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Authors: John McKillen, Francis McNeilly, Catherine Duffy, Michael McMenamy, Irene McNair, Bernt Hjertner, Andrena Millar, Karen McKay, Paula Lagan, Brian Adair, Gordon Allan

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Isolation in cell cultures and initial characterisation of two novel bocavirus species from swine in Northern Ireland


*Corresponding author.

aVeterinary Sciences Division, Agri-Food and Biosciences Institute, Stormont, Belfast BT4 3SD, United Kingdom

bSchool of Biological Sciences, Queen’s University Belfast, 97 Lisburn Road, Belfast, BT9 7BL, United Kingdom

*Corresponding author. Tel.: +44 (0)28 90525752: +44 (0)28 90525823

E-mail address: john.mckillen@afbini.gov.uk (John McKillen).

Correspondence address: Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stoney Road, Stormont, Belfast BT4 3SD, United Kingdom
Abstract

We report the isolation in cell cultures of two novel bocavirus species in pigs from farms in Northern Ireland with clinical postweaning multisystemic wasting syndrome (PMWS). We have designated the isolates as porcine bocavirus-3 (PBoV3) and porcine bocavirus-4 (PBoV4). To date 5082 and 4125 bps of PBoV3 and PBoV4 have been sequenced, respectively. PBoV3 and PBoV4 show nucleotide homology to other known bocaviruses in swine and other organisms. Open reading frame (ORF) analysis has shown that these viruses have a third small ORF, equivalent to the NP1 ORF that distinguishes the bocaviruses from other paroviruses.

A panel of porcine field sera was screened by indirect immunofluorescence against both viruses. Of the 369 samples analysed, 32 (8.7%) and 35 (9.5%) sera were seropositive for PBoV3 and PBoV4 respectively, thus providing serological evidence of the exposure of swine in the field to bocavirus-like viruses. To date, the clinico-pathological significance of these novel swine bocaviruses, as primary pathogens or as immunosuppresive triggers for other infectious agents, is undetermined.

Key Words
Swine, bocavirus, virus isolation

1. Introduction

Parvoviruses are widespread pathogens causing a variety of diseases in animals. The family parvoviridae is sub-divided into two sub-families; Parovirinae which infect
vertebrates and Densovirinae which infect arthropods. Further subdivision of Parvovirinae into genera has not been well classified but currently at least five genera have been described; Parvovirus, Erythrovirus, Dependovirus, Amdovirus and Bocavirus. Two other genera have recently been proposed; Hokovirus (Lau et al., 2008) and Cnvirus (Hijikata et al., 2001; Wang et al., 2010). Paroviruses are small non-enveloped viruses and have a single stranded DNA genome of approximately 5 kb. The genus Bocavirus, currently consists of bovine parovirus-1 (BVP-1) (Abinanti and Warfield, 1961), canine minute virus (CMV) (Carmichael, 2004), gorilla bocavirus (Kapoor et al., 2010a), four genotypes of human bocavirus (HBoV) (Kapoor et al., 2010b) and several species of porcine bocavirus. Recently 1879 base pairs (bps) of a novel bocavirus referred to as porcine boca-like virus (PBo-likeV) has been sequenced from porcine lymph nodes (Genbank accession no. FJ872544) (Blomström et al., 2009 and 2010) using next-generation sequencing. Another virus called porcine parovirus 4 (PPV4) has been detected in a pig in the USA. This virus is related to bovine parovirus 2, but is similar to the genus bocavirus in terms of genomic arrangement (Genbank accession no. GQ387499) (Cheung et al., 2010). In China a further two putative bocavirus species have been detected and named porcine bocavirus 1 (PBoV1), (Genbank accession no. HM053693) and porcine bocavirus 2 (PBoV2) (Genbank accession no. HM053694). The same researchers have also identified two other partial bocavirus-like sequences, referred to as porcine bocavirus isolates 6V and 7V (Genbank accession nos. HM053672 and HM053673). This manuscript describes the isolation and initial characterization of two novel porcine bocavirus isolates (Genbank accession nos. JF512472 and JF512473) that are distinct.
from each other and from other porcine boca-like viruses previously reported. The viral
genomes are compared to the other swine bocaviruses, the other known parvoviruses and
to representative members of the sub-family *Parvovirinae*.

