Comparison of Different Antigen Preparations as Substrates for Use in Passive Hemagglutination and Enzyme-Linked Immunosorbent Assays for Detection of Antibody Against Bovine Enteric Coronavirus†

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Received 8 November 1982/Accepted 31 March 1983

Purified coronavirus, detergent extracts of purified coronavirus, and virus-infected Madin-Darby bovine kidney cells were evaluated as antigen substrates in enzyme-linked immunosorbent assay (ELISA) and passive hemagglutination systems. Only detergent-extracted and -unextracted, purified viruses were reactive as antigen substrates in ELISA, whereas all three antigen preparations could be used for sensitization of erythrocytes in the passive hemagglutination assay. The passive hemagglutination system with infected cell extracts exhibited a similar level of sensitivity and specificity to the ELISA system employing purified coronavirus but enabled 300 times more tests to be performed per volume of virus-infected cell culture.

Coronaviruses have been associated with diarrhea in a wide variety of mammals and have been isolated from clinically ill calves (3, 10), sheep (12), pigs (5, 13, 18), foals (1), dogs (2), cats (6), mice (15, 17), and humans (4), as well as from turkeys with enteritis (11). Experimentally, coronavirus infections of calves cause a severe enteritis with involvement of the small and large intestines (9), and preliminary serological investigation has revealed that bovine enteric coronavirus is widespread in western Canada and may, therefore, be an important pathogen (14).

Successful serological screening programs require a simple, rapid, sensitive, and inexpensive assay method, and the introduction of enzyme-linked immunosorbent assay (ELISA) systems in recent years has fulfilled this need, enabling sero-epidemiological studies on a variety of pathogens.

An alternative assay method, passive hemagglutination (PHA), has been used for serodiagnosis of a wide variety of microbial infections but to date has not been evaluated for coronaviruses. This paper describes a comparison of these two methods for the detection of antibody against bovine enteric coronavirus and evaluates different antigen preparations for use in each system.

MATERIALS AND METHODS

Cells and virus growth. Madin-Darby bovine kidney cells were cultured by standard methods in Eagle minimal essential medium supplemented with 10% fetal bovine serum. Confluent cell monolayers in 150-cm² tissue culture flasks (Corning Glass Works) were washed once with phosphate-buffered saline (PBS) before inoculation with 2.0×10^5 PFU of bovine coronavirus (originally obtained from S. Dea, Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, St. Hyacinthe, Quebec, Canada). After adsorption for 1 h at 37°C, the inoculum was removed, and 50 ml of Eagle minimal essential medium plus 2% fetal bovine serum was added. The cells were then incubated at 37°C for 3 days.

Preparation of antigen substrates. Infected cell extracts were prepared by pelleting cells from the tissue culture fluid at $2,000 \times g$ for 20 min (MSE chilspin) and adding this back to the remaining adherent cells previously removed from the flask by scraping into a small volume of PBS with a rubber policeman. The infected cells were washed three times, suspended in 1 ml of PBS per 150-cm² flask, extracted with 0.5% Nonidet P-40 (NP-40), and stored at -70°C.

Purified virus (approximately 1.5×10^9 PFU) was prepared from tissue culture flasks (6 by 150 cm²) by subjecting the cultures to three cycles of freezing and thawing and by clarifying the tissue culture fluid by centrifugation at $3,500 \times g$ for 20 min. Virus that was present in the supernatant fluid was pelleted at $54,500 \times g$ for 4 h (Beckman L5-65) and suspended in 1 to 2 ml of TNE buffer (0.05 M Tris-hydrochloride, 0.1 M NaCl, and 1 mM EDTA), pH 7.6. This suspension was carefully layered onto 30 ml of a 20 to 60% (wt/vol)

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linear sucrose gradient prepared in TNE buffer and centrifuged at $81,500 \times g$ for 2 h. The visible virus band was collected and diluted in PBS, and the virus was pelleted at $54,500 \times g$ for 4 h. This pellet was suspended in 1 ml of PBS and stored at -70°C . Whenever necessary, the purified virus was extracted with 0.5% NP-40.

Control antigens were prepared from mock-infected cells by the methods described above.

Preparation of antisera. Standard positive antisera were prepared by hyperimmunization with coronavirus of gnotobiotic calves which had previously been orally infected with the same virus. Control sera possessing no reactivity against bovine coronavirus were obtained from noninfected gnotobiotic calves.

