Veterinary Microbiology 152 (2011) 39–45

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

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

John McKillen [a,*], Francis McNeilly [a], Catherine Duffy [b], Michael McMenamy [b], Irene McNair [a], Bernt Hjertner [b], Andrena Millar [b], Karen McKay [a], Paula Lagan [a], Brian Adair [a], Gordon Allan [a]

[a] Veterinary Sciences Division, Agri-Food and Biosciences Institute, Stoney Road, Stormont, Belfast BT4 3SD, United Kingdom
[b] School of Biological Sciences, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom

ARTICLE INFO

Article history:
Received 3 August 2010
Received in revised form 29 March 2011
Accepted 14 April 2011

Keywords:
Swine
Bocavirus
Virus isolation

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 parvoviruses.

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.

1. Introduction

Parvoviruses are widespread pathogens causing a variety of diseases in animals. The family *Parvoviridae* is sub-divided into two sub-families; *Parvovirinae* 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). Parvoviruses are small non-enveloped viruses and have a single stranded DNA genome of approximately 5 kb.

The genus *Bocavirus*, currently consists of bovine parvovirus-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, 2010) using next-generation sequencing. Another virus called porcine parvovirus 4 (PPV4) has been detected in a pig in the USA. This virus is related to bovine parvovirus 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).

* Corresponding author. Tel.: +44 028 90525752/823.
E-mail address: john.mckillen@afbini.gov.uk (J. McKillen).

0378-1135/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetmic.2011.04.013

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 subfamily *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 1-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 h and thereafter at daily intervals. After 6 days the cultures were subjected to three freeze/thaw cycles and the cell lysates (200 μl) 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 were extracted from 200 μl of culture material using the Qiagen DNA Blood Mini Kit (Qiagen, Crawley, UK) 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, UK) 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 with PCV2. 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 (Mc Nair et al., 2004). The sera were pooled and screened by IIF (1/100 dilution) against coverslip cultures of primary pig 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 and Carmichael (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 dH_2O and boiled for 10 min. Two μl of this was used for PCR using M13 primers. Colonies with PCR products of greater than 500 bps 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, UK), 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 boca-