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Detection of novel porcine bocaviruses in fecal samples of asymptomatic pigs in Cameroon

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ABSTRACT

Improvements and widespread use of nucleic acid amplification and sequencing methods have led to the recognition of new virus diversity in various domestic animals, including pigs. In this study we utilized either virus species specific or broadly reactive PCR assays to describe the occurrence and genetic diversity of selected DNA viruses belonging to families *Adenoviridae*, *Circoviridae*, *Anelloviridae* and *Parvoviridae* in Cameroonian pigs. Fecal specimens were collected during spring of 2011. No adenoviruses, circoviruses and anelloviruses were detected, however, high prevalence and remarkable genetic diversity within the identified parvoviruses and, particularly, within bocaviruses was observed. PPV4 was the most prevalent virus (20%), followed by PBoV3 (18%), PBoV4 (18%), PBoV5 plus 6V/7V (16%), and PBoV1 plus PBoV2 (6%). The frequency of mixed infections with various combinations of these virus species reached 20%. Genetic analysis of the identified viruses showed that the capsid gene of PBoV1 and PBoV2 strains shared up to 91% and 94% nt sequence similarities to reference PBoV1 and PBoV2 strains, respectively. The identified PBoV3 and PBoV4 strains shared ⩽95% and ⩽98% nt identities with reference PBoV3 and PBoV4 strains, respectively, along the NS gene, whereas the PBoV5 strains shared 86% nt identities with Hungarian and 87% nt identities with Chinese PBoV5 strains along the capsid gene. In addition, a single PBoV5-like strain shared ⩽71% nt sequence identity with other PBoV5 strains. This is the first study to report evidence of the circulation of bocaviruses in Africa and contributes to our understanding of the impact of globalization on the dispersal of new and emerging viruses.

1. Introduction

Pig industry is an important sector of the agriculture worldwide including African countries in the sub-Saharan area. Productivity is threatened by several factors, including the continuous circulation and resurgence of well characterized old viruses and the emergence of new viral diseases.

Improved nucleic acid amplification and sequencing technologies have led to the description of numerous new DNA viruses in swine during the past few years. A handful of these novel DNA viruses belong to the *Parvovirus* and the *Bocavirus* genera (*Parvovirinae* subfamily, *Parvoviridae* family) (Hijikata et al., 2001; Blomstrom et al., 2009; Blomström et al., 2012; Lau et al., 2008; Wang et al., 2010; Cheung et al., 2010; Cheng et al., 2010; McKillen et al., 2011; Li et al., 2012). Both genera posses a single stranded DNA genome of ~5–5.5 kb in length and encode two or three open reading frames (ORFs). Most species within the *Parvovirus* genus encode two major ORFs; these are the non-structural protein (NS) and the capsid proteins (VP1/2). One exception is PPV4, which similarly to members of the *Bocavirus* genus, has an additional protein coding region located between ORF1 and ORF2 (Allander et al., 2005; Cheung et al., 2010). However, this third ORF of bocaviruses is not homologous to other parvovirus genes.

Among newly described parvoviruses of swine, porcine parvovirus 2 (PPV2), was discovered in pig sera in Myanmar as a novel, genetically divergent member of *Parvovirinae* (Hijikata et al., 2001). Later, genetically related parvoviruses were discovered in

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serum samples of pigs affected by 'high fever' disease (PRRS associated disease) and postweaning multisystemic wasting syndrome (Wang et al., 2010), forming a distinct genetic cluster (Wang et al., 2010) within *Parvovirinae*. In 2008, a new porcine parvovirus, named porcine hokovirus (PHoV) belonging to the newly proposed genus *Hokovirus* was identified in Hong Kong (Lau et al., 2008). Analysis of the genome sequences showed that PHoV strains were closely related to human parvovirus 4 and bovine HoV, and formed a distinct cluster within the family (Lau et al., 2008). Based on a recent classification, PHoV was renamed as porcine parvovirus 3 (PPV3; Cheung et al., 2010). Another novel porcine parvovirus, PPV4 was detected in 2005 from porcine circovirus-associated disease, which affected swine herds in the USA (Cheung et al., 2010) and was subsequently reported in China (Huang et al., 2010; Zhang et al., 2011). The detection rates of PPV4 infection in swine herds were reported low worldwide (range, 2.1%–6.6%; Cheung et al., 2010; Zhang et al., 2011; Cságola et al., 2012).

The history of bocaviruses dates back to the 1960s (Abinanti and Warfield, 1961; Binn et al., 1970). The first porcine bocavirus was described only in 2009 in swine feces with postweaning multisystemic wasting syndrome in Sweden and it was designated as porcine boca-like virus (PBo-likeV) (Blomstrom et al., 2009). In addition to Europe, PBo-likeV has also been identified in China and have been named porcine bocavirus (PBoV) (Zhai et al., 2010; Zeng et al., 2011) or porcine bocavirus 1 (Shan et al., 2011a; Zhang et al., 2011). At the same time other porcine bocaviruses, genetically different from PBo-likeV, were characterized from the feces of swine in China and were tentatively named PBoV1, PBoV2, 6V and 7V (Cheng et al., 2010). Also, two highly divergent bocaviruses, provisionally named PBoV1-H18 and PBoV2-A6 were detected by using random PCR amplification (Shan et al., 2011a). Among these, PBoV1-H18 was very closely related to porcine boca-like virus (PBo-LikeV) reported in the Swedish study (Blomstrom et al., 2009), whereas PBoV2-A6 was very closely related to PBoV1 and PBoV2 published from China (Shan et al., 2011a). Simultaneously, a number of new, genetically distinct porcine bocaviruses designated as PBoV3, PBoV4 and PBoV5 were also described (McKillen et al., 2011; Lau et al., 2011; Shan et al., 2011b; Li et al., 2012; Yang et al., 2012). Renaming of different PBoVs, mainly PBoV1, PBoV2 and 6V/7V (Cheng et al., 2010) was proposed by independent research groups (Zhang et al., 2011; Shan et al., 2011b). However, given that all these reports were published during the past 2 years or so, the nomenclature of the newly recognized PBoVs remains unresolved.

