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**TITLE (Max 160 characters with spaces):**

Cross-reactive and cross-neutralizing activity of human mumps antibodies against a novel mumps virus from bats

**RUNNING TITLE (Max 40 characters with spaces):**

Cross-neutralization of bat mumps virus

**AUTHORS:**

Shannon M. Beaty<sup>1</sup>, Raffael Nachbagauer<sup>1</sup>, Ariana Hirsh<sup>1</sup>, Frederic Vigant<sup>1</sup>, James Duehr<sup>1</sup>, Kristopher Azarm<sup>1</sup>, Alice J. Stelfox<sup>2</sup>, Thomas A. Bowden<sup>2</sup>, W. Paul Duprex<sup>3</sup>, Florian Krammer<sup>1</sup>, Benhur Lee<sup>1</sup>

**AFFILIATIONS:**

<sup>1</sup> Department of Microbiology, Icahn School of Medicine at Mount Sinai, New York, United States, <sup>2</sup> Division of Structural Biology, Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom, <sup>3</sup> Department of Microbiology, Boston University School of Medicine, Boston, United States

**ABSTRACT:**

A mumps-like virus that is conspecific with human mumps virus (MuV) was recently identified in African bats (bat mumps virus, BMV). In order to evaluate its antigenic relatedness to MuV, we cloned and rescued a recombinant EGFP-reporter MuV vaccine strain (JL5 strain) bearing the F and HN glycoprotein genes of the BMV substituted into the homologous open reading frames. This chimeric rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> exhibited a hypofusogenic phenotype but grew to higher titers in Vero cells compared with the parental rMuV<sup>JL5</sup>. Cross-reactivity against recombinant soluble F and HN glycoproteins and cross-neutralization were demonstrated using hyperimmune mouse sera as well as a curated panel of human sera from donors in the U.S. All mouse and human

25 sera that were able to neutralize rMuV infection also had cross-neutralizing activity against  
26 rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>. Our data suggests that people who have neutralizing antibodies against MuV  
27 might be protected from infection by BMV.

28 **Abstract word count = 144 (Limit: 150)**

29 **KEYWORDS (3-10):**

30 Neutralizing antibodies; Mumps virus; Viral envelope proteins; Zoonoses; Africa; Cross-reactive  
31 antibodies; Sequence homology.

## BACKGROUND

Mumps virus (MuV) is a contagious virus of the *Rubulavirus* genus that typically causes painful swelling of the parotid and other salivary glands (1). Live attenuated mumps vaccines were introduced in the 1960's and 1970's, with vaccine effectiveness ranging from 91% to 94.6% after 2 doses (2). Consequently, the incidence of mumps morbidity has been greatly reduced in countries where high vaccine coverage has been achieved (3). Recently however, the epidemiology of mumps has changed and it is re-emerging in the developed world, possibly as a result of waning immunity and/or a lack of broadly protective immunity.

MuV was thought to be an exclusive human pathogen with no animal reservoir until recently, when the complete genomic sequence of a conspecific mumps-like virus was obtained from an African bat (BMV) (4). It is not currently known if BMV is capable of infecting humans, but the discovery of a possible animal reservoir for MuV raises concerns that elimination of circulating virus and subsequent cessation of vaccination might leave humans susceptible to disease from such a reservoir-borne virus. The genetic proximity of these two viruses suggests that antibodies against MuV might provide protection from zoonotic spillover events; thus, it is critical for risk assessment to evaluate the BMV-neutralizing activity of antibodies from MuV-seropositive individuals.

Evidence of functional and antigenic relatedness between BMV and MuV has been demonstrated by syncytia formation resulting from heterotypic expression of the fusion (F) and attachment (HN) envelope glycoproteins in tissue culture and the presence of MuV-reactive antibodies in serum from African bats (4,5). In this study, we used hyperimmune mouse sera and a panel of human sera to characterize the cross-reactive and cross-neutralizing activity of antibodies against the F and HN glycoproteins of MuV and BMV.

## METHODS

**Cloning & rescue of recombinant viruses.** Each of the chimeric viruses and the parental virus (rMuV) were rescued in one well of a 6-well plate of BSRT7 cells by co-transfection of 5 µg of the anti-genomic plasmid, 2 µg of a plasmid encoding a codon-optimized T7 polymerase (T7opt), and 0.3 µg, 0.1 µg and 0.2 µg of T7-driven support plasmids encoding the N, P, and L proteins, respectively. For efficient rescue, we used the maximal T7 promoter to drive our antigenomic transcript and a hammerhead ribozyme (HhRbz) sequence in the transcribed 5'-end of antigenome (6) (**Supplemental Figure 1**). Transfection was performed using LipofectamineLTX (Invitrogen) according to the manufacturer's instructions. At 7 days post-transfection, supernatants were collected from the rescue wells and amplified by two sequential passages on DF-1 cells (ATCC CRL 12203) and clarified supernatants were stored at -80°C.

**Generation of mouse hyperimmune serum.** Purified stocks of rMuV and rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> were inactivated by treatment with 0.03% formalin at 4°C for 48 hours and delivered intramuscularly into the hindlimb of 6-8 week-old female BALB/c mice, adjuvanted with polyIC (5µg per injection, *Invivogen*). Blood samples were collected at 3 weeks post vaccination and serum was prepared by centrifugation at 12,000 rpm for 5 minutes.

