Detection by Radioimmunoassay and Enzyme-Linked Immunosorbent Assay of Coronavirus Antibodies in Bovine Serum and Lacteal Secretions

L. RODAK,1,2† L. A. BABIUK,1,2* AND S. D. ACRES2

Department of Veterinary Microbiology1 and Veterinary Infection Disease Organization,2 University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0

Received 11 January 1982/Accepted 18 March 1982

Coronaviruses are a group of single-stranded RNA viruses with characteristic club-shaped projections on the surface of their lipid envelope. In addition to sharing biochemical and biophysical properties, they also appear to share an affinity for mucosal surfaces, particularly those of the respiratory and gastrointestinal tracts (10-12, 14). Most bovine coronavirus isolates grow poorly in tissue culture. Bovine coronavirus is often a cause of gastroenteritis in animals between 5 and 30 days of age (2, 16, 17). Even though bovine coronavirus is often difficult to isolate directly from feces, a number of isolates have been adapted to grow in vitro and to produce various degrees of cytopathic effects (3, 13, 16, 18). Using one of these isolates has allowed us to compare the sensitivity of two immunodiagnostic tests, radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), with serum-neutralizing assays (SN) to detect antibody titers in the serum, colostrum, and milk of cattle. Additionally, we correlated the sensitivity of the tests with the quality of reagents used in the respective immunoassays.

It has been reported for coronaviruses of other species that ELISA can be used for detection of virus (8) and that it is more sensitive than SN (9). The present report demonstrates the prevalence of coronavirus antibody in cattle after natural infection and after vaccination with a commercially available, modified live vaccine. The use of RIA and ELISA as rapid diagnostic and epidemiological tools for studies of coronaviruses in cattle is discussed.

MATERIALS AND METHODS

Cells and viral antigens. Georgia bovine kidney cells were cultured in Eagle minimal essential medium as described previously (1). Each liter of medium was supplemented with 2 mmol of glutamine, 10 ml of nonessential amino acids (GIBCO Laboratories, no. 114), 3.5 mg of gentamicin, and 2.5 g of sodium bicarbonate. For growth, the medium contained 10% fetal bovine serum. Confluent cultures were washed twice with Hanks balanced salt solution before infection with bovine coronavirus (obtained from D. Brian, University of Tennessee, Knoxville). After adsorption of virus for 1 h at 37°C, Eagle medium plus 2% bovine serum albumin fraction V was added. When cytopathic effects were evident, 3 to 4 days later, the cultures were subjected to three freeze-thaw cycles before removal of the cells by centrifugation at 2,500 × g for 20 min. The virus present in the supernatant fluid was pelleted at 82,500 × g for 2.5 h at 4°C. The pelleted virus antigen was suspended in phosphate-buffered saline (PBS) and stored at -70°C. Control antigen was prepared in an identical manner from uninfected cells. Before being used for coating plates, the antigen was

† Permanent address: Veterinary Research Institute, 621 32 Brno, Czechoslovakia.
diluted 200-fold in 0.1 M carbonate-bicarbonate buffer (pH 9.6).

Preparation of 125I-labeled rabbit antihuman immunoglobulin. Commercially available immunoglobulin G (IgG) fraction of rabbit antihuman IgG (Cappel Laboratories, lot number 15205) containing 25% protein with anti-IgG activity was used. For use in RIA, 1 mg of protein was labeled with 125I by the chloramine-T method (6), and unbound 125I was removed by passing the material through Sephadex G-25. In the stock solution of labeled protein, 98.4% of the activity was precipitable with 10% trichloroacetic acid. The final test solution of 125I-labeled antibody was prepared by diluting the stock solution with PBS containing 5% normal rabbit serum and 0.2% Tween 20 to a final activity of 5 x 10^5 cpm/ml (8.3 x 10^5 dpm/ml).

Peroxidase-labeled conjugates and substrate. Two conjugates were used. One was purified by affinity chromatography, and the other was not. The affinity-purified peroxidase-conjugated rabbit antihuman IgG (Zymed Laboratories, lot number 10723) was diluted 1:1,000 in PBS containing 5% normal rabbit serum and 0.2% Tween 20 before use. The other conjugate of non-purified rabbit antihuman IgG (Cappel Laboratories, lot number 14984) was diluted 1:200 in the same diluent before use.

