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Self-Assembly of the Recombinant Capsid Protein of a Swine Norovirus into Virus-Like Particles and Evaluation of Monoclonal Antibodies Cross-Reactive with a Human Strain from Genogroup II[▽]

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Noroviruses (NoVs) are responsible for the majority of gastroenteritis outbreaks in humans. Recently, NoV strains which are genetically closely related to human genogroup II (GII) NoVs have been detected in fecal specimens from swine. These findings have raised concern about the possible role of pigs as reservoirs for NoVs that could infect humans. To better understand the epidemiology of swine NoVs in both the swine and the human populations, rapid immunoassays are needed. In this study, baculovirus recombinants were generated to express the capsid gene of a swine NoV GII genotype 11 (GII.11) strain which self-assembled into virus-like particles (VLPs). Subsequently, the purified VLPs were used to evoke monoclonal antibodies (MAbs) in mice. A panel of eight promising MAbs was obtained and evaluated for their ability to bind to heterologous VLPs, denaturated antigens, and truncated capsid proteins. The MAbs could be classified into two groups: two MAbs that recognized linear epitopes located at the amino-terminal half (shell domain) of the swine NoV GII.11 VLPs and that cross-reacted with human GII.4 NoV VLPs. The other six MAbs bound to conformational epitopes and did not cross-react with the human GII.4 VLPs. To our knowledge, this is the first report on the characterization of MAbs against swine NoVs. The swine NoV VLPs and the MAbs described here may be further used for the design of diagnostic reagents that could help increase our knowledge of the prevalence of NoV infections in pigs and the possible role of pigs as reservoirs for NoVs.

Caliciviruses cause a variety of diseases in humans and animals. Members of this family are small, nonenveloped icosahedral viruses with a nonsegmented, positive-sense, polyadenylated RNA genome (22). The genome is 7.3 to 8.3 kb long and has two or three open reading frames (ORFs) that encode a polyprotein that undergoes protease processing to produce several nonstructural proteins, including an RNA-dependent RNA polymerase. The major capsid protein (VP1) and a minor capsid protein (VP2) are encoded by ORF1 and ORF2, respectively (in the genera *Lagovirus* and *Sapovirus*), or by ORF2 and ORF3, respectively (in the genera *Vesivirus* and *Norovirus*). On the basis of phylogenetic variation and genome organization, the family *Caliciviridae* has been divided into four distinct genera: *Norovirus*, *Sapovirus*, *Vesivirus*, and *Lagovirus*. Two new genera have been proposed for viruses infecting calves and rhesus monkeys, respectively (19, 46, 56).

Noroviruses (NoVs) are a widespread cause of nonbacterial gastroenteritis. In humans, NoVs are considered the major cause of acute gastroenteritis in adults and have been identified as the cause of approximately half of all gastroenteritis outbreaks worldwide (for recent reviews, see references 1, 17, 62, and 70). On the basis of the phylogenetic analysis of the capsid gene, NoVs have been classified into five genogroups (genogroup I [GI] to GV) that are further subdivided into 8, 19, 2, 2, and 1 genotypes, respectively (43, 68, 77). NoVs infecting humans fall into GI, GII, and GIV, with viruses in GII being the most prevalent (21). In fact, strains belonging to GII genotype 4 (GII.4) have accounted for the great majority of NoV outbreaks worldwide during the last decade (8, 36, 41).

NoVs have also been described in animals (for reviews, see references 54 and 67). Bovine and murine NoVs are classified separately into GIII and GV, respectively, while swine NoVs are grouped into at least three genotypes (GII.11, GII.18, and GII.19) together with human strains into GII (68). Recently, a lion NoV related to GIV NoVs has been reported (43).

The first methods of finding NoV in animals were based on oligonucleotide primers targeting human NoVs and yielded low prevalence values (60, 66). More recent studies with more specific and sensitive detection reagents have shown that NoVs are frequent in husbandry animals worldwide (14, 18, 48, 49, 53, 65, 68, 73). The existence of animal NoVs is relevant in several ways. (i) As in humans, infections can cause enteric disease in farm animals, resulting in economic losses for the livestock industry (25, 54, 72). (ii) Animals could be infected by and act as reservoirs for human NoVs. Conversely, animal

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NoVs could be zoonotic and could infect humans. In addition, homologous recombination between the human NoV and the animal NoV genomes could give rise to variants with completely new pathogenic and host tropism characteristics. These concerns are especially important for swine NoVs, since they are very closely related genetically to the most prevalent (GII) human NoVs. (iii) Animal NoVs could provide a much needed animal model for the study of the pathogenesis of NoV and immune responses to NoV. The swine model of NoV infection would make an excellent candidate, considering the physiological and immunological similarities between the human and the pig species (57, 58).

With the exception of the recently discovered mouse strains, NoVs cannot be easily grown in cell culture (16), although a complex three-dimensional system for the culture of human NoVs has recently been described (59). In early studies, NoVs had to be obtained from fecal specimens from experimentally infected human volunteers (64). A major breakthrough in NoV research was the finding that when VP1 of Norwalk virus (the prototypic strain of the genus *Norovirus*) was expressed in insect cells and other recombinant systems, it self-assembled into virus-like particles (VLPs) that are morphologically and antigenically identical to the native viral particle (24, 31). Subsequently, VP1 from a number of different human and animal NoV genotypes have been expressed in insect cells (4, 15, 23, 26, 30, 32, 39).

