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Original article

Molecular studies on pig cryptosporidiosis in Poland

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Abstract

Cryptosporidium intestinal parasites have been detected in farmed pigs worldwide. Infections are usually asymptomatic with a low number of oocysts shed in pig feces. This makes the recognition of infection difficult or unsuccessful when microscopic methods are used. The aim of this study was molecular identification of *Cryptosporidium* species in pig herds raised in Poland with regard to the occurrence of zoonotic species. In total, 166 pig fecal samples were tested. The examined pigs were aged 1 to 20 weeks. Overall, 39 pig farms were monitored for parasite presence. The detection and identification of *Cryptosporidium* DNA was performed on the basis of PCR-RFLP and nucleotide sequence analysis of the amplified 18 SSU rRNA and COWP gene fragments. Infected animals were housed in 21 (53.8%) of the pig farms monitored. The presence of *Cryptosporidium* was confirmed in 46 (27.7%) samples of pig feces. Among positive fecal samples, 34 (29.3%) were collected from healthy animals, and 12 (24%) from diarrheic pigs. Most infected animals (42.1%) were 2 to 3 months old. The following parasite species were detected: *C. scrofarum*, *C. suis* and *C. parvum*. Indeed, asymptomatic infections caused by *C. scrofarum* were observed in the majority of the herds. Mixed infections caused by *C. suis* and *C. scrofarum* were not common; however, they were observed in 8.6% of the positive animals. *C. parvum* DNA was found only in one sample collected from a diarrheic pig. The application of molecular diagnostic tools allowed for detection and identification of *Cryptosporidium* species in pigs. The sporadic findings of *C. parvum* are subsequent evidence for the contribution of pigs in the transmission of cryptosporidiosis from animals to humans.

Key words: *Cryptosporidium*, PCR-RFLP, pigs, mixed infections

Introduction

Protozoan parasites belonging to the *Cryptosporidium* genus have been detected in over 150 species of vertebrate animals (Fayer 2008). The use of molecular tools utilizing nucleic acid amplification fol-

lowed by DNA sequence analysis allowed for identification of *Cryptosporidium* species and genotypes (Carreno et al. 2001, Smith et al. 2006). More recently, these methods have been widely used for detection and identification of *Cryptosporidium* isolates originating from pigs raised in Europe (Mišić et al. 2003,

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Hajdusek et al. 2004, Maddox-Hyttel et al. 2006, Hamnes et al. 2007, Langkjaer et al. 2007, Suárez-Luengas et al. 2007, Zintl et al. 2007, Kváč et al. 2009a), Asia (Chen and Huang 2007), North America (Jenkins et al. 2010) and Australia (Ryan et al. 2003, Johnson et al. 2008). Despite differences observed in the extensiveness of the infection between swine herds, *Cryptosporidium* pig genotype II was predominantly detected. Recently, based on oocyst morphology molecular data and experimental infection studies, *Cryptosporidium* pig genotype II was raised to a species level and renamed as *C. scrofarum* (Kváč et al. 2013). Another species frequently infecting pigs is *C. suis* (Suárez-Luengas et al. 2007, Santín and Trout 2008, Kváč et al. 2009a). Except specific *Cryptosporidium*, adapted to a swine host, other species or genotypes such as *C. parvum*, *C. muris*, *Cryptosporidium* mouse genotype I, *C. felis* and *Cryptosporidium* rat genotype have been occasionally detected in pigs (Chen and Huang 2007, Zintl et al. 2007, Kváč et al. 2009b, Jenkins et al. 2010). The sporadic findings of *C. parvum* may indicate that pigs can also serve as an animal reservoir for human cryptosporidiosis (Maddox-Hyttel et al. 2006, Zintl et al. 2007, Kváč et al. 2009b). Although the studies on *Cryptosporidium* occurrence in farm animals in Poland have been widely conducted, the presence of this parasite in pigs has received a little attention (Bajer 2008, Raś-Noryńska and Sokół 2013).

The aim of this study was molecular identification of *Cryptosporidium* species in pigs raised in Poland with regard to the occurrence of zoonotic species.

Materials and Methods

The study was conducted on 39 pig commercial farms located in 12 out of 16 administrative provinces in Poland. From each province 1 to 7 farms were randomly chosen for sampling without previous information on parasite occurrence. They represented different administrative locations across each province. Animals were housed indoors using one of the two the most popular pig husbandry management systems in Poland (slatted floor or straw bedding). In total, 166 fecal samples were collected between May and September 2008. Animals were aged 1 week to 5 months. 116 samples were derived from healthy pigs, whereas 50 samples were from diarrheic ones. Feces were placed individually into plastic containers without fixatives, labeled, and sent to the laboratory. Before analysis they were stored for a maximum of one week at 4°C, or at – 20°C if processing was delayed more than one week.

