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## ***Cryptosporidium scrofarum* n. sp. (Apicomplexa: Cryptosporidiidae) in domestic pigs (*Sus scrofa*)**

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### **Abstract**

We describe the morphological, biological, and molecular characteristics of *Cryptosporidium* pig genotype II and propose the species name *Cryptosporidium scrofarum* n. sp. to reflect its prevalence in adult pigs worldwide. Oocysts of *C. scrofarum* are morphologically indistinguishable from *C. parvum*, measuring 4.81–5.96 μm (mean = 5.16) × 4.23–5.29 μm (mean = 4.83) with a length to width ratio of 1.07 ± 0.06 (n = 400). Oocysts of *C. scrofarum* obtained from a naturally infected pig were infectious for 8-week-old pigs but not 4-week-old pigs. The prepatent period in 8-week-old *Cryptosporidium*-naive pigs was 4–6 days and the patent period was longer than 30 days. The infection intensity of *C. scrofarum* in pigs was generally low, in the range 250–4000 oocysts per gram of faeces. Infected pigs showed no clinical signs of cryptosporidiosis and no pathology was detected. *Cryptosporidium scrofarum* was not infectious for adult SCID mice, adult BALB c mice, Mongolian gerbils (*Meriones unguiculatus*), southern multimammate mice (*Mastomys coucha*), yellow-necked mice (*Apodemus flavicollis*), or guinea pigs (*Cavia porcellus*). Phylogenetic analyses based on Small subunit rRNA, actin, and heat shock protein 70 gene sequences revealed that *C. scrofarum* is genetically distinct from all known *Cryptosporidium* species.

### **Keywords**

*Cryptosporidium scrofarum*; Taxonomy; Morphology; Molecular analyses; Transmission studies; *Cryptosporidium* pig genotype II

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## 1. Introduction

Porcine cryptosporidiosis, which was first described in the USA (Bergeland, 1977; Kennedy et al., 1977), has been reported to occur worldwide. *Cryptosporidium parvum* was initially considered to be the causal agent based on experimental infection studies undertaken in the 1980s (Moon and Bemrick, 1981; Tzipori et al., 1982; Vítovec and Koudela, 1992). Subsequent molecular epidemiology and experimental infection studies revealed the susceptibility of pigs to a number of species including *C. parvum*, *C. hominis*, *C. suis*, *C. felis*, and *C. meleagridis*. *Cryptosporidium muris* and *C. tyzzeri* also have been isolated from pigs; however, infectivity could not be confirmed experimentally (Kvá et al., 2012). In addition to the named species, genotypes including *Cryptosporidium* sp. Eire w65.5, *Cryptosporidium* rat genotype, *C. suis*-like, and *Cryptosporidium* pig genotype II, have been reported in pigs (Langkjær et al., 2007; Zintl et al., 2007). Among these species and genotypes, only *C. suis* (Morgan et al., 1999; Ryan et al., 2004) and *Cryptosporidium* pig genotype II appear to be adapted to pigs. Ryan et al. (2003) identified *Cryptosporidium* pig genotype II based on its unique small subunit rRNA (SSU) gene sequence; however, no biological data have been reported.

*Cryptosporidium* oocysts have been detected in the faeces or slurry of pigs in Australia (Johnson et al., 2008; McCarthy et al., 2008; Ryan et al., 2003), Brazil (Fiuza et al., 2011), Canada (Budu-Amoako et al., 2012; Farzan et al., 2011; Guselle et al., 2003; Olson et al., 1997; Sanford, 1987), China (Chen et al., 2011; Wang et al., 2010; Yin et al., 2011), Croatia (Bilic and Bilkei, 2006), Czech Republic (Jeníková et al., 2011; Kvá et al., 2009a,c; Vítovec et al., 2006), Denmark (Langkjær et al., 2007; Maddox-Hyttel et al., 2006), England (Featherstone et al., 2010), Germany (Epe et al., 2004; Wieler et al., 2001), Ireland (Zintl et al., 2007), Italy (Canestri Trotti et al., 1984), Japan (Izumiyama et al., 2001), Korea (Rhee et al., 1991), Nigeria (Yatswako et al., 2007), Norway (Hamnes et al., 2007), Spain (Nunez et al., 2003; Quilez et al., 1996; Reinoso and Becares, 2008; Villacorta et al., 1991), Switzerland (Ebeid et al., 2003), Taiwan (Hsu et al., 2008), Trinidad and Tobago (Adesiyun et al., 2001), and USA (Atwill et al., 1997; Bergeland, 1977; Kennedy et al., 1977; Tacal et al., 1987; Xiao et al., 1994; Xiao et al., 2006). *Cryptosporidium* pig genotype II was reported in domestic pigs in nine countries. In addition, wild boars, calves, marine fish, and humans have been reported as natural hosts of this genotype (Table 1) (Kvá et al., 2009a,b,c; N mejc et al., 2012; Ng et al., 2011; Reid et al., 2010).

