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

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The sensitivity of a radioimmunoassay (RIA), an enzyme-linked immunosorbent assay (ELISA), and a serum neutralization assay (SN) for detecting antibodies to bovine coronavirus in serum and colostrum were compared. Although there proved to be a good correlation among all three assays ($r = 0.915$ and 0.964 for RIA with SN and ELISA, respectively), RIA and ELISA proved to be at least 10 times more sensitive than neutralization tests. By using these techniques, it was possible to detect a time-dependent decrease in antibody levels in bovine colostrum after parturition. Using ELISA, we demonstrated that 12 of 12 herds in Saskatchewan, and 109 of 110 animals tested, had antibody to bovine coronavirus. There was no elevated antibody response in serum or lacteal secretions of cows vaccinated once or twice with a commercially available modified live rota-coronavirus vaccine. In addition to being more sensitive than SN, ELISA and RIA proved to have other advantages for measuring antibody levels to bovine coronavirus and therefore warrant wider use as tools in diagnostic virology.

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 detec-

tion 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

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diluted 200-fold in 0.1 M carbonate-bicarbonate buffer (pH 9.6).

Preparation of ^{125}I-labeled rabbit antibovine immunoglobulin. Commercially available immunoglobulin G (IgG) fraction of rabbit antibovine 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 ^{125}I by the chloramine-T method (6), and unbound ^{125}I 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 ^{125}I-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 5×10^5 cpm/ml (8.3×10^3 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 antibovine 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 antibovine 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% H_2O_2 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 ^{125}I-labeled rabbit antibovine 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 $P \leq 0.001$) 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 antibovine 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 ^{125}I-labeled or peroxidase-conjugated antibodies. This 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

Sample no.	Titer[a]		
	RIA[b]	ELISA	
		Purified IgG	Unpurified IgG
Serum 71	109,350	36,450	1,350
Serum 73	12,150	12,150	1,350
Colostrum 72	450	150	50
Colostrum 4	1,350	450	50

[a] Values represent the highest dilution which pro-
vided a V/C ratio of greater than 2.

[b] Antibody used for RIA was unpurified and ob-
tained 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 unpuri-
fied antibody demonstrated that RIA was at
least 10 times more sensitive than ELISA. How-
ever, 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. Through-
out 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 colos-
trum 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
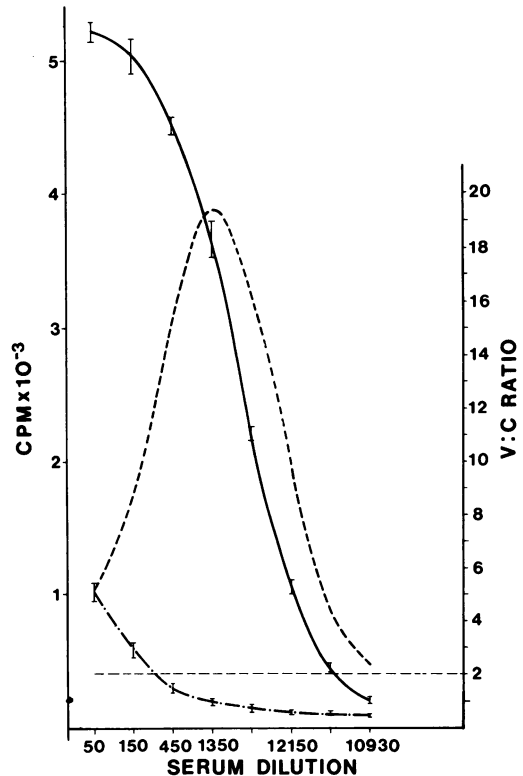considered negative for coronavirus antibody.

FIG. 1. Detection of coronavirus antibodies in bo-
vine serum by RIA. (———) Activity bound to corona-
virus positive-antigen-coated plates; (—·—) activity
bound to control plates; (-----) ratio of virus to control
plates.

