Veterinary Parasitology: Regional Studies and Reports 22 (2020) 100473

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Short Communication

Molecular characterization of *Cryptosporidium* spp. from domestic pigs in Argentina

Lorena A. De Felice[a], Gastón Moré[a,b,*], Javier Cappuccio[b,c], María C. Venturini[a], Juan M. Unzaga[a]

[a] Laboratorio de Inmunoparasitología (LAINPA), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, 60 y 118 (1900), La Plata, Argentina
[b] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Rivadavia 1917 (C1033AAJ), Buenos Aires, Argentina
[c] Estación Experimental Agropecuaria (EEA) Marcos Juarez, Instituto Nacional de Tecnología Agropecuaria (INTA), Ruta 12 Km.3 (2580), Marcos Juarez, Córdoba, Argentina

ARTICLE INFO

Keywords:
Cryptosporidium suis
Cryptosporidium scrofarum
Piglets
Oocysts
Microscopy
Nested PCR

ABSTRACT

Cryptosporidiosis in pigs is caused by different *Cryptosporidium* species or genotypes, with *C. suis* and *C. scrofarum* considered porcine specific species. There is scarce information on *Cryptosporidium* infection in pigs in South America. A total of 520 individual faecal samples were obtained from 1, 2, 3 and 4 week old piglets (n = 130 from each age group), from 13 Argentinean intensive pig farms. The diagnosis of species of *Cryptosporidium* combined microscopy and molecular techniques. Genotyping from samples with *Cryptosporidium* oocysts at microscopy was performed by genus-specific and species-specific nested PCR targeting 18S rRNA gene fragments, and sequencing. Microscopic analysis detected *Cryptosporidium* oocysts in 47/520 (9%) faecal samples from 11/13 (85%) farms, with farm infection rates between 0 and 17.5%. Presence of *Cryptosporidium* oocysts was associated with diarrhea. The proportion of microscopically positive samples was not associated with piglet age. A total of 15/47 (32% of samples with oocyst compatible structures) were positive by genus and species-specific nested PCR. Species-specific PCR and sequencing showed presence of *C. suis*, *C. scrofarum*, and both species in 3, 8 and 4 samples, respectively. The proportion of positive samples on each specific PCR was similar between age groups, being *C. suis* proportion slightly higher in 4 week old piglets. The use of molecular tools allowed the confirmation of *C. suis* and *C. scrofarum* infection in Argentinean pigs. Cryptosporidiosis was widely distributed in the main pig husbandry area from Argentina, with a low to moderate intra farm infection rate.

1. Introduction

The intracellular parasites of the genus *Cryptosporidium* infect a wide variety of vertebrate hosts (fish, amphibians, reptiles, birds and mammals) including humans (Fayer, 2010). These apicomplexan protozoans have an important impact on animal and human health, producing waterborne outbreaks and causing asymptomatic to severe intestinal infections (Němejc et al., 2013; Dellarupe et al., 2016).

Cryptosporidiosis can cause significant neonatal morbidity and productive loses in farmed livestock. Cryptosporidiosis in pigs has been reported worldwide with infection rates ranging between 0 and 87.5% (Kváč et al., 2009; Jeniková et al., 2011). Clinical signs due to natural cryptosporidiosis in pigs are infrequent; however, experimental infections show a wide range of responses from subclinical to severe clinical manifestations, such as diarrhea, vomiting and anorexia (Tzipori et al., 1982). Pigs can be infected by different *Cryptosporidium* species or genotypes (*Cryptosporidium parvum*, *C. muris*, *C. tyzzeri* and others), however, most of these species or genotypes occurred infrequently (Morgan et al., 1999; Ryan et al., 2003, 2004). Two porcine specific species have been described: *C. suis* and *C. scrofarum*. *Cryptosporidium suis* infects all age categories, being detected more frequently in pigs younger than 5 weeks old (Ryan et al., 2004), whereas *C. scrofarum* infections occur mostly in weaned piglets, older than 4 weeks of age (Jeniková et al., 2011; Nguyen et al., 2012; Kváč et al., 2009; Kváč et al., 2013). Even though both *C. suis* and *C. scrofarum* species have been identified in human infections, *C. suis* was reported mostly affecting patients with previous immunosuppressive diseases (Xiao et al., 2002; Wang et al., 2013) as well as concomitant digestive tract diseases (Leoni et al., 2006; Bodager et al., 2014). Conversely, there is only one report of *C. scrofarum* infection in an immunocompetent patient with a clinical history of diarrhea (Kváč et al., 2013).

