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Detection of a respiratory coronavirus from tissues archived during a pneumonia epizootic in free-ranging Pacific harbor seals *Phoca vitulina richardsii*

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ABSTRACT: In June 2000, 21 adult harbor seals *Phoca vitulina richardsii* were found dead along a localized section of the central California coast. Necropsy of 5 fresh carcasses revealed pulmonary congestion, consolidation, and hemorrhage. Histopathological changes in lungs from 2 of these seals included a necrotizing lymphocytic and histocytic lobar pneumonia with intra-lesional bacteria. A coronavirus (CoV) was detected in archived tissues from 1 of the 5 seals via a degenerate PCR for nidoviral RNA-dependent RNA polymerase (RdRp), and was subsequently confirmed via specific PCR. Based on the partial RdRp sequence, the CoV was identified as a novel, divergent member of the CoV group 1a. The virus is tentatively named harbor seal coronavirus (HSCoV). The clinical significance of HSCoV and its involvement in the etiology of the epizootic pneumonia and deaths of the harbor seals is uncertain.

KEY WORDS: Harbor seal · *Phoca vitulina richardsii* · Coronavirus · Pneumonia

INTRODUCTION

Since the emergence and discovery of the coronavirus (CoV) associated with human severe acute respiratory syndrome (SARS) in 2002, CoVs have gained considerable notoriety. The SARS virus is thought to be of zoonotic origin from a wild animal reservoir (Li et al. 2005). This has led to a renewed interest in CoVs of animals, and a need for a better understanding of CoV transmission among species and clarification of pathogenesis of CoV infections (Saif 2004). Since 2002, a number of novel animal CoVs have been detected from both domesticated animals and wildlife (Carrington et al. 2008, Decaro & Buonavoglia 2008, Woo et al. 2009).

CoVs are a genus of enveloped viruses with a linear positive stranded RNA genome. They are classified together with the genus *Torovirus* in the family *Coronaviridae* of the order *Nidovirales*. The genus *Coronavirus* has been divided into 3 groups (González et al. 2003). It has been proposed that Group I be subdivided into Groups 1a and 1b (Woo et al. 2007); Group 1a has only been found in laurasiatherian mammal hosts, primarily those from the Carnivora, whereas Group 1b has been found in both laurasiatherian and euarchontoglire mammal hosts. It has been proposed that Group 2 be divided into the following groups: Group 2a, which has been found in both laurasiatherian and euarchontoglire mammal hosts; Group 2b, which contains SARS and viruses from microchiropteran bats; Group 2c, from mi-

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crochiropteran bat hosts; and Group 2d, from megachiropteran bat hosts (Woo et al. 2007). It has been proposed that Group 3 be divided as follows: Groups 3a, from avian hosts; Group 3b, which contains a coronavirus from a beluga whale *Delphinapterus leucas*, the only coronavirus identified in a marine mammal to date; and Group 3c, which contains viruses from diverse avian hosts as well as one from an Asian leopard cat *Prionailurus bengalensis* that may represent recent host switching (Woo et al. 2009).

In late May and early June 2000, 21 adult harbor seals *Phoca vitulina richardsii* were found dead along a localized 10 mile (16 km) section of the central California coast at Point Reyes National Seashore. Upon postmortem examination of 5 relatively fresh carcasses, all seals had grossly abnormal lungs, and histological examination of 3 cases revealed severe pneumonia. A similar event with similar gross necropsy findings had occurred in 1997, when approximately 90 seals were found dead in the same area, but no conclusive etiology for the event was identified. In both events, a viral infection with secondary bacterial pneumonia was suspected. CoVs are recognized causes of enteric and respiratory infections that are often fatal in young animals. Here we report on the identification of a novel respiratory coronavirus from one of these harbor seals. This novel coronavirus of wildlife is a novel member of Group 1a and is distinct from, but most closely related to the CoVs of ferrets, cats, dogs, and swine.

MATERIALS AND METHODS

Animals and samples. On 26 May 2000, a harbor seal (HS D62) was found dead at McClure's Beach, Point Reyes National Seashore, in central California, and a field necropsy was conducted. Tonsil, stomach, cerebellum, brain stem, and lymph node were collected in 10% formalin for histological analysis. An additional 4 adult harbor seals were found dead on 6 June 2000. Two harbor seals, designated as HS 4 and 5, were fresh dead. Complete sample sets were collected in 10% formalin for histological examination, and the lung was frozen at –80°C. Two other seals, HS 1 and 6, were moderately decomposed, and the lung only was collected for archiving at –80°C . On 8 June, a sixth fresh dead seal was found, HS 17, and tissues were collected for histology in 10% formalin, and lung archived at –80°C.

