Molecular Detection of Parvovirus in Manchurian Chipmunks (*Tamias sibiricus asiaticus*) Captured in Korea

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Abstract
Cross-species transmission of viral diseases alarms our global community for its potential of novel pandemic events. Of various viral pathogens noted recently, parvoviruses have posed public health threats not only to humans but also to wild animals. To investigate the prevalence of parvoviruses in wild Manchurian chipmunks, here we detected genetic fragments of the nonstructural protein of parvovirus by polymerase chain reaction in wild Manchurian chipmunk specimens captured in the central and southern regions of South Korea and compared their sequence homology with references. Of a total of 348 specimens examined, chipmunk parvovirus (ChpPV)-specific gene fragments were detected with a 31.32% rate (109 chipmunks of 348) in their kidney, liver, lung, and spleen samples, and the chipmunks captured in Gangwon Province exhibited the highest positive rate (45.37%), followed by Gyeongsang (35.29%), Gyeonggi (31.03%), Chungcheong (20.00%), and Jeolla (19.70%). When compared with the reference sequences, a partial ChpPV sequence showed 97.70% identity to the previously reported Korean strain at the nucleic acid level. In the phylogenetic analysis, ChpPV exhibited closer relationship to primate parvoviruses, erythroviruses, and bovine parvovirus than to adeno-associated viruses. Despite limited sample size and genetic sequences examined in this study, our results underline the prevalence of ChpPV in Korea and emphasize the need of close surveillance of parvoviruses in wild animals.

Introduction
Parvoviruses are members of the family *Paroviridae*, which include 2 different subfamilies of the parvoviruses (*Parovirinae*) that infect vertebrates including humans and the invertebrate-infecting densoviruses (*Densovirinae*) [1, 2]. The subfamily *Parovirinae* is currently subdivided into 8 established genera: *Amdoparvovirus*, *Aevparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Depenparvovirus*, *Erythroparvovirus*, *Protoparvovirus*, and *Tetraparvovirus* [1]. They are small, nonenveloped viruses and retain a single-stranded DNA genome that encodes nonstructural (NS)1 protein, nucleoprotein 1, and viral protein (VP)1 and VP2 [1]. In susceptible hosts, the parvoviruses can cause clinical and subclinical diseases...
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[1, 3], such as reproductive failure, enteritis, panleukopenia, hepatitis, erythrocyte aplasia, immune complex-mediated vasculitis, and cerebellar ataxia [4–6]. The parvoviruses also cause clinically silent in rodents and, sometimes, persistent infections that are associated with prolonged virus release [7]. Given the increasing threat of parvovirus infection to laboratory and wild rodents [8–10], it is of great importance to comprehend the distribution and host range of the parvoviruses in nature.

Chipmunk parvovirus (ChpPV), a tentative member of the genus Erythroparvovirus [11], was initially identified from hepatitis B surface antigen-positive sera collected from Manchurian chipmunks in Korea [12]. The nucleotide and amino acid sequences of its genome showed high homology to those of human parvovirus B19 (B19V) and simian parvovirus, but ChpPV appeared to be distinct from other mammalian autonomous parvoviruses or adeno-associated viruses in a phylogenetic analysis [12, 13]. It was also described that the transcription profile of ChpPV in COS-7 cells showed unique features, compared with those of the Erythroparvovirus members [13]. However, like the NS1 protein of the B19V, the ChpPV NS1 was found to be a potent inducer of apoptosis [13], and protoparvoviruses, which include the rodent parvoviruses, are also known to induce cell deaths by the interaction with other cellular activators [14–18]. Unfortunately, however, only a limited number of ChpPV genomic sequences have been identified, and our understanding of ChpPV still remains limited. In this study, we investigated the prevalence of ChpPV in Korea using tissue samples of the wild chipmunks captured and discussed the phylogenetic relationship of ChpPV with other parvoviruses.

Materials and Methods

Specimens

A total of 348 wild Manchurian chipmunks (Tamias sibiricus asiaticus) were captured in the central and southern mountain regions of Korea in 2000: Chungcheong (CC, n = 70 chipmunks from 2 sites), Gyeonggi (GG, n = 87 from 5 sites), Gangwon (GW, n = 108 from 7 sites), Jeolla (JL, n = 66 from 5 sites), and Gyeongsang (GS, n = 17 from 1 site) provinces (Fig. 1). The kidney, liver, lung, and spleen were collected from the chipmunks after euthanasia and autopsy procedures and have been kept frozen at −70°C. The kidney, liver, lung, and spleen were collected from 7 sites), Jeolla (JL, n = 108 from 2 sites), Gyeonggi (GG, n = 87 from 5 sites), Gangwon (GW, n = 108 from 7 sites), Jeolla (JL, n = 66 from 5 sites), and Gyeongsang (GS, n = 17 from 1 site) provinces (Fig. 1). The kidney, liver, lung, and spleen were collected from the chipmunks after euthanasia and autopsy procedures and have been kept frozen at −70°C.