In contrast, by using milk obtained 5 days after
parturition when the level of immunoglobulin is
lower, it was possible to observe only the de-
creasing 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 mea-
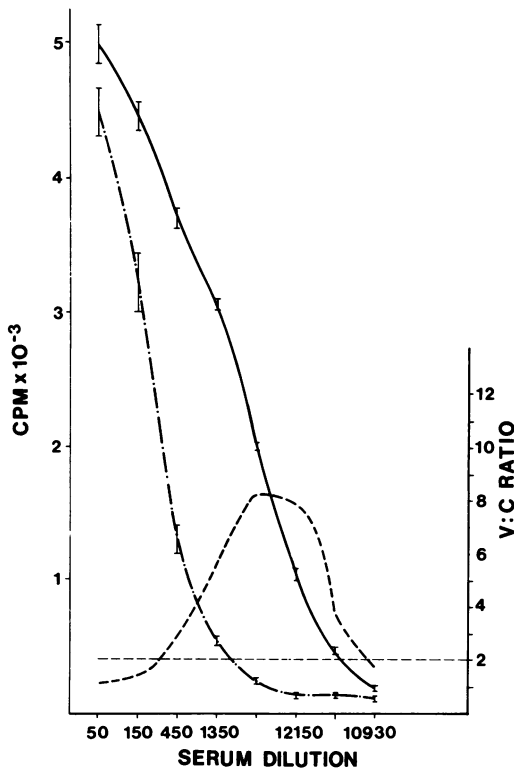suring specific antibody to coronavirus was ob-

FIG. 2. Coronavirus antibody titers, as determined by RIA, present in colostral whey of cattle at parturition. (——) Activity in virus coated plates; (—·—) activity in control plates; (-----) ratio of virus to control plates.
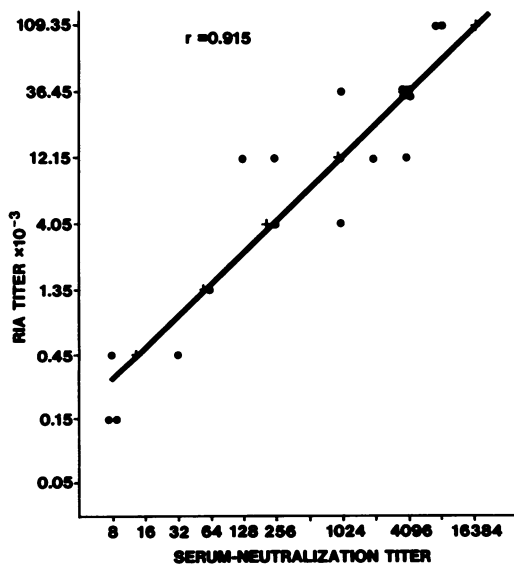

FIG. 3. Correlation between RIA and SN results in measuring antibody levels to coronavirus. Symbols: individual serum samples (●) and correlation (×).

tained 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).

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
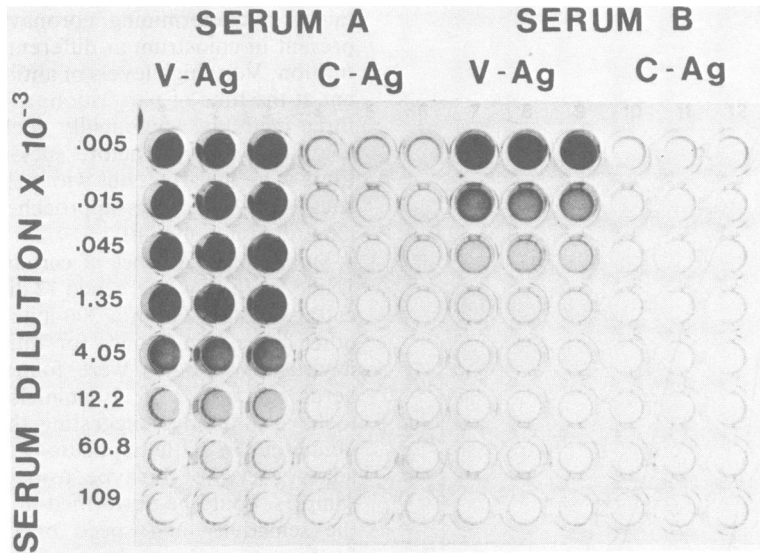
FIG. 4. Detection of coronavirus antibody by ELISA. Plates were coated with virus antigen (V-Ag) or control antigen (C-Ag) incubated with various dilutions of serum as described in the text.

