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Serologic Cross-reactivity of Murine Parvovirus Capsid Antigens

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Genomic sequence analysis of autonomous parvoviruses within the genus *Protoparvovirus* generates 2 groups that are principally of mouse origin: the minute virus of mice (MVM) strains (MVMp, MVMi, MVMc, MVMm) and the mouse parvovirus (MPV)–like strains (MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, LuIII). Baculovirus-expressed recombinant capsid protein (rVP2) from each of these 11 parvovirus strains were produced, purified, and demonstrated to form virus-like particles. Each rVP2 preparation was then used as antigen in a multiplex fluorescent immunoassay and to immunize 5 different strains of mice. Sera from immunized mice, mice experimentally monoinfected with various MVM or MPV isolates, and mice naturally infected with murine parvoviruses were evaluated with the multiplex fluorescent immunoassay rVP2 panel. Results for sera from immunized mice indicate that homologous antigen–antisera interactions produced the strongest seroreactivity. All MVM antigens were highly cross-reactive with heterologous MVM antisera, while more variability was observed in heterologous antigen–antisera reactions among the MPV-like strains. MPV-1, MPV-3, HaPV, and LuIII were highly cross-reactive with each other, MPV-2 and MPV-5 were highly cross-reactive with each other, and MPV-4 displayed modest cross-reactivity with certain MPV-like strains. Serologic cross-reactivity patterns similar to those in immunized mice were observed in mice experimentally infected with MVMp, MVMm, MPV-1, MPV-5, or HaPV, and in sera from mice naturally infected with MVM and MPV. Serologic cross-reactivity spectrums suggest a small panel of rVP2 antigens (MVM, MPV-1, MPV-2, MPV-4) combined with the generic murine parvovirus recombinant nonstructural protein 1 (rNS1) antigen are sufficient for qualitative detection of currently known MVM and MPV-like strains.

Abbreviations and Acronyms: AUC, area under curve; HaPV, hamster parvovirus; mfi, median fluorescence intensity; MFI, multiplex fluorescent immunoassay; MPV, mouse parvovirus; MVM, minute virus of mice; NS1, nonstructural protein 1; rNS1, recombinant NS1; ROC, receiver operating characteristic; rVP2, recombinant VP2; VLP, virus-like particle; VP2, viral capsid protein 2

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Introduction

Minute virus of mice (MVM) and mouse parvovirus (MPV) continue to be detected in contemporary laboratory mouse colonies despite advances in diagnostic testing and management since they were first discovered.[2,38,48] Both MVM and MPV can have deleterious effects on research due to in vitro and in vivo immunomodulatory effects,[11,20,36,41–43] tumor suppression,[27,28,36,41,42] and contamination of cell cultures and tissues originating from mice.[11,15,17,24,25,41,45] Potential transmission of MVM and MPV between research facilities remains a concern due to their high degree of environmental stability[30] and their potential to induce persistent infection in mice and cell lines.[5,21,32,53] Once detected, murine parvoviruses can incite significant management and operational costs to animal care programs and investigators. Collectively, these concerns indicate that further investigation to improve detection and management is warranted.

Numerous strains of murine parvoviruses within the genus *Protoparvovirus* have been identified. MVM was first isolated in 1966 from a preparation of adenovirus, with this prototypic strain designated MVMp.[16] MVMi was isolated from contaminated EL4 lymphocytes[11] and was later shown to be immunosuppressive in vitro.[20,43] MVMc was isolated 3 decades ago as a contaminant of BHK-21 cells.[7] MVMm was isolated from NOD mice displaying growth retardation, reduced fecundity, and premature deaths.[44] MVMm is the most common strain of MVM detected in naturally infected laboratory mice (91% of MVM-infected mice), with MVMc accounting for the remaining positive mice.[9]

MPV-1 was originally isolated from cultures of cytolytic T lymphocytes and splenocytes.[41] Initial genomic sequence analysis indicated that MPV-1 was genetically most closely related to LuIII, a parvovirus of unknown host species origin isolated as a cell culture contaminant.[29] Investigation of mice naturally infected with parvovirus revealed 2 novel MPV genotypes designated MPV-2 and MPV-3,[9] and subsequent investigations identified 2 additional genotypes designated MPV-4 (not yet isolated) and MPV-5.[22,33] Hamster parvovirus (HaPV) was isolated from Syrian hamsters with clinical disease, displays 98% nucleotide sequence homology with MPV-3, and can productively infect laboratory mice.[6,9,14] MPV-1 is the most common MPV strain detected in laboratory mice (78% of MPV-infected mice), followed by MPV-2 (21%) and MPV-3 (1%).[9]

Rodent chaphamaparvovirus 1, also known as mouse kidney parvovirus or murine chapparvovirus, is the most recently identified rodent parvovirus. It is associated with inclusion

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body nephropathy, persistent interstitial nephritis, and fibrosis in laboratory mice.[19,26,46,51] This virus has been placed into the newly recognized genus *Penstyldensovirus* with genetically similar viruses identified in other animal species.[52] It has an approximately 10% smaller genome than members of the genus *Protoparvovirus*, and highly heterologous capsid gene sequences as compared with MVM and MPV, so it was not evaluated in the current serologic cross-reactivity studies. Similarly, rat parvoviruses were excluded from the current studies due to highly heterologous capsid gene sequences as compared with MVM, MPV, HaPV, and LuIII.[7,9]

Examination of genomic DNA sequence alignments of the murine parvovirus strains within the genus *Protoparvovirus* reveals a common genetic organization with conservation of the promoter regions, splice junctions, and translation start and stop codons.[7,9] Amino acid alignments of the major nonstructural protein (NS1) show a high degree of homology, which is expected since this protein is functionally critical for parvovirus replication and transcriptional regulation. More diversity exists among the major capsid protein (viral capsid protein 2 [VP2]) amino acid sequences, which indicate that the murine parvoviruses form 2 distinct groups: the MVM strains (MVMp, MVMi, MVMc, MVMm) and the MPV-like strains (MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, LuIII).[9] The three-dimensional structure of the MVM capsid has been determined, and phenotypic characteristics displayed by MVM have been mapped to specific surface structures of the virus capsid.[1,39] Changes at different amino acid loci could induce minor structural alterations that alter conformational epitopes important for antibody recognition, and would also impact linear epitopes.

Serology is commonly used to detect parvovirus infections in laboratory mice, with the multiplex fluorescent immunoassay (MFI) preferred for its high-throughput multiplex format that enables simultaneous detection of multiple murine pathogens from microliter quantities of sera.[35] Recombinant MVM NS1 (rNS1) antigen serves as a generic rodent parvovirus diagnostic antigen,[50] but host factors such as age and strain can limit seroconversion to rNS1 in MPV-infected mice.[10,23] Subsequently, recombinant major capsid proteins (rVP2) for MVMp and MPV-1 were developed and are now in widespread use as serologic screening assays.[3,37] Two reports indicate limited serologic cross-reactivity between MPV-1 and MPV-2.[18,31] Laboratories have therefore incorporated both MPV-1 and MPV-2 capsid antigens in their mouse serologic screening profiles to enhance MPV detection. However, it remains unknown if the currently used rVP2 antigens are capable of sensitive serologic detection of currently known MVM and MPV strains. The goals of the current studies were to determine the relative serologic cross-reactivity among recombinant capsid proteins (rVP2) for the various murine parvoviruses within the genus *Protoparvovirus*, and to subsequently identify a subset of rVP2 antigens that could be used in a MFI to improve serologic detection of these murine parvovirus infections.

