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Development and application of full-length and polymerase chain reaction-derived partial-length VP4- and VP7-specific nucleic acid probes for the differentiation of porcine rotavirus serotypes

Rosen, Blair Ira, Ph.D.
The Ohio State University, 1993

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DEVELOPMENT AND APPLICATION OF FULL-LENGTH AND POLYMERASE CHAIN REACTION-DERIVED PARTIAL-LENGTH VP4- AND VP7-SPECIFIC NUCLEIC ACID PROBES FOR THE DIFFERENTIATION OF PORCINE ROTAVIRUS SEROTYPES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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****

The Ohio State University

1993

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To My Parents
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INTRODUCTION

Rotaviruses are double-shelled nonenveloped viruses in the family Reoviridae. The virus has a double-stranded RNA genome consisting of 11 gene segments that produce a characteristic pattern after electrophoretic separation and staining in polyacrylamide gels (125,270). Numerous investigations of viral gastroenteritis in children, and the young of many mammalian and avian species have implicated rotaviruses as important etiological agents (72,127). Rotaviruses have been documented as a primary pathogen of newborn and weaned pigs, and serological studies have indicated infections among swine herds may reach nearly 100% (27,30,34,139,156,157). Neonatal diarrhea of swine results in economic losses due to mortality, increased veterinary costs, and decreased productivity of survivors (53,127,289). Antigenic characterization of rotaviruses has revealed several morphologically identical but antigenically distinct serogroups (A to G) of viruses (29,211,235). Four serogroups of rotavirus (A, B, C, and E) are known to infect swine (235). Group A rotaviruses, which have been the most extensively characterized, are the subject of the discussions and investigations in this study.

Methods for the characterization of the antigenic specificities of rotavirus have traditionally been performed using serological reagents. Initial studies using hyperimmune polyclonal antibodies distinguished several different serotype specificities (95,131,272). Subsequent studies indicated these assays were based on the immunodominant epitopes on the outer capsid glycoprotein VP7 (118,122,152,262). At least 11 and potentially 14 serotypes of rotaviruses have been classified in human beings and animals on the basis of VP7 or G (glycoprotein) serotype specificities (38,39,68,261).
Serotype G4 and 5 rotaviruses, represented by the prototype strains Gottfried and OSU, respectively, were initially detected 9 to 16 years ago in the U.S. and have recently been detected in other countries (30,56,133,173,248,266). Two additional G serotypes of rotavirus have been isolated from swine and characterized. These include the YM rotavirus (G11) isolated in Mexico and several strains of G3 rotaviruses isolated in Australia (7,133,187,229). Other G serotypes of rotavirus have been reported in swine but have not been characterized (17,209). Few studies have reported on the antigenic diversity of porcine rotaviruses in the U.S. following the discovery of the Gottfried and OSU strains (146,209).

Genetic and monoclonal antibody-based assays revealed that the neutralization specificities of rotaviruses are determined by a second outer capsid protein, VP4 (199,202,247). Experiments with naturally occurring or laboratory produced reassortant rotaviruses determined that neutralization epitopes on both outer capsid proteins may be important in eliciting immunological protection, indicating the need for a two serotype classification systems for distinguishing G types (VP7) and P types (VP4) (130,204). The diversity and distribution of P type specificities in porcine rotaviruses have not been systematically characterized.

Information related to the serotype diversity and prevalence of rotaviruses in swine may have direct implications for the development of effective vaccines. Rotavirus vaccine trials in children have suggested that induction of protective immunity may be dependent on the serotypes of rotaviruses prevalent in a particular region (87,182,214). Several vaccine strategies in humans have involved the use of vaccines that include two or more serotypes of rotaviruses for the induction of protective immunity (87,214). Similar strategies have been used in the development of porcine rotavirus vaccines (286). The efficacy of these vaccines in field trials have been variable (87,126,214,286).

Serological-based enzyme-linked immunosorbent assays using monoclonal antibodies specific for rotaviruses of different G serotype specificities have been used for the
characterization of human and porcine rotaviruses (2,25,116,188,262). These assays are specific and can be used for the analysis of large numbers of samples. Problems, however, are often encountered because of genetic variability that prevents the detection of antigenic variants, and large numbers of untypable samples due to the loss of the rotavirus outer capsid proteins (25,52,116,288).

An alternative method for the differentiation of rotavirus serotypes involves the use of nucleic acid probes. Hybridization studies with probes prepared from the gene segments coding for VP4 and VP7 of human rotaviruses have been used for the differentiation of rotaviruses of different serotype specificities (81,150). The identification of the nucleic acid regions coding for the neutralization epitopes on VP4 and VP7 have enabled refinements in the development of probes (68,104,107,108,150). These regions are conserved among rotaviruses of the same serotype and are variable among rotaviruses of different serotypes. This information has allowed the development of smaller specific probes that encompass the variable regions and eliminate large areas of conserved nucleic acid sequences.

In the following series of studies, nucleic acid probes were developed and applied for the detection and characterization of porcine rotaviruses. The main objectives of the studies were to develop specific reagents capable of detecting porcine rotaviruses with homologous serotype specificities, and to apply the probes for the analyses of rotaviruses in porcine field samples. A side benefit of these reagents were their potential for identifying unreactive and therefore new serotypes of rotavirus in swine. Primary emphasis was given towards the development of probes prepared from the VP4 and VP7 coding genes of porcine strains Gottfried (G4, P6) and OSU (G5, P7). These strains were chosen because of the reported predominance of rotaviruses with similar serotype specificities in swine in the U.S. during previous studies by researchers in our department (30,206). Hybridization studies were initially conducted with probes prepared from full-length gene 9 (coding for VP7) cDNA. When nucleic acid sequence information became available partial-length gene 9 probes were
produced that encompassed smaller regions of sequence diversity. The production of the partial-length probes was facilitated by the use of the polymerase chain reaction. Similar studies were later conducted using Gottfried and OSU full and partial-length gene 4 probes. The development of the probes used in the following studies occurred concurrently with similar probes used for the characterization of human rotaviruses. The lack of reactivity of many of the rotavirus-positive field samples with the Gottfried and OSU probes prompted the development and use of VP4- and VP7-specific probes from the porcine rotavirus YM (G11) and VP7-specific probes from the human rotaviruses Wa (G1), DS-1 (G2) and P (G3).
CHAPTER I
LITERATURE REVIEW

INTRODUCTION

Rotaviruses are nonenveloped, double-stranded RNA viruses that constitute one genus in the family Reoviridae. The virus is ubiquitous and has been isolated or serologically detected in human beings, birds, and nearly all domestic and wild mammalian species (59,102). Rotaviruses are causative agents of acute diarrheal disease that is typically manifested in infants and the young of many animal species. Studies implicating rotaviruses as gastrointestinal pathogens of swine followed similar reports in studies of human infants, calves, and mice (1,126,127,145,146,182,242,245). Clinical signs of infection in pigs include diarrhea, anorexia, and depressed growth rates, although subclinical infections also occur (16,20,74). Porcine rotaviruses are now recognized as a widespread pathogen associated with diarrheal diseases in both nursing and newly weaned pigs (20,23,110,124,245). Serological and RNA electrophoretic analyses have determined the presence of several antigenically distinct groups (serogroups A, B, C, and E) or rotavirus in swine (22,174,196,226). The focus of this literature review and the subsequent chapters will be on group A porcine rotavirus.

MORPHOLOGY AND STRUCTURAL PROTEINS

The morphology of porcine rotaviruses is identical to the morphology described for rotaviruses isolated from human beings and other animal species. Rotaviruses are nonenveloped viruses that possess icosahedral symmetry. The complete and infectious form of the virus is composed of two concentric protein shells or capsids with a diameter of 76-77
nm (55,199). The name 'rotavirus,' derived from the Latin word rota, a wheel, is due to the spoke-like appearance of the capsomeres and the well-defined outer layer that are visible when phosphotungstate stained double-capsid particles are viewed by electron microscopy (35,65,199). Both double- and single-shelled (70.5 nm) virus particles may be found in samples obtained from rotavirus-infected cell culture and fecal preparations (145,199,230). A third type of particle, a rotavirus core (50 nm), may be found in some rotavirus preparations and can be produced by chemical disruption of single-shelled particles (17,55). Although extracellular virus particles are nonenveloped, particles observed in the cisternae of the rough endoplasmic reticulum of infected cells may possess a temporary membrane (35,161,199).

The RNA coding assignments for the structural proteins have been studied for rotaviruses isolated from human beings and many animal species (55,138,143). As a result the following correlation between the RNA segments and the rotaviral structural proteins have been determined: VP1 (core protein) coded by RNA segment 1; VP2 (core protein) coded by RNA segment 2; VP3 (core protein) coded by RNA segment 3; VP4 (outer capsid protein) coded by RNA segment 4; VP6 (inner capsid protein) coded by RNA segment 6; and VP7 (outer capsid protein) coded by RNA segment 7, 8, or 9. The remaining 5 gene segments (including two segments of the 7, 8, and 9 triplet depending on the virus strain) code for nonstructural proteins that are not incorporated in the mature virion (55,138,143).

The outer capsid of rotaviruses is composed of the polypeptides VP4 (formerly called VP3) and VP7 (55,132). VP4 is a nonglycosylated protein (molecular weight approximately 88,000) associated with viral virulence, infectivity, hemagglutination, growth restriction in cell culture, protease-enhanced plaque formation, and possesses serotype specific and cross-neutralizing epitopes (55,58,98,114,136). Virus infectivity is enhanced by cleavage of VP4 by trypsin into two polypeptides, VP5 (molecular weight approximately 60,000) and VP8 (molecular weight approximately 28,000) (54,58). The glycosylated outer capsid protein VP7
is the most abundant outer capsid protein, and the second most abundant protein in the virion (55). The carbohydrates associated with VP7 are of the high-mannose type and are attached by N-linked glycosylation (55,191). This glycoprotein (molecular weight approximately 38,000) possesses the major antigenic sites associated with virus neutralization (51,189,217). Double-shelled rotavirus particles treated with EDTA or other calcium chelating agents result in the loss of the outer capsid proteins and the production of single-shelled rotavirus particles (70,102,201).

Analysis of the three-dimensional structure of rotavirus has determined that VP4 exists as a 120 Å spike on the surface of the rotavirus particle (4,178). Each spike consists of a dimer of VP4 proteins with a bilobed globular domain at the end. Cryo-electron microscopic examination of the interaction of the spikes with the Fab fragments of neutralizing monoclonal antibodies indicate that the VP4 antigenic sites are located at the distal ends of the spikes.

The major structural protein of rotaviruses is VP6 (molecular weight approximately 45,000) located on the outer surface of single-shelled particles (166,195). The protein exists as trimeric units that may associate into hexameric structures held together by disulphide bridges (133,195). Treatment of single-shelled particles with chaotropic agents (e.g. 1M CaCl₂, sodium thiocyanate) results in the removal of VP6 and the production of rotavirus cores (17,201). Antigenic specificities associated with VP6 include the serogroup and subgroup antigenic determinants.

Rotavirus core particles are composed of three viral proteins, VP1 (molecular weight approximately 124,000), VP2 (molecular weight approximately 102,000), and VP3 (molecular weight approximately 98,000) (55,166). The VP1 protein makes up approximately 2% of the mass of virions (55). Comparisons of the deduced amino acid sequence of VP1 with protein databases have revealed similarities of one specific region (GDD consensus sequence) and other regions with RNA polymerases of other RNA viruses including the reovirus lambda 3
protein (amino acids 585 to 739) and the bluetongue virus P1 protein (amino acids 667 to 769) (55). Recent experiments demonstrating the interaction of radiolabeled, photoreactable nucleotide analogs with the VP1 protein of single-shelled rotavirus particles has lent support to the conclusion that VP1 is involved in the RNA-dependent RNA polymerase activity of rotaviruses (238).

The VP2 protein is the second most abundant protein in single-shelled rotavirus particles (24,55). Experiments in vitro have revealed that VP2 and a nonstructural rotavirus protein (molecular weight approximately 31,000) can bind rotavirus RNA (24). The VP3 protein (molecular weight approximately 98,000) is a minor structural protein (12,55). Comparisons of the deduced amino acid sequences of this protein with other RNA polymerases suggest that VP3 may also be involved in RNA replication (55).

MORPHOGENESIS

Studies of the morphogenesis of porcine and other animal and human rotaviruses indicate virus entry into cells may occur by two routes; receptor-mediated endocytosis or direct penetration of the cell membrane (12,134,179). Uncoating or removal of the outer protein shell of rotaviruses following virus entry was suggested in several studies to be due to low levels of intracellular Ca$^{2+}$ (102,134). The influence of Ca$^{2+}$ levels on uncoating, however, has not been firmly established.

Viral precursor proteins and subviral particles assemble in viroplasmic inclusions in the cytoplasm adjacent to the RER (12,142,161,199). The subviral particles consist of structural and nonstructural viral proteins; in addition, distinct replication intermediates have been detected (76). Single-shelled particles produced in the viroplasmic inclusions acquire the outer capsid proteins VP4 and VP7 during or following the budding of virus particles into the cisternae of the RER (12,35,161,199). Although double-shelled rotaviruses accumulate in the luminal space of the RER, single-shelled rotaviruses and core particles have also been
reported in vitro and in vivo (12,35,145,161,199). The release of rotavirus particles occurs following lysis of the membranes of rotavirus-infected cells (199).

Tubules composed of rotavirus proteins have been observed in fecal material, and in the cytoplasm or nucleus of infected cells during the course of rotavirus replication (12,59,144,199). Tubules and other aberrant structures observed in infected cells are postulated to be composed of VP6, because of the observation that recombinant baculovirus-expressed VP6 protein can aggregate into similar structures (56).

**PHYSICOCHEMICAL PROPERTIES**

The nonenveloped double protein layer surrounding the rotavirus genome is relatively stable, and enables the virus to survive in soil and water in the environment (92,241,209). Experimental studies conducted 1 year apart on the survival time of rotavirus in estuarine water showed a 1000-fold reduction in virus titer in a time interval ranging from 2 to 14 days at a temperature of 20°C, indicating nonseasonal factors may affect virus survival (107). Similar studies on rotavirus in chlorinated tap water at 4 and 20°C showed no decrease or a 2 log10 decrease in virus titers, respectively, during a 64 day interval (180). These studies and others indicate the virus may persist for weeks or months under certain environmental conditions, and waterborne transmission may be of importance in the epidemiology of rotavirus (107,180,209,241).

In fecal material the virus may survive for 7-9 months at 18-20°C (241). At higher temperatures the survival time of infective virus is reduced, but survival may vary depending on the presence of stabilizing factors (102). Rotaviruses may also survive for several hours to days on animate or inanimate surfaces, and may absorb to soil particles and be released in an infectious state by rain to the groundwater (3,92,241). For these reasons, porcine rotavirus is impossible to eliminate from the farm environment, but may be
controlled by preventing the accumulation of fecal material in conjunction with the use of selected disinfectants.

The most effective disinfectants against rotaviruses are ethanol preparations at concentrations of 70% or greater (59,202,241). Other disinfectants that have been found effective against rotavirus include formaldehyde (3.7% or greater), chlorinated phenolic compounds, chloramine T (Multichlor), and 0.01% iodine (1% Wescodyne) (59,102,202,241).

Investigations of rotavirus morphology and RNA polymerase activities have determined that infectious double-shelled rotaviruses are stabilized by the addition of relatively low concentrations of CaCl₂ in viral diluents (0.15 to 1.5 mM), and that complete viral particles contain Ca^{2+} (17,38,203,207). This information has led to the development of methods for the dissociation of complete virions to single-shelled particles for the activation of the RNA polymerase capabilities of rotaviruses in vitro and for the production of nucleic acid probes (17,38,67,72,137). Alternatively, efforts to maintain the integrity of rotavirus particles for subsequent serological analysis may be accomplished by the addition of CaCl₂ in viral diluents (63).

GENOMIC PROPERTIES

The rotavirus genome consists of 11 segments of double-stranded RNA that range in size from 667 (segment 11) to 3,302 (segment 1) base pairs (55,102). Sequence data from the gene segments from different virus strains indicate a total genome of approximately 18,522 base pairs (55).

The nucleic acid and deduced amino acid sequences of the genomic segments from several strains of porcine rotavirus have been determined. Primary emphasis has been given to the genomic segments coding for the outer capsid proteins VP4 (P type) and VP7 (G type) that contain the neutralization determinants. The nucleic acid sequences for the genomic segment coding for VP7 have been published for eight porcine rotavirus strains representing
serotypes 3, 4, 5, and 11 (87,89,105,190). Several features of the VP7 gene have been conserved in all porcine rotaviruses. All eight porcine rotaviruses possess a VP7 gene 1,062 bases long with two potential in phase initiation codons (AUG) at nucleotides 49-51 and 136-138. The nucleotide sequences encode VP7 proteins of either 297 or 326 amino acids. One potential glycosylation site is present in all porcine rotaviruses at amino acid residue 69. This glycosylation site is conserved in all rotavirus strains except for the bovine rotaviruses NCDV and RF (55). The eight cysteine residues present in all rotavirus VP7s are conserved in porcine rotaviruses.

Although the VP7 of most porcine rotaviruses are characterized by a single potential glycosylation site, analysis of the predicted amino acid sequence of a single G serotype 3 porcine rotavirus isolated in Australia revealed two potential glycosylation sites (156). The second glycosylation site was detected at amino acid position 238. Rotaviruses with two potential glycosylation sites have previously been reported only among human and bovine rotaviruses (106,156).

Nucleic acid and amino acid homology studies of human rotaviruses have demonstrated a high degree of homology among the VP7 genes of rotavirus strains of the same G serotype (96). Similar levels of homology (generally >90% and >84% nucleic and amino acid homology, respectively) have also been observed between the VP7 genes of human and porcine rotaviruses or among porcine rotaviruses of the same G serotypes isolated on different continents (89,105). The high levels of homology determined by sequencing data between human and porcine rotaviruses have also been confirmed by nucleic acid hybridization studies with porcine rotavirus gene 9 probes (186). Comparisons between porcine rotaviruses of serotypes G4 and 5 have demonstrated nucleic and amino acid homologies of <76% and <77%, respectively (89,105). Notably higher homologies, however, have been observed between porcine rotaviruses of serotypes G3, 5, and 11 (79-83% and 86-89% nucleic and amino acid homologies, respectively) (105,190).
The nucleic acid and deduced amino acid sequences of the genomic segment 4, coding for VP4, of the Gottfried and OSU porcine rotaviruses have been determined (90,165). The Gottfried genomic segment 4, like the Gottfried VP7 coding gene, shows a high degree of homology with the corresponding genomic segments of certain human rotaviruses. Comparisons of the Gottfried VP4 gene with the five distinct VP4 genes of human rotaviruses (78,88) shows a higher homology (87.1 to 88.1% amino acid homology) with rotaviruses of "asymptomatic strains" (M37, 1076, McN13, and ST3) (69,90). This homology has been confirmed by nucleic acid hybridization studies (184). Amino acid homologies of the Gottfried VP4 with the VP4 of other animal rotaviruses (Rhesus rotavirus, SA11, Nebraska calf diarrhea virus, and the OSU porcine rotavirus) ranges from 71.5 to 75.2% (90).

The genomic relationships of porcine rotaviruses with rotaviruses isolated from human beings or nonporcine animal species was reported in a study by Flores et al., (68). In this study, genomic RNA probes consisting of all 11 gene segments were prepared by the in vitro transcription of purified rotavirus particles. Northern blot hybridizations with probes prepared from bovine, simian, canine, porcine, and human rotaviruses revealed a high degree of homology between rotavirus isolates from homologous human or animal species. Investigations of heterologous hybridization reactions indicated a greater degree of genomic homology between a human rotavirus (strain Wa) and two porcine rotaviruses (strains OSU and EE) than with rotavirus strains from the other animal species investigated.

Genomic rearrangements have been reported in porcine rotaviruses as well as rotaviruses from human beings and other animal species (14,175,216,223). Most genomic rearrangements involve genomic segment 11 that results in shortened or so-called "supershort" electrophoretic patterns (55,139). The effect of gene arrangements on the biological properties of the viruses are unknown. Two porcine rotaviruses characterized by a shortened electrophoretic pattern were reported in a study by Bellinzoni et al. (14). Molecular and genetic analyses of both isolates revealed a rearrangement in genomic
segment 11 resulting from a partial duplication and deletion of the genes with retention of the normal 5' to 3' orientation of the duplicated section (86,139). Despite the altered gene structure, the apparent molecular weight of the protein products encoded by the genomic segments was unaffected. Differences in the biological properties of one of the altered porcine isolates was determined in a study by Mattion et al. (139). The altered isolate produced larger plaques in MA-104 cell monolayers and outgrew a nearly identical but electrophoretically normal porcine strain isolated from the same fecal sample. Previous studies on the coding assignments of the rotavirus genome have identified the protein product of genomic segment 11 as a nonstructural protein (55).

ANTIGENIC RELATIONSHIPS

Rotaviruses are serologically defined by three major antigenic specificities, a common or serogroup-specific antigen, a subgroup-specific antigen, and the serotype-specific antigens. A common antigen that defines rotavirus serogroups is located on VP6 and is the major antigen involved in polyclonal antibody-based assays for rotavirus detection (11,26,102,215,244). All rotaviruses isolated from human beings and animals were originally thought to possess a common group antigen (49). However, seven groups (serogroups A to G) of antigenically distinct rotaviruses that differ in their RNA electrophoretic patterns have been isolated from human beings and animals (53,174,196). Four of these antigenically distinct serogroups (A, B, C, and E) of rotaviruses have been detected in swine (22,174,196,226).

Group A rotavirus serotypes were initially established based on polyclonal antibody-based serum neutralization assays (77,104). The serotypes defined by these assays were based on the antigenic specificities of the outer capsid protein VP7 (97,99,113). A widely accepted serotyping scheme based on the VP7 or glycoprotein (G) neutralization specificities was proposed by Hoshino et al. (104). In this study seven G serotypes of rotavirus were established using a plaque reduction serum neutralization assay. Currently at least 11 and
potentially 14 G serotypes of rotaviruses have been identified in human beings and animals (30,31,55,218).

Three of the seven G serotypes of rotavirus defined by Hoshino et al. (104) have been detected in swine (serotypes G3, 4, and 5). Serotype G4 and 5 porcine rotaviruses, represented by the prototype strains Gottfried and OSU, respectively, were originally isolated in the U.S. (23). The Gottfried and OSU strains of porcine rotavirus have been extensively characterized both antigenically and genetically (87,89,90,104,165). Other strains of serotype G4 and 5 porcine rotaviruses have been isolated or detected in the U.S., as well as several other countries (23,45,105,140,206). Serotype G3 porcine rotaviruses have been isolated in Australia, and there is serologic evidence of infection in pigs in Thailand and Argentina (15,153,177). Serotype G3 and 4 porcine rotaviruses are antigenically related to human rotaviruses of the same VP7 serotype.

A fourth serotype of rotavirus, strain YM, was originally isolated from swine in Mexico (190). Porcine rotaviruses of the same serotype have also been detected in Venezuela and the U.S. (131,183). Molecular and antigenic characterization of strain YM indicated this rotavirus was antigenically unique, and was not serologically related to any other previously established group A rotavirus G serotype (6,190). Strain YM was subsequently classified as a group A rotavirus serotype G11 (55). Two other studies have indicated the possible presence of other strains/serotypes of porcine rotavirus. These include serotype 1 and 2 porcine rotaviruses detected in Argentina using monoclonal antibodies (MAbs) produced against human rotavirus serotypes (15), and the ISU-64 and ISU-65 porcine rotaviruses detected in the U.S. (172). Further antigenic, molecular, and epidemiologic studies are needed to confirm the serological or strain relationships of these latter viruses and to determine their geographical distribution.

