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Porcine Enteric Caliciviruses: Genetic and Antigenic Relatedness to Human Caliciviruses, Diagnosis and Epidemiology

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Abstract

Porcine enteric caliciviruses include sapoviruses and noroviruses. Porcine sapoviruses infect pigs of all ages and cause diarrhea in young pigs, whereas porcine noroviruses were detected exclusively from adult pigs without clinical signs. Importantly, certain porcine norovirus strains were genetically and antigenically related to human noroviruses. This raises public health concerns that pigs may be reservoirs for emergence of epidemic human norovirus strains. This article reviews the discovery of porcine noroviruses and sapoviruses, their classification, diagnosis, epidemiology and genetic and antigenic relatedness to human caliciviruses.

Keywords

calicivirus; porcine; norovirus; sapovirus

Viruses in the family *Caliciviridae* are nonenveloped, polyadenylated single-stranded positive-sense RNA viruses [1]. They are 27–35 nm in diameter and contain a genome of 7 to 8 kb. Caliciviruses were classified into 4 genera in 2002 by the International Committee on the Taxonomy of Viruses (ICTV): *Norovirus*, *Sapovirus*, *Vesivirus* and *Lagovirus* [2]. Later, a bovine enteric calicivirus strain, Bo/NB/80/US, was identified as forming a potential new genus in *Caliciviridae* [3] with the Bo/Newbury1/76/UK strain subsequently proposed as another member [4], but the classification of both viruses has not yet been assigned by ICTV. These enteric caliciviruses including noroviruses (NoVs), sapoviruses (SaVs) and the unclassified bovine NB and Newbury1 caliciviruses cause gastroenteritis in humans or animals. The vesiviruses and lagoviruses cause systemic disease. Vesicular exanthema of swine virus (VESV), a vesivirus, causes vesicular exanthema in pigs, and the rabbit hemorrhagic disease virus (RHDV), a lagovirus, causes a fatal hemorrhagic disease in rabbits [1]. This review focuses on the diagnosis, epidemiology of porcine NoVs and SaVs and their genetic and antigenic relatedness to human caliciviruses.

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I. History of enteric caliciviruses and the discovery of porcine NoVs and SaVs

Noroviruses

The first virus that was discovered to cause human diarrhea was the prototype NoV, Norwalk virus. It was discovered in 1972 by Kapikian et al. [5] in stools of school children with diarrhea in Norwalk, Ohio by using immune electron microscopy (IEM) and it was described as having an indistinct surface morphology. Because Norwalk virus did not replicate in cell culture or animals except for primates, its detailed characterization was impeded and it remained unclassified until 1993 when molecular cloning and sequencing of the viral genome identified it as a member of the family *Caliciviridae*. Subsequently, many small, round-structured viruses (SRSV), morphologically similar to Norwalk virus, were initially referred to as Norwalk-like viruses in the *Caliciviridae* [6]. In 2002, Norwalk and Norwalk-like viruses were assigned to the genus *Norovirus* [2]. Since the discovery of Norwalk virus, NoVs have emerged as the most common pathogen causing food- and water-borne viral gastroenteritis in humans including both outbreaks and sporadic cases worldwide [7–10]. The human NoVs have been listed as class B biological pathogens by the National Institutes of Health/Biodefense Program.

Soon after the discovery of Norwalk virus in humans, scientists identified NoVs from diarrheic calves [11,12]. The first animal NoV strain, Bo/Newbury2/1976/UK, was detected from calves with enteritis in the UK. Later, another distinct genotype of bovine NoV, Bo/Jena/78/GEN, was identified in Germany. However, no information on NoV in pigs was available. In 1998, NoV RNA was first detected from adult pig cecal samples in Japan, and later in Europe and the US [13–15]. Subsequently, NoVs were found to be widely distributed in healthy adult pigs [16]. Because NoVs alone may not cause clinical signs in adult pigs, they may have been overlooked previously. Although their role in pig diarrhea is still undefined, porcine NoVs may play an important role in calicivirus evolution. For example, pigs might be a reservoir for the emergence of new caliciviruses potentially transmissible to humans.

Recently, a NoV was detected in mice [17]. However, it causes a systemic infection in mice that is distinct from other human and animal NoVs. The clinical signs included encephalitis, vasculitis of the cerebral vessels, meningitis, hepatitis and pneumonia after inoculation via different routes. Therefore, MNV-1 is not a typical enteric NoV.

Sapoviruses

Sapoviruses are a second distinct group of enteric caliciviruses in the *Caliciviridae* family. They were assigned to the genus *Sapovirus* in 2002 and are associated with gastroenteritis in humans and animals [18–21]. They were first found in stools of diarrheic children in 1976 by direct EM [22,23]. Sapoviruses have typical caliciviral cup-shaped depressions on the viral surface. The prototype SaV is the Sapporo strain detected during a gastroenteritis outbreak in an infant center in Sapporo, Japan in 1977 [18]. In 1980, the first porcine SaV, the Cowden strain, was identified together with rotavirus and astrovirus particles in the fecal samples of diarrheic piglets by EM [24]. In 2001, SaV RNA and viral particles were detected from diarrheic mink by RT-PCR and IEM, respectively [21]. Sapoviruses are now recognized as emerging pathogens causing diarrhea in humans, especially children and the elderly, mink and swine [1,2,19–21].

