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Development of a Biotin-Streptavidin-Enhanced Enzyme-Linked Immunosorbent Assay Which Uses Monoclonal Antibodies for Detection of Group C Rotaviruses†

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A biotin-streptavidin-enhanced enzyme-linked immunosorbent assay (ELISA) which uses monoclonal antibodies (MAbs) for the detection of group C rotaviruses was developed. An assay in which plates were coated with three pooled MAbs and biotinylated polyclonal immunoglobulin G (IgG) (polyclonal antibody [PAb]) was used as the detector (MAb capture-PAb detector) was found to be the most sensitive and specific of the assays when it was compared with assays in which plates were coated with polyclonal antiserum and detection was done with either biotinylated polyclonal antiserum (PAb capture-PAb detector) or biotinylated pooled MAbs (PAb capture-MAb detector). The MAb capture-PAb detector ELISA detected 83% of samples confirmed to be positive for group C rotaviruses, whereas the PAb capture-PAb detector assay detected 63% of positive samples and the PAb capture-MAb detector assay detected 65% of positive samples. All three procedures detected both of the bovine and the two human group C rotaviruses, but none of the three procedures detected fecal samples containing group A and B rotaviruses or fecal samples negative for group C rotaviruses used in this study. The sensitivity of the MAb capture-PAb detector ELISA was determined by serially diluting fecal group C rotaviruses; antigens were detected in maximal positive dilution ranges of 1:1,000 to 1:3,000 for the samples tested. On the basis of the cell culture immunofluorescence assay infectivity titer of semipurified cell culture-passaged Cowden group C rotavirus, the sensitivity of the MAb capture-PAb detection ELISA for detection of homologous group C rotavirus was 53 fluorescent focus units per ml. Epitope mapping by use of the biotinylated MAbs in a competition assay suggested that our MAbs may bind to three different but overlapping epitopes. These results suggest that the MAb capture-PAb detector ELISA can be used to study the epidemiology of group C rotaviruses in humans and animals.

Rotaviruses are important etiological agents of acute gastroenteritis in human infants and neonatal animals worldwide (11, 15, 30, 33, 38). Their genomes consist of 11 segments of double-stranded RNA. Rotaviruses belong to the family *Reoviridae* and have been further subdivided into at least seven serogroups (serogroups A to G) on the basis of their distinct antigenicities and the electropherotype migration patterns of their genomes in polyacrylamide gels (15, 16, 25, 26). Members within each serogroup share common antigens that are thought to reside on the major inner capsid protein (2, 3, 8, 18, 28). Serogroups B to G, or non-group A rotaviruses, are more fastidious in their in vitro growth requirements (30, 33). However, one group C porcine (Cowden) strain and one bovine (Shintoku) strain have been propagated in a rhesus monkey kidney cell line, MA104 (32, 37, 39). In contrast, group A rotaviruses grow more readily in cell culture with the aid of proteolytic enzymes, and this has facilitated diagnosis of infections with this serogroup by serological techniques (11, 15, 19, 38). In comparison with the diagnosis of group A rotaviruses, simple assays such as enzyme-linked immunosorbent assays (ELISAs) for the detection of non-group A rotaviruses are lacking primarily because of a lack of appropriate and easily obtainable reagents. Non-group A rotaviruses are detected by immune electron microscopy by using polyclonal antiserum to purified fecal virus or convalescent-phase serum and by polyacrylamide gel electrophoresis analysis of double-stranded RNA (1, 22, 31). Recently, a monoclonal antibody (MAb) to human group B rotavirus was developed and used in an ELISA to detect group B rotaviruses in human fecal samples (5). Although the importance of non-group A rotaviruses in acute gastroenteritis has yet to be determined conclusively, the association of the group C rotaviruses with diarrhea in different animals species, including humans, from several countries (4, 6, 7, 9, 10, 21–23, 27) confirms a need for the development of detection assays to improve surveillance of this rotavirus serogroup.

Recently, we characterized MAbs to the porcine group C rotavirus 41-kDa protein (VP6 protein) (24). These MAbs cross-reacted in immunofluorescence tests with a bovine group C rotavirus and field strains of porcine group C rotaviruses (24, 39). In this report, we describe the development of a streptavidin-biotin-enhanced ELISA that uses the cross-reacting MAbs to detect porcine group C rotaviruses in fecal samples and tissue cultures. We also evaluated its use as a diagnostic test for the detection of group C rotaviruses from different species (bovine and human).

MATERIALS AND METHODS

Virus strains. The group C rotaviruses used in this study included the fecal and tissue culture (TC)-adapted porcine

