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Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States

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Abstract. A respiratory variant of transmissible gastroenteritis virus (TGEV), designated PRCV-Ind/89, was isolated from a swine breeding stock herd in Indiana. The virus was readily isolated from nasal swabs of pigs of different ages and induced cytopathology on primary porcine kidney cells and on a swine testicular (ST) cell line. An 8-week-old pig infected oral/nasally with the respiratory variant and a contact pig showed no signs of respiratory or enteric disease. These pigs did not shed virus in feces but did shed the agent from the upper respiratory tract for approximately 2 weeks. Baby pigs from 2 separate litters (2 and 3 days old) also showed no clinical signs following oral/nasal inoculation with PRCV-Ind/89. In a third litter, 5 of 7 piglets (5 days old) infected either oral/nasally or by stomach tube developed a transient mild diarrhea with villous atrophy. However, virus was not isolated from rectal swabs or ileal homogenates of these piglets, and viral antigen was not detected in the ileum by fluorescent antibody staining even though the virus was easily recovered from nasal swabs and lung tissue homogenates. Swine antisera produced against PRCV-Ind/89 or enteric TGEV cross-neutralized either virus. In addition, an anti-peplomer monoclonal antibody, 4F6, that neutralizes TGEV also neutralized the PRCV-Ind/89 isolate. Radioimmunoassays with a panel of monoclonal antibodies indicated that the Indiana respiratory variant and the European PRCV are antigenically similar.

A porcine respiratory coronavirus (PRCV) has recently been identified as enzootic in most, if not all, countries of western Europe and in East Germany.[1,8,9,14,15,21] This new coronavirus of pigs was first recognized in Belgium in the spring of 1984 when a serologic survey of slaughterhouse sows showed a marked increase in the prevalence of antibodies to transmissible gastroenteritis virus (TGEV).[14] This increase in the number of seropositive animals occurred in the absence of TGEV vaccination in Belgium and without a noticeable increase in the incidence of clinical transmissible gastroenteritis (TGE) the previous winter. Since this initial observation, the virus has spread rapidly in the European swine population and in one case the virus was transmitted 20 miles by air currents, infecting susceptible swine in a closed, well-managed herd (P. Hare, personal communication).

Pigs experimentally inoculated with PRCV exhibited no clinical signs of disease but developed TGEV neutralizing antibodies. Pathogenesis studies have shown that PRCV replicated to high titers in the respiratory tract but only to a very low degree in the gut of infected pigs.[12,13] Additional studies have shown that the European PRCV grows easily in the porcine continuous cell lines ST and PD5 without extensive adaptation.[13] Both TGEV and PRCV are fully neutralized at comparable titers by antisera prepared against the classical enteric TGEV. Likewise, convalescent antisera from PRCV-infected swine cross-neutralize TGEV.

Despite the antigenic relatedness of the European PRCV and classical TGEV, they can be differentiated with monoclonal antibodies (MAbs).[2,7,10,15] Whereas most neutralizing MAbs directed against the peplomer glycoprotein recognize both viruses, certain nonneutralizing epitopes on TGEV are absent on the PRCV peplomer. Thus, antigenic determinants on the peplomer glycoprotein of TGEV are modified or absent in PRCV. Some nonneutralizing MAbs are employed in blocking assays to distinguish serologically between a TGEV or PRCV infection.[3,7,15] In this assay, TGEV is incubated with either TGEV or PRCV antiserum followed by the distinguishing MAb.

The purpose of this report is to describe a pneumotropic coronavirus isolated from swine in the United States. The US isolate (PRCV-Ind/89) is similar in pathogenicity, tissue distribution, and antigenicity to the European PRCV.

Materials and methods

Infected herd study. A swine herd in Indiana that exports breeding stock experienced an unexplained seroconversion to TGEV during the winter of 1988-1989. Pigs in this herd

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had not been vaccinated or knowingly exposed to TGEV. There had been no clinical evidence of either an enteric or a respiratory infection.

Because the serologic, vaccinal, and clinical history of this US herd was reminiscent of western European farms infected with PRCV, attempts to isolate virus were initiated in cell cultures and in seronegative pigs. Twenty-six nasal swabs were obtained from different groups of weaned pigs that were 4, 5, 6, 7, and 8 wk old.