Differences in the age of pigs infected with *C. suis* and *Cryptosporidium* pig genotype II have been reported recently. *Cryptosporidium suis* appears to infect all age categories of pigs, though prevalence is lower in older pigs. Conversely, *Cryptosporidium* pig genotype II appears to be specific for older pigs (Jeníková et al., 2011; Kvá et al., 2009a,c; Langkjær et al., 2007; N mejc et al., 2012), with a much lower prevalence observed in younger age categories, primarily in pre-weaned piglets (Table 1).

No other names have been used for *Cryptosporidium* pig genotype II in publications or GenBank. There are 49 sequences for 6 genes available in GenBank (43, 2, 1, 1, 1, and 1 for the SSU, actin, HSP70 (heat shock protein 70), COWP (*Cryptosporidium* oocyst wall protein), 15 kDa and 23 kDa sporozoite surface antigen, respectively).

We undertook this study to examine the experimental transmission, oocyst morphology, and molecular characteristics of *Cryptosporidium* pig genotype II. Based on the collective data from this and other studies, which show that *Cryptosporidium* pig genotype II is genetically distinct from known *Cryptosporidium* species and uniquely adapted to adult pigs, we propose the species name *Cryptosporidium scrofarum* n. sp.

## 2. Materials and Methods

### 2.1. Source of oocysts for transmission studies

Faecal samples from 3–15-week-old pigs kept on a commercial farm were screened for *Cryptosporidium* by microscopic examination of faecal smears stained with aniline-carbol-methyl violet (ACMV) (Milá ek and Vítovec, 1985) and PCR. The naturally infected 9-week-old pigs (*Sus scrofa*) were purchased and separately housed. Faecal specimens were collected daily and stored in a 2.5% potassium dichromate solution. *Cryptosporidium* oocysts were purified for morphology, phylogeny, and infectivity analyses using sucrose gradient (Arrowood and Sterling, 1987) and caesium chloride gradient centrifugation (Kilani and Sekla, 1987). Purified oocysts were stored for up to 4 weeks in PBS with antimycotics and antibiotics at 4°C in darkness. The parasite was confirmed to be the *Cryptosporidium* pig genotype II by sequence analysis of the SSU gene using previously described methods (Jenková et al., 2011; Jiang et al., 2005). The number of oocysts administered to animals was determined by hemacytometer counting. The viability of oocysts was examined using an assay previously described by Kvá et al. (2007a). Briefly: the viability of oocysts was determined by propidium iodide (PI) staining (Sauch et al., 1991). Examined oocysts were washed in distilled water (DW;  $1 \times 10^5$  oocysts in 100  $\mu$ l) and mixed with 10  $\mu$ l of PI (1% solution, SIGMA). After 30 min of incubation at room temperature in the dark, the oocysts were washed twice with DW. Oocyst viability was examined using fluorescence microscopy (filter 420 nm, Olympus IX70). A total of  $5 \times 10^6$  oocysts was counted.

### 2.2. Oocyst morphology

Oocysts were examined using differential interference contrast (DIC) microscopy, brightfield microscopy following ACMV staining, and epifluorescence microscopy following labelling with genus-specific FITC-conjugated antibodies (*Cryptosporidium* IF Test, Cryptocel, Medac) (Olympus IX70 microscope; Olympus CZ, Czech Republic). Morphology and morphometry were determined using digital analysis of images (M.I.C. Quick Photo Pro v.1.2 software; Optical Service, Czech Republic) collected using an Olympus Camedia C 5060 WIDEZOOM 5.1 megapixel digital camera (Optical Service). A 20  $\mu$ l aliquot containing 100,000 purified oocysts was used for each measurement. Length and width of oocysts ( $n = 100$ ) were measured under DIC at  $\times 1,000$  magnification. The shape index and length-to-width ratio of each oocyst was calculated. As a control, the morphometry of *C. parvum* ( $n = 100$ ) from a naturally infected 30-day-old Holstein calf was measured by the same person using the same microscope. A photomicrograph of *C. scrofarum* oocysts observed by DIC, ACMV and IFA was deposited as a phototype at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic.

### 2.3. DNA extraction and molecular analyses

Total DNA was extracted from 200 mg of faeces, 50  $\mu$ l of purified oocysts, or 200 mg of tissue by bead disruption for 60 s at 5.5 m/s using 0.5 mm glass beads in a FastPrep®–24 Instrument (MP Biomedicals, CA, USA) followed by isolation/purification using a commercially available kit in accordance with the manufacturer's instructions (QIAamp! DNA Stool Mini Kit or DNeasy! Blood & Tissue Kit, Qiagen, Hilden, Germany). Purified DNA was stored at  $-20^\circ\text{C}$  prior to being used for PCR.