There is scarce information about *Cryptosporidium* infection in domestic pigs in South America and its potential productive and zoonotic implications. The aim of this study was to identify and characterize the

* Corresponding author at: Universidad Nacional de La Plata, Facultad de Ciencias Veterinarias, Calle 60 y 118 (Zip code: 1900) La Plata, Argentina.
E-mail address: gastonmore@fcv.unlp.edu.ar (G. Moré).

https://doi.org/10.1016/j.vprsr.2020.100473
Received 19 June 2020; Received in revised form 15 September 2020; Accepted 18 September 2020
Available online 20 September 2020

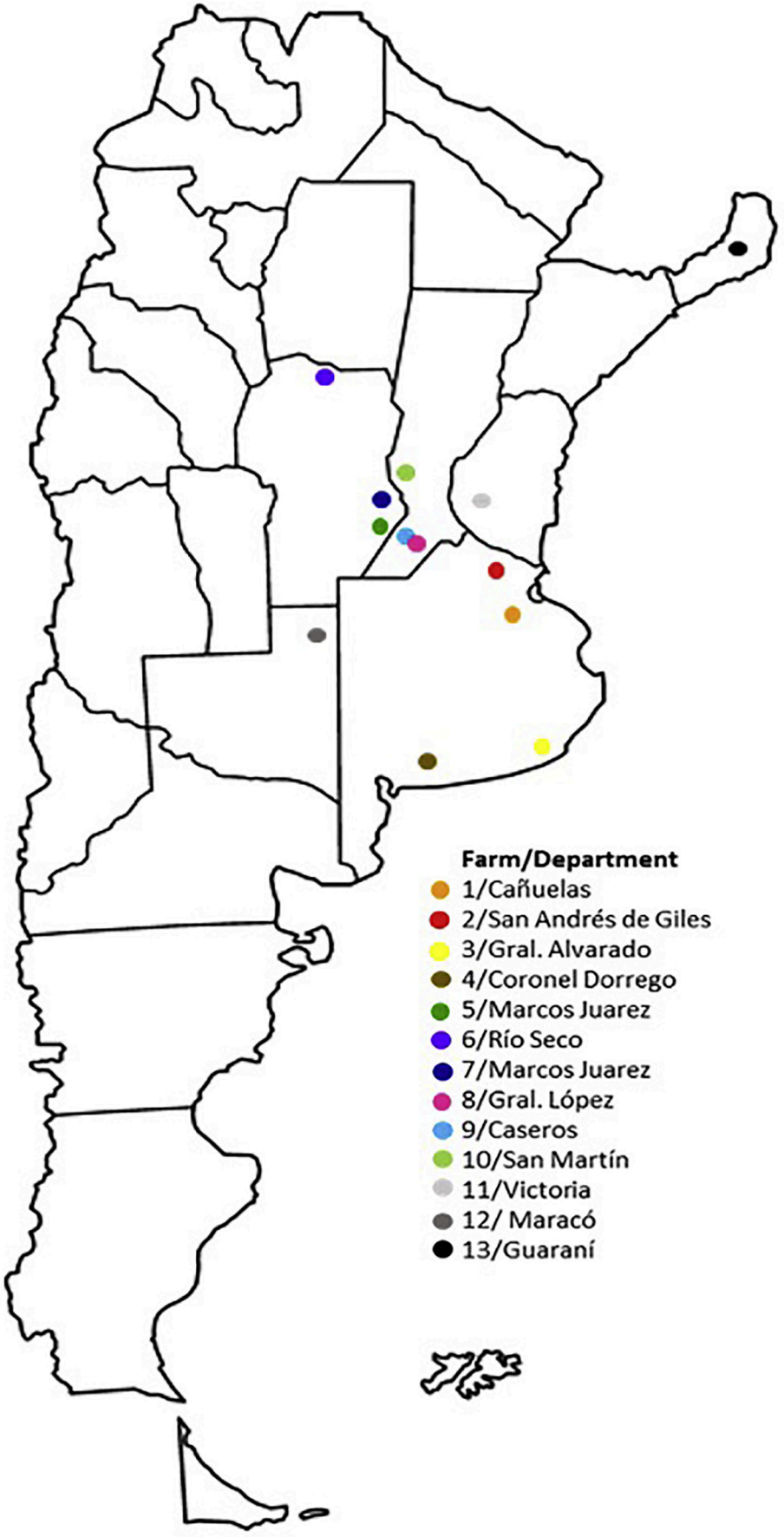

Fig. 1. Sampled pig farms location. Argentina map.