**Quantification of seroreactivity by flow cytometry.** Vero cells were infected with rMuV or rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> at an MOI of 0.75 and incubated at 37°C for 24 hours. Infected cells were incubated in PBS with 10mM EDTA at 4°C for 20 minutes before being scraped and collected. Cells were pelleted by centrifugation at 1250 rpm for 5 minutes, washed with PBS and resuspended in PBS + 2% FBS. Cells were resuspended in hyperimmune mouse serum diluted 1:200 in PBS + 2% FBS and incubated at 4°C for 1 hour, washed twice with PBS + 2% FBS and incubated with a fluorescent secondary antibody (goat anti-mouse AlexaFluor647, 1:1000 dilution) at 4°C for 1 hour. Cells were washed twice with PBS + 2% FBS, resuspended in PBS + 2% paraformaldehyde, and fluorescence intensity was measured by flow cytometry.

**Study subjects and serum samples.** Serum samples were purchased from Innovative Research (Michigan, USA) as de-identified research reagents. Serum was collected between August and November 2014 in Michigan, USA from donors aged 18-64 years old. The serum was heat-inactivated by incubation at 56°C for 30 minutes and stored at -20°C.

**Generation of purified F and HN glycoproteins.** Recombinant baculoviruses were used to produce the various soluble F and HN glycoproteins as described previously (7), and protein preps were purified by metal-resin affinity chromatography as described by Margine et al. (2013) (8).

**Mumps IgG ELISA.** Purified soluble F and HN glycoproteins were used to coat 96-well plates (ThermoScientific #3855) and reactivity was quantified by ELISA as described previously (9). For comparison, sera were also tested using a commercially available mumps IgG ELISA kit (Sigma-Aldrich #SE120093) according to manufacturer's instructions.

**Seroneutralization assay.** For each serum, serial dilutions were made in 200 µL plain DMEM and pre-incubated with 1000 infectious units of either rMuV, rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>, or Newcastle disease virus (rNDV) for 1 hour at 37°C before transferring the inoculum to 4 × 10<sup>4</sup> Vero cells that had been seeded the day before infection. Infected cells were incubated at 37°C for 24 hours, fixed with formalin, and EGFP-expressing cells were quantified using a plate reader. For each serum tested, the dilution series for neutralization was performed in triplicate.

## RESULTS

The F and HN glycoproteins of MuV play critical roles in determining the viral host range, tissue tropism, and contain the primary antigenic determinants involved in virus neutralization. Thus, to assess the risk for zoonotic transmission of BMV, we first performed phylogenetic analysis of the F and HN amino acid sequences using sequences from BMV and representative MuV strains covering all 10 currently recognized genotypes. For both the F and HN

glycoproteins, BMV is more distantly related to all MuV strains than any of the MuV genotypes are to each other (**Supplemental Figures 2A-B and 3**). Previous studies have shown that cross-neutralizing activity can vary across the distinct MuV genotypes (10), so cross-neutralization of the more distantly related BMV would not necessarily be expected *a priori*.

#### *Syncytia formation and growth kinetics of chimeric rMuV<sup>JL5</sup>-BMV viruses*

We generated a series of isogenic chimeric viruses that would allow for testing the cross-neutralizing and cross-reactive relationship between MuV and the distantly related BMV. We cloned the F and HN genes of BMV into the homologous open reading frames of a recombinant EGFP-reporter MuV (rMuV) encoding the Jeryl Lynn 5 (JL5) vaccine strain. Three chimeric viruses were generated by substituting either the F gene only (rMuV<sup>JL5</sup>-F<sup>BMV</sup>), the HN gene only (rMuV<sup>JL5</sup>-HN<sup>BMV</sup>), or both F and HN genes (rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>) (**Supplemental Figure 4A**). The parental rMuV and all three chimeras were rescued successfully and formed syncytia in the rescue well, demonstrating a functional relationship between heterotypic envelope glycoproteins for the rMuV<sup>JL5</sup>-F<sup>BMV</sup> and the rMuV<sup>JL5</sup>-HN<sup>BMV</sup> viruses. While the heterotypic complementation previously observed by Kruger et al. (2015) illustrates functional compatibility of the F- and HN glycoproteins in the absence of other viral proteins (5), the viral replication and virus-induced syncytia observed in this study further show that fusion-competent heterotypic F-HN complexes are incorporated into viral particles and that heterotypic complementation can occur in the context of multi-cycle viral replication.

In order to characterize the fusion activity and replication kinetics of the chimeric rMuV<sup>JL5</sup>-BMV viruses, Vero cells were infected with the MuV-BMV chimeras or rMuV. Very large syncytia were observed in cells infected with rMuV; however, fusion was dramatically reduced in cells infected with rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> or rMuV<sup>JL5</sup>-F<sup>BMV</sup> and an intermediate fusion phenotype was observed in cells infected with rMuV<sup>JL5</sup>-HN<sup>BMV</sup> (**Supplemental Figure 4B**), consistent with previous findings (5). Reduced syncytia formation in cells infected with rMuV<sup>JL5</sup>-F<sup>BMV</sup> and

rMuV<sup>JL5</sup>-HN<sup>BMV</sup> might reflect some degree of inefficiency in cell-cell fusion driven by heterotypic F-HN complexes. The hypofusogenic phenotype of the rMuV<sup>JL5</sup>-F<sup>BMV</sup> virus compared with rMuV<sup>JL5</sup>-HN<sup>BMV</sup> suggest that the BMV F glycoprotein might be responsible for a reduction in syncytia formation.