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(Cowden, L1049) (1, 33, 37) and bovine (Shintoku) strains (39) and two human group C strains (88-182 and 88-261) provided as diarrheic stool samples from children with gastroenteritis in Japan (kindly supplied by M. Oseto and Y. Yamashita, Institute of Public Health, Ehime, Japan). The titers of the cell culture-passaged porcine and bovine group C rotaviruses, which were determined in a cell culture immunofluorescence assay (CCIF), were 3×10^5 and 3×10^4 fluorescent focus units (FFU)/ml, respectively (37, 39). The Cowden TC group C rotavirus was semipurified as described previously (18) for quantitation by ELISA. This semipurified virus pool had a final titer of 10^5 FFU/ml as determined by CCIF (37, 38). Several non-cell culture-adapted field strains of porcine group C rotaviruses derived from the stools of diarrheic pigs in Ohio (designated Ah, HF, KH, NB, and WH) and from Mississippi (designated Wi) used in this study have been described previously (17). Each sample was passaged in gnotobiotic pigs, and the intestinal contents were collected aseptically at the onset of diarrhea (1, 37). Four additional field strains from diarrheic pigs in herds from Ohio (herds BS and FS) and the Ohio Agricultural Research and Development Center swine herd (herds D and SB), which have not been described previously, were also propagated in gnotobiotic pigs, and their intestinal contents were collected (1, 37). Two fecal samples (samples Z1858 and Z1859 containing the MV strain) collected from diarrheic weaned pigs in the Ohio State University (OSU) swine herd and confirmed to be positive for group C rotavirus were also included in the study. Immunofluorescence, immune electron microscopy, and electropherotyping were used to confirm the presence of group C rotaviruses in these samples (1, 31).

The other rotavirus serogroups used in this study included group A rotaviruses passaged in gnotobiotic pigs (porcine OSU and Gottfried and human Wa strains), porcine group B rotavirus (Ohio strain), and bovine group A (designated NCDV strain) and group B (designated ATI strain) rotaviruses passaged in gnotobiotic calves. The titers of all group A rotaviruses in intestinal contents, determined by CCIF, were at least 10^6 FFU/ml (1, 37, 39). In addition, the gnotobiotic pig-passaged porcine enteric coronavirus (transmissible gastroenteritis virus [TGEV]) and group C rotavirus-negative (gnotobiotic and conventional) fecal samples (samples 1 to 6) from pigs were also included as negative controls. Samples (feces, intestinal contents, cell culture lysates, or semipurified virus) were diluted for the test in buffer containing 1% non-fat dry milk (Carnation Co., Los Angeles, Calif.), 2% fetal calf serum (Intergen; Cell Culture Laboratories, Cleveland, Ohio), and phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). Samples were screened at initial dilutions of 1:25 for samples from fecal or intestinal contents and 1:2 or 1:10 for viruses from cell culture. Selected rotavirus from fecal or intestinal contents or semipurified virus (Cowden) samples from TC were serially diluted twofold from 1:10 to 1:3,000 for endpoint titration by ELISA.

MAbs and antiserum. The MAbs (RC8B4, RC9E5, and RC15B7) used in this study have been described previously (24). These MAbs were of the immunoglobulin G1 (IgG1) (MAbs RC8B4 and RC15B7) and IgG3 (MAb RC9E5) isotypes and immunoprecipitated a 41-kDa protein (VP6 protein) of porcine group C rotavirus. They failed to recognize serogroup A and B rotaviruses but recognized a group antigen common to the homologous Cowden rotavirus, other porcine group C rotaviruses, and a bovine group C rotavirus in immunofluorescence tests (24). The polyclonal anti-group C rotavirus serum was prepared by hyperimmunizing gnotobiotic pigs with the purified Cowden strain of group C rotavirus (1). Serum from uninfected gnotobiotic pigs and ascites from a mouse inoculated with SP2/0 mouse myeloma cells were included as negative controls.

Purification and biotinylation of antiserum and MAbs. The MAbs and polyclonal antiserum to group C rotavirus were purified through a protein A-Sepharose (Zymed Laboratories, South San Francisco, Calif.) column after equilibration with either high salt buffer (20 mM Tris-HCl, 3.0 M NaCl [pH 8.9]) or Tris-buffered saline (20 mM Tris-HCl, 0.15 M NaCl [pH 8.2]). The MAb ascites and antiserum were clarified by centrifugation at maximum speed in an Eppendorf centrifuge (Beckman, Palo Alto, Calif.). Supernatants were diluted 1:6 in either high salt buffer (IgG1 MAbs) or Tris-buffered saline (antiserum and IgG3 MAb) before they were applied to the column. Immunoglobulins were then eluted with 0.1 N acetic acid (pH 3.0), and 0.5-ml fractions were collected into tubes which contained 100 μl of 0.5 M Tris buffer (pH 8.9). After elution, fractions were tested, and those containing proteins were pooled and dialyzed against 0.1 M $NaHCO_3$ (pH 8.5) overnight at 4°C. Protein concentrations of the purified MAbs and polyclonal IgG were determined and adjusted to approximately 1.0 mg/ml and were then reacted with 150 μl of biotinylation reagent (Enzotin; Enzobiochemicals, New York, N.Y.) per ml dissolved in dimethyl sulfoxide for 4 h at 25°C. Thereafter, solutions were dialyzed overnight against Tris buffer at 4°C, divided into aliquots, and stored at 4°C (12, 34, 35).