Cells and viruses. Swine testicular (ST) cells[11] were grown in modified Eagle's minimal essential medium (MEM)[a] supplemented with fetal bovine serum (FBS) (10%), sodium bicarbonate (0.22%), lactalbumin hydrolysate (0.25%) sodium pyruvate (0.01%), and gentamicin sulfate (50 µg/ml). Primary pig kidney cells were grown in modified Eagle's MEM that also contained 2 µg/ml amphotericin B.[b]

The Purdue strain (115e) and the pig-passaged Miller strain (p439 +) of TGEV[c] were used as avirulent and virulent control viruses, respectively, to infect baby pigs. The Purdue strain, passage 115, was passed 3 additional times on ST cells before inoculating pigs. the Miller strain was administered to pigs as a 1:1,000 dilution of homogenized intestinal contents.[16]

The Miller TGEV strain passaged 60 times on ST cells[20] and feline infectious peritonitis virus (FIPV) strain UCD-1[17] were used as viral antigens for radioimmunoassays.

Virus isolation from secretions and excretions of naturally or experimentally infected pigs. Swabs of the anterior nasal cavity, tonsils, and rectum were immersed in 2 ml of virus transport media and frozen at -70 C. Virus transport medium was the supplemented Eagle's MEM maintenance medium containing 2% FBS, penicillin (25 units/ml), streptomycin (25 µg/ml), neomycin sulfate (25 µg/ml), bacitracin (0.25 units/ml), and gentamicin sulfate (50 µg/ml). Samples were thawed, and swabs were squeezed with sterile forceps prior to low speed centrifugation to remove debris. Sample supernatants (400 µ1) were inoculated onto confluent ST cells grown in 60- × 15-mm tissue culture dishes or primary kidney cells. After 1 hr incubation at 37 C, the inoculum was removed, and 5 ml of virus transport medium containing amphotericin B (5 µg/ml), anti-rotavirus sera (1 ml/liter), and anti-enterovirus sera (1 ml/liter) was added. In samples with the respiratory coronavirus (PRCV-Ind/89), cytopathic effect (CPE) was apparent in 1 or 2 days. Samples not showing CPE were blind passaged on ST cells 3 times before being considered negative.

Plaque-reduction assay. A 50% plaque-reduction test was used for estimating the virus neutralization (VN) antibody titer of swine sera and of mouse ascites fluid containing MAb 4F6.[20]

Experimental infection of neonatal piglets. Three separate litters of pigs were given different dosages of PRCV-Ind/89. Each litter was subdivided into experimentally infected and control piglets. All piglets, seronegative for TGEV, were housed in individual Plexiglas isolators in a room maintained at 34 C and fed an SPF-LAC ration.[d] In the first experiment, principals (PRCV-Ind/89 infected) and control piglets (TGEV and cell culture medium inoculated) were housed in isolators in 2 different rooms for additional protection against cross contamination.

The first litter (litter A) consisted of 10 5-day-old conventional pigs. Five of these piglets were inoculated oral/nasally with 5 ml of PRCV-Ind/89 that was passaged 2 times on ST cells (titer = 3.5×10^7 PFU/ml). Two pigs were also inoculated via a stomach tube with 5 ml of the same virus inoculum. The remaining 3 pigs served as controls and were treated as follows: a negative control pig was inoculated with cell culture medium, another pig was inoculated oral/nasally with 5 ml of virulent Miller strain TGEV (3×10^3 PFU/ml), and a third pig was infected oral/nasally with 5 ml of the avirulent Purdue strain of TGEV (7.5×10^7 PFU/ml).

Beginning on the second day postinfection (DPI), the piglets were swabbed daily (nasal, tonsillar, rectal) to monitor virus shedding. Inoculated pigs were euthanized and necropsied according to the following schedule: the negative control pig and the Miller strain-infected pig (at 1 DPI); the avirulent Purdue strain-infected pig (at 3 DPI); the piglets infected oral/nasally with the respiratory isolate (at 3, 4, 5, 7, and 8 DPI). The 2 pigs given PRCV-Ind/89 via a stomach tube were swabbed but not euthanized.