A nested PCR approach was used to amplify a region of the SSU (~ 830 bp; Jiang et al., 2005; Xiao et al., 1999) and HSP70 genes (~ 325 bp; Morgan et al., 2001). A semi-nested PCR was used to amplify the actin gene (~ 619 bp; this study). Three primers were designed using the pig genotype II sequence in Genbank (EF012374). The forward primer for both primary and secondary reactions was ScrofActinFA (TGT AGG TGA CGA GGC TCA ATC CAA). ScrofActinRA (ATC GAT TGG AAA GTG GTC TCG CCA) and

ScrofActinRB (TTC TGG GCA CCT AAA TCT CTC GCT) were used as reverse primers in the primary and secondary reactions, respectively. The primary PCR reactions were carried out in a volume of 100 µl containing 1 µl of DNA template (or water as a negative control), 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 200 µM dNTPs, 0.2 µM of each primer, and 2.5U of Taq DNA polymerase (Promega GoTaq Flexi). Secondary reactions were carried out under similar conditions with the exception that 2 µl of primary product was used as template. PCR reactions were run in a thermo cycler (Eppendorf Mastercycler gradient) with an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min. A final elongation step of 72°C for 10 min was included to ensure complete extension of amplified products. Conditions were the same for both primary and secondary reactions. In order to distinguish *C. scrofarum* from *C. suis* in mixed infections, a *C. scrofarum*-specific forward primer was used in secondary PCR amplifications (Jeníková et al., 2011). DNA from *C. scrofarum* and *C. hominis* were used as positive and negative controls, respectively. Secondary PCR products were detected by agarose gel (1.5%) electrophoresis, visualized by ethidium bromide staining (0.2 µg/ml), and extracted using QIAquick! Gel Extraction Kit (Qiagen). Purified secondary products were 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 identity of *C. parvum* used for morphological analyses was verified using sequencing of SSU (data not shown).

#### 2.4. Phylogenetic analyses

The nucleotide sequences of each gene obtained in this study were edited using the programme ChromasPro 1.5 (Technelysium, Pty, Ltd.) and were aligned with each other and with reference sequences from GenBank using ClustalX 2.0.6. Alignment adjustments were made manually to remove artificial gaps using BioEdit. Phylogenetic analyses were performed using the software MEGA5 (Tamura et al., 2011). Neighbour joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) trees were constructed. All ambiguous positions were removed for each sequence pair. The reliability of branches in trees was assessed using the bootstrap analysis with 1000 pseudo-replicates, with values above 50% reported. Phylograms were drawn using the MEGA5 and were manually adjusted using CorelDrawX5. SSU, actin, and HSP70 sequences have been deposited in GenBank under the accession numbers JX424840, JX424841, and JX424842, respectively.

#### 2.5. Transmission studies

**2.5.1. Animals**—Five 8-week-old adult SCID mice (strain C.B-17), BALBc mice (Charles River, Germany), Mongolian gerbils (*Meriones unguiculatus*) (Charles River, Germany), southern multimammate mice (*Mastomys coucha*), yellow-necked mice (*Apodemus flavicollis*), guinea pigs (*Cavia porcellus*) (Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, Czech Republic), and 4- and 8-week-old pigs (*Sus scrofa*) (3 animals per group; purchased from the farm operated by the Faculty of Agriculture, University of South Bohemia in České Budějovice, Czech Republic) were used for experimental infection studies.

**2.5.2. Experimental design**—To prevent environmental contamination with oocysts, laboratory rodents were housed in plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM, Znojmo, Czech Republic) with high-efficiency particulate air filters. The mice were supplied with a sterilized diet (TOP-VELAZ, Prague, Czech Republic) and water *ad libitum*.

Pigs were individually housed in 5 m<sup>2</sup> pens with concrete walls and floor in an isolated building. Water and feeding mixture were available *ad libitum*. Rodents (five animals per

group) were each inoculated orally by stomach tube with 1,000,000 purified oocysts suspended in 200 µl of dH<sub>2</sub>O. Pigs (three animals in each category) received the same infection dose in a 20 ml suspension via syringe.

Faecal samples from all experimental animals were collected daily for 30 days post infection (DPI). Samples were stained with aniline-carbol-methyl violet (Milá ek and Vítovec, 1985) and the presence of *Cryptosporidium* specific DNA was confirmed using nested PCR targeting the SSU gene. Infection intensity was reported as the number of oocysts per gram (OPG) of faeces as previously described (Kvá et al., 2007b).

**2.5.3. Clinical and pathomorphological examinations**—A complete examination of all gastrointestinal organs was conducted at necropsy. Tissue specimens of the gastrointestinal tract were sampled and processed for histology according to Kvá and Vítovec (2003) and for PCR analyses (see chapter 2.3.). Histology sections were stained with haematoxylin and eosin (HE), Wolbach's modified Giemsa stain, alcian blue, and genus-specific FITC conjugated monoclonal antibodies targeting *Cryptosporidium* oocyst wall antigens (*Cryptosporidium* IF Test, Crypto cel, Medac).

**2.5.4. Animal care**—Animal caretakers wore new disposable coveralls, shoe covers, and gloves every time they entered the buildings. All wood-chip bedding, pig faeces, and disposable protective clothing were sealed in plastic bags, removed from the buildings and incinerated. All housing, feeding, and experimental procedures involving pigs, laboratory mice of different strains, Mongolian gerbils, southern multimammate mice, yellow-necked mice, and guinea pigs were conducted under protocols approved by the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic and Institute and National Committees (Protocol No. 071/2010).