On the basis of the X-ray crystallographic structure of Norwalk virus, capsids are composed of 90 dimers of VP1 and exhibit $T = 3$ icosahedral symmetry (52). Each VP1 monomer (530 amino acids [aa]) contains a short N-terminal region (aa 1 to 49), followed by a shell (S) domain (aa 50 to 225) and a protruding (P) domain that can be divided into two subdomains: P1 (aa 226 to 278 and 406 to 520) and P2 (aa 279 to 405). The N-terminal/shell (N/S) domain forms the inner core of the capsid and is the most conserved part of VP1, while the P domain forms the protruding arches of the capsid and is more diverse. The P2 subdomain, which is located at the surface of the capsid, contains the highest degree of variability in the genome among NoV strains. It contains the determinants of strain specificity, receptor binding (11, 63), and potential neutralizing antibody recognition sites (10, 40).

In this paper, we describe the cloning and expression of recombinant GII.11 swine NoV VLPs, the use of these VLPs to obtain swine NoV-specific monoclonal antibodies (MAbs), and the primary characterization of these antibodies. To our knowledge, this is the first report of MAbs against a swine NoV.

MATERIALS AND METHODS

Viruses and cells. Derivatives of *Autographa californica* nuclear polyhedrosis virus containing the full-length capsid protein gene of swine NoV strain SwVA34 (Sw/NV/VA34/1998/NL; GenBank accession no. AY077644) (66), the different fragments of the gene (the N/S, P, and P2 domains), and the full-length capsid protein gene of a human NoV from a GII.4 strain (Ast6139/01/Sp; GenBank accession no. AJ583672) (5) were propagated in insect cell lines grown in suspension or monolayer cultures at 28°C in TNM-FH medium (Sigma) supplemented with 5% fetal calf serum (Gibco). *Spodoptera frugiperda* (SF9) cells were used for the generation of recombinant baculoviruses, plaque assays, and the preparation of high-titer viral stocks. *Trichoplusia ni* (H5) cells were used for the high-level expression of recombinant proteins.

RT-PCR cloning of swine NoV capsid nucleotide sequences. The swine GII.11 NoV strain Sw/NoV/34/1998/NET (SwVA34) was obtained from fecal samples as part of a surveillance study in The Netherlands and has already been described (66). To amplify a 3-kb reverse transcription-PCR (RT-PCR) product that includes the VP1 major capsid protein, 2.5 μl of SWVA34 RNA was reverse transcribed with 200 U of Superscript III (Invitrogen) and primer $(T)_{25}$VN (37). RNA was removed from the RNA-DNA hybrids by treatment with RNase H for 20 min at 37°C. Long template PCR was performed with primer swine POL fw (Table 1) and primer $(T)_{25}$VN by using Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland), according to the manufacturer's instructions.

TABLE 1. Oligonucleotides used for cloning

Oligonucleotide name	Sequence (5′–3′)[a]
Swine POL fw	TGGTGATTTCCGCATCTCTA
swNV-F	AAATAGATCTATAAATA**TG**AAGATGG CGTCTAATGACGCTTCC
swNV-R	GGTACCAGATC**TCA**ACGAACTCTCCT GCGCCCACTGCC
swNVS-R	CAACGGTACC**TCA**CACCAGAAA-CATA AACTCAAAGTC
SwNVP-F	TAGAAGATCTATAAATA**TG**AAGCCCC CCACTGTTGAGTCTAAAAC
SwNVP-R	GTACGGTACC**TCA**ACGAACTCTCCTG CGCC
SwNVP2-F	ATAGAGATCTATAAATA**TG**AAGACCA CACAGCTTGTGTCTTG
SwNVP2R	TCACGGTACC**TCA**AAGG-TTGGTGTTG TTCG

[a] Restriction site sequences are underlined. Start and stop codons are shown in boldface.

The 3-kb products were excised from the agarose gel with UV light from a Dark Reader (Clare Chemical Research Inc., Dolores, CO), cloned by using a TA cloning kit (Invitrogen, Leek, Netherlands), and sequenced by using an ABI Prism BigDye Terminator cycle sequencing reaction kit (Perkin-Elmer, Nieuwerkerk a/d IJssel, The Netherlands) on an automated sequencer (model 377; Applied Biosystems, Nieuwerkerk a/d IJssel, Netherlands). From a total of 10 colonies picked, seven clones had an insert of the expected size. Four of these clones were sequenced, and one clone (clone pSw1POL_CAP) with a nucleotide sequence that exactly matched the ORF2 sequence of strain SwVA34 reported previously (GenBank accession no. AY077644) was selected for the subsequent construction of the recombinant baculovirus.

