Published in final edited form as:

Vaccine. 2016 February 10; 34(7): 950–956. doi:10.1016/j.vaccine.2015.12.045.

Immunogenicity and efficacy of alphavirus-derived replicon vaccines for respiratory syncytial virus and human metapneumovirus in nonhuman primates

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Abstract

Human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are major causes of illness among children, the elderly, and the immunocompromised. No vaccine has been licensed for protection against either of these viruses. We tested the ability of two Venezuelan equine encephalitis virus-based viral replicon particle (VEE-VRP) vaccines that express the hRSV or hMPV fusion (F) protein to confer protection against hRSV or hMPV in African green monkeys. Animals immunized with VEE-VRP vaccines developed RSV or MPV F-specific antibodies and serum neutralizing activity. Compared to control animals, immunized animals were better able to control viral load in the respiratory mucosa following challenge and had lower levels of viral genome in nasopharyngeal and bronchoalveolar lavage fluids. The high level of

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immunogenicity and protective efficacy induced by these vaccine candidates in nonhuman primates suggest that they hold promise for further development.

INTRODUCTION

Human respiratory syncytial virus (hRSV) and human metapneumovirus (hMPV) are negative-sense RNA viruses and members of the family *Paramyxoviridae* [1]. Each virus is a significant cause of severe lower respiratory tract infection [2–5]. The very young, the elderly, and immunocompromised populations are particularly at risk, although symptomatic infection also occurs in the healthy adult population [6].

Infection with hRSV [7, 8] or hMPV does not confer sterilizing immunity, and an effective vaccine has not yet been licensed for either virus. Trials conducted in the 1960s of a formalin-inactivated RSV vaccine resulted in significantly enhanced respiratory disease among vaccinees [9–12] and in the death of two trial participants upon natural infection [10]. Similar effects of immunization with formalin-inactivated RSV have since been observed in animal studies [13, 14]. Preclinical studies with many non-replicating RSV vaccine candidates have stalled because of concerns about enhanced disease in animal models.

Immunization with live attenuated RSV strains or with other viruses that express RSV antigens does not result in enhanced disease in NHP [15], although in some instances, immunization of mice with chimeric viruses that express RSV antigens can result in enhanced disease [16]. However, no live vaccine has been approved for RSV or MPV. Two important differences between immunization with live vaccines and inactivated or subunit vaccines are the *in vivo* production of native antigen and the activation of the intracellular innate immune response by live virus infection. Notably, both of these differences are thought to contribute to the failure of the formalin-inactivated RSV vaccine. We have previously developed and tested virus replicon particle (VRP) vaccines against hMPV and hRSV in mice and cotton rats [17, 18]. Others have demonstrated the ability to elicit RSV-neutralizing antibodies with VRP in macaques [19], but their protective efficacy has never been tested in nonhuman primates. Like live vaccines, the VRP vaccines activate innate immune pathways [20, 21] and elicit adaptive immune responses to glycoprotein antigens expressed *in vivo* [22]. Parenteral injection of VRP vaccines also stimulates a mucosal immune response [22, 23]. Here we extend our previous work in rodents and show that VRP-based RSV and MPV vaccines are also effective at stimulating protective mucosal immunity in non-human primates.

2. MATERIALS AND METHODS

2.1 VEE replicon constructs and generation of virus replicon particles (VRPs) containing genes encoding hRSV F or hMPV F

Venezuelan equine encephalitis VRPs encoding hRSV F (designated VRP-RSV.F) or hMPV F (VRP-MPV.F) proteins were produced, as previously described [22]. Briefly, the hRSV or hMPV F genes were inserted into a VEE-based replicon cDNA, pVR21, which was derived from mutagenesis of a cDNA clone of the Trinidad donkey strain of VEE. The heterologous

genes were cloned into pVR21 downstream of the subgenomic 26S promoter via a two-step PCR and ligation process. For generation of VRPs, capped RNA transcripts of pVR21 containing hRSV or hMPV F genes were generated *in vitro* with the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). Similarly, helper transcripts that encoded the VEE capsid and glycoproteins genes were generated *in vitro*. Baby hamster kidney (BHK) cells then were co-transfected by electroporation with the pVR21 and helper RNAs and culture supernatants were harvested at 30 hours after transfection. VRPs were partially purified and concentrated by pelleting through 20% (w/v) sucrose in phosphate-buffered saline (PBS), then re-suspended in endotoxin-free PBS.