Materials and Methods

Virus. MVMp, MVMi, MVMc, MVMm, MPV-1b, HaPV, and LuIII were propagated as described previously.[7,9] DNA was extracted from each virus using a MagneSil KF genomic DNA extraction kit (Promega, Madison, WI) and a KingFisher robotic extraction station (Thermo Fisher Scientific, Waltham, MA) per the manufacturers' recommendations. MPV-2, MPV-3, and MPV-5 DNA were obtained from samples acquired during an epidemiologic survey of currently circulating field parvovirus strains.[9] MPV-4 DNA and the MPV-5 isolate were kindly provided by Dr. Ken Henderson (Charles River, Wilmington, MA). The genomic sequences for MPV-4 and MPV-5 were determined as previously described[7,9] and were assigned the following GenBank accession numbers: FJ440683 for MPV-4 and FJ441297 for MPV-5.

Recombinant viral protein generation and purification. The rNS1 baculovirus stock was kindly provided by Dr. Lela Riley (University of Missouri, Columbia, MO). Each baculovirus that expressed rVP2 was prepared as previously described,[37] but without histidine tags. The VP2 gene for each of the 11 parvovirus strains was amplified by PCR using Platinum Taq DNA polymerase high fidelity (Thermo Fisher Scientific). Primers MVM 2794–2812 forward (5′-gcatatgtcgacggatccATGAGTGATGGCACCAGCC-3′) and MVM 4557–4522 reverse (5′-gcggtaccctcgaggTTAGTAAG TATTTCTAGCAACAGGTCTTGTTATAAG-3′) were used for the MVM strains (MVMp, MVMi, MVMc, MVMm), and MPV 2655–2673 forward (5′-gcatatgtcgacggatccATGAG TGATGGCACCGAGC-3′) and MPV 4418–4385 reverse (5′-gcggtaccctcgaggTTAGTAAGTATTTCTAG CAACAGGTCTAGAAAGC-3′) were used for the MPV-like strains (MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, LuIII). The lowercase letters in the forward primer sequence represent an additional sequence that includes restriction endonuclease sites for SalI and BamHI, whereas the lowercase letters for the reverse primers represent an extra sequence that includes restriction enzyme sites for KpnI and XhoI. Reactions were performed in a 50-µl volume in a PerkinElmer model 2,400 thermocycler. Each reaction mixture contained 5 µl of template DNA, 200 nM for each oligonucleotide primer, and 45 µl of Platinum PCR SuperMix high fidelity (Thermo Fisher Scientific). Thermocycle parameters were 30 s of denaturation at 94 °C followed by 45 cycles of 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C, and 105 s of elongation at 72 °C. Amplicons generated were resolved by agarose gel electrophoresis, and the target band was eluted with a QIAquick kit (Qiagen, Germantown, MD) according to the manufacturer's instructions. The 1,764-bp products and the pFastBacI vector (Thermo Fisher Scientific) were then digested for 4 h with the restriction enzymes BamHI and XhoI (Promega), DNA digests were electrophoresed on a 1.5% NuSieve agarose gel, and the target bands were eluted with a QIAquick kit. The PCR products were then ligated into the pFastBacI vector and amplified in TOP10 competent cells (Thermo Fisher Scientific). Recombinant plasmid DNA was purified from the transformed cells using the Qiagen QIAprep miniprep kit, and the DNA was sequenced by the University of Arizona Genetics Core Facility to confirm the DNA insert sequence and orientation. Each recombinant plasmid was then used to transform MAX Efficiency DH10Bac competent cells (Thermo Fisher Scientific), and the resulting recombinant bacmid DNA was extracted following the manufacturer's recommendations. The DNA was again sequenced by the University of Arizona Genetics Core Facility to confirm the DNA insert sequence and orientation. Bacmid DNA was used to transfect SF9 cells using Cellfectin reagent (Thermo Fisher Scientific), and the recombinant baculoviral stock was collected and quantified by plaque assay.

The rNS1 and each recombinant major capsid protein (rVP2) were expressed in High Five insect cells. For the rVP2 proteins, 250-mL spinner flasks containing 150 mL of 8×10^5 High Five cells per mL in Express Five SFM media (Thermo Fisher Scientific) supplemented with 16.5 mM L-glutamine were infected with recombinant baculovirus at a multiplicity of infection between 0.1 and 0.5. The cells were pelleted after 72 h, resuspended in sterile PBS containing 2 mM Pefabloc SC (Roche Diagnostics, Indianapolis, IN), and freeze-thawed 3 times. A freon extraction

was performed and cellular debris was removed by centrifugation at 10,000 × *g*. The resulting supernatant was centrifuged at 330,000 × *g* in a Beckman ultracentrifuge using a type 70 Ti rotor for 30 min at 4 °C to pellet the recombinant protein. The pellet was resuspended in PBS and then added to a 10% to 40% CsCl step gradient and centrifuged at 120,000 × *g* in a Beckman centrifuge using a SW 41 Ti rotor for 16 h at 4 °C. The rVP2 protein band was removed and dialyzed overnight with PBS in a 10 kDa membrane (Spectrum Chemical Mfg. Corp., Gardena, CA). Purified protein was stored at −80 °C until use. For rNS1 protein production, 50% confluent flasks of High Five cells were infected at a multiplicity of infection of 0.15. Cells were collected at 72 h postinfection, washed twice with PBS, resuspended in lysis buffer (20 mM HEPES-KOH [pH 7.5], 5 mM KCl, 7.5 mM $MgCl_2$) with 2 mM Pefabloc SC, and then sonicated for 8 cycles of 30 s on full power. KCl was added to a final concentration of 0.3 M and the lysate was incubated for 30 min on ice. Cell debris was pelleted by high-speed centrifugation at 27,000 × *g* for 10 min at 4 °C, and 10 mM imidazole was added to the supernatant. The His-tagged rNS-1 protein was purified from the lysate with TALON metal affinity resin (Takara Bio, San Jose, CA) per the manufacturer's protocol, and 10% v/v glycerol was added to the purified rNS1 with storage in liquid nitrogen until use. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich, St. Louis, MO). Protein purity was assessed by PAGE of 5 µg of each preparation with bands visualized by Coomassie blue staining. A Kodak Gel Logic 100 system (Eastman Kodak, Rochester, NY) and its accompanying software were used to digitize the gel image and determine molecular mass and density of each protein band.

Electron microscopy. Each rVP2 preparation was visualized with assistance from the University of Arizona Imaging Core Facility to confirm virus-like particle (VLP) formation. Each purified recombinant capsid protein was diluted to 0.08 µg/µl in sterile PBS. Four microliters of each sample was sandwiched between a carbon film and a mica substrate. The carbon films with the adhered capsids were floated onto 50 µl of 2% phosphotungstic acid (pH 7.4) and then picked up on 200 mesh grids. Each sample was then visualized using a JEOL 100 CX II transmission electron microscope (JEOL, Peabody, MA).

