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In vitro detection of Shiga toxin using porcine alveolar macrophages

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Abstract. Porcine alveolar macrophages were found to be highly susceptible to the cytolytic effects of a toxin (Shiga toxin [Stx]) produced by certain strains of *Escherichia coli* and sometimes associated with clinical disease in pigs and other animals. In comparison with the cells that are most commonly used for Stx detection and titration in vitro (namely, Vero cells), porcine alveolar macrophages appeared to be generally more sensitive and test results could be obtained in less time. Moreover, unlike Vero cells, porcine alveolar macrophages need not be continuously propagated to ensure immediate availability. They can simply be removed from a low-temperature repository, thawed, seeded, and shortly thereafter exposed to the sample in question. These characteristics suggest that porcine alveolar macrophages may be useful in developing a highly sensitive and timely diagnostic test for Stx.

On October 7, 1999, a request was made to assist in determining the cause of illness affecting a group of 3,600 approximately 50-lb pigs. The pigs had recently been moved several hundred miles from nurseries to grow/finish facilities comprising 3 separate buildings (1,200 pigs/building) at the same site. The predominant clinical sign was moderate to severe, and sometimes fatal, diarrhea. During the acute stage of the disease, many pigs were also recumbent. Others were ambulatory but uncoordinated. By the time assistance was requested, clinical signs had almost subsided, and although several possible causes were discussed, no further diagnostic assistance was provided.

On February 1, 2000, a second request was made for diagnostic assistance. Another group of approximately 50-lb pigs at the same site was affected with what was believed to be the same clinical problem. The following day, samples were collected from 10 pigs of different pens within the same building. Pigs in each of the 3 buildings at the site were similarly affected, but the group from which samples were collected had most recently developed clinical signs.

A blood sample, nasal swab, rectal swab, and tonsil swab were obtained from each of the 10 pigs. Because of the extensive diarrhea, all pigs were covered with feces and it was noted during sample collection that every nasal swab, and to a lesser extent tonsil swabs, had some level of fecal contamination. Each swab was submerged in 2 ml of Earle's balanced salt solution supplemented with a high concentration of antibiotics in a 13- × 100-mm screw-capped tube. The type and concentration of antibiotics were as previously described for maintaining cultures of porcine alveolar macrophages.[3] All samples were placed on ice and immediately taken to the National Animal Disease Center, Ames, Iowa, for further treatment.

Serum was obtained from each of the blood samples. Swab contents were dispersed into the surrounding 2 ml of transport medium by vigorous agitation. Swabs were discarded, an additional 2 ml of transport medium was added to each tube, and the contents of each tube were filtered through a 450-μm filter. Aliquots of serum and of the filtered medium from each swab tube were added to the nutrient medium of each of 4 types of cell cultures, namely, porcine alveolar macrophages, established lines of monkey kidney (MARC-145) and porcine testicle (McClurkin) cells, and fetal porcine kidney cells. In each case, 50 μl of sample was added to the nutrient medium (1 ml) of a 2 cm^2 monolayer of the appropriate cell type in a 24-well cell culture plate. Serum was also tested for porcine reproductive and respiratory syndrome virus (PRRSV) by a nested-set polymerase

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Table 1. Isolation of viruses from serum and swab samples collected from pigs believed to be naturally affected with edema disease.

	Pig number									
Virus*	1	2	3	4	5	6	7	8	9	10
PRRSV	+†	+	+	+	+	+	+	+	+	+
PRCV	+	−	−	−	−	−	+	−	−	+
PEV	+	+	+	+	+	−	+	+	+	−

* PRRSV = porcine reproductive and respiratory syndrome virus; PRCV = porcine respiratory coronavirus; PEV = porcine enterovirus. No other viruses were identified.

† + = virus identified; − = virus not identified. Viruses were identified by isolation in 1 or more types of cell cultures (see text) except for identification of PRRSV in 3 pigs (serum) only by PCR.

chain reaction (PCR).[8] Three blind passages were made in MARC-145, porcine testicle, and fetal porcine kidney cells before a sample was considered virus-free.