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 cornaviruses 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
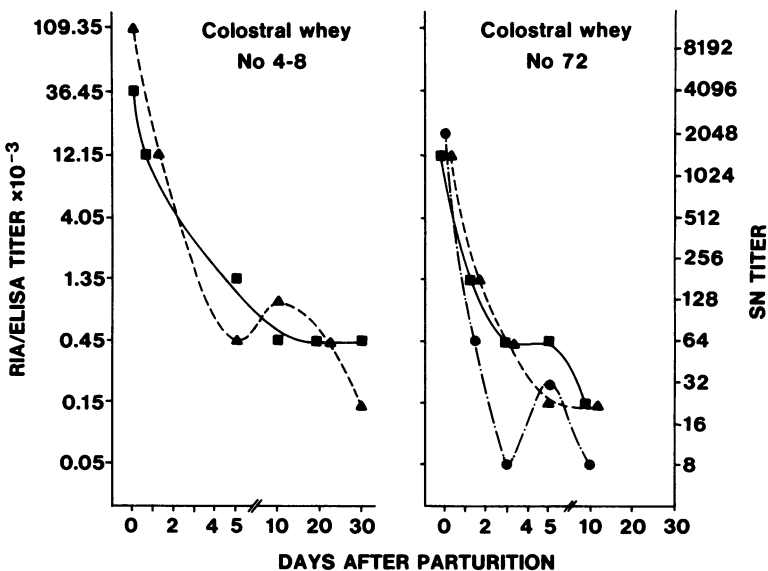

FIG. 5. Coronavirus antibodies in milk at different times postparturition as measured by RIA (■), ELISA (▲), and SN (●).

TABLE 2. Coronavirus antibody titer (by ELISA) in serum and lacteal secretions of cows given a modified live virus vaccine

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

[a] GMT, Geometric mean titer.

fact that these techniques do not require sterile samples. Thus, samples that are submitted to regional diagnostic laboratories in unsterile vials can still be assayed by RIA and ELISA. Furthermore, if samples are not processed properly before submission, they may be toxic to tissue culture cells. The use of RIA and ELISA can also alleviate this problem. Finally, the procedures can be automated so that numerous samples can be assayed at once and the results are available within a few hours whereas SN for coronavirus takes at least 6 days for results to be obtained. Because of automation, the reproducibility of the results by ELISA and RIA is much greater than for SN. Since it is difficult to read the rather nonspecific endpoints in SN tests with bovine coronavirus, titers may not be reproducible; such is not the case with nonsubjective tests such as RIA and ELISA.

The prevalence of coronavirus antibodies in cattle suggests that this agent is very widespread and may be an important pathogen in cattle in western Canada. By using sensitive methods of detection for both virus antigen and antibody, it should be possible to understand more fully the epidemiology of coronavirus and its role in neo-natal diarrhea as well as to determine the level of coronavirus antibody in colostrum and milk that will protect calves from enteric disease under different systems of management. Furthermore, these assays will greatly assist us in the evaluation of serological responses to vaccination. The commercial modified live vaccine failed to cause significant increases in coronavirus titers of the serum, colostrum, or milk of cows which were given one or two doses of vaccine, and antibody titers declined just as rapidly in vaccinated as in control cows. These results indicate that this vaccine does not stimulate the development of protective antibody titers in the milk of cows and may be of limited value in preventing coronavirus diarrhea in the field.

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.

ACKNOWLEDGMENT

LITERATURE CITED

1. **Babiuk, L. A., R. C. Wardley, and B. T. Rouse.** 1975. Defense mechanisms against bovine herpesvirus: relationship of virus-host cell events to susceptibility to antibody-complement cell lysis. Infect. Immun. **12:**958–963.

