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Development of specific nucleic acid probes for the differentiation of porcine rotavirus serotypes

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ABSTRACT

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A dot blot hybridization assay is described for the detection and differentiation of porcine rotavirus serotypes. Recombinant complementary DNA (cDNA) representing gene 9 (the gene encoding the neutralization antigens in VP7 glycoprotein) from OSU (porcine rotavirus serotype 1) and Gottfried (porcine rotavirus serotype 2) strains were used to determine the optimal hybridization conditions which allow specific detection of group A porcine rotaviruses. Probes were prepared by excision of the inserts from the recombinant plasmids and radiolabeling of cDNA with ³²P by the random primer extension method. Probes were hybridized at various stringencies with viral RNA from different rotavirus serotypes bound to nylon membranes. Hybridization at low stringency (26% base pair mismatch for stable hybrid formation) had high sensitivity but low specificity. Hybridization at high stringency (16% base pair mismatch for stable hybrid formation) produced high specificity but decreased the sensitivity observed at low stringency. Probes were specific for rotavirus at both stringencies and did not hybridize with nucleic acids from other porcine viruses. Subgenomic gene 9 fragments were then tested to provide more specific probes. A 322 bp fragment from OSU gene 9 between nucleotides 382 and 704 and a 266 bp fragment from Gottfried gene 9 between nucleotides 230 and 496 were found to be specific as hybridization probes. These studies demonstrated the feasibility of the dot blot hybridization assay using subgenomic fragments of gene 9 to detect and differentiate serotypes of porcine rotavirus. Additional studies are warranted to further evaluate the sensitivity and the capability of these probes to detect porcine field isolates of the same serotype.

INTRODUCTION

Rotaviruses, members of the family Reoviridae, are a major cause of viral diarrhea in many animals and man (Woode et al., 1974; Flewett and Woode, 1978; Estes et al., 1983). Rotaviruses have been grouped into five antigenically distinct groups (A to E) with group A probably being the most prevalent (Pedley et al., 1986). Group A rotaviruses share a common group antigen (Woode et al., 1976) on protein VP6, an inner capsid protein (Greenberg et al., 1983b). Group A rotaviruses have been further classified into two subgroups (I and II) based on the subgroup antigen also located on VP6

(Greenberg et al., 1983b; Estes et al., 1983). The majority of animal rotaviruses possess the subgroup I antigen while the majority of human rotaviruses have the subgroup II antigen (Hoshino et al., 1984). Porcine group A rotavirus strain OSU has a subgroup I antigen and the Gottfried strain of group A rotavirus has a subgroup II antigen (Hoshino et al., 1984). Neutralization antigens VP4, formerly VP3 (Liu et al., 1988), and VP7 are associated with the outer capsid of rotavirions (Greenberg et al., 1983c).

The viral genome of group A rotaviruses consists of 11 segments of double stranded RNA that migrate in a characteristic electrophoretic pattern in polyacrylamide gels (Kalica et al., 1978). The group A rotavirus common and subgroup antigens on VP6 are coded for in gene segment 6 (Estes et al., 1983; Greenberg et al., 1983a). Neutralization antigens of VP4 and VP7 are encoded by gene segments 4 and either 8 or 9, respectively (Kalica et al., 1981; Greenberg et al., 1983a). At least seven serotypes of group A rotaviruses have been reported in accordance with the unified scheme of serotyping rotaviruses from animals and man (Hoshino et al., 1984). In swine, two serotypes of group A rotaviruses have been recognized (Bohl et al., 1984). OSU and Gottfried strains have been designated as the prototype strains for porcine rotavirus serotypes 1 and 2 (Bohl et al., 1984). According to the unified serotyping scheme established for rotaviruses occurring in animals and man, OSU has been classified as serotype 5 and Gottfried as serotype 4. Recently, two new strains of porcine rotavirus were isolated in Iowa (Paul et al., 1988a). These new strains, designed ISU-64 and ISU-65, were shown by neutralization tests to be new porcine rotavirus serotypes. Two additional rotavirus serotypes have been reported in Mexico (Ruiz et al., 1988) and Australia (Nagesha and Holmes, 1988).

The most common methods for detection of rotavirus are: electron microscopy, enzyme-linked immunosorbent assay (ELISA) and polyacrylamide gel electrophoresis (PAGE). Serotyping of rotaviruses has been traditionally done by tissue culture isolation followed by plaque reduction neutralization (Hoshino et al., 1984) or fluorescent focus inhibition assays that use hyperimmune antisera to each of the rotavirus serotypes (Woode et al., 1983). Recently, several groups of investigators have developed serotype-specific monoclonal antibodies for use in ELISA for serotyping of rotavirus field isolates (Shaw et al., 1985; Coulsen et al., 1987; Taniguchi et al., 1987). While this approach appears promising, studies have shown that monoclonal antibody based ELISA may not detect all rotavirus isolates belonging to the same serotype (Coulson et al., 1987; Taniguchi et al., 1987). As an alternative, nucleic acid hybridization procedures for detection of rotavirus have been used (Pedley and McGrae, 1984; Lin et al., 1987). Similar techniques for differentiating rotavirus serotypes in humans using recombinant cDNA hybridization probes specific for gene segment 9 were demonstrated by Dimitrov et al. (1985) and Lin et al. (1987). In the present study, a dot blot hybridization

assay is described for the detection and serotypic differentiation of porcine rotavirus utilizing hybridization probes prepared from recombinant cDNA representing gene 9 from OSU and Gottfried strains (porcine rotavirus serotypes 1 and 2, respectively).

MATERIALS AND METHODS

Cells

Rhesus monkey kidney cells, MA-104 (National Veterinary Services Laboratory, Ames, IA), were used to propagate rotaviruses. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% Serum Plus (Hazelton Research Products, Inc., Lenexa, KS). The cell monolayers were washed twice with DMEM prior to inoculation with virus. The virus inoculum was pretreated with 10 μ g of trypsin (type IX; Sigma Chemical Co., St. Louis, MO) per ml for 1 h at 37°C. The inoculum was absorbed onto cultures of MA-104 cells for 1.5 h in roller bottles and the culture media was replaced with DMEM.