Analysis of VP7 with monoclonal antibodies have identified 4 to 5 overlapping neutralization epitopes (121,151,210). One epitope is immunodominant and critical for virus
neutralization (121,192,210). Further analyses have determined the presence of homotypic and heterotypic neutralization determinants (42,135,194). Nucleic acid sequence analysis of the VP7 coding gene of rotavirus mutants resistant to neutralization with monoclonal antibodies identified three amino acid regions associated with neutralization (51). Comparative analyses of the deduced amino acid sequences of different G serotypes of animal and human rotaviruses confirmed the presence of these three regions and identified three additional regions of clustered sequence divergence (85,87,96). The six regions (designated A to F and encompassing nucleotides 39 to 50, 87 to 101, 120 to 130, 143-152, 208 to 221, and 233 to 242) were highly conserved among rotaviruses of the same G serotype and were variable among rotaviruses of different G serotypes (96). The serotype of rotaviruses of different G serotype specificities was subsequently shown to be predictable based on comparisons of the nucleic acid sequence of regions B and E of the VP7 coding gene (97).

A second viral protein associated with the induction of neutralizing antibodies is VP4, a protease-sensitive outer capsid protein that determines the P serotype specificity (55,99,167,205). Topographical analyses of the epitopes on VP4 have identified antigenic sites associated with P serotype-specific and heterotypic neutralization responses (136,217). Epitopes involved in cross-reactive neutralization responses have been located in VP5, the 60,000 MW polypeptide resulting from the cleavage of VP4 with trypsin (136,217). The serotype-specific epitopes are located in VP8, the smaller 28,000 MW cleavage product of trypsin (120,136,217). Nucleic acid sequence comparisons of the gene 4 segments of human rotaviruses of different P serotypes confirmed the serological studies and identified regions of greatest variability or conservation between amino acids 106 to 192 and 246 to 538, respectively (55,88,119).

Group A porcine rotaviruses have recently been characterized according to their VP4 or P specificities. The Gottfried (serotype G4) porcine rotavirus VP4 protein is more closely related serologically to the VP4 of human rotaviruses than to animal rotaviruses (90,115). In
addition, nucleic acid and amino acid homology studies have shown the Gottfried VP4 protein to be more closely related to "asymptomatic" human rotavirus strains of serotypes 1 to 4 (M37, 1076, McN13, and ST3) than to these same serotypes of "symptomatic" human rotavirus strains (Wa, DS-1, P, and VA70) (90). This high degree of relatedness suggests a common evolutionary ancestor between the Gottfried and human rotaviruses (89,90,115).

Serological comparisons of the OSU (serotype G5) porcine rotavirus VP4 with rotaviruses of human and animal origin were reported in a study by Liprandi et al. (132). In this study, one-way serum neutralization assays with hyperimmune antiserum against recombinant baculovirus-expressed OSU VP4 distinguished at least 2 VP4 serotypes among porcine rotavirus strains of serotypes G3, 4, 5, and 11. The criterion for distinction of VP4 serotypes in the study was based on serum neutralization titer differences of eightfold or more. Porcine rotaviruses designated porcine VP4 serotype 1 included the porcine strains OSU, ISU-64, ISU-65, YM, and several other strains isolated in the U.S., Venezuela, and Argentina. The Gottfried rotavirus was classified as porcine VP4 serotype 2. The antigenic variability of porcine rotavirus VP4s was also investigated with a panel of eight OSU VP4-specific neutralizing MAbs (131). Analysis of 20 strains of porcine rotavirus by an immunofluorescent assay identified 5 groups (or monotypes) of porcine rotavirus that differed in their reactivity to one or more of the monoclonal antibodies. Four monotypes were identified among porcine rotaviruses of VP4 serotype 1. The fifth monotype, characterized by viruses that failed to react with any of the eight monoclonal antibodies, included the Gottfried rotavirus (serotype G4) and two strains of rotavirus from Venezuela (both serotype G5). MAbs produced against the OSU VP4 were specific for porcine rotaviruses and failed to neutralize selected human, bovine, and simian rotavirus strains.

Serotypic relationships of the Gottfried and OSU porcine rotavirus VP4s with the VP4s of serotype G4 and 5 porcine rotaviruses, respectively, isolated in the United States (23,104) have been determined by nucleic acid hybridization studies (184). The analyses were
performed using partial-length PCR-derived gene 4 probes encompassing serotype-specific coding regions of genomic segment 4. The P serotype specificities of the serotype G4 SB2, SB3, and SB5 porcine rotavirus strains were similar to the Gottfried rotavirus. Analysis of the serotype G5 EE and A580 strains of porcine rotaviruses revealed P serotype specificities similar to the OSU rotavirus. Serotypic relationships of the Gottfried and OSU VP4s were also determined with selected rotavirus strains of human and animal (nonporcine) origin. The OSU gene 4 probe hybridized with the equine H1 rotavirus indicating shared P serotypic specificities. Previous analyses of the OSU and equine H1 rotaviruses with OSU gene 9 probes and hyperimmune antisera (104,186) indicated shared P and G serotype specificities between these two strains of animal rotaviruses. The specific hybridization of the Gottfried gene 4 probe with "asymptomatic" human rotaviruses, as opposed to "symptomatic" human rotaviruses, confirmed previous serological and nucleic acid and amino acid homology studies (88,90).

Two strains of porcine rotavirus (SB-1A and MDR-13) have been isolated that exhibit serological specificities of two different rotaviruses. In each case the underlying genetic factor was different. The SB-1A porcine rotavirus was originally isolated by Bohl and Saif (unpublished) and further characterized by Hoshino et al., (104). This virus exhibited serotype specificities characteristic of Gottfried and OSU when analyzed by a plaque reduction-neutralization assay using hyperimmune antisera. The genetic basis of the two serotype specificities was revealed by serological and nucleic acid hybridization studies by Hoshino et al. (103) and Midthun et al. (149) with reassortant viruses (103,149). In these studies the SB-1A rotavirus was found to be a naturally occurring reassortant that possessed a genomic segment 9 (coding for VP7) similar to the Gottfried porcine rotavirus, and a genomic segment 4 (coding for VP4) similar to the OSU porcine rotavirus. Rotavirus strains possessing dual serotype specificities analogous to SB-1A have also been observed in another porcine rotavirus isolate and in a human rotavirus strain (103,149,155). In contrast to the
SB-1A porcine rotavirus strain, the serotype G3 and 5 serological specificity of the MDR-13 porcine rotavirus was due strictly to the VP7 viral protein (157). Genomic sequence analysis of MDR-13 revealed similar amino acid homology between serotype G3 and 5 porcine rotaviruses in the VP7 antigenic regions A, B, and C (157). Previous analysis of rotavirus variants by Dyall-Smith et al. (51) have associated the A, B, and C regions of the VP7 genomic segment with the serotype specific neutralization sites (51).

The subgroup specificity of rotaviruses is defined by an antigenic determinant(s) located on VP6, and has been used as an epidemiologic marker to distinguish the antigenic properties of different strains of rotavirus (2,98,113,116,224). The subgroup specificities of rotaviruses have, in general, segregated with human and porcine rotaviruses of specific serotypes or RNA electrophoretic patterns (2,79,102,104,112,116). Most human rotaviruses of serotypes G2 and 8, and many porcine rotaviruses including serotype G3, 5, and 11 are subgroup 1 (79,104,113,116,153,177,190). Human rotaviruses with subgroup 1 specificity are generally characterized with "short" RNA electrophoretic patterns, in that gene segments 10 and 11 migrate slower by polyacrylamide gel electrophoresis (PAGE) relative to the "long" electrophoretic patterns characteristic of subgroup 2 rotaviruses. Subgroup 2 rotaviruses include human rotaviruses of serotypes G1, 3, 4, and 9, and the G4 porcine rotaviruses Gottfried and SB-1A (80,104,116). Human rotavirus strains that do not conform with the general association of subgroup 1 and 2 specificities and RNA electrophoretic patterns have been reported (79,81,83). A serotype G4 porcine rotavirus with subgroup 1 specificity has also been detected (104). Several studies have implicated the existence of other subgroup specificities among human and porcine rotaviruses (81,140,213,229,236).

Subgroup specificity has primarily been associated with the major inner capsid protein VP6 (98,113). Studies with MAbs produced against human and porcine rotaviruses, however, have revealed another subgroup-like epitope on the inner capsid protein VP2 (214,219). The antigenic reactivity patterns of VP2-specific MAbs with human rotaviruses correlated almost
identically with human rotavirus strains bearing subgroup 2-specific epitopes on VP6 (219). However, studies of porcine rotaviruses revealed independent segregation of VP2 and VP6 subgroup-specific antigens (214). The subgroup-like epitopes on VP2 proteins of human and porcine rotaviruses have not been detected in other animal rotavirus strains (214).

**PATHOGENESIS AND PATHOGENICITY**

The pathogenesis of porcine rotaviruses has been extensively studied in conventional, colostrum-deprived, and gnotobiotic pigs (43,124,145,161,222). The virus causes acute diarrhea and other clinical symptoms including depression, anorexia, weight loss, dehydration, and occasionally vomiting (126,145,222,245). Transmission of the virus is by the fecal-oral route. Historically the disease caused by porcine rotavirus resembles syndromes previously referred to as milk scours, white scours, colibacillosis, serum-modified TGE, l'enterite colibacillaire de la troisieme semaine, Drei-Wochen-Durchfall, nutritional scours, feed scours, 3-week scours, 3-week-enteritis, and weaning diarrhea (21,122,124).

Rotaviral diarrhea has been observed in nursing piglets less than 1 week to 5 weeks of age and in pigs weaned at 3 to 8 weeks of age (21,126,145). The disease in weaned pigs often occurs within 3 to 5 days following weaning (21,101,124,245). Reports on mortality are variable, with ranges of 7 to 20% in nursing pigs and 3 to 50% in weaned pigs (21,241). Infected pigs generally recover within 1-10 days (21,74,101,124,245). Although rotavirus is widespread among pig herds, many rotavirus infections are subclinical (21,74,101,241).

Electron and immunofluorescent microscopy has shown that replication of porcine rotavirus occurs in the villous epithelium of the small intestine (43,126,161,199,222). In general, clinical signs correlate with the detection of rotavirus in villous epithelium and the appearance of pathological lesions of the mucosa (43,161,222). Diarrhea, however, often precedes gross histopathological lesions (142,222).
Histopathologic studies have shown disruption and desquamation of villous epithelium due to virus replication and lysis. This results in villous shortening and fusion, and lengthening of the crypts (39,43,142,173,222). The distribution of damaged villi throughout the small intestine is variable. In some cases transverse sections of intestinal sections contain atrophied villi on one side and normal villi in adjacent sections (142,173). In all studies the ileum and jejunum sustained the most villus damage (43,142,173,222,233).

Damaged and lysed epithelial cells result in the release of large amounts of virus into the feces of infected animals (43,222). The damaged epithelium is replaced by flat to cuboidal shaped cells with uneven or incomplete microvillar borders (142,161,173,222). The immature cuboidal cells from the crypts lack the membrane-bound lactase and other enzymes characteristic of differentiated mature epithelial cells (147). As a result, an osmotic diarrhea due to carbohydrate malabsorption develops (94,246).

The reduction of clinical signs and recovery of infected pigs correlate with the regeneration of the villi and a decrease in rotavirus antigen in villous epithelium as determined by immunofluorescent microscopy (43,142,222). In some cases recovery from rotavirus infections was biphasic, with the reoccurrence of rotavirus antigen in the tips of the villi (40,43). The secondary round of virus replication in the villi was accompanied by an increase in virus titer in the intestinal lumen (43).

Although the tissue tropism of porcine rotavirus is primarily the villous epithelium in the small intestine, several studies have detected rotavirus particles or antigens in other tissues. Studies by Theil et al. (222) detected rotavirus antigen by immunofluorescence in the epithelial cells of the colon of 2 gnotobiotic pigs at 24 hours post infection (222). The rotavirus infection in the colon did not cause histopathologic changes and was transitory, since it was not detected later during the course of the disease. Indications of rotavirus infection and replication in dome M cells was reported during a study of M cell function in pigs (33). The piglet used in the study was asymptomatically infected with a group A porcine
rotavirus and a porcine enteric adenovirus. Villi adjacent to domes showed characteristic rotavirus lesions that included degenerate and necrotic cells and scattered desquamation of villi tips. Rotaviruses were also detected between luminal surface projections of M cells and in lymphocytes and mononuclear leukocytes in central hollow regions.

Extramucosal infections by porcine rotavirus have been reported in pigs by one research group (108,109,204). In their studies, colostrum-deprived or colostrum-fed pigs were infected per os with cell culture propagated porcine rotavirus, enterovirus, or both enterovirus and rotavirus (108,109,204). Porcine rotavirus was detected in sample preparations collected from lung, spleen, brain, and from the small intestine of both mono- and dual-infected pigs as determined by immunofluorescent infectivity assays (109,204). Histopathological lesions, however, were not detected in any of the extramucosal locations (108). The extramucosal spread of rotavirus has been reported in other animal species. In mice, rotavirus was detected in the liver following oral infection with a heterologous animal rotavirus (234). Rotavirus was also detected in the lung, spleen, brain, and other organs of mice in an investigation of the virus responsible for epidemic diarrhea of infant mice (117). In another study, a rotavirus viremia was reported following inoculation of a one-day old colostrum-deprived calf reported to be prostrated from post-operative shock (118). Studies concerning the extramucosal detection of rotavirus in pigs and other animal species are limited, and are exceptions rather than the rule.

Porcine rotaviruses, like most animal and human rotaviruses, are primarily species specific. Limited studies of experimental cross-species infections have demonstrated the establishment of a subclinical infection in a gnotobiotic lamb after one passage with a porcine rotavirus isolate (232). Another porcine rotavirus isolate, however, failed to infect a gnotobiotic lamb, and both porcine isolates failed to infect gnotobiotic calves after one passage (232). Of all the experimental animals that have been used for cross-species infections, the natural host of porcine rotaviruses, the pig, has been the most universal for
establishing infections or disease with rotaviruses of human and animal origin (29,44,77,148, 228,232,243,247).

Low infective doses can establish rotavirus infections or clinical disease in pigs. This was demonstrated in numerous studies of the pathogenesis of porcine rotavirus in that the administration of 0.5 to 1 ml volumes of 10 to 33% fecal or intestinal suspensions of rotavirus to piglets were sufficient for establishing rotavirus infections or disease (20,145,173,199,245). In some cases 2 ml volumes of 10% suspensions of fecal material from rotavirus-negative specimens (determined by electron microscopy) established rotaviral diarrhea in susceptible pigs (231). The administration of virus preparations adjusted to contain 100 to 1000 fluorescent forming units of virus have also been shown to cause piglet death (233).

The virulence of porcine rotaviruses and the pathological damage they cause in susceptible hosts can be attenuated by passage of rotaviruses in cell culture. In general, porcine rotaviruses passaged repeatedly in cell culture often produce less severe clinical symptoms in piglets (23,93,204), although exceptions occur (204). Comparative studies of attenuated porcine rotaviruses with virulent parental strains have shown reduced villous damage and the retention of normal lactase activity in piglets infected with attenuated strains (233). The decreased pathological damage associated with attenuated porcine rotavirus has been associated with reduced rates of infection of enterocytes along the length of the small intestine over time (233). Although porcine rotavirus strains attenuated by high cell culture passage may show reduced clinical disease upon initial infection of piglets, restoration of full virulence may occur upon repeated animal passage (93).

**DIAGNOSIS**

A variety of assays and techniques are currently available for the diagnosis of porcine rotavirus infections. These assays may be broadly classified into the categories of direct particle visualization, serology, and nucleic acid studies. The direct visualization of rotavirus
by electron microscopy played an important role in the early studies of porcine rotavirus and is used in many research laboratories today (172,177,182,197,245). Electron microscopy allows the detection of other viral agents in clinical samples, and can be modified with immunological reagents to increase the specificity and sensitivity of the technique (28,82,163,197,198). Although electron microscopy remains an important method for diagnosis, often serving as the "gold standard," its use for screening large numbers of samples is cumbersome. Several new and sensitive diagnostic assays are now available (48,50,130,188).

Immunofluorescence assays based on rotavirus infectivity are another method that were used in initial investigations of porcine rotavirus infections (3,4). These assays are based on the use of fluorescein isothiocyanate-labeled antibodies to detect rotavirus antigens in infected intestinal sections or cell monolayers inoculated with suspensions from clinical samples (23,28,153,199,221,222,245). The advantages of these assays in comparison to other cell culture based assays are that adaptation of rotaviruses to serial propagation in cell culture is not required, and infectious virus can be detected and quantitated (8,32,75,233). Modifications of these assays using polyclonal and monoclonal antibodies have enabled the development of fluorescent focus neutralization tests for the differentiation of rotavirus serotypes (16,30,51,131). The immunofluorescence technique is also used to study the pathogenesis of porcine rotavirus infections by detecting rotavirus in intestinal sections or smear preparations, and to determine the serotypes of rotavirus isolates in fluorescent focus reduction tests (21,75,115,157,172).

One of the most widespread serological assays for the detection of rotaviruses, applicable for analysis of large numbers of samples, is the enzyme-linked immunosorbent assay (ELISA) (52,84,177,220). Modifications of the antibody reagents used in the ELISA has enabled the distinction of porcine rotavirus subgroups and serotypes (116,130,131,140,154,214). Monoclonal antibodies have been particularly useful in overcoming cross-reactivity
problems associated with the use of polyclonal serum (227). Difficulties, however, have been encountered because of the extreme specificity of monoclonal antibodies, and the occurrence of genetic variability among rotaviruses of the same serotype (19,41,95,217). Efforts to overcome these problems may require the use of a panel of pooled monoclonal antibodies against each serotype (95).

Although serological assays enable the detection and antigenic characterization of porcine rotavirus strains, they often cannot discriminate between specific rotavirus isolates recovered from the same host species. This information may be obtained by examining the patterns of the 11 segments of rotavirus double-stranded RNA (dsRNA) following their electrophoretic separation and staining in polyacrylamide gels (225). The electropherotypes observed by the migration of the dsRNA are often characteristic for different isolates and may be used to determine the circulation of particular rotaviruses in disease outbreaks (37,57,101,181,193). This generalization, however, may not be true for all rotavirus isolates since the migration of dsRNA segments by PAGE does not directly correlate with molecular weight (57). Consequently dsRNA segments of different rotaviruses with similar mobilities may differ in their nucleotide sequence and code for structural proteins with different antigenic specificities (10,57,71). Electrophoresis has been particularly useful for the identification of genetic anomalies (14,141), a potential indicator of human rotavirus subgroup specificity (79,112), and the detection or differentiation of group A and non-group A porcine rotaviruses (22,36,110,174,226), mixed infections (193), and rotaviruses from different animal species that exhibit distinct electropherotypes (57).

Another nucleic-acid based assay for the detection and characterization of rotaviruses involves the reverse transcription of rotavirus RNA followed by a two-step amplification procedure using conserved or serotype-specific oligonucleotide primers and the polymerase chain reaction (PCR) (78,91,160). In this procedure, the published nucleic acid sequences of rotavirus genes coding for VP4 and VP7 were used to produce oligonucleotide primers specific
for several G and P serotypes of rotaviruses. Analysis of the molecular size of the amplified gene segments produced using different primer pairs enabled the differentiation of the G or P serotype specificities of rotaviruses amplified from stool samples. Comparisons of the molecular typing procedures with conventional serologic methods resulted in complete correlation. The PCR-based assays developed provided an alternative or complementary procedure for serological studies with monoclonal antibodies.

A third procedure for the detection or differentiation of rotavirus serotypes based on rotavirus RNA analyses involves the use of nucleic acid probes. This information will be presented in a subsequent section on nucleic acid probes for rotavirus detection.

DETECTION AND CHARACTERIZATION WITH NUCLEIC ACID PROBES

The development and use of nucleic acid probes for the analysis of rotaviruses opened a new era in rotavirus research and provided alternative reagents to assays based on serological analyses. Two of the earliest studies that described the use of probes for the detection of rotavirus RNA in clinical samples were reported by Flores et al., (72) and Pedley and McCrae (176). The source, type of nucleic acid, and selection of rotavirus gene segments used for the production of probes differed in each investigation.

In studies by Flores et al. (72), RNA probes were prepared by in vitro transcription of the 11 genome segments from purified single-shelled human, bovine, and porcine rotavirus particles. All of the probes detected rotavirus RNA extracted from human stool samples by dot hybridizations. Differences in the hybridization signals, however, resulted in the need for longer exposures of the autoradiographs with some probes in order to obtain equivalent hybridization signals. The RNA probe-based assay was reported to be 10 to 100 times more sensitive (maximum sensitivity was 8 pg of homologous RNA) for rotavirus detection than an ELISA. In studies by Pedley and McCrae (176), DNA probes were prepared by nick translation of plasmids containing individually cloned bovine rotavirus gene segments. Dot
hybridizations of the single gene-specific probes with bovine field isolates and laboratory
generated reassortants resulted in hybridization signals of variable intensities. Although the
gene-specific probes were useful in the detection of field isolates of bovine rotavirus, the
investigators in this study pursued the development of the probe-based assay as a method for
the detection of RNA segment reassortment in rotavirus-infected cell culture and field
sample preparations.

Further developments in the use of nucleic acid probes for the diagnosis of rotavirus in
clinical samples centered on the use of probes prepared from individual gene segments
(50,128,129). The probes were used singly or in conjunction with other gene-specific
segments. Initial investigations by Lin et al. (128) with a mixture of probes prepared from
the Wa human rotavirus indicated nucleic acid hybridization was a sensitive and specific
assay that compared favorably with other rotavirus detection techniques. During the course
of their investigation, differences in the intensity of hybridization signals were noted between
homologous reactions with the Wa rotavirus (G1, P8) and the heterologous bovine rotavirus
NCDV (Nebraska calf diarrhea virus) (G6, P1). Hybridizations were subsequently conducted
using probes prepared from individual Wa rotavirus gene segments. Nucleic acid probes
prepared from gene segments encoding the ribonucleoprotein viral core (segments 1, 2, 3, and
6) and the nonstructural proteins (gene segments 5, 10, and 11) were equivalent in their
ability to detect human rotavirus clinical isolates that possessed RNA electrophoretic
patterns similar to the Wa rotavirus. In contrast, probes prepared from gene segments 4 and
9 (coding for the immunologically relevant outer capsid proteins VP4 and VP7, respectively)
produced variable hybridization signals. Further studies with probes prepared from gene
segments 4, 9, and 6 (the latter coding for the inner capsid protein VP6 that contains the
common serogroup and subgroup antigenic determinants) with rotaviruses of human and
animal origin demonstrated the ability of the respective probes to differentiate rotaviruses
according to their antigenically defined serotype and subgroup specificities (128,129).
The serotyping and subgrouping capabilities of probes prepared from gene segments 9 and 6 were confirmed in similar studies by Dimitrov et al. (50). In this study, many of the parameters affecting probe specificity were investigated in carefully designed experiments. One parameter investigated involved comparisons of the binding capacity of filters used for the immobilization of rotavirus RNA. The higher binding capacity of nylon membranes compared to nitrocellulose resulted in the use of nylon membranes in this study and many subsequent hybridization studies on rotaviruses (13,73,119,170,185). Another set of parameters involved the correlation between the serotype and subgroup specificities of rotavirus-specific probes with the stringency conditions used in the hybridizations. In general, high temperatures (52 to 65°C) and the addition of formamide (7 to 13%) to hybridization solutions increased the serotype or subgroup specificities of the probes.

