Induction of Protective Immunity in a Syrian Hamster Model Against a Cytopathogenic Strain of Andes Virus

Valeria Paula Martinez* and Paula Julieta Padula
Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud “Dr. C. G. Malbrán”, Argentina

Andes virus (ANDV) is responsible for the Hantavirus Pulmonary Syndrome cases in Argentina and neighboring countries, with moderate to high case-fatality rates. ANDV has some particular features, which make it unique among other members of the Hantavirus genus such as person-to-person transmission and causing a disease similar to Hantavirus Pulmonary Syndrome in the hamster as an animal model. The kinetics of replication in Vero E6 cells of an ANDV strain isolated in Argentina, called Andes/ARG, was studied. Cytotoxic effect and the formation of clear plaques were observed and therefore Andes/ARG could be quantified by classic plaque assay. The Andes/ARG strain was found to be highly lethal in Syrian hamsters allowing experiments to demonstrate the protective potential of vaccines. A recombinant nucleocapsid protein of ANDV induced a long lasting antibody response and protective immunity against a homologous challenge, but to a lower extent against heterologous challenge by the Seoul virus. J. Med. Virol. 9999:1–9, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: hantavirus pulmonary syndrome; vaccine candidate; Mesocricetus auratus

**INTRODUCTION**

The genus Hantavirus belongs to the family Bunyaviridae, which comprises trisegmented single-stranded RNA viruses. Hantaviruses are rodent-borne viruses of worldwide distribution that cause human diseases. Old World and New World hantaviruses have been associated with Hemorrhagic Fever with Renal Syndrome and Hantavirus Pulmonary Syndrome, respectively.

Andes virus (ANDV), characterized from the Hantavirus Pulmonary Syndrome cases in Argentina [Lopez et al., 1996] and several neighboring countries [Johnson et al., 1997; Johnson et al., 1999; Padula et al., 2000a], causes high case-fatality rates [Martinez et al., 2010]. ANDV had shown some particular features which make it unique among other members of Hantavirus genus, such as its ability to establish person-to-person transmission, which has only been described in Argentina and Chile [Padula et al., 1998; Martinez et al., 2005; Ferres et al., 2007]. In addition, it was the only pathogenic hantavirus that could cause illness in an animal model reproducing a disease similar to Hantavirus Pulmonary Syndrome in hamsters [Hooper et al., 2001]. Other pulmonary hantaviruses, such as Sin Nombre and Choclo, failed to cause illness in hamsters [Hooper et al., 2001; Wahl-Jensen et al., 2007; Eyzaguirre et al., 2008]. Furthermore, hantaviruses are very difficult to isolate, only three pathogenic agents from South America have been successfully isolated in cell culture: Laguna Negra, Araucaria, and ANDV [Johnson et al., 1997; Padula et al., 2002; Machado et al., 2010].

To date there is no specific treatment for hantavirus infections, efforts have been focused on the development of hantavirus vaccines. Because Hantavirus Pulmonary Syndrome is a relatively rare disease, most vaccine attempts have been directed to Hemorrhagic Fever with Renal Syndrome. Because of the lack of suitable disease models for Hemorrhagic Fever with Renal Syndrome, protection has been usually measured by the ability to protect from infection. Several of these approaches include conventional vaccines, such as rodent brain and cell culture-derived inactivated vaccines, and molecular vaccines [Schmaljohn, 2009]. The nucleocapsid protein expressed by different
systems has been used to protect rodents from infection with Hemorrhagic Fever with Renal Syndrome agents with different efficacy [Lundkvist et al., 1996; Dargevicuie et al., 2002; de Carvalho Nicacio et al., 2002; Klingstrom et al., 2004]. A successful approach for the protection of bank voles consisting in recombinant chimeric virus-like particles carrying a segment of the nucleocapsid protein of Puumala virus [Ulrich et al., 1998] helped to identify the protective regions within the nucleocapsid protein [Koletzki et al., 2000]. For Hantavirus Pulmonary Syndrome few vaccine approaches have been evaluated for Sin Nombre virus and ANDV Chile-971786 strain. DNA vaccines based on M and S segment of Sin Nombre and Chile-971786 viruses were proved in animal models [Bharadwaj et al., 1999; Bharadwaj et al., 2002; Custer et al., 2003; McElroy et al., 2004]. A DNA vaccine based on ANDV M segment resulted neither immunogenic nor protective in the ANDV Chile-971786/hamster lethal model of disease [Custer et al., 2003]. However, non-replicating adenovirus vectors expressing the nucleocapsid protein of ANDV Chile-971786, and both glycoproteins administered to hamsters individually or in combination, elicited a robust immune response that protected hamsters from disease [Safronetz et al., 2009].

In this study, an Argentinean strain of ANDV, Andes/ARG, showed cytopathic effect and formation of clear plaques in Vero E6 cells. Hamster's susceptibility to infection with this strain was evaluated in order to develop a model of disease useful for protection studies. The potential efficacy in protection of a recombinant vaccine based on the nucleocapsid protein expressed in *Escherichia coli* was analyzed with the Andes/ARG-hamster model.

