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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 10
ACT: 519 PRESENTACION	

Abogado(a)/Señor(a)
JUAN CARLOS CUESTA QUINTERO

Asunto:	Radicación:	11-82854- -10
	Trámite:	11
	Evento:	378
	Actuación:	519
	Oficio:	14707
	Folios:	10

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2009/055470				
Fecha de Presentación Internacional	02/12/2009				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

Descripción:	11-082854-00000-0000	01/Julio/2011
Reivindicaciones:	11-082854-00000-0000	01/Julio/2011
	11-082854-00009-0000	29/Julio/2013
Dibujos:	11-082854-00000-0000	01/Julio/2011
Secuencias:	11-082854-00000-0000	01/Julio/2011
Respuesta del solicitante:	11-082854-00007-0000	06/Mayo/2013
Prioridad:	US 61/119.058	02/Diciembre/2008

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Modificaciones aceptadas (Art 34 D 486 y Art 19, 22 y 34 PCT)

Se aceptan las modificaciones a las reivindicaciones en el radicado N° 11-082854-00009-0000 ya que no amplían la materia inicialmente presentada, y sobre dicho capítulo se realiza el análisis de patentabilidad.

4. Presentación de solicitudes Fraccionarias (Art 36 D 486)

Se acepta para examen la solicitud fraccionaria presentada e identificada como 2013-112934, porque no implica ampliación de la materia contenida en la solicitud inicial.

5. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada. En la presente solicitud se estudiarán las 8 reivindicaciones en el radicado No. 13-112934-00009-0000.

Título de la solicitud: "Protozoo modificado que expresa al menos dos proteínas variables de superficie (VSP)"

El problema planteado en esta solicitud consiste en la necesidad suministrar vacunas útiles para el tratamiento de infecciones producidas por protozoarios.

Para resolver este problema técnico la solicitud en estudio proporciona protozoos modificados que expresan más de una proteína variable de superficie, vacuna que lo comprende, línea de hibridoma, anticuerpo monoclonal que reconoce las proteínas, procedimientos, usos y métodos.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 61 K 39/002; C 12 N 1/11.

6. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

Las reivindicación 8 no es patentable, de acuerdo con el Art 14, puesto que hace referencia al uso del protozoo modificado para la fabricación de un medicamento.

7. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara puesto que:

- No proporciona el número de depósito del microorganismo
- No identifica las proteínas de superficie que son expresadas en la superficie del protozoo
- No identifica el mecanismo mediante el cual los genes de las enzimas Dicer y RdRP son silenciados. A partir de la descripción se entiende que los genes son silenciados mediante la introducción de iRNA, sin embargo el capítulo reivindicatorio es muy general y no señala dicha característica esencial junto con las secuencias útiles para el silenciamiento de los genes. El solicitante deberá indicar si el silenciamiento se hace por dicho mecanismo, y cuales secuencias de

iRNA silencian cada gen, esto con el fin de hacer que el capítulo reivindicatorio sea claro sin hacer referencia a la descripción. Dichas observaciones aplican también para las reivindicaciones 3 a 7.

La reivindicación 2 (antigua reivindicación 1) no es clara puesto que presenta una falsa dependencia de la reivindicación 1, dicha reclamación es general y no presenta las características esenciales del protozoo, es decir el número de depósito y las modificaciones presentadas dentro de dicho microorganismo.

La reivindicación 6 no es clara porque no especifica cual es el “mecanismo de variación antigénica”, el solicitante debe proporcionar las características esenciales del microorganismo reclamado, tal como ya se mencionó.

Descripción (Art 28 D 486)

Debido a que la solicitud se refiere a material biológico, es necesario presentar el Certificado de Depósito para el protozoo modificado.

8. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: 02/Diciembre/2008, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D2	Nature 456, 750-754	Antigenic variation in Giardia lamblia is regulated by RNA interference	11/Diciembre/2008

El documento D2¹ la modificación de Giardia intestinalis (Pág. 750 Resumen), y los efectos del silenciamiento de los genes Dicer y RdRP en la expresión de más de una proteína variable de superficie Resumen Pág. 750.

9. Examen de Patentabilidad (Art 14 D486)

Novedad (Art16 D486)

La reivindicación 1 consiste en “Protozoo parasito modificado, caracterizado por que comprende la expresión simultánea y permanente en su superficie de más de una proteína variable de superficie (VSP) y comprende la expresión reducida de las enzimas seleccionadas del grupo comprendido por Dicer, ARN polimerasa RNA dependiente (RdRP) y ambas”

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D2
Protozoo parasito	Giardia intestinalis (Pág. 750 Resumen)
Expresión simultánea y permanente en su superficie de más de una proteína variable de superficie (VSP) y comprende la expresión reducida de las enzimas seleccionadas del grupo comprendido por Dicer y/o ARN polimerasa RNA dependiente (RdRP)	Silenciamiento de genes para Dicer y RdRP y la expresión de más de una proteína variable de superficie (Pág. 752 Col. 2 Párr. [04] y Figura 3b Pág. 753)

Las características esenciales de la reivindicación 1 habían sido divulgadas previamente en el documento D2, el cual enseña el parasito protozoario Giardia intestinalis (Pág. 750 Resumen) que presenta el silenciamiento de genes para Dicer y RdRP y la expresión de más de una proteína variable de superficie (Pág. 752 Col. 2 Párr. [04] y Figura 3b Pág. 753) En consecuencia, la reivindicación 1 no es nueva según lo divulgado en el documento D1.