We sought to compare the replication kinetics of the chimeric MuV-BMV viruses with that of rMuV by multi-cycle growth curves. While rMuV<sup>JL5</sup>-HN<sup>BMV</sup> replication was delayed relative to rMuV, it reached a similar peak titer (**Supplemental Figure 4C**). In contrast, both chimeras containing the F glycoprotein from BMV (rMuV<sup>JL5</sup>-F<sup>BMV</sup> and rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>) grew to significantly higher titers than rMuV. Thus, it appears that substitution of the MuV F glycoprotein with the F glycoprotein of BMV confers an increase in viral replication, regardless of which HN glycoprotein is encoded in the viral genome.

#### *Cross-reactivity in mouse & human serum*

In order to evaluate the serological relationship between rMuV and rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>, sera from mice immunized with each virus were tested for cross-reactivity. Vero cells infected with either virus were stained with mouse hyperimmune serum and seroreactivity was quantified by flow cytometry. While naïve mouse sera were non-reactive to rMuV-infected cells, sera from all immunized mice were highly reactive to cells infected with either rMuV or rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> (**Supplemental Figure 5A-B**). These results suggest that standard MuV vaccination in humans might elicit a cross-reactive response that could provide protection from BMV. Reactivity and cross-reactivity were also evaluated in a panel of 54 sera from human donors. For each serum, direct binding of reactive antibodies to viral envelope glycoproteins was quantified by an enzyme-linked immunosorbent assay (ELISA) using purified soluble versions of the envelope glycoproteins (sBMV-F, sBMV-HN, sMuV-F, sMuV-HN), as depicted in **Figure 1A**. Human sera were reactive to the F and HN glycoproteins from both viruses and we found a highly significant cross-reactive relationship between homologous glycoproteins (**Fig 1B-C**). The HN-specific

reactivity was significantly greater than F-specific reactivity for both BMV and MuV, suggesting that mumps vaccination or natural infection might elicit a stronger antibody response against the HN glycoprotein than the F glycoprotein (**Fig 1D**).

#### *Cross-neutralization in human serum*

Next, we sought to determine if the cross-reactive human sera also exhibited cross-neutralizing activity between MuV and BMV. Thus, we performed a neutralization assay on the same panel of human serum samples (**Fig 2A**). Of the 54 sera that were tested, 39 samples (72.2%) were neutralizing against rMuV. The mean neutralizing dilution against rMuV, rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> and rNDV was 85.6, 75.3, and 4.5, respectively (threshold for seropositivity = 10). All of the rMuV-positive sera were cross-neutralizing against rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> and the positive correlation between the neutralizing activity against rMuV and rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> was highly significant (**Fig 2B**). These results demonstrate a strong antigenic relationship between BMV and MuV and underscore the potential for cross-protective immunity by MMR vaccination.

## **DISCUSSION**

Taken together, cross-complementation of the envelope glycoproteins, cross-reactivity, and cross-neutralization between the two mumps viruses implies significant structural and functional overlap in the F and HN glycoproteins of MuV and BMV. As the F and HN glycoproteins are the primary determinants of viral entry and antigenicity, their high conservation provides a structural basis for the antigenic relationship that allows for cross-neutralization of the two viruses. Strikingly, mapping of the sequence conservation between the envelope glycoproteins of MuV and BMV onto the structure of MuV F or -HN glycoproteins shows very few surface exposed areas that are not identical or similar and absolute conservation of all 6 residues known to be involved in sialic acid-binding (**Supplemental Figure 6A-B**) (11). Furthermore, while previously identified neutralizing epitopes on helices  $\alpha 3$  (N329-F340) and  $\alpha 4$  (G352-R360) of the HN



glycoprotein have only 58% and 66% sequence identity between MuV and BMV, respectively, the neutralizing epitope on helix  $\alpha 2$  (T265-D266) is completely conserved, which could indicate an important role in cross-neutralization (**Supplemental Figure 6C-D**) (12).

The results of this study demonstrate a strong antigenic relationship between MuV and BMV. All mouse and human sera used in this study that were found to have rMuV-reactive or rMuV-neutralizing antibodies also have some degree of activity against rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup>, which supports that the single serotype of MuV might extend to include BMV. In the course of preparing this manuscript, Kato et al. (2016) published similar information showing evidence of cross-neutralizing activity between MuV and BMV (13). Our results confirm and extend theirs by showing cross-reactivity and direct binding of human serum to MuV and BMV envelope glycoproteins. Although cross-protective immunity is difficult to determine, the cross-reactive antibody response against rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> in all rMuV-immunized mice suggests that standard mumps vaccination may confer some degree of protection against BMV.

**Manuscript text word count = 1,996**

*[max 2000 words, 15 references, 2 inserts (tables and figures, with no more than 4 panels per figure)]*

## FOOTNOTES

**Acknowledgments.** We thank all of the serum donors whose contribution made this study possible.

**Previous presentation of information in this study.** Some of the information in this study was presented at the Negative Strand Virus meeting in Siena, Italy in June 2015 (Abstract #276).

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**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

**Corresponding author contact information.** Correspondence and requests should be addressed to Dr. Benhur Lee, M.D. Address: Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, #1124, New York, NY 10029. Telephone: 212-241-2552. Email address: benhur.lee@mssm.edu.

