Brazilian Journal of Microbiology 45, 2, 695-698 (2014)
ISSN 1678-4405

Short Communication

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

Marcos Michel de Souza[1], Livia Regina Manzine[1], Marcos Vinicius G. da Silva[2], Jefferson Bettini[3], Rodrigo Vilares Portugal[3] Angela Kaysel Cruz[2], Eurico Arruda[2], Otavio Henrique Thiemann[1]

[1]Departamento de Física e Informática, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil.
[2]Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP, Brazil.
[3]Laboratório Nacional de Nanotecnologia, Centro Nacional de Pesquisa em Energia e Materiais, Campinas, SP, Brazil.

Submitted: May 22, 2013; Approved: September 09, 2013.

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 (Macabeth anda Patterson, 1995; Scheffter *et al.*, 1995; Schefter, 1994; Suh, 1996).

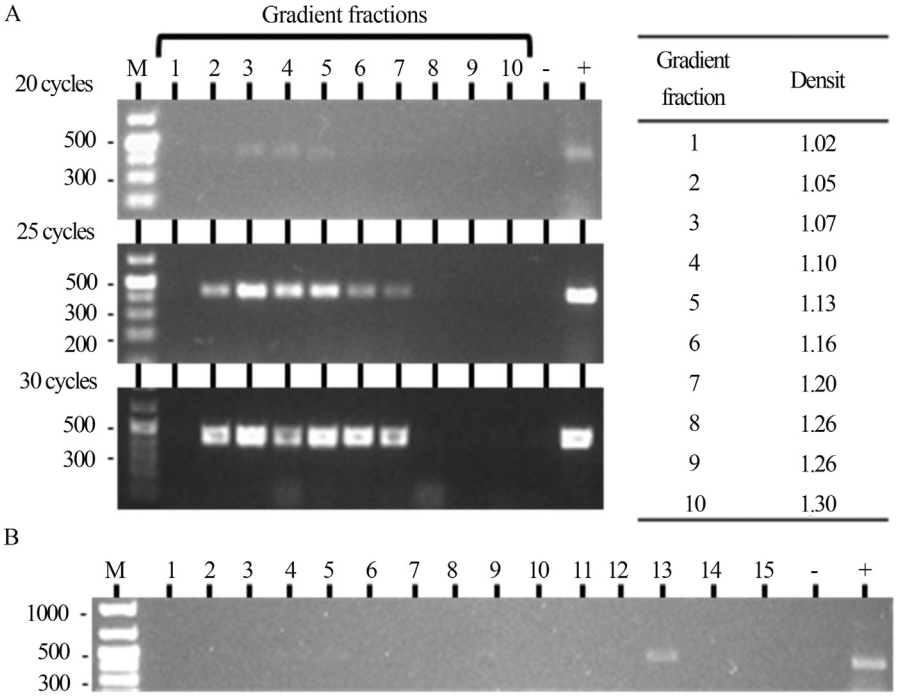
Several aspects of leishmaniasis are still the subject of intense investigation. Leishmaniasis is predominantly a rural disease of serious public health concern affecting 88 countries on 4 continents with an estimated 1.6 million new cases annually (WHO, 2010). One poorly understood aspect of the infection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

Send correspondence to O.H. Thiemann. Departamento de Física e Informática, Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos, SP, Brazil. E-mail: thiemann@ifsc.usp.br.

presently reported enabled observation of LRV1-4 particles associated with filaments associated with the capsid vertices, 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% Opitiprep (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 (Opitiprep) 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.



Gradient fraction	Densit
1	1.02
2	1.05
3	1.07
4	1.10
5	1.13
6	1.16
7	1.20
8	1.26
9	1.26
10	1.30

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.

