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# Identification of a novel coronavirus possibly associated with acute respiratory syndrome in alpacas (*Vicugna pacos*) in California, 2007

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**Abstract.** Alpaca respiratory syndrome (ARS) was first recognized in California in October 2007. This syndrome is characterized by acute respiratory signs, high fever, and occasional sudden death, and has mostly been observed in pregnant alpacas (*Vicugna pacos*), although all signalments have been affected. A similarity in clinical signs to cases located on the East Coast of the United States was observed; however, a causative agent had not been identified. Preliminary diagnostic submissions to the California Animal Health and Food Safety Laboratory System (CAHFS) were negative for known bacterial, parasitic, fungal, and viral pathogens, as well as for toxins, making the etiology of this disease unknown. However, based on pathologic findings, a viral or toxic etiology was strongly considered. A novel coronavirus was recovered from lung tissue of a clinical case submitted to CAHFS. The coronavirus identity was confirmed in tissue culture by transmission electron microscopy and by sequence analysis of a conserved region within the viral genome. Statistical analysis calculating a serologic association between the serum virus neutralization antibody titer and coronavirus, the presence of exposure history on 40 animals with a history of ARS, and 167 controls provided an odds ratio of 121 (95% confidence interval: 36.54 and 402.84; $P < 0.0001$). The findings indicate that the ARS-associated coronavirus described is distinct from the previously reported gastrointestinal-associated coronavirus identified in alpaca herds.

**Key words:** Alpacas; camelids; coronavirus; respiratory infection.

Coronaviruses (CoVs; order *Nidovirales*, family *Coronaviridae*) are large, enveloped, single-stranded RNA viruses. Based on genotypic and serologic characterization, CoVs are classified into 3 groups[2] that are species and cell-type specific. Species specificity is determined at the level of viral binding and penetration.[4] Because of a unique mechanism for viral replication, a high frequency of recombination is possible.[6,7,10] Some strains of CoVs replicate well in cell culture, whereas others are very difficult to isolate. The challenge of growing CoVs in cell cultures has been widely reported.[9] Commonly described diseases caused by CoVs are associated with respiratory, enteric, hepatic, and neurologic signs in different species. Coronavirus infection associated with group 2 CoVs in New World camelids was first identified in 1998 in llamas (*Llama glama*) and alpacas (*Vicugna pacos*) showing signs of severe diarrhea.[3,5]

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Between October 2007 and December 2007, respiratory disease, with a sometimes fatal outcome, was observed in alpacas in California. Signs of clinical disease ranged from mild upper respiratory disease with influenza-like presentation to severe respiratory disease resulting in death. A total of 11 necropsy cases were submitted to the California Animal Health and Food Safety Laboratory (CAHFS).

The gross and histopathologic findings revealed a similar pattern of changes in all cases, with slight variations reflecting a presumed variation in the time course from onset to death. Gross findings consisted of severe pulmonary congestion and edema, often with marked pleural effusion. Histologically, there was severe pulmonary congestion and edema with a marked, diffuse, acute to subacute, interstitial to bronchointerstitial pneumonia that was most pronounced around terminal airways and adjacent central acinar alveoli but which often extended out diffusely into the alveolar parenchyma. Salient features included free fibrin deposition within the lumen of terminal airways and alveoli, often with hyaline membrane formation. In several cases, this edema and fibrin deposition was accompanied by a variable degree of epithelial necrosis and regenerative hyperplasia, which was most prominently seen at the junction between terminal airways and alveolar ducts, and was accompanied by light infiltrates of macrophages within the septa and free in airway and alveolar lumen (Fig. 1). In select cases, there were also mild, interstitial, lymphocytic infiltrates, centered around

