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Cryptosporidium and *Giardia* in Danish organic pig farms: seasonal and age-related variation in prevalence, infection intensity and species/genotypes

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Highlights

- High *Cryptosporidium* and *Giardia* prevalence in organic pigs throughout the year
- Piglets predominantly hosted *C. suis*, while older pigs mainly hosted *C. scrofarum*

- The intensity of infection was >6 x higher for *C. suis* compared to *C. scrofarum*
- *Crypto/Giardia* infected pigs excreted fewer oocysts compared to mono-infected pigs
- Public health risk of *Cryptosporidium/Giardia* in Danish organic pigs seems negligible

Abstract

Although pigs are commonly infected with *Cryptosporidium* spp. and *Giardia duodenalis*, including potentially zoonotic species or genotypes, little is known about age-related infection levels, seasonal differences and genetic variation in naturally infected pigs raised in organic management systems. Therefore, the current study was conducted to assess seasonal and age-related variations in prevalence and infection intensity of *Cryptosporidium* and *Giardia*, evaluate zoonotic potential and uncover correlations between species/genotypes, infection intensity and faecal consistency. Shedding of oocysts and cysts ((oo-)cysts) was monitored at quarterly intervals (September 2011 to June 2012) in piglets (n=152), starter pigs (n=234), fatteners (n=230) and sows (n=240) from three organic farms in Denmark. (Oo-)cysts were quantified by immunofluorescence microscopy; and 56/75 subsamples from *Cryptosporidium* infected pigs were successfully analysed by PCR amplification and partial sequencing of the small subunit (SSU) 18S rRNA and *hsp70* genes, while 13/67 *Giardia* subsamples were successfully analysed by amplification and partial sequencing of the 18S rRNA and the *gdh* genes. Altogether, *Cryptosporidium* or *Giardia* infections were observed in 40.9% (350/856) and 14.0% (120/856) of the pigs, respectively, including 8.2% (70/856) infected with both parasites. Prevalence, intensity of infections and presence of *Cryptosporidium* species varied significantly between age-groups; 53.3% piglets, 72.2% starter pigs, 40.4% fatteners and 2.9% sows were infected with *Cryptosporidium*, whereas 2.0% piglets, 27.4% starter pigs, 17.8% fatteners and 5.0% sows were infected with *Giardia*. The overall prevalence was stable throughout the year, except for dual-infections that were more prevalent in September and

December ($p < 0.05$). The infection intensity was age-related for both parasites, and dual-infected pigs tended to excrete lower levels of oocysts compared to pigs harbouring only *Cryptosporidium*. Likewise, pigs infected with *C. scrofarum* excreted fewer oocysts (mean CPG: $54,848 \pm 194,508$ CI: 9085–118,781) compared to pigs infected with *C. suis* (mean OPG: $351,035 \pm 351,035$ CI: 67,953–634,117). No correlation between faecal consistency and (oo-)cyst excretion levels was observed.

Of the successfully genotyped isolates, 38/56 (67.9%) were *C. scrofarum* and 18/56 (32.1%) were *C. suis*, while the livestock specific *G. duodenalis* Assemblage E was detected in 11/13 (84.6%) isolates and the potentially zoonotic Assemblage A was identified in 2/13 (15.4%) isolates. Piglets exclusively hosted *C. suis*, with one exception, while starter pigs and fatteners predominantly hosted *C. scrofarum*. As organic pigs are partly reared outdoors, environmental contamination with *Cryptosporidium* and *Giardia* is inevitable. Nevertheless, the present data indicate that the potential public health risk associated with both of these parasites in Danish organic pig production seems to be negligible.

Keywords: *Cryptosporidium*; *Giardia duodenalis*; organic pigs; prevalence; molecular typing.

1. Introduction

Protozoan parasites belonging to the genera *Cryptosporidium* and *Giardia* are prevalent in a variety of animal species, including cattle, pigs and humans (Current and Garcia, 1991; Maddox-Hyttel *et al.*, 2006). Both parasites can cause severe symptoms, primarily diarrhoea, e.g. in young calves and humans. In addition, giardiasis is known to induce chronic malnutrition and growth retardation with subsequent risk of impaired cognitive function in humans (Berkman *et al.*, 2002). Moreover, post-giardiasis complications such as fatigue and abdominal symptoms years after infection have been observed (Morch *et al.*, 2009, 2013; Naess *et al.*, 2012). Natural porcine infections with either of the parasites are typically asymptomatic (Quilez *et al.*, 1996; Maddox-Hyttel *et al.*, 2006; Kváč *et al.*, 2009a; Nemejc *et al.*, 2013; Zhang *et al.*, 2013), whilst watery diarrhoea, anorexia and increased mortality have been described in piglets experimentally infected with *C. parvum* (Tzipori *et al.*, 1982; Enemark *et al.*, 2003).

Both parasites can be transmitted directly through contact with infected individuals or indirectly by consumption of faeces contaminated food or water (Meinhardt *et al.*, 1996; Fayer, 2004). Livestock, in particular cattle, has been implicated as a major source of surface water contamination with *Cryptosporidium* and *Giardia* (oo-)cysts (Bodley-Tickell *et al.*, 2002; Thurston-Enriquez *et al.*, 2005). Moreover, *Cryptosporidium* contamination of water sources through surface run-off from faeces deposited on agricultural land has repeatedly been documented (Tate *et al.*, 2000; Davies *et al.*, 2004; Thurston-Enriquez *et al.*, 2005). In addition, Petersen *et al.* (2012) demonstrated that *C. parvum* oocysts present in pig slurry applied to soil can leach and potentially contaminate groundwater. Cryptosporidiosis and giardiasis are widespread and debilitating diseases in calves, and cattle often host the zoonotic *C. parvum* and to a lesser degree also *G. duodenalis* Assemblage A. Thus, cattle have frequently been the

subject of *Cryptosporidium* and *Giardia* surveys (Appelbee *et al.*, 2003; Maddox-Hyttel *et al.*, 2006; Silverlås *et al.*, 2013). The opposite applies for pigs, where cryptosporidiosis and giardiasis generally have been disregarded. Additionally, pigs are mostly reported to host *C. suis* and *C. scrofarum* (Ryan *et al.*, 2003; Langkjaer *et al.*, 2007; Yin J.H. *et al.*, 2013; Nemejc *et al.*, 2013; Nguyen *et al.*, 2013; Zhang *et al.*, 2013), that are considered relatively host specific; and *G. duodenalis* Assemblage E (Langkjaer *et al.*, 2007; Armson *et al.*, 2009; Sprong *et al.*, 2009), which is restricted to livestock. Recently, the awareness of porcine infections with these protozoan parasites has, nevertheless, increased worldwide. This has led to occasional observations of the zoonotic *C. parvum* and *G. duodenalis* Assemblage A in pigs (Xiao *et al.*, 2006; Leoni *et al.*, 2006; Langkjaer *et al.*, 2007; Armson *et al.*, 2009; Kváč *et al.*, 2009b), as well as *C. andersoni* (Hsu *et al.*, 2008), *C. muris* (Zintl *et al.*, 2007), *C. tyzzeri* (Budu-Amoako *et al.*, 2012), *G. duodenalis* Assemblage C, D, E and F (Langkjaer *et al.*, 2007; Armson *et al.*, 2009; Minetti *et al.*, 2014), all of which have been documented sporadically in humans (Leoni *et al.*, 2006; Sprong *et al.*, 2009; Raskova *et al.*, 2013). However, none of these studies provided a comprehensive description of seasonal and age-related differences in prevalence or genetic variation of *Cryptosporidium* and *Giardia* in outdoor reared pigs. Detailed knowledge of potential zoonotic transmission is furthermore hampered because only few studies in pigs have characterised specimens by sequence analysis of molecular markers (Spong *et al.*, 2009).