1URL: <http://www.nature.com/nature/journal/v456/n7223/abs/nature07585.html>

Las reivindicaciones dependientes 2 a 7 no presentan características técnicas adicionales, por lo cual se considera tampoco cumplen con el requisito de novedad.

10. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D2	Nature 456, 750-754	1 a 7	Novedad

11.Respuesta a los argumentos del señor solicitante

Argumentos con respecto la calidad, concisión y sustento

La solicitante argumenta que las objeciones de claridad se ven superadas puesto que el nuevo capítulo reivindicatorio incluye las secuencias ahora reclamadas en la reivindicación 3. Sin embargo, las modificaciones del capítulo reivindicatorio en el radicado N° 11-082854-00009-0000 del 29/Julio/2013 no contienen dichas secuencias. Se aclara a el solicitante, que las las objeciones a la claridad y sustento de la solicitud persisten, puesto que el capítulo reivindicatorio no define el mecanismo de silenciamiento de los genes mencionados, y por otra parte dicho protozoo no se encuentra correctamente caracterizado, puesto que no se proporciona el numero de depósito del protozoo modificado, ni cuáles son las modificaciones estructurales realizadas sobre el mismo, es decir cuáles son las proteínas (con sus respectivas secuencias) que se están expresando simultáneamente el parásito de manera que presente la actividad antigénica deseada.

Argumentos con respecto a la novedad de la solicitud

La solicitante afirma que la característica esencial que provee novedad a la solicitud es el silenciamiento de los genes RdRP y/o el gen Dicer, de manera que el parásito expresa una variedad de proteínas de superficie de manera simultánea y permanente. Las modificaciones al capítulo reivindicatorio realizadas en el radicado N° 11-082854-00009-0000 del 29/Julio/2013 superan la objeción previamente presentada por esta oficina, sin embargo tal como se señala en el análisis de patentabilidad, dicho concepto no es nuevo frente al documento D2, el cual divulga todas las características esenciales de las reivindicaciones presentadas por el solicitante.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-08-13

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ARTICLES

Antigenic variation in *Giardia lamblia* is regulated by RNA interference

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Giardia lamblia (also called *Giardia intestinalis*) is one of the most common intestinal parasites of humans. To evade the host's immune response, *Giardia* undergoes antigenic variation—a process that allows the parasite to develop chronic and recurrent infections. From a repertoire of ~190 variant-specific surface protein (VSP)-coding genes, *Giardia* expresses only one VSP on the surface of each parasite at a particular time, but spontaneously switches to a different VSP by unknown mechanisms. Here we show that regulation of VSP expression involves a system comprising RNA-dependent RNA polymerase, Dicer and Argonaute, known components of the RNA interference machinery. Clones expressing a single surface antigen efficiently transcribe several VSP genes but only accumulate transcripts encoding the VSP to be expressed. Detection of antisense RNAs corresponding to the silenced VSP genes and small RNAs from the silenced but not for the expressed *vsp* implicate the RNA interference pathway in antigenic variation. Remarkably, silencing of Dicer and RNA-dependent RNA polymerase leads to a change from single to multiple VSP expression in individual parasites.

Clonal phenotypic variation of surface-exposed antigenic determinants (antigenic variation) is a major evasion process developed by several microorganisms, which use different mechanisms to switch the expression of their surface antigens to maintain chronic infections under the continuous immune pressure generated by their hosts¹. The protozoan *G. lamblia* also undergoes antigenic variation^{2,3}. This binucleated, flagellated parasite not only is one of the most common causes of human intestinal disease but also is of great biological interest because it derives from the earliest branch of the eukaryotic line of descent^{2,4}. In *Giardia*, antigenic variation accounts for the variable and/or persistent course of some infections as well as for the propensity for multiple re-infections, and involves a family of VSPs^{2,3}. VSPs cover the entire surface of the trophozoite and are the main antigens recognized by the host immune system³. VSPs vary in size from 30 kDa to 200 kDa; they possess a variable cysteine-rich amino-terminal region and a conserved carboxy-terminal region that includes a hydrophobic transmembrane domain and a short cytosolic tail³. The parasite genome encodes a repertoire of about 190 VSP genes⁴, but only one VSP is expressed at any given time on the surface of individual trophozoites^{2–8}. Switching to the expression of another VSP occurs once every 6–13 generations, even in the absence of any immunological pressure⁷. Similar to most other *G. lamblia* genes, VSP genes have no introns and their 5' upstream regions are relatively short and have been found to have limited or no sequence conservation. Moreover, there are no typical eukaryotic promoters present in these regions. The 3' downstream regions from *Giardia* genes, including VSP-coding genes, also tend to be short, typically 0–30 nucleotides long. So far, neither gene-rearrangement processes nor promoter-dependent switch-on/switch-off mechanisms have been demonstrated to be involved in *Giardia*'s antigenic switching^{2,3,7}.