Collectively, PRRSV, porcine respiratory coronavirus (PRCV), and porcine enteroviruses (PEV) were identified by virus isolation in 1 or more types of cell cultures. More specifically, PRRSV was isolated in MARC-145 cells from the serum of 7 pigs, and it was identified by PCR in the sera of all 10 pigs. Although PRRSV was not identified by cytopathic effects in any of the other types of cell cultures, it was detected in the culture medium of 2 macrophage cultures by adding medium from macrophage cultures to cultures of MARC-145 cells 4 days after the macrophage cultures had been exposed to the field samples. The PRCV and PEV were isolated from 3 and 8 pigs, respectively. The PRCV was isolated only from nasal swabs and only in porcine testicle cells. The PEV was isolated from rectal swabs (8 pigs), nasal swabs (5 pigs), and a tonsil swab (1 pig). All of these 14 swabs were positive in porcine testicle cells, most were also positive in fetal porcine kidney cells, and a few were also positive in MARC-145 cells. The identity of PRRSV and PRCV was confirmed by PCR using specific primers. Portions of the PRCV genome were also sequenced. The PRRSV isolates were further characterized by restriction fragment length polymorphism (RFLP). The RFLP pattern was 1-4-1 (7 pigs) and 1-3-1 (3 pigs). These RFLP patterns indicated that the isolates were field strains of PRRSV, i.e., not vaccine or vaccine-derived strains.[4] The identity of PEV was confirmed by indirect immunofluorescence with specific antisera kindly provided by the National Veterinary Services Laboratories, Ames, Iowa (J. Landgraf). A summary of the viruses isolated from each of the pigs is presented in Table 1.

Table 2. Shiga toxin titer of selected samples.

Sample*	Titer†
123	<1
S1191	≥2,048
2R	81,920
3R	640
5R	≥81,920
4N	10,240
5N	1,280
2T	2,560
3T	1,280

* Samples 123 and S1191 were culture supernatant fluids from nontoxin (123) and Shiga toxin-producing (S1191) strains of *E. coli*. The remaining samples (R = rectal, N = nasal, T = tonsil) were from pigs (2, 3, 4, or 5) involved in the clinical episode under investigation.

† Samples 123 and S1191 were tested in 2-fold dilutions from undiluted to 1:2,048; the remaining samples were tested in 2-fold dilutions from 1:40 to 81,920.

The isolation of virus from rectal, nasal, and tonsil swabs in cultures of porcine alveolar macrophages was precluded because overnight incubation of cultures exposed to these samples resulted in partial to complete cytolysis. In general, damage was most severe in cultures exposed to rectal-swab samples, but cultures were affected regardless of the type of swab sample. This observation and the knowledge that all nasal swabs and perhaps all tonsil swabs were contaminated with feces suggested that porcine alveolar macrophages might be highly sensitive to some type of enterotoxin.

A cytolytic role for enterotoxin became even more plausible when a probable diagnosis of edema disease was subsequently made at the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) by histologic and bacteriologic examination of additional samples submitted from the same clinical episode. This suggested that the putative toxin we had identified was a Shiga toxin (Stx) produced by *Escherichia coli*. If so, it appeared that porcine alveolar macrophages might be a highly sensitive cell type for detecting Stx. A series of steps was taken to test this hypothesis.

The first step was to confirm the identity of the toxin in the field samples by neutralizing its activity with a bovine polyclonal antiserum for Stx 1, Stx 2, and Stx 2e.[6] Field samples randomly selected for testing were fluids from 2 rectal swabs, 2 nasal swabs, and 1 tonsil swab. The toxin preparation used as a positive control was supernatant fluid from an Stx-positive culture of *E. coli*, strain S1191.[2] Vero cells were used as the indicator of toxin activity.[1] In all cases, the toxic activity was neutralized by the polyclonal antiserum but not by fetal calf sera.

The second step was to determine the titers of toxin in a representative group of field samples and compare them with the titer of supernatant fluid from a culture of *E. coli* strain S1191. The comparison was done using Vero cells as the indicator system. The samples selected for comparative testing and their corresponding Stx titers are presented in Table 2.

The third step was to compare titers of toxic activity determined in porcine alveolar macrophages with those determined in Vero cells. In this case, culture fluids from *E. coli*

Table 3. Comparison of porcine alveolar macrophages and Vero cells for titration of Shiga toxin (Stx).