As pigs have the capability to excrete extremely high numbers of oocysts and cysts per gram of faeces (OPG and CPG) (Fayer *et al.*, 2006; Maddox-Hyttel *et al.*, 2006), fertilisation of agricultural land with slurry or faecal deposition directly on the ground from infected free-range pigs can eventually be a source of human waterborne cryptosporidiosis and giardiasis. Organic pigs, contrary to conventionally reared pigs, have access to outdoor areas throughout most of their lives. Therefore, this study was conducted to: 1) assess seasonal- and age-related

variations in prevalence and infection intensity of *Cryptosporidium* and *Giardia* in organic pig farms, 2) to evaluate the zoonotic potential of these infections through DNA sequence analyses, and 3) to uncover any correlation between species/genotypes hosted by the pigs, infection intensity and faecal consistency in the pigs.

2. Material and methods

2.1 Study farms

Three commercial Danish organic pig farms with laboratory confirmed *Cryptosporidium* and *Giardia* infections were selected for the study based on the owner's willingness to participate. The farms were visited at quarterly intervals from September 2011 to June 2012. In all farms, piglets were born outdoor on farrowing pastures and weaned at seven to nine weeks of age. The farrowing pastures were subdivided into smaller paddocks, with one to five sows separated by a single electrical wire; in practice, allowing the piglets free access to the entire area. Post-weaning, the starter pigs were housed indoor in concrete pens with access to a partially roofed outdoor area. Moreover, all weaned pigs were provided with straw bedding up to 25 cm deep.

On farm 1, weaned pigs from several sows were randomly allocated into pens accommodating 20–25 pigs. At 35 kg (\approx 15 weeks of age) the group was split in two, and half of the pigs were relocated to a new pen. The stable was partly roofed with no clear distinction between indoor and outdoor areas. Bedding was deepest underneath the roofed part of the stable and gradually decreased towards the open part where the floor was partially slatted. On farm 2 and 3, starter pigs and fatteners were kept in separate, fully roofed units with separate outdoor runs and separate feeding areas.

2.2 Sample collection

Individual faecal samples were collected rectally at quarterly intervals from September 2011 to June 2012. At each visit, we aimed at taking 20 samples from sows (10 pregnant and 10 lactating), fatteners (80–100 kg) and starter pigs (20–30 kg corresponding to 9–14 weeks of age), respectively. In addition, samples were collected immediately after defecation from 9–19 suckling piglets (≤ 7 weeks) in the farrowing paddocks. The samples were transported on ice to the laboratory and stored at 5°C until processing.

2.3 Faecal examination

Faecal consistency was assessed on a scale from one to five (1: normal; 2: soft, 3: liquid; 4: watery; 5: watery with blood and/or intestinal tissue). *Giardia* cysts and *Cryptosporidium* oocysts ((oo-)cysts) were isolated and enumerated as previously described by Maddox-Hyttel *et al.* (2006). Briefly, 1 g faeces was suspended by vortexing in 3.5 mL 0.01% Tween 20 and filtered through multilayered 20-thread gauze (a 5 cm \times 5 cm piece placed in a 10 mL syringe and held in place by a piece of wire). Another 3.5 mL 0.01% Tween 20 was added and the fluid pressed through the gauze with the syringe piston. The filtrate was under layered with 3.5 mL flotation fluid (saturated saline with glucose (50 g in 100 mL) diluted 1:1 with MQ water (final specific gravity = 1.13 g mL⁻¹) and centrifuged at 53 $\times g$ for 3 min. The supernatant was transferred to a clean tube and washed three times using MQ water and subsequently centrifuged at 1540 $\times g$ for 10 min to obtain a final sample volume of 2 mL. For each sample, a 10 μ L subsample was placed in a well on a 3-well Teflon printed diagnostic slide (Immuno-Cell Int., Belgium) with 10 μ L MQ water already added to the well. The slide was air-dried overnight, fixed with acetone and left to dry for 5 min before adding 25 μ L of anti-*Giardia*/ and anti-*Cryptosporidium* fluorescein isothiocyanate (FITC)-labelled antibody mix (Crypto/*Giardia*-CEL IF, Cellabs, Australia) according to the manufacturer's instructions. The (oo-)cysts were

quantified by epifluorescence microscopy at 200× or 400× magnification and expressed as oocysts or cysts per g (OPG, CPG). The theoretical detection limit was 200 (oo-)cyst per gram of faeces corresponding to (oo-)cysts counted in 10 µL of an initial 2 mL of processed suspension.

2.4 Molecular and phylogenetic analyses

Purified (oo-)cysts were stored at 5°C for approximately one year prior to DNA extraction using a QIAamp® DNA Stool Mini Kit (QIAGEN GmNH, Hilden, Germany) according to the manufacturer's directions, except that the lysis temperature was increased to 95°C, and the DNA was eluted with only 100 µl Buffer AE, and subsequently stored at -20°C.

When possible, samples from the two pigs containing the highest OPG and CPG were selected for molecular and phylogenetic analyses from each farm, age-group and season. Furthermore, all samples from pigs with CPG≥1000 were selected ($n=32$). Thus, for some groups at particular time points, faeces from more than two animals were included in the molecular analyses.

Partial nucleotide sequences of the *Cryptosporidium* isolates were obtained by polymerase chain reaction (PCR) and subsequent sequencing of the small subunit ribosomal RNA gene (18S SSU rDNA locus) and the heat shock protein (*hsp*) 70 gene, whereas molecular typing of *G. duodenalis* was performed by PCR and partial sequencing of the 18S rRNA and the glutamate dehydrogenase (*gdh*) genes (Langkjaer *et al.*, 2007).