Table 1
Distribution of *Cryptosporidium* prevalence by staining-microscopy and molecular diagnosis for each sampled farm and piglets age in relation with average of diarrheic samples.

Farm	Diarrhea	Farm prevalence		Age (week)			
	% (n/40)	MIC % (n/40) PCR % (n/40)		Prevalence MIC/PCR % (n/10)			
				1	2	3	4
1	60	17.5	5	0/0	20/0	40/10	10/10
2	40	0	0	ND	ND	ND	ND
3	40	0	0	ND	ND	ND	ND
4	80	5	0	20/0	0/0	0/0	0/0
5	62.5	10	7.5	0/0	10/0	0/0	30/30
6	47.5	7.5	0	0/0	20/0	10/0	0/0
7	50	7.5	0	0/0	10/0	20/0	0/0
8	57.5	15	2.5	20/0	0/0	0/0	40/10
9	60	15	12.5	20/10	20/10	20/20	0/0
10	55	15	5	30/20	30/0	0/0	0/0
11	70	15	2.5	20/10	30/0	0/0	10/0
12	27.5	2.5	0	0/0	0/0	0/0	10/0
13	72.5	7.5	5	10/10	10/10	0/0	10/0
Total	55.6 (289/520)	9 (47/520)	2.9 (15/520)	9.2/3.8	11.5/1.5	6.9/2.3	8.5/3.8

MIC = staining and microscopy. PCR = molecular diagnosis (species-specific nPCR). ND = not determined. Note: the total prevalence in regard to age was calculated over n = 130 (total samples from each category).

infection with *Cryptosporidium* spp. in domestic suckling and recently weaned piglets from Argentina.

2. Materials and methods

2.1. Sample collection and examination

Individual faecal samples (n = 520) were randomly obtained from 13 pig farms under the intensive breeding system. Farms were selected without previous knowledge of parasitological status, and all of them were located in the most important geographical area of pig husbandry under intensive management systems in Argentina (Fig. 1). One (n = 130), 2 (n = 130) and 3 (n = 130) week old suckling piglets, and 4 week old recently weaned piglets (n = 130) were sampled. Ten samples were collected from each age group in each farm (n = 40). Samples were collected from the floor immediately after deposition into individually labelled nylon bags and stored at 4 °C until analysis in the laboratory. Faeces were classified as normal (faeces maintained the original shape), or diarrheal (faeces took the form of the container) (Němejc et al., 2013). Samples were concentrated by sedimentation-flotation technique as previously described for other protozoan oocysts (Schares et al., 2005) and stained by modified Ziehl-Neelsen technique according to Henriksen and Pohlenz (1981). Samples with 4–5 μm diameter oocysts observed in a light microscope at 1000× magnification were identified as positive for *Cryptosporidium* spp. Aliquots from concentrated material were preserved in 1.5 ml DNase free microtubes at −20 °C until PCR analysis.

2.2. DNA extraction, PCR and sequencing

DNA was extracted from ~250 μl concentrated faecal samples with *Cryptosporidium* spp. oocyst compatible structures at microscopy, using the ZR faecal DNA kit following the manufacturer's instructions (Zymo, USA). The purified DNA samples were stored at −20 °C until PCR amplification.

Cryptosporidium spp. DNA amplification was performed by nested PCR (nPCR) which amplified fragments of the 18S rRNA gene. First, a set of genus-specific primers were used for the primary nPCR protocol (Xiao et al., 2001; Jiang et al., 2005). Positive samples on the genus-specific PCR were subjected to a second nPCR to identify *C. suis* and *C. scrofarum* with species-specific primers (Jeniková et al., 2011). The PCR products were analysed on 1.5% agarose gel and stained with SYBR safe (Invitrogen, USA). The amplicons obtained were purified with Wizard SV Gel and PCR Clean-Up System (Promega, USA) and submitted for sequencing (http://dna.macrogen.com) in both senses, with the primers used for each specific nPCR. Nucleotide sequences were aligned and edited with GENEIOUS software (R9 version). Consensus sequences obtained were compared with others available in GenBank by Mega-Blast analysis from NCBI (htpps://blastn.ncbi.nlm.nih.gov).