ELISA. Polystyrene, 96-well plates (Nunc-Immuno Plate MaxiSorp; Nunc Inc., Naperville, Ill.) were coated with 2.0 μg of purified single MAbs or pooled MAbs (RC8B4, RC9E5, and RC15B7) per ml (100 μl per well) or with hyperimmune antiserum and were diluted in carbonate buffer (pH 9.6). Plates were incubated overnight at 4°C. As controls, purified immunoglobulin from the sera of uninfected gnotobiotic pigs and ascites from SP2/0 cells were used at the same concentrations. Plates were washed three times with PBS-T and blocked with 5% non-fat dry milk for the polyclonal antibody (positive coating) and uninfected pig (negative coating) serum at 25°C for 1 h or were not blocked for the MAb (positive coating) and SP2/0 (negative coating) systems. The test samples were diluted (1:25) in buffer containing 1% non-fat dry milk and 2% fetal calf serum in PBS-T, added in duplicate to wells with both the positive and negative coatings, and incubated at 37°C for 90 min. After plates were washed four times with PBS-T, 0.5 μg of biotinylated MAbs (individual or pooled) or polyclonal antiserum diluted in biotin buffer (0.1 M bicarbonate buffer, 0.01 M NaCl, and 0.02 M KCl [pH 8.2]) per ml was added, and the plates were then incubated at 37°C for 1 h. Plates were again washed four times with PBS-T, and horseradish peroxidase-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaitherburg, Md.) diluted 1:4,000 in PBS-T was added. Plates were incubated at 25°C for 30 min, and after a final wash (four times with PBS-T), 2,2′-azino-bis(3-ethylbenzthiazoline)sulfonic acid (Sigma Chemical Co., St. Louis, Mo.) in 0.1 M sodium citrate (pH 4.2) containing 0.15% H_2O_2 substrate was added; the plates were then incubated for 30 min at 25°C. The reaction was stopped with 50 μl of 5% sodium dodecyl sulfate per well, and the A_{414} was read by using a Titertek Multiscan plate reader (Flow Laboratories Inc., McLean, Va.). Samples were considered positive if their mean absorbance values on antibody-coated wells were greater than the mean + 3 standard deviations of the absorbance values of the negative control samples on

wells with positive and negative coatings and had a positive/negative (P/N) ratio of ≥1.2. The P/N ratio was calculated by dividing the mean absorbance of duplicate samples on antibody-coated wells by the mean absorbance + 3 standard deviations of the negative samples (non-group C rotaviruses). The P/N ratio cutoff value of ≥1.2 was calculated as the mean P/N value for all the negative samples + 3 standard deviations.

MAb competition assays. MAb competition assays were performed. They were similar to the ones described previously (5, 35). Microtiter plates were coated with 2.0 μg of purified polyclonal antiserum per ml. A fecal sample from a gnotobiotic pig infected with the Cowden strain was diluted 1:20 in 1% non-fat dry milk–2% fetal calf serum in PBS-T and was then added to the plates; the plates were incubated for 90 min at 37°C as described above for the regular ELISA. Unlabeled competitor MAbs diluted in PBS-T to give final concentrations of 0.33 to 9 μg/ml were added (50 μl per well) to the captured virus on the plates. Saturating amounts of biotinylated MAbs (previously determined to be 0.5 μg/ml) were diluted in PBS-T, and 50 μl was added to the unlabeled competitor MAbs or to controls (50 μl of PBS-T). The virus-MAb (labeled and unlabeled) mixture was then incubated at 37°C for 2 h. Plates were washed, and the procedure was continued as described above for the ELISA.

TABLE 1. Reactivities of non-group C rotaviruses and virus-negative control samples in ELISAs for group C rotaviruses

Virus serogroup/host[a]/strain and virus-negative controls	A_{414} (P/N ratio) for the following capture Ab-detector Ab[b]:		
	PAb-PAb	PAb-MAb	MAb-PAb
A/Po/Gottfried	0.090 (0.84)	0.175 (0.91)	0.104 (0.81)
A/Po/OSU	0.039 (0.36)	0.139 (0.72)	0.083 (0.68)
A/Bo/NCDV	0.031 (0.29)	0.138 (0.72)	0.088 (0.69)
A/Hu/Wa	0.040 (0.37)	0.162 (0.84)	0.099 (0.77)
B/Po/Ohio	0.047 (0.44)	0.157 (0.75)	0.060 (0.47)
B/Bo/ATI	0.050 (0.47)	0.137 (0.71)	0.078 (0.61)
Coronavirus/Po/TGEV	0.094 (0.89)	0.165 (0.87)	0.080 (0.63)
Virus-negative pig feces			
1	0.044 (0.36)	0.131 (0.42)	0.064 (0.28)
2	0.036 (0.29)	0.228 (0.74)	0.057 (0.25)
3	0.044 (0.36)	0.218 (0.71)	0.169 (0.73)
4	0.036 (0.29)	0.199 (0.65)	0.057 (0.25)
5	0.044 (0.36)	0.238 (0.77)	0.078 (0.45)
6	0.055 (0.52)	0.229 (0.67)	0.082 (0.47)

[a] Po, porcine; Bo, bovine; Hu, human.

[b] Capture Ab-detector Ab represents antibody (Ab) used for capture and the detector antibody. PAb, polyclonal antibody; MAb, pooled MAbs. Values are mean absorbances of samples. The P/N ratio (in parentheses) was obtained by dividing the mean absorbance of duplicate samples on antibody-coated wells by the mean absorbance + 3 standard deviations of the duplicate samples on the negative coatings. A P/N ratio of ≥1.2 for a sample was considered positive.

