

## Experimental reproduction of pneumonia in gnotobiotic pigs with porcine respiratory coronavirus isolate AR310

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**Abstract.** The pathogenicity of porcine respiratory coronavirus (PRCV) isolate AR310 was determined for gnotobiotic pigs. PRCV-AR310 was isolated from the intestines of a nursery pig from a herd with endemic transmissible gastroenteritis. The AR310 isolate was plaque purified and cell culture propagated, passed once in a gnotobiotic pig, then used as inoculum for a gnotobiotic pig pathogenicity study. Eight pigs were inoculated oronasally with  $2 \times 10^6$  plaque-forming units of PRCV-AR310. Eight pigs served as controls and received cell culture medium. Two pigs from each group were necropsied at 3, 5, 10, and 15 days postinoculation (DPI). There was moderate multifocal to coalescing reddish tan consolidation of 60% of the lung by 10 DPI. Microscopic examination revealed a necrotizing and proliferative bronchointerstitial pneumonia characterized by necrosis, squamous metaplasia, dysplasia, proliferation of airway epithelium, mononuclear cell infiltration of alveolar septa, mild type II pneumocyte proliferation, and lymphohistiocytic alveolar exudation. The microscopic lesions were mild by 3 DPI, moderate by 5 DPI, severe by 10 DPI, and mostly resolved by 15 DPI. No lesions were observed in the intestines of these pigs. There was no clinical respiratory disease. Control pigs remained normal and had no lesions. PRCV was isolated from the lungs but not from the intestines of inoculated pigs. PRCV was not isolated from the lungs or intestines of control pigs. PRCV was also isolated from the nasal and rectal swabs of inoculated but not of control pigs.

The first isolation of porcine respiratory coronavirus (PRCV) was reported in 1986 from pigs in Belgium,<sup>13</sup> and now PRCV is enzootic in Europe. An increase in Seroprevalence of antibodies to transmissible gastroenteritis virus (TGEV) in Europe without clinical evidence of enteric disease suggested that infection with a related coronavirus had occurred and researchers subsequently isolated PRCV. This virus replicated to high titers in the respiratory tract in experimental pigs, and those pigs developed antibodies that neutralized TGEV. There is disagreement among researchers as to the pathogenicity of PRCV isolates in Europe. Some researchers have reported mild to moderate bronchointerstitial pneumonia in neonatal pigs; however, the pigs remained clinically normal.<sup>3,11</sup> In another study, a serious and sometimes fatal catarrhal bronchopneumonia was reproduced in 5-week-old specific-pathogen-free (SPF) pigs with intranasal inoculation of PRCV.<sup>16</sup> There are additional reports of fever and dyspnea in 25kg SPF pigs inoculated intratracheally with PRCV.<sup>6</sup>

Porcine respiratory coronavirus was first isolated in the USA in 1989.<sup>7</sup> This isolate was designated as ISU-1-PRCV or Ind-89. Subsequently, ISU-2-PRCV was isolated from a herd in North Carolina.<sup>7,20</sup> The herds

involved seroconverted to TGEV without showing clinical signs of respiratory or enteric disease.<sup>7,20</sup> ISU-1-PRCV was replicated in the lungs of experimentally inoculated neonatal pigs but did not cause significant respiratory lesions.<sup>20</sup>

Another PRCV (PRCV-AR310) has since been isolated from pigs in the USA.<sup>17</sup> All PRCV isolates examined thus far exhibit a characteristic deletion in the 5' end of the S gene when compared with TGEV. The PRCV-AR310 isolate has a 621-bp deletion in the S protein<sup>12</sup> that is different from the 681-bp deletion of ISU-1-PRCV/Ind-8919 and the 672-bp deletion of the French PRCV isolate RM 4.<sup>15</sup> The pathogenicity of PRCV-AR310 in 3-day-old gnotobiotic pigs is described in this report.

### Materials and methods

**Source farm.** A farrow-to-feeder pig herd in Arkansas was investigated because of a history of endemic TGE problems in nursery pigs. Clinical signs, microscopic enteric lesions, and repeated positive TGEV fluorescent antibody tests on intestines had previously confirmed the diagnosis.

**Virus stock.** PRCV-AR310 was isolated from homogenates of intestinal tracts by inoculation of swine testis (ST) cell line as previously described.<sup>12</sup> Virus was plaque purified 3 times, and gnotobiotic pigs were inoculated oronasally at the 10th cell culture passage. Lungs from a gnotobiotic pig at 3 days after infection were homogenized, clarified, and filtered through a 0.22- $\mu$ m filter and served as virus stock for this experiment. The lung filtrate virus stock had a titer of  $1 \times 10^7$  plaque-forming units (PFU)/ml.

