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Novel Avulaviruses in Penguins, Antarctica

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We identified 3 novel and distinct avulaviruses from Gentoo penguins sampled in Antarctica. We isolated these viruses and sequenced their complete genomes; serologic assays demonstrated that the viruses do not have cross-reactivity between them. Our findings suggest that these 3 new viruses represent members of 3 novel avulavirus species.

Avian paramyxovirus (APMV) belongs to the genus *Avulavirus*, family *Paramyxoviridae*. There are 13 recognized *Avulavirus* species, each with 1 member, called avian paramyxovirus 1–13 (APMV-1–APMV-13) (*1*). A putative APMV-14 also has been recently described but not yet formally recognized (*2*).

In the past decade, APMV-10 through APMV-14 have been reported because of the intensification of surveillance of avian influenza A viruses (*3*–*6*). Most of the avulaviruses have been detected in wild birds associated with mild or no clinical disease; only Newcastle disease virus (a strain of APMV-1), APMV-2, and APMV-3 might cause substantial disease in poultry (*7*). Previous studies have described the presence of APMV-1, APMV-3, APMV-7, APMV-8, and other as-yet uncharacterized avulaviruses in Antarctic penguins (*8*). As a part of avian influenza surveillance expeditions in Antarctica during 2014–2016, we identified 3 novel avulaviruses in Gentoo penguins.

Cloacal, fecal, and serum samples were collected from Gentoo penguins (*Pygoscelis papua*) and Adélie penguins (*P. adeliae*), at 7 Antarctic locations (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/7/17-0054-Techapp1.pdf) during 2014–2016. Diagnostic tests, virus isolation, and serologic assays confirmed the identity of these paramyxoviruses (online Technical Appendix).

We successfully isolated virus from 12 cloacal samples from Gentoo penguins on Kopaitic Island; these viruses showed positive hemagglutination titers ranging from 4 to 128 hemagglutination units. From these 12 isolates, only 5 were further confirmed by reverse transcription PCR and Sanger sequencing (*9*), suggesting the presence of new avulaviruses. All PCR-positive isolates were pooled and submitted for next-generation sequencing by using MiSeq 250 paired cycle run (Illumina, San Diego, CA, USA) (*10*).

By using next-generation sequencing, we obtained the genomic sequences of 3 novel avulaviruses that were

named as follows: Antarctic penguin virus A (APVA), Antarctic penguin virus B (APVB), and Antarctic penguin virus C (APVC) (GenBank accession nos. KY452442–KY452444). Genome lengths of the 3 new avulaviruses ranged from 14,926 to 15,071 nt. The 6 genes for avulaviruses (coding for the nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and RNA-dependent RNA polymerase protein) were identified in these virus genomes (online Technical Appendix Figure 2, panel A). The sequence assembly was validated by coverage mapping (online Technical Appendix Figure 2, panel B). The genomes described here are coding-complete; future experiments are needed to sequence the absolute terminus of the nontranslating region.

The 3 avulaviruses reported in this study showed 57%–60% genome-wide nucleotide identities to all other avulaviruses, as well as 32%–50% protein identities in the hemagglutinin-neuraminidase protein gene and 31%–48% in the fusion protein gene (online Technical Appendix Figure 2, panel C). These new avulaviruses have 64%–67% genome-wide identity among each other. Accordingly, phylogenetic analyses (whether conducted by using genomes or specific genes) revealed that the new viruses form a monophyletic cluster with APMV-1, APMV-9, APMV-12, and APMV-13 (Figure; online Technical Appendix Figure 3). Recently, a cutoff of ≤60% identity of nucleotide distance on whole genome has been proposed to differentiate avulaviruses (*3*); however, APMV-12 and APMV-13 and these 3 newly discovered viruses have higher identity. Thus, we suggest that this criterion requires further validation.

Phylogenetic analysis and pairwise comparison suggests that APVA, APVB, and APVC might each represent novel avulavirus species, which we recommend naming *Avian avulavirus 15*, *16*, and *17*, respectively (pending approval by the International Committee on Taxonomy of Viruses). We performed a hemagglutination inhibition assay by using APMV-1, APMV-2, APMV-3, APVA, and APVC antisera against isolates confirmed. No cross-reactivity was observed between APVA, APVB, and APVC. These viruses also did not show cross-reactivity against APMV-1, APMV2, and APMV-3 antisera. Antigenic results support the idea that novel viruses are 3 distinct species.

We observed cytopathic effects during infection of MDBK cells and Vero cells in all isolates evaluated. These effects were characterized by cell rounding and detachment of the monolayer, but syncytia were not evident (online Technical Appendix Figure 4).