PCR products were purified either directly from the PCR reaction, or following electrophoresis and extraction of specific DNA fragments from the agarose gel using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). The purified PCR products were sequenced in both directions on an ABI 3130-4 Genetic Analyzer (Applied Biosystems, Europe BV, Danish branch in Nærum, DK) using Big Dye1 Terminator V3.1 cycle sequencing kit (Applied Biosystems). Alignment of selected representative fragments (one for

each species and each molecular marker) and reference sequences from GenBank were made with Clustal W using the software MEGA6 (Tamura *et al.*, 2013). Phylogenetic relationships were determined by neighbour joining analyses using 1000 bootstrap replicates, and the evolutionary distances were computed using the Jukes-Cantor method. In addition, the average evolutionary divergence over sequence pairs was determined for each of the gene fragments to estimate the number of nucleotide substitutions per site from averaging over all sequence pairs within each species (Tamura *et al.*, 2013).

2.5 Statistical analyses

Mono-infection with *Cryptosporidium* or *Giardia* and dual infection with both parasites were binary response variables, and differences between age-groups, farms and sampling seasons were evaluated using a logistic regression model allowing findings from the same farm to be correlated. The model was estimated using restricted pseudo-likelihood estimation (SAS Glimmix procedure).

The OPG were $\ln(x)$ transformed, while CPG were square root transformed to ensure a normally distributed interval dependent variable. The differences in OPG means between mono/dual infected pigs and age-groups were analysed by linear regression to test whether the mean number of (oo-)cysts excreted per pig differed by age-group, mono/dual infection or if there was any interaction between these. The normality assumption was validated by quantile-quantile plots and Shapiro–Wilk test, and variance homogeneity was validated by residual plot. Thereafter, the non-significant effects were removed by stepwise backward model reduction on a 5% significance level. The final model for excreted oocysts (OPG) included farm number, season and age-group as fixed factors. The differences in CPG were square root transformed and analysed using a generalized linear regression model assuming a

gamma error distribution and an inverse link function. The distributional assumptions were validated by inspecting the residual distribution. One extreme CPG value was omitted from the analysis. All mean data \pm standard deviation (S.D.) are stated as arithmetic means calculated from the raw non-transformed data. All statistical analyses were done in SAS, version 9.3 (SAS institute Inc., Cary, NC).

The *Cryptosporidium* spp., *C. suis* or *C. scrofarum*, were a binary response variable, and differences between age-groups, farms and sampling seasons were evaluated using a logistic regression model allowing findings from the same farm to be correlated. The model was estimated using restricted pseudo-likelihood estimation (SAS Glimmix procedure).

3. Results

3.1 Prevalence

Faecal samples were collected from 240 sows, 230 fatteners, 234 starter pigs and 152 piglets, yielding a total of 856 samples. Based on microscopical examination, 46.7% (400/856) of the sampled pigs tested positive for *Cryptosporidium*, *Giardia* or both parasites. Of these, 40.9% (350/856) were infected with *Cryptosporidium* and 14.0% (120/856) with *Giardia*. Of the *Cryptosporidium*-positive pigs, 20.0% were dual-infected with *Giardia*, while 58.3% of the *Giardia*-positive pigs were concomitantly infected with *Cryptosporidium*, corresponding to a total of 8.2% (70/856) dual-infected pigs. The theoretical expected prevalence of dual-infections was 5.7% (0.409×0.140). Pigs infected with *Giardia* thus had an increased relative risk of being concomitantly infected with *Cryptosporidium* (relative risk= 2.02; CI 1.45–2.83; $p < 0.0001$) and vice versa (relative risk= 1.53; CI 1.28–1.83; $p < 0.0001$).

The prevalence of *Cryptosporidium* infections was significantly age-related both on study level ($p=0.0002$) and within each farm ($p<0.05$) (Table 1). Infections were markedly more prevalent in starter pigs (72.2%) compared to any other age-group ($p<0.0001$) and least prevalent in sows (2.9%). Likewise, the overall prevalence of *Giardia* was significantly age-related ($p=0.0017$) with higher prevalence in starter pigs (27.4%) and lower prevalence in piglets (2.0%) compared to other age-groups. Yet, the prevalence of *Giardia* mono-infections was similar in starter pigs (7.3%), fatteners (9.6%) and sows (4.6%), whereas no piglets (0.0%) were mono-infected with this parasite (Table 1). In agreement with the overall high prevalences observed in starter pigs, dual-infections were significantly ($p<0.0001$) more prevalent in this age-group (20.1%) compared to any other age-group.

Generally, the prevalences of both parasites were unaffected by season at study level and within each farm and age-group. Yet, fatteners and starter pigs tended to have more *Giardia* infections during autumn and winter, and dual-infections were more prevalent in September and December ($p<0.05$).

3.2 Oocyst and cyst excretion levels

In general, regardless of age, farm, season and species, *Cryptosporidium* were excreted at considerably higher levels compared to *Giardia*. The intensity of infection ranged 200–10,551,200 OPG with a mean of $99,062 \pm 35,151$ OPG and median of 2100 OPG for *Cryptosporidium* infected pigs, and 200–21,400 CPG with a mean of $1,285 \pm 261$ CPG and median of 400 CPG for *Giardia* infected pigs. In addition, the excretion levels of *Cryptosporidium* were markedly lower for dual-infected pigs (all ages) compared to mono-infected pigs (all ages) (mean \pm S.E., and median, dual-infected: $11,157 \pm 3,776$ OPG and 1,600 OPG; mono-infected: $121,038 \pm 43,846$ OPG and 2200 OPG), although not statistically

significant ($p>0.05$). Moreover, only 1.4% of the dual-infected pigs (all ages) excreted 10^4 – 10^5 OPG while the corresponding number was 8.6% for *Cryptosporidium* mono-infected pigs (all ages), and with one exception (a dual-infected piglet), excretion levels $>10^5$ OPG were only observed in *Cryptosporidium* mono-infected piglets. Moreover, only three of 152 (4.3%) piglets were dual infected, while 78 out of 152 (51.3%) were mono-infected with *Cryptosporidium*.

At all four sampling seasons, some pigs did not excrete detectable numbers of (oo-)cysts, while others excreted extreme quantities. The average excretion levels for each age-group are presented in Figure 1, demonstrating an age-related excretion pattern, where the infection intensity decreased with increasing age. Piglets excreted, on average, 1,177 times more OPG and 8 times more CPG than sows. Infection intensity was independent of season and farm.

Mean oocyst excretion for *C. suis* infected pigs was 351,035 OPG (range: 2.4×10^3 – 1.6×10^6 ; S.D. 351,035; 95% CI: 67953–634117) while pigs infected with *C. scrofarum* excreted 54,848 OPG (range: 0.6×10^3 – 1.2×10^6 ; S.D. 194,508; 95% CI: -9085–118781). The average excretion levels of *C. suis* and *C. scrofarum* are presented for each age-group in Table 2. Scouring animals (faecal scores ≥ 3) were not detected among the sampled animals and infection intensity was unrelated to faeces consistency ($p>0.05$).