To delve into the molecular mechanisms involved in *Giardia*'s antigenic variation, we initially performed nuclear run-on experiments to determine whether regulation of VSP expression is controlled at the transcriptional or at the post-transcriptional level. Then, we searched for the existence of sense and antisense VSP RNAs in trophozoites by polymerase chain reaction with reverse transcription (RT–PCR) and

for the activity of enzymes involved in the synthesis and degradation of double-stranded (ds)RNAs in some eukaryotes, such as RNA-dependent RNA polymerase (RdRP), Dicer and Argonaute. The characterization involved cloning and expressing these genes as well as the analysis of small RNAs generated from VSP dsRNAs. Additionally, expression of different VSPs was evaluated after silencing the components of the *Giardia* RNA interference (RNAi) pathway.

Transcription of VSPs in *Giardia*

Transcription of VSP genes was analysed by nuclear run-on assays using RNA isolated from trophozoites of clone WB9B10 (Fig. 1a), which only express VSP9B10 (GenBank accession number AAK97086) on their surface (Supplementary Fig. 1). The results indicate that most of the VSP genes were simultaneously transcribed. In contrast, when total RNAs extracted from two different *Giardia* clones (WB9B10 and WB1267) were probed with an oligonucleotide corresponding to the conserved 3' end of VSP genes, only one transcript of the molecular size corresponding to the VSP expressed on the surface of these clones was detected (Fig. 1b). Additionally, very low-molecular-weight bands, suspected to be degradation products, were seen. Accumulation of only one VSP transcript was observed in different *Giardia* clones using *vsp*-specific probes (reviewed in ref. 3). These results raise the question of why only one VSP transcript accumulates if more than one *vsp* is transcribed at the nuclei of the parasite. One possible explanation is that the non-translated VSP messenger RNAs are specifically degraded, a feature of a post-transcriptional gene silencing (PTGS) model^{9,10}.

Post-transcriptional gene silencing in *Giardia*

A key step of PTGS is the production of dsRNAs that share homology with the silenced gene^{9,10}. RT–PCR assays designed to specifically amplify sense or antisense VSP products revealed, after cloning and sequencing these fragments, that RNAs of both strands are present in trophozoites (Supplementary Fig. 2). To evaluate the possible simultaneous transcription of sense and antisense RNAs for VSP-coding

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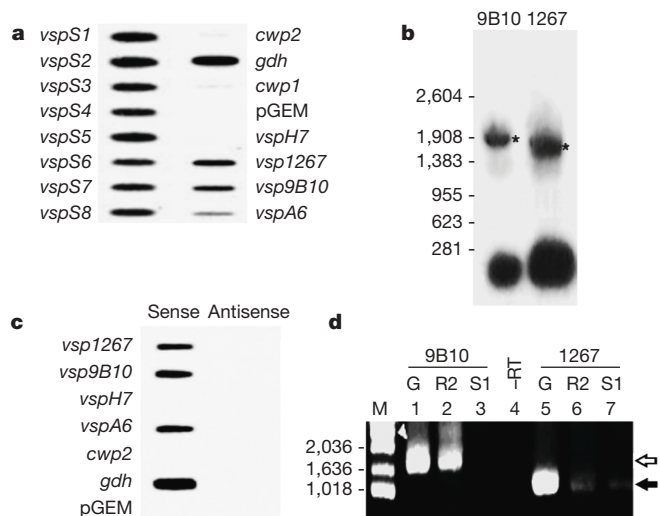


Figure 1 | Several VSP genes are simultaneously transcribed in *Giardia*.

a, Nuclear run-on using freshly isolated *Giardia* nuclei transcribed *in vitro* in the presence of [32 P]UTP. Total RNA from clone WB9B10 was used to probe a membrane on which different DNA products were slot blotted. Isolated *vsp* fragments (*vspS1*–*S8*, Supplementary Fig. 2) are on the left, whereas specific genes (*vsp9B10*, *vsp1267*, *vspA6*, *vspH7*, *cwp1*, *cwp2* and *gdh*) and the cloning vector pGEM, used as negative controls, are on the right. *cwp1* and *cwp2* were not transcribed in non-encysting trophozoites, and neither was *vspH7*, which is not present in the genome of the WB isolate, whereas *gdh* showed active transcription. **b**, Northern blot analysis on total RNA extracted from clones WB9B10 and WB1267 probed with the 3' conserved region of *vsp* (Supplementary Fig. 2). In addition to very-low-molecular-weight species, a single transcript of the size corresponding to the mRNA encoding the clone-specific VSP was found (asterisks). Size markers in nucleotides are displayed on the left. **c**, Sense and antisense *in-vitro*-transcribed genes (*vsp9B10*, *vsp1267*, *vspA6*, *vspH7*, *cwp2* and *gdh*) were slot blotted and hybridised with the products of nuclear run-on under the same conditions as in Fig. 1a. Only sense transcripts were detected. **d**, Comparison among PCR products generated from clone WB9B10 trophozoites by specific primers (9B10F/9B10R and 1267F/1267R) on genomic DNA (lanes 1 and 5), or cDNAs generated with either reverse primer R2 (lanes 2 and 6) or sense primer S1 (lanes 3 and 7). Lane 4 is a control of 7 without RT. The white arrow indicates the *vsp9B10* fragment, which is present in genomic DNA and in the sense cDNA but not in the antisense cDNA. The black arrow indicates the *vsp1267* fragment, which is present in genomic DNA, in the sense cDNA and in the antisense cDNA. M, molecular marker.

