Prevalence of Hepatitis E Virus Antibodies in Canadian Swine Herds and Identification of a Novel Variant of Swine Hepatitis E Virus

DONGWAN YOO,† PHILIP WILLSON, YANLONG PEI, M. ANTHONY HAYES, ANNE DECKERT, CATHERINE E. DEWEY, ROBERT M. FRIENDSHIP, YUNGHO YOON, MARCELO GOTTSCALK, CARMENCITA YASON, and ANTONIO GIULIVI

Departments of Pathobiology and Population Medicine, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1, Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan S7N 5E3, College of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Quebec J2S 7C6, Atlantic Veterinary College, University of Prince Edward Island, Charlottetown, Prince Edward Island C1A 4P3, and Bureau of Infectious Diseases, Centre for Infectious Disease Prevention and Control, Health Canada, Ottawa, Ontario KIA 0L2, Canada, and Department of Animal Science, ChungAng University, Anseong, Korea

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Swine hepatitis E virus is a newly identified potentially zoonotic virus from pigs of particular concern for possible direct transmission to a human xenotransplant recipient by organ transplantation. In the present study, prevalence of serum antibodies to hepatitis E virus was examined in Canadian swine herds. A total of 998 serum samples collected from 6-month-old healthy slaughter hogs were examined by enzyme immunoassay and Western blot analysis for antibodies to the recombinant open reading frame 3 (ORF3) protein of hepatitis E virus expressed in Escherichia coli. These samples represented more than 80 different swine production units from five major swine-producing provinces across Canada. From this study, 594 samples (59.4%) were found to be positive for hepatitis E virus antibody. The seroprevalence was higher in Quebec (88.8%) and Ontario (80.1%) than in Alberta and Saskatchewan (38.3%). By PCR using a pair of oligonucleotide primers deduced from the ORF2 sequence of human hepatitis E virus, a specific hepatitis E virus sequence was recovered from feces of pigs. The nucleotide sequence identity between the U.S. swine hepatitis E virus and the Canadian isolate (SK3) was only 85.8%, suggesting that genotypic variations may exist in swine hepatitis E virus in North America. Among 165 serum samples collected from humans in Saskatchewan, 2.4% were found to be positive for antibodies to the hepatitis E virus ORF3 protein. Our data indicate that hepatitis E virus is highly prevalent in commercial swine populations in Canada and support the suggestion that the swine hepatitis E virus may be an important zoonotic agent for humans.

Hepatitis E is one of the five recognized types (A, B, C, D, and E) of human hepatitis caused by viral agents. Among these five types, hepatitis E is known as non-A non-B, food-borne, or waterborne hepatitis (2, 34), whereas hepatitis B, C, and D are known as serum hepatitis since they are mainly transmitted through blood. Hepatitis G virus (HGV) has been identified and characterized. HEV is a small nonenveloped virus with a single-stranded positive-sense RNA genome of approximately 7.2 kb. The full-length genomic sequence has been determined using strains isolated from different geographical regions, and two distinct genotypes are known to exist: Asian type and Mexican type (37). HEV was initially considered to be a member of the family Caliciviridae. However, on the basis of comparative phylogenetic analysis, it was recently removed from the Caliciviridae family and assigned as an unclassified genus of “hepatitis E-like virus” (4, 29).

Hepatitis E is endemic in the areas of Asia, South America, the Middle East, and Northern and Western Africa. In contrast to those countries, HEV has been considered absent in Canada, the United States, and countries in Western Europe. However, individuals in the countries where hepatitis E is nonendemic with no known history of traveling to areas of endemity have contracted the disease (6, 13, 23, 38, 40), suggesting that HEV may be more widespread than previously recognized. Recently in the United States, two distinct HEVs have been isolated from two patients with acute hepatitis E (designated US-1 and US-2) (18, 32). The US-1 sequence is substantially divergent from other known human HEVs, with a fatality rate up to 20%, especially during the third trimester (11, 14).

The etiological agent for human hepatitis E has recently been identified and characterized. HEV is a small nonenveloped virus with a single-stranded positive-sense RNA genome of approximately 7.2 kb. The full-length genomic sequence has been determined using strains isolated from different geographical regions, and two distinct genotypes are known to exist: Asian type and Mexican type (37). HEV was initially considered to be a member of the family Caliciviridae. However, on the basis of comparative phylogenetic analysis, it was recently removed from the Caliciviridae family and assigned as an unclassified genus of “hepatitis E-like virus” (4, 29).