During the past 30 years, significant progress has been made in studies of enteric caliciviruses. Major breakthroughs include the following. The genomes of representative strains of NoVs and SaVs were characterized and the structure of Norwalk virus was established by x-ray crystallography of baculovirus-expressed Norwalk virus-like particles (VLPs) [25–28]. Primers for reverse transcription (RT)-PCR were developed and RT-PCR became widely used for diagnosis [29]. Host histo-blood group antigens (HBGA) were discovered as possible host

cell receptors or co-receptors for NoV infection [30]. The porcine SaV Cowden strain was adapted to growth in a continuous porcine kidney cell line [31,32] and bile acids and a signal transduction pathway was shown to influence its replication *in vitro* and potentially *in vivo* in the duodenum [33]. The murine NoV MNV-1 strain was subsequently shown to replicate in primary murine dendritic cells and macrophages [34]. The Cowden and MNV-1 strains remain the only cultivable SaV and NoV strains, respectively, in the world. The pathogenesis of porcine SaV Cowden strain was delineated in gnotobiotic pigs [19,20]. Most recently, reverse genetic systems were generated for the porcine Cowden SaV and the human Norwalk NoV, in which infectious viral particles were rescued after replication of the viral genomic RNA [35, 36]. In spite of these advances, most NoVs and SaVs remain refractory to growth in cell culture and extensive efforts to propagate them *in vitro* have failed [37]. Animal models that mimic the diarrheal disease seen in humans are lacking, but a gnotobiotic pig model for human NoV has recently been described [38]. Consequently, knowledge of the origin of new emerging epidemic strains in humans, the pathogenesis of NoVs, immune responses and immunity to enteric caliciviruses is limited.

II. Genetic organization and classification of NoVs and SaVs

The first complete genome of a NoV was described for Norwalk virus in the early 1990s [25] (Fig. 1). Presently, more than 20 complete genomes of human, 1 of bovine and 1 of murine NoVs have been characterized. The NoV genome is 7.3–7.7 kb long and includes 3 open reading frames (ORF) that encode a non-structural polyprotein, a major capsid protein (VP1 or capsid protein), and a minor capsid protein (VP2 or small basic protein). The polyprotein undergoes protease processing to produce several nonstructural proteins [1,39,40]. The capsid protein is composed of a conserved shell (S) domain and hypervariable protruding (P) domain, with the latter subdivided into P1A, P2 and P1B subdomains based on x-ray crystallography of Norwalk virus VLPs [27].

The first genome of SaVs was determined for Hu/Manchester/93/UK [28,41] (Fig. 1). Since then, the full length genomes of 4 human and 2 porcine SaVs have been characterized [42–45]. The genome is 7.1–7.5 kb long and unlike NoVs, it contains two main ORFs based on sequence analysis. The ORF1 encodes a polyprotein that undergoes protease processing to produce several non-structural proteins and a capsid protein. The capsid protein gene of SaVs is contiguous with ORF1, whereas the capsid protein of NoVs is encoded by a separate ORF2 (Fig. 1). This is the major difference in genome organization between NoVs and SaVs. The ORF2 of SaVs encodes a small basic protein of unknown function. For some human SaVs (genogroup I, IV and V, see classification below), another ORF3 overlapping the 5'-end of the capsid gene was proposed because there is a conserved translation initiation motif *GCAAUGG* at the 5'-end of this ORF3 [46,47].

Because there is no cell culture system or small animal model for NoVs and SaVs except for the porcine SaV Cowden and the murine NoV MNV-1 strains [19,31], antigenic classification of these viruses by two-way cross-neutralization tests is not yet possible. Genetic classification of these viruses based on phylogenetic analysis of the complete capsid sequences is the generally accepted method [46,48,49]. Katayama et al. [48] investigated which region of the genome was most suitable to classify the genotypes and genogroups of NoVs based on similarity plot analysis, bootstrap value and pairwise distances of the entire genomes of 18 strains, the complete sequences of ORF1, ORF2 and ORF3, and segments of ORF1 and ORF2. They found that the full-length genome, the complete ORF1 or ORF2, and the capsid N-terminal/S domain could segregate viruses into genus, genogroup and genotypes (also called genetic clusters). However, the RNA-dependent RNA polymerase (RdRp) could not be used to clearly identify genotypes; the P domain of the capsid protein could not be used to assign genogroups; and the VP2 could not distinguish genus. There is no such detailed investigation

of classification for SaVs. Sequence analysis of the complete capsid region is a generally accepted method for classification of SaVs into genogroups and genotypes. The full name of a NoV or SaV strain is organized as follows: Host species from which the virus was obtained/genus/genogroup-genotype/strain name/year of occurrence/country of origin [1]. The classification and GenBank accession numbers of representative NoV and SaV strains were summarized in two published papers [15,50].

III. Classification, recombination of porcine NoVs and SaVs, and their genetic relatedness to human caliciviruses

Genetic classification of NoVs

Noroviruses are genetically highly diverse viruses. Recently, Zheng et al. [51] analyzed the complete capsid proteins of 164 NoV strains and divided them into 29 genotypes within 5 genogroups: 8 clusters in genogroup (G) I (GI/1–8), 17 in GII (GII/1–17), 2 in GIII (GIII/1–2), and 1 each in GIV and GV. For this classification of NoVs, the authors collected large numbers of genetically variable NoV strains, generated an amino acid sequence alignment based on the predicted 3D structures of the capsid proteins, and evaluated the uncorrected pairwise amino acid distances by maximum likelihood pairwise distance analysis. The uncorrected pairwise amino acid distances of the capsid proteins of the 164 NoVs within genotypes, between genotypes and between genogroups were 0–14.1%, 14.3–43.8% and 44.9–61.4%, respectively.