2. Materials and Methods

2.1 Origin of bocavirus-like virus isolates.

Two studies were carried out in 2004.

*Farm A*: A longitudinal study was conducted on a pig farm in Northern Ireland with an
ongoing problem with post-weaning multisystemic wasting syndrome (PMWS).

Nasopharyngeal swabs, faecal swabs and clotted blood samples were taken from 51
piglets derived from five sows when the youngest litter was one week old and thereafter
at days 19, 29, 40, 49, 57, 68 and 93 post farrowing.

*Farm B*: In a separate study, tissue samples were taken from three 3-week old, two 6-
week old and two 9-week old pigs submitted from a farm with an ongoing problem with
PMWS.

2.2 Virus isolation

Tonsil, or faecal swab suspensions and tissue homogenates were inoculated into roller
tube cultures of semi-confluent primary pig kidney cell lines using standard methods.

Cultures were examined for signs of cytopathic effect (CPE) after 72 hours and thereafter
at daily intervals. After six days the cultures were subjected to three freeze/thaw cycles
and the cell lysates (200µl) were inoculated into fresh primary pig kidney cells.
Cultures that exhibited an evident CPE after two passages were expanded into cell culture flasks of primary pig kidney, frozen and thawed as before and the lysates stored at -80°C. Unidentified cultures of particular interest were purified by either sucrose gradient purification alone or sucrose coupled with CsCl gradient purification using standard methods. Viral-containing aliquots were identified by electron microscopy and by immunofluorescent assay using pooled sera from the five maternal sows from farm A.

2.3 Testing of isolates for common porcine viruses

Cell lysates (200 μl) of cultures that exhibited a CPE or were positive by IFA with the sow sera were screened by PCR or reverse-transcriptase PCR (RT-PCR) for common porcine viruses. DNA and RNA was extracted from 200 μl of culture material using the Qiagen DNA Blood Mini Kit (Qiagen, Crawley, U.K.) and QIAamp RNA Blood Mini Kit (Qiagen), respectively, according to the manufacturer’s instructions. PCV1, PCV2 (Ouardani et al., 1999), PPV (Wilhelm et al., 2006) and adenovirus PCR assays were carried out using HotStarTaq Master Mix (Qiagen) according to the manufacturer’s instructions. Enterovirus CPE groups 1, 2 and 3 (Palmquist et al., 2002, Krumbholz et al., 2003) and reovirus (Leary et al., 2002) RT-PCR assays were carried out using Superscript III SuperScript™ One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Paisley, U.K.) according to the manufacturer’s instructions.

2.4 Production of swine antisera

Six 26-day old snatch farrowed colostrum deprived piglets were inoculated oro-nasally
Five days later each animal was infected oro-nasally with a 4 ml tissue culture pool of unidentified isolates from both farm studies. The piglets were euthanized 32 days later. Blood samples were taken immediately prior to euthanasia. PCV2 antibodies were detected by IPMA as previously described (McNair et al., 2004). The sera were pooled and screened by IIF (1/100 dilution) against coverslip cultures of primary kidney cells infected with the unidentified viruses, using an anti-swine FITC conjugate (1/30). The pooled serum was subsequently used as an immunoreagent to detect the unidentified isolates in cell culture.

2.5 Cloning and sequencing of PBoV3 and PBoV4

An aliquot of nucleic acid (100 µl), extracted from a CsCl purified fraction (1.2 ml) of one of the isolates of interest (PBoV3) using a standard phenol/chloroform method (Russell and Sambrook, 2001), was analysed on a 1% TAE agarose gel. The single stranded or double stranded status of the resulting bands was confirmed by acridine orange staining using a variation of the method described by McMaster et al., 1977.

