HHS Public Access

Author manuscript

Vet Parasitol. Author manuscript; available in PMC 2015 May 19.

Published in final edited form as:

Vet Parasitol. 2013 November 8; 197(0): 504–508. doi:10.1016/j.vetpar.2013.07.003.

Cryptosporidium suis and *Cryptosporidium scrofarum* in Eurasian wild boars (*Sus scrofa)* in Central Europe

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Abstract

From 2011 to 2012, to identify *Cryptosporidium* spp. occurrence in Eurasian wild boars (*Sus scrofa*) 29 randomly selected localities (both forest areas and enclosures) across the Central European countries of Austria, the Czech Republic, Poland, and the Slovak Republic were investigated. *Cryptosporidium* oocysts were microscopicaly detected in 11 out of 460 faecal samples examined using aniline-carbol-methyl violet staining. Sixty-one *Cryptosporidium* infections, including the 11 infections that were detected by microscopy, were detected using genus- or species-specific nested PCR amplification of SSU rDNA. This represents a 5.5 fold greater sensitivity for PCR relative to microscopy. Combining genus-and species-specific PCR tools significantly changes the perspective on the occurrence of *Cryptosporidium* spp. in wild boars. While RFLP and direct sequencing of genus specific PCR-amplified products revealed 56 *C. suis* (20) and *C. scrofarum* (36) monoinfections and only 5 mixed infections of these species, species-specific molecular tools showed 44 monoinfections and 17 mixed infections with these species. PCR analysis of the gp60 gene did not reveal any other *Cryptosporidium* infections.

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Similar to domestic pigs, *C. scrofarum* was detected as a dominant species infecting adult Eurasian wild boars (*Sus scrofa*). *Cryptosporidium* infected wild boars did not show signs of clinical disease. This report is perhaps the most comprehensive survey of cryptosporidial infection in wild boars.

Keywords

Cryptosporidium suis; *Cryptosporidium scrofarum*; Eurasian wild boar; Central Europe; SSU; PCR

1. Introduction

Protozoa of the genus *Cryptosporidium* are apicomplexan parasites that inhabit the digestive and respiratory systems of birds, fish, reptiles, and mammals, including humans (O'Donoghue, 1995; Xiao et al., 2004; Fayer, 2010). In domestic pigs, *Cryptosporidium* infections were first described by Bergeland (1977) and Kennedy et al. (1977) in the USA. Although at least 11 different *Cryptosporidium* species or genotypes (*C. felis*, *C. hominis*, *C. melaegridis*, *C. muris*, *C. parvum*, *C. scrofarum*, *C. suis*, *C. tyzzeri*, *Cryptosporidium sp. Eire w65.5*, *Cryptosporidium* rat genotype, unknown *Cryptosporidium* genotype from pig slurry), have been considered to be infectious for pigs (Morgan et al., 1999; Ryan et al., 2003, 2004; Xiao et al., 2006; Chen and Huang, 2007; Zintl et al., 2007; Kváč et al., 2013), recent studies clearly show that *Cryptosporidium suis* and *Cryptosporidium scrofarum* are host specific including age specificity of *C. scrofarum* (Ryan et al., 2004; Jeníková et al., 2011; Kváč et al., 2012, 2013).

The epidemiology of cryptosporidial infections of domestic pigs (*Sus scrofa domesticus*) has been thoroughly reported worldwide during the last decade. However, the current data regarding *Cryptosporidium* and cryptosporidiosis in wild boars are limited. Using immunofluorescence assays, Atwill et al. (1997) and Castro-Hermida et al. (2011) reported the presence of *Cryptosporidium* spp. in feral pigs in western California (USA) and wild boars in Galicia (NW, Spain), respectively. Only García-Presedo (2013) reported *C. scrofarum*, *C. suis* and *C. parvum* in wild boars in Galicia (NW, Spain) based on PCR results. Although Atwill et al. (1997) reported the DNA sequences of cryptosporidia from feral pigs, and Pereira et al. (1998) subsequently showed that feral pig isolates differed from those infecting livestock and humans by 1.0 to 1.2%, the causative species/genotype was not reported. More recently, Němejc et al. (2012) reported the molecular epidemiology of *Cryptosporidium* spp. in Eurasian wild boars.

It has been shown that wild animal populations, including wild boars, can serve as an environmental reservoir of *Cryptosporidium* spp. that are transmitted to domestic animals and humans (Waldron et al., 2010; Rašková et al., 2013). The aim of this study was to examine the occurrence of *Cryptosporidium* spp. in Eurasian wild boars living in their natural habitat in selected Central European areas and to identify *Cryptosporidium* species/genotypes using molecular methods. The data were compared with previous reports from both domestic pigs and wild boars.

