Serological Characterization of Bovine Rotaviruses Isolated from Dairy and Beef Herds in Argentina

RODOLFO C. BELLINZONI,1,2* JORGE O. BLACKHALL,1 NORA M. MATTION,1,2 MARY K. ESTES,2 DAVID R. SNODGRASS,3 JOSE L. LATORRE,1 AND EDUARDO A. SCODELLER1

Centro de Virología Animal, 1414 Capital Federal, Argentina1; Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 770302; and Moredun Research Institute, Edinburgh EH97 7JH, Scotland, United Kingdom3

Received 8 May 1989/Accepted 25 July 1989

Bovine rotaviruses isolated from beef and dairy herds in Argentina were serotyped by the immunoperoxidase focus reduction assay as previously described (G. Gerna, M. Battaglia, G. Milanesi, N. Passarani, E. Percivalle, and E. Cattaneo, Infect. Immun. 43:722–729, 1984). Three strains from beef herds were related to the UK and NCDV bovine rotavirus strains defined as serotype 6 (Y. Hoshino, R. G. Wyatt, H. B. Greenberg, J. Flores, and A. Z. Kapikian, J. Infect. Dis. 149:694–702, 1984). Two other strains from dairy herds were classified as bovine viruses related to the bovine B223 strain reported by Wood and co-workers (G. N. Wood, N. E. Kelso, T. F. Simpson, S. K. Gaul, L. E. Evans, and L. Babiuk, J. Clin. Microbiol. 18:358–364, 1983) in the United States. A serotyping antibody-capture enzyme-linked immunoassay to detect serotype 6 rotavirus using a serotype 6-specific monoclonal antibody was developed and evaluated for strain characterization. Characterization of 72 group A rotavirus-positive fecal samples from beef herds and 43 fecal samples from dairy herds showed a predominance of serotype 6 rotavirus in beef herds but both serotype 6 and non-serotype 6 rotaviruses in dairy herds. Analysis of genomic double-stranded RNA by polyacrylamide gel electrophoresis showed that when outbreaks were caused by one serotype only a single electropherotype was present in all samples.

Rotaviruses have been reported as the main infectious cause of diarrhea in young calves in the United Kingdom (25, 27), and they are an important etiologic agent of diarrhea in the United States and other countries (4, 9), as well as in Argentina (2). Recently, an inactivated rotavirus vaccine administered in an oil adjuvant to pregnant cows was shown to be effective in the passive prevention of calf diarrhea in beef herds in Argentina (1). The vaccine was developed by using a local strain of bovine rotavirus that belonged to serotype 6. This serotype has been reported to be the most common in the United States (32), in the United Kingdom (24, 26), and in preliminary studies in Argentina (R. Bellinzoni, unpublished data).

This paper reports the analysis of the antigenic variation of rotavirus strains isolated from beef and dairy herds in Argentina. These studies were done using a conventional focus reduction neutralization assay and a serotyping enzyme-linked immunosassay (ELISA) which allowed a more rapid analysis of a large number of samples. In addition, the genomic double-stranded RNA patterns from different rotavirus strains were studied by polyacrylamide gel electrophoresis (PAGE). The antigenic variation of rotaviruses isolated from both beef and dairy herds was evaluated because this information is important for the development of all-inclusive calf diarrhea prevention programs. Specifically, we wanted to be able to determine whether vaccination affords heterotypic protection in calves and whether it results in the appearance of new rotavirus serotypes.

The cultivable bovine rotaviruses, UK, B678, NCDV, and B223 were used. Argentinian strains of bovine rotavirus (T14, T15, T18, T21, T26, T47, T51, T67, T82, T86, and T149) were isolated from fecal samples obtained from diarrheic calves. Viruses were adapted and propagated in MA104 cells as previously described (2). Before characterization, each strain was purified by two cycles of passage in cells at terminal dilution. All tissue-culture-adapted viruses were shown to be the same type as those in the original fecal sample by PAGE of viral RNA. Guinea pig hyperimmune sera against strains UK, NCDV, B223, B678, T21, T26, and T82 were produced as described by Gaul et al. (12).

