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Genomic analysis of a pneumovirus isolated from dogs with acute respiratory disease

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ABSTRACT

A previously unrecognized virus belonging to the subfamily *Pneumovirinae* and most closely related to murine pneumovirus (MPV) was identified in domestic dogs in 2 related animal shelters. Additional diagnostic testing yielded 3 new viral isolates and identified 6 additional PCR positive dogs from other USA locations indicating that its distribution is not geographically limited. Nucleotide sequences encompassing 9 of the 10 genes were compared to the only 2 available MPV strains, 15 and J3666. Several features distinguished the canine pneumovirus (CnPnV) from the murine strains. Two regions of diversity were identified in the amino-proximal region of P and the overlapping P2 ORF was only 54 amino acids (aa) compared to 137 aa in MPV. The G protein had an amino-terminal cytoplasmic tail 18 aa longer than in the MPV strains. The CnPnV SH protein showed the highest divergence with only 90.2% aa identity when compared to MPV strain 15. Like strain 15, the CnPnV SH ORF coded for a protein of 92 aa while J3666 has a 114 aa variant. Comparison of CnPnV isolates at culture passages 4 and 17 revealed 7 nt differences within the 8598 nt sequenced. Of note was a substitution at nt 364 in G resulting in a termination codon that would produce a truncated G protein of 122 aa. Analysis of early passage and *ex vivo* samples showed the termination codon in G to be predominant after 6 days in culture indicating rapid selection of the mutation in A72 cells.

1. Introduction

A previously unrecognized pneumovirus isolated from dogs was recently reported (Renshaw et al., 2010). It was identified during a diagnostic assessment to define the pathogens in an ongoing occurrence of acute respiratory disease in 2 associated urban animal shelters in the northeastern US. Sequence analysis of three regions of the genome obtained using degenerate primers indicated the virus was closely related to murine pneumovirus (MPV), classically known as pneumonia virus of mice (PVM).

urine pneumovirus is classified in the *Pneumovirinae* subfamily in the family *Paramyxoviridae*. The subfamily contains two genera, *Pneumovirus* and *Metapneumovirus*. Human RSV is the type species in the *Pneumovirus* genus which also includes the closely related ruminant species, bovine RSV (BRSV) and ovine RSV, in addition to MPV. The *Metapneumovirus* genus includes human metapneumovirus (HMPV), and avian metapneumovirus (AMPV) subtypes (A–D) (Fauquet and International Committee on Taxonomy of Viruses, 2005). The gene arrangement of viruses in the *Pneumovirus* genus is NS1-NS2-N-P-M-SH-G-F-M2-L (Fields et al., 2007). The non-structural accessory proteins NS1 and NS2 are unique to the viruses in the *Pneumovirus* genus. In MPV, NS2 was found to be a strong antagonist of the type I interferon response (Buchholz et al., 2009) but in the same study the role of NS1 as a virulence factor was less clear as an NS1deletion mutant reduced virulence in BalbC mice but

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there was no increase in interferon production or signaling. The phosphoprotein (P) of paramyxoviruses associates with the large (L) protein to form the core RNA-dependent RNA polymerase complex which in association with the N protein makes up the complete nucleocapsid. The M or matrix protein is a structural component that promotes association of the nucleocapsid with the membrane through hydrophobic interactions. Embedded in the outer membrane are the attachment (G), fusion (F), and small hydrophobic (SH) proteins. The function of the latter is not well understood but in RSV it has been implicated in the blocking of apoptosis (Fuentes et al., 2007) and is also believed to be a viroporin that mediates membrane permeability (Gan et al., 2008). The M2 gene of MPV contains two open reading frames (ORFs) coding for the M2-1 and M2-2 proteins that act as regulators of transcription and RNA genome replication (Dibben et al., 2008). Non-translated regions (NTR) are located between the protein coding regions. Within the NTR are the gene start (GS) and gene end (GE) sequences that define the transcriptional boundaries on the negative strand template. Short, non-transcribed intergenic regions (IGR) lie between the GS and GE.