2. Material and Methods

Origin of faecal samples

A total of 460 faecal samples from adult European wild boars (*Sus scrofa*) were collected at 29 randomly selected localities (including forest areas and enclosures (50–350 ha)) in Austria (3 localities, 44 samples), the Czech Republic (14 localities, 231 samples), Poland (9 localities, 129 samples), and Slovakia (3 localities, 56 samples) during the period 2011–2012.

Fresh faecal samples were taken directly from the rectum of hunted animals (n=21) or from the ground (n=439) at feeding places in forests where the concentration of animals was very high. Each sample was placed into an individual sterile plastic container without fixative, transported to the laboratory in a cool box and stored at 4°C until processing.

Microscopical examination

All samples were microscopically analyzed within 24 h using the aniline-carbol-methyl violet staining method (Miláček and Vítovec, 1985). Faecal consistency was noted at the time of sampling and DNA was isolated up to a week after collection. The infection intensity was determined from the microscopic examination as number of oocysts per gram (OPG) according to Kváč et al. (2007).

DNA isolation

Two hundred milligrams of each faecal sample was homogenized by bead disruption using FastPrep-24 (Biospec Products, Bartlesville, OK, USA) for 60 s at a speed of 5.5 $m.s^{-1}$ in 800 µl of lysis buffer of QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) with 0.5 mm glass beads (Biospec). Total DNA was extracted following the manufacturer's instructions and was kept frozen at –20°C until PCR amplification.

Molecular detection of Cryptosporidium spp

The presence of *Cryptosporidium* species and genotypes was determined by two approaches targeting SSU rDNA. The first used a genus specific nested PCR followed by digestion of amplified fragments with *Ssp* I and *Vsp* I (Fermentas, St. Leon-Rot, Germany) and restriction fragment length polymorphism analysis, as previously described by Xiao et al. (2001) and Jiang et al. (2005). The second used *C. suis* and *C. scrofarum* specific primers, as previously described by Jeníková et al. (2011). The nested PCR protocol by Alves et al. (2003) was used to amplify a fragment of the gp60 gene. *Cryptosporidium hominis* DNA was used as a positive control for genus-specific PCR (SSU rDNA and GP60), and *C. suis* and *C. scrofarum* DNA were used as positive controls for species-specific PCR. The amplicons and products of PCR/RFLP analyses were electrophoresed in 2% agarose gels supplemented with 0.2 mg/ml of ethidium bromide and visualized under ultraviolet light. In addition, to confirm the identity of *Cryptosporidium* spp., randomly selected secondary PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and sequenced in both directions with an ABI 3130 genetic analyser (Applied Biosystems, Foster City, CA) using the secondary PCR primers and the BigDye1 Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, California) in 10 µl reactions. The

nucleotide sequences obtained in this study were aligned with reference sequences retrieved from GenBank using the ClustalX 2.012 programme (ftp://ftpigbmc.ustrasbg.fr/pub/ClustalX/).

3. Results

A total of 460 faecal samples of Eurasian wild boars from 29 localities of four Central European countries were examined by both microscopical and molecular tools. Eleven (2.4%) were microscopically positive for *Cryptosporidium* spp. oocysts. Low infection intensity, ranging from 500 to 1,500 oocysts per gram, was detected in these samples. Using *Cryptosporidium* genus-specific, *C. suis*-specific, and *C. scrofarum*-specific PCR assays amplifying fragments of SSU rDNA, *Cryptosporidium* spp. were detected in 61 faecal samples (13.3%). All microscopically positive samples were confirmed using molecular tools. *Cryptosporidium* spp. was not detected at 9 enclosures (4 in Poland, 3 in the Czech Republic, 1 in Austria, and 1 in the Slovak Republic; Table 1). At least one positive faecal sample was detected at all other localities. *Cryptosporidium* prevalence recorded at the locality level reached up to 35.3%. RFLP and direct sequencing of genus specific PCR-amplified products revealed 56 *C. suis* (20) and *C. scrofarum* (36) monoinfections and only 5 mixed infections of these species. Species-specific PCR revealed 18 *C. suis* mono-infections (3.9%), 26 *C. scrofarum* mono-infections (5.7%) and 17 mixed infections of *C. suis* and *C. scrofarum* (3.7%). *Cryptosporidium suis* was not found at 20 localities and maximum prevalence reached 14.3% at 1 enclosure in the Czech Republic. Sixteen were found to be *C. scrofarum* free whereas the maximum prevalence reached 33.3% at 1 enclosure in the Czech Republic. Mixed infections of *C. suis* and *C. scrofarum* were found at 8 localities only (Table 1). The maximum prevalence of mixed infections was 15.8% at one enclosure in the Czech Republic.