Group A rotavirus-positive fecal samples were collected for a previous survey in which different diarrheic etiologic agents were studied in beef and dairy herds (R. Bellinzoni, J. Blackhall, N. Auza, H. Terzolo, N. Mattion, A. Moreira, A. Casaro, J. LaTorre, and E. A. Scodeller, submitted for publication). Analysis of genomic double-stranded RNA from different rotaviruses was performed as previously described (28).

A serotyping ELISA using polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) was developed with a procedure modified from Burns et al. (5). The wells of the plates were coated overnight with mouse monoclonal antibody (MAb) (ascitic fluid diluted 1:10,000 in phosphate-buffered saline solution [PBS]) directed to a neutralizing epitope on VP7 of the bovine UK strain (serotype 6) or with an MAb directed to a common nonneutralizing epitope on VP7, which was purified in a protein A column and diluted in PBS to a final concentration of 2 μg/ml. After an overnight incubation with the MAb, the ELISA plates were washed twice with 0.1% Tween 20 in PBS and blocked with 1% bovine serum albumin in PBS for 2 h at 37°C. The fecal samples were diluted 20% (wt/vol) in PBS containing 1% bovine serum albumin and 10% fetal bovine serum, homogenized, and clarified by centrifugation at 3,000 × g for 15 min. The supernatant was added to duplicate wells and was incubated overnight at 4°C. The plates were washed five times with 0.1% Tween 20 in PBS.

The detector antibody used was a guinea pig hyperimmune serum to CsCl-purified double-shelled particles of the UK strain, diluted 1:5,000 in PBS containing 1% bovine serum.
albumin and 10% fetal bovine serum. The detector serum was incubated for 1 h at 37°C, and the wells were then washed five times before the conjugate (horseradish peroxidase- 
goat-anti-guinea pig serum [Bioydea, Israel]) was 
added at a dilution of 1:5,000 (in PBS with 1% bovine serum 
albumin and 10% fetal bovine serum). The conjugate was 
incubated for 1 h at 37°C, the wells were washed, and 
the substrate ABTS-H₂O₂ [2,2'-azinobis(3-ethylbenzthiazoline-
α sulfonic acid); Sigma Chemical Co., St. Louis, Mo.] was 
added. The absorbance (optical density) was determined 
at 414 nm (OD₄₁₄) in a micro ELISA reader (LabSystem Inc., 
Chemetron, Buenos Aires, Argentina).

A fecal sample was considered to be positive for a 
serotype 6 virus if the OD₄₁₄ was at least three times 
the mean of the OD₄₁₄ obtained with non-serotype 6 rotavi-
ruses. Samples were considered untypable when they were 
negative with the serotype 6 MAb and when the OD₄₁₄ 
obtained with the common VP7 MAb failed to be at least 
three times the mean OD₄₁₄ obtained with rotaviruses 
treated with 50 mM EDTA (single-shelled particles).

Neutralization assays were done by using the immunoper-
oxidase focus reduction assay (IPFRA), previously 
described by Gerna et al. (13). This assay was performed 
in 96-well tissue culture microtiter plates (NUNC, Roskilde, 
Denmark). Confluent MA104 cell monolayers were washed 
three times with Eagle medium lacking fetal bovine serum. 
Viruses were pretreated with 10 μg of trypsin per ml and 
than diluted in serum-free medium to yield approximately 
200 focus-forming units per 0.1 ml. Equal volumes of diluted 
virus suspension and serial twofold dilutions of serum (or 
serum-free Eagle medium for the virus control) were incu-
bated for 2 h at 37°C, and then 0.1 ml of each mixture was 
added to duplicate wells. The plates were incubated for 18 h 
at 37°C, fixed with 80% acetone, and stained by IPFRA (13). 
Tetra-hydrochloride 3,3′-diaminobenzidine (Sigma) was 
used as the substrate. The neutralization titer of each serum 
was determined as the reciprocal of the highest dilution of 
sample giving an 80%-or-greater reduction in the number of 
stained cells, compared with the virus control wells. Neu-
tralization titers with a 20-fold-or-greater two-way difference 
in titer were used as the criteria for serotype distinction 
between different viruses (16, 24).

The serotypes of 11 rotavirus strains (5 from dairy herds 
and 6 from beef herds) adapted to growth in MA104 cells 
were determined with hyperimmune guinea pig serum pre-
pared against the UK bovine strain. The six virus strains 
isolated from beef herds cross-reacted with the UK strain, 
and the five strains isolated from dairy herds were not 
neutralized by this serum (data not shown).

