Rotavirus-Like, Calicivirus-Like, and 23-nm Virus-Like Particles Associated with Diarrhea in Young Pigs†

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Virus particles morphologically similar to caliciviruses and rotaviruses were detected by electron microscopy (EM) in the intestinal contents of a 27-day-old diarrheic nursing pig. A third small spherical 23-nm virus-like particle was also observed. Calicivirus-like particles averaged 33 nm in diameter. Similar to rotaviruses, rotavirus-like particles were present as single-capsid 55-nm forms or double-capsid 70-nm particles. Most gnotobiotic pigs orally exposed to samples containing these three viruses developed diarrhea and villous atrophy of the small intestine, and all shed the three viruses in their intestinal contents. Attempts to propagate these viruses in cell culture were unsuccessful. The antigenic relationship of the rotavirus-like particles to known rotaviruses was explored by immune EM and immunofluorescent staining. By these techniques, the rotavirus-like particles did not cross-react with antisera to porcine, bovine, or human rotaviruses or to reovirus type 3. Antisera from gnotobiotic pigs exposed to all three viruses had enzyme-linked immunosorbent assay and virus neutralization titers of <4 against porcine rotavirus. Previous infection of gnotobiotic pigs with the mixture containing rotavirus-like particles failed to protect them against a subsequent challenge with porcine rotavirus. The antigenic relationship of the calicivirus-like particles to known caliciviruses was investigated by immune EM and virus neutralization. By these tests, the calicivirus-like particles did not react with antisera against feline calicivirus strain 255 or M-8. In a study conducted at Plum Island Animal Disease Center, antiserum against the three combined agents did not specifically neutralize any serotype of swine vesicular exanthema virus.

Electron microscopy (EM) of feces from humans or animals with diarrhea has led to the discovery of a number of virus or virus-like particles whose role in the causation of diarrhea is currently being evaluated (4, 5, 10–12, 15, 16, 20, 21, 27, 29). Because many of these viruses have not been cultivated in cell culture, EM remains the primary method for their detection. One such group of morphologically characteristic viruses initially detected in diarrheic stools by EM is the rotaviruses (7, 32). They are now well recognized as a cause of diarrhea in young pigs (2, 13, 19, 30) as well as in the young of numerous other species, including humans (7, 32). To date, all rotaviruses have been shown to be antigenically related through common group-specific antigens. Thus, antisera prepared against rotaviruses from one species cross-react with rotaviruses from other species as demonstrated by immune electron microscopy (IEM), immunofluorescence, complement fixation, and enzyme-linked immunosorbent assay (ELISA) tests (9, 23, 31, 33). Rotaviruses from different species are indistinguishable morphologically. Two structural types are commonly seen by EM: complete double-capsid particles, 70 nm in diameter, and smaller incomplete single-capsid particles about 55 nm in diameter with spoke-like surface projections (6, 8).

Recently, particles morphologically similar to feline caliciviruses have been observed in fecal samples from babies with a history of enteritis (5, 16) and also from calves with diarrhea (29). However, the role of caliciviruses in the etiology of these diarrheas is presently undefined.

In the present report, three different virus-like particles were detected by EM in the gut contents from a single diarrheic nursing pig. This study characterizes the morphology of these particles and examines their combined pathogenicity for gnotobiotic pigs. In addition, the relationship of these virus-like particles to known rotaviruses and caliciviruses was investigated.

MATERIALS AND METHODS

Field specimen. The rotavirus-like particles (RVLP), calicivirus-like particles (CVLP), and 23-nm
virus-like particles were detected in the intestinal contents of a 27-day-old conventional nursing pig from an Ohio swine herd. The only clinical sign that the pig exhibited was diarrhea. Contents of the large intestine were processed for IEM as described previously (23).