Serology. The MFI format was used to evaluate sera for the presence of recombinant capsid-specific antibodies. Each purified rNS1 and rVP2 antigen was covalently coupled to carboxylated polystyrene microspheres (Luminex, Austin, TX) at a coupling concentration of 25 µg of protein per 5×10^6 microspheres according to the manufacturer's recommended protocols. Ovalbumin, $A9_{2L}$ mouse fibroblast cell lysate, and High Five insect cell lysate were each similarly coated to microspheres to serve as negative control antigens, and purified goat anti-mouse IgG + IgM (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) was also coated to microspheres as a control to verify addition of sera to each well in the MFI plate. Microspheres were stored at 4 °C in the dark until use. Master mixes containing all test and control antigens were prepared, and mouse sera were evaluated with a LiquiChip workstation (Qiagen, Valencia, CA) as described previously.[5] Briefly, antigen-coated microspheres were incubated for 60 min with diluted sera, washed twice, incubated with phycoerythrin-conjugated $F(ab')_2$ fragment goat anti-mouse immunoglobulin G (H+L) secondary antibody (Jackson ImmunoResearch Laboratories), washed twice, and resuspended in stop solution containing formalin. The microplate was then shaken for 5 min and analyzed on the LiquiChip workstation. Each sample was run in duplicate. Results are reported as the median fluorescent intensity (mfi) observed within the total of 50 antigen-coated microspheres analyzed per well.[13] Baselines were determined by receiver operating characteristic (ROC) AUC analysis, with mfi results >3,000 considered positive for each rVP2 antigen and >300 considered positive for the rNS1 antigen. None of the sera exceeded the mfi baseline of 300 mfi for any of the negative control antigens.

Mouse immunization. Female Hsd:ICR, Hsd:ND4 Swiss Webster, BALB/cAnNHsd, C3H/HeNHsd, and C57BL/6NHsd mice were obtained (Inotiv, Indianapolis, IN). All mice were specified to be free of murine viruses (mouse hepatitis virus, minute virus of mice, mouse parvovirus, mouse rotavirus, encephalomyelitis virus, pneumonia virus of mice, Sendai virus, lymphocytic choriomeningitis virus, murine norovirus, ectromelia virus, Hantaan virus, mouse adenovirus, mouse cytomegalovirus, respiratory enteric virus III, K virus, lactic dehydrogenase elevating virus, polyoma virus, and mouse thymic virus), pathogenic bacteria, and endoparasites and ectoparasites by the vendor. Each mock or experimental group was housed separately in sterilized static microisolation cages on aspen chip bedding. Teklad NIH-31 diet (Inotiv) and hyperchlorinated water were provided ad libitum, and all animal manipulations were performed in a class IIA biologic safety cabinet using standard microisolation technique. Animals were housed in a biocontainment facility at a temperature of 22 to 24 °C, humidity of 30% to 70%, 12 to 15 air exchanges per hour, and a 12-h light/12-h dark cycle. The University of Arizona IACUC approved all animal experiments for all experiments described, which met humane care and use regulatory standards in compliance with the NIH *Guide for the Care and Use of Laboratory Animals* and the University's AAALAC accreditation.

Each strain of mice was immunized with each of the 11 recombinant proteins or PBS (mock immunized mice). Two commonly used outbred stocks (ICR, SW) were chosen to represent immune responses in heterogeneous populations, and 3 commonly used inbred strains (C57BL/6, C3H, BALB/c) were chosen to represent the differing immune responses observed within each of these homogeneous strains.[10,12] At least 2 male and 2 female mice per mouse strain were inoculated with each immunogen to provide an equivalent sex ratio for each strain/immunogen group. Inocula were prepared by adding a 1:1 dilution of 0.4 µg/µl of each recombinant protein in PBS and TiterMax (TiterMax USA, Norcross, GA). An emulsification was formed by repeated aspiration of the mixture through a blunt 18-gauge needle attached to a 1-mL all plastic syringe for ~2 min. Mice were injected subcutaneously with 25 µl of the emulsified mixture with a 27-gauge needle at 2 different sites (total = 10 µg) in the interscapular region and near the tail base. After 4 wk, mice were euthanized by carbon dioxide inhalation, blood samples were collected by cardiocentesis, and the resulting sera was stored at −80 °C until use. Each serum sample was then evaluated by MFI in duplicate, and the mean of the duplicate results was used for analysis. Homologous seroreactivity for each rVP2 antigen was determined as the mean of MFI results for sera from all mice immunized with the same rVP2 antigen (n = 18 to 22 per antigen). Sera were considered nonresponsive to immunization if their homologous seroreactivity was <50% as compared with the group mean, and these samples (n = 14) were excluded from further analysis. Percent reactivity of each heterologous antigen/antisera combination was then determined relative to the homologous group mean for each antigen.

Experimental mouse infections. Sera obtained from mice experimentally infected with individual parvovirus isolates were evaluated by MFI. All sera were previously demonstrated to be seropositive to the inoculated parvovirus by other serologic

Table 1. Percent amino acid identity among rodent parvovirus isolates for VP2 as determined by GCG Best Fit program

	MVMp	MVMi	MVMc	MVMm	MPV-1b	MPV-3	HaPV	LuIII	MPV-2	MPV-5	MPV-4
MVMp	**100**	98.0	95.6	95.7	74.1	74.4	73.7	73.9	74.7	73.5	74.6
MVMi		**100**	96.3	95.9	73.7	73.7	73.2	73.5	74.1	72.9	73.9
MVMc			**100**	96.1	73.7	73.9	73.4	73.4	74.9	73.5	73.7
MVMm				**100**	73.7	73.5	73.0	73.0	73.7	72.4	73.9
MPV-1					**100**	92.9	92.0	83.5	89.1	90.1	92.3
MPV-3						**100**	98.5	84.9	93.2	94.6	90.6
HaPV							**100**	84.4	92.3	93.7	89.8
LuIII								**100**	83.3	84.5	84.9
MPV-2									**100**	95.6	91.5
MPV-5										**100**	90.0
MPV-4											**100**

Bold type is used to highlight 100% percent VP2 amino acid sequence identity between identical strains; e.g., MVMp on the column is 100% identical to MVMp on the top row.

assays (IFA, ELISA, and/or MFI). Various ages (1 d old to 6 wk old) of male and female mice (ICR, SW, BALB/c, C3H, C57BL/6) were infected by various methods (oral, oronasal, gastric gavage, intraperitoneal) in experiments performed during the course of several years, all of which were also approved by the University of Arizona IACUC.[5,8,10,12,14,49] Each mouse was infected with at least 1 ID_{50} or 5×10^3 $TCID_{50}$ of MVMp, MVMm, MPV-1b, HaPV, or MPV-5. Most mice were euthanized by carbon dioxide inhalation between 3 and 8 wk postinfection ($n = 90$), with the remaining mice euthanized at 1 or 2 wk postinfection ($n = 17$). After euthanasia blood was collected by cardiocentesis and the resulting sera were stored at −80 °C until evaluated by MFI.