**MATERIALS AND METHODS**

**Virus and Hamsters**

A strain of ANDV, called Andes/ARG, was isolated from the lung of an *Oligoryzomys longicaudatus* captured in the Patagonian Forests ecoregion [Padula et al., 2002]. The isolate was obtained in October 2000; it was confirmed by immunofluorescence with rabbit serum raised against ANDV nucleocapsid protein. The stock used in this study was the 9th viral passage in Vero E6 cells (ATCC, CRL 1586), and its infectious titer was 2 × 10^8 plaque forming units (PFU)/ml. Undiluted supernatants or dilutions in PBS were utilized as inoculums in challenge experiments and to infect Vero E6 cell monolayers. Twenty-one-week-old male Golden Syrian Hamsters (*Mesocricetus auratus*) were utilized in either infection or immunization experiments. They were provided by the National Institute of Biologic Production from the National Administration of Health Institutes and Laboratories “Dr. C. G. Malbrán”. Animals were anesthetized by an intramuscular injection of ketamine (approximately 3 mg/100 g of body weight) prior inoculation.

**Establishment of Virus Replication Kinetics**

Culture tubes were seeded with 2 × 10^4 Vero E6 cells per tube in 1 ml of Eagle's minimal essential medium containing 10% heat-inactivated fetal bovine serum (FBS), 10 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml amphotericin B (10% FBS cEMEM) and incubated at 37°C in an humidified atmosphere containing 5% CO2 for 48 hr. Confluent monolayers were infected with Andes/ARG at a multiplicity of infection (moi) of 0.5 and maintained with 2% FBS cEMEM; mock-infected cells (PBS) were used as controls. Cells and supernatant were collected daily to perform plaque assay and RNA extraction.

**Cell Viability Assay**

Confluent monolayers infected in the same manner and simultaneously with the cells infected for the growth curve were used to determine cell viability by trypan blue dye exclusion assay. Cells adherent to culture tubes were treated with a solution of 0.25% trypsin and 1 mM EDTA, once detached were combined with the floating cells collected from the culture
media, washed and resuspended in PBS and mixed with an equal volume of 0.4% trypan blue. The cells that excluded the dye (viable cells) and the stained cells (death cells) were counted under a microscope using a hemocytometer chamber.

**Plaque Assay and Plaque Reduction Neutralization Test (PNRT)**

Dilutions of infected cell supernatants were added to 24-well plates containing 7-day-old Vero E6 cell monolayers. After adsorption of 1 hr, the wells were overlaid with 0.5% agarose in 10% PBS cEMEM. Plates were incubated 11 to 12 days at 37 °C in a humidified atmosphere containing 5% CO₂ and cells were then fixed with 10% formaldehyde solution and stained with 0.2% crystal violet.

Neutralization activity of serum against Andes/ARG was analyzed by PNRT. Hamster serum samples previously heated for 30 min at 56 °C, were serially diluted (twofold) and mixed with an equal volume containing 100 PFU of virus per 100 µl. The mixtures were incubated overnight at 4 °C, inoculated into wells of 24-well plates containing 7-day-old Vero E6 cell monolayers and plaque assay was performed as described above. An >80% reduction in number of plaques was selected as criterion for virus neutralizing titers.

**RNA Extraction and Real-Time RT-PCR**

Fluids of infected monolayers were centrifuged to pellet floating cells and samples of 200 µl of supernatant were subjected to RNA extraction. Cell monolayers were trypsinized and resuspended in 200 µl of PBS; 100 µl of cell suspension were subjected to RNA extraction. RNA was extracted using Trizol (Invitrogen) and purified by the RNAid kit (Bio 101) following both manufacturer’s recommendations. For ANDV RNA quantification real-time RT-PCR was performed using a MyiQ single color RT-PCR detection system (BioRad). Primers and probe were designed to amplify the conserved region of the S-segment of ANDV (AH1 strain) cloned in pGem (Promega) and purified hamsters challenged at different time points were calculated by paired t-test. The efficiency of ANDV-rNP as vaccine was calculated by comparing survival between the experimental group (n = 6) and the control group (n = 5) challenged 22 weeks after vaccination was assessed by Fischer's exact test.
RESULTS

Andes/ARG Infection Produces Dramatic CPE on Vero E6 Cells

Vero E6 cells infected with Andes/ARG appeared normal in morphology during the first 2 days post infection (p.i.). At day 3, many cells were rounded up and had detached from the tube wall (Fig. 1). The proportion of detached cells increased up to day 7 p.i. in which almost all cells had detached from the tube. The uninfected control monolayer remained unaltered along the entire experiment as shown in Figure 1. This observation prompted a study to determine the ability of Andes/ARG to form visible plaques in Vero E6 cells by plaque assay. Infected monolayers stained with crystal violet showed clearly visible plaques (Fig. 2). This technique was used in the subsequent titration experiments of Andes/ARG.

Andes/ARG Replication Kinetics in Cell Culture

In order to analyze whether the CPE on Vero E6 infected monolayers was associated to Andes/ARG infection, cell viability, infectious virus, and level of viral genome were measured since days 1 to 7 p.i. As shown in Figure 3A, there was a gradual increment of infectious virus in supernatant up to day 5 p.i., day in which the maximum level was reached (3.4 × 10^4 PFU/ml). The infectious viral titer obtained in the present curve was less than one log lower than the titer of the viral stock used in this assay. The percentage of infected cells on day 2 and 6 was 10% and 90%, respectively (data not shown). An inverse correlation between cell viability and virus RNA accumulation within cells was found (Pearson r: −0.96; P = 0.0006) (Fig. 3B). Maximum molecule number of S-segment was reached at day 7 p.i. probably due to viral RNA accumulated within or associated with death of cells.