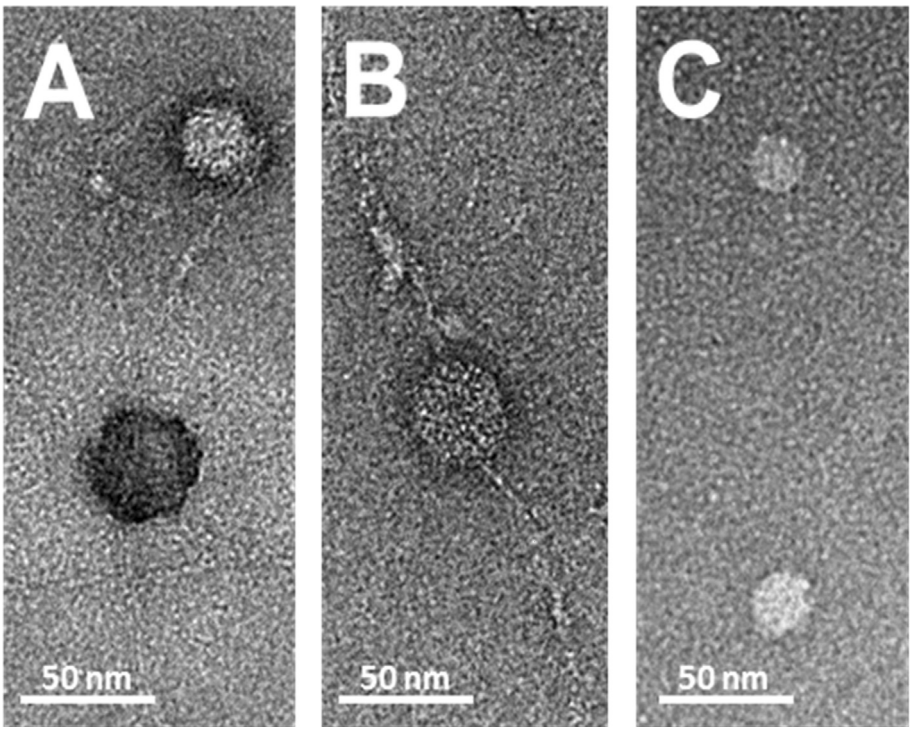


Figure 2 - Negative stain electron microscopy images of LRV1-4 present in fraction 13 of the size-exclusion chromatography. A and B) LRV1-4 capsid particles, with diameter ranging from 37 to 44 nm showing the icosahedral structure of the particles. In A a darker particle is present that could represent 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 μL 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

This work was supported in part by research grant 98/14138-2 from the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP). M. M. S. was supported by a CAPES and L.R.M. by a FAPESP student fellowship. E.A. is recipient of a long term research scholarship from CNPq. We wish to thank the members of the Protein Crystallography and Structural Biology Group (IFSC-USP) for helpful discussions in the course of this work, and to express our gratitude to Dr. Susana A. Sculaccio, José A. L. da Costa and José G. Catarino for technical help during this study.

References

Gupta V, Deep A (2007) An insight into the *Leishmania* RNA virus. Indian J Med Microbiol 1:7-9.

Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, Schutz F, Zangger H, Revaz-Breton M, Lye LF, Hickerson SM, Beverley SM, AchaOrbea H, Launois P, Fasel N, Masina S (2011) *Leishmania* RNA virus controls the severity of mucocutaneous leishmaniasis. Science 6018:775-778.

Macbeth J, Patterson JL (1995) The short transcript of *Leishmania* RNA virus is generated by RNA cleavage. J Vir 6:3458-3464.

Ro YT, Kim EJ, Hyun IL, Saiz M, Carrion R, Patterson JL (2004) Evidence that the fully assembled capsid of *Leishmania* RNA virus 1-4 possesses catalytically active endoribonuclease activity. Exp Mol Med 2:145-156.

Scheffter S, Widmer G, Patterson JL (1994) Complete sequence of *leishmania* RNA Virus 1-4 and identification of conserved sequences Virology 199:469-483.

Scheffter SM, Ro YT, Chung IK, Patterson JL (1995) The complete sequence of *Leishmania* RNA virus LRV2-1, a virus of an old-world parasite strain. Virology 212:84-90.

Suh JJ, Lee GE, Kim YM, Lee JH, Kim DM, Suh JW, Chung IK (1996) Genomic structure of a double-stranded RNA virus in the parasite *Leishmania major*. Mol Cells 6:40-44.

Tamarra LC, Patterson JL (2003) Synthesis of viruslike particles by expression of the putative capsid protein of *Leishmania* RNA virus in a recombinant baculovirus expression system. J Vir 68:358-365.

Tarr PI, Aline RF, Smiley BL, Scholler J, Keithly J, Stuart K, 1988. LR1: A candidate RNA virus of *Leishmania*. Proc Natl Acad Sci USA 85:9572-9575.

Weeks R, Aline RF, Myler PJ, Stuart K, 1992. LRV1 viral particles in *Leishmania guyanensis* contain double-stranded or single-stranded RNA. J Vir 66:1389-1393.

WHO - World Health Organization (2010) Working to overcome the global impact of neglected tropical diseases: First WHO report on neglected tropical diseases. Available at: http://whqlibdoc.who.int/publications/2010/9789241564090_eng.pdf. Accessed 22 May 2013.