Ileum and lung tissue specimens were collected from each piglet for virus isolation. Ileal tissues were also processed for histopathology and fluorescent antibody (FA) studies. For histopathology, tissue samples were fixed in phosphate buffered 10% formalin, embedded in paraffin, sectioned at 4 µm thickness, and stained with hematoxylin and eosin (HE). Samples for FA studies were immersed in embedding medium[e] and frozen (-70 C). For virus isolation, tissue specimens were frozen on dry ice and stored (-70 C) until processing. Tissue homogenates, a 20% (w/v) suspension in cold phosphate buffered saline (PBS), pH 7.2, were thawed, minced, and disrupted in a polytron tissue homogenizer.[f] Tissue debris was removed by low speed centrifugation, and the supernatant virus titer was determined on ST cells.

The second litter (litter B) consisted of 10 3-day-old hysterectomy-derived colostrum-deprived (HDCD) piglets. These piglets were subdivided into 4 groups. Pigs in group I (n = 3) were given via stomach tube 5 ml of a 20% lung homogenate from pig #5 (litter A), which was inoculated with PRCV-Ind/89 and showed signs of diarrhea at necropsy. The virus in the lung homogenate titered 8.5×10^5 PFU/ml. Pigs in group II (n = 3) were given via stomach tube 5 ml of a 20% ileal homogenate from pig #5 of litter A. This ileal homogenate yielded no virus and was subsequently filtered (0.22-µm membrane) to remove bacteria before inoculating the HDCD piglets. Pigs in group III (n = 2) received a filtered 20% homogenate of ileal tissue from the negative control pig #10 (litter A). Pigs in group IV (n = 2) received 5 ml of PBS via stomach tube.

The third litter (litter C) consisted of 10 2-day-old conventional pigs divided into 2 groups. The uninfected control group (n = 5) remained in Plexiglas isolation cages; littermates (n = 5) were infected oral/nasally with 5 ml of the respiratory virus passed twice on ST cells (titer = 1×10^6 PFU/ml). The 5 inoculated pigs were swabbed on 7 DPI. All 10 pigs were swabbed and bled on 8 DPI.

Radioimmunoassay (RIA). The reactivity of sucrose gradient purified viral antigens TGEV, PRCV-Ind/89, and FIPV were compared with a panel of MAbs[g] that was selected to define antigenic sites A, B, C, and D.[4] Site D MAb 40.1 has

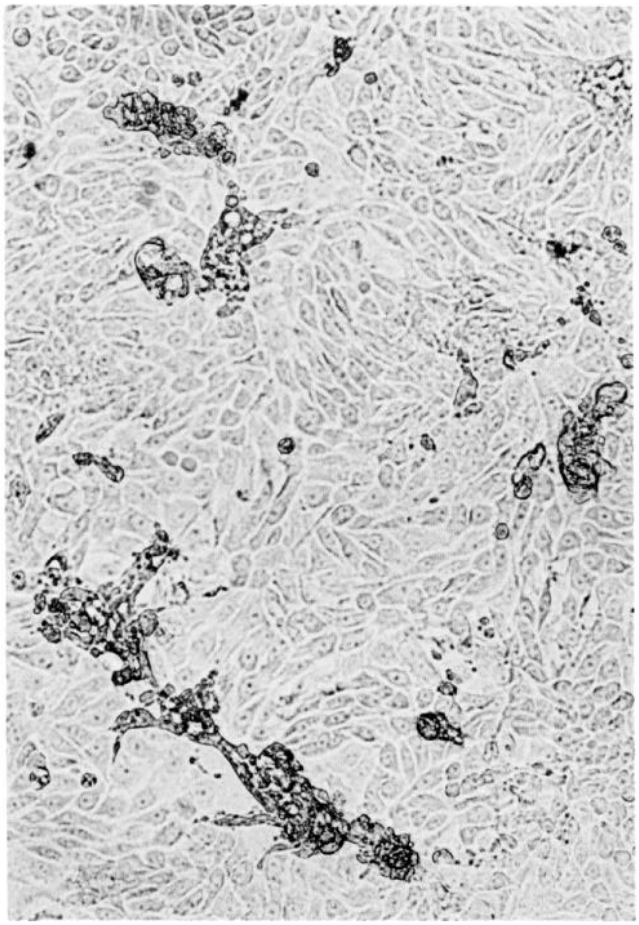

Figure 1. Photomicrograph showing early syncytia formations in a monolayer of ST cells infected with the Indiana respiratory coronavirus.

been shown to distinguish between PRCV and TGEV.[10] RIAs were carried out as described previously using ^{125}I-labeled anti-mouse $F(ab')_2^h$ as the indicator antibody.[20]

Results

Virus isolation. Virus was isolated from 9 of 26 nasal swab samples from weaned pigs (4-8 weeks old) in a swine breeding herd in Indiana. The virus replicated directly in either primary porcine kidney cells or in ST cells without adaptation. On ST cells, virus-induced cytopathology was indicated by the formation of large, often elongated, syncytia (Fig. 1). The syncytia would detach from the intact cell sheet, and often these floating fused cells were the first evidence of virus isolation. In approximately 2 days, the entire cell monolayer would be destroyed.