Refinements in the stringency conditions necessary for the differentiation of rotaviruses with VP7-specific probes led to the development of several hybridization protocols for the serotypic differentiation of human and porcine rotaviruses (66,111,185,186,248). In these studies, full- or partial-length gene 9 DNA probes were prepared directly by reverse transcription of newly synthesized mRNA (66,248), or indirectly by nick translation or random primer extension of cloned gene 9 cDNA segments (111,185,186). Hybridization reactions of the probes with previously characterized rotavirus strains of different G serotype specificities resulted in the specific detection of rotaviruses that were serologically related to the strain from which the probes were derived. The broad serotyping capabilities of the probes were particularly evident in hybridization reactions with porcine rotavirus gene 9 probes (186). In this study, probes prepared from the Gottfried (G4) and OSU (G5) porcine rotavirus strains produced hybridization signals with human or animal (nonporcine) rotaviruses of homologous G serotype specificities. The specificities of the human rotavirus gene 9 probe were demonstrated in investigations of rotavirus-positive clinical samples. The
results of the dot hybridizations were similar to those obtained with a monoclonal antibody serotyping assay (66) or with RNA PAGE analyses and serological results (248).

Hybridization studies pertaining to the use of rotavirus-specific probes for the differentiation of rotavirus serotypes preceded sequencing studies on the gene segments coding for VP4 and VP7. The identification of the variable regions of the genes that coded for the neutralization determinants (55,85,87,88,96,119) provided both an explanation for the hybridization reactivities that had been observed, and information that facilitated the production and preparation of serotype specific nucleic acid probes.

One method for the preparation of large quantities of specific cDNA segments involved amplification by the polymerase chain reaction (PCR). This procedure was pioneered in DNA amplification studies on the b-globin gene of human beings (152,200). In this procedure, a DNA template was incubated in the presence of a mixture of a large molar excess of specific oligonucleotide primers and the four deoxyribonucleoside triphosphates. The primers were complementary to different DNA strands of the template and bordered the region of the double-stranded DNA to be amplified. One cycle of the reaction consisted of exposure of the DNA template mixture to three different temperatures to promote template denaturation, annealing or hybridization of the oligonucleotide primers to the template, and extension of the primers by the addition and action of a DNA polymerase. Consecutive repetitions of the cycle resulted in multifold increases in the target DNA sequence.

The application of the PCR procedure to the production of serotype-specific partial-length VP4- and VP7-specific probes was reported in studies on human, bovine, and porcine rotaviruses (73,119,169,170,184,187). The specific length and sequence of the probes differed in individual studies. In general, however, the VP7-specific probes encompassed three of six variable regions (A, B, and C) as defined by Green et al. (1987), and the VP4-specific probes encompassed variable sequences (nucleotides 211 to 612) near the 5' end of gene segment 4 (55,120,135,189). The specificity of the VP7-specific probes for rotaviruses of homologous
serotypes were demonstrated in hybridization reactions with heterologous serotypes of human, bovine, and porcine rotaviruses (73,187). The porcine VP7-specific probes were also determined to be specific in hybridization reactions against homologous and heterologous serotypes of rotaviruses from human beings and other animal (nonporcine) species (187). Similar hybridization studies with the human, bovine, and porcine VP4-specific probes confirmed the ability of these probes to detect rotaviruses with homologous P serotype specificities (119,170,184).

Hybridization procedures for the detection of rotaviruses with nucleic acid probes have traditionally relied on the use of radioactive isotopes for probe labeling. Several alternative labeling and detection methodologies have recently been introduced and have been applied in studies on rotaviruses. One method, introduced by a commercial probe manufacturer (SNAP probes; Molecular Biosystems, Inc., San Diego, Calif.), involved the production of a 26 base pair synthetic oligodeoxyribonucleotide probe (SNAP) labeled with alkaline phosphatase (5,168). The alkaline phosphatase was conjugated to the probes through a 21-carbon spacer arm at the C-5 position of a thymidine base (168). The hybridization time of the SNAP probes was 15 minutes, and was considerably shorter than the 16 to 48 hour hybridization times with traditional radioactive labeled probes (50,66,129). The hybridization signals were detected by a colored precipitate that formed after incubation of the hybridized blots with the substrates Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Two research groups reported the use of SNAP probes for the detection of group A rotaviruses in human clinical samples (5,168). The sensitivity of the probes were reported to be equivalent to rotavirus analysis by PAGE and several commercial ELISA-based assays. Direct comparisons were not performed, however, with radioactive isotope-labeled probes.

Another alternative probe labeling and detection procedure involves the use of biotinylated nucleic acid probes. Two research groups have reported on the use of biotinylated probes for the detection of group A rotaviruses; however, the composition,
length, and source of the probes differed in each study (13,60). In one study, a 40 base pair synthetic oligodeoxynucleotide was produced that corresponded to conserved nucleotides (nucleotides 33 to 72) of the VP7 coding gene of the bovine rotavirus strain NCDV (60). The probe was labeled at the 3' end with biotin-7-dATP. Application of the probes in dot hybridizations of human clinical samples indicated the probes were more sensitive than analyses by PAGE and ELISA. In another study, biotinylated RNA probes were produced by reverse transcription of full length clones of gene segment 6 of the simian rotavirus SA11 (13). Biotinylation was accomplished by the inclusion of biotin-11-UTP in the nucleotide mixture used during reverse transcription. Dot and Northern blot hybridizations of this probe with established human and animal rotavirus strains revealed sensitivities equivalent to probes labeled with $^{32}$P.

**EPIDEMIOLOGY**

Porcine rotaviruses are enzootic in swine herds throughout the world. Prevalence studies based on viral particle or antigen detection have indicated that greater than 60% of swine herds are infected with porcine rotavirus (21,47,130,211,212). Serological studies have indicated infections among herds may be nearly 100% (23,27,61,125).

Although rotavirus shedding has been reported in some litters less than 3 days old (46), the incidence of rotavirus infections in nursing pigs less than one week old is generally low (74,130,211,237). Rotavirus shedding and clinical disease are more often observed initially in 1 to 2-week-old pigs (21,74,130,237). In countries where farm management practices promote the nursing of pigs for 4-6 weeks, the number of rotavirus infections in nursing pigs generally increases with age, with the highest rate of infection in 3 to 5-week-old pigs (74,130,211,237). Nearly all litters of pigs excrete rotavirus in the feces by 5-6 weeks of age (47,74). Although the incidence of rotavirus infection in pigs is high, many infections in nursing pigs are subclinical (46,47,211). Serological data on pigs of various age groups
indicate that rotavirus infections may reach nearly 100% among swine populations (47,211,237,245).

The duration of rotavirus excretion in individual nursing pigs was observed in one study to vary between 1-12 days (74). In this same study several pigs excreted rotavirus intermittently at intervals of between one and eleven days (74). Intermittent excretion of rotavirus by infected pigs and aggregation of rotavirus in immune complexes (40) may account for the failure of some researchers to isolate rotavirus concurrently from animals with clinical disease (46,47). In another study, rotavirus excretion by nursing pigs was observed for periods of from 1-3 weeks, with re-excretion of rotavirus in the feces at 3-4 weeks following the prior episode (46,47). Rotavirus shedding by weaned pigs may also follow a previous episode of rotavirus shedding during the nursing period (74,101). Although rotavirus shedding in sows has not been detected in studies using ELISA (46,47,74), rotavirus has been detected by the concentration of fecal samples and detection of virus by immunofluorescence in cell culture (16). Rotavirus excretion may occur in sows seropositive for rotavirus and has been detected in the feces of sows 5 days prior to and 2 weeks after farrowing (16). Further indirect evidence for rotavirus shedding in sows was reported in studies by Lecce et al. (122,123,125), in that pigs farrowed in sanitary environments and with initial contacts limited to the sow were shown to develop clinical disease and shed rotavirus (122,123,125).

Several studies have demonstrated a higher rate of rotavirus infections in pigs born to gilts than to sows (7,74,211). The prevalence rate of rotavirus infections during the first week of life for litters from gilts compared to sows was shown in one study to be 37.5% and 3.0%, respectively (211). Litters from gilts have also been shown to excrete virus for longer periods of time and to suffer more severe diarrhea (74). The higher rates of infection in gilt litters has been proposed to be due to lower levels of colostral antibody (211). In addition,
observations on the general conditions of the animals have indicated gilts were dirtier than sows particularly on their abdomens and udders (74).

The incidence of porcine rotavirus infections in reference to changes in climatic conditions was reported in a study by Utrera et al. (237) during epidemiological investigations in Venezuela. Temperature was not an important factor in this study since the tropical weather conditions in this country remained relatively constant throughout the year. Two sharp fluctuations were noted, however, during comparisons with other climatic variables. These involved a decrease in porcine rotavirus infections during the tropical rainy season and an increase in rotavirus infections during periods of low rainfall and humidity. The increase in porcine rotavirus infections noted during periods of low humidity were correlated with results of studies in human beings (25,171,237). In both situations low relative humidity was suggested to promote an increase in aerosol formation, particularly in reference to virus laden dust from fecally contaminated surfaces, that could result in increased spread of the infection to a greater number of susceptible individuals (25,237).

The incidence of porcine rotavirus infections in comparison to other enteropathogenic agents has been the subject of several investigations. In one study in the U.S., the enteropathogens rotavirus, E. coli, transmissible gastroenteritis virus, Isospora suis, and Clostridium perfringens, isolated alone or in combination in 148 and 24 cases of preweaning and postweaning diarrhea, respectively, were identified (62). Rotavirus (which included rotavirus serogroups A, B, and C) was the most frequently detected pathogen as determined by PAGE and silver staining (62). Rotavirus was detected in 33% and 38% (alone) and 46% and 77% (in combination with other agents) of the total cases of preweaning and postweaning diarrhea, respectively (62). In a similar study of neonatal diarrhea of pigs in Canada involving many of the same pathogens, group A rotavirus was detected in 8% of 749 diarrheic pigs and 9.2% of 325 outbreaks of diarrhea, and was one of the least frequently detected agents (150). Rotavirus detection in the Canadian study was performed by electron
microscopy and fluorescent antibody staining of small intestine sections, however, and did not detect non-group A rotaviruses (62,150). In another study in Sweden, the incidence of rotavirus and *Isospora suis* infections in litters of pigs 3-27 days old and characterized with steatorrhoea was investigated (164). Both agents were detected in 15% of 41 litters and 24% of 17 selected herds with problems of steatorrhoea (164). Coinfections with both agents occurred in only one sample, and neither pathogen was detected in herds without problems of steatorrhoea (164).

The characterization of porcine rotavirus VP6-specific subgroup antigenic specificities, and the geographical distribution of porcine rotavirus subgroups, have been reported in several studies. In North America, analysis of 56 fecal and intestinal samples collected from diarrheic pigs sampled in the midcentral U.S. and Canada identified 41% as subgroup 1 and 25% as subgroup 2, with 34% of the samples not classifiable (116). The geographical distribution of subgroup 1 and 2 porcine rotaviruses, respectively, was 60% and 40% from Ohio, 63% and 37% from other midwestern states, and 67% and 33% from Canada (116). The higher prevalence of porcine rotaviruses with subgroup 1 antigenic specificity noted in North America has also been observed in other countries, although in all studies the number of samples examined were limited. In Thailand, subgroup analysis of 13 porcine samples positive for group A rotavirus identified all specimens as subgroup 1 (177). Seven of the 13 subgroup 1 porcine samples were also characterized as serotype 3 rotaviruses using monoclonal antibodies produced against human rotaviruses (177). The subgroup specificity of 28 group A rotavirus-positive samples collected from pig herds in one region of Venezuela identified 26 specimens as subgroup 1 and two as subgroup 2 (130). In another study conducted in Argentina, subgroup analysis of 32 group A rotavirus positive samples identified 13 specimens as subgroup 1 and two specimens as subgroup 2 (140). Of the remaining 17 samples, 2 specimens possessed both subgroup 1 and 2 specificities, and 15 specimens were neither subgroup 1 nor 2 (140).
In contrast to the epidemiology studies reported on porcine rotavirus subgroups, limited studies have been conducted on the distribution of porcine rotavirus serotypes. Where possible, information concerning the epidemiology of porcine rotavirus serotypes has been presented in the section on porcine rotavirus antigenic relationships.

Initial studies of human and animal rotaviruses indicated that under natural conditions rotaviruses are primarily species specific (59). Several studies, however, have recently presented evidence of cross-species infections (158,159,235). In one study, genetic evidence of cross-species infections of human beings with a rotavirus of porcine or bovine origin was reported in a serological survey of rotaviruses isolated from stool samples from patients in Chiang Mai, Thailand (235). In this study, three human rotavirus isolates with unusual RNA electrophoretic patterns and subgroup specificities that were characteristic of animal rotaviruses were identified. Analysis of the genomic relatedness of two of the three human rotavirus isolates by Northern blot hybridizations revealed a greater degree of homology with porcine rotaviruses than with rotaviruses of human or nonporcine animal species. Analysis of the third human rotavirus isolate revealed serological and genomic similarities with rotaviruses of bovine origin. Genetic evidence of cross-species infections of human beings with rotaviruses of feline origin have also been reported (158,159).

**PREVENTION AND CONTROL**

Efforts to increase the protection provided by lactogenic immunity and to stimulate active immunity in pigs prior to weaning, has led to the development and marketing of two porcine rotavirus vaccines in the U.S. (239). One of the federally licensed vaccines is a killed vaccine while the other is a modified live virus (64,239,240). Investigations of the efficacy of the vaccines by one commercial company showed little protection offered by killed rotavirus vaccines administered intraperitoneally or by oral and intramuscular routes in limited studies with colostrum-deprived pigs 5 to 7 days old (239). Although studies with the
modified live vaccine showed increased protection of nursing and weaned pigs under experimental conditions (64,239), its efficacy in field situations has been questioned by research groups (101,240). In all of the commercial studies, efforts have concentrated on the protection of piglets against porcine rotaviruses of G serotypes 4 and 5. The isolation of porcine rotaviruses with other antigenic specificities as previously noted may partially account for the variability in protection observed with the vaccines in field trials. Antigenic variability, however, was not an factor in one vaccine study in which the field strain of porcine rotavirus isolated was the same serotype as the vaccine strain (101).

The incidence and severity of clinical disease caused by porcine rotavirus may be influenced by management procedures on farms. One management procedure involves the use of an all-in, all-out system for both farrowing units and nurseries. This system allows time for the cleaning and fumigation of rooms between litters and prevents the build-up of rotavirus and other enteropathogenic agents in the environment that is characteristic of continuous use facilities, which may increase the incidence of diarrheal disease (18,122,125). Production systems used in Scandinavian countries with more traditional farrowing pens, with loose sows on concrete floors with straw, were shown in one epidemiologic study to have lower prevalence rates of rotavirus infection in suckling pigs than modern restrictive farrowing pens, with crated sows and wire mesh or slotted floors (211). The lower incidence of infection in traditional farrowing pens was postulated to be due to the greater access of sows to fecal material than crated sows. This could result in repeated subclinical (booster) infections of sows with may prolong the protective levels of milk antibodies (211).

Rearing regimens involving the weaning of pigs within one week of birth pose problems due to the high incidence of rotavirus infection and diarrhea (9,125,162). Early weaned pigs lack the protection normally provided by lactogenic immunity, and their survival may be jeopardized unless reared in specially designed nurseries where sanitation is rigorously maintained (125).
Diet may play an important role in the control of diarrheal disease in weaned pigs. Animals restricted in the quantity of food fed on an hourly or daily basis during the first few days after weaning may have a decreased incidence of rotaviral disease (123,100). Similar findings were shown in a study that limited the percentage of solids in feed mixtures fed at specified time intervals to newly weaned pigs (123).

Oral rehydration therapy has been used successfully for the treatment of dehydration caused by *E. coli* and rotavirus infections in pigs and calves (34,208). Fluid replacement therapy using isotonic electrolyte solutions containing glucose and glycine are particularly effective (34,208). Experimental studies with rotavirus and *E. coli*-infected gnotobiotic and colostrum-deprived pigs showed rehydration therapy was effective in reducing mortality and weight loss associated with diarrhea (34,208). Rehydration studies in conventionally raised pigs have showed reduced mortality in weaned pigs, and increased weight gains in pigs with post-weaning diarrheic episodes lasting longer than 3 days (34,208). However, the practicality of this procedure for treating large numbers of pigs is questionable.

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CHAPTER II

HYBRIDIZATION PROBES FOR THE DETECTION AND DIFFERENTIATION OF TWO SEROTYPES OF PORCINE ROTAVIRUS

INTRODUCTION

Rotaviruses are intestinal pathogens of virtually all avian and mammalian species including humans. The viral genome consists of 11 segments of double-stranded RNA (dsRNA) and is surrounded by two concentric protein shells. Analysis of gene-product relationships have established the presence of a common or subgroup antigen among group A rotaviruses on VP6, an inner capsid protein, coded by gene segment 6 (12,16). Rotavirus serotypes, defined by neutralization of viral infectivity, have been defined primarily by the outer capsid glycoprotein VP7, coded for by gene segments 8 or 9 (12,16). Additional neutralization epitopes have recently been demonstrated on the outer capsid protein VP4, coded by gene segment 4 (12,21,24).

Because of their importance in diarrheal diseases, a variety of assays have been developed to detect and differentiate rotaviruses. These assays, that are based on our knowledge of the morphological and biochemical make-up of rotaviruses, fall under the general categories of serology, biochemistry, and electron microscopy. Recently a genetic approach involving the use of nucleic acid probes has been reported for the study of rotaviruses. Probes have been used for the characterization of human and animal rotavirus reassortants, as well as the detection of human rotavirus in clinical specimens (6,19,27). By the selection of probes corresponding to gene segments 6 and 9, coding for the immunologically relevant proteins VP6 and VP7, respectively, human rotaviruses have also been distinguished according to subgroup and serotype specificities (5,20).
Studies of various human and animal rotaviruses have revealed at least seven different serotypes (15). Four of the serotypes have been detected in rotaviruses in humans (serotypes 1 to 4) and five serotypes have been detected in rotaviruses in animals (serotypes 3 to 7) (15). Two different rotavirus serotypes have been identified in swine in the U.S., and four potentially new serotypes have been reported in Australia, Mexico, and the U.S. (2,23,26,28). Of the endemic U.S. strains, the Gottfried and OSU rotaviruses have been extensively characterized, and represent the prototype group A porcine rotavirus serotypes 1 and 2, respectively (2). The Gottfried and OSU porcine rotaviruses have additionally been classified as serotypes 4 and 5, respectively, according to the unified serotyping scheme of Hoshino et al. (15).

In this study, a dot hybridization assay was developed for the detection and characterization of porcine rotaviruses in cell culture and intestinal specimens. Radiolabeled probes were prepared from cloned cDNA corresponding to OSU and Gottfried porcine rotavirus gene 9 segments, that code for the major neutralizing glycoprotein VP7.

**MATERIALS AND METHODS**

**Viruses**

Cell culture-adapted rotaviruses used in this study were the OSU (prototype virus), EE (OSU-serotype rotavirus), and Gottfried (prototype virus) (2,30). The tissue culture rotaviruses were grown in monkey kidney (MA104) cells in roller bottles (2). The cells were grown in Eagle minimal essential medium supplemented with 0.5% lactalbumin hydrolysate (LAH), 0.14% NaHCO₃, and 10% fetal bovine serum. The cells were washed with glucose-potassium chloride-sodium chloride (GKN Saline) solution to remove fetal calf serum prior to infection. Viruses were passaged in serum-free cell culture medium containing 0.05% pancreatin (4X NF, 2.5% [10x], GIBCO Laboratories, Grand Island, New York).
Viral RNA extracted from feces or intestinal contents were obtained following passage of virus in gnotobiotic pigs as previously reported (29). Rotavirus negative samples as determined by immunodiagnostic assays were obtained from porcine field specimens. Intestinal viruses used in this study that have been described previously were: OSU prototype virus and the serotype 5 A580 strain; Gottfried prototype virus and the serotype 4 porcine rotavirus strains SB-2, SB-3, and SB-5; the naturally occurring porcine rotavirus reassortant SB-1A (possessing serotype 4 (gene 9) and 5 (gene 4) characteristics); and porcine enteric calicivirus-like virus (2,7,15).

**RNA extraction**

Rotavirus double-stranded RNA (dsRNA) was extracted from intestinal contents by modification of the procedures of Herring et al. (13). Intestinal contents (0.5 g) were diluted 1:6 in 0.1 M Tris-10 mM CaCl₂ buffer. The virus samples were vortexed and then centrifuged at 1200 x g for 30 min. The supernatant was removed, and sodium dodecyl sulfate (SDS) and sodium acetate were added to a concentration of 1.0% and 0.1 M, respectively. The supernatant was extracted with an equal volume of a 3:2 (vol/vol) phenol solution-chloroform mixture. The phenol solution consisted of 26.56 M phenol, 3.23 M m-cresol, and 0.017 M 8 hydroxyquinoline. Following removal of the upper aqueous phase, 4 M sodium acetate and ethanol were added to a final concentration of 0.1 volume and 67%, respectively. The RNA was precipitated overnight at -20°C, and pelleted by centrifugation at 12,000 x g. The RNA was suspended in Tris-NaCl-EDTA buffer (0.01 M Tris, 0.1 M NaCl, 5 mM EDTA, pH 8.0) and frozen at -20°C until used.

Extraction of rotavirus RNA from cell culture virus was performed by clarification of infected cell culture fluids by centrifugation at 430 x g for 20 min. The virus in the resulting supernatant was semi-purified by high speed centrifugation at 122,000 x g for 3 h at 15°C through a 40% sucrose cushion containing 10 mM CaCl₂. The virus pellets were
suspended in 1 ml of 0.1 M sodium acetate buffer (pH 5) containing 1% SDS. The virus suspension was mixed with an equal volume of phenol solution-chloroform mixture, and rotavirus RNA was extracted as mentioned above for fecal virus.

**Nucleic acid quantitation**

The nucleic acid concentration in extracted samples was estimated by absorbance readings at 260 nm in a spectrophotometer using the absorbance values of double-stranded DNA. The nucleic acid concentration calculated represented the total nucleic acid in the extracted samples and did not reflect only rotavirus-specific dsRNA.

**RNA electrophoresis**

Rotavirus RNA in extracted samples was confirmed using electrophoresis with the discontinuous buffer system of Laemmli (17). Rotavirus double-stranded RNA was resolved in 12.5% polyacrylamide separation gels with 4% stacking gels. The RNA bands were visualized using the silver staining method of Herring et al. (13).