### 3. Results

#### 3.1 Experimental transmission studies

*Cryptosporidium scrofarum* was not detected by microscopy or PCR in the faeces of 4-week-old pigs following experimental infection. Conversely, sporadic oocyst shedding was detected in 8-week-old pigs beginning at 6–7 DPI and continuing for the duration of the study. *Cryptosporidium scrofarum* DNA was detectable in faeces by PCR beginning at 3 DPI and continuing for the duration of the study. Infection intensity was generally less than 2000 oocysts per gram of faeces (Figure 1). Infected pigs showed no clinical signs of disease and pigs necropsied at 30 DPI showed no macroscopic signs of intestinal cryptosporidiosis. Histological examination of the gastrointestinal tract of animals infected with *C. scrofarum* revealed the presence of developmental stages primarily attached to the microvillar border along small intestine with a predilection for the jejunum and ileum. The ratio of infected to non-infected glands in the jejunum and ileum was approximately 1:200. The proportion of infected glands was much lower or developmental stages were absent in other parts of the small and large intestine. However, *C. scrofarum* DNA was detected by PCR (Table 2). No pathological changes were observed.

*Cryptosporidium scrofarum* was not detected by microscopy or PCR in the faeces of *Mus musculus* (SCID and BALB/c), *Meriones unguiculatus*, *Mastomys coucha*, *Apodemus flavicollis*, or *Cavia porcellus* following experimental infection. Histological and molecular examination of gastrointestinal tract tissue from these rodents did not reveal the presence of *Cryptosporidium* developmental stages.



### 3.2. Molecular characterization

Isolates from naturally and experimentally infected pigs shared 100% sequence identity at the SSU, actin, and HSP70 loci. SSU and HSP70 sequences were identical to GenBank sequences EU331243 and DQ833282, respectively. The actin sequence was identical to a sequence from a pig isolate in Norway (GenBank: EF012374) with the exception of three polymorphisms, one of which was non-synonymous. SSU, actin, and HSP70 sequences from the faeces of positive pigs were 100% identical to sequences from isolates used for infection. Neighbour joining, maximum likelihood, and maximum parsimony trees constructed from SSU, actin, and HSP70 sequences support the placement of *C. scrofarum* in a cluster with *C. ryanae*, *C. xiaoi*, and *C. bovis* (Figure 2).

### 3.3. Oocyst morphology

Oocysts of *C. scrofarum* were similar in size to *C. parvum*, measuring 4.81–5.96  $\mu\text{m}$  (mean = 5.16)  $\times$  4.23–5.29  $\mu\text{m}$  (mean = 4.83) with a length-to-width ratio of  $1.07 \pm 0.06$  (n = 4 $\times$ 100) (Fig. 3A and Table 3). Oocysts of *C. scrofarum* recovered from infected pigs were morphologically similar to those used for infection (Table 3). Oocysts in faecal smears showed typical *Cryptosporidium* ACMV staining characteristics (Fig. 3B). Fixed *C. scrofarum* oocysts labelled with FITC conjugated anti-*Cryptosporidium* oocyst wall antibody and examined by epifluorescence microscopy displayed typical apple green, halo-like fluorescence (Fig. 3C).

## 4. Discussion

Based on morphology, *C. scrofarum* oocysts obtained from naturally and experimentally infected pigs in the present study were indistinguishable from *C. parvum* and larger than oocysts of *C. bovis* (4.89  $\times$  4.63  $\mu\text{m}$ ), *C. ryanae* (3.16  $\times$  3.73  $\mu\text{m}$ ), and *C. xiaoi* (3.94  $\times$  3.44  $\mu\text{m}$ ) (Fayer and Santin, 2009; Fayer et al., 2005, 2008). Other *C. scrofarum* oocyst characteristics including wall thickness and inner structure were not different from the *C. parvum* oocysts examined in this or other studies (Upton and Current, 1985; Vítovec et al., 2006).

It is unclear whether *C. scrofarum* can be distinguished from *C. suis*, the other pig-adapted species, based on oocyst morphology alone. Ryan et al. (2004) reported that *C. suis* oocysts had a similar morphology to *C. parvum*; however, Vítovec et al. (2006) found that *C. suis* oocysts were larger than *C. parvum* and, by extension, would be larger than *C. scrofarum*.

*Cryptosporidium scrofarum*, previously named *Cryptosporidium* pig genotype II, has been detected in domestic pigs and wild boars in at least 19 countries (see introduction). Most reported infections have been in pigs, with the exception of sporadic infections in a human, a calf, and Western school whiting (Kvá et al., 2009b; Ng et al., 2011; Reid et al., 2010). We have shown that a number of rodents are not susceptible to experimental infection with *C. scrofarum*. These data collectively support a conclusion that *C. scrofarum* is relatively specific for pigs. Furthermore, experimental data from the present study show that 8-week-old but not 4-week-old pigs are susceptible to infection. These data verify the age specificity hypothesis that was predicated on molecular epidemiology studies (Jeníková et al., 2011; Johnson et al., 2008; Kvá et al., 2009a,c; Langkjær et al., 2007).