A double stranded DNA band of approximately 5 kb was excised from the gel, digested using a selection of common restriction enzymes, cloned into pUC19 vector, transformed into One Shot® TOP10 cells (Invitrogen) and incubated on agar containing X-gal. Seventy five white colonies were selected for further analysis. Half of each colony was scrapped of using a pipette tip, placed in 10 µl of dH₂O and boiled for 10 minutes. Two µl of this was used for PCR using M13 primers. Colonies with PCR products of greater than 500bps were selected and plasmid minipreps were prepared using QIAprep Spin Miniprep Kit (Qiagen). The plasmid preparations were used as
template for sequencing reactions using BigDye Terminator Kit version 3.1 (Applied Biosystems, Warrington, U.K.), primed with M13 forward and reverse primers. The resulting sequences were assembled, trimmed and analysed using Vector NTI software (Invitrogen).

These sequences or consensus sequences from fragments that aligned with each other were submitted for nucleotide (blastn) query and translated nucleotide query (blastx) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

PCR primers were designed for each sequence or alignment that showed homology to the known bocaviruses by blastx (Oligo 5.0 software, Molecular Biology Insights).

Combinations of these primers were then used to amplify the areas between the clones using Expand Long Template PCR System (Roche Diagnostics L.T.D., Burgess Hill, U.K.). The PCR amplicons were sequenced by primer walking.

An alignment was made of complete genome sequences of HBoV, CMV and BPV1. A number of short degenerate reverse primers were designed as near as possible to the 3’ of the three genomes based on regions of homology. These primers were then used to amplify extracts of PBoV using already existing forward primers also located near the 3’ end of the known sequence PBoV3.

Amplicons produced by both these sequencing strategies were sequenced and analysed in Vector NTI and blastn as described.

All the existing primers designed during the sequencing of PBoV3 were used for preliminary sequencing and analysis of a second isolate of interest (PBoV4).

2.6 Genomic and phylogenetic analyses of PBoV3 and PBoV4 nucleotide sequences
The assembled sequences of PBoV3 and PBoV4 were compared to those of HBoV, BVP1 and CMV and the other swine bocaviruses (Table 1). The sequences were converted to Fasta format and analysed using ClustalW1.81 (http://align.genome.jp/) for nucleotide similarity (Table 1). Included in the analysis is a range of other paroviruses. A phylogenetic tree was created using MEGA version 4 (Tamura et al., 2007) (bootstrapped neighbour joining analysis) with parovirus complete genome or almost complete genome sequences from GenBank, along with the PBoV3 and PBoV4 partial sequences.

The assembled sequences of PBoV3 and PBoV4 were analysed for potential open reading frames (ORFs) using ORF finder software on the National Centre for Biotechnology Information (NCBI) website, http://www.ncbi.nlm.nih.gov/gorf/gorf.html.

2.7 Swine sera survey
A panel of 369 miscellaneous swine sera (1/50 and 1/250 dilutions) were screened by IIF using an anti-swine FITC conjugate (1/30) against coverslip cultures of primary pig kidney inoculated with the unidentified isolates. These sera dated from 1985-2007 but the majority of samples were collected in 2004 and 2007.

3. Results
3.1 Virus isolation
As expected, a number of common porcine viruses including porcine adenovirus, enteroviruses and reoviruses, circovirus and porcine parovirus were isolated from animals in both farm studies. Two unidentified isolates that were recovered were subject
In the longitudinal study, the faecal suspension from piglet 41 at the fourth sampling exhibited an evident CPE in the form of rounded cells after four passages in primary pig kidney cell culture. The isolate was subsequently referred to as PBoV4.

In the second study, a primary pig kidney cell culture inoculated with a homogenate of small intestine (SI3) from a six-week old piglet also exhibited a CPE in the form of rounded cells. Post-mortem analysis reported that this animal was in poor condition and had pneumonia and severe diarrhoea. This virus is designated as PBoV3.