Cloning of the swine NoV full-length capsid gene and different gene fragments into baculovirus transfer vector. The baculovirus transfer vector chosen for expression of the swine NoV capsid protein was plasmid pBacPAK8XB (2). The primers used for PCR amplification are summarized in Table 1. The full-length capsid gene of SwVA34 was subcloned in pBacPAK8XB by using the cloned cDNA (pSw1POL_CAP) as the template for PCR and primer pair swNV-F and swNV-R. After PCR amplification and digestion with BglII, the capsid gene sequence was cloned into the unique BglII restriction site in plasmid pBacPAK8XB, resulting in plasmid pSwNV1.

The gene fragments containing the N/S domain (aa 1 to 216), the P domain (aa 217 to 547), and the P2 subdomain (aa 275 to 425) of the SwVA34 capsid protein were subcloned in pBacPAK8XB by using plasmid pSwNV1 as the template for the PCRs, resulting in plasmids pSwNV-NS, pSwNV-P, and pSwNV-P2, respectively. The primer pairs used were swNV-F and swNVS-R to amplify the N/S construct, SwNVP-F and SwNVP-R to amplify the P construct, and SwNVP2-F and SwNVP2R to amplify the P2 construct. The amplified DNA fragments were digested with the BglII and KpnI restriction enzymes and were inserted into pBacPAK8XB. All the inserted sequences in the resulting recombinant plasmids were verified by sequence analyses.

Generation of recombinant baculoviruses. All recombinant baculoviruses were produced with the BacPAK baculovirus expression system (Clontech), as described previously (2). Briefly, monolayers of SF9 insect cells were cotransfected with recombinant transfer vectors and Bsu36I triple-cut *A. californica* nuclear polyhedrosis virus DNA (35) by using lipofectamine (Invitrogen). Recombinant baculoviruses were selected on the basis of their LacZ-negative phenotypes, plaque purified, and propagated as described elsewhere (33).

Expression and purification of the recombinant swine NoV VLPs. H5 insect cell monolayers were infected with the recombinant baculovirus at a multiplicity of infection of 10. After incubation (6 to 7 days, 28°C), the infected cells were scraped into the medium. The culture medium was then clarified by centrifugation (at 10,000 rpm for 10 min with a GSA rotor), and the supernatant was

TABLE 2. Amino acid sequence identities within VP1 protein of SwVA34 with sequences within VP1 proteins of representative NoV strains of the different GII genotypes

Genotype	Strain	GenBank accession no.	Capsid length (aa)	% Amino acid sequence identity		
				VP1	N/S domain	P domain
GII.11	SwVA34	AY077644	547	100.0	100.0	100.0
	SwK5/JP	AB221132	547	98.0	99.5	96.9
	Sw43	AB074892	547	97.6	98.6	96.9
	QW48	AY823303	547	97.1	99.1	95.7
	Sw918	AB074893	547	96.5	98.6	95.1
GII.19	QW170	AY823306	548	81.3	90.5	75.1
	QW218	AY823307	548	81.3	90.5	75.1
GII.3	G5175/AUS	DQ379713	548	71.3	81.4	64.5
	Toronto	U02030	548	70.8	81.0	63.9
	Mexico	U22498	548	70.4	80.5	63.6
	Arg320	AF190817	548	70.4	81.9	62.7
GII.6	Miami/292	AF414410	547	70.6	82.8	62.4
	SU17-JPN	AB039779	547	70.4	82.8	62.1
	Baltimore	AF414408	550	69.9	82.3	62.0
GII.18	QW101	AY823304	557	69.4	81.9	60.5
	QW125	AY823305	557	69.2	81.9	60.2
GII.14	M7	AY130761	536	67.5	82.8	57.5
GII.8	Amsterdam	AF195848	537	67.5	81.9	58.0
GII.17	CSE1	AY502009	540	67.4	77.8	60.5
GII.7	Leeds	AJ277608	540	66.5	81.9	56.8
GII.10	Erfurt	AF427118	548	66.4	80.5	57.1
GII.2	Melksham	X81879	542	66.4	80.1	57.2
GII.12	Chitta	AB032758	535	66.4	76.9	59.9
GII.1	Hawaii	U07611	535	66.2	77.8	58.4
GII.5	Hillingdon	AJ277607	540	66.1	80.1	57.0
GII.9	VA97207	AY038599	537	66.1	81.4	55.3
GII.4	Bristol	X76716	539	64.7	79.6	54.7
GII.16	Tiffin	AY502010	540	64.6	77.8	55.8
GII.13	Fayetteville	AY113106	542	63.2	76.5	54.0
GII.15	J23	AY130762	555	60.6	75.8	50.6
GI.1	Norwalk	M87661	530	44.7	56.9	36.7

centrifuged at 26,000 rpm for 2 h with a Beckman SW28 rotor. The pelleted material was resuspended in 0.2 M phosphate-buffered saline (PBS; 0.2 M sodium phosphate, 0.1 M NaCl, pH 6.0) for VLPs (PBS-V), extracted twice with Vertrel XF extraction buffer, and subjected to centrifugation (at 35,000 rpm for 2 h with a Beckman SW55 rotor) through a 20% sucrose cushion of 1.5 ml made with PBS-V. Subsequently, the pellet was suspended in a solution of CsCl (0.42 g/ml) and subjected to isopycnic gradient centrifugation at 35,000 rpm for 18 h in a Beckman SW55 rotor. The visible opalescent band in the CsCl gradient was collected by micropipetting, diluted in PBS-V, and pelleted by centrifugation at 26,000 rpm for 2 h in a Sorvall TH-641 rotor to remove CsCl. The pellet was finally resuspended in PBS-V containing protease inhibitors (Complete; Roche) and stored at 4°C. The protein concentrations of the VLP preparations were determined with a bicinchoninic acid protein assay kit (Pierce).