Andes/ARG is Highly Lethal in Hamsters

In a preliminary susceptibility testing of the hamsters, none of the five Andes/ARG inoculated animals presented any signs of disease up to day 8 p.i. but they were found all dead at day 10 p.i., while control animals remained healthy. Based on these results, a second study was then performed to determine the lethal doses needed to kill 50% of the animals (LD50). Five groups of four hamsters were infected with different viral doses beginning with 2 × 10^3 PFU (10-fold dilutions). All animals died with the exception of one hamster inoculated with 2 × 10^3 PFU, so LD50 was estimated to be lower than 2 PFU. Signs of illness appeared evident 1 or 2 days before death and the time of survival increased with the decreasing input viral load. The only survivor, infected with 2,000 PFU, developed a robust IgG response (final titer >6,400 at day-35 p.i.).

The Nucleocapsid Protein Resulted Highly Immunogenic in Hamsters

The hamsters vaccinated with 40 μg of ANDV-rNP developed humoral response before challenge as it was evidenced by the IgG ELISA test. The response to

<table>
<thead>
<tr>
<th>Hamster no.</th>
<th>Immunogen</th>
<th>Viral doses (PFU) at indicated time (weeks p.v.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h28-h31</td>
<td>ANDV-rNP</td>
<td>4 × 10^2</td>
</tr>
<tr>
<td>h25-h27</td>
<td>PBS</td>
<td>2 × 10^3</td>
</tr>
<tr>
<td>h63-h68</td>
<td>ANDV-rNP</td>
<td>1 × 10^4</td>
</tr>
<tr>
<td>h57-h62</td>
<td>rCP</td>
<td>1 × 10^4</td>
</tr>
<tr>
<td>h33-h35</td>
<td>ANDV-rNP</td>
<td>2 × 10^3</td>
</tr>
<tr>
<td>h36-h38</td>
<td>PBS</td>
<td>2 × 10^3</td>
</tr>
</tbody>
</table>

p.v., post vaccinated.

<table>
<thead>
<tr>
<th>Hamster no.</th>
<th>IgG α-SEOV-NP titer b.ch.</th>
<th>IgG α-ANDV-NP titer b.ch.</th>
<th>Survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>h103</td>
<td>&lt;100</td>
<td>ND</td>
<td>Yes</td>
</tr>
<tr>
<td>h104</td>
<td>&lt;100</td>
<td>ND</td>
<td>No</td>
</tr>
<tr>
<td>h109</td>
<td>&lt;100</td>
<td>409600</td>
<td>Yes</td>
</tr>
<tr>
<td>h111</td>
<td>&lt;100</td>
<td>&gt;409600</td>
<td>Yes</td>
</tr>
</tbody>
</table>

b.ch., before challenge; p.ch., post challenge.

Hamsters were immunized with 40 μg of SEOV-rNP.

Doses of Andes/ARG were 1 × 10^5 FFU; challenge was performed 22 weeks p.v.
the nucleocapsid protein was significantly higher in the group challenged 22 weeks post vaccination (p.v.) (paired t-test, \( P = 0.017 \)) (Fig. 5). ANDV-rNP did not elicit ANDV neutralizing antibodies (nAbs) in the hamsters (Fig. 6; mean nAb titer = 20; \( N = 12 \)). The hamsters vaccinated with SEOV-rNP or rCP showed negative titers against ANDV-rNP. The four hamsters vaccinated with SEOV-rNP developed high IgG titers to SEOV antigen (Table II).

**ANDV-rNP Prevented the Lethal Disease Caused by Andes/ARG in Hamsters**

Immunized hamsters of the homologous challenge group were divided in three groups to be challenged at different time after immunization and with two different doses (Table I). Almost all hamsters immunized with ANDV-rNP survived challenge without any apparent sign of illness, 12/13 (>92%) (Fig. 4). From

---

**Fig. 1.** ANDV infection in Vero E6 cells monolayers. **A:** non-infected control monolayer 3 days p.i.; **(B–D)** infected monolayes at 3, 5, and 6 days p.i (moi = 0.5), respectively.

**Fig. 2.** Visualization of plaques in Andes/ARG infected Vero E6 infected monolayers. After incubation of 11–12 days infected monolayers were stained with the crystal violet procedure. The inoculum used to infect the left well was 1/10 of that used for the right well.

*J. Med. Virol. DOI 10.1002/jmv*
the group challenged 4 weeks p.v., only one immunized hamster died at day 15 post challenge (p.ch.). The survivors of this group were challenged again with the highest viral dose (10⁴ PFU) 43 weeks p.v. and they all survived (N = 3). Long duration of immune response could be achieved: the three hamsters challenged once at 43 weeks after immunization survived without any manifestation of illness. All hamsters from the control groups died, the mean day of death was 11. The challenge heterologous group was utilized as control to prove specificity of ANDV-rNP to Andes/ARG but only one of the four hamsters immunized with SEOV-rNP died (day 14 p.ch.); the remaining three hamsters showed signs of illness (lack of movement, no interest in food, breathing faster) but they recovered and survived.

