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Argentine hemorrhagic fever vaccines

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Argentine hemorrhagic fever (AHF), an acute disease caused by Junin virus (JUNV, Arenaviridae), has been an important issue to public health in Argentina since the early 1950s. The field rodent *Calomys musculinus* is JUNV natural reservoir and human disease is a consequence of contact with infected rodents. A steady extention of AHF endemic area is being observed since the first reports of the disease. Important achievements have been made in: (a) improvement of methods for the etiological diagnosis; (b) implementation and validation of therapeutical measures; (c) development of vaccines to protect against AHF. Reference is made to different research strategies used to obtain anti-AHF vaccines in the past and anti-arenaviral diseases in the present. Information is updated on features and field performance of Candid #1 vaccine, a live attenuted vaccine currently used to prevent AHF. This vaccine was developed through a joint international effort that envisioned it as an orphan drug. With transferred technology, Argentine government was committed to be Candid #1 manufacturer and to register this vaccine as a novel medical product under the Argentine regulatory authority. Candid #1 vaccine is the first one used to control an arenaviral hemorrhagic fever, the first live viral vaccine to be manufactured and registered in Argentina, reaching its target population through governmental effort.

Introduction

Argentine hemorrhagic fever (AHF) is an acute viral infection caused by Junin virus (JUNV), one of six agents known to be pathogenic for man, belonging to the Genus Arenavirus, integrated by at least 23 recognized agents.[1]

The virions of the Arenaviridae Family are lipid-enveloped, pleomorphic particles, surrounded by an electron-dense unit membrane with external surface projections. In the interior, the nucleocapside appears as a close, circular structure involving two predominant size classes of viral genome RNA, named as S (short) and L (large) RNA. Virions have three main protein components: nucleocapside protein (NP), the envelope proteins GP1 and GP2, derived from the cleavage of a common precursor GPC and the nonglicosilated protein Z.[2-5] Also, electron-dense granules are usually present, and have been identified as host-cell-derived ribosomes of unknown significance.

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Like most arenaviruses, JUNV has a primary rodent species as a natural reservoir, the field mouse *Calomys musculinus*.[6-8] Humans usually become infected through direct contact with infected rodents or by inhaling infectious rodent excreta or secretions, during labor or recreational exposure. Consequently, the AHF endemic area is mainly determined by the natural cycle of JUNV in its natural host, sub-summing the extent of geographical distribution of *C. musculinus*, widely spread in central and northwestern Argentina.[9]

The Disease

Seroepidemiological surveys demonstrated a low prevalence of inapparent or subclinical human infections with JUNV,[10,11] but the regular consequence of human infection with JUNV is the development of signs and symptoms to allow the presumptive diagnosis of FHA, a disease originally described by Arribalzaga in 1955.[12] Confirmatory diagnosis is made by JUNV isolation and/or specific serological conversion.[13-15]

AHF epidemics occur yearly involving a variable number of cases from historical 3,500 to current 30 to 50 confirmed cases per year.

The incubation period for AHF is estimated to be between 7 and 14 days, and starts with a gradual onset of asthenia, anorexia, myalgia and fever through several days, followed by further constitutional, gastrointestinal, neurological and cardiovascular symptoms and signs. Back pain, epigastric pain, headache, retro-orbital pain, photophobia, dizziness, constipation and mild diarrhea may occur. Frequently, vascular phenomena like flushing of the chest, neck and face, conjuntival injection, congestion and even bleeding of the vessels bordering the gums are present, as well as petechiae and/or tiny vesicles over an erythematous palate and fauces. In most cases, cutaneous petechiae will be found, mainly in the axillary region. Neurological signs are also very common. At the end of this phase the patient may be irritable, lethargic and with a fine tremor of the hand and tongue. Moderate ataxia, cutaneous hyperesthesia and a decrease in deep tendon reflexes and muscular tonicity are present.[16,17] Superimposed oral candidiasis is frequently found at the end of this phase.

Worsening of vascular and/or neurological symptoms has a fatal prognosis and account for 15% to 20% mortality from AHF.

