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■ BRIEF COMMUNICATION

# Evaluation of α-Tubulin as an Antigenic and Molecular Probe to Detect *Giardia lamblia*

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**Abstract:** The α/β-tubulin heterodimer is the basic subunit of microtubules in eukaryotes. Polyclonal antibodies specific to recombinant α-tubulin of *Giardia lamblia* were made, and found effective as a probe to specifically detect *G. lamblia* by immunofluorescence assays. Nucleotide sequences of α-tubulin genes were compared between *G. lamblia* WB and GS strains, prototypes of assemblage A and assemblage B, respectively. A set of primers was designed and used to amplify a portion of the α-tubulin gene from *G. lamblia*. PCR-RFLP analysis of this α-tubulin PCR product successfully differentiated *G. lamblia* into 2 distinct groups, assemblages A and B. The results indicate that α-tubulin can be used as a molecular probe to detect *G. lamblia*.

Key words: *Giardia lamblia*, α-tubulin, immunofluorescence assay, PCR-RFLP

*Giardia lamblia* is a significant cause of diarrheal outbreaks worldwide, and infects a variety of mammalian hosts, including humans [1]. Therefore, the development of a precise classification system for this organism is required to monitor possible sources of outbreaks as well as to identify the routes of infections. Two major lineages have been defined for human-derived *G. lamblia*: assemblages A and B [2] (otherwise referred to as groups I- II and groups III-IV [3,4] or as the 'Polish' and 'Belgian' genotypes [5], respectively). Two antigenically distinct *G. lamblia* strains, WB and GS, were established via an infection model using gerbils, and served as prototypes for assemblage A and B, respectively [6]. Later, *G. lamblia* GS was found as the most potent strain in infection of mice [7]. In addition to these 2 groups, 3 additional assemblages of *G. lamblia* were identified from animals: assemblages C and D from dogs [8], and assemblage E from livestock [9]. Furthermore, phylogenetic analysis of 4 housekeeping genes revealed that *G. lamblia* includes at least 7 different lineages, assemblage A to G [10].

Diverse genetic markers that successfully detect or classify *G. lamblia* have been reported. Two sets of primers were designed based on the SSU-rDNA region of *G. lamblia*: JW1 and JW2 [11], and RH11 and RH4 [12]. Primers specific to the *tim* gene encoding triose-1-phosphate isomerase have been shown to differentiate assemblage A from assemblage B [13]. Primers GGL and GGR were made for the unique *G. lamblia* gene encoding giardin [14]. In addition, we have designed a set of primers specific for *GLORF-C4*, which allows classification of *G. lamblia* into 2 assemblages, assemblage A and B [15].

In the present study, we developed tools to detect *G. lamblia* using one of the abundant cytoskeletal components of this eukaryote, i.e., microtubules. We aimed to make an antigenic probe to detect *G. lamblia*, and a molecular tool not only to detect but also differentiate 2 representative *G. lamblia* assemblages. Most of all, α-tubulin comprising microtubules was used as an antigenic probe to detect *G. lamblia*. Searching of the *G. lamblia* genome database identified 2 genes for α-tubulin of *G. lamblia* WB, OFR#112079 and ORF#103676. Comparison of the nucleotide sequences of these ORFs revealed 6 synonymous mutations but no change in the amino acid sequence. Therefore, we used the ORF#103676 for further studies.

*G. lamblia* WB (ATCC#30957; American Type Cellular Collection, Manassas, Virginia, USA) strain was axenically cultivated using modified TYI-S-33 media [16]. A 1,365 bp DNA fragment encoding α-tubulin was amplified from *G. lamblia* WB using α-tubulin-ERI-F (5′-ATTGAATTCATGCGTGAGTGCATCTC-3′: underlined bases denote an EcoRI site) and α-tubulin-NotI-R (5′-GCGCGGCCGCTCGTAGGCGTCGTCCTCC-3′: underlined bases indicate a NotI site) primers, and then cloned into pGEX-4T-1 (Amersham Pharmacia, Buckinghamshire, UK) to produce pZhu30. GST-tagged recombinant α-tubulin protein (GST-α-tubulin) was expressed in *Escherichia coli* BL21 (DE3), and then

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used to immunize Sprague-Dawley rats (3 times at 2 week-intervals, 200 $\mu$g for each immunization).

