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Canine pneumovirus replicates in mouse lung tissue and elicits inflammatory pathology

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Abstract

Canine pneumovirus (CnPnV) was recently isolated from the respiratory tracts of shelter dogs and shares sequence similarity with the rodent pathogen, pneumonia virus of mice (PVM). We show here that CnPnV replicates in and can elicit local proinflammatory cytokine production and neutrophil recruitment to lung tissue and the airways. In contrast to PVM J3666 infection, fatal CnPnV infections are observed only in response to high titer intranasal inocula (>67 $TCID_{50}$ units). Sera from mice that recover from CnPnV infection contain antibodies that cross-react with PVM antigens; these mice are protected against lethal PVM infection. Given these findings, it will be intriguing to determine the relative role(s) of CnPnV and PVM in eliciting respiratory symptoms in susceptible canine species.

Keywords

Inflammation; Neutrophil; Cytokine; Respiratory virus

Introduction

Canine pneumovirus (CnPnV) was identified by Renshaw *et al.* (2010) as part of a search for novel respiratory virus pathogens in dogs confined to animal shelters. The novel infectious agent was detected by a unique cytopathic effect induced in A72 fibroblast cultures, and was subsequently identified with pooled monoclonal antibodies against the human pneumovirus pathogen, respiratory syncytial virus (RSV). Sequence fragments of the virus L polymerase and N nucleoprotein genes initially suggested a close relationship between CnPnV and the murine pneumovirus pathogen, pneumonia virus of mice (PVM; Easton *et al.*, 2006; Krempl *et al.*, 2005). Recent publication of virus genome sequence indicates substantial homology with some distinct differences between CnPnV and the characterized PVM J3666 and 15 strains; these include an extended CnPnV G (attachment) protein amino-terminus and sequence divergence among the SH (small hydrophobic) genes (Renshaw *et al.* 2011).

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PVM is a natural rodent pathogen (family *Paramyxoviridae*, genus Pneumovirus) that was first isolated and characterized by Horsfall and Curnen (1946). While PVM is among the viruses recognized as contaminant pathogens of research and commercial rodent colonies (Miyata *et al.*, 1995; Zenner, Regnault, 2000), little is known regarding its prevalence among wild rodents. PVM has been used to model of severe respiratory virus infection in inbred strains of mice (Domachowske *et al.*, 2000; Domachowske *et al.*, 2002; Garvey *et al.*, 2005; Anh *et al.*, 2006; Gabryszewski *et al.* 2011; Rosenberg and Domachowske, 2008). In response to a minimal inoculum, the PVM J3666 strain undergoes robust replication and elicits profound granulocyte recruitment to the airways and lung tissue in response to soluble proinflammatory mediators; this condition can progress to pulmonary edema and acute respiratory failure, similar to the more severe forms of RSV experienced by hospitalized human infants (Tregoning, Schwarze, 2010; Hall *et al.*, 2009). The PVM model of acute respiratory virus infection has been used for the *in vivo* exploration of both therapeutic and preventive antiviral strategies (Bonville *et al.*, 2004; Gabryszewski *et al.*, 2011).

Given its recognized similarity to the PVM pathogen, we explored the BALB/c mouse as a target host species for CnPnV infection *in vivo*.

Methods

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 inoculations (50 μL) while under light anaesthesia (20% halothane in mineral oil) and were likewise anaesthetized prior to sacrifice via cervical dislocation. All protocols were evaluated and approved as per the National Institute 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) was maintained by mouse passage. CnPnV from tissue culture supernatant from infected canine fibroblast A72 cells ((Renshaw *et al.*, 2010; 3×10^6 $TCID_{50}$ units / mL) was used for initial intranasal inoculation of BALB/c mice to prepare mouse-passaged stocks. Virus replication was detected in lung tissue from mice inoculated with unmanipulated tissue culture supernatant; no replication was detected in mice receiving heat-inactivated tissue culture supernatant, i.e., three serial freeze (dry-ice) and heat (95°C) cycles, a process previously shown to inactivate PVM (Gabryszewski *et al.*, 2011). Stocks of mouse-passaged CnPnV were generated by blade homogenization of excised mouse lung tissue in complete tissue culture medium (Iscove's Modified Dulbecco's medium with 10% fetal calf serum) followed by centrifugation and storage of the virion-containing clarified supernatant in liquid nitrogen. The titer of mouse-passaged CnPnV stock, assayed as described below, was 4×10^5 $TCID_{50}$ units / mL.

Virus quantification by $TCID_{50}$ analysis

$TCID_{50}$ analysis was used to evaluate PVM J3666 as well as CnPnV. Briefly, A-72 (canine tumor fibroblast ATCC CRL1542) maintained in standard culture, were plated at 1.6×10^5 cells/mL, 0.2 mL / well in 2% FBS, and incubated overnight at 37°C, 5%CO_2. Lung homogenates from PVM J3666 or CnPnV-infected mice were dialyzed (50K MWCO tubing) against DMEM to remove cell-activating and cytotoxic mediators. Serial 10-fold dilutions were added to cells, which were then incubated at 37°C; cytopathic effect (CPE), which including rounding and piling of cells, was recorded at day 3 after inoculation.

