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Novel pneumoviruses (PnVs): Evolution and inflammatory pathology

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ABSTRACT

A previous report of a novel pneumovirus (PnV) isolated from the respiratory tract of a dog described its significant homology to the rodent pathogen, pneumonia virus of mice (PVM). The original PnV–Ane4 pathogen replicated in and could be re-isolated in infectious state from mouse lung but elicited minimal mortality compared to PVM strain J3666. Here we assess phylogeny and physiologic responses to 10 new PnV isolates. The G/glycoprotein sequences of all PnVs include elongated amino-termini when compared to the characterized PVMs, and suggest division into groups A and B. While we observed significant differences in cytokine production and neutrophil recruitment to the lungs of BALB/c mice in response to survival doses (50 $TCID_{50}$ units) of representative group A (114378-10-29-KY-F) and group B (7968-11-OK) PnVs, we observed no evidence for positive selection ($dN > dS$) among the PnV/PnV, PVM/PnV or PVM/PVM G/glycoprotein or F/fusion protein sequence pairs.

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Introduction

A novel pneumovirus (PnV), originally called canine pneumovirus, was isolated by Renshaw et al. (2010, 2011) as a part of a larger effort to identify respiratory pathogens from dogs. The original isolate (Ane4) elicited a unique cytopathic effect in A72 canine fibroblasts, showed reactivity with pooled monoclonal antibodies against human respiratory syncytial virus (hRSV), and was found to be highly homologous (> 90% overall nucleotide sequence homology) to the rodent pneumovirus pathogen, pneumonia virus of mice (PVM). While relatively little is known about rodent pneumovirus infection in the wild (Kaplan et al., 1980; Greenwood and Sanchez, 2002; Smith et al., 1993; Becker et al., 2007; Descoteaux and Mihok, 1986; Drexler et al. 2012), PVM has been reported with significant frequency in research and commercial rodent colonies in both mouse and rat sentinels (Liang et al., 2009; Zenner and Regnault 2000; Miyata et al., 1995). PVM strain J3666 has been developed as a model for the study of acute pneumovirus infection in vivo (Rosenberg and Domachowske, 2008; Bem et al., 2011), as it is highly virulent for almost all inbred mouse strains (Anh et al., 2006) and elicits clinical symptoms and pathophysiology similar to severe hRSV disease in humans (Rosenberg and Domachowske, 2012). In contrast, the two PVM strain 15 s (str15/warwick and str 15/atcc) differ somewhat in terms of responses elicited in vivo (reviewed in (Dyer et al., 2012)). PVM Y is a fully independent isolate, originally derived from a spontaneous infection in athymic mice (Weir et al., 1988; Roths, et al., 1993).

The contributions of PnVs to respiratory tract disease in canine species have not yet been fully explored. However, PnV–Ane4 fulfills Koch's postulates for a microbial pathogen in rodent species, specifically: (1) isolated PnV–Ane4 can be introduced into healthy BALB/c mice where it elicits disease, (2) PnV–Ane4 can be isolated in abundance from the lungs of diseased BALB/c mice and identified *ex vivo*, in tissue culture, and (3) isolated PnV–Ane4 grown from tissue culture can be re-introduced into new, healthy BALB/c mice, where they again elicit disease (Percopo et al., 2011; data not shown).

In this manuscript, we examine 10 new PnVs isolated from the respiratory tracts of both canine and feline species from various locations in the continental United States. An evaluation of PnV G/glycoprotein-encoded amino acid sequence led us to define PnV groups A and B, which are phylogenetically distinct from the currently characterized PVMs. Given this finding, we have examined virus replication and cellular and biochemical inflammation elicited by representatives from each of the two PnV groups in order to ascertain whether there are specific responses to potentially distinct viral determinants.

Results and discussion

Sequence comparisons between PVMs and PnVs

This analysis includes the encoded (full-length) amino acid sequences of the G/glycoproteins of the 11 independent PnVs,