Degenerate and specific PCR. In 2008 RNA was extracted from the archived frozen lungs of the 5 harbor seals (HS 1, 4, 5, 6, and 17) using the RNeasy Mini Kit (Qiagen). RT-PCR for a conserved region of the coronaviral RNA-dependent RNA polymerase (RdRp) was performed using the OneStep RT-PCR Kit (Qiagen) using previously described primers 2Bp and 4Bm (Stephensen et al. 1999) on all 5 RNA extracts. The mixtures were amplified in a thermal cycler (PX2, Thermo Hybaid) with an initial reverse transcription at 40°C for 45 min, 95°C for 15 min, followed by 5 cycles of denaturation at 94°C for 1 min; annealing at 40°C for 2 min, DNA extension at 72°C for 1 min, then 40 cycles of denaturation at 94°C for 1 min; annealing at 46°C for 1.5 min, DNA extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR products were resolved in 1% agarose gels. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed using the Big-Dye Terminator Kit (Perkin-Elmer) and the above second-round primers, and analyzed on ABI 3130 automated DNA sequencers at the University of Florida Interdisciplinary Center for Biotechnology Research Sequencing Facilities. All products were sequenced at least 3 times in both directions. Primer sequences were edited out prior to further analyses. Based on this edited sequence, the specific forward primer harbor seal coronavirus (HSCoV)-F1 5′-CCA AGG CTA GGG CTC GCA CT-3′ and specific reverse primer HSCoV-R1 5′-CAT TAT CTA CGC CTA AAG TGA G-3′ were designed. Specific RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen) using the specific primers HSCoV-F1 and HSCoV-R1 on all 5 RNA extracts. The mixtures were amplified with an initial reverse transcription at 40°C for 45 min, 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 1 min; annealing at 51°C for 1 min, DNA extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR products were resolved in 1% agarose gels. The bands were excised and purified using the QIAquick gel extraction kit (Qiagen). Direct sequencing was performed as above.

In addition to CoVs, all 5 samples were tested using PCR assays for other virus groups that are known to induce syncytial cell formation. Primers and protocols for the broad detection of herpesviruses, paramyxoviruses, and reoviruses were used as previously described (Vandevanter et al. 1996, Nollens et al. 2007, Wellehan et al. 2009).

Phylogenetic analysis. The sequences generated using primers 2Bp and 4Bm were compared to those in GenBank (National Center for Biotechnology Information), EMBL (Cambridge, UK), and DDBJ (Mishima, Shizuoka, Japan) databases using BLASTN (Altschul et al. 1997). Homologous 208 to 220 nucleotide sequences and predicted amino acid sequences of nidoviral RdRp were aligned using 3 methods: ClustalW2 (Larkin et al. 2007), T-Coffee (Notredame et al. 2000), and MUSCLE (Edgar 2004). Bayesian analyses of the predicted nucleotide alignment were performed using MrBayes 3.1 (Ronquist & Huelsenbeck 2003) with gamma distributed rate variation and a proportion of invariant sites,

and a general time reversible model. The first 10% of 1 000 000 iterations were discarded as a burn in. Maximum likelihood (ML) analyses of each alignment were performed using PHYLIP (Phylogeny Inference Package, version 3.66) (Felsenstein 1989), running each alignment using the DNAml program with global rearrangements, 5 replications of random input order, and gamma plus invariant rate distributions. The values for the gamma distribution were taken from the Bayesian analysis. Equine torovirus (GenBank accession no. X52374), a member of the family *Coronaviridae* outside of the genus *Coronavirus*, was designated as the outgroup. The alignment was used to create data subsets for bootstrap analysis to test the strength of the tree topology (200 re-samplings) (Felsenstein 1985).

RESULTS

Gross and histological findings

HS 1, 5, 6, and 17 were adult males; HS 4 and 62 were adult females. Blubber depths ranged from 12 to 20 cm, indicating moderate nutritional status. Each seal had severely congested lungs, with areas of consolidation and hemorrhage and blood-tinged foam in the trachea and bronchi. The mediastinal, tracheobronchial, and submandibular lymph nodes and tonsils were grossly enlarged and hemorrhagic. The gastrointestinal tracts were empty, with traces of feces in the rectum. The histopathological findings in HS D62 included mild multifocal ulcerative gastritis, acute congestion of the cerebellum and brainstem, germinal center depletion and/or histiocytosis with syncytial cell formation in the tonsils, and a peripheral lymph node (Fig. 1). The lung was not submitted for histologic examination. Syncytial cells did not have either intranuclear or intracytoplasmic inclusion bodies. HS 4, 5, and 17 had acute severe necrotizing lymphocytic and histiocytic lobar pneumonia without syncytia, fibrinoid vasculitis, thrombosis, septal edema, and intralesional mixed gram-positive and gram-negative bacteria. In addition, HS 17 had evidence of gram-negative bacterial sepsis. *Pseudomonas aeruginosa* was cultured from all 3 of these seals (Gaffney et al. 2008). As was the case in D62, germinal centers in lymphoid tissues were often hyalinized, histiocytic or depleted. Rare syncytia were observed in the lymph nodes of HS 5, but not HS 4 or HS 17.

