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Immunogenicity of mumps virus vaccine candidates matching circulating genotypes in the United States and China

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ABSTRACT

Mumps virus (MuV) causes acute infection in humans with characteristic swelling of the parotid gland. While vaccination has greatly reduced the incidence of MuV infection, there have been multiple large outbreaks of mumps virus (MuV) in highly vaccinated populations. The most common vaccine strain, Jeryl Lynn, belongs to genotype A, which is no longer a circulating genotype. We have developed two vaccine candidates that match the circulating genotypes in the United States (genotype G) and China (genotype F). We found that there was a significant decrease in the ability of the Jeryl Lynn vaccine to produce neutralizing antibody responses to non-matched viruses, when compared to either of our vaccine candidates. Our data suggests that an updated vaccine may allow for better immunity against the circulating MuV genotypes G and F.

1. Introduction

MuV is an enveloped, non-segmented, negative-sense RNA virus in family *Paramyxoviridae* and genus *Rubulavirus*. MuV is present in the saliva of infected patients [1] and can be transmitted between individuals through the upper respiratory tract or conjunctiva by droplet transmission. The characteristic symptom of mumps infection is the swelling of the parotid gland [2,3]. Although mumps is classically considered a disease of children, there are also many cases of infections in post-pubertal individuals, causing orchitis occurs in many males [4,5]. Oophoritis and mastitis are less common, but can occur in post-pubertal females [6,7]. Although sterility due to orchitis is considered rare [8], there are many cases of sterility or decreased sperm count described [9] and the cause of sterility is being investigated [10].

There is currently no effective treatment of mumps, with administration of mumps-specific immunoglobulins having limited success [11]. The best way to prevent mumps disease is vaccination. The current mumps vaccines are live attenuated viruses. The most common vaccine worldwide was generated from the Jeryl Lynn strain, a geneotype A virus. The vaccine was developed over 50 years ago by serially passaging virus isolated from a patient in hen's eggs and chick embryo cell culture [12]. There have been other vaccines produced by similar methods, including Leningrad-3 and later L-Zagreb [13] in the former Soviet Union, Rubini in much of Europe [14], and Urabe in Japan, Europe, and Canada [15].

Although the MuV vaccines have been very effective in decreasing the total number of mumps cases in vaccinated populations, there continues to be cases in these highly-vaccinated populations. In 2000, the United States Department of Health and Human Services set a goal set to eliminate indigenous mumps cases by the year 2010 [16]. But, after numerous large outbreaks in the United States, the goal is to have under 500 reported cases per year [17]. However, in 2016, there were over 4000 cases in the United States [18]. In North America and parts of Europe, the most common circulating MuVs are genotype G, while the most common vaccine strain, Jeryl Lynn, is genotype A [19,20].

There is evidence in other countries that vaccination with unmatched MuV strains fails to provide protection against MuV infection. In Korea, where the Jeryl Lynn (genotype A) and Urabe AM9 (genotype B) strains are used for vaccination, the most common circulating MuV are genotype H [21]. The genotype C virus circulating in India may not be neutralized effectively after immunization with their L-Zagreb vaccine (genotype N) [22]. In China, there is still circulating MuV in the population, even with widespread vaccination [23], most of which appears to be of genotype F [24]. The vaccine used in China is S_{79} strain (genotype A) [25].

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Previously in our lab, a MuV vaccine candidate based on a genotype G virus was generated [26]. This vaccine was based on a clinical isolate from a large outbreak in Iowa in 2006. Attenuation was introduced by preventing the transcription of the V gene and deleting the SH ORF. This vaccine was shown to be safe and effective in generating an immune response in mice [26]. In this work, we further characterize this vaccine, as well as produce and test a chimeric vaccine to match the genotype F viruses circulating in China. The immunogenicity and antigenicity were assessed among the Jeryl Lynn vaccine and our genotype G and F vaccines.

2. Methods

2.1. Phylogenetics

All available full length genome sequences were obtained from the Virus Pathogen Resource and a representative set was selected [27]. Using MEGA 7, the Maximum Likelyhood method based on the JTT matrix-based model was used to generate trees for the F and HN protein sequences.

2.2. Plasmids and cells

All plasmids were constructed using standard molecular cloning techniques. Plasmid sequences were based on virus isolated in Iowa from 2006 (GenBank: JN012242.1). MuV NP, P, and L were previously cloned into the pCAGGS expression vector. The plasmid encoding the MuV(ΔVΔSH) virus sequence was previously generated [26]. The MuV(ΔVΔSH, gen-F) rescue plasmid was generated by replacing the F and HN ORFs with the sequence from PZH0804 genotype F strain. The DNA for PZH0804 replacement was generated by gene synthesis (GenScript).