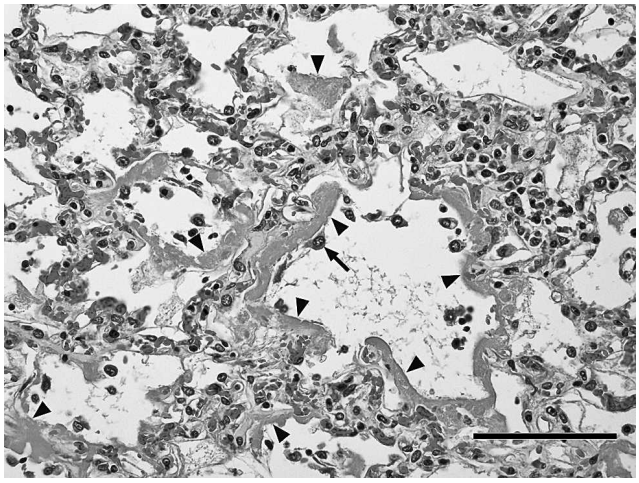

**Figure 1.** Lung from a 9-year-old, female, postpartum alpaca (*Vicugna pacos*). The lung is congested with expanded edematous alveolar septa in which there are mild, scattered interstitial infiltrates of mixed mononuclear inflammatory cells consisting of macrophages with a single focal aggregate of lymphocytes and plasma cells. Prominent "hyaline" membranes (fibrin) are pasted along the surface of several alveoli (arrowheads), which also contain small numbers of free mononuclear cells (primarily macrophages) and granular to lacey proteinaceous material compatible with edema fluid. The alveolar epithelium is mildly hypertrophied and hyperplastic with isolated regenerative epithelial cells lying on the surface of hyaline membranes (arrow). Bar = 100 μm.

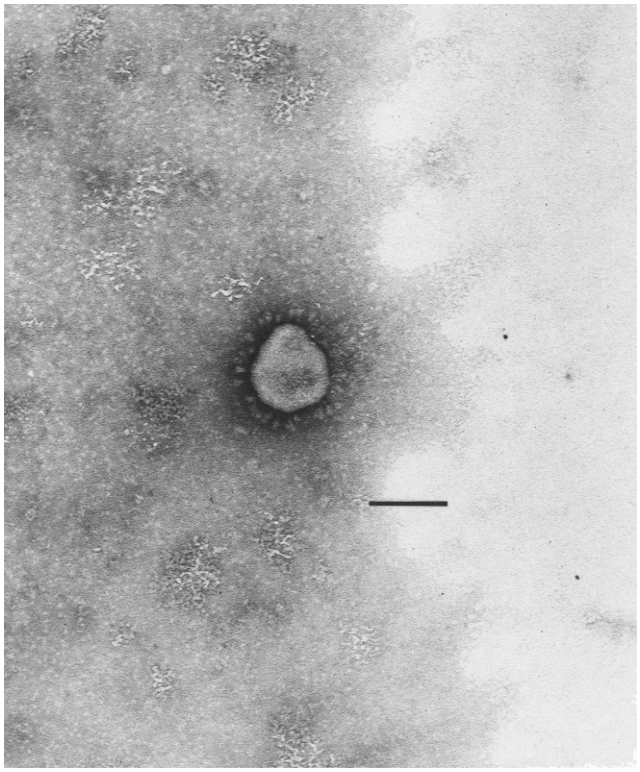

**Figure 2.** Electron microscopy shows a 120-nm range with morphology typical for coronavirus. Negative staining. 50,000× magnification. Bar = 100 nm.

the terminal airways, with multifocal interstitial fibrosis of terminal airways noted in one animal.

Multiple cell lines were inoculated with lung tissue homogenates from the necropsy cases onto primary and continuous bovine, equine, and alpaca cell lines. A CoV was successfully isolated out of lung tissue of one animal using a continuous feline renal cell line (Crandell–Rees feline kidney [CRFK][1]). Cytopathogenic effects (CPE), detected as viral plaques, were observed 6 days postinoculation on the lung tissue in the CRFK cell line. The first passage showed CPE at 3 days, followed by additional passages with consistent CPE at 48 hr. A CRFK cell line was obtained from an independent source, and the same lung tissue was inoculated onto this cell line. An identical CPE pattern was observed. A stock of a 2× plaque-purified virus was prepared for future test development. Concentrated virus was examined by transmission electron microscopy, revealing enveloped viral particles of approximately 120 nm, demonstrating the morphology typically observed for CoVs (Fig. 2).