Virus detection by quantitative PCR

We have developed a dual standard curve method for determining virus recovery in absolute copies from infected mouse lung tissue (PVM_{SH}/GAPDH; Percopo *et al.*, 2009; Gabryszewski *et al.*, 2011). This assay has been altered to target the sequence of the CnPnV *SH* gene, including cpv-F-primer 5'-GCT GTT ATC AAC ACA GTG TGT G-3', cpv-R-primer 5'-GCC TGA TGT AGC AAT GCT C-3'and probe: 6FAM-CGC TGA TAA TGG CCT GCA GCA-TAMRA. The GAPDH standard curve includes serial dilutions (10^7, 10^6, 10^5, 10^4, 10^3 molecules / reaction) of mouse GAPDH coding sequence in pCMV Sport 6 (American Type Culture Collection cat no. 10539385). The CnPnV *SH* standard curve includes serial dilutions (10^6, 10^5, 10^4, 10^3, 10^2 molecules per reaction) of the full-length CnPnV *SH* gene cloned into pCR 2.1. Experimental triplicate data points are interpolated to linear standard curves over the concentration ranges indicated.

Preparation of anti-PVM N protein polyclonal antibody and Western blot

Recombinant glutathione-S-transferase-PVM N fusion protein was generated using the vector pGEX-5X-3. GST-PVM-N protein was purified on glutathione agarose and used to inoculate rabbits via a standard protocol with Freund's complete and incomplete adjuvants (Spring Valley Laboratories, Sykesville, MD). The antibody fraction was purified by ammonium sulfate precipitation followed by affinity-isolation on protein A agarose and stored at 1.2 mg/mL in phosphate buffered saline (PBS) with 0.1% BSA. Clarified homogenates from infected mouse lung tissue were diluted 1:1 with reducing sample buffer (Invitrogen), heated to 65°C and run on 14% trisglycine acrylamide gels, blotted to nitrocellulose and blocked with 5% non-fat dry milk in tris-buffered saline with 0.1% Tween (T-TBS). The anti-PVM N primary antibody was used at a concentration of 12 μg / mL (1:100) in T-TBS with 1% gelatin. Secondary antibody was a 1:1000 dilution of alkaline-phosphatase conjugated goat anti-rabbit IgG, followed by NBT/BCIP developing reagents (BioRad, Richmond, CA). Rabbit anti-beta actin was purchased from Cell Signaling (Millipore Corporation) and used at a 1:500 dilution.

Seroconversion

Anti-PVM antibodies in mouse sera were detected by ELISA (Biotech Trading Partners, catalog #SMART-M12, El Cerrito, CA).

Detection of Proinflammatory Cytokines

Lungs from control (uninfected) mice, mice challenged with heat-inactivated CnPnV and mice infected with actively-replicating CnPnV were blade homogenized into 1 mL IMDM with 10% heat-inactivated fetal calf serum and the supernatants clarified by centrifugation. Clarified homogenates were evaluated by ELISA (R&D Systems, Minneapolis, MN) and values obtained were normalized for total protein (BCA assay, Pierce, Rockford, IL). Values were controlled for matrix effects by diluting 5 μL of various concentrations of standards in 45 μL of buffer provided or 45 μL of lung tissue from an uninfected mouse, blade homogenized and clarified as above.

Lung histopathology and immunohistochemical detection of virus antigens

Prior to excision from the chest cavity, excess blood was eliminated by perfusion via the right ventricle, and the lungs were inflated trans-tracheally with 10% phosphate buffered formalin. The heart and lungs were excised, fixed in 10% phosphate buffered formalin, and mounted in butterfly formation. Hematoxylin and eosin stained tissue was prepared by Histoserv, Inc. (Germantown, MD). Tissue sections were also probed with purified anti-PVM N antibody (1:200 dilution) or control rabbit IgG followed by peroxidase-conjugated

anti-Ig and developing reagents for immunohistochemical localization of virus antigen (Histoserv).

Statistical Methods

All experiments were repeated at least twice with number of mice (n) as indicated. Data were evaluated by Mann-Whitney U test (non-parametric, for small samples) and Kruskal–Wallis one-way analysis of variance for multiple samples.

Results and Discussion

Canine pneumovirus (CnPnV) infects BALB/c mice

We demonstrate that CnPnV, a novel pneumovirus with sequence similarity to PVM, replicates in lung tissue and can cause a lethal infection in wild-type BALB/c mice. We evaluated survival in response to intranasal inoculation with CnPnV [Figure 1A]. All mice inoculated with 67 $TCID_{50}$ units / 50 μL survived through day 30, as did 40% of the mice inoculated with 200 $TCID_{50}$ units / 50 μL. All surviving mice seroconverted, documenting recovery from active infection. All mice inoculated with 400 $TCID_{50}$ units / 50 μL died between days 7 and 14 ($t_{1/2}$ = 8.7 days). In contrast, these mice are much more susceptible to the lethal sequelae of PVM J3666 infection [Figure 1B]. There were no survivors in response to intranasal inocula as low as 1.7, 0.5 or 0.17 $TCID_{50}$ units / 50 μL ($t_{1/2}$ = 5.9, 8.0, and 9.9 days, respectively). If one interpolates from the inverse correlation between $TCID_{50}$ units administered vs. $t_{1/2}$ for PVM J3666 infection, the survival ($t_{1/2}$) of 8.7 days in response to 400 $TCID_{50}$ units CnPnV is similar to the response one would anticipate from 0.4 $TCID_{50}$ units of PVM J3666, or a difference of approximately 1000-fold.

Virus replication in lung tissue

Virus replication was assessed in response to a non-lethal inoculum (35 $TCID_{50}$ units / 50 μL; [Figure 2]). CnPnV genome copies, measured on a log scale via a dual standard curve qRT-PCR assay targeting the virus *SH* gene ($CnPnV_{SH}$/GAPDH), was detected in lung tissue as early as day 3 after initial challenge, with increases in virus recovery measured at all time points thereafter. No CnPnV was detected in uninfected mice, nor was CnPnV detected in lung tissue in mice challenged with an equivalent inoculum of heat-inactivated virions (data not shown).