BSR-T7 cells were maintained in DMEM supplemented with 10% FBS, 1% P/S, 10% tryptose phosphate broth, and 400 μg/ml G418. Vero cells were maintained in DMEM with 10% FBS and 1% P/S. All cells were cultured at 37 °C and 5% CO_2.

2.3. Virus rescue

BSR-T7 cells (1 day, 60–80% confluent, 6-well plate) were transfected with pCAGGS-NP (100 ng), pCAGGS-P (160 ng), pCAGGS-L (2000 ng), and full length genome (2500 ng) using JetPRIME (Polyplus). After 48–72 h, cells were co-cultured with Vero cells at a ratio of 1:5 in a 10-cm dish until CPE was observed. Single plaques were isolated and expanded in Vero cells. Titer was determined by plaque assay. Virus was confirmed to match plasmid sequence by RTPCR and sequencing. Primer sequences are available upon request.

2.4. Viruses

The genotype G vaccine is based on a strain isolated from a patient during the 2006 outbreak in Iowa. This virus was attenuated through insertion of two nucleotides in the RNA editing region of P/V to prevent V expression and deletion of the SH ORF, as previously described [26]. The Jeryl Lynn vaccine strain was isolated from the measles, mumps, and rubella vaccine, as previously described [26]. The genotype F vaccine was generated by rescuing an Iowa/06(ΔVΔSH) virus that has the F and HN ORFs replaced with that of PZH0804.

2.5. Western blotting

To measure total protein, cells were infected at an MOI of 0.1. After 48 h, cells were lysed with 2× Laemmli Sample Buffer (Bio-Rad), and heated at 95 °C for 5 min. Samples were then resolved on a 10% acrylamide gel by SDS-PAGE and transferred to Amersham Hybond LFP PVDF membranes (GE). Immunoblotting was performed by incubating the membranes with anti-NP and anti-P or anti-F mAbs generated in our lab and mouse anti-actin (Sigma) in 5% milk + PBS + 0.1% Tween 20 (PBST) followed by incubation with Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch). The blot was visualized on the Typhoon FLA 7000 (GE Healthcare Life Sciences) and the densitometry analysis was performed using ImageQuant TL software (GE).

2.6. Growth curves

Vero cells in 6-well plates were infected with MuV at an MOI of 0.01 in 1 ml of DMEM + 2% FBS + 1% P/S for 1 h in triplicate. Cells were washed with PBS and 2 ml of DMEM + 2% FBS + 1% P/S was added to the cells. Samples were collected at 24, 48, 72, and 96 h post-infection. Virus titers were determined by plaque assay on Vero cells. Significance was determined by two-way ANOVA using the Holm-Sidak posttest.

2.7. Mouse experiments

6–8 week old BALB/c mice were used for all experiments. Mice were infected intranasally (i.n.) with 10^5 PFU of each vaccine candidate in 100 μl. At day 21 post vaccination, mice were boosted. At day 14 post-boost, mice were euthanized, blood was for ELISA and neutralization assays, and splenocytes were isolated for ELISpot. All animal studies were conducted under guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

2.8. ELISA

ELISA antigen was generated by infecting Vero cells with MuV strains. At 48 h, cells were lysed by freeze and thaw cycles and sonication. Protein amounts were standardized to the amount of F protein. Immulon high binding polystyrene plates (Thermo Scientific) were coated overnight at 4 °C. Plates were washed and blocked with 300 μl of diluent/blocking solution (KPL) with 5% nonfat milk for 1 h at room temperature. Serum was diluted in KPL diluent/blocking solution and added to each plate. Secondary goat anti-mouse HRP antibody (SouthernBiotech) was used at 1:1000. Plates were developed with SureBlue Reserve TMB substrate (KPL). Absorbance was determined using the BioTek Epoch reader. Titers are reported for the highest serum dilution that had an absorbance greater than 0.5 at 450 nm.

2.9. Interferon-gamma ELISpot

ELISpot was performed using the Mouse IFN-gamma BD ELISpot Set using the manufacturer's protocol. In short, plates were coated with IFN-gamma-specific capture antibody. Plates were blocked with RPMI containing 10% FBS. Spleens were removed from mice and cells were isolated through mechanical disruption followed by red blood cell lysis using ACK lysis buffer. Cells were resuspended in RPMI containing 10% FBS at 250,000 cells per 100 μl. Cell were stimulated with lysates used during ELISA, PMA/ionomycin, or media only for 48 h. Plates were developed using an anti-IFN-gamma antibody conjugated to biotin, followed by incubation with Streptavidin-HRP. An AEC substrate set (BD) was sued to develop. Spots were visualized using the Cellular Technology Ltd. IMMUNOSPOT reader.

