

Short Communication

An improved purification procedure for *Leishmania* RNA virus (LRV)

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Abstract

Leishmania RNA Virus (LRV, *Totiviridae*) infect *Leishmania* cells and subvert mice immune response, probably promoting parasite persistence, suggesting significant roles for LRV in host-parasite interaction. Here we describe a new LRV1-4 purification protocol, enabling capsid visualization by negatively stained electron microscopy representing a significant contribution to future LRV investigations.

Key words: *Leishmania guyanensis*, *Leishmania* RNA virus, LRV, virus isolation, electron microscopy.

In 1960 the first protozoa virus was discovered in *Entamoeba histolytica*, opening the possibility of investigating similar virus in unicellular eukaryotes (Gupta and Deep, 2007). Almost three decades later, in 1988, the first *Leishmania* infecting virus was reported as a *Leishmania* RNA virus or LRV (Tarr *et al.*, 1988).

Leishmania RNA viruses are currently classified as belonging to the *Totiviridae* family, composed of virus particles with capsids of approximately 40 nm in diameter and a 5,280-nucleotide double-stranded RNA genome (Schefter, 1994). LRV genome contains two open reading frames encoding a capsid protein with endoribonuclease activity and a RNA polymerase (Macabath and Patterson, 1995; Scheffter *et al.*, 1995; Scheffter, 1994; Suh, 1996).

Several aspects of leishmaniasis are still the subject of intense investigation. Leishmaniasis is predominantly a rural disease of serious public health concern affecting 88 countries on 4 continents with an estimated 1.6 million new cases annually (WHO, 2010). One poorly understood aspect of the infection is the mechanisms involved in lesion development in mucocutaneous leishmaniasis. However, it

has been shown that a strain of *L. guyanensis* infected with LRV1-4 induced more metastasizing lesions in mice than a virus-free one. These observations suggest that the presence of LRV1-4 rendered the animals more susceptible to disseminated infection, perhaps enabling *Leishmania* to subvert host immune response and promote parasite persistence (Ives *et al.*, 2011). This observation brings to light a potential involvement of LRV in *Leishmania* pathogenesis. However, to further advance the investigation of LRV1 interaction with the parasite, it is paramount to obtain pure LRV particles. Previous work has described the production of “recombinant” LRV particles in *Leishmania* (Ro *et al.*, 2004) or baculovirus cells (Tamarra and Patterson, 2003). These experiments greatly advanced the study of LRV, however, as they result in empty capsid virions, since only the capsid protein is introduced into the expression system, a functional study of the virus is not possible. The seminal work that established the existence of LRV (Tarr *et al.*, 1988) involved the isolation of native viral particles. Those experiments resulted in cumbersome protocols and apparently low virion yield. The LRV1-4 purification protocol

presently reported enabled observation of LRV1-4 particles associated with filaments associated with the capsid verticils, suggesting extruded RNA.

LRV was purified from *Leishmania (Viannia) guyanensis* strain MHOM/BR/75/M4147, hereby referred to simply as M4147, which is infected with LRV1-4. Promastigotes were maintained in medium M199 at 26 °C supplemented with 10% heat inactivated fetal bovine serum, 2% human male urine, 100 mM adenine, 10 mg/mL hemin, 40 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES, pH 7.4), 50 mg/mL penicillin and 50 mg/mL streptomycin. *Leishmania* cells were grown to a density of 10^5 parasites/mL, after which they were centrifuged at 3000 x g for 10 min (4 °C) and suspended in buffer A containing 10 mM Tris-HCl (pH7.5), 150 mM NaCl, 10 mM DTT and 0.1% Triton X-100. The cell suspension was lysed by sonication in a 550 Sonic Dismembrator (Fisher Scientific) and monitored by observation in the microscope, then clarified by centrifugation at 3000 x g for 10 min, filtered through a 22 µm filter and subject to ultracentrifugation in a 10% to 50% Oritiprep (Accurate Chemical Corp.) step gradient of 400 µL volume layers of 10% (1.06 g/mL density), 20% (1.11 g/mL density), 30% (1.17 g/mL density), 40% (1.22 g/mL density) and 50% (1.27 g/mL density) iodixanol in buffer A. The gradient was centrifuged for 3 h at 350.000 x g at 4 °C and fractionated in 10 fractions (200 µL each) were separated from the gradient. Gradient fractions were analyzed for LRV1-4