Inoculation of gnotobiotic pigs. Two 5-day-old gnotobiotic pigs were inoculated by the oral route with 1 to 2 ml of a 10% suspension of filtered (0.22-μm filter, Millipore Corp., Bedford, Mass.) large-intestine contents from the diarrheic conventional pig. Exposed pigs were killed shortly after the onset of diarrhea. Mucosal smears were made of sections of the small intestine for fluorescent microscopy examination (2). The large-intestine contents were collected aseptically, and samples were processed for IEM or used to orally infect additional gnotobiotic pigs. The entire small intestine was examined microscopically for evidence of villous atrophy as described previously (2). Two gnotobiotic pigs (pigs 1 and 2), 8 and 19 days of age, respectively, were orally inoculated with a filtrate containing the three viruses. The pigs were allowed to recover from the diarrhea and then orally challenged with porcine rotavirus, pig 1 at 19 days postexposure and pig 2 at 29 days postexposure. Both pigs were observed for diarrhea and then sacrificed.

Antisera to RVLP, CVL, and 23-nm virus-like particles. Convalescent antisera against the three combined viruses were collected from gnotobiotic pigs 3 to 4 weeks after oral exposure to the virus mixture. Hyperimmune serum against a mixture of the three viruses was prepared in a gnotobiotic pig by oral inoculation with the three viruses followed by intramuscular injection of the virus mixture after 1 month. The pig was bled 1 week after the intramuscular injection and the serum was collected. This antiserum, which was used for the IEM studies, had an optimal IEM titer of 200 against the three viruses (optimal IEM titer, reciprocal of serum dilution which gave maximal clumping of each of the three viruses).

Rotaviruses and rotavirus antisera. The porcine rotavirus used in this study was from the contents of the large intestine of a gnotobiotic pig inoculated with an Ohio State University isolate (23). Convalescent porcine rotavirus antisera from a gnotobiotic pig inoculated 30 days previously had a virus neutralization (VN) titer of 410 and an optimal IEM titer against porcine rotavirus of 50.

Bovine rotavirus was from the large-intestine contents of a gnotobiotic calf exposed 18 h previously to the Nebraska calf diarrhea virus bovine rotavirus. Antiserum to bovine rotavirus, from a gnotobiotic calf inoculated orally and twice intramuscularly with Nebraska calf diarrhea virus, had a VN titer of 5,100 and an optimal IEM titer of 500 against bovine rotavirus.

Antisera to human rotavirus types 1 and 2. Antisera to human rotavirus produced in gnotobiotic calves were kindly supplied by R. G. Wyatt (National Institutes of Allergy and Infectious Diseases, Bethesda, Md.) and were used for IEM at a dilution of 1:25.

A pooled mouse anti-arbovirus ascitic fluid, also obtained from Dr. Wyatt, contained antibody to arboviruses of the Kemerovo group, palyam group, and no. 8 group. It was used for IEM at a 1:20 dilution.

Reovirus type 3 and reovirus type 3 antisera. Reovirus type 3, Abney strain (American Type Culture Collection, Rockville, Md.), was passaged in a gnotobiotic pig, and the large-intestine contents were harvested as the source of reovirus type 3. Antiserum against this reovirus was produced in a gnotobiotic pig. Its optimal IEM titer against reovirus was 100.

Antiserum to feline calicivirus. Specific pathogen-free cat antiserum against feline calicivirus strain 255 was supplied by J. H. Gillespie (Cornell University, N.Y.). This antiserum had a reported titer of 500 50% serum-neutralizing doses per 0.025 ml. It was used for IEM at a dilution of 1:25. A hyperimmune rabbit antiserum against the M-8 strain of feline calicivirus was provided by Eldon Davis (Norden Laboratories, Lincoln, Nebr.). It was used for IEM at a 1:50 dilution.