**Preparation of 32P-labeled probes**

Full length cDNA copies of gene 9 from OSU and Gottfried porcine rotaviruses were provided by Mario Gorziglia, National Institutes of Health, Bethesda, Maryland (8,9). The HB101 strain of *E. coli* containing the recombinant plasmids was cultivated in 1 liter of ampicillin or tetracycline-containing medium. Plasmids containing cDNA clones of OSU and Gottfried gene 9 were extracted from cleared lysates as previously described (1). The plasmids were purified by centrifugation in cesium chloride-ethidium bromide gradients, and the lower DNA band was collected and dialyzed against Tris-EDTA buffer at 4°C for 72 h. Probes were prepared from plasmids by nick translation (BRL nick translation system, Bethesda Research Laboratories, Gaithersburg, Maryland) using 32P
deoxycytidine-5'-triphosphate (ICN Biomedicals Inc., Irvine, Calif., specific radioactivity 650 Ci/mmole). Probes were also prepared from the OSU and Gottfried gene 9 cDNA that was excised from the plasmids. Rotavirus-specific cDNA was excised from the plasmids following digestion with Pst I or Pst I and Eco RI. The cleavage products were separated on 6% polyacrylamide gels, and the inserts were electroeluted and concentrated by ethanol precipitation. The gene 9 cDNA was suspended in sterile water that was treated with diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Missouri) and probes were prepared by nick translation as described above.

**RNA sample application and hybridization**

Rotavirus RNA was denatured at 100°C in a water bath for 5 min followed by cooling in ice for 5 min. The RNA was dotted onto 0.45 μm nylon membranes (Bio-Rad Laboratories, Richmond, Calif.) in volumes of 5 μl or less with a micropipette. In some cases samples were applied twice in order to apply the desired amount of nucleic acid. The nylon membranes were air dried, baked for 2 h at 80°C in a vacuum oven, and then stored at 4°C until used. Prehybridization was performed in Seal-A-Meal bags (Dazey Corporation, Industrial Airport, Kansas) for 4 h at 42° or 52°C in 10 ml of solution containing: 50% formamide, 5X SSC, 50 mM phosphate buffer pH 6.5, 0.2% SDS, 2X Denhardt's solution, and 100 μg/ml of yeast tRNA. Hybridization was performed in a similar solution that in addition contained 4.5% dextran sulfate and 2 X 10⁶ counts per minute (CPM) of denatured probe (= 1.1 x 10⁷ CPM/ug DNA template). After hybridization at 42° or 52°C for 16-24 h, the membranes were washed 4 times at room temperature in 2X SSC, 0.1% SDS, followed by 2 washes at the temperature of hybridization in 0.4X SSC, 0.1% SDS. The membranes were rinsed in distilled water, blotted until damp, and exposed to Kodak XAR film (Rochester, New York) with intensifying screens at -70°C for 1-4 days.
RESULTS

Optimization of hybridization conditions and sensitivity

Rotavirus RNA preparations extracted from cell culture and intestinal contents were examined using electrophoresis to confirm the presence of rotavirus dsRNA (data not shown). Samples negative for rotavirus RNA (negative controls) and positive for rotavirus RNA were selected for use in the dot hybridization assays.

Hybridization studies were initially conducted using $^{32}$P-labeled recombinant plasmids containing the gene 9 cDNA inserts. Hybridizations conducted with these probes were characterized by relatively poor signal strength and excessive background problems with rotavirus negative field specimens, that was postulated to be due to bacteria containing plasmids homologous to the recombinant plasmids (data not shown). As a result, all further development of the assay involved excision and purification of the cDNA inserts from the recombinant plasmids prior to radiolabeling.

Hybridizations with the radiolabeled gene 9 probes were initially performed at 42°C. In Fig. 1, reactions of the Gottfried gene 9 probe with homologous cell culture virus and recombinant plasmid were significantly stronger than heterologous reactions with both the OSU cell culture virus and recombinant plasmid. A small amount of cross-reactivity however was observed at higher RNA concentrations. In order to overcome this, the stringency of the reaction was increased by increasing the temperature of hybridization from 42°C to 52°C. In Fig. 2, reaction of the Gottfried gene 9 probe at 52°C resulted in reduced cross-reactivity with the OSU cell culture virus, and retained its ability to detect Gottfried rotavirus in fecal samples. The weak hybridization signal observed between the Gottfried gene 9 probe and the OSU recombinant plasmid at 52°C, was believed to be due to the retention of small amounts of plasmid sequences following initial excision and preparation of the insert for use as probes. Similar observations concerning the increased
specificity of the gene 9 probes at 52°C were observed for the OSU gene 9 probe (data not shown).

The gene 9 probes were estimated to detect at least 2 ng of rotavirus RNA. This was shown in Figs. 1 and 2 with reactions of the Gottfried gene 9 probe against serial dilutions of cell culture rotavirus RNA preparations. The actual sensitivity of the probes was considerably higher, since the nucleic acids quantitated and serially dotted onto the nylon membranes were crude, and were not purified in this assay to eliminate MA104 cell nucleic acids.

Detection and characterization of porcine rotavirus strains

Following establishment of the hybridization conditions necessary to achieve specificity, the gene 9 probes were reacted against a variety of porcine rotavirus RNA extracts of cell culture and intestinal origin. The reaction of the OSU gene 9 probe against OSU prototype and serotype 5 porcine rotavirus RNA samples from intestinal specimens obtained from 8 different gnotobiotic pigs is shown in Fig. 3 (samples 1 to 8). The OSU gene 9 probe detected virus in 6 of 8 samples examined. Positive controls used in the assay consisted of OSU cell culture virus preparations (OSU prototype viruses and the serotype 5 EE strain) and the OSU gene 9 recombinant plasmid. The OSU gene 9 probes proved to be highly specific, with only a small amount of cross-reactivity with the Gottfried gene 9 recombinant plasmid and cell culture virus. The cross-reactivity observed was negligible when compared to the OSU cell culture virus and recombinant plasmid positive controls. Hybridization was not observed with the rotavirus negative field specimens and the MA104 cell extract negative controls.

The reaction of the Gottfried gene 9 probe with Gottfried prototype and other serotype 4 porcine rotavirus RNA samples from intestinal specimens obtained from 7 different gnotobiotic pigs is shown in Fig. 4. The Gottfried gene 9 probe detected virus in 7 of 7
samples examined that included the Gottfried prototype virus, and the serotype 4 porcine rotavirus strains SB-1A, SB2, SB3, and SB5. The Gottfried gene 9 probe reacted strongly with the Gottfried cell culture virus and recombinant plasmid positive controls. A small amount of cross-reactivity was observed with the OSU cell culture and fecal virus, and the OSU recombinant plasmid. Hybridization was not observed with the negative controls, that consisted of rotavirus negative field specimens, porcine enteric calicivirus-like virus, and MA104 cell extract.

The specificity of the OSU and Gottfried gene 9 probes was confirmed by reaction of the probes with heterologous rotavirus RNA samples. This is shown in Figs. 5 and 6 with reaction of the OSU and Gottfried gene 9 probes with duplicate sets of Gottfried and OSU RNA samples, respectively, dotted onto a second pair of nylon membranes.

**DISCUSSION**

The use of nucleic acid probes as a diagnostic tool for the detection of rotavirus in fecal samples was first reported by Flores et al. (4) and Pedley and McCrae (27). Further developments in the technique and selection of specific gene segments as probes has demonstrated the ability to distinguish human rotavirus according to subgroup and serotypic specificities (3,5,20). We report in this study the application of these techniques towards the development of a dot hybridization assay for the detection and serotypic characterization of porcine rotaviruses.

Through the use of relatively high stringency conditions (52°C, 50% formamide, 5X SSC), radiolabeled gene 9 probes from Gottfried or OSU porcine rotaviruses proved useful in the detection and discrimination of porcine rotavirus RNA extracts from cell culture and intestinal specimens. These included the OSU and Gottfried prototype viruses, as well as several previously described serotype 4 and 5 porcine rotavirus strains (2,30). Of particular interest was the exclusive reaction of the Gottfried gene 9 probe with the serotype
4 porcine rotavirus SB-1A (Fig. 4, sample 7). This strain is a naturally occurring reassortant originally isolated in our laboratory, and shares neutralization specificity with serotype 4 and serotype 5 rotaviruses (15). The basis for this dual classification is due to the high degree of homology in gene segment 9 coding for VP7 with Gottfried (serotype 4 rotavirus), and gene segment 4 coding for VP4 with OSU (serotype 5 rotavirus) (14,22). The hybridization of the Gottfried gene 9 probe and the lack of any cross-reactivity with the OSU gene 9 probe (Fig. 5, sample 7) confirmed the specificity of the probes used in this study, and indicated a high degree of homology between the Gottfried and SB-1A rotavirus gene 9 segments. This observation confirmed previous extensive studies indicating a serotype 4 porcine rotavirus as the parental origin for the SB-1A gene 9 segment (14,22).

The reaction of the Gottfried gene 9 probe with a variety of serotype 4 porcine rotavirus strains resulted in the detection of 7 of 7 samples examined. The ability of the probes to detect a variety of rotavirus strains of the same serotype correlates with previous studies indicating 91-99% amino acid homology among the VP7 polypeptides of rotaviruses of the same serotype (10). In contrast, the OSU probe failed in the detection of two samples both of the serotype 5 porcine rotavirus A580 strain, despite previous confirmation using electrophoresis of rotavirus RNA in all samples dotted on the nylon membranes. The failure of the OSU probe to detect virus in these two samples may have been due to: 1) mislabeling or cross-contamination of laboratory samples; 2) low levels of rotavirus-specific RNA out of the total nucleic acid quantitated and dotted onto the nylon membranes; or 3) classification of the A580 strain as a serotype 5 rotavirus due to antigenic characteristics of the VP4 polypeptide. Further studies are currently underway to determine the basis for the results obtained.

The probes used in this study consisted of full length cDNA copies of the OSU and Gottfried gene 9 segments. As expected, some degree of cross-reactivity between serotypes was observed due to highly conserved regions in the gene 9 segments and an overall 74%
nucleotide identity (9,10). Under conditions of relatively high stringency, however, cross-reactivity was minimized, and significant differences were observed between signal strengths of homologous and heterologous hybridization reactions. The problem of cross-reactivity as it applies to the distinction of weak positives and false negatives may seem to limit the general usefulness of this assay system. This phenomena, however, is not unique to nucleic acid hybridization, and as with other assay systems such as the enzyme-linked immunosorbent assay (ELISA), it points to the importance of using appropriate positive and negative controls.

Further improvements in the development of the dot hybridization assay system are currently being investigated by our laboratory and others. These include the use of smaller probes representative of the variable regions (serotype-specific) of the gene 9 segments, as well as the use of nonradioactive labeling (5,18,25). The application of the porcine rotavirus gene 9 segments toward the serotypic classification of field specimens, and studies on the prevalence and distribution of porcine rotavirus serotypes as determined by monoclonal antibodies, are currently being conducted by our laboratory. Recent studies indicating the presence of neutralizing epitopes on the VP4 polypeptide, and the occurrence of both human and animal rotavirus reassortants possessing shared intertypic characteristics, point for the need for a series of probes for the serotypic classification of rotaviruses (12,21,22,24). With the continued development of new probes representative of other virus serotypes and gene segments, nucleic acid probes may provide a useful new tool for the detection and serotypic classification of porcine rotaviruses.
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FIG. 1. Dot hybridization of the Gottfried gene 9 probe at 42°C. The Gottfried probe was reacted against serial dilutions of the Gottfried (GOTT. P) and OSU (OSU P) recombinant plasmids, and RNA extracted from Gottfried (GOTT. T/C) and OSU (OSU T/C) cell culture viruses. A small amount of cross-reactivity was observed with the OSU recombinant plasmid and cell culture virus RNA extract.
FIG. 2. Dot hybridization of the Gottfried gene 9 probes at 52°C. The Gottfried probe was reacted against serial dilutions of the Gottfried (GOTT. P) and OSU (OSU P) recombinant plasmids, and RNA extracted from Gottfried (GOTT. T/C) and OSU (OSU T/C) cell culture viruses, and Gottfried fecal viruses (GOTT. F) passaged through gnotobiotic pigs. The cross-reactivity of the Gottfried gene 9 probe with OSU cell culture virus RNA was significantly reduced.
FIG. 3. Dot hybridization of the OSU gene 9 probe. Serial dilutions of OSU prototype (samples 1-4, 6, and 8) and the serotype 5 A580 strain (samples 5 and 7) viral RNA extracted from intestinal contents of individual gnotobiotic pigs are shown in the top panel. Control samples included in the assay (bottom panel, left-to-right) were: OSU T/C (OSU prototype cell culture virus [left and center], and the serotype 5 EE strain of cell culture virus); G. T/C (Gottfried cell culture virus); G. FECAL (Gottfried fecal virus passaged through gnotobiotic pigs [left and right]); FIELD (rotavirus negative field specimens [left and right]); and P-MA (Gottfried recombinant plasmid [top], OSU recombinant plasmid [center], and MA104 cell nucleic acid extract [bottom]).
FIG. 4. Dot hybridization of the Gottfried gene 9 probe. Serial dilutions of Gottfried prototype (samples 1 and 2) and the serotype 4 porcine rotaviruses SB2 (sample 3), SB3 (samples 4 and 5), SB5 (sample 6), and SB-1A (sample 7) viral RNA extracted from intestinal contents of individual gnotobiotic pigs are shown in the top panel. Control samples included in the assay (bottom panel, left-to-right) were: G. T/C (Gottfried cell culture virus); OSU (OSU cell culture virus); OSU FECAL (OSU fecal virus passaged through gnotobiotic pigs [left and right]); FIELD (rotavirus negative field specimens [left and right]); PEC (porcine enteric calicivirus-like virus); and P-MA (Gottfried recombinant plasmid [top], OSU recombinant plasmid [center], and MA104 cell nucleic acid extract [bottom]).
### FIG. 5. Dot hybridization of the OSU gene 9 probe with Gottfried RNA samples.

Samples dotted on the nylon membranes were identical to those shown in Fig. 4.

Samples dotted on the nylon membrane were identical to those shown in Fig. 3.
CHAPTER III

SEROTYPIC DIFFERENTIATION OF GROUP A ROTAVIRUSES WITH PORCINE ROTAVIRUS GENE 9 PROBES

INTRODUCTION

Rotaviruses are important causative agents of acute viral gastroenteritis in humans and the young of many mammalian and avian species (16,17). The virus is nonenveloped, and possesses a double-stranded RNA (dsRNA) genome composed of 11 segments surrounded by two capsid layers. Rotavirus infections are commonly associated with diarrhea in suckling and recently weaned pigs (7,32,50). In swine in the United States, serotype 4 and 5 porcine rotaviruses, represented by the prototype strains Gottfried and OSU, respectively, have been well characterized (9,22,23,25), and two potentially new serotypes of porcine rotavirus have been isolated (39). Serotype 4 and 5 porcine rotaviruses have also been detected in several other countries, and several new serotypes of porcine rotavirus have been reported in Australia, Mexico, and Argentina (2,4,35,38,44).

Nucleic acid probes have recently been used for the detection of group A rotaviruses. Initial investigations using radiolabeled probes prepared from a combination of rotavirus gene segments proved successful in the detection of all serotypes of group A rotaviruses (33,40). Through the manipulation of hybridization stringency conditions and selection of specific gene segments, subgroup or serotypic specificities of human rotaviruses have been distinguished (19,34,54).

Nucleic acid probes have also recently been applied to the detection and serotypic characterization of porcine rotaviruses (43). Under relatively stringent hybridization conditions (52°C, 50% formamide, 5X SSC), gene 9 cDNA from OSU and Gottfried porcine
rotaviruses, coding for the immunologically relevant protein VP7, differentiated between serotype 4 and 5 group A rotaviruses isolated from swine.

Although nucleic acid probes for the serotypic differentiation of swine (serotypes 4 and 5) and human (serotypes 1 to 4) group A rotaviruses have been developed, no extensive studies have been conducted to confirm the serotypic specificity of the probes when tested against rotaviruses from animal species other than the target species for which the test was devised. Prior to acceptance of nucleic acid probes as a method for serotypically characterizing rotaviruses, nucleic acid probes should be evaluated for their ability to detect and discriminate between rotaviruses of similar and different serotypes or subgroups. In this study, radiolabeled cDNA probes prepared from full length Gottfried and OSU porcine rotavirus gene 9 segments were investigated in a dot hybridization assay. The probes were used to detect and differentiate homologous and heterologous serotypes of known group A rotaviruses of human and animal origin under low and high stringency conditions. In addition, the specificity of the probes against other enteric viruses of porcine origin was determined by reaction of the probes with non group A porcine rotaviruses (groups B and C), porcine enteric calicivirus, and a porcine coronavirus, transmissible gastroenteritis virus. Diagnostic field samples from pigs positive for group A rotaviruses were also examined for serotype 4 or 5 specificity using the probes.

MATERIALS AND METHODS

Viruses

Cell culture-adapted animal and human group A rotaviruses of known serotypes were used in the assays. These included the following: Wa (serotype 1); DS-1 (serotype 2); M, SA11, and RRV (serotype 3); ST3, VA70, and Gottfried (serotype 4); OSU and Equine H1 (serotype 5); NCDV (serotype 6); and a second bovine rotavirus serotype, B223, tentatively classified as serotype 10 (9,21,28,51,53) (See Table 1). Cell culture-adapted rotaviruses were
propagated in monkey kidney (MA104) cells in roller bottles or tubes, and stationary flasks. The cells were grown in Eagle minimal essential medium supplemented with 0.5% lactalbumin hydrolysate, 0.14% NaHCO₃, and 10% fetal bovine serum. Prior to infection, the cells were washed with glucose- potassium chloride-sodium chloride saline solution to remove fetal calf serum. Viruses were passaged in confluent monolayers of MA104 cells maintained in serum-free cell culture medium containing 0.05% pancreatin (4X NF, 10x concentrated, GIBCO Laboratories, Grand Island, New York).

In addition to the cell culture-passaged rotaviruses, viruses passaged in gnotobiotic pigs were also tested. These viruses were as follows: serotype 4 (OSU), serotype 5 (Gottfried), and porcine rotavirus SB-1A (intertypic rotavirus, serotype 4 via VP7 and serotype 5 via VP4) group A rotaviruses (See Table 1), porcine group B rotavirus, porcine group C rotavirus, porcine enteric calicivirus (PEC), and a coronavirus, transmissible gastroenteritis virus (TGEV) (8,20,28,45,49).

Field Specimens

Nucleic acid was extracted from feces or intestinal contents of 17 pigs characterized with diarrhea. The samples were obtained from pigs in California (submitted by Dr. A. Castro, California Veterinary Diagnostic Laboratory Services, Davis), Illinois (submitted by Dr. G. B. Fritz, Animal Disease Laboratory, Dept. of Agriculture, Galesburg), South Dakota (submitted by Dr. D. Benfield, South Dakota Veterinary Diagnostic Laboratory, Brookings), and Ohio. The field specimens were classified as positive or negative for group A rotavirus based on cell culture immunofluorescence (48), immune electron microscopy (8), subgroup-specific ELISA (30), or dsRNA polyacrylamide gel electrophoresis (26). Three field specimens determined to be negative by the above mentioned tests were used as negative controls.
Although antibody serotyping data on the 14 rotavirus positive field specimens was not available, five field samples selected for hybridization analysis were previously tested by a subgroup-specific ELISA in our laboratory (30). Results from the subgroup-specific ELISA identified samples 1, 2, 4, and 5 as subgroup 2, and sample 3 as subgroup 1.

**RNA extraction and nucleic acid quantitation**

Rotavirus dsRNA was extracted from virus infected cells, feces, and intestinal contents as previously described (43). Briefly, cell culture-adapted rotaviruses were cultivated in MA-104 cells until a cytopathic effect was observed. The virus cultures were frozen and thawed, and the culture medium clarified by centrifugation at 430 \( x \) \( g \) for 20 minutes. The virus in the resulting supernatants was semi-purified by high speed centrifugation (122,000 \( x \) \( g \) for 3 hours) through a 40% sucrose cushion containing 10 mM CaCl\(_2\). The virus pellets were suspended in 1 ml of 0.1 M sodium acetate buffer (pH 5) containing 1% sodium dodecyl sulfate (SDS) and deproteinized by phenol-chloroform extraction. Rotavirus dsRNA was precipitated with ethanol overnight at -20°C. Precipitated RNA was pelleted by centrifugation at 12,000 \( x \) \( g \), suspended in Tris-NaCl-EDTA buffer (0.01 M Tris, 0.1 M NaCl, 5 mM EDTA, pH 8.0), and stored frozen at -20°C.

Extraction of viral RNA from intestinal contents and feces was performed by diluting samples (0.5 g) 1:6 in 0.1 M Tris-10 mM CaCl\(_2\) buffer, pH 7.4. The virus suspensions were clarified by centrifugation at 1200 \( x \) \( g \) for 30 minutes. The supernatant was collected, and SDS and sodium acetate were added to a concentration of 1.0% and 0.1 M, respectively. The virus suspensions were deproteinized by phenol-chloroform extraction, and processed as described above for cell culture virus.

The concentration of nucleic acid in rotavirus positive and negative samples was estimated by \( A_{260} \) readings in a spectrophotometer. The nucleic acid concentration calculated represented the total nucleic acid in the extracted samples, and did not represent
RNA Electrophoresis

Rotavirus RNA in extracted samples was confirmed using polyacrylamide gel electrophoresis. Electrophoresis was performed using the discontinuous buffer system of Laemmli (31). Rotavirus double-stranded RNA was resolved in 12.5% polyacrylamide separation gels with 4% stacking gels. Electrophoresis was performed at 20 mA for 24 hours. The RNA bands were visualized using the silver staining method of Herring et al. (26).

Preparation of $^{32}$P-labeled probes

Full length cDNA copies of gene 9 from OSU and Gottfried porcine rotavirus inserted into the plasmid vectors pBR322 and pTZ18R, respectively, were provided by Mario Gorziglia, National Institutes of Health, Bethesda, Maryland (22,23). The HB101 strain of E. coli containing the recombinant plasmids was cultivated in ampicillin or tetracycline-containing medium. Plasmids containing cDNA clones of OSU and Gottfried gene 9 were extracted from cleared lysates as previously described (6). The plasmids were purified by centrifugation at 336,445 x g for 19 hours in cesium chloride-ethidium bromide gradients, and the lower DNA band was collected and dialyzed against Tris-EDTA buffer at 4°C for 72 h. The OSU and Gottfried gene 9 cDNA were excised from the plasmids by digestion with Pst I or Pst I and Eco RI. The cleavage products were separated on 6% polyacrylamide gels and stained with ethidium bromide, and the inserts were excised, electroeluted, and concentrated by ethanol precipitation. In some cases when DNA isolation was difficult in polyacrylamide gels, the cleavage products were separated on 1% agarose gels, excised, electroeluted, and extracted with phenol-chloroform solution and chloroform-isoamyl alcohol prior to ethanol precipitation. Probes were prepared from the gene 9 cDNA inserts.
by nick translation (BRL nick translation system, Bethesda Research Laboratories, Gaithersburg, Maryland) using $^{32}$P deoxycytidine 5'-triphosphate (ICN Biomedicals Inc., Irvine, Calif., specific activity 650 Ci/m mole).