2.2 VRP titration

Serial dilutions of VRP-RSV.F or VRP-MPV.F were used to inoculate BHK cells in eight-chamber slides (Nunc) for 20 hours at 37 °C. Infected BHK cells were fixed and immunostained for VEE nonstructural proteins. Infectious units then were calculated from the number of stained cells per dilution and converted to infectious units (IU) per milliliter.

2.3 Vaccination and challenge of African green monkeys

African green monkeys aged 9 months to 2 years that tested seronegative for exposure to hRSV and hMPV were purchased from the Wake Forest Primate Facility (Winston-Salem, NC) and transferred to the Wisconsin National Primate Research Center (Madison, WI), where all experiments were conducted. Animals were segregated into groups as shown in Table 1. On day 0, animals were anesthetized and vaccinated intradermally in both arms with 10^8 infectious units (IU) of VRP-RSV.F or VRP-MPV.F. Animals were boosted with a second dose of the same vaccine and dose on day 28. Blood was drawn to provide samples for serology on days 0, 28, 36, 56 and 84. On day 56, animals were anesthetized and intubated before simultaneous inoculation by the intranasal and intratracheal routes with a 1 mL inoculum per site containing 10^6 PFU of hMPV strain A2 or hRSV strain A2 in Opti-MEM I medium (Invitrogen). Nasopharyngeal swabs and bronchoalveolar lavages were performed before challenge on day 56 and then every other day until day 68. Swabs and bronchoalveolar lavage fluids were frozen at stored at −80 °C until analyzed for viral titer. All experiments were conducted after receiving approval of the Wisconsin National Primate Research Center Institutional Animal Care and Use Committee.

2.4 ELISA of virus-specific serum IgG

Recombinant soluble trimeric forms of hRSV [24] or hMPVF [25, 26] proteins were produced in 293F cells and purified by FPLC as described previously. 384-well plates (Nunc) were coated with 2 μg/mL of recombinant hRSV F or hMPV F protein and incubated overnight at 4 °C. The plate was washed 3x, incubated with blocking buffer for 1 h at room temperature, and washed once more. Serum samples were diluted serially in blocking buffer and applied to the plate in triplicate. Following overnight incubation at 4 °C, the plate was washed 4x. A 1:4,000 dilution of alkaline phosphatase-conjugated anti-monkey IgG (Fitzgerald Industries, Acton, MA) was applied to the plate and incubated for one hour at room temperature. The plate was washed 4x and then incubated with 4-nitrophenyl phosphate disodium salt hexahydrate substrate solution (Sigma) for thirty minutes.

Absorbance was measured at 405 nm using a BioTek Power Wave HT plate reader. All washes were performed using a BioTek EL406 plate washer. Antigen-specific titer was determined by calculating the maximum inverse dilution that resulted in an absorbance of 0.2 greater than pre-immunization sera at the same dilution.

