Noroviruses (NVs) are important human pathogens that cause acute gastroenteritis. Genetically related animal enteric NVs have also been described, but there is no evidence of interspecies transmission of NVs. In this study we characterized antibody prevalence among domestic pigs by using recombinant capsid antigens of two human NVs (Norwalk and Hawaii) and one swine NV (SW918) that is genetically related to GII human NVs. Recombinant SW918 capsid protein expressed in baculovirus self-assembled into virus-like particles (VLPs) that were detected by antibodies against GII (Hawaii and Mexico), but not G1 (Norwalk and VA115), human NVs. NVs recognize human histo-blood group antigens as receptors, but SW918 VLPs did not bind to human saliva samples with major histo-blood group types. Seventy-eight of 110 (71%) pig serum samples from the United States and 95 of 266 (36%) pig serum samples from Japan possessed antibodies against SW918. Serum samples from pigs in the United States were also tested for antibodies against human NVs; 63% were positive for Norwalk virus (G1) and 52% for Hawaii virus (GII). These results indicate that NV infections are common among domestic pigs; the finding of antigenic relationships between SW918 and human NVs and the detection of antibodies against both G1 and GII human NVs in domestic animals highlights the importance of further studies on NV gastroenteritis as a possible zoonotic disease.

Seroprevalence of Noroviruses in Swine

Tibor Farkas,1,2* Setsuko Nakajima,3 Masaaki Sugieda,4 Xiao Yun Deng,1 Weiming Zhong,1 and Xi Jiang1,2

Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center,1 and Department of Pediatrics, University of Cincinnati College of Medicine,2 Cincinnati, Ohio, and Department of Virology, Medical School, Nagoya City University, Aichi,3 and Shizuoka Prefectural Institute of Public Health and Environmental Science, Shizuoka,4 Japan

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Noroviruses (NVs) are important human pathogens that cause acute gastroenteritis. Genetically related animal enteric NVs have also been described, but there is no evidence of interspecies transmission of NVs. In this study we characterized antibody prevalence among domestic pigs by using recombinant capsid antigens of two human NVs (Norwalk and Hawaii) and one swine NV (SW918) that is genetically related to GII human NVs. Recombinant SW918 capsid protein expressed in baculovirus self-assembled into virus-like particles (VLPs) that were detected by antibodies against GII (Hawaii and Mexico), but not G1 (Norwalk and VA115), human NVs. NVs recognize human histo-blood group antigens as receptors, but SW918 VLPs did not bind to human saliva samples with major histo-blood group types. Seventy-eight of 110 (71%) pig serum samples from the United States and 95 of 266 (36%) pig serum samples from Japan possessed antibodies against SW918. Serum samples from pigs in the United States were also tested for antibodies against human NVs; 63% were positive for Norwalk virus (G1) and 52% for Hawaii virus (GII). These results indicate that NV infections are common among domestic pigs; the finding of antigenic relationships between SW918 and human NVs and the detection of antibodies against both G1 and GII human NVs in domestic animals highlights the importance of further studies on NV gastroenteritis as a possible zoonotic disease.

Caliciviridae consists of four genera, Norovirus (NV), Sapovirus (SV), Lagovirus, and Vesivirus (7), from which NV and SV mainly cause acute gastroenteritis in humans; therefore, these two genera also are called human caliciviruses (HuCVs). Recently several animal enteric CVs genetically closely related to NV or SV have been reported (1, 9, 10, 21, 23). The porcine enteric CV is the only cultivable enteric CV that is closely related to HuCVs, representing a distinct genogroup within SV (9). The bovine enteric CVs (the Jena and Newbury viruses) represent two distinct clusters of genogroup III (GIII) NV (1, 21), while the swine enteric CVs that are closely related to human NVs represent a distinct cluster within genogroup II (22, 23). The discovery of these animal CVs raised the question about CV gastroenteritis as a zoonotic disease.