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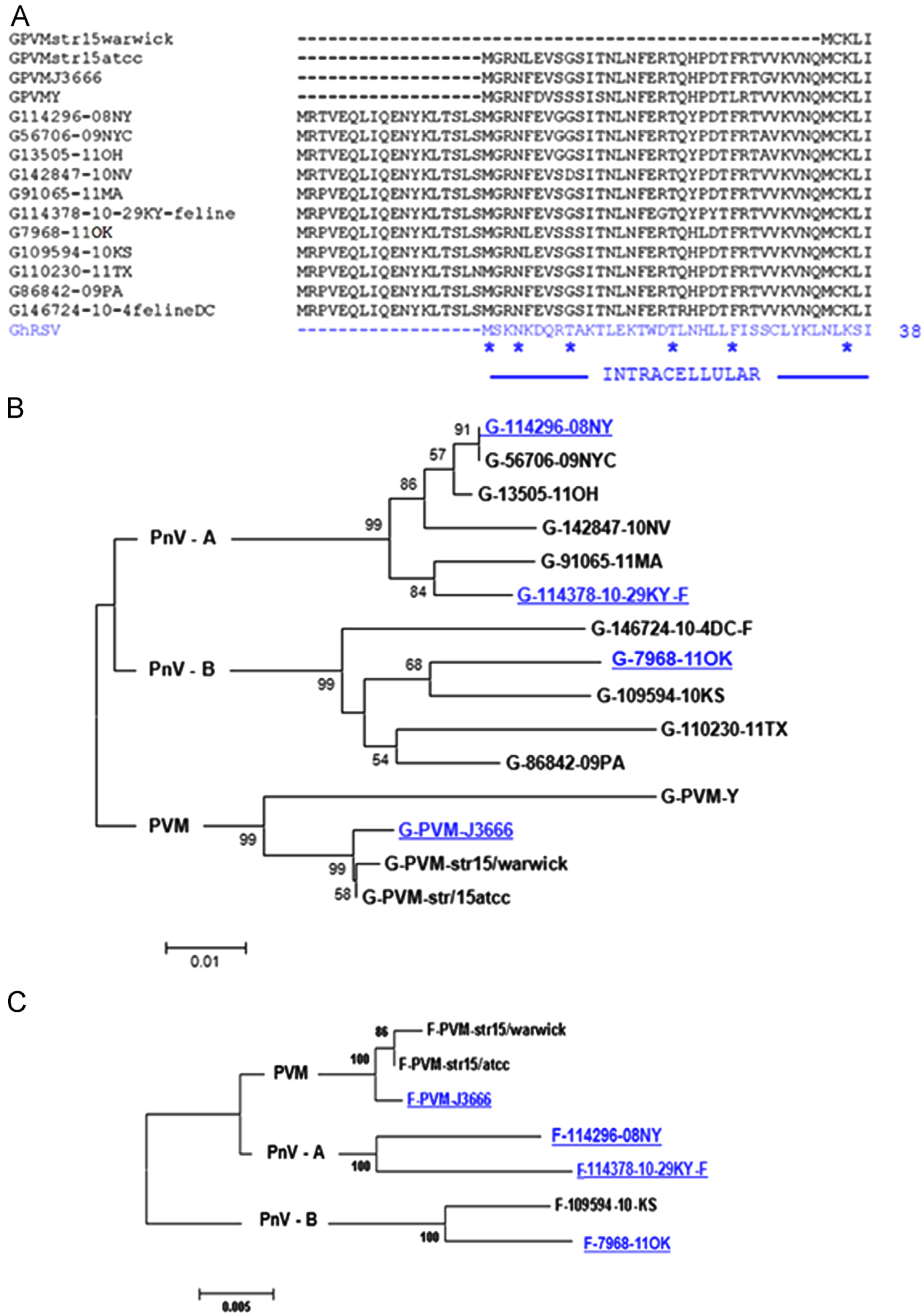

Fig. 1. Novel PnV sequences. (A) Amino termini of G/glycoprotein sequences of pneumonia virus of mice (PVM; strain 15/warwick, strain 15/atcc, J3666, Y, Genbank nos. AY743910, AY729016, NC_00657, and JQ899033, respectively) and pneumoviruses (PnV, 11 independent isolates, Genbank acc. no. KC495958-KC495967; see also Supplemental Table 1). In blue, the intracellular domain of the G/glycoprotein sequence of human respiratory syncytial virus (hRSV strain 19, Genbank FJ614813). Asterisks indicate points of full sequence identity between all viruses shown. **(B)** Unrooted Neighbor-joining tree (Nei-Gojobori, 2000 bootstrap replicates) with full-length G/glycoprotein amino acid sequences. Three independent clusters, PnV—group A, PnV—group B and PVM are defined as shown. **(C)** Parallel analysis of F/fusion protein sequences (Genbank acc. nos. KC603765–KC603767) supports the cluster divisions generated in (B). PVM and PnVs shown in blue and underlined are featured in this manuscript or in Percopo et al. (2011).

including the original isolate, PnV–Ane4 (in this manuscript, denoted by its original code, 114296-08-NY) and the 4 characterized G/glycoprotein sequences from the known PVMs. Of note, G/glycoprotein sequence divergence of the human pneumovirus pathogen, hRSV, defines the epidemiologic groups A and B and provides the basis for most molecular studies (reviewed in (Johnson et al., 1987; Garcia et al., 1994; Venter et al., 2001)). Likewise, the G/glycoproteins include most of the sequence

divergence between PVM J3666 and the two PVM strain 15 s (Randhawa et al., 1995; Thorpe and Easton, 2005; Krempl et al., 2005).

Overall, the homology between the G/glycoproteins of the PVMs and the PnVs is high. Comparison of amino acid sequences of 44 independent full-length pairs (4 from the PVMs vs. 11 from the PnVs) yields a value of 90.3 $\pm$ 1.7% sequence identity.