*Cryptosporidium scrofarum* has a prepatent period of 4–6 DPI, which is longer than the prepatent periods observed in its closest relatives including *C. xiaoi* (7–8 DPI), *C. ryanae* (11 DPI), and *C. bovis* (16 DPI) (Fayer and Santin, 2009; Fayer et al., 2008; Fayer et al., 2005). The reported prepatent period of *C. suis* in pigs ranges from 2–9 days (Enemark et al., 2003; Vítovec et al., 2006; Xiao et al., 1994). In agreement with previous observations, pigs infected with *C. scrofarum* showed no clinical signs of disease. Similar to *C. ryanae*, *C.*

*scrofarum* infections are characterised by shedding of a low number of oocysts (Fayer et al., 2008).

Phylogenetic analyses based on SSU, actin and HSP70 gene sequences show that *C. scrofarum* is genetically distinct from *C. suis* and forms a cluster with *C. ryanae*, *C. bovis* and *C. xiaoi*. At the SSU locus, *C. scrofarum* is 97.3%, 97.4%, and 97.6% similar to *C. ryanae*, *C. xiaoi*, and *C. bovis*, respectively. In contrast, *C. scrofarum* and *C. suis* are only 92.9% similar at the SSU locus. These similarities are less than the similarities between recognized *Cryptosporidium* species including *C. andersoni* and *C. muris* (99.1% at the SSU locus), and *C. parvum* and *C. tyzzeri* (99.0% at the SSU locus). At the actin locus, *C. scrofarum* was 85.3%, 87.0%, 87.2%, and 86.6% similar to *C. suis*, *C. ryanae*, *C. xiaoi*, and *C. bovis*, respectively. This compares to the 99.0% similarity between *C. andersoni* and *C. muris* and 99.1% similarity between *C. parvum* and *C. tyzzeri*. At the HSP70 locus, *C. scrofarum* was 81.1%, 86.6%, 88.2%, and 87.3% similar to *C. suis*, *C. ryanae*, *C. xiaoi*, and *C. bovis*, respectively. This compares to the 98.1% similarity between *C. andersoni* and *C. muris* and the 99.3% similarity between *C. parvum* and *C. tyzzeri* at this locus.

In conclusion, oocyst morphology, nucleotide sequence, and infectivity data demonstrate significant biological and taxonomic differences between *Cryptosporidium* pig genotype II and recognized *Cryptosporidium* species. According to ICZN and criteria for naming *Cryptosporidium* species (Xiao et al., 2004) we propose the name *Cryptosporidium scrofarum*.

## Taxonomic summary

### *Cryptosporidium scrofarum* n. sp.

**Diagnosis:** Oocysts are shed in faeces fully sporulated. Sporulated oocysts ( $n = 4 \times 100$ ) measure  $4.81\text{--}5.96\ \mu\text{m}$  (mean = 5.16)  $\times$   $4.23\text{--}5.29\ \mu\text{m}$  (mean = 4.83) with a length-to-width ratio of  $1.07 \pm 0.06$ . Four sporozoites are present in each oocyst. Endogenous stages are unknown.

**Type host:** Pig (*Sus scrofa*).

**Other natural hosts:** Human (*Homo sapiens*) (Kvá et al., 2009b), calf (*Bos taurus*) (Ng et al., 2011), wild boar (*Sus scrofa*) (N mejc et al., 2012), and Western school whiting (*Sillago vittata*) (Reid et al., 2010).

**Experimental transmission:** Attempts to infect common laboratory mice (*Mus musculus*), Mongolian gerbils (*Meriones unguiculatus*), southern multimammate mice (*Mastomys coucha*), yellow-necked mice (*Apodemus flavicollis*), and guinea pigs (*Cavia porcellus*) were unsuccessful.

**Prepatent period:** 4–6 days (this study).

**Patent period:** More than 30 days (this study).

**Age specificity:** *Cryptosporidium scrofarum* n. sp. is infectious for post-weaned piglets only (this study).

**Type locality:** Czech Republic.

**Other localities:** Worldwide.

**Site of infection:** Duodenum, jejunum, ileum, caecum, and colon.

**Material deposited:** A phototype, description of oocysts and DNA are deposited at the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic.

DNA sequences: partial sequences of SSU, actin, and HSP70 genes were submitted to GenBank under the accession numbers JX424840, JX424841, and JX424842, respectively.

**Etymology:** this species was first named *Cryptosporidium scrofarum* from the Latin noun “scrofa” (meaning a breeding sow) according to ICZN Article 11.9.1–3 as a plural in the genitive case; as it appears to be adapted to adult pigs.