Naturally infected mice. Sera ($n = 332$) were obtained from Charles River Laboratories Research Animal Diagnostic Services (Wilmington, MA) and IDEXX BioAnalytics (Columbia, MO), which provide health monitoring services to research animal facilities internationally. Collectively these sera represent a random sampling of different strains, ages, and sexes of mice. Sera were from mice previously determined to be serologically positive for MVMp, MPV-1, and/or MPV-2. Original serologic results were interpreted according to the parameters used routinely by these labs, with an adjusted score of 3 (equivalent to an mfi of 3,000) or higher considered positive.[13,31] Sera were stored frozen at −80 °C until evaluated by the complete rVP2 MFI panel.

Statistical analysis. Serologic cross-reactivity between immunized mouse groups was first evaluated by pairwise correlations to determine correlation coefficients. Values were calculated on logscale to reduce the effects of heavily skewed distributions. Based on these pairwise correlations, each antigen was placed into one of 4 serogroups. For a comparative summary, ROC curve methods were used with AUC as a summary statistic. The AUC provides a global indication of an assay's ability to discriminate between 2 serogroups. AUC values range from 0.5 (no discriminatory ability) to 1.0 (perfect discrimination). An AUC threshold of 0.8 was targeted for each serogroup since AUC values around 0.8 have been considered acceptable for biomarkers of human disease. A distribution curve was produced for each group and a baseline was established that would accurately discriminate between each specific antigen group compared with the other groups.

Results

Sequence analysis. The amino acid sequences of the predicted proteins for MVMp, MVMi, MVMc, MVMm, MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, and LuIII were translated from genomic DNA sequences, aligned, and the percent identity for each protein comparison was calculated. All strains exhibit a high percentage NS1 protein identity, from 92.2% to 99.3%, consistent with previous reports.[4,7,9,50] Comparison of the predicted VP2 amino acid sequences suggests categorization of murine parvovirus strains into 2 subgroups: the MVM strains, and the MPV-like strains, which include HaPV and LuIII (Table 1). The MVM strains exhibit high VP2 amino acid sequence identity (95.6% to 98.0%), whereas the MPV-like strains exhibit more divergent VP2 amino acid identities (83.3% to 98.5%).

Baculovirus production and analysis. Baculovirus-expressed recombinant capsid protein (rVP2) was produced and purified for MVMp, MVMi, MVMc, MVMm, MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, and LuIII. To assess the purity of each protein as well as normalize protein concentrations, 5 µg of each was run on a polyacrylamide gel and the resulting protein bands were visualized using Coomassie blue (Figure 1A). A single band of the predicted ~64 kDa size was seen for each VLP with no contaminating protein bands. Kodak Gel Logic 100 software was used to determine the net intensity value for each band, which was then used to normalize protein concentrations. Each rVP2 preparation was also visualized by transmission electron microscopy (Figure 1B). All protein preparations displayed VLP formation and contained minimal contaminating debris.

Seroreactivity of immunized mice. Immunized mouse sera were evaluated with a bead master mix containing all test and control antigens on a Qiagen LiquiChip workstation. Mean homologous antisera reactivity was determined for each group of mice immunized with the same rVP2 antigen for all mouse strains combined ($n = 17$ to 22 antisera per antigen). Individual serum samples were considered nonresponsive to immunization if their homologous reaction had <50% reactivity as compared with the mean from the other mice immunized with the homologous antigen. A total of 2 ICR, 8 BALB/c, and 4 C57BL/6 mice immunized with 7 different antigens were determined to be nonresponsive and were excluded from further analysis. Percent reactivity of each heterologous antiserum was determined relative to the mean homologous mfi for each antigen (Figure 2). Heterologous sera displaying reactivity >25% of mean homologous mfi, equivalent to the 3,000 mfi baseline determined by ROC AUC, were interpreted as significant cross-reactivity to each rVP2 antigen. Mean mfi values for all antisera from each group of immunized mice were also compared with visualized seroreactivity trends for each rVP2 antigen (Figure 3).

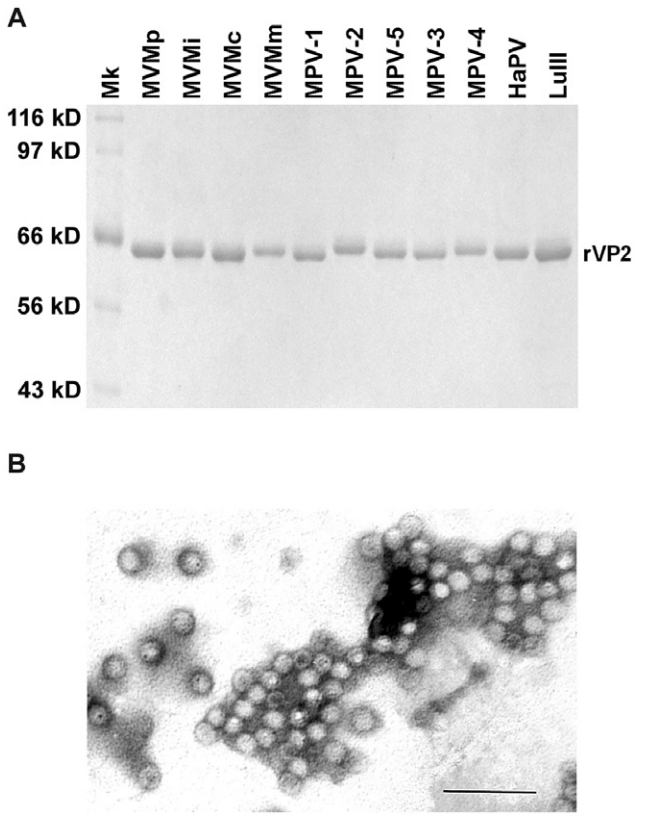

Figure 1. (A) Purity and normalized protein concentrations for each rVP2 protein (5 µg) as demonstrated by Coomassie-stained polyacrylamide gel. (B) Transmission electron micrograph of negative stained VLP formed from purified MVMp rVP2. Similar images were observed for other rVP2 preparations. Scale bar, 100 nm. Mk, marker.

Evaluation of individual heterologous serologic reactions indicate the 11 rVP2 antigens form 4 serogroups (Figure 2). Sera from mice immunized with the 4 MVM immunogens cross-reacted with each of the 4 MVM serologic antigens, with 87.8% of heterologous sera displaying reactivity greater than 25% of mean homologous mfi. Within the MPV-1/MPV-3/HaPV/LuIII group 82.4% of heterologous sera displayed reactivity >25% of mean homologous mfi, and within the MPV-2/MPV-5 group 81.8% of heterologous sera displayed reactivity >25% of mean homologous mfi. Heterologous sera reacted with MPV-4 antigen at >25% mean homologous mfi in only 9% of samples, indicating that it is a distinct serogroup. Interestingly, a moderate level of one-way cross-reactivity was observed for certain groups of antisera to antigens in other serogroups. MPV-1 antigen reacted with antisera from mice immunized with MPV-4 (45.0%). MPV-2 antigen reacted with antisera from mice immunized with MVMi (55.6% of sera samples) and MVMc (66.7%). MPV-5 antigen reacted with antisera from mice immunized with MVMm (44.4%), MPV-1 (36.8%), MPV-3 (44.4%), HaPV (29.4%), and LuIII (35.0%). Antisera from mice immunized with MPV-5 reacted with MPV-1 antigen (45.5%), MPV-3 antigen (40.9%), HaPV antigen (31.8%), and LuIII antigen (40.9%). Comparison of mean mfi for each group of immunized mice for each rVP2 antigen also indicates 4 main serogroups, and likewise indicates one-way reactivity between certain antisera groups and rVP2 antigen combinations (Figure 3).