RESULTS

Comparison of different ELISA systems. The optimal concentrations of purified MAbs and antiserum used for the coating and detection of antibodies were established by checkerboard titration. Different procedures were initially investigated by using individual MAbs, but the final three procedures compared for sensitivity and specificity included the following: (i) use of plates coated with polyclonal antiserum (polyclonal antibody [PAb]) (capture) and biotinylated polyclonal IgG as detector (PAb-PAb); (ii) use of plates coated as described for i, but with biotinylated pooled MAbs used as the detector (PAb-MAb); and (iii) use of plates coated with pooled MAb (capture) and with biotinylated polyclonal IgG used as the detector (MAb-PAb). Table 1 summarizes the absorbance values and P/N ratios of the control samples (non-group C rotaviruses and group C virus-negative pigs). Although background signals for the PAb-MAb assay were higher than those for the other two procedures, positive reactions were not observed for pig fecal samples containing non-group C rotaviruses, another enteric virus (TGEV), or no virus. On the other hand, all three procedures detected the homologous Cowden group C rotavirus in both cell culture and gnotobiotic pig fecal samples. There was good agreement in the sensitivities of the three assays for detecting seven samples (those containing strains Cowden, Ah, NB, WH, D [K519] MV [Z1858], and SB [K556]) confirmed to be positive for porcine group C rotavirus. Similarly, the procedures detected the group C rotavirus in samples from humans, but strain 88-261 could not be tested in the PAb-MAb assay because of an insufficient amount of sample. Higher concentrations of the bovine Shintoku strain were detected by all three tests; however, with lower concentrations (diluted cell culture virus), antigen was detected only by the MAb-PAb assay (Table 2). In general the MAb-PAb ELISA procedure was more sensitive (83%) than the PAb-MAb and PAb-PAb ELISA procedures, which had similar sensitivities (63 to 65%).

Specificity and determination of virus titers by MAb-PAb ELISA. Representative samples of fecal or intestinal contents were used to compare the relative titers of group C rotavirus and the specificity of the MAb-PAb assay. Five samples—homologous Cowden (fecal and TC), Ah (R1215), D (K519), bovine (Shintoku), and human (88-182)—which are known to contain group C rotaviruses and four samples belonging to group A (OSU [porcine], Wa [human], and NCDV [bovine]) and group B (Ohio porcine) rotaviruses were titrated in the ELISA. Reciprocal dilutions of the viruses which gave 50% maximal absorbance signals for the different group C rotaviruses were as follows: D (K519), 1,000; Ah (R1215), 500; Cowden (fecal), 250; Cowden (TC), 200; bovine (Shintoku), 120; and human (88-182), 100 (Fig. 1). The endpoint titers of the Cowden semipurified fecal virus (initial titer by CCIF, 8×10^5 FFU/ml) and Cowden TC virus (initial titer by CCIF, 1×10^5 FFU/ml) detected by ELISA were 348 and 53 FFU/ml, respectively. None of the negative control samples reacted positively (<1:10) (Fig. 1).

Competition binding assays. Competition binding assays were performed to determine whether the MAbs recognized the same epitopes. Each MAb competed strongly with itself, as was expected (Fig. 2). MAbs RC8B4 and RC15B7 had similar binding affinities, competing strongly in a one-way competition reaction (Fig. 2A) and moderately in the other competition reaction (Fig. 2C). There was a reciprocal lack of competition between MAbs RC8B4 and RC9E5 (Fig. 2A and B), whereas MAb RC15B7 competed moderately in a one-way manner with RC9E5 (Fig. 2B and C). This suggests that MAbs RC8B4 and RC9E5 bind to different but overlapping sites, with RC15B7 binding closer to RC8B4. Figure 3 summarizes the relative binding affinities of the MAbs.

DISCUSSION

The application of rapid and sensitive techniques such as nucleic acid hybridization, immunofluorescence, and ELISA

TABLE 2. Reactivities of group C rotaviruses in three ELISAs

Virus source, virus serogroup/host[a]/strain, and designation	A_{414} (P/N ratio) for the following capture Ab-detector Ab[b]:		
	PAb/PAb	PAb/MAb	MAb/PAb
Porcine			
C/Po/Cowden			
L1049	1.364 (12.75)	1.341 (6.38)	0.854 (6.72)
U1131	0.320 (2.99)	0.603 (3.14)	1.379 (8.95)
L1049[c]	1.453 (13.8)	1.632 (4.80)	1.016 (7.94)
TC, 1:2			
TC, 1:10	0.455 (4.25)	1.225 (6.38)	0.592 (4.63)
C/Po/Ah			
Z639	1.256 (11.74)	0.351 (1.83)	0.802 (6.27)
R1215	0.741 (6.93)	1.461 (7.61)	0.902 (7.05)
C/Po/HF, Z208	0.032 (0.30)	0.155 (0.81)	0.104 (0.81)
C/Po/KH, Z13	0.095 (0.89)	0.193 (1.01)	0.080 (0.63)
C/Po/NB Z1078	0.201 (1.88)	0.705 (3.67)	0.733 (5.65)
C/Po/WH P1048	1.417 (13.24)	1.044 (5.44)	0.443 (3.46)
C/Po/Wi			
P1116	0.072 (0.67)	0.233 (1.21)	0.468 (3.66)
Z1081	0.100 (0.94)	0.155 (0.81)	0.280 (2.19)
C/Po/BS, N608	0.046 (0.38)	0.120 (0.23)	0.112 (0.66)
C/Po/D, K519	0.954 (7.95)	0.846 (6.48)	1.855 (10.85)
C/Po/FS, W1970	0.086 (0.72)	0.132 (0.24)	0.374 (2.19)
C/Po/SB			
K249	0.087 (0.73)	0.122 (0.23)	0.256 (1.50)
K556	0.703 (5.86)	0.887 (6.48)	1.681 (9.83)
C/Po/MV[d]			
Z1858	0.225 (1.88)	0.753 (4.60)	0.941 (5.50)
Z1859	0.102 (0.86)	0.166 (0.92)	0.168 (0.98)
Human			
C/Hu/88-182	0.678 (5.78)	0.409 (2.67)	0.287 (2.35)
C/Hu/88-261	0.805 (6.71)	ND[e]	0.705 (4.12)
Bovine			
C/Bo/Shintoku	0.314 (2.94)	0.392 (2.05)	0.230 (1.80)
C/Bo/Shintoku	0.185 (1.73)	0.312 (1.63)	0.365 (2.10)
TC, 1:2			
TC, 1:10	0.072 (0.67)	0.191 (1.00)	0.140 (1.20)
% Positive	63	65	83