Antiserum to SVE virus. A pool was made from hyperimmune sera prepared in conventional swine against swine vesicular exanthema (SVE) types A48, B51, C52, and J56. A similar pool was made from convalescent conventional pig antiserum against SVE types D30, E54, F55, H54, I53, and K54. These antiseras were supplied by J. C. Callis (Plum Island Animal Disease Center [PIADC], Greenport, N.Y.).

IEM. IEM was conducted by incubating virus samples with antiserum as described in a previous report (23). In tests for cross-reactivity against heterologous viruses, antiseras were used at dilutions two- to fourfold lower than their optimal IEM dilutions against homologous virus. Negatively stained grids were examined at 80 kV in a Philips 201 electron microscope (23).

Immunofluorescent staining. Immunofluorescent staining was performed on small intestinal mucosal smears as described previously by using fluorescein-conjugated pig antiserum to porcine rotavirus or an anti-reovirus fluorescein conjugate (2). For staining of the three agents, an indirect technique was used using the hyperimmune antiserum against the three combined agents and fluorescein-conjugated antiserum to porcine immunoglobulin G (IgG) (Miles Laboratories, Elkhart, Ind.).

ELISA and VN test. Convalescent and hyperimmune antiseras against the three combined agents were tested for VN antibodies against porcine rotavirus by a plaque reduction test, similar to a technique previously described (18). Non-neutralized virus was detected on an embryonic rhesus monkey kidney cell line, MA104, by a procedure in which the agar overlay contained pancreatic (0.15% pancreatic 4× N.F., GIBCO Laboratories, Grand Island, N.Y.), and diethylnitrovinyl ether (100 μg/ml). Virus neutralization titers were expressed as the reciprocal of the sample dilution resulting in 80% reduction in plaques.

These antiseras were also tested for antibodies against porcine rotavirus and bovine rotavirus by an ELISA test performed by a modification of the technique described by Voller (28). Briefly, it involved coating microtiter plates (Dynatech 220-24) with cell culture-propagated porcine or bovine rotavirus or uninfected control cell culture fluid followed by the addition of the following reagents (with washes and incubation periods between each reagent as described (28)): test sera, rabbit antiserum to porcine IgG, goat antiserum to rabbit IgG conjugated to alkaline phosphatase, and the substrate, p-nitrophenylphosphate (Sigma 104-5). Titers were expressed as the reciprocal...
of the highest dilution which had an optical density at 400 nm greater than that of the uninfected control plus 2 standard deviation units.

**Virus isolation attempts.** Suspensions of large-intestine contents from gnotobiotic pigs infected with the combined three agents were inoculated onto primary porcine kidney and MA104 cell cultures. Cells were examined for a cytopathic effect during three successive blind passages. The large-intestine inocula were also assayed for plaque-forming viruses by overlaying the inoculated MA104 cells with agar containing pancreatin and diethylaminoethyl-dextran at the same concentrations as those used in the VN tests. At the PIADC laboratory, porcine kidney, pig testicle, and Vero cell lines were used in attempts to isolate a cytopathogenic virus from the virus mixture.

**RESULTS**

**Morphology of the virus particles.** Three different viruses were detected by EM in the intestinal contents collected from a 27-day-old conventional diarrheic pig. These included CVLP, which averaged 33 nm in diameter (total of 100 particles measured, range of 28 to 38 nm) and were morphologically similar to feline caliciviruses. As shown in the electron micrograph in Fig. 1, some particles had a hollow six-pointed star appearance (single arrows), whereas in others 10 peripheral spoke-like projections were evident (double arrow). All particles had cup-like surface depressions.

Similar to rotaviruses, RVLP were present in two morphological forms. Larger particles averaged 70 nm in diameter and had a double outer-capsid layer with a smooth outer periphery (Fig. 2A). The smaller particles averaged 55 nm in diameter and had a single outer-capsid layer composed of spokelike tubular capsomers (Fig. 2B). The larger particles were often predominant in many preparations. Morphologically the RVLP appeared to be identical to porcine rotavirus, which is shown for comparison in Fig. 2C.

