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Microtiter Tests for Detecting Antibody in Bovine Serum to Parainfluenza 3 Virus, Infectious Bovine Rhinotracheitis Virus, and Bovine Virus Diarrhea Virus

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Microneutralization tests for detection of antibody in bovine serum to three bovine viruses are described. The Madin-Darby bovine kidney cell line was used with parainfluenza 3 virus (PI 3), whereas serially cultivated bovine embryonic kidney cells were used for infectious bovine rhinotracheitis virus and bovine virus diarrhea virus. Comparison of micro-hemagglutination-inhibition (HI) with micro-serum-neutralization (SN) tests for PI 3 showed the SN test to be more sensitive, more specific, and therefore more useful than the HI test for detecting antibody. Although the effect of trypsin-periodate treatment of serum was to reduce the HI titer of numerous sera by a twofold dilution, sufficient evidence could not be found to indicate that nonspecific HI inhibitors to PI 3 are present in bovine sera.

This report is part of an investigation of the shipping fever complex of cattle in which parainfluenza 3 virus (PI 3), bovine virus diarrhea virus (BVD), and infectious bovine rhinotracheitis virus (IBR) have been incriminated. Since antibody to PI 3 is measured primarily by hemagglutination-inhibition (HI) tests whereas antibody to IBR and BVD is measured by the more time-consuming serum-neutralization tests (SN), much more serological data have been obtained for PI 3 than for the latter two viruses. The miniaturization of the HI test by Takatsy (14) and Sever (12) and the development of suitable microtiter equipment have increased the ease with which the HI test can be performed. Recently the utility of the microtiter system has been further increased by its adaptation to SN tests (10, 11, 13).

The purpose of this research was to develop micro-SN tests for measuring antibody to BVD and IBR and to investigate the micro-HI test for PI 3. Although nonspecific HI inhibitors to PI 3 have not been found in bovine serum (4, 8), the significance of HI titers of 20 or less has been questioned (1). Our initial results with the micro-HI test also led us to question the significance of titers of 10 and 20. Since others have reported that treatment of bovine serum with kaolin, bentonite, trypsin, receptor-destroying enzyme (RDE), or periodate did not produce a reduction in the HI titer (4, 8), we investigated the effect of treatment of serum with trypsin and periodate. We also compared the micro-SN with the micro-HI test for detecting antibodies to PI 3.

Henceforth, HI and SN will refer to micro-HI and micro-SN, respectively, when used in reference to the work performed in our experiments.

MATERIALS AND METHODS

Cell cultures. The Madin-Darby bovine kidney (MDBK) cell line and bovine embryonic kidney (BEK) cells between the 20th and 40th passage were used. Cultures for SN tests were grown in a yeast extract-Eagle medium-lactalbumin hydrolysate-peptone (YELP) medium (9) with 10% fetal bovine serum, 200 units of penicillin per ml, 200 μg of streptomycin per ml, and 100 μg of neomycin per ml.

Viruses. The following viruses were used in HI and SN tests and were obtained from G. H. Frank of the National Animal Disease Laboratory, Ames, Iowa: (i) IBR, (ii) the SF-4 strain of PI 3, and (iii) the NADL strain of BVD.

Calves. All calves were between 6 and 10 months of age and were from Auburn University and two Auburn University Agricultural Experiment Sub-stations.

Immunization. Three New Zealand White rabbits were given 2×10^7 $TCID_{50}$ of the SF-4 strain of PI 3 intranasally four times biweekly. Three calves (no. 46-48) were given 8×10^7 $TCID_{50}$ of the SF-4 strain of PI 3 intranasally followed by an intramuscular injection with the same amount of virus 2 weeks later. The calves were kept in isolation after immunization

was started. Three calves (no. 49, 50, and 109) from the same herd were kept as controls in another isolation room.

Thirty calves from a single herd were divided so that 15 calves were given a combined live BVD-IBR-PI 3 commercial vaccine (Pitman-Moore, Indianapolis, Ind.) intramuscularly twice biweekly; 15 calves were kept as contact controls. This sera were tested for neutralizing antibody to BVD and IBR.