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**Gnotobiotic pigs.** Gnotobiotic pigs were obtained by closed hysterectomy and maintained in gnotobiotic isolators as previously described.<sup>9</sup> The isolators were maintained at an ambient temperature of 30 C, and pigs were fed commercial milk substitute.<sup>a</sup> Fecal swabs were collected from each pig at the time of necropsy prior to removal from the isolators to check bacterial sterility.

**Experimental transmission.** Sixteen 3-day-old gnotobiotic pigs were randomly allotted into 4 isolators containing 4 pigs each. Eight pigs served as mock-infected controls. Eight pigs were challenged intranasally (3 ml) and orally (1 ml) with solution containing a total of  $2 \times 10^6$  PFU. Intranasal inoculations were made by slowly dripping the material into the nares, and oral inoculations were made by injecting the material into the back of the mouth. The pigs were monitored for signs of enteric and respiratory disease. Two control pigs and 2 PRCV-AR310-inoculated pigs were euthanized by electrocution 3, 5, 10, and 15 days postinoculation (DPI). Nasal and rectal swabs were collected daily from each pig. Virus isolation was attempted from the lungs, intestines, and nasal and rectal swabs.

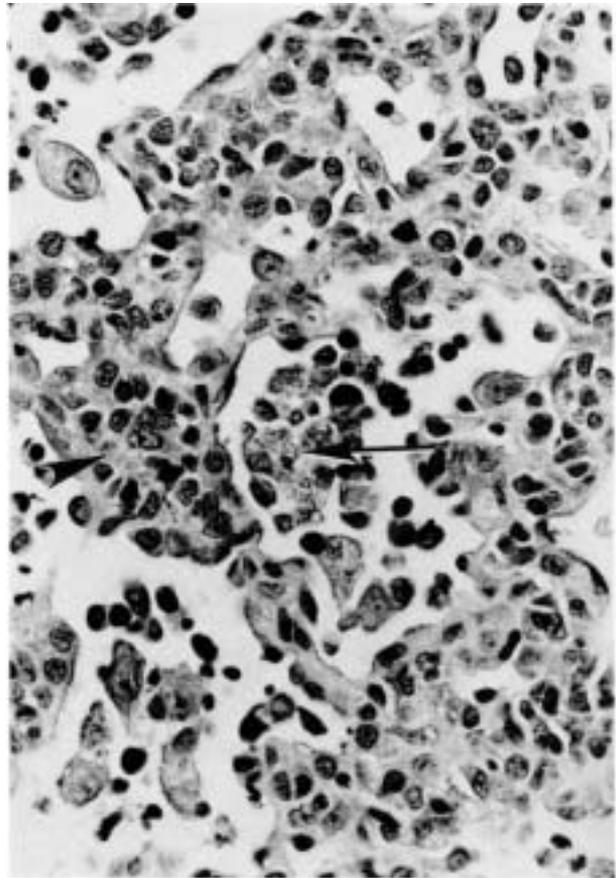
**Virus isolation and titration from experimental pigs.** Lungs or small intestines were homogenized in Eagle's minimal essential medium (20% w/v), clarified at  $1,000 \times g$  for 10 min, and filtered through a  $0.22 \mu\text{m}$  filter. Confluent 3-5 day-old monolayers of ST cells in  $25\text{cm}^2$  flasks were inoculated with 0.2 ml of filtrate. After 1 hr incubation at 37 C, the monolayer was washed and new culture medium was added. Cells were observed for cytopathic effect. If no cytopathic effect was observed, the cells in the flasks were frozen and thawed 3 times, and cell lysates were inoculated onto new monolayers. Samples were passed 3 times. The presence of TGEV was confirmed by indirect immunofluorescence using gnotobiotic pig antisera to the Miller strain of TGEV and mouse anti-TGEV-S monoclonal antibodies.<sup>21</sup> Virus titration was performed by inoculation of ST monolayers with 10-fold concentrations of tissue filtrates.

**Microscopic examination.** Brain, nasal turbinate, heart, lung, duodenum, jejunum, ileum, colon, liver, and kidney of all pigs were collected in 10% neutral buffered formalin, embedded in paraffin, sectioned at  $4 \mu\text{m}$ , and stained with hematoxylin and eosin (HE). Lungs were inflated with formalin at the time of necropsy by injection into the trachea, and then the trachea was ligated and the entire lung immersed in formalin. Histologic lesions were evaluated by light microscopy.