Genetic classification of porcine NoVs

Among the 164 NoVs, the first detected porcine NoV Sw43/1997/JP strain was classified as GII/11 [52]. Recently, porcine NoVs QW101/2003/US and QW170/2003/US were identified from US swine [15]. At the amino acid level, their complete capsid proteins share 71% sequence identity to each other, and share 61–71% and 62–81% identity, respectively, to other genotypes of NoVs including the GII-11 porcine NoV Sw43 strain. Together with their topology on the Neighbor-joining phylogenetic tree (Fig. 2), the capsid proteins of these 2 strains represent 2 new genotypes in genogroup II: GII-18/QW101 and GII-19/QW170. More interestingly, the GII-18 porcine NoVs share the highest amino acid identity with the human GII-3/Mexico strain. Unlike bovine NoVs that belong to a distinct genogroup III within the *Norovirus* genus, the porcine NoVs were classified into the same genogroup II as the most prevalent human NoVs [53], especially the QW101 strain that is genetically most closely related to the human NoVs.

Genetic classification of SaVs

Similar to NoVs, SaVs are genetically variable viruses and have been classified into at least 6 genotypes in 5 genogroups based on the complete capsid sequences: 3 each in genogroup I and II (GI/1–3, GII/1–3), one each in genogroup III, IV and V [46,47]. The pairwise amino acid distances of SaVs within genotypes, between genotypes and between genogroups were 1–8%, 9–29% and 51–63%, respectively. The range of the distance between genogroups (9–29%) is relatively smaller compared to that of NoVs (14.3–43.8%). This is probably due to much fewer strains characterized for SaVs than for NoVs.

Genetic classification of porcine SaVs

Presently, porcine SaVs have been reported from the US, Venezuela, South Korea and Japan [44,45,50,54,55]. Most strains are Cowden-like (GIII) SaVs based on sequence in the capsid or RdRp region except for QW19/02/US, JJ681/00/US, LL26/02/US and K7/JP strains. Interestingly, the QW19 strain shared the highest amino acid identity (66.3%) with human GII SaVs Mc10, C12 and Cruise ship, but not with any porcine SaV strains, in a sequence fragment of 95 amino acid (aa) in the RdRp region [50]. Although it is impossible to accurately classify

the QW19 strain as a certain genotype based only on the 95 aa RdRp sequence, it likely represents a new genogroup because the overall intragenogroup amino acid identity of the 95 aa RdRp region of SaVs is 80–100% [50]. The complete capsid protein of JJ681 strain has been sequenced [50]. It shares only 32–36% identity to each SaV genogroup (GI–GV); thus, it represents a new SaV genogroup (GVI?). The complete genome of K7/JP strain was recently determined [45]. It shares 95% and 42% sequence identity with the LL26 and QW19 strains, respectively, in the 95 aa RdRp region; it showed only 32–37% amino acid identity with the other SaV strains in the complete capsid region, except for the JJ681 strain with which it shares 52% amino acid identity. Thus, the K7 and LL26 strains together likely form another new genogroup (VII?) of porcine SaVs. In summary, porcine SaVs comprise at least 3 genogroups (GIII, JJ681-like GVI? and LL26/K7-like GVII?) and possibly a further potentially new genogroup (QW19-like), more similar to human SaVs.

Recombination of porcine NoVs and SaVs

The detection of an RNA molecule of ~2 kb in stool samples from a human volunteer infected with NoV Norwalk strain and detection of a 2.3 kb RNA from murine NoV MNV-1-infected RAW 264.7 cells strongly suggests that NoVs produce subgenomic RNA molecules [25,34]. Similarly, a 2.2 kb RNA was detected by an *in vitro* replication assay with the replication complex of SaV Cowden strain extracted from virus-infected cells suggesting that SaVs also generate subgenomic RNA during virus replication [33].

Co-infection with multiple genotypes, even genogroups (GI and GII), of NoVs is not a rare event in humans [56–58]. The RdRp-capsid junction region of caliciviruses contains a highly conserved ~20 nucleotide (nt) motif in genomic and subgenomic RNA molecules that is suggested to be a transcription start signal [1,48]. This conserved nucleotide motif is almost identical within each genogroup of NoVs and SaVs, probably facilitating homologous recombination during co-infection of a cell by different genotypes of NoVs or SaVs within the same genogroup. In fact, recombinant NoVs having a recombination site at the RdRp-capsid junction region were identified in humans, calves and pigs [15,48,59–63]. Two porcine NoVs, QW170 and QW218 strains, which share 99% nt identity at the 3'-end 3kb sequence of the genome, were reported as potential recombinants [15]. They showed high sequence identity to GII-11/Sw43 strain in the RdRp region but the identity dropped abruptly at the RdRp-capsid junction region. In the RdRp-phylogenetic tree, they were grouped together with the Sw43 strain, whereas they segregated into a new cluster (GII-19) on the capsid-phylogenetic tree. However, it is unclear which strain, the QW170 (or QW218) and the Sw43, was the parent or progeny strain. To date, this type of recombination occurs exclusively between NoVs in the same genogroup (GI, GII and GIII) although co-infection by genogroup I and genogroup II NoVs occurred in humans.

A recent study [64] revealed that NoV recombination probably also occurs in the capsid gene. Seven potential recombinants [GI/1 (n = 1), GII/1 (n = 1), GII/3 (n = 1), GII/4 (n = 3), and GII/5 (n = 1)] were detected from 94 human NoV strains examined. Further analysis showed that recombination sites were mainly located between the putative P1A and P2 domains and/or within the P2 domain. These putative recombination regions showed common features, such as sequence length and composition (upstream and downstream GC- and AU-rich sequences, respectively). Also, the predicted RNA secondary structure of this region demonstrated characteristics of homologous recombination activators.