Total RNA was extracted from infected cell culture using a commercial extraction kit[a] according to the manufacturer's recommendation. Viral RNA amplification was based on methods described previously,[8] using 2 degenerate oligonucleotide primers, 2Bp and 4Bm, targeting a conserved region within the CoV genome. The following modifications were made to the polymerase chain reaction (PCR) protocol: the master mix contained 2.5 μl of manufacturer's buffer, 7.5 mM of MgCl, 0.25 μM of each primer, 750 μM of deoxyribonucleotide triphosphate mix,[b] 20 U of RNase inhibitor,[c] 10 U of reverse transcriptase,[d] and 2.5 U of Platinum Taq[d] in a total reaction volume of 25 μl. Polymerase chain reaction was performed on an automated real-time PCR system[e] using the following protocol: 42°C for 900 sec, 95°C for 120 sec, and 5 cycles of 98°C for 30 sec, 40°C for 120 sec, and 72°C for 60 sec. This amplification round was followed by 35 cycles of 98°C for 30 sec, 50°C for 90 sec, and 72°C for 60 sec. The PCR products were verified on a 4% agarose gel and observed for a 251-bp product.

Using PCR centrifugal filter devices,[f] the PCR product was purified and sent to a commercial sequencing facility.[g] Sequence data were analyzed using Chromas 2.33[h] software and compared with published sequences using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Analysis revealed a high similarity (nucleic acid similarity, 176 of 196, 89.8%; expectation value [E value]: 3e-68; GenBank accession no. GB398771) to CoV isolates grouped as subtype 1b.

Because CoV was recovered only from lung tissue of 1 affected alpaca, it was necessary to demonstrate a relationship between the presence of the virus or viral antibody and a positive ARS clinical history. "Positive ARS history" was defined as animals that originated from a farm with reported, recent respiratory problems compatible with ARS in addition to a recent history of travel to a local alpaca show in Monterey, CA. Samples collected from animals housed on a ranch with no recent respiratory

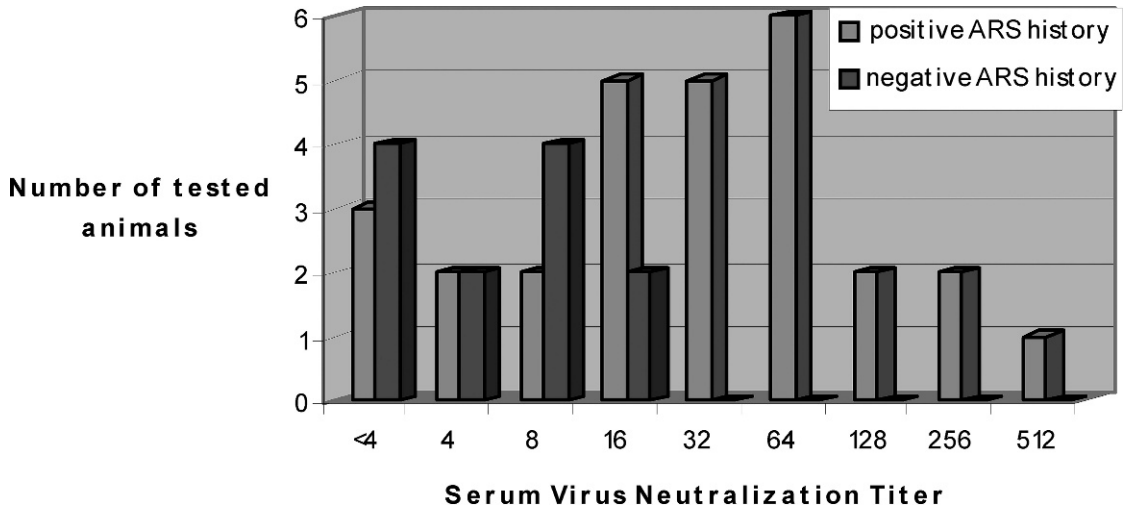


**Figure 3.** Serum virus neutralization results originated from animals with a known exposure to alpaca respiratory syndrome (ARS) determined by recent travel and ranch history.