Molecular Detection of ChpPV

Chipmunk specimens were homogenized using TissueLyser II (Qiagen, Hilden, Germany), and genomic DNAs were extracted using the Exgene SV mini kit (Geneall, Seoul, Republic of Korea) following the manufacturer’s protocol. The eluted solutions of extracted DNAs were kept at −20°C and were directly used in polymerase chain reaction (PCR). All the samples were screened with 2 different sets of primers specific to the NS protein gene of ChpPV to confirm parvovirus infection. The primer sets were designed using the highly conserved regions of the NS protein in the genome, which has significant homology with those of other parvoviruses [12], and their sequences are as follows: PV-f1 forward primer 5′-AGC ACA CTA CAC CAG GC-3′ and PV-f1 reverse primer 5′-GAG ACC CCT GTT AGT TGC CC-3′ that detect the NS nucleotide regions 2,242–2,411 (169-bp fragment) and PV-f2 forward primer 5′-GGA CTC CGT CAC GTG CTT TA-3′ and PV-f2 reverse primer 5′-TTG GCT AGC ACC CAT TAG GC-3′ that detect the NS nucleotide regions 897–1,527 (630-bp fragment). Two independent PCR reactions with the PV-f1 and PV-f2 primer sets were performed in a final volume of 50 μl reaction mixture containing 2 μl of the extracted chipmunk DNAs, 5 μl of 10X Taq reaction buffer, 200 μM dNTP, 0.4 μM each primer (forward and reverse), and 1.25 μl of DiasterTaq polymerase (Solgent, Seoul, Republic of Korea) with nuclease-free distilled water. After the mixtures had been initially incubated at 95°C for 5 min, amplification was performed for 40 cycles (1 cycle includes denaturation at 95°C for 20 s, annealing at 55°C for 45 s, and elongation at 72°C for 5 min). The PCR products were separated and inspected by electrophoresis in 1.5% TBE agarose gel.

Sequencing and Genetic Analysis of ChpPV

The PCR products were purified using Expin™ Gel SV (Geneall) and commercially sequenced using BigDye™ Terminator v3.1 Cycle Sequencing Kit and ABI 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). The nucleotide sequences of each DNA fragment (169 bp and 630 bp, respectively) of ChpPV were investigated using online Blast Search on National Centre for Biotechnology Information, US National Library of Medicine (Bethesda, MD, USA). Using extension primers designed, a partial ChpPV sequence was obtained (1,389 nts in length) (online suppl. material; for all online suppl. material, see www.karger.com/doi/10.1159/000520388), and it was compared with the prototypic, reference sequences (GenBank Accession Nos. U86868 and GQ200736) using the ClustalW method implemented in MegAlign of DNASTAR (v5.03; Madison, WI, USA).

Phylogenetic Analysis of ChpPV

To compare the phylogenetic relationship of ChpPV with other parvoviruses, genomic sequences of the other Parovirinae viruses were collected from the GenBank database, based on the study by Chen et al. [13]. Twelve reference sequences (GenBank Accession Nos. AF043303, AF221122, AF406967, AY064476, AY386330, DQ000496, DQ335246, DQ335247, FJ214110, NC_004295, NC_006152, and U26342) were first aligned with the ChpPV sequences (ChpPV-1999 and ChpPV-2000) using MAFFT (v7.310) [19], and the 2 most common regions (region 1, between nucleotide residues 3,540 and 3,874, and region 2, between nucleotide residues 3,907 and 4,087 in the U86868 genome) in the NS protein gene were chosen for the phylogenetic analysis. For each NS region, a best-fit nucleotide substitution model (for region 1, 1,000 iteration).

10X Taq reaction buffer, 200 μ M dNTP, 0.4 μ M each primer (forward and reverse), and 1.25 U of DiasterTaq polymerase (Solgent, Seoul, Republic of Korea) with nuclease-free distilled water. After the mixtures had been initially incubated at 95°C for 5 min, amplification was performed for 40 cycles (1 cycle includes denaturation at 95°C for 20 s, annealing at 55°C for 45 s, and elongation at 72°C for 5 s) in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The final elongation reaction was performed at 72°C for 5 min. The PCR products were separated and inspected by electrophoresis in 1.5% TBE agarose gel.

