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Novel bocaparvoviruses in rabbits

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ABSTRACT

Bocaparvovirus is a newly established genus within the family *Parvoviridae* and has been identified as a possible cause of enteric, respiratory, reproductive/neonatal and neurological disease in humans and several animal species. In this study, metagenomic analysis was used to identify and characterise a novel bocaparvovirus in the faeces of rabbits with enteric disease. To assess the prevalence of the novel virus, rectal swabs and faecal samples obtained from rabbits with and without diarrhoea were screened with a specific PCR assay.

The complete genome sequence of the novel parvovirus was reconstructed. The virus was distantly related to other bocaparvoviruses; the three ORFs shared 53%, 53% and 50% nucleotide identity, respectively, to homologous genes of porcine bocaparvoviruses. The virus was detected in 8/29 (28%) and 16/95 (17%) samples of rabbits with and without diarrhoea, respectively. Sequencing of the capsid protein fragment targeted by the diagnostic PCR identified two distinct bocaparvovirus populations/sub-types, with 91.7–94.5% nucleotide identity to each other. Including these novel parvoviruses in diagnostic algorithms of rabbit diseases might help inform their potential pathogenic role and impact on rabbit production and the virological profiles of laboratory rabbits.

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Introduction

Bocaparvovirus is a newly established genus within the family *Parvoviridae* and includes a genetically diverse group of parvoviruses first reported in humans and other animal species in the 1960s[1]. Common features of the bocaparvovirus genome include the approximately 4.9–5.3 kilonucleotide (kn) long single-stranded DNA genome with complex hairpin structures at the 5′ and 3′ ends. The genome is predicted to encode three or four proteins, NS1, NP1 and VP1/VP2. Bocaparvoviruses have been identified as a possible cause of enteric, respiratory, reproductive/neonatal and neurological disease, in humans and several animal species, but infection is frequently asymptomatic (Allander et al., 2005; Schildgen et al., 2008; Mitui et al., 2012; Knipe and Howley, 2013; Bodewes et al., 2014).

Rabbits are important livestock, wild, pet and laboratory animals. Exploring the diversity of the rabbit virome could yield important information for the development of specific diagnostic tools to preserve their health and welfare and to prevent unexpected outcomes during animal experiments. In this study we investigated the identification and genomic characterisation of a novel bocaparvovirus detected in the faeces of rabbits with enteric disease. In addition, screening with specific primers was performed to assess the prevalence of this novel parvovirus in rabbit faecal and swab specimens.

Materials and methods

Sample collection

Faecal samples (numbered 160/01) were collected in Northern Italy in 2001 from a rabbit with enteritis and pooled into a single specimen. Additionally, two specimen collections (A and B) were analysed. Collection A included samples obtained from symptomatic rabbits during 2005–2008. The samples were pooled by combining small and large intestine tracts and/or intestinal contents from two to five rabbits (35–55 days of age) with gastrointestinal signs. Collection B included rectal swabs collected from asymptomatic animals (30–35 days of age) from 11 commercial rabbit breeding enterprises located in three different regions of Northern Italy (Lombardia, Emilia Romagna and Veneto) during 2008. The study was conducted with the approval (1/2015) of the Ethical Committee of the University of Bari.

Next generation nucleotide sequencing (NGS)

Viral nucleic acid was extracted from 10% faecal homogenates of rabbit sample 160/01 using the RNeasy Mini Kit (Qiagen). Nucleic acids were amplified by a random-primed RT-PCR assay, and used as template for next generation sequencing (NGS) using the Ion Torrent platform as described in detail elsewhere (Mihalov-Kovács et al., 2015). The sequence data were evaluated by the CLC Genomic Workbench[2]. Contigs were prepared by combining de novo assembly and reference mapping steps. As a

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[1] See: International Committee on Taxonomy of Viruses http://www.ictvonline.org (accessed 2 August, 2015).

[2] See: CLC bio http://www.clcbio.com (accessed 2 August, 2015).