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sequence identity of only 74% (32). In parallel with this study, an HEV has been identified from pigs in Illinois (26), and surprisingly, this swine isolate of HEV is genetically and antigenically close to the US-1 strain of human HEV. The sequence similarity between the US-1 human and swine HEV genomes is approximately 97 and 93% in open reading frame 2 (ORF2) and ORF3, respectively (26, 32). Furthermore, swine HEV is able to infect primates under experimental conditions (25). These findings implicate a possible transmission of the virus from pigs to humans. The potential for HEV to cause disease in swine has not been adequately evaluated.

Swine has been considered to be a donor animal species for xenotransplantation, and organ transplantation might directly transmit swine viruses to a human recipient who may pose further xenozoonotic risks in the community (41). Also, HEV from swine might sometimes be transmitted to humans through environmental contact. Accordingly, we have serologically surveyed Canadian swine herds for hepatitis E. In this communication, we report that swine HEV is highly prevalent in Canadian swine herds and describe a novel genotypic variant of swine HEV isolated from Canadian pigs.

MATERIALS AND METHODS

Sample specimens. Serum samples were collected from 6-month-old, healthy slaughter hogs in the Canadian provinces of Alberta, Saskatchewan, Ontario, Quebec, and Prince Edward Island. These pigs represented major pig production areas in Canada. Human serum samples were obtained from patients admitted to the Royal University Hospital in Saskatoon, Saskatchewan, Canada, during the period of 1998 to 2000. These human serum samples represented a randomized collection from individuals who tested negative for human immunodeficiency virus and hepatitis C. Both swine and human serum samples were stored at −70°C until tested for antibody.

Enzyme-linked immunosassays. Antibodies to HEV in human sera were determined using a commercial hepatitis E antibody detection kit according to the manufacturer’s instructions (Hepatitis E [rDNA] Antigen Abbott HEV EIA kit; Abbott GmbH, Biesbade-Delkenheim, Germany). Antibodies to HEV in swine sera were also tested using the commercial kit but with slight modifications.

Brieﬂy, pigs were bled in phosphate-buffered saline at 1:400 and incubated for 60 min at 40°C with a polystyrene bead coated with the recombinant HEV antigen provided with the kit. The bead was washed four times with 4 mL of distilled water per wash. The bead was transferred to a fresh tube and incubated for 1 h at 40°C with a 1:400 dilution of rabbit anti-swine antibody coupled to horseshadish peroxidase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The bead was washed four times again with 4 mL of water each and transferred to a new tube. Substrate was prepared by adding 2.56 mg of o-phenylenediamine−2HCl per mL of citrate phosphate buffer containing 0.02% hydrogen peroxide. Three hundred microliters of the substrate solution was added per well. The reaction was terminated by adding 1 mL of 1 N sulfuric acid per well. The mixture was sonicated at the highest scale (model W-385; Ultra- sonics Inc., Farmingdale, N.Y.) and centrifuged at 12,000 rpm for 10 min in a microcentrifuge. The suspension was analyzed for absorbance at 500 nm with a Microplate reader (model MR-5; Dynatech Laboratories, Alexandria, Va.). The cutoff value, including the negative grey area (ΔAbs2 × cutoff value = 10% of the positive control), was considered HEV negative.

Subcloning of ORF3 gene of HEV. The coding sequence for the ORF3 protein of HEV was kindly provided by Burton Beames (Southwest Foundation for Biomedical Research, San Antonio, Tex.). The ORF3 gene was originally derived from a Barmay isolate of human HEV. The ORF3 gene was amplified with a pair of oligonucleotides (forward primer, 5′-CGGGATCCATGAAATAA CATGTCTTTTGC-3; reverse primer, 5′-GGCGATCTTCAGGCGCCAGCC CGACC-3′) by PCR. The PCR amplification was conducted as follows: denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min for 30 cycles, followed by a 3 min of additional extension time. The ORF3 PCR fragment was digested with BamHI and inserted into the BamHI site of pGEX-2T (Amersham Pharmacia Biotech) downstream of the glutathione S-transferase (GST) gene to generate pGEX-HEVF3. Cloning and manipulation of DNA were performed according to the standard procedures (30).