Specificity of the anti-$\alpha$-tubulin polyclonal antibodies was then examined by western blot analysis. *E. coli* extracts expressing GST-$\alpha$-tubulin were incubated with polyclonal goat anti-GST (Amersham Pharmacia) or rat anti-$\alpha$-tubulin antibodies, and incubated with alkaline phosphatase (AP)-conjugated anti-goat IgG (Sigma, St. Louis, Missouri, USA) and AP-conjugated anti-rat IgG (Sigma), respectively. The immunoreactive protein was visualized using the nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) system (Promega, Madison, Wisconsin, USA). GST-$\alpha$-tubulin was recognized as an immunoreactive protein of 77 kDa by both anti-GST and anti-$\alpha$-tubulin antibodies (Fig. 1A). Incubation of *E. coli* lysates expressing GST-$\alpha$-tubulin with anti-$\alpha$-tubulin antibodies resulted in the appearance of an immunoreactive, non-specific band as indicated with an asterisk in Fig. 1A. Native $\alpha$-tubulin was also detected as a protein of 50 kDa in lysates of *Giardia* trophozoites by western blot using anti-$\alpha$-tubulin antibodies (Fig. 1B).

The polyclonal antibodies against GST-$\alpha$-tubulin were then used for an immunofluorescence assay (IFA) of *Giardia* trophozoites as well as other protozoa, such as *Trichomonas vaginalis*, *Entamoeba histolytica*, and *Naegleria fowleri* (Fig. 2). Trophozoites of *T. vaginalis* T016 were grown in a TYM medium [17]. An axenic culture of *E. histolytica* HM1 : IMSS (ATCC#30459) was grown in TYI-S-33 medium [18], whereas *N. fowleri* Carter NF69 (ATCC#30215) was cultured in CGV medium [19]. Trophozoites were attached to glass slides coated with L-lysine in a humidified chamber. *G. lamblia* and *T. vaginalis* were then fixed with chilled 100% methanol at -28°C for 10 min, and permeabilized with PBS/0.5% Triton X-100 for 10 min. *E. histolytica* and *N. fowleri* were fixed with 10% formalin for 30 min, incubated with 1 M NaOH for 5 min, and then permeabilized with 20% Tween 20 for 5 min at room temperature. After 1 hr-incubation in blocking buffer containing 5% goat serum and 3% bovine serum albumin (BSA), the cells were reacted with mouse anti-$\alpha$-tubulin monoclonal antibodies specific to chicken $\alpha$-tubulin (1 : 300 dilution; Sigma T9026, monoclonal anti-$\alpha$-tubulin antibody produced in mouse clone DM 1A with microtubules from chicken embryo brain used as the immunogen) or rat anti-GST $\alpha$-tubulin polyclonal antibodies (1 : 200 dilution) at 4°C overnight. The cells were incubated with TRITC-conjugated anti-rat IgG or FITC-conjugated anti-mouse IgG (1 : 200 dilution; Jackson ImmunoResearch Lab, West Grove, Pennsylvania, USA) at 37°C for 1 hr. As a control, the fixed trophozoites were incubated with mouse preimmune serum instead of commercial anti-$\alpha$-tubulin antibodies or with rat preimmune serum for polyclonal anti-$\alpha$-tubulin antibodies. The slides were reacted with 1 $\mu$g/ml 4'6-diamidino-2-phenylindole (DAPI; Sigma), and mounted with anti-fade mounting medium (Vectashilde; Vector, Burkingame, California, USA). The slides were then observed using a fluorescence microscope (Zeiss, Oberkochen, Germany).