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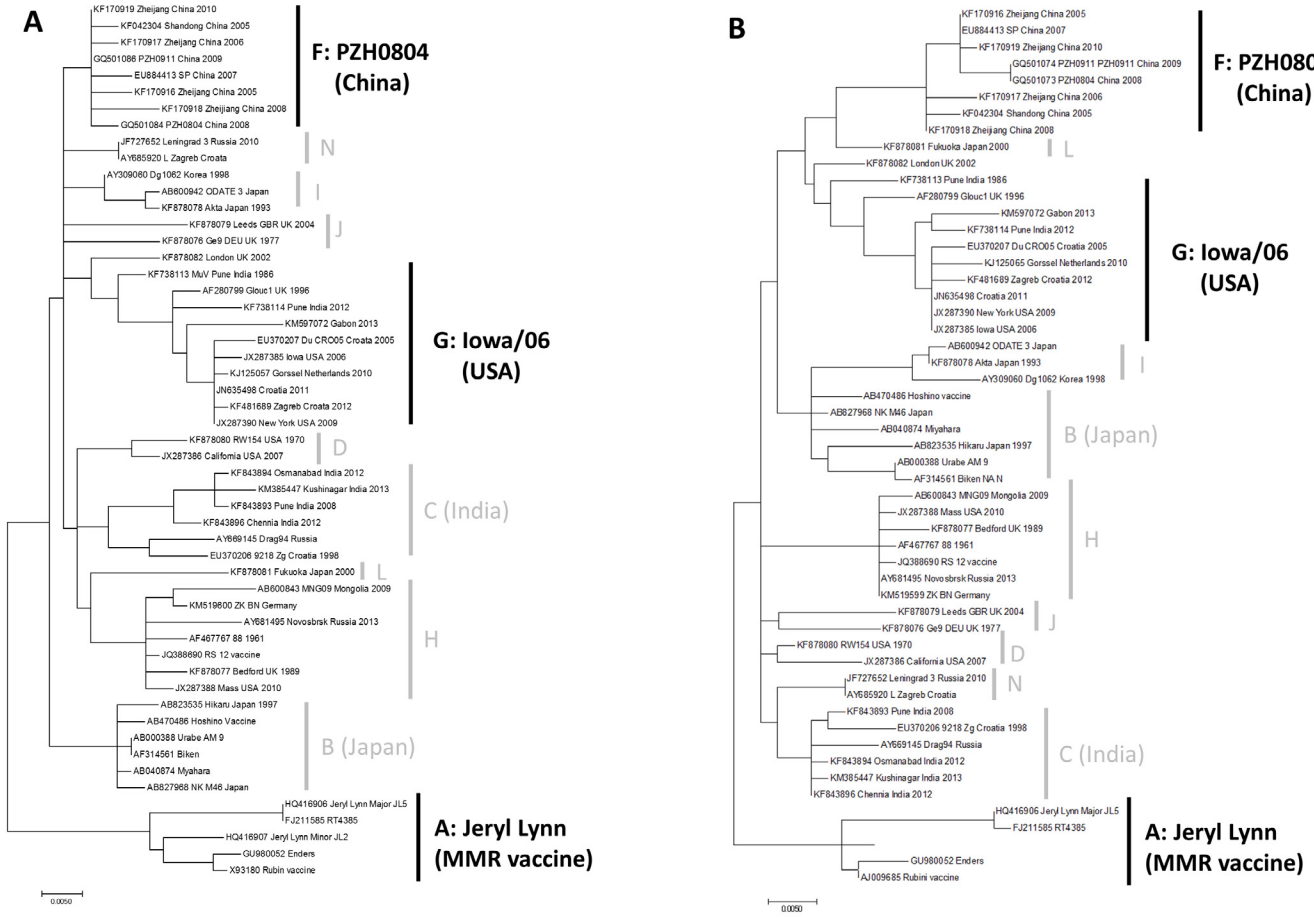
2.10. Plaque reduction neutralization assay

Heat-inactivated serum was diluted in DMEM. Diluted serum was incubated with 80 PFU of virus in 100 μl of DMEM for 1 h at 37 °C. The mixture was used to infect Vero cells in a 6-well plate. Cells were incubated at 37 °C for 1 h. Media was removed and overlaid with DMEM + 2% FBS + Penicillin/Streptomycin + 1% Low-melt agarose. The number of plaques was determined at 6–7 days