Protein expression and partial purification. Escherichia coli JM109 cells transformed with pGEX-HEVF3 were grown at 37°C in Luria-Bertani medium with vigorous shaking. When the culture reached an optical density of 0.6 at 600 nm, isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 2 mM, and the culture was further incubated for an additional 6 h. Cells were collected by low-speed centrifugation at 500 × g for 20 min, and the cell pellet was resuspended in 4 mL of 25% sucrose in 50 mM Tris-HCl (pH 8.0). After two freeze-thaw cycles, 10 mg of lysosyme was added and the suspension was incubated for 10 min on ice followed by addition of 30 mL of 2× RIPA-TET buffer (5; 2× RIPA is 20 mM Tris-HCl [pH 8.0], 300 mM NaCl, and 2% sodium deconcholate; TET is 100 mM Tris-HCl [pH 8.0], 50 mM EDTA, and 2% Triton X-100). The mixture was sonicated at the highest scale (model W-385; Ultras- onics Inc., Farmingdale, N.Y.) and centrifuged at 12,000 rpm for 15 min using an SS34 rotor in the high-speed centrifuge (Sorvall RC-5B; Du Pont Instruments, Wilmington, Del.). The pellet as insoluble aggregates was resuspended in water and stored at −20°C.

SDS-PAGE and Western blot analysis. The partially purified protein preparation (insoluble aggregates) was resolved by polyacrylamide gel electrophoresis (PAGE) on a sodium dodecyl sulfate (SDS)−12% polyacrylamide gel. The gel was either stained with Coomassie blue (R-250) for direct visualization or transferred to nitrocellulose membrane for Western blot analysis. For Western blotting, approximately 1 μg of protein was loaded on a gel and the transferred membrane was cut into 10 longitudinal strips so as to contain 100 ng of protein per strip. Membrane strips were blocked overnight with 1% bovine serum albumin (fraction V) in 20 mM Tris-HCl (pH 7.5)−500 mM NaCl, followed by three washes for 5 min each with TTBS (Tris-HCl 7.5, 500 mM NaCl, 0.05% Tween 20). The strips were incubated with serum diluted in the blocking solution for 2 h at room temperature and washed three times with TTBS. The strips were then incubated for 1 h at room temperature with a 1:3,000 dilution of secondary antibody (goat anti-rabbit antibody conjugated with alkaline phosphatase [Kirkegaard & Perry Laboratories]). The strips were washed again with TTBS and developed for color reaction by adding a mixture of 3 mg of nitroblue tetrazolium chloride and 1.5 mg of BCIP (5-bromo-4-chloro-indolylphosphate p-toluidine salt), both prepared in 70% N,N′-dethylethamidylformide.

Viral RNA extraction. The U.S. isolate of swine HEV, kindly provided by Suzanne Emerson (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland), was used as a positive control. Formaldehyde-fixed air-dried tissues were homogenized in 1 mL of 50 mM Tris-HCl (pH 8.0) containing 20 mg/mL heparin (Sigma Chemical Co., St. Louis, Mo.) by sonication. A 100-μl aliquot of the 10% fecal suspension was mixed with Trizol (GIBCO BRL Canadian Life Technologies; Mississauga, Ontario, Canada) and extracted once with chloroform. The upper phase was transferred to a fresh tube, and RNA was precipitated by adding 0.4 volume of isopropanol with 20 μg of glycogen as a carrier (Boehringer Mannhein, Laval, Quebec). The RNA precipitate was collected by centrifugation at 12,000 rpm for 10 min in a microcentrifuge (Eppendorf International Co., Hamburg, Germany) and washed once with 70% ethanol. The RNA was resuspended in water, and the entire amount of RNA was subjected to cDNA synthesis.