### 3.2 Testing of isolates for common porcine viruses

PCR and RT-PCR analyses established that the CPE or IFA staining with sow sera from PBoV3 and PBoV4 isolates were not due to the presence of PCV1, PCV2, PPV, porcine enterovirus types 1, 2 and 3, porcine adenovirus or porcine reovirus.

Indirect immunofluorescence analyses using specific sera against porcine adenovirus, PCV1, PCV2 and PPV also proved negative. The isolates therefore remained unidentified.

### 3.3 Swine antiserum production

Following experimental infection with PCV2 and a selection of the unidentified isolates of interest, a pool of terminal sera was produced. This pool was used to detect the unidentified isolates in cell culture by indirect immunofluorescence. Strong nuclear and faint cytoplasmic staining was observed in PBoV3 infected cell cultures (Fig. 1a). A similar pattern of staining was detected against isolate PBoV4 (Fig. 1b).
3.4 Electron microscopy

Viral-containing fractions from sucrose gradient and CsCl gradient purification were identified by electron microscopy (Fig. 2a and Fig. 2b). Virus particles were clearly evident in the samples. Virus particles of both isolates were estimated to be 25-30nm in diameter and similar in size to other known members of the sub-family *Parovirinae*.

3.5 Cloning and sequencing of PBoV3 and PBoV4

By sequencing cloned restriction fragments as well as the amplicons from PCR generated by amplifying between the clones and by consensus PCR, 5082 bps of PBoV3 and 4125 bps of PBoV4 were sequenced. Initial partial sequencing of PBoV3 lower band produced an identical sequence to that of the upper band. Partial PBoV3 and PBoV4 sequence was submitted to GenBank (Acc. No. JF512472-JF512473).

3.6 Phylogenetic comparisons of PBoV3 and PBoV4 nucleotide sequences to existing bocaviruses

Table 1 is a table of similarities (ClustalW 1.81) of the sequences of PBoV3 and PBoV4 compared to each other and to the sequences of the known bocavirus sequences and representatives of the sub-family parovirinae. PBoV3 and PBoV4 are 84% homologous. PBoV3 is 41-49% homologous to non-porcine bocaviruses (HBoV1-4, CMV and BVP1) PBoV4 is 36-46% homologous to these viruses.
Among the swine bocaviruses PBoV3 and PBoV4 partial sequences show least similarity to PBoV-like virus from Sweden, with 42% and 11% homology respectively. The two viruses are more similar to the Chinese viruses (PBoV1 and 2), showing homologies of 50% and 51% (PBoV3) and 41% and 51% (PBoV4). PBoV3 is 73% and 78% homologous to the partial Chinese sequences 6V and 7V respectively, whereas PBoV4 is 52% homologous to both.

Figure 3 shows the Mega version 4 generated phylogenetic tree of PBoV partial sequences and complete or nearly complete genome sequences of other parvoviruses. With both viruses there were three putative ORFs on the positive strand over 200 amino acids in size. These corresponded closely in size and position to the NS1, NP1 and (partial) VP1/2 ORFs of the other known bocaviruses according to ORF analysis of their NCBI Genbank files. The putative ORFs corresponding to NS1 in PBoV3 and PBoV4 were considerably different in size at 800 and 667 amino acids respectively.

3.7 Swine sera survey

Of the 369 sera analysed, 32 (8.7%) and 35 (9.5%) samples were seropositive for PBoV3 and PBoV4, respectively.