The epidemiology and prevalence of animal NVs are not well understood. In The Netherlands, 31.6% of pooled stool specimens from veal calf farms and 4.2% of individual stool specimens from dairy cattle were positive for NVs related to the Newbury virus (25). By using an enzyme-linked immunosorbent assay specific for the Jena virus, a study in Germany showed that 9% of the diarrhea stool samples and 99% of the serum samples collected from dairy cows were positive for Jena virus antigens or antibodies, respectively (2). SW918, a prototype strain of swine NVs that shares 64 to 69% amino acid identity with other cluster representatives within GII NVs, was first detected in the cecum contents of healthy pigs in Japan by reverse transcription-PCR (RT-PCR) in 1997 (22). The antigenic identity of SW918 remains unknown due to a lack of reagents for diagnosis. The detection rate of swine NVs in Japan was low (0.35%), and a similar low detection rate (2%) was reported in The Netherlands (26). Recently, Wang et al. reported the detection of swine NVs in 5 of 275 fecal samples collected from six pig farms in the United States and by redesigning their original primers the detection rate improved to 23% (63/275) (27). The role of NVs in causing disease in pigs remains unclear.

In this study we describe the prevalence of antibody against a swine NV (SW918) in pigs in Japan and the United States by an enzyme immune assay (EIA) based on baculovirus-expressed SW918 virus-like particles (VLPs). We also characterized the antigenic identity of SW918 by using type-specific antibodies raised against human NVs. Furthermore, we investigated the prevalence of antibody among pigs against human NVs to address if cross-species transmission of NVs can occur between animals and humans. Although our results did not have the answer, the data presented in this study highlighted the necessity of studying animal NVs in the future.

MATERIALS AND METHODS

Serum and stool samples. A total of 110 serum samples collected from pigs in the United States were tested for antibodies against SW918 and human NVs (Norwalk and Hawaii). Seventy-six of these were collected from Iowa, 2 from Oregon, and 22 from Texas. No other data were available on these samples. Ten serum samples were collected in Kentucky in 2003 in a slaughterhouse from pigs between 6 to 12 months of age. A total of 266 serum samples collected from 6-month-old pigs between 1997 and 2003 in Shizuoka prefecture in Japan were tested for antibodies against SW918. Thirty-eight of these samples were collected in 1997, 50 in 1998, 100 in 2002, and 78 in 2003. A total of 104 stool specimens, 100 collected from healthy pigs and 4 from pigs with diarrhea that were less than 3 months old from a farm in Ohio, and 13 intestinal contents collected from the large intestines of pigs between 6 to 12

* Corresponding author. Mailing address: Division of Infectious Diseases, Cincinnati Children’s Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229-3039. Phone: (513) 636-0131. Fax: (513) 636-7655. E-mail: Tibor.Farkas@chcmc.org.
months of age in a slaughterhouse in Kentucky in 2003 were tested for the presence of NVs by RT-PCR and EIA. Stool and serum specimens were stored at −20°C.

Detection of NVs in stool specimens by RT-PCR. RNA was extracted from stool specimens by using the Trizol reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer’s instructions. Primer set p290H-I/p290H-I,J,K was targeted to the RNA polymerase gene, was used to detect NVs in stool specimens (6, 14). These primers produce a 319-bp amplification for NVs and are also able to detect SVTs with a 331-bp product. To amplify the capsid gene of SW918, p455 including the start codon (underlined) of ORF2 and containing an overhang with SpeI and Sall restriction enzyme sites (CCACTAGTCGAC-GGTGTGAATGAGATGCGGCCT) and p457 including the stop codon (underlined) of ORF2 with an overhang containing a NotI site (GAGCGGCCGC-TCAACGAGCCCGCC TGC) have been utilized. RT-PCR was performed as described previously (4).

Human stool samples positive for NVs were included in the extraction and RT-PCR as positive controls. For amplification of the capsid cDNA, the extension time was extended to 3 min.

