

Isolation and characterization of a coronavirus from pigeons with pancreatitis

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Objective—To identify and partially characterize a coronaviruslike virus isolated from naturally infected pigeons.

Animals—50 specific pathogen-free (SPF) embryonated chicken eggs, 30 White Leghorn SPF chickens, and 12 clinically normal pigeons.

Procedures—Pancreatic tissue specimens from sick pigeons were inoculated into SPF embryonated chicken eggs for viral isolation and investigation of morphologic and hemagglutinating properties of the isolate, called PSH050513. Furthermore, virulence studies in SPF chickens and experimental pigeons were performed. The spike (S) glycoprotein gene of PSH050513 was further sequenced and analyzed.

Results—PSH050513 was isolated and identified from the experimentally infected pigeons by a routine method, which was in accordance with Koch's postulates. The complete S protein (1,167 amino acids) was compared with published S protein sequences of other avian and mammalian coronaviruses. A high degree of sequence identity (79.3% to 99.6%) was observed between the S protein sequence of PSH050513 and published sequences of avian infectious bronchitis virus (IBV); only limited identity (< 37.8%) was observed with turkey coronavirus and mammalian coronaviruses. Furthermore, when the virus was inoculated into SPF chickens, pancreatitis developed.

Conclusions and Clinical Relevance—PSH050513 has been tentatively identified as a novel member of group 3 coronaviruses that have close genetic relationships with IBV strains. (*Am J Vet Res* 2006;67:1575–1579)

The Coronaviridae is a large and diverse family of enveloped positive-stranded RNA viruses.¹ The family comprises 2 genera, Coronavirus and Torovirus, which share similarities in morphology, genome organization, and genome expression.² At approximately 30,000 nucleotides, their genome is the largest found in any of the RNA viruses.^{2,3} The Coronaviridae family has a broad host range, infecting many mammalian and avian species and causing upper respiratory tract, gastrointestinal, hepatic, and CNS diseases. In humans and birds, coronaviruses primarily cause upper respiratory tract infections, whereas porcine and bovine coronaviruses establish enteric infections that result in severe economic loss.^{2,4}

Received October 17, 2005.

Accepted February 21, 2006.

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Supported by a grant from the anti-SARS Science Foundation of Shanghai Jiao Da Onlly Company, Limited (grant No. SARS03-03).

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ABBREVIATIONS

IBV	Infectious bronchitis virus
TCoV	Turkey coronavirus
SPF	Specific pathogen free
HA	Hemagglutination
RT	Reverse transcription
S gene	Spike glycoprotein gene
PEDV	Porcine epidemic diarrhea virus
HCoV	Human coronavirus
SARS	Severe acute respiratory syndrome
TGEV	Porcine transmissible gastroenteritis virus
FCoV	Feline infectious peritonitis virus
MHV	Murine hepatitis virus
CCoV	Canine coronavirus
BCoV	Bovine coronavirus

Coronaviruses have been subdivided into 3 major antigenic groups on the basis of antigenic differences identified by serologic analyses, and these findings have been substantiated by nucleotide sequence analyses.⁵ Groups I and II contain several mammalian viruses, including those that affect humans, pigs, cows, dogs, horses, cats, and rodents. Group III contains only avian viruses, including IBV that affects chickens and TCoV.⁶ Infectious bronchitis virus primarily causes upper respiratory tract infections in chickens, whereas TCoV causes an acute enteric disease in turkeys.⁷ Results of recent investigations reveal that avian coronaviruses infect other avian species, including peafowl, partridge, teal, greylag geese, feral pigeons, and mallards.⁷⁻¹¹ However, coronaviruses that infect poultry are antigenically similar and phylogenetically related.⁷ For example, IBV has also been isolated from a peafowl, teal, and partridge in a recent coronavirus screening of domestic birds^{9,10} as well as from a flock of racing pigeons.¹⁰

In May 2005, a disease outbreak of a coronavirus infection occurred in a flock of pigeons in Shanghai, China. Affected pigeons had hyperemic pancreases that were swollen. The purpose of the study reported here was to isolate the causative coronavirus from pancreatic tissues of experimentally infected pigeons. Clinical signs and pathologic changes that were similar to those found in affected pigeons in the disease outbreak under field conditions were reproduced in clinically normal pigeons, and the virus isolate was molecularly characterized.

Materials and Methods

Chicken embryos and animals—The study was approved by the Animal Use and Care Committee of Shanghai Jiaotong University. Specific pathogen-free embryonated chicken eggs,^a White Leghorn SPF chickens,^b and clinically normal pigeons^a were used in this study.

