Recombination analysis based on the complete genome of bocavirus

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Abstract
Bocavirus include bovine parvovirus, minute virus of canine, porcine bocavirus, gorilla bocavirus, and Human bocaviruses 1-4 (HBoVs). Although recent reports showed that recombination happened in bocavirus, no systematical study investigated the recombination of bocavirus. The present study performed the phylogenetic and recombination analysis of bocavirus over the complete genomes available in GenBank. Results confirmed that recombination existed among bocavirus, including the likely inter-genotype recombination between HBoV1 and HBoV4, and intra-genotype recombination among HBoV2 variants. Moreover, it is the first report revealing the recombination that occurred between minute viruses of canine.

Introduction
Members of the genus *Bocavirus* are non-enveloped single-stranded DNA (ss-DNA) virus, which belong to the Parvoviridae family. The bocavirus genome is not segmented and contains a single molecule of linear, positive- or negative-sense DNA of 4,000-6,000 nucleotides in length [1]. Known members of bocavirus include bovine parvovirus (BPV), minute virus of canine (MVC), porcine bocavirus (PBoV), gorilla bocavirus (GBoV), and Human bocaviruses 1-4 (HBoV1-4).

The MVC genome shares about 43% identity with BPV over the genome level [2,3]. BPV was first identified in 1961 in samples from calves with diarrhea [4], while MVC was first isolated from canine fecal samples in 1970 [5]. HBoV was first described in pooled nasopharyngeal aspirates from children with respiratory infections in 2005, and was provisionally categorized into the genus bocavirus [1]. Subsequently, HBoV2, HBoV3, and HBoV4 were discovered, sharing a mean similarity of 80% with HBoV1, and all have been categorized into the genus bocavirus [6,7]. Recently, new bocavirus species were isolated from gorilla and swine, and most closely related to HBoV [8,9].

Although recently reports showed that recombination happened in bocavirus [8], no study has systematically investigated the recombination among bocavirus strains. In the present study, therefore, we analyzed the available complete bocavirus genome sequences in GenBank to elucidate the recombination among bocavirus strains.

Methods

**Sequences**
The study sequences comprised all the 121 available complete genome sequences of bocavirus from GenBank dated September 2010. Sequences were firstly screened to exclude patented and artificial mutants, and then aligned in the ClustalW program [10]. The alignment was manually adjusted for the correct reading frame. Sequences showing less than 1% divergence from each other were considered as the same. The remaining 54 BoV genomes included one GBoV, one BPV, two PBoVs, three MVCs, and 47 HBoVs.

**Phylogenetic analysis**
Before phylogenetic analysis, multiple-alignment was performed in the ClustalW program. Phylogenetic trees were constructed using the neighbor-joining method and evaluated using the interior branch test method with Mega 4 software [11]. Percent bootstrap support was indicated at each node. GenBank accession no. was indicated at each branch.
Recombination Detection

The remaining 54 bocavirus genomes were re-aligned in the ClustalW program. Detection of potential recombinant sequences, identification of potential parental sequences, and localization of possible recombination break points were determined using the Recombination Detection Program (RDP)[12], GENECONV [13], BOOTSCAN [14], MaxChi [15], CHIMAERA [16], and SISCAN [17] methods embedded in RDP3 [18]. A Multiple-comparison-corrected P-value cutoff of 0.05 was used throughout.

Results and Discussion

Based on the 54 complete genomes, a phylogenetic tree was constructed (Figure 1). The genotypes of these bocavirus showed in the phylogenetic tree were consistent with the genotype information from the original sources. From the phylogenetic tree, we can see that HBoV2 and HBoV4 closely related and formed into one cluster; HBoV1 and HBoV3 clustered separately, and GBoV showed more related to HBoV1 than to the other HBoVs. Six potentially significant recombination events were found with a high degree of confidence (p value ≤ 4.4 × 10^-3) judged by the above-mentioned six recombination detection methods.

