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ORIGINAL PAPER

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Use of a novel DNA melting profile assay for the identification of PCR-amplified genomic sequences encoding for variant-specific surface proteins from the clonal GS/M-83-H7 line of *Giardia lamblia*

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Abstract During infections, *Giardia lamblia* undergoes a continuous change of its major surface antigens, the variant-specific surface proteins (VSPs). Many studies on antigenic variation have been performed using *G. lamblia* clone GS/M-83-H7, which expresses surface antigen VSP H7. The present study was focused on the identification and characterization of *vsp* gene sequences within the genome of the clonal *G. lamblia* GS/M-83-H7 line. For this purpose, we applied a PCR which specifically amplified truncated sequences from the 3′-terminal region of the *vsp* genes. Upon cloning, most of the *vsp* gene amplification products were shown to be approximately identical in size and thus could not be distinguished from each other by conventional gel electrophoresis. In order to pre-estimate the sequence complexity within the large panel of *vsp* clones isolated, we elaborated a novel concept which facilitated our large-scale genetic screening approach: PCR products from cloned DNA molecules were generated and then subjected to a DNA melting profile assay based on the use of the LightCycler Instrument. This high-throughput assay system proved to be well suited to monitor sequence differences between the amplification products from closely related *vsp* genes and thus could be used for the primary, sequence-related discrimination of the corresponding clones. After testing 50 candidates, *vsp* clones could be divided into five groups, each characterized by an individual DNA melting profile of the corresponding amplification products. Sequence analysis of some of these 50 candidates confirmed data from the aforementioned assay in that clones were demonstrated to be identical within, but different between, the distinct groups. The nucleotide and deduced amino acid sequences of five representative *vsp* clones showed high similarities both among each other and also with the corresponding gene segment of the variant-specific surface antigen (VSP H7) expressed by the original GS/M-83-H7 variant type. Furthermore, three of the genomic *vsp* sequences turned out to be identical to *vsp* sequences that represented previously characterized transcription products from in vivo- or in vitro-switched GS/M-83-H7 trophozoites. In conclusion, the DNA melting profile assay seems to be a versatile tool for the PCR-based genotyping of moderately or highly diversified sequence orthologues.

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Introduction

Giardia lamblia is an intestinal protozoan parasite of humans and various animals. Manifestation of the disease varies from asymptomatic carriage to severe diarrhoea and malabsorption. Several studies revealed that the immune response to a *G. lamblia* infection is strongly influenced by the parasite's ability to continuously change its major surface antigen, the variant-specific surface protein (VSP) (Nash 1992). Surface antigen alterations have been extensively studied in *G. lamblia* clone GS/M-83-H7, which originally expressed surface antigen VSP H7 (Müller and Gottstein 1998).

In a recent overall view based on a large selection of in vivo- and in vitro-derived *G. lamblia* GS/M-83-H7 populations, highly diversified *vsp* gene expression could be demonstrated on antigenic switching of the parasites (Bienz et al. 2001). In this study, antigenic variation of the parasite in the mouse infection model was revealed to be a continuous process associated with the consecutive appearance of relatively distinct sets of *vsp* transcripts.

In the present study, we performed a *vsp*-gene specific PCR on genomic parasite DNA to identify further *vsp* gene sequences from *G. lamblia* GS/M-83-H7. This PCR-based screening included a fluorometric DNA melting profile assay which allowed primary sequence-related discrimination of cloned *vsp* gene amplification

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products in a large format. The *vsp* sequences identified proved to be closely related to *vsp H7* and similar other *vsp* segments previously identified within *G. lamblia* GS/M-83-H7 (Bienz et al. 2001).

Materials and methods

Parasites

The origin, axenization and cloning of the *G. lamblia* clone GS/M-83-H7 have been described by Aggarwal et al. (1989). This clone expresses a major 72 kDa antigen (VSP H7) on its surface which is recognized by monoclonal antibody G10/4. Trophozoites from a clonal GS/M-83-H7 line of *G. lamblia* were cultivated in modified TYI-S-33 medium with antibiotics as previously described (Keister 1983).