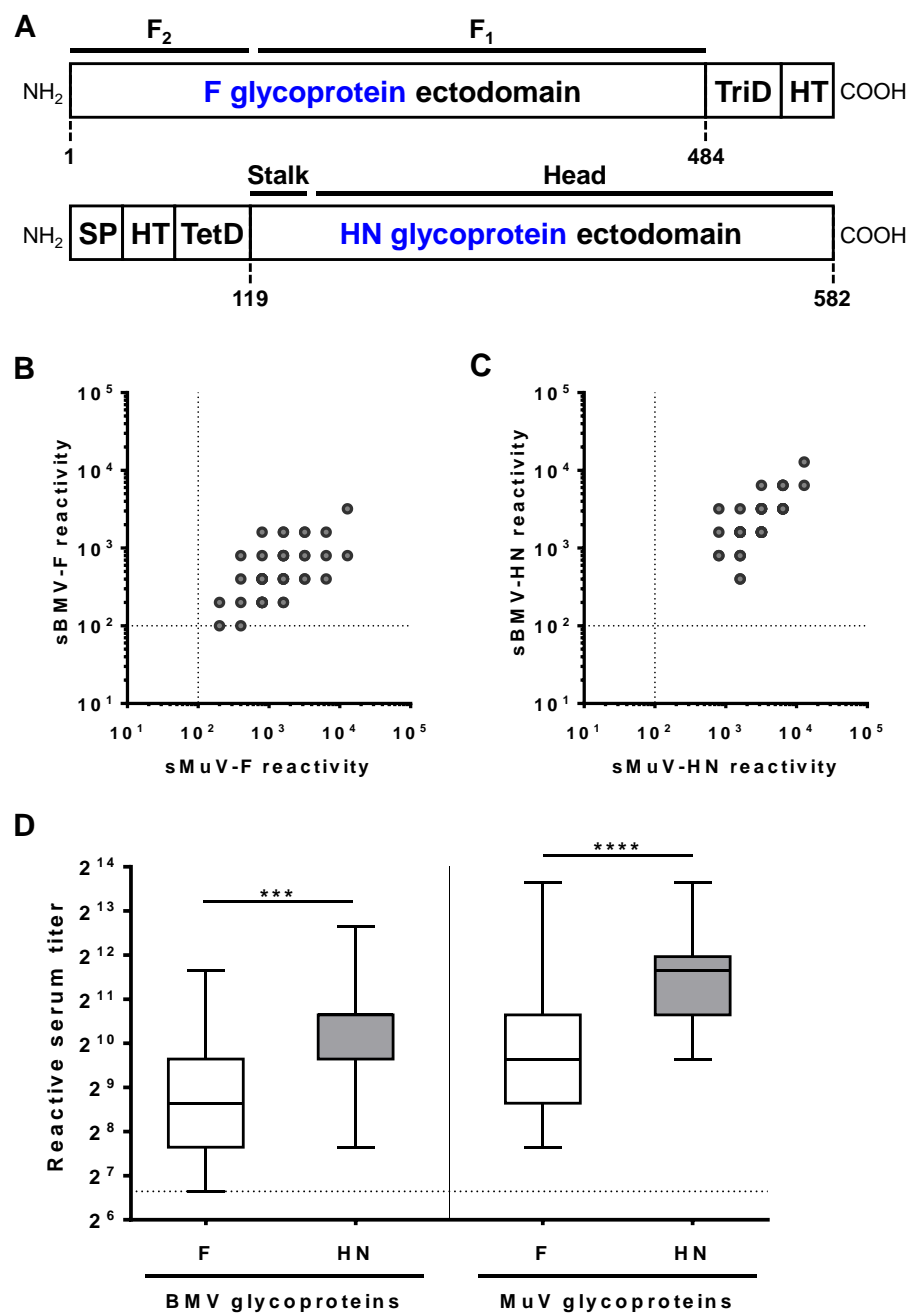
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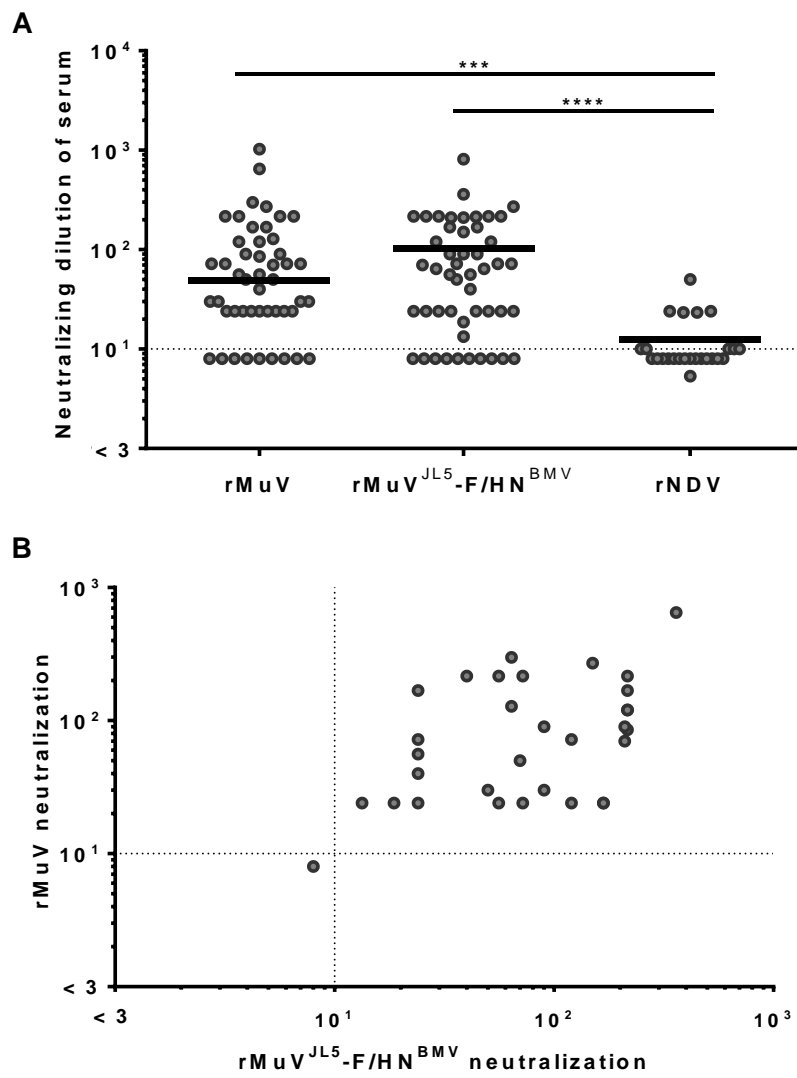