C

F

	Iowa/06	Jeryl Lynn
Jeryl Lynn	1518/1617 (93.9%)	
PZH0804	1543/1617 (95.4%)	1512/1617 (93.5%)

F

	Iowa/06	Jeryl Lynn
Jeryl Lynn	513/539 (95.2%)	
PZH0804	521/539 (96.7%)	512/539 (95.0%)

HN

	Iowa/06	Jeryl Lynn
Jeryl Lynn	1623/1749 (92.8%)	
PZH0804	1651/1749 (94.4%)	1628/1749 (93.1%)

HN

	Iowa/06	Jeryl Lynn
Jeryl Lynn	555/583 (95.2%)	
PZH0804	567/538 (97.3%)	560/583 (96.1%)

Fig. 1. Phylogenetic tree of mumps virus (MuV) fusion (F) and hemagglutinin (HN) amino acid sequences from available full length sequences. Full length MuV sequences were obtained from the Virus Pathogen Resource (viprbrc.org), and duplicate or highly similar sequences were removed. The remaining sequences were used to compare the protein sequences for F (A) and HN (B) proteins. We generated Maximum Likelihood trees using a method based on the JTT matrix-based model [1]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA7 [2]. Labels for the established MuV genotypes were added to the right of the trees. The genotypes studied in the paper (A, G, and F) are highlighted. (C) Comparison of F and HN gene and protein sequences for the vaccine viruses. The number of matched nucleotides or amino acids in the coding sequence for F and HN ORFs was determined for each of the vaccine viruses, and the % similarity was calculated.

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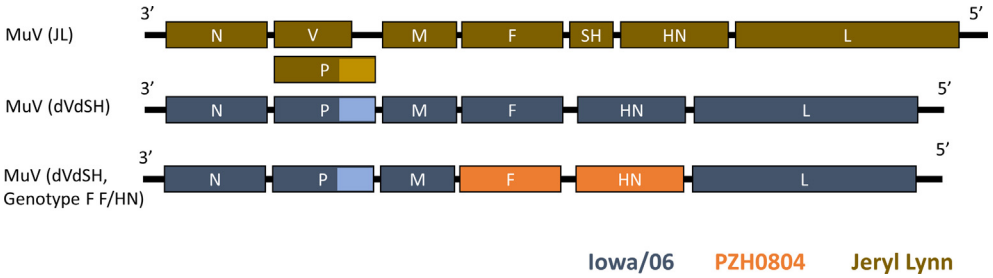

Fig. 2. Schematics of vaccines viruses. NP, nucleoprotein; V, V protein; P, phosphoprotein; M, matrix protein; F, fusion protein; SH, small hydrophobic protein; HN, hemagglutinin-neuraminidase protein; L, large protein/RNA-dependent polymerase. Vaccine candidates are based on sequences for Jeryl Lynn vaccine (genotype A), Iowa/USA/2006 (genotype G), and PZH0804/China/2008 (genotype F). The Iowa/USA/2006 vaccine candidate was generated by inserting two alanine residues in the RNA editing site to prevent V transcription and by removing the SH ORF. The Jeryl Lynn vaccine strain was isolated from the measles, mumps, and rubella (MMR) vaccine.