Recombination at the RdRp-capsid junction region also occurs among SaVs. Porcine SaV QW270 was a potential recombinant strain between a JJ259-like strain (RdRp region) and a Cowden-like strain (capsid, after RdRp) of the same genotype in SaV genogroup III [50]. A recombination event probably occurred for porcine SaV MM280 and a Cowden-like strain, although a strain carrying a similar RdRp but different capsid from that of the MM280 strain

was not detected. In humans, not only intragenogroup but also intergenogroup (between GII and GIV) SaV recombinants have been identified [43,65].

In summary, genetically diverse and potentially recombinant porcine NoVs and SaVs were identified. Porcine NoVs were classified into 3 genotypes in genogroup II: GII-11, -18 and -19, with the GII-11 genetically most closely related to human NoVs. Porcine SaVs comprise at least 3 genogroups (GIII, GVI? and GVII?). The QW19 porcine SaV (G?) is genetically closest to human SaVs and may represent a new genogroup of SaV.

IV. Diagnosis of porcine NoVs and SaVs

Laboratory diagnosis of NoVs and SaVs focuses on the detection of viral antigen, viral RNA and serum antibody responses. The EM and antigen-enzyme-linked immunoassay (ELISA) detect intact virions and viral antigens, respectively; reverse transcription (RT)-PCR and real-time PCR detect viral RNA; and recombinant VLPs have been widely used as antigens for antibody-ELISA to detect serum antibodies. Except for real-time PCR, all the above methods have been used for the detection of porcine NoVs and SaVs.

Electron microscopy

The porcine SaV prototype Cowden strain, and the human NoV and SaV prototype Norwalk virus and Sapporo virus, respectively, were first detected by EM or IEM [5,18,24]. Direct EM is not a very sensitive technique with a detection limit of 10^6 particles per ml of stool for enteric viruses [29]. Compared to SaVs, NoVs do not have the distinctive calicivirus morphology (cup-shaped surface depressions) and they are harder to differentiate from other small round viruses including enteroviruses, astroviruses and picornaviruses in stool specimens [6,66]. The IEM can usually increase the sensitivity of EM up to 10 or more-fold and one can observe both viral morphology and the antigen-antibody reaction [5,67]. The specificity of IEM depends on the quality of the antiserum used in the experimental procedures. The disadvantages of EM and IEM include the need for highly skilled experts and expensive equipment as well as the time consuming process to examine individual specimens. Therefore, it is not a practical method for investigation of large numbers of samples.

RT-PCR

Nowadays, RT-PCR is the primary assay for detection of porcine NoVs and SaVs [13–15, 45,50,54,55,68]. Two strategies have been used: (1) RT-PCR using porcine NoV- or SaV-specific primer sets to detect porcine NoVs and SaVs, but not strains from humans or other animal species [68]; (2) RT-PCR using calicivirus-, NoV- or SaV-conserved primer sets followed by sequencing confirmation to screen for porcine NoVs and SaVs, and other new caliciviruses [13–15,45,50].

Several factors affect the sensitivity and specificity of RT-PCR assays, including the quality of the sample, the methods used for RNA extraction and purification, the primers, RT-PCR conditions and the methods for detection of virus-specific amplicons. The primers mainly determine the specificity of the RT-PCR reactions. Although nested RT-PCR can increase sensitivity up to 10–1,000 times, it is less used because of the high risk of cross-contamination [29]. Real-time RT-PCR is more sensitive and it has become an alternative method for detection of low amounts of virus, especially from environmental samples [69,70].

Extraction of RNA—Two major aspects should be considered in the selection of an RNA extraction method: efficiency of RNA recovery and ability to remove RT-PCR inhibitors. At present, the most accepted RNA extraction method for fecal samples is a mixture of guanidinium thiocyanate (GTC), phenol and chloroform extraction and precipitation of RNA

by alcohol [15,29,50]. The principal reagent is commercially available from several companies, such as TRIzol from Invitrogen (Carlsbad, CA).

Antibody capture of NoV and then heating to release the viral RNA from captured virus particles is a simple method. It can concentrate and purify virus particles from large volumes of environmental samples containing low amounts of viruses [71,72]. The limitation of this method is similar to that of ELISA: NoV hyperimmune serum is usually highly specific and only detects homologous strains. With NoV common epitope-specific monoclonal antibodies (MAb) available in the future, this method may be evaluated in more detail and applied to field studies.

Currently, some laboratories are using various robots, such as NucliSens Extractor based on bioMérieux's patented Boom silica technology, for RNA extraction and purification from fecal samples.

Primer selection—The genetic diversity of porcine NoVs and SaVs has made it very difficult to select a single primer pair to detect all circulating strains. The majority of specific primers have been designed based on the most conserved RdRp region of the genome [29,68]. However, sequence analysis of the polymerase region of a wide range of virus strains indicated that this region is also variable with the nucleotide identity as low as 53% between strains of different genogroups and 60–64% within genogroup [59].