CnPnV infection can elicit production of proinflammatory cytokines but minimal histopathology

At a survival inoculum (35 $TCID_{50}$ units / 50 μL), CnPnV infection results in local production of proinflammatory mediators [Figure 3; Supplemental Figure 2], including CCL3 (MIP-1α), CCL2 (MCP-1), CXCL10 (IP10), CXCL1 (KC), and IFNγ, all cytokines associated with disease pathology in the PVM infection model (Gabryszewski *et al.*, 2011; Bonville *et al.*, 2006). Interestingly, despite the presence of these proinflammatory mediators, we observe only minimal histopathology in response to CnPnV at this inoculum [Figures 4A and 4B]. The neutrophils that are recruited are found primarily in focal perivascular infiltrates [Figure 3C and 3D] rather than dispersed throughout the parenchyma, as is observed in severe pneumovirus infection (Rosenberg, Domachowske, 2008); neutrophils can also be detected in bronchoalveolar lavage (BAL) fluid [Figure 4E] but not as prominently or in as substantial numbers as in the acute, fatal PVM J3666 infection (Bonville *et al.*, 2006). It is intriguing to find limited neutrophil recruitment and dispersion in the presence of prominent proinflammatory cytokine expression. Interestingly, we have observed similar limited neutrophil recruitment despite relatively normal levels of proinflammatory cytokine expression in PVM-infected interferon alpha-beta receptor

(IFNαβR$^{-/-}$) mice (Garvey *et al.* 2005) and likewise in the absence of interferon-gamma-mediated signaling (Bonville *et al.* 2009). It will be intriguing to determine if there are any connections between these findings and what we have observed here.

CnPnV is cross-reactive and cross-protective with pneumonia virus of mice (PVM)

Renshaw *et al.* (2010, 2011) reported high sequence similarity between CnPnV and PVM strains 15 and J3666. In Figure 5A, a Western blot with clarified lung-tissue homogenates from CnPnV-infected and naïve mice were probed with polyclonal anti-PVM N (nucleoprotein) antibody. A 43 kDa PVM N protein was detected clarified homogenates from CnPnV-infected mouse lungs; a protein of indistinguishable electrophoretic mobility was detected in the homogenate from PVM-infected mouse lungs [Supplemental Figure 2]. Virus was detected in epithelial cells lining the bronchioles in lungs of CnPnV infected mice [Figure 5B], similar to what was observed previously for mice infected with PVM strain J3666 (Bonville *et al.*, Virology 2006). Similarly, we found that sera from CnPnV-inoculated mice that had recovered from infection (day 21) contain antibodies that cross-react with PVM antigens by ELISA [Figure 5C]; no cross-reacting antibodies were detected in control mice, nor were any cross-reacting antibodies detected in sera from mice that were inoculated with heat-inactivated CnPnV. Furthermore, mice that recovered from CnPnV infection were protected against subsequent lethal PVM J3666 infection; no protection is observed in mice previously inoculated with heat-inactivated, non-replicating CnPnV [Figure 5D]. Claassen *et al.* (2007) have explored the role of CD4^{+} and CD8^{+}T cell PVM G protein protective epitopes in promoting immunity against PVM infection *in vivo*; Frey *et al.* (2008) likewise reported noted the contributions of both T cell subsets in promoting virus clearance. This is an intriguing and important field for continued study, as human pneumovirus infection (respiratory syncytial virus) is notorious for its inability to elicit long-term immunity *in vivo*. Models such as these will assist with the examination of long-lasting immunity to pneumovirus infection. The full nature of potential protective responses (or lack thereof) generated in response to acute infection can be determined via experiments such as those in Figure 5D, carried out in both in B, T cell and subset deficient, as well as various appropriate gene-deleted mouse models, with the second pneumovirus challenge delayed for weeks to months.

Of note, while heat-inactivated CnPnV is a foreign antigen that will likely irritate lung tissue and elicit a cytokine response if inoculated repeatedly at high concentration, it is not an effective immunogen when administered at this dosage via the intranasal route when compared to the active virus. We observed similar results when C57BL/6 mice that recovered from infection with attenuated PVM strain 15 were then challenged with PVM J3666 (Ellis *et al.*, 2007)

Relationships among mammalian pneumoviruses

Shown in Figure 5 is a phylogenetic tree featuring the *SH* sequences of human RSV, bovine RSV, two isolates of PVM (strain J3666 and strain 15) and CnPnV (Renshaw *et al.* 2011). CnPnV shares significant sequence homology with both strains of PVM, including 97% amino acid sequence homology shared with both F (fusion) proteins. In contrast, the SH (small hydrophobic) protein is the most divergent among the three viruses. SH is a pneumovirus structural protein found on the virus surface; recently Fuentes *et al.* (2007) found that the SH protein from RSV had a protective, immunomodulatory function, and could inhibit proinflammatory signaling by tumor necrosis factor (TNF). The CnPnV SH gene encodes a protein with 90 and 96% amino acid sequence identity to its counterparts in PVM strain J3666 (highly virulent, mouse passaged) and PVM strain 15 (less virulent, tissue culture maintained), although the highly virulent J3666 SH sequence is somewhat longer than that of either strain 15 or CnPnV (114 vs. 92 amino acids). Another point of significant

divergence is the G protein; by inference to other pneumoviruses, this protein is essential for virus attachment (reviewed in Easton *et al.*, 2006) although this has not been formally evaluated for PVM. CnPnV G protein is 92% identical to the G proteins from both PVM J3666 and PVM strain 15, but includes an amino terminal cytoplasmic extension that may have an impact on function and virulence.