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result, three contigs of 1702, 1627, and 458 nucleotides (nt) in length were obtained. The contigs were most similar to porcine bocaparvoviruses (48–55% aa) by BlastX (Altschul et al., 1990). The specimen also yielded a large number of reads for other well-known rabbit enteric viruses, including 1906 reads for astrovirus and 1879 reads for rotavirus.

Full-length genome sequencing of lapine bocaparvovirus

The whole genome of the novel bocaparvovirus was reconstructed using a primer walking approach. The 1702-, 1627-, and 458-nt long contigs were mapped using a reference porcine bocaparvovirus genome. Specific primers were designed using the software Primer3 (Koressaar and Remm, 2007) to fill the gaps between the various contigs. Long PCRs were carried out using the La Taq DNA polymerase (Takara Bio). Also, the 5′ and 3′ ends of the genome were determined by a previously described 5′ RACE protocol (Scotto-Lavino et al., 2007), with minor modifications, using the kit 5′ RACE System for rapid amplification of cDNA Ends (Version 2.0, Life Technologies). The amplicons were purified and cloned using a TOPO XL Cloning Kit (Life Technologies). Consensus sequences were generated by sequencing at least three clones for each PCR fragment. The sequence of the genome of the lapine bocaparvovirus was deposited in GenBank under the accession number KP729195.

Phylogenetic analyses

Sequence and phylogenetic analyses were carried out using a commercially available software package (Geneious version 7.1.8, Biomatters). Genome sequences of bocaparvovirus strains were retrieved from GenBank and aligned using Clustal W (Larkin et al., 2007). For phylogenetic analysis, the nucleotide alignments were analysed using Mr Bayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

PCR-based screening

To estimate the prevalence of the novel bocaparvovirus in rabbit breeding enterprises, stool samples, rectal swabs, and intestinal contents obtained from rabbits (collections A and B) were tested using PCR primers designed specifically to detect lapine bocaparvovirus. The primers for the diagnostic PCR assay, 1257for (5′-TAACTGCCGGAGTGCACATC-3′) and 1260rev (5′-TCCTGTTCTGAGTACTTGGTGA-3′), were designed to amplify a 257 base pair (bp) fragment at the 3′ end of ORF2. The AccuPrime Taq DNA polymerase (Life Technologies) was used for PCR amplification. Cycling thermal conditions included initial activation of the polymerase at 94 °C for 2 min, and 35 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s, followed by final extension at 72 °C for 10 min.

Sequence analysis based on diagnostic region of ORF2 of rabbit bocaparvoviruses

A total of 24 amplicons generated in the PCR screening of rabbit samples from collections A and B were selected on the basis of DNA concentration (measured using a quantitative standard) and subjected to direct Sanger sequencing. The amplicons were purified after gel-excision using the kit Qiaquick gel extraction (Qiagen). Sequencing was performed at Eurofins Genomics laboratories.

Virus cultivation

Faecal samples (sample 160/01 and an additional five samples that tested positive using PCR) were homogenised in 10% Dulbecco's modified Eagle's medium and centrifuged at 15,000 *g*. The supernatant was filtered with 0.22 µm filters (Jet Biofil), and inoculated onto freshly seeded RK-13 (rabbit kidney) cell lines, incubated at 37 °C in 5% CO_2. Viral growth was evaluated in the serial passages by monitoring the onset of cellular cytopathic effect and by testing the supernatant in PCR with primers 1257for and 1260rev.

Results

Genome analysis and protein prediction

The 5020 nt long genome of lapine bocaparvovirus displayed short inverted repeats at the 5′ and 3′ genome terminations, consistent with hairpin structures typical of parvoviruses (Knipe and Howley, 2013). Three viral proteins were predicted (Fig. 1). ORF1 was 2157 nt long (97–2253) and encoded a 718 amino acid (aa) long NS1 homologue, whereas ORF2 was 2175 nt long (2676–4850) and encoded the capsid protein VP1 of 724 aa in length and its potential derivatives (VP2 and VP3). A third ORF (ORF3) was 615 nt long (2075–2689) and encoded the NP1 protein, which was 204 aa in length. The estimated molecular weights of these proteins were 81.6, 80.2 and 23.3 kd, respectively. By BlastN (Altschul et al., 1990), the three ORFs shared the highest nt identity (53%, 53% and 50%) with porcine bocavirus 1 (HM053693), p 2 (HM053694) and p 4-1 (NC_016032), respectively. By BlastX (Altschul et al., 1990), the three ORFs shared the highest aa similarity (50%, 50% and 43%, respectively), with porcine bocaparvovirus 2 (ADI60258.1).