Embryonated chicken eggs were used for isolation of field isolates and re-isolation attempts of coronavirus from pancreatic tissues of the experimentally infected pigeons. White Leghorn SPF chickens and clinically normal pigeons were used for the pathogenicity experiment.

All of the animals used in this study were housed in stainless steel isolation cabinets with negative-pressure and high-efficiency particulate air-filtered ventilation. The cabinets were under an automatically controlled light-dark cycle during the experiments. The animals had free access to feed and water. Euthanasia was conducted by inhalation of 6% carbon monoxide.

Origin of virus—PSH050513 was isolated from the swollen pancreases of sick pigeons that had distinct clinical signs of an upper respiratory tract infection. Naturally infected pigeons had clinical signs of depression, weakness, wheezing, watery eyes, and tracheal rales. At necropsy, the prominent lesion was a swollen pancreas with severe congestion. Pancreatic tissue specimens from sick pigeons were extracted and homogenized. After being subjected to 3 freeze-thaw cycles, the suspension was centrifuged at $6,000 \times g$ at 4°C for 15 minutes, filtered through $0.45\text{-}\mu\text{m}$ pore-diameter membrane,^c and then passaged in 10-day-old SPF embryonated eggs by the intra-allantoic route for virus isolation.

Virus isolation, propagation, and morphology—Ten-day-old SPF embryonated eggs were inoculated with 0.3 mL of the suspension via the allantoic route and candled daily; embryos that died after 24 hours of incubation were discarded. After 72 hours of incubation, allantoic fluid from some of the embryonated eggs was harvested and used as inoculum for further serial passages (0.2 mL/embryo). Remaining embryonated eggs were incubated for 120 hours at 37°C for observation of pathologic changes in embryos. The infected allantoic fluid was then negatively stained with 2% sodium phosphotungstate and examined by electron microscopy.

HA properties—Hemagglutination properties of the virus isolate were examined by use of the routine mini HA test. The virus isolate was directly treated with the phospholipase C_1 ^d for 2 hours at 37°C , with a final enzyme concentration of 1 U/mL. A 1% suspension of chicken RBCs was used for HA testing. Saline (0.9% NaCl) solution and allantoic fluid without virions were used as negative controls.

Pathogenicity test—Twelve clinically normal 30-day-old pigeons were randomly assigned to 1 of 2 groups with 6 pigeons in each group. Each pigeon in group 1 was inoculated intratracheally and IM with 0.2 mL of the allantoic fluid containing PSH050513. Pigeons in group 2 were mock inoculated with sterile allantoic fluid and served as negative controls. Inoculated pigeons were observed for clinical signs of disease. Pancreases were collected and processed as described previously for virus re-isolation after the euthanasia was performed. Pancreases were evaluated grossly for pathologic changes.

Viral RNA extraction and RT-PCR amplification of the S gene—The harvested allantoic fluids containing PSH050513 isolated from pigeons originally infected under field conditions and experimentally infected pigeons were used to prepare viral RNA. A total of 250 μL of PSH050513 was dissolved in 800 μL of RNA isolation reagent,^e and RNA was isolated according to the descriptions of the manufacturer. The RNA obtained was resuspended in 20 μL of ribonuclease-free water and heated at 58°C for 10 minutes.

The oligonucleotide primers used to amplify the entire S gene coding sequence of PSH050513 were designed from the published sequence of the S gene of IBV strains, with a few modifications (GenBank accession Nos. DQ075323 and

NC_001451). The sequence of primers is as follows (positions calculated from the 66th nucleotide in the front of the start codon of the S gene): first upstream primer, 5'-TGAAACTGAACAAAAGA-3' (positions 1 to 18); first downstream primer, 5'-CATAACTAACATAAGGGC-3' (382 to 399); second upstream primer, 5'-ACCGTTCCTGGTATGTCTT-3' (339 to 358); second downstream primer, 5'-GGGCAACTTGTTACATTTTCAC-3' (1699 to 1721); third upstream primer, 5'-GTTGCCCTTAIGTTAGTTA-3' (1715 to 1733); and third downstream primer, 5'-TTGTATTAGTTGTTGGAGC-3' (3684 to 3702). The genomic RNA was analyzed by use of a 2-step RT-PCR method. Synthesis of cDNA was performed with an RNA PCR assay kit^e according to the manufacturer's protocol, with 20 pmol of the various downstream primers. The RT reaction was incubated at 48°C for 30 minutes, heated for 5 minutes at 99°C , and then incubated for 5 minutes at 5°C to stop the reaction.