Degenerate and specific PCR

PCR amplification using the degenerate primer pair 2Bp and 4Bm resulted in a 208 bp product, after editing out of primer sequences, in 1 of the 5 harbor seal lung

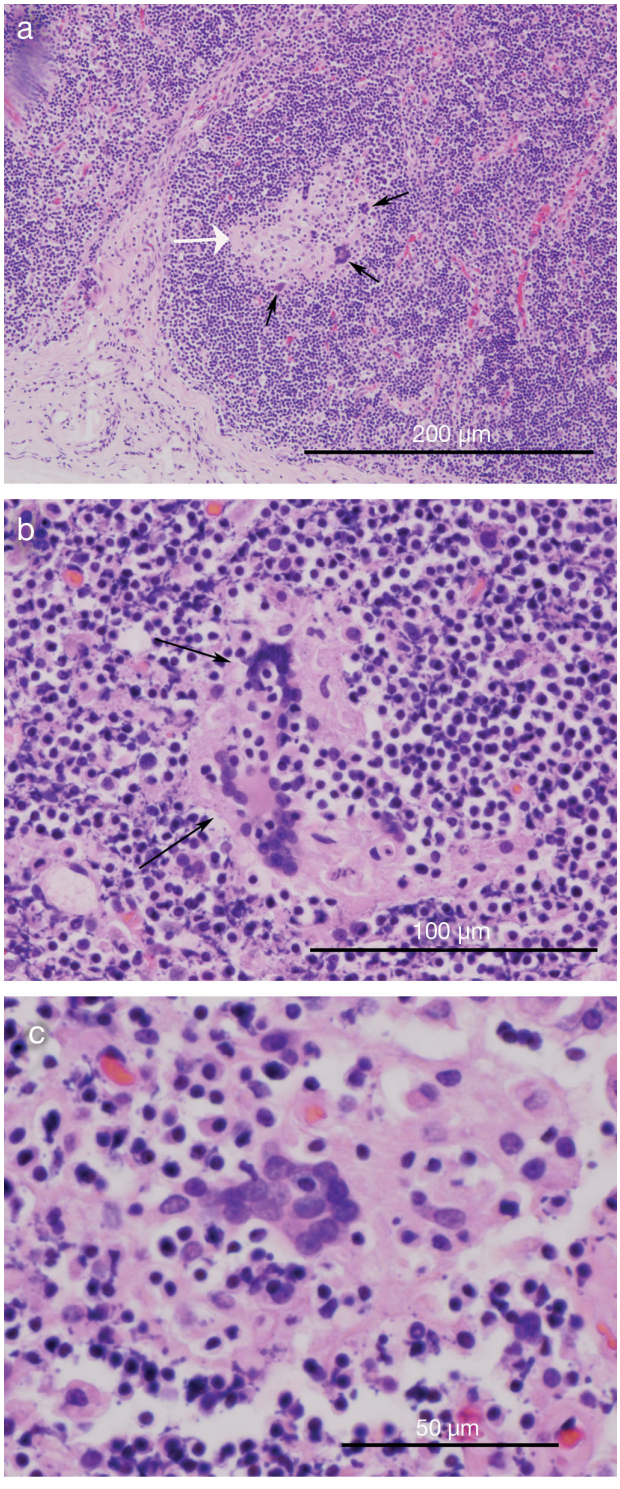

Fig. 1. *Phoca vitulina richardsii*. (a) Spleen from a harbor seal (HS D62) that died during the 2000 outbreak. Note the germinal center depletion (white arrow) and the presence of multinucleated syncytial cells (black arrows) (H&E). (b) Tonsil from HS D62. Note the multinucleated syncytial cells (arrows) within a germinal center (H&E). (c) Tonsil from HS D62. Note the multinucleated syncytial cell in the depleted germinal center of a secondary follicle (H&E)