First-strand cDNA synthesis and PCR. The extracted RNA was mixed with 500 ng of reverse primer (5′-CTACAGAGGCGCCAGCTGGATGTC-3′) and incubated at 70°C for 10 min followed by immediate transfer to ice. The first-strand cDNA was synthesized at 42°C for 2 h by 200 U of Superscript II RNase H− Reverse Transcriptase (GIBCO BRL) in the presence of 0.6 mM deoxy- nucleoside triphosphates (dTTP, dATP, dCTP, and dGTP), 4 mM dithio- threitol, 1 U of RNasin (Promega Biotech, Madison, Wis.), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl2. The reaction was terminated by incubating at 95°C for 5 min and chilled on ice. A first-round PCR was performed in a volume of 50 μL using 1.5 U of Taq DNA polymerase (GIBCO BRL) and the primers: 5′-AGCTTCTTACGTTGATG TGCAGTCT-3′, a 0.4 mM concentration of each deoxynucleoside triphosphate (dTTP, dATP, dCTP, and dGTP), 2 mM MgCl2, 20 mM Tris-HCl (pH 8.4), and 50 mM KCl. The PCR profile was as follows: 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min for 35 cycles, followed by an additional incubation at 72°C for 10 min.
To increase the detection sensitivity, a second-round PCR was performed using 3 µl of the first-round PCR product. The reaction conditions for second-round PCR were identical to those of the first-round PCR except that the following primers were used: forward primer, 5'-GCTCAGGTATCTGCGGTCGAG-3'; reverse primer, 5'-GGGCTGAACAAATCTGACATC-3'. After the second-round PCR, 3 µl was analyzed by agarose gel electrophoresis.

**Nucleotide sequencing.** Nucleotide sequences were determined by the PCR-based automatic dideoxy cycle sequencing (Perkin-Elmer, Norwalk, Conn.) at the Guelph Molecular Supercentre, University of Guelph. Sequences were analyzed using the software package of the Genetics Computer Group via the Seqweb 1.2 interface at http://www.cbr.nrc.ca.

**Statistical analysis.** Each pig was exclusively classified as seropositive or seronegative after enzyme-linked immunoassay based on the criteria described above. The serological data were analyzed with the aid of a statistical software program (Prism 2.01; GraphPad Software Inc., San Diego, Calif.). The chi-square statistic was calculated from the two-by-four contingency table, and significance was set at a $P$ of <0.05.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this study has been deposited into the GenBank database under accession number AF347692.

## RESULTS

**Expression of the HEV ORF3 protein and cross-reactivity with swine HEV antibody.** For detection of HEV infection in humans, we used an immunoassay-based diagnostic kit that is commercially available but not currently available as a diagnostic tool in North America. In order to corroborate results with this assay, we developed an additional assay for the ORF3 protein of HEV that was expressed in *E. coli*. The full-length ORF3 coding sequence derived from human HEV was subcloned and expressed as a fusion protein with GST under the tac promoter. The recombinant fusion protein of approximately 35 kDa was expressed as insoluble aggregates upon induction with IPTG. The insolubility of the expressed protein facilitated partial purification of the protein (Fig. 1, lane 3). The level of protein expression was estimated to be 2 mg/100 ml of the bacterial culture.

Since the expressed recombinant ORF3 protein was derived from a human isolate (Burmese genotype) of HEV, the cross-reactivity of this antigen with HEV antibodies in pigs was examined. According to the deduced amino acid sequences of the ORF3 protein of HEV, the sequence identity between Burmese isolate and the swine HEV appears to be 81% (GenBank accession numbers M73218, D10330, and AF082843), suggesting the likelihood of immunologic cross-reaction. Preliminary experiments showed that a 25% positivity. Contingency table analysis showed that the effect of geographic region on rate of seropositivity was highly significant ($P < 0.001$). Some of the tested samples appeared to be strongly positive, with optical density readings of more than 2.0, and this portion accounted for 5.4% of samples. Five hundred and four samples were determined to have an $A_{492}$ of more than 1.0, indicating that more than 50% of pigs became strongly seroconverted for HEV antibody by 6 months of age.
Identification of HEV sequence from pig. To confirm the presence of HEV or HEV-like virus in swine herds, attempts were made to identify the HEV-specific sequence from pigs. Fecal samples were randomly obtained from 8-week-old pigs from different pens in Saskatchewan and examined for viral sequence by reverse transcriptase PCR. A specific band of a 289-bp fragment was amplified after two rounds of PCR amplification from three out of six fecal samples examined (Fig. 3A, lanes 2, 3, and 4). Sample number 4 exhibited a relatively weak signal, but it was considered a definite positive (lane 4). The DNA in the amplified fragment, designated HEV-SK3, was sequenced to confirm the specificity. The sequence was found to represent a region of ORF2 of HEV (nucleotide positions 5649 to 5860 with respect to the sequence of the swine HEV U.S. isolate) (Fig. 3B). When the HEV-SK3 sequence was compared to that of swine HEV isolated in the U.S., the sequence identity appeared to be only 85.8%. It was interesting, however, that the identity of the predicted amino acid sequence between the two swine HEV isolates was nearly 100% for this particular region. The identification of specific HEV sequence from pigs demonstrates that a swine form of HEV exists in Canadian pigs which has maintained near identity at the protein level, although the nucleotide sequence has diverged from the strain identified in the United States.