We also performed a hemagglutination inhibition assay by using APVA and APVC viruses. Three serum samples from Adélie penguins from Kopaitic Island reacted against APVC (titers 10–40), and 1 reacted against APVA (titer 40) (online Technical Appendix Table). This result suggests that these novel avulaviruses can also infect Adélie penguins.

We report the successful virus isolation and whole-genome sequencing of avulaviruses in Antarctic penguin populations. Our analyses show that these viruses are genetically and antigenically divergent, indicating that Antarctic

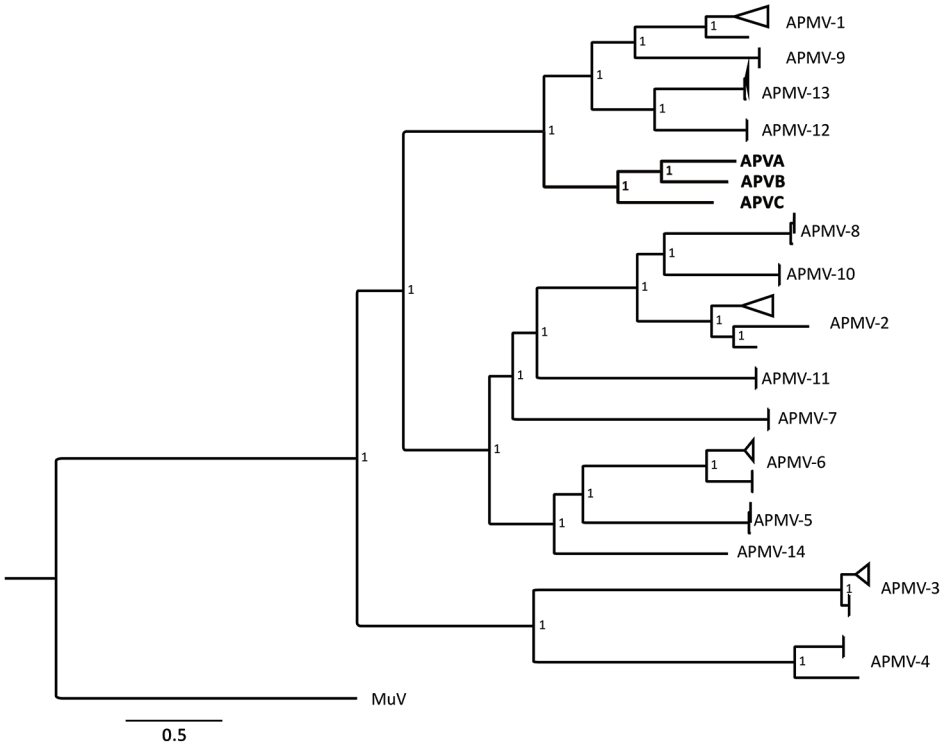

Figure. Bayesian phylogenetic tree based on concatenated nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and RNA-dependent RNA polymerase protein gene sequences of 80 avulaviruses analyzed in a study of avulaviruses in penguins, Antarctica. Mumps virus was used as outgroup. Bold indicates the 3 novel viruses isolates in this study. The best-fit model of nucleotide substitution was generalized time reversible plus gamma plus invariant sites. The analysis was considered complete if the average SD of the split frequencies was <0.01 and effective sample size was >200. The values represent the posterior probabilities of each node. Scale bar indicates nucleotide substitutions per site. APMV, avian paramyxovirus; APVA, Antarctic penguin virus A; APVB, Antarctic penguin virus B; Antarctic penguin virus C; MuV, mumps virus.

penguins harbor multiple avulaviruses. An important limitation is that the new viruses were not tested serologically against APMV-4 through APMV-13; however, genetic and antigenic differences between the new viruses support the idea that they are new species.

These data suggest that in Antarctica a much greater diversity of avulaviruses exists than previously recognized. Therefore, additional studies to evaluate the presence of these new viruses in other birds in Antarctica are needed to better understand the ecology and transmission of avulaviruses in this pristine environment.

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Rickettsia sibirica mongolitimonae Infection, Turkey, 2016

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In 2016, *Rickettsia sibirica mongolitimonae* was diagnosed for a man in Turkey. He had been bitten by a *Hyalomma marginatum* tick, from which PCR detected rickettsial DNA. Sequence analysis of the DNA identified *R. sibirica mongolitimonae*. Immunofluorescence assay of patient serum indicated *R. conorii*, which cross-reacts. PCR is recommended for rickettsiosis diagnoses.