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           1                                                          60
EqToro     ATTGTCAGCTAAAGCAAGAGCAAGAACAGTTTCCTCATGCTCATTTATAGCATCAACAAT
WhBreamV   AAGACAAAAGAAAACCCGCGTCCGCACACTAGGAGGTTCATCATTCATCACATCATCAAT
HSCoV      CGTCTCTGCCAAGGCTAGGGCTCGCACTGTTGGTGGCGTGTCACTCCTGTCCACAATGAC
FelCoV     TATTTCTGGAAAGGCAAGAGCTCGTACTGTCGGAGGAGTGTCGCTTCTCTCAACAATGAC
FerECoV    CATATCAGGTAAGGCACGAGCTCGTACTGTTGGTGGTGTGTCACTTTTGTCAACTATGAC
MuniaCoV   GATTTCAGCAAAAGATCGTGCTCGCACTGTTGCAGGTGTTTCTATTATAAGCACCATGAC
TGEV       TATTTCTGGTAAGGCAAGAGCTCGTACAGTAGGAGGAGTTTCACTTCTTTCTACCATGAC
HuCoVNL    TATTAGTGGTAAAGAACGTGCTAGAACTGTTGGTGGTGTTTCTCTGTTGTCCACAATGAC
CanCoV     TATTTCTGGAAAGGCTAGAGCTCGTACAGTAGGAGGAGTTTCACTCTTTTCTACTATGAC
GooseCoV   TATATCTGCAAAGAATAGGGCTCGTACCGTTGCAGGTGTATCTATATTATCTACTATGAC
IBV        CATATCCGCGAAAAATAGAGCGCGTACAGTGGCAGGTGTGTCTATCCTTTCTACTATGAC
PigeonCoV  AATTTCTGCTAAAAATAGAGCAAGAACTGTTGCTGGTGTTTCAATCCTTTCTACTATGAC
MuHV       TATTAGTGCTAAGAATAGGGCCCGCACCGTTGCTGGTGTCTCTATTCTCAGTACTATGAC
BelugaCoV  CATTTCTGCTAAGTCACGTGCACGTACTGTTGCAGGTGTGTCAATTCTTAGCTCGATGAC
BovCoV     TATTAGTGCTAAGAATAGAGCCCGCACTGTTGCTGGTGTTTCCATACTTAGTACTATGAC
BatCoV512  CATTAGTGGTAAAGATCGAGCACGCACTGTTGGTGGTGTTTCACTCTTGTCTACCATGAC
ALC        AATTTCAGCCAAGGATCGTGCTCGCACTGTGGCAGGAGTGTCTATAATTAGCACCATGAC
BatCoVHKU6 TATTAGTGGCAAAGAACGTGCACGTACTGTTGGTGGTGTTTCCTTGCTCTCCACTATGAC
BatCoVHKU9 TATAAGTGCTAAAAATCGCGCTCGCACTGTAGCTGGTGTTTCTATAGCATCTACTATGAC
BatCoVHKU4 TATTAGTGCTAAGAATAGAGCACGTACTGTAGCAGGCGTGTCTATACTTAGCACAATGAC
BatCoVHKU5 TATTAGTGCTAAGAATAGAGCCCGGACTGTTGCCGGTGTGTCGATTCTTAGTACAATGAC
HumCoVHKU1 TATCAGTGCTAAGAATAGAGCTCGCACTGTAGCAGGTGTTTCTATTCTTAGTACTATGAC
SARSCoVAS  CATTAGTGCAAAGAATAGAGCTCGCACCGTAGCTGGTGTCTCTATCTGTAGTACTATGAC
PEDV       TATTAGTGGCAAAGAACGTGCACGCACAGTGGGTGGTGTCTCGCTTTTGTCAACCATGAC