**Nucleic acid sample application and hybridization**

Nucleic acid samples were denatured at 100°C for 5 minutes followed by cooling at 0°C for 5 min. The samples were dotted onto 0.45 μm nylon membranes (Zetaprobe; Bio-Rad Laboratories, Richmond, CA) in volumes of 5 μl or less with a micropipette until the desired quantity of nucleic acid was applied. In some cases, field samples were applied to the nylon membranes using a filtration manifold (HybriDot Manifold; Bethesda Research Laboratories, Gaithersburg, Maryland). Following sample application, the nylon membranes were air dried, baked for 2 hours at 80°C in a vacuum oven, and stored at 4°C.

Hybridization of the OSU and Gottfried gene 9 probes at 52°C, and the subsequent wash steps, were performed as previously described (43). The membranes were given a final rinse in distilled water, blotted, and exposed to Kodak XAR film (Eastman Kodak Company, Rochester, New York) with intensifying screens at -70°C for 1 to 7 days.

**RESULTS**

**Specificity of the Gottfried and OSU gene 9 probes**

The serotype specificity of the Gottfried and OSU porcine rotavirus gene 9 probes were determined by dot hybridization with dsRNA extracted from rotaviruses of known serotype of human and animal origin. Previous studies in our laboratory established that under relatively high stringency conditions (52°C, 50% formamide, 5X SSC), hybridization reactions of the Gottfried and OSU gene 9 probes with serotype 4 and 5 porcine rotaviruses were serotype specific (43). Under relatively high stringency conditions, hybridization reactions of the Gottfried and OSU gene 9 probes were serotype specific, with little or no
cross-reactivity with heterologous group A rotavirus serotypes, or other viruses (Fig. 7 and 8). Homologous and heterologous hybridization reactions of the Gottfried gene 9 probe are shown in Figure 7. The Gottfried gene 9 probe (serotype 4) produced strong hybridization signals with Gottfried cell culture virus, as well as with Gottfried intestinal virus and the intertype virus SB-1A (VP7 serotype 4 and VP4 serotype 5). The probe also produced strong hybridization signals with the human serotype 4 rotaviruses VA70 and ST3 originally recovered from symptomatic and asymptomatic infections, respectively. Little or no cross-reactivity could be detected with group A human rotavirus serotypes 1 to 3 (Wa, DS-1, and M), simian rotaviruses SA11 and RRV, bovine rotaviruses NCDV and B223, the equine rotavirus H1, and the porcine rotaviruses OSU and EE (serotype 5). Hybridization was not observed with group B and C porcine rotaviruses, or with PEC and TGEV. Hybridization of the Gottfried gene 9 probe with control samples produced a strong hybridization signal with the Gottfried recombinant plasmid (positive control), and no cross-reactivity with the negative controls including the OSU recombinant plasmid, MA104 cell nucleic acid extract, and the rotavirus negative field sample.

Homologous and heterologous hybridization reactions of the OSU gene 9 probe under relatively high stringency conditions are shown in Figure 8. The OSU gene 9 probe produced hybridization signals with homologous OSU cell culture virus, the serotype 5 EE strain of cell culture virus, and the OSU intestinal virus. In addition, the OSU gene 9 probe produced a strong hybridization signal with the serotype 5 equine H1 rotavirus. Hybridization was not observed with Gottfried cell culture and intestinal viruses, and the intertype virus SB-1A. Similarly, little or no cross-reactivity was detected with heterologous group A rotavirus serotypes (human, simian, and bovine), group B and C porcine rotaviruses, PEC, and TGEV. The OSU gene 9 probe produced a strong hybridization signal with the OSU recombinant plasmid (positive control), and demonstrated no cross-reactivity with the negative controls including the Gottfried
recombinant plasmid, MA104 cell nucleic acid extract, and rotavirus negative field sample.

**Cross-reactivity under relatively low stringency conditions**

Under conditions of relatively low hybridization stringency (52°C, no formamide, 5X SSC), the Gottfried and OSU gene 9 probes showed broad cross-reactivity with human and animal rotaviruses (Figures 9 and 10). Homologous and heterologous hybridization reactions of the Gottfried gene 9 probe are shown in Figure 9. The Gottfried gene 9 probe, as demonstrated in Figure 7, produced strong hybridization signals with Gottfried cell culture and intestinal viruses, intertype virus SB-1A, and the serotype 4 human rotaviruses VA70 and ST3. In addition, the Gottfried gene 9 probe (serotype 4) detected heterologous group A human, simian, bovine, and porcine (serotype 5) rotavirus serotypes. Despite the broad cross-reactivity with group A rotaviruses, hybridization was not observed with group B and C porcine rotaviruses, PEC, and TGEV. Similarly, cross-reactivity was not observed with the MA104 cell nucleic acid extract and the rotavirus negative field sample negative controls.

Hybridization reactions of the OSU gene 9 probe under conditions of relatively low stringency are shown in Figure 10. The OSU gene 9 probe (serotype 5) showed broad cross-reactivity with homologous and heterologous group A rotaviruses, reacting with human, simian, bovine, and porcine (serotypes 4 and 5) rotaviruses. Cross-reactivity was not observed with group B and C porcine rotaviruses, PEC, and TGEV. Although the OSU gene 9 probe did not hybridize with the rotavirus negative field sample, a weak hybridization signal was observed with the MA104 cell nucleic acid extract.
Hybridization with porcine field samples

Application of the Gottfried and OSU gene 9 probes for the analysis of porcine rotavirus field samples are shown in Figures 11 and 12. The Gottfried and OSU gene 9 probes collectively detected rotaviruses in 6 of 14 group A rotavirus positive samples tested. The reactions of the Gottfried gene 9 probe are shown in Figure 11. The Gottfried gene 9 probe detected serotype 4 rotaviruses in 5 of 14 samples 1, 2, 4, 5, and 10. A weak hybridization signal was observed between the Gottfried gene 9 probe and sample 6, as well as the OSU cell culture and intestinal virus negative controls. Hybridization was not observed with the three rotavirus negative field samples. The OSU gene 9 probe detected serotype 5 rotavirus in 1 of 14 samples (sample 3), with no cross-reactivity with the Gottfried gene 9 cell culture and intestinal virus negative controls, and the three rotavirus negative field samples (Figure 12). No samples reacted with both probes.

Rotavirus RNA Electrophoresis

The dsRNA electrophoretic patterns of the group A cell culture-adapted rotaviruses, the SB-1A group A rotavirus, and the porcine group B and C intestinal viruses agreed with previously published dsRNA patterns (Fig. 13) (9,28,29,37,46,49,52). Electrophoresis of the rotavirus dsRNA confirmed the presence of viral RNA in the extracted samples, and in particular the 7, 8, and 9 gene segments of each virus. The presence or absence of rotavirus dsRNA in samples extracted from the porcine serotype 4 and 5 intestinal viruses, the rotavirus negative samples (PEC, TGEV, MA104 cell nucleic acid extract), and the rotavirus field samples, were also confirmed by electrophoresis (data not shown).

DISCUSSION

Recent investigations of rotavirus epidemiology have focused on the serotype specificities of rotavirus in field isolates and clinical samples (3,5,28,35,38,53). This
information is needed to determine the prevalence and distribution of rotavirus serotypes in order to develop effective vaccine strategies. Traditional methods for the distinction of rotavirus serotypes have involved plaque reduction and fluorescent focus neutralization tests (9,10,28). More recently, serotyping ELISAs employing monoclonal antibodies have been developed which permit the rapid evaluation of many samples, and overcome problems involved in cell culture adaptation encountered in previous assays (5,11,47). A second recent approach for the serotypic characterization of rotaviruses has involved the development of radiolabeled probes. Through the selective use of probes prepared from gene 6 segments, human rotaviruses have been distinguished according to subgroup specificities (34). Alternatively, probes prepared from gene 9 segments have enabled the serotypic distinction of human and porcine rotaviruses (19,34,54,43).

Although porcine and human gene 9 nucleic acid probes have been shown to serotypically distinguish rotaviruses isolated from porcine and human sources, respectively, no extensive studies have been performed to determine the specificity of these probes against rotavirus serotypes and serogroups from other animal species. In practice, before confidence or acceptance of a new assay for the serotypic discrimination of rotaviruses can be established, the performance of VP7 specific probes against rotavirus isolates from various species should be investigated. In this study, porcine rotavirus gene 9 probes were used to serotypically characterize group A rotaviruses of human and animal origin in a dot hybridization assay. Under relatively stringent hybridization conditions (52°C, 50% formamide, 5X SSC), gene 9 probes from the Gottfried (serotype 4) and OSU (serotype 5) porcine rotaviruses showed a high degree of specificity, with little or no cross-reactivity with the heterologous porcine serotype, as well as other serotypes of group A rotaviruses. In addition, the specificity of the probes, using the nucleic acid extraction and hybridization procedures in this study, was confirmed by the lack of cross-reactivity with group B and C porcine rotaviruses, and other enteric viruses (PEC and TGEV) which may
potentially be present in porcine clinical samples.

The ability of radiolabeled porcine rotavirus gene 9 probes to specifically detect rotaviruses of the same serotype was demonstrated by hybridization of the Gottfried gene 9 probe with the serotype 4 human rotaviruses ST3 and VA70. These results confirm previous studies indicating a high degree of nucleic acid homology between these viruses (23,24), and correlate with previous limited studies indicating the ability of VP7 gene probes from one animal species (simian SA11, serotype 3) to specifically detect rotaviruses of the same serotype in another species (human Yo, serotype 3) (54). In addition, the Gottfried gene 9 probe demonstrated its usefulness in the characterization of reassortant rotaviruses by its exclusive reaction with the naturally occurring porcine rotavirus reassortant SB-1A. This virus possesses serotype 4 and 5 characteristics due to a high degree of homology of gene segment 9 coding for VP7, with Gottfried (serotype 4 rotavirus), and gene segment 4 coding for VP4 with OSU (serotype 5 rotavirus) (27,36).

Under conditions of relatively low stringency (52°C, no formamide, 5X SSC), porcine rotavirus gene 9 probes proved useful in the detection of all serotypes of group A rotaviruses. Of the two preparations of porcine gene 9 probes used in this study, the Gottfried gene 9 probe produced stronger hybridization signals with heterologous serotypes of group A rotaviruses. Although the total quantity of RNA placed on the filters was standardized, the different intensities of signals may reflect different quantities of viral RNA present in the samples tested. The broad cross-reactivity observed has been documented by other investigators (12,54), and is due to approximately 74% sequence homology between the VP7 genes of different serotypes of group A rotavirus (23,42).

Detection of group A rotaviruses by dot hybridization has previously been performed using a mixture of ^32P-labeled cDNA's representing the 11 rotavirus segments, or a full length probe of the rotavirus gene 6 segment (12,18,33). Under relatively low hybridization stringency conditions, we have demonstrated that porcine rotavirus gene 9 probes may also
be used for group A rotavirus detection.

Despite the broad cross-reactivity of the group A porcine rotavirus gene 9 probes under low stringency conditions, hybridization was not observed with group B or C porcine rotaviruses, as well as a porcine enteric calicivirus or coronavirus, using the nucleic acid extraction and hybridization procedures presented in this study. The lack of hybridization with the group B or C rotaviruses suggests a low level of nucleic acid homology between the gene 9 segments of these viruses. Similar results have been reported by Zheng et al. (54) concerning the failure of gene 9 probes from 4 serotypes of group A rotaviruses to hybridize under relatively stringent hybridization conditions with an adult rotavirus strain (group B). In hybridization studies with other genomic segments, cDNA probes prepared from gene 3 of the infectious diarrhea of infant rat (IDIR) strain of group B rotavirus failed to hybridize with human and simian group A rotavirus (15). The low level of nucleic acid homology among group A, B, and C rotaviruses presented in these studies is supported by immunological analyses which have established antigenic differences among rotaviruses of each serogroup (8,14,41,49).

The Gottfried and OSU gene 9 probes were applied to the serotypic differentiation of 14 group A porcine rotavirus positive field samples. The probes collectively detected and serotypically differentiated 6 of 14 samples. Support for the serotypic identity of the field isolates tested is provided by the following observations. (i) The Gottfried and OSU gene 9 probes have previously been demonstrated to selectively hybridize with rotaviruses of the same serotype under the hybridization conditions used. (ii) The Gottfried and OSU gene 9 probes did not react nonspecifically with nucleic acids found in fecal preparations (as evidenced by the lack of hybridization with the rotavirus negative fecal preparations). (iii) Subgroup classification by ELISA of 5 of the 14 samples resulted in the correlation of subgroup 2 positive samples with serotype 4 positive samples (Gottfried) and the subgroup 1 positive sample with the serotype 5 positive sample (OSU) (30). Subgrouping analyses of
rotaviruses have previously correlated subgroup 2 rotaviruses only with Gottfried (serotype 4 rotavirus) (13). Subgroup 1 specificities, however, have been associated with serotype 3, 5, or other serotypes of porcine rotaviruses (13,38,39).

The Gottfried gene 9 probe produced a weak but detectable hybridization signal with field sample number 6. Although the hybridization signal produced with sample 6 was similar to signals produced with the OSU cell culture and intestinal virus negative controls, the OSU gene 9 probe failed to hybridize with this sample. The hybridization signal observed may therefore be due to a low titer of serotype 4 rotavirus, or due to a low level of cross-reactivity with a porcine rotavirus of another serotype. The Gottfried and OSU gene 9 probes failed to serotypically differentiate the remaining 7 field samples. Recent studies, however, have identified other serotypes of rotavirus in swine (38,39,44), and the prevalence of these porcine rotavirus serotypes in the United States has not been determined.

A small amount of cross-reactivity was observed between the OSU gene 9 probe and the MA104 cell nucleic acid extract (negative control) under low stringency conditions. Hybridization of viral probes to cellular rRNA's have previously been reported (1,13). Despite this cross-reactivity, the OSU and Gottfried gene 9 probes failed to hybridize with nucleic acid extracted from rotavirus negative fecal samples, indicating that the concentration of rRNA's from bacterial and animal sources in field samples may not pose a problem.

The technique of virus detection through the use of hybridization probes is a rapidly developing field of study, which holds potential as a new method for serotypic analyses of viruses in clinical specimens. Although traditional tests involving serum neutralization will remain the standard for the establishment and confirmation of new rotavirus serotypes, hybridization probes may provide a supplementary method for the screening of large numbers of samples to determine serotype prevalence. In addition, nucleic acid
probes may overcome the problem of antigenic variants within serotypes which fail to react with monoclonal antibodies used in serotyping assays (11).

REFERENCES


FIG. 7. Composite of the dot hybridizations of the Gottfried gene 9 probe at relatively high stringency conditions (52°C, 50% formamide, 5X SSC). The amount of nucleic acid sample applied to the membranes were as follows: the group A human simian, bovine, and porcine (serotype 4 and 5 and the intertype virus SB-1A), the MA104 cell nucleic acid extract, and nucleic acid extracted from the rotavirus negative fecal sample, diluted twofold and applied at amounts from 1 ug to 250 ng; group A equine H1 rotavirus, diluted fivefold and applied at amounts from 500 ng to 20 ng; the OSU and Gottfried recombinant plasmids, applied at 325 ng; and the porcine group B and C rotaviruses, porcine enteric calicivirus, and the transmissible gastroenteritis virus, applied at an initial amount of 1 to 5 ug and serially diluted twofold. Nucleic acid samples in the top panel were, from left to right: human group A rotaviruses (Wa, DS-1, M, VA70, and ST3); simian group A rotaviruses (SA11 and RRV); bovine group A rotaviruses (NCDV and B223); the porcine rotaviruses (group B rotavirus-like virus [Gp B RVLV] and group C pararotavirus [Gp C PaRV]), and the group A equine H1 rotavirus. Nucleic acid samples in the bottom panel were, from left to right: porcine enteric calicivirus (PEC); transmissible gastroenteritis virus (TGE); MA104 cell nucleic acid extract (MA-104); OSU group A rotavirus samples [OSU (S5)] - OSU prototype cell culture virus (T/C), serotype 5 EE strain of cell culture virus (EE T/C), and OSU prototype virus passaged through a gnotobiotic pig (GUT); Gottfried group A rotavirus samples [Gott (S4)] - Gottfried prototype cell culture virus (T/C), and Gottfried prototype virus passaged through a gnotobiotic pig (GUT); intertype virus SB-1A (S4/5); rotavirus-negative sample obtained from a diarrheic pig (NEG FIELD); and the Gottfried and OSU recombinant plasmids [top and middle, respectively] (REC P). Where possible, the serotype of the group A rotaviruses was designated (S1 to S6)
FIG. 7.
FIG. 8. Composite of the dot hybridizations of the OSU gene 9 probe at relatively high stringency conditions (52°C, 50% formamide, 5X SSC). The samples probed were the same as in Figure 7.
FIG. 9. Dot hybridization of the Gottfried gene 9 probe at relatively low stringency conditions (52°C, no formamide, 5X SSC). The samples probed were the same as in Figure 7 with the exception of the group A equine H1 rotavirus.
FIG. 10. Dot hybridization of the OSU gene 9 probe at relatively low stringency conditions (52°C, no formamide, 5X SSC). The samples probed were the same as in Figure 7 with the exception of the group A equine H1 rotavirus.
FIG. 11. Serotypic differentiation of porcine field samples with the Gottfried gene 9 probe. The samples were probed under relatively high stringency conditions (52°C, 50% formamide, 5X SSC). Rotavirus positive field samples are numbered 1 to 14. Rotavirus negative field samples are numbered 15 to 17. Positive control samples included the OSU (O) and Gottfried (G) fecal (F) and cell culture (T/C) preparations. The field samples and the OSU and Gottfried fecal preparations were applied in triplicate in volumes of 40 ul, 20 ul, and 10 ul (top to bottom). The OSU and Gottfried cell culture preparations were diluted fivefold from 250 ng to 10 ng (top to bottom).
FIG. 12. Serotypic differentiation of porcine field samples with the OSU gene 9 probe.

The samples, sample volumes, and hybridization conditions used were the same as in Figure 11.
FIG. 13. The dsRNA electrophoretic patterns of the cell culture-adapted rotaviruses and the group B, group C, and SB-1A intestinal viruses. Lane A, human rotavirus Wa; lane B, human rotavirus DS-1; lane C, human rotavirus M; lane D, human rotavirus VA70; lane E, human rotavirus ST3; lane F, simian rotavirus SA11; lane G, simian rotavirus RRV; lane H, bovine rotavirus NCDV; lane I, bovine rotavirus B223; lane J, equine rotavirus H1; lane K, porcine group B rotavirus; lane L, porcine group C rotavirus; lane M, porcine rotavirus OSU; lane N, porcine rotavirus Gottfried; lane O, porcine rotavirus SB-1A; and lane P, porcine rotavirus EE. The rotavirus dsRNA electrophoretic patterns shown were resolved in 3 separate polyacrylamide gels.
TABLE 1. Cell culture-adapted rotaviruses and the SB-1A intestinal rotavirus used for nucleic acid hybridization with Gottfried and OSU gene 9 cDNA probes

<table>
<thead>
<tr>
<th>Rotavirus</th>
<th>Origin of virus</th>
<th>Serotype</th>
<th>Designation in Figures 1 to 4</th>
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<tbody>
<tr>
<td>Wa</td>
<td>Human</td>
<td>1</td>
<td>Human (S1) Wa</td>
</tr>
<tr>
<td>DS-1</td>
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<td>Human</td>
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<tr>
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<td>Human</td>
<td>4</td>
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<td>Simian</td>
<td>3</td>
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</tr>
<tr>
<td>RRV</td>
<td>Simian</td>
<td>3</td>
<td>Simian (S3) RRV</td>
</tr>
<tr>
<td>NCDV</td>
<td>Bovine</td>
<td>6</td>
<td>Bovine (S6) NCDV</td>
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<tr>
<td>B223</td>
<td>Bovine</td>
<td>10 (?)</td>
<td>Bovine (?) B223</td>
</tr>
<tr>
<td>OSU</td>
<td>Porcine</td>
<td>5</td>
<td>OSU (S5) T/C</td>
</tr>
<tr>
<td>EE</td>
<td>Porcine</td>
<td>5</td>
<td>OSU (S5) EE T/C</td>
</tr>
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<td>Porcine</td>
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<td>Gott (S4) T/C</td>
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<tr>
<td>SB-1A</td>
<td>Porcine</td>
<td>VP7(4)</td>
<td>S4/5</td>
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<td></td>
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CHAPTER IV
CHARACTERIZATION OF FULL-LENGTH AND POLYMERASE CHAIN REACTION-DERIVED PARTIAL-LENGTH GOTTFRIED AND OSU GENE 4 PROBES FOR SEROTYPIC DIFFERENTIATION OF PORCINE ROTAVIRUSES

INTRODUCTION

Group A rotaviruses are enteric pathogens of children, swine, and many other animal species (11,21). The viral genome consists of 11 segments of double-stranded RNA which is surrounded by two capsid layers. Group A rotavirus serotypes were initially established by polyclonal antibody-based serum neutralization assays (13,25). The serotypes defined by these assays were based on the antigenic specificities of VP7 (17,18,31,54). Genetic and monoclonal antibody-based assays later determined that the neutralization specificities of rotaviruses were determined by two outer capsid proteins, VP4 and VP7, which are capable of eliciting neutralizing antibodies (40,42,51). Currently, at least 11 and potentially 14 serotypes of rotaviruses have been classified in human beings and animals on the basis of VP7 or G type specificities (5,6,10,53). Four G types infect swine, and other potential G types have been reported (1,3,36,45).

The outer capsid protein VP4 is protease sensitive and is cleaved by trypsin into two polypeptides (VP5 and VP8) which play a major role in viral infectivity and virulence (9,43). Serological analyses of rotavirus variants with monoclonal antibodies or bacterially expressed VP5 and VP8 subunit proteins with hyperimmune antisera have identified distinct neutralization regions located primarily on VP8 (30,34). Studies on immunity and cross-protection have shown that VP4 may be as important as VP7 in eliciting rotavirus neutralizing antibodies and heterotypic immunity (22,24,44). Genomic
segment 4, coding for VP4, segregates independently of the genomic segment coding for VP7 (24). As a result, gene reassortment can occur, and interserotype bridging due to VP4 and VP7 has been reported for human and porcine isolates (23,24,39). Characterization of VP4 serotypes (P types) is as important as VP7 for the development of potential vaccines. Despite the importance of VP4, antigenic relationships among the VP4s of different porcine rotaviruses have only recently been investigated (16,32,33,38).

Two assays have been developed for the direct detection and differentiation of porcine rotavirus G serotypes. One is based on the detection of G type-specific epitopes with monoclonal antibodies in an enzyme-linked immunosorbent assay (37). The second assay involves hybridization of specific nucleic acid probes to differentiate rotavirus serotypes on the basis of differences in the nucleic acid sequences of the VP7 encoding gene (27,46,47). Variability of certain regions of the VP7 encoding gene has been associated with serotypic differences among rotavirus strains (8,17). The advantage of nucleic acid hybridization is its potential for detecting variants which could be missed by monoclonal antibody-based assays (7,32).