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Results

For the molecular detection of ChpPV, the kidney, liver, lung, and spleen samples of 348 wild chipmunks (*Tamias sibiricus asiaticus*) (Fig. 1a) were investigated by PCR. If ChpPV genomic sequences were identified in at least 1 organ specimen, that individual chipmunk was considered as positive for parvovirus infection. As shown in Table 1, 109 chipmunks (31.32%) were positive for ChpPV, and the chipmunks captured in Gangwon (GW) Province exhibited the highest positive rate (45.37%), followed by those in Gyeongsang (GS, 35.29%), Gyeonggi (GG, 31.03%), Chungcheong (CC, 20.00%), and Jeolla (JL, 19.70%). Of the 109 ChpPV-positive specimens, 56 exhibited double positive for both primer sets (Fig. 1b). ChpPV was detected highest in the spleen (26.44% and
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13.79% for the primer sets 1 and 2, respectively), whereas only 18.10% and 8.91% of the lung samples were positive for the primer sets 1 and 2, respectively (Fig. 1c). The average positive detection rates for the primer sets 1 and 2 were 24.07% and 12.50%, respectively (Fig. 1c). More than 88% (97 out of 109 chipmunks) were found to be ChpPV positive in at least 2 different organs and 42.20% (46 out of 109 chipmunks) were positive for ChpPV in all the 4 tested organs (Fig. 1d).

Based on the ChpPV genetic sequences identified, a partial NS protein gene sequence (ChpPV-2000) was recovered (online suppl. material), and its sequence homology was compared with the prototypic ChpPV reference sequences registered in the GenBank database (Accession Nos. U86868 and GQ200736) [12, 13]. At the nucleic acid level, ChpPV-2000 exhibited 97.70% homology to both references, and only 2 nucleotides were found to be deleted (at 3,566 nucleotide residues in the references) or inserted (between 4,924 and 4,925 nucleotide residues in the references) (Table 2). Previously, the NS1 and VP1 of ChpPV exhibited distinct phylogenetic relationships to those of other parvoviruses [13]. In our ML trees (Fig. 2a, b), the NS gene sequences of ChpPV-1999 and ChpPV-2000 were also clustered separately from those of other parvoviruses. Given the phylogenetic grouping patterns, ChpPV appeared to be more closely related to primate parvoviruses (RhMPV and SPV), erythroviruses (A6 and V9), and bovine parvovirus (BPV) than with the adeno-associated viruses (AAV2, AAV5, and AAVGo) and canine minute virus (CMV). However, the ChpPV NS gene sequences showed less relationships to those of primate bocaparvovirus (PBoPV1) and bovine parvovirus 3 (BPV3) (Fig. 2a, b).

Discussion/Conclusion

Among DNA viruses, the parvoviruses exhibit the highest mutation rate, almost comparable to RNA viruses, and they are extremely recombination-prone viruses [22]. Given a wide variety of hosts of the parvoviruses, the genetic diversity of the parvoviruses is therefore extensive, and there might be often low homology between the different parvoviruses at nucleotide or protein levels. It might be associated why the various parvoviruses exhibit a wide range of subclinical or clinical diseases in their hosts [18]. Furthermore, host-switching events have been reported in the parvoviruses [23], and co-divergence of parvoviruses and their hosts has been also noticed [11]. For these reasons, it is of great importance to search further for new natural variant parvoviruses with altered genetic and antigenic properties.

The parvovirus was described in Manchurian chipmunks inhabiting in Korea, and their complete genome sequence (ChpPV-1999) was determined in 1999 [12]. Given the results of Yoo et al. [12], ChpPV-1999 exhibited 47% identity to the B19 virus. The putative capsid protein of ChpPV-1999 showed a >34% homology with those of the B19 virus and SPV, but only <20% with those of other parvoviruses [12]. Based on these results, we tried to investigate how ChpPV would be diverged from other parvoviruses. We first investigated the prevalence of ChpPV using the internal organ specimens of 348 wild Manchurian chipmunks and detected ChpPV DNA sequences. As presented in Table 1, the infection rate of ChpPV was shown as high as 31.32% (109/348) (Fig. 1).

