Parasitol Res (2009) 105:775–779
DOI 10.1007/s00436-009-1454-5

ORIGINAL PAPER

Examination for double-stranded RNA viruses in *Trichomonas gallinae* and identification of a novel sequence of a *Trichomonas vaginalis* virus

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Received: 1 April 2009 / Accepted: 20 April 2009 / Published online: 12 May 2009

Abstract To determine if double-stranded RNA (dsRNA) viruses exist and are potential virulence factors in *Trichomonas gallinae*, virus purification via ultracentrifugation was attempted for 12 *T. gallinae* isolates recovered from wild birds. Following purification, virus-like particles were not observed by transmission electron microscopy, nor were dsRNA segments visualized in agarose gels after electrophoresis of extracted RNA from any of the 12 *T. gallinae* isolates. However, virus particles and dsRNA segments were detected from a previously determined virus-infected *T. vaginalis* isolate as a control using identical purification procedures. Subsequent reverse transcription-polymerase chain reaction analysis of the dsRNA of the virus in this isolate revealed a novel sequence of the RNA-dependent RNA polymerase gene of *T. vaginalis* viruses.

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Introduction

Avian trichomonosis, caused by the protozoan *Trichomonas gallinae*, has been reported from several continents and is considered a major disease for numerous avian species in the orders Columbiformes and Falconiformes (Stabler 1954; Forrester and Spalding 2003; Villanua et al. 2006; Gerhold et al. 2007). Previous investigations with *T. gallinae* disclosed a wide spectrum of virulence ranging from subclinical to highly virulent. With highly virulent isolates, a bird can succumb to infection within 14 days of inoculation with a single trichomonad, whereas with avirulent isolates, a bird may fail to seroconvert after inoculation with 1×10^6 organisms (Stabler and Kihara 1954; Honigberg 1979).

The factors affecting the virulence of *T. gallinae* have not entirely been identified; however, previous research disclosed that an avirulent *T. gallinae* isolate exposed to a nucleic acid extract from a clinically virulent isolate demonstrated increased infectivity in pigeons (Honigberg et al. 1971). The transformed avirulent isolate was able to maintain lasting infections in pigeons, whereas the untransformed original avirulent isolate caused a transient infection (Honigberg et al. 1971). Further research suggested that RNA may be partially responsible for the transformation effect (Honigberg 1979); however, the particular type of RNA was not determined. Furfine and Wang (1990)

successfully isolated a double-stranded RNA (dsRNA) virus from the protozoan *Giardia lamblia* (GLV) and subsequently infected virus-free *G. lamblia* with a single-stranded RNA (ssRNA) intermediate of GLV. The investigators disclosed that the ssRNA was a competent replicative intermediate for the GLV dsRNA virus, demonstrating that transfection of RNA from virus-positive to virus-free protozoans could initiate a productive infection.

Numerous investigations on the influence of virulence factors in the biology of *T. vaginalis*, a sexually transmitted protozoan and the cause of human trichomonosis, have been conducted (Alderete et al. 1986; Provenzano et al. 1997; Alderete 1999). Approximately one half of all *T. vaginalis* isolates are infected with dsRNA viruses or virus-like particles (VLPs) described as heterogeneous populations of icosahedral, filamentous, cylindrical, and/or spherical virus particles ranging from 33–200 nm in diameter (Wang and Wang 1985; Benchimol et al. 2002a, b). Similar dsRNA viruses or VLPs have been found in several other protozoa including *Cryptosporidium parvum* (Kniel et al. 2004), *G. lambia* (Wang et al. 1993), and *Leishmania braziliensis* (Widmer et al. 1989).

The presence of dsRNA viruses or VLPs within *T. vaginalis* is associated with expression of immunogenic proteins on the trichomonad surface, variations in protozoal phenotypes, and upregulation of certain proteins, including known virulence factors (Alderete et al. 1986; Wang et al. 1987; Provenzano et al. 1997; Alderete 1999). Although the exact roles of the dsRNA viruses and VLPs are not known, the upregulation of proteins and phenotypic change associated with virus infection suggest that intracellular viruses may be associated with virulence in *T. vaginalis*. Given the reported nucleic acid transformation of *T. gallinae* (Honigberg) and to determine if intracellular viruses exist and are associated with virulence in *T. gallinae*, we investigated the presence of intracellular viruses in both clinically virulent and avirulent *T. gallinae* isolates. During the course of this investigation, a unique sequence encoding the RNA-dependent RNA polymerase of the *T. vaginalis* dsRNA virus was found.