Relative seroreactivity among different mouse stocks/strains was determined. The mean mfi value for all homologous antisera–antigen reactions for each of the 5 different mouse stocks/strains was calculated to evaluate generic strain response

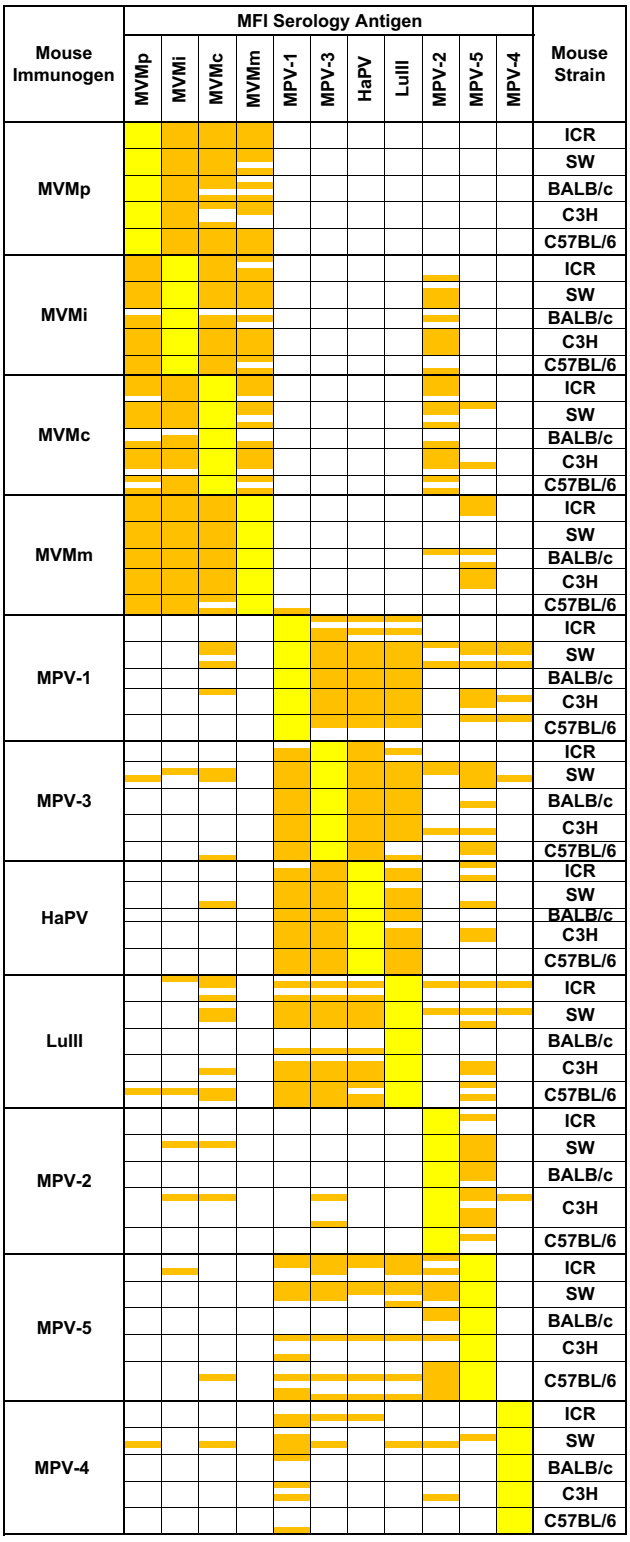

Figure 2. Semiquantitative analysis of seroreactivity across 5 mouse strains immunized with each purified rVP2 capsid antigen. Each immunogen is indicated in the left-hand column, and each serologic MFI antigen is indicated in the 11 middle columns. The reactivity of antisera obtained from each individual mouse to each serologic antigen is colorimetrically indicated in each intersecting rectangle as homologous (yellow), or heterologous with seroractivity >25% (orange) or <25% (white) as compared with the mean homologous seroreactivity.

to immunization. Outbred stocks were similar in serologic response following immunization (ICR mean = 15,118 mfi, Swiss mean = 14,166 mfi), while inbred strains generated increased

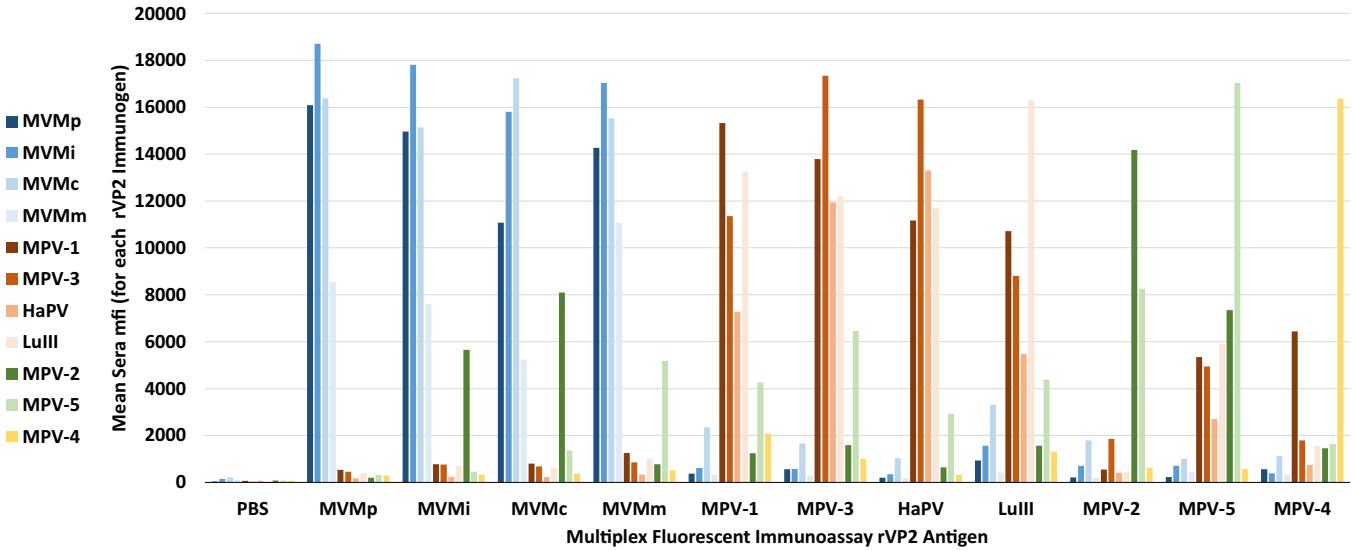

Figure 3. Homologous and heterologous seroreactivity across all mice immunized with each rVP2 purified antigen, expressed as mfi. Serum reactivity to each antigen for each serogroup is colorimetrically indicated as follows: MVM serogroup (blues), MPV-1 serogroup (reds), MPV-2 serogroup (greens), and MPV-4 (yellow).

responsiveness from BALB/c (mean mfi = 12,879) to C3H (mean mfi = 16,382) to C57BL/6 (mean mfi = 19,542).