mRNA by semi-quantitative RT-PCR. The cDNA synthesis was performed with RevertAid M-MuLV Reverse Transcriptase and 8 nucleotide long random primers from total RNA isolated from each gradient fraction using Trizol (Invitrogen) reagent following the manufacturer instructions. PCR amplification of the LRV1-4 specific cDNA was accomplished with sequence-specific primers (LRV1-4a 5'-ATGTGATGGCCCCGTGGTATTGG-3' and LRV1-4b 5'-AACTCCGCCGGGTGAAACAGGTC-3') in rounds of 20, 25 or 30 amplification cycles, generating a PCR product of 442 bp. The 25-cycle regimen resulted in the best estimate of the RNA content, whereas 30 amplification cycles resulted in the saturation of the signal for each LRV1-4-containing sample, whereas 20 cycles resulted in low signal, underestimating the amount of LRV1-4 RNA in each fraction (Figure 1A). The presence of LRV1-4 specific RNA was detected in samples 2 to 7, and a higher concentration of the LRV1-4 RNA was observed in samples 3 to 5.

The iodixanol (Oritiprep) reagent was removed from the samples by size exclusion chromatography. The gradient fraction 4 (Figure 1A) was loaded into a Superdex 200 HR 10/300 (GE) size exclusion column in buffer A at a flow rate of 0.5 mL/min and monitored at 280 nm. The LRV1-4 RNA was detected by RT-PCR in fraction 13 (Figure 1B). Samples of 1 mL were pooled together and concentrated in a 100 kDa concentrator.

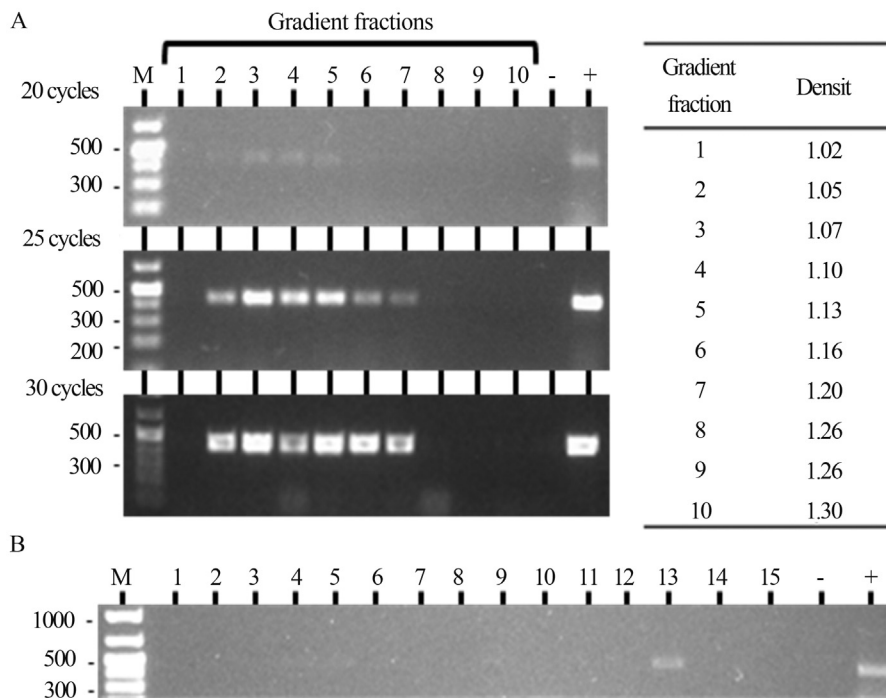


Figure 1 - LRV1-4 purification steps. A) Detection of LRV1-4 specific RNA fragment by RT-PCR in the gradient fractions 1 to 10. The semi-quantitative amplification is shown by 20, 25 and 30 amplification cycles were the relative abundance of LRV1-4 is shown. B) Detection of LRV1-4 specific RNA fragment by RT-PCR in the size-exclusion chromatography fractions 1 to 15. The virus is present only in fraction 13.