2. **Dea, S., R. S. Roy, and M. E. Begin.** 1980. Physicochemical and biological properties of neonatal calf diarrhea coronaviruses isolated in Quebec and comparison with Nebraska calf coronavirus. Am. J. Vet. Res. **41:**23–29.

3. **Dea, S., R. S. Roy, and M. E. Begin.** 1980. Bovine coronavirus isolation and cultivation in continuous cell lines. Am. J. Vet. Res. **41:**30–38.

4. **Forghani, B., N. J. Schmidt, and E. H. Lennette.** 1975. Solid phase radioimmunoassay for typing herpes simplex viral antibodies in human sera. J. Clin. Microbiol. **2:**410–418.

5. **Gilman, S. C., and J. J. Docherty.** 1977. Detection of antibodies specific for herpes simplex virus in human sera by the enzyme linked immunoassay. J. Infect. Dis. **136:**286–293.

6. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89:**114–123.

7. **Karber, G.** 1931. Beitrag, zurkillektiren Behanduny, Pharmakoloischer Reinhenversuche. Arch. Exp. Pathol. Pharmakol. **162:**480–483.

8. **Kraaijeveld, C. A., M. H. Madge, and M. R. Macnaughton.** 1980. Enzyme-linked immunosorbent assay for coronaviruses HCV229E and MHV3. J. Gen. Virol. **49:**83–89.

9. **Kraaijeveld, C. A., S. E. Reed, and M. R. Macnaughton.** 1980. Enzyme-linked immunosorbent assay for detection

of antibody in volunteers experimentally infected with human coronavirus strain 229E. J. Clin. Microbiol. **12:**493–497.

10. **Langpap, T. J., M. E. Bergeland, and D. E. Reed.** 1979. Coronaviral enteritis of young calves: virologic and pathologic findings in naturally occurring infections. Am. J. Vet. Res. **40:**1476–1478.

11. **Macnaughton, M. R., B. J. Thomas, H. A. Davies, and S. Patterson.** 1980. Infectivity of human coronavirus strain 229E. J. Clin. Microbiol. **12:**462–468.

12. **Mebus, C. A., L. E. Newman, and E. L. Stair.** 1975. Scanning electron, light, and immunofluorescent microscopy of intestine of gnotobiotic calf infected with calf diarrheal coronavirus. Am. J. Vet. Res. **36:**1719–1725.

13. **Mebus, C. A., E. L. Stair, M. B. Rhodes, and M. J. Twiehaus.** 1973. Neonatal calf diarrhea: propagation, alteration and characteristics of a coronavirus-like agent. Am. J. Vet. Res. **34:**1719–1725.

14. **Mebus, C. A., E. L. Stair, M. B. Rhodes, and M. J. Twiehaus.** 1973. Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. Vet. Pathol. **10:**45–64.

15. **Pronovost, A. D., A. Baumgarten, and G. D. Hsiung.** 1981. Sensitive chemiluminescent enzyme-linked immunosorbent assay for quantification of human immunoglobulin G and detection of herpes simplex virus. J. Clin. Microbiol. **13:**97–101.

16. **Sharpee, R. L., C. A. Mebus, and E. P. Bass.** 1976. Characterization of a calf diarrheal coronavirus. Am. J. Vet. Res. **37:**1031–1041.

17. **Stair, E. L., M. B. Thodes, R. G. White, and C. A. Mebus.** 1972. Neonatal calf diarrhea: purification and electron microscopy of a coronavirus-like agent. Am. J. Vet. Res. **33:**1147–1156.

18. **Stott, E. J., L. H. Thomas, J. C. Bridger, and N. J. Jebbett.** 1976. Replication of a bovine coronavirus in organ cultures of foetal trachea. Vet. Microbiol. **1:**65–69.

19. **Voller, A., A. Bartlett, and D. E. Bidwell.** 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Pathol. **31:**507–520.

20. **Ziegler, D. W., H. D. Hutchinson, J. P. Køplan, and J. H. Nakano.** 1975. Detection by radioimmunoassay of antibodies in human smallpox patients and vaccines. J. Clin. Microbiol. **1:**311–317.