The first case of human infection with *Rickettsia sibirica mongolitimonae* was reported in France in 1996 (*1*). The infection is called lymphangitis-associated rickettsiosis

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Novel Avulaviruses in Penguins, Antarctica

Technical Appendix

Materials and Methods

Collection Samples

A total of 262 cloacal swabs, 83 fecal samples, and 138 blood samples were collected from two penguin species, Gentoo (*Pygoscelis papua*) and Adélie penguin (*P. adeliae*) (INACH RT 12–13), located in seven Antarctic stations during three scientific expeditions from 2014 to 2016. Blood samples were centrifuged at 800 g by 10 min and obtained sera were used for the hemagglutination inhibition assay (HIA) using isolated viruses from the present study. All samples tested negative to Influenza A virus (FLUAV) and Newcastle disease virus (NDV) using Real-Time Quantitative RT-PCR protocol. All protocols were approved by the Animal Ethics Committees of University of Chile (Pro. No. 150430002 and No. 02–2016) and University of Concepcion (Protocol No. CBE-48–2013).

Laboratory Tests

Cloacal and fecal samples were inoculated in SPF embryonic chicken eggs, and propagated Vero and MDBK (Madin Darby Bovine Kidney) cells to evidence cytopathic effect. Hemagglutination assay (HA) was used to detect hemagglutinant agents.

Cross reactivity was evaluated by hemagglutination inhibition assay (HIA) using APA, APC, APM-1, APMV-2 and APM-3 antisera. Briefly, APVA and APVC viruses were propagated and inactivated vaccines were prepared. To generate the antisera 2 guinea pigs per virus were immunized. Sera were collected after day 28. APMV-1, APMV-2 and APMV-3 antisera were kindly provided by Dr. Goyal at University of Minnesota.

Specific HIA was done with Antarctica penguin virus A and C isolated and tested against the sera obtained from Adélie penguins from Kopaitic Island.

Phylogenetic Analysis

Sequence alignment was performed by using MAFFT (*1*). Nucleotide sequence from each protein were aligned and then concatenated for further genetic and phylogenetic analyses. Maximum likelihood and Bayesian phylogenetic trees were inferred from sequence alignments by using PhyML 3.0 (*2*) and MrBayes v3.2.5 (*3*), respectively. The best-fit model of nucleotide substitution was identified by jModelTest (*4*). The robustness of ash analyses was assessed by bootstrap resampling process of 1,000 replications and when the average standard deviation of the split frequencies form the Markov chain Monte Carlo analysis was <0.01.

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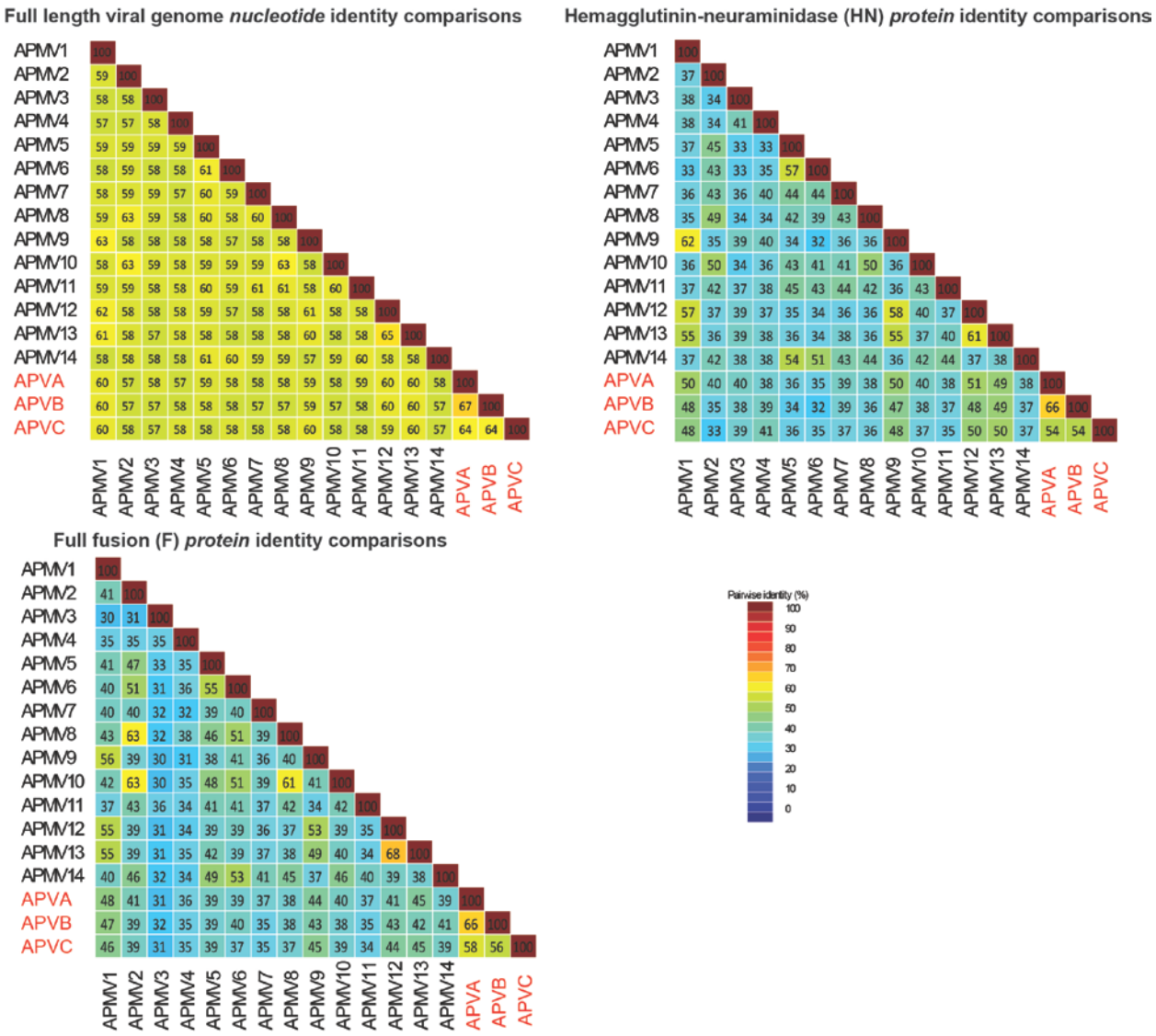
Technical Appendix Table. Sample sites and occurrence of avulaviruses in Antarctic penguins