IFA using commercial $\alpha$-tubulin antibodies or polyclonal anti-$\alpha$-tubulin antibodies showed strong fluorescence mainly against cytoskeletal structures, such as the 8 flagella and a median body of *Giardia* trophozoites (Fig. 2A). In contrast, incubation with mouse preimmune serum or rat preimmune serum did not demonstrate any fluorescence in *Giardia* trophozoites. The 2 antibodies were also tested for IFA with another flagellated protozoan, *T. vaginalis* (Fig. 2B). Reaction of *T. vaginalis* with the monoclonal antibodies against $\alpha$-tubulin generated green fluorescence at the flagella, but no red fluorescent signal was detected in *T. vaginalis* upon incubation with polyclonal antibodies made against GST-$\alpha$-tubulin. In contrast, IFA of *E. histolytica* (Fig. 2C) as well as *N. fowleri* (Fig. 2D) failed to produce any fluorescence using either of the 2 $\alpha$-tubulin antibodies or the preimmune sera.

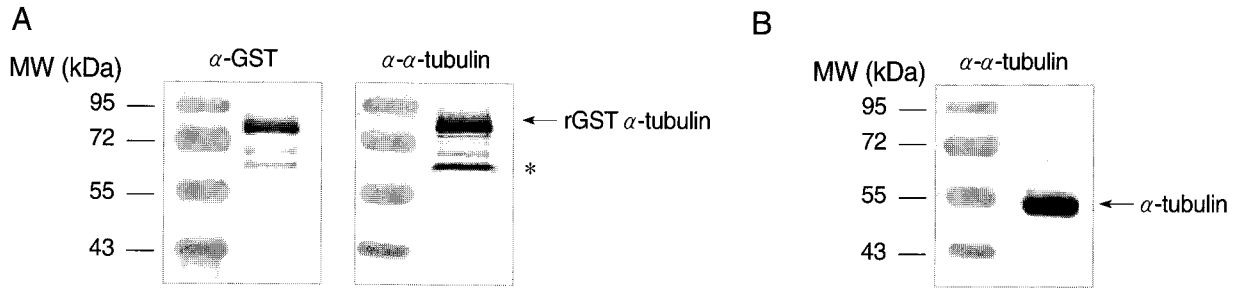


Fig. 1. Specificity of polyclonal antibodies against GST-$\alpha$-tubulin of *G. lamblia*. (A) Extracts of *E. coli* expressing GST-$\alpha$-tubulin were incubated with anti-GST or anti-GST-$\alpha$-tubulin antibodies. (B) Native $\alpha$-tubulin was detected only in *Giardia* trophozoites reacted with anti-GST-$\alpha$-tubulin antibodies. Incubation of *E. coli* lysate expressing GST-$\alpha$-ubulin with anti-GST-$\alpha$-tubulin antibodies resulted in an immunoreactive and non-specific band as indicated by an asterisk.

The results indicate that α-tubulin can be used as an antigenic probe to detect *G. lamblia*. Polyclonal antibodies against α-tubulin of *G. lamblia* did not show any cross-reactivity to other protozoa, *T. vaginalis*, *E. histolytica*, and *N. fowleri*. On the contrary, commercial monoclonal antibodies made against chicken microtubules reacted only with *T. vaginalis* and *G. lamblia*, suggesting that the epitope recognized by these monoclonal antibodies is also present in these 2 flagellates, however, it is absent in other 2 protozoa without flagella. The antigenic region working for these monoclonal antibodies may be more obviously presented in flagellates than amoebic protozoa. Reaction of polyclonal anti-α-tubulin antibodies only with *G. lamblia*, but not with other protozoa, suggests the presence of significant difference in composition of these tubulins.

We next examined whether the α-tubulin gene could be used to categorize *G. lamblia* by PCR-RFLP. Genomic DNA was prepared from axenically cultivated *G. lamblia* WB, GS (ATCC#-50581), and K1 [20] strains. Trophozoites of *T. vaginalis*, *E. histolytica*, and *N. fowleri* were used a source of genomic DNA. In addition, *E. coli* DH5α and *Vibrio vulnificus* (ATCC#29307) were propagated in Luria Bertani broth with different saline concentrations (1 and 2% NaCl, respectively). DNAs of the above strains were prepared by phenol extraction as described [20].