Treatment of sera used in HI test. All sera, unless otherwise indicated, were treated with trypsin and periodate as described by Davenport and Minuse (3), which includes heating at 56 C for 30 min, and subsequently adsorbed with guinea pig red blood cells (RBC). Untreated sera were heated at 56 C for 30 min and adsorbed with guinea pig RBC.

Sera used for comparing HI and SN titers to PI 3. Two hundred sixty-seven serum samples were obtained from 61 unimmunized calves over a period of 2 months. Some animals had clinical signs of respiratory infection for 1 or 2 weeks, whereas others appeared healthy during the interval sera were collected.

HI test. The HI test described by Sever (12) was performed as follows. Phosphate-buffered saline (*p*H 7.2) was used as diluent. Dropper pipettes and dilution loops calibrated to deliver 0.025 ml were used with "U" bottomed Lucite plates (Cooke Engineering Co., Alexandria, Va.). Duplicate wells were used for each dilution of serum. A drop of serial twofold dilutions of serum was incubated with a drop of the SF-4 strain of PI 3 containing 4 to 8 units of hemagglutinin at ambient temperature for 1 hr. Two drops of guinea pig RBC, standardized to contain 5×10^7 cells/ml as recommended by Hierholzer and Suggs (6), were added, and the plates were held at 5 C overnight. The HI titer was considered the reciprocal of the highest dilution of serum in which complete agglutination did not occur. The lowest dilution of serum tested was 1:10.

SN test. The SN test was performed with the same equipment used in the HI test except MicroTest II plates and lids (Falcon Plastics, Los Angeles, Calif.) were used instead of those used in the HI test. Fetal bovine serum was pretested for antibodies to BVD, IBR, and PI 3 before use in growth medium. The general procedure for the SN test has been described (10, 11, 13). Duplicate wells were used for each dilution of serum. A drop of serial twofold dilutions of serum was incubated with a drop containing 50 to 100 $TCID_{50}$ of IBR, PI 3, or BVD at 37 C for 1 hr. A drop containing 200,000 cells per ml was added to each well, and the cultures were incubated at 37 C. MDBK cells were used for PI 3 tests, and BEK cells were used for IBR and BVD tests. Titers were determined for PI 3 on the 6th day, for IBR on the 4th day, and for BVD on the 7th day. Titers were taken as the reciprocal of the highest dilution of serum to protect completely cells in both wells. The lowest dilution of serum tested was 1:4. With continuous use, the microtiter dilution loops became toxic and prevented growth of cells. Considerable care in washing and flaming loops was necessary to prevent toxicity from developing.

RESULTS

Effect of trypsin-periodate treatment on HI titer to PI 3. Two hundred forty-one serum samples obtained from 153 calves were tested both untreated and trypsin-periodate-treated for antibody to PI 3. Thirty-seven sera had titers of less than 10 in both the untreated and treated samples. One hundred fifteen sera had their titer reduced after treatment, whereas 89 sera had the same titer whether treated or untreated (Table 1). No instance was found in which a treated sample had a higher titer than an untreated sample. No bovine serum sample had its titer reduced more than a twofold dilution. Sera obtained from

TABLE 1. *Effect of trypsin-periodate treatment of bovine serum on hemagglutination-inhibition titers*

Hemagglutination-inhibition titers with		Frequency
Untreated serum	Treated serum	
<10	<10	37
10	<10	31
10	10	52
20	10	48
20	20	32
40	20	31
40	40	5
80	40	4
160	80	1

TABLE 2. *Correlation between hemagglutination-inhibition (HI) and serum-neutralization (SN) titers to parainfluenza 3 virus*

HI titer	No. of samples with SN titer of										
	<4	4	8	16	32	64	128	256	512	1024	2048
<10	16	1	0	2	3	0	0	0	0	0	0
10	20	10	26	43	30	13	1	0	0	0	0
20	1	5	2	14	17	30	13	3	1	0	0
40	0	0	0	0	0	0	7	4	0	1	0
80	0	0	0	0	0	0	1	0	2	1	0

TABLE 3. *Comparison of hemagglutination-inhibition (HI) and serum-neutralization (SN) tests for detecting antibody to parainfluenza 3 virus*[a]