Although pneumoviruses are species-limited pathogens, bovine and human RSV are similar to one another and maintain numerous cross-reactive epitopes (Pastey, Samal, 1997); likewise, antibodies cross-reactive to bovine RSV have been identified in numerous non-bovine species, including cats and dogs (van der Poel, *et al.*, 1995). As such, given the findings presented here, it will ultimately be important to determine the extent to which this emerging mammalian pneumovirus pathogen can replicate and elicit respiratory symptoms in the canine population. Likewise, it will be intriguing to determine whether or not the previously-identified PVM pathogens can do likewise.

In conclusion, we have shown that CnPnV, a novel pneumovirus isolated from domesticated dogs, replicates effectively in mouse lung tissue and can lead severe respiratory sequelae, although mice can survive in response to a 1000-fold increased virus inoculum compared to PVM J3666. Virus replication leads to cytokine production but only minimal recruitment of neutrophils; as such, this virus strain may ultimately provide important insights into which proinflammatory mediators are truly crucial, and likewise, which mediators are supportive or redundant, for progression to irreversible inflammatory pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Anh DB, Faisca P, Desmecht DJ. Differential resistance/susceptibility patterns to pneumovirus infection among inbred mouse strains. Am J Physiol Lung Cell Mol Physiol. 2006; 291:L426–L435. [PubMed: 16556725]

Bonville CA, Lao V, DeLeon JM, Gao JL, Easton AJ, Rosenberg HF, Domachowske JB. Functional antagonism of chemokine receptor CCR1 reduces mortality in acute pneumovirus infection *in vivo*. J Virol. 2004; 78:7984–7989. [PubMed: 15254170]

Bonville CA, Bennett NJ, Koehnlein M, Haines DM, Ellis JA, DelVecchio AM, Rosenberg HF, Domachowske JB. Respiratory dysfunction and proinflammatory chemokines in the pneumonia virus of mice (PVM) model of viral bronchiolitis. Virology. 2006; 349:87–95. [PubMed: 16563455]

Bonville CA, Percopo CM, Dyer KD, Gao J, Prussin C, Foster B, Rosenberg HF, Domachowske JB. Interferon-gamma coordinates CCL3-mediated neutrophil recruitment *in vivo*. BMC Immunol. 2009; 10:14. [PubMed: 19298652]

Claassen EA, van Bleek GM, Rychnavska ZS, de Groot RJ, Hensen EJ, Tijhaar EJ, van Eden W, van der Most RG. Identification of a CD4 T cell epitope in the pneumonia virus of mice glycoprotein and characterization of its role in protective immunity. Virology. 2007; 368:17–25. [PubMed: 17632195]

Domachowske JB, Bonville CA, Gao JL, Murphy PM, Easton AJ, Rosenberg HF. The chemokine MIP-1α and its receptor CCR1 control pulmonary inflammation and antiviral host defense in paramyxovirus infection. J. Immunol. 2000; 165:2677–2682. [PubMed: 10946298]

Domachowske JB, Bonville CA, Easton AJ, Rosenberg HF. Differential expression of pro-inflammatory cytokine genes *in vivo* in response to pathogenic and nonpathogenic pneumovirus infection. J Infect Dis. 2002; 186:8–14. [PubMed: 12089656]

Easton, AJ.; Domachowske, JB.; Rosenberg, HF. Pneumonia virus of mice. In: Cane, P., editor. Perspectives in Medical Virology. Vol. vol. 12. 2006. p. 299-319.

Ellis JA, Martin BV, Waldner C, Dyer KD, Domachowske JB, Rosenberg HF. Mucosal inoculation with an attenuated mouse pneumovirus protects against virulent challenge in wild type and interferon gamma receptor-deficient mice. Vaccine. 2007; 25:1085–1095. [PubMed: 17052820]

Frey S, Krempl CD, Schmitt-Gräff A, Ehl S. Role of T cells in virus control and disease after infection with pneumonia virus of mice. J Virol. 2008; 82:11619–11627. [PubMed: 18815308]

Fuentes S, Tran KC, Luthra P, Teng MN, He B. Function of the respiratory syncytial virus small hydrophobic protein. J Virol. 2007; 81:8361–8366. [PubMed: 17494063]

Gabryszewski SJ, Bachar O, Dyer KD, Percopo CM, Killoran KE, Domachowske JB, Rosenberg HF. *Lactobacillus*-mediated priming of the respiratory mucosa protects against lethal pneumovirus infection. J. Immunol. 2011; 186:1151–1161. [PubMed: 21169550]

Garvey TL, Dyer KD, Ellis JA, Bonville CA, Foster B, Prussin C, Easton AJ, Domachowske JB, Rosenberg HF. Inflammatory responses to pneumovirus infection in IFNαβR gene-deleted mice. J Immunol. 2005; 175:4735–4744. [PubMed: 16177121]

Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. N. Engl. J. Med. 2009; 360:588–598. [PubMed: 19196675]

Horsfall FL, Curnen EC. Studies on pneumonia virus of mice (PVM): I. The precision measurements in vivo of the virus and antibodies against it. J. Exp. Med. 1946; 83:25–42.