Cryptosporidium DNA was extracted from 0.1 g (100 µl) of animal feces using a previously described method (Rzeżutka and Kaupke 2013). Extracts containing parasite DNA were used directly for molecular analyses or stored at – 20°C until use. The

18 SSU rRNA nested-PCR was used for the detection of parasite DNA. The genus specific set of primers and reaction conditions were described by Xiao et al. (1999). Only minor modifications were introduced to the PCR mix: a higher concentration of *Taq* polymerase (2.5 U) and the addition of 20 µg of bovine serum albumin per 50 µl of each reaction mixture. This allowed an increase in amplification efficiency and a reduction of reaction inhibition. DNA extracts positive at the 18 SSU rRNA gene locus were further investigated using species-specific COWP-PCR assay (Homan et al. 1999) with modified temperature profile and reaction conditions (Rzeżutka and Kaupke 2013). All steps of the molecular analysis were controlled by inclusion of the following set of controls: positive and negative extraction control consisting of *Cryptosporidium* – free pig feces spiked with *C. parvum* oocysts (Waterborne™, Inc., New Orleans, LA, USA) at a concentration of 10⁴ cells and all reagents containing distilled water instead of the template. At the PCR level a positive control of *Cryptosporidium* DNA (lysate of *C. parvum* oocysts or DNA of *C. hominis*) and negative control (a reaction mixture containing water instead of the DNA template) were set up.

Cryptosporidium species were determined by RFLP analyses of the 18 SSU rRNA gene fragments using restriction enzymes *NdeI*, *SpeI/BcuI* (Zintl et al. 2007), *SspI* (Xiao et al. 1999, 2006) and *TaqI* (Homan et al. 1999) for COWP-PCR amplicons (for fragment sizes see Table 1). For digestion, 4 or 8 µl of nested-PCR products were incubated with 1 FDU (*NdeI*, *TaqI*, *SpeI/BcuI*) or 10U (*SspI*) of restriction enzymes (Fermentas, Vilnius, Lithuania) and with 1 µl of the appropriate reaction buffer FastDigest (*NdeI*, *TaqI*) or buffer G. Reactions were carried out at 37°C for 15 minutes (*SpeI/BcuI*), 1 hour (*NdeI*) and 3 hours (*SspI*). After the digestion, enzymes were inactivated under conditions recommended by the supplier. PCR products and their restriction fragments were analysed in 1.7% or 2.5% agarose gels stained with ethidium bromide.

To confirm correct RFLP identification of detected species, sequencing of selected 18 SSU rRNA (8 samples) and COWP PCR amplicons (2 samples) was performed. PCR products were excised from the agarose gel and purified using a Gel Extraction Kit (Qiagen, Hilden, Germany), according to the manufacturer's recommendations. They were subjected to directional sequencing on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, California). A consensus sequence was established with the use of a nucleotide sequence editor (Sequence Navigator, Applied Biosystems, Foster City, California). The sequences were aligned with published sequences from the GenBank database using a NCBI-BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Table 1. Restriction fragments obtained after digestion of *Cryptosporidium* 18 SSU rRNA and COWP gene fragments.

Enzyme	Restriction site	Target gen	Restriction fragments (bp)	<i>Cryptosporidium</i> spp.	Reference
<i>Nde</i> I	CA↓TATG GTAT↑AC	18 SSU rRNA	545, 280	<i>C. parvum</i>	Zintl et al. 2007
			552, 280	<i>C. suis</i>	
			not cutting	<i>C. scrofarum</i> *	
<i>Spe</i> I/ <i>Bcu</i> I	A↓CTAGT TGATC↑A	18 SSU rRNA	701, 131	<i>C. suis</i>	Xiao et al. 1999, 2006
			not cutting	<i>C. scrofarum</i>	
			not cutting	<i>C. parvum</i>	
<i>Ssp</i> I	AAT↓ATT TTA↑TAA	18 SSU rRNA	449, 254, 108, 12, 11	<i>C. scrofarum</i>	Homan et al. 1999
			453, 365, 11, 9	<i>C. suis</i>	
			450, 267, 108, 12, 11	<i>C. parvum</i>	
<i>Taq</i> I	T↓CGA AGC↑T	COWP	374, 266	<i>C. parvum</i> genotyp 2 (<i>C. parvum</i>)	Homan et al. 1999
			470, 170 or 374, 266	<i>C. parvum</i> genotyp 1 (<i>C. hominis</i>)	

* previously known as *Cryptosporidium* pig genotype II

Table 2. Prevalence of *Cryptosporidium* spp. in tested pigs.