Qualitative serology results for each immunized mouse group were established using the MFI baseline of 3,000 mfi established by ROC AUC (Table 2). Heterologous reactions showed consistently strong cross-reactivity for sera among mice immunized with the various MVM strains (66.7% to 100% of mice positive), among the MPV-1/MPV-3/HaPV/LuIII group (70.0% to 100% positive), and between MPV-2 and MPV-5 (72.7% to 77.3% positive). MPV-4 serum displayed low cross-reactivity with most heterologous rVP2 antigens, but was detected by MPV-1 antigen (60% positive). All mock immunized mice were negative by MFI for all antigens evaluated.

Seroreactivity of infected mice. Parvovirus-positive sera from several experimental monoinfection studies were evaluated qualitatively by the MFI rVP2 antigen panel (Table 3). Sera from mice experimentally infected with MVMp or MVMm were seropositive to heterologous MVM antigens in 33% to 100% of samples. Sera from mice experimentally infected with MPV-1 or HaPV were seropositive to heterologous MPV-1/MPV-3/HaPV/LuIII antigens in 35% to 100% of samples. Sera from mice experimentally infected with MPV-5 were seropositive to MPV-2 antigen in 93% of samples. Although sera from mice infected with MVMp and MVMm were not detected by non-MVM rVP2 antigens, there are several examples of one-way serologic cross-reactivity among the MPV serogroups. Sera from mice infected with MPV-1 or HaPV were seropositive to MPV-2, MPV-5, or MPV-4 antigens in up to 18% of samples. Sera from mice infected with MPV-5 were seropositive to heterologous MPV-1/MPV-3/HaPV/LuIII antigens in up to 71% of samples and to MPV-4 antigen in 14% of samples.

Sera from mice naturally infected by MVM and/or MPV ($n = 332$) were obtained from 2 commercial diagnostic laboratories and evaluated by an MFI panel comprised of the rNS1 antigen and the 11 rVP2 antigens (Table 4). Each sample was initially identified by the commercial laboratories as seropositive to one or more of the NS1, MVMp, MPV-1, and/or MPV-2 antigens. All sera were positive for the rVP2 antigen homologous

Table 2. Qualitative serology results for rVP2 immunized mice

	Multiplex fluorescent immunoassay rVP2 antigens										
Antisera	MVMp	MVMi	MVMc	MVMm	MPV-1	MPV-3	HaPV	LuIII	MPV-2	MPV-5	MPV-4
Mock	0/20[a]	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
MVMp	**20/20**	20/20	18/20	14/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
MVMi	17/18	**18/18**	17/18	14/18	0/18	0/18	0/18	1/18	10/18	0/18	0/18
MVMc	13/18	17/18	**18/18**	12/18	0/18	0/18	0/18	0/18	12/18	2/18	0/18
MVMm	18/18	18/18	17/18	**18/18**	1/18	0/18	0/18	1/18	1/18	10/18	0/18
MPV-1	0/19	2/19	4/19	0/19	**19/19**	17/19	15/19	15/19	3/19	7/19	5/19
MPV-3	1/18	1/18	3/18	0/18	17/18	**18/18**	18/18	15/18	5/18	8/18	2/18
HaPV	0/17	0/17	1/17	0/17	17/17	17/17	**17/17**	15/17	1/17	6/17	0/17
LuIII	2/20	3/20	9/20	0/20	15/20	14/20	14/20	**20/20**	6/20	9/20	2/20
MPV-2	0/22	2/22	3/22	0/22	0/22	3/22	0/22	1/22	**22/22**	16/22	1/22
MPV-5	0/22	1/22	1/22	0/22	10/22	11/22	7/22	11/22	17/22	**22/22**	0/22
MPV-4	1/20	1/20	1/20	0/20	12/20	3/20	1/20	3/20	2/20	1/20	**20/20**

[a]Number of mice positive/total tested, with positive defined as mfi >3,000.
Bold type is used to highlight homologous antigen:antisera results, e.g. MVMp antisera tested against MVMp antigen, MPV-1 antisera tested against MPV-1 antigen, etc.

Table 3. Qualitative serology results for mice experimentally infected with parvovirus isolates

	Multiplex fluorescent immunoassay rVP2 antigens										
Mouse sera	MVMp	MVMi	MVMc	MVMm	MPV-1	MPV-3	HaPV	LuIII	MPV-2	MPV-5	MPV-4
MVMp	**3/3**[a]	1/3	1/3	3/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
MVMm	22/22	22/22	22/22	**22/22**	0/22	0/22	0/22	0/22	0/22	0/22	0/22
MPV-1	0/34	0/34	0/34	0/34	**34/34**	15/34	12/34	17/34	0/34	4/34	5/34
HaPV	0/34	0/34	0/34	0/34	24/34	34/34	**34/34**	26/34	0/34	6/34	0/34
MPV-5	0/14	1/14	0/14	0/14	8/14	10/14	5/14	10/14	13/14	**14/14**	2/14

[a]Number of mice positive/total tested, with positive defined as mfi >3,000.
Bold type is used to highlight homologous antigen:antisera results, e.g. MVMp antisera tested against MVMp antigen, MPV-1 antisera tested against MPV-1 antigen, etc.

to the corresponding commercial laboratory VP2 antigen, and 90% of the sera were also positive to the NS1 antigen. Sera positive to the MVMp antigen were also positive to the MVMi (98% of samples), MVMc (100%), and MVMm (90%) antigens. Sera negative to MVMp antigen but positive to MPV-1 and/or MPV-2 were positive at low levels to MVMi (1%) or MVMc (4%). Sera positive to MPV-1 antigen were positive to the MPV-3 (91%), HaPV (79%), and LuIII (67%) antigens. Sera negative to MPV-1 antigen but positive to MVMp and/or MPV-2 were positive to MPV-3 (21%) at a modest level. Interestingly, most sera positive to MPV-1 but negative to MPV-2 were positive to MPV-5 (62%) and MPV-4 (60%). Sera positive to MPV-2 antigen were positive to the MPV-5 (93%) antigen. Sera negative to MPV-2 antigen but positive to MVMp alone or to both MVMp and MPV-1 were positive to MPV-5 (11%) and MPV-4 (17%) antigens.

