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Stable expression of green fluorescent protein mediated by GCV *in Giardia canis*

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Abstract

Giardia canis can be infected with a double-stranded RNA virus, that is giardiavirus (*G. canis* virus, GCV). In this study, green fluorescent protein (GFP) was stably expressed in *G. canis* mediated by GCV. The plasmid *pNEO/GDH/MCS/GFP*, containing the neomycin phosphotransferase (NEO) encoding region flanked by the 636 nt of 5′-terminus and the 2174 nt of 3′-terminus from GCV positive strand RNA, was constructed by inserting GFP gene into downstream from the NEO gene and glutamate dehydrogenase (GDH) 5′-terminus uncoding regions on a single plasmid, and its *in vitro* transcript was introduced into GCV-infected *G. canis* by electroporation. The transfectants expressed GFP persistently under G418 selection. This stable transfection system should provide a valuable tool for genetic study of *G. canis*.

Keywords: *Giardia canis*; GCV; Green fluorescent protein; Neomycin phosphotransferase

1. Introduction

Giardia, a parasitic protozoan, is one of the flagellated unicellular eukaryotic microorganism whose effect on the host can range from asymptomatic infection to chronic nutritional malabsorption and diarrhea throughout the world. Infection occurs in following ingestion of cysts which excyst in the small intestine to form trophozoites that are responsible for the symptoms of the illness [1]. It is the most common cause of outbreaks of waterborne and occasionally food-borne diarrhea in developed countries and there is a very high prevalence and incidence of infection in developing countries [2]. There are currently six recognized species of *Giardia*, but only *Giardia lamblia* is known to infect multiple host species [3,4]. *G. lamblia* is considered as a species complex, whose members, albeit morphologically identical, can be assigned to at least seven distinct Assemblages based on genetic analyses [5]. Only Assemblages A and B have been detected in humans and in a wide range of other mammalian hosts, whereas the remaining Assemblages (C to G) are likely to be host-specific [3,6]. Assemblages C and D are dog-specific [7]. The majority of *Giardia* infections in dogs are asymptomatic, but some infected dogs may suffer from acute or chronic diarrhea, weight loss, poor weight gain despite a normal appetite, and, less commonly, vomiting and lethargy [8].

Phylogenetic analyses of its small ribosomal RNA sequence, transcriptional factors and various protein coding sequences have placed *Giardia* as one of the earliest diverging lineages in the eukaryotes [9,10]. Some of *Giardia*'s most interesting features, including polyploid dual nuclei and asexual lifecycle, have made it impossible to use traditional genetic tools in the study of *Giardia*. The inability to perform genetic analysis on this parasitic protozoan has been a major impediment to study *Giardia*, and the way of performing sufficiently stable genetic transformation of *Giardia* should be of significant value [9–11]. *Giardia* virus is a double-stranded RNA (dsRNA) virus of the *Totiviridae* family that infects only *Giardia* [12]. By expressing the dicistronic viral

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transcripts in transfected *Giardia*, the giardiavirus can be developed into an effective transfecting and expressing vector of *Giardia*, which has been turned out to be particularly useful for studying the mechanisms of translation in this primitive eukaryote.

Giardias has been isolated from dogs in changchun, China, whose genetic characterization and sequence comparison showed that the strain belongs to Assemblage D. In addition, *Giardia canis* virus (GCV), harboring a double-stranded genome of 6276 bp, was isolated from the *G. canis* strain [13]. In this study, a stable transfection system has been established based on the GCV and stable express of green fluorescent protein in *G. canis*.

2. Materials and methods

2.1. Parasite and cell culture

G. canis (*Changchun* strain) trophozoites infected with GCV were cultured as described before [14].

2.2. Plasmids constructions

The plasmids pGCV636 and pGCV2174 were derivatives of pGCV carrying the 6276-bp GCV cDNA (Fig. 1A). The GCV 5′-terminus 636-bp and 3′-terminus 2174-bp fragments were generated by PCR using pGCV as template and oligonucleotides (GCV-636F: 5′-GAGCTCTAATACGACTCACTATAGGAAG-GAGTGCCAGGCCATTACC-3′; GCV-636R: 5′-GTCGACCTCGCTGCCGCCCAGCGCGTGATTA-3′; GCV-2,174F: 5′-GGATCCATGGTCGATCTACGGAGCTTAT-3′ and GCV-2,174R: 5-AAGCTTCGACCCCCTCG-TACGCTGCCTCCTAC-3′. The underlined portion was T7 promoter core sequence.) as primers, and cloned into pMD18-T vector (Takara, Dalian, China), producing pGCV636 and pGCV2174. The NEO gene was generated from pGFP-C1 (Clontech) by PCR with primers (NEO-F: 5′-GCGTCGACAT-GATTGAACAAGATGGATTGC-3′; NEO-R: 5′-CCCGGGTCAGAAGAACTCGTCAAGAAGGC-3′) and