2.3. Statistical analysis

Statistical analysis was performed using WinEpi 1.0, software for Windows, with 95% confidence intervals (CI) and probability level ($p < 0.05$). The general *Cryptosporidium* infection rate as well as the intra farm infection rate was calculated. Chi-square test was used to determine the association between samples with *Cryptosporidium* oocyst and presence of diarrhea. The differences among proportions (age /microscopy positive samples; age /species-specific nPCR positive samples) were calculated by Chi-square test (differences among percentages).

3. Results

A total of 47/520 (9%, CI95% 6.57% -11.50%) faecal samples showed *Cryptosporidium* oocysts by Ziehl-Neelsen staining. *Cryptosporidium* spp. positive samples were detected in 85% (11/13) of farms. The infection rate within farms was between 0 and 17.5% (CI95% 0.00% - 29.28%). A total of 289/520 (55.57%) samples were diarrheal stools (Table 1). Of the 47 samples that were positive for microscopy, 33 were diarrheic stools (70.21%). The presence of *Cryptosporidium* oocysts was associated with diarrhea (p = 0.0342). *Cryptosporidium* oocysts were detected in piglets from all age groups. Twelve (9.23%), 15 (11.53%), 9 (6.92%) and 11 samples (8.46%) were positive in 1, 2, 3 and 4 week old piglets, respectively. The proportion of microscopy and PCR positive samples was not associated with age ($p > 0.05$).

Genus specific nPCR allowed the detection of *Cryptosporidium* DNA in 15/47 samples (32% of samples with oocyst compatible structures). The 15 samples (10 suckling and 5 recently weaned piglets) were positive by species-specific nPCRs: 3 were positive for *C. suis* (~ 482 bp amplicon), 8 samples were positive for *C. scrofarum* (~ 443 bp amplicon) and 4 were positive for both *Cryptosporidium* species and identified as mixed infections. The prevalence of *Cryptosporidium* spp. by staining-microscopy and nPCR for each sampled farm and piglets' age, in relation with average of diarrheic samples by farm is shown in Table 1. A total of 17 purified products (with a gel estimated concentration higher than 40 ng) from the 15 positive faecal samples were submitted for sequencing. The positive PCRs and sequencing results concerning the region and piglets' age are shown in Table 2. A total of 13 sequences were obtained; 4 samples failed or had low-quality sequencing results. Five consensus sequences were 100% identical among them and revealed 100% identity with *C. suis* sequences reported in GenBank. Eight sequences evidenced 99.70–100% identity among them and 99.77–100% sequence identity with *C. scrofarum* sequences reported in GenBank. All the sequences identified as *C. suis* had a sequence identity of 91.17–96.32% with *C. scrofarum* sequences.

The species-specific PCRs allowed to confirm *Cryptosporidium* infection in 3.8% of 1-week old (0.76% *C. suis* and 3.07% *C. scrofarum*), 1.53% in 2 week old (1.53% *C. scrofarum*), 2.3% in 3 week old (1.53% *C. suis* and 2.3% *C. scrofarum*) and 3.84% in 4 week old piglets (3.07% *C. suis* and 2.3% *C. scrofarum*). The proportion of positive samples on each specific nPCR was similar among piglet age groups, being *C. suis* proportion slightly higher in the recently weaned piglets ($p \geq 0.05$).

Table 2
Specific nPCRs and sequencing results in regard to piglets age and location.

Farm/Sample ID	Age (weeks)	Province/Department	Specific nPCR	Sequencing	GenBank
				BLAST identity (#)	ID
11/259	1	Entre Ríos/Victoria	*C. suis*	100% *C. suis*	MT124675
13/214	1	Misiones/Guaraní	*C. scrofarum*	ND	
9/510	1	Santa Fé/Caseros	*C. scrofarum*	100% *C. scrofarum*	MT124670
10/547	1	Santa Fé/San Martín	*C. scrofarum*	ND	
10/554	1	Santa Fé/San Martín	*C. scrofarum*	100% *C. scrofarum*	MT124672
13/221	2	Misiones/Guaraní	*C. scrofarum*	100% *C. scrofarum*	MT124669
9/526	2	Santa Fé/Caseros	*C. scrofarum*	100% *C. scrofarum*	MT124671
9/534	3	Santa Fé/Caseros	*C. scrofarum*	ND	
1/107	3	Buenos Aires/Cañuelas	*C. suis*	ND	
			C. scrofarum	100% *C. scrofarum*	MT124665
9/528	3	Santa Fé/Caseros	*C. scrofarum*	100%*C. scrofarum*	MT124667
			C. suis	100% *C. suis*	MT124674
1/365	4	Buenos Aires/Cañuelas	*C. suis*	100% *C. suis*	MT124676
5/506	4	Córdoba/Marcos Juarez	*C. suis*	100% *C. suis*	MT124677
8/177	4	Santa Fé/Gral. López	*C. scrofarum*	100% *C. scrofarum*	MT124668
5/503	4	Córdoba/Marcos Juarez	*C. suis*	ND	
			C. scrofarum	ND	
5/504	4	Córdoba/Marcos Juarez	*C. scrofarum*	99.77% *C. scrofarum*	MT124666
			C. suis	100% *C. suis*	MT124673

