Feral swine virome is dominated by single-stranded DNA viruses and contains a novel Orthopneumovirus which circulates both in feral and domestic swine

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Feral swine are known reservoirs for various pathogens that can adversely affect domestic animals. To assess the viral ecology of feral swine in the USA, metagenomic sequencing was performed on 100 pooled nasal swabs. The virome was dominated by small, ssDNA viruses belonging to the families Circoviridae, Anelloviridae and Parvovirinae. Only four RNA viruses were identified: porcine kobuvirus, porcine sapelovirus, atypical porcine pestivirus and a novel Orthopneumovirus, provisionally named swine orthopneumovirus (SOV). SOV shared ~90% nucleotide identity to murine pneumonia virus (MPV) and canine pneumovirus. A modified, commercially available ELISA for MPV found that approximately 30% of both feral and domestic swine sera were positive for antibodies cross-reactive with MPV. Quantitative reverse transcription-PCR identified two (2%) and four (5.0%) positive nasal swab pools from feral and domestic swine, respectively, confirming that SOV circulates in both herds.

Besides causing damage to the environment, agriculture and wildlife, feral swine harbour a number of pathogens which can infect livestock. Pseudorabies virus (PRV) was eradicated from domestic animals from the USA in 2004; however studies have found evidence of widespread circulation in feral swine (Gaskamp et al., 2016; Pedersen et al., 2013; Müller et al., 2011). While not a concern for human health, PRV transmission from feral swine to other species has been documented (Cramer et al., 2011). Molecular and serological surveys have found that feral swine are variably infected with porcine circovirus type 2 (PCV2), porcine reproductive and respiratory syndrome virus, vesicular stomatitis virus, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Lawsonia intracellularis, Salmonella, Streptococcus suis, Brucella suis and Brucella abortus (Gaskamp et al., 2016; McGregor et al., 2015; Baroch et al., 2015; Stephenson et al., 2015; Corn et al., 2009; Rodriguez et al., 2000). For PCV2, feral swine appear to be an important reservoir for genetic diversity (Franzo et al., 2015; Fabisiak et al., 2012). Feral swine are also commonly infected with influenza A virus and the pandemic H1N1 virus of 2009 was identified in feral swine in Texas, representing a potential host and reservoir for domestic swine and zoonotic infections (Feng et al., 2014; Corn et al., 2009; Clavijo et al., 2013).

In order to assess the ecology of viruses infecting feral swine in the USA, we performed viral metagenomic sequencing on 600 nasal swabs assembled into 100 pooled samples. Pools of six nasal swabs were assembled from nasal swabs collected from the same state and county in 2011 and 2012. Samples originated from AL (8 pools), TX (13 pools), OK (63 pools), KS (4 pools), NH (1 pool), NC (10 pools) and MO (1 pool). Metagenomic sequencing was performed as previously described using three separate MiSeq runs (Mitra et al., 2016). Reads were mapped to the host (Sus scrofa) and unmapped reads were assembled de novo using CLC Genomics. Contigs were analysed by BLASTn to identify viruses using previously described criteria (Mitra et al., 2016).
A total of 16 different viruses were identified by BLASTN analysis. The feral swine virome was dominated by small, ssDNA viruses in the families Circoviridae and Anelloviridae. Torque teno virus (Anelloviridae) was the most commonly detected virus and was identified in 73 % of the samples. Reads mapping to other diverse anelloviruses were the second most common group of viruses detected, with 26 % of samples positive. PCV2 was identified in 13 % of the samples. Members of the family Parovirinae were also frequently detected, with porcine parvovirus 1, porcine parvovirus 2, porcine parvovirus 5, porcine parvovirus 6, porcine bocavirus and porcine hokovirus detected in 2, 2, 4, 8, 4 and 1 % of the pools, respectively. Other DNA viruses detected include porcine cytomegalovirus in 5 %, porcine adenovirus in 1 % of pooled samples.

Only four RNA viruses were detected. Porcine sapelovirus and porcine kobuvirus were each detected in a single pool. The recently described atypical porcine pestivirus was also identified in a single pool (Hause et al., 2016). Consequently detected, with porcine parvovirus 1, porcine parvovirus 5, porcine parvovirus 6, porcine bocavirus and porcine hokovirus detected in 2, 2, 4, 8, 4 and 1 % of the pools, respectively. Other DNA viruses detected include porcine cytomegalovirus in 5 %, porcine papillomavirus in 1 % and porcine adenovirus in 1 % of pooled samples.

