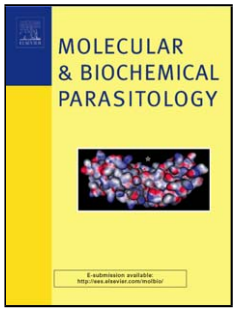

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Transient transfection of *Cryptosporidium parvum* using green fluorescent protein (GFP) as a marker

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Abbreviations: CPV, *Cryptosporidium parvum* virus; GFP, Green fluorescent protein; UTR, untranslated region; nt, nucleotide; bp base pair; ORF, open reading frame; dsRNA, double-stranded RNA; RT, reverse transcription; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; ELISA, enzyme linked immunosorbent assay.

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Abstract

Cryptosporidium parvum is a protozoan parasite that infects a variety of mammals. The parasite has been shown to harbor a dsRNA virus (CPV) and in the present study, we have developed a CPV transient transfection system for this parasite by using green fluorescent protein (GFP) to replace the partial gene encoding region of the larger dsRNA (CPV-L) and the smaller dsRNA (CPV-S) virus. Two viral RNA-mediated transfection vectors: pCPVL-GFP and pCPVS-GFP were successfully constructed and both in vitro transcripts were electroporated into oocysts and sporozoites. Transient expression of GFP was detected in *C. parvum* oocysts and excysted sporozoites by fluorescence microscopy and by RT-PCR detection of GFP mRNA and antisense RNA in transfected *C. parvum* oocysts. Our study provides a new approach for studying gene expression and regulation in *C. parvum* and will hopefully lead to the construction of a stable CPV transfection system in the future.

Key words: *Cryptosporidium parvum*; transfection; green fluorescent protein.

1. Introduction

Cryptosporidium is a ubiquitous intestinal parasite, which can cause diarrheal disease in a wide range of hosts around the world [1,2]. The parasite causes a self-limiting diarrheal illness in immunocompetent hosts, but in immunocompromised hosts, cryptosporidiosis can develop into a severe, chronic and life-threatening infection [3]. To date, no effective vaccine and drugs have been developed for this ubiquitous parasite, particularly in immunocompromised hosts. Lack of successful therapies is partly due to the difficulties in culturing the parasite in vitro and lack of a transfection system for genetic manipulation [4]. The development of effective transfection methods, will result in a better understanding of this parasite's biology and its relationship with the host and will also lead to more efficient molecule-based vaccines and drugs [5,6].

Double-stranded RNA (dsRNA) viruses, belonging to the family Totiviridae, have been identified in several protozoan parasites, including *Cryptosporidium* [7,8]. Protozoan viruses have great potential as genetic manipulation instruments, as some of them are small, easily modified and highly infectious to the parasite host, as well as being present in numerous copies in host cells.

Cryptosporidium parvum has been shown to harbor a dsRNA virus (CPV) with two diverse constituents: large (L) and small (S) extra-chromosomal viral double-stranded RNA (dsRNA) segments (CPV-L and CPV-S), which are 1,786 nt and 1,374 nt respectively in length with only a single long open reading frame (ORF) each [7,8].

DNA-mediated and/or viral RNA-mediated transfection research has been successfully carried out in many other protozoon parasites [9-42]. However, to date no studies have been conducted on viral RNA-mediated transfection in *C. parvum*. In the present study we have demonstrated transient viral RNA-mediated transfection in *C. parvum* using GFP as a marker.

2. Materials and methods

2.1. Parasites and animals

Cryptosporidium parvum oocysts (Changchun isolate, Jilin province, China, genotyped as *C. parvum*-cattle genotype) were purified from naturally infected calf faeces by sucrose and CsCl gradient centrifugation [43]. Oocysts (10^7) were inoculated per os into 2-6-day-old newborn calves, which had not had colostrum. Three days PI, oocysts were collected and purified from faeces.