Electron microscopy. Samples (approximately 5 μl) were applied to glow-discharged carbon-coated grids for 2 min. and negatively stained with 2% (wt/vol) aqueous uranyl acetate. Micrographs were recorded with a Jeol 1200 EXII electron microscope operating at 100 kV at a nominal magnification of ×40,000.

Sequence analysis. The amino acid sequences of the complete capsid protein and the N/S and P domains of swine NoV strain SwVA34 were compared with those of swine and human NoV strains representative of the different genotypes of GII. The classification and the GenBank accession numbers of the NoVs strains are listed in Table 2. The capsid subdomains were determined on the basis of previously reported data (52). The Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST) was used to find homologous hits. Multiple-sequence alignment was performed with the ClustalW program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = /NPSA/npsa_clustalw.html) (12).

Production of MAbs. To produce MAbs, four female BALB/c mice were inoculated intraperitoneally with 50 μg of purified swine NoV VLPs in complete Freund's adjuvant. The animals were 5 weeks old at the time of the first inoculation. After that, the mice were boosted twice, at 3-week intervals, with 50 μg of purified VLPs in incomplete Freund's adjuvant. After the second boost, sera were collected from the tail vein and checked for antibodies specific for swine NoV capsid protein by enzyme-linked immunosorbent assay (ELISA). The two mice with higher antibody titers were further immunized twice with purified VLPs and no adjuvant 5 and 3 days, respectively, before they were sacrificed.

Hybridomas were obtained by fusion of splenocytes with myeloma SP2 cells in the presence of polyethylene glycol, as described elsewhere (6, 20). The supernatants of the individual hybridoma clones were screened by ELISA. Supernatants that recognized purified VLPs but that did not react with extracts from wild-type (wt) baculovirus-infected H5 insect cells were selected. All positive hybridomas were subcloned at least twice before expansion.

The Mouse Typer isotyping panel from Bio-Rad Laboratories was used to determine the immunoglobulin (Ig) isotypes of the MAbs, and the conditions recommended by the manufacturer were used.

ELISA. ELISA plates (Polysorp; Nunc, Rosklide, Denmark) were coated with 0.1 μg/well of purified VLPs (swine NoV or human GII.4 VLPs) diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were blocked with PBS–5% skim milk for 2 h at 37°C, washed five times with PBS-0.05% Tween 20 (PBS-T), and incubated with serial dilutions of the hybridoma supernatants. Each hybridoma supernatant sample was analyzed in parallel in a well lacking antigen to determine the level of background binding. After several washes with PBS-T, the plates were incubated at 37°C for 1 h with horseradish peroxidase-conjugated goat anti-mouse Ig (Bio-Rad) at a 1/1,000 dilution. Finally, the plates were extensively washed with PBS-T and the color reaction was developed with the peroxidase substrate (4 mg/ml *o*-phenylenediamine and 0.08% H_2O_2 [30%] in 0.05 M phosphate-citrate buffer [pH 5.0]). The reaction was stopped by addition of 3 N H_2SO_4, and color development was recorded at 492 nm. The reaction of a supernatant sample with the antigen was considered positive when the absorbance value was over threefold the background level (the absorbance of the well lacking antigen).

SDS-PAGE and Western blot analyses. Samples were boiled for 5 min in protein dissociation buffer containing 5% (vol/vol) β-mercaptoethanol, 2% (wt/vol) sodium dodecyl sulfate (SDS), 10% (vol/vol) glycerol, 80 mM Tris-HCl (pH 6.8), and 0.01% (wt/vol) bromophenol blue. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and were visualized by Coomassie brilliant blue staining.

For Western blot analyses, proteins were transferred from gels onto polyvinylidene difluoride membranes by standard blotting procedures. The membranes were saturated (overnight, 4°C) with PBS–5% (wt/vol) nonfat dry milk and incubated (1 h, 37°C) with the primary antibody, as indicated. After several washes with PBS-T, the membranes were incubated (1 h, 37°C) with horseradish peroxidase-conjugated goat anti-mouse Ig (Bio-Rad). The membranes were washed extensively with PBS-T and developed with the peroxidase substrate (0.05% 4-chloro-1-naphthol, 0.08% H_2O_2 [30%], and 20% methanol in PBS).

Flow cytometry. H5 insect cells were infected with the indicated recombinant baculoviruses. Three days postinfection, the cells were detached and fixed for 15 min in 10% formalin (Sigma). For labeling of the cells, the cells were permeabilized in PBS-T for 10 min. The cells were then incubated for 1 h at 4°C with hybridoma supernatant diluted 1:5 in PBS-T. After three washes with PBS-T the cells were incubated for 30 min at 4°C in PBS-T containing R-phycoerythrin-conjugated anti-mouse antibody (Dako) at a 1:100 dilution. After three washes, the labeled cells were analyzed by flow cytometry with a FACScallibur cytometer and CellQuest software.

