Veterinary Parasitology 202 (2014) 330–334

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Short Communication

Age related susceptibility of pigs to *Cryptosporidium scrofarum* infection

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ARTICLE INFO

Article history:
Received 1 November 2013
Received in revised form 30 January 2014
Accepted 8 February 2014

Keywords:
Cryptosporidium scrofarum
Molecular analyses
Transmission studies
Susceptibility
Infection
Pigs

ABSTRACT

Piglets from 4 to 8 weeks of age originated from a *Cryptosporidium*-free research breed were orally inoculated with 1×10^6 infectious oocysts of *Cryptosporidium scrofarum*. The number of shed oocysts per gram of faeces served to describe the infection intensity and prepatent period. In addition, faecal samples collected daily and tissue samples of the small and large intestine collected at 30 days post-inoculation were examined for the *C. scrofarum* small subunit ribosomal RNA gene using PCR. The piglets inoculated at 4-weeks of age remained uninfected, whereas 5-week-old and older animals were fully susceptible with a prepatent period ranging from 4 to 8 days. Susceptible pigs shed oocysts intermittently, and shedding intensity, reaching a mean maximum of 6000 oocysts per gram, did not differ significantly among infected animals. This study demonstrates that pigs become susceptible to *C. scrofarum* infection as late as 5-weeks of age. The mechanisms of age related susceptibility remain unknown.

1. Introduction

Many species show changes in susceptibility to infectious agents that correlate with their ontogenesis. Effective host defence against disease-causing agents depends upon a combination of multiple mechanisms that include both natural and immune defensive measures (Murphy, 1991). Until recently, two major host groups were recognized based on their age related susceptibility to infection with *Cryptosporidium* species. In the first group, susceptibility is not dependent on host age. For example, both juvenile and mature cattle are susceptible to *Cryptosporidium andersoni* infection (Santín et al., 2004). The second group represents hosts susceptible to infection only as juveniles, e.g. laboratory BALB/c mice and cattle are not susceptible to *Cryptosporidium parvum* as adults. In contrast, humans of all ages are susceptible to *C. parvum* (Nichols, 2008). Field studies have shown that the pig adapted *Cryptosporidium* species *Cryptosporidium suis* and *Cryptosporidium scrofarum* differ in the age of pigs that they infect. *C. suis* appears to infect all age categories, though prevalence is lower in older pigs. In contrast, *C. scrofarum* appears to be specific for older pigs (e.g. Jeníková et al., 2011; Langkjær et al., 2007). Predicated on observations by Kváč et al. (2013), it was

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http://dx.doi.org/10.1016/j.vetpar.2014.02.012
0304-4017/

hypothesized that *C. scrofarum* is infectious for older pigs but not piglets. This hypothesis was tested using experimental infections in the present study.

2. Materials and methods

C. scrofarum oocysts were purified from naturally infected 7-week-old pigs using a caesium chloride gradient (Arrowood and Donaldson, 1996), and were stored for a maximum of 4 weeks in PBS with antimycotics and antibiotics at 4 °C. *C. scrofarum* was confirmed in purified oocyst suspensions by a nested PCR amplifying a partial fragment of the small ribosomal subunit rDNA (SSU). Oocyst viability was determined using propidium iodide (PI) staining (Sauch et al., 1991) and fluorescence microscopy (filter 420 nm, Olympus IX70).

Groups of 4-, 5-, 6-, 7- and 8-week-old naïve pigs (*Sus scrofa*) (3 animals per group; purchased from the *Cryptosporidium* free farm operated by the University of South Bohemia in České Budějovice, Czech Republic) were inoculated via syringe per os with a dose of 1×10^6 viable oocysts of *C. scrofarum* per animal in 20 ml distilled water. Each pig was individually housed in 5 m^2 pens with concrete walls and floor in an isolated building. Sterile water and feeding mixture were provided ad libitum. Faecal samples from all experimental animals were cleared away and collected daily. The presence of *Cryptosporidium* oocysts and specific DNA was confirmed using examination of smears stained with aniline-carbol-methyl violet (ACMV) (Miláček and Vítovec, 1985) and nested PCR targeting the SSU gene (Jiang et al., 2005), respectively. Infection intensity was reported as the number of oocysts per gram (OPG) of faeces as previously described (Kváč et al., 2007). All experiments were terminated 30 days post-inoculation (DPI), each animal was sacrificed, and tissue specimens of the gastrointestinal tract were sampled and processed for PCR analyses.