Substrate was prepared by dissolving 8 mg of 5-aminosalicylic acid in 10 ml of distilled water. The solution was adjusted to pH 5.0 with 0.1 M NaOH, and 1 ml of 0.05% H2O2 in 0.1 M phosphate-citric acid buffer (pH 5.0) was added before use. RIA procedure. Flat-bottomed polystyrene wells (Removastrip Imulon, Dynatech Laboratories, Inc.) placed in a Removawell strip holder (Dynatech) were coated with 0.1 ml of diluted viral or control antigen and incubated overnight at room temperature. The wells were then washed three times with PBS and incubated with 0.1-ml volumes of diluted test sera or colostral whey. Each sample dilution was added to triplicate wells coated with viral or control antigen or both. After incubation for 2 h at room temperature in a humidified chamber, the wells were washed three times with PBS and incubated an additional 2 h with 0.1-ml volumes of 125I-labeled rabbit antihuman IgG. After a final three washes in PBS, the radioactivity in counts per minute bound in individual wells was determined with an automatic gamma counter (Searle Radiographics, Inc., model 1185). The average activities and standard deviations of triplicate wells coated with viral or control antigen and incubated with the same sample dilution were determined, and a V/C ratio (ratio of average activities bound in wells with viral and control antigens) was calculated. Samples were considered positive up to the highest dilution giving a V/C ratio greater than 2. The V/C ratio obtained after incubation without serum or with negative serum was always approximately 1. Under such conditions, V/C ratios higher than 2 are highly significant (usually P ≤ 0.001) by Student's t test.

ELISA procedure. The preparation of coated plates and antibody dilutions for ELISA were identical to those for RIA. After incubation with antibody, 0.1-ml volumes of peroxidase-labeled rabbit antihuman IgG were added to each well. After a 2-h incubation, the plates were washed three times and incubated for an additional 60 min with 5-aminosalicylic acid. The enzyme-mediated reaction was measured at 450 nm with the aid of an ELISA plate reader (Titertek, Flow Laboratories, Inc.). The average absorbance, standard deviation, and V/C ratio of triplicate wells coated with viral or control antigen were calculated. The highest sample dilution giving a V/C ratio greater than 2 was considered positive. Reaction of the coronavirus antigen with monospecific bovine antibody to bovine herpesvirus and rotavirus always gave a V/C ratio less than 2.

SN procedure. Neutralization titers were obtained by preparing serial dilutions of the serum of the colostral whey and reacting 0.5-ml volumes with 0.5 ml of coronavirus containing 1,000 50% tissue culture infective dose units for 1 h at 37°C. The virus-serum or virus-whey mixture was then added to quadruplicate wells of a microtiter plate containing Georgia bovine kidney cells. After adsorption for 1 h, the fluids were removed and replaced with fresh Eagle medium plus 2% bovine serum albumin and incubated an additional 4 days. Since the cytopathic effect seen is generally not specific, the endpoints were determined by hemadsorption. This was achieved by layering a 1% suspension of fresh mouse erythrocytes on the monolayer for 60 min. Erythrocytes were removed by washing, and the antibody titers were computed by the Karber method (7), using the dilution that reduced hemadsorption in excess of 90% as the endpoint.

Evaluation of a commercial coronavirus vaccine. A modified live-virus vaccine for prevention of diarrhea caused by bovine coronavirus is available commercially (Calf-Guard, Norden Laboratories); however, no serological studies on the vaccine have been reported. We used the ELISA procedure developed here to measure the immune response of cows to the vaccine. Pregnant beef cows were vaccinated intramuscularly with 3 ml of vaccine according to the manufacturer's instructions. One group of cows was vaccinated once approximately 6 to 9 weeks before parturition. A second group was given two vaccinations 3 weeks apart during the last trimester of gestation. A third group of unvaccinated cows served as controls. Cows were bled at the time of the first vaccination and 3 and 6 weeks later. Cows in the control and single-vaccination groups were milked within 1 h, 24 h, 5 days, and 20 days after calving. Cows in the double-vaccinated group were milked within 1 h, 36 h, 5 days, and 21 days after calving. Whey was prepared by using commercial rennin and stored at −20°C until used for analysis.

RESULTS

Specificity and sensitivity of assays. Viral and control antigens for RIA and ELISA assays are suitable only if the activities or absorbances result in a V/C ratio of approximately 1 after reaction with negative serum in the case of viral antigen, or with any serum reacted with control antigen. In our preliminary experiments, the antigens were prepared to achieve this goal (data not shown).