Pooled nasal swab samples were also inoculated oral/nasally into an 8-week-old TGEV-seronegative pig. A second seronegative pig was added to the isolation room 4 days later. Virus was recovered from the infected pig and the contact pig beginning at 7 DPI and 3 days postcontact, respectively (data not shown). The virus was isolated more readily from nasal than from tonsillar swabs. Virus was shed from these pigs up to and apparently beyond 10 days after exposure. After 14 days, no virus was recovered from either animal.

Virus (4×10^5 PFU/ml after a single passage on ST cells) from a nasal swab of the contact pig was the seed virus (PRCV-Ind/89) for additional experiments.

Serologic relationship to TGEV. In cross-neutralization studies, both TGEV and the respiratory isolate were neutralized by antisera prepared in gnotobiotic pigs against the classical enteric TGEV (Table 1). In addition, an anti-peplomer site A-specific MAb, 4F6, produced against TGEV neutralized both TGEV and PRCV-Ind/89. Convalescent antisera from an experimentally infected pig and from weaned pigs of the Indiana herd cross-neutralized classical TGEV even more efficiently than the homologous respiratory virus.

Experimentally infected neonatal pigs. The PRCV-Ind/89-infected and control piglets from 3 separate litters were observed for clinical signs of enteric or respiratory infection. No clinical signs were observed in piglets of litters B and C, which were given the lower virus dosages. These piglets were infected with the respiratory virus because 2 of 3 group I piglets in litter B seroconverted by DPI 17, and all of the group I piglets (n = 3) showed partial protection against a TGEV challenge. In addition, all 5 experimentally infected piglets of litter C seroconverted by 8 DPI and were shedding virus when monitored at 7 and 8 DPI (data not shown). Piglets from litter A, which received a higher virus dose (2×10^8 PFU), were also without respiratory signs, but 5 of 7 respiratory virus-infected piglets in this litter showed a mild diarrhea of approximately 3-day duration.

Piglets from litter A were swabbed to determine the shedding pattern of the respiratory virus. Virus was recovered from nasal and tonsillar swabs of respiratory virus-infected pigs but not from rectal swabs (Table 2). Only once, at 3 DPI, was virus recovered by rectal swab from piglet #2 inoculated with PRCV-Ind/89 by stomach tube. However, a single sampling of a Purdue virus-infected control littermate (piglet #9) with scours at 3 DPI yielded virus from nasal, tonsillar, and rectal swabs.

Fluorescent antibody and histopathology studies were conducted on ileal tissues taken from respiratory isolate-infected piglets at 3, 4, 5, 7, and 8 DPI. Control samples consisted of ileal tissues from littermates that were given cell culture medium (piglet #10) or a virulent Miller strain of TGEV and euthanized on the following day (piglet #8) or ileum from a litter-mate given the avirulent Purdue virus and euthanized at 3 DPI (piglet #9). In the PRCV-Ind/89-infected group, distinct villous atrophy was observed in 3 of the 5 piglets. Both of the control piglets infected with the

Table 1. Plaque-reduction neutralization titers of sera and ascites fluid to homologous and heterologous viruses.

Sera or ascites	Viruses	
	PRCV-Ind/89	TGEV
Convalescent serum (25 DPI) from pig experimentally infected with PRCV-Ind/89	158*	620
Convalescent serum from Indiana herd pig #17	28	256
Convalescent serum from Indiana herd pig #24	1,000	1,500
Anti-TGEV serum from gnotobiotic pig	5,200	13,200
Anti-peplomer monoclonal antibody 4F6	3.4×10^5	2.1×10^6

* Reciprocal of dilution resulting in a 50% plaque reduction.

Miller or the Purdue strain of TGEV showed marked villous atrophy accompanied by fusion of adjacent villi. Direct FA staining of cryostat sections of ileum showed that TGEV antigen was detected with virulent and avirulent control samples, but no FA-positive enterocytes were apparent in ileal tissues of PRCV-Ind/89-infected piglets.