Statistical analysis showed that ANDV-NP constitutes an efficient vaccine in the group challenged at 22 weeks p.v. (Fisher’s exact test, P = 0.0022). For the other groups (challenged 4 and 43 weeks p.v.) in order to detect statistically significant differences in the proportion of animals vaccinated with nucleocapsid protein versus control, higher number of treated and control animals would be necessary.

Humoral immune responses were analyzed in serum samples of hamsters which survived to virus challenge. Changes in antibody titers to the nucleocapsid protein and the development of nAbs was determined. In the group challenged 4 weeks p.v., a significant increment in IgG titers to the nucleoprotein was observed (paired t-test P = 0.001), while this was not evidenced in the group challenged 22 weeks p.v. (Fig. 5). There were no detectable nAbs in hamsters prior to challenge, but moderate titers were found after challenge in all survivors tested, this increment could be consistent with Andes/ARG replication. Although two hamsters developed high nAb titers, there were no significant differences between groups (Fig. 6).

**Evaluation of Lower Doses**

The success in protection with 40 μg of ANDV-rNP prompted us to evaluate the immunization with lower doses of protein (Table III). Immunization with 4 and 0.8 μg of ANDV-rNP induced a robust response. Both doses resulted efficient in protection of hamsters to Andes/ARG challenge.

**DISCUSSION**

American hantaviruses constitute a growing group in the Americas. Unlike Hemorrhagic Fever with
Renal Syndrome, which causes 1,50,000 cases annually in Eurasia but with low lethality, Hantavirus Pulmonary Syndrome has shown lesser incidence but higher lethality [Khaiboullina et al., 2005]. Several hundred cases of Hantavirus Pulmonary Syndrome are reported annually on the American continent. Consequently, there is a need for effective prevention of hantaviral infections.

One of the hallmarks of in vitro hantavirus infection is the absence of cytolysis or marked cytopathic effect and many investigators have reported difficulty in plaqueing hantaviruses [Summers and McClain, 1999]. In the present study, as a consequence of the observation of a rapid loss of viability of Andes/ARG infected Vero E6 cell culture, a classic staining procedure with crystal violet on plaque assay was optimized and clear formation of plaques was obtained. Therefore, an accurate method for virus titration was established for this strain. In general for hantavirus plaque assay, the use of immunostaining was recommended for virus quantification. Other investigators could quantify the Chilean strain of ANDV by immunocytochemistry [Tischler et al., 2005; Valdivieso et al., 2006]. Visualization of ANDV strain Chile-9717869 plaques stained with neutral red was possible but difficult, and it was suggested that it could be a consequence of an existing disturbance of the functions of the virus in the infected cells, which lead them to take-up neutral red in a different way than uninfected cells do [Wahl-Jensen et al., 2007]. For Andes/ARG, it could be possible to perform a staining procedure as is usual for cytolytic viruses.

Cell death could be triggered by the high rates of virus replication. In a previous study, ANDV strain Chile-9717869 infections tend to produce higher levels of RNA and do so more rapidly than Sin Nombre infections [McElroy et al., 2004]. In our study, an inverse correlation between cell viability and Andes/ARG RNA level within cells was found. Virus RNA could remain stable in non-infectious virus particles accumulated within or associated to unviable cells. The long stability of hantaviral particles has been reported; Hantaan virus could maintain its infectiousness up to 8 days at 37°C under humid conditions [Hardestam et al., 2007]. Future studies will be necessary to find out the viral mechanism which led cells to death.

No vaccines are available for any Hantavirus Pulmonary Syndrome agent and therefore, there is an urgent need for well-characterized hantavirus vaccine candidates. As it was proved for the Chilean strain [Hooper et al., 2001], Andes/ARG was highly lethal in hamsters. LD₅₀ was estimated to be lower than 2 PFU. In the present study, it was found that ANDV-rNP was able to avoid death and illness in Andes/ARG challenge experiments using hamster as an animal model. The protection was long-lasting; it was proved up to 43 weeks after immunization. As low as 0.8 µg of ANDV-rNP were enough to provide protection. The development of moderate titers of nAbs only after challenge could mean that the virus could establish an asymptomatic infection indicating that the immunization was not sterile. A protective effect of non-nAb has been found in animal models for

![Fig. 6. nAbs in immunized hamsters before and after challenge. Final titers of nAbs in hamsters challenged 4, 22, and 43 weeks after the immunization procedure, from left to right, respectively. One animal from the first group died after challenge.](image)

<p>| TABLE III. Effect of ANDV-rNP Doses in Immunogenicity and Protection |
|--------------------------|--------------------------|--------------------------|--------------------------|</p>
<table>
<thead>
<tr>
<th>No. of hamsters</th>
<th>Immunization</th>
<th>IgG α-Nucleocapsid protein titer (mean)</th>
<th>Number of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>40 µg ANDV rNP</td>
<td>256100</td>
<td>2/2</td>
</tr>
<tr>
<td>2</td>
<td>4 µg ANDV rNP</td>
<td>1638400</td>
<td>2/2</td>
</tr>
<tr>
<td>2</td>
<td>0.8 µg ANDV rNP</td>
<td>409600</td>
<td>2/2</td>
</tr>
<tr>
<td>2</td>
<td>40 µg rCP</td>
<td>&lt;100</td>
<td>0/2</td>
</tr>
</tbody>
</table>

ND, not determined; h.ch., before challenge; p.ch., post challenge.