Swine testicular (ST) cells (American Type Culture Collection, Rockville, MD) were used to propagate transmissible gastroenteritis (TGE) virus and porcine parvovirus (PPV). Cells were maintained in Eagle's minimum essential medium (Hazelton Research Products, Inc., Lenexa, KS) supplemented with lactalbumin hydrolysate (0.5%) (Sigma Chemical Co., St. Louis, MO) and 2% Serum Plus. Virus inoculum was absorbed onto monolayers of ST cells for 1 h prior to addition of fresh medium.

Virus samples

Rotavirus strains were cultivated in MA-104 cells. The strains used were as follows: rhesus rotavirus (RRV), representing serotype 3; bovine rotavirus strain NCDV, representing serotype 6 and genetic reassortant rotaviruses RRV \times D and RRV \times DS-1 containing 10 genes from RRV and the VP7 gene from human rotavirus serotypes 1 and 2, respectively (obtained from Harry Greenberg, Veterans Administration Hospital, Palo Alto, CA); porcine OSU strain (serotype 5, porcine rotavirus serotype 1) originally isolated by Bohl et al. (1984) (obtained from Gerald Woode, Texas A & M University, College Station, TX); porcine Gottfried strain (serotype 4, porcine rotavirus serotype 2) originally isolated by Bohl et al. (1984) (obtained from Linda Saif, OARDC, Wooster, OH); porcine rotavirus isolates ISU-64 and ISU-65 (Paul et al., 1988a); porcine rotavirus serotype 1 field isolates 7360, and 7325 from Iowa; porcine rotavirus serotype 1 isolates 6167R, 6418, and 10986 (Janke et al., 1988) from infected pigs in Missouri (provided by Robert Solorzano, University of Missouri, Columbia, MO). Additional non-rotavirus samples included TGE virus Miller strain (ATCC, Rockville, MD) pseudorabies vi-

rus (PRV) strain K (Paul et al., 1982) and porcine parvovirus (PPV) strain NADL-2 (Paul and Mengeling, 1980).

Fecal specimens were collected from 3-day-old gnotobiotic piglets 1 day post-infection with rotaviruses. Pigs were infected with OSU-infected cell lysates, group C rotavirus Cowden strain (ATCC, Rockville, MD) or group B rotavirus (obtained from Ken Thiel, OARDC, Wooster, OH).

Purification of viruses and extraction of nucleic acids

After extensive cytopathic effect, virus infected cell cultures were frozen and thawed twice and cells and supernates were concentrated by centrifugation at $100\ 000\times g$ for 2 h. The pellets were resuspended in PBS and extracted with trifluorotrichloroethane (Genetron; E.I. du Pont de Nemours and Co., Wilmington, DE) and clarified by centrifugation at $800\times g$ for 20 min. Each viral suspension was pelleted through 30% sucrose at $100\ 000\times g$ for 3 h. The virus pellet was resuspended in PBS and virus particles were further purified by isopycnic centrifugation in cesium chloride density gradients (McCrae, 1985). The virus band was collected, virions pelleted at $100\ 000\times g$ for 1 h and the virus pellet resuspended in 1 ml of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Rotavirus double-stranded RNAs (dsRNAs) were obtained by extractions with an equal volume of phenol-chloroform (1:1) followed by a second extraction of the supernatant with an equal volume of chloroform. RNA in the supernatant was precipitated with ethanol by overnight incubation at -20°C followed by centrifugation at $13\ 000\times g$ in a micro-centrifuge. The RNA pellet was resuspended in TE buffer and stored at 4°C . Confirmation of viral RNA was achieved by electrophoresis in Laemmli 0.75 mm-thick slab gels (Laemmli, 1970) with 10% resolving and 3.5% stacking gels. Electrophoresis was performed at a constant current of 9 mA for 16 h at 4°C , and gels were stained with silver (Herring et al., 1982).

Viral RNA was extracted from fecal samples as previously described (Paul et al., 1988b). Briefly, 0.2 g fecal samples were diluted with 0.6 ml of sodium acetate buffer, sonicated and clarified by centrifugation at $13\ 000\times g$ for 1 min. The supernatants were harvested, extracted with 0.5 ml of phenol-chloroform and clarified by centrifugation at $13\ 000\times g$ in a micro-centrifuge for 1 min. The presence of viral RNA was confirmed by polyacrylamide gel electrophoresis.

Viral RNA was obtained from TGEV infected cell cultures by extraction with acid guanidium thiocyanate and phenol-chloroform as described previously (Chomczynski and Sacchi, 1987).

Porcine parvovirus DNA was isolated from ST cells infected with PPV by phenol-chloroform extraction (Davis et al., 1986). Pseudorabies virus DNA was isolated from porcine kidney cell line, MVPK, infected with PRV and purified on sodium iodine gradients (Paul et al., 1982). Cellular DNA from

uninfected MA-104 cells was obtained by previously described procedures (Davis et al., 1986).

Quantitation of nucleic acids

Rotavirus dsRNA extracted from cesium chloride purified virions was quantitated spectrophotometrically at a wavelength of 260 nm. Assuming dsRNA has an optical density (OD) similar to double stranded DNA, an OD of 1 corresponds to approximately 50 $\mu\text{g}/\text{ml}$ (Maniatis et al., 1982). Rotavirus dsRNA concentration was additionally confirmed by visual comparison of various dilutions of the virus dsRNA with dilutions of lambda DNA (BRL, Bethesda, MD) standards in 2% agarose gels stained with ethidium bromide. TGEV RNA, PPV DNA, PRV DNA, and MA-104 cellular DNA were quantitated spectrophotometrically.

Application of nucleic acids onto nylon membranes

Various concentrations of rotavirus dsRNAs were heat denatured (100°C, 5 min) in 200 μl 5 mM Tris-HCl (pH 7.5), 5 mM EDTA (Lin et al., 1987), quenched on ice, and further diluted with an equal volume of cold 20 \times SSC (1 \times SSC=0.15 M NaCl, 0.015 M sodium citrate). The denatured samples were filtered onto Zeta-Probe blotting membranes (Bio-Rad Laboratories, Richmond, CA) that had been presoaked with 20 \times SSC by using a 96-well filtration manifold (Schleicher and Schuell, Inc., Keene, NH). After air-drying, the membranes were baked in a vacuum oven for 2 h at 80°C. In addition to rotavirus dsRNAs, negative control nucleic acids were spotted onto the membranes. These included uninfected MA-104 cell DNA, yeast tRNA (Bethesda Research Laboratories, Bethesda, MD), *E. coli* 1625 DNA (provided by Michael Wannemuehler, Iowa State University, Ames, IA), TGEV RNA, PPV DNA and PRV DNA.