Virus isolations were attempted from lung and intestinal homogenates of infected litter A piglets. No virus was recovered from intestinal homogenates of the 5 experimental piglets inoculated with PRCV-Ind/89, whereas virus was recovered from lung homogenates in 4 of 5 experimental piglets, and lung homogenate titers ranged from 4×10^3 to 4×10^6 PFU/g. In contrast, TGEV was recovered from intestinal homogenates of the Miller and the Purdue virus-infected piglets at 4×10^6 and 2×10^4 PFU/g, respectively, and from lung homogenates of these piglets at 10^3 and 3×10^6 PFU/g, respectively. No virus was recovered from either lung or intestine of the medium-inoculated negative control piglet.

Monoclonal antibody binding patterns. Results of RIAs with MAbs that recognize antigenic sites A, B, C, and D on the TGEV peplomer glycoprotein are shown in Fig. 2. Site A, sites A and B, site B, and site C determinants were present on TGEV, PRCV-Ind/89, and FIPV. Site D-specific MAb 40.1 bound to TGEV showed only limited and altered binding to PRCV-Ind/89, and did not bind to FIPV.

Discussion

A TGEV variant that causes an inapparent respiratory infection in neonatal and weaned pigs was isolated from a swine breeding stock herd in Indiana. The virus was easily recovered from the upper respiratory tract during the 2 weeks following exposure of an experimentally infected and a contact pig, but infectious virus was not detected in the feces. The virus caused no respiratory or enteric signs of disease in weaned pigs. Most neonatal piglets were asymptomatic. Two litters of piglets inoculated oral/nasally with the virus (4×10^6 and 5×10^6 PFU) were subclinically infected. Five of 7 piglets from a third litter given a larger dose of virus (2×10^8 PFU) exhibited mild diarrhea for 3 days, and villous atrophy was observed on histologic examination. However, unlike animals with acute TGEV, these piglets were alert, were neither gaunt nor dehydrated, and recovered from the diarrhea. In addition, the piglets with mild diarrhea were neither excreting infectious virus in feces nor could virus be isolated from ileal tissue homogenates, and viral antigen was not observed in ileal specimens by FA staining. In contrast, the TGEV variant virus was readily recovered in the upper respiratory airways and in ho-

Table 2. Virus shedding in 5-day old piglets given PRCV-Ind/B9, virulent TGEV, avirulent TGEV, or cell culture medium.

	Piglet number†																					
	1			2			3			4			5			6			7			8			9			10		
DPI*	N	T	R‡	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R	N	T	R
1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	−	−	−	...	...	...	−	−	−
2	+	+	−	−	+	−	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−	...	...	...	...	...	...	...	...	...
3	+	+	−	+	+	+	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−	...	...	...	+	+	+	...	...	...
4	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−	+	+	−	...	...	...	...	...	...	...	...	...	...	...	...
5	+	+	−	+	+	−	+	+	−	+	+	−	...	...	...	+	+	−	...	...	...	...	...	...	...	...	...	...	...	...
7	+	+	−	+	+	−	+	+	−	+	+	−	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
8	+	+	−	+	−	−	+	−	−	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

* DPI = days postinoculation.

† Piglets 1 and 2, respiratory virus via stomach tube; piglets 3–7, respiratory virus oral/nasal; piglet 8, Miller strain TGEV oral/nasal; piglet 9, Purdue strain TGEV oral/nasal; piglet 10, cell culture medium-inoculated control oral/nasal.

‡ N = nasal swab, T = tonsillar swab, R = rectal swab. + = cultures with virus cytopathic effect, − = cultures that were considered negative and remained negative after 3 blind passages on ST cells.