Renshaw and colleagues (2011) reported that the G/glycoprotein encoded by the initial isolate, 114296-08-NY, included an extended amino terminus when compared to PVM J3666 and PVM str15/atcc. We show here that these 18 additional N-terminal amino acids are found in all 11 PnV variants (Fig. 1A). By analogy with the characterized structure of the hRSV G/glycoprotein, these amino termini extend the cytoplasmic/intracellular region of this protein. Interestingly, there is clearly some inherent plasticity at this site, as the amino terminus of PVM strain 15/warwick is substantially truncated compared to the other PVMs and PnVs (Krempl and Collins, 2004; Krempl et al., 2007).

Shown in Fig. 1B is an unrooted Neighbor-joining tree displaying the relationships among the PnV and PVM G/glycoproteins. Overall, this analysis provides strong statistical support for division of PnVs into two groups, which we have named groups A and B, each distinct from one another and from a third group that includes all currently characterized PVMs. Group A includes the two isolates from New York together with independent isolates from Ohio, Nevada, Massachusetts and Kentucky. Group B includes new isolates from Pennsylvania, Texas, Oklahoma, Kansas and Washington, D.C. Comparable results were obtained from the analysis of available PnV and PVM F/fusion protein gene sequences (Fig. 1C). The divergence of the PVMs from the PnVs might be anticipated, as all currently characterized PVMs were originally isolated from laboratory mice, 20 and ~70 years ago (Horsfall and Hahn, 1940; Weir et al., 1988). In contrast, there are no obvious reasons that explain why the recently-characterized PnVs segregate into two distinct groups. Group A and group B PnVs were identified at multiple locations in the U.S. and throughout the study period (Supplemental Table 1). The specific sites of amino acid sequence divergence that distinguish the three groups (PVM vs. PnV group A vs. PnV group B) are distributed over the full length of the G/glycoprotein polypeptide, although they are at highest density near the carboxy terminus (Supplemental Fig. 1).

Responses of BALB/c mice to inoculation with CnPnVs 114378-10-29-KY-F and 7698-11-OK

Given these observations, our intent was to compare the responses of BALB/c mice to parallel challenge with a representative group A (114378-10-29-KY-F) and group B (7968-11-OK) PnV. Similar to our initial findings with PnV–Ane4 (114296-08-NY from group A; Percopo et al., 2011), all of the mice challenged with 50 $TCID_{50}$ units or fewer of either PnV survive (Fig. 2) and seroconvert (data not shown). PnV 7968-11-OK is somewhat more virulent than PnV 114378-10-29-KY-F (LD_{90}s calculated at 180 and 800 $TCID_{50}$ units, respectively). However, both PnVs are dramatically less virulent than PVM J3666, in which inocula of 0.5 and 0.17 $TCID_{50}$ units (100- to 300-fold fewer than those used here) promote a uniformly fatal infection in BALB/c mice (Percopo et al., 2011).

Virus replication and production of proinflammatory mediators in lung tissue were assessed in response to sublethal inocula (50 $TCID_{50}$ units/50 μL) of each PnV. Virus replication was detected as early as day 3 after initial intranasal challenge, with peak levels observed at day 6 post-inoculation, determined using a dual standard curve quantitative PCR method that provides absolute calibration of both the virus SH gene and the cellular GAPDH (Gabryszewski et al., 2011; Fig. 3). No virus was recovered from

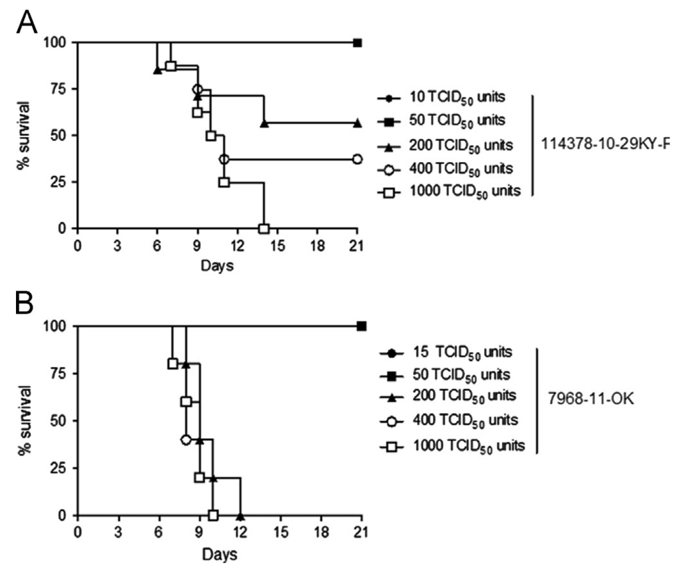

Fig. 2. Survival analysis. (A) Survival of mice inoculated with PnV group A 114378-10-29-KY on day 0, n=7–8 mice per group. **(B)** Survival of mice inoculated with PnV group B 7968-11-OK on day 0, n=5 mice per group. All inoculations were in a 50 μL volume; $TCID_{50}$ assay as described in Materials and methods.