RESULTS

Construction and expression of swine NoV recombinant VLPs. The full-length capsid protein gene derived from a swine NoV strain, strain SwVA34, collected in The Netherlands (66) was cloned into a baculovirus transfer vector, which was then used to obtain recombinant baculovirus SwNV1, which expressed the swine NoV VP1 protein. Cultures of H5 insect cells were infected with wt or SwNV1 baculovirus to analyze the expression of the recombinant protein. A major polypeptide band with the expected molecular mass of ~60 kDa was identified in lysates of SwNV1-infected H5 cells after analysis by SDS–10% PAGE and staining with Coomassie blue (Fig. 1A).

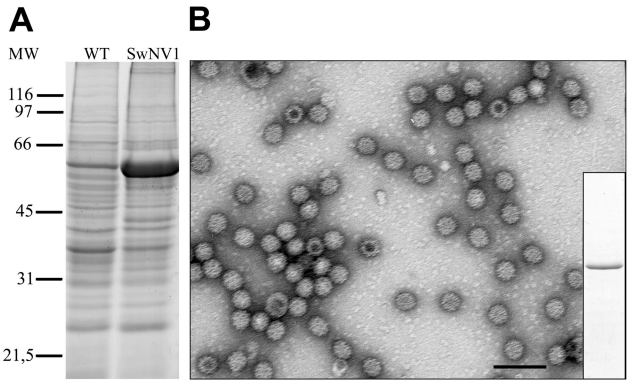

FIG. 1. Expression of swine NoV VLPs. (A) H5 cells were infected with wt or SwNV1 recombinant baculovirus, and equal amounts of infected cell lysates were analyzed by SDS–10% PAGE and Coomassie blue staining. Molecular weight markers (MW; in 10^3 Da) are given on the left. (B) Electron microscopy of negatively stained purified swine NoV VLPs. Purified particles were analyzed for protein content by SDS–10% PAGE and stained with Coomassie brilliant blue (inset). Scale bar, 100 nm.

To determine whether the recombinant swine NoV VP1 was able to assemble into VLPs, supernatants from infected H5 cell cultures were subjected to CsCl-gradient centrifugation and were characterized by electron microscopy. Negatively stained fractions enriched in the swine NoV VP1 (Fig. 1B, inset) revealed VLPs that were morphologically similar to authentic NoV virions (Fig. 1B) and that were approximately 40 nm in diameter (18).

Sequence comparison. The amino acid identities of the predicted complete, N/S, and P domains of the capsid protein of porcine NoV strain SwVA34 with other reported porcine NoVs and representative human NoV strains of GII are summarized in Table 2. On the basis of phylogenetic analysis of the complete VP1 amino acid sequence, strain SwVA34 shared 96.5 to 98% amino acid identity with other porcine strains of GII.11 (strains SwK5/JP, Sw43, and QW48), including prototype strain Sw918 (61), and 81.3% amino acid identity with porcine strains of GII.19 (strains QW170 and QW218). The human strains most closely related to strain SwVA34 belonged to GII.3 (70.4 to 71.3% amino acid identities) and GII.6 (69.9 to 70.6% amino acid identities). The amino acid identities to porcine NoV strains of GII.18 (strains QW101 and QW125) ranged from 69.2 to 69.4%. The amino acid identity with the Bristol strain, the prototype strain of GII.4, which is responsible for the majority of human NoV outbreaks worldwide, was 64.7%. Within GII, the lowest amino acid identity (60.6%) was observed with the human NoV strain of GII.15. Comparison of the amino acid identities of the N/S and P domains showed similar relationships. The amino acid identities of the N/S domain of SwVA34 with the N/S domains of NoV strains of different GII genotypes ranged from 75.8 to 99.5%, and the amino acid identities of the P domain ranged from 50.6 to 96.9%.

Production of swine NoV-specific MAbs. Purified, recombinant SwVA34 VLPs were used to immunize mice and obtain swine NoV-specific MAbs, as described in Materials and Meth-

TABLE 3. Reactivities of the eight swine NoV-specific MAbs

MAb name[a]	Reactivity					
	SwVA34 VLPs[b]	SwVA34 VLPs[c]	GII.4 VLPs[d]	N/S domain[e]	P domain[e]	P2 domain[e]
SwNV3A10	+	+	+	+	−	−
SwNV6E11	+	+	+	+	−	−
SwNV2C1	+	−	−	−	−	−
SwNV6C9	+	−	−	−	−	−
SwNV8C12	+	−	−	−	−	−
SwNV8E12	+	−	−	−	−	−
SwNV8F10	+	−	−	−	−	−
SwNV8B10	+	−	−	−	−	−

[a] All MAbs were of the IgG1 isotype.
[b] Recognition of the swine NoV VLPs of strain SwVA34 by ELISA.
[c] Recognition of the swine NoV VLPs of strain SwVA34 by Western blotting.
[d] Recognition of the human NoV VLPs of GII.4 (strain Ast6139/01/Sp) by ELISA.
[e] Recognition of the N/S, P, or P2 domain of the SwVA34 capsid protein by ELISA.

ods. After the hybridoma cell lines were screened, eight positive hybridoma cell lines derived from two different immunized mice were selected. The eight MAbs obtained were determined to be isotype IgG1. A summary of the characteristics of the eight MAbs described in this paper is shown in Table 3.