2.2. Purification of oocysts

Oocysts were concentrated from feces using a one-step ethyl ether method. Briefly, 50 ml of fresh feces suspension was mixed with 20 ml of ethyl ether and mixed vigorously for 15 min and then centrifuged at 1000 × g for 15 min at 4 °C. The pellet was washed three times in PBS, pH 7.2, by centrifugation at 1,500 × g for 10 min at 4 °C. Oocysts were further purified using discontinuous Sheather's gradient centrifugation and cesium chloride gradient centrifugation as previously described [43]. Purified oocysts were suspended in PBS containing 0.01% Tween 20, 100 U of penicillin per ml, 100 µg

of streptomycin per ml, and 100 µg of gentamicin per ml and stored at 4 °C until they were used for transfection. The total numbers of oocysts purified from faeces were counted using a haemocytometer. Oocysts were resuspended in acidic water (pH 2.5–3) containing 0.5% trypsin/EDTA to stimulate excystation and incubated at 37 °C for 90 min with mixing every 5 min to stimulate excystation. Thereafter, the excystation suspension was centrifuged at 2000 × g for 8 min and oocysts were resuspended in maintenance media. The excystation rate was determined by microscopic observation.

2.3. Cell culture

Cryptosporidium was cultured using the human ileocecal adenocarcinoma cell line HCT-8, in RPMI 1640 medium (Invitrogen Co., GIBCO^{TM}, Grand Island, N.Y., US) supplemented with 4 mM L-glutamine, 30 mM HEPES (pH 7.3), 100 U of penicillin per ml, 100 µg of streptomycin per ml, 100 µg of kanamycin per ml, and 0.25 µg of amphotericin B per ml. Cell culture growth medium was supplemented with 10% fetal bovine serum (FBS) (Sijiqing Co., Hangzhou, China) for maintenance of uninfected cells and with 2% FBS for maintenance of cells following inoculation with oocysts. Cells were incubated in a humidified incubator at 37 °C in an atmosphere containing 5% CO_2.

2.4. Transfection constructs

2.4.1 DNA extraction and purification

Oocyst suspensions were centrifuged at 2000 × g for 10 min and the pellets were resuspended in 1000 µl of lysis buffer (4 M urea, 200 mM Tris, 20 mM NaCl, 200 mM EDTA, pH 7.4) and 7 µl of proteinase K (40 mg/ml). Samples were then subjected to

three cycles of freezing in liquid nitrogen for 5 min and thawing at 65 °C for 5 min and finally incubated for 2 h at 65 °C to release nucleic acids. Total nucleic acids were extracted by phenol extraction and isopropanol precipitation in the presence of 1 M ammonium acetate, dissolved in TE water and used as templates in PCR.

2.4.2 PCR amplification

The entire cDNA sequences of the larger and smaller dsRNAs were amplified by PCR using high fidelity proofreading Pfu DNA Polymerase (Tiangen Co., Beijing, China). Each PCR reaction system contained 5 ng of template, 15 pmol of relevant forward and reverse primers (see Table 1A), 0.5 U Pfu DNA polymerase, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.2 mM dNTPs. Cycling parameters were 1 cycle for 5 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 58 °C (for primers LF1/LR1), 65 °C (for primers LF2/LR2), 55 °C (for primers SF/SR) and 2 min at 72 °C and a final 10 min extension step at 72 °C.

2.4.3 Plasmid Constructions

Four plasmids, pCPVL1, pCPVL2, pCPVS and pGFP-C1 were used as the basis for the construction of all transfection plasmids. pCPVL1 and pCPVL2 are both pMD18-T vectors (TaKaRa) into which 489 bp and 548 bp of 5' and 3' flanking sequences of CPV-L were cloned. pCPVS is also a pMD18-T vector that included the entire sequence of CPV-S. The pGFP-C1 (Clontech) plasmid contained the GFP gene, encoding domain.

Approximately 489 bp and 548 bp of 5' and 3' flanking sequences of CPV-L were

respectively amplified by PCR using pCPVL1 and pCPVL2 plasmids as templates and primer pairs L_1F/L_1R and L_2F/L_2R (Table 1B). Approximately 459 bp and 293 bp of 5' and 3' untranslated region (UTR) of CPV-S were respectively amplified by PCR using pCPVS plasmid as templates and primer pairs S_1F/S_1R and S_2F/S_2R (Table 1B). The GFP coding gene was amplified from the pGFP-C1 plasmid by PCR with primers GFP-F and GFP-R (Table 1B). The five amplified segments were retrieved from agarose gels and cloned into pMD18-T vectors. This resulted in the intermediate vectors pCPVL489, pCPVL548, pCPVS459, pCPVS293 and pGFP.