Materials and methods

Parasite isolation

Seven virulent and five avirulent isolates of *T. gallinae* were acquired from oral swabs from several avian species (Table 1). Clinically virulent isolates were cultured from birds with trichomonad-associated lesions (e.g., caseous oral cankers), whereas clinically avirulent isolates were cultured from birds lacking corresponding gross lesions. The isolates initially were cultured in In-Pouch™ TF kits (BioMed Diagnostics, White City, OR, USA), incubated at 37°C, and examined for five consecutive days for trichomonad growth. Further subcultures were performed using Diamond's media (pH 7.0; Diamond 1957) supplemented with 10% heat-inactivated horse serum (HIHS; Sigma–Aldrich, St. Louis, MO, USA). Initially, cultures were supplemented with antibiotics as previously described (Diamond 1957). Once axenic cultures were established, the use of antibiotics was discontinued. Axenic isolates in late logarithmic growth were harvested by centrifugation (750×*g* for 10 min) and cryopreserved in liquid nitrogen using HIHS supplemented with 8% dimethyl sulfoxide (Sigma–Aldrich) until further use.

Virus purification

Approximately 2×10^9 trichomonads from each of the 12 *T. gallinae* isolates and one virus-positive *T. vaginalis* isolate used as a control were harvested by centrifugation at 750×*g* for 20 min at room temperature, resuspended in approximately 30 ml TNM buffer (150 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris, pH 7.5), and subjected to three freeze-thaw cycles followed by sonication for 2 min. The lysate was clarified by centrifugation at least twice at 10,000×*g* for 20 min at 4°C. The supernatant was then pelleted through a 20% sucrose cushion prepared in TNM buffer at 100,000×*g* for 2 h at 4°C. The sediment was resuspended in approximately 5 ml of TNM buffer, equilibrated to a density of 1.35 g/ml with CsCl and centrifuged at 100,000×*g* for 24 h at 4°C. One-milliliter fractions were collected from the bottom of each tube and concentrated and desalted with TNM buffer using Amicon ultra-15 centrifugal filter devices (Millipore Co., Billerica, MA, USA) per the manufacturer's instructions.

Molecular identification

RNA was extracted from 140 µl of the Amicon filter retentate using QIAamp® viral RNA mini kit (Qiagen Inc., Valencia, CA, USA) per the manufacturer's instructions. Extracted RNA was separated by gel electrophoreses using a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. Fragments were excised and the RNA purified using a QIAquick® Gel Extraction kit (Qiagen Inc.). Reverse transcription-polymerase chain reaction (RT-PCR) was performed on gel extracted RNA (*T. vaginalis* positive control) as well as directly from the filter retentate extracted RNA (*T. gallinae* and *T. vaginalis* positive control) using the random decamer primers OPD-1 (5′-ACCGCGAAGG-3′), OPD-2 (5′-GGA CCCAACC-3′), OPD-3 (5′-GTCGCCGTCA-3′), OPD-4 (5′-TCTGGTGAGG-3′), and OPD-5 (5′-TGAGCG GACA-3′). The RT-PCR reaction included 2.5 µl of RNA

Table 1 *Trichomonas gallinae* isolates obtained from free-ranging birds included in the virus purification attempts

Isolate/Host	State of origin (USA)	Clinical virulence
Band-tailed pigeon 1 (*Columba fasciata*)	California	Virulent
Broad-winged hawk 1 (*Buteo platypterus*)	Florida	Virulent
Cooper's hawk 4 (*Accipiter cooperii*)	Arizona	Virulent
Common ground-dove 1 (*Columbina passerina*)	Texas	Avirulent
House finch 1 (*Carpodacus mexicanus*)	Kentucky	Virulent
Mourning dove (*Zenaida macroura*) ATCC 30095[a]	Pennsylvania	Virulent
Mourning dove 11	Georgia	Avirulent
Mourning dove 18	Georgia	Virulent
Mourning dove 21	Kentucky	Virulent
Rock pigeon 20 (*Columba livia*)	Georgia	Avirulent
Rock pigeon 28	Georgia	Avirulent
Rock pigeon ATCC 30230[a]	Undetermined	Avirulent

[a] Obtained from American Type Culture

in a 50-µl reaction containing 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 0.01% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, Madison, WI, USA), 0.75 U *Taq* DNA polymerase (Promega), 0.75 U of AMV reverse transcriptase (Promega) , and 1 µM of primer. Cycling parameters for the amplification were 25°C for 5 min, 42°C for 1 h, 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 36°C for 60 s, 72°C for 90 s, and a final extension at 72°C for 10 min. Amplicons were purified from 2% agarose as per RNA segments. Extracted DNA was then cloned and transformed into competent *Escherichia coli* using a Qiagen PCR Cloning Plus kit per the manufacturer's instructions (Qiagen Inc.). Selected recombinant colonies were cultured overnight in Luria Bertani (LB) broth supplemented with 100 µg/ml ampicillin at 37 C with shaking. Plasmid DNA was purified using a QIAprep® Spin Miniprep kit (Qiagen Inc.) according to the manufacturer's instructions. DNA inserts from recombinant plasmids were sequenced using universal T7 and SP6 primers. Sequences were obtained using an ABI PRISM® 3100 Genetic Analyzer at the Integrated Biotechnology Laboratories, University of Georgia (Athens, GA, USA).

Electron microscopy

To further investigate the presence of viruses, Formvar-coated nickel grids were floated on 40 µl of the Amicon filter retentate. Negative staining was performed using 2% uranyl acetate and grids were observed in a JEOL 1210 electron microscope at 120 kV.