*Dose of Andes/ARG was 2 × 10⁵ PFU; challenge was performed 4 weeks p.v.*

hantavirus and other viruses [Schmaljohn et al., 1990; Takita-Sonoda et al., 1993; Yoshimatsu et al., 1993; Safronetz et al., 2009]. Cell-mediated immune responses such as antibody-dependent cellular cytotoxicity or direct cellular cytotoxicity have been considered as the most plausible mechanisms involved in the protection. Monoclonal antibodies to antigenic sites of the nucleoprotein inhibited the spread of Hantaan virus in cell culture, and it has been suggested that a humoral immune response alone should be considered a protective mechanism [Yoshimatsu et al., 1996].

In a previous work, hamsters administered with non-replicating adenovirus vector expressing the nucleoprotein of ANDV strain Chile-9717869 were successfully protected against 154 focus forming units (FFU) of the Chilean strain in challenge experiments [Safronetz et al., 2009]. In the present work, immunized hamsters survived at least to viral inoculums as high as 10,000 PFU. The immunization with recombinant nucleocapsid protein could have been avoided due to the asymptomatic infection in challenge experiments, if lower viral doses than 2,000 PFU had been used. In fact, it is not known which is the minimal viral input required to establish natural infections with ANDV, but it is probably lower than viral doses used in this study. SEOV-rNP was utilized as control to prove specificity of ANDV-NP but it also provided high degree of protection to Andes/ARG challenge. However, unlike ANDV-NP, SEOV-NP protein did not protect hamsters from illness, suggesting a poor cross-protective immunity for Hantavirus Pulmonary Syndrome.

A vaccinia virus-vectored Hantaan virus vaccine protected hamsters from infection against closely related, Murindae-borne Seoul virus but not against more distantly related Cricetidae-borne hantavirus Puumala [Chu et al., 1995]. Different degree of cross protection could also be achieved with recombinant nucleoproteins from several hantaviruses in bank voles challenged with Puumala virus [de Carvalho Nicacio et al., 2002]. However, pre-existing cross-nAbs did not contribute to the protective immunity elicited by prior hantavirus infection [Hooper et al., 2001]. Furthermore, a study performed in Hantavirus Pulmonary Syndrome survivors from different geographic origin demonstrated the absence of heterologous nAb titers [Valdivieso et al., 2006]. These findings could be considered in broadly vaccine designs against hantavirus infections.

The present study has demonstrated the protective efficacy of a recombinant protein against the lethal ANDV Argentinean strain. The difficulties of in vitro hantaviruses propagation in cell culture constitute a disadvantage to obtain live vaccines. Producing large quantities of a Hantavirus Pulmonary Syndrome virus for inactivation would require specialized high-containment facilities. Molecular approaches to vaccines for Hantavirus Pulmonary Syndrome and Hemorrhagic Fever with Renal Syndrome circumvent problems associated with live vaccines such as rodent brain and cell culture-derived vaccines. [Schmaljohn, 2009]. Furthermore, the potential capacity of persistent infection of ANDV [Manigold et al., 2010] discourages the use of live vaccines for American hantaviruses. A vaccine designed and based on a defined and single peptide presents advantages because its production and control are simpler and safer. Extensive efficacy testing and the evaluation of additional components will be necessary in Hantavirus Pulmonary Syndrome vaccine designs.

ACKNOWLEDGMENTS

We gratefully acknowledge Silvia A. Girard for her excellent technical assistance, Dr. M. Amoroso for veterinary medical care and Dr. E. Pasquinelli for providing the facility of exclusive use for these experimental procedures with animals. Research was conducted in compliance with institutional guidelines and the national law no.: 14,346, that regulates experiments involving animals, and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996 [Grossblatt, 1996].

REFERENCES


Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions.

- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please refer to the proof mark-up symbols guidelines. If manually writing corrections on your proof and returning it as a scanned pdf via email, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

<table>
<thead>
<tr>
<th>Query No.</th>
<th>Query</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>As per the journal style maximum limit of 48 characters (including space) is allowed for short title. Please reduce the short title accordingly.</td>
<td></td>
</tr>
<tr>
<td>Q2</td>
<td>Please check the section heading has been changed from 'TEXT' to 'INTRODUCTION'.</td>
<td></td>
</tr>
<tr>
<td>Q3</td>
<td>Please give manufacturer information for this product: town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q4</td>
<td>Please give manufacturer information for this product: town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q5</td>
<td>Please give manufacturer information for this product: town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q6</td>
<td>Please give manufacturer information for this product: company name, town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q7</td>
<td>Please give manufacturer information for this product: town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q8</td>
<td>Please give manufacturer information for this product: town, state (if USA), and country.</td>
<td></td>
</tr>
<tr>
<td>Q9</td>
<td>Please check the insertion of ‘to’ here.</td>
<td></td>
</tr>
<tr>
<td>Q10</td>
<td>Please provide the surname for the second editor name.</td>
<td></td>
</tr>
</tbody>
</table>
USING E-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required Software

Adobe Acrobat Professional or Acrobat Reader (version 7.0 or above) is required to e-annotate PDFs. Acrobat 8 Reader is a free download: http://www.adobe.com/products/acrobat/readstep2.html. For help with system requirements, go to: http://www.adobe.com/support/.