Presence of HEV antibody in humans. Canada is considered a country of nonendemicity for HEV. Since Canadian pigs were found to contain high levels of HEV-specific antibodies, it was of interest to examine if humans could also have anti-HEV antibodies. One hundred sixty-five serum samples were obtained from humans in Saskatchewan and tested for HEV antibody. Four individuals (2.4%) were found to be positive. One individual was strongly positive, with an $A_{492}$ of 1.46 (cutoff value, 0.6), and another individual was weakly positive, with an $A_{492}$ of 0.8 (cutoff value, 0.45), but was a definite positive. The other two individuals showed a lower signal, with $A_{492}$ of 0.8 and 0.5 (cutoff values, 0.6 and 0.45, respectively), but it was concluded that these were definite positives after repeat of the test. Clinical correlation with seropositive individuals was not possible to ascertain.

**DISCUSSION**

We employed the commercial HEV immunoassay and the Western immunoblot to study the prevalence of HEV antibodies in pigs. Both diagnostic approaches utilized recombinant antigens from human HEV isolates. The enzyme immunoassay was based on two recombinant antigens (ORF2 and ORF3 proteins of Burmese HEV [SG-3 and 8-5 strains]) expressed in *E. coli* as a fusion protein with CMP-2-keto-3-deoxyoctulosonic acid synthetase (27), and this test has successfully been utilized to detect swine HEV antibody in pigs (5, 12, 24). Recently, the recombinant antigen-based anti-HEV antibodies were reevaluated, and the sensitivity and specificity for immunoglobulin G were demonstrated to be 86.7 and 92.1%, respectively (20). These values were comparable to those previously

![FIG. 2. Immunoreaction of the recombinant ORF2 protein of human HEV with swine sera. One microgram of the HEV3-GST fusion protein was loaded into a wide preparative well and electrophoresed by SDS-PAGE. The gel was transblotted to nitrocellulose membrane, and the membrane was cut into 10 longitudinal strips so as to contain 0.1 µg of the antigen per strip. The individual strips were subjected to Western blot immunoassay with various dilutions of swine serum.](image-url)
reported (9, 19, 22). For our studies, we also used a Western blot assay to supplement the enzyme immunoassay and demonstrated the cross-reactivity of swine antibody to the human HEV GST-ORF3 antigen. With both diagnostic approaches, HEV antibodies were reliably assayed in a broad sample from the Canadian pig populations.

Susceptibility of pigs to HEV was initially identified in an attempt to develop pigs as an animal model for HEV infections (3). Subsequently, domestic pigs were found to have been infected with human HEV (7). Following the isolation of HEV from pigs, several studies were conducted in various countries to examine the prevalence of HEV antibodies in pigs. Those studies revealed that pigs in both countries where human HEV is endemic and countries where human HEV is nonendemic were positive for HEV antibodies. Pigs in Australia, New Zealand, Taiwan, Korea, Canada, and the United States were seropositive to HEV (all countries where human HEV is nonendemic) (5, 12, 24, 26; Olga Garkavenko [Auckland Hospital, Wellington, New Zealand], personal communication), as were pigs in China, Thailand, and Nepal (countries where human HEV is endemic) (7, 24).

In the present study, a large-scale serological survey was conducted to assess the degree of prevalence of anti-HEV antibodies in swine herds across Canada. Pigs examined for this study were all healthy, 6-month-old pigs and represent major pig-producing areas of the country. The prevalence of HEV antibodies in these pigs varied significantly by geographic region ($P < 0.001$) and was surprisingly high, up to 89% in Quebec followed by 80% in Ontario (Table 1). In a previous study, the prevalence of 15 to 38% was observed in sows in Ontario and Quebec, respectively (24). This difference may have been attributed to the age of the animals and the sample size plus the number of farms tested. Alberta and Saskatchewan showed a relatively lower prevalence, 38%. In the Western Provinces, swine production units are generally spatially dispersed, while Quebec and Ontario farms are rather clustered, and thus the density of pig farms and the geographical conditions may have contributed to the different levels of antibody prevalence in those areas.