Preparation of ³²P-labeled probes

OSU gene 9 cDNA cloned in pBR322 plasmid (Gorziglia et al., 1983) was excised from the plasmid by *Pst*I cleavage. Gottfried gene 9 cDNA cloned in plasmid pTZ18R (Gorziglia et al., 1988) was excised from the plasmid by digestion with restriction enzymes *Hind*III and *Eco*RI. The cleavage products were separated on 0.8% agarose gels, the insert excised from the gel, separated from the gel matrix by phenol-chloroform extraction and concentrated by ethanol precipitation (Maniatis et al., 1982). Purified gene 9 cDNA inserts were resuspended in TE in appropriate volumes and stored at 4°C. Five subfragments were excised from gene 9 from both OSU and Gottfried cDNA inserts (Fig. 1 and Table 1). The five subfragments (A-E) from OSU gene 9 were obtained by digestion of the 1062 bp gene 9 cDNA insert with *Sau*3AI, *Taq*I, and *Bal*I restriction enzymes. The five subfragments (F-J) from Gottfried gene 9 cDNA were obtained by digestion with *Sau*3AI, and double

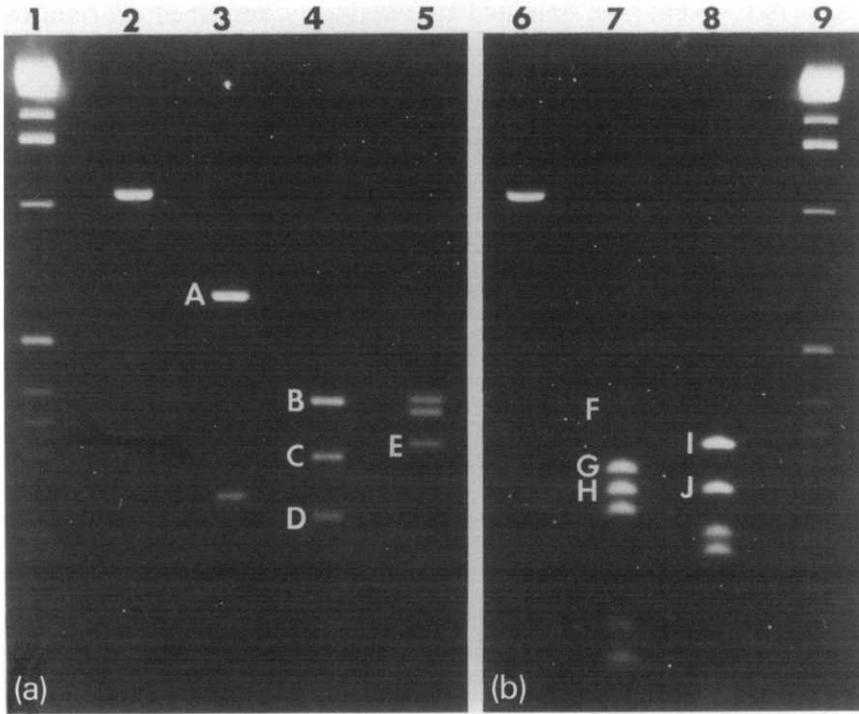


Fig. 1. Electrophoretic patterns of OSU gene 9 cDNA (panel A) and Gottfried gene 9 cDNA (panel B) cleaved with restriction endonucleases to produce cDNA subfragments (A–J) used as probes. OSU gene 9 cDNA (lane 2) was digested with *Sau3AI*, *TaqI* and *BamI* (lanes 3, 4 and 5 respectively). Gottfried gene 9 cDNA (lane 6) was digested with *Sau3AI* (lane 7) and with *AluI* and *BamHI* (lane 8). 1 kb DNA ladder was used as a marker for molecular size (lanes 1 and 9). Cleaved cDNA was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Subfragment F was not readily apparent in this photograph.

digestion with *AluI* and *BamHI*. The 322 bp subfragment (E) lying between nucleotides 382 and 704 of OSU gene 9 cDNA was excised with restriction enzyme *BamI*. The 266 bp subfragment lying between nucleotides 230 and 496 was excised with restriction enzymes *AluI* and *BamHI*. The subfragments were isolated in agarose gels and purified as previously described. The whole gene 9 cDNA inserts and the cDNA subfragments were labeled by the random primer extension method (Amersham, Arlington Heights, IL) using ^{32}P -dCTP (3000 Ci/mmol) according to the manufacturer's protocol. Specific activities of the radiolabelled probes ranged between 1×10^8 and 1×10^9 cpm/ μg of DNA.

Molecular hybridization

Several different parameters were chosen to vary the stringency conditions for hybridization. The stringency was adjusted by changing the temperature

TABLE I

OSU and Gottfried gene 9 subfragment probes

OSU gene 9 subfragments	Restriction enzyme	Digestion sites	Size (bp)
A	<i>Sau3AI</i>	230, 868	638
B	<i>TaqI</i>	284, 671	387
C	<i>TaqI</i>	284	283
D	<i>TaqI</i>	855	208
E	<i>BalI</i>	382, 704	322
Gottfried gene 9 subfragments			
F	<i>Sau3AI</i>	611, 986	375
G	<i>Sau3AI</i>	230, 517	287
H	<i>Sau3AI</i>	229	229
I	<i>AluI</i> and <i>BamHI</i>	496, 824	328
J	<i>AluI</i> and <i>BamHI</i>	230, 496	266