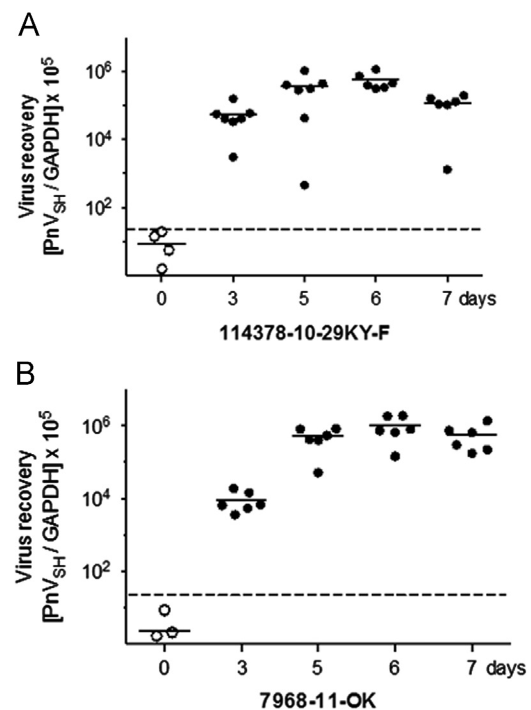

Fig. 3. Virus recovery. Virus recovery from mice inoculated with 50 $TCID_{50}$ units (A) PnV 114378-10-29-KY or (B) PnV 7698-11-OK on day 0. Recovery on days 3, 5, 6 and 7 after inoculation determined by dual standard curve qRT-PCR (Gabryszewski et al., 2011); dashed horizontal line is limit of detection of the assay, n=6–7 mice per time point.

lung tissue of mice challenged with an equivalent inoculum of heat-inactivated virions (data not shown).

Virus replication was associated with local production of proinflammatory cytokines, including CCL3 (MIP-1α), CXCL1 (KC), CXCL-10 (IP-10), CCL2 (MCP-1), and IFNγ (Fig. 4). Proinflammatory cytokines were detected over background levels at day 5 to 6, and remained elevated through day 7 after inoculation. Interestingly, we found that cytokine responses to PnV-7968-11-OK were significantly diminished (pg/mg lung protein) overall compared to