Molecular studies of genomic segment 4 have determined variable nucleic acid sequences located in the VP8 encoding region of VP4 (10,34). The variable regions have been associated with serotypic differences among VP4 proteins (P types) (30,34,48). In this study, full-and partial-length VP4 probes were produced and investigated to determine their ability to differentiate genomic segment 4 among group A porcine rotaviruses. The specificities of the probes were tested against rotaviruses isolated from humans and animals. The objective of the study was to develop reagents suitable for the routine P typing of porcine rotavirus field samples.
MATERIALS AND METHODS

Viruses

Human and animal cell culture-adapted group A rotaviruses were propagated in MA104 cells as previously described (47). The rotavirus strains of known serotype (as defined by their VP7 specificities) used in the assays included the following: Wa and M37 (serotype 1); DS-1 and 1076 (serotype 2); rhesus rotavirus, SA11, McN13, and M (serotype 3); VA70, ST3, SB-1A, and Gottfried (serotype 4); OSU, EE, A580, and equine H1 (serotype 5); Nebraska calf diarrhea virus (serotype 6); and B223 (serotype 10) (Table 2). The human rotavirus strains selected for this study possessed three of the four VP4 gene alleles currently documented among human rotaviruses (15). They included: (i) the symptomatic human rotaviruses Wa, M, and VA70, which represent genetic group 1; (ii) the symptomatic human rotavirus DS-1, which represents genetic group 2; and (iii) the asymptomatic human rotaviruses M37, 1076, McN13, and ST3, which represent genetic group 3. The naturally occurring porcine rotavirus reassortant SB-1A was selected for study because of its dual serotype specificities (23,24): its VP7 protein is similar to that of Gottfried rotavirus (G serotype 4), while its VP4 is similar to that of OSU rotavirus (G serotype 5).

Several porcine rotaviruses were propagated by passage through gnotobiotic pigs (49). The animal-passaged rotaviruses used were as follows: Gottfried, SB2, SB3, SB5, and SB-1A rotaviruses (G serotype 4); OSU rotavirus (G serotype 5); porcine serogroup B rotavirus; and porcine serogroup C rotavirus (Table 2).

Nucleic acid extraction and quantitation

Rotavirus double-stranded RNA (dsRNA) was extracted from virus-infected MA104 cells and intestinal samples by modifications of procedures previously described (46). Briefly, virus-infected cell culture supernatants and diluted intestinal samples were
clarified by centrifugation for 10 to 20 min at 430 x g or 1,200 x g, respectively. Rotaviruses in cell culture supernatants were semipurified by centrifugation at 122,000 x g for 3 h through a 40% sucrose cushion. Sodium dodecyl sulfate and sodium acetate were added to the clarified or semipurified virus preparations to concentrations of 1.0% and 0.1 M, respectively. The virus suspensions were deproteinized with phenol-chloroform, and rotavirus dsRNA was precipitated with ethanol overnight at -20°C. The precipitated dsRNA was suspended in sterile water treated with diethyl pyrocarbonate (Sigma Chemical Co., St. Louis, Mo.) and stored at 4°C.

Nucleic acid was extracted from negative control samples, which included mock-infected MA104 cells and intestinal samples from rotavirus-negative gnotobiotic pigs. The procedures described above for rotavirus dsRNA extraction were used for extraction of negative controls. Positive controls, which consisted of recombinant plasmids containing Gottfried or OSU genomic segment 4 cDNA inserts, were used. Positive control samples were extracted by the procedures described below for preparation of 32P-labeled probes.

The concentration of nucleic acid in rotavirus-positive and -negative samples was determined in a spectrophotometer at 260 nm. The absorbance readings of rotavirus-positive samples were used as estimates of rotavirus dsRNA concentrations for dot hybridization experiments. The spectrophotometer measurements did not discriminate between rotavirus dsRNA and nucleic acid from cells or other sources. In Northern (RNA) blot experiments, the amount of rotavirus dsRNA used was adjusted on the basis of A260 readings and the staining intensity of rotavirus RNA segments when visualized in polyacrylamide gels.

RNA electrophoresis and Northern blot

The presence or absence of rotavirus RNA in cell culture preparations, intestinal samples, and the negative control samples (nucleic acid extracted from mock-infected
MA104 cells and the intestinal contents of rotavirus-negative pigs) was confirmed by polyacrylamide gel electrophoresis. The dsRNA was resolved in 12.5% polyacrylamide separation gels (1.5 mm thick) by the discontinuous buffer system of Laemmli (29). The silver staining method of Herring et al. (20) was used for detection of rotavirus dsRNA in extracted samples. Rotavirus dsRNA for Northern blot studies was electrophoresed in 10% polyacrylamide gels (0.75 mm thick) and stained with ethidium bromide. The dsRNA segments were electrophoretically transferred to nylon membranes (Nytran, Schleicher & Schuell, Keene, New Hamp.) as previously described (52).

**Preparation of $^{32}$P-labeled probes**

Plasmid vectors pTZ18R containing full-length cDNA copies of Gottfried and OSU porcine rotavirus gene 4 segments were used (16,41). The DH5α strain of *Escherichia coli* containing the recombinant plasmids was cultivated in medium containing ampicillin. Extraction of the recombinant plasmids from cleared lysates of *E. coli* was performed by the procedure of Birnboim and Doly (2). Purification of the plasmids and excision of the genomic segment 4 cDNA inserts were performed as previously described (47), except *Bam* HI was used for excision of the cloned inserts. Probes were prepared and labeled by using nick translation and $^{32}$P-dCTP (47).

Two partial length gene 4 cDNAs were produced and amplified by using specific oligonucleotide primers and the polymerase chain reaction (PCR) in a DNA thermal cycler (Perkin Elmer Cetus Corp., Norwalk, Conn.). The partial length cDNAs encompassed portions of the variable region (nucleotides 213 to 612) in genomic segment 4, which encodes VP8 (10,14). Purified recombinant plasmids containing Gottfried and OSU genomic segment 4 cDNA inserts were used as templates. The primers used for production of the partial length Gottfried gene 4 segment were 5'CCATATCAGCCAACGAGT3', complementary to nucleotides 211 to 228, and 5'TTACTACTTCTACATCAGGT3',
complementary to nucleotides 607 to 588. The partial length OSU gene 4 segment was produced with primers 5'CATAACCAACCAACCACCTTTC3' and 5'TGATGTCATATTATACGTG3', complementary to nucleotides 212 to 231 and 612 to 593, respectively. The PCR was terminated after 30 cycles of denaturation (94°C for 1 min), primer annealing (42°C for 1.5 min), and primer extension (72°C for 3 min). To confirm the production of cDNA of the appropriate length, the PCR products were analyzed by agarose gel electrophoresis and compared with molecular size markers following ethidium bromide staining. Fragments of the expected size were purified by excision from 1% low-melting-point agarose (Bethesda Research Laboratories, Gaithersburg, Md.) gels and extracted with phenol (50). Partial length ³²P-dCTP labeled PCR-derived probes were prepared from purified PCR products as described for full length genomic segment 4 cDNA inserts.

Nucleic acid sample preparation and hybridization conditions

Nucleic acid samples for dot hybridizations were heat denatured and dotted onto nylon membranes (Zeta Probe; Bio-Rad Laboratories, Richmond, Calif.) (46). The samples were applied to the membranes in volumes of 5 ul or less, and sample application was repeated when necessary until the desired quantity was applied. Samples for dot and Northern blot hybridizations were fixed to the nylon membranes by baking for 0.5 and 1 h, respectively, at 80°C in a vacuum oven (National Appliance Company, Tualatin, Oreg.) and stored at 4°C.

The hybridization procedure, solutions, and final wash steps were performed as previously described (46) with minor modifications. Hybridization temperatures (37 to 52°C) and formamide concentrations (25 to 50%) were varied to determine the optimum hybridization conditions for full- and partial-length gene 4 probes. The hybridization stringency conditions necessary for optimal probe specificity and sensitivity were determined by visual inspection of the intensity and specificity of the reactions. Optimal
stringency conditions for dot and Northern blot hybridizations were determined to be 42°C and 50% formamide concentration for the full length Gottfried and OSU gene 4 probes and the Gottfried PCR-derived probe. The optimal stringency conditions for the OSU PCR-derived probe were 42°C and 40% formamide concentration. Approximately 4 x 10^6 cpm of denatured probe was added per 10 ml of hybridization solution.

Following hybridizations and autoradiography, disassociation of nucleic acid probes from target sequences on selected nylon membranes was performed according to the manufacturer's instructions (Bio-Rad Laboratories). The nucleic acid samples fixed on nylon membranes were then rehybridized with a second probe by the hybridization procedures described above.

RESULTS

Specificities of the full length Gottfried and OSU gene 4 probes

The specificity of the Gottfried probe with rotaviruses of human and animal origin was tested by dot hybridization (Fig. 14). The Gottfried probe produced strong hybridization signals with the homologous Gottfried cell culture-propagated rotavirus and recombinant plasmid and a weak hybridization signal with the ST3 human rotavirus. Moderate to weak hybridization signals were also observed, however, with the Wa strain of human rotavirus, the OSU strain of porcine rotavirus, and the recombinant plasmid containing an OSU genomic segment 4 cDNA insert. Hybridization was not observed with nucleic acid extracted from other human and animal group A or porcine group B and C rotaviruses or with the negative controls (mock-infected MA104 cells and rotavirus-negative intestinal samples).

Hybridization reactions of the OSU gene 4 probe with human and animal rotavirus strains are shown in Fig. 15. The OSU probe produced strong hybridization signals with OSU porcine rotavirus and recombinant plasmid and with equine H1 rotavirus. Weak
hybridization signals were observed with SB-1A animal-passaged rotavirus, Gottfried cell culture-propagated rotavirus, and the Gottfried recombinant plasmid. Positive results were not observed with other rotavirus specimens and the negative controls.

The presence of rotavirus nucleic acid on the membranes was confirmed by disassociation of the Gottfried gene 4 probe followed by hybridization of the membrane with a full-length Gottfried gene 9 probe under low stringency conditions (42°C, 0% formamide, and 5X SSC [1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) (Fig. 16). Previous studies in our laboratory have shown this probe to be cross-reactive with heterologous serotypes of rotaviruses under low stringency conditions (47) as documented in Fig. 16.

Serological and deduced amino acid sequence analyses of the VP4 polypeptide of Gottfried porcine rotavirus and the asymptomatic human rotaviruses have indicated a high degree of homology (16). We investigated the homology and the rotavirus gene segment specificity of the Gottfried gene 4 probe with the asymptomatic human rotaviruses of G serotypes 1 to 4 by Northern blot hybridization (Fig. 17). The Gottfried probe produced moderate to strong hybridization signals with the genomic segment 4 of Gottfried porcine rotavirus and the asymptomatic human rotaviruses M37 (G serotype 1), 1076 (G serotype 2), McN13 (G serotype 3), and ST3 (G serotype 4). Comparatively weak hybridization signals were observed with the symptomatic human rotaviruses Wa and VA70, while no hybridization signal was observed with OSU porcine rotavirus.

Specificities of the partial length Gottfried and OSU gene 4 PCR-derived probes

The specificity of the Gottfried probe with rotaviruses of human and animal origin is shown in Fig. 18. The Gottfried PCR-derived probe was more specific than the full length Gottfried probe and hybridized only with the homologous Gottfried porcine rotavirus and recombinant plasmid. Hybridization was not observed with other human and animal rotavirus samples, the OSU recombinant plasmid, and the negative controls.
Hybridization reactions of the OSU PCR-derived probe with human and animal rotavirus strains also showed a higher degree of specificity in comparison with the full length OSU probe (Fig. 19). The OSU PCR-derived probe produced strong hybridization signals with OSU cell culture-propagated rotavirus, OSU recombinant plasmid, and equine H1 rotavirus. A weak hybridization signal was also observed with the homologous SB-1A animal-passaged rotavirus. Hybridization signals were not observed with other human and animal rotavirus samples (including Gottfried cell culture-propagated rotavirus and recombinant plasmid) and the negative controls.

The sensitivity of the OSU PCR-derived probe for the detection of other strains of animal rotaviruses with OSU-like VP4 segments and the specificity of the probe for genomic segment 4 were examined by dot and Northern blot hybridization (Fig. 20). The OSU probe hybridized with the genomic segment 4 of the cell culture-adapted OSU, EE, A580, and SB-1A strains of porcine rotaviruses and equine H1 rotavirus by Northern blot hybridization. Similar hybridization reactivities were observed with these porcine rotavirus strains by dot hybridization. As in the dot hybridizations in Fig. 19, positive signals were not observed by Northern blot hybridization with the NCDV, SA11, and Gottfried strains of cell culture-propagated rotaviruses (Fig. 20).

Dot hybridization reactions of the Gottfried PCR-derived probe with animal-passaged G serotype 4 porcine rotaviruses possessing Gottfried-like VP4 segments are shown in Fig. 21. The Gottfried probe hybridized with the animal-passaged Gottfried (samples 1 to 3), SB2 (sample 4), SB3 (samples 5 and 6), and SB5 (sample 7) strains of G serotype 4 porcine rotaviruses and with the positive controls (Gottfried cell culture-propagated rotavirus and recombinant plasmid). Weak hybridization signals were observed with the OSU control samples (OSU cell culture-propagated rotavirus and recombinant plasmid), however, the signals were not as strong as those observed for the animal-passaged G serotype 4 rotaviruses and Gottfried positive controls. Hybridization was not observed with the
animal-passaged G serotype 4 reassortant rotavirus SB-1A (sample 8) or with the negative controls (nucleic acid extracted from mock-infected MA104 cells and the intestinal contents from three rotavirus-negative gnotobiotic pigs, samples 9 to 11).

Hybridization reactions of the OSU PCR-derived probe with animal-passaged OSU rotaviruses were similar to the homologous reactions observed with the Gottfried PCR-derived probe. However, cross-reactivity was not observed with the Gottfried cell culture-propagated rotavirus and recombinant plasmid (data not shown).

DISCUSSION

In this study, full-and partial-length gene 4 probes were produced from Gottfried and OSU genomic segment 4 cDNA. The probes were prepared for use as potential diagnostic reagents for the differentiation of the genomic segment 4 of porcine rotaviruses. To determine the hybridization stringency conditions necessary for optimal probe specificity and sensitivity, the probes were tested against a diverse group of human and animal rotaviruses of heterologous group A serotypes and against representative group B and C porcine rotaviruses.

The full length Gottfried and OSU gene 4 probes produced strong hybridization signals with the homologous porcine rotaviruses and recombinant plasmids containing Gottfried or OSU gene 4 cDNA inserts. Moderate to weak hybridization signals were observed, however, with some heterologous rotavirus strains. In general, rotaviruses of homologous P serotypes could be differentiated from heterologous P serotypes of rotaviruses by using cell culture-adapted rotavirus strains in the dot hybridization assays. The heterologous hybridization signals, however, are a potential source of problems for differentiating low-titer rotaviruses which give weak homologous reactions from high-titer rotaviruses which give strong heterologous reactions in field samples. Consequently, the partial-length PCR-derived gene 4 probes, which were more specific and equally sensitive, were better
candidates for use as diagnostic reagents. Hybridization signals were not observed with either full-or partial-length gene 4 probes with group B or C rotaviruses.

Hybridization reactions of the full length Gottfried gene 4 probe with human and animal rotaviruses produced moderate to weak hybridization signals with the asymptomatic human rotaviruses (M37, 1076, McN13, and ST3) in dot or Northern blot hybridization assays. These results agreed with previous deduced amino acid sequence comparisons indicating a relatively high degree of homology (87.1 to 88%) between the VP4s of these rotavirus strains (16). Unexpected hybridization signals were observed with the heterologous symptomatic human rotavirus Wa and the porcine rotavirus OSU in dot hybridizations. Comparisons of the deduced amino acid sequences of VP4 between Gottfried rotavirus and symptomatic human rotaviruses (e.g., Wa) have revealed 77.5 to 77.8% amino acid sequence homology (16). Similar comparisons between the Gottfried and OSU porcine rotaviruses have determined 72.1% VP4 amino acid sequence homology (16). A possible explanation for the cross-reactivity observed with the Wa and OSU rotavirus strains was revealed by hybridization of one membrane a second time with a full length Gottfried gene 9 probe at low stringency (Fig.16). Although attempts were made to dot equivalent amounts of rotavirus dsRNA based on A260 readings, differences in rotavirus titers in cell culture preparations resulted in the dotting of a larger quantity of Wa and OSU rotavirus RNA. The heterologous hybridization signals observed with these two rotavirus strains may have been due to the larger quantity of rotavirus RNA dotted and interactions of the conserved regions of the full length Gottfried gene 4 probe with the OSU and Wa rotavirus RNA samples. This conclusion is supported by results from the Northern blot hybridization studies. In this assay, the amount of rotavirus RNA used was based on A260 readings and the intensity of ethidium bromide stained rotavirus RNA visualized in polyacrylamide gels. Hybridization reactions of the full-length Gottfried gene 4 probe with rotavirus RNA preparations of approximately equivalent staining
intensity resulted in moderate to strong hybridization signals with the genomic segment 4 of Gottfried porcine rotavirus and the asymptomatic human rotaviruses, comparatively weak hybridization signals with the symptomatic human rotaviruses Wa and VA70, and no hybridization signals with OSU porcine rotavirus.

The hybridization of both full length and PCR-derived OSU gene 4 probes with the cell culture-propagated G serotype 5 equine H1 rotavirus was not expected, since previous dot hybridization studies by our laboratory demonstrated hybridization of an OSU gene 9 probe with equine H1 rotavirus (47). These results indicate shared P and G serotype specificities, which further corroborates previous serological studies indicating shared neutralization specificities between G serotype 5 porcine rotaviruses and equine H1 rotavirus (25,26). The genetic homology shared between other RNA segments of these two rotaviruses has not been determined. Equine rotaviruses with G serotype 5 specificities have rarely been detected in serological surveys of horses in the United States and England (4,19). Equine H1 rotavirus, originally isolated from a foal in England, may represent a unique laboratory strain.

The Gottfried and OSU PCR-derived gene 4 probes were designed by using specific oligonucleotide primers to encompass areas of major sequence diversity among rotavirus serotypes in the VP8 coding region and to eliminate large areas of conserved nucleic acid sequences. Although hybridization results with the PCR-derived probes were more specific than those with the full-length gene 4 probes, equivalent hybridization signals were observed with the homologous porcine rotaviruses. Dot hybridizations of the Gottfried PCR-derived gene 4 probe with the pig-passaged G serotype 4 porcine rotaviruses produced hybridization signals with the SB2, SB3, and SB5 strains of rotavirus. These results paralleled previous dot hybridization studies in our laboratory indicating sequence homology between the VP7 coding genes of Gottfried rotavirus and the SB2, SB3, and SB5 rotavirus strains (46). Although the VP4 and VP7 coding genes of the SB2, SB3, and SB5
strains of rotavirus are similar to those of Gottfried rotavirus and indicate shared P and G serotype specificities, serological analyses have indicated that these porcine rotavirus strains are not identical (3,25,28).

The dot hybridization of the PCR-derived Gottfried gene 4 probe, in contrast to that of full-length gene 4 probe, did not produce a hybridization signal with the asymptomatic human rotavirus ST3. Comparisons of the deduced amino acid sequence encoded by the PCR-derived Gottfried gene 4 cDNA with the corresponding region of the related asymptomatic human rotavirus 1076 indicated an amino acid homology of only 70%. The lower percentage of amino acid homology suggests a lower nucleic acid homology between the PCR-derived gene 4 probe and the asymptomatic human rotaviruses, which could account for this change in probe reactivity.

The ability of the OSU PCR-derived gene 4 probe to detect porcine rotaviruses with similar gene 4 segments was demonstrated by dot and Northern blot hybridizations with SB-1A, EE, A580, and OSU cell culture-adapted porcine rotaviruses. These results confirmed previous RNA-RNA hybridization studies indicating a high degree of homology between the gene 4 segments of OSU, EE, and SB-1A porcine rotaviruses (12,35). Although genetic relationships between the gene 4 segments of the OSU and A580 strains of porcine rotavirus have not previously been established, serological studies in our laboratory have indicated cross-neutralization (unpublished data). The OSU PCR-derived probe was effective in the detection of OSU rotavirus extracted from intestinal contents of infected gnotobiotic pigs in addition to the cell culture-propagated rotavirus strains.

Extensive characterization of the partial length PCR-derived OSU and Gottfried gene 4 probes developed in this study has shown them to be specific and sensitive reagents for the differentiation of porcine rotaviruses. The hybridization reactivity of the probes with porcine rotaviruses corresponded with results of previous serological and genetic studies. The gene 4 probes, in conjunction with our previously developed Gottfried and OSU gene 9
probes, currently provide a full complement of reagents for determining the prevalence of the Gottfried and OSU P and G serotypes among field specimens. The analysis of rotaviruses from swine herds with both gene 4 and gene 9 probes should provide information for determining which vaccine strains in relation to P and G neutralization specificities should be developed and administered for controlling rotavirus diarrheal disease. An additional benefit will be the capability to detect new or previously unrecognized P or G rotavirus serotypes in swine herds. Investigations are currently underway in our laboratory to develop nonradioactive detection procedures for the potential adaptation of these reagents for use in diagnostic laboratories.

REFERENCES


FIG. 14. Dot hybridization of the full-length Gottfried gene 4 probe with human and animal rotaviruses. The G and G/P serotype designations of the group A rotaviruses are shown in parentheses above the strain designations. Nucleic acid samples were diluted fivefold and applied to the membranes as follows: (i) cell culture-adapted group A rotaviruses (human, simian, bovine, equine, and porcine OSU and Gottfried [Gott]), and mock-infected MA104 cells, applied in amounts from 500 to 20 ng; (ii) OSU and Gottfried recombinant plasmids (OSU P and Gott P, respectively) applied in amounts from 250 to 10 ng; and (iii) nucleic acid extracted from the intestinal contents of rotavirus-infected gnotobiotic pigs, group B rotavirus (Gp B RVLV), group C rotavirus (Gp C PaRV), the SB-1A group A rotavirus, and rotavirus negative [Rota (-)] gnotobiotic pigs, applied in amounts from 1000 to 40 ng.
FIG. 15. Dot hybridization of the full length OSU gene 4 probe with human and animal rotaviruses. The samples probed were the same as those in Fig. 14.
FIG. 16. Rehybridization of the membrane shown in Fig. 14 with a full length Gottfried gene 9 probe. The full length Gottfried gene 4 probe was stripped from the nylon membrane as described in the text. The membrane was reprobed with a Gottfried gene 9 probe under low-stringency conditions (42°C, 0% formamide, and 5X SSC) to confirm the fixation of rotavirus dsRNA samples on the membrane.
FIG. 17. Northern blot hybridization of the full length Gottfried gene 4 probe. The G serotype designations of the group A rotaviruses are shown in parentheses following the strain designations. (Left) dsRNA electrophoretic patterns of eight group A human and animal rotaviruses resolved in a 10% polyacrylamide gel and stained with ethidium bromide; (right) autoradiogram depicting the hybridization of the Gottfried gene 4 probe with the eight group A rotaviruses following electrophoretic transfer of the rotavirus RNA to a nylon membrane.
FIG. 18. Dot hybridization of the Gottfried PCR-derived gene 4 probe with human and animal rotaviruses. The samples probed were the same as those in Fig. 14.
FIG. 19. Dot hybridization of the OSU PCR-derived gene 4 probe with human and animal rotaviruses. The samples probed were the same as those in Fig. 14.
FIG. 20. Northern and dot hybridizations of the OSU PCR-derived gene 4 probe. The G and G/P serotype designations of the group A rotaviruses are shown in parentheses following or above the strain designations. (Top left) dsRNA electrophoretic patterns of eight group A animal rotaviruses resolved in a 10% polyacrylamide gel and stained with ethidium bromide; (top right) autoradiogram depicting the hybridization of the OSU probe with the eight group A rotaviruses following electrophoretic transfer of the rotavirus RNA to a nylon membrane; (bottom) dot hybridization of the OSU probe with cell culture-adapted porcine rotaviruses and nucleic acid extracted from mock-infected MA104 cells. The amount of sample applied was the same as in Fig. 14, except for the OSU sample on the far right, which was applied at a single undetermined amount to aid in blot orientation.
FIG. 21. Dot hybridization of the Gottfried PCR-derived gene 4 probe with gnotobiotic pig-passaged G serotype 4 porcine rotaviruses. Samples 1 to 3, Gottfried rotavirus; sample 4, SB2 rotavirus; samples 5 and 6, SB3 rotavirus; sample 7, SB5 rotavirus; sample 8, SB-1A rotavirus; samples 9 to 11, nucleic acid extracted from the intestinal contents of rotavirus-negative gnotobiotic pigs; MA104, nucleic acid extracted from mock-infected MA104 cells; OSU C/C and Gott. C/C, OSU and Gottfried cell culture-adapted rotaviruses, respectively; OSU P and Gott. P, OSU and Gottfried recombinant plasmids, respectively. Nucleic acid samples were diluted fivefold and applied to the membranes as follows: samples 1 to 11, applied in amounts from 1,000 to 40 ng; all other samples, applied in amounts from 250 to 10 ng.
TABLE 2. Cell culture-adapted and gnotobiotic pig-passaged rotaviruses used for nucleic acid hybridization with Gottfried and OSU full- and partial-length gene 4 probes.

