

## BRIEF COMMUNICATIONS

### Alveolar macrophages as a diagnostic sample for detecting natural infection of pigs with porcine reproductive and respiratory syndrome virus

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In a previous series of studies focused on the diagnosis of porcine reproductive and respiratory syndrome (PRRS), we tested sera, alveolar macrophages, and various tissues for their suitability as diagnostic samples when collected from acutely and persistently infected pigs.<sup>3,5</sup> The results of these studies indicated that the causative virus (PRRS virus [PRRSV]) could usually be isolated from any of several kinds of samples (serum, tonsils, lungs, and alveolar macrophages) during acute infection but from only alveolar macrophages during later stages of infection. However, during a recent experiment that was initially unrelated to our investigations of PRRS, we found that alveolar macrophages may sometimes be the diagnostic sample of choice even during acute infection. Details of this fortuitous observation follow.

A group of 20 8-week-old pigs was delivered from a commercial swine herd to our laboratory for use in an experiment on pseudorabies (PR). On the following day (day 0) we euthanized and necropsied 2 of the 20 pigs as noninfected controls for the PR experiment (not described here) and found small areas of consolidation in the lungs of both. Although we suspected that these lesions were the result of a bacterial pneumonia, we tested alveolar macrophages of both of the pigs for PRRSV. We also tested the sera collected on day 0 from these and the remaining 18 pigs for both PRRSV and homologous antibody. The detection of PRRSV and antibody in some of these initial samples led us to test additional samples collected at later times (Table 1). The methods used to identify PRRSV and homologous antibody are outlined briefly in the following paragraphs. Additional details can be found in previous reports.<sup>5,2</sup>

For detection of virus, alveolar macrophages and sera were tested in MARC-145 cells.<sup>1</sup> Alveolar macrophages were collected by aspiration, dispensed to 60-mm Petri dishes, and maintained at 37 C in a humid atmosphere of 5% CO<sub>2</sub>. A 1% concentration of porcine antiserum for PR (neutralization titer = 4,096) was included in the macrophage culture (nutrient) medium to neutralize any PR virus present in the culture. After 72 hours of incubation, a sample (0.2 ml) of the nutrient medium from each macrophage culture was added to the nutrient medium (1 ml) of a correspondingly identified confluent monolayer (2 cm<sup>2</sup>) of MARC-145 cells. Se-

rum was tested for PRRSV in the same way except that 0.05 ml of serum was added to the nutrient medium of MARC-145 cells because larger volumes were sometimes cytotoxic. After 24 hours of incubation, the inoculum-nutrient medium was aspirated and fresh medium was added. The MARC-145 cell cultures were examined daily for at least 7 days for the characteristic cytopathic changes induced by PRRSV. Irrespective of whether cytopathic changes were detected, 1

**Table 1.** Identification of porcine reproductive and respiratory syndrome virus (PRRSV) and homologous antibody in samples collected from naturally infected pigs.

Pig no.	Necropsy day†	Virus isolation*				Antibody titer‡	
		Serum		Lung	Culture medium	Day 0	Necropsy
		Day 0	Necropsy				
1	0	-§	-	...	+	<4	<4
2	0	-	-	...	+	64	64
3	1	-	-	...	+	64	256
4	1	-	-	...	+	<4	<4
5	3	-	-	...	+	<4	<4
6	3	+	+	...	+	<4	<4
7	5	-	-	...	-	16	16
8	5	-	-	...	-	<4	<4
9	7	-	-	...	-	<4	4
10	7	-	-	...	+	256	1,024
11	9	-	-	...	-	<4	<4
12	9	-	-	+	+	<4	16
13	14	-	-	-	+	<4	<4
14	14	-	-	-	+	16	1,024
15	15	-	-	-	-	<4	<4
16	15	-	+	+	+	<4	256
17	16	-	-	+	+	<4	64
18	16	-	-	-	+	<4	<4
19	16	-	-	-	+	<4	256
20	16	-	-	-	+	<4	256

\* From serum collected at times indicated (PRRSV was not isolated from sera of any of the live pigs bled on days 7 and 14), from lung collected at necropsy, and from culture medium collected from a 72-hour culture of alveolar macrophages collected at necropsy.

† Day 0 = the day on which all pigs except pigs 1 and 2 were exposed to pseudorabies virus in an unrelated experiment and the day after all of the pigs were delivered to the National Animal Disease Center from their farm of origin.