pCPVL489 and pCPVS459 were then respectively digested with restriction enzyme Bam HI and Sac I to release the 489 bp and 459 bp fragments. pGFP was digested with Sac I and Hind III to release the GFP coding region. A pPloyII/SfiNot vector (Promega) was digested with Bam HI and Hind III. Two 5' flanking sequences from pCPVL489 and pCPVS459 and the GFP coding sequence were serially cloned into the pPloyII/SfiNot backbone using a concentration ratio of 3:3:1 with T4 DNA ligase (TaKaRa) at 16 °C overnight. This resulted in two new recombinant plasmids: pCPVL489/GFP and pCPVS459/GFP.

pCPVL489/GFP was digested with Bam HI and Hind III, and pCPVL548 was digested with Hind III and Xho I. 1209 bp and 548 bp segments were retrieved from agarose gels and serially cloned into vector pPloyII/SfiNot digested with Bam HI and Xho I using a concentration ratio of 3:3:1. The pCPVL459/GFP vector was also digested with Bam HI and Hind III and pCPVS293 was digested with Hind III and Pst I. 1179 bp and 293 bp segments were retrieved from agarose gels and mixed with vector pPloyII/SfiNot digested with Bam HI and Pst I at a concentration ratio of 3:3:1. The two

mixtures were ligated with T4 DNA ligase at 16 °C overnight. The final recombinant plasmids were pCPVL-GFP and pCPVS-GFP. The final plasmid constructs are illustrated in Fig. 1.

2.5. Parasite transfection by electroporation

2.5.1. Transfection buffers

Direct transfection of both oocysts and excysted sporozoites was attempted. *Cryptosporidium* was transfected using cytomix as the electroporation buffer previously described for *Eimeria* transfection [41]. Freshly purified oocysts and excysted sporozoites were washed twice in incomplete cytomix (10 mM K_2HPO_4/KH_2PO_4, pH 7.6, 120 mM KCl, 0.15 mM $CaCl_2$, 25 mM HEPES, 2 mM EGTA and 5 mM $MgCl_2$). The washed pellet was then resuspended in complete cytomix (incomplete cytomix supplemented with 2 mM ATP and 5 mM glutathione).

2.5.2. In Vitro Transcription

Plasmids pCPVL-GFP and pCPVS-GFP were linearized at their 3' ends by digestion with Xho I and Pst I. The digested products were recovered from agarose gels and quantitated using a SmartSpec Plus Spectrophotometer (BioRad). Transcription reactions were performed using the T7 RiboMAX^{TM} Express large scale RNA production system (Promega). The synthesized RNA was purified by phenol extraction and isopropanol precipitation in the presence of 1M ammonium acetate, and dissolved in diethy1 pyrocarbonate-treated water. The purified RNA was analyzed by 1.0% agarose-formaldehyde gel electrophoresis and used in the electroporation experiments.

2.5.3. Oocyst Electroporation

For oocyst electroporation, five cuvettes (BTX, 0.4 cm) were prepared for transfection and filled with 800 µl (1×10^7) of oocyst suspension each. 200 µg each of linearized in vitro transcription plasmids pCPVL-GFP and pCPVS-GFP were added to three cuvettes. These cuvettes were numbered 1-3 and were immediately electroporated using a voltage of 3000 V/cm and an average pulse time of 8 ms. The fourth cuvette, which was a negative control and did not contain linearized transcription plasmids was also electroporated. The fifth cuvette was not electroporated and was incubated at room temperature. The four electroporated cuvettes and the fifth non-electroporated cuvette were placed on ice for 15 min and then centrifuged for 10 min at 2000 × g. The pellet was then mixed with 1 ml of excystation buffer and incubated at 37 °C for 90 min as described in section 2.2. The sample containing unexcysted oocysts and sporozoites was centrifuged for 10 min at 2000 × g, resuspended in PBS buffer and inoculated onto 80 to 100% confluent HCT-8 monolayers in 35 mm × 10 mm cell culture wells (BD Falcon™, Boston, US) and incubated for 18 h in a 37 °C incubator with 5% CO_2.