Baculovirus expression of swine NV capsid protein. The original capsid sequence of SW918 was determined by sequencing overlapping PCR products or cDNA clones covering parts of the gene (23). To express the SW918 capsid protein in baculovirus, the entire capsid gene of SW918 was amplified from the original stool specimen. The identity of the cDNA clone was verified by sequencing of both ends. In order to construct recombinant baculoviruses, the ~1.6-kbp SW918 capsid gene product was gel purified, digested with Sall and NotI, and cloned into Sall/NotI-digested pFastBac1 vector plasmid. Recombinant baculoviruses were generated using the Bac-To-Bac baculovirus expression system (Gibco BRL, Life Technologies) according to the manufacturer’s instructions. Recombinant baculovirus-infected Sf9 cells, cloned with high levels of expression were selected, and viral stocks were prepared. For large-scale expression of the SW918 capsid protein, S9 cultures were infected with 5 to 10 multiplicity of infection (MOI) recombinant baculoviruses. Cultures were harvested at 4 to 5 days postinfection. Cells were collected by centrifugation at 3,200 ×g for 15 min (Beckman GPR table-top centrifuge) and freeze-thawed 3 times. Both cell medium and cell lysates were centrifuged at 10,000 ×g for 20 min to remove cell debris and baculovirus particles. Supernatants were under-layered with 5 ml of 20% sucrose and centrifuged at 27,000 rpm in a SW28 rotor for 2 h at 4°C in a Beckman L90 K centrifuge. Pellets were collected and layered onto 10 to 50% discontinuous sucrose gradients. Gradients were run at 100,000 ×g for 2.5 h, fractionated, and analyzed on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels. Peak fractions, containing the ~60-kDa capsid protein band, were diluted with 4 volumes of phosphate-buffered saline (PBS) and centrifuged at 100,000 ×g for 2.5 h, and the pellets were resuspended in PBS and stored at −70°C. The protein concentration of VLP preparations was determined by measuring the optical density at 280 nm (OD280) and visually by running aliquots on SDS 10% polyacrylamide gels containing bovine serum albumin standards.

Antibody detection EIA. EIAs were used to measure specific antibodies in serum samples from pigs, and antigens used in these EIAs were as follows: GI/1, GI/2, GI/3, GI/4, GI/5 from genogroup I and Hawaii (GI/1I) and SW918 from genogroup II NV (8, 15, 17, 19). Equal amounts (50 ng/well) of recombinant VLPs were coated and EIAs were performed as described elsewhere (16, 18). Briefly, proteins were coated onto 96-well microtiter plates (Immulon 2HB; Thermolabsystems, Franklin, Mass.) in PBS overnight at 4°C. Wells coated with mock-infected S9 cell culture material served as negative controls. After blocking with 5% BLOTTO (Carnation nonfat milk) in PBS, serum samples diluted in 1% BLOTTO-PBS were added and the plates were incubated for 1 h at 37°C. Horseradish peroxidase-conjugated (HRP)-conjugated rabbit anti-swine immunoglobulin (ICN, Aurora, Ohio) was added at 1:5,000 dilution in 1% BLOTTO-PBS, and the plates were incubated for 1 h at 37°C. Between each step the plates were washed five times with PBS–0.5% Tween 20. TMB substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) was added and the color reaction was developed for 10 min at room temperature. After adding 100 μl of 1 M H2PO4 solution per well, results were read on a microtiter plate reader at OD450. The cutoff point of the test (OD450 > 0.2; positive/negative ratio > 5) was originally established for human stool samples (P. W. Huang, unpublished data).

Western blot analysis. SW918 capsid proteins separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) were electro-blotted onto nitrocellulose membranes (Osmonics NitroBind; GE Osmonics Labstore, Minnetonka, Minn.). Membranes were blocked with 5% BLOTTO-PBS overnight, stained with first and HRP-labeled secondary antibodies diluted in PBS–0.2% Tween 20 (PBS–T)–1% BLOTTO. Signals were detected with an ECL detection system (Amersham Biosciences Corp., Piscataway, N.J.) according to the manufacturer’s instruction. All wash steps were done by PBS-T.

Saliva binding assays. Saliva samples from healthy human subjects with known ABO, Lewis, and secretor histo-blood types derived from previous studies were used in the saliva binding EIAs to determine if SW918 also recognizes human histo-blood group antigens (HBGAs) (12). Briefly, human saliva samples were diluted to 1:1,000 in PBS (pH 7.4), boiled for 10 min, centrifuged at 10,000 ×g, and supernatants were coated onto 96-well microtiter plates (Immunol 2HB; Thermolabsystems) overnight at 4°C. Plates were blocked with 5% BLOTTO-PBS and recombinant SW918 VLPs at 1 μg/ml were added. After incubation at 37°C for 1 h, VLP binding was detected by the pooled anti-NV guinea pig hyperimmune serum obtained by cross-immunization and a HRP conjugate as previously described (12). Between each step the plates were washed five times with PBS–0.5% Tween 20. Recombinant capsid proteins from human NVs MOH (GI/5), Norwalk (GI/1), VA207 (GI/9), and VA387 (GI/4) (3, 17, 19), representing different receptor binding patterns of NV, were used as positive controls in the EIAs (3, 12). To test if addition of human or swine stool samples can make a difference in the binding, boiled and clarified stool samples were added with the SW918 VLPs in a final concentration of 1%. Six human stool samples, of which four enhanced the binding of the Hawaii virus VLPs to synthetic oligosaccharides, and 10 pig stool samples were tested on 12 saliva samples representing all the major HGBA types.