In contrast to the species-specific PCR approach, endonuclease digestion of SSU rDNA amplicons revealed 20 mono-infections with *C. suis*, 36 mono-infections with *C. scrofarum* and only 5 mixed infections with both porcine specific cryptosporidia No other *Cryptosporidium* species or genotypes were detected by sequencing of randomly selected samples or PCR targeting the gp60 gene. No diarrhoea was observed in the examined faecal samples.

Generally, the occurrence of *C. scrofarum* was more frequent than *C. suis*, with the exception of the Slovak Republic. In total, 35 and 43 cases of *C. suis* and *C. scrofarum* were detected, respectively (Table 1).

4. Discussion

Wild boars (*Sus scrofa*) represent one of the problematic species of free living animals due to successful adaptation to landscape conditions and owing to growing of corn and other energetic crop reproduce rapidly. In USA, where the wild boar has been imported, is considered to be invasive species. Linked to increased quantity of wild boar in nature huge damage in agricultural crops and elevated transmission of zoonotic diseases occur (Schley and Roper, 2003).

While both porcine *Cryptosporidium* species, *C. suis* and *C. scrofarum*, have been reported worldwide in domestic pigs (e.g. Guselle et al., 2003; Maddox-Hyttel et al., 2006; Vítovec et al., 2006; Hamnes et al., 2007; Langkjaer et al., 2007; Suárez-Luengas et al., 2007; Jeníková et al., 2011; Kváč et al., 2009b, 2012, 2013), data on the occurrence of *Cryptosporidium* spp. in wild boars is less extensive (see Introduction). We found that 13.3% of the screened Eurasian wild boars from four Central European countries had *Cryptosporidium* infection. These data correspond with our preliminary findings from the Czech Republic (16.5%, Němejc et al., 2012). Atwill et al. (1997) and Castro-Hermida et al. (2011) found a slightly lower prevalence of *Cryptosporidium* spp. in feral pigs in the USA (5.4%) and in wild boar in Spain (7.4–11.5%), respectively. These differences could be due to the different tools used for detection and the different environmental conditions in which the animals live. However, studies consistently show a relatively low prevalence of *Cryptosporidium* spp. in wild pigs. In contrast, *Cryptosporidium* prevalence in domestic pigs kept under different management systems on commercial farms can reach more than 70% (see Němejc et al., 2013). In general, however, the infection rate of animals is reduced with increasing age on pig farms. Thus, these data support the theory that adult pigs are parasitized to a lesser extent (Sanford, 1987; Quílez et al., 1996; Guselle et al., 2003; Maddox-Hyttel et al., 2006; Vítovec et al., 2006; Hamnes et al., 2007).

Naturally occurring cryptosporidial infection in pigs did not show clinical signs (Guselle et al., 2003; Vítovec et al., 2006; Langkjaer et al., 2007; Suárez-Luengas et al., 2007; Kváč et al., 2009a,b). Similar to previous reports from studies of domestic pigs, we did not find any association between diarrhoea and the presence of either *C. suis* or *C. scrofarum* in Eurasian wild boars. In addition, we detected only 2.4 % of positive samples using microscopy-based techniques. These results are consistent with previous studies (Němejc et al., 2013). Absence of clinical signs, pathological course of infection and low infection intensity could be linked to previous infection history, age, immune status of individuals but could also support the hypothesis that parasite virulence for a specific host can decrease during the co-evolution (Lambrechts et al., 2006; Carval et Ferriere, 2010). The co-evolution of *C. suis* and *C. scrofarum* with pigs could support this model.

Kváč et al. (2013) and Jeníková et al. (2011) experimentally and empirically demonstrated the age specificity of both *C. suis* and *C. scrofarum*. Although the present study did not show the prevalence of *Cryptosporidium* spp. in all age categories of wild boars, our findings are in agreement with other studies that show the dominant occurrence of *C. scrofarum* in adult pigs (Hamnes et al., 2007; Langkjaer et al., 2007; Johnson et al., 2008; Kváč et al., 2009a,b; Jeníková et al., 2011).

The greater number of detected *C. scrofarum* infections using PCR-RFLP could suggest a higher infection intensity of this species compared to *C. suis*. Generally, using genus-specific primers is limited by the tendency of PCR to preferentially amplify the most abundant target (Tanriverdi et al., 2003). Thus, *C. scrofarum* was more often diagnosed using PCR-RFLP. All these data support the hypothesis that porcine cryptosporidia are age specific, but the susceptibility of wild boar piglets to *C. scrofarum* needs to be verified in future studies.