The sensitivity of RIA and ELISA methods generally depends not only on the quality of viral and control antigens, but also on the quality of 125I-labeled or peroxidase-conjugated antibodies. It was illustrated in our studies by the use of two different commercially available peroxi-
TABLE 1. Comparison of reagents for detection of antibodies to coronavirus

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>RIA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 71</td>
<td>109,350</td>
<td>36,450</td>
<td>1,350</td>
</tr>
<tr>
<td>Serum 73</td>
<td>12,150</td>
<td>12,150</td>
<td>1,350</td>
</tr>
<tr>
<td>Colostrum 72</td>
<td>450</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>Colostrum 4</td>
<td>1,350</td>
<td>450</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent the highest dilution which provided a V/C ratio of greater than 2.

<sup>b</sup> Antibody used for RIA was unpurified and obtained from the same company as unpurified IgG for ELISA.

dase-labeled rabbit antibovine IgG conjugates (Table 1). With a conjugate purified by affinity chromatography, we obtained titers 3 to 25 times higher than those obtained with an unpurified conjugate.

A comparison of the RIA and ELISA methods using the same commercially available unpurified antibody demonstrated that RIA was at least 10 times more sensitive than ELISA. However, the sensitivity of ELISA approached that of RIA if chromatographically purified reagent was used in the ELISA and unpurified antibody was used in the RIA (Table 1). Similar results were obtained on numerous occasions, even after the serum was frozen and thawed three times, indicating that the test was reproducible and could use sera that had been subjected to a number of freeze-thaw cycles.

All samples to be examined were diluted threefold in the range of 50 to 100,000. Throughout the positive range, the V/C ratio for each dilution was higher than 2, but the actual V/C ratio values gave a sigmoidal curve similar to a prozone effect often observed in immunological assays (Fig. 1). These results demonstrate that the approximate titer cannot be extrapolated from one V/C value obtained using a low serum dilution, since at high concentrations of immunoglobulin there is higher nonspecific binding and thus lower V/C ratios. Furthermore, the actual shape of the curve is influenced by the starting concentration of immunoglobulins in the sample. In the case of serum, concentrations of 1:50 and higher allowed a complete analysis of the sigmoidal curve (Fig. 1), whereas in colostrum taken at the time of parturition, in which the concentration of total immunoglobulin was high, the positive part of the curve became evident only after dilution of the colostrum beyond 1:450 (Fig. 2). Therefore, if only lower dilutions were tested, the colostrum might be considered negative for coronavirus antibody.

In contrast, by using milk obtained 5 days after parturition when the level of immunoglobulin is lower, it was possible to observe only the decreasing part of the curve.

**Comparison of RIA, ELISA, and SN titers in serum and colostrum.** For a comparison of the three assays, a total of 32 serum and colostrum samples were examined by ELISA, RIA, or SN. A high degree of correlation between SN and RIA titers (correlation coefficient r = 0.915) (Fig. 3) and between RIA and ELISA (r = 0.964) (data not shown) was achieved. Although the actual values used for ELISA to determine the correlation coefficient were obtained with the aid of an ELISA reader, comparable results could be obtained by visual examination (Fig. 4). The actual titer for serum A as determined with the ELISA reader was 12,150; for serum B it was 450 (Fig. 4). Thus, the advantage of ELISA over the other two assays is the speed with which the assay can be performed and read without the need for expensive equipment.

Further evidence that the tests were all measuring specific antibody to coronavirus was ob-
Survey of prevalence of coronavirus in cattle. Using ELISA, we screened 12 different herds of cattle situated within a 300-mile radius of each other in western Canada for antibodies to coronavirus. Antibodies were found in all of the herds, and only 1 of 110 animals was found not to have antibody, suggesting that the virus is ubiquitous in cattle populations in western Canada. A survey of this type, using over 100 serum samples, could be performed in one day. Thus, the sensitivity and speed of ELISA warrant further use of this technique in diagnostic laboratories.

Serological and lactogenic response to vaccination. Serum antibody titers to coronavirus did not change in cows given one or two injections of the modified live-virus vaccine (Table 2). In addition, there were no significant differences in colostrum or milk titers between cows in either of the vaccinated groups and the control group. Similarly, SN tests on serum and lacteal secretions from cows in the double-vaccination group showed no increase in titer as compared with control cows (data not shown).

DISCUSSION

RIA and ELISA are widely used methods for diagnosis of various infections, especially viral diseases of humans and animals. These methods generally exceed the sensitivity of conventional methods used in diagnostic laboratories, but specificity and sensitivity are very dependent on the quality of the antigens. Any attempts to use these assays must take this into consideration. For this reason, these assays were first introduced for use with viruses which grow well in culture (4, 5, 20).