The cDNA (10 μL) was added to 50 μL of a PCR reaction and amplified by use of a Taq DNA polymerase.^e The same primers were used as already described. The concentration of Mg^{2+} in the reaction was 1.5 mM. The amplification program of the different pairs of primers consisted of an initial 4-minute step at 94°C , followed by 40 cycles of denaturation at 94°C for 40 seconds; annealing at 48° , 46° , and 40°C for 40 seconds each; and extension at 72°C for 50 seconds, 90 seconds, and 2 minutes. A final elongation step at 72°C for 10 minutes was performed, followed by chilling to 4°C .¹² Polymerase chain reaction products were analyzed on a 1.0% agarose gel.

DNA sequencing and sequence analysis—Polymerase chain reaction products were excised from 1% agarose gels and purified by use of an agarose gel purification kit^e according to the manufacturer's protocol. Purified PCR products were subcloned into the cloning vector^e and transformed into competent cells.^d Cells that had a recombinant plasmid were selected on agar plates^d containing ampicillin and X-gal staining solution. Plasmid DNA for sequencing was prepared by use of a plasmid preparation kit.^e Plasmid DNA was digested by *Eor*I and *Pst*I restriction enzymes^e and electrophoresed on 1% agarose gel to confirm the size of the insert. Sequencing was performed with M13 forward and M13 reverse primers. Sequencing of DNA was performed by use of a DNA analyzer.^f The alignment and phylogenetic analysis of the deduced amino acid sequences of the S gene were performed by use of the cluster method (Appendix).⁸

Virulence studies in chickens—Thirty White Leghorn SPF chickens were housed in stainless steel isolation cabinets with negative-pressure and high-efficiency particulate air-filtered ventilation. At 15 days of age, 2 groups of 10 chickens were inoculated intranasally with 0.2 mL of the allantoic fluid containing PSH050513. The 10 chickens in the remaining group were mock inoculated with sterile allantoic fluid and served as a control. The chickens were examined daily for signs of infection for 30 days after inoculation.

Results

Virus isolation and morphology—The SPF embryonated chicken eggs were inoculated with the pancreatic suspension containing PSH050513, and the allantoic fluids were harvested for further passage in embryos after 72 hours of incubation at 37°C . Other embryonated eggs were incubated for 120 hours to observe the gross lesions in embryos. In the third passage, the presence of pathologic changes on embryos that were similar to those found with infectious bronchitis was observed. Gross lesions of infected embryos included hemorrhage on the legs, mottled necrosis of

the liver, and swelling of the kidneys. Affected embryos had stunted growth (Figure 1). On electron



Figure 1—Photograph of pathogenic changes caused by PSH050513 in SPF chicken embryos. The control chicken embryo is pictured on the left, and the chicken embryo inoculated with PSH050513 is pictured on the right.

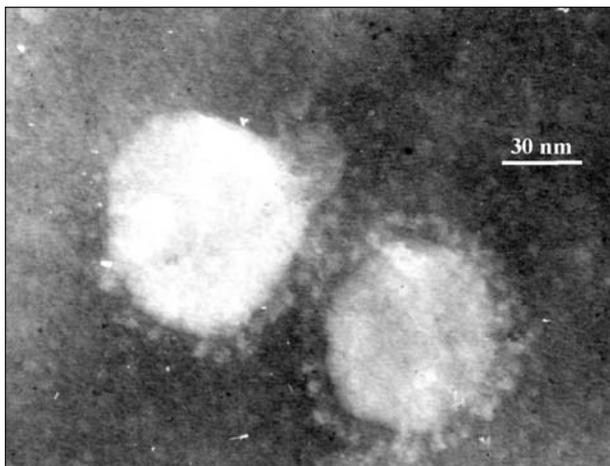


Figure 2—Electron micrograph of PSH050513 after negative staining with 2% sodium phosphotungstate. Bar = 30 nm

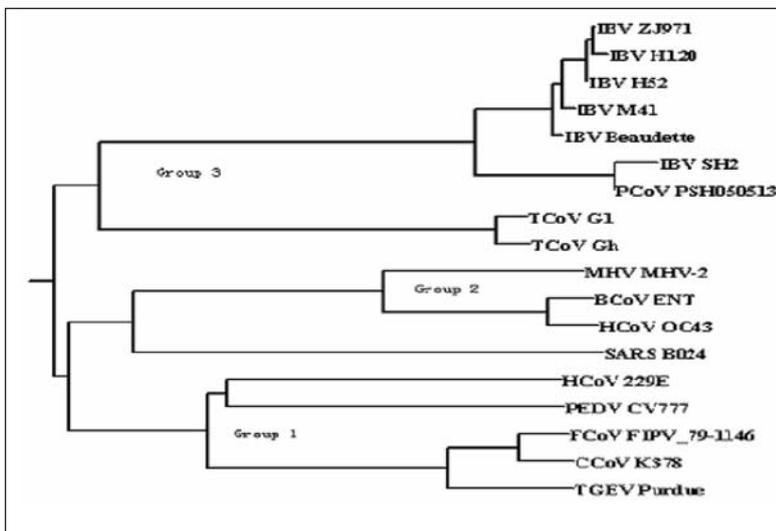


Figure 3—Phylogenetic tree constructed on the basis of the deduced amino acid sequence of the spike glycoprotein of PSH050513.