           61                                                        120
EqToro     ATTTAGATTTGCACATAAACCAGTAACATCTAAAATGGTTGAAGTAGCACAGAATTCAGG
WhBreamV   CTTCCGCATGCTCCACAAACCAGTCACCAACAAAATGGTCCAAACAGCCCAAGCAAACAT
HSCoV      TACCAGACAGTACCATCAAAAGCACCTCAAGTCAATCGCAGCTGCGCGTAATGCTA----
FelCoV     AACGAGACAGTACCATCAGAAGCATTTAAAATCCATCGCTGCTACACGTAATGCCA----
FerECoV    CACAAGACAGTATCATCAGAAACACTTAAAGCCTATTGCCGCCATGCGTAACGCTA----
MuniaCoV   TAACAGGCAGTACCATCAGAAGATTCTGAAATCGATTTCACTTGCACGCAATCAGA----
TGEV       TACGAGACAATATCATCAGAAGCATTTGAAGTCAATTGCTGCAACACGCAATGCTA----
HuCoVNL    CACAAGACAATACCATCAAAAACATCTTAAATCCATTGTTAATACACGCAATGCCA----
CanCoV     TACGAGACAATACCACCAGAAGCATTTAAAGTCAATTGCTGCAACACGCAATGCCA----
GooseCoV   TAATAGACAGTATCACCAGAAGGTGCTTAAGTCTATTGTTAACACACGTAATGCAG----
IBV        TAATAGGCAGTTTCATCAGAAGATTCTTAAGTCTATAGTCAACACTAGAAACGCTC----
PigeonCoV  CAATAGGCAATATCATCAAAAGGTGCTTAAGTCTATTGTTAATACTAGAAATGCTC----
MuHV       TGGCAGAATGTTTCATCAAAAGTGTCTAAAGAGTATAGCAGCTACTCGCGGTGTTC----
BelugaCoV  AAACCGCCAATTTCATCAGAAGTGCCTCAAATCTATTGTGAACACGCGGAATGCAA----
BovCoV     TGGCAGAATGTTTCATCAAAAATGTTTGAAAAGTATAGCAGCTACACGTGGTGTTC----
BatCoV512  TACTAGACAATACCACCAGAAACACCTGAAATCTATTGTCAACACTAGAGGTGCTT----
ALC        TAACAGGCAGTACCATCAAAAGATGCTGAAATCTATTTCACTTGCACGCAATCAGA----
BatCoVHKU6 CACTAGACAGTATCACCAGAAGCATTTGAAGTCTATTGTCAACACGCGCGGTGCGT----
BatCoVHKU9 AAATAGACAATTTCATCAGAAGATGCTTAAGTCTATAGCTGCAGCACGTGGTGCAT----
BatCoVHKU4 TAATCGTCAATATCACCAGAAGATGTTGAAGTCTATGGCTGCAACGCGTGGAGCCA----
BatCoVHKU5 TAATCGTCAATACCACCAGAAAATGCTTAAGTCTATGGCTGCTACGCGGGGTTCCA----
HumCoVHKU1 AGGCCGAATGTTCCATCAAAAATGTTTGAAGAGTATAGCAGCTACCCGAGGTGTTC----
SARSCoVAS  AAATAGACAGTTTCATCAGAAATTATTGAAGTCAATAGCCGCCACTAGAGGAGCTA----
PEDV       CACTCGGCAGTATCATCAGAAACACCTTAAATCCATAGTTAATACTAGGGGCGCTT----
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Fig. 2. Nucleotide alignment of the partial harbor seal coronavirus (HSCoV; 208 bp; in **bold**) and homologous coronavirus RNA-dependent RNA polymerase (RdRp) sequences. Conserved nucleotides are indicated. Sequences retrieved from GenBank include Equine torovirus (EqToro; DQ310701), White bream virus (WhBreamV; DQ898157), Feline coronavirus (FelCoV; GenBank accession no. DQ848678), Ferret enteric coronavirus (FerECoV; DQ340560), Munia coronavirus (MuniaCoV; FJ376622), Transmissible gastroenteritis virus (TGEV; DQ811789), Human coronavirus NL (HuCoVNL; AY518894), Canine coronavirus (CanCoV; EU856362), Goose coronavirus (GooseCoV; AJ854123), Infectious bronchitis virus (IBV; AY851295), Pigeon coronavirus (PigeonCoV; AY851295), Murine hepatitis virus (MuHV; AF208067), Beluga whale coronavirus (BelugaCoV; NC_010646), Bovine coronavirus (BovCoV; DQ811784), *Scotophilus* bat coronavirus 512 (BatCoV512; DQ648858), Asian leopard cat coronavirus (ALC; EF584908), *Myotis* bat coronavirus HKU6 (BatCoVHKU6; DQ249224), *Rousettus* bat coronavirus HKU9 (BatCoVHKU9; EF065513), *Tylonycteris* bat coronavirus HKU4 (BatCoVHKU4; DQ249214), *Pipistrellus* bat coronavirus HKU5 (BatCoVHKU5; DQ249217), Human coronavirus HKU1 (HumCoVHKU1; AY597011), SARS coronavirus (SARSCoVAS; AY427439), Porcine epidemic diarrhea virus (PEDV; AF353511)