(#) Reference sequences on BLASTn comparison (GenBank accession numbers): *C. scrofarum* (JX424840, KP704557, MG516763) and *C. suis* (JF710255, KT22302, AF108861). ND = no data.

4. Discussion

Cryptosporidium infection has been found in pigs in several countries in all age categories. In the present study, *Cryptosporidium* infection rate was 9% (6.57%–11.50%) by staining and microscopy from concentrated faecal material. This result was similar to previous reports from Czech Republic (Němejc et al., 2013) and Slovakia (Danišová et al., 2016). Worldwide, the *Cryptosporidium* prevalence on farm level varies widely between 1 and 100% (Ryan et al., 2003; Suárez-Luengas et al., 2007; Zheng et al., 2019). These differences may be due to many factors such as age/category, the diagnostic techniques applied and the different management systems and practices.

This study was carried out on intensive swine housing system farms, with slatted floor, where the slurry is collected to minimize contact with the pigs. Most sampled farms (85%) resulted positive by microscopic diagnosis, whereas *Cryptosporidium* infection rates on the farm level were 0–17.5%. This range was slightly higher than that obtained in a study on farms in China (Zheng et al., 2019), but lower than in the Czech Republic (Němejc et al., 2013). Both mentioned studies were also performed in farms with an intensive breeding system, however, in addition to the suckling and nursery categories, they included older pigs.

The relationship of *Cryptosporidium* infection in pigs with the outcome of clinical signs is controversial. On one hand, previous studies described anorexia and voluminous watery diarrhea in pre-weaned piglets experimentally infected with *C. suis* (Enemark et al., 2003). Mišič et al. (2003) observed diarrhea in all *Cryptosporidium* positive nursing piglets; furthermore, Nguyen et al. (2012) reported the association between the occurrence of diarrhea and the level of *Cryptosporidium* oocyst excretion. On the other hand, Jeniková et al. (2011) and Němejc et al. (2013) found no diarrhea in pigs naturally infected with both pig-specific species. In our study, the presence of diarrhea was associated with the *Cryptosporidium* positive result by microscopy. However, not all the *Cryptosporidium* positive pigs showed diarrheic faeces, suggesting different infection susceptibility among individuals. In addition, the proportion of diarrheal samples in the sampled piglet population was high (around 55%), and since other agents associated with diarrhea in piglets were not evaluated, the potential synergy could not be assessed and ruled out.

The distribution of *Cryptosporidium* infection rates in different pig age/categories are not fully understood. The highest risk of infection and developing clinical signs have been associated with weaning, an immune and dietary critical step for piglets (Thompson et al., 1996). Nguyen et al. (2012) reported that suckling piglets are at the highest risk for *Cryptosporidium* infection followed by weaned piglets, sows and finishing pigs. Němejc et al. (2013) identified a peak of infection in pre-weaned piglets (up to 5 weeks old). However, Yui et al. (2014) and Zheng et al. (2019) among others, observed the highest rates of *Cryptosporidium* infection in weaned piglets (4 to 8 weeks old). In this study, we detected *Cryptosporidium* oocysts in samples from all age groups (1 to 4 weeks old), without differences in the proportion of positive samples among age groups. The age distribution of *Cryptosporidium* infection in different farms may be related to several factors, including different weaning time, dietary changes, as well as the health status and possible concomitant diseases (Enemark et al., 2003; Zheng et al., 2019). Our results suggest a constantly low level of exposure to *Cryptosporidium* in suckling piglets from intensive management farms in Argentina.