2.5 Neutralization of hRSV by antibodies in serum

The RSV neutralizing activity of antibodies in sera harvested from animals immunized with VRP was measured by a plaque reduction assay using HEp-2 cell culture and the wild-type hRSV strain A2. Diluted RSV strain A2 suspended to yield 50 plaques per well was mixed with 1:4 dilutions of sera, and incubated at 37 °C for 1 hr. Cell monolayers in 24-well tissue culture plates at 80–90% confluency were inoculated in duplicate by replacing the medium in each well with 75 μL of virus-serum mixture. After incubation at 37 °C for 1 hr, virus solution was aspirated from the wells, and cell monolayers then were overlaid with 0.75% methylcellulose in Opti-MEM I (Invitrogen) supplemented with 2% FBS, 320 μg/mL l-glutamine, 2.7 μg/mL amphotericin B, and 45 μg/mL gentamicin. Cultures were incubated for 4 days at 37 °C in 5% CO_2, after which the overlay was removed and the monolayers were fixed in 80% cold methanol. Plaques were stained and quantified by an immunoperoxidase staining procedure, as described [14]. Plaques for each sera dilution were counted, duplicate values averaged, and sera dilution versus plaque number plotted. The activity of serum was calculated as the inverse dilution that resulted in 60% reduction in the number of plaques. The 60% plaque reduction neutralizing activity was determined by regression curve analysis.

2.6 Neutralization of hMPV by antibodies in serum

MPV neutralization was performed as previously described [18, 27]. Briefly, a suspension of live hMPV was diluted to yield 50 plaques per well and mixed with 1:1 with dilutions of sera, and incubated at room temperature for one hour. Virus-serum mixtures or mock treated control virus were inoculated onto LLC-MK2 cell culture monolayers, adsorbed for 1 hour at room temperature, overlaid with 0.75% methylcellulose in Opti-MEM I medium with 5 μg/mL trypsin-EDTA, and incubated at 37°C with 5% CO_2 for 4 days. Cell culture monolayers were fixed and stained by immunoperoxidase using hMPV-specific antibodies to identify plaques. Calculation of the serum neutralizing activity for hMPV was the same as described above for RSV.

2.7 RSV titer in the respiratory tract following live virus challenge

RSV titer in lavage fluid and fluid recovered from nasal swabs was measured by directly plating on HEp-2 cell monolayer cultures. Clindamycin and levofloxacin were added to the overlay to prevent bacterial contamination of the culture by flora in the respiratory tract samples. HEp-2 cell cultures were grown and developed, and plaques were stained and quantified as described above.

2.8 RT-PCR assay to quantitate hRSV or hMPV titer in respiratory tract samples

Samples were thawed and RNA was extracted using the MagMax-96 Viral Isolation kit (Applied Biosystems) and stored at −80 °C until further use. Real-time RT-PCR was

performed in 25 μL reaction mixtures containing 5 μL of extracted RNA on an ABI StepOnePlus Real-Time PCR System (Life Technologies/Applied Biosystems) using the AgPath-ID One-Step RT-PCR kit (Applied Biosystems/Ambion). Primers and probe targeting the hRSV matrix (M) gene [28] or hMPV N gene [29] were used, as previously described. Cycling conditions were 50 °C for 30 min, followed by an activation step at 95 °C for 10 min and then 45 cycles of 15 sec at 95 °C and 30 sec at 60 °C. Samples with cycle threshold (Ct) values less than 40 were considered positive.

RESULTS

3.1 Serum RSV F- and MPV F-specific IgG

We have shown previously that immunization of mice and cotton rats with VRP-RSV.F and VRP-MPV.F vaccine constructs results in a protective immune response against hRSV and hMPV [17, 18]. To determine if similar levels of protection could be achieved in non-human primates, we immunized 16 African green monkeys with VRP encoding hRSV F (Table 1) or hMPV F (Table 2) and measured F-specific antibodies at four time points following immunization. Immunization with VRP-RSV.F resulted in significant levels of RSV F binding antibodies by day 28 following immunization, prior to boost. The titer in serum from each animal in the VRP-RSV.F group increased slightly in the eight days following boost, but titers on day 45 were not significantly different from titers on day 28. Sera from animals immunized with VRP-MPV.F or with media did not contain detectable levels of RSV F-specific IgG on day 28. Serum collected on day 36 (eight days following boost) contained low levels of RSV F-specific antibodies, though the titer in these animals waned by day 45. Control animals that were not immunized with VRP-RSV.F seroconverted following challenge on day 56.