Since a partial sequence has only been available in this study, we attempted to sequence the full genome of ChpPV-2000. Among the double-positive ChpPV specimens, we selected several samples to extend sequence reads of ChpPV, and viral DNA fragments identified in each step were used as templates for the design of next primers. However, we could not obtain consistent sequencing results, and only a 1,379-nucleotide ChpPV-2000 sequence

| Table 1. Detection rates of the ChpPV genomic sequences from wild chipmunks |
|-------------------------------|------------------|-------------------------|
| Region            | Sites, n | Captured chipmunks, n | % rate of ChpPV detection, % (n) |
| Chungcheong (CC) | 2        | 70                      | 20.00 (14)                   |
| Gyeonggi (GG)    | 5        | 87                      | 31.03 (27)                   |
| Gangwon (GW)     | 7        | 108                     | 45.37 (49)                   |
| Jeolla (JL)      | 5        | 66                      | 19.70 (13)                   |
| Gyeongsang (GS)  | 1        | 17                      | 35.29 (6)                    |
| Total            | 20       | 348                     | 31.32 (109)                  |

| Table 2. Sequence homology of ChpPV with the references |
|-----------------------------|------------------|------------------|
| GenBank Accession No.        | Sequence identity, n/N (%) | Gap, n/N (%) |
| U86868                      | 1,357/1,389 (97.70) | 2/1,389 (0.14) |
| GQ200736                    | 1,357/1,389 (97.70) | 2/1,389 (0.14) |
Fig. 2. Phylogenetic relationship of ChpPV and other parvoviruses. Using the sequence sets of 2 different NS gene regions (nucleotide residues between 3,540 and 3,874 (a) and between 3,907 and 4,087 in the ChpPV-1999 genome (b)), the ML phylogenetic trees of ChpPV and other parvoviruses were obtained. Bootstrap values were indicated next to the branches, and branch lengths were estimated by the number of substitutions per site. ChpPV sequences were indicated with red circles. The name of each virus was indicated with the GenBank accession numbers. Virus name abbreviations: A6, erythrovirus A6; AAV2, adeno-associated virus 2; AAV5, adeno-associated virus 5; AAVGo, adeno-associated virus Go.1; B19, B19 virus; BPV, bovine parvovirus; BPV3, bovine parvovirus 3; CMV, canine minute virus; PBoPV1, primate bocaparvovirus 1; RhMPV, rhesus macaque parvovirus; SPV, simian parvovirus; V9, human erythrovirus V9.
was recovered (online suppl. material). Using this sequence, we compared a sequence identity of ChpPV-2000 with the references, and based on the sequence homology analysis, ChpPV-2000 showed 97.70% sequence homology to both reference sequences (Table 2). In the phylogenetic analysis, it was also suggested that ChpPV might be more closely linked to the primate parvoviruses, erythroviruses, and BPV than with the adeno-associated viruses and CMV (Fig. 2). However, our recovered ChpPV-2000 is a partial sequence of the NS gene, which may be rather conserved than other genetic regions, and given the fact of high mutation rates of the family Paroviridae, compared to double-stranded DNA viruses, further investigation using the full-genome sequences of ChpPV should be done in the near future.

In this study, we investigated the prevalence of parvovirus infection in wild Manchurian chipmunks in Korea. Despite the limited sample size and genetic sequences analyzed, our results suggest that ChpPV may be widespread in wild chipmunks, which emphasizes the need of close surveillance of ChpPV and other parvoviruses in wild animals.

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**Statement of Ethics**

Experiments using wild Manchurian chipmunk specimens that were released for research purposes from the Korea Bank for Pathogenic Viruses were exempted from the research ethical review of the Institutional Animal Care and Use Committee (IACUC) of the Korea University College of Medicine because neither national laws (regulations) nor formal IACUC guidelines had been established at the time that the chipmunks were captured, and their collected internal organs were stored after autopsy. Nevertheless, we conducted the experiments using the protocols of small animal models in accordance with the recommendations from the Guide for the Care and Use of Laboratory Animals of the Animal and Plant Quarantine Agency of Korea.

**Conflict of Interest Statement**

The authors have no conflicts of interest to declare.

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**Author Contributions**

K.-J.S. contributed to the conception of the idea and design of this study. J.I.K. and K.-J.S. contributed to data collection and drafting and writing of the manuscript. K.P., H.S., and S.M.C. contributed to the laboratory studies and data collection and participated in the experiments. J.I.K. and K.-J.S. participated in data analysis and critical revision of the manuscript. All the authors read and approved the final manuscript.

**Data Availability Statement**

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

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