Once you have Acrobat Reader on your PC and open the proof, you will see the Commenting Toolbar (if it does not appear automatically go to Tools>Commenting<Commenting Toolbar). If these options are not available in your Adobe Reader menus then it is possible that your Adobe version is lower than 7 or the PDF has not been prepared properly.

PDF Annotations (Adobe Reader version 7 or 8) – Commenting Toolbars look like this:

(PC, Adobe version 7)

(PC, Adobe version 8, right-click on title bar (Comment & Markup) to show additional icons)

(Mac)

PDF Annotations (Adobe Reader version 9)

If you experience problems annotating files in Adobe Acrobat Reader 9 then you may need to change a preference setting in order to edit.

The default for the Commenting toolbar is set to ‘off’ in version 9. To change this setting select ‘Edit | Preferences’, then ‘Documents’ (at left under ‘Categories’), then select the option ‘Never’ for ‘PDF/A View Mode’. (the Commenting toolbar is the same as in version 8).
TO INDICATE INSERT, REPLACE, OR REMOVE TEXT

- Insert text

Click the ‘Text Edits’ button on the Commenting toolbar. Click to set the cursor location in the text and simply start typing. The text will appear in a commenting box. You may also cut-and-paste text from another file into the commenting box. Close the box by clicking on ‘x’ in the top right-hand corner. It can be deleted by right clicking (for the PC, ctrl-click on the Mac) on it and selecting ‘Delete’.

- Replace text

Click the ‘Text Edits’ button on the Commenting toolbar. To highlight the text to be replaced, click and drag the cursor over the text. Then simply type in the replacement text. The replacement text will appear in a commenting box. You may also cut-and-paste text from another file into this box. To replace formatted text (an equation for example) please Attach a file (see below).

- Remove text

Click the ‘Text Edits’ button on the Commenting toolbar. Click and drag over the text to be deleted. Then press the delete button on your keyboard. The text to be deleted will then be struck through.

HIGHLIGHT TEXT/MAKE A COMMENT

Click on the ‘Highlight’ button on the commenting toolbar. Click and drag over the text. To make a comment, double click on the highlighted text and simply start typing.

ATTACH A FILE

Click on the ‘Attach a file’ button on the commenting toolbar. Click on the figure, table or formatted text to be replaced. A window will automatically open allowing you to attach a file. To make a comment, go to ‘General’ and then ‘Description’ in the ‘Properties’ window. A graphic will appear indicating the insertion of a file.

LEAVE A NOTE/COMMENT

Click on the ‘Note Tool’ button on the commenting toolbar. Click to set the location of the note on the document and simply start typing. Do not use this feature to make text edits.

REVIEW

To review your changes, click on the ‘Show’ button on the commenting toolbar. Choose ‘Show Comments List’. Navigate by clicking on a correction in the list. Alternatively, double click on any mark-up to open the commenting box.

UNDO/DELETE CHANGE

To undo any changes made, use the right click button on your mouse (for PCs, Ctrl-Click for Mac). Alternatively click on the ‘Edit’ in the main Adobe menu and then ‘Undo’. You can also delete edits using the right click (Ctrl-Click on the Mac) and selecting ‘Delete’.

SEND YOUR ANNOTATED PDF FILE BACK TO WILEY VIA jmvprom@wiley.com

Save the annotations to your file and return as an e-mail. Before returning, please ensure you have answered any questions raised on the Query form that you have inserted all the corrections: later inclusion of any subsequent corrections cannot be guaranteed.

Note: Comprehensive instructions are provided within your PDF file: to access these instructions please click on the Comments and Markup menu in the main tool bar, or click on Help.
COPYRIGHT TRANSFER AGREEMENT

Date: ____________________ Contributor name: ____________________

Contributor address: ___________________________________________

Manuscript number (Editorial office only): __________________________

Re: Manuscript entitled __________________________________________

for publication in _____________________________________________

published by _________________________________________________

Dear Contributor(s):

Thank you for submitting your Contribution for publication. In order to expedite the editing and publishing process and enable Wiley-Blackwell to disseminate your Contribution to the fullest extent, we need to have this Copyright Transfer Agreement signed and returned as directed in the Journal's instructions for authors as soon as possible. If the Contribution is not accepted for publication, or if the Contribution is subsequently rejected, this Agreement shall be null and void. Publication cannot proceed without a signed copy of this Agreement.

A. COPYRIGHT

1. The Contributor assigns to Wiley-Blackwell, during the full term of copyright and any extensions or renewals, all copyright in and to the Contribution, and all rights therein, including but not limited to the right to publish, republish, transmit, sell, distribute and otherwise use the Contribution in whole or in part in electronic and print editions of the Journal and in derivative works throughout the world, in all languages and in all media of expression now known or later developed, and to license or permit others to do so.

2. Reproduction, posting, transmission or other distribution or use of the final Contribution in whole or in part in any medium by the Contributor as permitted by this Agreement requires a citation to the Journal and an appropriate credit to Wiley-Blackwell as Publisher, and/or the Society if applicable, suitable in form and content as follows: (Title of Article, Author, Journal Title and Volume/Issue, Copyright © [year], copyright owner as specified in the Journal). Links to the final article on Wiley-Blackwell’s website are encouraged where appropriate.