The response to immunization was similar among animals immunized with VRP-MPV.F. All animals immunized with VRP-MPV.F had significant levels of MPV F-specific serum IgG 28 days following primary immunization and prior to boost. The boost immunization stimulated an increase in MPV F-specific IgG, and challenge with MPV stimulated a further increase. MPV F-specific antibodies were not detected in serum from control animals until after challenge on day 56.

3.2 Serum RSV and MPV neutralizing activity

Virus-neutralizing antibodies in serum have been identified as an important correlate of protection against acute LRI by RSV [30–32]. To determine if immunization with VRP-RSV.F stimulated the production of virus neutralizing antibodies, we measured the RSV virus neutralizing activity of serum from AGM immediately prior to immunization and at several time points following immunization (Table 3). Sera collected immediately prior to immunization did not contain detectable virus neutralizing activity. However, 28 days following immunization, sera from three of four animals immunized with VRP-RSV.F contained RSV-neutralizing activity. Following boost, neutralizing titers increased in all four animals. Challenge with RSV on day 56 resulted in a further increase in serum neutralizing antibodies by day 84 in three of four animals in the VRP-RSV.F group.

Immunization with VRP-MPV.F stimulated the production of serum MPV-neutralizing antibodies by 28 days following the primary immunization (Table 4). The boost immunization and challenge with virus stimulated increases in the level of MPV-neutralizing activity in serum in all animals in the VRP-MPV.F group. Virus neutralizing activity was not detected in serum from the control groups from either arm of the study until after challenge with virus.

3.3 Challenge RSV titer in BAL and nostril

On day 56 following primary immunization, animals were challenged via intranasal and intratracheal routes with a 1 mL inoculum per site containing 10^6 PFU of hMPV strain A2 or hRSV strain A2 in Opti-MEM I medium. BAL fluid and nostril swab samples were collected on day 58 before challenge and every other day until day 68. The numbers of RSV PFU in samples from the RSV arm of the study were measured by direct plaque assay of samples on HEp-2 monolayers. RSV plaques were developed by immunoperoxidase staining. Immunization with VRP-RSV.F was effective in limiting RSV levels in BAL and nasal samples (Fig 1A). The levels of RSV detected in the VRP-RSV.F group were lower than levels in the control groups and were cleared sooner. RSV PFU/ml for all data points when RSV was detected in the VRP-RSV.F samples ranged from five to 65. One animal in the VRP-RSV.F group did not have detectable RSV in BAL at any time point following challenge. All animals in the negative control groups had detectable virus in BAL following challenge, and the number of PFU/mL ranged from 20 to 7850 PFU for all data points when virus was detected. RSV in the BAL from animals in the negative control groups persisted past the time point when virus was no longer detectable in the any animal from the VRP-RSV.F group.

Animals immunized with VRP-RSV.F were also better able to control RSV in upper respiratory track (Fig 1B). RSV was detected in a nostril swab sample at only one time point from one animal in the VRP-RSV.F group. RSV was detected in three out of four of the animals in the two negative control groups at three time points.

3.4 RT-PCR detection of virus

In addition to measuring live virus in BAL fluid and nostril swab samples, we also measured viral genome in the samples using RT-PCR. In this assay, detection of PCR product at a low cycle number indicates relatively more viral genome than detection of PCR product at a higher cycle number. Detection of product below 40 cycles is considered positive, and failure to detect product by 45 cycles is considered negative. Cycle thresholds between 40 and 45 cycles are ambiguous.