Calf	Serological test	Titer at				
		Week 0	Week 1	Week 2	Week 3	Week 4
46	HI	10	20	20	20	40
	HI, tryp-per[b]	10	20	10	20	20
	SN	8	8	16	64	64
47	HI	20	20	20	20	40
	HI, tryp-per	10	10	10	20	20
	SN	8	8	16	64	64
48	HI	10	10	20	20	20
	HI, tryp-per	<10	10	20	20	20
	SN	<4	4	16	128	64
49	HI	10	10	10	10	10
	HI, tryp-per	10	10	10	10	10
	SN	<4	<4	<4	<4	<4
50	HI	<10	<10	<10	<10	<10
	HI, tryp-per	<10	<10	<10	<10	<10
	SN	<4	<4	<4	<4	<4
109	HI	<10	<10	10	<10	<10
	HI, tryp-per	<10	<10	<10	<10	<10
	SN	<4	<4	<4	<4	<4

[a] Calves 46 to 48 were immunized with parainfluenza 3 virus on week 0 and 2. Calves 49, 50, and 109 were not immunized.

[b] Serum treated with trypsin-periodate.

TABLE 4. *Comparison of hemagglutination-inhibition (HI) and serum-neutralization (SN) titers for antibody to parainfluenza 3 virus in immunized rabbits*[a]

Rabbit	Serological test	Preimmunization titer	Titer at weeks after 1st immunization	
			4 weeks	7 weeks
1	HI	20	80	320
	HI, tryp-per[b]	<10	40	320
	SN	<4	128	2,048
2	HI	20	80	160
	HI, tryp-per	<10	40	160
	SN	<4	128	4,096
3	HI	20	160	320
	HI, tryp-per	<10	80	160
	SN	<4	256	2,048

[a] Rabbits were immunized with parainfluenza 3 virus at weekly intervals.

[b] Serum treated with trypsin-periodate.

calves at different times did not respond consistently to trypsin-periodate treatment.

Correlation between HI and SN titers to PI 3. HI titers of trypsin-periodate-treated sera were compared with SN titers of the same sera. Of the sera with HI titers of either less than 10 or 10, 74 and 14%, respectively, had SN titers of less than 4. When HI titers were at least 10, SN titers averaged higher than corresponding HI titers (Table 2).

Development of antibody to PI 3 after immunization. A comparison between the HI and SN tests for measuring the antibody response to PI 3 was made. The response of calves to immunization with PI 3 was not noticeable with the HI test, whereas the response was very obvious with the SN test (Table 3). Rabbits responded with considerably higher titers than calves, and their response was measurable by both HI and SN. As with bovine serum, rabbit serum yielded higher titers by SN than HI (Table 4).

Serological diagnosis of PI 3 infection in calves. A comparison was made between the HI and SN tests for diagnosing PI 3 infection in calves. Seroconversion was judged by a fourfold or greater rise in titer between different serum samples. Biweekly serum samples from 69 calves were taken over a period of 2 months and tested. Eleven calves showed no seroconversion by either test. Of 31 calves which showed seroconversion by HI, all showed seroconversion by SN. An additional 27 calves showed seroconversion only by SN. Thus, of 69 calves, the HI test detected seroconversion in 45% of the calves, and the SN test detected seroconversion in 84% of the calves.

SN test for detecting antibody to IBR. Of the 15 calves given the combined BVD-IBR-PI 3

TABLE 5. *Distribution of infectious bovine rhinotracheitis virus serum-neutralization titers in vaccinated calves*

Titer	No. of calves with titer at week[a]				
	0	2	4	6	8
<4	15	7	0	3	5
4	0	5	5	5	8
8	0	3	7	7	2
16	0	0	3	0	0

[a] Calves were vaccinated on week 0 and 2.

TABLE 6. *Distribution of bovine virus diarrhea virus serum-neutralization titers in vaccinated calves*

Titer	No. of calves with titer at week[a]				
	0	2	4	6	8
<4	15	15	1	0	0
4	0	0	4	0	0
8	0	0	6	6	5
16	0	0	3	7	4
32	0	0	1	2	5
64	0	0	0	0	1

[a] Calves were vaccinated on week 0 and 2.

vaccine, none had prevaccination titers to IBR. Antibody was first detected in the serum of 8 of the 15 calves by 2 weeks. Maximum titers were detected 6 to 8 weeks after starting immunization and then declined. All vaccinated calves developed antibody to IBR. The distribution of the SN titers is shown in Table 5. Titration end points were usually sharp so that the transition from complete neutralization to no neutralization occurred within two or three dilutions. None of the control animals had antibody to IBR.