of hybridization, varying the salt concentration, and by the addition of different amounts of formamide. Increasing the temperature of the hybridization minimizes interaction of the probe with partially homologous sequences. As the salt concentration increases, the rate of hybridization increases. Conversely, the rate of hybridization decreases as formamide concentration increases. Formamide acts to lower melting temperature (T_m), the temperature at which nucleic acids are 50% denatured. Therefore, low temperatures (37–42°C) are usually used with high salt concentration (4× to 5×SSC) with the inclusion of 50% formamide. Hybridization performed at high temperatures (52 to 65°C) require low salt concentration (0.5× to 2.5×SSC) and may include formamide. Initial studies utilized the conditions reported by Dimitrov et al. (1985). Membranes with the immobilized nucleic acids were prehybridized in heat sealed plastic bags for 2 h at either 42°C or 56°C. For some membranes, prehybridization was carried out at 56°C with 13% formamide. For all membranes, the prehybridization solution consisted of 6×SSC, 4×Denhardt's solution (1×Denhardt's=0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll), 100 mM Tris (pH 7.5), 2 mM EDTA, 0.3% sodium dodecyl sulfate (SDS), and 200 µg/ml of sonicated, denatured salmon testes DNA (Sigma Chemical Co., St. Louis, MO). Hybridization was performed for 18–24 h in a solution (50–100 µl/cm² of membrane) of 4×SSC, 2×Denhardt's solution, and Tris, EDTA, SDS and salmon testes DNA as in prehybridization solution, 13% deionized formamide when required and ³²P-labeled, denatured probes.

Additional studies were performed utilizing a different set of stringency conditions. The prehybridization solution contained 2.5×SSC or 5×SSC, 100 mM Tris (pH 7.5), 7% SDS, 0.02% bovine serum albumin, 0.02% polyvinyl-

pyrrolidone, 0.02% Ficoll, 2 mM EDTA, 500 $\mu\text{g}/\text{ml}$ of salmon testes DNA/ml, and 50% deionized formamide. Prehybridization was carried out for 4 h at either 42°C in 5 \times SSC (0.75 M Na⁺) or at 52°C in 2.5 \times SSC (0.375 M Na⁺) (Flores et al., 1986). Hybridization was conducted for 18–24 h with the ³²P-labeled probes using the same reagents as in the prehybridization.

Following hybridization, membranes were washed by one of two procedures. Initial studies utilized the method described by Dimitrov et al. (1985) as follows: three times in 3 \times SSC–0.2% SDS, twice in 1.5 \times SSC–0.2% SDS and twice in 3 \times SSC. These washes were performed at the same temperature as the hybridization. Membranes hybridized in 50% formamide were washed according to the method reported by Flores et al. (1986). Membranes were washed four times at room temperature in 1 \times SSC–0.1% SDS, twice in 2 \times SSC–0.1% SDS, and twice in 1 \times SSC. The last two washes were done at the temperature of hybridization. The air-dried membranes were exposed to Kodak XAR film (Rochester, NY) with intensifying screens at –70°C. The exposure time of the autoradiographs varied between 4 and 24 h. The exposure time was considered correct when 1 ng of homologous dsRNA and 100 ng of heterologous dsRNA were both detected.

RESULTS

Optimization of hybridization conditions

Homology between pBR322 plasmid DNA sequences and *E. coli* DNA sequences was examined by hybridization of a ³²P-labeled pBR322 DNA probe to phenol–chloroform extracted porcine fecal samples (data not shown). The probe reacted with a number of the fecal samples whether positive or negative for the presence of rotavirus. These results strongly indicated the presence of homologous sequences in the genomes of pBR322 and *E. coli*. Because the strong signal sometimes created by this apparent homology could diminish its specificity to discriminate rotavirus serotypes, all future hybridizations were performed with the gene 9 cDNA inserts or selected regions from these inserts without the plasmid.

Rotavirus cDNA probes corresponding to gene 9 from OSU strain were tested for reactivity with heterologous RNA representing rotaviral serotypes 1 through 6 (Fig. 2A). Under conditions of low stringency (42°C, 4 \times SSC) with 43% base pair (bp) mismatch, reactions of each probe with homologous RNA from cell culture propagated virus was visibly stronger than reactions with heterologous RNA. However, under these conditions, some reactivity with RNA from heterologous rotavirus serotypes did occur. This reactivity was especially evident at the 100 ng level and most notable between OSU (porcine serotype 1) and Gottfried (porcine serotype 2). Increasing the temperature of hybridization to 56°C (33% bp mismatch) and 56°C with 13%

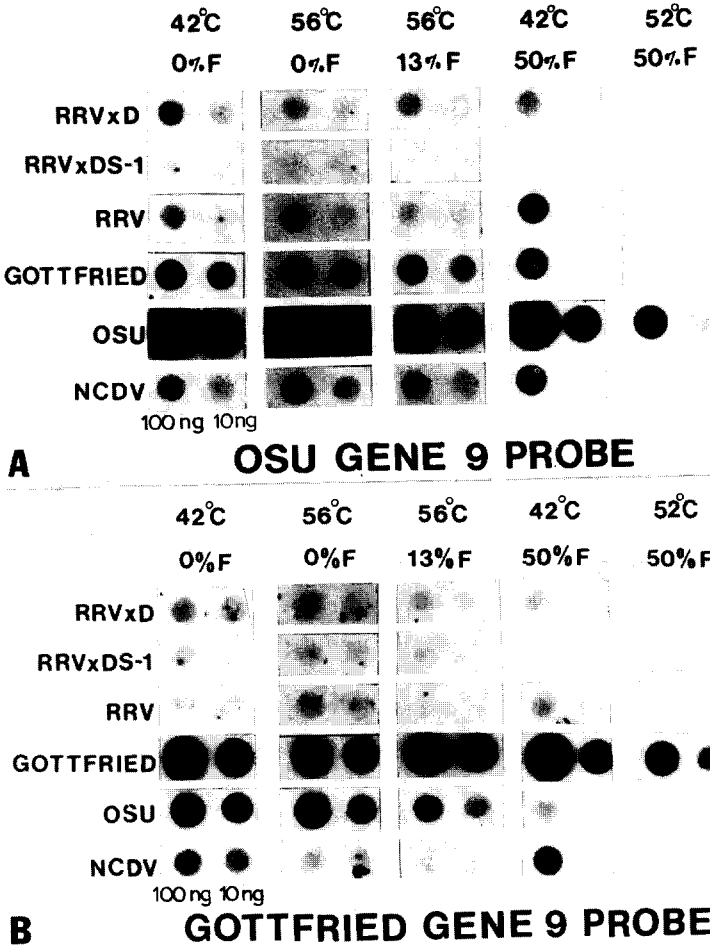


Fig. 2. Optimization of stringency conditions for the dot blot hybridization assay. Rotaviral RNA representing serotype 1 (RRV×D), serotype 2 (RRV×DS-1), serotype 3 (RRV), serotype 4 (Gottfried), serotype 5 (OSU), and serotype 6 (NCDV) were spotted at two concentrations (100 ng and 10 ng) and hybridized to OSU gene 9 (A) and Gottfried gene 9 (B) probes under various conditions of stringency ranging from low (42°C, 4×SSC, 0% formamide) to high (52°C, 2.5×SSC, 50% formamide).