The protective effects of immunization with VRP-RSV.F as measured by levels viral genome were generally similar to the outcome based on levels of live virus. The average cycle threshold for BAL samples from the VRP-RSV.F group was 31.6 for all time points when virus was detected compared with an average cycle threshold 28.6 for samples from the negative control groups (Fig 2A). Additionally, animals in the VRP-RSV.F group had detectable viral genome on an average of three days following challenge compared with an average of six days for animals in the negative control groups. Results from nostril swab

samples were similar. The average cycle threshold of all time points at which virus was detected in samples from the VRP-RSV.F group was 34.3 compared with 30.0 for samples from the negative control groups. VRP-RSV.F immunized animals had detectable viral genome in nostril swab samples on average of two days compared with an average of four days for samples from animals in the negative control groups (Fig 2B).

MPV genome was present at lower levels in BAL fluid and nostril swab samples from VRP-MPV.F immunized animals than in samples from negative control animals. The average cycle threshold in BAL samples from VRP-MPV.F immunized animals was 28.5 and viral genome in those samples was detected on three days, compared with an average cycle threshold of 26.4 and presence of genome on six days for samples from animals in the control groups (Fig 3A). MPV genome was similarly reduced in nostril swab samples (Fig 3B). Samples from VRP-MPV.F animals had an average cycle threshold of 28.7 for all time points at which viral genome was detected compared with an average cycle threshold of 25.4 for nostril swab samples from control groups. Viral genome was detected in nostril swab samples from animals in the VRP-MPV.F group on an average of five days compared with an average of six days for samples from animals in the negative control groups.

DISCUSSION

We showed here that VEE VRP vaccines that encode hRSV or hMPV F proteins can elicit neutralizing antibodies and limit the magnitude and duration of virus shedding in infected nonhuman primates. VRP-based vaccines have been shown to be effective at stimulating a potent adaptive immune response in other experimental systems [21, 22, 33].

We observed that animals immunized with VRP-RSV.F produced low levels of MPV F-specific serum following immunization (Table 1). Monoclonal antibodies that recognize conserved epitopes on hRSV and hMPV have been reported [27, 34], suggesting there is some minimal antigenic similarity in the F proteins. Epitopes that are capable of eliciting cross-reactive, neutralizing antibody responses are particularly attractive candidates for vaccine antigens. Recent advances in the computational design of antigens have demonstrated that neutralizing antibodies can be elicited in NHP following immunization with de novo designed antigen [35]. While epitopes that can elicit responses specific for multiple pathogens are of great interest to computational scientists, our observation of MPV F-specific antibodies following immunization with VRP-RSV.F raises the possibility that immunization with native antigen in the right context also might elicit cross-reactive, neutralizing antibodies.

The elicitation of cross-reactive neutralzing antibodies as a vaccine design goal is desirable, but the relevance of cross reactive, non-neutralizing antibodies is less certain. Altered ratios of RSV binding and neutralizing antibodies have been noted in previous studies of inactivated RSV vaccines, and indeed some antibodies of this non-neutralziing type enhance disease following certain viral infections [36, 37]. We observed the highest levels of RSV virus in BAL in animals that were immunized with the VRP-MPV.F vaccine. The small group sizes in this study did not allow for a high level of statistical power to detect significant differences, so we cannot confidently say if this difference is significant. These

animals cleared the virus and did not exhibit any obvious clinical differences compared to other control animals. Nevertheless, the possibility of antibody-enhanced disease in animals immunized with VRP-MPV.F should not be overlooked.

Generation of neutralizing antibodies is an accepted correlate of immunity for the many viral infections [38], although other immune mechanisms could contribute to protection. The protective effect of palivizumab, an RSV-neutralizing humanized murine monoclonal antibody administered to at-risk infants, demonstrates that neutralizing antibodies are sufficient to mediate at least partial protection against RSV disease [39]. Furthermore, antibody-mediated neutralization of RSV correlates with the association rate of the antibody [40, 41]. VRPs have been demonstrated previously to induce serum neutralizing antibodies to RSV [19], but here we also show that they induce protection against challenge with live virus and they do so for either RSV or MPV. Interestingly, animals immunized with VRP-RSV.F had lower viral titers in BAL and nostril and lower titers of serum neutralizing antibodies. This observation is consistent with T cell mediated control of RSV or with the occurrence of virus-specific sIgA that could limit the infection. The VRP vaccine platform has been shown previously to stimulate a virus specific CD8+ T cell response and virus-specific IgA in the respiratory mucosa of rodents [17]. These effector mechanisms also likely contribute to viral control in NHPs.