Treatment of the disease consists of early transfusion of immune plasma from AHF convalescent people, administered in standardized doses of neutralizing antibodies.[18] Around 10% of the cases treated with immune plasma develop a late neurologic syndrome (LNS). This LNS begins after a period free of symptoms, and is characterized by febrile symptoms, cerebellar

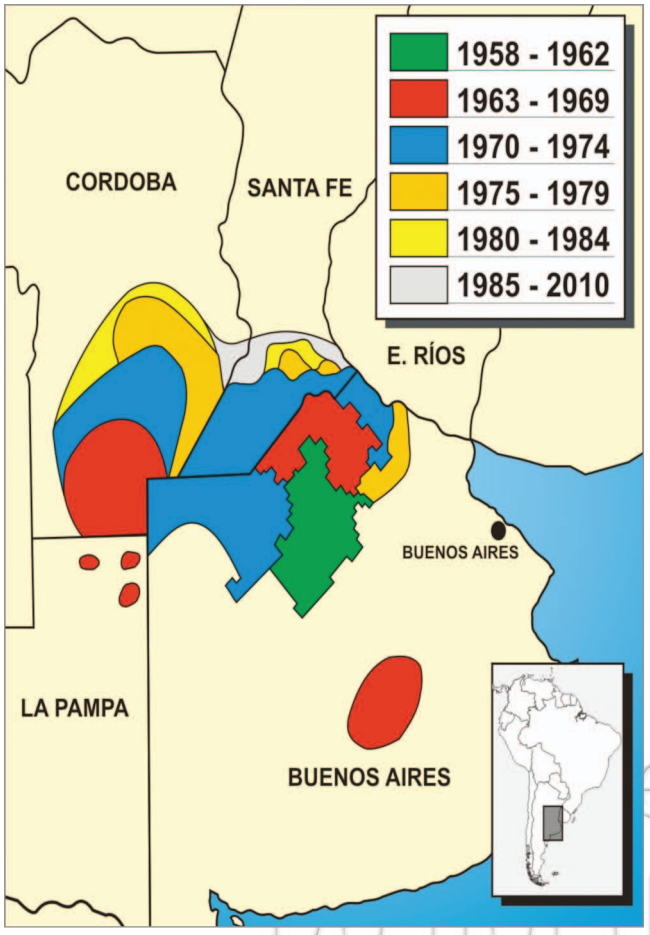

Figure 1. Progressive extension of Argentine hemorrhagic endemic area.

signs and cranial nerve palsies.[19] LNS had been exclusively registered among immune plasma treated patients, the pathogenesis is unclear and is not related to donor, severity of the disease, antibody titer in the transfused plasma or other variables examined.[20] LNS patients recover with symptomatic treatment. Surviving cases experience a prolonged, protracted convalescence that lasts from one to three months.

Retrospective studies show the continuous changing in both incidence and distribution of AHF. When the disease was first reported in the early 1950s, the extension of the affected area was about 16,000 km^2 involving an estimated number of 270,000 at-risk people. As shown in **Figure 1**, cases were recorded in an steadily growing area up to present, when AHF has spread over 150,000 km^2, inhabited by more than three million people.[21] No AHF cases have been detected outside the Argentine areas depicted in **Figure 1**, though previous studies on JUNV activity in rodent populations indicate a likely further extension of the endemic area toward northwest, in a trend to follow geographical range of *C. musculinus*. Cases occur in clusters varying in both time and space, being factors in emergency and re-emergency of AHF poorly understood, though previous research indicates that epidemiological features of AHF are strongly influenced by the JUNV activity in variably dense populations of *C. musculinus*.[7,8]

Since the first reports on AHF and the natural cycle of JUNV in field rodents, it was understood that reservoirs of JUNV as well as their possible contact with humans cannot be eliminated, and main effort was directed to therapeutics and/or preventive strategies to protect at-risk population. This was addressed by three main approaches: (1) to count on reliable AHF incidence records, for which the availability of methods for viral diagnostic of the disease was greatly improved; (2) to assist in AHF cases, for which the efficacy of treatment of AHF patients with convalescent plasma was demonstrated;[19] (3) to coordinate research to obtain an effective antigen for human immunization against JUNV. For the last, attempts started in 1959, along four research strategies, reaching different development stages, as depicted in **Table 1**.

Killed JUNV antigens, obtained by different inactivation methods, resulted in unsuitable products. Main troubles were found in the huge amounts of antigen required for immune stimulation, the need for repeated inoculation and the transient protection conferred by these viral preparations. Actual improved culture and purification methods plus the use of adjuvants might lead to improvements in yield, purity and immunogenicity of inactivated JUNV immunogens.