formamide (28% bp mismatch) the reactivity with heterologous RNA was slightly decreased. These results led to the imposition of additional changes in the stringency conditions. Under conditions of low stringency (42°C, 5×SSC, 50% formamide) the allowable bp mismatch is 26% and the results were similar to those observed at 56°C, 13% formamide (28% bp mismatch). The OSU gene 9 probe did react with 100 ng of Gottfried RNA as well as with RNA from serotype 1 (RRV×D), serotype 3 (RRV), and serotype 6

(NCDV) rotavirus. However, the reactions of the Gottfried gene 9 probe (Fig. 2B) with heterologous RNA decreased appreciably with only minimal reaction at the 100 ng level with OSU and serotypes 1 (RRV×D) and 3 (RRV). A slight increase in reactivity with the Gottfried gene 9 probe and NCDV was observed under these conditions. Increasing the conditions of stringency (52°C, 2.5×SSC, 50% formamide) the allowable bp mismatch was decreased to 16%. Under these conditions, reactivity of both the OSU and Gottfried gene 9 probes with RNA from heterologous serotypes was eliminated. However, under these conditions, there was also a substantial decrease in the signal generated by hybridization of the probes with homologous RNA. The strong signal generated at the 10 ng level at low stringency (26% bp mismatch) was diminished substantially and barely apparent with the OSU gene 9 probe and homologous RNA at high stringency. Based on these observations, additional studies for differentiating rotavirus serotypes were performed at stringencies permitting 26% bp mismatch (42°C, 5×SSC, 50% formamide) and 16% mismatch (52°C, 2.5×SSC, 50% formamide).

Specificity and sensitivity of complete gene 9 cDNA probes

The specificity of the dot blot hybridization assays was further evaluated with the inclusion of rotavirus RNA from porcine field isolates used as positive controls, and nucleic acid from several other sources used as negative controls. Under conditions of low stringency (26% bp mismatch), the OSU gene 9 probe reacted very intensely with homologous RNA, as well as with RNA from porcine field isolates 7360, 10986, and RNA from a fecal sample taken from a gnotobiotic pig that had been infected with OSU (Fig. 3A). Moderate reactivity was also observed with RNA from porcine field isolates 7325, 6167R, 6418, and new porcine rotavirus serotype ISU-64. The diminished signal with these samples indicates a reduction in the homology of the nucleotide sequence of gene 9 from that with OSU. These differences were further amplified under conditions of high stringency (16% bp mismatch). Rotaviruses 7360, 10986, 7325, 6167R and 6418 have been serotyped as porcine rotavirus serotype 1. Hybridization of the OSU gene 9 at high stringency was only indicated in those samples with the strongest signal at low stringency (Fig. 3B). These included field isolates 7360, 10986, the gnotobiotic fecal sample, and, of course, homologous OSU RNA. In addition, reaction with RNA from heterologous serotype 1 (RRV×D), serotype 3 (RRV), serotype 6 (NCDV) and isolate ISU-64 seen at low stringency (Fig. 3A) were absent at high stringency (Fig. 3B). Field isolates 7325, 6167R, and 6418 did not react with the OSU probe under these conditions. However, as indicated previously, the signal generated with homologous OSU RNA was decreased from the 1 ng level to the 10 ng level. Specificity of the OSU gene 9 probe was further confirmed by the lack of hybridization with 100 ng of negative control nucleic acids from uninfected MA-104 cell DNA, *E. coli* DNA, yeast tRNA,