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           121                                                       180
EqToro     TGGCTTTTGCCTCATTGGTGTATCCAAGTATGGTTTAAAGTTTTCAAAGTTTTTGAAAGA
WhBreamV   CGGCCCCTTTCTAATTGGAATTTCAAAATTCAACCTCGGATTTCACAAATACCTCTCCGC
HSCoV      -----CAGTAGTCATAGGGACCACAAAGTTCTATGGCGGCTGGGAYGCCATGTTGAAAAA
FelCoV     -----CTGTTGTTATAGGAACTACCAAGTTCTATGGTGGCTGGGATAACATGTTAAAGAA
FerECoV    -----CAGTTGTCATTGGTACAGCCAAGTTTTACGGCGGATGGGACGATATGTTAAAGAA
MuniaCoV   -----CTATAGTAATAGGCACTACCAAGTTTTATGGTGGTTGGGATAATATGTTACGTAG
TGEV       -----CTGTGGTCATTGGTTCAACCAAGTTTTATGGTGGTTGGGACAATATGCTTAAAAA
HuCoVNL    -----CTGTTGTTATTGGTACTACCAAATTTTATGGTGGTTGGAATAATATGTTGCGTAC
CanCoV     -----CTGTGGTCATTGGTTCAACCAAGTTTTATGGTGGTTGGGATAACATGCTTAAAAA
GooseCoV   -----CTGTTGTTATCGGTACATCCAAGTTCTATGGAGGCTGGGACAACATGTTGCGCAA
IBV        -----CTGTAGTTATTGGAACAACCAAGTTTTATGGCGGTTGGGATAACATGTTGAGAAA
PigeonCoV  -----CTGTTGTTATAGGAACTACCAAGTTTTATGGTGGTTGGGATAACATGTTAAGAAA
MuHV       -----CTGTAGTTATAGGCACCACGAAGTTCTATGGCGGTTGGGATGATATGTTACGCCG
BelugaCoV  -----CCGTTGTAATAGGCACTACTAAGTTTTATGGAGGATGGGATAATATGCTCCGCAA
BovCoV     -----CTGTTGTTATAGGCACCACTAAGTTTTATGGCGGCTGGGATGATATGTTACGTCG
BatCoV512  -----CTGTTGTTATTGGAACCACTAAGTTCTATGGTGGTTGGGATAATATGCTCAAAAC
ALC        -----CCATCGTGATTGGAACAACCAAATTCTATGGTGGTTGGGACAACATGTTACGACG
BatCoVHKU6 -----CTGTTGTTATAGGTACTACAAAATTTTATGGTGGCTGGGATAATATGCTTAAGAC
BatCoVHKU9 -----CTGTTGTTATAGGTACCACTAAATTTTATGGAGGGTGGAATCGCATGCTTCGCAC
BatCoVHKU4 -----CGTGTGTCATAGGCACAACTAAATTTTATGGTGGTTGGGACTTTATGTTAAAAAC
BatCoVHKU5 -----CGTGCGTCATAGGGACCACTAAGTTCTATGGTGGTTGGGACTTTATGTTAAAAAC
HumCoVHKU1 -----CTGTTGTTATAGGAACCACTAAATTTTATGGTGGTTGGGACGATATGTTACGTCA
SARSCoVAS  -----CTGTGGTAATTGGAACAAGCAAGTTTTACGGTGGCTGGCATAATATGTTAAAAAC
PEDV       -----CGGTTGTTATTGGTACTACTAAGTTTTATGGTGGTTGGGACAATATGCTTAAGAA

           181                                  220
EqToro     TAAGTAC---GGTGCTATTGAGGGTTTTGATGTGTTTGGT
WhBreamV   TCACCATCCAAACGGAATAGAAGACTGTCAAGTTATGGGT
HSCoV      TCTCACT---TTAGGCGTAGATAATGGTTGCCTTATGGGT
FelCoV     TTTAATG---CGTGATGTAGACAATGGTTGTTTGATGGGA
FerECoV    TTTGATG---CGTGACGTTGATAATGGCTGTCTTATGGGT
MuniaCoV   GCTGATG---CACAATATCAACAATCCCATATTAGTGGGC
TGEV       TTTAATG---CGTGATGTTGATAATGGTTGTTTGATGGGA
HuCoVNL    TTTAATT---GATGGTGTTGAAAACCCTATGCTCATGGGT
CanCoV     TTTAATG---CGTGACGTTGATAATGGTTGTTTGATGGGA
GooseCoV   TCTTATT---GGTGGCGTTGATAATCCTATGCTTATGGGC
IBV        CCTTATT---CAGGGTGTTGAAGACCCGATTCTTATGGGT
PigeonCoV  CCTCATT---AAAGGTGTTGATGACCCCATTCTTATGGGA
MuHV       CCTTATT---AAAGATGTTGATAGTCCTGTACTCATGGGT
BelugaCoV  CCTTATG---CGCGGTGTGGAGGATCCTGTTCTGATGGGG
BovCoV     CCTTATT---AAAGATGTTGATAATCCTGTACTTATGGGT
BatCoV512  ACTTATT---AAGGATGTTGAAAACCCCCATTTAATGGGA
ALC        ACTGATG---TGTAATATCAACAATCCCATTTTAGTGGGT
BatCoVHKU6 ACTTATT---GGTGATGTGGATAACCCGAATCTTATGGGT
BatCoVHKU9 TTTGTGT---GAAGGTGTAGAAAATCCACATTTGATGGGT
BatCoVHKU4 CTTATAT---AAGGATGTTGAGAGTCCACATTTAATGGGT
BatCoVHKU5 CTTGTAT---AAGGATGTAGATAATCCTCACTTGATGGGT
HumCoVHKU1 TCTTATA---AAGGATGTTGACAACCCTGTTCTTATGGGT
SARSCoVAS  TGTTTAC---AGTGATGTAGAAACTCCACACCTTATGGGT
PEDV       CCTTATT---GATGGTGTTGAAAATCCGTGTCTTATGGGT
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Fig. 2 (continued)

samples (HS 1). Specific PCR on the same sample using the specific primers HSCoV-F1 and HSCoV-R1 yielded an amplicon of 148 bp, after editing out of primers. The 208 bp sequence was submitted to GenBank under accession no. FJ766501. No coronaviral RNA was amplified from the other 4 harbor seals (HS 4, 5, 6, and 17) with either the degenerate or the specific PCR. No paramyxoviral or reoviral RNA was detected in any of the samples.