Research lines toward development of live antigens yielded more promising results. In several previous studies, heterologous live Tacaribe virus (another member of Arenaviridae family) was demonstrated to protect experimental animals from JUNV challenge, facing the drawbacks of short termed immunity. Tacaribe virus was proposed as a candidate for a heterologous vaccine against AHF, though few data on eventual consequences of human infection with this virus are available. Live attenuated JUNV $XJCl_3$ strain was derived from a highly passaged XJ prototype strain cloned in MA-111 cell line rendering attenuated for guinea pigs and eliciting long lasting immunity with high titers of neutralizing antibodies. Following these findings a seed vaccine was prepared in suckling mouse brain to be used in the first trial in humans. Along a two-year period, up to 636 healthy volunteers from the endemic area were inoculated with $XJCl_3$, most of whom developed a subclinical infection with mild symptoms. Presence of neutralizing antibodies was demonstrated in 90% of these volunteers up to nine years post-vaccination. Clinical trials with this vaccine were interrupted due to the fact that this antigen was prepared in suckling mouse brain and cloned in a heteroploid cell line. Published records on clinical trials with $XJCl_3$ have been of great value as base data to compare the performance of latter developments on AHF prevention.

XJ0, another JUNV attenuated strain, derived from a common parental line with $XJCl_3$, was used in experimental animals. XJ0 had not been replicated in heteroploid cells, and it was reported to confer protection against pathogenic challenge in guinea pigs. These studies were discontinued when persistent JUNV was isolated from lymphohemopoietic organs of guinea pigs up to 60 days post-inoculation of XJ0 strain.

Live attenuated JUNV Candid #1 (C#1) strain was developed as the result of a joint effort started in 1979 involving Argentine Ministry of Health and Social Action, US Army

Table 1. Summary of developments in argentine hemorrhagic fever vaccines

Vaccine development strategy	Inactivation procedure immunogen	Pre -linical assays results	Clinical trial	Citation number
Killed virus	Formalin inactivation	Mainly protective	Yes	22–24
	Photoinactivation	Mainly protective	No	25–28
Viral subunits	Capside glycoprotein G38	Immunogenic and protective	No	29–30
Heterologous live virus	Tacaribe virus	Immunogenic and protective	No	31–39
	Animal and tissue culture passage Junin $XJCl_3$	Immunogenic and protective	Yes	40–45
Homologous live attenuated virus	Animal and tissue culture passage Junin XJ_0 strain	Immunogenic and protective	No	46–49
	Animal passage and tissue culture cloning Junin Candid #1	Immunogenic and protective	Yes	50–53

Immunogenic, triggers a detectable immune response; Protective, protects against a challenge with pathogenic JUNV or AHF.

Medical Research Institute of Infectious Diseases, under auspices of United Nations Development Program and Pan American Health Organization.[54] C#1 was derived from parental strain XJ 44 through final dilution and pseudo-single burst cloning in FRhL-2 certified diploid cell line (ATCC-CCL 160). C#1 has a well defined passage history, and extensive preclinical assays in mice, guinea pigs and Rhesus monkeys demonstrated this viral strain to be a safe and immunogenic vaccine candidate. Master seed and secondary seed were harvested after two additional passages in FRhL-2 cells. As a vaccine, C#1 history includes one passage in guinea pig, 44 passages in suckling mice brain and 19 passages in FRhL-2 cells.

C#1 is a vaccine for which the target population is small enough to make it commercially unappealing, since private provision would not compensate the costs for elaboration and distribution of the product. These circumstances, which define C#1 as an orphan drug,[55] were envisioned since the begining of the development task, when Argentine government committed itself to be the manufacturer and sponsor of C#1 vaccine.