Although clinical cases of hepatitis E have been rare in Western Europe and North American countries, antibodies to HEV have been found in human populations in those countries (36). One study indicated that 1 to 2% of blood donors in the United States were seropositive to HEV (17), and another study demonstrated that 1.2 to 1.4% of 5,000 blood donors were seropositive in Northern California (21). Many (31 to 38%) of the seropositive individuals involved in that study had no history of international travel (21). In Brazil (a country where HEV is nonendemic), 2.6 to 3% of healthy blood donors were found to have HEV antibodies (10). Similarly, the presence of HEV antibodies was reported in countries where HEV is nonendemic in Europe, including Spain, Sweden, Germany, Greece, England, Finland, Italy, and The Netherlands (1, 15, 16, 42, 43). Similar data were observed in human populations in Ontario (M. Fearon [Ontario Ministry of Health, Toronto, Ontario], personal communication). In our study, it was found that 2.4% of the human population in Saskatchewan was positive to HEV, which is similar to the reported data in other countries where HEV is nonendemic.

However, in the absence of the virus, there was no evidence that the anti-HEV antibodies reflected subclinical HEV infections in humans. Although some cases were reported to have been associated with travel to regions of endemicity, the reason for a relatively consistent rate of anti-HEV antibodies in these countries of nonendemicity was unknown. In New Zealand, a hepatitis E case was reported in a young male who had not
traveled overseas within the previous 2 years and had not been in contact with any overseas travelers before he was clinically ill (6). In the United States, at least three cases of hepatitis E were reported in Minnesota, Tennessee, and California (8, 32, 38). In the United Kingdom, four cases of acute hepatitis E were reported without established history of traveling to areas of endemicity or contacts with patients with diagnosed cases of HEV (23). HEVs were isolated from the two U.S. patients (US-1, US-2), and their genomic sequences were found to be divergent from two major known types of HEV (Asian and Mexican types) by 16 to 17%. Independently from these studies, an HEV was recently isolated from a pig in the United States (26). While the U.S. human HEV was only 73 to 74% identical to the genomic sequences of two major types of human HEV (Asian and Mexican), the sequence identities between US-1 and swine HEV were strikingly high, with 97% identity for ORF2 and an overall identity of 91% for the entire genome (8, 26, 32).

In the present study, in addition to the prevalence of anti-HEV antibodies, we demonstrated the presence of a specific viral sequence directly from pigs from three of the six tested pens of pigs (Fig. 3A). The identification of HEV-specific viral sequence suggests that this pig virus might be responsible for the anti-HEV antibodies identified in Canadian pigs. Three of the six tested pens of pigs appeared to have actively been infected, suggesting that HEV may be as highly prevalent in swine in Canada as elsewhere in the world.

In human HEV, at least eight different genotypes have been reported: Burmese genotype 1 (35), Mexican genotype 2 (13), U.S. genotype 3 (18, 32), Chinese/Taiwanese genotype 4 (12, 39), European genotypes 5 to 7 (Greek [33], Italian [44], Australian [40]), and Argentinian genotype 8 (31). Currently, HEV (of swine origin) sequences have been made available from Spain, Taiwan, and the United States. The Spanish sequence was identified indirectly from sewage of swine origin (28), whereas the Taiwanese and the U.S. sequences were obtained directly from pigs (12, 26). While the entire genome has been sequenced for U.S. swine HEV, only a small portion of the viral genome has been sequenced for either the Spanish or the Taiwanese isolates. Furthermore, the latter sequences do not represent the same region of the genome. Thus, we were only able to compare the Canadian HEV-SK3 sequence to the prototype U.S. swine HEV sequence. According to the sequence comparisons and the phylogenetic analysis (Fig. 4), the swine HEV-SK3 recovered in a Saskatchewan farm appears to be distinct from the U.S. swine HEV by 14.2%. This limited available information suggests that a parallel genetic divergence may also exist in swine HEV, as with human HEV. Determination of the full-length genomic sequence of the swine HEV-SK3 isolate and identification of additional HEV isolates of swine origin will help us better understand the genetic divergence of swine HEV, and such studies are currently in progress.

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