Age		Number of pigs		Prevalence (%)
		not diarrheic	diarrheic	
<1 month	pre-weaners	13 (2)	20 (0)	6
>1 to 2 month		29 (11)	14 (3)	32.5
>2 to 3 months	weanlings	30 (11)	8 (5)	42.1
>3 to 5 months		44 (10)	8 (4)	26.9
Total		116 (34)	50 (12)	27.7

Values in brackets represent numbers of positive animals detected

Results

The specific amplicon corresponding to the 18 SSU rRNA gene fragment was successfully obtained for 46 (27.7%) out of 166 analysed pig feces. In the group of positive samples, 34 (29.3%) were collected from healthy animals, while 12 (24%) originated from diarrheic pigs (Table 2). In the case of two samples, positive amplicons were obtained for both 18 SSU rRNA and COWP gene fragments. For three samples positive at the 18 SSU rRNA gene locus the amount of generated PCR product was insufficient to perform further molecular species identification.

RFLP analysis of 43 DNA samples (18 SSU rRNA amplicons) revealed the presence of *C. scrofarum* (40 samples), *C. suis* (7 samples) and mixed infections of *C. scrofarum*/*C. suis* (4 samples). Two samples were positive at the COWP PCR locus and subsequent RFLP analysis revealed a band pattern consistent with *C. parvum* only for one sample. The restriction profile

of the second sample did not correspond to *C. parvum*, *C. hominis* or *C. meleagridis*. Sequence analysis of this strain showed a low 89% identity to *C. meleagridis*. The *Cryptosporidium* sequences were deposited in the GenBank under accession numbers KF597530-34 (*C. scrofarum*), KF597528-29 (*C. suis*), and KF597535 (*C. parvum*). Species identity established on the basis of molecular analysis showed that 34 pigs carried *C. scrofarum*, 3 pigs *C. suis* and 6 had mixed infections with *C. suis*/*C. scrofarum*, *C. scrofarum*/*C. parvum* or *C. scrofarum*/*Cryptosporidium* sp.

The infected animals were housed in 21 (53.8%) out of 39 monitored farms. The most frequently identified *Cryptosporidium* species was *C. scrofarum*, which was present in 20 (51.2%) of farms. *C. suis* was found in 5 (12.8%) and *C. parvum* only in one farm (2.5%). The occurrence of parasite species and their distribution by age categories are presented in Table 3. In the pig population aged up to 5 months,

Table 3. Occurrence and distribution of *Cryptosporidium* spp. in particular age groups of pigs.

Age	Not diarrheic				Diarrheic		
	<i>C. scrof.</i>	<i>C. suis</i>	<i>C. parvum</i>	<i>C. sp</i>	<i>C. scrof.</i>	<i>C. suis</i>	<i>C. parvum</i>
<1 month	2	–	–	–	–	–	–
>1 – 2 months	11 ^a	2 ^a	–	1	1	2	–
>2 – 3 months	11 ^b	2 ^b	–	–	4	1	–
>3 – 5 months	8	–	–	–	3	–	1 ^c
Total	32	4	–	1	8	3	1

C. scrof. – *Cryptosporidium scrofarum*

C. sp – *Cryptosporidium* sp.

^a Mixed infection *C. scrofarum*/*C. suis* (2 samples)

^b Mixed infection *C. scrofarum*/*C. suis* (2 samples)

^c Mixed infection *C. scrofarum*/*C. parvum* (1 sample)

40 (24%) of animals carried *C. scrofarum*, with its prevalence among the monitored farms at a level of 51.2%. In addition, *C. scrofarum* was associated with the occurrence of asymptomatic infections. Also this parasite was frequently reported in diarrheic pigs. *C. suis* was detected in 7 (4.2%) animals aged 1-3 months. These invasions appeared also as mixed infections of *C. suis*/*C. scrofarum*. Zoonotic *C. parvum* was found only in one diarrheic pig. Additionally, in one sample originating from a weaning pig *Cryptosporidium* species was not identified.

Discussion

Currently, the methods used for diagnosing *Cryptosporidium* infection not only rely on microscopic but also upon molecular tests, allowing precise identification of parasite species/ genotype. Furthermore, they remain the method of choice during diagnostics of subclinical infections which frequently occur in pigs. *Cryptosporidium* species infecting animals are readily differentiated at the 18SSU rRNA and COWP loci, using selective amplification and subsequent digestion of generated PCR amplicons. For example, the PCR-RFLP assay of the COWP gene locus is characterised by a unique specificity in the amplification of DNA only from selected parasite species (Homan et al. 1999). It amplifies and distinguishes oocysts of *C. parvum*, *C. hominis* and *C. meleagridis* (Jiang and Xiao, 2003). In this study, for one sample positive at COWP PCR, the RFLP pattern was not identical to the pattern obtained for *C. parvum*, *C. hominis* nor *C. meleagridis*. It is of note that this sample gave a negative result when analysed using Lib 13 and GP 60 PCR (data not shown). Although the sample contained a mixture of *Cryptosporidium* DNA originating from two different species, the presence of *C. scrofarum*

was not shown when 18SSU rRNA PCR was used. During the course of amplification, not all broadly reacting PCR tools discriminate DNA matrixes obtained from related species. A DNA template derived from a dominant species present in the sample is efficiently amplified in yielding a positive signal on the gel (Xiao 2010). The inability to identify parasite species clearly shows the need to use several molecular tools targeting different regions within the parasite genome. This approach is especially recommended when analysed samples originate from animals which are known to harbour several *Cryptosporidium* species or genotypes, or when concurrent infections are common.