EM. Drops of sucrose gradient purified VLPs were adsorbed onto Formvar/ carbon 200 mesh copper grids (Electron Microscopy Sciences, Fort Washington, Pa.). Drops were air dried with 1% ammonium-molybdate, and examined using a Zeiss EM 10 transmission electron microscope (EM).

RESULTS

Baculovirus-expressed SW918 capsid proteins self-assemble into VLPs. VLP formation was first suggested by the fact that the recombinant SW918 capsid proteins concentrated in the middle (fraction 6) of the sucrose gradients (Fig. 1). Direct evidence was obtained by EM examination. VLPs with typical NV morphology were observed in negative-stained EM grids prepared from the peak fractions containing the 60-kDa SW918 capsid proteins (Fig. 2).

SW918 is antigenically related with human NV. SW918 VLPs were detected in the antigen detection EIA by the pooled rabbit and guinea pig polyvalent antibodies produced by cross-immunization with recombinant capsid proteins of multiple human NVs. To further determine the antigenic identity of SW918, Western blot analyses were performed using antibodies raised against individual human NVs. To ensure comparable results, all serum samples, except the pooled serum, were used in a dilution adjusted for their titer against the
homologous antigen. Among the four antibodies tested, the SW918 capsid protein reacted with antibodies specific for both GII (Hawaii and Mexico) but neither GI (Norwalk and VA115) NVs (Fig. 3). This result is consistent with the phylogenetic grouping of SW918 belonging to GII of NV.

Search for NVs in swine stools by EIA and RT-PCR. Since the SW918 capsid protein was detected by the cross-immunization sera against human NVs (both rabbit and guinea pig), we screened pig stools for the presence of NVs by using an antigen detection EIA developed for the detection of human NVs in our laboratory (18). The original stool specimen containing the prototype SW918 virus and originally negative pig stool samples seeded with SW918 VLPs gave positive reactions in the test. When stool samples collected from pigs less than 3 months old were tested, 2 of the 104 samples, both collected from healthy pigs, gave OD values significantly higher (0.45 and 1.5) than the cutoff value of the test (OD450/H11022 0.2; P/N H11022 5). However, neither EM nor RT-PCR demonstrated the presence of NV in these stools. We hypothesize that the EIA signals either were nonspecific or that the stool specimens may contain diverse strains missed by the primers originally designed to detect human NVs. None of the 104 stool specimens and 13 intestinal contents collected from pigs were positive in the RT-PCR. Future study to test these stool specimens with additional primers is necessary.

SW918 does not recognize human histo-blood group antigens. Human NVs are known to recognize human HBGAs as receptors. To address the issue of interspecies transmission of NVs, we tested SW918 VLPs for their ability to bind to human HBGAs. Among 52 human saliva samples representing the major ABO, Lewis, and secretor HBGAs types tested, none reacted with SW918 VLPs even when higher concentrations of both saliva and SW918 VLPs were used (data not shown). Addition of human or pig stool samples did not promote binding. This result indicates that SW918 does not share the same receptors with the four representative binding patterns of NVs described in our previous studies (12).

High prevalence of antibodies against SW918 was detected in pigs raised in the United States or Japan. Seventy-eight of the 110 (71%) serum samples collected from pigs in the United States reacted with an OD450 reading of >0.2 at a dilution of 1:100 and 13% at a dilution of 1:1,000 in the SW918 EIA. The OD ranged from 0.2 to 2.7 with a mean OD of 0.7 for the positive samples at 1:100 dilution (Table 1).