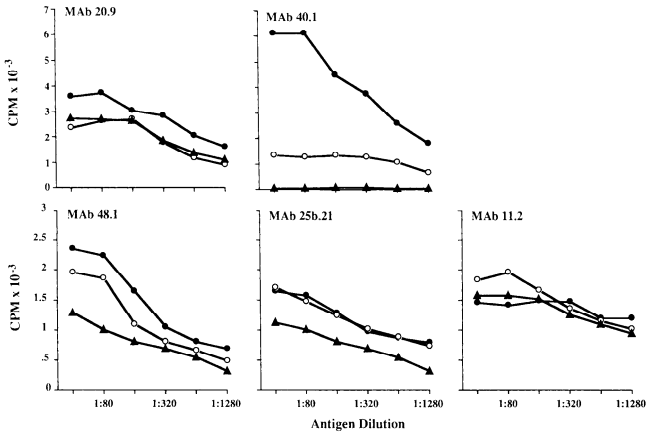

Figure 2. Solid-phase binding of peplomer-specific MAbs to TGEV (●), PRCV-Ind/89 (○), and FIPV (▲). A series of viral antigen dilutions were incubated with unlabeled MAbs that define antigenic site A (MAb 20.9), sites A and B (MAb 48.1), site B (MAb 25b.21), site C (MAb 11.2), and site D (MAb 40.1). Anti-mouse immunoglobulin, ^{125}I-labeled F(ab')$_2$, was used to quantitate the level of binding by each MAb.

mogenates of lung tissue from these piglets. These features and the serologic cross-neutralizing activity to TGEV suggest that a respiratory coronavirus similar to the European PRCV is present in the United States.

It has not been possible to import the European PRCV into the United States for comparative studies, but MAbs that were previously used to distinguish between TGEV and the European PRCV are available.[10] These MAbs identify 4 distinct epitopes (sites A, B, C, and D) on the peplomer glycoprotein of TGEV.[4] The major neutralizing MAbs recognize sites A and B. Recent studies[10] using indirect immunofluorescence assays have demonstrated that MAbs 20.9 (site A), 48.1 (sites A and B), 25b.21 (site B) and 11.2 (site C) reacted with TGEV, European PRCV isolates, and FIPV, whereas MAb 40.1 (site D) bound only to TGEV. By RIA, these MAbs bind to the PRCV-Ind/89 isolate and to the European PRCV isolates in a similar manner. That is, all MAbs except site D MAb 40.1 showed similar binding patterns for both TGEV and PRCV-Ind/89, whereas site D MAb 40.1 bound to TGEV but bound only marginally and with different binding characteristics to the PRCV-Ind/89 isolate. Thus, specific MAb binding patterns further indicate that the PRCV-Ind/89 isolate is a respiratory coronavirus with antigenic characteristics similar to European PRCV isolates.

Other coronaviruses that might infect pigs have properties distinct from PRCV-Ind/89. Examples include FIPV and canine coronavirus (CCV) that could possibly cause a low level seroconversion to TGEV, at least in young pigs,[17,18] but do not adapt readily to ST cell culture on primary isolation[19] as was seen with PRCV-Ind/89. The porcine coronaviruses hemagglutinating encephalomyelitis virus (HEV) and porcine epidemic diarrhea virus (PEDV) will infect neonatal and weaned pigs but do not produce antisera in pigs that cross-neutralize TGEV. In addition, TGEV can persist in a swine herd as an enzootic infection. However, enzootic TGEV does not replicate readily in ST cell cultures on primary isolation and does not lose enteric tropism. A virulent Japanese strain of TGEV was attenuated by serial passage in cell culture.[5] Following attenuation, this strain, TO-163, almost completely lost the ability to replicate in enteric tissues but retained respiratory tropism.[6] Thus, laboratory strain TO-163 has the same pathogenicity and tissue distribution properties as both the European and US PRCVs.

The VN test is used in the United States to detect TGEV antibody and to confirm a diagnosis of TGE. Unfortunately, antibodies produced by the PRCV-Ind/89 and TGEV are indistinguishable by the VN test. This situation creates a significant problem for US swine producers with respiratory coronavirus-infected herds who wish to export TGEV-free pigs. In Europe, a blocking enzyme-linked immunosorbent assay test is used to distinguish between swine infected with TGEV or with the European PRCV. Such an assay needs to be developed in the United States. If the respiratory coronavirus spreads in the United States as it has in Europe, reagents to differentiate these viral infections will be required to certify pigs for export as TGEV-free.

In the spring of 1989, 2 additional virus isolations were made from swine herds in Minnesota and North Carolina. The herd histories and cell culture characteristics of these viruses were the same as those of PRCV-Ind/89. All 3 respiratory virus isolates are antigenically closely related to TGEV but can be differentiated from other classical TGEV isolates by hybridization assays with specific cDNA probes (Wesley, unpublished data). However, no new herds infected with the respiratory coronavirus have come to our attention during the winter of 1989-1990. Currently, the prevalence of the respiratory coronavirus in US swine is unknown.

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Sources and manufacturers

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