Two of the bovine strains (T21 and T26) that did not react 
with the UK hyperimmune serum and one strain (T82) 
that did react were selected for further analysis in reciprocal 
nutralization assays. Other prototype bovine serotype I 
strains (UK and NCDV) as classified by Woode et al. (32) 
and Ojeh et al. (24), a bovine serotype II strain (B223) 
classified in the United States by Woode et al. (32), and 
a bovine virus (B678) not related to the NCDV and UK strains 
described in the United Kingdom by Ojeh et al. (24) were 
selected for the reciprocal tests. In addition, two Argentinian 
strains related to the UK bovine strain were analyzed in 
one-way cross-neutralization assays with the prototype bo-
viruses strains. Each of the selected test strains was isolated 
from a different outbreak of diarrhea and showed a distinct 
RNA electropherotype when its genomic RNA was analyzed 
by PAGE.

Table 1 shows the neutralization assay results obtained. 
The T82 strain showed a two-way cross-reaction with both 
UK and NCDV strains (serotype 6). Strains T51 and T67 
showed relatedness with T82, UK, and NCDV in one-way 
analysis and were classified as serotype 6. None of these 
three strains cross-reacted with the B223 and B678 strains. 
The non-serotype 6 strains, T21 and T26, showed no cross-
reactivity with the NCDV or UK strains. Both T21 and T26 
were related by one way cross-reaction, and both were 
related to the B223 strain in two-way cross-reactions.

The T26 strain showed a one-way cross-reaction with the 
B678 strain. None of the other strains analyzed reacted with 
B678. When the strains of different origins were compared, 
one of the dairy herd strains showed cross-reactions with 
serotype 6, whereas all samples from beef herds were 
classified as serotype 6. More strains must be analyzed to 
determine whether this is a general finding.

Serotyping by the neutralization focus reduction assay is 
time-consuming, labor-intensive, and not practical for 
analyzing a large number of samples. Therefore, we also 
developed and evaluated an ELISA using an MAb specific for 
a neutralizing epitope on VP7 of the UK strain (serotype 6) 
to allow more rapid characterization of serotype 6 viruses.

First we determined the specificity of the serotyping 
ELISA by testing prototype human Wa (serotype 1) and 
DS-1 (serotype 2), simian SA11 (serotype 3), and porcine 
Gottfried (serotype 4) and OSU (serotype 5) strains (data not
TABLE 2. Specificity of serotyping ELISA with bovine prototype viruses and test strains

<table>
<thead>
<tr>
<th>Virus strain* (species)</th>
<th>ELISA* OD$_{414}$</th>
<th>Designated serotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serotype 6-</td>
<td>Common VP7 MAb</td>
</tr>
<tr>
<td></td>
<td>specific MAb</td>
<td></td>
</tr>
<tr>
<td>UK (Bo)</td>
<td>1.1</td>
<td>+2</td>
</tr>
<tr>
<td>NCDV (Bo)</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>B223 (Bo)</td>
<td>0.01</td>
<td>0.1</td>
</tr>
<tr>
<td>B678 (Bo)</td>
<td>0.07</td>
<td>0.2</td>
</tr>
<tr>
<td>T14 (Bo) Arg-D</td>
<td>0.1</td>
<td>+2</td>
</tr>
<tr>
<td>T15 (Bo) Arg-D</td>
<td>0.07</td>
<td>+2</td>
</tr>
<tr>
<td>T18 (Bo) Arg-D</td>
<td>0.07</td>
<td>+2</td>
</tr>
<tr>
<td>T21 (Bo) Arg-D</td>
<td>0.06</td>
<td>+2</td>
</tr>
<tr>
<td>T26 (Bo) Arg-D</td>
<td>0.015</td>
<td>0.3</td>
</tr>
<tr>
<td>T47 (Bo) Arg-B</td>
<td>1.1</td>
<td>1.8</td>
</tr>
<tr>
<td>T51 (Bo) Arg-B</td>
<td>0.5</td>
<td>1.9</td>
</tr>
<tr>
<td>T67 (Bo) Arg-B</td>
<td>0.8</td>
<td>1.7</td>
</tr>
<tr>
<td>T82 (Bo) Arg-B</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>T86 (Bo) Arg-B</td>
<td>1.3</td>
<td>1.7</td>
</tr>
<tr>
<td>T149 (Bo) Arg-B</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>UK + EDTA (Bo)*</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>NCDV + EDTA (Bo)*</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Strains UK, NCDV, B223, and B678 were tissue-culture-adapted samples. All other strains were stool samples. Bo, Bovine; Arg, Argentinian; D, dairy herd; B, beef herd.
* ELISA was performed as explained in the text.
* Strains T14 to T149 were classified as serotype 6 or non-serotype 6 by IPFRA performed with hyperimmune serum against UK strain. UK and NCDV strains were classified as serotype 6 by Hoshino et al. (16) and Estes (in press). B223 and B678 were described as non-serotype 6 by Woode et al. (32) and Oje et al. (24).
* NC, Not classified with a serotype number at this time.
* Strains and NCDV strains were tested with 50 mM EDTA to determine the reactivity of single-shell particles with the serotype 6 MAb and the common VP7 MAb.