Statistical analysis. Baselines for each MFI assay were determined by ROC AUC analysis, with mfi results >3,000 determined to be positive for each rVP2 antigen and >300 determined to be positive for the rNS1 antigen. These baselines were used for subsequent qualitative analyses. Pairwise correlations between the mfi results of mice immunized with each of the 4 MVM rVP2 immunogens and heterologous MVM antigens reveal strong correlations of 0.9 (Table 5). Pairwise correlations between members of the MPV-1/MPV-3/HaPV/LuIII serogroup also show strong correlation coefficients of 0.9. Correlations between the MPV-2/MPV-5 serogroup display modest correlation of 0.6. MPV-4 shows correlation coefficients of 0.4 to 0.5 across the MPV-like group. Pairwise correlations between members of the MVM compared with the 3 MPV serogroups were near zero (correlation coefficients = –0.2 to 0.4). Four serogroups were established based on these correlations: the MVM serogroup, the MPV-1/MPV-3/HaPV/LuIII serogroup, the MPV-2/MPV-5 serogroup, and the MPV-4 serogroup. The summary AUC statistic was established for each assay within each group (Table 6). The AUC value within the MVM group members revealed a strong ability to distinguish MVM from other immunogens (AUC = 0.93 to 0.98), regardless of the MVM strain used as rVP2 antigen. MVMp or MVMi provide the greatest ability to distinguish MVM immunized mice from all non-MVM immunized mice (AUC = 0.96 to 0.98). The MPV-1/MPV-3/HaPV/LuIII group also performs well in distinguishing mice inoculated with other rVP2 antigens within this group (AUC = 0.85 to 0.90). MPV-1 and LuIII perform slightly better than the other assays in this task (AUC = 0.88 to 0.90). The MPV-2/MPV-5 group adequately distinguishes this group from other rVP2 antigens (AUC = 0.79 to 0.83). The MPV-4 antigen displayed the highest AUC (0.72) toward the MPV-2/MPV-5 group.

Discussion

Detection of murine parvovirus infections in laboratory mouse colonies has improved significantly during the past few decades due to advances in both serology and PCR. This has led to decreased prevalence of MVM and MPV to <1% among random samples submitted to commercial diagnostic laboratories according to recent surveys.[2,33,40] The current studies focused on assessment of various MVM and MPV serologic antigens to further enhance identification of infected colonies, which in turn could help ensure that eradication efforts are successful. Improved detection could also help reduce the prevalence of MVM and MPV in contemporary mouse colonies and thereby minimize their impact on research.

Serologic detection of murine parvoviruses utilizes multiple antigens. While the NS1 proteins of all MVM and MPV strains exhibit a high percentage of amino acid similarity,

Table 4. Qualitative serology results for naturally infected mice

Commercial lab positive	NS1	MVMp	MVMi	MVMc	MVMm	MPV-1	MPV-3	HaPV	LuIII	MPV-2	MPV-5	MPV-4
MVMp	22/22[a]	**22/22**[b]	22/22	22/22	20/22	0/22	0/22	0/22	0/22	0/22	0/22	0/22
MPV-1	206/229	0/229	4/229	7/229	0/229	**229/229**	211/229	187/229	155/229	0/229	146/229	140/229
MPV-2	27/34	0/34	0/34	0/34	0/34	0/34	12/34	1/34	0/34	**34/34**	31/34	1/34
MVMp and MPV-1	13/13	**13/13**	13/13	13/13	13/13	**13/13**	8/13	5/13	8/13	0/13	4/13	6/13
MVMp and MPV-2	1/1	**1/1**	1/1	1/1	1/1	0/1	0/1	0/1	0/1	**1/1**	0/1	0/1
MPV-1 and MPV-2	19/21	0/21	0/21	3/21	0/21	**21/21**	20/21	16/21	11/21	**21/21**	21/21	13/21
MVMp, MPV-1, and MPV-2	12/12	**12/12**	11/12	12/12	9/12	**12/12**	11/12	8/12	9/12	**12/12**	11/12	7/12

Positive sera were initially identified by 2 commercial laboratories using a panel of rNS1, MVMp, MPV-1, and MPV-2 rVP2 antigens. Bold type is used to highlight homologous antigen:antisera results, e.g. MVMp antisera tested against MVMp antigen, MPV-1 antisera tested against MPV-1 antigen, etc.
[a]Number of sera positive/total tested, with positive defined as mfi >3,000 for rVP2 antigens, >300 for rNS1 antigen.
[b]Qualitative results for rVP2 antigens homologous to commercial lab antigens in bold.

Table 5. Correlation coefficients between each pairing of rVP2 antigens

	Multiplex fluorescent immunoassay										
Antisera	MVMp	MVMi	MVMc	MVMm	MPV-1	MPV-3	HaPV	LuIII	MPV-2	MPV-5	MPV-4
MVMp	**1.0**[a]	0.9	0.9	0.9	0.0	0.0	−0.1	0.0	0.2	0.1	0.2
MVMi		**1.0**	0.9	0.9	−0.2	−0.1	−0.2	−0.1	0.2	0.0	0.1
MVMc			**1.0**	0.9	0.0	0.1	0.0	0.1	0.4	0.2	0.3
MVMm				**1.0**	−0.1	0.0	−0.2	0.0	0.3	0.1	0.2
MPV-1					**1.0**	0.9	0.9	0.9	0.2	0.5	0.5
MPV-3						**1.0**	0.9	0.9	0.3	0.6	0.5
HaPV							**1.0**	0.9	0.2	0.5	0.4
LuIII								**1.0**	0.2	0.5	0.4
MPV-2									**1.0**	0.6	0.5
MPV-5										**1.0**	0.5
MPV-4											**1.0**

[a]Results determined by pairwise correlations calculated on logarithmic scale.
Bold type is used to highlight homologous antigen:antisera results, e.g. MVMp antisera tested against MVMp antigen, MPV-1 antisera tested against MPV-1 antigen, etc.

seroconversion to NS1 does not always occur in mice exposed to MPV.[10,23,50] As a result, rNS1 antigen is commonly used in combination with 2 or more rVP2 antigens for rodent parvovirus serologic testing. The amino acid sequences of rodent parvovirus (VP2) capsid proteins reveal more variation as compared with the NS1 protein. The MVM strains (MVMp, MVMi, MVMc, MVMm) exhibit high VP2 amino acid sequence identity, whereas the MPV-like strains (MPV-1, MPV-2, MPV-3, MPV-4, MPV-5, HaPV, LuIII) display more divergence.

Serological responses across 5 different strains of mice were evaluated for each rVP2 antigen. Each cohort of mice was immunized with a normalized amount of rVP2 protein emulsified in adjuvant for each of the 11 murine parvovirus strains, each serum sample was run in duplicate against all 11 antigens using an MFI assay, and the relative immune responsiveness was determined. Overall, the vast majority of mice across all 5 strains generated a similar immune response regardless of immunogen, with mfi values >10,000. Interestingly, the 2 outbred stocks displayed similar mfi response levels across all rVP2, while the 3 inbred strains displayed consistent antibody responses across all rVP2 that varied between each strain, most likely due to genetic differences that impact humoral immune response between each strain.[31,47] A few mice were deemed nonresponsive to immunization and subsequently excluded from further analysis.

Table 6. ROC AUC values for antisera groups

	Antisera groups		
MFI assay	MVMp, MVMi, MVMc, MVMm	MPV-1, MPV-3, HaPV, LuIII	MPV-2, MPV-5
MVMp	0.96[a]		
MVMi	0.98		
MVMc	0.93		
MVMm	0.95		
MPV-1		0.88	
MPV-3		0.86	
HaPV		0.85	
LuIII		0.90	
MPV-2			0.83
MPV-5			0.79
MPV-4			0.72

[a]AUC values range from 0.5 (no discriminatory ability) to 1.0 (perfect discrimination), with 0.8 considered good discrimination.