## Results

**Experimental reproduction of lesions.** All control and PRCV-inoculated pigs remained clinically normal throughout the experiment. The pigs consumed all their milk, and no elevations in body temperatures were seen.

At 3 DPI, 1 of 2 PRCV-infected pigs had gross pneumonic lesions characterized by 20% multifocal to coalescing reddish tan consolidation of the lung involving parts of all lobes. Both PRCV-infected pigs killed at 5 DPI had gross lesions similar to those in the other pigs at 3 DPI but with more interlobular edema present.

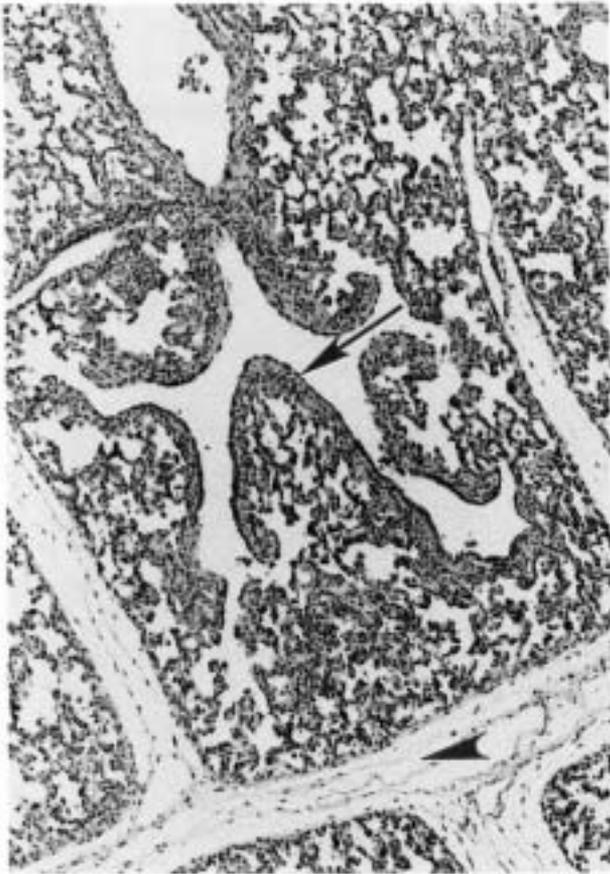


**Figure 1.** Microscopic appearance of a PRCV-AR310-inoculated gnotobiotic pig lung 10 days postinoculation. Note the interstitial thickening with mononuclear cells (arrow head) and lymphoplasmacytic and macrophagic alveolar exudate (arrow).

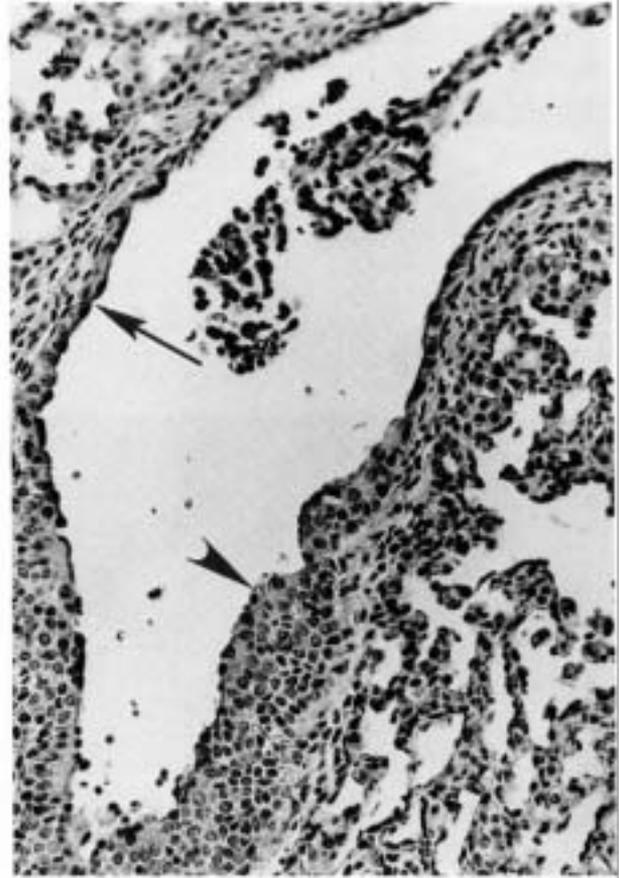
At 10 DPI, there was bilateral multifocal reddish-tan consolidation of 60% of the lungs of both pigs. At 15 DPI there were no visible gross lesions in the infected pigs. There were no gross lesions in control pigs.