microscopy, the size of PSH050513 was 80 to 130 nm in diameter. The virions were coronal, pear shaped, and covered with an envelope connected to pedunculate projections (Figure 2).

HA properties—A 1% suspension of chicken RBCs was used for HA testing. Saline solution and allantoic fluid without virions were used as negative controls. Infected allantoic fluid could not directly agglutinate a 1% suspension of chicken RBCs, although RBCs were agglutinated after the addition of phospholipase C₁.

Pathogenicity and virus reisolation—No distinct clinical signs were observed in clinically normal pigeons inoculated with PSH050513 during the first 18 days after inoculation. However, from day 20 after inoculation, clinical signs of respiratory tract infection were observed and pigeons began to drink more water while eating less feed. Other effects such as ruffled feather and clinical signs of depression were also observed. Overall, infected pigeons had 16.6% mortality rate. Necropsy examination revealed that pigeons had excess mucus in the trachea with distinct hemorrhage and a hyperemic pancreas that was swollen as well as pulmonary lesions. Clinical signs of infection and pathologic changes were not found in control group pigeons. After chicken embryos were inoculated with 0.3 mL of the pancreatic suspension from experimentally infected pigeons, embryos had lesions similar to those of chicken embryos inoculated with material from pigeons originally infected under field conditions.

Analysis of S gene sequence—Polymerase chain reaction products were composed of 3,504 nucleotides, coding a polypeptide of 1,167 amino acid residues (GenBank accession No. DQ160004). The S gene sequence of the PSH050513 that was reisolated from experimentally infected pigeons shared 100% identity with that of material from pigeons originally infected under field conditions.

Compared with those of other avian and mammalian coronaviruses¹³ recorded in GenBank, the deduced amino acid sequences of the isolated PSH050513 were most similar to that of IBV strains SH2 (99.6%), M41 (84.4%), H52 (84.4%), Beaudette (84.0%), ZJ971 (83.8%), and vaccine strain H120 (79.3%). The isolated PSH050513 had only limited identities with the following coronaviruses: TCoV (strain G1; 37.8%), TCoV (Gh; 37.4%), PEDV (CV777; 26.3%), HCoV (229E; 25.6%), SARS (B024; 24.1%), TGEV (Purdue; 24.1%), FCoV (FIPV 79-1146; 24%), MHV (MHV-2; 23.9%), CCoV (K378; 23.3%), BCoV (ENT; 22.3%), and HCoV (OC43; 22.1%).

A phylogenetic tree was prepared to further examine the relationship between spike glycoprotein sequences of PSH050513 and published sequence data for selected avian and mammalian coronaviruses (Appendix). It revealed that

PSH050513 was more closely related to group 3 coronaviruses (IBV and TCoV) than to members of group 1 (TGEV, PEDV, FCoV, CCoV, and HCoV strain 229E) and group 2 (HCoV strain OC43, BCoV, and MHV) coronaviruses. In addition, higher identity could be observed between PSH050513 and IBV strains, compared with TCoV strains (Figure 3).

Virulence studies—All of the young chickens inoculated with PSH050513 had signs of respiratory tract infection 3 days after inoculation. Clinical signs such as gasping, nasal discharge, depression, lethargy, and huddling were also observed 4 to 6 days after inoculation. But only 1 of the 10 chickens died during the experiment. Gross lesions in organs of the dead chicken were also confined mainly to the pancreas. The pancreatic parenchyma of the dead chicken was distinctly swollen and hemorrhagic. Clinical signs of the surviving chickens resolved gradually and were absent by day 14 after inoculation. No overt clinical signs or pathologic changes were observed in the control group.

Discussion

Coronaviruses have been recently identified in several avian species besides chickens and turkeys.⁷⁻¹¹ In our study, a novel avian coronavirus associated with pancreatitis in a flock of pigeons was serially propagated by SPF embryonated chicken egg inoculation and partially characterized. The virus was identified as a coronavirus on the basis of virion size and morphology, HA properties, and genetic relationship to IBV strains, as determined by S gene sequence analysis.¹⁴ The virus is tentatively identified as a group 3 coronaviruses on the basis of the origin of the virus.