problems and owners that did not participate in alpaca shows were considered "negative ARS history." Forty serum samples taken from alpacas with a known history of ARS were compared by titration against the plaque-purified CoV using a serum virus neutralization (SVN) assay. Serum virus neutralization was performed by testing a 2-fold serial dilution of alpaca serum using 100–300 as the median tissue culture infective dose ($TCID_{50}$) of the recovered CoV as antigen. Titers are shown in Figure 3. A cutoff value of ≥1:16 was established, emphasizing high specificity. An additional 167 serum samples, collected in multiple states from alpacas with unknown disease histories that were sent in for routine testing, were subjected to the SVN assay, and results were transformed into dichotomous results using a previously determined cutoff value. Results from these 167 animals were included into the "negative/unknown history" control-group category for calculating the odds ratio. The statistical association of the 207 serum samples revealed a significant odds ratio of 121.43 (95% confidence interval [CI]: 36.539 and 402.841; $P \leq 0.001$) among the presence of antibody to the CoV, a history of being housed on a ranch with recent history of travel to a local alpaca show, and recent respiratory outbreaks (Table 1). Statistical analysis was performed by using a commercial spreadsheet[i] to calculate odds ratios and 95% CIs.

Previous reports of CoVs in alpacas have focused on the identification and characterization of serogroup 2 CoV, associated with diarrhea.[5] Based on preliminary sequencing results on the CoV isolated from lung tissues of the CAHFS necropsy case and using a short PCR fragment targeting the polyprotein 1a, the novel virus can be grouped in serogroup 1 and is, therefore, different from the virus isolated from alpaca feces as previously reported.[5] Whole-genome sequencing is currently in progress to provide a full comparison between the 2 viruses. To date, sequence data obtained from the spike gene, the nucleoprotein gene, and the membrane gene underline the observations that the isolated CoV belongs in serogroup 1 (data not shown).

Unfortunately, the CoV was recovered from a single set of submitted tissues, despite efforts to recover the virus from additional cases by the authors and from partner diagnostic laboratories in the United States. Factors such as the time between death and collection of samples, the sample quality, and whether the animals underwent prolonged or intensive veterinary care before death might have interfered with the ability to recover the virus from affected animals. Coronaviruses have a reputation of being difficult to detect in a diagnostic environment. With the exception of a small number of prototype strains, very few wild CoV strains grow in cell culture, which may explain why many attempts to isolate a virus by various laboratories using different primary and continuous cell cultures have been unsuccessful. Alternatively, this virus may have represented a spurious finding.

Serologic results, however, demonstrate a strong association between the presence of antibody to the novel CoV and the exposure to ARS, according to travel history or ranch exposure. The significance of the statistical association is underlined by the 95% CI and the $P$ value. It is possible that the unique CoV was detected as the cause of disease but that it has not continued to circulate or has yet to be exposed to susceptible populations of alpacas. An alternate consideration would be that the high prevalence of titers to the novel CoV result from population selection bias because all animals in the positive ARS history group had attended the same show and were exposed to the same environment, allowing that ARS might have been caused

**Table 1.** A 2 × 2 table for odds ratio calculation and determination of the 95% confidence interval.*

| Antibody titer | Positive ARS history | Negative/unknown ARS history |
|---|---|---|
| ≥1:16 | 25 | 5 |
| <1:16 | 7 | 170 |

* ARS = alpaca respiratory syndrome; odds ratio = (25 × 170)/(7 × 5) = 121.429; $P < 0.0001$; 95% confidence interval: 36.539 and 402.84.

by a different agent or a combination of the novel coronavirus and other factors. Although pathologic, histologic, and serologic results indicate an association between the novel CoV and the outbreak of ARS in alpacas, there is still a need to fulfill Koch postulates to prove a true association between the virus and ARS.

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## Sources and manufacturers

a. Qiagen Inc., Valencia, CA.
b. Applied Biosystems, Foster City, CA.
c. Ambion®, Applied Biosystems/Ambion, Austin, TX.
d. Superscript® II Reverse Transcriptase, Invitrogen Corp., Carlsbad, CA.
e. SmartCycler® II, Cepheid Inc., Sunnyvale, CA.
f. Montage®, Millipore Corp., Billerica, MA.
g. Davis Sequencing, Davis, CA.
h. Copyright © 2003–2008, Technelysium Pty Ltd., Tewantin, Queensland, Australia.
i. Microsoft Office Excel® 2003, Microsoft Corp., Redmond, WA.

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