Results

Double-stranded RNA viruses or VLPs were not detected in any of the 12 *T. gallinae* isolates by gel electrophoresis of RNA extractions, RT-PCR, or negative-staining electron microscopy following the virus purification attempts. Not unexpectedly, a ~4.5-kb fragment was detected on gel electrophoresis of the RNA extracted Amicon filter retentate from the *T. vaginalis* virus-infected positive control. Subsequent sequence analysis of a cloned RT-PCR product generated from the 4.5-kb RNA fragment (GenBank FJ997643) using OPD-5 revealed an 81% to 84% nucleotide and an 86% to 90% deduced amino acid identity to four *T. vaginalis* virus RNA-dependent RNA polymerase partial sequences by BLAST analysis (GenBank accessions U08999.1, DQ270032.1, DQ528812.1, and U57898.1). Electron microscopic examination of the Amicon filter retentate of the *T. vaginalis* virus purified material revealed numerous 33-nm diameter icosahedral VLPs (Fig. 1).

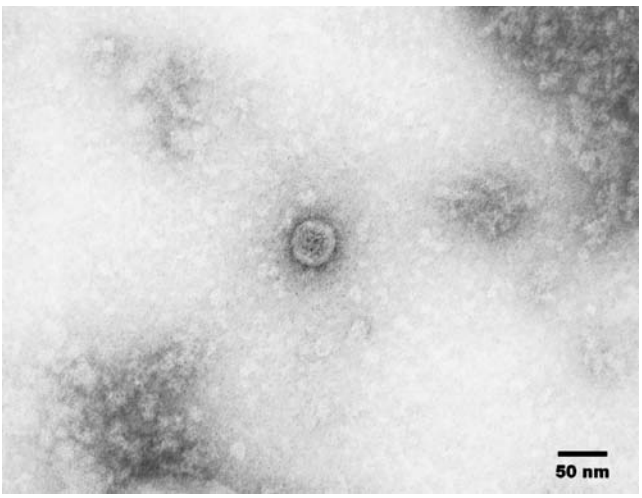

Fig. 1 A virus-like particle from *Trichomonas vaginalis* negatively stained with 2% uranyl acetate and visualized by electron microscopy

Discussion

The inability to detect intracellular dsRNA viruses in the twelve examined *T. gallinae* isolates could be due to the lack of dsRNA viruses within the protozoa or, alternatively, to unique properties in a *T. gallinae* dsRNA virus that does not permit detection using the chosen techniques. Virus purification by ultracentrifugation was successfully performed with a positive control *T. vaginalis* isolate in our laboratory and has been shown to have a high success rate in detecting viruses in other organisms (Benchimol et al. 2002a, b; Poulos et al. 2006). Virus-like particles were detected in *Tritrichomonas fetus* by electron microscopy only after the trichomonads were treated with cytoskeleton-affecting chemicals including colchicine, vinblastine, taxol, nocodazole, and griseofulvin (Vancini and Benchimol 2005). Future attempts to detect viruses in *T. gallinae* should include such protocols. Our findings suggest that intracellular viruses are not associated with nor modulate the virulence of *T. gallinae*. Further research is possibly needed to confirm the presence and determine a role, if any, of currently undetectable viruses in the virulence of *T. gallinae*.

The partial RNA-dependent RNA polymerase sequence of the *T. vaginalis* dsRNA virus identified in this investigation is unique compared to other *T. vaginalis* virus sequences in GenBank. It has been established that the identity between *T. vaginalis* dsRNA virus sequences is variable, which may be expected given *T. vaginalis* isolates are infected with several different dsRNA viruses (Khoshnan and Alderete 1993; Benchimol et al. 2002b). The virus particles observed on electron microscopic examination from the *T. vaginalis* ultracentrifugation retentate are consistent with the size and shape of viruses previously detected in the isolate (Benchimol et al. 2002a).

Acknowledgements This investigation was supported primarily by Cooperative Agreement No. 2001-96130032-CA, Veterinary Services, APHIS, USDA; Cooperative Agreement 01ERAG0013, United States Geological Survey, Biological Resources Division, USDI; and sponsorship of SCWDS by the fish and wildlife agencies of Alabama, Arkansas, Florida, Georgia, Kentucky, Kansas, Louisiana, Maryland, Mississippi, Missouri, North Carolina, Ohio, Puerto Rico, South Carolina, Tennessee, Virginia, and West Virginia. Support from the states to SCWDS was provided in part by the Federal Aid to Wildlife Restoration Act (50 Stat. 917). Additional support was given by Quail Unlimited's Dove Sportsman Society. We thank Danny Watson of the Kentucky Department of Fish Wildlife Resources, Autumn Smith of the Caesar Kleberg Research Institute-Texas A&M University, Jeff Cann from the California Department of Fish and Game, and Bill Mannan from the School of Renewable Natural Resources, University of Arizona for collection of isolates. Additionally, we thank Mary Ard, University of Georgia, for assistance with the electronic magnification.

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