Ninety-five of the 266 (36%) serum samples collected in Japan reacted with an OD 450 higher than the cutoff value at a dilution of 1:100 and 17% at a dilution of 1:1,000 in the SW918 EIA. The OD ranged from 0.2 to 2.7 with a mean OD of 0.7 for the positive samples at 1:100 dilution (Table 1).

High prevalence of antibodies in pigs was also detected against the prototype Norwalk virus. Surprisingly, 63% of the

<table>
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<th>Location and year</th>
<th>No. of serum samples tested</th>
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<td>10^{-2}</td>
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<tr>
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<td>38</td>
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<tr>
<td>1998</td>
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<td>Unknown</td>
<td>110</td>
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FIG. 1. Electrophoresis of recombinant SW918 VLPs. Discontinuous sucrose gradient (10 to 50%) fractions (f1 to f12) of Sf9 cultures infected with recombinant SW918 baculovirus were collected and analyzed for the presence of SW918 capsid proteins. Equal amounts (5 ul) of fractions (f3 to f10) were separated by SDS-10% PAGE and protein bands were visualized by Coomassie blue staining. The arrow indicates the 60-kDa full-length capsid protein.

FIG. 2. Negatively stained SW918 VLPs. Grids were stained with 1% ammonium-molybdate and examined using a Zeiss EM 10 transmission electron microscope. Arrows indicate VLPs with typical morphology.

FIG. 3. Western blot immunostaining of SW918 capsid protein. SW918 VLPs (300 and 150 ng) were separated by SDS-10% PAGE, blotted onto nitrocellulose membranes, and stained with anti-NV antibodies raised in guinea pigs. A: preimmunization antibody; B: pooled, cross-immunization antibody (GI + GH); C: anti-Norwalk virus antibody (GI); D: anti-VA115 antibody (GI); E: anti-Mexico virus antibody (GH); F: anti-Hawaii virus antibody (GI). Arrows indicate the 60-kDa full-length capsid protein.
110 pig serum samples collected in the United States reacted with the Norwalk viral antigen at a dilution of 1:100 and 1% at a dilution of 1:1,000, but only 52 and 0% reacted with the Hawaii antigen, respectively (Table 2). The OD values at 1:100 dilution ranged between 0.2 and 0.8 with a mean of 0.37 for Norwalk and between 0.2 and 0.6 with a mean of 0.3 for Hawaii. Since SW918 and Norwalk belong to different genetic groups of NVs and represent different antigenic types, it is unlikely that the observed antibodies against Norwalk virus were induced by SW918-like strains. This possibility also is excluded by the fact that several pigs had high titers of antibodies against SW918 but low titers or no detectable antibodies against Norwalk virus. Fifty-two of the 110 (47%) serum samples were positive for antibodies against all three antigens tested. Three sera were positive only for antibodies against the SW918 antigen and one for antibodies against the Norwalk antigen. All Hawaii antigen-positive serum samples were also positive for SW918.

**DISCUSSION**

This study was initiated for the characterization of the antigenicity of a swine NV (SW918) that is genetically closely related to GII human NVs. Following the expression of the SW918 capsid proteins in baculovirus, we further demonstrated that SW918 VLPs were antigenically related with human GII NVs, confirming the genetic classification. This result raised several questions related to the origin of the strain and the possibility of cross-species transmission of NVs between animals and humans. Because these questions are important in understanding the epidemiology, immunology, and host range of NVs, we performed further studies to address them.

First we investigated whether SW918 or SW918-like viruses can be detected in pigs raised in the United States. By taking advantage of the cross-reactivity between SW918 and human NVs, we used a well-established human NV antigen detection EIA in an attempt to find antigenically related NVs in pig specimens. We also screened pig stool specimens by RT-PCR with a broadly reactive primer set to increase the chance of finding NVs. However, none of these attempts ended with a conclusive result. Some possible explanations of the negative result are the following: (i) the majority of the samples tested were nondiarrhea stool specimens and the samples came from one pig farm at one time point; (ii) our primers were designed for the detection of human NVs and they might have limitations detecting swine NVs; and (iii) the presence of RT-PCR inhibitors in the pig stools cannot be excluded. Future studies with larger collection of samples particularly of diarrhea stool samples and samples collected from pigs at different ages are necessary and confirmation of the two EIA positive samples by RT-PCR with different primers is a possibility.

