Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 38 (2008) 1051–1055
www.elsevier.com/locate/ijpara

Fecundity of *Cryptosporidium parvum* is correlated with intracellular levels of the viral symbiont CPV

M.C. Jenkins [a,*], J. Higgins [b], J.E. Abrahante [c], K.E. Kniel [d], C. O'Brien [a], J. Trout [b], C.A. Lancto [c], M.S. Abrahamsen [c], R. Fayer [b]

[a] *Animal Parasitic Diseases Laboratory, ARS, USDA, Beltsville, MD, USA*
[b] *Environmental Microbial Safety Laboratory, ARS, USDA, Beltsville, MD, USA*
[c] *Department of Veterinary and Biomedical Sciences, University of Minnesota, USA*
[d] *Department of Animal & Food Sciences, University of Delaware, USA*

Received 18 September 2007; received in revised form 9 November 2007; accepted 13 November 2007

Abstract

Differences in the virulence and fecundity of *Cryptosporidium parvum* isolates have been observed by several researchers studying cryptosporidiosis. The purpose of the present study was to determine if there was a correlation between intracellular levels of the viral symbiont CPV in *C. parvum* and fecundity of two isolates of the parasite, namely *C. parvum* Beltsville (B) and *C. parvum* Iowa (I). Dairy calves infected with 10^6 *C. parvum*-B excreted 5-fold more oocysts compared with calves infected with the same number of *C. parvum*-I oocysts. The increased fecundity of the former strain was corroborated by semi-quantitative PCR assay of DNA isolated from cell cultures infected with either *C. parvum*-B or *C. parvum*-I. Quantitative reverse transcriptase-PCR analysis of viral RNA revealed a 3-fold greater number of CPV in *C. parvum*-B compared with *C. parvum*-I oocysts. These findings may indicate a role for CPV in fecundity and possibly virulence of *C. parvum*.

Keywords: *Cryptosporidium parvum*; Oocysts; Fecundity; Viral symbiont; Virus

1. Introduction

Viruses have been found in a number of protozoa, including *Cryptosporidium* (CPV; Khramtsov et al., 1997, 2000; Xiao et al., 2001), *Giardia* (GLV; Wang and Wang, 1986a; De Jonckheere and Gordts, 1987; Miller et al., 1988a), *Trichomonas* (TVV; Wang and Wang, 1986b; Tai et al., 1993; Benchimol et al., 2002a,c), *Leishmania* (LRV; Tarr et al., 1988; Widmer et al., 1990), *Eimeria* (Revets et al., 1989; Lee and Fernando, 1999; del Cacho et al., 2001) and *Babesia* (Johnston et al., 1991). In general, the viruses are ~30 nm in diameter and either icosahedral or spherical in shape. A capsid surrounds a double- or single-stranded 5–7 kb RNA genome that codes for two proteins- a viral RNA-dependent RNA polymerase and a 80–100 kDa capsid protein (Tarr et al., 1988; Wang and Wang, 1991; Wang et al., 1993; Khoshnan et al., 1994; Su and Tai, 1996; Chung et al., 1998; Bessarab et al., 2000; Zamora et al., 2000). While virus-free isolates of *Giardia* (De Jonckheere and Gordts, 1987), *Cryptosporidium* (Khramtsov et al., 2000), *Trichimonas* (Weber et al., 2003) and *Leishmania* (Tarr et al., 1988) have been identified, there have been several descriptions of two or more distinct double-stranded RNA (dsRNA) viruses within a single population (Tai et al., 1996; Benchimol et al., 2002a,b,c). The function of RNA viruses in protozoa is unknown, however, a few reports described a relationship between parasite phenotype and viral presence. For example, cultured *Trichomonas vaginalis* harboring TVV express a surface

* Corresponding author. Tel.: +1 301 504 8054; fax: +1 301 504 5306.
E-mail address: Mark.Jenkins@ars.usda.gov (M.C. Jenkins).

0020-7519/$34.00
doi:10.1016/j.ijpara.2007.11.005

immunogen and undergo phenotypic variation that is not observed in virus-free parasites (Wang et al., 1987). Also, infection of virus-free strains of *Giardia lamblia* with GLV causes lower reproductive rates of the protozoan (Miller et al., 1988a). Different isolates of *Cryptosporidium parvum* differ in the number of oocysts required to establish an infection. For example, 10-fold differences in the 50% infectious dose (ID_{50}) of *C. parvum* strains for humans have been reported (Okhuysen et al., 1999; Teunis et al., 2002). The reason for the observed differences in infectivity is unknown, but it has been speculated that virulence factors such as proteins involved in attachment or modulation of the immune response are involved (Okhuysen and Chappell, 2002). In our studies, dairy calves infected with identical numbers of *C. parvum* Beltsville (B) or *C. parvum* Iowa (I) oocysts consistently produce 5- to 10-fold greater numbers of *C. parvum*-B than *C. parvum*-I oocysts (unpublished observations). *C. parvum* virus (CPV) was found in both isolates. The purpose of the present study was to determine whether a correlation existed between the level of CPV and fecundity of the *C. parvum*-B and *C. parvum*-I strains in dairy calves.