4. Discussion

The application of molecular methods to the discovery of new viruses has led to the identification of several new members of the sub-family Parvovirinae. Two new paroviruses named as hokoviruses have been isolated recently from pigs and cattle and phylogenetic analysis of these has determined them to be related to the human parvovirus
Parv4 (Lau et al., 2008). These viruses may eventually be assigned to a new genus, *Hokovirus*. A virus referred to as porcine parovirus 2 (PPV2) has been isolated from swine in Myanmar (Hijikata et al., 2001) and has been subsequently reported in China (Wang et al., 2010). It has been suggested that this virus could be designated as another genus called Cn virus. Nucleic acids from another virus, referred to as porcine parovirus 4 (PPV4) has been sequenced from a lung lavage taken from a diseased pig co-infected with PCV2. PPV4 is most closely related to bovine parovirus 2 in terms of genomic homology, yet it has the three ORF genomic arrangement associated with bocaviruses. As well as the known primate (Kapoor et al., 2010a+b), bovine (Abinanti and Warfield, 1961) and canine bocaviruses (Carmichael, 2004), a number of bocavirus-like sequences have recently been detected in pig samples. One of these was isolated from the lymph nodes of a PMWS-affected pig in Sweden using next generation sequencing (Blomström et al., 2009). Investigation of the prevalence of this virus found that it was almost twice as prevalent in PMWS affected than non-PMWS affected pigs sampled in Sweden from 2003-2007 (Blomström et al., 2010). This virus has subsequently been found to be widespread in pigs in China where it has been reported to have a statistically significant association with respiratory disease (Zhai et al., 2010), and the full coding sequence has been described (Zeng et al., 2011). Two further putative bocavirus-like species detected in China (PBoV1 and PBoV2), (Genbank accession nos. HM053693 and HM053694) also have the characteristic three ORF genomic arrangement associated with bocaviruses. Two partial sequences identified by the same researchers (isolates 6V and 7v, Genbank accession nos. HM053672 and HM053673) will likely represent another two novel swine bocavirus species. The two bocavirus isolates described in this manuscript (PBov3 and
PBoV4) also represent two distinct bocavirus species and to date are the only swine bocaviruses isolated that have been adapted to grow in tissue culture. These viruses were isolated as part of two swine farm studies conducted in Northern Ireland, were grown in cell cultures and using a variety of molecular strategies 5082 nucleotides of PBoV3 and 4125 nucleotides of PBoV4 were sequenced. Clustal W analysis of the nucleic acid sequences, phylogeny and the ORF analysis confirmed the designation of the two isolates as bocaviruses. At a nucleic acid level the similarities of PBoV3 and PBoV4 with the other known bocaviruses are consistent with the similarities among the known bocaviruses. According to the International Committee for the Taxonomy of Viruses the criteria for defining a new species in the bocavirus genus is less than 95% homology in the non-structural genes (http://www.ictvdb.org/). It is likely therefore that PBoV1-4, PBo-likeV and viruses 6V and 7V represent seven distinct species of swine bocavirus, although more sequencing of 6V and 7V will be required as well as more specific analysis of the non-structural genes of the other viruses. Mega 4 analysis produced a phylogenetic tree confirming that the PBoV3 and 4 isolates are related to the other bocavirus. Phylogentic analyses of the protein sequences of the three genes in the bocaviruses will be carried out after full sequencing of PBoV3 and PBoV4. Agarose gel electrophoresis of nucleic acid isolated from purified viral particles showed one double and one single stranded DNA band. Parvoviruses vary regarding the amount and sense of DNA that is encapsidated. For example, bovine parvovirus 1 encapsidates approximately 10 to 15% of the plus sense strand (Chen et al., 1988). Therefore the gel electrophoresis pattern observed could be consistent with a parvovirus that encapsulated
both positive and negative sense strands in different amounts / proportions. The sequences were subjected to ORF analysis using NCBI ORF finder. Bocaviruses are known to have three major open reading frames encoding two non-structural proteins and a nested set of structural proteins. The 5' ORF codes for a large non-structural protein NS-1, the smaller middle ORF encodes another non-structural protein and the right hand ORF encodes a nested set of structural proteins. ORF analysis found that both PBoV3 and PBoV4 have three potential ORFs over 200 amino acids in size that correspond with those of the known bocaviruses. Analysis of the NS1 ORF shows for PBoV4 that two nucleotide changes coding for putative amino acid 668 result in termination of the NS-1 ORF at this point, significantly shorter than the 800 aa size of the PBoV3 counterpart. However, both the NS1 and NP1 ORFs from both PBoV3 and PBoV4 fit into the range of sizes of the ORFs of the other bocaviruses. No stop codon was found for the PBoV VP1/2 sequence using NCBI ORF Finder, but comparison with the other bocaviruses would suggest that this is very close to being the complete VP1/2 coding sequence. To date, the clinico-pathological significance of these novel swine bocaviruses has not been determined. The initial association of PBo-likeV with respiratory infections and increased prevalence in PMWS-affected pigs is of significance. Indeed in this study, PBoV3 was recovered from a piglet that presented with pneumonia and severe diarrhoea. Even if PBoV3 and PBoV4 are not directly associated with any disease they may have the potential to function as immunosupressive triggers for other infectious agents. An initial serological survey detected that 32 out of 369 sera tested were seropositive for PBoV3 and 35 were seropositive for PBoV4. With the exception of two serum samples dating from 1990, all of the seropositive sera were sampled in either 2004 or 2007.
However, given the relatively low levels of antibody detected in positive samples, it cannot be discounted that negative results were a reflection of antibody degradation over time. Nevertheless, these results confirmed that swine in the field had been exposed to bocavirus-like viruses. Further serological investigation will be carried out on archival samples. Optimised real-time PCR based assays will be developed to detect the viral genome. It is likely that these viruses are widespread in pig populations. It is intended that the pathogenicity of the virus will be determined by experimental infection in colostrum deprived pigs and monoclonal antibodies produced for use as a serological tool.