<table>
<thead>
<tr>
<th>Rotavirus Strain</th>
<th>Origin of virus</th>
<th>Serotype (Defined by G type) or group</th>
<th>Serotype Defined by P typea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wa^b</td>
<td>Human</td>
<td>1</td>
<td>8 (1)</td>
</tr>
<tr>
<td>M37^c</td>
<td>Human</td>
<td>1</td>
<td>6 (3)</td>
</tr>
<tr>
<td>DS-1^b</td>
<td>Human</td>
<td>2</td>
<td>4 (2)</td>
</tr>
<tr>
<td>1076^c</td>
<td>Human</td>
<td>2</td>
<td>6 (3)</td>
</tr>
<tr>
<td>M^b</td>
<td>Human</td>
<td>3</td>
<td>--</td>
</tr>
<tr>
<td>McN13^c</td>
<td>Human</td>
<td>3</td>
<td>-- (3)</td>
</tr>
<tr>
<td>SA11</td>
<td>Simian</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>RRV</td>
<td>Simian</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>VA70^b</td>
<td>Human</td>
<td>4</td>
<td>8 (1)</td>
</tr>
<tr>
<td>ST3^c</td>
<td>Human</td>
<td>4</td>
<td>6 (3)</td>
</tr>
<tr>
<td>Gottfried</td>
<td>Porcine</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>SB-1A^d</td>
<td>Porcine</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>SB2</td>
<td>Porcine</td>
<td>4</td>
<td>6e</td>
</tr>
<tr>
<td>SB3</td>
<td>Porcine</td>
<td>4</td>
<td>6e</td>
</tr>
<tr>
<td>SB5</td>
<td>Porcine</td>
<td>4</td>
<td>6e</td>
</tr>
<tr>
<td>OSU</td>
<td>Porcine</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>EE</td>
<td>Porcine</td>
<td>5</td>
<td>7e</td>
</tr>
<tr>
<td>A580</td>
<td>Porcine</td>
<td>5</td>
<td>7e</td>
</tr>
<tr>
<td>H1</td>
<td>Equine</td>
<td>5</td>
<td>7e</td>
</tr>
<tr>
<td>NCDV^f</td>
<td>Bovine</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>B223</td>
<td>Bovine</td>
<td>10</td>
<td>--</td>
</tr>
<tr>
<td>--</td>
<td>Porcine</td>
<td>Group B</td>
<td>--</td>
</tr>
<tr>
<td>--</td>
<td>Porcine</td>
<td>Group C</td>
<td>--</td>
</tr>
</tbody>
</table>

^aAccording to the tentative P serotyping scheme for animal and human rotaviruses proposed by Estes and Cohen (10). Serotypes in parentheses are according to the P serotyping scheme for human rotaviruses proposed by Gorziglia et al. (16). --, not determined.

^bSymptomatic strains of human rotaviruses.

^cAsymptomatic strains of human rotaviruses.

^dNaturally occurring reassortant rotavirus: VP7 is similar to that of Gottfried porcine rotavirus; VP4 is similar to OSU porcine rotavirus.

^eP serotype as determined using the Gottfried and OSU gene 4 PCR-derived probes in this study and classified according to the serotyping scheme of Estes and Cohen (10).

^fNCDV, Nebraska calf diarrhea virus.

^gNot determined.
CHAPTER V
SEROTYPIC DIFFERENTIATION OF PORCINE ROTAVIRUSES IN FIELD SAMPLES FROM DIARRHEIC PIGS USING PORCINE VP4- AND HUMAN AND PORCINE VP7-
SPECIFIC NUCLEIC ACID PROBES

INTRODUCTION

Rotaviruses are eleven-segmented double-stranded RNA viruses that are intestinal pathogens of infants and the young of many animal species. In swine and in other animal species, rotaviruses cause intestinal malabsorption resulting in diarrhea and sometimes death (11,34,64). Rotaviral diarrhea has been noted primarily in nursing and newly weaned pigs (7,33,64).

Serological characterization of group A rotaviruses is based on neutralization determinants located on the two outer capsid proteins, VP4 (a protease sensitive protein which determines the P serotype specificity) and VP7 (a glycoprotein which determines the G serotype specificity). Two G serotypes of rotavirus have been characterized in swine in the United States (8,57). These viruses, represented by the prototype Gottfried (G4, P5) and OSU (G5, P7) strains, were initially reported 9 to 16 years ago (8,57). Rotaviruses with similar G serotype specificities have been detected in swine in several other countries (9,29,38,55). The genes coding for the P and G neutralization determinants may segregate independently in mixed infections. Evidence for this is based on serological and hybridization studies of a porcine rotavirus field isolate (SB-1A) with Gottfried- and OSU-like serological specificities originally isolated by our laboratory (Saif, L. J., and E. H. Bohl, unpublished) and characterized by Hoshino et al. (27) and Midthun et al. (40), and a porcine isolate with a similar mixed serotype specificity reported from Australia (42).
Recently two other G serotypes of rotavirus were isolated from swine and characterized. These include the YM rotavirus (serotype G11) isolated in Mexico and several strains of G3 rotaviruses isolated in Australia (1,29,41,52). Serological evidence of G11 and G3 infections in swine have been reported in Venezuela and in Thailand and Argentina, respectively (4,35,48). Genetic and serological analyses of the YM rotavirus confirmed that the G serotype specificity of the virus is distinct from other known animal and human rotaviruses (52), while the deduced amino acid sequence of its gene segment 4 is similar to the OSU rotavirus (36). Other serotypes of rotavirus have been detected in swine but have not been fully characterized (4,46).

Information related to the serotype diversity and prevalence of rotaviruses in swine have direct implications for the development of effective vaccines. Porcine rotavirus vaccines currently in use in the U.S. have been designed using representative strains of serotype G4 and G5 porcine rotaviruses (62). Investigators studying porcine rotavirus vaccines have reported variable efficacies (26,62). Although efforts are being made to design rotavirus vaccines that offer heterotypic protection (30), most current rotavirus vaccine strategies have been directed towards the induction of homotypic immunity to serotypes of rotavirus prevalent in a particular area (13,39,47).

Our laboratory has previously used nucleic acid probes directed against the genes coding for VP7 and VP4 for the characterization of porcine and bovine rotavirus serotypes (44,45,49,50,51). The specificity of these probes and similar probes prepared from human rotaviruses have been demonstrated in dot and Northern blot hybridization studies against homologous and heterologous serotypes of rotaviruses (12,31,44,45,49,51). In this study VP4 (P)- and VP7 (G)-specific probes prepared from the Gottfried (G4, P6) and OSU (G5, P7) strains of porcine rotaviruses were used to examine and evaluate the serotype specificities of circulating strains of rotaviruses in fecal or intestinal samples collected from diarrheic pigs obtained from the U.S. and to a limited extent, Canada. Nucleic acid probes prepared
from the VP7 coding genes of the human rotaviruses, Wa (G1), DS-1 (G2), P (G3), and the porcine rotavirus YM (G11) were also used to determine the presence of rotaviruses with similar serological specificities in swine.

MATERIALS AND METHODS

Viruses

Human and animal cell culture-adapted rotaviruses of known serotype were used to confirm the serotype specificity of the probes. The rotavirus strains used in the assays included the following: Wa (G1); DS-1 (G2); M, SA11, and rhesus rotavirus (RRV) (G3); VA70, ST3, and Gottfried (G4); OSU and equine H1 (G5); Nebraska calf diarrhea virus (NCDV) (G6); and B223 (G10). The viruses were propagated in MA104 cells as previously described (51).

Porcine rotavirus strains propagated by passage in gnotobiotic pigs (53) were as follows: Gottfried, SB2, SB3, SB5, and SB-1A (G4); OSU (G5); porcine serogroup B rotavirus; and porcine serogroup C rotavirus.

Field Samples

A total of 216 porcine fecal or intestinal samples submitted to our laboratory from several states in the U.S. and Canada or collected locally from herds in Ohio were analyzed for rotaviruses. Fifty seven samples, collected between the years 1985 to 1992, were determined to be group A rotavirus-positive by RNA electrophoresis. The 57 group A rotavirus-positive samples were obtained from pigs in California (n=1, submitted by A. Castro, California Veterinary Diagnostic Laboratory Services, Davis); Canada (n=3, submitted by R. Magar, Food Production and Inspection Branch, Agriculture Canada, Quebec, and n=1, submitted by S. Carmen, Ontario Ministry of Agriculture and Food, Veterinary Laboratory Services, Guelph, Ontario); Illinois (n=2, submitted by G. B. Fritz,
Rotavirus RNA extracted from the 57 rotavirus-positive field samples were analyzed by hybridization with the Gottfried and OSU gene 9 probes. Because of limited quantities of some fecal and intestinal samples, the following number of samples were analyzed with the remaining porcine and human probes: 1) 52 samples were analyzed with the Gottfried (P6) and OSU (P7) gene 4 probes, and the YM (G11) gene 9 probe; and 2) 25 samples were analyzed with the Wa (G1), DS-1 (G2), and P (G3) gene 9 probes.

**Rotavirus nucleic acid extraction**

The extraction and deproteinization of rotavirus dsRNA from cell culture-passaged rotaviruses, rotavirus-positive and -negative fecal and intestinal samples, and mock-infected MA104 cells were performed as previously described (49,50).

**Nucleic acid electrophoresis and rotavirus RNA quantification**

Nucleic acid extracted from cell culture-passaged rotaviruses and fecal or intestinal samples were examined by PAGE in 12.5% polyacrylamide gels (49). The RNA electrophoretic patterns of rotavirus-positive samples were visualized by staining with silver nitrate (25).
The relative amounts of rotavirus dsRNA extracted from cell culture-passaged rotaviruses were estimated by comparisons of the intensity of silver stained viral dsRNA following analysis by PAGE. Estimates of the amounts of rotavirus dsRNA was performed to enable dotting of equivalent amounts of RNA for dot hybridizations in experiments to determine the specificity of the probes. Nucleic acid extracted from the intestinal contents of rotavirus infected and noninfected gnotobiotic pigs, and recombinant plasmids, were applied to the membranes as previously described (49).

**Preparation of $^{32}$P-labeled probes**

Plasmid vectors containing full-length cDNA copies of gene segments 4 and 9 (coding for VP4 and VP7, respectively) of the Gottfried, OSU, and YM porcine rotavirus strains were used for the preparation of partial-length probes. The plasmid vectors and bacterial hosts containing the Gottfried and OSU VP4 and VP7 coding genes were provided by Mario Gorziglia, National Institutes of Health, Bethesda, Maryland (20-22, 43). The YM gene 4 and gene 9 segments cloned in pGEM3Z plasmids were used to transform the DH5α strain of *Escherichia coli*. The recombinant plasmids were extracted from cultures of transformed *E. coli* by the alkali lysis minipreparation procedure of Sambrook et al. (54).

Partial length clones of the VP7 coding gene (gene 9) of the human rotaviruses Wa, DS-1, and P cloned in PUC 13 plasmids were used as templates for subsequent polymerase chain reaction (PCR) amplifications. Individual cultures of the DH5α strain of *E. coli* containing one of the human rotavirus recombinant plasmids were prepared in medium containing ampicillin. The recombinant plasmids were extracted from the bacterial hosts by the mini-prep procedure described above.

Partial-length gene 4 cDNAs were produced from the Gottfried, OSU, and YM gene 4 recombinant plasmids using specific oligonucleotide primers and PCR as previously described (49). The partial-length cDNAs encompassed nucleotides 211 to 607 (Gottfried)
and 212 to 612 (OSU and YM) of the gene 4 segments. The variable nucleic acid sequences in this region were selected for amplification because of previous associations of this region with the neutralization determinants responsible for serotype differences among VP4 proteins (10,32,37). Partial length gene 4 PCR products of the expected length were confirmed by agarose gel electrophoresis and comparison with molecular size markers. The gene 4 PCR products were partially purified by centrifugation through Centricon 30 microconcentrators (Amicon Division, W. R. Grace & Co., Conn.) prior to labeling with 32P-dCTP.

The recombinant plasmids containing gene 9 cDNA segments of the Gottfried, OSU, and YM porcine rotaviruses and the human rotaviruses Wa, DS-1 and P were used as templates for the production and amplification of partial-length gene 9 cDNA by PCR. The strategy used for production of the gene 9 PCR products was similar to that used for the gene 4 segments described above. The partial-length gene 9 cDNA's encompassed nucleotides 51 to 392 of the porcine and human rotavirus gene 9 segments. Previous nucleic acid sequence analysis studies have determined that this region contains three of six variable regions involved in determining serotype specificity (18,20,23). The specific oligonucleotide primers used in the PCR reaction were identical to primers described for the production of human rotavirus gene 9 probes (14), except the primer complementary to nucleotides 51 to 71 was 5'GTATGGTACTGAATATACCAC3'. The PCR reaction was performed as previously described (49). PCR products of the expected size as determined by agarose electrophoresis were semi-purified by centrifugation through Centricon 30 microconcentrators. Partial-length gene 4 and 9 PCR-derived probes were prepared by nick translation (nick translation system; GIBCO BRL, Gaithersburg, Md.) with 32P-dCTP (ICN Biomedicals Inc., Irvine, Calif.; specific activity 650 Ci/mmol).
Nucleic acid sample preparation and hybridization conditions

The nucleic acid samples were analyzed by dot hybridization. Nucleic acid samples selected for analyses were denatured at 100°C for 5 min and then cooled at 0°C for 5 min. Ribonucleic acid samples extracted from cell culture-passaged rotaviruses were dotted onto nylon membranes (Zetaprobe; Bio-Rad Laboratories, Richmond, Calif.) in volumes of 5 µl with a micropipette. Nucleic acid samples extracted from field samples were applied to the nylon membranes with a filtration manifold (HybriDot Manifold; Bethesda Research Laboratories). The nucleic acid samples were fixed to the nylon membranes by baking for 0.5 h at 80°C in a vacuum oven (National Appliance Company, Tualatin, Oreg.) and stored at 4°C.

The hybridization procedure, solutions, and final wash steps were performed as previously described (50), except dextran sulfate was omitted from the hybridization solution since its contribution in shortening the hybridization time was not deemed essential in this study. The hybridizations of the PCR-derived gene 9 probes were initially conducted at 42°C or 52°C, 50% formamide, and 5X SSC (1X SSC is 0.15M NaCl plus 0.015M sodium citrate) to determine the stringency conditions necessary for optimal probe specificity and sensitivity. Visual inspection of the intensity and specificity of the signals on the autoradiographs resulted in later hybridizations being conducted at 42°C. The stringency conditions necessary for optimal probe specificity of the PCR-derived Gottfried and OSU gene 4 probes were previously established and determined to be 42°C, 50% formamide, 5X SSC for the Gottfried probe and 42°C, 40% formamide, 5X SSC for the OSU probe (49). The optimal stringency conditions for the YM gene 4 probe were similar to the OSU probe. Approximately 1 X 10^6 cpm of heat denatured probe was added per milliliter of hybridization solution.
RESULTS

Nucleic acid electrophoresis

Nucleic acid extracted from cell culture-passaged rotaviruses and fecal or intestinal samples were analyzed by polyacrylamide gel electrophoresis (data not shown). The RNA electrophoretic patterns of the rotavirus strains of known serotype used in this study were identical to RNA electropherotypes previously published (28,51). Fifty seven samples out of a total of 216 porcine field samples analyzed had dsRNA patterns characteristic of group A rotaviruses. The group A-positive samples were selected for further study with the human and porcine nucleic acid probes. Seven group A-positive field samples were characterized with more than 11 dsRNA segments indicating multiple rotavirus strains. The 159 group A rotavirus-negative field samples contained nucleic acid patterns characteristic of group B rotavirus (n=1), group C rotavirus (n=3), or no recognizable or visible nucleic acid (n=151). Each of the four remaining samples contained 2 segments of nucleic acid with electrophoretic patterns similar to those previously observed in fecal preparations from diarrheic cattle or swine (15,60).

Specificity of the PCR-derived gene 9 probes

The specificity of the PCR-derived gene 9 probes was determined by hybridization reactions with dsRNA extracted from rotaviruses of human and animal origin. Dot hybridizations of the Gottfried PCR-derived gene 9 probe are shown in Figure 22. The Gottfried probe was specific and produced strong hybridization signals with the Gottfried cell culture-passaged rotavirus and the recombinant plasmid containing the Gottfried gene 9 cDNA insert. A hybridization signal was also observed with the gnotobiotic pig-passaged SB-1A rotavirus which has previously been determined to have a gene 9 segment similar to the Gottfried rotavirus (40). Weak hybridization signals were observed with nucleic acid extracted from the heterologous OSU cell culture-passaged rotavirus and the
OSU recombinant plasmid which contained the OSU gene 9 cDNA insert. The intensity of these signals, however, were significantly weaker than the hybridization reactions with the corresponding homologous samples. Hybridization signals were not observed with other group A human and animal rotavirus samples, the porcine group B and C rotaviruses, and the negative controls (mock-infected MA104 cells and rotavirus-negative intestinal samples).

The Gottfried PCR-derived gene 9 probe, contrary to our full-length probe (51), did not produce hybridization signals with the serotype G4 human rotaviruses, VA70 and ST3. The overall objectives of this study were to develop diagnostic probes capable of characterizing rotaviruses of similar G serotype specificities in swine. In order to confirm this capability, the Gottfried PCR-derived probe was hybridized with several animal-passaged porcine rotaviruses previously demonstrated to be serologically related to Gottfried (Fig. 23). The Gottfried probe produced hybridization signals with the homologous Gottfried rotavirus and the SB2, SB3, SB5, and SB-1A strains of serotype G4 porcine rotaviruses.

Hybridization reactions of the OSU PCR-derived gene 9 probe with human and animal rotavirus strains revealed the OSU probe was also specific, with hybridization signals detected only with the OSU (G5) and equine H1 (G5) rotavirus strains, the OSU recombinant plasmid, and the porcine rotavirus strains A580 (G5) and EE (G5) (data not shown). The hybridization signals with the A580 strain, however, were weak compared to hybridization signals with the OSU and EE rotaviruses. The specificity of the PCR-derived probes prepared from the human rotaviruses Wa, DS-1, and P were confirmed by hybridization reactions with homologous and heterologous serotypes of rotaviruses of human and animal origin (data not shown). Investigations of the specificity of the YM PCR-derived gene 9 probe (G11) were conducted in a similar manner (Fig. 24). In this case, however, we did not have access to the YM porcine rotavirus strain (due to importation
restrictions) or another serologically confirmed G11 rotavirus to use as a positive control. The recombinant plasmid containing the YM gene 9 cDNA insert was used in this assay as the positive control. In order to correlate studies of the specificity of the YM gene 9 probe with the other gene 9 probes investigated, hybridizations were conducted with a mixture of the YM and OSU gene 9 probes (Fig. 24A). The exposure time of the autoradiograph following the hybridization and subsequent wash steps was adjusted to allow sufficient time for the detection of hybridization signals with RNA extracted from the OSU cell culture-passaged rotavirus. Hybridization reactions of the YM and OSU porcine rotavirus gene 9 probes with rotaviruses of human and animal origin produced strong signals with the OSU (G5) and equine H1 (G5) rotaviruses and the YM recombinant plasmid (containing a YM gene 9 cDNA insert). A weak hybridization signal was observed with the Gottfried rotavirus (G4) while no hybridization signals were observed with the other heterologous rotavirus strains. The hybridization reaction of the YM gene 9 probe alone did not produce hybridization signals with the OSU, equine H1, or Gottfried rotavirus strains (Fig. 24B).

**Specificity of the PCR-derived gene 4 probes**

The specificity of the OSU and Gottfried PCR-derived gene 4 probes with heterologous serotypes of rotaviruses have previously been demonstrated (49). Hybridization reactions of the YM gene 4 probe with rotaviruses of human and animal origin are shown in Figure 25. The strategy used for the determination of the specificity of the YM PCR-derived gene 4 probe was similar to that used for the YM gene 9 probe. In this study, however, cohybridizations were conducted with the YM and Gottfried gene 4 probes (Fig. 25A). The Gottfried and YM gene 4 probes produced hybridization signals with the equine H1, OSU, and Gottfried rotavirus strains and the YM recombinant plasmid (containing a YM gene 4 cDNA insert). The hybridization of the YM gene 4 probe alone produced signals with the
Analysis of porcine field samples

Hybridization analyses were performed on porcine field samples determined to be group A rotavirus-positive by PAGE. The hybridization results of the Gottfried gene 9 probe with 15 representative porcine field samples are shown in Figure 26. Hybridization signals were observed with the homologous gnotobiotic pig-passaged Gottfried rotavirus and field samples 1, 12, and 13.

The OSU gene 4 probe was hybridized with a replicate membrane that contained the same samples shown in Figure 26 (Fig. 27). The OSU probe produced hybridization signals with the gnotobiotic pig-passaged OSU rotavirus and field sample 12. As noted, the Gottfried gene 9 probe and the OSU gene 4 probe produced hybridization signals with field sample 12 (isolated from a pig in Ohio). These results indicated the detection of another porcine rotavirus isolate with VP4 and VP7 coding genes similar to the SB-1A rotavirus. The hybridization reactions of the Gottfried and OSU probes with the rotavirus-positive porcine field samples identified the following number of serologically related viruses: Gottfried gene 9 probe (G4), 15.8% (9 of 57); Gottfried gene 4 probe (P6), 3.8% (2 of 52); OSU gene 9 probe (G5), 7.0% (4 of 57); and OSU gene 4 probe (P7), 13.5% (7 of 52).