Identification and molecular analysis of genomic *vsp* clones

Selective PCR amplification of genomic *vsp* gene sequences was achieved using previously described (Bienz et al. 2001) *vsp* gene-specific forward primer, *vsp-forward* (5′-GCAGATCTC*ACIA-AYGGIGTITGYACIGC*-3′; positions I = deoxyinosine, Y = C,T) containing a Bgl2 restriction site (underlined) followed by nucleotides [nts] 1147–1166 (in italics) of the *vsp H7* gene and a *vsp* gene-specific reverse primer *vsp-rev1* (5′-CGGCGGCCGC**TCA**-*GAACCACCAGCAGAGGAA*-3′). The latter contained a *Not*1 restriction site (underlined) as anchor sequence including the stop codon (in bold) and a sequence complementary to nts 1636–1653 (in italics) of the *vsp H7* gene. PCR was performed in five cycles comprising 94°C, 30 s; 38°C, 30 s; 72°C, 150 s and subsequently 30 cycles comprising 94°C, 30 s; 60°C, 30 s; 72°C, 150 s with a final 15 min extension at 72°C. Introduction of artificial mutations during amplification was minimized by using the Expand High Fidelity PCR System (Roche Diagnostics, Basel, Switzerland) for all PCRs.

All recombinant DNA methods, unless otherwise stated, were those of Sambrook et al. (1989). Amplified DNA was cloned into the pGEMeasy vector (Promega, Madison, Wis.) by using *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) as bacterial host. Sizes from cloned DNA inserts were assessed as follows: bacterial colonies harbouring plasmid clones were touched with a sterile pipette tip and bacterial cells adhering to the tip were transferred into 100 µl TNN lysis buffer (10 mM NaOH, 0.5% Tween 20, 0.5% Nonidet P40). After a 5 min boiling step, 1 µl of the resulting bacterial lysates (containing recombinant plasmids) was used for PCR (50 µl reactions) as described above. Respective PCR products representing cloned inserts were purified by using Concert Nucleic Acid purification system (GibcoBRL, Basel, Switzerland) and amplicon sizes were determined by electrophoresis in 2% agarose gels.

Additionally, amplicons (solubilized in distilled H_2O) were subjected to a DNA melting profile assay which permits sequence-related characterization of the PCR products by their melting behaviour. The assay was performed in the LightCycler real-time, on-line PCR system (Roche Diagnostics, Basel, Switzerland) which had been developed to fluorometrically quantitate PCR amplification products (Wittwer et al. 1997). DNA quantification in this system includes the staining of amplicons with DNA double-strand-specific fluorescent dye SYBR Green 1 (Molecular Probes, Leiden, The Netherlands). The DNA melting profiles of amplicons are acquired by the gradual heating of the samples and the continuous measurement of the loss of fluorescence caused by melting double-stranded DNA and the concomitant release of SYBR Green 1 (Ririe et al. 1997). The shape of such a DNA melting profile is a function of the GC/AT ratio of a given nt sequence. The DNA melting profile assay was done as follows: 50 ng of purified *vsp* gene amplification product was dissolved in 20 µl 0.5×PCR buffer containing 1.5 mM $MgCl_2$ and 0.5×SYBR Green 1 and then incubated for 20 s at 99°C (denaturation of PCR product). Subsequently, the temperature was reduced to 30°C (renaturation of PCR products and binding of SYBR Green 1) and finally gradually increased to 95°C at a temperature transition rate of 0.1°C/s with continuous acquisition (determination of the melting profile by measuring loss of fluorescence at 520 nm). Data from the DNA melting profile assay were processed by using the LightCycler standard software (version 3.5.3) of the LightCycler Instrument and were graphically illustrated by showing rates of change in fluorescence as a function of temperature (–d[F1]/dT).