[a] Po, porcine; Hu, human; Bo, bovine.
[b] Capture Ab-detector Ab represents antibody (Ab) used for capture and the corresponding detector antibody. PAb, polyclonal antibody; MAb, pooled MAbs. Values are mean absorbances of samples. The P/N ratio (in parentheses) was obtained by dividing the mean absorbance of the samples (duplicates) on antibody-coated wells by the mean absorbance + 3 standard deviations of all negative samples (non-group C rotaviruses) in Table 1. A P/N ratio of ≥1.2 for a sample was considered positive.
[c] TC, virus in tissue culture (diluted 1:2 or 1:10).
[d] The MV strains were group C rotavirus-positive fecal samples from diarrheic pigs.
[e] ND, not done because of insufficient fecal sample size.

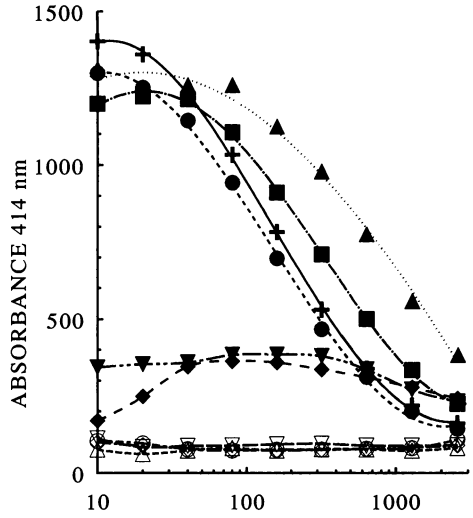

FIG. 1. Titration of representative fecal samples (and Cowden TC) positive for rotaviruses in the MAb-PAb ELISA for group C rotavirus. Group C rotavirus Cowden (fecal) (●), Cowden (TC) (+), Ah (R1215) (■), and D (K519) (▲); group A rotavirus OSU (○); and group B rotavirus Ohio (△) from pigs; group C rotavirus Shintoku (▼) and group A rotavirus NCDV (▽) from cows; and group C rotavirus 88-182 (◆) and group A rotavirus Wa (◇) from humans were tested. The porcine Cowden fecal group C rotavirus had an initial titer of 8×10^5 FFU/ml by CCIF and was positive by ELISA at an endpoint titer of approximately 348 FFU/ml. Similarly, the Cowden TC group C rotavirus had an initial titer of 10^5 FFU/ml by CCIF and was positive by ELISA at an endpoint titer of approximately 53 FFU/ml. Note the overlapping baseline absorbance values for the following controls: group B rotavirus, Ohio; group A rotavirus, NCDV and Wa. A_{414} values should be multiplied by 0.001.

for the detection of the serogroup A rotaviruses has assisted immensely in the diagnosis of these viruses in cases of acute gastroenteritis in humans and animals (13, 19, 20, 29, 38). MAb capture ELISAs have been developed for detecting and defining the antigenic nature of the different proteins of the serogroup A rotaviruses (14, 36). Group C rotaviruses are increasingly incriminated in outbreaks of gastroenteritis in humans (6, 8, 21, 27), but they are difficult to propagate in cell culture (30, 33), making a rapid and sensitive assay for their detection imperative. Recently, a MAb capture ELISA was developed for the detection of group B rotaviruses in human infants (5). One of the advantages of a MAb ELISA is the generation of a sufficient quantity of reagents with little or no variation between batches. Hitherto, the unavailability of reagents for the detection of group C rotaviruses in human and animal species has hampered epidemiological studies. We developed an ELISA to detect group C rotaviruses from porcine and bovine fecal or cell culture samples and two human fecal samples.

Three different ELISA procedures (PAb-PAb, PAb-MAb, and MAb-PAb) were evaluated, and their specificities were shown by a lack of reactivity with serogroup A and B rotaviruses, TGEV, and virus-negative samples. Although the sensitivities, specificities, and background signals for the control samples varied among the three procedures, the MAb-PAb procedure was the most sensitive and specific. It detected 83% of the known positive samples, whereas the