ELISA procedure. ELISA tests were performed in microplates (19) by using a modification of the method of Engvall and Perlmann (7).

Round-bottomed, 96-well microtiter plates (Immunon 2; Dynatech Laboratories, Inc.) were sensitized with 100 μl of an optimal concentration of antigen preparation per well (determined by titration) diluted in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C . Before use, the sensitized plates were washed six times with distilled water (see Results). Test samples were diluted 4- or 10-fold from an initial dilution of 1:10 in 0.01 M PBS containing 0.05% Tween 20, and 100 μl of each dilution was added to duplicate wells of the sensitized plate. Positive and negative control sera were included in each series of tests. After incubation for 1 h at room temperature, the plates were washed six times with distilled water (see Results) and further incubated for 1 h after the addition of 100 μl of an optimal dilution of affinity-purified, peroxidase-conjugated rabbit anti-bovine immunoglobulin G (IgG) (H + L) (Zymed Labs) per well. Finally, the plates were again washed six times with distilled water (see Results), and bound conjugate was reacted with chromagen and enzyme substrate (100 μl of recrystallized 5-aminosalicylic acid [Aldrich Chemical Co.] per well diluted to 1 mg/ml in 0.01 M phosphate buffer containing 0.01 M Na_2EDTA [pH 5.95] to which 0.005% hydrogen peroxide was added immediately before use [8]). After 30 min at room temperature, the absorbance (450 nm) of each well was determined with a micro-ELISA reader (Dynatech Laboratories). Negative controls had an optical density of less than 0.005 U. Endpoint titers were therefore defined as the highest dilution of test sample giving an optical density of greater than 0.05 U.

Passive hemagglutination. (i) **Sensitization of turkey erythrocytes.** Turkey erythrocytes (TRBCs) were fixed with formaldehyde by the method of Sequeira and

Eldridge (16) and then treated with tannic acid, followed by sensitization with an optimal dilution of antigen preparation.

(ii) **Passive hemagglutination tests.** Test sera were titrated in 4- or 10-fold dilutions from an initial dilution of 1:20 or 1:10, respectively, in V-well microtiter plates (Dynatech Laboratories) with 0.1 M PBS (pH 7.2) containing 1% heat-inactivated normal turkey serum. A 1% suspension (25 μl) of the sensitized TRBCs in 0.1 M PBS (pH 7.2) containing 1% heat-inactivated normal rabbit serum was added to each well. After gentle agitation, the plates were covered and allowed to stand at room temperature for 30 min before endpoint agglutination titers were recorded.

RESULTS

Microtiter plates and TRBCs were each sensitized with a range of dilutions of the different antigen preparations. Titrations of standard positive and negative sera were then tested on several occasions for reactivity in both systems to determine the dilution of each antigen preparation which gave maximum sensitivity and specificity in ELISA and PHA. Table 1 shows a typical set of results. Preliminary experiments indicated that washing plates with distilled water was as effective as using either saline or PBS with or without Tween 20.

There was no difference in the sensitivity or specificity of TRBCs optimally sensitized with each of the three antigen preparations and reacted with standard positive or negative antisera. The concentration of NP-40-extracted preparations necessary for optimal sensitization of TRBCs was, however, 10-fold lower than the concentration of purified virus. In contrast, similar concentrations of all three antigen preparations proved optimal for sensitization of ELISA plates. Sensitizing plates with purified virus, however, provided a considerable increase in sensitivity of the ELISA system with no loss of specificity. Purified virus was, therefore, used as antigen substrate for further ELISA studies, whereas the NP-40 extract of virus-infected Madin-Darby bovine kidney cells was used for sensitization of TRBCs.

The reproducibility of each assay system was evaluated by testing three sera in a fourfold dilution series a minimum of five times on separate occasions. The $\log_{10}$ mean titers of the three

TABLE 1. Dilution endpoints of sera, positive or negative, for anticoronavirus activity by PHA or ELISA by using different antigen substrates at optimum concentration

Antigen substrate	PHA			ELISA		
	Optimal antigen dilution	Positive serum	Negative serum	Optimal antigen dilution	Positive serum	Negative serum
NP-40-infected cell extract	160	5,120	<20	160	800	<50
Purified virus	10	1,280	<20	100	64,000	<50
NP-40-purified virus extract	160	2,560	<20	100	1,000	<50

FIG. 1. Distribution of endpoint antibody titers against coronavirus obtained with ELISA and PHA. Samples were titrated by 10-fold dilutions from an initial dilution of 1:10. Symbols: $\times$, sera; $\circ$, whey.

sera were 3.9, 3.4, and <1.7 by ELISA, and the corresponding titers by PHA were 3.3, 2.8, and <1.3 , respectively. The range of titers obtained for the five replicates did not exceed one four-fold dilution for any of the three sera tested. Sensitizing different batches of ELISA plates or TRBCs with optimal dilutions of antigen substrates prepared on different occasions gave identical results. The absence of nonspecific reactivity was confirmed by parallel tests with plates or TRBCs sensitized with control antigens.