The variation in host range and tissue tropism of coronavirus is largely attributable to variations in the spike glycoprotein. In our study, the complete S gene of PSH050513 was obtained by use of an RT-PCR method. A high degree of identity (79.3% to 99.6%) was observed between the spike glycoprotein sequence of PSH050513 and spike glycoprotein sequences of IBV strains. In contrast, it had substantially lower identity (< 37.8%) with spike glycoprotein sequences of TCoV and group 1 (TGEV, PEDV, FCoV, CCoV, and HCoV strain 229E) coronaviruses and group 2 (HCoV strain OC43, BCoV, and MHV) coronaviruses.

It is known that coronaviruses can be mutated frequently because of the absence of a proofreading mechanism of RNA polymerases during replication, which could lead to nucleotide insertions, deletions, or point mutations in the S gene, especially in the S1 segment of the genome. Additionally, evidence exists that some coronaviruses may have developed by genetic recombination, mutation, or a combination of mutation and recombination.¹⁵ Compared with the spike glycoprotein sequences of IBV strains from GenBank, most sequence variations of the spike glycoprotein of PSH050513 were observed between residues 3 to 26, 51 to 85, 121 to 148, and 273 to 327. Regions between 51 to 85 and 121 to 148 that had high amino acid variations herein were similar to hypervariable region 1 (residues 56 to 69) and hypervariable region 2 (residues 117 to 131) of IBV. Hypervariable regions are

associated with 2 separate viral- neutralizing and conformationally dependent epitopes.^{16,17} Amino acid variation region of residues 273 to 388 was similar to region III (residues 274 to 387) associated with a neutralizing epitope. The cleavage site sequence of spike glycoprotein for PSH050513 in Arg-Arg-Phe-Arg-Arg (RRFRR) was similar to the pattern of most domestic IBV strains, RRF, and SRR. These findings indicate that PSH050513 has more similarity with IBV strains than other coronaviruses.

The phylogenetic tree constructed on the basis of spike glycoprotein sequences revealed that PSH050513 formed a cluster with IBV strains and shared 78.5% to 96.6% identity with each other. In contrast, PSH050513 had 37.4% and 37.8% identity with TCoV strains G1 and Gh. These findings indicate that PSH050513 is more closely related to IBV strains than to TCoV strains and genetically distinct from TCoV. In addition, PSH050513 formed a new subcluster with IBV strain SH2, which was recently isolated from sick chickens with nephritis in Shanghai, China; PSH050513 and IBV strain SH2 shared an identity of 99.6%. However, results of a virulence study¹⁸ in chickens revealed differences between the 2 viruses, indicating that the relationship between them needs further investigation. It is well known that avian pathogens can be easily transmitted between chickens and other species. For example, avian pathogenic influenza viruses were recently reported in migratory birds as well as in chickens.¹⁹ Perhaps avian coronaviruses have also been transmitted between chickens and migratory birds. However, our study was based on a single isolate and only a relatively small portion of the coronavirus genome (3.5 kilobases); additional studies are needed to confirm these findings.

Although we have identified a coronavirus or coronaviruslike virus in pigeons with severe pancreatitis, the pathogenicity of the virus and its etiologic role in upper respiratory tract infections have not been fully examined. Additional studies are needed to determine the transmitting mechanisms of PSH050513 and the prevalence of coronavirus infections in pigeons.

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- b. Shanghai Jiaotong University Laboratory Animal Technology Co Ltd, Shanghai, China.
- c. Shenergy Biocolor Co Ltd, Shanghai, China.
- d. Sigma Chemical Co, St Louis, Mo.
- e. TaKaRa BIO Inc, Dalian, China.
- f. Applied Biosystems, Foster City, Calif.
- g. DNAMAN, Lynnon Biosoft Co, QC, Canada.

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Appendix

Viruses and GenBank accession sequence numbers used in this study.

Isolate (strain)	Accession No.
IBV (M41)	X04722
IBV (H52)	AF352315
IBV (ZJ971)	AF352311
TCoV (Gh)	AY342356
HCoV (229E)	X16816
TGEV (Purdue)	NC_002306
BCoV (ENT)	NC_003045
CCoV (K378)	X77047
SARS (B024)	AY687360
IBV (Beaudette)	AJ311362
IBV (H120)	M21970
IBV (SH2)	DQ075324
TCoV (G1)	AY342357
HCoV (OC43)	NC_005147
MHV (MHV-2)	AF107212
PEDV (CV777)	NC_003436
FCoV (FIPV 79-1146)	NC_007025