**Figure 1. Reactivity of human serum to purified F and HN glycoproteins. (A)** Schematic of soluble F and HN glycoproteins that were produced in a baculovirus system. To mimic the oligomeric structure of the envelope glycoproteins, a T4 fibrin trimerization domain and a hexahistidine tag was appended to the C-terminus of the F ectodomain (aa 1-484) whereas a baculovirus gp64 signal peptide, a hexhistidine tag, and a human vasodilator-stimulated phosphoprotein tetramerization domain was appended to the N-terminus of the HN ectodomain (aa 119-582) (14). Thrombin cleavage sites were placed between the glycoprotein reading frames and the oligomerization domains and tags in both constructs. TriD, trimerization domain; HT, hexahistidine tag; SP, signal peptide; TetD, tetramerization domain. Numbers below each glycoprotein diagram indicate the amino acid residues that correspond to the full-length glycoprotein. The sBMV-F glycoprotein could not be recovered in significant quantities, so the native proteolytic cleavage site (RRRKR) was mutated to a linker sequence (GSGSG), which allowed for the expression and recovery of higher quantities of sBMV-F. **(B)** and **(C)** Reactivity represent the endpoint dilution of serum for reactivity against purified F or HN glycoproteins from MuV or BMV. Dashed lines represent the threshold limit of detection. Panel B, F glycoprotein: Pearson  $r = 0.6160$ ,  $P < 0.0001$ . Panel C, HN glycoprotein: Pearson  $r = 0.8001$ ,  $P < 0.0001$ . **(D)** Box-and-whisker plots indicate human serum reactivity to purified F and HN glycoproteins, as measured by ELISA endpoint serum dilution. The difference in reactivity to F and HN glycoproteins was evaluated by a two-tailed paired t-test. \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ . Dashed lines represent the threshold limit of detection.



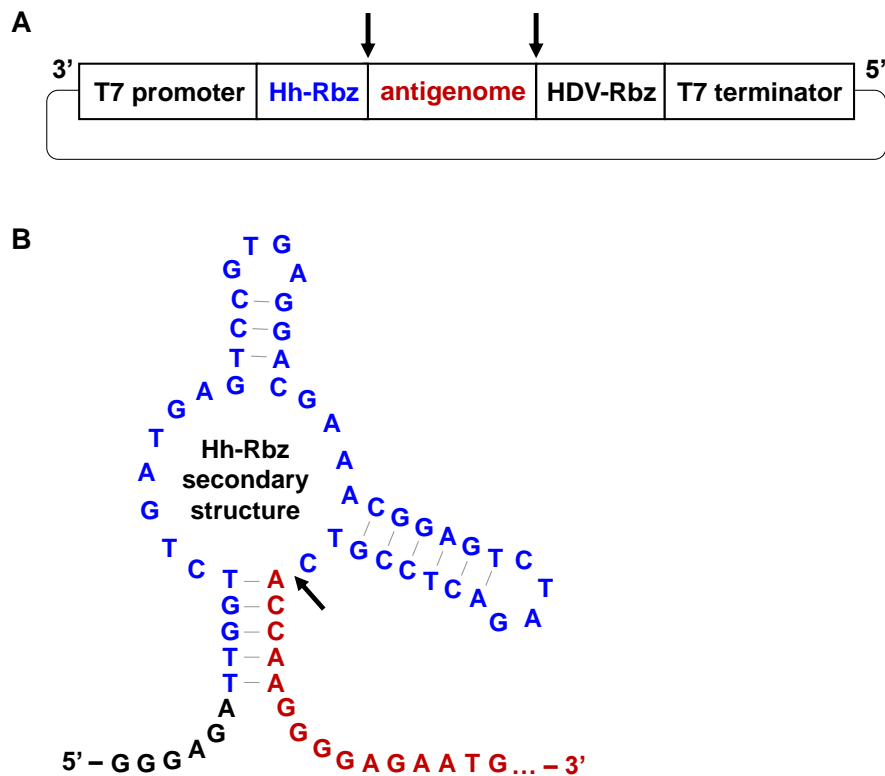
**Figure 2. Neutralization activity in a panel of human sera.** Each data point represents the average neutralizing dilution of serum from three experimental replicates (error bars not shown for the purpose of clarity in the figure). **(A)** The bold horizontal lines indicate the geometric mean neutralization values of each dataset and differences between mean neutralization values were evaluated by a one-way ANOVA; \*\*\*\*  $P < 0.0001$ . The inhibitory dilution was determined as the lowest dilution in each series with less than 50% infection. Dashed lines represent the threshold of seropositivity. A threshold inhibitory dilution of 1:10 was used as a cutoff for seropositivity, based on several factors: (a) the limit of detection of our assay was 1:8, (b) apparent nonspecific inhibition was observed at serum concentrations greater than 1:10, (c) the vast majority of sera had an inhibitory dilution less than 1:10 for the negative control group (rNDV). **(B)** Pearson's correlation was used to determine the significance of the cross-neutralizing activity; Pearson's  $r = 0.5793$ ,  $P < 0.0001$ . Dashed lines represent the threshold of seropositivity as defined above. Sera that were able to neutralize rNDV in addition to rMuV and rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> were considered cytotoxic or nonspecific (5 out of 59 sera), and as such were removed from further analyses.