Krempl CD, Lamirande EW, Collins PL. Complete sequence of the RNA genome of pneumonia virus of mice. Virus Genes. 2005; 30:237–249. [PubMed: 15744580]

Miyata H, Kishikawa M, Kondo H, Kai C, Watanabe Y, Ohsawa K, Sato H. New isolates of pneumonia virus of mice (PVM) from Japanese rat colonies and their characterization. Exp. Anim. 1995; 44:95–104. [PubMed: 7601231]

Pastey MK, Samal SK. Analysis of the bovine respiratory syncytial virus fusion protein (F) using monoclonal antibodies. Vet. Microbiol. 1997; 58:175–185. [PubMed: 9453129]

Percopo CM, Qiu Z, Phipps S, Foster PS, Domachowske JB, Rosenberg HF. Pulmonary eosinophils and their role in immunopathologic responses to formalin-inactivated pneumonia virus of mice. J. Immunol. 2009; 183:604–612. [PubMed: 19542471]

Renshaw RW, Zylich NC, Laverack MA, Glaser AL, Dubovi EJ. Pneumovirus in dogs with acute respiratory disease. Emerg. Infect. Dis. 2010; 16:993–995. [PubMed: 20507755]

Renshaw R, Laverack M, Zylich N, Glaser A, Dubovi E. Genomic analysis of a pneumovirus isolated from dogs with acute respiratory disease. Vet. Microbiol. 2011; 150:88–95. [PubMed: 21324612]

Rosenberg HF, Domachowske JB. Pneumonia virus of mice: severe respiratory virus infection in a natural host. Immunol. Lett. 2008; 118:6–12. [PubMed: 18471897]

Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 2007; 24:1596–1599. [PubMed: 17488738]

Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. Clin. Microbiol. Rev. 2010; 23:74–98. [PubMed: 20065326]

van der Poel WHM, Langedijk JPM, Kramps JA, Middel WGJ, Brand A, van Oirschot JT. Bovine respiratory syncytial virus antibodies in non-bovine species. Arch. Virol. 1995; 140:1549–1555. [PubMed: 7487487]

Zenner L, Regnault JP. Ten-year long monitoring of laboratory mouse and rat colonies in French facilities: a retrospective study. Lab. Anim. 2000; 34:76–83. [PubMed: 10759370]

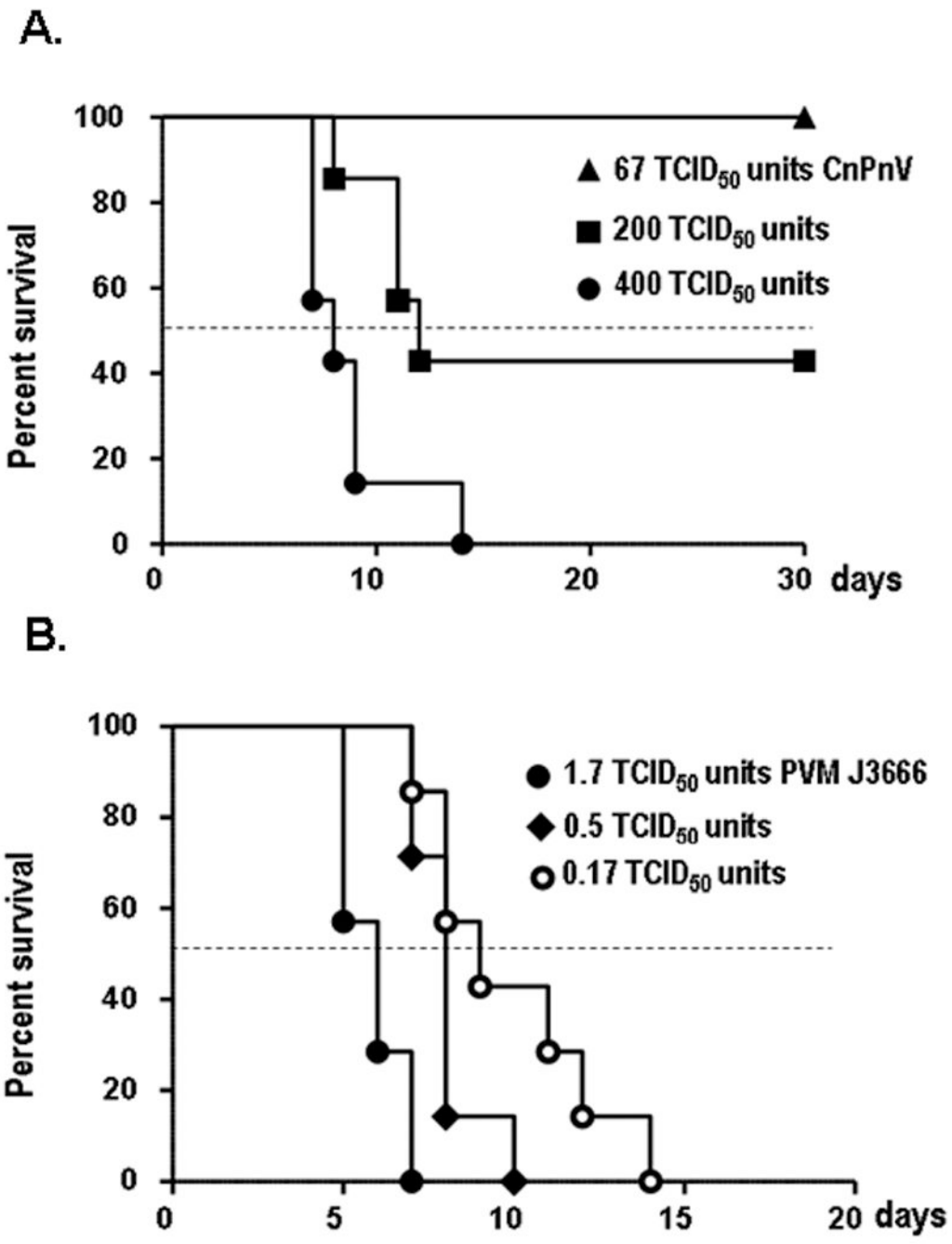

Figure 1. Survival of mice inoculated intranasally with CnPnV or PVM strain J3666