SN test for detecting antibody to BVD. Of the 15 calves given the combined BVD-IBR-PI 3 vaccine, none had SN antibody for BVD in their prevaccination sera or in sera collected 2 weeks after starting vaccination. One month after the first injection, SN antibody was present in all vaccinated calves. Antibody persisted throughout the duration of the experiment and gradually increased. Distribution of the antibody titers is shown in Table 6. Titration end points with BVD were not always sharp as with PI 3 and IBR. The transition from complete neutralization to no neutralization often extended over several dilutions. None of the control calves had antibody to BVD.

DISCUSSION

Several investigators have shown that nonspecific HI inhibitors for PI 3 are either infrequent or absent in bovine serum. Ketler et al. (8) based their conclusions on several observations. First, they found that 27 of 75 calves between 3 to 4 months old had HI titers of less than 10. Second, they found a close correspondence between HI and SN titers. However, this involved only eight sera with HI titers of less than 10; these all had SN titers of less than 5. Furthermore, no calves with HI titers of 10 or 20 were compared. Of the eight sera with HI titers of 40 or greater, all had neutralizing antibody. Third, treatment of sera with RDE or kaolin did not reduce HI titers. Dawson (4) obtained similar results. He found a good corresponce between HI and SN titers. However, only 7 sera with HI titers of 32 or less and 13 sera with HI titers greater than 32 were tested for neutralizing antibody. He also found that treatment of sera with either RDE, trypsin, periodate, kaolin, or bentonite failed to reduce HI titers from those of the corresponding untreated sera.

We found that treatment of bovine serum with trypsin and periodate frequently reduced the titer of a serum by a twofold dilution. However, there was no correlation between the titer of a serum sample and its titer after treatment. Low-titered sera as well as high-titered sera were equally affected by trypsin-periodate treatment. Furthermore, biweekly serum samples from individual calves varied in their response to trypsin-periodate treatment. Our data do not provide sufficient evidence to contradict the prevalent opinion that nonspecific HI inhibitors to PI 3 are either absent or infrequent in bovine serum.

The significance of low HI titers is independent of the existence of HI inhibitors. Low HI titers may merely reflect an artifact of the HI test. Abinanti et al. (1) examined the sera of 788 cattle and concluded that HI titers of 20 or greater could be considered positive for antibody. This agrees with our results. Although several investigaors who have used tube tests have found the HI test to be more sensitive than the SN test for detecting antibody to PI 3 (1, 5, 7), our results with microtiter tests have been the opposite. We found the SN test was more sensitive than the HI test as judged by (i) its ability to detect antibody production in immunized animals (Tables 3 and 4), (ii) its ability to detect seroconversion more frequently than by use of the HI test, and (iii) by its ability to attain titers higher than those attained in the HI test (Table 2), except for instances at low dilutions in which no antibody could be found by SN when a positive reaction was obtained by HI. We found the SN test more specific than the HI test as judged by

the fact that it demonstrated neutralizing antibody in all sera with HI titers above 20, but it did not demonstrate comparable neutralizing antibody in every serum which had an HI titer below 20. Considering the SN test was found to be more sensitive than the HI test and that HI and SN antibodies to parainfluenza viruses are probably directed against the same antigen(s), since they have the same specificities (2), it is likely that the presence of low HI titers without corresponding SN titers cannot be attributed to reactions against different antigens.

SN tests for PI 3 and IBR both gave sharp titration end points. BVD end points often extended over more than three dilutions. Improvement in the test for BVD might be achieved by using fewer cells since the BEK cells used were more susceptible to BVD during stages of active growth than when confluent, especially when infected with small amounts of virus. In these experiments, SN end points were calculated as the last dilution of serum which gave complete protection of cells. Since BVD end points often extended over several dilutions, when the SN test is used for diagnostic purposes, it would be advisable to use more replicates per serum-dilution and one of the standard methods of calculating 50% SN end points to increase the accuracy of the test.

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