genes, as suggested to occur for other genes in *Giardia* by a relaxed mechanism controlling transcription¹¹, we performed a second nuclear run-on experiment using specific sense and antisense probes (Fig. 1c). In this assay, we were unable to detect VSP-antisense RNAs, indicating that those molecules could be generated post-transcriptionally. We also analysed PCR products generated from clone WB9B10 using *vsp9B10*- and *vsp1267*-specific primers (Fig. 1d). The band corresponding to *vsp9B10* was present in genomic DNA and sense complementary DNA but poorly in antisense cDNA. In contrast, *vsp1267* (GenBank accession number M63966), which is not expressed on the surface of clone WB9B10, could be amplified from genomic DNA, and equally amplified from both sense and antisense cDNAs. These results demonstrate that VSPs are transcribed simultaneously (further supporting the results of the nuclear run-on experiments), and that there is an low abundance of antisense transcripts for the VSP that is expressed and a presence of antisense RNAs for VSPs that are transcribed but not translated.

Components of the *Giardia* RNAi machinery

RdRP-mediated, unprimed production of dsRNAs from aberrant mRNAs and primed/unprimed production of dsRNA guided by short interfering RNAs (siRNAs) is necessary for triggering RNAi in some organisms^{12–14}. It has been suggested that this enzyme could

be acting as 'an aberrant-RNA sensor'¹⁵. A *Giardia* homologue of RdRP was identified. This RdRP gene encodes a basic protein of 155,257 Da that shares high homology with other eukaryotic RdRPs and greatly differs from the one encoded by the *Giardia* virus¹⁶, indicating the protozoal nature of the identified gene. Analysis of the *Giardia* RdRP sequence indicates that this protein lacks signal peptide and transmembrane domains, but contains a ribosomal protein S2 signature-1 motif, which has been associated with mRNA binding¹⁷. *Giardia* RdRP transcription was verified by RT-PCR and northern blotting, and its localization assessed by haemagglutinin-tagged expression. RdRP of different organisms have cytosolic or nuclear localization, but our results show that *Giardia* RdRP is probably associated to ribosomes present on the cytoplasmic side of the endoplasmic reticulum. Moreover, the enzyme is active in trophozoites, because it was capable of forming high-molecular-weight RNAs *in vitro* in the presence of homologous VSP RNAs (Supplementary Fig. 3b).

A characteristic of RNAi is the degradation of dsRNA into ~21–25-nucleotide siRNAs by the dsRNA-specific RNase Dicer¹⁸. Previously, a *Giardia* Dicer homologue was identified, its structure solved, and the *in vitro* Dicer activity of the recombinant protein demonstrated¹⁹. Here we show that this enzyme is constitutively expressed during the entire life cycle of *Giardia* and verify its cytoplasmic localization (Supplementary Fig. 3). To evaluate the activity of *Giardia* Dicer, *in vitro* assays using radiolabelled dsRNA were exposed to a post-nuclear *Giardia* extract. Our results (Fig. 2a) demonstrated that, regardless of the gene and the strand that is labelled (sense, antisense, or both), dsRNAs are processed into small RNA fragments of 20–30 nucleotides (similar to those shown for the recombinant enzyme in ref. 19), and that this processing is favoured, as in other eukaryotes²⁰, by the presence of ATP (Fig. 2b). In addition, small RNAs obtained from those experiments were able to be cloned similar to siRNAs that have 5'-P and 3'-OH ends. Sequencing of those siRNAs indicated that they derived from the input VSP genes and that they are 22–25 nucleotides in length (Supplementary Table 1).

Argonaute proteins are essential players in gene-silencing pathways guided by small RNAs. These proteins are part of the RNA-induced silencing complex that recognizes the target mRNA and guide post-transcriptional gene silencing either by destabilization of the mRNA or by translational repression²¹. We were able to identify in *Giardia* a single Argonaute protein (*Giardia* AGO) that has PIWI and PAZ domains. Its expression was evaluated by northern blotting and its cellular localization determined by expressing an haemagglutinin-tagged version of the protein. Similar to that observed in other cells²¹, *Giardia* AGO localizes to the cytoplasm (Supplementary Fig. 3).