B. RETAINED RIGHTS

Notwithstanding the above, the Contributor or, if applicable, the Contributor’s Employer, retains all proprietary rights other than copyright, such as patent rights, in any process, procedure or article of manufacture described in the Contribution.

C. PERMITTED USES BY CONTRIBUTOR

1. Submitted Version. Wiley-Blackwell licenses back to the Contributor the following rights with respect to the final published version of the Contribution:

   a. After publication of the final article, the right to self-archive on the Contributor’s personal website or in the Contributor’s institution/employer’s institutional repository or archive. This right extends to both intranets and the Internet. The Contributor may not update the submission version or replace it with the published Contribution. The version posted must contain a legend as follows: This is the pre-peer reviewed version of the following article: FULL CITE, which has been published in final form at [Link to final article]

   b. The right to transmit, print and share copies with colleagues.

2. Accepted Version. Re-use of the accepted and peer-reviewed (but not final) version of the Contribution shall be by separate agreement with Wiley-Blackwell. Wiley-Blackwell has agreements with certain funding agencies governing reuse of this version. The details of those relationships, and other offerings allowing open web use, are set forth at the following website: http://www.wiley.com/go/funderstatement. NIH grantees should check the box at the bottom of this document.

3. Final Published Version. Wiley-Blackwell hereby licenses back to the Contributor the following rights with respect to the final published version of the Contribution:

   a. Copies for colleagues. The personal right of the Contributor only to send or transmit individual copies of the final published version in any format to colleagues upon their specific request provided no fee is charged, and further-provided that there is no systematic distribution of the Contribution, e.g. posting on a listserve, website or automated delivery.

   b. Re-use in other publications. The right to re-use the final Contribution or parts thereof for any publication authored or edited by the Contributor (excluding journal articles) where such re-used material constitutes less than half of the total material in such publication. In such case, any modifications should be accurately noted.

   c. Teaching duties. The right to include the Contribution in teaching or training duties at the Contributor’s institution/place of employment including in course packs, e-reserves, presentation at professional conferences, in-house training, or distance learning. The Contribution may not be used in seminars outside of normal teaching obligations (e.g. commercial seminars). Electronic posting of the final published version in connection with teaching/training at the Contributor’s institution/place of employment is permitted subject to the implementation of reasonable access control mechanisms, such as user name and password. Posting the final published version on the open Internet is not permitted.

   d. Oral presentations. The right to make oral presentations based on the Contribution.

4. Article Abstracts, Figures, Tables, Data Sets, Artwork and Selected Text (up to 250 words).

   a. Contributors may re-use unmodified abstracts for any non-commercial purpose. For on-line uses of the abstracts, Wiley-Blackwell encourages but does not require linking back to the final published versions.

   b. Contributors may re-use figures, tables, data sets, artwork, and selected text up to 250 words from their Contributions, provided the following conditions are met:

      (i) Full and accurate credit must be given to the Contribution.

      (ii) Modifications to the figures, tables and data must be noted. Otherwise, no changes may be made.

      (iii) The reuse may not be made for direct commercial purposes, or for financial consideration to the Contributor.

      (iv) Nothing herein shall permit dual publication in violation of journal ethical practices.
D. CONTRIBUTIONS OWNED BY EMPLOYER

1. If the Contribution was written by the Contributor in the course of the Contributor's employment (as a "work-made-for-hire" in the course of employment), the Contribution is owned by the company/employer which must sign this Agreement (in addition to the Contributor's signature) in the space provided below. In such case, the company/employer hereby assigns to Wiley-Blackwell, during the full term of copyright, all copyright in and to the Contribution for the full term of copyright throughout the world as specified in paragraph A above.

2. In addition to the rights specified in paragraph B above and the rights granted back to the Contributor pursuant to paragraph C above, Wiley-Blackwell hereby grants back, without charge, to such company/employer, its subsidiaries and divisions, the right to make copies of and distribute the final published Contribution internally in print format or electronically on the Company's internal network. Copies so used may not be resold or distributed externally. However, the company/employer may include information and text from the Contribution as part of an information package included with software or other products offered for sale or license or included in patent applications. Posting of the final published Contribution by the institution on a public access website may only be done with Wiley-Blackwell's written permission, and payment of any applicable fee(s). Also, upon payment of Wiley-Blackwell's reprint fee, the institution may distribute print copies of the published Contribution externally.

E. GOVERNMENT CONTRACTS

In the case of a Contribution prepared under U.S. Government contract or grant, the U.S. Government may reproduce, without charge, all or portions of the Contribution and may authorize others to do so, for official U.S. Government purposes only, if the U.S. Government contract or grant so requires. (U.S. Government, U.K. Government, and other government employees: see notes at end)

F. COPYRIGHT NOTICE

The Contributor and the company/employer agree that any and all copies of the final published version of the Contribution or any part thereof distributed or posted by them in print or electronic format as permitted herein will include the notice of copyright as stipulated in the Journal and a full citation to the Journal as published by Wiley-Blackwell.