First pilot lots of C#1 were elaborated at The Salk Institute, Swiftwater, PA USA, to be used in phases I, II and III clinical trials involving over 6,500 volunteers, inoculated in randomized, double-blind, placebo-controlled studies performed on adult males, in Argentina. These trials demonstrated that the protective efficacy of C#1 against AHF was 95% (95% CI: 82–99%).[16]

Production of Candid #1 Vaccine in Argentina

The accomplishment of Argentine commitment to be the C#1 manufacturer required the building of new facilities to fulfill GMP requirements.[56] These included four independent units: a specific pathogen free breeding mice colony, tissue culture banking area, vaccine production and vaccine quality control/quality assurance areas. A 3,267 square meters plant, including technical over ceiling, was built as part of Instituto Nacional de Enfermedades Virales Humanas (INEVH-ANLIS) in Pergamino city, Province of Buenos Aires, in Argentina. The four units, each with independent ventilation system and restricted/unidirectional circulation for operators, materials and wastes, were sequentially started between 1994 and 1999. Concomitantly, the technology for production and quality control of C#1 vaccine was transferred from the The Salk Institute to Argentine manufacturers and every procedure was performed following a GMP (Good Manufacturing Practice) quality system. The facility was inspected and approved by ANMAT, the Argentine regulatory authority, as a plant to manufacture viral live attenuated vaccines for human use (ANMAT Disposition N° 3775/01).

C#1 Master and Work Seeds were furnished as a part of the original international agreement that led to the development of this vaccine.

The new facility was started by elaborating small consistency lots of C#1 vaccine. Lot N° 3 was used in a preclinical assay to compare the performance of this vaccine with that of USA manufacturers in protecting guinea pigs against a challenge with a virulent JUNV strain. Results were comparable between vaccine from both manufacturers and with those in original C#1 research/development data.[53,57]

Lot N° 7A was the first up scaled lot of Argentine C#1 vaccine, elaborated after a number of consistency lots, used in a human trial. This was done following a clinical assay protocol due to the change in the vaccine manufacturer laboratory. A bridging clinical study was designed to compare C#1 vaccine manufactured in Argentina with the vaccine produced in USA that had been previously used. Nine hundred forty six human volunteers older than 15 years were inoculated in a randomized, double-blind fashion. Comparison was made on the basis of immunogenicity as a subrogating indicator of efficacy. The general events considered related to the vaccines were not clinically relevant and disappeared either spontaneously or with symptomatic treatment. Rates of adverse events were not significantly different between vaccines from both manufacturers, whilst the immunogenicity, as measured by detection of anti-JUNV neutralizing antibody, resulted in equivalent mean titers (XG 148 vs. 176; seroconversion 97.1% vs. 99.5%).[58] These results entitled the Instituto Nacional de Enfermedades Virales Humanas (INEVH) to register the vaccine produced in Argentina under the National Regulatory Authority (ANMAT, Resolution N° 4882, August 2006).

Candid #1 Vaccine Features

Safety. C#1 vaccine safety was consistently demonstrated from pre-clinical and clinical assays performed along development

and registration of this vaccine. Inoculation of C#1 in Rhesus monkeys, rabbits and guinea pigs in different ages, weights, dosage and routes induced no changes in the healthy original status of the animals.

In humans, initial trials involving 83 volunteers, randomized in different cohorts that received C#1 in a variety of dosage and inoculation routes, the vaccine was clinically well tolerated along follow up periods from one month to two years.

In Phase II clinical trial, none of 55 new volunteers developed significant signs and/or symptoms and similar results were found in Phase III trial: out of 3,255 volunteers that received C#1, only six (1.1%) had one or more reported adverse effects, mainly consisting of headache and minor constitutional symptoms.[16]

Records on adverse effects in all the above clinical assays were based on a passive reporting system. When assessing the comparability of C#1 manufactured in US with the vaccine manufactured in Argentina, a clinical bridging study was designed to emphasize the search for adverse effects by means of an active follow up system. From 946 volunteers vaccinated with C#1 no severe adverse events related to the vaccines were reported. The general events considered related to C#1 vaccine were not clinically relevant and ceased either spontaneously or with symptomatic treatment.[58]

Viral strain attenuation. Glycoproteins GP1 and GP2 have been identified as the structural components of the spikes on the arenaviruses envelope, being GP1 the most variable among these viral species. These data point them as major targets of the host immune response: NT antibodies are elicited only against the glycoproteins, and so seems to happen with T lymphocytes involved in the cellular immune response to experimental arenaviral infection. Furthermore, in the context of the various components of the immune response, the expression of the arenavirus glycoproteins would play a major role in viral pathogenesis and, transitively, can be expected to influence over attenuation features of viral strains.