The third virus-like particles observed were small spherical particles which averaged 23 nm in diameter (average of 100 particles, range 19 to 25 nm) (Fig. 3). For all three of the different virus-like agents, empty particles (particles with dark-staining centers) were also apparent (Fig. 1-3).

**Cell culture studies.** No cytopathic effects or plaques were observed in cell cultures inoculated with samples containing the three combined agents, either in our laboratory or at the PIADC laboratory.

**Transmission and pathogenicity studies.** Results of the transmission and pathogenicity studies conducted with these three viruses are summarized in Table 1. When the field specimen containing the three different virus-like particles was used to orally infect two 5-day-old gnotobiotic pigs, both pigs developed a profuse tan diarrhea 2 to 4 days postexposure and were sacrificed. No other clinical signs were noted. All three viruses were detected by IEM of the large-intestine contents. Examination of the small intestine revealed villous atrophy in the duodenum of both pigs.

Contents of the large intestine containing the three viruses were used to subsequently infect 12 other gnotobiotic pigs (three additional passages), and results are summarized in Table 1. A total of 11 of these 12 pigs developed diarrhea at an average of 3.5 days postexposure (range of 2 to 7 days postexposure). All three viruses were detected by IEM in the intestinal contents of all pigs. Ten pigs had villous atrophy in the small intestine. Indirect immunofluorescent staining of mucosal smears with the hyperimmune antiserum prepared against the three viruses revealed varying numbers of immunofluorescent epithelial cells. However, there was no immunofluorescence of epithelial cells when stained with the porcine rotavirus antiserum conjugate.

**Relationship of the RVLP to known ro-
rotavirus was detected by IEM in the intestinal contents. Epithelial cells in small-intestine mucosal smears had specific immunofluorescence when stained with the porcine rotavirus antiserum conjugate.

(ii) In vitro cross-reactivity studies. Results of studies of the cross-reactivity of the RVLP with rotavirus and reovirus by IEM are summarized in Table 2. By IEM, porcine and bovine rotaviruses reacted with both homologous and heterologous rotavirus antisera. Figure 2C illustrates the reaction between porcine rotavirus and its homologous antiserum. The porcine RVLP did not react by IEM with any of the rotavirus antisera tested or with an antiserum to reovirus type 3 or a mouse antiserum to arbovirus (Table 2). These heterologous antisera did not react by IEM with either the 55- or 70-nm forms of RVLP. Similarly, antisera against the three combined agents reacted with the RVLP (Fig. 2A), but not with rotaviruses or reovirus type 3.

Mucosal smears from pigs infected with the three agents showed immunofluorescence in the indirect test only with homologous antisera.
against the three combined agents and not with porcine rotavirus or reovirus type 3 fluorescein antibody conjugates. In addition, mucosal smears from pigs infected with porcine rotavirus did not show immunofluorescence when reacted in the indirect test with antisera against the three combined agents.

Convalescent and hyperimmune antisera against the three combined agents were also tested for rotavirus antibodies by ELISA and a virus neutralization plaque reduction test. By both tests, these antisera had titers of <4 against porcine rotavirus. In the ELISA test, the antisera also had titers of <4 against bovine rotavirus.

**Relationship of the CVLP to known caliciviruses.** When the field specimen which contained large numbers of CVLP was reacted with specific pathogen-free cat antisera to feline calicivirus or hyperimmune rabbit antisera to feline calicivirus, no IEM clumping was observed. However, incubation of the CVLP with its homologous antisera produced large virus-antibody aggregates similar to those depicted in Fig. 1. Convalescent and hyperimmune gnotobiotic pig antisera against the three combined agents were also sent to Donald Kahn (Pittman Moore, Washington, N.J.) and tested in a VN test against the feline calicivirus strain 255. He found no neutralization of the feline calicivirus by these antisera (personal communication).