Specificities of the MAbs. To analyze the specificities and cross-reactivities of the MAbs, we performed ELISAs in which serial dilutions of the hybridoma supernatants were assayed against different baculovirus-expressed recombinant VLPs. As expected, none of the MAbs reacted with extracts of mock-infected or wt baculovirus-infected H5 insect cells (data not shown). The eight MAbs reacted strongly with SwVA34 VLPs (Fig. 2A, upper panel). In addition, two of the MAbs (MAbs SwNV3A10 and SwNV6E11) recognized heterologous VLPs derived from a Spanish NoV strain (strain Ast6139/01/Sp) grouped in GII.4 (5), although the reactivities of both MAbs to the heterologous VLPs were clearly lower than the reactivity exhibited against SwVA34 VLPs (Fig. 2A, lower panel). None of the MAbs showed any reactivity against VLPs derived from rabbit hemorrhagic disease virus (genus *Lagovirus*) (2) or feline calicivirus (genus *Vesivirus*) (data not shown).

To further characterize the nature of the epitopes recognized by the MAbs, we analyzed their reactivities in Western blots. The two MAbs shown to cross-react with GII.4 VLPs (MAbs SwNV3A10 and SwNV6E11) by ELISA also reacted in Western blot assays with both the SwVA34 and the GII.4 VLPs (Fig. 2B). This result indicates that these MAbs recognize linear epitopes of the NoV capsid protein. The other six MAbs did not recognize SwVA34 or GII.4 VLPs in Western blot assays (data not shown), suggesting that these MAbs recognize conformational epitopes.

In agreement with the results mentioned above, we found that MAbs SwNV3A10 and SwNV6E11 reacted strongly in ELISAs against the denatured SwVA34 capsid protein (treated with 8 M urea), while the other six MAbs did not recognize the denatured capsid protein in ELISAs (data not shown).

Physical mapping of the epitopes recognized by the MAbs. In order to localize the regions of the capsid protein recognized by the MAbs, we prepared three additional recombinant baculoviruses that expressed the N/S domain (aa 1 to 216), the P domain (aa 217 to 547), and the P2 subdomain (aa 275 to 425) of the strain SwVA34 VP1 protein, respectively. Expression of recombinant polypeptides of the expected sizes was confirmed by SDS–12% PAGE analysis (Fig. 3A). The recombinant proteins were recognized by an SwVA34 VLP-specific rabbit polyclonal antiserum by ELISA and Western blotting (data not shown). The P domain was expressed at high levels, similar to those exhibited by the full-length capsid protein, whereas the N/S and P2 domains were expressed at notably lower levels.

Cell extracts prepared from H5 insect cell cultures that had been infected with the recombinant baculoviruses expressing the different VP1 protein fragments were assayed by indirect ELISA and Western blotting with the MAbs. The two cross-reactive MAbs, MAbs SwNV3A10 and SwNV6E11, reacted strongly with the full-length VP1 protein and the N/S truncated

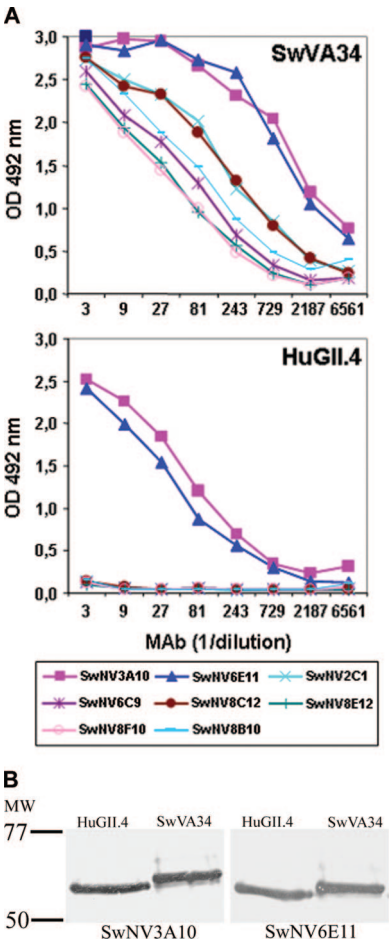

FIG. 2. (A) Reactivities of the MAbs with SwVA34 and human GII.4 (HuGII.4) NoV VLPs. The wells of ELISA plates were coated with equal amounts of purified SwVA34 or human GII.4 VLPs, and serial dilutions (threefold) of the eight hybridoma cell culture supernatants were used to detect antigens (OD, optical density). (B) Reactivities by Western blotting of MAbs SwNV3A10 and SwNV6E11 against SwVA34 and human GII.4 capsid proteins. Purified VLPs were resolved by SDS–10% PAGE and transferred to membranes which were reacted with the indicated hybridoma cell culture supernatants. Molecular weight markers (MW; in 10^3 Da) are given on the left.