$TCID_{50}$ units of **A.** CnPnV or **B**. PVM as indicated inoculated in a constant 50 μL volume; n = 7 mice per group; dotted line indicates 50% survival (t½).

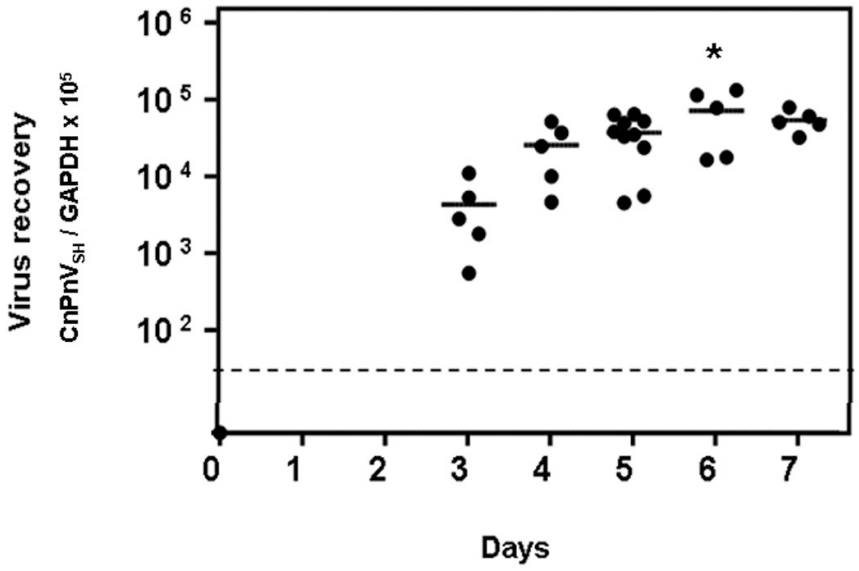

Figure 2. Replication of CnPnV in mouse lung tissue

Mice were challenged at day 0 with a sublethal inoculum (35 $TCID_{50}$ units in 50 μL) and virus recovery was evaluated at time points thereafter (note log scale); n = 5 – 10 mice per time point; *p < 0.05 vs. day 3; dotted line, lower limit of detection.