An attempt was made to assess the antigenic relationship of the CVLP to SVE by IEM. When the CVLP were reacted with pooled convalescent or hyperimmune conventional pig antisera against the various SVE serotypes, some virus clumping was evident at low dilutions of the antisera. However, since these antisera were prepared in conventional pigs, it was not possible to determine whether this reaction was specific for SVE antibodies or whether it reflected antibodies in these sera specific for CVLP due to a previous exposure to this virus. In support of the latter, when these three agents were tested by IEM against five different adult swine sera, clumping of all three agents by each serum was evident, suggestive of antibodies present from prior exposure to these viruses. Therefore, to further exclude any antigenic relationship of CVLP to SVE, antisera were tested at PIADC by a VN test against the 11 SVE serotypes (A-K). No specific virus neutralization occurred.

**DISCUSSION**

Detection of three different virus-like particles

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**Table 1. Transmission and pathogenesis of calicivirus-like, rotavirus-like, and 23-nm virus-like agents in gnotobiotic pigs**

<table>
<thead>
<tr>
<th>Virus inoculum</th>
<th>Age (days) of pigs at infection</th>
<th>No. with diarrhea/no. inoculated</th>
<th>No. with virus in gut content/no. examined</th>
<th>Avg incubation period (days)</th>
<th>No. with intestinal villous atrophy/no. examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Field specimen</td>
<td>5</td>
<td>2/2</td>
<td>2/2</td>
<td>3 (2-4)</td>
<td>2/2</td>
</tr>
<tr>
<td>1st gnotobiotic pig-passaged virus</td>
<td>3-11</td>
<td>6/6</td>
<td>6/6</td>
<td>3.5 (2-7)</td>
<td>4/6</td>
</tr>
<tr>
<td>2nd gnotobiotic pig-passaged virus</td>
<td>3-4</td>
<td>4/4</td>
<td>4/4</td>
<td>3.5 (2-7)</td>
<td>4/4</td>
</tr>
<tr>
<td>3rd gnotobiotic pig-passaged virus</td>
<td>9</td>
<td>1/2</td>
<td>2/2</td>
<td>3.5 (3-4)</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate range.

**Table 2. Cross-reactivity of RVLP with known rotaviruses and reovirus by IEM**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Porcine RVLP&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reovirus type 3</th>
<th>Porcine rotavirus</th>
<th>Human rotavirus</th>
<th>Bovine rotavirus</th>
<th>Arbovirus&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine RVLP</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Porcine rotavirus</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bovine rotavirus</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Reovirus type 3</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hyperimmune antiserum to the three combined agents: RVLP, CVLP, and 23-nm virus-like particles.

<sup>b</sup> Supplied by Richard Wyatt, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

<sup>c</sup> IEM positive (+), ≥ 90% of virus particles in virus-antibody aggregates; IEM negative (-), ≤ 2% of virus particles in aggregates.

<sup>d</sup> ND, Not determined.
in intestinal contents reveals the plethora of agents which may be encountered in a single outbreak of diarrhea from an individual animal. To our knowledge none of these agents has previously been identified in intestinal contents of pigs or associated with diarrhea in pigs. These experiments confirmed that all three agents were transmissible to gnotobiotic pigs and that the mixture of the three agents consistently caused diarrhea and villous atrophy within the small intestine of exposed pigs. None of these viruses was cultivable in cell culture either in this laboratory or at the PIADC laboratory.

Future studies are under way to separate these three agents and determine their individual pathogenicities in gnotobiotic pigs. Preliminary studies employing virus ultrafiltrates separated by membranes of very small pore diameter suggest that the RVLP itself and the CVLP plus the 23-nm virus-like particle may separately cause diarrhea (E. H. Bhol, L. J. Saif, K. W. Theil, and R. F. Cross, unpublished data).