2. Materials and methods

2.1. Comparison of fecundity of C. parvum isolates

To compare isolate fecundity, six dairy calves (1–2 days of age, three calves/strain) were each orally inoculated with 10^6 *C. parvum*-B or *C. parvum*-I strain oocysts. The oocysts used in the challenge infection were derived from standard propagation procedures (O'Brien and Jenkins, 2007) and enumerated by counting in triplicate with the aid of a hemacytometer. The calves were obtained at birth from the Beltsville Agricultural Research Centre (BARC) dairy and were handled according to animal care guidelines as approved by the BARC Animal Care and Use Committee. The calves were housed in metabolic crates from days 4–10 after infection to allow for daily 24 h collection of fecal material in 20-L containers. The volume of feces was measured to allow for calculation of total oocyst output. Oocyst counts were performed by removing three 50 ml samples from each container after thorough mixing to ensure heterogeneity of the excreted fecal material. The samples were processed for *C. parvum* oocysts using CsCl centrifugation (Kilani and Sekla, 1987). The concentration of oocysts was determined by immunofluorescent staining using a commercial *Cryptosporidium* detection kit (Mer*IF*luour, Meridian Diagnostics) and counting three 10 µl aliquots using epifluorescence microscopy. Mean daily and total oocyst production of calves infected with *C. parvum*-B were compared with mean daily or total oocyst production of calves infected with *C. parvum*-I in an unpaired one-way *t*-test for significance using the GraphPad In-Stat (GraphPad Software, San Diego, CA).

2.2. RNA extraction from C. parvum oocysts

Aliquots of 10^9 *C. parvum*-B or *C. parvum*-I oocysts were suspended in 100 µl sterile water containing 2 U RNase inhibitor (Invitrogen, Gaithersburg, MD) and subjected to three cycles of freezing and thawing at −70 and 50 °C to lyse the oocysts. The lysate was subjected to RNA extraction using the Qiagen viral RNA kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA was treated with DNase (Invitrogen), by incubation at 37 °C for a minimum of 3 days, followed by DNase inactivation and storage at −70 °C until used as a template for reverse transcriptase (RT)-PCR.

2.3. Quantitative RT-PCR for viral symbiont CPV RNA and C. parvum 18S rRNA

CPV RNA levels were compared between *C. parvum*-B and *C. parvum*-I oocysts using quantitative RT-PCR. To control for slight differences in oocyst numbers, the CPV RT-PCR signal was normalized to the 18S rRNA signal generated by RT-PCR using *C. parvum*-specific primers. Comparative quantitative PCR was performed using a Stratagene Mx3000 P quantitative instrument. The iScript One-Step RT-PCR with SYBR Green kit (BioRad, Hercules, CA) was utilized for quantitative experiments. In brief, 25 µl reactions contained a mix consisting of 12.5 µl 2× SYBR master mix, 2.5 µl of template, 0.5 µl of iScript enzyme, 6.5 µl of water and 1.5 µl of each primer (2.5 µM forward and reverse CPV primers, or 0.5 µM forward and reverse rRNA primers). Primers for the viral dsRNA were: virsymbFor 5′ TGG TTC CGA TTT TAC CGG AA 3′ and virsymbRev 5′ AAC GAC AAT TAG GAC TCA AAT GAC C 3′ which targeted highly conserved nucleotides 702–721 and 1029–1052 of the CPV dsRNA in *C. parvum* KSU isolate (Genbank Accession No. U95996). Alignment of the target CPV sequence from the KSU, Beltsville (B) and Iowa (I) strains provides sufficient genotypic variation at six loci to distinguish between the three *C. parvum* strains (Fig. 1). Primers for the *C. parvum* 18S rRNA gene were: 995F 5′ TAG AGA TTG GAG GTT GTT CCT 3′ and 1206R 5′ CTC CAC CAA CTA AGA ACG GCC 3′. After the initial cDNA synthesis step at 50 °C for 10 min, an activation/denaturation step at 95 °C for 5 min proceeded, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing/extension at 59 °C for 30 s. Fluorescence was read after the end of each annealing cycle during the PCR phase. After the amplification cycles, a melting curve was run to confirm the existence of a single product. Melting was performed by increasing the temperature in 0.2° increments starting at 55 °C until the temperature reached 95 °C. cDNA made from RNA harvested from *C. parvum*-B or *C. parvum*-I was run in duplicate reactions with CPV or 18S rRNA primers. CPV levels were compared between the two *C. parvum* isolates by linearizing the C_t values (expressed in log scale) of each replicate. The ratio of expression for each