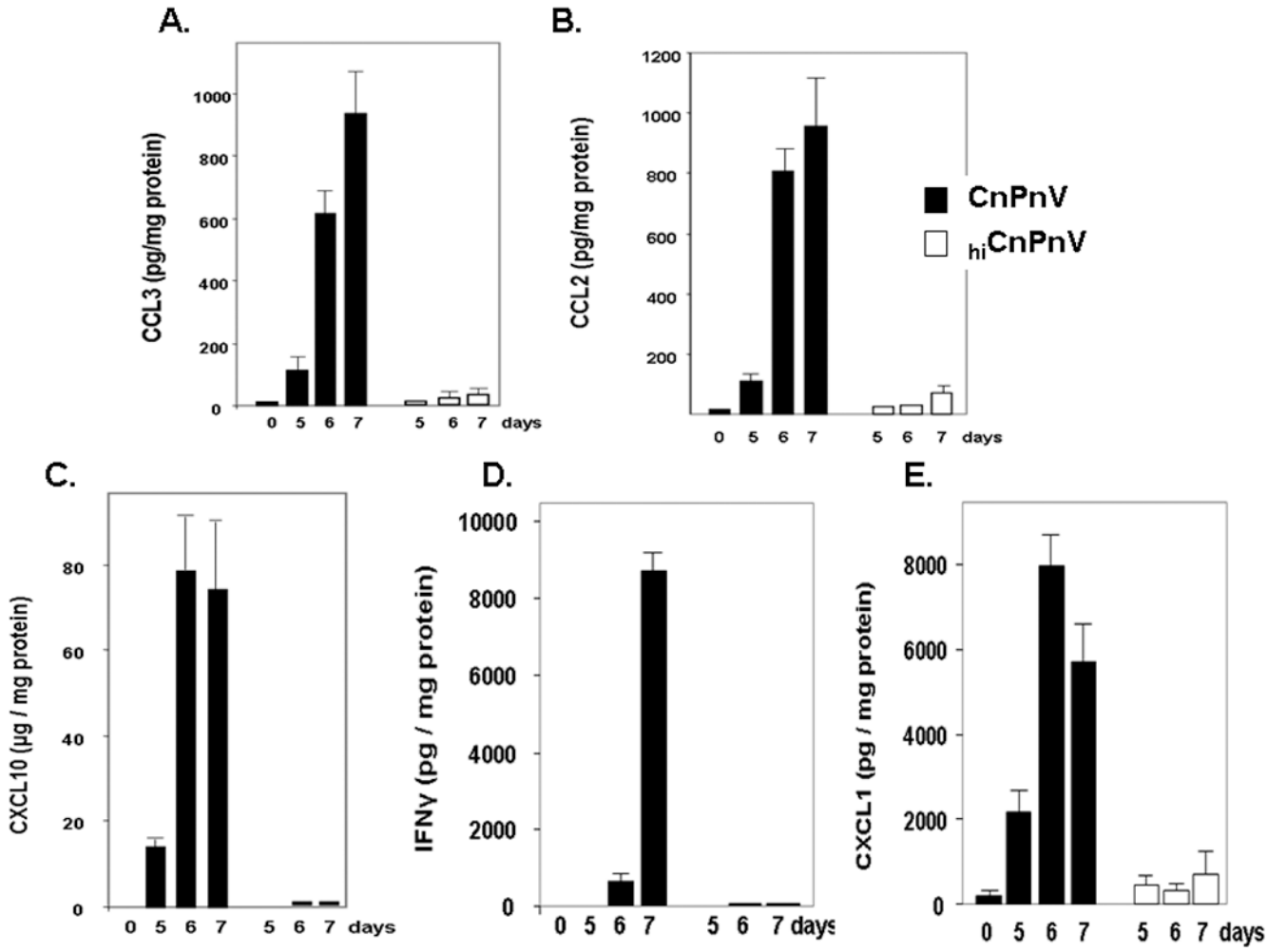

Figure 3. CnPnV infection elicits production of proinflammatory cytokines

Cytokines detected in clarified lung tissue homogenates at day 7 in response to the sublethal inoculum (35 $TCID_{50}$ units in 50 µL) including **A.** CCL3 (MIP-1α) **B.** CCL2 (MCP-1), **C.** CXCL10 (IP-10), **D.** IFNγ, and **E.** CXCL1 (KC); hi, heat-inactivated; n = 4 – 5 mice per time point, see also Supplemental Figure 1.

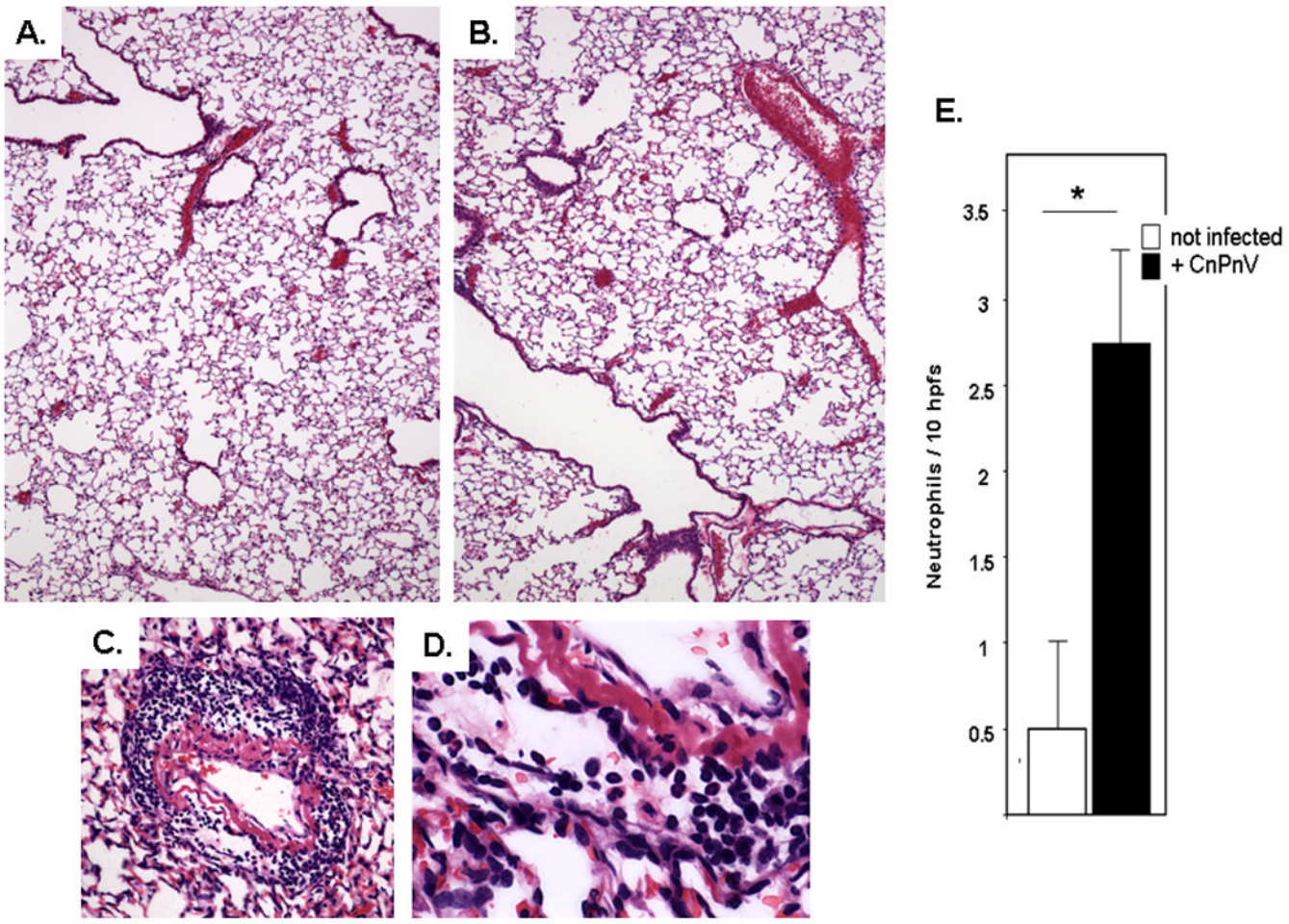

Figure 4. Lung histopathology

A. Normal mouse lung tissue. **B**. Lung tissue (day 7) from mouse inoculated with CnPnV (35 $TCID_{50}$ units). **C.** An example of one of the few focal perivascular inflammatory infiltrates with **D.** neutrophils as the predominant cell type. **E.** Neutrophils, albeit few in number, are also detected in bronchoalveolar lavage (BAL) fluid; hpf, high power (40X) field; n = 5 mice per point.