The conserved motifs of the Ca^{2+} binding loop (YXGXG) and the catalytic centre (HDXXY) of phospholipase A_2 were identified in the VP1 of the lapine bocaparvovirus (Zádori et al., 2001). On phylogenetic analysis based on the whole genome, the rabbit bocaparvovirus was most closely related to porcine bocaviruses 1 and 2 and it was distantly related to bocaparvoviruses of primate, ungulate, pinniped and carnivore origin (Fig. 2). Phylogenetic trees constructed by NS1, VP1 and NP1 region yielded similar topology (Appendix: Supplementary Figs. S1–S3).

PCR-based screening

In the PCR-based screening, 8/29 (28%) specimens collected from symptomatic rabbits (collection A) and 16/95 (17%) of those collected from asymptomatic animals (collection B) were positive for lapine bocaparvovirus. In the asymptomatic group, bocaparvovirus DNA was detected in 7/11 (63%) rabbit colonies, with prevalence rates of 20–40% in each colony.

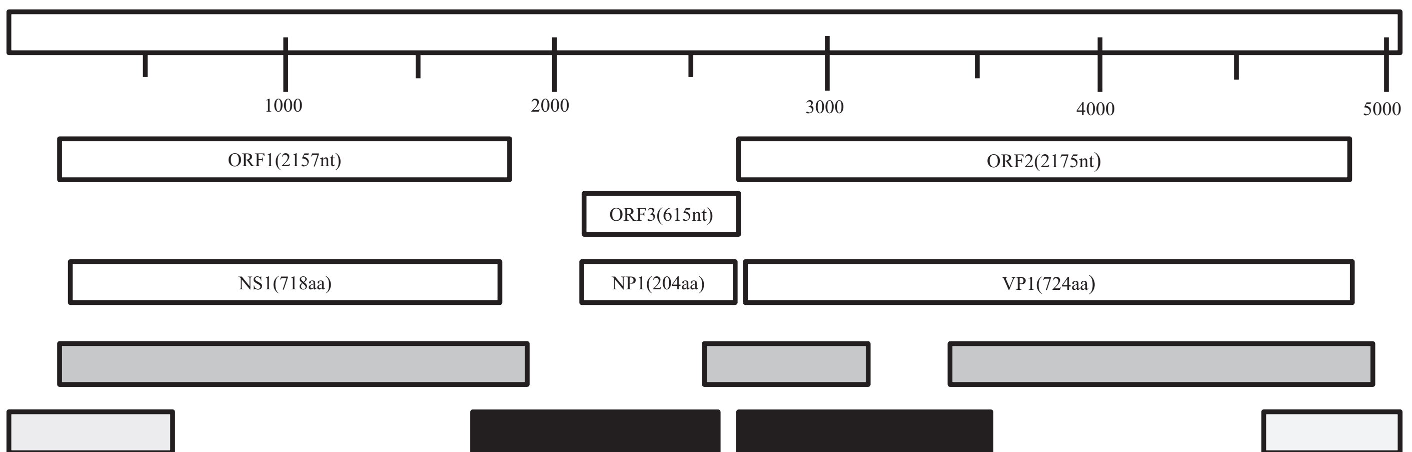

Fig. 1. Genome map and sequence reconstruction of the rabbit bocaparvovirus. The position of the three open reading frames (ORFs) and the proteins predicted are indicated. The contigs 1–3 coloured in grey were generated in next generation sequencing. The fragments coloured in light grey at the 5′ and 3′ ends were generated with a 5′ RACE protocol. The fragments coloured in black were generated by a primer walking strategy.

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