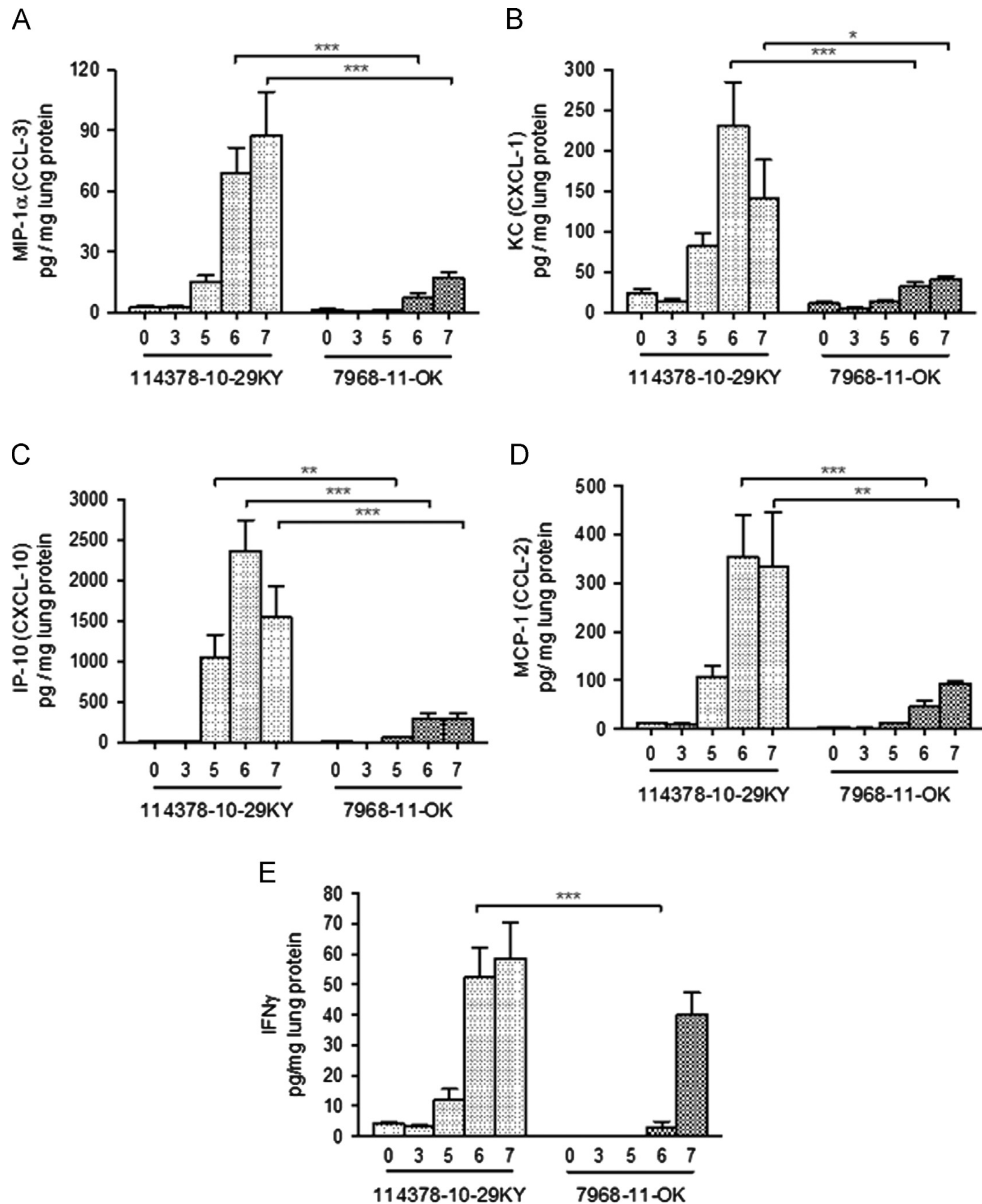

Fig. 4. Detection of proinflammatory cytokines. Lung homogenates were prepared from mice inoculated with 50 TCID$_{50}$ units PnV 114378-10-29-KY or PnV 7698-11-OK and subjected to ELISA, with values corrected for total protein to detect **(A)** MIP-1α/CCL3 **(B)** KC/CXCL1 **(C)** IP-10/CCL10 **(D)** MCP-1/CCL2 and **(E)** IFNγ; $n = 6–7$ mice per time point. Shown are the means +/− SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two way ANOVA with Bonferroni's multiple comparisons test.

those detected in response to PnV-114378-10-29-KY at this virus inoculum. No significant cytokine production was detected in lung tissue from mice challenged with an equivalent inoculum of heat-inactivated virions (data not shown). As shown in Fig. 5, transcription of IFN-β was induced by both PnVs, although no immunoreactive IFN-β protein was detected at any time point. Expression of IFNβ transcript in the absence of detectable protein was observed in mice infected with PVM J3666, while immunoreactive interferon-alpha remained prominent (Garvey et al., 2005). Pneumovirus nonstructural proteins NS-1 and NS-2 have been explored as virus-mediated interferon-antagonists (Schlender et al., 2000; Spann et al., 2004; Heinze et al., 2011), although the precise mechanism remains uncertain.

PnV infection was associated with mild multifocal pneumonia (Fig. 6A and B), with peribronchiolar and perivascular cellular infiltrates, composed mainly of neutrophils (Fig. 6C and D). No hemorrhage or edema was observed. Neutrophils were detected in bronchoalveolar (BAL) fluid, a greater percentage detected in response to PnV 114378-10-29-KY vs. PnV 7968-11-OK (Fig. 6E) correlating with the differential cytokine responses.

Thus, both representative group A and group B PnVs selected for study replicate in mouse lung tissue and elicit cytokine production and neutrophil recruitment to the airways. We observed some differential responses between representative group A and group B PnVs that merit further consideration, although the general patterns observed were consistent with those reported previously in

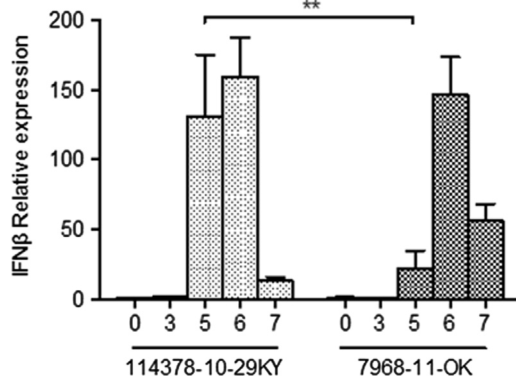

Fig. 5. Expression of transcripts encoding IFNβ. RNA from lungs of mice inoculated with 50 $TCID_{50}$ units PnV 114378-10-29-KY or PnV 7698-11-OK were evaluated by qRT-PCR for IFNβ; $n=6$–7 mice per time point, $^{**}p<0.01$, two-way ANOVA with Bonferroni's multiple comparisons test.

response to inoculation with the original isolate, PnV–Ane4/114296-08-NY (Percopo et al., 2011). Overall, PnVs as a group elicit a relatively mild inflammatory response with substantially less mortality per $TCID_{50}$ unit compared to PVM J3666.