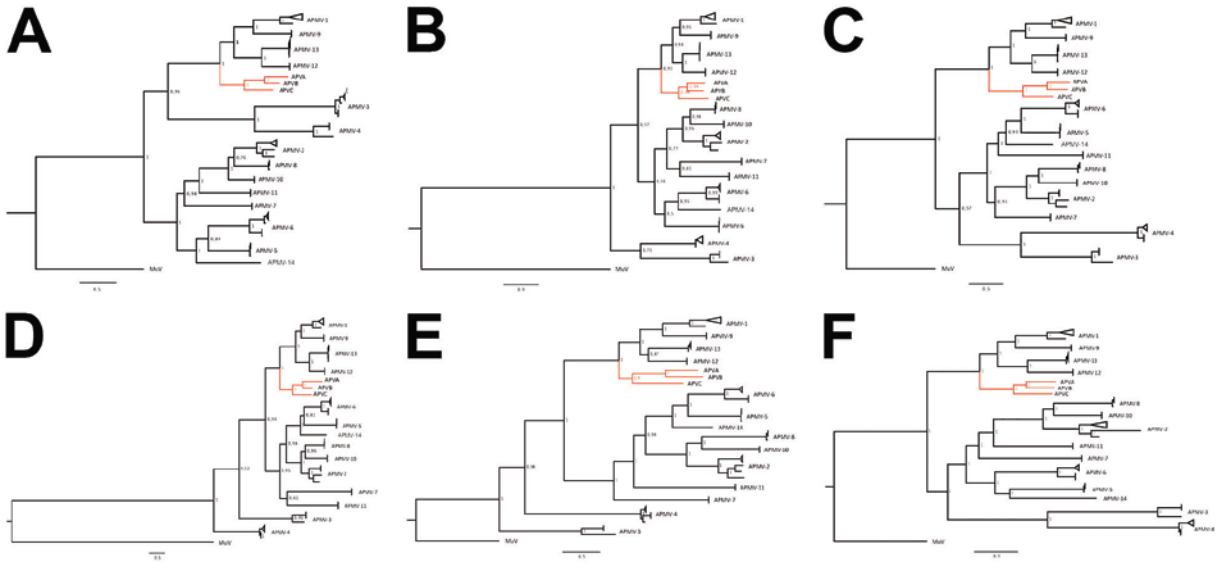
	Gentoo penguin samples				Adélie penguin samples		
Location	Cloacal swab	Environmental sample	HA positive	RNA detected	Sera	HI Virus A	HI Virus C
Ardley Island	–	–	–	–	28	–	–
Arctowski Base	–	–	–	–	29	–	–
Kopaitic Island	132	–	12	5	81	3	1
Dorian Bay	–	22	–	–	–	–	–
Pleneau Island	–	44	–	–	–	–	–
Brown Base	–	17	–	–	–	–	–
*G.G.V. Base	130	–	–	–	–	–	–
Total	262	83	12	5	138	3	1

*Gabriel Gonzalez Videla.