Regulation of VSP expression

The occurrence of multiple, homologous VSP transcripts could direct the generation of antisense RNAs by *Giardia* RdRP after transcription of several VSP genes took place. Moreover, the presence and activity of Dicer, and probably of AGO, suggests that an RNAi-like mechanism might be involved in regulation of the expression of surface antigenic variants in *Giardia*. In this cell in particular, a post-transcriptional regulatory system lying in the cytoplasm might be beneficial to synchronize both transcriptionally active nuclei of the parasite².

To test this hypothesis, we analysed whether the *Giardia* PTGS machinery could discriminate the presence of different VSP mRNAs by mixing *Giardia* cytoplasmic extracts with one, two or three different VSP transcripts generated *in vitro*. When two or more labelled VSP mRNAs were incubated with trophozoite extracts containing the RNAi machinery, small VSP RNAs were produced, with an identical pattern to the Dicer activity assay products (compare Fig. 2a and Fig. 2c). In contrast, whenever a single transcript was incubated, no mRNA degradation took place. Additionally, if the unrelated genes *cwp2* (which encodes the cyst wall protein 2; not shown) or *gdh* (which encodes glutamate dehydrogenase) were added to a single radiolabelled VSP mRNA, no degradation to small

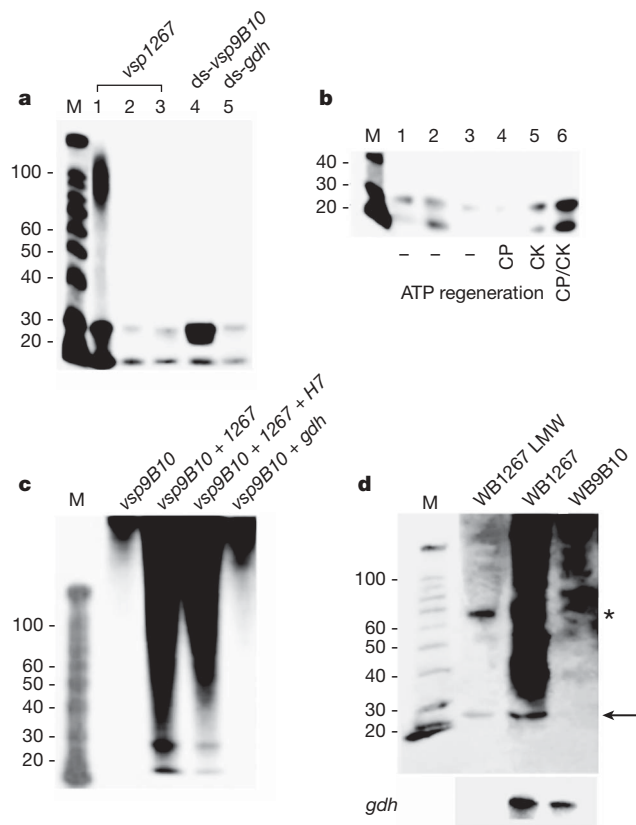


Figure 2 | Dicer activity and detection of VSP small RNAs in *Giardia*.

a, Generation of small RNAs from dsRNAs by *Giardia* extracts demonstrated Dicer-like activity. For *vsp1267* dsRNA, both strands (lane 1), only the sense RNA strand (lane 2) or only the antisense RNA strand (lane 3) were radioactively labelled. For *vsp9B10* and *gdh* both strands were labelled. Double-stranded RNAs were incubated with a *Giardia* WB9B10 extract at 37 °C for 1 h; next, total RNA was isolated and electrophoresed. In all cases, small RNAs were produced. **b**, Effect of the presence of ATP on dsRNA processing by *Giardia*. Lanes 1 and 2 show untreated controls: incubation of *vsp1267* dsRNA with *Giardia* cytoplasmic lysates without ATP depletion (1 h and 3 h of incubation, respectively). ATP was depleted with 2 mM glucose and 0.1 U μl^{-1} hexokinase (lane 3) and then regenerated using phosphocreatine (CP, lane 4), creatine kinase (CK, lane 5) or both (lane 6). **c**, Generation of small RNAs by incubation of *vsp* riboprobes with *Giardia* extracts from clone WB9B10. One, two or three different VSP mRNAs (*vsp9B10*, *vsp1267*, *vspH7*) were mixed and incubated with *Giardia* extracts. [^{32}P]-labelled small RNAs were produced whenever more than one transcript was present. RNA size markers in nucleotides are on the left. **d**, Total RNA from clone WB9B10 and clone WB1267 trophozoites as well as low-molecular-weight (LMW) RNAs from the clone WB1267 were electrophoresed, blotted and probed using partially digested *in-vitro*-transcribed *vsp9B10* RNA. In clone WB9B10, no small RNAs were found. In contrast, small RNAs from *vsp9B10* were present in clone WB1267 (arrow), which does not express VSP9B10. Interestingly, ~70-nucleotide-long RNAs (asterisk) that might represent incompletely digested mRNAs can also be observed. *gdh* was used as a loading control.