In nature, infection of rodents with MPV is believed to be subclinical or latent. Kaplan et al. (1980) observed that an epizootic outbreak of MPV coincided with a significant decline in a population of mice but high mortality associated with infection remains unproven. The study of naturally occurring infections has largely been limited to estimations of prevalence based on serological surveys. Four species of wild voles and mice surveyed in the UK all had antibodies to MPV with an overall prevalence of 42% (126/297) (Kaplan et al., 1980) and 3 of 15 grey squirrels in North Wales, UK had antibodies that recognized MPV (Greenwood and Sanchez, 2002). In contrast, two serological surveys of wild *Mus domesticus* in Australia and the UK did not identify antibodies to MPV in any of the animals tested (Smith et al., 1993; Becker et al., 2007). Likewise, a survey in Canada failed to find any antibodies to MPV in the sera of 485 voles (Descoteaux and Mihok, 1986). While members of the order *Rodentia* are generally assumed to be the natural hosts of MPV, the natural host range of pneumoviruses is largely unknown. Serological evidence of natural infection of a few non-rodent species was described by Horsfall and Curnen (1946) who found neutralizing activity to MPV in rabbits and non-human primates. Infection of humans with MPV or an antigenically-related virus was suggested by serological data, but there is limited evidence for involvement in clinical disease (Horsfall and Hahn, 1940; Horsfall and Curnen, 1946; Pringle and Eglin, 1986).

As in wild rodents, unintended infections of laboratory mice by MPV are usually not associated with clinical signs. An exception to this is the development of fatal pneumonia in unintentionally infected athymic mice (Weir et al., 1988; Richter et al., 1988). In contrast, experimental infections with the only two available strains of MPV, strain 15 and J3666, can be lethal even at low inoculum doses (Rosenberg and Domachowske, 2008). Strain 15 was the first isolate that was described in 1938 and it originated from apparently healthy mice (Horsfall and Hahn, 1939). Following the first passage of lung homogenates in mice, strain 15 was considered to be avirulent, however, virulence developed with successive passages in mice with fatal pneumonia developing as early as the third passage. It is anecdotally reported that strain 15 has since been repeatedly passaged in cell culture over time. Specific information regarding the isolation and propagation of strain J3666 is unavailable although it has reportedly been passaged entirely in mice and may have originated from the same isolate as strain 15 (Thorpe and Easton, 2005). The full-length sequences of both the Warwick and American Type Culture Collection (ATCC) isolates (Thorpe and Easton, 2005; Krempl et al., 2005) of strain 15 have been published. The two variants of strain 15 differ at only 12 nt positions and are therefore greater than 99.9% identical. Differences in the pathogenicity of the two strain 15 isolates in different mouse strains have been reported and may be due to a truncated G protein in the Warwick isolate (Krempl and Collins, 2004). The genome sequence of J3666 is 99.7% identical to the strain 15 isolates.

Domestic dogs confined together, as in shelters, boarding kennels, or breeding facilities, are often affected with acute respiratory infections (kennel cough) (Buonavoglia and Martella, 2007). The disease is transmitted rapidly and is difficult to eliminate as new animals are continually introduced. A spectrum of agents may produce a complex syndrome of multiple and sequentially overlapping infections that can make diagnosis and treatment challenging. Affected dogs have clinical signs ranging from mild dry cough and nasal discharge to pneumonia and death in severe cases. As one of the major health issues affecting domestic dogs, it is important to classify all of the infectious agents that may be involved. This report provides compelling evidence that the newly identified pneumovirus in dogs is found throughout the USA and that it can be routinely isolated and identified by PCR in animals with acute respiratory disease. Based on the widespread distribution and data showing transmission among dogs held in close confinement (manuscript in preparation), we propose the provisional naming of this virus as canine pneumovirus (CnPnV).

2. Materials and methods

2.1. Virus isolation

Nasal and pharyngeal specimens were collected from mixed breed dogs with respiratory disease signs using moist swabs and were processed within 24 h of receipt at the Animal Heath Diagnostic Center at Cornell University. Prior to inoculation, the swabs were immersed in 3 ml minimum essential medium (MEM) with Earle's salts (Gibco 10370, Invitrogen, Carlsbad, CA, USA), 0.5% bovine serum albumin, penicillin (200 U/ml), streptomycin (200 μg/ml), and fungizone (2.5 μg/ml) for 30 min and then mechanically agitated for 10 s. Aliquots of the nasal and pharyngeal swab extracts, 0.5 ml each, were combined and used to inoculate a single T25 flask of semi-confluent A72 cells (Binn et al., 1980) (CRL-1542, American Type