Evolutionary constraints promoting diversity among PnVs and PVMs

As a means of assessing the evolutionary constraints promoting divergence of the PnVs from one another and the PnVs from the PVMs, the rates of nonsynonymous ($dN=n/N$) and synonymous substitution ($dS=s/S$) were evaluated between all nucleotide sequence pairs encoding the G/glycoproteins (Fig. 7A). Save for the few pairs with minimal inter-sequence divergence (i.e., both d*N* and $dS<0.01$), dN/dS was below unity (<1.0) throughout. As such, the analysis provides no evidence for positive selection, i.e., there are no apparent constraints serving to promote the rate of non-synonymous nucleotide substitution over that of synonymous (silent) substitution between any of these sequence pairs. The same result ($dN/dS<1.0$) was found in a smaller comparison of pairs of nucleotide sequences encoding the F/fusion protein (Fig. 7B).

Conclusions

Our work characterizes novel pneumovirus variants (PnVs) that are phylogenetically distinct from the four characterized variants of pneumonia virus of mice (PVM). These PnVs were originally isolated from the respiratory tracts of dogs and cats, although the nature of the relationship between these hosts and the PnV pathogen remains under investigation. Interestingly, all PnVs evaluated thus far can elicit disease in BALB/c mice, although they are substantially less virulent per tissue-culture infectious dose ($TCID_{50}$) unit when compared to inflammatory pathology and mortality elicited by PVM J3666.

Materials and methods

Isolation and sequencing of the G/glycoprotein genes of PnV variants

Virus culture and RNA extraction were as described (Renshaw et al. (2011)). Reverse transcription PCR (RT-PCR) was performed in a one-step reaction (Qiagen #210212) according to the manufacturer's instructions in a 25 μL volume with 1 μL of total RNA that had been diluted 1:5–1:10 and 10 pmol of each primer (Supplemental Table 2). Reaction conditions were reverse transcription at 50 °C for 30 min, inactivation/denaturation at 95 °C for 15 min, and 40 cycles of 30 s at 95 °C, 30 s at 52 °C, and 2 min at 72 °C. Products were examined by agarose gel electrophoresis and sequenced using an Applied Biosystems 3730 DNA analyzer. G/glycoprotein sequences were submitted to Genbank under accession numbers KC495958-KC495967.

Isolation and sequencing of the F/fusion genes of PnV variants

RNA was prepared from infected lung mouse tissue as previously described (Gabryszewski et al., 2011). Reverse transcription was performed using a first-strand cDNA synthesis kit (Invitrogen, kit #11146-016) with random primers. Nested PCR amplification reactions were done according to the manufacturer's instructions in a 50 μL volume (Invitrogen, kit #11708-13). Primers sets used for amplification include forward and reverse primers 5′-TGT GCA GAC TCA GCT GGC CTA G-3′and 5′-AGC AAC GCA GAG GAC GTC AAT T-3′ and forward and reverse nested primers 5′-ACC CCC GCA CCT AGG AGG TC-3′ and 5′-TCA AGG GTT TTG TTC CAG AGG GAG G-3′; all were designed based on PVM J3666 F gene sequence (GenBank NC006579). Cycling parameters included a hold at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 50 °C for 30 s, and 68 °C for 2.5 min and 2 min for the first and second reactions, respectively, followed by a 6-min hold at 68 °C. PCR products were sequenced using an Applied Biosystems 3130 DNA analyzer. Primers used to determine the complete sequence were based on PnV-Ane4/114296-08-NY sequence (GenBank HQ734815). Overlapping PCR products were generated to cover gaps and binding regions. All regions were sequenced in both directions. F/fusion sequences were submitted to Genbank under accession numbers KC603765–KC603767.

Mice

Eight to ten week old BALB/c mice (Division of Cancer Therapeutics, National Cancer Institute, MD) were used in all experiments. Mice received intranasal inocula (50 μL) at various titers (determined by $TCID_{50}$ assay (Percopo et al., 2011)) while under isoflurane anesthesia and were likewise anesthetized prior to sacrifice via cervical dislocation. All protocols were evaluated and approved as per the National Institutes of Allergy and Infectious Diseases Animal Study Protocol LAD 8E and carried out in accordance with the Institute's Animal Care and Use Committee Guidelines.

Viruses

PVM strain J3666 (10^5 $TCID_{50}$ units/mL; Supp. Fig. 2) was maintained by mouse passage. PnV–Ane4/114296-08-NY (Renshaw et al., 2010, 2011) and new PnVs were isolated from respiratory secretions of mixed-breed dogs or cats with signs and symptoms of respiratory disease (Supplemental Table 1). Mouse-passaged stocks of 114378-10-29 KY-F, and 7968-11-OK were prepared and were evaluated by $TCID_{50}$ assay as previously described (Percopo et al., 2011), with titers determined to be 2×10^5 and 5×10^4 $TCID_{50}$ units/mL, respectively. Virus heat-inactivation was achieved by three serial freeze (dry-ice) and heat (95 °C) cycles, a process previously shown to inactivate PVM (Gabryszewski et al., 2011).