Unlike many pathogens, infection with hRSV [7, 8] or hMPV does not confer durable, protective immunity against recurrent infection. Consequently, the immune response to a successful hRSV or hMPV vaccine likely will differ significantly from the response to natural infection. Human RSV and hMPV have evolved mechanisms to suppress key components of the innate immune response. RSV has been shown to inhibit activation of interferon pathways [42–47], STAT pathways [47], SOCS pathways [43], and activation of NF-κB [44]. Although less well-studied, hMPV also has been shown to inhibit activation of Type I interferon signaling pathways [48, 49]. These mechanisms of immune evasion prevent maturation and activation of pulmonary dendritic cells [50, 51] and are likely factors in the ability of hRSV and hMPV to infect without stimulating a protective immune response.

Numerous live attenuated hRSV and hMPV vaccine candidates have been tested. Historically these vaccines have been developed by cold passaging the virus and subsequently identifying attenuating mutations by sequencing, although more recently reverse genetic techniques have enabled a greater degree of rational design [52, 53]. Recently Meng and colleagues generated a hRSV live vaccine candidate in which the NS1 and NS2 genes were codon-deoptimized [54]. Codon-deoptimization of these genes resulted in significantly lower levels of protein translation and consequently reduced immune evasion and enhanced neutralizing antibody titer in mice. Similarly, the efficacy of the VEE-VRP likely rests on the ability of these vectors to present the native F antigens in a context that differs from the immune evasion milieu stimulated by wild-type pneumovirus infection. The presentation of structurally correct viral antigen to the immune system in combination with activation of Type I interferon pathways [55, 56] and DC maturation [57] by the VEE-VRP vaccine platform appear to induce an immune response that is superior to that elicited by inactivated virus and possibly conventional live attenuated viruses. The level of efficacy

that we observed following immunization with VRP vaccines might be increased by inserting genes for additional adjuvant components into the replicon particle.

The existence of pre- and post-fusion forms of RSV F has complicated the development of an RSV F based subunit vaccine for RSV. Recent advances in understanding the structural biology of RSV F [58, 59] and the computational design of a scaffold antigen that elicits RSV neutralizing antibodies [35] have increased the potential for success with RSV subunit vaccines. However, any successful subunit vaccine will need to include an effective adjuvant. Currently alum is the only adjuvant that is approved for widespread use in the United States. Additionally, subunit vaccines are typically inefficient at stimulating MHC Class I-restricted CD8+ T cell responses. The intrinsic adjuvant activity of VRP particles and their ability to stimulate a potent CD8+ T cell response [60, 61] are significant strengths of the VRP-platform and likely contribute to their effectiveness in stimulating an effective immune response in NHPs against hRSV and hMPV.

Acknowledgments

We thank the Wisconsin National Primate Research Center Scientific Protocol Implementation unit for performing the monkey experiments; and the support of the Wisconsin National Primate Research Center, P51 RR000167/P51 OD011106.

This study was supported by NIAID, NIH through an ARRA administrative award for supplemental funding to U54 AI 057157, the Region IV Southeast Regional Center of Excellence in Emerging Infections and Biodefense and by NIH grant R01 AI088250.