Because the excluded mice were immunized with 7 different rVP2 antigens and represented 3 different mouse strains, immunization error is considered the most likely cause for their lack of responsiveness.

Serological results from immunized, experimentally infected, and naturally infected mice indicate 4 serogroups, reflective of the VP2 amino acid sequence analysis. Sera from mice immunized or infected with MVM consistently displayed strong serologic cross-reactivity with heterologous MVM antigens. Predicted VP2 amino acid sequence analysis indicates that the MVM serogroup members are highly homologous with few VP2 amino acid differences, and thus linear and conformational epitopes should be highly conserved with strong serologic cross-reactivity. The MPV-like serogroups display more heterology among VP2 amino acid sequences, and thus would be expected to have less conserved epitopes and more variable serologic cross-reactivity. This variability was displayed with cross-reactivity primarily observed among mouse sera within the MPV-1/MPV-3/HaPV/LuIII and within the MPV-2/MPV-5 serogroups, with MPV-4 forming a fourth serogroup. Most of the viruses within each of these serogroups display a high amino acid identity with each other as compared with other murine parvoviruses. The exception is LuIII, which displays only 84% amino acid sequence identity with other MPV-like parvoviruses but shows high cross-reactivity with MPV-1, MPV-3, and HaPV. Because LuIII was discovered as a cell culture contaminant and its host species origin is unknown, this finding suggests that LuIII may have originated from a rodent species. One-way serologic cross-reactivities were also observed between various antigen/antisera combinations across the 3 MPV serogroups. One-way serologic cross-reactivity has been reported in other virus families and is thought to occur when common epitopes are selectively displayed during infection of different hosts.[34] Because the VLPs developed in this study are noninfectious, this one-way reactivity may be due to differences in humoral immune response among the mouse strains.

Although sera were only available from mice experimentally infected with 5 of the 11 viruses evaluated in this study, the correlation between the serologic results between immunized and experimentally infected mouse sera for these 5 viruses suggests that these findings should extrapolate to sera obtained from mice naturally infected with the various murine parvoviruses. Unfortunately, the sera obtained from naturally infected mice only reflect those mice detected by the

MVMp, MPV-1, and MPV-2 antigens currently used by commercial diagnostic laboratories, so mice infected with the other 8 viruses may not be represented at their actual prevalence in contemporary laboratory mouse populations. Regardless, the serologic data from naturally infected mice are consistent with the data observed in the immunized and experimentally infected mice. Sera cross-reacted within the MVM serogroup antigens, within the MPV-1/MPV-3/HaPV/LuIII serogroup antigens, and between the MPV-2/MPV-5 serogroup antigens, with one-way cross-reactivity observed primarily among the 3 MPV serogroups. As expected, the seroprevalence of mice naturally infected with MVM, MPV-1, and/or MPV-2 was consistent with prior epidemiologic prevalence data,[9] with most samples seropositive to MPV-1 antigen (83%) and a lower number of samples seropositive to MVMp (14%) and MPV-2 (20%) antigens. Seropositivity to 2 or more parvoviruses was observed in 14% of samples, and importantly 90% of samples from naturally infected mice were seropositive to rNS1 antigen, confirming its utility as a generic murine parvovirus antigen. Unfortunately, sera from mice experimentally or naturally infected with MPV-4 were not available, so we could not directly assess their serologic reactivity to other rVP2 antigens. It would be interesting to evaluate sera from mice that were serologically positive to NS1 antigen, but negative to MVMp, MPV-1, and MPV-2 antigens, to determine whether a percentage of these mice are seropositive to MPV-4 rVP2 antigen. It would also be interesting to further investigate MPV-4 immunized mice at multiple time points after immunization to determine whether time postinfection would improve detection of MPV-4 by other murine parvovirus rVP2 antigens, in particular by MPV-1 antigen, to which 45% of MPV-4 antisera cross-reacted after a single immunization. This finding would be consistent with a recent study in which seroconversion to MPV-2 rVP2 antigen was observed in mice experimentally monoinfected with MPV-1e, but only after day 28 postinfection.[31]

Statistical analysis confirmed the results from the qualitative MFI data analyses. The viruses were separated into the MVM serogroup, the MPV-1/MPV-3/HaPV/LuIII serogroup, and the MPV-2/MPV-5 serogroup on the basis of correlation values of 0.6 or above for serologic cross-reactivity. MPV-4 most closely aligned with the MPV-2/MPV-5 serogroup with a 0.5 correlation value, but also displayed correlation values of 0.4 to 0.5 with the MPV-1/MPV-3/HaPV/LuIII serogroup. Statistically it was determined that MVMi, LuIII, and MPV-2 would be the optimal antigens to detect all antisera for each corresponding group, on the basis of the ROC AUC values for each rVP2 antigen within each of these groups. However, all antigens in each group were deemed to sufficiently distinguish antisera from other groups since all have AUCs of 0.8 or above, the statistically significant threshold for adequate discrimination between each of the 4 serogroups. In practice, MVMp rVP2 is the most common diagnostic antigen used to detect the MVM group and should reliably detect the most common circulating strains MVMm and MVMc, given the high correlative values of 0.9 to 1.0 across MVM rVP2 antigens. MPV-1 is considered the most appropriate antigen to use for detection of the MPV-1/MPV-3/HaPV/LuIII group, as there is negligible difference between the AUC for MPV-1 and LuIII, and because MPV-1 is the most commonly detected MPV in prior epidemiologic studies.[9] Either MPV-2 or MPV-5 rVP2 antigen would be appropriate to detect the MPV-2/MPV-5 strains. Inclusion of the MPV-4 rVP2 antigen to murine parvovirus serologic testing profiles may be warranted, although MPV-4 antisera from infected mice would likely be detected by rNS1 and potentially by MPV-1 rVP2, as MPV-4 immunized mouse antisera showed cross-reactivity to MPV-1 rVP2 antigen in 45% of samples.

These results indicate that homologous antigen–antisera interactions produce the strongest seroreactivity. MVM rVP2 antigens were highly cross-reactive with heterologous MVM antisera, while more variability was observed in heterologous antigen–antisera reactions among the MPV-like strains. MPV-1, MPV-3, HaPV, and LuIII were highly cross-reactive with each other, MPV-2 and MPV-5 were highly cross-reactive with each other, and MPV-4 displayed modest cross-reactivity with heterologous MPV-like rVP2 antigens. Using the rNS1 antigen as a generic indicator of murine parvovirus infection assists with confirming positive rVP2 serology results and also with detecting novel murine parvovirus infections. In conclusion, serologic cross-reactivity spectrums suggest that a small panel of antigens (rNS1, MVMp, MPV-1, MPV-2, MPV-4) should enable qualitative detection of known MVM and MPV-like strains. Fortunately, these antigens are commonly used in MFI assays by commercial rodent diagnostic laboratories, with the possible exception of MPV-4, and should detect the vast majority of MVM and MPV-like viruses circulating in contemporary laboratory mouse colonies.

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Conflict of Interest

Kenneth S. Henderson is an employee of Charles River Laboratories, a company that produces and distributes research models and provides diagnostic services. The other authors have no conflicts of interest to declare.

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