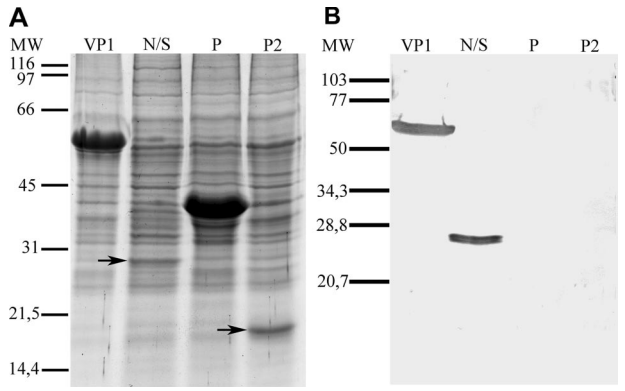

FIG. 3. (A) Analysis of MAb SwNV3A10 binding to the fragments of VP1. H5 cells were infected with recombinant baculoviruses expressing the swine NoV full-length VP1 or the protein domains: N/S, P, and P2. Equal amounts of infected cell lysates were analyzed by SDS–12% PAGE and Coomassie blue staining. Arrows indicate the positions of the recombinant polypeptides N/S and P2. Molecular weight markers (MW; in 10^3 Da) are given on the left. (B) Reactivity in Western blots of MAb SwNV3A10 against the VP1 fragments. Infected cell lysates were resolved by SDS–12% PAGE and transferred to membranes, which were reacted with the hybridoma cell culture supernatant of MAb SwNV3A10. Molecular weight markers (MW; in 10^3 Da) are given on the left.

protein by both ELISA (Table 3) and Western blotting assays. Figure 3B shows the reactivity of MAb SwNV3A10 with the swine NoV VP1 protein fragments in Western blots and shows that the reactivity was identical to that of MAb SwNV6E11 (data not shown). This result indicates that the epitopes recognized by these MAbs localize within the first 216 aa of the SwVA34 capsid protein. In contrast, the other six MAbs, shown to recognize conformational epitopes, recognized only the full-length VP1 protein in ELISAs and did not react with any of the truncated versions of the SwVA34 capsid protein (Table 3).

Finally, the specificities of the MAbs were further confirmed by flow cytometry. H5 insect cells were infected with wt or recombinant baculoviruses expressing SwVA34 VLPs, GII.4 VLPs, and the truncated versions of the SwVA34 capsid protein. Fixed cells were labeled with the MAbs and were analyzed by flow cytometry. The results obtained precisely matched our ELISA and Western blot assay results (Table 3). Figure 4 shows the results obtained with MAbs SwNV2C1 and SwNV3A10, representative of the two patterns of reactivity exhibited by the panel of MAbs specific for the swine NoV capsid protein reported in this study.

DISCUSSION

In this study, we expressed swine NoV capsid proteins in a baculovirus expression system and used the recombinant VLPs to generate and evaluate a panel of MAbs.

Of the eight MAbs selected, MAbs SwNV3A10 and SwNV6E11 recognized linear epitopes in the conserved N/S domain of swine NoV VP1 and cross-reacted with VLPs derived from an NoV strain of GII.4, which is the genotype responsible for the majority of NoV outbreaks in humans (36). The six other MAbs recognized conformational epitopes and did not cross-react with GII.4 VLPs.

The epitopes recognized by the non-cross-reactive MAbs could not be mapped to the N/S, P, or P2 domain because they did not recognize any of the truncated forms of recombinant VP1 protein expressed. One possible explanation for this lack of reactivity is that the conformational epitopes recognized by these MAbs are not correctly expressed in the truncated versions of the recombinant capsid protein. Another possibility is that the MAbs recognize epitopes made up of sequences from both the N/S and the P domains or from two adjacent capsid monomers so that none of the truncated proteins contains the mature epitopes. Further studies of these MAbs will be necessary to understand the exact locations of these epitopes.

Several groups have reported MAbs against human, bovine, and murine NoVs (28, 29, 34, 47, 64, 71, 75, 76) which have