shown) and different bovine rotaviruses (Table 2). The serotype 6-specific MAb reacted strongly with the UK, NCDV, and UK-related Argentinian strains, and no reaction was observed with non-serotype 6 strains. An MAb to a common epitope on VP7 was used in the ELISA to determine that all virus strains tested contained the outer capsid. Several strains (B223, B678, and T26) showed a low OD$_{414}$ reading, probably because they lacked sufficient VP7 to give a strong reaction. However, it is also possible that these viruses lacked the common epitope on VP7 detected by this MAb.

One hundred fifteen fecal samples from diarrheic calves containing group A rotaviruses were assayed using the serotyping ELISA. The results are shown in Table 3.

![FIG. 1. RNA electrophoretic patterns of bovine rotaviruses associated with diarrhea outbreaks.](http://jcm.asm.org/)

An outbreak of diarrhea (Fig. 1, lane 1) from which all other samples showed a different RNA electrophoretic pattern (lanes 2 to 6). The RNA patterns of serotype 6-related rotaviruses (lanes 1 and lanes 7 to 18) were distinct from those of non-serotype 6 rotaviruses (lanes 2 to 6).

This paper reports the serologic characterization of bovine rotaviruses isolated in Argentina. Two methods of serotyping, a standard neutralization assay (IPFRA) and a serotyping ELISA, were used for virus characterization. The standard neutralization assay has been widely used in serotyping human strains and strains from other mammalian or avian species (6, 16, 21, 24, 32). Serotype specificity in group A rotaviruses is defined by the two outer capsid proteins: VP7, the glycoprotein, and VP4, a protease-sensitive protein which is the hemagglutinin in many virus strains (15, 23, 29; M. K. Estes, in B. N. Fields, ed., *Virology*, in press). Serotype classification based on neutralization assays using hyperimmune serum probably is mediated by VP7 and to a lesser extent by VP4. The most common method used to produce hyperimmune antisera for serotyping assays is to hyperimmunize animals with CsCl-purified viral particles. The amounts of VP4 present in such preparations may be small since VP4 is present in small amounts in viral particles (17) and often is not stable when viruses are purified in CsCl gradients (5). Some authors previously used convalescent-phase sera obtained from animals primed orally with virulent viruses. Neutralization assays done with these sera may better evaluate the role of both proteins, but these also show less-distinct reactivities (11, 32). The typing sera used in this study were obtained from guinea pigs that were not primed orally, so most of the antibodies probably were directed to VP7. An interesting result of the IPFRA was that two bovine strains (T21 and T26) showed two-way relatedness with the B223 strain isolated in the United States by Woode et al. (32). These strains showed different RNA electropherotypes in PAGE and were isolated from dairy herds located about 800 km apart. T21 was isolated from a herd in which non-serotype 6 strains predominated, and it showed the same RNA electropherotype observed in four other non-serotype 6 strains detected in the same herd (Fig. 1, lanes 2 to 6). These data suggest that the serotype represented by B223 may be another bovine serotype which shows wide geographic distribution similar to that previously noted for NCDV and UK.