Phylogenetic analysis

BLASTN results showed the highest score with Feline coronavirus (GenBank accession no. DQ848678). Results of all 3 alignment methods were identical, with no indels present when either nucleotide or predicted amino acid *Coronavirus* sequences were aligned. When additional nidoviruses equine torovirus and white bream virus (GenBank accession no. DQ898157)

were added, predicted amino acid alignments were identical by all 3 methods, and the nucleotide alignment based on the predicted amino acid alignment was used for analysis (Fig. 2). The Bayesian tree is shown in Fig. 3. Bootstrap values from ML analysis are shown on the tree.

DISCUSSION

Here we report on the identification of a novel respiratory CoV from a Pacific harbor seal. Our phylogenetic analysis places this virus as a basally divergent Group 1a CoV. It is distinct from, but most closely related to, the CoVs of ferrets, cats, dogs, and swine. While inclusion of this virus in Group 1a would create greater diversity within this one subgroup than is seen in any other subgroup, this clustering of HSCoV with the CoV of ferrets, cats, dogs, and swine is supported. There does not appear to be a clear demarcation for separating it into distinct subgroups.

Because this is a retrospective investigation and due to the incomplete sampling at the time of the outbreak, the clinical significance of HSCoV cannot fully be determined. However, the involvement of HSCoV in the etiology of the epizootic pneumonia and resulting die-off of the harbor seals is suspected. Several CoV have a known tropism for the lower respiratory tract, and porcine, bovine, canine, and human respiratory CoVs have demonstrated the ability to cause epizootics and the potential to cause fatalities (Erles et al. 2003, Costantini et al. 2004, Decaro et al. 2008). The reported histopathological changes, including the formation of syncytia or giant cells, is consistent with those associated with other CoV, such as porcine respiratory coronavirus and SARS virus (Kusanagi et al. 1992, Franks et al. 2003). However, HSCoV was only detected in 1 of 5 analyzed lung samples, and no samples were collected from HS D62 for molecular analysis. The intralesional bacteria and *Pseudomonas* sp. likely represent opportunistic overgrowth and secondary invaders, suggesting that the lung samples were not collected during the acute phase of the infection, although this cannot be confirmed from the samples available. The window of detection of CoV in respiratory infections can be short. Only 1 in 3 piglets sheds detectable porcine respiratory CoV levels by 5 d following first clinical signs (Costantini et al. 2004). Similarly, shedding of the human SARS virus peaks 7 to 10 d after onset of clinical signs (Poon et al. 2004). It is possible that 4 HSCoV negative harbor seals had cleared the initial HSCoV infection or that the virus load in those lung samples had tapered beyond the detection limit of both our degenerate and specific PCR assay. A similar event had occurred 3 yr earlier, in 1997, when approximately 90 seals were found dead in the same area.