G. CONTRIBUTOR'S REPRESENTATIONS

The Contributor represents that the Contribution is the Contributor's original work, all individuals identified as Contributors actually contributed to the Contribution, and all individuals who contributed are included. If the Contribution was prepared jointly, the Contributor agrees to inform the co-Contributors of the terms of this Agreement and to obtain their signature to this Agreement or their written permission to sign on their behalf. The Contribution is submitted only to this Journal and has not been published before. (If excerpts from copyrighted works owned by third parties are included, the Contributor will obtain written permission from the copyright owners for all uses as set forth in Wiley-Blackwell’s permissions form or in the Journal’s Instructions for Contributors, and show credit to the sources in the Contribution.) The Contributor also warrants that the Contribution contains no libelous or unlawful statements, does not infringe upon the rights (including without limitation the copyright, patent or trademark rights) or the privacy of others, or contain material or instructions that might cause harm or injury.

CHECK ONE BOX:

☐ Contributor-owned work

ATTACH ADDITIONAL SIGNATURE PAGES AS NECESSARY

Contributor’s signature

Date

Type or print name and title

Co-contributor’s signature

Date

Type or print name and title

☐ Company/Institution-owned work

(made-for-hire in the course of employment)

Company or Institution (Employer-for-Hire)

Date

Authorized signature of Employer

Date

☐ U.S. Government work

Note to U.S. Government Employees

A contribution prepared by a U.S. federal government employee as part of the employee’s official duties, or which is an official U.S. Government publication, is called a “U.S. Government work,” and is in the public domain in the United States. In such case, the employee may cross out Paragraph A.1 but must sign (in the Contributor’s signature line) and return this Agreement. If the Contribution was not prepared as part of the employee’s duties or is not an official U.S. Government publication, it is not a U.S. Government work.

☐ U.K. Government work (Crown Copyright)

Note to U.K. Government Employees

The rights in a Contribution prepared by an employee of a U.K. government department, agency or other Crown body as part of his/her official duties, or which is an official government publication, belong to the Crown. U.K. government authors should submit a signed declaration form together with this Agreement. The form can be obtained via http://www.opsi.gov.uk/advice/crown-copyright/copyright-guidance/publication-of-articles-written-by-ministers-and-civil-servants.htm

☐ Other Government work

Note to Non-U.S., Non-U.K. Government Employees

If your status as a government employee legally prevents you from signing this Agreement, please contact the editorial office.

☐ NIH Grantees

Note to NIH Grantees

Pursuant to NIH mandate, Wiley-Blackwell will post the accepted version of Contributions authored by NIH grant-holders to PubMed Central upon acceptance. This accepted version will be made publicly available 12 months after publication. For further information, see www.wiley.com/go/nihmandate.
COLOR REPRODUCTION IN YOUR ARTICLE

These proofs have been typeset using figure files transmitted to production when this article was accepted for publication. Please review all figures and note your approval with your submitted proof corrections. In the event that color figure were included with the final manuscript files that we received for your article, this form must be completed and returned with your corrected proofs.

Because of the high cost of color printing, we can only print figures in color if authors cover the expense. Please indicate if you agree to pay charges for color figures to be printed in color (see table below for prices) or prefer black and white reproduction.

You will be invoiced for color charges once the article has been published in print.

Failure to return this form with your article proofs may delay the publication of your article.

JOURNAL OF MEDICAL VIROLOGY

JOURNAL ______________________ MS. NO. ____________ NO. COLOR PAGES ____________

MANUSCRIPT TITLE __________________________________________

AUTHOR(S) ________________________________________________

<table>
<thead>
<tr>
<th>No. Color Pages</th>
<th>Color Charge</th>
<th>No. Color Pages</th>
<th>Color Charge</th>
<th>No. Color Pages</th>
<th>Color Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$950</td>
<td>5</td>
<td>$3400</td>
<td>9</td>
<td>$5850</td>
</tr>
<tr>
<td>2</td>
<td>$1450</td>
<td>6</td>
<td>$3900</td>
<td>10</td>
<td>$6350</td>
</tr>
<tr>
<td>3</td>
<td>$1950</td>
<td>7</td>
<td>$4400</td>
<td>11</td>
<td>$6850</td>
</tr>
<tr>
<td>4</td>
<td>$2450</td>
<td>8</td>
<td>$4900</td>
<td>12</td>
<td>$7350</td>
</tr>
</tbody>
</table>

***Contact jmvprod@wiley.com for a quote if you have more than 12 pages of color***

☐ Please print my figures color ☐ Please print my figures in black and white

☐ Please print the following figures in color __________________________

and convert these figures to black and white __________________________

Approved by __________________________

Billing Address __________________________________________

E-mail __________________________________________

Telephone __________________________________________

Fax __________________________________________
Additional reprint and journal issue purchases

Should you wish to purchase additional copies of your article, please click on the link and follow the instructions provided: https://caesar.sheridan.com/reprints/redir.php?pub=10089&acro=JMV

Corresponding authors are invited to inform their co-authors of the reprint options available.

Please note that regardless of the form in which they are acquired, reprints should not be resold, nor further disseminated in electronic form, nor deployed in part or in whole in any marketing, promotional or educational contexts without authorization from Wiley. Permissions requests should be directed to mailto: permissionsus@wiley.com

For information about ‘Pay-Per-View and Article Select’ click on the following link: http://wileyonlinelibrary.com/ppv