3.2 Molecular characterization of *Cryptosporidium* and *Giardia*

Of the 350 *Cryptosporidium* positive samples, we attempted to analyse 75 samples by partial sequencing of the 18S rDNA locus and the *hsp70* gene; 55 samples from *Cryptosporidium* mono-infected pigs and 20 samples from dual-infected pigs. Of these, 40 samples from mono-infected pigs and 16 from dual-infected pigs were successfully sequenced revealing 38 (67.9%) *C. scrofarum* and 18 (30.9%) *C. suis*. Of the 16 successfully sequenced samples from dual-

infected pigs, 14 were *C. scrofarum* and two were *C. suis*. Seasonal- and age-related prevalences of *Cryptosporidium* species on the three farms are presented in Table 3.

The *Cryptosporidium* species infecting the pigs was determined by age. Piglets hosted exclusively *C. suis* (except for one piglet infected with *C. scrofarum*), while starter pigs and fatteners were infected with *C. suis* as well as *C. scrofarum*; the latter species occurring most frequently. Species distribution appeared unaffected by season or farms. The sows excreted few oocysts (below 1,000 OPG), and no typing results could be obtained from this age-group. Both *C. suis* and *C. scrofarum* were detected on all three farms during all seasons.

For those *Cryptosporidium* isolates where typing results were obtained with both molecular markers (n=35), the results were consistent. Phylogenetic analyses of the *Cryptosporidium* 18S rDNA (Fig. 2) and *hsp70* gene sequences (Fig. 3) were largely consistent, and the sequences were 95-100% identical to those previously published for porcine isolates of *C. suis* and *C. scrofarum* from GenBank. Estimates of the number of base substitutions per site from averaging over all sequence pairs within each group involved 44 18S rDNA nucleotide sequences (13 *C. suis*, 31 *C. scrofarum*) with a total of 476 base pairs in the final dataset, and 47 *hsp70* sequences (13 *C. suis*, 34 *C. scrofarum*) with a total of 354 base pairs in the final datasets. Rates of nucleotide substitutions were generally higher in the *hsp70* sequences compared to 18S rDNA sequences, but while *hsp70* sequences of *C. suis* (0.046) were more variable relative to *C. scrofarum* (0.020) the opposite was the case for 18S rDNA sequences (*C. suis*: 0.001; *C. scrofarum*: 0.004). Selected representative sequences (B14, B26, B35, B37) from the present study have been uploaded to GenBank under the Accession numbers: KT223028–29 and KT716819–20.

Of the 120 *Giardia* positive samples, we attempted to analyse 67 by partial sequencing of the 18S rDNA locus and the *gdh* gene; 31 samples from *Giardia* mono-infected pigs and 36 samples from dual-infected pigs. None of the dual-infected pigs were successfully sequenced, while genotypes were successfully obtained from 13 of the mono-infected isolates, revealing 11 (84.6%) *G. duodenalis* Assemblage E and two (15.4%) Assemblage A. Assemblage E was detected in samples from all seasons and was found in pigs from all three farms, whereas Assemblage A was only detected in pigs from farm 2 and 3 in June and September, respectively (Table 4). *Giardia* species distribution was only analysed to calculate the relative risk of a co-infection with both parasites, while too few data were obtained from the screening to do logistic regression.

4. Discussion

4.1 Prevalence

Cryptosporidium and *Giardia* infected pigs were identified on all three farms, at all seasons and from pigs of all ages. Nevertheless, young age was strongly associated with prevalence and intensity of infections, particularly for *Cryptosporidium*, which had very high prevalence rates in piglets and starter pig whereas *Giardia* infections were more prevalent only in starter pigs compared to any other age-group. The prevalences of both parasites were unaffected by season, while the prevalence of dual-infections peaked in September and December. In accordance with previous studies in pigs, sheep and cattle (Maddox-Hyttel *et al.*, 2006; Santin *et al.*, 2007; Siwila and Mwape, 2012; Budu-Amoako *et al.*, 2012), *Cryptosporidium* infections were approximately three times more prevalent than *Giardia* infections. Moreover, the dual-infections constituted a substantial part of the infections: 20.0% of the *Cryptosporidium* and 58.3% of the *Giardia* positive pigs. Hence, pigs infected with one parasite appeared more likely

to be simultaneously infected with the other. Most previous studies of *Cryptosporidium* infection in pigs did not include *Giardia*, and in the few studies that did, dual-infected animals were not reported (Budu-Amoako *et al.*, 2012; Farzan *et al.*, 2011). However, studies in cattle, sheep and horses identified *Cryptosporidium/Giardia* dual-infected young animals by PCR or immunofluorescence assay with prevalences ranging 7–26% (Xiao *et al.*, 1993; Xiao and Herd, 1994; Castro-Hermida *et al.*, 2006; Hamnes *et al.*, 2006; Sweeny *et al.*, 2011). None of these studies compared the intensity of the infections, while others have reported cryptosporidiosis to be a risk factor for giardiasis in children and vice versa (e.g. Wang *et al.*, 2013). The relatively high prevalence of dual-infections reported here as well as in other livestock, indicates that dual-infections are fairly common. This may be the result of identical infection routes and infectivity of the two parasite species, rather than one being a risk factor for the other. Yet, solid data, e.g. experimental infection studies, to support this hypothesis is lacking.

Weaning of piglets may have triggered the high prevalence in starter pigs observed in this study and elsewhere, since weaning is associated with significant social, environmental and nutritional changes resulting in dramatic alterations of gut structure, microbiology and function (Montagne *et al.*, 2007). However, two studies in Vietnam demonstrated that the prevalence of *Cryptosporidium* in pre-weaned pigs (< 1 month) was significantly higher than in post-weaned pigs (1–2 month) (Nguyen *et al.*, 2012, 2013). This discrepancy, compared to our study where the highest prevalences were detected post weaning, was probably related to differences in weaning age, and diagnostic methods. Pigs in our study were weaned at 7–9 weeks of age, i.e. approximately one month later than pigs in the Vietnamese study. Further, the Vietnamese study used the Modified Ziehl-Neelsen technique, a method known to have low sensitivity. Thus, post weaning samples with low intensity might have remained undetected.