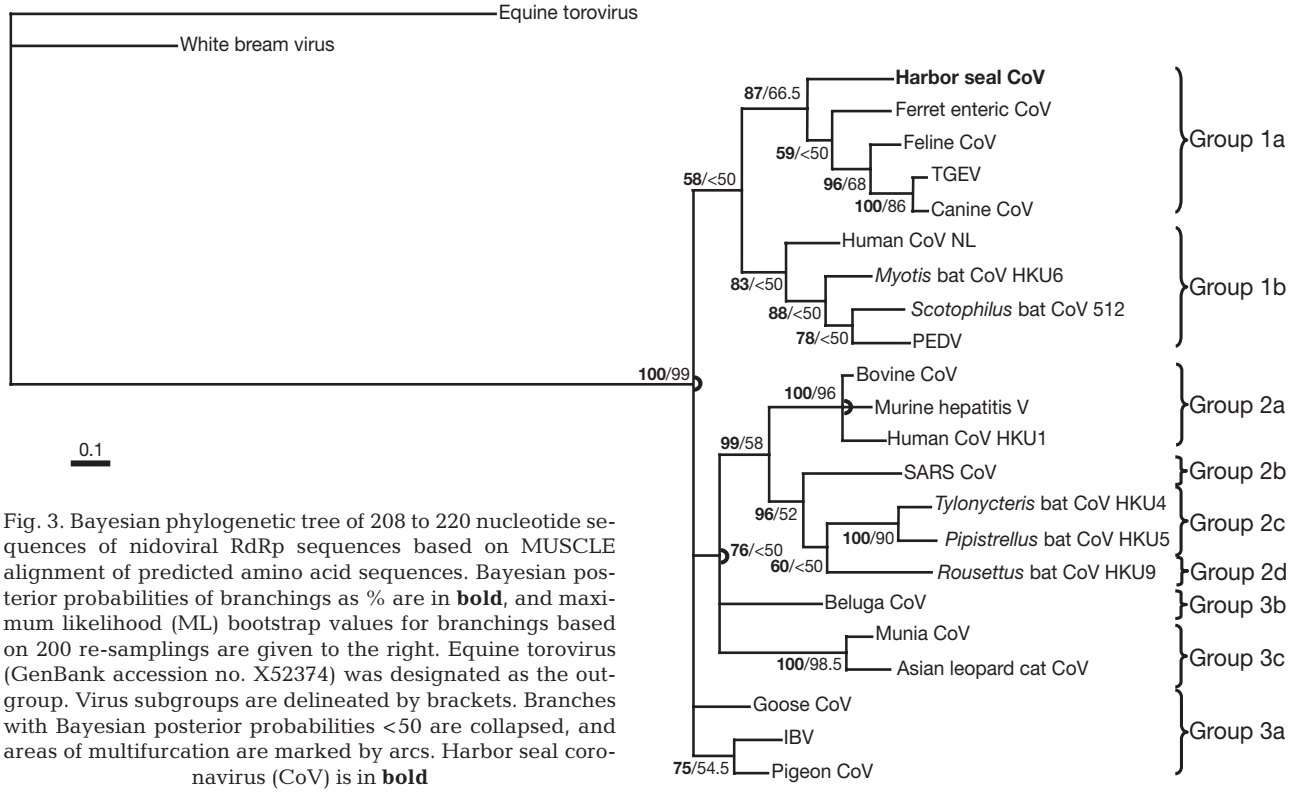

Fig. 3. Bayesian phylogenetic tree of 208 to 220 nucleotide sequences of nidoviral RdRp sequences based on MUSCLE alignment of predicted amino acid sequences. Bayesian posterior probabilities of branchings as % are in **bold**, and maximum likelihood (ML) bootstrap values for branchings based on 200 re-samplings are given to the right. Equine torovirus (GenBank accession no. X52374) was designated as the outgroup. Virus subgroups are delineated by brackets. Branches with Bayesian posterior probabilities <50 are collapsed, and areas of multifurcation are marked by arcs. Harbor seal coronavirus (CoV) is in **bold**

Necropsy and histopathological analysis of 7 of these seals yielded similar gross and histopathological presentations (authors' unpubl. data) to the 5 cases included in this study. During this earlier epizootic, syncytia formation was observed in the lungs of 3 animals and in the adrenal, liver, and salivary gland of 2 other animals. The causative agent for the 1997 respiratory infections and fatalities was never determined. The involvement of HSCoV in the 1997 epizootic is a distinct possibility. However, no suitable samples are available for retrospective HSCoV testing from this event. Thus, archiving of tissues from wildlife involved in die-offs is highly recommended, so later retrospective investigations using more modern techniques can be employed.

We were able to generate only limited genomic HSCoV sequence (208 bp), and the exact phylogenetic placement of HSCoV is therefore provisional. Attempts to amplify flanking sequences using combinations of specific and degenerate primers were unsuccessful. However, the overall topology and branching order of the phylogenetic tree (Fig. 3) based on the 208 bp HSCoV and CoV homologous sequences are consistent with the generally accepted phylogenetic branching order of the CoVs (Decaro & Buonavoglia 2008, Woo et al. 2009). We do note that our phylogenetic analysis does not support the monophyly of the proposed Subgroups 3b and 3c with 3a, and finds weak support of monophyly of these subgroups with Group 2. Previous analyses have found that Subgroups 3b and 3c were most closely related to the Group 3a viruses (Mihindukulasuriya et al. 2008, Woo et al. 2009). These differences may be due to the inclusion of non-*Coronavirus* nidoviral outgroup rooting. If the ancestral coronavirus node were within the proposed Group 3, then lack of an appropriate outgroup for rooting may cause basal viruses to appear monophyletic. Other analyses that have used non-*Coronavirus* nidoviral outgroup rooting have found that Group 3a CoVs appear fairly basal (Schütze et al. 2006, Wise et al. 2006), and this would make sense if there was an early divergence between viruses using mammalian hosts in Groups 1 and 2 and those using avian hosts in Group 3. However, these other analyses did not include viruses in the proposed Subgroups 3b and 3c. The major limitation of this approach is that there are limited regions that can be reliably aligned with non-*Coronavirus* nidoviruses. Examination of these further regions and additional taxa may confirm or refute monophyly of these subgroups.

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