Bovine coronaviruses generally are difficult to adapt to grow in cell culture and produce recognizable cytopathic effects (2, 3, 13, 18). We were able to obtain an isolate of a bovine coronavirus that grew in culture and thus could be used in RIA and ELISA for diagnosis of this agent. To compare the sensitivity of these assays with that of conventional assays, we compared the antibody levels in serum and colostrum obtained by RIA and ELISA with those obtained by SN. The results demonstrated a high correlation between the tests ($r = 0.915$ for RIA-SN; $r = 0.964$ for RIA-ELISA). In most cases, ELISA titers were obtained by determining coronavirus antibodies present in colostrum at different times postparturition. Very high levels of antibody were present at the time of parturition, as detected by all three methods, and rapidly declined thereafter (Fig. 5). Similarly, before suckling, calf serum titers were negative, but within 24 h of birth and suckling, these titers approached those of the dam (data not shown).
FIG. 4. Detection of coronavirus antibody by ELISA. Plates were coated with virus antigen (V-Ag) or control antigen (C-Ag) incubated with various dilutions of serum as described in the text.

higher than those obtained by SN. Furthermore, using the same antibody in the indicator system, the RIA gave titers at least 10 times higher than ELISA titers. This is comparable to what has been reported in other systems (19). However, once the proper antigens are available, the sensitivity of the assays can be increased even more by using chromatographically purified antibody (Table 1), and a further increase in sensitivity can be achieved by using luminometry with ELISA (15). Although the sensitivity of RIA and ELISA in our study was only 10 to 100 times that of SN, it should be pointed out that the sensitivity of the SN test for coronaviruses is relatively high compared with that for other viruses.

Another advantage of RIA and ELISA over SN, in addition to sensitivity and speed, is the

FIG. 5. Coronavirus antibodies in milk at different times postparturition as measured by RIA (■), ELISA (▲), and SN (●).
TABLE 2. Coronavirus antibody titer (by ELISA) in serum and lacteal secretions of cows given a modified live virus vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>Cow</th>
<th>Serum, at time after injection of vaccine</th>
<th>Lacteal secretions, at time after calving (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 3 wk 6 wk Part-</td>
<td>0 1-1.5 5 20-21</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>4,050 4,050 4,050</td>
<td>36,450 4,050 4,050 150</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4,050 4,050 4,050</td>
<td>12,150 4,050 450 150</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4,050 4,050 1,350</td>
<td>12,150 4,050 450 150</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4,050 4,050 4,050</td>
<td>36,450 12,150 1,350 150</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4,050 4,050 4,050</td>
<td>12,150 12,150 1,350 150</td>
</tr>
<tr>
<td>GMT*</td>
<td></td>
<td>4,050 4,050 3,263</td>
<td>18,855 6,286 1,082 150</td>
</tr>
<tr>
<td>Single vaccination</td>
<td>6</td>
<td>1,350 1,350 1,350</td>
<td>12,150 12,150 150 50</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4,050 4,050 4,050</td>
<td>36,450 12,150 1,350 150</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4,050 4,050 1,350</td>
<td>12,150 4,050 450 450</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>4,050 4,050 4,050</td>
<td>12,150 1,350 450 150</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4,050 4,050 4,050</td>
<td>12,150 4,050 150 150</td>
</tr>
<tr>
<td>GMT</td>
<td></td>
<td>3,263 3,263 2,616</td>
<td>15,040 5,035 450 150</td>
</tr>
<tr>
<td>Double vaccination</td>
<td>11</td>
<td>4,050 4,050 4,050</td>
<td>36,450 4,050 150 50</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>4,050 4,050 4,050</td>
<td>12,150 12,150 150 0</td>
</tr>
<tr>
<td></td>
<td>13</td>
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<td>12,150 450 450 50</td>
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<tr>
<td></td>
<td>14</td>
<td>1,350 1,350 1,350</td>
<td>12,150 450 150 50</td>
</tr>
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<td></td>
<td>15</td>
<td>4,050 4,050 4,050</td>
<td>36,450 1,350 450 50</td>
</tr>
<tr>
<td>GMT</td>
<td></td>
<td>2,616 2,616 2,616</td>
<td>18,855 1,679 233 24</td>
</tr>
</tbody>
</table>

* GMT, Geometric mean titer.

The finding that RIA was the most sensitive assay for detection of coronavirus antibody makes it the most suitable assay system for research laboratories equipped with gamma counters and where a high degree of sensitivity is required to measure small amounts of antigen or antibody. In contrast, ELISA, although less sensitive, can be carried out in every laboratory since the results can be evaluated even with the naked eye. Furthermore, the fact that all serum
samples that were positive by RIA were also positive by ELISA should make the ELISA the test of choice in routine laboratory testing of sera for bovine coronavirus.

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LITERATURE CITED