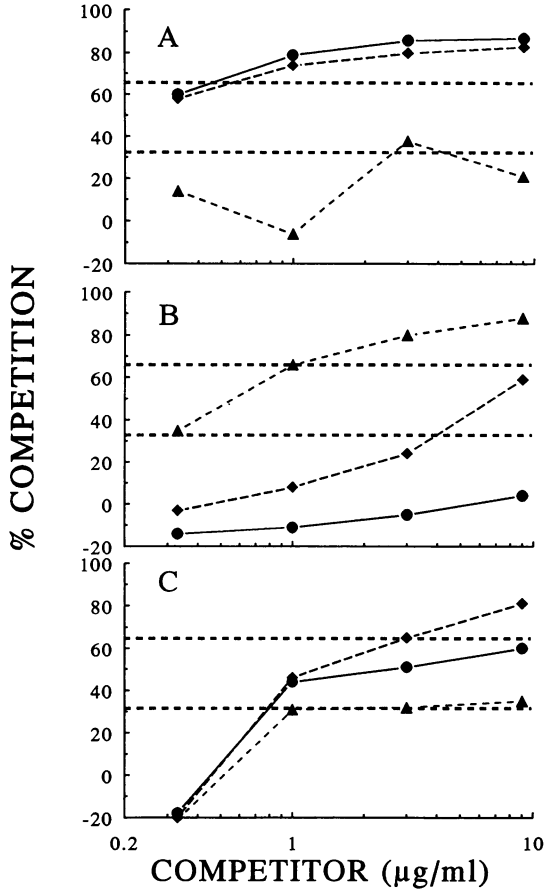

FIG. 2. Competition ELISA with unlabeled purified MAbs against biotinylated MAbs. Unlabeled competitor MAbs were serially diluted threefold starting at 9.0 μg/ml and going to 0.33 μg/ml, and saturating levels of labeled MAbs were added. Plates were coated with purified polyclonal antiserum (2.0 μg/ml), and the fecal Cowden virus diluted 1:20 was bound to the coated plates. (A) Competition of RC8B4 (●) against itself, RC9E5 (▲), and RC15B7 (◆). (B) Competition of RC9E5 (▲) against itself, RC8B4 (●), and RC15B7 (◆). (C) Competition of RC15B7 (◆) against itself, RC8B4 (●), and RC9E5 (▲).

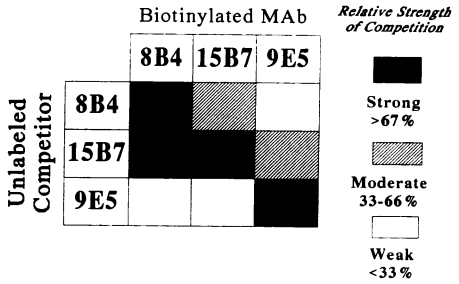

FIG. 3. Relative competition of MAbs.

PAb-PAb and the PAb-MAb assays detected 63% and 65% of known positive samples, respectively. The sensitivity of the MAb-PAb ELISA for detecting group C rotaviruses in intestinal contents or feces ranged from maximal positive dilutions of 1:1,000 to 1:3,000 or 50% maximal absorbance signals of 1:100 to 1:1,000. On the basis of CCIF infectivity titers, the sensitivity of the ELISA for the detection of the homologous Cowden fecal and semipurified TC group C rotaviruses was 348 and 53 FFU/ml, respectively. The lower sensitivity of the ELISA for the detection of group C rotavirus in feces may reflect some interference in antigen binding by extraneous proteins that are present in feces. However, this sensitivity level should be more reflective of the actual sensitivity of the assay for routinely detecting group C rotaviruses in feces.

We observed a pattern of reactivity which enabled us to categorize the porcine group C rotaviruses studied by these assays into (i) strains (Cowden, Ah, NB, WH, D, SB-K556, and MV-Z1858 strains) that were detected by all three assays, (ii) those strains (Wi, FS, and SB-K249) that were detected by at least one of the assays, and (iii) those strains (BS, HF, KH, and MV-1859) that were not detected by any of the assays. This reactivity pattern may be due to variations in the amount of virus in the samples, especially for the MV-1859 strain, since another isolate from the same outbreak (isolate MV-Z1858) was reactive. Other workers (17, 18a) characterized seven of the viruses (strains Cowden, Ah, NB, HF, KH, WH, and Wi) included in this study for their genetic diversity by using a gene 5 (VP6 gene) cDNA probe prepared from Cowden porcine group C rotavirus. They noted by dot hybridization that strains Cowden, Ah, and NB reacted strongly; strains WH and Wi reacted moderately; and strains HF and KH reacted weakly with the gene 5 probe. Our findings are similar to their observations, in that strains which reacted either strongly or moderately with the gene 5 probe reacted positively in the three ELISAs and the two strains (HF and KH) that reacted weakly with the gene 5 probe were negative in all three ELISAs. Similarly, the weaker signals obtained in the ELISA with the bovine group C rotavirus correlates with the weaker reactions obtained with the gene 5 probe (18a). Jiang et al. (18a) speculated that the reasons for the weaker reactions might relate to the occurrence of subgroups of group C rotaviruses or simply lower viral double-stranded RNA concentrations in some samples. The overall similarity of the Cowden gene 5 probe results and our ELISA results are not surprising, since our MAbs were directed to the 41-kDa protein (protein VP6) of Cowden porcine group C rotavirus, which is encoded for by gene 5 (2, 3, 8, 18, 24). The competition data suggest that the MAbs recognized three different epitopes on the 41-kDa protein (protein VP6) of porcine group C rotavirus. Two of the MAbs, RC8B4 and RC9E5, bound to two overlapping sites, with the third MAb, RC15B7, binding to a site closer to RC8B4 (Fig. 2 and 3). This finding is consistent with our observations (data not shown) that the use of single MAbs as capture or detector antibodies in the ELISA were not as sensitive as the use of the pool of MAbs.