Of the 47 *Cryptosporidium* positive samples identified by microscopy, only 15 resulted positive by genus and species-specific nPCRs. It has been suggested that the oocyst burden in a sample could influence the PCR results as well as the DNA extraction technique (Xiao et al., 2001). In the present study, a faecal concentration technique was applied to increase the oocyst number on the DNA extraction sample (Schares et al., 2005), and a previously established DNA extraction technique for Apicomplexan protozoan oocysts, with efficient control for inhibitors, was used (Herrmann et al., 2011). It is known that no staining method is completely effective for the detection of *Cryptosporidium* oocysts in stool samples, however, the modified Ziehl-Neelsen technique is still one of the most used in laboratories due to its low cost and relative simplicity (Casemore et al., 1985; Uppal et al., 2014). Due to the low specificity of acid-fast staining and microscopy, presence of other similar protozoans producing *Cryptosporidium* oocyst-like structures in some samples (Aghamolaie et al., 2016) cannot be ruled out, and could partly explain the poor agreement between the microscopy and the nPCR.

Many studies have shown that pigs are naturally and frequently infected with *C. suis* and *C. scrofarum* which are considered pig adapted species (Ryan et al., 2004; Kváč et al., 2013). However, other *Cryptosporidium* species were also identified in pigs with lower prevalence and sometimes as mixed infections (Morgan et al., 1999). Also, several studies identified an age-specific distribution of *Cryptosporidium* spp. in

pigs, where *C. suis* was more prevalent and predominantly detected in pre-weaned piglets, while C. *scrofarum* was only detected in weaned piglets and older age categories (Suárez-Luengas et al., 2007; Jeniková et al., 2011; Kváč et al., 2009, 2013; Němejc et al., 2013; Zheng et al., 2019). Nevertheless, Kváč et al. (2013) reported that pigs infected with *C. scrofarum* start to shed oocysts in the range of 6–7 days post-infection. In accordance with previous reports, we detected only pig-adapted *Cryptosporidium* spp. as follows: *C. suis* in 3 samples, *C. scrofarum* in 8 samples and *C. suis/C. scrofarum* mixed infection in 4 samples. Although we found a slightly higher proportion of *C. suis* in recently weaned piglets, both *C. suis* and *C. scrofarum* species were identified in piglets from all age groups. Similar results were published by Wang et al. (2010) and Danišová et al. (2016) as they detected at least 1 sample positive for *C. scrofarum* in piglets younger than 1 month. Our results showed a greater distribution of *C. scrofarum* being detected in all age categories and with higher frequency than *C. suis*.

Cama et al. (2006) reported that conventional genus-specific nPCR sequencing is a limited method for identification of *Cryptosporidium* mixed infection because the dominant species or genotype in the samples becomes preferentially amplified. In this study, we used genus-specific nPCR as screening, followed by two nPCRs using the species-specific primers designed by Jeniková et al. (2011). Using this approach, we were able to identify mixed infections in 4 samples. Interestingly, these mixed infections appear only in 3 and 4 week old piglets, suggesting an increased risk of infection with different species along with age. Further studies with a higher sample number are required to confirm this observation. All genus-specific nPCR positive samples were also positive in at least one specific nPCR. Nevertheless, the presence of other concomitant *Cryptosporidium* spp. was not completely ruled out. The two identified *Cryptosporidium* species have zoonotic potential, and as such, special measures should be considered to improve the sanitary and safe disposal of pig faeces, among other measures, to minimize environmental contamination.

Our results suggest the species-specific nPCRs as useful tools to improve molecular *Cryptosporidium* infection diagnosis in pigs. However, other protozoans should be considered when species-specific nPCRs resulted in negative and *Cryptosporidium* oocyst-like structures are observed.

In conclusion, *C. suis* and *C. scrofarum* infection were confirmed by molecular tools in Argentinean pigs. The infection rate obtained by staining and microscopy was low to moderate and was widely distributed in the main pig husbandry area from Argentina. Further surveys should be carried out in farms with different breeding systems and include more ages/categories, in order to better understand the epidemiology of *Cryptosporidium* infection in Argentina.

Ethical statement

All authors concur with this submission. The material is original and is not under consideration for publication elsewhere.

Declaration of Competing Interest

All the authors declare no conflict of interests.

Acknowledgements

We would like to thank Isidoro Ercoli for his excellent technical assistance and Dra. Magdalena Rambeaud for the language revision of the manuscript.

This research was funded by the Research Grant PICT 2016-3117 from *Agencia Nacional de Promoción Científica y Tecnológica*, FONCyT, Argentina.