Pigs raised on straw bedding have previously been shown to be more commonly infected with *Cryptosporidium* than pigs raised on slatted floors (Nemejc *et al.*, 2013). Yet, the prevalences of *Cryptosporidium* (72.2%) and *Giardia* (27.4%) in starter pigs in the present study, where the pigs had access to straw bedding, were similar to those described in an earlier Danish study of conventional pig farms, where the animals were raised on slatted floor (Maddox-Hyttel *et al.*, 2006). The *Cryptosporidium* prevalences in our study were, however, considerably higher than the prevalence of 33–48% for starter pigs reported elsewhere (Budu-Amoako *et al.*, 2012; Yui *et al.*, 2014). It should be noted though, that prevalence of *Cryptosporidium* in piglets (53.3%) prior to weaning was noticeably higher than formerly described both in Denmark (Maddox-Hyttel *et al.*, 2006) and abroad (Budu-Amoako *et al.*, 2012; Yui *et al.*, 2014). Outdoor rearing and late weaning as practiced in organic pig production probably contributed to these differences. In Danish organic pig production, suckling piglets have unrestricted access to large pasture areas with accumulation of manure and contact with several sows (Serup, 2010). This is in contrast to conventional pig production where lactating sows and their piglets are housed in individual, indoor pens with slatted floors (Nielsen & Norgaard, 2010). Our results are in agreement with a study of pigs in Western Australia where *Cryptosporidium* was found to be more prevalent in outdoor pig herds (17.2%) than in indoor herds (0.5%) (Ryan *et al.*, 2003). Additionally, stress related to changing weather conditions may have contributed to the high prevalence and infection levels observed in our study of outdoor reared animals. Due to the cross-sectional study design with repeated sampling in each farm, but without repeated sampling of individual pigs, we were unable to demonstrate if the high prevalence in piglets was caused by inability to clear the infection or due to repeated re-infection. In contrast, hardly any piglets had detectable *Giardia* infections, whereas the parasite was relatively prevalent among starter pigs and fatteners.

As cold and humid conditions, occurring during winter, are known to favour (oo-)cyst survival (Robertson *et al.*, 1992; Olson *et al.*, 1999), prevalence was expected to vary in outdoor raised pigs due to seasonality. However, the prevalence of *Cryptosporidium* was relatively constant during this one-year study, demonstrating a continuous infection pressure and environmental contamination throughout the year; supported by the presence of susceptible piglets due to farrowing at all seasons. *Giardia* cysts are more sensitive than *Cryptosporidium* oocysts to adverse environmental conditions (Olson *et al.*, 1999), which might account for the fluctuation of *Giardia* prevalence observed in starter pigs and fatteners between sampling seasons in the present study.

4.2 Infection intensity

As for the prevalence, the infection intensity of both parasites was age related with the highest *Cryptosporidium* intensity in piglets, while the highest *Giardia* intensity appeared in starter pigs. Nonetheless, the dual infected piglets excreted more *Giardia* cysts than the dual infected starter pigs (Fig. 1). The *Cryptosporidium* intensities are in accordance with previously reported data (Yui *et al.*, 2014; Jeniková *et al.*, 2011). The lower *Cryptosporidium* excretion levels in starter pigs compared to piglets (Fig. 1) may have been the result of acquired immunity or parasite factors, as this age-group, in agreement with e.g. Yin *et al.* (2013), was predominantly infected with a *Cryptosporidium* species different from that observed in the piglets (Table 3). Nevertheless, excretion levels $>10^3$ OPG were seen in 68.6% of the infected starter pigs, demonstrating relatively high infection intensities in this age-group despite development of immunity and predominant infection with *C. scrofarum*. Pigs concomitantly infected with *Cryptosporidium* and *Giardia* excreted lower levels of (oo-)cysts compared to mono-infected pigs. Hence, our results may suggest that dual-infections prevent pigs from excreting

extremely high numbers of (oo-)cysts. Yet, the differences were insignificant, probably due to relatively few dual-infected pigs, and large variation in (oo-)cyst excretion within the age-groups. To our knowledge, this is the first study to compare infection intensity of dual- and mono-infected pigs, and, although a few studies have documented prevalence of mono- and dual-infections in cattle and analysed infection intensity, they did not compare the infection intensity between mono- and dual-infected animals (O'Handley *et al.*, 1999; Hamnes *et al.*, 2006). Accordingly, more studies are needed to reveal whether these parasite infections may have antagonistic effects or whether the lower infection intensity observed in dual-infected pigs was mainly caused by *Giardia* infections occurring later in life compared to *Cryptosporidium*, and thus the majority of the dual infected pigs are more immunologically mature compared to the young piglets. This study revealed that the intensity of *Cryptosporidium* infections in pigs was linked to species and age, as *C. suis* infected pigs excreted above 6 times more oocysts than *C. scrofarum* infected animals, and piglets exclusively hosted *C. suis*. This is in agreement with results of Kváč *et al.* (2009a), who found that pigs infected with *C. suis* shed statistically higher numbers of oocysts compared with pigs infected with *C. scrofarum*. Nevertheless, as our study was not specifically designed to analyze species-specific differences in oocyst excretion, firm conclusions regarding variation in reproductive potential of the porcine *Cryptosporidium* species needs further scrutiny.

Based on scoring of the faecal samples the infections were subclinical over the course of the study and no correlation could be found between faecal consistency and (oo-)cyst excretion levels. A recent Japanese study showed that piglets with signs of diarrhoea shed more *Cryptosporidium* oocysts than piglets with normal or loose stools (Yui *et al.*, 2014); but the study did not exclude co-infection with other pathogens. In contrast, and in agreement with our findings, most other studies demonstrated a subclinical course of infection (Xiao, 1994;

Quilez *et al.*, 1996; Guselle *et al.*, 2003; Ryan *et al.*, 2003; Vitovec *et al.*, 2006; Suarez-Luengas *et al.*, 2007; Jeníková *et al.*, 2011). This suggests limited clinical importance of *Cryptosporidium* and *Giardia* in naturally infected pigs, but does not preclude an impact on productivity.

Furthermore, co-infection with other commonly occurring pathogens may result in clinical disease, e.g. rotavirus has previously been demonstrated to enhance pathogenicity leading to severe disease and mortality in pigs with cryptosporidiosis (Enemark *et al.*, 2003).

4.3 Species composition

Cryptosporidium suis, *C. scrofarum*, *G. duodenalis* Assemblage E and the zoonotic Assemblage A were identified in the present study. *Cryptosporidium scrofarum* was the most commonly isolated species on all three farms, and the species predominantly identified in starter pigs and fatteners. In contrast, piglets exclusively hosted *C. suis* with a single exception. This finding is congruent with results seen in other studies where piglets under the age of four weeks were refractory to *C. scrofarum* infection (Langkjaer *et al.*, 2007; Johnson *et al.*, 2008; Kváč *et al.*, 2009a; Jeníková *et al.*, 2011; Yin J.H. *et al.*, 2013; Kváč *et al.*, 2014). Likewise, *C. suis* and *C. scrofarum* were the most common species in pigs according to studies by others (Xiao *et al.*, 2006; Langkjaer *et al.*, 2007; Zintl *et al.*, 2007; Johnson *et al.*, 2008; Kváč *et al.*, 2009b; Yin J.H. *et al.*, 2013), while infections with species known to be highly zoonotic such as e.g. *C. parvum* have only occasionally been demonstrated (Budu-Amoako *et al.*, 2012; Kváč *et al.*, 2009; Zintl *et al.*, 2007; Featherstone *et al.*, 2010). Our results may, however, be biased by selection of samples for genotyping only from the two pigs with the highest excretion levels from each farm, age-group and season, rendering them unrepresentative of the entire population. Molecular analyses of samples containing few oocysts might have revealed less host adapted species or other species with lower reproductive capacity.