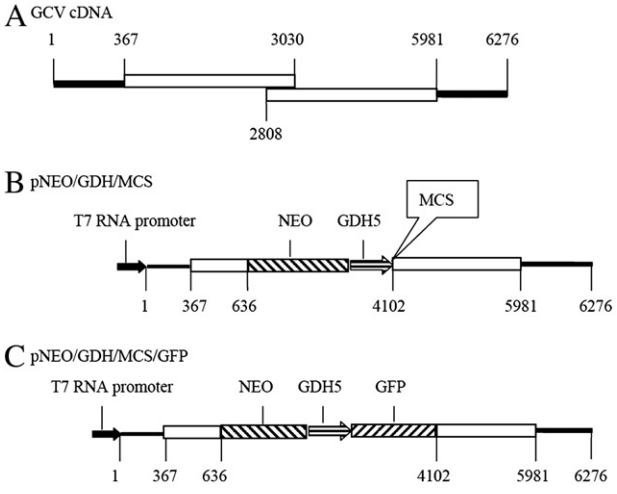

Fig. 1. Schematic diagrams of transfection vectors. The cassette, containing the NEO and GDH gene promoter flanked by the 5′- and 3′-portions of GCV cDNA, were located downstream from the T7 RNA promoter. MCS was located at the downstream from GDH gene promoter.

cloned into pMD18-T vector, producing pNEO. The GDH5 which include glutamate dehydrogenase core promoter was amplified from *G. canis* genome DNA by PCR with the primers (GDH5-F: 5′-GCCCCGGGCACGTCGTCGTTCTCG-3′; GDH5-R: 5′-GGATCCATCGATCATATGTCGCGACTG-CAGCCGCGGAGTACTCATTTTAAAATCTGGGGC-3′. The underlined portion was "multiple cloning sites" (MCS) including *Sca*I, *Sac*II, *Pst*I, *Nru*I, *Nde*I, *Cla*I and *Bam*HI restriction enzyme sites.) and cloned into pMD18-T vector, resulting in pGDH5. The recombinant pGCV636 was digested with *Sac*I/*Sal*I, pNEO was digested with S*al*I/S*ma*I, pGDH5 fragment was digested with *Sma*I/B*amH*I, pGCV2174 was digested with B*amH*I/H*ind*III, and they were subcloned into the S*ac*I/H*ind*III site of pUC18 (TaKaRa), generating the stable transfection vector pNEO/GDH/MCS (Fig. 1B). The GFP genes amplified from pGFP-C1 with the primers (GFP-F: 5′-CTGCAGGGTAAAGGAGAA-GAACTTTTCA-3′; GFP-R: 5′-GGATCCAATTCTTTATC-CATGCCATGTGTAATC-3′), initially cloned into pMD18-T vector, resulting in pGFP. The pGFP fragment cut with *Pst*I/B*amH*I and subcloned into the same site of pNEO/GDH/MCS, giving rise to the plasmid pNEO/GDH/MCS/GFP (Fig. 1C).

2.3. In vitro transcription

The plasmid pNEO/GDH/MCS/GFP was linearized at the 3′-terminus with *Hind*III and the transcription reactions with T7 RNA polymerase (Promega) were performed as described previously [15]. The synthesized RNA was purified by phenol extraction and isopropanol precipitation in the presence of 1 M ammonium acetate and dissolved in diethy pyrocarbonate-treated water. The purified RNA was analysized by 0.8% agarose-formaldehyde gel eletrophoresis and used in the electroporation experiments. At the same time, linearized plasmid *pNEO/GDH/MCS* was dealt with the same method as mock-transfected control.

2.4. Electroporation and G418 selection

The *in vitro* transcript was introduced into a GCV-infected strain of *G. canis* trophozoites by electroporating as described previously [15–17]. Approximately 5×10^6 trophozoites were transfected with 100 μg of the in vitro transcript.

Drug selection began one day after the electroporation at 150 μg/ml G418, and progressed through serial passages at 1:12 into a medium containing 200 μg/ml and then 300 μg/ml, gradually increased G418 concentrations to 750 μg/ml, which has regarded as the selection concentration in *Giardia* [17]. Then these trophozoites were continuously subcultured.

2.5. Detection mRNA of NEO gene in transfected G. canis

At the 1st, 7th, 14th, 21st, 28th day after the electroporation, detection mRNA of NEO gene in transfected *G. canis* was performed. The electroporated *G. canis* trohpozoites were pelleted and washed twice in PBS. Total RNA was extracted and used as templates in RT-PCR. Primer NEO-R was used to prime the RT reaction, following denaturation of the reverse transcriptase at 95 °C, and primer NEO-F was added to complete the PCR.