References

Aghamolaie, S., Rostami, A., Fallahi, Sh., Tahvildar Biderouni, F., Haghighi, A., Salehi, N., 2016. Evaluation of modified Ziehl–Neelsen, direct fluorescent-antibody and PCR assay for detection of *Cryptosporidium* spp. in children faecal specimens. J. Parasit. Dis. 40 (3), 958–963.

Bodager, J.R., Parsons, M.B., Wright, P.C., Rasambainarivo, F., Roellig, D., Xiao, L., Gillespie, T.R., 2014. Complex epidemiology and zoonotic potential for *Cryptosporidium suis* in rural Madagascar. Vet. Parasitol. 207, 140–143.

Cama, V.A., Gilman, R.H., Vivar, A., Ticona, E., Ortega, Y., Bern, C., Xiao, L., 2006. Mixed *Cryptosporidium* infections and HIV. Emerg. Infect. Dis. 12, 1025–1028.

Casemore, D.P., Armstrong, M., Sands, R.L., 1985. Laboratory diagnosis of cryptosporidiosis. J. Clin. Pathol. 38 (12), 1337–1341.

Danišová, O., Valenčáková, A., Petrincová, A., 2016. Detection and identification of six *Cryptosporidium* species in livestock in Slovakia by amplification of SSU and GP60 genes with the use of PCR analysis. Ann. Agric. Environ. Med. 23, 254–258.

Dellarupe, A., Unzaga, J.M., Moré, G., Kienast, M., Larsen, A., Stiebel, C., Rambeaud, M., Venturini, M.C., 2016. *Cryptosporidium varanii* infection in leopard geckos (*Eublepharis macularius*) in Argentina. Open Vet. J. 6, 98–101.

Enemark, H.L., Ahrens, P., Bille-Hansen, V., Heegaard, P.M., Vigre, H., Thamsborg, S.M., Lind, P., 2003. *Cryptosporidium parvum*: infectivity and pathogenicity of the 'porcine' genotype. Parasitology 126, 407–416.

Fayer, R., 2010. Taxonomy and species delimitation in *Cryptosporidium*. Exp. Parasitol. 124, 90–97.

Henriksen, S.A., Pohlenz, J.F., 1981. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. Acta Vet. Scand. 22, 594–596.

Herrmann, D.C., Maksimov, A., Pantchev, N., Vrhovec, M.G., Conraths, F.J., Schares, G., 2011. Comparison of different commercial DNA extraction kits to detect *Toxoplasma gondii* oocysts in cat faeces. Berl. Munch. Tierarztl. Wochenschr. 124, 497–502.

Jeniková, M., Nemejc, K., Sak, B., Kvetonova, D., Kvác, M., 2011. New view on the age-specificity of pig *Cryptosporidium* by species-specific primers for distinguishing *Cryptosporidium suis* and *Cryptosporidium* pig genotype II. Vet. Parasitol. 176, 120–125.

Jiang, J., Alderisio, K.A., Xiao, L., 2005. Distribution of *Cryptosporidium* genotypes in storm event water samples from three watersheds in New York. Appl. Environ. Microbiol. 71, 446–4454.

Kváč, M., Hanzlikova, D., Sak, B., Kvetonova, D., 2009. Prevalence and age related infection of *Cryptosporidium suis*, *C. muris* and *Cryptosporidium* pig genotype II in pigs on a farm complex in the Czech Republic. Vet. Parasitol. 160, 319–322.

Kváč, M., Kestranov, M., Pinkovac, M., Kvetonova, D., Kalinova, J., Wagnerova, P., Kotkova, M., Vitovec, J., Ditrich, O., Mc Evoyd, J., Stengerd, B., Sak, B., 2013. *Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*). Vet. Parasitol. 191, 218–227.

Leoni, F., Amar, C., Nichols, G., Pedraza-Díaz, S., Mc Lauchlin, J., 2006. Genetic analysis of *Cryptosporidium* from 2414 humans with diarrhoea in England between 1985 and 2000. J. Med. Microbiol. 55, 703–707.

Mišič, Z., Katič-Radivojevič, S., Kulišič, Z., 2003. *Cryptosporidium* infections in nursing, weaning and post-weaned piglets and sows in the Belgrade district. Acta Vet. (Belograd) 5–6, 361–366.

Morgan, U.M., Buddle, R., Armson, A., Elliot, A., Thompson, R.C.A., 1999. Molecular and biological characterisation of *Cryptosporidium* in pigs. Aust. Vet. J. 77, 44–47.