Quantitative RT-PCR

RNA was prepared from lung tissue from infected mice as previously described (Gabryszewski et al., 2011). Virus copy number per copy GAPDH was determined by a dual standard curve quantitative PCR method (Renshaw et al., 2011; Gabryszewski et al., 2011) modified so that primers and probe would detect all PnV isolates in use in our experiments: pnv-F-primer 5′-GTT ATC AAC ACA GTG TGT GCG C-3′, pnv-RV-primer 5′-CTG ATG TAG CAA

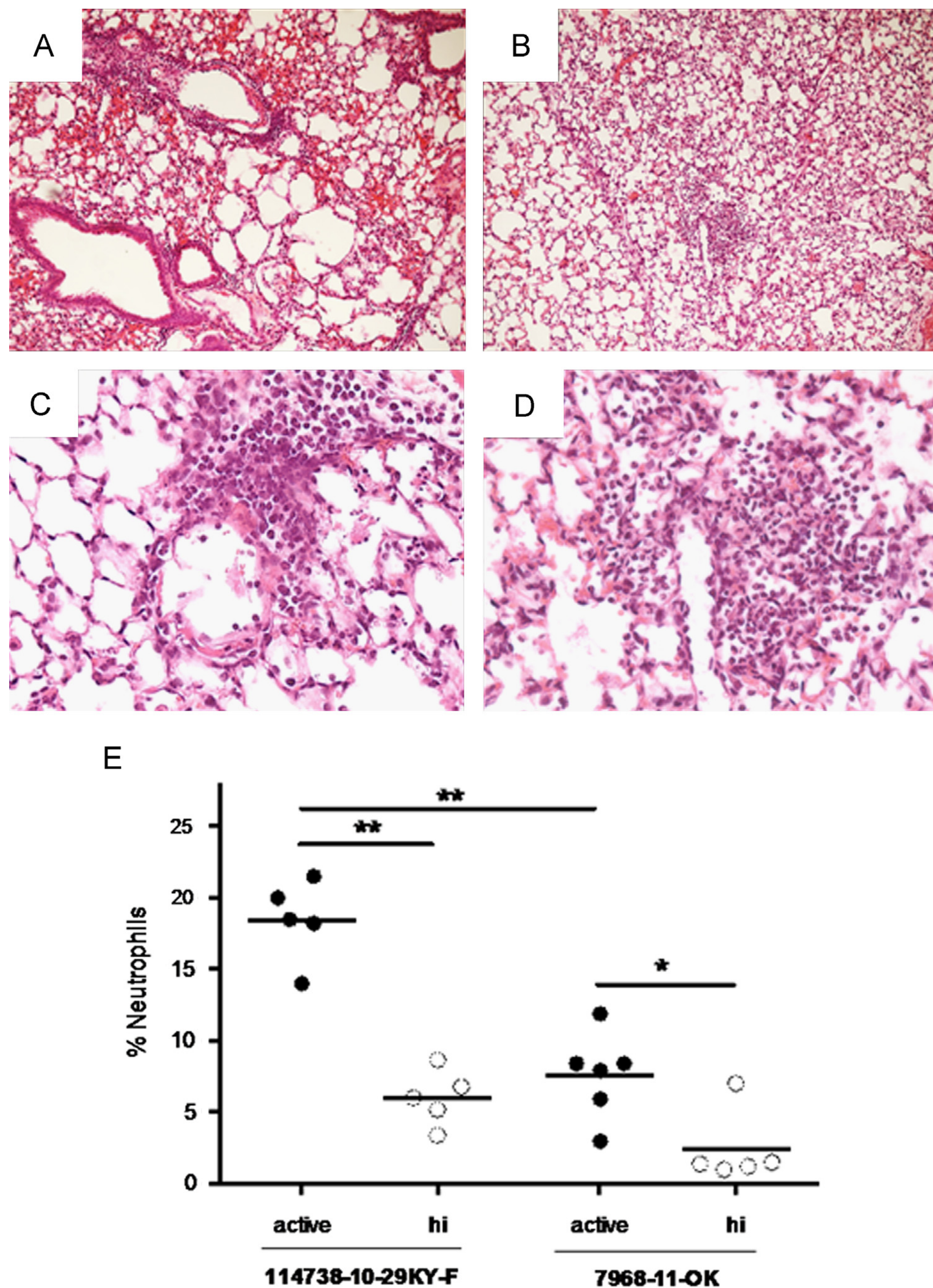

Fig. 6. Histopathology and neutrophil recruitment. ((A)–(D)) Hematoxylin and eosin (H&E) stained fixed lung tissue from mice at day 7 after inoculation with 50 $TCID_{50}$ units PnV 114378-10-29KY-F ((A), (C)) or 90 $TCID_{50}$ units PnV 7968-11-OK ((B), (D)) at original magnifications 10 × ((A), (B)) or 40 × ((C), (D)). (E) Percent (%) neutrophils in bronchoalveolar lavage (BAL) fluid from mice inoculated with 50 $TCID_{50}$ units active or heat-inactivated PnV 114378-10-29KY-F or PnV 7968-11-OK at day 7 after inoculation, $n = 5$–6 mice per group, $^{*}p < 0.05$, $^{**}p < 0.01$ Mann–Whitney *U*-test.