2.5.4. Sporozoite Electroporation

For sporozoite electroporation, five cuvettes were filled with 800 µl (1×10^7) of sporozoite suspension each. 200 µg each of linearized in vitro transcripton plasmid pCPVL-GFP and pCPVS-GFP were added to three cuvettes labelled 6-8 and electroporated using a voltage of 3000 V/cm and an average pulse time of 8 ms. The ninth cuvette, which contained no linearized in vitro transcripton plasmids was also

electroporated as a negative control. The tenth cuvette was not electroporated. Electroporated cuvettes, 6-9 and the non-electroporated control were then centrifuged for 10 min at 2000 × g and the sporozoites resuspended in cell culture media and inoculated onto 80 to 100% confluent HCT-8 monolayers in 35 mm × 10 mm cell culture dish (BD Falcon™, Boston, US) and incubated for 18 h at 37 °C with 5% CO_2.

2.6. Detection by Fluorescence microscopy

After 12 h post incubation in a 37 °C CO_2 incubator, cells were examined and again at 18 h. Before observation by fluorescence microscopy (Olympus BX51, Tokyo, Japan), cultures in culture dishes were first incubated at 4 °C for an hour to allow GFP maturation. Cells were then washed (centrifuged for 10 min at 2000 × g) in ice-cold PBS and fixed in cold 2% formaldehyde. The number of fluorescent oocysts were counted using a fluorescent microscope. Where large numbers of oocysts were present, further dilutions were required. The transfection efficiency was calculated as a percentage as follows: {Number of oocysts expressing GFP in 5 ml sample ÷ 5 × Total volume of oocyst suspension (ml) / Number of total oocysts collected from faeces} × 100

2.7. Detection of GFP mRNA and antisense RNA in transfected *C. parvum*

The electroporated *C. parvum* oocysts were pelleted and washed twice in PBS. Total nucleic acid was extracted by phenol extraction and isopropanol precipitation in the presence of 1M ammonium acetate, dissolved in TE water and used as templates in RT-PCR. For amplification of GFP sense mRNA, the primer GFP-R was used to prime the RT reaction. Following denaturation of the reverse transcriptase at 95 °C, primer

GFP-F was added to complete the PCR. For amplification of antisense RNA of the GFP gene, primer GFP-F was used to initiate the RT reaction and primer GFP-R was used to complete the PCR.

2.8. Detection of GFP expression levels

After 12 h post incubation, the survival rate of oocysts was estimated by haemocytometer. Oocysts, which accounted for approx. 4×10^5 of cells, were pelleted, suspended with 1 ml PBS buffer and the cells lysed by sonication. The resultant pellet was utilized as an antigen, blocked with 10% fetal bovine serum and GFP expression levels detected using polyclonal rabbit antiserum to GFP (Invitrogen) in an indirect ELISA.

3. Results

3.1. Transient expression of GFP in transfected *C. parvum* oocysts

Strong transient expression of GFP was observed in electroporated *C. parvum* oocysts and subsequently excysted sporozoites by fluorescence microscopy after 18 h post incubation (cuvettes 1-3) (Fig. 2A and B). No fluorescence was detected in negative controls (cuvettes 4-5). The results indicate that the in vitro transcripts of linearized pCPVL-GFP and pCPVS-GFP plasmids successfully expressed GFP in electroporated *C. parvum* oocysts and the resulting excysted sporozoites.

3.2. Lack of GFP expression in transfected *C. parvum* sporozoites

After 18 h post incubation, no green signal was observed in cuvettes 6-10. Thus electroporated sporozoites did not generate green fluorescence and express GFP, indicating that the direct sporozoite-transfection system failed.

3.3. Detection of GFP mRNA and antisense RNA in transfected *C. parvum* oocysts

An RT-PCR assay was used to monitor the presence of the mRNA and antisense RNA of the GFP gene. For the *C. parvum* oocyst transfection system, which was transfected with the transcripts of pCPVL-GFP, pCPVS-GFP and the mixture of both, the 720-bp DNA fragment, representing the coding region of the GFP gene, was amplified with both the sense and antisense primers using RT-PCR (Fig. 3A, B and C), suggesting that both the sense (lanes 2, 4, 7) and antisense (lanes 3, 5, 8) strands of the transcripts of the GFP gene were present in the electroporated *C. parvum* oocysts. There was no amplification of the GFP gene in negative controls or in transfected sporozoites.