The α-tubulin gene from *G. lamblia* strain GS was amplified by using primers, α-tubulin-ERI-F and α-tubulin-NotI-R, and then sequenced using an automatic DNA sequencer (Applied Biosystems, Foster City, California, USA) to confirm difference in the genes encoding α-tubulins of 2 *G. lamblia* strains, WB and GS. Nucleotide sequences encoding α-tubulin of the GS strain were submitted to GenBank under the accession number, FJ-232540. Alignment of α-tubulin from the GS strain with the WB strain (ORF#103676) indicated 97% and 99% identity in their nucleotide and amino acid sequences, respectively (data not shown).

A search for restriction enzyme sites in the α-tubulin gene revealed that a recognition site for AisSI is present only in the

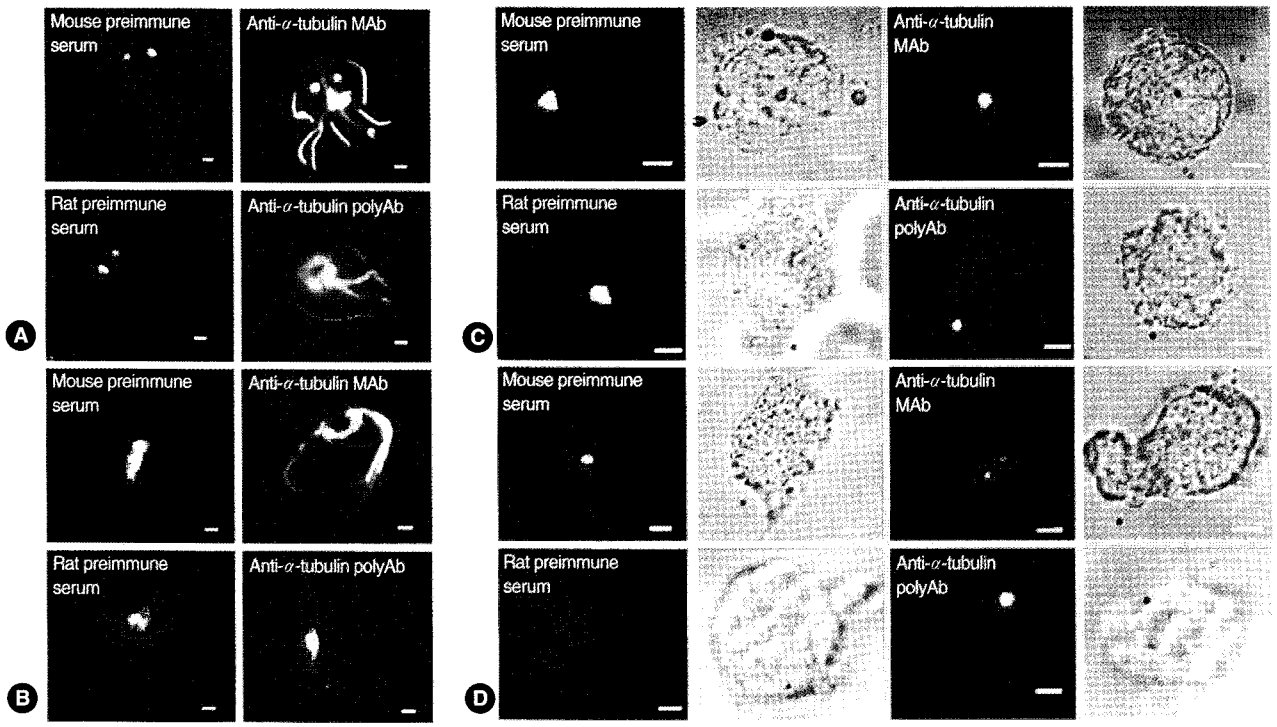


Fig. 2. IFA of *G. lamblia* (A), *T. vaginalis* (B), *E. histolytica* (C), and *N. fowleri* (D) using anti-α-tubulin antibodies. Trophozoites of *G. lamblia* and *T. vaginalis* were fixed with chilled 100% methanol at -20°C for 10 min, and permeabilized with PBS/0.5 % Triton X-100 for 10 min. *E. histolytica* and *N. fowleri* were fixed with 10% formalin for 30 min, incubated with 1 M NaOH for 5 min, and then permeabilized with 20% Tween 20 for 5 min at room temperature. Cells were then reacted with commercially available monoclonal antibodies against chicken α-tubulin (1 : 300 dilution) or with polyclonal antibodies against GST-α-tubulin of *G. lamblia* (1 : 200 dilution). Fixed trophozoites were also reacted with mouse preimmune or rat preimmune sera as controls for commercial monoclonal antibodies or polyclonal GST-α-tubulin antibodies, respectively. The slides were subsequently incubated with either TRITC-conjugated anti-rat IgG or FITC-conjugated anti-mouse IgG (1 : 200 dilution). To visualize nuclei, the cells were treated with 1 μg/ml 4′6-diamidino-2-phenylindole (DAPI), mounted with anti-fade mounting medium (Vectashilde; Vector), and then observed with a Zeiss fluorescent microscope (Zeiss). The bars represent 2 μm.