More than two thirds of recognized *Cryptosporidium* species have been detected in wild animals, which suggest that wild animals serve as natural reservoirs for transmission of zoonotic cryptosporidia to domestic animals and humans. Both porcine *Cryptosporidium* species, *C. suis* and *C. scrofarum*, have been previously reported to be human pathogenic (Xiao et al., 2002; Kváč et al., 2009c). Recent studies showed the presence of both swine cryptosporidia in source water (Ryan et al., 2005; Feng et al., 2011; Xiao et al., 2012). Monitoring and characterization of the sources of oocyst contamination in watersheds will help in the development and implementation of the most appropriate watershed management policies to protect the public from the risks of waterborne *Cryptosporidium* (Ryan et al., 2005). However, neither *C. suis* nor *C. scrofarum* currently represent a major risk to public health.

Acknowledgements

This study was supported by projects of the Grant Agency of the University of South Bohemia (022/2010/Z and 11/2013/Z) and NIH Grant Number 2P20 RR015566 from the National Center for Research Resources.

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Table 1

The distribution of *Cryptosporidium suis* and *Cryptosporidium scrofarum* among examined wild boars in Austria, the Czech Republic, Poland, and the Slovak Republic based on the result of PCR using species-specific primers

Country	Locality	No. of screened samples	Microscopy positive	PCR positive	Prevalence [%]	Molecular characterisation of *Cryptosporidium* spp.					
						Mono-infection				Mixed infection	
						C. suis		*C. scrofarum*		*C. suis* + *C. scrofarum*	
						Positive	Prevalence [%]	Positive	Prevalence [%]	Positive	Prevalence [%]
Austria	1	20	1[a]	5	25.0	2	10.0	1	5.0	2	10.0
	2	16	1[b]	3	18.8	0	-	2	12.5	1	6.3
	3	8	0	0	-	0	-	0	-	0	-
Total		***44***	***2***	***8***	***18.2***	***2***	***4.5***	***3***	***6.8***	***3***	***6.8***
Czech Republic	4	19	0	5	26.3	2	10.5	0	-	3	15.8
	5	17	2[b]	6	35.3	2	11.8	4	23.5	0	-
	6	7	0	1	14.3	1	14.3	0	-	0	-
	7	14	0	0	-	0	-	0	-	0	-
	8	18	0	1	5.6	0	-	0	-	1	5.6
	9	31	1[a]	6	19.4	4	12.9	0	-	2	6.5
	10	6	0	0	-	0	-	0	-	0	-
	11	10	0	2	20.0	0	-	2	20.0	0	-
	12	7	0	0	-	0	-	0	-	0	-
	13	9	0	1	11.1	0	-	1	11.1	0	-
	14	26	1[a]	5	19.2	2	7.7	1	3.9	2	7.7
	15	34	0	6	17.7	2	5.9	0	-	4	11.8
	16	3	0	1	33.3	0	-	1	33.3	0	-
	17	30	2[b]	5	16.7	0	-	5	16.7	0	-
Total		***231***	***6***	***39***	***16.9***	***13***	***5.6***	***14***	***6.1***	***12***	***5.2***
Poland	18	12	1[b]	3	25.0	0	-	3	25.0	0	-
	19	15	0	1	6.7	0	-	1	6.7	0	-

Country	Locality	No. of screened samples	Microscopy positive	PCR positive	Prevalence [%]	Molecular characterisation of *Cryptosporidium* spp.					
						Mono-infection				Mixed infection	
						C. suis		*C. scrofarum*		*C. suis* + *C. scrofarum*	
						Positive	Prevalence [%]	Positive	Prevalence [%]	Positive	Prevalence [%]
	20	13	2[b]	4	30.8	1	7.7	3	23.1	0	-
	21	18	0	0	-	0	-	0	-	0	-
	22	12	0	0	-	0	-	0	-	0	-
	23	14	0	0	-	0	-	0	-	0	-
	24	15	0	1	6.7	0	-	1	6.7	0	-
	25	15	0	2	13.3	0	-	0	-	2	13.3
	26	15	0	0	-	0	-	0	-	0	-
Total		***129***	***3***	***77***	***8.5***	***7***	***0.8***	***8***	***6.2***	***2***	***1.6***
	27	10	0	1	10.0	0	-	1	10.0	0	-
Slovak Republic	28	26	0	2	7.7	2	7.7	0	-	0	-
	29	20	0	0	-	0	-	0	-	0	-
Total		***56***	***0***	***3***	***5.4***	***2***	***3.6***	***1***	***1.8***	***0***	-
Overall total		**460**	**11**	**61**	**13.3**	**18**	**3.9**	**26**	**5.7**	**17**	**3.7**

[a] determined by following PCR as *C. suis*;

[b] determined by following PCR as *C. scrofarum*

Vet Parasitol. Author manuscript; available in PMC 2015 May 19.