Rotaviruses are a well-recognized cause of diarrhea in the neonates of many species (7, 32). To date all rotaviruses have been shown to be antigenically related through common group-specific antigens (9, 23, 31, 33). This is the first report of an agent recovered from the gut contents of a diarrheic pig which is morphologically similar to rotavirus but antigenically unrelated by all the tests employed (in vivo cross-protection, IEM, immunofluorescent staining, VN, and ELISA). Nor does this virus appear to be antigenically related to reoviruses by IEM and immunofluorescent staining tests. The RVLP was initially detected as antigenically distinct from rotavirus by IEM by using monospecific antisera to porcine rotavirus prepared in gnotobiotic pigs. By using this technique, neither the 70-nm nor the 55-nm forms of RVLP were clumped, thus suggesting that the RVLP and porcine rotavirus do not share common group-specific antigens within the inner-capsid layer as has been reported for the different human rotavirus serotypes (1 and 2) or for rotaviruses from different species (3, 6, 34). The IEM findings also demonstrate the importance of using monospecific reagents for the detection and diagnosis of rotavirus and emphasize the role of IEM in assuring that particles morphologically similar to rotaviruses by EM are also antigenically related. Separation, purification, and physicochemical studies of RVLP are necessary to further investigate its relationship to known rotaviruses.

Caliciviruses are well-known animal pathogens with the prototype viruses restricted in host range to pigs (SVE virus), cats (feline calicivirus), and pinnipeds (San Miguel sea lion virus) (26), although a recent report indicates that monkeys may be infected with San Miguel sea lion virus as well (24). Other recent studies have implicated caliciviruses as possible causes of diarrhea in humans (5, 16) and calves (29), although the calf virus differs somewhat in morphological appearance from the prototype caliciviruses.

Caliciviruses are a major cause of respiratory disease in cats (26). In swine, SVE is an acute febrile disease characterized by formation of vesicles on the snout, within the oral cavity, and on the feet (17). Previous reports have indicated that a broad cross-reactivity exists among different isolates of feline caliciviruses (22, 26). Smith et al. (25) demonstrated a broad cross-reactivity between the SVE and San Miguel sea lion caliciviruses by IEM but only a limited cross-reactivity between SVE virus and a feline calicivirus (F-9).

The CVLP detected in this report were shown to be antigenically unrelated to SVE virus types A–K by VN tests. By both IEM and VN tests, no antigenic relationship was detected between the CVLP and feline caliciviruses, strains 255 and M-8. Further evidence for the distinctiveness of the calici-like viruses from the prototype caliciviruses was their failure to replicate in porcine cell cultures, since both feline and SVE caliciviruses are cytopathic for susceptible cell cultures (26). Also, clinical signs resembling those reported for SVE (17) were not observed in either conventional or experimentally infected pigs.

Morphologically the CVLP resembled the prototype caliciviruses both in size (average of 33 nm in diameter for CVLP compared with a range of 30 to 37 nm for feline caliciviruses) (1) and surface structure. Cuplike surface depressions were readily observed, as were some particles with a hollow six-pointed star appearance and others with 10 evenly spaced surface projections, all frequently described characteristics of known caliciviruses (26). Similar morphological descriptions have been reported for calicivirus particles associated with cases of human diarrhea (5, 14). Future tests will be conducted to determine whether the CVLP is antigenically related to those caliciviruses reported to cause diarrhea in humans (5) and calves (29). Attempts will also be made to isolate this virus and study its pathogenicity in gnotobiotic pigs and eventually to assess the prevalence of this virus, the RVLP, and the 23-nm virus within the swine population. In preliminary tests, all five adult swine sera tested by IEM were positive for antibodies to these three agents.

Possible identity of the 23-nm virus-like particle which had no recognizable surface structure is unknown. Whether it causes diarrhea alone or
has any relationship to some small, round, non-
cultivable viruses shown to cause diarrhea in
humans, such as Norwalk Agent (10) and other
parvovirus-like particles (11, 21), is undeter-
mined.

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