RNAs was detected (Fig. 2c), indicating that the silencing machinery specifically processes homologue RNAs. Similar results were obtained when using cell extracts from different *Giardia* clones (WBA6, WB1267 or GSH7): *vsp9B10* RNA was processed to small RNAs only when combined with other homologue VSP genes but not when it was the only *vsp* added to the reaction (Supplementary Fig. 4). It is clear that, under the conditions used in these experiments (excess of exogenous RNAs, short incubation times), the presence in the trophozoite extracts of endogenous siRNAs and various sense and antisense VSP RNAs certainly does not interfere with the silencing process. Because the VSP mRNAs used in these experiments were synthesized *in vitro*, it is obvious that the silencing mechanism can

discriminate among homologous mRNAs in the absence of any possible post-transcriptional RNA modification.

Considering that sense and antisense VSP transcripts were found in *Giardia* and that Dicer activity was demonstrated experimentally, we looked for small RNAs resulting from *vsp* dsRNA degradation. Using partially a digested *vsp9B10* probe for northern blot assays in *Giardia* clones WB9B10 and WB1267, we were able to detect small RNAs for VSP9B10 in the clone that does not express that VSP (WB1267 in this case). On the other hand, no small RNAs for VSP9B10 were found in clone WB9B10, which expresses VSP9B10 on the parasite's surface (Fig. 2d)

These results raise the question of how a single VSP transcript bypasses this silencing process and is translated and expressed at the surface of the parasite. In some organisms it is known that RNAi correlates with methylation of homologous DNA sequences, which in turn modulates transcription²². Nonetheless, DNA methylation has been reported to be absent in this parasite². Moreover, methylated nucleotides could not be detected in *Giardia* DNA by high-performance liquid chromatography (Supplementary Fig. 5). In this context, it is reasonable that after the transcription of the *vsp* repertoire, differences in concentration of each VSP transcript might constitute an exclusion factor that allows the temporally most abundant VSP mRNA to saturate the silencing machinery and be expressed at the parasite surface.

To test whether the concentration of different VSP transcripts has a role in antigenic switching, we unbalanced VSP expression *in vivo* by expressing in clone WB9B10 either *vspH7* (GenBank accession number, AAA18202) or constructs containing antisense regions of *vsp9B10* under the control of a strong promoter such as that of the α -tubulin gene^{23,24}. VSPH7 showed variable expression even under the pressure of the selection drug, and VSP9B10 also decreased over the time (Supplementary Fig. 6). Besides, when VSP9B10 was knocked down, its expression on the parasite's surface diminished slightly faster than in the control (Supplementary Fig. 7). These results suggest that the promoter region of *vsps* has little or no influence on VSP expression and, therefore, that a PTGS mechanism must be involved in *Giardia*'s antigenic variation. Additional *in vitro* experiments in which the concentration of different VSP transcripts was unbalanced demonstrated that mRNA concentration may be relevant for a given VSP to circumvent the silencing machinery and be translated (Supplementary Fig. 8).

Silencing of *Giardia* RdRP, Dicer and AGO

Because specific gene knockouts are not possible in *Giardia* owing to its polyploidy and the presence of two nuclei², we sought for direct evidence supporting the involvement of the characterized RNAi components during antigenic variation by knocking down the expression of *Giardia* *dicer*, *rdrp* and *ago* by constitutive expression in trophozoites of part of their antisense transcripts²³. When a reduction of the expression of *rdrp* (RdRP-AS) or *dicer* (Dicer-AS) took place (Supplementary Fig. 9), trophozoites that express more than one VSP in their surface were generated, as determined by immunofluorescence assays using specific monoclonal antibodies (Fig. 3a and Table 1), flow cytometry (Fig. 3b) and western blotting (Fig. 3c). Silencing of *Giardia* *ago* did not produce any viable clone, suggesting that this molecule is essential for the parasite, probably by maintaining the integrity of the genome by controlling the action of transposons²⁵. Cells in which Dicer or RdRP were knocked down proliferate and encyst in culture as normal, and no deleterious effect on VSP regulation caused by the procedure was observed (Table 1).

Our results are consistent with a major role for the cellular PTGS machinery itself in the selection of one VSP transcript destined to be expressed in a single trophozoite. Although this PTGS mechanism resembles gene silencing phenomena controlling the expression of foreign genes¹², in *Giardia* it targets a family of endogenous genes, as recently demonstrated in mouse oocytes^{26,27}. It has been shown that antisense transcripts for CWP1 also appear early during *Giardia*