5. Acknowledgments:

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6. References:


Lau SK, Woo PC, Tse H, Fu CT, Au WK, Chen XC, Tsoi HW, Tsang TH, Chan JS, Tsang DN, Li KS, Tse CW, Ng TK, Tsang OT, Zheng BJ, Tam S, Chan KH, Zhou B, Yuen KY. Identification of novel porcine


**Figure Captions**

**Fig. 1** Indirect immunofluorescence detection of (a) PBoV3 and (b) PBoV4 in primary pig kidney cells with polyclonal swine antiserum (1/100 dilution). Strong nuclear staining is evident and faint cytoplasmic staining can also be seen.

**Fig. 2a** Electron microscopy of virus (a) PBoV3 and (b) PBoV4.

**Fig. 3** Mega version 4 bootstrapped neighbour joining phylogenetic tree of PBoV3 and PBoV4 partial sequences with full or nearly full genome sequences of representatives of the Parvovirinae. Bootstrapping was carried out with 1000 replicates.
Table 1. Clustal W analysis showing % nucleic acid homologies of the Northern Ireland PBoV isolates compared to representatives of the other bocaviruses, other porcine parvovirus and bovine parvovirus 2. Sequence lengths and accession nos. for known bocaviruses are: Porcine Bocavirus-SX-CHN (4786 bps, HQ223038), human bocavirus-1 ST1 (5217 bps, DQ000495), human bocavirus-2 PK-2255 (5134 bps, FJ170279), human bocavirus-3 W855 (5164 bps, FJ948861), human bocavirus-4 NI-385 (5104 bps, NC_012729), bovine parvovirus-1 Abinanti (5515 bps, DQ335247), canine minute virus GA3 (5402 bps, FJ214110), porcine parvovirus-4 (5905 bps, GQ387499), bovine parvovirus-2 (5610 bps, NC_006259), porcine hokovirus HK1 (5043 bps, EU200671), porcine parvovirus-2 (Myanmar erythroivirus, 5118 bps, AB076669), porcine parvovirus NADL-2 (5075 bps, NC_001718), porcine bocavirus-1_Chn (PBoV1, 5173bps, HM053693), porcine bocavirus-2_Chn (PBoV2, 5186bps, HM053694), porcine bocavirus 6V Chn (2407bps, HM053672), porcine bocavirus 7V Chn (2434bps, HM053673). PBoV3 and PBoV4 sequences are 5082 and 4125 bps respectively.

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Figure 3