GS strain, but absent from the WB strain (Fig. 3A). We therefore designed a PCR-RFLP analysis of the α-tubulin gene to distinguish assemblage A from assemblage B of *G. lamblia*. Two primers specific to the α-tubulin gene, genotype F and genotype R, were used to amplify the 3′ portion of α-tubulin from *G. lamblia* WB, GS, and K1 strains. The 2 primers were used to amplify a portion of the α-tubulin genes from 3 different *G. lamblia* strains (WB, GS, and K1) as a PCR product of 754 bp (Fig. 3B). These primers are specific for the α-tubulin gene from *G. lamblia*, because PCR using the same set of primers failed to produce any DNA fragment from genomic DNA of other protozoa (*N. fowleri*, *E. histolytica*, and *T. vaginalis*), or from bacteria (*E. coli* and *V. vulnificus*).

The α-tubulin gene was also evaluated as a probe to differentiate assemblages A and B in *G. lamblia* by PCR-RFLP analysis (Fig. 3C). The resultant PCR products were digested with AisSI (New England Biolab, Ipswich, Massachusetts, USA), which is present only in the PCR product derived from the GS strain, an assemblage B. As expected from the unique presence of the AsiSI site in the α-tubulin gene of the GS strain, the 754 bp PCR product derived from the GS strain was digested into 2 DNA fragments (551 bp and 203 bp), whereas the size of the α-tubulin PCR product from the WB strain was not affected by digestion with AsiSI.

Detection and classification of *G. lamblia* is still an important problem, as this organism has attracted worldwide attention as a source of diarrheal outbreaks. Advanced technologies have been developed to detect *G. lamblia*, which include labeling by fluorescent in situ hybridization and quantum dots-labeled IFA [21], as well as real-time PCR [22]. IFA coupled with immunomagnetic separation is currently one of the main methods to detect *Giardia* [23]. In addition, PCR-RFLP had been used frequently in field investigations on the occurrence of *Giardia* among primary school children [24] or in wild animals [25]. Therefore, the identification and characterization of new markers is needed to improve current detection methods for *G. lamblia*. Our