To our knowledge, members of the genus Orthopneumovirus have not been previously identified in swine; however a serological survey of pigs in Ireland using a bovine respiratory syncytial virus (BRSV) antigen found that 41 % of sera contained antibodies that cross-reacted with BRSV (Allan et al., 1998). De novo assembly of one of the orthopneumovirus-positive samples generated a 14 885 bp contig comprised of 19 672 reads mapping which was 93 and 91 % identical to MPV and CPV by BLASTN, respectively, suggesting this virus represents a novel swine orthopneumovirus (SOV). ORF analysis determined that the genome was organized similar to members of Orthopneumovirus, with genes NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2 and L oriented from the 3'-end of the single, negative sense RNA molecule (Wang et al., 2011). Similar to MPV, ORF overlap was identified for M2-1 and M2-2 only (Krempl et al., 2005). Other members of Orthopneumovirus, human respiratory syncytial virus (HRSV) and BRSV, differ in having ORF overlap between M2 and L in addition to M2-1 and M2-2 (Wang et al., 2011). The genome sequence for SOV strain 57 was deposited in Genbank under accession number KX364383.

Table 1. ORF composition and length for swine orthopneumovirus strain 57 (SOV), murine pneumonia virus strain PVM3666 (MPV) and canine pneumovirus strain dog/Bari/100-12/ITA/2012 (CPV). Pairwise amino acid identity between SOV ORFs and counterparts in PVM and CPV are shown

<table>
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<tr>
<th>ORF</th>
<th>Swine orthopneumovirus strain 57</th>
<th>Murine pneumonia virus PVM3666</th>
<th>Canine pneumovirus dog/Bari/100-12/ITA/2012</th>
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*aa, Amino acids.
BLASTN and BLASTP analysis of the 11 ORF nucleotide and predicted proteins of SOV, respectively, found ≥90% identity to MPV and CPV with the exception of the attachment glycoprotein (G) which only showed 81–88% amino acid identity to MPV and CPV. This is lower than the 90.3 ±1.7% identity between G amino acid sequences of MPV and CPV (Glineur et al., 2013). Pairwise amino acid alignment of the ORFs for SOV, CPV, and MPV (Table 1) found greater than 90% identity between the three viruses except for NS1 (83.3–86.0% identity) and G (84.1–88.1% identity). Similar to CPV, the G protein of SOV is 18 aa longer than its counterpart in MPV (Table 1) (Renshaw et al., 2011; Glineur et al., 2013). Variability in protein length has also been observed for the SH protein. Like CPV, the SH protein of SOV is predicted to have 92 aa residues (Renshaw et al., 2011). MPV in contrast has SH proteins of 92, 96 and 114 aa residues depending on the isolate (Thorpe & Easton, 2005; Renshaw et al., 2011). The L protein of SOV contained a 2 aa deletion, S2955–N1956 relative to MPV and CPV. All other protein lengths were conserved between MPV, CPV and SOV.

To explore the evolutionary relationship between SOV and other members of Orthopneumovirus, phylogenetic analysis was performed using MEGA 6.0 (Afonso et al., 2016). Complete genome nucleotide sequences were aligned by ClustalW and phylogeny was inferred using the maximum likelihood algorithm using the best fitting General Time Reversible model with data gamma distributed. Tree topology was verified by 1000 bootstrap replicates. SOV was closely related to MPV and CPV (Fig. 1a), and the clade formed by SOV, CPV and MPV was distantly related to BRSV and HRSSV. Phylogeny was also explored using a conserved region of the L gene in order to include a sequence from a recently detected bat pneumovirus (Drexler et al., 2012). SOV occupied an ancestral position to MPV and CPV and together formed a sister clade to one composed of BRSV and HRSSV along with an ancestral bat pneumovirus (Fig. 1b). Phylogenetic analysis was also performed on the gene encoding the G protein because it is the most variable region of the genome (Glineur et al., 2013). Similar to a previous report, CPV and genetically similar feline pneumoviruses (FPVs) clustered into two distinct clades which were separate from a clade formed by MPV (Fig. 1c) (Glineur et al., 2013). SOV occupied an ancestral position on the tree most closely related to MPV.

To assess the frequency of SOV infection in swine, a commercially available ELISA designed to detect antibodies to MPV (XpressBio, Frederick, MD) was modified by replacement of the secondary anti-mouse peroxidase antibody with anti-swine peroxidase antibody using the same 1:5000 dilution. While it is unknown whether anti-SOV antibodies will cross-react with MPV antigen, previous work found cross-reactivity between an mAb to HRSSV and CPV (Renshaw et al., 2010). Given the close genetic relationship between MPV and SOV, we hypothesized that cross-reactivity was likely. Sera from 42 feral swine collected across the USA in 2010 and 2011 were analysed by ELISA and 13 (31%) were antibody positive according to the manufacturer’s specifications of a sample net absorbance between the positive viral antigen well and negative viral antigen well greater than 0.3, along with positive and negative mouse control serum net absorbance of greater than 0.6 and less than 0.25, respectively. Positive samples’ net absorbances ranged from 0.38 to 2.43, with positive and negative mouse control serum net absorbances of 2.80 and 0.00, respectively. Likewise, 46 domestic swine serum samples originally submitted to Iowa State University in 2014 for unrelated diagnostic testing were analysed by ELISA and 15 (33%) were positive (net absorbances 0.76–2.18). In addition, samples from two different sow farms located in North Carolina were analysed by ELISA. Sera from farm 1 were all negative (n=10), while 13 of 14 (93%) samples from farm 2 were positive (net absorbances 0.51–1.53). Twenty-one sera collected from high health-specific pathogen free pigs were analysed as negative controls and were negative. These results suggest that SOV circulates in both feral and domestic swine, however further confirmatory serological testing using SOV antigen is needed to verify these results.