‡ Titer determined by indirect immunofluorescence microscopy.

§ + and - = PRRSV was or was not isolated, respectively.

|| ... = lung was not collected for testing.

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additional passage was made on day 4 by collecting 0.2 ml of nutrient medium from the culture exposed to the sample inoculum and adding it to the nutrient medium of another culture of MARC-145 cells. This additional passage strategy was continued throughout the study even though cytopathic changes were never evident only in the second culture of MARC-145 cells.

For detection of antibody, sera were tested by indirect immunofluorescence microscopy. Confluent monolayers (surface area/monolayer = 0.0133 cm<sup>2</sup>) of MARC-145 cells grown in 60-well microwell plates at 37 C in a humid atmosphere containing 5% CO<sub>2</sub> were infected with about 20 plaque-forming units of PRRSV. After an additional 20-24 hours of incubation at 37 C in 5% CO<sub>2</sub> (when plaques comprised about 20 infected cells), monolayers were rinsed once with phosphate-buffered saline (PBS, pH 7.2) and then fixed in 90% ethanol. Fixed monolayers were air-dried and then either used immediately or stored at 4 C until needed. Each serum tested for antibody was added (10 µl) to an infected, fixed monolayer, and the monolayers were reacted with serum for 30 minutes at 37 C in a humid atmosphere. Following incubation with serum, monolayers were rinsed twice with PBS, once with distilled water, and air-dried. They were next reacted with conjugate (fluorescein isothiocyanate-labeled goat anti-porcine immunoglobulin, 10 µl/well, that had been digested with papain<sup>6</sup>) and incubated for 30 minutes at 37 C in a humid atmosphere. Following incubation with conjugate, monolayers were again rinsed with PBS and distilled water and air-dried. Finally, a 1:1 mixture of glycerine and PBS was added to each well (10 µl/well), and monolayers were examined (13 x objective) by fluorescence microscopy for the presence of infected (fluorescent) plaques.

In addition to tests for PRRSV and homologous antibody, the lungs of 4 pigs (pigs 17, 18, 19, 20) were submitted to the Diagnostic Bacteriology Laboratory of the National Veterinary Services Laboratories (US Department of Agriculture, Animal and Plant Health Inspection Service (APHIS)) in Ames, Iowa, to test for bacterial pathogens.

Porcine reproductive and respiratory syndrome virus was isolated from the day-0 serum sample of 1 pig (pig 6) and from alveolar macrophage cultures of both pigs euthanized and necropsied on day 0 (pigs 1, 2). Antibody for PRRSV was identified in the day-0 serum of 5 pigs (pigs 2, 3, 7, 10, 14). Titers ranged from 16 to 256. Over the 16-day interval during which pigs were tested for PRRSV and homologous antibody, PRRSV was isolated from sera of 2 of the 20 pigs (pigs 6, 16), from alveolar macrophage cultures of 15 of the 20 pigs (pigs 1-6, 10, 12-14, 16-20), and from the lungs of 3 of 9 pigs.

The difference in the success rates between isolating PRRSV from serum and from alveolar macrophages (i.e., 2/20 vs. 15/20) is magnified by the fact that alveolar macrophages were collected once (at necropsy), whereas blood was collected from most of the pigs 2 or more times during the study; i.e., 18 pigs were bled at least 2 times (day 0 and at necropsy), 10 pigs were bled at least 3 times (days 0, 7 and at necropsy), and 6 pigs were bled 4 times (days 0, 7, 14 and at necropsy). Porcine reproductive and respiratory syndrome virus was isolated from only 4 of the 54 corresponding serum samples, from pig 6 on days 0 and 3 and from pig 16 on days 14 and 15.

A total of 11 of the 20 pigs had serum antibody (titers  $\geq 4$ ) for PRRSV by the time they were euthanized. Virus was isolated from alveolar macrophage cultures of all but 2 (pigs 7, 9) of these. The collective results of virus isolation and serology indicated that at least 17 of the 20 pigs were infected with PRRSV either shortly before (discussed in a subsequent paragraph) or during the 16-day interval of the experiment. Antibody titers for PRRSV increased for 3 of the 4 pigs that were seropositive on day 0 and then bled and tested again at a later time.