For sequencing of cloned *vsp* gene segments, DNA preparations from corresponding recombinant plasmids were done by using E.Z.N.A. Plasmid Midi Kit system (Peqlab Biotechnologies, Erlangen, Germany). Analysis was done using a commercial sequencing service (Solvias, Basle, Switzerland) and was based on the use of M13(-20) and M13rev primers as recommended by the manufacturer of the pGEMeasy vector system.

Sequence alignments

Alignment of nucleotide sequences (see Fig. 2A) and comparison of the corresponding amino acid (aa) sequences (see Fig. 2B) from the *vsp* clones isolated were done using MultAlin and the ESPript1.9 computer software, available at the ExPASy Molecular Biology Server using the Blosum62 symbol comparison table (Henikoff and

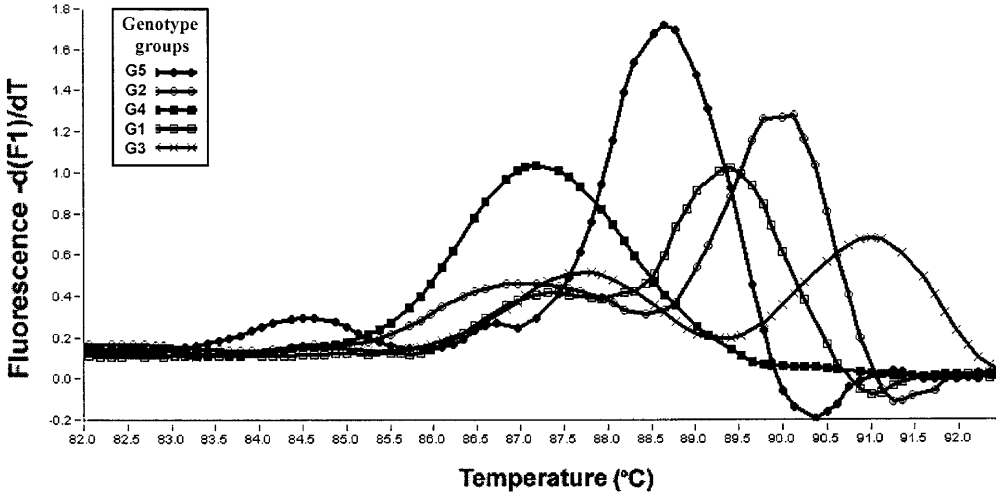

Fig. 1 LightCycler-based determination of DNA melting profile from cloned *vsp* amplification products. Profiles from *vsp* clones *G1–G5*, each representing a distinct group of clones with identical DNA melting profiles, are shown by giving rates of change in fluorescence as a function of temperature (rates given as – d[F1]/dT)