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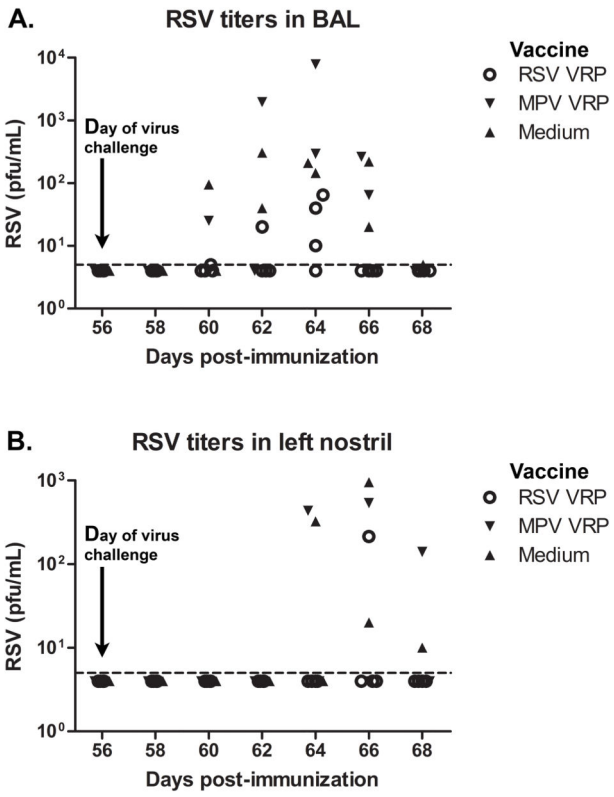

Figure 1.

Detection of hRSV in BAL (A) or nostril (B) following challenge. The level of viable RSV was measured in each compartment by direct plaque assay of samples on HEp-2 cell monolayer cultures. Plaques were visualized by immunoperoxidase staining.

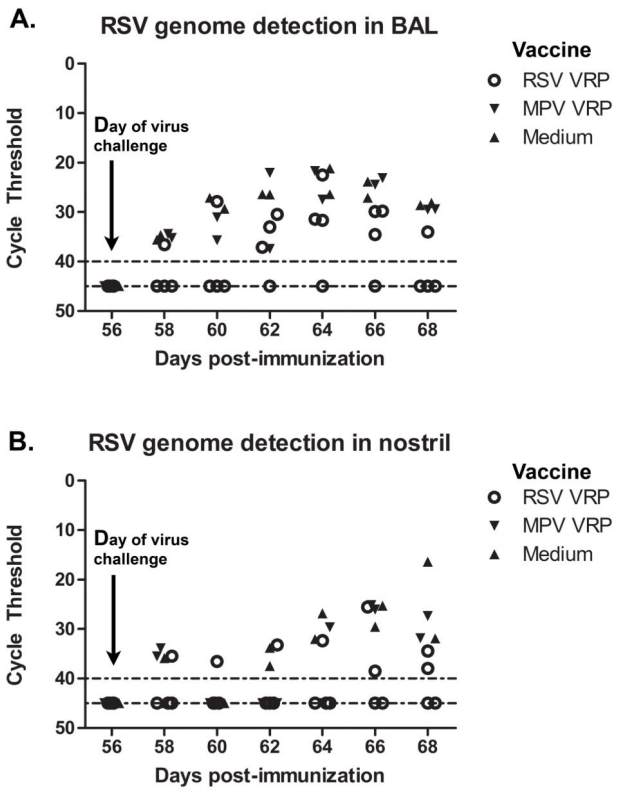

Figure 2.

Detection of RSV genome in BAL (A) or nostril (B) following challenge. Primers and probe targeting the hRSV matrix (M) gene were used to detect hRSV genome. Samples with a cycle threshold value less than 40 were considered positive.