No typing results were obtained from sows. Low OPG or differences in species and genotypes infecting the sows compared to other age groups may have contributed to this unsuccessful molecular typing. Nevertheless, other studies detected both *C. suis* and *C. scrofarum* in sows (Zintl *et al.*, 2007; Budu-Amoako *et al.*, 2012).

We were unable to evaluate any relationship between the presence of *G. duodenalis* Assemblages and age, as only 13 of 67 *Giardia*-positive samples were successfully genotyped. The low success rate was probably caused by inadequate storage of the semi-purified cysts, which were stored in MQ water at 5°C for approximately one year prior to DNA extraction and PCR analysis. This was unintended and due to a mistake, and probably resulted in degradation of the DNA (Nechvatal *et al.*, 2008) which could have been prevented by preservation in e.g. ethanol (Wilke and Robertson, 2009) or freezing (Kohli *et al.*, 2008). Nevertheless, the zoonotic Assemblage A was detected in two pigs: a starter pig and a fatterer, while the positive isolates containing Assemblage E were obtained from starter pigs, fatteners and sows, from all three farms and at all four seasons.

The phylogenetic analysis showed that Danish isolates grouped together with *C. scrofarum* and *C. suis* isolates from GenBank. Yet, intraspecies variations within the *hsp70* gene fragments were demonstrated particularly for *C. suis* where an evolutionary distance of 0.046 was revealed between the isolates. This is considerably higher than observed for *C. scrofarum*. In comparison, little variation was present within the 18S rDNA fragments, but interestingly, *C. scrofarum* was more variable than *C. suis* within this gene locus. In accordance with our results, actin gene variants of *C. suis* and *C. scrofarum* were recently described in Japan (Yui *et al.*, 2014). However, few studies have analysed genetic variation within porcine

Cryptosporidium species and further epidemiological studies are needed to fully understand the extent and significance of this variation.

No simultaneous infection with several *Cryptosporidium* species/genotypes was observed, which corresponds with earlier studies, where only few have reported mixed *Cryptosporidium* infection (Kváč *et al.*, 2009b). Diagnosis of mixed infections is limited by one species usually predominating. Thus, as most PCR assays only amplify the dominant type (Tanriverdi *et al.*, 2003), other species may remain undetected.

In conclusion, the study of three organic, Danish pig farms including examination of 856 faecal samples collected from different age-groups throughout one year revealed high prevalences of *Cryptosporidium* and *Giardia* infections leading to an inevitable risk of environmental contamination. However, the identified *Cryptosporidium* species were all of the pig-specific species and while the zoonotic *G. duodenalis* Assemblage A was present, it was rare. Although, sporadic cases of *C. suis* and *C. scrofarum* are reported in humans (Xiao *et al.*, 2002; Cama *et al.*, 2003; Bodager *et al.*, 2015), the contribution from organic pigs in this study to the epidemiology of cryptosporidiosis and giardiasis in humans is apparently of minor importance. Accordingly, our findings suggest that the public health risk associated with *Cryptosporidium* and *Giardia* originating from organically outdoor reared pigs seems to be negligible in Denmark.

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Figure captions:

Figure 1: Intensity of infection in organic pigs naturally mono-infected with *Cryptosporidium* (n=280) and *Giardia* (n=50), or dual-infected with both parasites (n=70). Mean \pm SEM (oo-)cysts per gram of faeces (OPG/CPG) per age-group. Based only on data from positive animals and merged across four seasons in a year. Please note the different scales on the Y-axes.

Figure 2: Phylogenetic relationships of porcine *Cryptosporidium* isolates inferred by Neighbour Joining analysis of 18S rRNA partial nucleotide sequences (628 base pairs in the final dataset). Percentage bootstrap support from 1,000 replicate samples is indicated at the left of each node. Accession numbers are shown in parentheses. The scale bar indicates 0.02 nucleotide substitution/site.

Figure 3: Phylogenetic relationships of porcine *Cryptosporidium* isolates inferred by Neighbour Joining analysis of partial nucleotide sequences of the *hsp70* gene (268 base pairs in the final dataset). Percentage bootstrap support from 1,000 replicate samples is indicated at the left of each node. Accession numbers are shown in parentheses. The scale bare indicate 0.02 nucleotide substitution/site.

Table 1: Seasonal- and age-related prevalence of *Cryptosporidium* and *Giardia* in mono-infected and dual-infected pigs determined by quantitative immuno-fluorescence microscopy of faecal samples from 856 pigs from three Danish organic pig farms, September 2011–June 2012.

	Total number of tested pigs	Piglets		Starter pigs		Fatteners		Sows		Total number of positive pigs. (%)
		Positive/n	Prev. (%)	Positive/n	Prev. (%)	Positive/n	Prev. (%)	Positive/n	Prev. (%)	
<i>Cryptosporidium</i> infection (combined dual- and mono-infections)										
Total	856	81/152	53.3	169/234	72.2	93/230	40.4	7/240	2.9	350 (40.9)
Farm 1	273	22/39	56.4	56/80	70.0	23/74	31.1	3/80	3.8	84 (30.8)
Farm 2	291	23/53	43.4	62/80	77.5	37/79	46.8	1/80	1.3	109 (37.5)
Farm 3	292	36/63	60.0	51/75	68.0	33/77	42.9	3/80	3.8	87 (29.8)
Sept	226	18/46	39.1	50/60	83.3	22/60	36.7	1/60	1.7	65 (28.8)
Dec	206	20/32	62.5	38/56	67.9	31/58	53.4	0/60	0.0	65 (31.6)
Mar	214	21/42	50.0	43/59	72.9	21/53	39.6	1/60	1.7	73 (34.1)
June	210	22/32	68.8	38/59	64.4	19/59	32.2	5/60	8.3	77 (36.7)
<i>Giardia</i> infection (combined dual- and mono-infections)										
Total	856	3/152	2.0	64/234	27.4	41/230	17.8	12/240	5.0	120 (14.0)
Farm 1	273	2/39	5.1	19/80	23.8	12/74	16.2	7/80	8.8	20 (7.3)
Farm 2	291	0/53	0.0	8/80	10.1	9/79	11.4	0/80	0.0	3 (1.0)
Farm 3	292	1/60	1.7	37/75	49.3	20/77	26.0	5/80	6.3	27 (9.2)
Sept	226	0/46	0.0	19/60	31.7	20/60	33.3	2/60	3.3	15 (6.6)
Dec	206	1/32	3.1	24/56	42.9	10/58	17.2	2/60	3.3	13 (6.3)
Mar	214	2/42	4.8	13/59	22.0	6/53	11.3	5/60	8.3	13 (6.1)
June	210	0/32	0.0	8/59	13.6	5/59	8.5	3/60	5.0	9 (4.3)