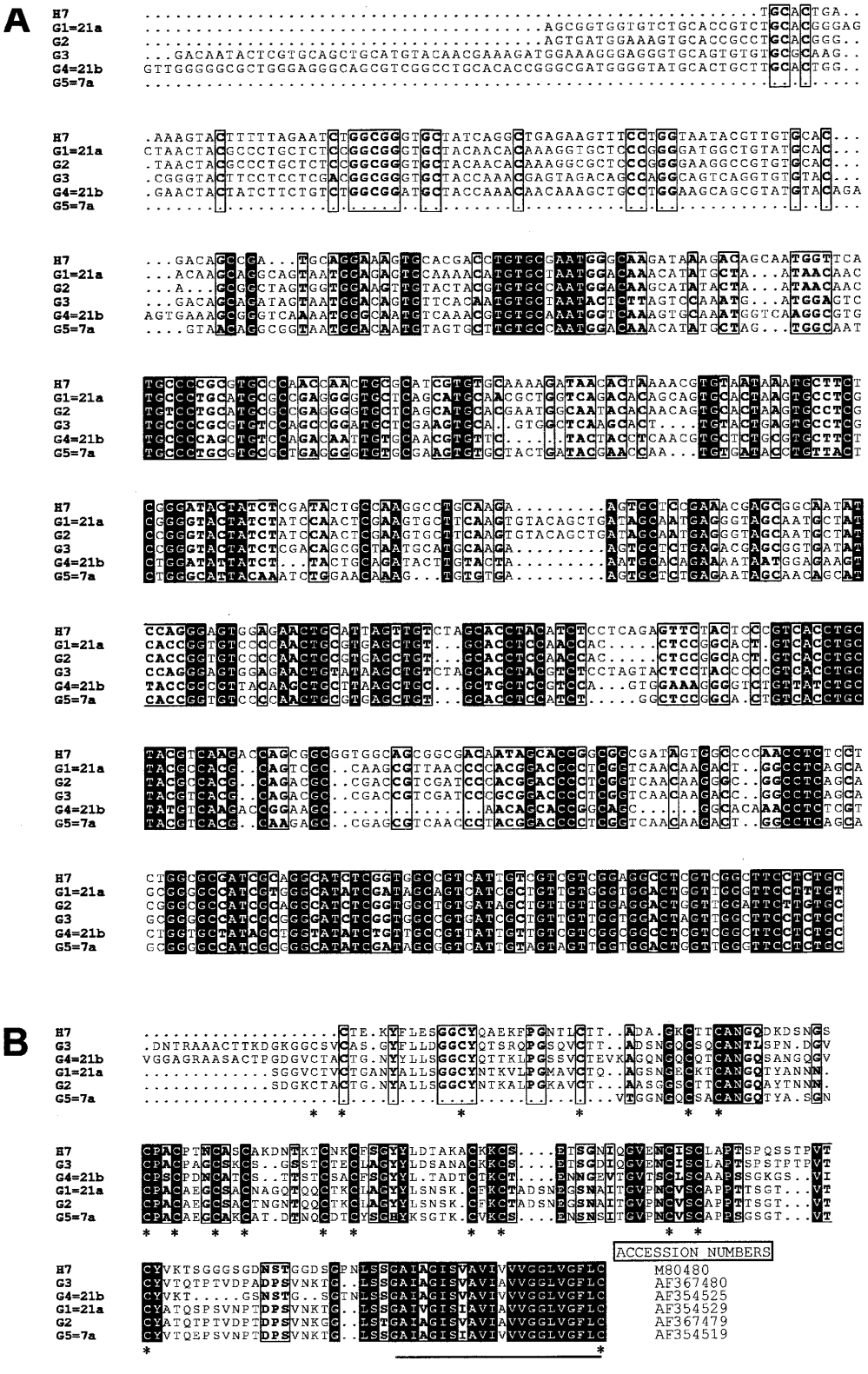

Fig. 2 Alignment of genomic nucleotide (nt) sequences from *vsp* gene segments *H7* (indicates *vsp H7*) and *G1–G5* (**A**), encoding amino acid (aa) sequences from C-terminal regions of H7 (indicates VSP H7) and subvariant VSPs G1–G5 (**B**) from *G. lamblia* clone GS/M-83-H7. Sequences *G1–G5* each represent a *vsp* clone from groups 1–5 as defined by DNA melting profile assay (see Fig. 1). Invariant positions are highlighted (*black background*). *Open boxes* indicate regions of high sequence similarities (nt/aa positions identical among the majority of the sequences are given in *bold*). *Dots* represent alignment gaps. In **B**, *asterisks* mark positions with frequent occurrence of cysteines and a *black horizontal bar* indicates the highly conserved transmembrane segment. In panel **B**, GenBank accession numbers are indicated behind the corresponding aa sequences

Henikoff 1992). Alignment of the above-mentioned sequences with the gene sequence *vsp H7* and the aa sequence VSP H7 (GenBank accession number M80480) was done with the ClustalX1.8.1 computer program using the Risler symbol comparison table (Risler et al. 1988).