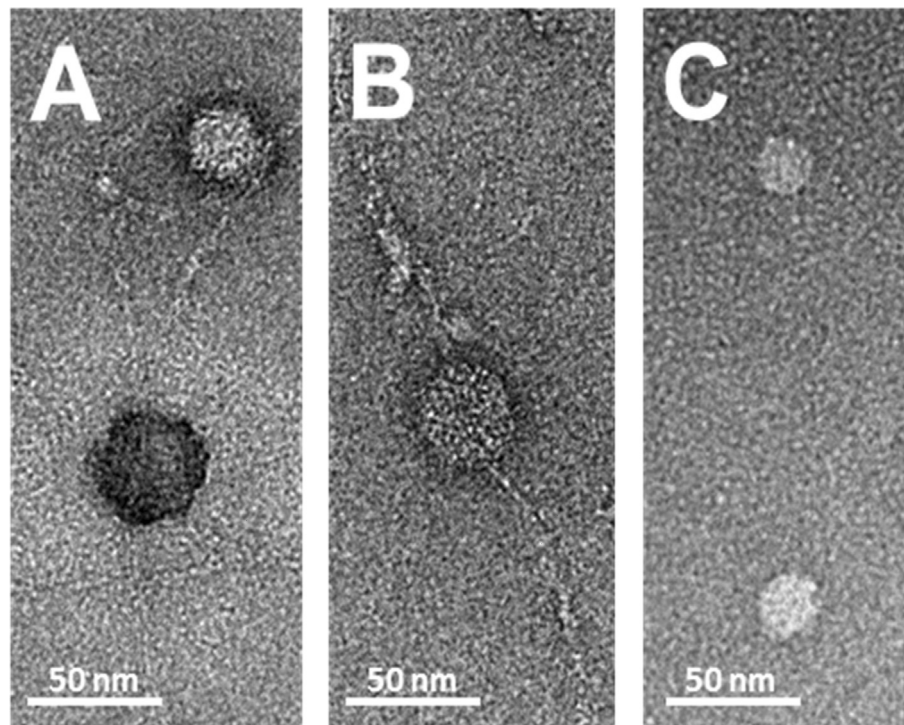


Figure 2 - Negative stain electron microscopy images of LRV1-4 present in fraction 13 of the size-exclusion chromatography. A and B) LRV1-4 capsid particles, with diameter ranging from 37 to 44 nm showing the icosahedral structure of the particles. In A a darker particle is present that could represent a damaged capsid filled with uranyl acetate. C) Sample of smooth-shaped and reduced diameter (approximately 25 nm) particles observed in fractions 3-5 of the size-exclusion chromatography. Other size particles of similar appearance can be seen.

For LRV1-4 visualization by negative stain, the concentrated pooled sample in buffer A was deposited onto holey carbon-coated grids, previously glow discharged for 25 s at 15 mA using an easiGlow system (PELCO). Sample was deposited for 30 s, followed by two washing steps, staining with 3 μ L of 2% uranyl acetate, blotting and air-drying. Images were recorded close to focus at x60,000 magnification using a Jeol JEM-2100 operating at 200 kV.

The sample contained LRV1-4 icosahedral particles in the size range expected for *Totiviridae*, with capsid diameters ranging from 37 to 44 nm (Figure 2A and 2B). A very faint RT-PCR amplification signal was detected in fractions 3 to 5 which, upon negative stain analysis revealed particles of about 25 nm diameter.

The smoother shape and reduced diameter (approximately 25 nm) of the particles in fractions 3-5 (Figure 2C) could raise the possibility for the existence of less dense, smaller capsid, perhaps even an inner shell that segregates at a different gradient density range. However, other possibilities exist, including the presence of unknown viruses within *Leishmania*, and that these smaller particles and their heterogeneous size distribution represent an artifact not yet determined. Confirmation of the actual nature of this finding awaits further studies. The yields of LRV1-4 cannot be estimated at this time, since comparison with previous work is not possible. The presently described purification protocol will allow further investigation on the

structural organization and replication process of *Leishmania* RNA viruses.

Acknowledgments

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