Sample*	Stx type†	Titer‡	
		Alveolar macrophages	Vero cells
DH5a	none	4	<4
DH5a/pCKS112	Stx 1	1,310,720	2,621,440
DH5a/pJES120	Stx 2	10,485,760	1,310,720
S1191	Stx 2e	10,240	2,560
123	none	<4	<4
5R	unknown	81,920	40,960

* Sample 5R was fluid from the rectal swab of pig 5; the remaining samples were culture supernatant fluid from the indicated strain of *E. coli*.

† The type of Stx produced by the indicated strain of *E. coli*; type-specific antisera were not available to determine the type, or types, of Stx in sample 5R.

‡ Samples DH5a and 123 were tested in 2-fold dilutions starting at 1:4; the remaining samples were tested in 2-fold dilutions starting at 1:160.

strains DH5a (pCKS112)[7] (Stx 1), DH5a (pJES120)[7] (Stx 2), and S1191 (Stx 2e) and fluid from the rectal swab of 1 of the field samples (pig 5) were used in the comparison. Negative controls were culture fluids from *E. coli* strain DH5a and nonpathogenic *E. coli* strain 123. Alveolar macrophages were 2-fold to 8-fold more sensitive than Vero cells for determining toxic activity in 3 of the 4 Stx-positive samples (Table 3). However, there were too few samples to reach a definitive conclusion in regard to relative sensitivity between these 2 cell types.

The fourth step was to determine if each of the 3 reference types of Stx (Stx 1, Stx 2, and Stx 2e) tested in step 3 would be neutralized with specific antisera (polyclonal antiserum for Stx 1, Stx 2, and Stx 2e) when porcine alveolar macrophages were used as the indicator system. In all cases, the polyclonal antisera, but not fetal calf serum, neutralized toxin activity.

On the basis of diagnostic testing done by others at the ISUVDL and the identification of Stx in the samples described in this report, a primary diagnosis of edema disease seems appropriate for the clinical episode in question. Moreover, in a retrospective study, genes for Stx 2e, enterotoxins STa and STb, and F18 fimbria were identified by PCR analysis of an *E. coli* strain that had been isolated from the same clinical episode and kept in the ISUVDL repository (Bosworth BT, Casey TA: 1997, Identification of toxin and pilus genes in porcine *Escherichia coli* using polymerase chain reaction [PCR] with multiple primer pairs. Gen Meet Am Soc Microbiol [abstr. B-509]). From this information, it seems possible that enterotoxin STa or STb or both caused the profuse diarrhea observed in most affected pigs. Conversely, the concurrent clinical effect, if any, of PRRSV, PRCV, and PEV is unclear.

There appear to be several attributes of porcine alveolar macrophages for detecting and titrating Stx. They are highly sensitive to Stx and may often provide titers of toxic activity equal to or greater than the commonly used Vero cells. Toxin can be added as soon as macrophages attach to the culture surface and nonadherent cells are rinsed away (this process is typically completed in less than 2 hours, with 1 hour for attachment and a few minutes to rinse away nonadherent cells and add fresh medium). Results are obtained in 16 hours or less (there is little or no change in the appearance of cultures after 16 hours of incubation). This contrasts with 48–72 hours of incubation required for Vero cell assays as they are commonly performed. Endpoints of toxic activity are relatively easy to determine because the amount of cytolysis usually changes abruptly. Unlike established cell lines, porcine alveolar macrophages need not be continuously propagated to ensure timely availability. They can simply be removed from a low-temperature repository, thawed, seeded, and used almost immediately. The toxins produced by *E. coli* that are common in ruminants and associated with foodborne human illness (Stx 1 and Stx 2) as well as the toxin associated with edema disease (Stx 2e) are all detected.

Although no clear differences were detected in the sensitivity of alveolar macrophages collected from different pigs during the course of this study (a total of 7 different donors were used to test this possibility), there is still a chance that at least subtle differences could be detected among pigs of different ages and different genetic backgrounds. If so, and if these in vitro differences reflect the corresponding pig's relative susceptibility to the specific enterotoxin-associated disease, alveolar macrophages might provide a phenotypic marker in attempting to select for disease resistance. The fact that alveolar macrophages can be collected relatively easily from live pigs[3] makes this hypothesis both testable and of potential practical significance.