Fifty-two sera and 92 whey samples from naturally infected cows were screened for anti-coronavirus activity with 10-fold dilutions (Fig. 1) in the ELISA and PHA systems described above. Parallel PHA tests were performed on whey samples, with tanned, nonsensitized TRBCs used as controls for nonspecific hemagglutination. In 11 of the 92 whey samples tested, the specific endpoint was masked by nonspecific agglutination. The results in Fig. 1 show little difference between the endpoints obtained with either assay, although ELISA appeared to be slightly more sensitive. For the majority of sam-

ples, the PHA titer was equal to or one 10-fold dilution less than that obtained by ELISA. The titer by PHA was significantly higher than that obtained by ELISA with only one sample (10^7 as opposed to 10^4). Anticoronavirus titers in consecutive whey samples collected at various times postparturition showed that levels of specific antibody were high at birth and then declined rapidly (Table 2).

DISCUSSION

The ELISA and PHA techniques described in this paper showed similar levels of sensitivity and specificity. The major difference between the systems lies in their requirements for different purities of antigen substrate to produce optimal sensitivity. For maximal sensitivity in ELISA, sensitization of the solid phase with purified whole virus was necessary, whereas in PHA, sensitization of TRBCs with a crude extract of infected cells was satisfactory. Purification of virus from infected tissue culture cells is relatively costly and time consuming in comparison with the preparation of an NP-40 extract of infected cells. Six 150-cm² tissue-culture flasks

TABLE 2. Decline of coronavirus antibody levels in colostrum or milk whey after parturition as measured by PHA and ELISA

Time post-parturition	Log ₁₀ titer whey antibody from animal:															
	A		B		C		D		E		F		G		H	
	PHA	ELISA	PHA	ELISA	PHA	ELISA	PHA	ELISA	PHA	ELISA	PHA	ELISA	PHA	ELISA	PHA	ELISA
0 h	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	4.0	3.0	7.0	4.0	4.0	4.0
36 h	2.0	3.0	2.0	3.0	1.0	2.0	3.0	3.0	2.0	2.0	3.0	3.0	2.0	3.0	2.0	3.0
3 days	1.0	2.0	2.0	3.0	1.0	1.0	2.0	3.0	2.0	2.0	3.0	2.0	2.0	2.0	2.0	2.0
5 days	1.0	2.0	<1.0	2.0	1.0	1.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	<2.0	2.0
10 days	<1.0	2.0	1.0	2.0	1.0	1.0	1.0	1.0	<1.0	2.0	<1.0	2.0	1.0	2.0	<2.0	2.0
21 days	<1.0	1.0	1.0	2.0	<1.0	<1.0	1.0	1.0	<1.0	1.0	1.0	2.0	<1.0	2.0	<2.0	2.0

yield 1 ml of purified virus, which would be sufficient for 480 ELISA tests. In contrast, the yield of infected cell extract from the same number of flasks would be sufficient for 153,600 PHA tests. Another advantage of PHA over ELISA is its rapidity; PHA can be read after 30 min, whereas ELISA requires a minimum of 150 min. The major disadvantage of PHA is the nonspecific agglutination found with certain whey samples; however, this can be readily detected by the use of tanned, nonsensitized control cells and therefore remains a problem only with whey samples possessing a low titer of specific antibody. Both of these assay systems are suitable for the screening or titration of large numbers of samples; however, the economy and speed of the PHA test suggests that this is the method of choice.

The rapid decline in the level of milk antibody after parturition has been previously reported for coronavirus antibody (14), and a similar situation is also found with rotavirus (20). This may explain the prevalence of such infections in neonatal calves, since these declining levels of antibody in milk would be insufficient to protect against the repeated high-challenge doses to which calves would be exposed under natural conditions.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Linda McDougall and Donna Dent, and thank Bonnie Neufeld for typing the manuscript.

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