Calicivirus “universal” primers based on the most conserved RdRp motifs have been designed. The primer pair p290 and p289 (or p110) targets the conserved motifs “DYSKWDST” and “YGDD” of the RdRp region of caliciviruses [73,74]. Compared to p289, p110 is 2 nt shorter at the 5'-end and it is degenerate. The primer pair p290/289 can amplify not only human GI and GII NoVs [73], but under low stringency conditions, bovine GIII NoVs and NB-like enteric caliciviruses [75], human, porcine and mink SaVs [21,47,73] and mink vesiviruses [21] are detected. New porcine NoVs, and porcine and human SaVs have been identified by using this primer pair [15,45,47,50]. Because these primers have nucleotide mismatches for both NoVs and SaVs, RT-PCR is performed at relatively low stringency conditions, such as low annealing temperature [73]. Although the detecting spectrums of these universal primers are wider, they are less sensitive for specific strains. For example, Wang et al. [68] found that the primer pair p290/110 was 250-fold less sensitive than the porcine GII NoV-specific primer pair PNV7/8 for detection of the porcine QW101 NoV. In addition, the specificity is lower because the reverse primer targets the YGDD motif, and the GDD motif especially, is also conserved among other RNA viruses [76]. A recent study reported that primer pair p290/289 also amplified rotaviruses [77]. Therefore, sequencing of the RT-PCR products of p290/289 (or p290/110) is necessary to prevent false-positive results.

A commonly accepted approach is to design and use several primer pairs for the detection of genetically diverse porcine NoVs and SaVs. Generally, primers designed based on the sequence of locally circulating strains have performed better than other primer pairs [78]; primers targeting non-polymerase regions of the viral genome are less broadly reactive; very specific primers are generally more sensitive than less specific primers for a specific strain.

Internal control (IC) RNA—The RT-PCR inhibitors are common and sample-dependent in food, shellfish and fecal samples [6,29] causing false-negative results. These inhibitors may include body fluids, food and bacterial cell constituents [79]. An IC RNA co-amplified with the target viral RNA can be used to monitor for inhibitors as well as technical errors. Diluting the extracted RNA of such samples or further purifying the extracted RNA and performing RT-PCR again usually decreases the false negative results. Diluting the extracted RNA is simplest and most samples diluted 1:10 or 1:50 will no longer inhibit the RT-PCR reaction

[6,68]. Stool samples from pigs infected with porcine SaV Cowden strain or porcine NoV QW101 strain have been shown to be positive at dilutions ranging from 1:5 to 1:125 and 1:5 to 1:12,500, respectively [68]. A similar dilution range (1:10 to 1:10,000) has also been found for human NoVs [80]. A drawback of this strategy is that dilution of weakly positive samples may exceed the detection limit of RT-PCR and therefore become negative.

There are two kinds of IC RNA molecules: competitive and noncompetitive. The competitive internal controls are suggested because the target virus RNA and IC RNA are amplified in the same RT-PCR condition. The approaches of how to generate such an IC RNA have been reviewed [81]. When using the competitive IC RNA, there are three possible outcomes of RT-PCR assays: (1) no product (the assay has failed-inhibition may be present); (2) the IC RNA, but not the virus fragment is obtained (the assay has worked, and the sample is most likely negative or viral RNA is below the detection limit); (3) the virus fragment, with or without the IC RNA fragment, is obtained (the assay has worked, and the sample is positive).

Confirmation of virus-specific RT-PCR amplicons—The simplest method for detection of RT-PCR products is agarose gel electrophoresis. If a sample shows a band having a similar size to that of the positive control amplicons, it will be considered positive. However, it can produce false-positive bands and such nonspecific bands are frequently seen by RT-PCR for NoVs and SaVs from fecal samples [29].

The use of a hybridization assay is probably the cheapest approach to interpret and confirm RT-PCR products. The most commonly used hybridization assays include dot or blot hybridization, liquid hybridization and Southern blot hybridization. The time required to perform a hybridization assay can be shortened without loss of sensitivity by using microwell hybridization assays [58,68,76]. Compared to sequencing of the amplicons, it is less time consuming and cheaper to confirm the specific amplicons, especially for testing large numbers of samples in epidemiological studies.

However, the limitation of hybridization assays for porcine NoVs and SaVs is that the genetic variability of these viruses makes it difficult to select a single probe to detect most if not all possible porcine NoV and SaV strains [68]. Although internal probes can detect strains with several nucleotide mismatches under hybridization conditions with lower stringency, strains with highly variable internal sequences may be missed. Consequently, gel electrophoresis followed by sequencing of RT-PCR products is still the gold standard to confirm NoV or SaV specific amplicons.

Real-time RT-PCR—In the past 2–3 years, various real-time RT-PCR methods, such as SybrGreen and TaqMan, have been used for detection of human NoVs from fecal and shellfish samples [69,70,82–87]. These may be used for detection of porcine NoVs and SaVs in the near future. Compared to conventional RT-PCR, the real-time RT-PCR is more sensitive, faster and quantitative if RT-PCR inhibitors are not present in the sample [84] and it also avoids the post RT-PCR confirmation steps, such as gel electrophoresis and hybridization. A SybrGreen real-time RT-PCR has been reported to be 10,000 times more sensitive than conventional RT-PCR, and the detection limit is 5 to 5×10^6 copies of RNA per reaction [69]. Previously, the disadvantage of real-time RT-PCR was mainly the expense. The prices of the instruments and reagents have decreased in the past few years and the methods are now being adopted by many laboratories although costs remain high compared to other methods for use in laboratories in developing countries.

Immunoassays

Immunoassays detect both viral particles and soluble antigens. An antigen-ELISA was developed for porcine Cowden strain [19]. Hyperimmune antisera from 2 species of animals

(pigs and guinea pigs) against porcine SaV Cowden strain were produced using purified virus from Cowden-infected pig fecal samples and recombinant virus-like particles (VLPs), respectively [88]. The antigen-ELISA has been used successfully for detection of the Cowden strain from experimentally infected Gn pigs or cell-culture supernatants [19,33].