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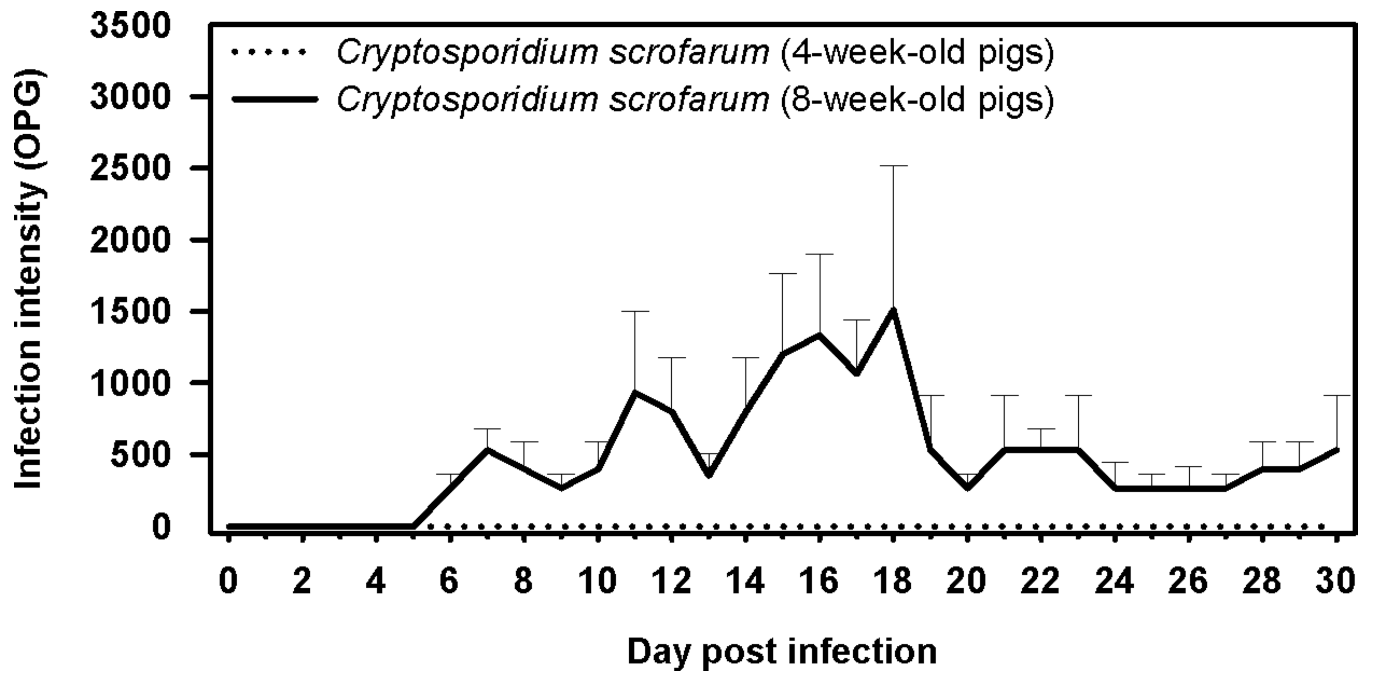
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