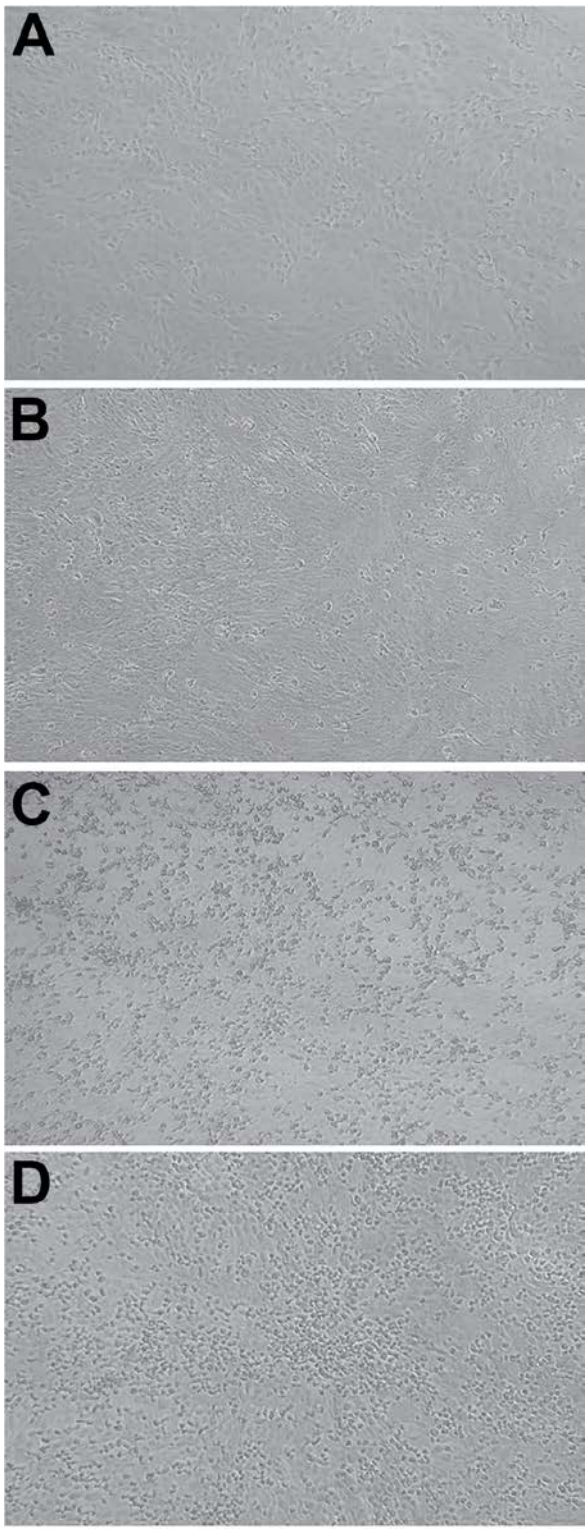
Technical Appendix Figure 1. Sampling locations on the Antarctica Peninsula. Orange dot indicate Kopaitic Island, location positive for Antarctic penguin avulaviruses.



Technical Appendix Figure 2. Genetic characteristics of penguin avulaviruses. Gene acronyms were described in the main text. A) Genome organization of penguin avulaviruses, Newcastle disease virus is include as an example B) Sequence coverage of the three penguin avulavirus coding-complete genomes obtained by next generation sequencing, no misalignment was detected in mapping because the sequence identities between all three viruses were substantial C) Pairwise comparisons between the penguin avulaviruses and other avulaviruses. Each number represents the pairwise nucleotide or amino acid identities between the corresponding taxa.



Technical Appendix Figure 3. Bayesian phylogenetic tree by gene using 80 avulaviruses reference sequences. The best-fit model of nucleotide substitution was GTR+I+G. The values represent the posterior probabilities of each node. Antarctic penguin viruses are colored in red. Mumps virus (MuV) was used as outgroup. A) Nucleoprotein gene B) Phosphoprotein gene C) Matrix gene D) Fusion protein gene E) Hemagglutinin-Neuraminidase gene and F) RNA-dependent RNA polymerase gene



Technical Appendix Figure 4. Cytopathogenic effect of MDBK cells with Antarctic penguin virus A and C in absence of trypsin (200x) A) MDBK cells mock infected B) MDBK cells infected by Antarctic penguin virus A at 1 dpi C) MDBK cells infected by Antarctic penguin virus C at 1 dpi D) MDBK cells infected by Antarctic penguin virus C at 3 dpi.