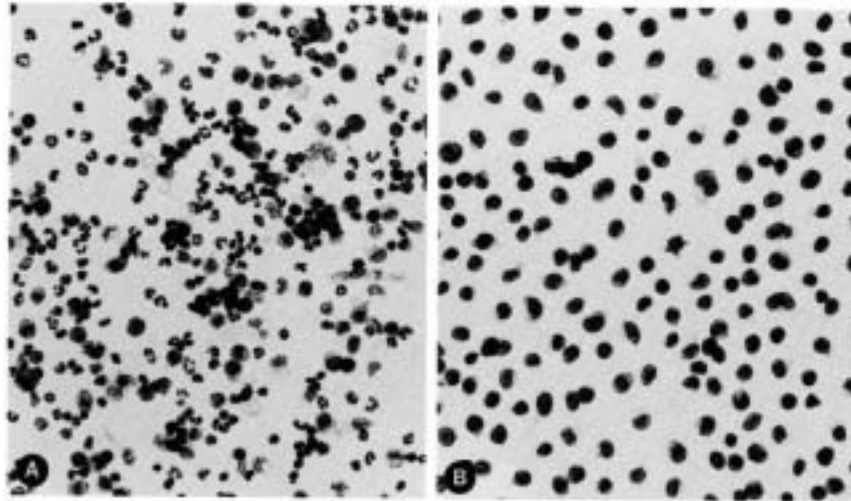
Macroscopic lesions of pulmonary consolidation were present in the lungs of all pigs. Depending on the pig in question, the severity and extent of these lesions ranged from small, discrete areas of consolidation to consolidation of most of an affected lobe. Neutrophils initially predominated in cultures prepared from the lungs of pig 2 (Fig. 1 A), but within the next 48 hours most of the neutrophils released into the culture medium. The remaining adherent cells were mostly alveolar macrophages and a few epithelial-like cells. Cultures prepared from the lungs of all other pigs were predominantly alveolar macrophages (Fig. 1B). Pathogenic bacteria were isolated from 2 of the 4 lungs submitted for testing, *Bordetella bronchiseptica* and *Haemophilus parasuis* from the lungs of pig 19 and *Haemophilus* sp. (similar to *H. parasuis*) from the lungs of pig 20.

The contribution, if any, of PRRSV to the pulmonary lesions is unknown. Our opinion that these lesions were the result of a bacterial infection is supported in part by the isolation of *Haemophilus* and *Bordetella* and the large number of neutrophils in lavage fluid collected from pig 2 (euthanized on day 0). On the other hand, since the completion of this work we have seen similar lesions in pigs kept in isolation and, to our knowledge, exposed only to a virulent strain of PRRSV.

Pseudorabies virus also may have caused some pulmonary damage. However, pulmonary lesions appeared no more severe or extensive in pigs euthanized a week or more after exposure to PR virus than in those euthanized earlier.

It has been our experience that serum antibody titers for PRRSV detected by indirect immunofluorescence microscopy are near or at their maximum by about 2 weeks postexposure.<sup>5,4</sup> Because most (15/20) of the pigs described here were free of antibody for PRRSV on day 0, i.e., the day after they were delivered to us from the commercial herd of origin, and because the antibody titer increased for 3 of the 4 pigs that were seropositive on day 0 and bled again at a later time, we assume that infection began when 1 or more members of the group were naturally exposed to PRRSV no more than about 2 weeks before they were delivered to us. If so, we collected samples from most, if not all, of the infected pigs during the acute stage of infection. Therefore, the detection of PRRSV in alveolar macrophages but not in the serum from many of these pigs suggests that alveolar macrophages may be the better sample from which to consistently isolate PRRSV, even during acute infection.

Our conclusion is that consideration should be given to testing alveolar macrophages, either as described in this report or by coculture with MARC-145 cells,<sup>3,5,7</sup> when other samples collected from pigs suspected of being infected with PRRSV fail to yield virus.



**Figure 1.** Cells flushed from the lungs of each of the 20 pigs were added to 60-mm plastic Petri dishes. One hour later nonadherent cells were rinsed away, and 1 of the replicate cultures prepared for each pig was fixed and subsequently stained for brightfield microscopy. **A.** A 1-hour culture prepared from pig 2. At 1 hour this culture comprised mostly neutrophils. However, with continued incubation of replicate cultures prepared from pig 2, most of the neutrophils released from the surface, leaving a sparse population of alveolar macrophages. **B.** A 1-hour culture prepared from pig 18. At 1 hour, and throughout the incubation interval, cultures prepared from pig 18 and from all other pigs except pig 2 comprised mostly alveolar macrophages.

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