Mono-infection										
<i>Cryptosporidium</i>	856	78/152	51.3	122/234	52.1	74/230	32.2	6/240	2.5	280 (32.7)
<i>Giardia</i>	856	0/152	0.0	17/234	7.3	22/230	9.6	11/240	4.6	50 (5.8)
<i>Cryptosporidium/Giardia</i> dual-infection										
Total	856	3/152	2.0	47/234	20.1	19/230	8.3	1/240	0.4	70 (8.2)
Farm 1	273	2/39	5.1	15/80	18.8	2/74	2.7	1/80	1.3	20 (7.3)
Farm 2	291	0/53	0.0	7/80	8.8	7/79	8.9	0/80	0.0	14 (4.8)
Farm 3	292	1/60	1.7	25/75	33.3	10/77	13.0	0/80	0.0	36 (12.3)
Sept	226	0/46	0.0	17/60	28.3	9/60	15.0	0/60	0.0	26 (11.5)
Dec	206	1/32	3.1	16/56	28.6	7/58	12.1	0/60	0.0	24 (11.7)
Mar	214	2/42	4.8	9/59	15.3	1/53	1.9	1/60	1.7	13 (6.1)
June	210	0/32	0.0	5/59	8.5	2/59	3.4	0/60	0.0	7 (3.3)

Table 2: Age-related oocyst excretion of *Cryptosporidium* species in pigs from three Danish, organic pig farms, September 2011–June 2012.

	Piglets		Starter pigs (%)		Fatteners (%)		Sows (%)		Total	
	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>
Mean OPG	513,919	1.21 x 10 ⁶	38,053	47,867	2,667	8,222	0	0	351,035	54,848
Min – Max	3,9 x 10 ⁴ – 1.6 x 10 ⁶	1.2 x 10 ⁶	7,6 x 10 ³ – 9,3 x 10 ⁴	1,0 x 10 ⁴ – 9,4 x 10 ⁴	2.4 x 10 ³ – 3.2 x 10 ³	600 – 3,5 x 10 ⁴	-	-	2.4 x 10 ³ – 1.6 x 10 ⁶	0.6 x 10 ³ – 1.2 x 10 ⁶

Table 3: Seasonal- and age-related prevalence of *Cryptosporidium* species in pigs, based on molecular analyses of 75 *Cryptosporidium* isolates from three Danish, organic pig farms, September 2011–June 2012.

	n ^a	Piglets (%)		Starter pigs (%)		Fatteners (%)		Sows (%)		Total number of positive pigs (%)		
		<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	<i>C. suis</i>	<i>C. scrofarum</i>	Total ^b	<i>C. suis</i>	<i>C. scrofarum</i>
Total	75	12 (92.3)	1 (7.7)	3 (13.6)	19 (86.4)	3 (14.3)	18 (85.7)	0	0	56 (74.7)	18 (32.1)	38 (67.9)
Farm 1	25	6	0	0	6	0	6	0	0	18 (72.0)	6 (33.3)	12 (66.7)
Farm 2	24	2	0	1	7	1	6	0	0	17(70.8)	4 (23.5)	13 (76.6)
Farm 3	26	4	1	2	6	2	6	0	0	21 (80.8)	8 (38.1)	13 (61.9)
Sept	19	5	0	1	4	0	6	0	0	16 (84.2)	6 (37.5)	10 (62.5)
Dec	16	0	1	0	6	2	3	0	0	12 (75.0)	2 (16.7)	10 (83.3)
Mar	18	3	0	0	6	1	4	0	0	14 (77.8)	4 (28.6)	10 (71.4)
June	22	4	0	2	3	0	5	0	0	14 (63.6)	6 (42.9)	8 (57.1)

Table 4: Seasonal and age-related prevalence of *Giardia duodenalis* Assemblages in pigs, based on molecular analyses of 67 *G. duodenalis* isolates from three Danish, organic pig farms, September 2011–June 2012.

	n	Piglets		Starter pigs		Fatteners		Sows		Total number of pos. animals (%)		
		Assemblage		Assemblage		Assemblage		Assemblage		Total	Assemblage	
		E	A	E	A	E	A	E	A		E	A
Total	67	0	0	9	1	1	1	1	0	13 (19.4)	11 (84.6)	2 (15.4)
Farm 1	25	0	0	3	0	0	0	1	0	4 (16.0)	4 (100)	0 (0.0)
Farm 2	13	0	0	1	1	0	0	0	0	2 (15.4)	1 (50.0)	1 (50.0)
Farm 3	29	0	0	5	0	1	1	0	0	7 (24.1)	6 (85.7)	1 (14.)
Sept	18	0	0	2	0	1	1	1	0	5 (27.8)	4 (80.0)	1 (20.0)
Dec	18	0	0	2	0	0	0	0	0	2 (11.1)	2 (100)	0 (0.0)
Mar	17	0	0	3	0	0	0	0	0	3 (21.4)	3 (100)	0 (0.0)
June	14	0	0	2	1	0	0	0	0	3 (21.4)	2 (66.7)	1 (33.3)