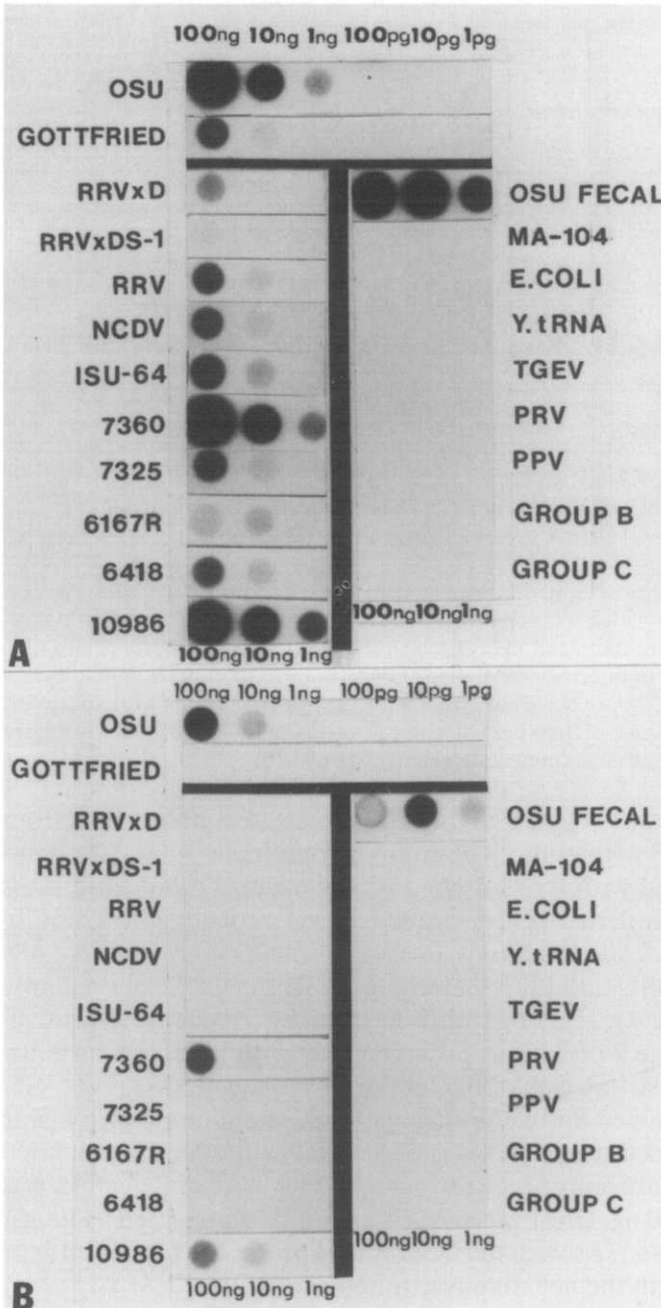


Fig. 3. Specificity and sensitivity of OSU gene 9 probe under conditions of low (A) and high stringency (B). Viral RNA from OSU and Gottfried were spotted in 10-fold dilutions (starting with 100 ng at the top left) on replicate membranes and hybridized to the OSU gene 9 probe. Positive control RNA (100 ng, 10 ng, and 1 ng) included porcine rotavirus isolates 7360, 7325, 6167R, 6418, and 10986. Negative control nucleic acid included uninfected MA-104 cell DNA, *E. coli* DNA, yeast tRNA, TGEV RNA, PRV DNA and PPV DNA. RNA from group B and group C rotavirus were spotted at three volumes of 100 μ l, 10 μ l and 1 μ l from phenol-chloroform extracts. In addition, RNA from rotavirus serotypes 1, 2, 3 and 6 were spotted along with ISU-64.

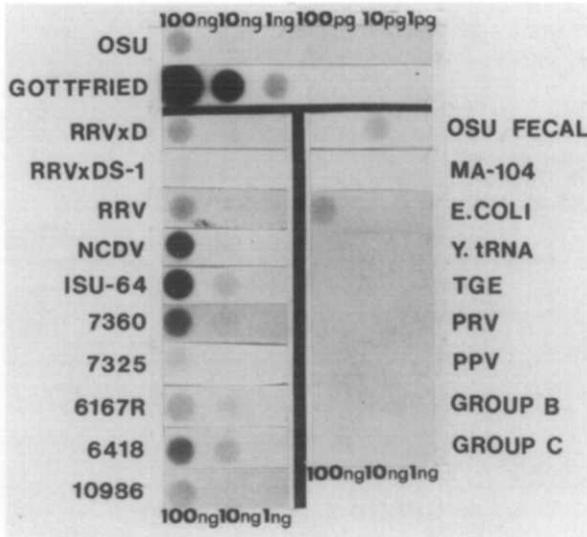


Fig. 4. Specificity and sensitivity of Gottfried gene 9 probe under conditions of low stringency. Viral RNA from OSU and Gottfried were spotted in 10-fold dilutions (starting with 100 ng at the top left) on replicate membranes and hybridized to the Gottfried gene 9 probe. Negative control nucleic acid included uninfected MA-104 cell DNA, *E. coli* DNA, yeast tRNA, TGEV RNA, PRV DNA, and PPV DNA. RNA from group B and group C rotavirus were spotted at three volume of 100 μ l, 10 μ l and 1 μ l from phenol-chloroform extracts. In addition, RNA from rotavirus serotypes 1, 2, 3, and 6 were spotted along with ISU-64.

TGEV RNA, PRV DNA, PPV DNA, and RNA from fecal samples taken from gnotobiotic pigs infected with group B or group C rotaviruses (Fig. 3B). Similar results were observed with the Gottfried gene 9 probe. Under conditions of low stringency, the Gottfried gene 9 probe reacted strongly with homologous Gottfried RNA and moderately with ISU-64, NCDV, 7360 and 6418 RNAs. Reaction with OSU and other heterologous RNAs was observed only at the 100 ng level (Fig. 4). Under conditions of high stringency, hybridization of the Gottfried gene 9 probe was observed only with homologous Gottfried RNA indicating a strong lack of nucleotide homology between the VP7 genes of prototype Gottfried and heterologous RNA (data not shown). The adverse effect of decreased sensitivity was observed again with the Gottfried gene 9 probe under conditions of high stringency. The level of detection was decreased from 1 ng to 10 ng. However, sensitivity could be increased by longer exposure (data not shown). As with the OSU gene 9 probe, the Gottfried gene 9 probe did not react with the negative control nucleic acids.

Selected regions of gene 9 as serotype specific hybridization probes

Results using complete gene 9 probes demonstrated the improved sensitivity (1 ng level) of the assay under conditions of low stringency. However,