Total DNA was extracted and purified from 200 mg of faeces, 200 mg of tissue, or 1,000,000 oocysts (inoculum) and fragments of SSU and *Cryptosporidium* 60-kDa glycoprotein (gp60) genes were amplified using genus and species specific primers, respectively, as previously described (Alves et al., 2003; Jeníková et al., 2011; Jiang et al., 2005). PCR amplicons were sequenced directly in both directions using an ABI 3130 sequence analyzer (Applied Biosystems, Foster City, CA). The identity of obtained sequences was verified by a BLAST search (www.ncbi.nlm.nih.gov/blast).

All housing, feeding, and experimental procedures involving 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).

The accumulated value of infection intensity was calculated as area under the curve (AUC) through the classical trapezoidal rule. More specifically, we calculated t_{max} (time at maximal intensity) and C_{max} (maximal intensity). Differences in the course of infection among groups of experimentally infected animals were tested using the *t*-test. Statistical analyses were performed using R (version 2.15.0), a software environment for statistical computing.

3. Results

Isolate of *C. scrofarum* obtained from a naturally infected pig and used as infectious inoculum shared 100% sequence identity at the SSU locus with isolates recovered from each experimentally infected pig (data not shown). Using PCR targeting the SSU and gp60 loci, no other *Cryptosporidium* spp. were detected either in the inoculum or in faecal samples from experimentally infected animals.

C. scrofarum was not detected by microscopy or PCR in the faeces of 4-week-old pigs following experimental infection. In contrast, oocyst shedding was detected in 5- to 8-week-old pigs. While pigs infected at 5- and 6-weeks of age began shedding oocysts detectable by microscopy from 8 DPI, oocysts were detected at 6–7 DPI in faeces of animals infected at 7- and 8-weeks of age. *C. scrofarum* DNA was detected in these groups one to two days before oocysts were detected by microscopy (Table 1, Fig. 1).

Intermittent shedding of oocysts was observed in all animals susceptible to infection using both microscopy and PCR analyses. Only 40–60% samples were *Cryptosporidium* positive based on PCR diagnostics. The sensitivity of detection by microscopy was 10% lower than that of PCR (Table 1). Mean infection intensity was generally less than 6000 OPG (Fig. 1). Any effect of animal age at the time of infection on the subsequent course of infection, including infection intensity expressed as AUC (Table 1), was not significant. Specific DNA of *C. scrofarum* was detected in tissue specimens of the duodenum, ileum and jejunum from pigs inoculated at 5- to 8-weeks of age. No *C. scrofarum* DNA was detected in any part of the gastrointestinal tract of 4-week-old piglets at the time of sacrificing.

4. Discussion

This study provides experimental evidence supporting the hypothesis that the susceptibility of pigs to *C. scrofarum* infection is age related. While animals under 4-weeks of age were not susceptible to infection, piglets older than 5 weeks demonstrated infection by shedding oocysts in their faeces. These data are consistent with the findings of Kváč et al. (2013) that 8-week-old pigs but not 4-week old pigs are susceptible to *C. scrofarum*. In field studies, there has been only one report of *C. scrofarum* in a pig younger than 1 month (Wang et al., 2010), and the first detection of this species is typically in pigs older than 6 weeks (e.g. Jeníková et al., 2011; Langkjær et al., 2007). Given that the prepatent period of *C. scrofarum* is 4–8 days in animals inoculated at 5-weeks of age, the experimental data from the present study are consistent with the observational data from field research.

A T-cell mediated immune response is necessary for self-curing of cryptosporidiosis and resistance against reinfection (e.g. Jalovecká et al., 2010; Miller and Schaefer, 2007). It is not known why young piglets are refractory to *C. scrofarum* infection; however, it is unlikely to be the result of acquired immunity since the naïve piglets used in the study were from a *Cryptosporidium*-free farm.

The phenomenon of infection resistance in younger animals is not unique and has been demonstrated in host relationships with other apicomplexan. Suckling rabbits