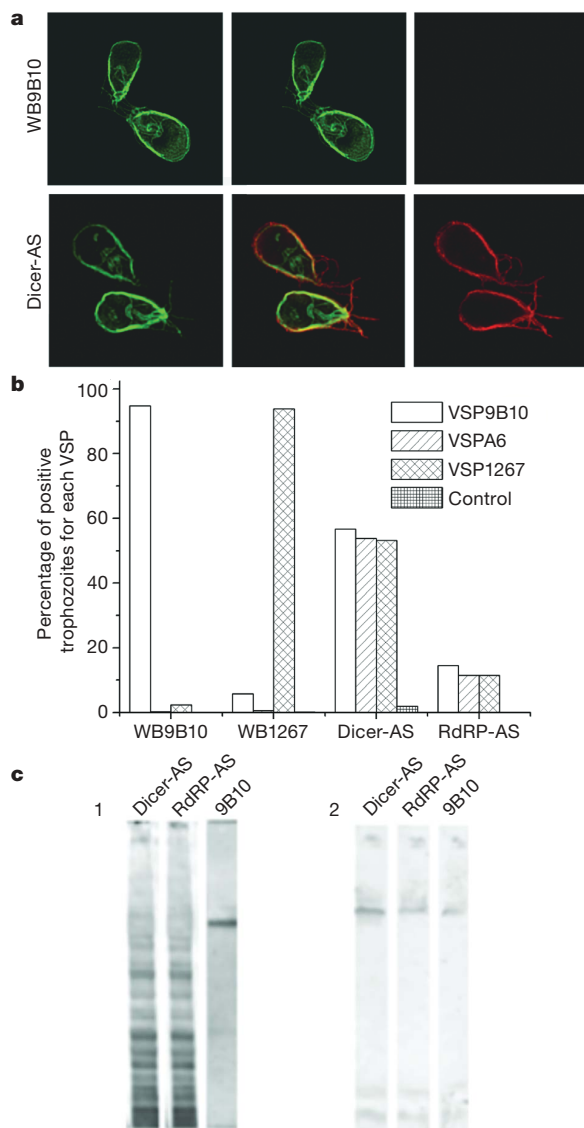


Figure 3 | Expression of different VSPs in *Giardia* rdp- and dicer-knockdown transgenic trophozoites. **a**, Direct immunofluorescence assay on mock-transfected (top panel) or Dicer-AS-transfected (bottom panel) trophozoites with FITC-conjugated monoclonal antibody 9B10 (VSP9B10; left panel) and TRITC-conjugated monoclonal antibody 5C1 (VSP1267; right panel). When *Giardia* Dicer expression was knocked down, trophozoites expressing VSP9B10 on the surface also expressed VSP1267 (merged image; middle panel). **b**, Percentage of *Giardia* trophozoites expressing a particular VSP as determined by flow cytometry assays using specific monoclonal antibodies (VSP9B10, monoclonal antibody 9B10; VSP1267, monoclonal antibody 5C1; VSPA6, monoclonal antibody 6A7) on clone WB9B10, clone WB1267, and with cells transfected with antisense constructs for *Giardia* RdRP (RdRP-AS) or *Giardia* Dicer (Dicer-AS). Goat anti-mouse immunoglobulins were used as a negative control. Clones display clear single-VSP expression patterns, whereas RdRP-AS and Dicer-AS show expression of more than one VSP per trophozoite. **c**, Western blot analysis of protein extracts from wild-type WB9B10 and WB9B10 trophozoites in which Dicer has been knocked down. After electrophoresis and transfer to nitrocellulose, the filters were hybridised with either (1) the monoclonal antibody 12F1 clone G3 (generated against the CRGKA domain conserved in all VSPs) or (2) monoclonal antibody 9B10 (specific for VSP9B10). Results show that the specific monoclonal antibody anti-VSP9B10 recognizes only one band in clone WB9B10 and in transgenic trophozoites, whereas the monoclonal antibody 12F1 reacts with many protein species in the transgenic cells, indicating that many VSPs are expressed simultaneously in *Giardia* when the RNAi pathway has been disrupted.

Table 1 | Quantitative analysis of VSP expression in *Giardia* rdp- and dicer-knockdowns

VSP	None	Mock	<i>Giardia</i> RdRP-AS	<i>Giardia</i> Dicer-AS
VSP9B10	99 ± 0.5	98 ± 1.2	90 ± 0.6	18 ± 2.0
VSP1267	0	0.5 ± 0.1	96 ± 0.2	22 ± 0.9
VSPA6	0	0	48 ± 2.3	17 ± 1.3
VSPS1	0	0	62 ± 4.1	36 ± 2.1
VSPS2	0	0	33 ± 1.1	28 ± 3.9
VSPS7	0	0	73 ± 0.3	65 ± 4.4

Percentage of *Giardia* trophozoites expressing a particular VSP as determined by indirect immunofluorescence assays using specific monoclonal antibodies (VSP9B10, monoclonal antibody 9B10; VSP1267, 5C1; VSPA6, 6A7; VSPS1, 1B2; VSPS2, 7B8; VSPS7, 6F8) on WB9B10 trophozoites transfected with the antisense construct of genes encoding *Giardia* RdRP and Dicer, with empty plasmid only (mock transfected) or non-transfected (none), after 5 days in culture. Goat anti-mouse immunoglobulins were used as a control and showed no reaction (not shown). Data represent the mean ± s.d. of three independent experiments performed in duplicate. Results indicate that many different VSPs are expressed in single trophozoites, as judged by addition of each percentage.

encystation²⁸. Because CWP genes also comprise a family of homologous genes, it is possible that, in addition to their transcriptional regulation²⁹, a post-transcriptional process may be affecting CWP mRNA stability.