Second, we investigated whether SW918 shares common host specificity with human NVs. Recently, human NV infection has been linked to HBGAs, and at least four different receptor recognition patterns have been identified according to the host blood types, including the secretor, ABO, and Lewis types (12). In this study, however, the SW918 VLPs did not bind to HBGAs present in human saliva, indicating it may not be able to target human cells. This result is not unexpected because it is known that most of the HBGAs are species specific, although common epitopes may exist. In our studies to measure the level of human HBGAs in saliva samples and test receptor binding, dried cow milk (BLOTTO) was used as the blocking reagent and we did not observe any interference in the test by the cow milk, indicating that cows do not share HBGAs with humans. This is also in accordance with the report by Hutson et al. (13) in which Norwalk virus that is known to bind to secretor A and O types was able to agglutinate red blood cells from humans and chimpanzees but not from other mammals.

However, we still cannot rule out the possible role of other ligands expressed in the intestine but not present in saliva in NV infection. Also, recent data suggested that stool components may enhance the attachment of NV VLPs to HBGAs (11). In our study, we tested human and swine stool samples for their ability to promote SW918 VLP binding to human saliva samples, but no such effect was observed (data not shown). Although these results do not suggest that SW918 can cause cross-species transmission to humans, the close genetic relationship of this strain to human NVs should alert us to the possible emergence of new strains with wider host ranges by either genomic RNA recombination or mutation during NV replication. In our recent studies mapping the receptor binding domain of NVs, a possible binding pocket on the P2 domain of the capsid has been identified and significant changes of receptor binding patterns of NVs have been observed by site-directed mutagenesis analysis within the pocket region even with a single amino acid change (24). Thus, it seems possible for an animal NV to jump to humans even if it is not native to humans. This is particularly true for strains like SW918 that are genetically close to human strains.

Third, we investigated how frequently SW918 causes infection in pigs. The high antibody prevalence observed in this study clearly indicated that infections of SW918 and/or SW918-like strains are common in pigs in both Japan and the United States. We tried to avoid the possibility of nonspecific reaction of the assays by using highly purified SW918 VLPs. This possibility also could be excluded by the fact that individual pigs revealed different levels of antibodies against the different human and swine NV capsid antigens. The high antibody prevalence is clearly contrary to the low detection rates of swine NVs in previous reports (22, 26) and the failure to detect any in this study by RT-PCR. As described above, multiple factors could result in the low detection rates and future studies about avoiding these factors are necessary.

We finally tested if antibody against human NVs can be detected in animals. Surprisingly, high levels of antibody prevalence against human NVs in pigs were detected. Even more interestingly, we observed a higher level of antibody prevalence

### TABLE 2. Prevalence of antibodies against Hawaii (GII) and Norwalk (GII) VLPs in pigs raised in the United States

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<th>Antigen</th>
<th>No. of serum samples tested</th>
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<tr>
<td></td>
<td></td>
<td>10^{-2}</td>
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<tr>
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<td>Norwalk</td>
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against a GI than a GII human NV. SW918 belongs to GII and we showed that it is antigenically distant from GI NVs, so the antibodies detected against Norwalk did not likely result from a cross-reaction with SW918 or SW918-like strains. These results also indicate that, as in humans, wide genetic and antigenic types of NVs may cocirculate in animals.

We realize that this study has limitations. For example, the antibody levels in pigs were not as high as the antibody levels against human NVs observed in human populations (20). One possibility is that most of the antibodies detected in this study probably resulted from heterologous responses against different antigenic types. Future studies with more recombinant capsid proteins representing different antigenic types in the ELAs may be necessary. Furthermore, the antibody detection experiments were performed in separate laboratories in the United States and Japan because of U.S. Department of Agriculture regulations for importing samples of animal origin. Although standard protocols were used in both laboratories, not all reagents were shared and variations of the results between the two laboratories remain possible. In conclusion, although this study did not provide the final answer of whether NVs can cause cross-species transmission, the findings of the antigenic relationship of SW918 with human NVs and the high seroprevalence against both animal and human NVs in pigs are significant. Future studies to look more closely on NV activities in the animal kingdom are necessary.

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