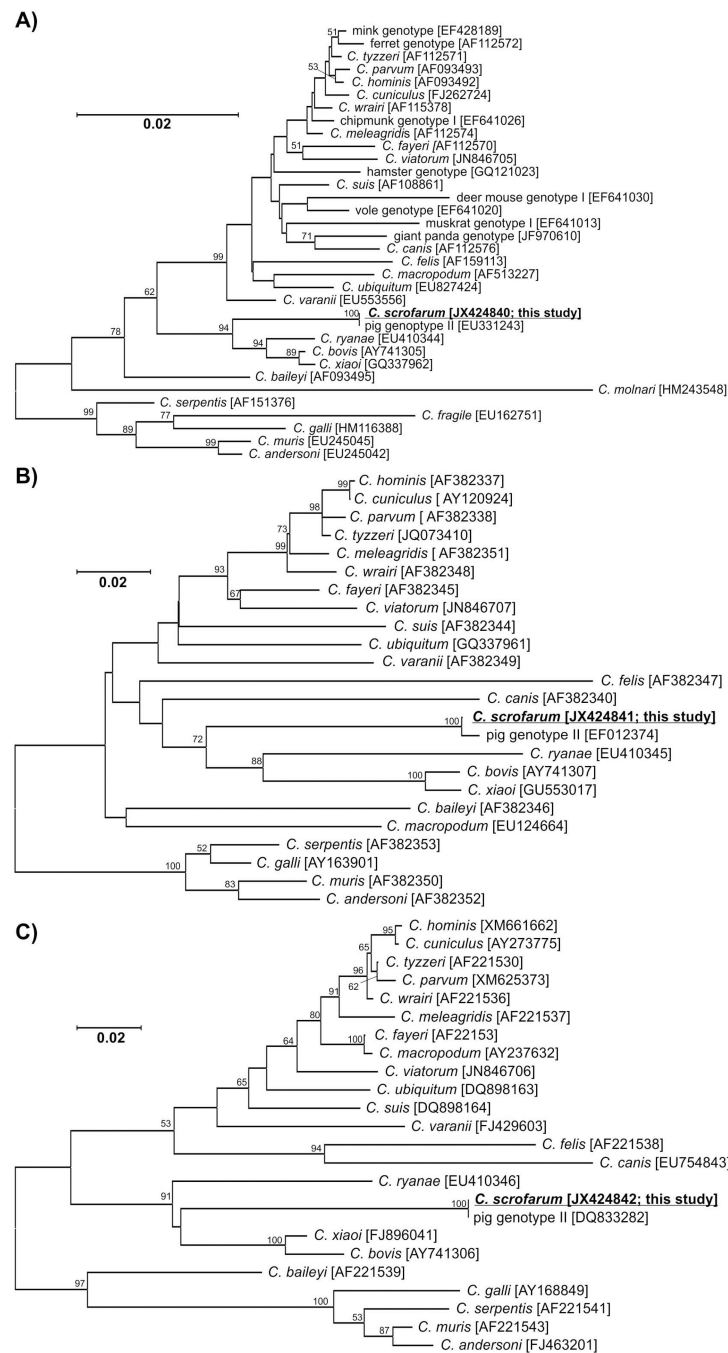
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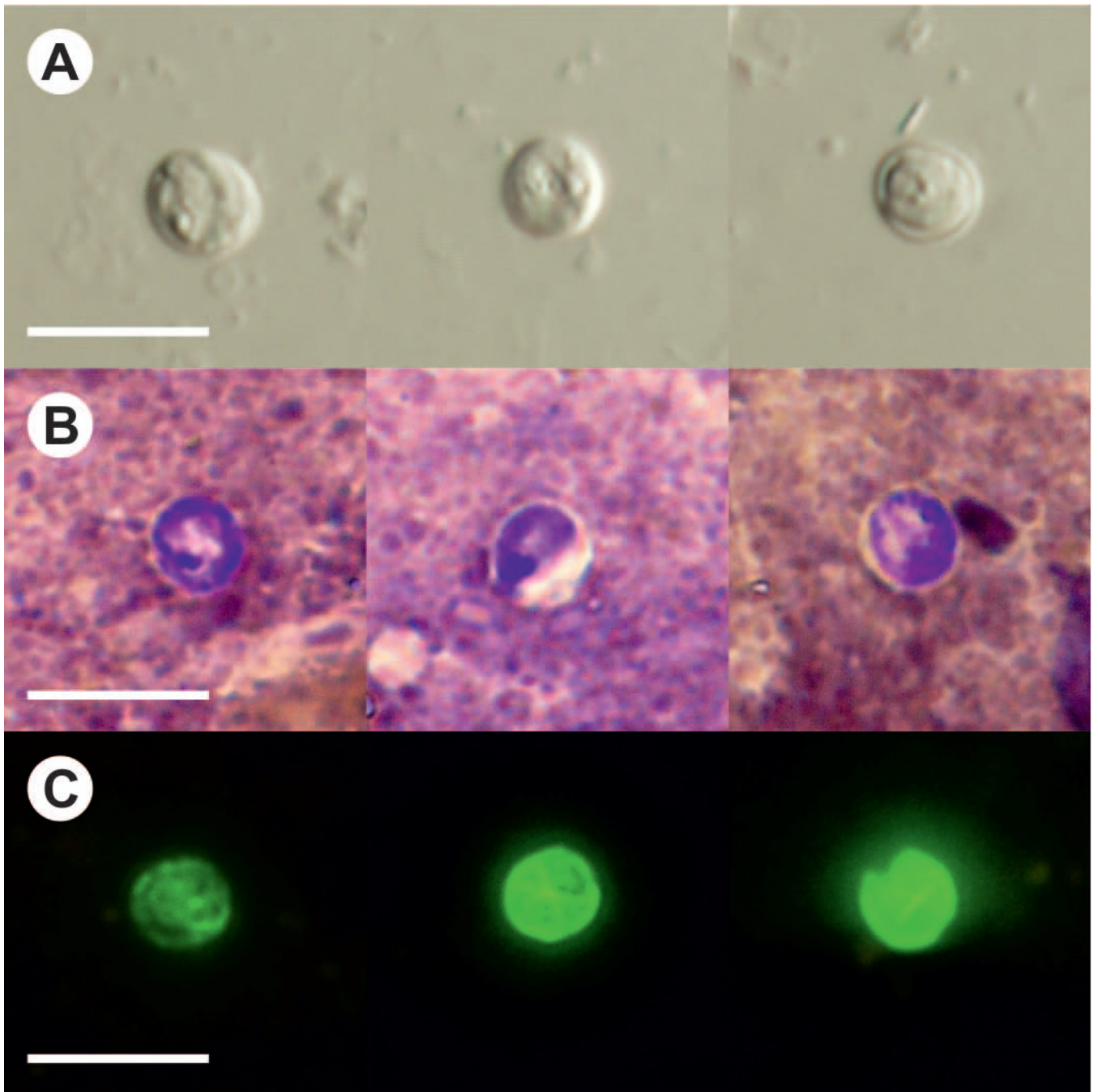