The antigen ELISA for detection of NoVs has proven to be sensitive with a detection limit of 0.025 ng of capsid protein [80,89] and 1:10,000 dilutions of viral antigen in the stools of volunteers still being detectable [80]. The sensitivity of ELISA was similar to that of traditional RT-PCR probably due to the large amounts of viral soluble proteins in stools [6,29].

Hyperimmune antiserum produced to VLPs is often highly specific to the homologous strain or viruses within the same genotype [80,89–93]. For example, the ELISA using hyperimmune serum against NoV GII-3/Mexico strain did not detect NoV GII-4/Grimsby virus, and vice versa [90]. The reason is probably that the most variable region, the P2 subdomain is on the surface of the VLPs and contains the immunodominant epitopes. The most conserved N-terminal/S domain is either buried inside and does not induce antibodies after inoculation of the animals with VLPs, or the S domain contains the common epitopes that are not immunodominant [6,27]. However, the high specificity of these ELISAs becomes a disadvantage during screening of NoV and SaV antigens in epidemiologic studies because the circulating strains are highly variable.

If all NoVs or SaVs, or at least each genogroup of NoVs or SaVs contains one or more common epitopes, the development of a broadly reactive antigen-ELISA will be possible. Many researchers have worked on this aim and recent findings are very promising. For example, broadly reactive MAbs against GI or GII NoVs have been generated [94]. Kitamoto et al. [95] generated 20 MAbs from mice immunized orally with either a single genotype of 4 NoV VLPs, one strain of SaV VLPs or with mixtures of two types of VLPs from different genogroups. Of interest, the first reported NoV common MAbs were obtained from mice immunized with only one type of NoV VLPs which means common epitopes exist among viruses within the *Norovirus* genus and can be used to develop a cross-reactive diagnostic assay for detection of NoVs. Two MAbs that reacted exclusively with SaV VLPs are SaV-specific and are the first reported MAbs for SaVs [95].

Recently, two commercial ELISA kits for detection of NoVs in stool samples have been evaluated with a panel of 103 stool samples containing 4 genotypes of GI and 10 genotypes of GII NoVs and 39 stool samples containing other enteric viruses [96]. The sensitivities and specificities of the two kits were tested. One kit had a high sensitivity (>70% for 10 of the 14 subgroups) but a specificity of only 69%, and the other kit had a low sensitivity (<30% for 6 GII subgroups) but a high specificity of 100%. However, they have not been tested for animal NoVs.

Immunoassays for detection of SaVs have a similar problem. The SaV antigen-ELISA was quite strain-specific using hyperimmune antiserum raised against the purified viruses from the stools of infected young children or VLPs produced by a baculovirus-expression system [97, 98].

Detection of serological responses

Antibody-ELISA assays for detection of antibodies against porcine GII NoVs have been developed recently using the VLPs or capsid proteins of porcine NoVs [99]. However, these antibody-ELISA assays are usually cross-reactive. For example, the VLPs of porcine NoV GII-11/Sw918 strain cross-reacted with antibodies against human GII NoVs (GII-1/Hawaii and GII-3/Mexico) [99].

Presently, the detecting antigens are generally baculovirus expressed VLPs. These antibody-ELISA assays are specific and sensitive for detection of NoV-specific antibodies and thus they have been used in several large-scale human epidemiologic studies [99–104]. However, these antibodies are usually cross-reactive, detecting antibody responses to both GI and GII NoVs in volunteers [80,105,106], making it impossible to identify the antigenic type of the infecting strain [107]. On the other hand, the seroprevalence may under-estimate the prevalence of NoV in a population. For example, in volunteer studies of Norwalk virus, individual serological responses are not in 100% agreement with virus infection: a number of volunteers who were clinically ill and shed virus did not seroconvert to Norwalk virus [108].

V. Epidemiology of porcine NoVs and SaVs

Prevalence of porcine NoVs

We recently reported the results of a prevalence study of porcine NoVs in the United States [16]. Fecal samples (n=621) were collected from swine of various ages from 7 swine farms and 1 slaughterhouse in 3 states (Ohio, Michigan and North Carolina) (Table 1). Three genotypes of porcine NoVs (GII-11, 18 and 19) were detected by RT-PCR using one pair of primers targeting the RdRp region. An IC RNA was used to monitor for RT-PCR inhibitors and the RT-PCR products were confirmed by microwell hybridization assays. Porcine GII NoVs were detected exclusively from finisher pigs (20 to 24 weeks of age) with an overall prevalence of 20% (range, 0–40% among farms and 3–40% among NoV-positive farms), although samples from other ages of pigs were also collected at the same time from the same farm when the finisher pigs tested NoV positive. In one farm, a NoV outbreak probably occurred because 70% (42/60) of finisher pigs were NoV-positive, whereas only 0% (0/30) and 7% (1/15) of finisher pigs tested NoV positive at 2 other time points. We sampled 98 nursing pigs on 5 different occasions and 124 post-weaning pigs at 7 different times, but still failed to detect porcine NoVs from either of these two age groups. One possibility is that younger pigs are not as susceptible to NoV infection, similar to NoV infections in humans. Noroviruses have been detected in young children between 2 months and 2 years of age [109], but no NoV infections were reported in infants less than 2 months of age. A seroprevalence study of antibodies to Norwalk virus showed that they are acquired gradually in childhood, then more quickly during the adult years and by 50 years of age about 50% of individuals have Norwalk virus antibodies [110]. Recently, researchers found that human NoVs bind to human histo-blood group antigens (HBGA) which were found to influence the genetic susceptibility of individuals to NoV infections [111–116]. Because the HBGAs are developmental antigens [117], they may not be expressed in high levels in the gut of younger children or similarly in younger nursing and post-weaning pigs. In addition, the alpha 2-linked fucosylated glycans, HBGA, are secreted in the milk in about 80% of human mothers and bind to NoV particles, thus inhibiting the binding of NoVs to their cell receptors in the gastrointestinal tract [118]. Thus, HBGA in milk could protect infants against NoV infections. By analogy, if porcine NoVs bind similarly to HBGAs expressed in the gut and in the sow milk, perhaps only mature pigs are most readily infected with NoVs. Another possibility is that the primer pair used in this study was not sensitive enough to detect low virus levels in younger pigs. Genotype-specific primers could be used to increase the sensitivity.