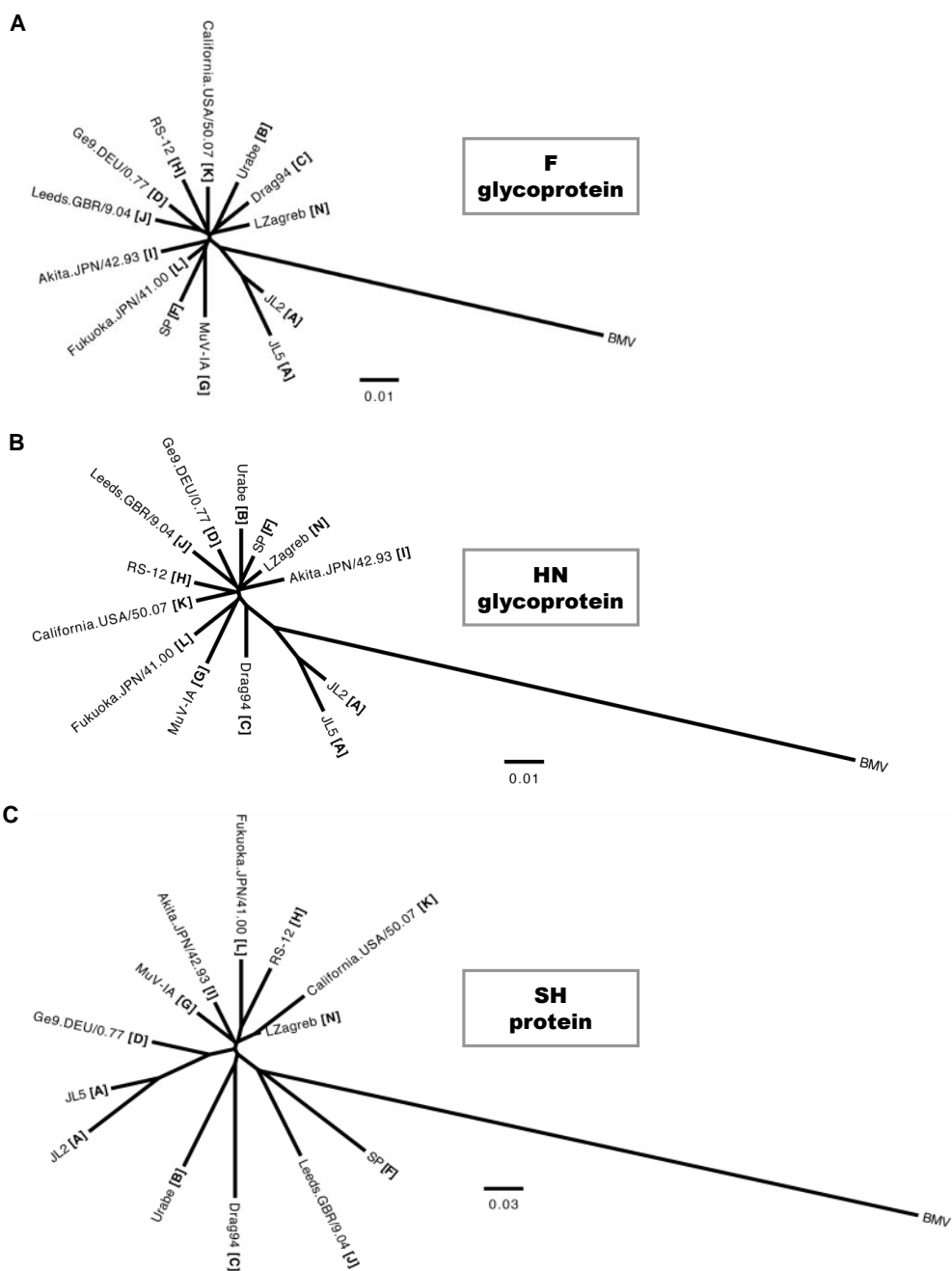


**Supplemental Figure 1. Schematic of rMuV antigenomic plasmid for reverse genetics. (A)**

Schematic of the antigenomic plasmid showing the context of the hammerhead ribozyme (Hh-Rbz) and hepatitis delta virus ribozyme (HDV-Rbz) sequences. Black arrows indicate sites of ribozyme autocleavage. **(B)** Secondary structure formed by the RNA transcript; hammerhead ribozyme sequence is shown in blue, the start of the viral antigenome is shown in red, and nucleotides derived from the optimal T7 promoter are shown in black. Black arrow indicates the sites of ribozyme autocleavage. Optimization of the rMuV reverse genetics system, including testing of the hammerhead ribozyme and other features that have improved rMuV rescue efficiency, is discussed in further detail by Beaty et al. (manuscript in preparation).