This marked heterogeneity of *Parvoviridae* in pigs is reshaping our understanding on virus species diversity in this host, making careful evaluation of the need for effective prevention and control strategies against these and other new viruses essential. Therefore, collection of comprehensive data about their occurrence, distribution, and genetic diversity, which together will shed light on relevant features and may help to understand the importance of these novel viruses, is required. Very limited studies have been published about prevalence and diversity of various porcine viruses in the central African region. In this study we sought to investigate the occurrence and genetic diversity of selected DNA viruses in Cameroonian pigs with established, presumed, or unknown disease associations by analyzing their fecal shedding.

2. Materials and methods

2.1. Specimens

Fecal samples were collected during 2011 predominantly from young, healthy domestic pigs reared in different regions of Cameroon. The samples were stored at −80 °C until processing.

2.2. Laboratory methods

Ten percent stool suspensions were made in 1 ml phosphate buffered saline (PBS) and then homogenized by vortexing. Total nucleic acid extraction from 100 μl suspensions collected after centrifugation (5 min, 16,000*g*) was carried out with an X-tractor Gene automated nucleic acid extraction robot (Corbett Robotics Pty. Ltd., Queensland, Australia) using Promega DNA and RNA Purification kit (Promega) following the manufacturer's instructions.

Virus specific PCR primers and protocols used to detect adenoviruses, porcine circovirus 2 (PCV2), porcine parvovirus (PPV) 1–4 (PPV1–PPV4), porcine boca-like virus (PBo-likeV) and porcine bocavirus (PBoV) 1, 2, 6V and 7V, torque teno sus virus (TTSuV) 1 and 2 have been previously described by Kiss et al. (1996), Fenaux et al. (2000), Cadar et al. (2012), Soares et al. (1999), Cságola et al. (2011), (2012), Zhai et al. (2010) and Segalés et al. (2008), respectively. Additional sets of oligonucleotide primers were designed to detect PBoV3 (BOCA3–355F 5′-GCACGGAGCTATTACTGGTT-3′, BOCA3–665R 5′-AGCTGTAGACCGGATTGTGA-3′; located, respectively, at sequence positions 355–374 and 646–665 of reference strain 64–1/N.Ireland/2004,), and PBoV4 (BOCA4–481F 5′-ACCTTGTGTGAGTCTGCTGA-3′; BOCA4–707R 5′-TAGTGCTTCCAGAGATCGAG-3′; located, respectively, at sequence positions 481–500 and 688–707 of reference strain F41/N.Ireland/2004). The oligonucleotide primers developed and utilized in our laboratories were designed based on whole genome sequences retrieved from GenBank. For diagnostic purposes conservative sequence regions were chosen with virus species specific variations regarding the target genomic regions.

The 50 μL reaction mixture for the PCR amplification consisted of 10 μL 10× My*Taq* Red Buffer (Bioline GmbH, Germany), 0.2 μL of My*Taq* DNA Polymerase (Bioline GmbH, Germany), 0.5 μL of 25 μM of forward and reverse primers, 1 μL of DNA template and ddH_2O up to 50 μL. The PCR conditions used for PBoV3/4 included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 3 min, and a final elongation step at 72 °C for 7 min. PCR products were run on 1.5% agarose gels stained with GR Safe Nucleic Acid Stain (Excellgen Inc., USA) and visualized with a Dark Reader blue light transilluminator (Chemical Research, Inc. USA). Amplicons of the expected sizes were subjected to direct nucleotide sequencing using the PCR primers.

2.3. Computer analyses

The obtained nucleotide sequences were evaluated, edited, assembled and then subjected to Blast search using Geneious v4.8.5 (Biomatters, Ltd). GenBank accession numbers of the sequences generated are shown in the figures. The nucleotide sequences characterized in the present study were compared to sequences available in the GenBank. Alignments were made using ClustalW as implemented in Geneious v4.8.5.

The evolutionary relationships of the viruses from this study with reference strains were determined using two different approaches, a Bayesian phylogenetic and a maximum likelihood analysis. For each data set we used jModeltest (Posada, 2008) to determine the models of nucleotide substitution that best fit the data. The Akaike's Information Criterion was chosen as the model selection framework and Hasegawa–Kishino–Yano model of sequence evolution with a proportion of invariant sites and gamma distributed rate heterogeneity (HKY+I+Γ) as the best model. Based on the selected nucleotide substitution model obtained, a Bayesian inference analysis was performed using the Markov chain Monte Carlo (MCMC) coalescent approach implemented in BEAST v1.6.2 package (Drummond and Rambaut, 2007). RaxML v7.0.3 (Stamatakis, 2006) was employed for maximum likelihood analyses using