It is still an open question how *Giardia* changes expression of one VSP for another. Because VSP switching occurs randomly, we favour the hypothesis that variations in general or local concentrations of different VSP transcripts, as occur in higher eukaryotes^{30,31}, may determine which one will circumvent the silencing system. It is then possible that during cell division each daughter switches to the expression of a different VSP. Alternatively, there may be differences in the level of transcription for each *vsp*. Consistently with this hypothesis, we have detected variations in individual *vsp* expression levels in nuclear run-on experiments performed as in Fig. 1. This sort of variation might depend of the chromatin state in different areas of the genome or the position of each gene within the genome. In this regard, it has been suggested recently that a possible epigenetic mechanism is involved in *Giardia*'s antigenic variation⁸.

In summary, a PTGS system (comprising at least RdRP and Dicer) is implicated in the regulation of surface antigen expression in *G. lamblia*. Because a humoral immune response in both experimental and natural *Giardia*-infected hosts coincides with the elimination of the original VSP, a functional role for cells and antibodies in the selection of phenotypic variants and on the course of infection was proposed³². Given that parasite protection against specific immune responses relies on switching the expression between immunologically distinct surface proteins^{2,3}, one way in which hosts can prevent infections with a specific immunological response is by producing antibodies against all surface antigenic determinants. In this report, we have shown that downregulation of components of the RNAi machinery leads to expression of more than one surface antigen in trophozoites. These cells could constitute a fundamental tool for generating a vaccine against this important human pathogen.

METHODS SUMMARY

Parasite culture and cloning. *Giardia* trophozoites were cultured in TYI-33 medium supplemented with adult bovine serum and bovine bile³³. Continuous cloning of trophozoites was carried out by limiting dilution and selection based on immunofluorescence assays using the corresponding VSP monoclonal antibody. Encystation was carried out as reported³³.

Nuclear run-on and RT-PCR. Nuclear run-on analyses on *Giardia* nuclear extracts were performed as described³⁴, with some modifications. Total RNA of trophozoites from different isolates was isolated using Trizol (Invitrogen), quantified spectrophotometrically, and treated with RQ1 RNase-Free DNase (Promega). First-strand cDNA was synthesized with Superscript III Reverse Transcriptase (Invitrogen).

Detection and cloning of small VSP RNAs. The detection of small RNA was performed according to ref. 35. [³²P]-labelled riboprobes were transcribed *in vitro* by T7 or SP6 RNA polymerase using VSP genes *vsp9B10*, *vsp1267* and *vspH7* cloned into p-GEM T-easy vector (Promega). Labelled RNA was partially hydrolysed by incubation at 60 °C in the presence of NaHCO₃ and Na₂CO₃. Isolation and cloning of small RNAs was performed essentially as described previously³⁶.

Endonuclease activity. Dicer activity was analysed by incubating dsRNA molecules with cytoplasmic extracts of different *Giardia* clones. VSP and control genes cloned into p-GEM T-easy vector (Promega) were transcribed *in vitro* to produce full-length sense [³²P]-labelled RNA probes. Pure or mixed VSP transcripts were incubated at 37 °C with *Giardia* extracts for different periods.

Transfection and immunofluorescence assays. The plasmid pTUBPac³⁷ was modified to introduce sense or antisense *Giardia* *dicer*, *rdp* and *ago* coding regions and the influenza haemagglutinin epitope²³. Transfection of *G. lamblia* trophozoites was done by electroporation as described previously³⁸. Indirect immunofluorescence assays were performed on non-encysting trophozoites by using an anti-haemagglutinin monoclonal antibody (Sigma)²³. For functional analysis of *Giardia* *dicer*, *rdp* and *ago*, gene knockdowns were confirmed by RT-PCR using gene-specific primers and by northern blotting.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions C.G.P. knocked down the expression of AGO and VSP9B10, expressed VSPH7 in WB strain trophozoites, performed confocal immunofluorescence assays, northern blots and quantitative RT-PCRs, and cloned and sequenced small RNAs; I.S. knocked down the expression of Dicer and RdRP, performed nuclear run-on and Dicer activity experiments, and cloned and sequenced RdRP, Dicer and VSP genes; R.Q. performed immunofluorescence assays, quantitative RT-PCRs and flow cytometry experiments; P.G.C. performed DNA methylation experiments; and F.D.R., E.V.E. and A.S. generated different monoclonal antibodies and performed immunofluorescence and immunoblotting assays. C.G.P., I.S. and H.D.L. wrote this manuscript. H.D.L. conceived and coordinated the project. All authors discussed the results and commented on the manuscript.

Author Information The GenBank accession numbers for *Giardia* Dicer, RdRP and AGO are AY142144, AF293414 and AY142143, respectively. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to H.D.L. (hlujan@ucc.edu.ar).