**Supplemental Figure 2. Amino acid sequence homology of the F, HN, and SH proteins from MuV and BMV.** An unrooted phylogenetic tree based on the amino acid sequence of the **(A)** F glycoprotein, **(B)** HN glycoprotein, and **(C)** SH protein was created using Clustal Omega Multiple Sequence Alignment with MBED-like clustering using a maximum of five iterations. The bracketed letter following the strain name at each branch denotes the genotype of that strain. The scale bars in panels **(A)** and **(B)** indicate 0.01 substitutions per residue; the scale bar in panel **(C)** indicates 0.03 substitutions per residue. Gene accession numbers used in this analysis are listed in Supplemental Figure 2.



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326 **Supplemental Figure 3. Strains of MuV used for phylogenetic analysis.** The name,

327 genotype and genbank accession number of the MuV strains used for phylogenetic analysis.

328 Accession number of BMV, HQ660095. According to recommendations by the World Health

329 Organization (WHO), genotype assignment for MuV strains is based on the small hydrophobic

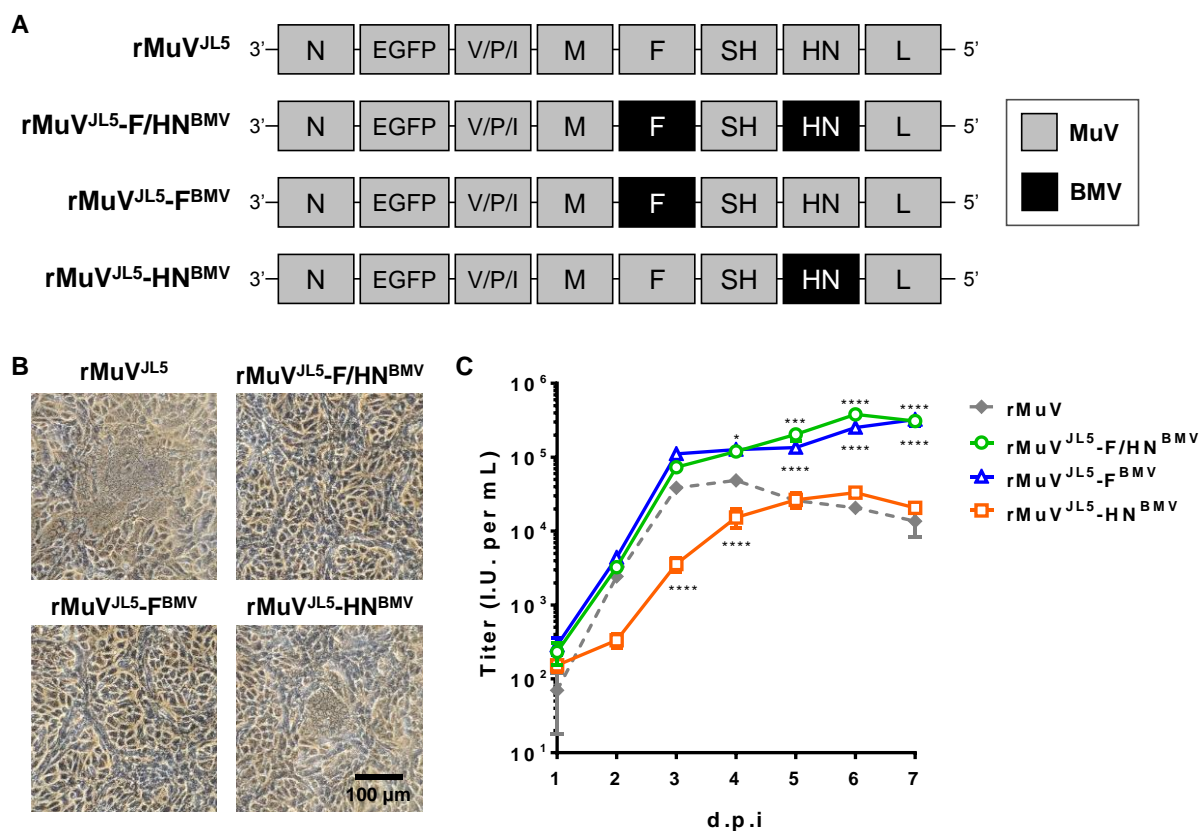
330 (SH) gene (15).

<b>MuV strain name</b>	<b>Genotype</b>	<b>Accession No.</b>
JL5	A	AF338106
JL2	A	AF345290
Enders	A	GU980052
Urabe	B	FJ375177
Drag94	C	AY669145
Ge9.DEU/0.77	D	KF878076
SP	F	EU884413
MuV-IA	G	JN012242
RS-12	H	JQ388690
Akita.JPN/42.93	I	KF878078
Leeds.GBR/9.04	J	KF878079
Cali50071	K	JX287386.1
Fukuoka.JPN/41.00	L	KF878081
Lzagreb	N	AY685921