TGC TCC TA-3′ and probe 6FAM-CGC TGA TAA TGG CCT GCA GCA-TAMRA. We have documented clear correlation between virus detection by this method and by $TCID_{50}$ (Supplemental Fig. 2). Detection of IFN-β was carried out using the 20X concentrated primer-probe set from ABI Assay by Design (Mm00439546_s1) used as per manufacturer's instructions (ABI, Columbia, MD). IFN-β expression was normalized to GAPDH as previously described (Gabryszewski et al., 2011).

Proinflammatory cytokine detection

At selected time points, lungs from mice challenged either with heat-inactivated or actively-replicating PnV variants were collected and blade-homogenized into 1 mL of PBS plus 0.1% BSA. Homogenates were clarified by centrifugation and supernatants were evaluated by ELISA (R&D Systems, Minneapolis, MN). Values obtained were normalized for total protein (BCA Assay, Pierce, Rockford, IL).

Neutrophil recruitment

On day 7 post-inoculation, neutrophils were evaluated in bronchoalveolar lavage (BAL) fluid from mice challenged either with heat-inactivated (hi) PnV or actively-replicating PnV variants using modified Giemsa staining (Diff-Quik; Fisher Scientific, Pittsburgh, PA). To prepare cells for staining, BAL fluids were subjected to centrifugation and resuspended in 100–200 μL PBS plus 0.1%

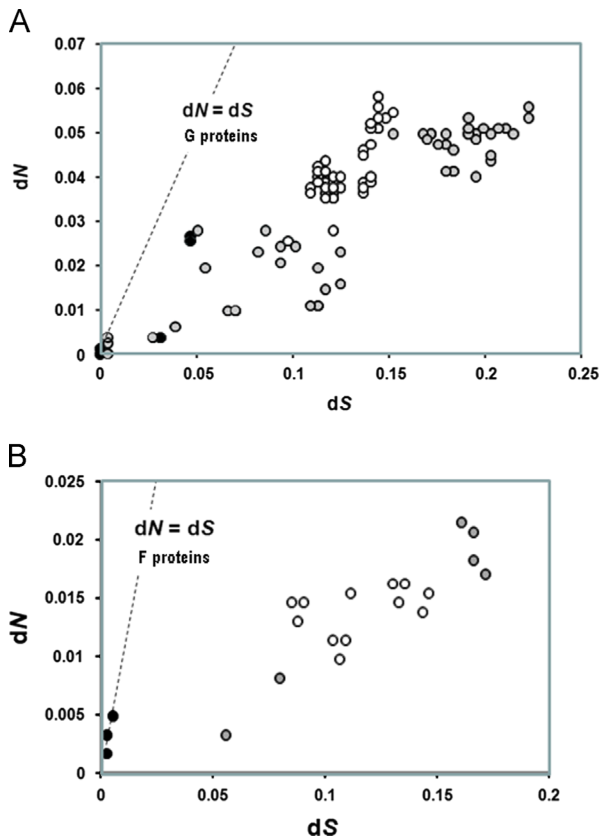

Fig. 7. Evolutionary analysis. Comparison of non-synonymous (d*N*) and synonymous (d*S*) substitution rates for individual PVM/PVM (black symbols), PnV/PnV (grey symbols) and PVM/PnV (white symbols); broken line, $dN/dS = 1.0$ as described in the text. **(A)** G gene and **(B)** F gene sequence pairs; Genbank nos. for all sequences listed in legend to Fig. 1.

BSA. Cells (10^5) were centrifuged onto slides using a Shandon Cytospin apparatus (Thermo-Electron, Pittsburgh, PA). Following staining and mounting of cells, 10 high-power fields were visually inspected by light microscopy.

Lung histopathology

Prior to excision of lungs from the chest cavity, excess blood was removed by perfusion via the right ventricle with PBS, and the lungs from mice challenged with either heat-inactivated PnV or actively-replicating PnV variants were inflated transtracheally using 250 μL 10% phosphate buffered formalin. The heart and the lungs were removed and fixed overnight in 10% phosphate buffered formalin at 4 °C. Samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (Histoserv, Germantown, MD).

Statistical, sequence and evolutionary analysis

Sequences were aligned with ClustalW (http://www.genome.jp/tools/clustalw/). Phylogenetic analysis was conducted using Molecular Evolutionary Genetics Analysis 4.0, which infers the evolutionary tree by the Neighbor-Joining (NJ) algorithm; details in the figure legends (Tamura et al., 2007). Data were evaluated using Mann–Whitney *U* test, Kruskal–Wallis one-way analysis of variance (ANOVA) with Dunn's multiple comparison test or two-way ANOVA with Bonferroni's multiple comparison tests, as appropriate. All statistical tests were included in the GraphPad Prism 5 software package (GraphPad Software, La Jolla, CA). Grubbs' test was performed to detect outliers.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2013.05.011.

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