Prevalence of porcine SaVs

We recently conducted a prevalence study of porcine GIII/Cowden-like, GVI?/JJ681-like, GVII?/LL26-like, and G?/QW19-like SaVs (Table 1) [16]. Among porcine SaVs, the GIII/Cowden-like SaVs were the most prevalent strains. This genogroup of porcine SaVs was detected from pigs of all ages with an overall prevalence of 62%. They were most prevalent among post-weaning pigs (83%) and least prevalent in nursing pigs (21%). Porcine GVI?/JJ681-like (4/621) and G?/QW19-like (5/621) SaVs were detected infrequently in pigs. The

GVII?/LL26-like SaVs were detected mainly in younger pigs with a prevalence of 20% and 9% in nursing pigs and post-weaning pigs, respectively, corresponding to the detection of K7 strain, which is genetically similar to LL26 strain, from a piglet with diarrhea in Japan [45].

Two more prevalence studies of porcine enteric caliciviruses by RT-PCR were reported recently [54,55]. Martinez et al. [54] detected 36 porcine enteric calicivirus-positives out of 204 (18%) fecal samples from piglets (0–9 weeks of age with or without diarrhea) in Venezuela using NoV- and SaV-conserved primers. Sequence analysis of the RT-PCR products of representative samples revealed that all viruses belonged to GIII/Cowden-like porcine SaVs. The lack of detection of porcine NoVs probably is due to the young age of piglets. However, the relatively low prevalence of porcine SaVs probably are due to the low sensitivity of their RT-PCR assays using broadly reactive primers as we discussed in the diagnosis section. Kim et al. in South Korea [55] detected 4.9% Cowden SaV-positives from nursing and weaned piglets out of 102 diarrheic pig fecal samples using 2 pairs of Cowden-specific primers targeting the RdRp and the capsid region, respectively [19]. The low prevalence in this study may be due to the relatively lower sensitivity of the RdRp primers PEC46/45 used compared to the primers PEC66/65 [68] and the high specificity of the capsid primers they used.

Co-infection of pigs by NoVs and SaVs, the latter mainly GIII/Cowden-like, was detected in 27% of 188 porcine SaV or/and NoV positive finisher pigs [16]. In humans, mixed infections of NoVs and SaVs were reported in 1 of 36 outbreaks [18] and mixed infections of NoVs and other enteric viruses, particularly rotaviruses, and less frequently adenoviruses and astroviruses, are also common [119,120]. Whether NoVs and SaVs are present as co-infections with other known pig pathogens, such as porcine rotaviruses, and whether such mixed infections exacerbate the clinical severity of diarrhea needs to be investigated.

Seroprevalence

There is only one seroprevalence study for porcine NoVs and no seroprevalence study for porcine SaVs. In the US, 71% of the pigs (n=110) were seropositive using VLPs of the Japanese porcine NoV/GII/Sw918 strain, 63% were seropositive using human NoV/GI/Norwalk VLPs and 52% were seropositive using human NoV/GII/Hawaii VLPs [99]. In Japan, the seroprevalence of the Sw918 strain in pigs was 36% (95/266).

In summary, these prevalence and seroprevalence studies show that porcine NoVs and SaVs circulate frequently in pigs worldwide. However, whether porcine NoVs and SaVs, except for Cowden SaV that has been confirmed to cause diarrhea and intestinal lesions in gnotobiotic pigs [19,20], are important causes of pig diarrhea in the field requires further investigation and testing of samples collected from both healthy and diarrheic pigs. Also needed are experimental studies of the infectivity of various strains of porcine enteric caliciviruses in seronegative pigs. Finally, whether the season influences the transmission and prevalence of porcine NoV and SaV infections in pigs is unknown.

Porcine NoVs and SaVs in shellfish

The genetic and antigenic relatedness between human and animal enteric caliciviruses suggests a possibility for interspecies transmission. Low infectious doses [1], prolonged asymptomatic shedding [80], environmental stability [121] and great strain diversity [47,51] increase the risk of infections by this virus family. Moreover, the identification of closely related animal enteric caliciviruses in pigs and cattle, and the existence of recombinants within human NoVs [48, 60], porcine NoVs [15], bovine NoVs [63,122], and also within the human and the porcine SaVs [43,50] raises concerns for possible infections or co-infections of humans with animal or with human and animal enteric caliciviruses, respectively. One possible source for mixed

occurrence of both animal and human enteric caliciviruses is via human consumption of contaminated raw shellfish.

In a recent survey of regional markets along the three U.S. coasts (East, West, and Gulf coasts), porcine NoV GII/18 were detected alone or simultaneously with human NoV GII/4 in 2 (4%) and 5 (11%), respectively, of 45 samples of oysters (each one collected at the same time from the same bay). The porcine SaV GIII/3 was also detected in 1/45 (2%) samples. Animal enteric caliciviruses were only detected in bays of the Washington and Oregon coasts in the western U.S., coinciding with the highest livestock production in these states among those included in this survey. This finding contrasts with that for human strains that were detected from each of the three coasts. Whether the presence of human NoVs in raw oysters represents a risk for gastroenteritis requires disease confirmation by outbreak investigation. Nevertheless the simultaneous detection of human and animal enteric caliciviruses in oysters raises concerns for possible human infections or co-infections with human and animal enteric caliciviruses, resulting in infections by animal NoVs or possible animal/human recombinants with emergence of new strains relevant for control of the disease.