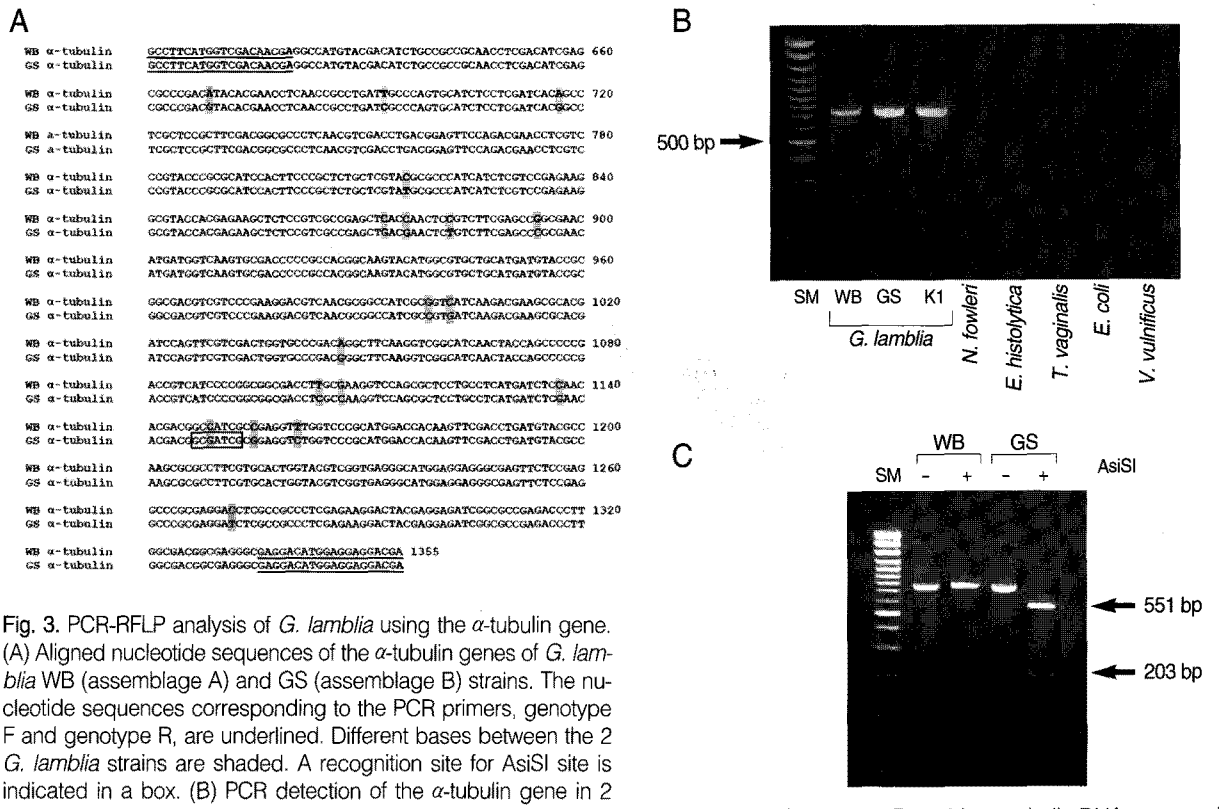


Fig. 3. PCR-RFLP analysis of *G. lamblia* using the α-tubulin gene. (A) Aligned nucleotide sequences of the α-tubulin genes of *G. lamblia* WB (assemblage A) and GS (assemblage B) strains. The nucleotide sequences corresponding to the PCR primers, genotype F and genotype R, are underlined. Different bases between the 2 *G. lamblia* strains are shaded. A recognition site for AsiSI site is indicated in a box. (B) PCR detection of the α-tubulin gene in 2 groups of *G. lamblia*, other protozoa and bacteria. Using 2 primers, genotype F and genotype R, 754 bp α-tubulin DNA was amplified from the following genomic DNAs; lane 1, DNA size marker, 100 bp ladder; lane 2, WB (assemblage A *G. lamblia*); lane 3, GS (assemblage B *G. lamblia*); lane 4, K1 (assemblage B *G. lamblia*); lane 5, *N. fowleri*; lane 6, *E. histolytica*; lane 7, *T. vaginalis*; lane 7, *E. coli* DH5 α and ; lane 7, *V. vulnificus*. (C) Grouping of *G. lamblia* using α-tubulin primers. Using genomic DNAs derived from *G. lamblia* WB and GS strains as templates, PCR was carried out with primers, genotype F and genotype R, and the resultant PCR products were digested with AsiSI endonucleases. lane 1, DNA size marker, 100 bp ladder; lane 2, PCR product of *G. lamblia* WB; lane 3, PCR product of *G. lamblia* WB digested with AsiSI; lane 4, PCR product of *G. lamblia* GS; and lane 5, PCR product of *G. lamblia* GS digested with AsiSI.

study extends the list of molecular markers which can be used to detect and classify *G. lamblia*.

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