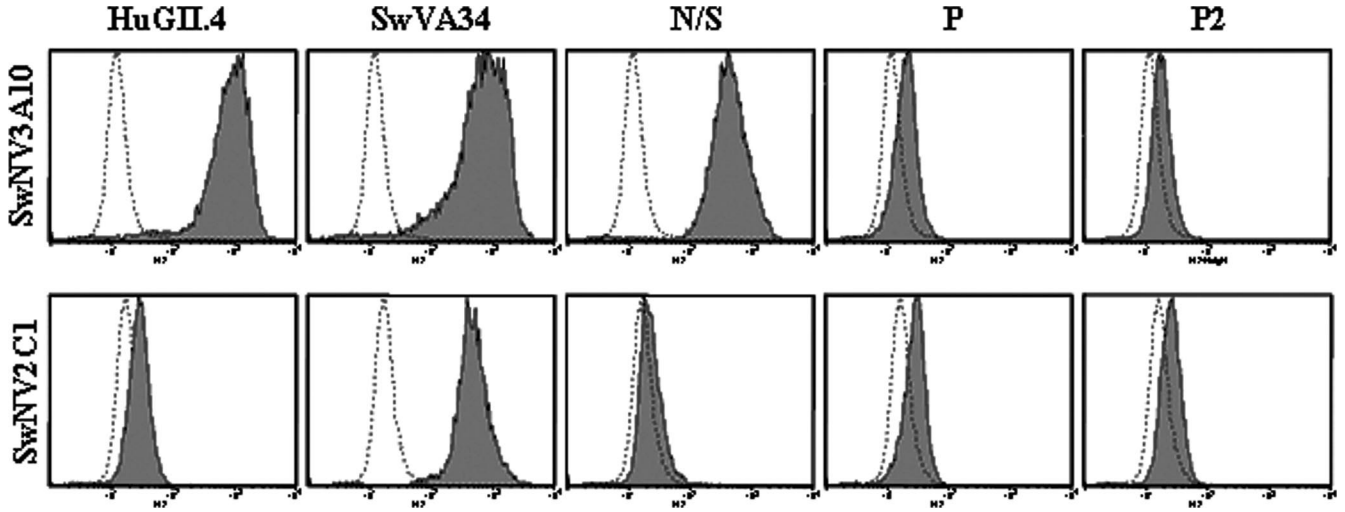

FIG. 4. Reactivities of MAbs SwNV3A10 and SwNV2C1 by flow cytometry. H5 insect cells infected with recombinant baculoviruses expressing the indicated NoV capsid proteins were incubated with hybridoma cell culture supernatants plus a R-phycoerythrin-conjugated anti-mouse antibody and analyzed by flow cytometry. The dashed line corresponds to the results for cells infected with wt baculovirus. HuGII.4, human GII.4.

been mapped to different regions of the VP1 protein. MAbs recognizing epitopes in the exposed P2 subdomain have been shown to neutralize murine NoV infection (38), and MAbs to GI.1 and GII.2 reference strains NV and Snow Mountain virus, respectively, blocked the binding of VLPs to CaCo-2 cells and inhibited hemagglutination (40). The epitope recognized by one of these antibodies (MAb 61.21) contained amino acids that are completely conserved among the various genogroups of NoV strains, including strains isolated from swine, bovine, and murine species (40). However, this MAb did not react to representative strains of GI and GII, including strains Southampton (GI.2), Desert Shield (GI.3), Toronto (GII.3), and Lordsdale (GII.4) (40).

Broadly reactive MAbs are of special interest, as they could be used for the development of simple, rapid, diagnostic assays for the detection of NoVs, including new previously unknown NoV strains, in epidemiological studies. Several NoV-specific MAbs exhibiting broad cross-reactivity (both intra- and intergenogroup cross-reactivity) have been described (3, 27, 34, 47, 55, 75), and the epitopes recognized by some of them have been precisely mapped to locations within the conserved N/S domain (3, 47, 74) or the C-terminal part of the P1 subdomain (50, 55, 75). However, none of these MAbs were tested with swine NoVs strains, and given the amino acid sequence diversity observed in the minimal binding regions reported for these MAbs, their reactivities with swine NoVs strains (of GII.11, GII.18, and GII.19) may not be readily anticipated (data not shown), although some of them would likely recognize swine strains.

In this respect, MAbs SwNV3A10 and SwNV6E11 seem promising. Since swine NoV strain VA34 (GII.11) and human strains of GII.4 are among the most divergent within GII (Table 2), we speculate that these MAbs might recognize NoVs belonging to most of the other genotypes of GII and could also recognize NoVs of other genogroups, as is the case for other MAbs which bind to epitopes located at the N/S domain (3, 47, 74). Work is in progress to further characterize the range of NoV strains that are recognized by these MAbs, with the focus on their usefulness as broad-range diagnostic reagents for NoV detection. On the other hand, MAbs specific for swine NoV strains would also constitute useful reagents. In follow-up studies, we will analyze the reactivity of our panel of MAbs with swine NoV strains of GII.18 and GII.19.

The fact that swine NoVs are genetically closely related to strains infecting humans has raised concerns about the possibility that these viruses might be able to cross the species barrier (18, 42, 66, 68, 69), which may result in a disease more serious than that typically associated with NoV infection (45).

Recombination between NoVs of different genotypes within GII has been reported (7, 51). Although it has not yet been reported, homologous recombination between swine and human NoV genomes could lead to new variants with altered tropisms and virulence characteristics. Recent findings indicate that such events could happen because (i) seroprevalence studies have shown that pigs' sera contain antibodies against human NoVs (18); (ii) human GII.4 NoVs can infect piglets experimentally (9) and human GII NoV genomic sequences have been detected in the manure of pigs (44); and (iii) surveillance studies with bivalve mollusks, a common source of food-borne NoV gastroenteritis, have demonstrated the simultaneous presence of both human and swine NoVs (13), leading to concerns about the risk of coinfection with human and animal NoV strains.

In summary, the relevance of NoV infections to human health, the close genetic relationship between human and swine NoV strains, and the prevalence of NoVs in swine highlight the need for the comprehensive surveillance of NoVs in pigs. The swine NoV VLPs and the MAbs reported in this study might eventually be used in diagnostic assays that could help increase our knowledge of the prevalence of NoV infections in pigs and the possible role of pigs as reservoirs for NoVs infecting humans.

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