Microscopically, interstitial septa were mildly thickened with mixed mononuclear cells. There was moderate lymphoplasmacytic and histiocytic bronchiolar and alveolar exudation (Fig. 1) and mild multifocal type II pneumocyte hypertrophy and proliferation. There was also moderate to severe airway epithelial necrosis, squamous metaplasia, dysplasia, and proliferation involving all sizes of airways (Figs. 2, 3). The pulmonary lesions were mild by 3 DPI, moderate by 5 DPI, severe by 10 DPI, and nearly resolved by 15 DPI. No lesions were detected in nasal turbinates. Microscopic lesions were not visible in the small or large intestines of the infected pigs. There were no microscopic lesions in lungs or intestines of control pigs (Fig. 4).

**Virus isolation from experimental pigs.** PRCV was not detected in the small intestines of any of the pigs. PRCV was isolated from the lungs of 2 PRCV-inoculated pigs at 5 DPI. Virus was also detected in the



**Figure 2.** Microscopic appearance of a PRCV-AR310-inoculated gnotobiotic pig lung 10 days postinoculation. Lesions are characterized by interlobular edema (arrow head), mild diffuse interstitial thickening with mononuclear cells, and squamous metaplasia of airway epithelium (arrow).



**Figure 3.** Bronchiole from a PRCV-AR310-inoculated gnotobiotic pig 10 days postinoculation, exhibiting proliferation (arrow head) and squamous metaplasia (arrow) of epithelium and mild exudate in airways.

nasal swabs at 3 and 4 DPI and rectal swabs at 1 and 2 DPI. PRCV was not detected in any of the control pigs.

### Discussion

In this report, PRCV-AR310 was pneumopathogenic for 3-day-old gnotobiotic pigs. PRCV-AR310 was isolated from the intestines of a nursery pig from a herd with an endemic TGE problem. Plaque-purified PRCV-AR310 caused moderate bronchiointerstitial pneumonia in gnotobiotic pigs following oronasal inoculation. No significant clinical respiratory disease was seen in the gnotobiotic pigs, probably because of the gnotobiotic environment. If PRCV-AR310 causes similar pulmonary lesions in conventional pigs, it might predispose them to secondary bacterial infections. A more practical and applicable model for studying this disease would be using conventionally reared 4-8-week-old pigs. Pigs of this age are the source of most of the isolates and are susceptible to clinical problems. A gnotobiotic pig model was chosen for this initial study

to characterize the lesions without the interplay of contaminating microorganisms.

PRCV replicates to high titer in the respiratory tract and to low titer in the gut,<sup>3,4</sup> which could explain the isolation of PRCV-AR310 from intestinal homogenates from the source herd in this study. PRCV was isolated from the rectal swabs collected from inoculated pigs which suggests that limited PRCV replication takes place in the intestinal tract. The presence of PRCV in the gut or rectal swabs may be due to ingestion of virus produced in the respiratory tract, viremia, or low-level replication in the small intestine. PRCV has also been previously isolated from plasma,<sup>3,4</sup> which may substantiate the viremia route of spread to the gut.

The genomic deletions in the S gene may contribute to the differences in pathogenicity between TGEV and PRCV isolates. It is clear that PRCV-AR310, with a 621-bp deletion in the S gene,<sup>12</sup> is pneumopathogenic and pneumotropic in gnotobiotic pigs. ISU-1/Ind-89 has a 681-bp deletion and is pneumotropic but not pneumopathogenic in gnotobiotic pigs.<sup>19,20</sup> The patho-