C#1 has been developed through final dilution and pseudo single burst cloning of the parental JUNV strain XJ44 in FRhL-2 diploid cells.[53] The cloned and sequenced GPC gene of C#1 was compared on ORF regions of SRNA of wild-type MC2 strain. Approximately 20 changes in the NH_2-proximal, and 19 in the COOH-proximal were found. But major changes were described in the GPC amino-terminal region that would determine important alterations in G_1, located in the virion surface and associated with the more internal G_2 protein.[59,60]

The attenuation phenotypic features are stable up to at least six passages in diploide cells, over the vaccine production level, but tends to be more variable through in vivo passages.

C#1 can be recovered from peripheral blood mononuclear cells (PBMC) of vaccine recipients up to 15 days pi. When PBMC are co cultivated on Vero cells JUNV can be found in the supernatants of these co cultures.[16] Dlb strain of JUNV, isolated from one individual ten days post-immunization with C#1, was inoculated i.m. into 21- to 30-day-old Hartley guinea pigs (weight 200 to 350 gr), to compare its attenuated phenotype with C#1 vaccine and parental strain XJ44. Rectal temperature, body weight and any other disease signs were recorded along the experiments. Groups of three inoculated animals were sacrificed on days 5, 8, 11 and 15. Three control guinea pigs, inoculated with viral diluent were incorporated, observed for 16 to 18 d pi and sacrificed as described. On each sacrifice day, three guinea pigs were killed and spleen, cerebrum and spinal cord were individually harvested. Organs from two of them were homogenized, washed and co-cultured on Vero cells in two 10-fold dilutions.[61] Spleen, brain and spinal cord from the third animal on each sacrifice day were formalin fixed and used in histopathological studies. Co. cultures were observed for 21 days and supernatants from each one were weekly harvested. JUNV was sought in these samples by PFU counting on Vero cells under agarose. **Table 2** summarizes the viral isolations from co-cultivated organs of guinea pigs inoculated with the studied JUNV strains and control animals. JUNV was found only in spleens of C#1 inoculated guinea pigs up to day 8 pi, while in animals inoculated with Dlb strain, virus was isolated from spleen up to day 11 pi and CNS (brain and spinal cord) up to 8 days pi. In contrast, JUNV was recovered from spleens, brains and spinal cords from every animal inoculated with the parental strain XJ44. No virus was detected in any of the control animals. In a simultaneous experiment to search for disease symptoms, as well as protective and lethal indexes of the same JUNV strains, no signs of disease were found in any guinea pig, and all those inoculated were protected against a challenge with a pathogenic JUNV strain (Gamboa G, personal communication). Histopathological examination in HE preparations showed no tisular alterations neither in inoculated nor control guinea pigs. Similar results were found when C#1 and XJ44 were inoculated in younger guinea pigs (8–10 days old), except that histopathological lesions were demonstrated in XJ44 infected animals and rear limb paralysis developed in 50% of them, from day 9 pi. Neither lesions nor signs of disease were found in C#1 inoculated and control animals (unpublished results). Lethal index for Dlb strain was >5.10, a higher value than 3.62, the reference lethal index for Candid #1.[53] The significance of this difference remains to be established, but preliminarily suggests that C#1 attenuated behavior is kept in Dlb-inoculated animals, though results shown herein demonstrate that in absence of clinical symptoms and/or visible histopathological alterations, the virus changed its neurotropism, reaching brain and spinal cord as soon as four days following ip inoculation, in a similar fashion that the less attenuated XJ44 parental strain. The only difference found was that Dlb strain became undetectable after eight days while XJ44 inoculated animals had virus in CNS al least for 15 days pi (**Table 2**). In previous unpublished results, no JUNV could be recovered by organ co-cultures from guinea pigs infected by any route, with any JUNV strain by day 30 pi. Taken together, these data suggest that human passage increases C#1 neurotropism, in accordance with previous studies that demonstrate the retention by C#1 of minimal neurotropism and some variation in the attenuated phenotype after in vivo passage.[53]

Immune response. The immune response to C#1 vaccine is indistinguishable from that found in a mild AHF, with a central role for humoral immune mechanisms. Specific anti-JUNV neutralizing antibodies become detectable from 15 days pi in

Table 2. Junin virus isolation by co cultivation of organs from infected and control guinea pigs on Vero cells