3.3. Expression level and duration of GFP expression in transfected *C. parvum* oocysts

After 12 h post incubation, approximately 40% of ooycsts were still intact. GFP expression peaked between 18 h to 24 h. Approximately 15% of oocysts and resultant sporozoites from each cuvette (cuvettes 1-3) yielded green fluorescence. There was no obvious difference in expression levels between plasmids pCPVL-GFP and pCPVS-GFP. These transcripts persisted in the transfected sporozoites for up to 7 days post-electroporation (Fig. 3D).

4. Discussion

Over the past decade, transfection systems have been developed and used for the genetic manipulation of Apicomplexan parasites. DNA-based transfection systems allow electroporation of circular or linear plasmid DNA into sporozoites or trophozoites. RNA virus-based transfection systems require electroporation of in vitro transcribed RNA into sporozoites or trophozoites. Transient or stable transfection was successfully obtained in many other protozoan parasites such as *Giardia lamblia*, *Toxoplasma gondii*, *Plasmodium falciparum* and *Entamoeba histolytica* using both DNA-mediated and viral RNA-mediated transfection systems [9-42]. The success of previous studies is due to a variety of factors including the relatively simple life cycles of some of the parasites and extensive previous research on strong promoter sequences, signal peptide sequences and drug resistance genes.

For example, genetic manipulation of *Giardia lamblia* was carried out by placing an exogenous reporter gene within the control of 5' and 3' flanking sequences of GDH or the luciferase gene [24,25]. Stable transfection assays for *Giardia lamblia* were implemented by using standard drug-resistance markers for selection in vitro in several studies [25,33,35]. High transfection efficiency has been achieved for *T. gondii,* which is easily cultured in vitro and as a result, has been utilized as a versatile genetic model organism to study the basic biology of other Apicomplexan parasites [36,37]. Successful transfection of *Eimeria* was achieved using a series of plasmids expressing selectable markers, including a panel of fluorescent reporter genes and a mutant *T. gondii* dihydrofolate reductase-thymidylate synthase (DHFR-TSm2m3) gene that conferred resistance to the drug pyrimethamine [41].

Very little research on *C. parvum* gene transfection has been conducted due to its complex life cycle and lack of effective selectable drug markers. However, the successful establishment of *E. tenella* DNA-based transient and stable transfection systems provided a new pathway to *C. parvum* due to their similar life cycle and genetic similarities. In the present study, we have used reverse genetic manipulation to develop a CPV–based RNA-mediated transient transfection method for *C. parvum*. In vitro transcripts of pCPVL-GFP and pCPVS-GFP introduced into *C. parvum* oocysts by electroporation resulted in the synthesis of both the sense and antisense GFP RNA, as well as the expression of GFP. The successful CPV-mediated transfection of *C. parvum* has several advantages (1). CPVs are easily purified and highly infectious to *Cryptosporidium*; (2). They are highly specific to their host and (3). Even at high infection doses (> 5,000 copies), there appeared to be no notable negative effect on oocyst morphology, growth and multiplication through the life cycle.

A considerable amount of time was spent optimizing the transfection conditions. Due to previous research, we assumed that electroporation efficiency would be highest for *C. parvum* life cycle stages as the oocyst stage is relatively dormant compared with sporozoites and merozoites. However, direct electroporation of sporozoites was not successful and therefore the oocyst stage, which was successfully transfected will be the choice for further study. Many different voltages and pulse times were tested to increase the electroporation efficiency. Optimal transfection was achieved using 3,000 V/cm and an average pulse time of 8 ms. A range of electroporation buffers were also tested and cytomix appears to be the most suitable buffer for transfection. As a result of optimizing all these conditions, we were able to obtain a transfection survival rate of up to 40%.

In the present study, a partial segment of the CPV cDNA open reading frame, was replaced by the GFP coding region, and the prokaryote T7 promotor was used for in vitro transcription. The construction of the CPV transfection vectors opens new avenues for genetic manipulation in *Cryproporidium*. The development of effective transfection methods will allow new approaches to understanding parasite biology and its relationship with the host.