To further assess whether MPV-like viruses circulate in swine, quantitative reverse transcription PCR (qRT-PCR) was performed using a previously described assay to detect MPV due to the high genetic similarity between MPV and SOV (Percopo et al., 2014). Screening of the 100 feral swine nasal swab pools by qRT-PCR identified two positives with C values of 31.8 and 32.5. The positive pools were the same ones identified as SOV positive by sequencing, validating the ability of the MPV PCR to detect SOV. We next designed a qRT-PCR targeting the SOV G gene (Forw, 5’-CAG AAT GCC ACA ACT CAG AAC-3’; Rev, 5’-CAT TTT GAC AGG CTT CGT GG-3’; Probe, 5’-FAM-AAC CAC TAG CCT ACC TCC CAC AGA-3’). The same two pools were again the only positive samples (C values 30.2 and 30.3). qRT-PCR was also performed on 80 nasal swab pools (three swabs per pool) collected from domestic pigs in 2015 with acute respiratory disease and submitted to Iowa State University for diagnostic testing. Using the MPV assay, one pool was positive with a C value of 33.7. The assay design based on SOV sequence identified the same positive sample as the MPV assay, as well as three additional positives, with C values 35.3–36.5. These results, in conjunction with the serological results, suggest that SOV circulates in both feral and domestic swine at low incidence, however more thorough epidemiological testing is needed to confirm this conclusion.

In order to investigate the genetic diversity of SOV, the G gene was amplified by PCR with primers designed to amplify the complete gene (Forw: 5’-CTA TCG GAA CGG AAT GAG AC-3’; Rev: 5’TGC CAG GAG CCA TAT TTG-3’). RT-PCR was performed for the six positive pools, however amplicons were only obtained from the two feral swine nasal swab pools. This is likely due to the very low amounts of SOV RNA present in four samples from domestic pigs. Sanger sequencing of the PCR products found that they were 99.9% identical.
MPV causes respiratory disease in rodents and has been used as a laboratory model for severe HRSV infections (Glineur et al., 2013). While initially identified in laboratory animals, MPV was recently identified in an African hedgehog with neurological disease (Madarame et al., 2014). Viruses genetically and antigenically similar to MPV have also been characterized from dogs and cats (Glineur et al., 2013). Investigation of an outbreak of respiratory

![Image of phylogenetic analysis](http://jgv.microbiologyresearch.org)

**Fig. 1.** Phylogenetic analysis of nucleotide sequences of (a) the complete genomes, (b) partial L gene and (c) the attachment glycoprotein (G). Phylogenetic trees were constructed by maximum likelihood analysis using the GTR+G model of nucleotide substitution with tree topology evaluated using 1000 bootstrap replicates. GenBank accession numbers are shown in parentheses. Recognized *Orthopneumovirus* species included murine pneumonia virus (pneumonia virus of mice), bovine respiratory syncytial virus and human respiratory syncytial virus (Afonso et al., 2016). Recognized *Metapneumovirus* species included were avian metapneumovirus and human metapneumovirus. Strain designation in the trees is as listed in GenBank and have not been corrected for changes in taxonomical nomenclature. Bars, 0.2 (a), 0.1 (b) and 0.02 (c) nucleotide substitutions per site.
disease in a dog shelter isolated CPV, which was genetically and antigenically similar to MPV (Renshaw et al., 2010). CPV has subsequently been identified in dogs with respiratory disease in Italy and the UK (Mitchell et al., 2013; Decaro et al., 2014). The finding of viruses closely related to MPV in multiple species with an etiological role in disease demonstrates the propensity of MPV-like viruses for interspecies transmission. Previous work found that another orthopneumovirus, HRSV, could infect and replicate in piglet tracheal organ cultures and caused death of ciliated epithelial cells (Fishaut et al., 1978). However, further research is needed to determine if SOV infection causes disease in swine. Importantly, MPV does not readily infect humans (Brock et al., 2012). The finding of SOV, a close relative of MPV, extends the host range to this group of viruses. Given the ability of MPV and CPV to cause respiratory disease, SOV should be considered for differential diagnostic testing for pigs with respiratory disease.

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