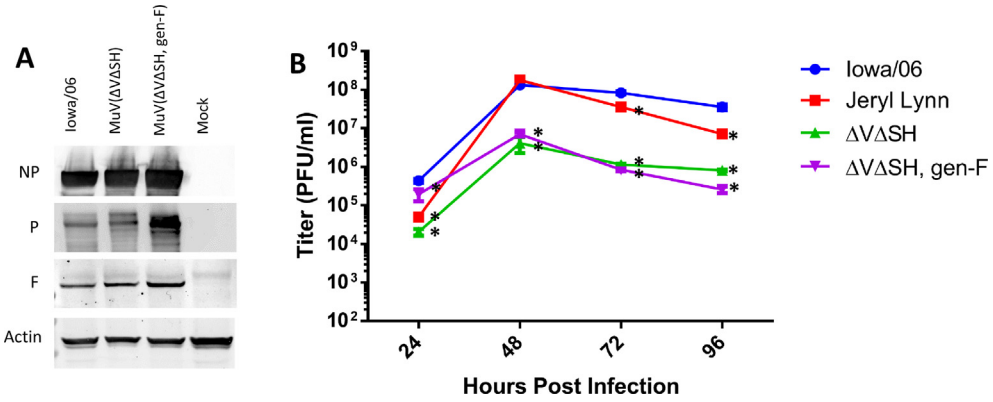

Fig. 3. Characterization of vaccine viruses. (A) Expression of viral proteins. Vero cells were infected at an MOI = 0.1 for 48 h with wild-type Iowa/USA/2006, MuV (ΔVΔSH), MuV (ΔVΔSH, gen-F), or mock infected. Cell lysates were analyzed by Western blotting after SDS-PAGE using antibodies specific for MuV NP, P, or F proteins. (B) Vaccine virus growth in cell culture. Vero cells were infected with viruses at an MOI = 0.01. The titer of virus in the media was determined at 24, 48, 72, and 96 h.p.i. The growth rate of each of the viruses was compared to the clinical isolate, Iowa/06. The titer of Jeryl Lynn was lower at 24, 72, and 96 h post infection (h.p.i), but there was no difference in the peak titer at 48 h.p.i. Both MuV(ΔVΔSH) and MuV(ΔVΔSH, gen-F) had decreased titers at all time points, and had about a 20-fold decrease in peak titer at 48 h post infection. (*n* is 3; 2-way ANOVA with Dunnett's multiple-comparison test comparing to Iowa/06; *, $P < 0.05$).

post infection. The plaque reduction titer was determined based on the dilution of serum that reduced the number of plaques by one half of the input virus control.

3. Results

3.1. Phylogenetic assessment of MuV isolates

To compare the genome sequences among virus isolates from different locations over time, full length virus sequences were obtained from the Virus Pathogen Resource [27]. There were 94 complete genomes available at the time of analysis. Sequences isolated from the same outbreak or vaccine strain were removed to avoid bias, leaving 54 sequences for further analysis. Maximum Likelihood trees were generated for F (Fig. 1A) and HN (Fig. 1B) protein sequences. We have selected three representative viruses to represent these groups: Jeryl Lynn (A), PZH0804/China/2008 (genotype F), which was isolated in Panzhihua in the Sichuan province of China in 2008 [28], and Iowa/USA/2006 (genotype G).

The similarity of the viruses chosen for the vaccine studies were assessed among the open reading frame (ORF) and encoded protein sequences (Fig. 1C). Iowa/06 and PZH0804 had the highest percent similarity. Jeryl Lynn still had a high degree of similarity for both the F and HN ORFs (92.8–93.9%) and protein sequences (95.2–96.1%).

3.2. Construction of chimera virus

The genotype G vaccine was previously generated in our lab [26]. It was attenuated through the removal of the immunomodulatory proteins V and SH, and will be referred to as MuV(ΔVΔSH). The genotype F vaccine was generated by replacing F and HN ORFs in the genotype G vaccine with those of the PZH0804/China/2008 virus. We chose to replace the F and HN ORFs because neutralizing antibodies against the surface antigens of MuV are thought the be important for protection [29]. This chimeric virus will be referred to as MuV(ΔVΔSH, gen-F) (Fig. 2).

3.3. Rescue and characterization of vaccine viruses

Vaccine viruses were rescued using a MuV reverse genetics system previously developed in our lab [30] and the sequence was confirmed by RT-PCR followed by sequencing. The protein expression of wild-type Iowa/06, MuV(ΔVΔSH), and MuV(ΔVΔSH, gen-F) in Vero cells was determined (Fig. 3A). Blotting for NP showed similar levels of expression, while there was increased expression of P for both vaccine strains. The increased expression of P is likely due to the lack of V transcription, as previously reported [31].

We characterized the growth of the vaccines in Vero cells using low MOI infection. MuV(ΔVΔSH) and MuV(ΔVΔSH, gen-F) had similar growth rates indicating that replacing the F and HN ORFs