Results and discussion

Vsp gene sequences were amplified by PCR from the genomic DNA of in vitro cultivated trophozoites from

clonal *G. lamblia* GS/M-83-H7 line. For DNA amplification, we used a degenerate forward primer, *vsp-forward*, targeting a relatively conserved region of *vsp H7* (nt positions 1147–1166) and a reverse primer, *vsp-rev1*, complementary to nt positions 1636–1653 inside the *vsp H7* region encoding for the highly conserved transmembrane stretch of the corresponding surface protein (Bienz et al. 2001). Amplified DNA molecules were cloned into the plasmid vector pGEMeasy and 50 *vsp* clones were further investigated.

In the next step, sizes of the 50 cloned DNA molecules were monitored by using the above-described PCR for the amplification of inserted DNA and subsequent analysis of amplicons by agarose gel electrophoresis (data not shown). In this analysis, amplicons from 45 clones turned out to be gel-electrophoretically quasi-indistinguishable in that they all migrated as bands of approximately 500 bp. The remaining five clones exhibited amplification products which all had a size of approximately 380 bp.

In order to enable a pre-estimation of the sequence complexity within the large group of genomic *vsp* clones isolated, a high-throughput and sequence-related examination of the DNA inserts was performed. For this purpose, PCR products representing the different inserts were subjected to a DNA melting profile assay (for examples see Fig. 1). All amplification products exhibited a rather complex melting profile consisting of at least two characteristic melting peaks in the higher temperature range (between about 83°C and 93°C). This indicated that, under the given conditions, the amplified DNA molecules did not melt at a unique temperature but contained individual sequence substructures which melted at different temperatures. Comparison of the complex melting profiles revealed that the panel of 50 *vsp* clones investigated subdivide into five distinct genotype groups, named *G1–G5* (Fig. 1).

Data from DNA sequencing (Fig. 2A) indicated that three representative clones from groups *G1* to *G5* contained *vsp* gene segments which were identical within, but distinct between, the individual groups. Sequences *G1–G5* encoded for aa sequences which exhibit between 59% (G3) and 33% (G5) identity to the corresponding region of VSP H7 and share typical structural elements of the C-terminal region of the *G. lamblia* VSPs (Fig. 2B). The most striking common features, a high content of cysteine residues partially located within 4-aa CXXC motifs and the transmembrane domain of these surface proteins, were identified. The aa sequences encoded by clones *G1–G4* also contained a 4-aa GGCY that could represent an additional marker sequence of the VSP family (Nash et al. 1995). Furthermore, genomic *vsp* sequences from clones *G1*, *G4* and *G5* proved to be identical to previously identified *vsp* sequences *21a*, *21b* and *7a* representing transcription products from trophozoites that had undergone antigenic variation during a *G. lamblia* clone GS/M-83-H7 infection in mice (Bienz et al. 2001).

In conclusion, we elaborated a DNA melting profile assay which was shown to be well suited for a primary sequence-related discrimination of gel-electrophoretically indistinguishable PCR amplification products. This assay allowed, for example, a clear discrimination of amplicons from clones *G1* and *G2* which turned out to be nearly identical in both size (495 bp [G1] versus 489 bp [G2]) and nt composition (approximately 87% sequence identity). Another examination (data not shown), not related to the present study, indicated that this assay is most likely not sensitive enough to detect single, or low-frequency, nucleotide differences within PCR-amplified, orthologous DNA molecules. Accordingly, our approach may not have discriminated between clones which contained nearly identical *vsp* sequences. Despite these putative experimental constraints, our data suggest that the DNA melting profile assay is a versatile tool for those investigations designed to demonstrate the genetic complexity within moderately, or highly, diversified gene families. For such purposes, this rapid test may be of greater practicability than established PCR-based genotyping methods such as the highly sensitive PCR/single-strand conformation polymorphism (PCR/SSCP) analysis (Miterski et al. 2000).

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