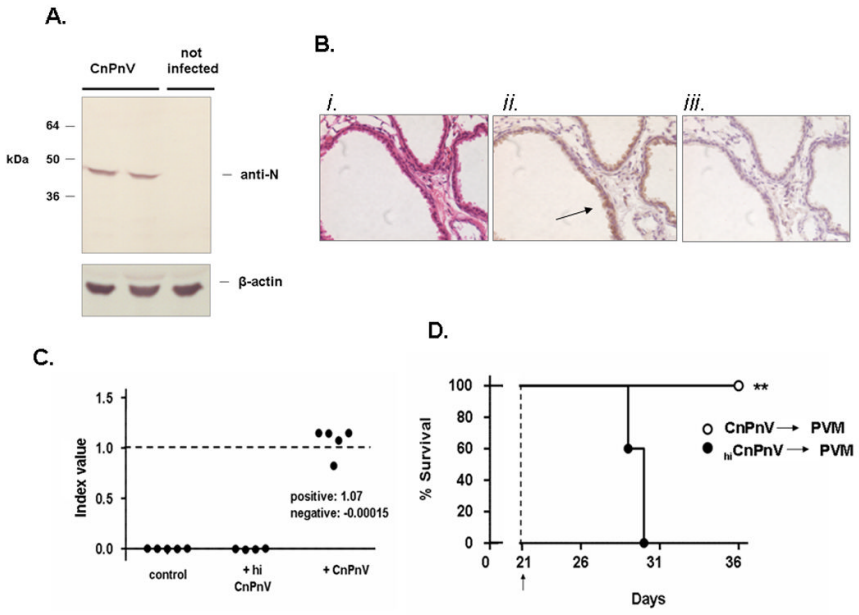

Figure 5. CnPnV is cross-reactive and cross-protective with pneumonia virus of mice (PVM) strain J3666

A. Clarified lung tissue homogenates from virus-infected mice probed with polyclonal anti PVM N protein antibody; see also Supplemental Figure 2. **B.** Immunoreactive CnPnV can be detected in bronchiolar epithelial cells from infected mice; (i) H&E stained lung tissue; (ii) tissue probed with polyclonal anti-PVM N protein; (iii) tissue probed with control rabbit antiserum. **C.** Detection of anti-PVM cross-reactivity in sera from mice recovered from acute CnPnV infection (t = 21 days after inoculation with 35 $TCID_{50}$ units CnPnV), but not control mice or mice inoculated with heat-inactivated (hi) CnPnV. **D.** Survival of mice challenged with a lethal inoculum of PVM strain J3666 (1.7 $TCID_{50}$ units) at t = 21 days after a first inoculum of CnPnV (35 $TCID_{50}$ units active or heat-inactivated virus); n = 5 mice per group, $^{**}p < 0.01$.

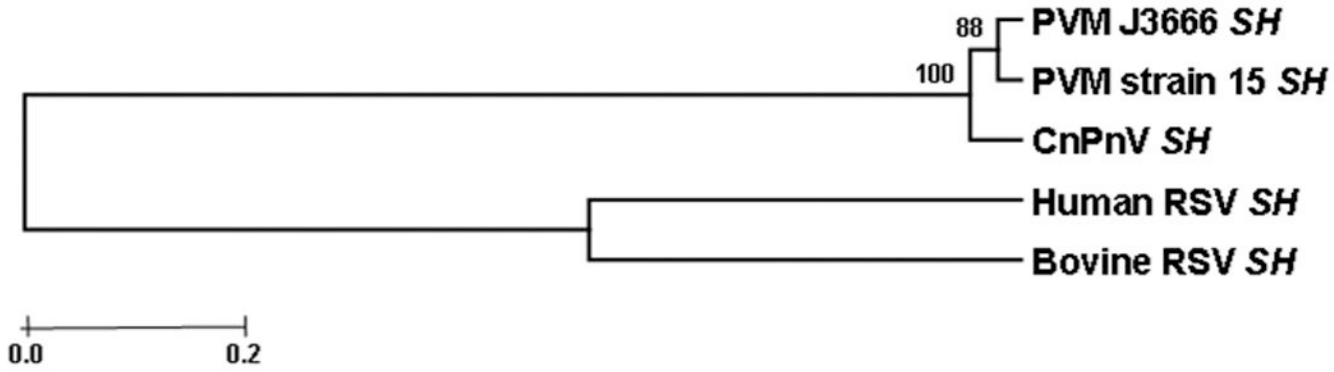

Figure 6. Phylogenetic relationships among pneumovirus pathogens

Tree constructed from nucleotide sequences of the pneumovirus SH genes using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method as per algorithms within MEGA 4.0 (Tamura *et al.*, 2007); 2000 bootstrap replicates, values > 50 as shown. GenBank reference numbers include: PVM J3666, AY573815; PVM strain 15 AY743910.1; bovine RSV (ATCC 51908), AF295543.1; human RSV, NC_001781.