5. Acknowledgements

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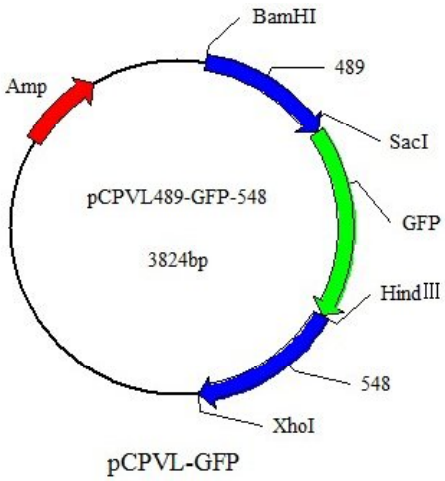
Table 1A. Primer sequences used in RT-PCR for amplification of CPV

Primer name	Location	Primer sequence
LF1	1-26	5'- GGAAAGAAGTATAGCTCAATTTTCGT -3'
LR1	1053-1074	5'- TAATCGCGACTACGCAAAGATG -3'
LF2	1051-1075	5'- CGCATCTTTGCGTAGTCGCGATTAT -3'
LR2	1767-1786	5'- AGGCTTCCCTCGGACCCTGC -3'
SF	1-23	5'- TGTCCTTCTAAAAGGATTTGAGC -3'
SR	1348-1374	5'- ATAAGAAACAAGTTCTAATGATTTCCT -3'

Table 1B. Primers used in the construction of the GFP recombination vectors

Primer name	Primer sequence
GFP-F	5'-GAGAGCTCTAAAGGAGAAGAACTTTTCA-3'
GFP-R	5'-GAAAAGCTTATTCTTAATCCATGCCATGTGTAATC-3'
L_1F	5'-GCGGATCCTAATACGACTCACTATAGGAAAGAAGTATAGCTCAAT-3'
L_1R	5'-GAGAGCTCGTACAACCGCTCTTAAAAGG-3'
L_2F	5'-GGGAAGCTTTTGACGATTTTGAAGTAAGATT-3'
L_2R	5'-GAGCTCGAGAGGCTTCCCTCGGACCCTG-3'
S_1F	5'-GCGGATCCTAATACGACTCACTATAGGTGTCCTTCTAAAAGGATTTG-3
S_1R	5'-GAGAGCTCATCAGCAGAAGGGTTCTATGA-3'
S_2F	5'-GAAAAGCTTCTCCGACGTCGAATCGAGTGC-3
S_2R	5'-GAGCTGCAGATAAGAAACAAGTTCTAATG-3

Figures and legends



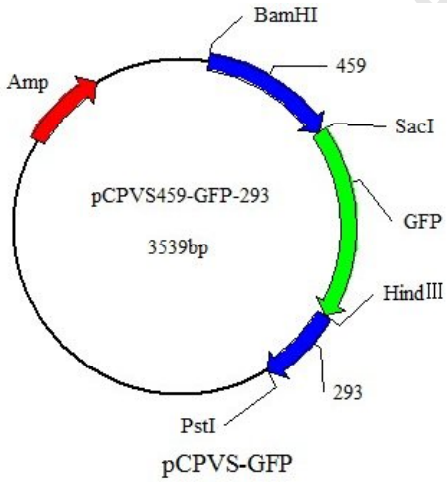
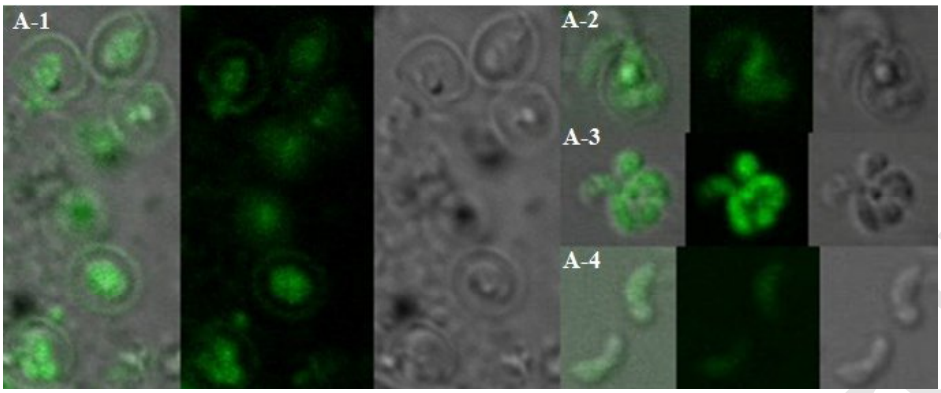


Fig. 1. Schematic representation of plasmids used for expression of GFP in *C. parvum*. The prokaryote T7 promoter was used for vitro transcription. The 5' flanking region of the virus gene (cDNA) was utilized to drive expression of the trans-genes.