Hybridization assays for the identification of group A (13, 20, 29) and group C (2, 3, 8, 18a, 22) rotaviruses by using specific cDNA probes have been developed. These assays proved to be sensitive and specific, but they have the disadvantage of not yet being widely applicable to large-scale investigations. Debouck and colleagues (8a) used a polyclonal sandwich ELISA to monitor the natural excretion of porcine group C rotavirus in closed swine herds, but the assay was not applied to the detection of group C rotaviruses from other species. Our MAb-based ELISA may prove useful for the detection of group C rotaviruses in humans and animals.

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REFERENCES

1. **Bohl, E. H., L. J. Saif, K. W. Theil, A. G. Agnes, and R. F. Cross.** 1982. Porcine pararotavirus: detection, differentiation from rotavirus, and pathogenesis in gnotobiotic pigs. J. Clin. Microbiol. **15:**312–319.
2. **Bremont, M. J., D. Chabanne-Vautherot, P. Vannier, M. A. McCrae, and J. Cohen.** 1990. Sequence analysis of the gene (6) encoding the major capsid protein (VP6) of group C rotavirus: higher than expected homology to the corresponding protein from group A virus. Virology **178:**579–583.
3. **Bremont, M. J., J. Cohen, and M. A. McCrae.** 1988. Analysis of structural polypeptides of porcine group C rotavirus. J. Virol. **62:**2183–2185.
4. **Bridger, J. C.** 1980. Detection by electron microscopy of calicivirus, astrovirus and rotavirus-like particles in faeces of piglets with diarrhoea. Vet. Rec. **107:**532–533.
5. **Burns, J. W., S.-K. W. Welch, S. Nakata, and M. K. Estes.** 1989. Characterization of monoclonal antibodies to human group B rotavirus and their use in an antigen detection enzyme-linked immunosorbent assay. J. Clin. Microbiol. **27:**245–250.
6. **Caul, E. O., C. R. Ashley, J. M. Darville, and J. C. Bridger.** 1990. Group C rotavirus associated with fatal enteritis in a family outbreak. J. Med. Virol. **30:**201–250.
7. **Chasey, D., and D. Davies.** 1984. Atypical rotaviruses in pigs and cattle. Vet. Rec. **114:**16.
8. **Cooke, S. J., P. R. Lambden, E. O. Caul, and I. N. Clarke.** 1991. Molecular cloning, sequence analysis and coding assignment of the major inner capsid protein gene of human group C rotavirus. Virology **184:**781–785.

8a. **Debouck, P., P. Callebaut, and M. Pensaert.** 1983. VIDO 4th International Symposium on Neonatal Diarrhea, p. 77–89. October 1983, Saskatoon, Saskatchewan, Canada. Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada.

9. **Dimitrov, D. H., M. K. Estes, S. M. Rangelova, L. M. Shindarov, J. L. Milnick, and D. Y. Graham.** 1983. Detection of antigenically distinct rotaviruses from infants. Infect. Immun. **41:**523–526.
10. **Espejo, R. T., F. Puerto, C. Soler, and N. Gonzalez.** 1984. Characterization of a human pararotavirus. Infect. Immun. **44:**112–116.
11. **Estes, M. K., and J. Cohen.** 1989. Rotavirus gene structure and function. Microbiology **53:**410–449.
12. **Ey, P. I., S. J. Prow, and C. R. Jenkin.** 1978. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using protein A-Sepharose. Biochemistry **15:**429–436.
13. **Flores, J., E. Boeggeman, R. H. Prucell, M. Sereno, I. Perez, L. White, R. G. Wyatt, R. M. Chanock, and A. Z. Kapikian.** 1983. A dot hybridization assay for detection of rotavirus. Lancet **i:**555–559.
14. **Greenberg, H. B., J. Valdesuso, K. Van Wyke, K. Midthun, M. Walsh, V. MacAuliffe, R. G. Wyatt, A. R. Kalica, J. Flores, and Y. Hoshino.** 1983. Production and preliminary characterization of monoclonal antibodies directed at two surface proteins of rhesus rotavirus. J. Virol. **47:**267–275.
15. **Holmes, I. H.** 1983. Rotaviruses, p. 259–423. *In* W. K. Joklik (ed.), The reoviridae. Plenum Press, New York.
16. **Hoshino, Y., R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian.** 1984. Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque reduction neutralization. J. Infect. Dis. **149:**694–702.
17. **Jiang, B. M., Y. Qian, H. Tsunemitsu, K. Y. Green, and L. J. Saif.** 1991. Analysis of the gene encoding the outer capsid glycoprotein (VP7) of group C rotaviruses by northern and dot blot hybridization. Virology **184:**433–436.
18. **Jiang, B. M., L. J. Saif, S. Y. Kang, and J. H. Kim.** 1990. Biochemical characterization of the structural and nonstructural polypeptides of a porcine group C rotavirus. J. Virol. **64:**3171–3178.

18a. **Jiang, B. M., et al.** Analysis of the genetic diversity of genes 5 and 6 among group C rotaviruses using cDNA probes. Arch. Virol., in press.