TABLE 3. Serologic characterization of bovine rotaviruses from dairy and beef herds by using the serotype 6-specific ELISA

<table>
<thead>
<tr>
<th>Rotavirus source</th>
<th>No. (%) of samples</th>
<th>Serotype 6</th>
<th>Non-serotype 6</th>
<th>Untypable*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef herds</td>
<td>61 (84.7)</td>
<td>1 (1.4)</td>
<td>10 (14)</td>
<td></td>
</tr>
<tr>
<td>Dairy herds</td>
<td>19 (44)</td>
<td>12 (28)</td>
<td>12 (28)</td>
<td></td>
</tr>
</tbody>
</table>

* Samples were considered untypable when they did not react in the ELISA with either the serotype 6 MAb or the common VP7 MAb.
strains (serotype 6). Only one strain (T26) showed a weak one-way cross-reaction with the B678 strain, which appears to be an unusual bovine serotype (24).

Serotype determination by neutralization assays is very time-consuming and labor-intensive and is not practical for analyzing large numbers of samples. Faster serotype characterization should be possible using recently characterized antibodies that recognize neutralizing epitopes on VP7 and VP4 (5, 10, 18–20, 29). The usefulness of serotyping ELISAs performed with serotype-specific MAbs directed to the glycoprotein VP7 has been reported (8, 14, 30, 31). We report here the specificity of an ELISA to detect rotavirus serotypes related to NCDV or UK strains (Table 1). Both strains have been classified as serotype 6 by Hoshino et al. (16) or as bovine serotype 1 by Woode et al. (32) and Ojeh et al. (24). With this serotyping ELISA, we were able to screen a large number of rotavirus-positive fecal samples from dairy and beef herds. Serotype 6 was predominant in beef herds (84.7%), in agreement with serotyping of bovine rotaviruses in other countries (24, 32). However, in dairy herds serotype 6 occurred less frequently (44%), and high percentages of samples were non-serotype 6 (28%) and untypable (28%). This observation may reflect the different types of management of calves in both kinds of herds. In dairy herds calves are handled in a crowded environment, and in most farms they are fed with lacteal substitutes. In beef herds calves are kept on spacious farms during all seasons, and they remain with their mothers until 7 months of age. Differences in the etiologies of diarrhea between dairy and beef herds have been observed by us in Argentina (Bellinzoni et al., submitted). It is also possible that some serotype 6 rotaviruses were not detected in the serotyping ELISA because the cutoff point OD540 to define positive results was high and also because of monotype variation between serotype 6 viruses. Viruses of the same serotype can escape detection by a single serotype-specific MAb because of monotype variation (7). However, this seems unlikely in our study since this variable should affect viruses from both kinds of herds in the same way.

In herds in which rotavirus was detected serotype 6 was present, and it was usually predominant when more than one serotype was detected in a single diarrhea outbreak. The observation that distinct serotypes possessed distinct viral RNA patterns in PAGE agrees with other recent reports of studies in which ELISA serotyping was used to characterize human rotavirus in combination with RNA electrophoresis. In these cases, changes in the VP7 antigen were associated with concurrent changes of genomic RNA electropherotypes (8, 22). However, additional typing is required to confirm this possibility.

Knowledge of the epidemiological behavior of rotaviruses in cattle will be helpful to elaborate strategies of disease prevention for calf diarrhea. One important observation of this study is the significant difference in serotype distributions between dairy and beef herds. In Argentina, an inactivated oil-adjuvant rotavirus vaccine has been shown to be effective in beef herds (1). The significant difference in the etiologies of diarrhea and the higher serotype variation in dairy herds suggest that vaccination on these farms may be less efficient than has been shown in beef herds. However, serotype variation does not seem to be a problem when rotavirus vaccines are administered to cows. Vaccination of cows with a single serotype induces heterotypic milk and serum neutralizing antibodies which protect calves by passive immunity (1, 3, 26). However, serotyping of rotavirus isolates from vaccinated animals would be useful to determine whether vaccination selects new rotavirus serotypes which are usually not common in the cattle population.

This work was partially supported by grants from the Programa Nacional de Biotecnología, Consejo Nacional de Investigaciones Científicas y Técnicas, Secretaría de Ciencia y Tecnica, and the Swedish Agency for Research Cooperation with Developing Countries and by National Science Foundation grant INT-8050282.

We thank G. Woode for the gift of NCDV and B223 bovine rotavirus and Andres Bellinzoni for technical assistance.

LITERATURE CITED