Němejc, K., Sak, B., Květoňová, D., Kernerová, N., Rost, M., Cama, V.A., Kváč, M., 2013. Occurrence of *Cryptosporidium suis* and *Cryptosporidium scrofarum* on commercial swine farms in the Czech Republic and its associations with age and husbandry practices. Parasitol. Res. 112, 1143–1154.

Nguyen, S.T., Honma, H., Geurden, T., Ikarash, M., Fukuda, Y., Huynh, V.V., Nguyen, D.T., Nakai, Y., 2012. Prevalence and risk factors associated with *Cryptosporidium* oocysts shedding in pigs in Central Vietnam. Res. Vet. Sci. 93, 848–852.

Ryan, U.M., Samarasinghe, B., Read, C., Buddle, J.R., Robertson, I.D., Thompson, R.C., 2003. Identification of a novel *Cryptosporidium* genotype in pigs. Appl. Environ. Microbiol. 69, 3970–3974.

Ryan, U.M., Monis, P., Enemark, H.L., Sulaiman, I., Samarasinghe, B., Read, C., Buddle, R., Robertson, I., Zhou, L., Thompson, R.C., Xiao, L., 2004. *Cryptosporidium suis* n. sp. (Apicomplexa: Cryptosporidiidae) in pigs (*Sus scrofa*). J. Parasitol. 90, 769–773.

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C., Conraths, F.J., 2005. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol. 35, 1525–1537.

Suárez-Luengas, L., Clavel, A., Quílez, J., Goñi-Cepero, M.P., Torres, E., Sánchez-Acedo, C., del Cacho, E., 2007. Molecular characterization of *Cryptosporidium* isolates from pigs in Zaragoza (northeastern Spain). Vet. Parasitol. 148, 231–235.

Thompson, P.B., Brewer, J., Brewer, E.E., 1996. Swine Care Handbook. National Pork Board, Des Moines, pp. 34.

Tzipori, S., Smith, M., Maken, T., Halpin, C., 1982. Enterocolitis in piglets caused by *Cryptosporidium* sp. purified from calf feces. Vet. Parasitol. 11, 1121–1126.

Uppal, B., Singh, O., Chadha, S., Jha, A.K., 2014. A comparison of nested PCR assay with conventional techniques for diagnosis of intestinal cryptosporidiosis in AIDS cases from northern India. Journal of Parasitology Research 2014 (Article ID 706105).

Wang, R., Qiu, S., Jian, F., Zhang, S., Shen, Y., Zhang, L., Ning, C., Cao, J., Qi, M., Xiao, L., 2010. Prevalence and molecular identification of *Cryptosporidium* spp. in pigs in Henan, China. Parasitol. Res. 107, 1489–1494.

Wang, L., Zhang, H., Zhao, X., Zhang, L., Zhang, G., Guo, M., Liu, L., Feng, Y., Xiao, L., 2013. Zoonotic *Cryptosporidium* species and *Enterocytozoon bieneusi* genotypes in HIV-positive patients on antiretroviral therapy. J. Clin. Microbiol. 51, 557.

Xiao, L., Singh, A., Limor, J., Graczyk, T.K., Gradus, S., Lal, A., 2001. Molecular

characterization of *Cryptosporidium* oocysts in samples of raw surface water and wastewater. Appl. Environ. Microbiol. 67, 1097–1101.
Xiao, L., Bern, C., Arrowood, M., Sulaiman, I., Zhou, L., Kawai, V., Vivar, A., Lal, A.A., Gilman, R.H., 2002. Identification of the *Cryptosporidium* pig genotype in a human patient. J. Infect. Dis. 185, 1846–1848.
Yui, T., Nakajima, T., Yamamoto, N., Kon, M., Abe, N., Matsubayashi, M., Shibahara, T., 2014. Age-related detection and molecular characterization of *Cryptosporidium suis* and *Cryptosporidium scrofarum* in pre- and post-weaned piglets and adult pigs in Japan. Parasitol. Res. 113, 359–365.
Zheng, S., Li, D., Zhou, C., Zhang, S., Wu, Y., Chang, Y., Chen, Y., Huang, J., Ning, C., Zhang, G., Zhang, L., 2019. Molecular identification and epidemiological comparison of *Cryptosporidium* spp. among different pig breeds in Tibet and Henan, China. Vet. Res. 15, 101.