Figure 2 indicated the inter-genotype recombination events that occurred between HBoV1 and HBoV4, which lead to the recombinant cluster HBoV2. The BOOTSCAN plot of this recombination event was showed in Figure 2A, which used the lineages represented by NC_012729 and EU984244 as the parental strains, leading to the daughter lineage represented by GU048665. To confirm this recombination event, the relevant strains were analyzed by neighbor joining trees using MEGA4. Figure 2B and 2C were two trees constructed on the non-recombinant region (position: 1-166 + 2967-end) and the recombinant region (position:167-2966), respectively. In Figure 2B, all HBoV2 strains clustered closely with HBoV3 and HBoV4, while Figure 2C showed discordant phylogenetic relationships compared with Figure 2B, where HBoV2 strains clustered with HBoV1. The phylogenetic analysis confirmed the recombination event that occurred between HBoV1 and HBoV4, leading to the recombinant HBoV2.

Figure 3A-E indicated the other 5 recombinants identified in the present study. The BOOTSCAN plot of each recombination event was showed on the left side, while phylogenetic tree based on recombination regions and non-recombination regions of parental strains in each recombination event were indicated on the right side. Figure 3A showed the recombinant occurred between two MVC strains, FJ899734[19] and AB158475 [20], leading to the recombinant NC_004442[3]. These recombination events provide the first evidence that recombination could happened among MVC strains. Figure 3B showed a recombination event happened intra-genotypely between two HBoV2 strains, FJ948860 and GU048662, leading to the recombinant FJ973558, another HBoV2 strain. FJ948860 and GU048662 were
Figure 2 Identification of recombination between HBoV1 and HBoV4, which led to the recombinant cluster of HBoV2. (A) BOOTSCAN evidence for the recombination origin on the basis of pairwise distance, modeled with a window size 200, step size 20, and 100 Bootstrap replicates; (B) Neighbor joining tree (2,000 replicates, Kimura 2-parameter distance) constructed using the non-recombinant region (Position 1-166 + 2967-end); (C) Neighbor joining tree (2,000 replicates, Kimura 2-parameter distance) constructed using the recombinant region (Position 167-2966).
Figure 3 Identification of the other 5 recombination events. The left part of each panel was BOOTSCAN evidence for the recombination origin on the basis of pairwise distance, modeled with a window size 200, step size 20, and 100 Bootstrap replicates. The right part of each panel were phylogenetic trees constructed based on recombination regions and non-recombination regions using Mega 4 software.
isolated from Australia in 2001 [7] and Thailand in 2007 according to the isolate information in GenBank, respectively, while FJ973558 was isolated from Tunisia in 2006 [8]. Figure 3C and 3D revealed two Bangpoo recombinants, GU048662 and GU048663, respectively. However, according to the isolate information in GenBank, GU048662, GU048663, and one of their parental isolates were isolated in the same lab in Thailand, therefore, it should be cared whether these recombination events non-naturally occurred by sequencing error and/or contamination in the lab. Figure 3E revealed recombination between the lineage represented by Nigeria HBoV2 isolate FJ973560 and Pakistan HBoV2 isolate lineage NC_012042, which led to a recombinant Pakistan isolate FJ170279. However, these three isolates were determined in the same lab [6,8], therefore, whether this recombination event non-naturally occurred by sequencing error and/or contamination should be confirmed by further study.

For RNA viruses, recombination has been shown to be an important feature of their evolution [21-23], and single-stranded DNA paraviruses have been shown to have a mutation rate approaching that of RNA viruses [24]. The HBoV sequences were previously considered to show very low protein and nucleotide sequence diversity [25-27]. Recently, however, other 3 different genotypes (HBoV2-4) were discovered, which reveals that the HBoVs own the property of high sequence diversity. The present study provide the evidence that recombination was observed through complete bacavirus genome analyses, including the likely inter-genotype recombination between HBoV1 and HBoV4, and intra-genotype recombination between HBoV2 variants. As a matter of fact, a recent study indicated HBoVs coinfection was detected [8], which will increase the chance of recombination between bocavirus strains.

**Conclusion**

Taken together, this study confirmed that recombination existed among bocavirus, including the likely inter-genotype recombination between HBoV1 and HBoV4, and intra-genotype recombination among HBoV2 variants. It is the first report revealing the recombination that occurred between minute virus strains of canine.

**Authors’ contributions**

WZ and XF conceived the study. WZ and XF wrote the paper. All authors performed recombination analysis and critically reviewed and approved the final manuscript.

**Competing interests**

The authors declare that they have no competing interests.

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