19. **Kapikian, A. Z., and R. M. Chanock.** 1990. Rotaviruses, p. 1353–1404. *In* B. N. Fields and D. M. Knipe (ed.), Virology, 2nd ed. Raven Press, New York.
20. **Lin, M., M. Imai, A. R. Bellamy, N. Ikegami, Y. Furuichi, D. Summers, D. L. Nuss, and R. Deibel.** 1985. Diagnosis of rotavirus infection with cloned cDNA copies of viral genome segments. J. Virol. **55:**509–512.
21. **Matsumoto, K., M. Hatono, K. Kobayashi, A. Hasegawa, S. Yamazaki, S. Nakata, S. Chiba, and Y. Kimura.** 1989. An outbreak of gastroenteritis associated with acute rotaviral infection in schoolchildren. J. Infect. Dis. **160:**611–615.
22. **Nagesha, H. S., C. P. Hum, J. C. Bridger, and I. H. Holmes.** 1988. Atypical rotaviruses in Australian pigs. Arch. Virol. **102:**91–98.
23. **Nicolas, J. C., J. Cohen, B. Fortier, M. H. Lourenco, and F. Bricout.** 1983. Isolation of a human pararotavirus. Virology **124:**181–184.
24. **Ojeh, C. K., B. M. Jiang, H. Tsunemitsu, S. Y. Kang, P. A. Weilnau, and L. J. Saif.** 1991. Reactivity of monoclonal antibodies to the 41-kilodalton protein of procine group C rotavirus with homologous and heterologous rotavirus serogroups in immunofluorescence tests. J. Clin. Microbiol. **29:**2051–2055.
25. **Pedley, S., J. C. Bridger, J. F. Brown, and M. A. McCrae.** 1983. Molecular characterization of rotaviruses with distinct group antigens. J. Gen. Virol. **64:**2093–2101.
26. **Pedley, S., J. C. Bridger, D. Chasey, and M. A. McCrae.** 1986. Definition of two groups of atypical rotavirus. J. Gen. Virol. **67:**131–137.
27. **Penaranda, M. E., W. D. Cubitt, P. Sinarachatanant, D. N. Taylor, S. Likanonsakul, L. J. Saif, and R. I. Glass.** 1989. Group C rotavirus infections in patients with diarrhea in Thailand, Nepal, and England. J. Infect. Dis. **160:**392–397.
28. **Qian, Y., L. J. Saif, A. Z. Kapikian, S. Y. Kang, B. Jiang, Y. Ishimaru, Y. Yamashita, M. Oseto, and K. Y. Green.** 1991. Comparison of human porcine group C rotaviruses by Northern blot hybridization analysis. Arch. Virol. **118:**269–277.
29. **Rosen, B. I., L. J. Saif, D. J. Jackwood, and M. Gorziglia.** 1990. Hybridization probes for the detection and differentiation of two serotypes of porcine rotavirus. Vet. Microbiol. **24:**327–339.
30. **Saif, L. J.** 1990. Nongroup A rotaviruses, p. 73–93. *In* L. J. Saif and K. W. Theil (ed.), Viral diarrheas of man and animals. CRC Press, Inc., Boca Raton, Fla.
31. **Saif, L. J., E. H. Bohl, K. W. Theil, R. F. Cross, and J. A. House.** 1980. Rotavirus-like, calicivirus-like and 23-nm virus-like particles associated with diarrhea in young pigs. J. Clin. Microbiol. **12:**105–111.
32. **Saif, L. J., L. A. Terrett, K. L. Miller, and R. F. Cross.** 1988. Serial propagation of porcine group C rotavirus (pararotavirus) in a continuous cell line and characterization of the passaged virus. J. Clin. Microbiol. **26:**105–111.
33. **Saif, L. J., and K. W. Theil.** 1985. Antigenically distinct rotaviruses of human and animal origin, p. 208–214. *In* S. Tzipori (ed.), Infectious diarrhoea in the young. Elsevier Science Publishing, Inc. New York.
34. **Seppala, I., I. Sarvas, F. Peterfy, and O. Makela.** 1981. The four classes of IgG can be isolated from mouse serum by using protein A-Sepharose. Scand. J. Immunol. **14:**335–342.
35. **Simkins, R. A., P. A. Weilnau, and L. J. Saif.** 1989. Epitope mapping and the detection of transmissible gastroenteritis viral proteins in cell culture using biotinylated monoclonal antibodies in a fixed-cell ELISA. **107:**179–190.
36. **Taniguchi, K., T. Urasawa, Y. Morita, H. B. Greenberg, and S. Urasawa.** 1987. Direct serotyping of human rotavirus in stools

by an enzyme-linked immunosorbent assay using serotype 1-, 2-, 3- and 4-specific monoclonal antibodies to VP7. J. Infect. Dis. **155:**1159–1166.

37. **Terrett, L. J., and L. J. Saif.** 1987. Serial propagation of porcine group C rotavirus (pararotavirus) in primary porcine kidney cell cultures. J. Clin. Microbiol. **25:**1316–1319.
38. **Theil, K. W.** 1990. Group A rotaviruses, p. 35–72. *In* L. J. Saif and K. W. Theil (ed.), Viral diarrheas of man and animals. CRC Press, Inc., Boca Raton, Fla.
39. **Tsunemitsu, H., L. J. Saif, B. Jiang, M. Shimizu, M. Hiro, T. Yamaguchi, T. Ishiyama, and T. Hirai.** 1991. Isolation, characterization, and serial propagation of a bovine group C rotavirus in a monkey kidney cell line (MA104). J. Clin. Microbiol. **29:**2609–2613.