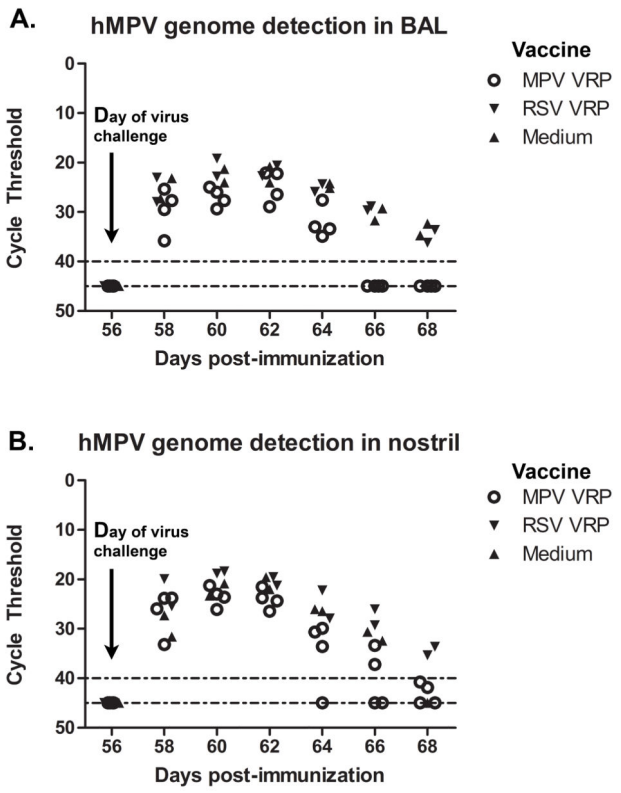

Figure 3.

Detection of hMPV genome in BAL (A) or nostril (B) following challenge. Primers and probe targeting the hMPV N gene were used to detect hMPV genome. Samples with a cycle threshold value less than 40 were considered positive.

Table 1

RSV F-specific serum IgG detected by ELISA

Group	Animal	Reciprocal titer (log_{10}) on indicated day of study			
		Day 28	Day 36	Day 45	Day 84
RSV F VRP	1	5.8	6.0	5.8	6.3
	2	5.7	5.9	5.6	6.0
	3	5.8	5.9	5.7	5.7
	4	5.7	5.9	5.8	6.3
MPV F VRP	5	<	2.2	<	6.1
	6	<	3.3	2.0	6.8
Medium	7	<	<	<	6.0
	8	<	<	<	6.0

< indicates binding antibodies not detected at lowest dilution tested (1:100).

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Table 2

MPV F-specific serum IgG detected by ELISA

Group	Animal	Reciprocal titer (log_{10}) on indicated day of study			
		Day 28	Day 36	Day 45	Day 84
MPV F VRP	9	3.9	4.9	4.9	5.6
	10	3.9	4.8	4.2	5.0
	11	4.0	4.8	4.7	5.6
	12	4.6	4.8	4.5	5.1
RSV F VRP	13	<	<	<	4.8
	14	<	<	<	4.7
Medium	15	<	<	<	5.0
	16	<	<	<	4.7

< indicates binding antibodies not detected at lowest dilution tested (1:100).

Table 3

Serum RSV neutralizing activity

Group	Animal	Reciprocal serum titer on indicated day of study				
		Day 0	Day 28	Day 36	Day 45	Day 84
RSV F VRP	1	<	20	53	224	1,233
	2	<	48	148	334	785
	3	<	<	892	430	337
	4	<	24	262	228	1,472
MPV F VRP	5	<	<	<	<	1,475
	6	<	<	<	<	4,749
Medium	7	<	<	<	<	2,219
	8	<	<	<	<	1,867

< indicates neutralizing activity was not detected even at the lowest dilution tested (1:20).

Table 4

Serum hMPV neutralizing activity

Group	Animal	Reciprocal titer on indicated day of study				
		Day 0	Day 28	Day 36	Day 45	Day 84
MPV F VRP	9	<	213	389	341	1,040
	10	<	267	955	359	>1,280
	11	<	242	764	516	>1,280
	12	<	90	292	136	1,034
RSV F VRP	13	<	<	<	<	600
	14	<	<	<	<	312
Medium	15	<	<	<	<	>1,280
	16	<	<	<	<	749

< indicates neutralizing activity not detected at lowest dilution tested (1:20).

>1,280 indicates serum neutralizing activity was present even at the highest dilution tested (1:1,280)

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