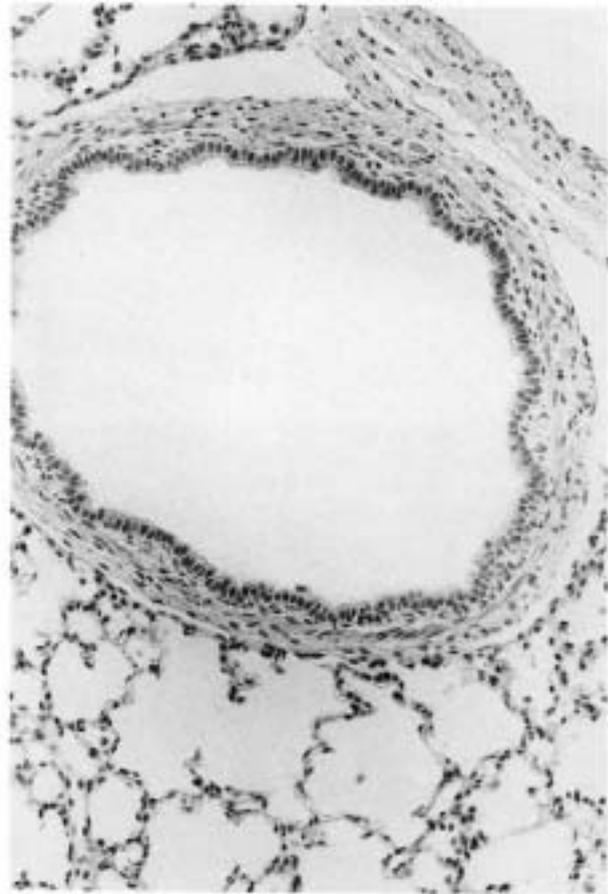
genicity reports of the European isolates are variable<sup>3,6,11,16</sup>

There has been much interest in the possibility of using PRCV as an immunogen for TGE. Because PRCV replicates to a limited extent in the intestine, the possibility of stimulating mucosal immunity against TGEV deserves consideration. Severe TGE outbreaks in neonatal pigs in the Belgian swine population have become rare since the appearance of PRCV.<sup>14</sup> However, outbreaks of TGE have occurred in herds previously infected with PRCV.<sup>8</sup> Researchers have shown partial protection against TGEV challenge by prior immunization of pigs with PRCV.<sup>14</sup> An enteropathogenic TGEV (AR302) was concurrently isolated from intestinal homogenates of another pig from the same source group of nursery pigs that PRCV-AR310 came from. The enteropathogenicity of that isolate was demonstrated in gnotobiotic pigs (P. G. Halbur, unpublished data). The isolation of both an enteropathogenic TGEV and a pneumopathogenic PRCV-AR310 from the same group of pigs as described in this study would suggest limited cross-protection in this herd.

PRCV should be suspected when there is mild to moderate respiratory disease in nursery age pigs with high titers to TGEV but no evidence of clinical TGE. Histopathologic identification of a necrotizing and proliferative bronchiointerstitial pneumonia would support the diagnosis. Virus isolation from nasal swabs of acutely affected pigs would confirm the diagnosis if other viral agents have been adequately ruled out.

The use of monoclonal antibodies for development of fluorescent antibody examination and immunohistochemical tests of clinical tissue samples should allow easier and more accurate diagnosis of this disease. Development of an accurate serologic test in the USA to differentiate TGEV from PRCV infection would be useful. A competitive inhibition enzyme-linked immunosorbent assay has been developed by researchers in Belgium and used for selective serodiagnosis. A complementary DNA probe has also been developed that will differentiate between enteric TGEV and a respiratory PRCV infection.<sup>18</sup>

Several PRCV isolates have been obtained from cases of moderate to severe respiratory disease in nursery pigs. High antibody titers to TGEV in these herds without diarrhea and with poor nursery pig performance were typical. Mixed infections were common. Porcine reproductive and respiratory syndrome virus (PRRSV) was also isolated from 1 herd. The losses from endemic respiratory disease in this herd were particularly severe. In most clinical cases, there was a mixture of bacteria isolated from the respiratory tract. *Haemophilus parasuis*, *Streptococcus suis*, and *Pasteurella multocida* were the most frequently isolated bacteria in conjunction with PRCV in the herds investigated.



**Figure 4.** Normal alveolar septa and bronchiolar epithelium of control gnotobiotic pig 10 days postinoculation.

The microscopic lesions are similar to those produced by swine influenza virus (SIV). Severe cases of SIV exhibit more extensive airway epithelial necrosis, a loose peribronchiolar cuff of mononuclear cells, migration of inflammatory cells into airways, and flooding of alveoli with serofibrinous exudate. The lesions also somewhat resemble those recently reported for a SIV variant,<sup>10</sup> except PRCV-AR310 does not produce hyalinization of alveolar septa, and the degree of type II pneumocyte proliferation seen with PRCV-AR310 is less. The PRRSV produces marked interstitial pneumonia in neonatal pigs characterized by mononuclear cell infiltration of alveolar septa<sup>2</sup> but does not cause airway epithelial damage as seen with PRCV-AR310.

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