**Figure 1.** Course of infection of *Cryptosporidium scrofarum* in 4- and 8-week-old pigs based on coprological examination of faeces.



**Figure 2.** Phylogenetic relationships between *Cryptosporidium scrofarum* (highlighted and underlined) and other *Cryptosporidium* spp. as inferred by a neighbour-joining analysis of **A)** the SSU (753 base positions in the final dataset), **B)** actin (586 base positions in the final dataset) and **C)** Heat Shock Protein 70 (323 base positions in the final dataset) genes. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates). Numbers at the nodes represent bootstrap values for the nodes gaining more than 50% support. Scale bar included in each tree.





**Figure 3.** *Cryptosporidium scrofarum* oocysts in **A**) differential interference contrast microscopy and stained by **B**) aniline-carbol-methyl violet and **C**) anti-*Cryptosporidium* FITC-conjugated antibody. Bar = 10  $\mu$ m.

Prevalence of *Cryptosporidium scrofarum* (*Cryptosporidium* pig genotype II) in various hosts including age-specificity and geographical locations. Genes used to characterise the genotype are presented, GenBank accession number were included where available

Table 1

Host	Location	No positive/ examined	Age / category	Gene (GenBank accession number)	Reference
		50/646	>9 weeks	SSU	Ryan et al., 2003
		0/123	>3 weeks		
	Australia	32/156	4 weeks- 6months	SSU	Johnson et al., 2008
		0/19	sows		
		2/?	unlisted	SSU	McCarthy et al., 2008
	Brazil	2/91	unlisted	SSU (GQ924104-05)	Fiuza et al., 2011
	Canada	46/122 1/2	unlisted >8 months	SSU, HSP70	Farzan et al., 2011
		43/81	1-3 months	SSU, HSP70	Budu-Amoako et al., 2012
		1/26	<1 month	SSU (GQ227704, GU254165- GU254170)	Wang et al., 2010
		10/77 3/5	1-2 month >6 month		
	China	16/94	unlisted	SSU (HQ844719-28; HQ844729- HQ844734)	Yin et al., 2011
<b>Domestic pig</b>					
		12/2323	unlisted	SSU (JF710244-52)	Chen et al., 2011
		36/123	5-6 months		Kvá et al., 2009a
		0/119	<4 weeks		
	Czech Republic	5/131	5-8 weeks	SSU	Kvá et al., 2009b
		17/123	>12 week		
		136/	<6 weeks		Jenková et al., 2011
	Denmark	4/29 129/356	<1 month >1 month	SSU, HSP70 (AY304993)	Langkjaer et al., 2007
		5/127	4-10 weeks		
	Ireland	5/121	SSU	10-24 weeks	Zintl et al., 2007
		0/94	adult		
	Norway	3/684	unlisted	SSU (EF012375), Actin (EF012372 and 74)	Hannes et al., 2007

Host	Location	No positive/ examined	Age / category	Gene (GenBank accession number)	Reference
	Spain	13/75 3/42	1-2 months 2-6 months	SSU (RFLP)	Suarez-Luengas et al., 2007
<b>Calf</b>	Australia	2/255	unlisted	SSU, Actin	Ng et al., 2011
<b>Western school whiting</b>	Australia	1/364	<4 months	SSU	Reid et al., 2010
<b>Wild Boar</b>	Czech Republic	7/193	adult	SSU	N meje et al., 2012
<b>Human</b>	Czech Republic	1/1	29-year-old	SSU (EU331243)	Kvá et al., 2009c

**Table 2**  
Presence of developmental stages in gastrointestinal tract of animals experimentally infected with *Cryptosporidium scrofarum*.

Host and category	Stomach		Duodenum		Jejunum		Ileum		Caecum		Colon		Rectum	
	HS	PCR	HS	PCR	HS	PCR	HS	PCR	HS	PCR	HS	PCR	HE	PCR
4-week-old pigs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8-week-old pigs	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SCID mice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BALB / c mice	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Mastomys coucha</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Apodemus flavicollis</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Cavia porcellus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Meriones unguiculatus</i>	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ presence of *Cryptosporidium* developmental stages or specific DNA; - absence of *Cryptosporidium* developmental stages or specific DNA; PCR - polymerase chain reaction; HS - histology sections.

Table 3

Morphometry of *Cryptosporidium scrofarum* and *C. parvum* oocysts

Isolate	Length (µm)		Width (µm)		Shape index (SD)
	Range	Mean (SD)	Range	Mean (SD)	
<i>Cryptosporidium scrofarum</i> naturally infected a 9-week-old pig	4.79–5.80	5.20 ±0.20	4.12–5.15	4.91 ±0.18	1.08 ± 0.05
<i>Cryptosporidium scrofarum</i> experimentally infected a 8-week-old pig no. 1	4.83–5.96	5.18 ±0.20	4.23–5.27	4.82 ±0.19	1.06 ±0.07
<i>Cryptosporidium scrofarum</i> experimentally infected a 8-week-old pig no. 2	4.76–5.89	5.16 ±0.23	4.19–5.29	4.83 ±0.21	1.07 ± 0.06
<i>Cryptosporidium scrofarum</i> experimentally infected a 8-week-old pig no. 3	4.89–5.71	5.12 ±0.22	4.21–5.31	4.85 ±0.22	1.08 ± 0.05
<i>Cryptosporidium parvum</i> from natural infection in a 30-day-old pig	4.42–6.06	5.35 ±0.33	4.21–5.58	4.86 ±0.39	1.11 ± 0.08