Pigs are natural hosts for both *C. suis* and *C. scrofarum* (previously known as *Cryptosporidium* pig genotype II) (Vítovec et al. 2006, Hammes et al. 2007). The infections caused by these parasites were reported in pigs in Europe with a herd prevalence from 1.4% to 62.5% depending on the age of the tested animals (Wieler et al. 2001, Mišić et al. 2003). In the current study, the overall prevalence of *Cryptosporidium* in the tested pig population was estimated at 27.7%. These results are similar to findings on the occurrence of *Cryptosporidium* in pig herds housed in Spain, the Czech Republic and Denmark, where the prevalence was estimated at 22.5%, 21.1% and 31.9% respectively (Maddox-Hyttel et al. 2006, Suárez-Luengas et al. 2007, Kváč et al. 2009a). The distribution of the parasite between age categories of the tested animals was not uniform. Most infected pigs were aged between 2 to 3 months (42.1%), followed by animals aged between 1 to 2 months (32.5%), 3 to 5 months (26.9%) and below 1 month (6%). It is of note that the majority of infected individuals were weaned pigs above the age of 1 month.

In this study, the following *Cryptosporidium* species were detected in pigs: *C. scrofarum*, *C. suis* and *C.*

parvum. The most prevalent *Cryptosporidium*, regardless of an animal's age and the monitored farm was *C. scrofarum*. It has been shown previously that the occurrence of *Cryptosporidium* species in pigs is related to the stage of pig production. *C. scrofarum* was identified in post-weaned pigs and *C. suis* in suckling piglets (Langkjaer et al. 2007, Suárez-Luengas et al. 2007, Kváč et al. 2009a, Němejc et al. 2013). Although, in some of the studies, *C. suis* was detected in pigs at a different age, i.e. in the pre- and post-weaning periods (Morgan et al. 1999, Guselle et al. 2003, Kváč et al. 2009a). The presence of *C. scrofarum* in post-weaned pigs corroborates our findings, where this parasite was often detected in animals over the age of one month. Our results have not fully confirmed the species distribution in relation to age, as the majority of *C. suis* infections were present in post-weaned animals (older than one month). From the studies conducted so far, the mixed infections caused by *C. suis* and *C. scrofarum* were not common; however, they were observed in our studies in 8.6% of the positive animals. Co-infections in pigs caused by these two parasites were previously described by Němejc et al. (2013). *C. parvum* is not considered as a frequent swine parasite, but it was occasionally found in pigs housed in Europe (Maddox-Hyttel et al. 2006, Zintl et al. 2007, Kváč et al. 2009b) and Canada (Budu-Amoako et al. 2012). In this study, *C. parvum* was detected once in a five-month-old finisher pig with diarrhoea. This animal was co-infected with *C. scrofarum*. A similar finding of mixed *C. scrofarum*/*C. parvum* infection in pigs was previously reported (Němejc et al. 2013). The identification of the subtype family of *C. parvum* has been undertaken (data not shown); however, due to the low amplicon concentration, sequencing for definitive subtype recognition was unsuccessful. The differences observed in *Cryptosporidium* prevalence and species distribution in pigs raised in Poland and other countries may come from diverse pig-farming operations and feeding conditions.

In conclusion, the application of molecular diagnostic tools allowed for the identification of *Cryptosporidium* species in pigs. The identified species were not different to those detected in other studies, and their distribution was comparable between diarrheic and not diarrheic pigs. Because the aetiology of pig diarrhoea was not elucidated by further testing of feces for bacterial and viral pathogens, the association of this symptom of the disease only with *Cryptosporidium* infection cannot be made. Although our findings comply with results on the occurrence of this protozoan parasite in pigs housed in other European countries, they have not fully confirmed the previously reported age related distribution pattern

of species in the pig host. The present study covered only small numbers of pigs compared to the whole animal population; nevertheless, its results increased our knowledge on the epidemiology of pig cryptosporidiosis. Moreover, the sporadic findings of *C. parvum* are subsequent evidence for the contribution of pigs in the transmission of cryptosporidiosis from animals to humans. In addition, it is the first report on the occurrence of *Cryptosporidium* species in pig herds housed in Poland.

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