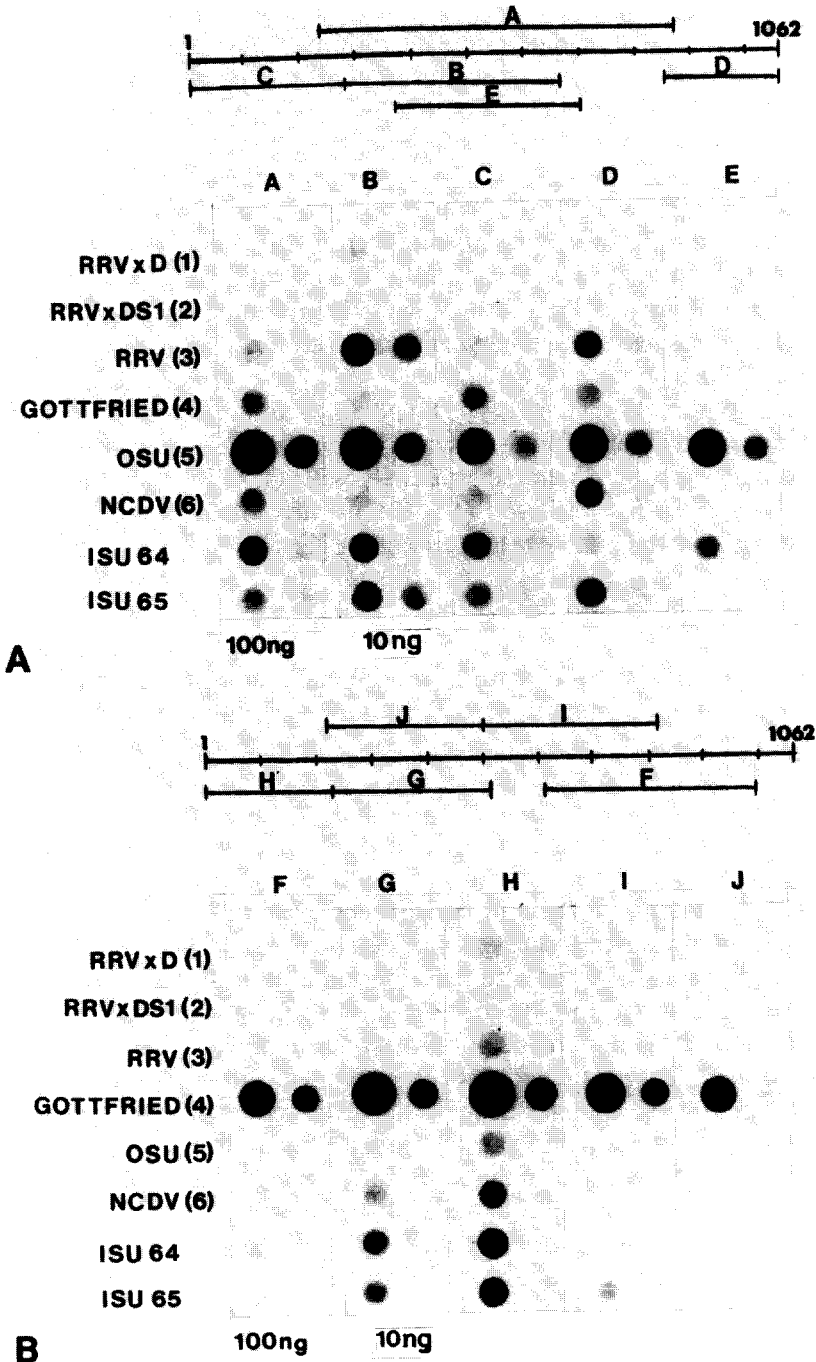


Fig. 5. Dot blot hybridization assay using probes prepared from selected regions of OSU (A) and Gottfried (B) gene 9 cDNA. Regions of gene 9 used as probes are depicted in schematic as subfragments A-J. Rotavirus RNA from infected cell cultures representing serotype 1 (RRV x D), serotype 2 (RRV x DS1), serotype 3 (RRV), serotype 4 (Gottfried), serotype 5 (OSU), serotype 6 (NCDV), and isolates ISU-64 and ISU-65 were spotted on replicate membranes at two concentrations (100 ng and 10 ng) and hybridized to the selected probes under low stringency hybridization conditions (42°C, 5xSSCD, 50% formamide). OSU and Gottfried subfragments F and I provided the most specific hybridizations.

under conditions of low stringency, the probes reacted with RNA from heterologous RNA, especially at higher concentrations. Therefore, we hypothesized that serotype specific probes prepared from selected regions of gene 9 and the utilization of low stringency conditions may improve the specificity while maintaining sensitivity. Five subfragments of gene 9 from OSU and Gottfried (Table 1) were ^{32}P -labeled and hybridized to heterologous rotavirus RNA under both high and low stringency conditions. Significant background was observed with subfragments A–D from OSU gene 9 and subfragments F–I from Gottfried gene 9 (Fig. 5). The 322 bp subfragment from OSU gene 9 cDNA (subfragment E) and the 266 bp subfragment from Gottfried gene 9 cDNA (subfragment J) were hybridized to rotavirus RNA from serotype 1 (RRV \times D), serotype 2 (RRV \times DS1), serotype 3 (RRV), serotype 4 (Gottfried), serotype 5 (OSU), serotype 6 (NCDV) and isolates ISU-64 and ISU-65 (Fig. 5). Under conditions of low stringency, the base pair mismatch is 26%. Subfragment probe E reacted primarily with OSU RNA but also reacted slightly with RNA from ISU-64 (Fig. 5A). The Gottfried subfragment probe J reacted only with Gottfried RNA (Fig. 5B).

DISCUSSION

Epidemiological studies to determine the prevalence and distribution of porcine rotaviruses have been hampered by the lack of suitable assays capable of both detection and differentiation of porcine rotavirus serotypes. Due to the involvement of two viral proteins, VP4, formerly VP3 (Liu et al., 1988), and VP7 in stimulating neutralizing antibodies, results from traditional methods can be ambiguous (Hoshino et al., 1987). Recently, a number of groups have developed VP7-serotype specific monoclonal antibodies (MAb) to be used with an enzyme-linked immunosorbent assay (ELISA) for the determination of human rotavirus serotypes (Coulson et al., 1987; Taniguchi et al., 1987). Although sensitive and efficient, these methods do have their limitations. Coulson et al. (1987) reported that rotavirus from some human stool specimens did not react with their specific MAb but did react with other MAbs to the same serotype. This non-reactivity of serotype-specific MAbs may have been caused by small base changes in the nucleotide sequence that codes for the neutralization epitopes within the VP7 gene (Dyall-Smith et al., 1986; Green et al., 1987) or possibly by changes in the proximity of the neutralization epitopes present in the protein's native form versus that of the linear molecule (Green et al., 1987; Morita et al., 1988). In another study, Taniguchi et al., (1987) reported the inability of a serotype-specific MAb based ELISA to determine the rotavirus serotype of 33% of infected human stool samples, possibly due to intraserotype antigenic variation. Dot blot hybridization assays have been utilized by a number of groups both for the detection of human rotavirus in clinical samples (Flores et al., 1983; Pedley and Mc-

Crae, 1984) and for the detection and characterization of enteric coronavirus infection of baby pigs (Shockley et al., 1987). Additional studies have been reported in which improved cDNA probes of gene-specific segments of the rotavirus genome were used to characterize human rotavirus by subgroup and serotype (Dimitrov et al., 1985; Lin et al., 1987; Flores et al., 1989).