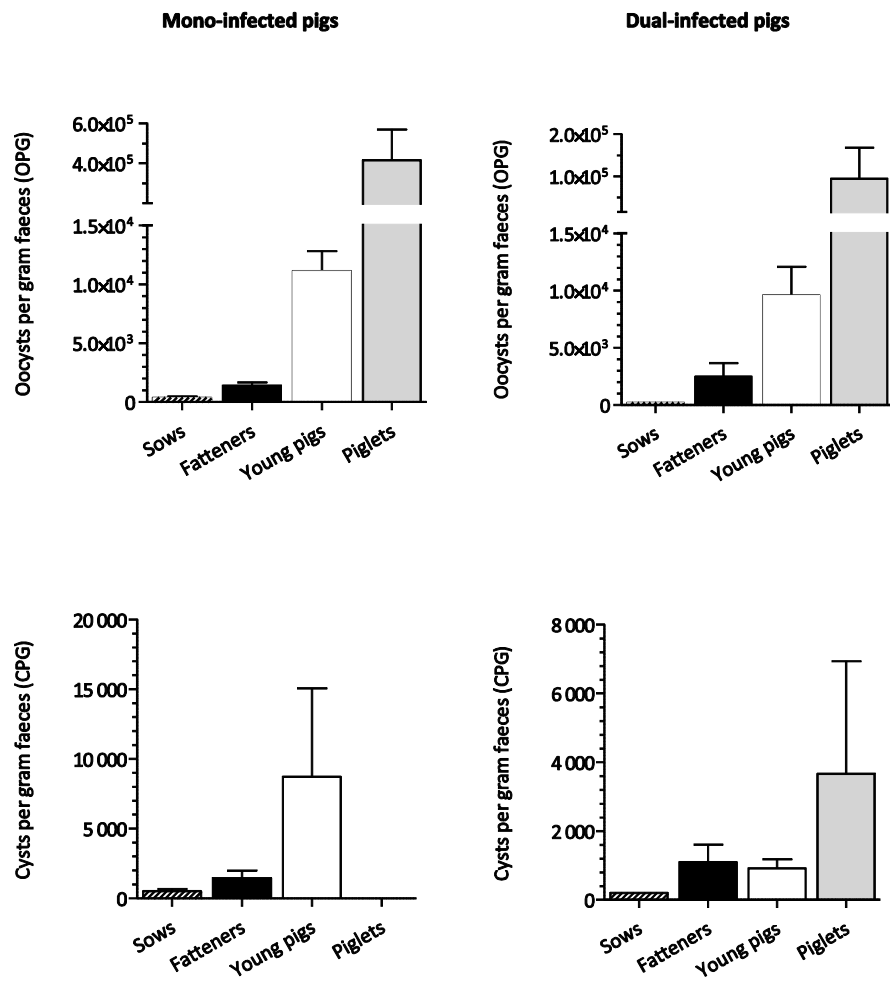


Fig. 1

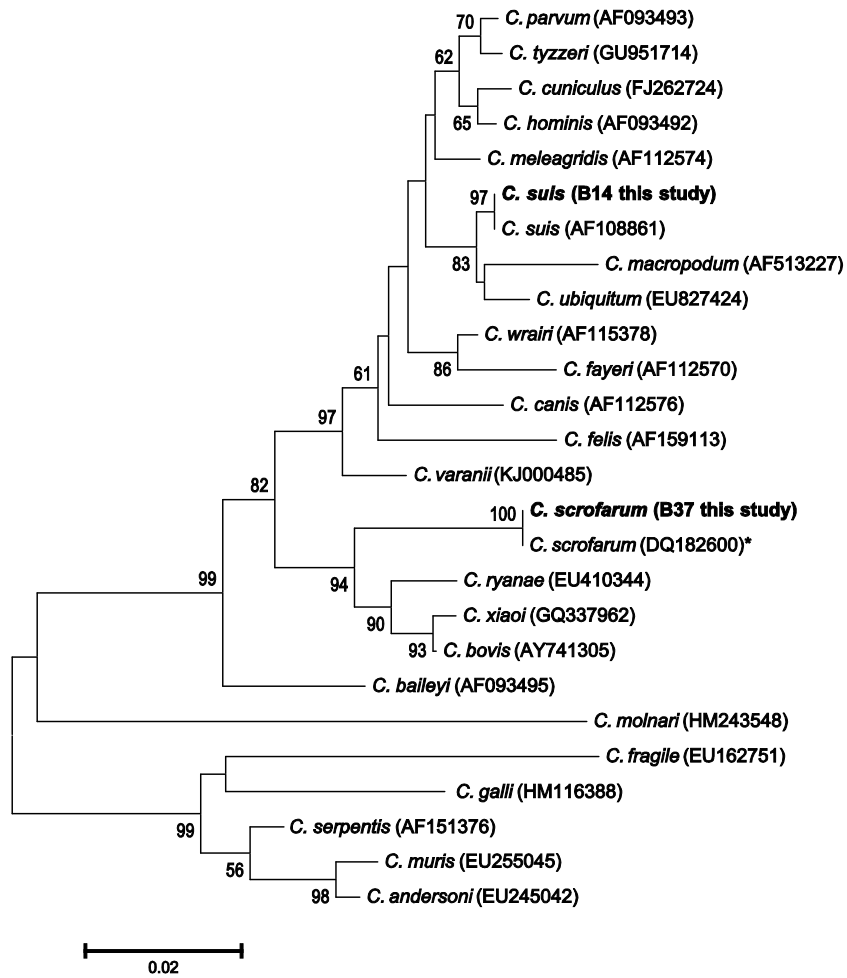


Fig. 2

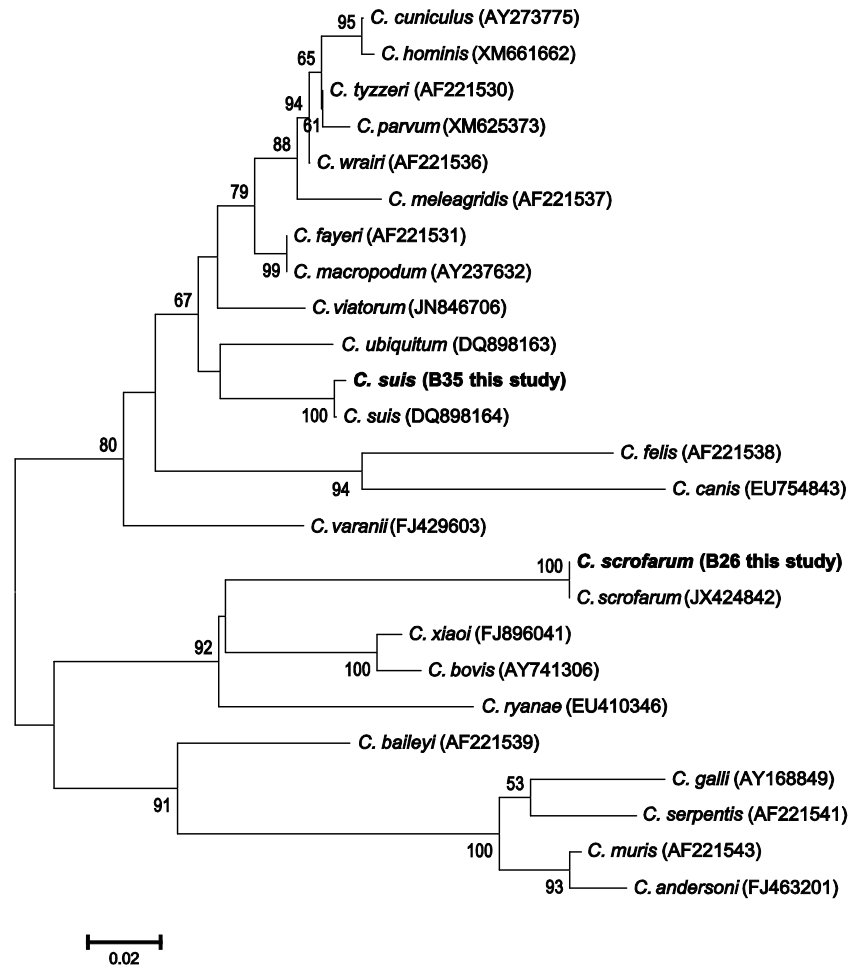


Fig. 3