VI. Antigenic relatedness to human caliciviruses

The antigenic relatedness between porcine and human NoVs was examined by Western blot analyses. The VLPs of porcine NoV GII-11/Sw918 strain cross-reacted with antibodies against human GII but not GI NoVs [123]. Gnotobiotic pig antiserum to GII-18/QW101-like porcine NoVs reacted with the capsid proteins of human NoVs, with the highest cross-reactivity to GII-3, 4, and 6 NoVs [15]. These findings coincide with the genetic distances between the porcine and human NoVs, whereby the QW101 strain also shares high amino acid identity with GII-3 (71%), GII-6 (71%), and GII-4 (63%) human NoVs. The extent of antigenic relatedness between porcine and human SaVs has not yet been tested or reported.

Summary

Diagnosis and epidemiologic studies of human enteric caliciviruses are frequently reported because these viruses cause gastroenteritis outbreaks in humans. Possible diarrhea outbreaks caused by porcine NoVs and SaVs have not been investigated because sensitive, validated assays for their routine detection were not available. No obvious clinical signs were observed for the GII NoV or GIII SaV positive adult pigs (sows and finisher pigs) but asymptomatic NoV and SaV infections are also seen in humans [1]. Moreover, some porcine NoV and SaV strains are genetically and antigenically closely related to human NoVs and SaVs, respectively, and the prevalence of these strains in the swine population is high. Instead of being homogeneous, RNA virus populations consist of closely related viral mutants and recombinants, referred to as quasispecies [124]. They are subject to continuous genetic variation, competition and selection during virus replication. When the virus population size increases, the expected number of viral genomes with more mutation sites also increases. Subsequently, this raises the risk of emergence of “new” variants with changes in host cell tropism or the ability to transmit or replicate in a different host species. Therefore, the high subclinical infection rate of NoVs and GIII SaVs in adult pigs is important for the persistence of these viruses in nature and poses a risk for emergence of strains that may transmit to humans. Although no inter-species recombinant strain has been found, an inter-genogroup human SaV recombinant has been described. Additional and large scale epidemiological studies should be performed in swine to monitor the antigenic and genetic variations among these viruses to better assess their prevalence and zoonotic potential.

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Figure 1.

The genome organization, open reading frames (ORF) and subgenomic RNA are shown for representative strains in the genus *Norovirus* (Hu/NoV/Norwalk/1968/US, GenBank accession number M87661) and genus *Sapovirus* (Hu/SaV/Manchester/1993/UK, GenBank accession number X86560). The size of each ORF is indicated as total amino acids (aa). The other numbers indicate nucleotide position. A poly(A) tail is shown as (A)n.

Fig. 2.

Unrooted Neighbor-joining phylogenetic tree of the capsid protein of caliciviruses by MEGA 3.1. The sequences are from GenBank. Four genera are indicated. The porcine enteric caliciviruses are in boldface; other animal caliciviruses are in italics and the remaining viruses are human caliciviruses. The Tree was tested by 500 bootstrap replicates.

Table 1
Prevalence of porcine GII NoVs, GIII and GVII?/LL26-like SaVs in pigs [No. positive/total (%)]

Swine farm or slaughterhouse	GII NoVs (Finisher)	GIII SaVs, GVII?:/LL26-like Sa Vs				
		Nursing	Post-weaning	Finisher	Sow	Total
MI farm A	2/61 (3)	NA ^a	NA	23/61 (37), 0/61 (0)	NA	23/61 (37), 0/61 (0)
NC farm A	2/8 (25)	NA	NA	8/8 (100), 0/8 (0)	NA	8/8 (100), 0/8 (0)
NC farm B	0/21 (0)	NA	NA	16/21 (76), 0/21 (0)	NA	16/21 (76), 0/21 (0)
OH farm A	5/22 (22)	4/14 (28), 3/14 (21)	12/12 (100), 7/12 (58)	20/22 (90), 0/22 (0)	13/13 (100), 0/13 (0)	49/61 (80), 10/61 (16)
OH farm B	43/105 (40)	7/61 (11), 15/61(25)	71/90 (78), 0/90 (0)	58/105 (55), 1/105 (1)	23/30 (76), 0/30 (0)	159/286 (55), 16/286 (6)
OH farm C	0/12 (0)	7/15 (46), 2/15 (13)	12/12 (100), 1/12 (8)	12/12 (100), 0/12 (0)	26/44 (59), 0/44 (0)	57/83 (68), 3/83 (4)
OH farm D	NA	3/8 (37), 0/8 (0)	9/10 (90), 3/10 (30)	NA	NA	12/18 (66), 3/18 (17)
OH slaughterhouse	12/83 (14)	NA	NA	65/83 (78), 0/83 (0)	NA	65/83 (78), 0/83 (0)
Total	64/312 (20)	21/98 (21) ^{c, b} , 20/98 (20)	104/124 (83) ^A , 11/124 (9)	202/312 (64) ^B , 1/312 (0)	62/87 (71) ^B , 0/87 (0)	389/621 (62), 32/621 (5)

^aNA = not available.

^bNumbers with different superscript letters (A, B and C) differ significantly ($P \leq 0.05$).