Our initial studies used stringency conditions reported by Dimitrov et al. (1985) in which serotyping of rotaviruses was achieved with the use of cloned cDNA of gene 6 (which codes for the subgroup antigen) and gene 9 (which codes for the neutralization antigen). They reported that rotavirus serotypes 2 and 3 could be differentiated with the gene 9 cDNA probe by using conditions of low stringency (56°C), and comparison with the results obtained with hybridization at 56°C, 13% formamide. In addition, a further increase in the stringency conditions to 65°C, 7% formamide allowed differentiation of serotypes 1 and 4 from serotype 3. Based on the equation $T_m (\text{DNA-RNA}) = 79.8 + 18.5 \log (\text{Na}^+) + 58.4 (\%G+C) + 11.8 (\%G+C)^2 - 820 / \# \text{ bp in duplex} - 0.5 (\% \text{ formamide})$ (Wahl et al., 1987) where (G+C) was given a value of 0.43 (Flores et al., 1986), the base pair mismatch allowed for the formation of stable hybrids was 33% at low stringency and 24% at high stringency. However, Green et al. (1987) reported 15–29% divergence in the amino acid sequences of the VP7 proteins of several different human rotavirus serotypes and 91 to 99% homology among rotaviruses of the same serotype. In addition, 74% nucleotide identity between OSU and Gottfried strains has been reported (Gorziglia et al., 1988) indicating that the conditions of stringency would have to be increased to differentiate porcine rotavirus serotypes 1 and 2. The conditions of stringency used by Flores et al. (1986) were found to be suitable for our hybridization assay. Under low stringency (42°C, 5×SSC, 50% formamide) the calculated base pair mismatch was 26%. As expected, under these conditions some reactivity was observed with the cDNA probes and heterologous RNA. Under these conditions, however, a sensitivity level of at least 1 ng was achieved with RNA from homologous rotavirus. In addition, RNA from isolates 7360 and 7325 from Iowa and 6167R, 6418, and 10986 from Missouri, all typed as porcine rotavirus serotype 1, could be identified with the OSU probe (Fig. 3A). Reactivity was also observed with RNA from ISU-64, recently reported to be a new serotype of porcine rotavirus. Similar reactivity of ISU-64 with the Gottfried probe was observed (Fig. 4). These data indicate that ISU-64 gene 9 may have higher homology with OSU and Gottfried than that observed with OSU or Gottfried with other rotavirus serotypes. Use of this new porcine serotype is suggested for the assessment of the specificity of OSU and Gottfried probes in the detection of porcine rotavirus serotypes. Under conditions of high stringency (52°C, 2.5×SSC, 50% formamide) the allowable base pair mismatch for the formation of stable hybrids was reduced to 16%. Under these conditions, a high level of specificity was achieved as indicated by the elimination of reactivity

with heterologous RNA. However, these conditions decreased the level of sensitivity for homologous RNA from 1 ng at low stringency to only 10 ng (Fig. 3B). In addition, the reactivity of isolates 7360 and 10986 was greatly reduced and the reactivity with isolates 7325, 6167R, and 6418 was eliminated (Fig. 3B). These results indicate sufficient diversity may exist within the VP7 gene among rotaviruses of the same serotype that, at conditions of high stringency, they may not be identified.

Results obtained with different stringencies indicated sensitivity was higher at low stringency but specificity was lower. Based on these observations, we concluded that probes prepared from selected regions of gene 9 that are serotype specific and hybridized to rotavirus RNA under low stringency conditions may provide the specificity and sensitivity achieved at two different stringencies. Recently, several groups have determined the nucleotide sequence of the VP7 gene of more than 35 rotavirus strains. Comparison of their deduced amino acid sequences have revealed six discrete divergent regions (A to F) among different serotypes (Green et al., 1987; Green et al., 1988): amino acids 39–50 (region A), 87–101 (region B), 120–130 (region C), 143–152 (region D), 208–221 (region E), and 233–242 (region F). A comparison of these same regions among rotaviruses belonging to the same serotype revealed 91–99% homology (Green et al., 1987). Furthermore, Dyall-Smith et al. (1986) identified three regions corresponding to regions B, D, and E as the antigenic sites involved in serotype-specific neutralization. Based on these reports, serotype-specific probes were prepared from nucleotide sequences corresponding to some of the previously described regions. The 322 bp subfragment from OSU gene 9 (fragment E) lies between bases 382 and 704 (Fig. 5A) and encompasses coding regions corresponding to region C–F. The 266 bp subfragment from Gottfried gene 9 (fragment J) lies between bases 230 and 496 (Fig. 5B) and encompasses coding regions corresponding to regions B–D. Under low stringency conditions (42°C, 5×SSC, 50% formamide) the probes were highly specific for RNA from homologous serotypes. These results showed that subfragments from the middle of gene 9 from both OSU (subfragment E) and Gottfried (subfragments I and J) were most specific for homologous rotavirus RNA. Sequences within these subfragments contained more divergent regions among rotavirus serotypes than the other subfragments. Surprisingly, subfragment G of Gottfried gene 9 was less specific than subfragment J although both subfragments were nearly identical in size and location within gene 9. Possible contamination of subfragment G with subfragment H could have occurred with excision of subfragment G from the agarose gel. These two subfragments are nearly identical in size (subfragment G was 287 bp and subfragment H was 229 bp) and subfragment H may have a large percentage of conserved sequences among rotavirus serotypes. However, this problem can be avoided in future studies by amplification of serotype-specific regions by polymerase chain reaction technology.

Sensitivity studies and further evaluation of the specificity of these probes are currently under investigation.

Practical application of the dot blot hybridization assay may require some modification. These include densitometry to minimize subjective comparisons of the reactions, the use of probes representing gene 4 that encodes VP4, and the construction of cDNA probes from selected regions in which highly conserved bases at the 5' end have been removed (Flores et al., 1989). Specific regions of DNA can now be amplified using synthetic oligonucleotides by polymerase chain reaction (PCR) and used as probes. Additionally, the use of nonradioactive labeled probes would eliminate the costly, cumbersome, and time consuming method of radioactive labeling. Recently, an enzyme-labeled oligonucleotide probe was developed to detect human rotavirus RNA in clinical samples using a dot blot hybridization assay (Olive and Sethi, 1989). The authors reported the assay to be highly sensitive and specific when compared with polyacrylamide gel analysis and enzyme-linked immunoassay.

Results of this study, combined with the availability of amplification techniques and alternative methods of labeling probes, offer the potential for nucleic acid probes to be used as versatile diagnostic tools for detection and serotyping of porcine rotaviruses, especially for the detection of noncultivable rotaviruses. Hybridization assays could also facilitate the collection of epidemiological information such as the distribution and prevalence of porcine rotavirus serotypes. Such information would be useful in formulating strategies for future development of efficacious vaccines for rotavirus-induced diarrhea in swine.

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