Because of the large number of samples which were unreactive with the Gottfried and OSU gene 4 and 9 probes, investigations were conducted to determine whether other serotypes of rotaviruses were present in swine. Hybridization analyses were conducted with PCR-derived gene 9 probes prepared from the human rotaviruses Wa (G1), DS-1 (G2), and P (G3), and the porcine rotavirus YM (G11). Hybridization reactions with the human rotavirus P and the YM gene 9 probes were of particular interest due to previous studies reporting serological evidence or the isolation of G3 and G11 rotaviruses from swine (4,35,41,48). Although G1 and G2 rotaviruses have not previously been isolated from swine,
one research group reported serological evidence for these serotypes based on serotyping
enzyme-linked immunosorbent assays with monoclonal antibodies specific for human G1
and G2 rotaviruses.(4).

No rotaviruses with homologous serotype specificities to the Wa (G1), DS-1 (G2) and P
(G3) rotaviruses were detected in hybridizations with the respective human rotavirus PCR-
derived gene 9 probes in 25 porcine field samples analyzed. In contrast, hybridization
signals were detected with 21.1% (11 of 52) of the field isolates analyzed with the YM gene 9
probe. The hybridization results of the YM gene 9 probe with 12 representative porcine field
samples that contained G11-positive field specimens is shown in Figure 28. The G11-
positive samples were collected (between the years 1989 to 1992) from swine from three
farms in Ohio and from swine maintained at the Ohio Agricultural Research and
Development Center. The G11-positive samples collected from two different locations were
obtained from outbreaks of diarrhea in nursing pigs. The G and P serotype specificities of
the typable field isolates identified in this study are summarized in Table 3. As noted,
G11-positive field samples were also identified in samples collected from swine in
California and Nebraska (Table 3, samples M, and Q). The P serotype specificity of only
one G11 rotavirus was determined to be OSU or YM-like (Table 3, sample V). None of the
samples had P specificities homologous to the Gottfried gene 4 probe.

Although a few field samples produced hybridization signals with more than one probe,
in all but two samples the serotype identity was determined from the stronger signal
intensities observed on autoradiographs with one particular probe. One porcine field
sample from Kansas that could not be characterized produced hybridization signals of
similar intensity with the Gottfried and YM gene 9 probes (Table 3, sample R). Analysis of
the RNA electrophoretic pattern of this sample by PAGE revealed only 11 discernible
segments of dsRNA. A second sample produced hybridization signals with both the
Gottfried and OSU gene 9 probes (Table 3, sample K). Analysis of the nucleic acid
extracted from this sample by PAGE revealed more than 11 segments of dsRNA (Table 3, sample K).

DISCUSSION

In this study, fecal and intestinal samples collected from group A rotavirus-infected swine from several states in the U.S. and to a limited extent Canada, were investigated to determine the prevalence and diversity of rotavirus P and G serotypes. The analyses were performed by nucleic acid hybridization, with initial efforts focused on the use of PCR-derived gene 4 and gene 9 probes prepared from the Gottfried (G4, P6) and OSU (G5, P7) porcine rotavirus strains. Because a relatively large number of samples (n=48, gene 9 probes; n=43, gene 4 probes) were unreactive with these probes, further studies were conducted with gene 9 probes prepared from the porcine rotavirus YM (G11) and the human rotaviruses Wa (G1), DS-1 (G2), and P (G3).

The PCR-derived gene 9 probes used in this study were similar to probes previously described in investigations of the G serotype diversity of human rotaviruses in clinical specimens (14). The rotavirus probes used in the human study were specific and their efficacy in determining the serotype specificities of rotaviruses was equal to monoclonal antibody-based typing assays. The PCR-derived gene 9 human and porcine probes used in this study were also extensively investigated and were effective in differentiating previously characterized, serologically defined rotaviruses. The hybridization stringency conditions for the gene 9 probes and our previously reported gene 4 probes (49) were adjusted to allow maximum sensitivity in detecting homologous serotypes of rotavirus, while maintaining serotype specificity.

Hybridization analysis of 57 and 52 group A rotavirus-positive porcine field samples with gene 9 and 4, respectively, Gottfried and OSU PCR-derived probes identified 17 samples with homologous VP4- or VP7- coding genes. Although there have been few
epidemiologic studies on rotavirus serotypes in swine in the U.S., one study by our laboratory on serum samples from breeding-age swine in Ohio reported neutralizing antibodies to the OSU strain of porcine rotavirus in 94% of 274 samples, and in each of 75 herds sampled (8). Although the reason for the failure to detect a higher proportion of OSU-like rotaviruses in the current study is unknown, several observations may partially account for this discrepancy. First, fluctuations in the prevalence of rotavirus serotypes have been documented in numerous studies on the temporal distribution of rotaviruses in children (2,5,19,63). Similar changes in the predominance of rotavirus serotypes may occur in animal populations, and thus may account for the decreased detection of OSU-like rotaviruses. Second, hybridization reactions of the OSU gene 9 probe with the A580 strain (G5) of porcine rotavirus in this study and a previous study (50) consistently produced weak hybridization signals compared to homologous reactions with the OSU rotavirus. Hybridization reactions of the OSU gene 4 probe, however, produced moderate to strong hybridization signals (49). The number of rotaviruses with VP4 and VP7 antigenic specificities similar to the A580 strain that may have contributed to the occurrence of neutralizing antibodies to OSU in the original serological survey is unknown. Similarly, the number of A580-like strains that could not be detected by hybridization analysis with the OSU gene 9 probe in this study could also not be assessed. Third, the high percentage of swine with neutralizing antibodies to OSU may also reflect neutralizing antibody responses induced by repeated infections with heterotypic rotaviruses (24,56,61,65).

Hybridization analyses of the rotavirus-positive field samples with the Gottfried and OSU probes detected one rotavirus with a VP7 coding gene similar to the Gottfried rotavirus (G4), and a VP4 coding gene similar to the OSU rotavirus (P7). The RNA electrophoretic pattern of the rotavirus in this sample was characterized by 11 RNA segments and did not indicate the presence of multiple strains. This virus possessed characteristics similar to the SB-1A rotavirus (a naturally occurring reassortant) that was originally isolated from
swine (Saif, L. J., and E. H. Bohl, unpublished). Rotaviruses with two G serotype specificities have also been isolated from a pig in Australia and from an asymptomatic human infant (40,42).

The large number of rotavirus-positive samples that were unreactive with the Gottfried and OSU probes suggested that rotaviruses with other P and G serotype specificities may be present in swine. Hybridization reactions with PCR-derived gene 9 probes prepared from the human rotaviruses Wa (G1), DS-1 (G2), and P (G3) did not detect rotaviruses with respective homologous serotype specificities in 25 of the 57 rotavirus-positive field samples of which sufficient quantity was available for analysis. Hybridization reactions with the porcine rotavirus YM (G11) gene 9 probe, however, produced hybridization signals with 11 of 52 porcine field samples analyzed. Ten of the G11-positive field samples identified were obtained from swine in Ohio (n=8), California (n=1), and Nebraska (n=1). Another field sample from Kansas produced hybridization signals with the YM and Gottfried gene 9 probes and could not be serologically distinguished. This study represents the first report of G11 rotaviruses in the U.S. The isolation of G11 rotaviruses from diverse regions of the U.S. indicates G11 rotaviruses may be widespread in many swine herds in the U.S. The discovery of G11 rotaviruses as the most frequently detected G-type among the swine samples we studied is of great importance to the swine industry, veterinary practitioners, and rotavirus vaccine producers in the U.S. Porcine rotavirus vaccines currently in use are specifically directed only against rotavirus serotypes G4 and G5 (26,62). The efficacy of these vaccines in stimulating immunological protection against G11 rotavirus strains is unknown.

The nucleic acid sequence of the YM gene 4 segment was previously reported in a study by Lopez et al. (36). In this study, comparisons of the deduced amino acid sequence of the YM VP4 protein with the reported VP4 sequences of rotaviruses of different serotype specificities indicated a high degree of homology with the OSU VP4 protein (97% overall
identity). Similar comparisons with the VP4 of other animal rotaviruses identified 82.6 and 81.9% identity with the SA11 and NCDV rotaviruses, respectively, while the lowest homology was observed with human rotavirus strains (68.5 to 70.7%). Hybridization reactions of the YM gene 4 probe with the OSU and equine H1 rotaviruses in our study confirmed previous amino acid homologies, and indicated the serotype specificity of the YM VP4 is P7. Furthermore, the hybridization stringency conditions used in our study were sufficient in preventing hybridization signals with other rotavirus serotypes of human and animal origin. An important observation pertaining to the hybridization reactions of the OSU gene 4 probe (essentially equivalent to the YM gene 4 probe) with the G11-positive field samples was that contrary to the original YM porcine isolate, only one field sample had a P serotype specificity similar to the YM VP4 gene (Table 3, sample V). This observation suggests the presence of other P serotype specificities in swine, and confirms previous observations concerning the independent reassortment of the genes determining VP4 and VP7 specificities (27,40).

The P and G serotype specificities of rotaviruses which were unreactive with the probes in this study are currently under investigation in our laboratory. Hybridization studies are also being conducted to determine the relationship of the A580 rotavirus strain with other serotype G5 rotaviruses, and to consider the inclusion of VP7-specific probes from this strain to detect rotaviruses with similar homologies in porcine field samples. The potential for detecting additional P and G serotypes in swine is likely, based on recent studies indicating the presence of G serotypes previously associated only with animal rotaviruses in humans and vice versa (3,6,17,41). Pigs, of all experimental animals that have been studied, have been demonstrated to be the most susceptible to infection or disease when infected with rotaviruses of human and animal (nonporcine) origin (16,58,66). The need for constant monitoring of the antigenic diversity of rotaviruses in swine is important for the development of effective porcine rotavirus vaccines and to identify new
rotavirus serotypes which may have the potential to cause zoonotic infections in humans (3,6,17,41,59).

REFERENCE


FIG. 22. Dot hybridization of the PCR-derived Gottfried gene 9 probe with human and animal rotaviruses. The G (S1 to S6, S4/5, S10) serotype specificities of the group A rotaviruses are shown in parentheses above the strain designations. The nucleic acid samples were diluted fivefold and applied to the membranes. The human, simian, bovine, equine, and porcine, OSU and Gottfried (Gott), were cell culture-passaged rotaviruses. Additional nucleic acid samples (bottom row) included for analysis were as follows: mock-infected MA104 cells; gnotobiotic pig-passaged serogroup B (Gp B RVLV) and C (Gp C PaRV) rotaviruses, two rotavirus negative pig samples [Rota (-)]; gnotobiotic pig-passaged SB-1A group A rotavirus; OSU recombinant plasmid (OSU P); and the Gottfried recombinant plasmid (Gott P).
FIG. 23. Dot hybridization of the PCR-derived Gottfried gene 9 probe with serotype G4 gnotobiotic pig-passaged rotaviruses. The nucleic acid samples were diluted fivefold and applied to the membranes. Samples 1 to 3, Gottfried rotavirus; sample 4, SB2 rotavirus; samples 5 and 6, SB3 rotavirus; sample 7, SB5 rotavirus; sample 8, SB-1A rotavirus; samples 9 to 11; nucleic acid extracted from the intestinal contents of rotavirus negative gnotobiotic pigs; nucleic acid extracted from mock-infected MA104 cells; OSU and Gott C/C, OSU and Gottfried cell culture-passaged rotaviruses, respectively; OSU and Gott. P, OSU and Gottfried recombinant plasmids, respectively.
FIG. 24. Dot hybridization of the PCR-derived YM and OSU gene 9 probes with human and animal rotaviruses. A). Hybridization of equivalent amounts (in terms of cpm) of the YM and OSU gene 9 probes with cell culture-passaged group A rotaviruses, nucleic acid extracted from mock-infected MA104 cells (MA104), and the homologous YM recombinant plasmid (YM P). The G serotype specificities (S1 to S6, S10) of the group A rotaviruses are shown in parentheses above the strain designations. B). Hybridization reactions of the YM gene 9 probe with group A animal rotaviruses. The amount of probe used (in terms of cpm) was equivalent to the amount of YM gene 9 probe used in panel A. The hybridization signals in panels A and B were produced simultaneously by exposure of the membranes to a single X-ray film.
FIG. 25. Dot hybridization of the PCR-derived YM and Gottfried gene 4 probes with human and animal rotaviruses. A). Hybridization of equivalent amounts (in terms of cpm) of the YM and Gottfried gene 4 probes with cell culture-adapted group A rotaviruses, nucleic acid extracted from mock-infected MA104 cells (MA104), and the homologous YM recombinant plasmid (YM P). The serotype specificities of the group A rotaviruses (S1 to S6, S10), as determined by their VP7 or G specificities, are shown in parentheses above the strain designations. B). Hybridization reactions of the YM gene 4 probe with group A animal rotaviruses. The amount of probe used (in terms of cpm) was equivalent to the amount of YM gene 4 probe used in panel A. The hybridization signals in panels A and B were produced as described for Figure 24.
FIG. 26. Hybridization of the Gottfried PCR-derived gene 9 probe with porcine field samples. The field samples were applied to the membranes in volumes of 50 ul, 20 ul, and 5 ul. Lanes 1 to 15, rotavirus-positive field samples; lanes 16 and 17, rotavirus-negative field samples. Positive controls included two individual samples of both the OSU and Gottfried (Gott) rotavirus strains extracted from the intestinal contents of rotavirus-infected gnotobiotic pigs.
FIG. 27. Hybridization of the OSU PCR-derived gene 4 probe with porcine field samples. The membrane was one of several replicates which were dotted with the same samples shown in Figure 26.
FIG. 28. Hybridization of the PCR-derived YM gene 9 probe with porcine field samples. The field samples were applied to the membranes in volumes of 50 μl and 15 μl. The samples shown in this autoradiogram differed from the field samples shown in Fig. 26. Lanes 1 to 12, rotavirus-positive field samples. Bottom row (left to right), two rotavirus-negative field samples, the OSU and Gottfried (Gott) rotavirus strains extracted from the intestinal contents of rotavirus-infected gnotobiotic pigs, the homologous YM recombinant plasmid containing a full-length YM gene 9 cDNA insert.
TABLE 3. The hybridization reactivities of the porcine rotavirus PCR-derived gene 9 (coding for the VP7 or G serotype specificity) and gene 4 (coding for the VP4 or P serotype specificity) probes with the group A rotavirus-positive field samples.

<table>
<thead>
<tr>
<th>Rotavirus Sample</th>
<th>Source of Sample</th>
<th>Gottfried VP4</th>
<th>Gottfried VP7</th>
<th>OSU VP4a</th>
<th>OSU VP7</th>
<th>YM VP7</th>
</tr>
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<tbody>
<tr>
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Fifty seven group A rotavirus-positive samples were analyzed with the Gottfried and OSU gene 9 probes, 52 samples were analyzed with the Gottfried and OSU gene 4 and YM gene 9 probes, and 25 samples were analyzed with the Wa (G1), DS-1 (G2), and P (G3) PCR-derived gene 9 probes. No hybridization signals were detected with the human rotavirus Wa, DS-1, and P gene 9 probes. Results of the probe-reactive samples are summarized above.
The hybridization reactivity of the OSU gene 4 probe was equivalent to the YM gene 4 probe. The YM gene 4 probe results were not shown.

Not determined due to insufficient quantity of field sample.

The samples exhibited multiple group A rotavirus RNA electrophoretic patterns when examined by PAGE followed by staining with silver nitrate.

The VP7 specificity could not be determined because of equivalent hybridization signals with two different probes.

Number of field samples positive with the respective probe.
The ability of probes prepared from specific gene segments to detect and differentiate rotaviruses of different serotype or subgroup specificities was first reported by Lin et al. (160) in investigations of human rotaviruses. Subsequent studies by this research group (161) and Dimitrov et al. (61) confirmed these initial findings, and determined that the specificity of the probes was dependent on the stringency conditions used during the hybridization. The serotype-specific probes used in these studies were prepared from the VP7 coding gene of the human rotavirus Wa and the simian rotavirus SA11. The production and application of probes from the VP7 coding gene from one or more rotavirus strains for the systematic characterization of the G serotype specificities of rotaviruses was not reported. Similarly, the potential and application of probes prepared from the VP4 coding gene for differentiating rotaviruses of different P serotype specificities was also not reported.

In this study, nucleic acid probes prepared from gene segments 4 and 9 coding for VP4 and VP7, respectively, were developed for the detection of porcine rotaviruses possessing P and G serotype specificities homologous to the parental strain from which the probes were derived. Initial investigations were conducted with probes prepared from cloned full length gene 9 cDNA inserts of the prototype Gottfried (G4) and OSU (G5) porcine rotaviruses. These strains were selected due to previous studies indicating the predominance of rotaviruses with similar G serotype specificities in swine (30,266). Although rotaviruses of different G serotype specificities generally possess 74% nucleotide identity in the VP7 coding gene (110,117), the stringency conditions selected for the hybridizations (52°C, 50%
formamide, and 5X SSC) were sufficient in minimizing heterologous cross-reactivity and optimizing hybridization signals with rotaviruses of homologous serotypes (224).

The full-length Gottfried and OSU gene 9 probes developed in this study were specific and were capable of differentiating RNA extracted from cell culture and gnotobiotic pig-passaged preparations of the respective rotaviruses. Further studies with other strains of porcine rotaviruses indicated the full-length Gottfried and OSU gene 9 probes could specifically hybridize with rotaviruses serologically related to Gottfried or OSU (224). Hybridization reactions of the Gottfried gene 9 probe with the serotype G4 porcine rotaviruses SB2, SB3, SB5, and SB-1A produced specific hybridization signals, with little or no cross-reactivity with the heterologous rotavirus OSU. A similar degree of specificity was observed in hybridization reactions of the full-length OSU gene 9 probe with the serotype G5 porcine rotaviruses OSU and EE, with little or no cross-reactivity with other G4 porcine rotaviruses. These results correlated with previous studies indicating 91 to 99% amino acid homology among the VP7 proteins of rotaviruses of the same serotype, and lower percentages of homology among rotaviruses of different serotypes (117).

The exclusive reaction of the Gottfried gene 9 probe with the naturally occurring porcine reassortant rotavirus SB-1A was of particular interest, due to previous studies indicating shared neutralization specificities with Gottfried and OSU (131). The basis of the dual serotype specificities of SB-1A was determined to be due to a high degree of homology of gene segment 9 with the Gottfried rotavirus (G4), and gene segment 4 with the OSU rotavirus (G5) (129,183). The nonreactivity of the OSU gene 9 probe with SB-1A confirmed the gene segment specificity of the Gottfried gene 9 probe, and indicated the potential of the probes to detect reassortant rotaviruses in field specimens.

The specificity of the full-length Gottfried and OSU gene 9 probes were later determined in studies with rotaviruses isolated from human beings and other animal (nonporcine) species. The Gottfried and OSU gene 9 probes specifically hybridized with rotaviruses of
homologous G serotype specificities regardless of the species of origin. Cross-species hybridization reactivities were observed with the Gottfried gene 9 probe with the homologous serotype G4 human rotaviruses VA70 and ST3, and the OSU gene 9 probe with the homologous G5 equine rotavirus strain H1. Little or no cross-reactivity was observed with heterologous serotypes of rotaviruses of human or animal origin.

The Gottfried and OSU PCR-derived gene 4 and 9 probes used in this study were produced following published reports of the regions of the gene coding for the VP4 and VP7 neutralization determinants (68,96,104,107,150,169,226). The generation of partial length cDNA by the polymerase chain reaction simplified the procedure necessary for the preparation of large quantities of specific cDNA for probe production.

An interesting observation in this study concerned the hybridization reactivity of the full-length OSU gene 9 probe with two strains of porcine rotaviruses with reported G5 serotype specificities. Although hybridization signals were detected with the serologically related EE strain of G5 porcine rotavirus, no hybridization signals were detected with the serotype G5 A580 strain (224). Subsequent studies with full-length and PCR-derived OSU gene 9 probes produced weak hybridization signals with the A580 rotavirus. In contrast, moderate to strong hybridization signals were observed in reactions with the OSU PCR-derived gene 4 probe (223). These results suggest that the A580 rotavirus may possess a VP7-coding gene that differs in nucleic acid sequence in some respects from the OSU gene 9 segment.

Although the hybridization reactivities of the full- and partial-length PCR-derived gene 9 probes produced in this study were identical in reactions with G4 porcine rotaviruses, differences in probe specificity were determined in hybridization reactions with human rotaviruses. These differences concerned the failure of the Gottfried PCR-derived probe to produce hybridization signals with the homologous G4 human rotaviruses ST3 and VA70. The partial-length porcine gene 9 probe developed in this study was identical in length and contained the same nucleotide regions (variable regions A, coding for amino acids 39 to 50; B,
coding for amino acids 87 to 101; and C, coding for amino acids 120 to 130) as partial-length gene 9 probes prepared for human rotaviruses (89). The hybridization specificity of the human rotavirus G4 probe with the Gottfried porcine rotavirus was not determined. The difference in reactivities of the Gottfried probes prepared from full or partial-length gene 9 segments should be considered in future studies. The inability of the Gottfried PCR-derived gene 9 probe to detect human rotaviruses with similar G serotype specificities indicate similar problems may be encountered in the analysis of rotaviruses in swine that possess antigenic and corresponding nucleic acid sequences that differ significantly from the Gottfried rotavirus.

The PCR-derived gene 4 probes developed and applied in this study represent the first systematic attempt to determine the P serotype specificities of rotaviruses in swine in the U.S. The PCR-derived gene 4 probes were more specific than full-length gene 4 probes and were required for the specific detection of rotaviruses with homologous P serotype specificities (223). Analyses of 52 rotavirus-positive field samples indicated that other P serotypes are present in rotaviruses in swine. Further work will be needed to determine the relationship of unreactive rotaviruses in the field samples with the P serotype specificities of previously established rotavirus strains.

Investigations of the SB2, SB3, and SB5 G4 porcine rotaviruses determined these strains possessed P serotype specificities homologous to the prototype Gottfried porcine rotavirus (serotype P6). These results agreed with the reactivity of VP4-specific monoclonal antibodies used for the characterization of distinct porcine and human rotavirus strains (145). Porcine rotaviruses with P serotype specificities homologous to the OSU rotavirus (P7) were identified in the G5 porcine rotavirus (EE), the A580 rotavirus, the G11 porcine rotavirus YM, and the naturally occurring G4 reassortant rotavirus SB-1A.

Hybridization analyses of rotavirus-positive porcine field samples with the Gottfried and OSU PCR-derived gene 9 probes determined a large number of unreactive samples. These
results prompted the development and use of gene 9 probes from several other G serotypes of rotaviruses. Hybridization analyses were subsequently conducted with gene 9 probes from the human rotaviruses Wa (G1), DS-1 (G2), and P (G3), and the porcine rotavirus YM (G11). The human rotavirus gene 9 probes were selected due to previous studies reporting the isolation or serological detection of rotaviruses with similar serotype specificities in swine (19,163,187,215). Hybridization analyses with the human rotavirus gene 9 probes did not detect any rotaviruses with homologous G serotype specificities. In contrast, hybridization signals were detected with 21.1% (11 of 52) field samples analyzed with the YM gene 9 probe. The G11-positive field samples identified in this study were obtained from swine in Ohio, California, and Nebraska. This study represents the first report of G11 rotaviruses in the U.S.

The hybridization protocols and nucleic acid probes developed in this study have provided the groundwork for an alternative method to monoclonal antibody-based assays for distinguishing rotavirus serotypes. Further refinements in the assay and the serotype-specific probes may provide a practical and complementary procedure to monoclonal antibody-based assays for the rapid differentiation of rotavirus isolates.
LIST OF REFERENCES