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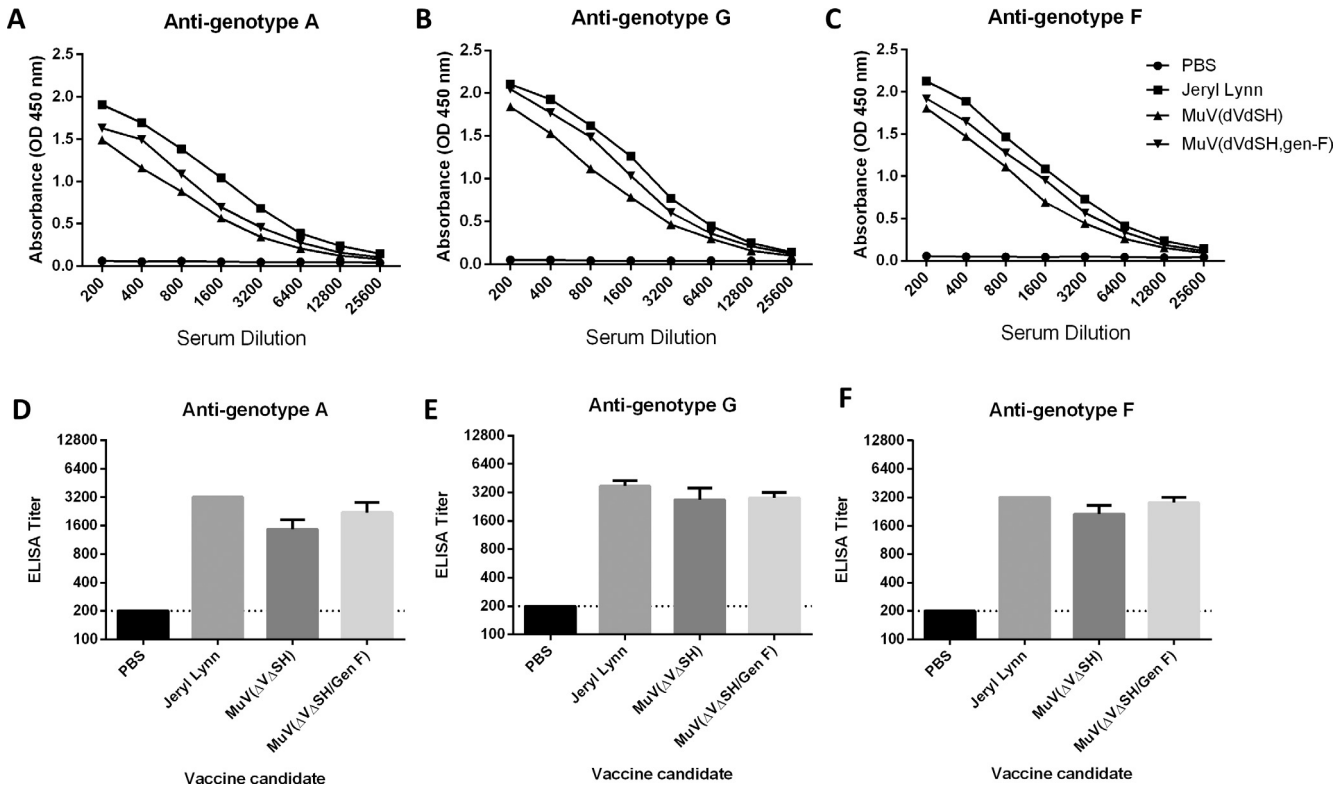

Fig. 4. Cross-reactive antibody titers in mice after immunization. Serum was collected from mice 14 days after boosting. Serum antibody titers were determined by ELISA. Dilution curves were generated using serum from each of the vaccination groups using plates coated with Jeryl Lynn (A), MuV (ΔVΔSH) (B), or MuV (ΔVΔSH, gen-F) (C). Titers were determined by using the dilution at which the OD at 450 nm was >0.5. The mean titer and SEM are reported for each vaccination group for plates coated with Jeryl Lynn (D), MuV (ΔVΔSH) (E), or MuV (ΔVΔSH, gen-F) (F). (n is 4–6; 1-way ANOVA with Fisher's LSD post hoc comparisons to homologous group; *, $P < 0.05$).