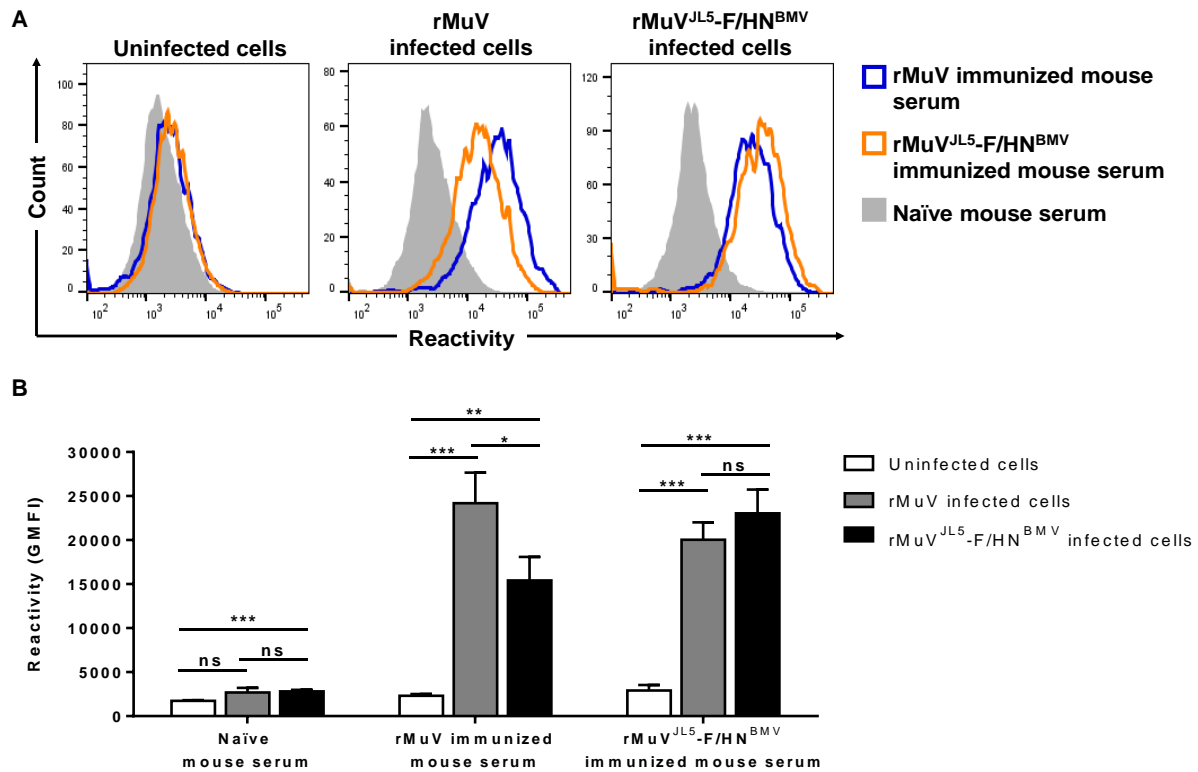
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**Supplemental Figure 2. Genomic design and growth of chimeric rMuV<sup>JL5</sup>-BMV viruses. (A)**

Schematic representation of the genome of the parental rMuV and chimeric rMuV<sup>JL5</sup>-BMV viruses. The different regions of the genome represented are not shown to scale. (B) Each recombinant virus was used to infect Vero cells at an MOI of 0.01. Supernatants were collected daily from infected cells, clarified of cell debris by centrifugation at 1250 rpm for 5 minutes and titrated on Vero cells using the EGFP reporter gene. All infections were performed in triplicate of triplicates and data points represent the mean of replicate infections; error bars show standard error. At each timepoint, titers of the chimeric viruses were compared with rMuV by a two-way ANOVA; \* P < 0.1, \*\*\* P < 0.001, \*\*\*\* P < 0.0001. (C) 20X images of virus-induced syncytia on infected Vero cells at 3 days post-infection (d.p.i). Scale bar in the bottom right corner indicates 100  $\mu$ m.



**Supplemental Figure 5. Cross-reactivity in hyperimmune mouse serum.** Serum from mice immunized with rMuV or rMuV<sup>JL5</sup>-F/HN<sup>BMV</sup> was used to stain Vero cells that were infected with the indicated virus and reactivity was quantified by flow cytometry. **(A)** Histogram plots show reactivity or cross-reactivity of the serum from a single representative mouse immunized with each virus. **(B)** Bars represent the average reactivity of sera from three mice. Error bars indicate one standard deviation; GMFI = geometric mean fluorescence intensity. The difference in reactivity was evaluated by a two-tailed unpaired t-test. \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001; ns, not significant.



**Supplemental Figure 6. Amino acid sequence homology of the F and HN glycoproteins and antibody epitope mapping. (A)** Sequence conservation between MuV-F and BMV-F mapped onto the closest homologous crystal structure, PIV5-F in the trimeric cleavage-activated prefusion state (shown in surface representation, PDB accession code: 4GIP). The solvent accessible surface is colored as follows: identical residues red, similar residues pink, and non-conserved residues white. **(B)** Sequence conservation between MuV-HN and BMV-HN mapped onto the surface of MuV-HN (surface representation, PDB accession code: 5B2D), colored as in panel A. To illustrate the expected 'heads-up/heads-down' configuration, MuV-HN dimers were superimposed onto the crystal structure of the tetrameric PIV5-HN ectodomain (PDB accession code: 4JF7). The expected position of the sialic acid binding site on the full-length ectodomain structure was determined by superposition of a sialylactose bound head-domain PIV5 HN glycoprotein structure (PDB accession code: 1Z4X) with sialylactose shown as yellow sticks, highlighted with dashed black circles. **(C)** A single MuV-HN protomer, shown in cartoon representation, with previously reported neutralizing antibody epitopes colored cyan. **(D)** Surface representation of a single MuV-HN protomer, colored as in panel A, with the neutralizing epitopes from panel C highlighted with cyan circles.