Strain	Spleen				Brain				Spinal cord			
	Day pi				Day pi				Day pi			
	4	8	11	15	4	8	11	15	4	8	11	15
C#1	+	+	−	−	−	−	−	−	−	−	−	−
Dlb	+	+	+	−	+	+	−	−	+	+	−	−
XJ44	+	+	+	+	+	+	+	+	+	+	+	+
Control Animals	−	−	−	−	−	−	−	−	−	−	−	−

+ Positive − Negative

an average of 18% of vaccinees, while 97% to 99% seroconvert by day 60 pi.[58] NT antibodies are known to confer protection against new infections with the same arenavirus, and for this reason NT antibody detection is used as a subrogate parameter in the determination of C#1 protective efficacy against AHF. Mean titers of NT antibodies elicited by C#1 are significantly lower than those raised by natural JUNV infection (X 773 vs. X 49; $p < 0.05$) probably due to different viral strains capabilities for replication and immune stimulation, but they persist for many years and probably for lifetime.[62] C#1 neutralizing antibody response consists of IgG, manly IgG1 subtype, which is frequently found in primary viral infections and has the most efficient complement-fixing activity, as well as high affinity binding to lymphocytes and macrophages.[63] This contributes to the importance of other immune functions, mainly directed to killing of infected cells.[64,65] Titers of α endogenous interferon, known to correlate with clinical severity and fatal outcome in AHF, remain at normal values in vaccinated individuals.[66,67]

Antibody-independent cellular mechanisms may also be important since JUNV-specific lymphocyte proliferation can be measured in vaccinated individuals whose NT antibodies are not detectable.[68,69] In this regard, it is worth to mention that about 19% of immunized people may become temporarily negative to anti-JUNV NT tests, and reappear as positive in later serum samples.[70] This could be attributed to low NT antibody titers edging test detection limits and/or new contacts with JUNV in nature, resulting in activation of memory cells, which role is emphasized by the low numbers of AHF cases among vaccinated people: along a 20 year period, only 12 cases of mild AHF have been recorded in approximately 257,000 immunized people (unpublished results).

Vaccination Campaigns to Prevent Argentine Hemorrhagic Fever

C#1 is being used as a lyophilized product to immunize males and females >15 years old at risk of having AHF. This vaccine, with a demonstrated protective efficacy of 95% against AHF,[16] was incorporated into the Argentine National Immunization Plan in 2007, and yearly campaigns are implemented during interepidemic season, aimed to a mainly rural population in the disease-endemic area. This intervention had a decisive impact on decreasing the magnitude of epidemic outbreaks, as well as on a relative increase in the AHF incidence in children younger than 15 years.[71]

As a biological product, C#1 is obtained by harvest of infected diploid cell cultures supernatants, by procedures widely proven in the manufacture of several massively used human vaccines. Stability studies demonstrated that C#1 can be stored at least six years at -20°C, which facilitates vaccine stock planning to face unexpected outbreaks of the disease. One disadvantage to be overcome is the thermal lability of C#1, since potency remains only 30 days at 2–8°C, and this is a limiting condition to store the vaccine close to the target population at rural sites.

Immunization campaigns seem to require the administration of a unique dose due to the long-lasting protection conferred by C#1. This protection boosters preexisting antibodies to JUNV and is not interfered by previous infection with other arenaviruses coexisting in AHF endemic area.[72] No data are available on simultaneous administration of C#1 with other vaccines, and its use is not recommended in immunologically compromised recipients and pregnant women, though no teratogenic effects have been proven.

Future Perspectives

Argentine hemorrhagic fever is currently been prevented by the administration of C#1, a live attenuated JUNV vaccine, that shows optimal balance between reactogenicity and immunogenicity. Experimental results in Rhesus monkeys have also demonstrated that C#1 protects primates from a challenge with Machupo virus, the etiological agent of Bolivian hemorrhagic fever. This cross protection is expectable given the genetical proximity of Junin and Machupo viruses, both belonging to Clade B of the New World subgroup of Arena virus.[73,74] Future studies are due on C#1 formulation to improve the heat stability of this vaccine in order to extend its availability at rural vaccination sites. On the other hand, new clinical trial protocols should address vaccination of children in the AHF endemic area. Novel strategies for cross protective vaccines for arenaviruses are being proposed that would circumvent the drawbacks related to risk for viral reversion and other limitations of attenuated viral vaccines once they have been up scaled and submitted to clinical trials.[75-77]

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