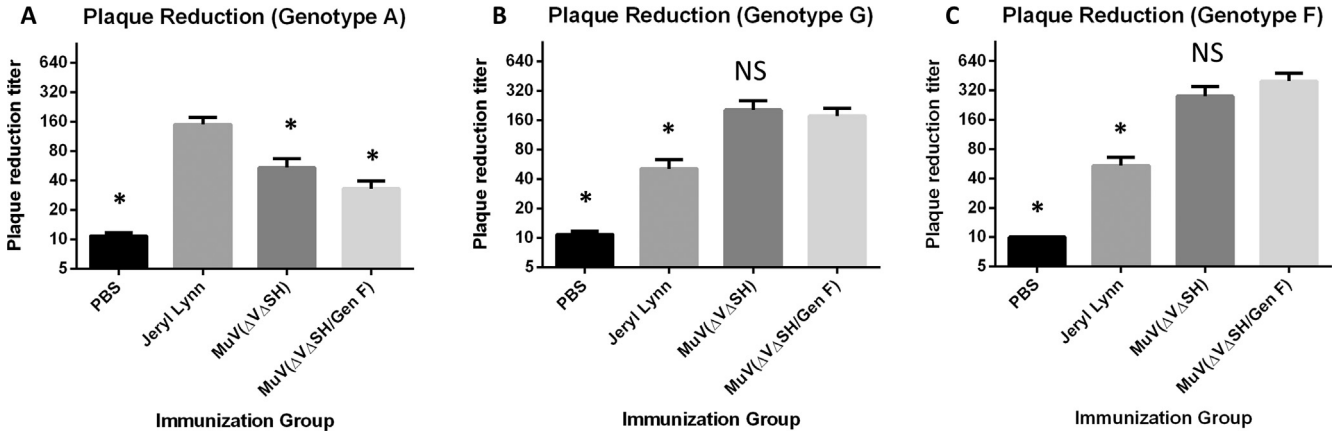

Fig. 5. Cross-neutralization titers in mice after immunization. Serum was collected from mice 14 days after boosting. A plaque reduction assay was used to determine the neutralization titer for each vaccination group. The plaque reduction titer for each vaccination group was determined using Jeryl Lynn (A), MuV (ΔVΔSH) (B), and MuV (ΔVΔSH, gen-F) (C). The mean 50% plaque reduction titer and SEM are reported. (n is 4–6; 1-way ANOVA with Fisher's LSD post hoc comparisons to homologous group; *, $P < 0.05$).

did not affect virus growth, although the titer for both viruses were lower than Jeryl Lynn (Fig. 3B).

3.4. Antibody responses after MuV vaccination

To compare immunogenicity of the vaccine candidates, BALB/c mice were immunized intranasally (i.n.) with 10^5 PFU of each vaccine candidate and boosted at 21 days post-vaccination. At day 14 post-boost, mice were euthanized and blood and spleens were collected.

To compare humoral immune responses, MuV-specific serum IgG titers were measured by ELISA. The average absorbance is reported for plates coated with antigen from Jeryl Lynn (Fig. 4A), MuV(ΔVΔSH) (Fig. 4B), and MuV(ΔVΔSH, gen-F) (Fig. 4C). The titer was determined as the highest serum dilution with an absorbance of 0.5 for each sample, and reported for Jeryl Lynn (Fig. 4D), MuV(ΔVΔSH) (Fig. 4E), and MuV(ΔVΔSH, gen-F) (Fig. 4F). The

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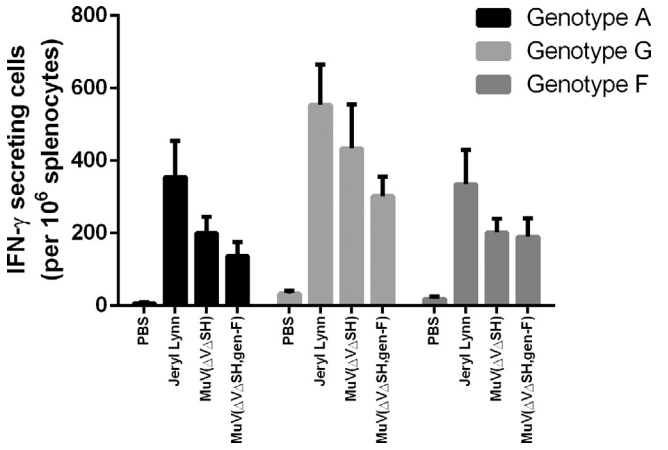

Fig. 6. Cellular immunity in mice after immunization. Spleens were removed from mice and splenocytes were isolated 14 days after boosting. ELISpot was used to determine the number of interferon-γ secreting cells. Splenocytes were stimulated with antigen from Jeryl Lynn, MuV (ΔVΔSH), or MuV (ΔVΔSH, gen-F). The mean and SEM were reported for the number of interferon-γ secreting cells per 10^6 splenocytes. (*n* is 4–6; 1-way ANOVA with Fisher's LSD post hoc comparisons to homologous group; *, $P < 0.05$).

ELISA titer trended higher for the Jeryl Lynn-vaccinated mice in each case, but the only significant difference was between titers from Jeryl Lynn and MuV(ΔVΔSH)-vaccinated groups against Jeryl Lynn ($p = 0.0016$). The results from the ELISAs suggest that there are similar amounts of antibody produced from vaccination with each of the vaccine candidates, and that there is little difference in antibody specificity among the genotypes.

