland [43], Germany [44], Italy [45] and Spain [46], as well as in Canada [47] and the USA [48]. The infection caused by this subtype was confirmed in goats in China [49], in 22 calves, 2 pigs and 47 humans in North Carolina, USA [50], and in humans in Australia with a prevalence as high as 47.6% [51].

The detection and identification of subtype *C. parvum* IIAA17G2R1 indicates another possible zoonotic potential, and draws attention to calves that can be an important source of infection causing cryptosporidiosis which, in turn, can affect the public health of humans. The repeated detection the *C. scrofarum* species in pigs in Strážske and *C. parvum* species in calves in Zemplínska Teplica points to persistent infection on the farms in Slovakia.

This is the first time that the *Cryptosporidium* species has been identified in livestock in Slovakia, and the identification and genotyping of this pathogen in that country completes the epidemiological situation in Europe.

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Ethical clearance

The study was approved by the Ethics Committee of University of Veterinary Medicine and Pharmacy, Kosice, Slovak Republic. All research met ethical and regulatory guidelines, including adherence to the legal requirements of the study country. All proposed methods for the handling and treatment of animals were in accordance with the Public Notice of the Slovak Republic (No. 23/2009 of 14 January 2009), concerning the requirements for the protection of animals used for experimental or other scientific purposes.

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