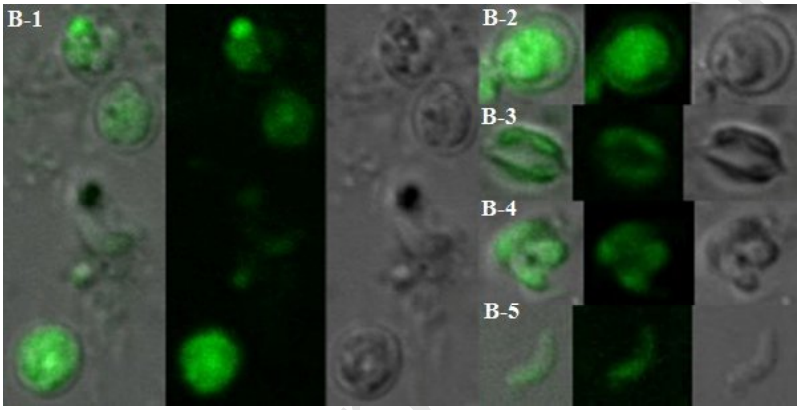


Fig. 2. Transgenic green fluorescent reporter-expression in *C. parvum* oocysts and sporozoites incubated for 18 h in HCT-8 cells (×1000). Figure A - transfection results for pCPVL-GFP and Figure B - transfection results for pCPVS-GFP. (A-1) Internal GFP expression in oocysts; (A-2) GFP expression in sporozoites escaping from partially broken oocyst wall; (A-3, A-4) GFP expression in sporozoites. (B-1) Internal GFP expression in oocysts; (B-2) GFP expression in sporozoites escaping from partially broken oocyst wall; (B-3 - B-5) GFP expression in sporozoites.

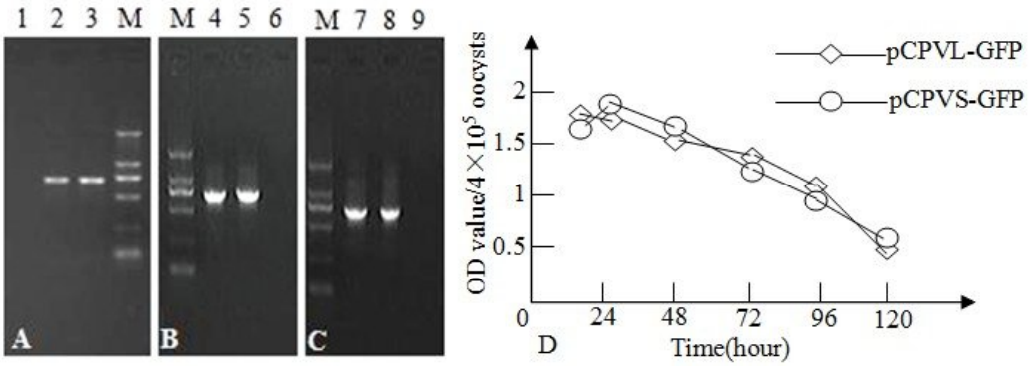


Fig. 3. Detection of the GFP mRNA and antisense RNA by RT-PCR of transfected *C. parvum* RNA. (A) pCPVL-GFP RT-PCR (B) pCPVS-GFP RT-PCR (C) RT-PCR analysis of a mixture of pCPVL-GFP and pCPVS-GFP. Lanes 1, 6 and 9 - Negative control, Lanes 2, 4 and 7 - Detection of GFP mRNA by RT-PCR. Lanes 3, 5 and 8, Detection of GFP antisense RNA by RT-PCR, M = DL2000 Marker. (D) Time course of GFP-expression levels in transfected *C. parvum* oocysts.