We next determined the cross-neutralizing antibody titers between the different genotypes. Mice immunized with Jeryl Lynn had neutralization titers against genotype A virus that were significantly greater than those produced with either the Iowa/06 or PZH0804 vaccines (Fig. 5A). Mice immunized with either MuV (ΔVΔSH) or MuV(ΔVΔSH, gen-F) produced significantly higher neutralizing titers against genotype F virus than Jeryl Lynn (Fig. 5B). A significantly lower neutralizing titer was produced in mice immunized with Jeryl Lynn, while mice immunized with MuV(ΔVΔSH) or MuV(ΔVΔSH, gen-F) produced similar neutralizing titers against genotype G virus (Fig. 5C). This data shows that there is a difference in neutralizing antibodies when comparing the genotype A virus to either the genotype F or G viruses.

3.5. Cellular immune response after MuV vaccination

Since cellular immunity may also be important in preventing disease caused by MuV [32,33], we measured the cellular immune responses after vaccination. The number of interferon-gamma (IFNγ)-secreting splenocytes of vaccinated mice was assessed at 14 days post-boost by ELISpot (Fig. 6). While we found that Jeryl Lynn consistently produced more IFNγ-secreting cells for each of the antigens, there was no significant difference. These results suggest that there is no genotype-specific difference in the cellular immune response between the vaccines.

4. Discussion

The MuV vaccine was able to greatly reduce the number of mumps cases after its introduction in the late 1960s. There was a resurgence of mumps cases in the late 1980s in the vaccinated population, which prompted the recommendation of a second dose of the MMR vaccine [34]. Two doses of the MMR vaccine reduced the number of mumps cases to under 300 annually in the United States from 2001 to 2005 [35]. However, mumps outbreaks have occurred in the United States in the last decade, including the large outbreak centered around a university in Iowa, which had over 5000 cases in 2006 [36–39]. Mumps outbreaks have also been reported in vaccinated populations in Europe and Asia [20–23,40,41]. None of these outbreaks were associated with a genotype A MuV, which was the Jeryl Lynn vaccine genotype.

Both of our vaccine candidates generated robust immune responses against the circulating genotype G and F viruses. While Jeryl Lynn vaccination generated a good immune response against genotype A virus, there was a significant decrease in the ability of serum from these mice to neutralize genotype G or F viruses. It is possible that this decrease may contribute to some of the recent outbreaks. It has been shown that there is variation in serum neutralizing antibodies among people over time [21,41–43]. Poor immune responses or waning immunity may result in some individuals dropping below a protective threshold. This may be exacerbated by a vaccine that does not match the circulating strains, since vaccinated individuals already start with a lower level or neutralizing antibodies against circulating strains.

Although there was a significant difference in neutralizing antibody titers between Jeryl Lynn and the two vaccine candidates, vaccination with Jeryl Lynn produced higher or similar serum ELISA titers and cellular immune responses. It is known that NP is an immunodominant antigen during MuV infection [44]. Antibodies against NP may mask a decrease in antibodies specific to F and HN proteins. The lack of differences in the cellular immune response may be due to NP or common T-cell epitopes in F and HN proteins. We do not currently know what the dominant T-cell epitopes are for MuV, but this data would suggest they are shared between these viruses.

Our results also allow us to start exploring the reasons for the differences in the neutralizing antibodies produced after vaccination. Since no antigenic difference between the genotype G and F vaccine candidates was observed using the neutralization assay, we propose that there is likely a common difference between the HN and/or F proteins of these viruses and Jeryl Lynn that accounts for the difference in neutralization titers. Using the structures of PIV5 F [45] and HN [46], we modeled the structure of MuV F and HN proteins. We compared common variations with sites that may be targeted by neutralizing antibodies [47,48]. We found that 7 sites in HN that differed in Iowa/06 and PZH0804 viruses compared to Jeryl Lynn. Two the differences, residues 354 and 356, have been shown to escape neutralization by neutralizing monoclonal antibodies [49]. The sites proposed to be important for F-specific neutralizing antibodies did not differ, but all epitopes may not have been mapped [48]. We also found many differences in the F and HN interacting domains. Antibodies recognizing this region may prevent F-HN interaction after receptor binding. Mutating these residues and testing their antigenicity will help with determining what sites are important for the differential neutralization between genotypes.

Although there are changes in the genotype, MuV is still considered to have only one serotype [50]. Previously, differences in neutralizing titer against a range of viruses was examined after a second vaccination with Jeryl Lynn [43]. Six weeks post-immunization, there was at least a 2-fold decrease in neutralizing titers against a genotype G virus compared with a homologous virus, consistent with our results. While the definitive role of antibody in protection has not been proven, it is possible that the observed decrease is responsible for the increase in infections seen in the United States over the past decade. Our Iowa/06-based vaccine produced a good immune response specific to both genotype F and G viruses. A vaccine matching the circulating genotypes may better protect against MuV infection.

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