The sensitivity of porcine alveolar macrophages to Stx also raises some interesting questions in regard to the pathogenesis of edema disease. Selected macrophage populations of murine peritoneal macrophages and human peripheral blood monocytes also have been tested for their susceptibility to Stx.[5,8] Although both populations were found to be resistant to the potential Stx-induced cytolytic effects, they responded to Stx exposure by producing proinflammatory cytokines that induced expression of endothelial cell receptors for Stx. It was speculated that this cascade of events predisposes to Stx-mediated vascular damage.[5,7] Whether the same or a similar phenomenon occurs in edema disease of swine remains to be determined.

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Detection of bovine herpesvirus-1 in peripheral blood mononuclear cells eight months postinfection

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Abstract. Peripheral blood mononuclear cells (PBMCs) from 5 calves (3 controls and 2 vaccinates) used in a bovine herpesvirus 1 (BHV-1) vaccine study with a BHV-1 Cooper strain challenge were collected 6 months after challenge. The PBMCs from the control animals were positive by immunofluorescence for the BHV-1 glycoprotein D (gD) while the vaccinates were negative. The PBMC samples from 4 of the 5 animals were examined for BHV-1 DNA by polymerase chain reaction (PCR) and for gD immunofluorescence at 8 months after challenge. The BHV-1 DNA and viral antigen were detected in PBMC samples at 8 months postinfection, but no virus was isolated.

Bovine herpesvirus-1 (BHV-1), an alphaherpesvirus,[15] is one of the most ubiquitous and important pathogens of cattle. Like other members of the alphaherpesvirus group,[13] BHV-1 grows rapidly in cell cultures, infects a wide range of cell types, and establishes latent infection in neurons of sensory and autonomic nerve ganglia.[2,12] The BHV-1 infection causes respiratory and reproductive tract diseases.[21] Cattle infected with BHV-1 exhibit clinical immunosuppression.[3,9] Animals with acute BHV-1 infection often develop secondary infection with *Manheimmia haemolytica*.[3] Immune suppression has also been seen in cattle vaccinated with modified live BHV-1 vaccine, as these animals have a reduced antibody response to a commercial *M. haemolytica* leukotoxin vaccine.[9] Following acute infection, BHV-1 antigen and DNA have been detected in spleen, prescapular lymph nodes, and peripheral blood mononuclear cells (PBMCs) through day 19 postinfection.[1] Other studies[18] have demonstrated BHV-1 antigen in pharyngeal, cervical, retropharyngeal and inguinal lymph nodes, and tonsil 7 days postinfection. They also demonstrated the presence of BHV-1 DNA and antigen in $CD4^+$ but not CD^+8 T cells in the lymph nodes and PBMCs. Recently, BHV-1 latency and reactivation has been demonstrated in tonsil 60 days postinfection.[19] The BHV-1 has also been detected in PBMCs of cattle vaccinated with a live BHV-1 gE-negative vaccine 4–5 months postvaccination using PCR.[7] In this study, BHV-1 glycoprotein D (gD) antigen and DNA were detected in PBMCs from calves 6 and 8 months after BHV-1 infection.

Five yearling beef calves (700–900 lbs) were used in this experiment. These calves were part of a vaccination-duration study (9 animals were in the original study). The other 4 animals were sold 4 months after the challenge. Prior to vaccination, the calves were seronegative (<2) by serum neutralization to BHV-1, bovine viral diarrhea virus (BVDV) types 1 and 2, and bovine respiratory syncytial virus. The animals were also PCR negative for BHV-1 and BVDV. The vaccinates and control animals were grouped together but kept in strict isolation throughout the vaccination, challenge, and postchallenge period. The vaccinated animals were given 2 doses of a commercial inactivated vaccine[a] containing BHV-1, 1 month apart, with the last dose given 8 months before challenge. All animals were challenged intranasally with 10^7 tissue culture infectious dose 50 ($TCID_{50}$) BHV-1 (Cooper strain, ATCC). Following challenge, BHV-1 virus was recovered from all calves. The vaccinated calves shed virus from day 1 to day 6, while the control calves shed virus day 1 through day 8 and the virus titers were consistently higher in the control group (2–3 log 10). There was no difference in clinical signs between control and vacci-

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